Toll-like receptor 4-dependent barrier dysfunction and its impact on irinotecan-induced gut toxicity and pain

A thesis submitted in fulfillment for degree of

DOCTOR OF PHILOSOPHY

in

The Discipline of Anatomy and Pathology

School of Medicine

The University of Adelaide

by

Hannah Rose Wardill

22/08/16
Declaration

“This work contains no material that has been accepted for the award of any other degree or diploma in any university or tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.”

I give consent for this copy of my thesis, when deposited in the University Library, being made available for loan and photocopy.

Hannah Rose Wardill

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Table of Contents

Declaration............................................................................................................................................ ii

Table of Contents ................................................................................................................................... iii

Acknowledgements ................................................................................................................................... x

Publications arising from this thesis .................................................................................................... xii

Contributions made by co-authors.......................................................................................................... xiv

Additional studies and publications ....................................................................................................... xxxii

Co-authored publications ....................................................................................................................... xxxiii

Thesis explanation ................................................................................................................................. xxxiv

Nomenclature ........................................................................................................................................... xxxv

Chapter 1 General introduction .............................................................................................................. 1

1.1 Chemotherapy-induced alimentary toxicity .................................................................................... 1

1.2 Pathobiology ..................................................................................................................................... 3

1.2.1 Common mechanisms underpin oral and gut toxicity ................................................................. 3

1.2.2 The 5-phase model of alimentary toxicity .................................................................................. 3

1.3 Irinotecan hydrochloride .................................................................................................................. 4

1.3.1 Irinotecan-induced diarrhoea ..................................................................................................... 5

1.3.2 Intestinal barrier dysfunction ..................................................................................................... 6

1.4 Gut dysbiosis: the catalyst for barrier dysfunction and innate immune activation ......................... 9

1.5 Toll-like receptors ............................................................................................................................. 10

1.5.1 TLR4-mediated glial activation and pain...................................................................................... 12

1.6 Hypotheses and aims ....................................................................................................................... 13

Chapter 2 Chemotherapy-induced mucosal barrier dysfunction: an updated review on the role of tight junctions .......................................................................................................................... 17

2.1 Abstract ........................................................................................................................................... 17
2.2 Introduction .................................................................................................................. 18
2.3 Mucosal barrier function ........................................................................................... 19
  2.3.1 Molecular structure of tight junctions.................................................................. 19
2.4 Are alterations in intestinal tight junctions pivotal to CIGT development?.............. 20
  2.4.1 Clinical studies .................................................................................................. 21
  2.4.2 Preclinical studies .............................................................................................. 22
2.5 Regulators of tight junctions constitute key steps in the pathophysiology of gut toxicity 25
  2.5.1 Proinflammatory cytokines disrupt barrier function .......................................... 25
  2.5.2 The extracellular matrix maintains mucosal homeostasis .................................. 26
2.6 Potential involvement of tight junctions in chemotherapy-induced diarrhoea .......... 27
2.7 Where to now? ......................................................................................................... 30

Chapter 3 Irinotecan disruptions tight junction proteins within the gut: implications for chemotherapy-induced gut toxicity ................................................................. 33
3.1 Abstract ...................................................................................................................... 33
3.2 Introduction ................................................................................................................ 34
3.3 Materials and Methods ............................................................................................. 36
  3.3.1 Animals and ethics ............................................................................................. 36
  3.3.2 Experimental design ......................................................................................... 36
  3.3.3 Clinical assessment of gut toxicity ...................................................................... 37
  3.3.4 Tissue preparation ............................................................................................. 37
  3.3.5 Real-time polymerase chain reaction (RT-PCR) ................................................ 38
  3.3.6 Statistical analysis .............................................................................................. 40
3.4 Results ......................................................................................................................... 41
  3.4.1 Irinotecan causes severe gut toxicity characterised by diarrhoea and weight loss .. 41
  3.4.2 Irinotecan causes severe histopathological damage in the small and large intestine.... 41
  3.4.3 Irinotecan causes molecular defects in intestinal tight junction proteins.................. 44
  3.4.4 RT-PCR efficiency and housekeeping gene stability (UBC) ................................. 48
Chapter 4 Tight junction defects are seen in the buccal mucosa of patients receiving standard dose chemotherapy for cancer ................................................................. 56

4.1 Abstract ........................................................................................................... 56
4.2 Introduction ...................................................................................................... 57
4.3 Materials and Methods .................................................................................... 60
  4.3.1 Patients ........................................................................................................ 60
  4.3.2 Clinical assessment of oral toxicity ............................................................. 61
  4.3.3 Histopathological analysis of the oral epithelium ...................................... 63
  4.3.4 Immunohistochemical analysis of tight junctions and inflammatory markers 63
  4.3.5 Statistical analysis ...................................................................................... 68
4.4 Results ............................................................................................................ 69
  4.4.1 Chemotherapy causes significant epithelial atrophy consistent with oral toxicity ...... 69
  4.4.2 Chemotherapy increases proinflammatory cytokines and alters MMP profiles ........ 72
  4.4.3 Tight junction defects are seen in the buccal mucosa following chemotherapy ....... 72
4.5 Discussion ....................................................................................................... 78
4.6 Conclusion ...................................................................................................... 82

Chapter 5 Toll-like receptor 4 signalling: a common biological mechanism of regimen-related toxicities – an emerging hypothesis for neuropathy and gastrointestinal toxicity .......... 85

5.1 Abstract ........................................................................................................... 85
5.2 Introduction ...................................................................................................... 86
5.3 Indirect neuromodulation through glial activation ............................................. 88
  5.3.1 The emerging role of glia in neuropathic pain ............................................. 88
  5.3.2 TLR4-mediated glial activation ................................................................. 91
  5.3.3 TLR4 in the central nervous system ............................................................ 92
5.4 TLR4 and neuropathic pain ............................................................................. 94
5.4.1 Peripheral tissue damage activates central TLR4 ................................................................. 94
5.5 Blood brain barrier disruption permits central pathology ....................................................... 96
5.6 Clinical translation ...................................................................................................................... 98
5.7 Conclusions and future directions ............................................................................................. 100

Chapter 6 Irinotecan-induced gastrointestinal dysfunction and pain are mediated by common
TLR4-dependent mechanisms ........................................................................................................104

6.1 Abstract ........................................................................................................................................104
6.2 Introduction ...................................................................................................................................105
6.3 Materials and Methods ................................................................................................................108
  6.3.1 Animal model and ethics ...........................................................................................................108
  6.3.2 Experimental design ................................................................................................................108
  6.3.3 Clinical assessment of gut toxicity .............................................................................................109
  6.3.4 Facial grimace criteria ...............................................................................................................109
  6.3.5 Tissue preparation ....................................................................................................................110
  6.3.6 Bacterial diversity profiling ......................................................................................................110
  6.3.7 Histopathological and immunohistochemical analysis ..............................................................111
  6.3.8 Assessment of in vivo intestinal permeability .........................................................................115
  6.3.9 Tissue cytokine protein quantification using the Luminex multiplex platform .................116
  6.3.10 Statistical analysis ................................................................................................................116
6.4 Results .........................................................................................................................................117
  6.4.1 Bacterial diversity profiling ......................................................................................................117
  6.4.2 BALB/c-Tlr4<sup>−/−</sup>billy mice have attenuated clinical manifestations of irinotecan-induced
gut toxicity .........................................................................................................................................121
  6.4.3 BALB/c-Tlr4<sup>−/−</sup>billy mice have improved histological architecture in the small intestine 121
  6.4.4 TLR4-dependent signalling contributes to intestinal barrier disruption .............................125
  6.4.5 BALB/c-Tlr4<sup>−/−</sup>billy mice exhibit a muted inflammatory response ..............................127
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.4.6</td>
<td>Irinotecan-induced pain is associated with TLR4-dependent astrocytic GFAP expression</td>
<td>130</td>
</tr>
<tr>
<td>6.4.7</td>
<td>Irinotecan increases blood brain barrier permeability to albumin</td>
<td>130</td>
</tr>
<tr>
<td>6.5</td>
<td>Discussion</td>
<td>134</td>
</tr>
<tr>
<td>6.6</td>
<td>Conclusions</td>
<td>139</td>
</tr>
<tr>
<td><strong>Chapter 7</strong></td>
<td><strong>TLR4-dependent claudin-1 internalisation and secretagogue-mediated chloride secretion regulate irinotecan-induced diarrhoea</strong></td>
<td><strong>142</strong></td>
</tr>
<tr>
<td>7.1</td>
<td>Abstract</td>
<td>142</td>
</tr>
<tr>
<td>7.2</td>
<td>Introduction</td>
<td>143</td>
</tr>
<tr>
<td>7.3</td>
<td>Materials and Methods</td>
<td>146</td>
</tr>
<tr>
<td>7.3.1</td>
<td>Animal model and ethics</td>
<td>146</td>
</tr>
<tr>
<td>7.3.2</td>
<td>Experimental design</td>
<td>146</td>
</tr>
<tr>
<td>7.3.3</td>
<td>Clinical assessment of gut toxicity</td>
<td>147</td>
</tr>
<tr>
<td>7.3.4</td>
<td>Tissue preparation</td>
<td>147</td>
</tr>
<tr>
<td>7.3.5</td>
<td>Tight junction analysis</td>
<td>147</td>
</tr>
<tr>
<td>7.3.6</td>
<td>Electrophysiological analysis using Ussing chambers</td>
<td>151</td>
</tr>
<tr>
<td>7.3.7</td>
<td>Statistical analysis</td>
<td>152</td>
</tr>
<tr>
<td>7.4</td>
<td>Results</td>
<td>153</td>
</tr>
<tr>
<td>7.4.1</td>
<td>Cytoplasmic redistribution of claudin-1 contributes to TLR4-dependent barrier disruption</td>
<td>153</td>
</tr>
<tr>
<td>7.4.2</td>
<td>Irinotecan increases chloride secretion in the distal colon via TLR4-independent mechanisms</td>
<td>160</td>
</tr>
<tr>
<td>7.5</td>
<td>Discussion</td>
<td>163</td>
</tr>
<tr>
<td>7.5.1</td>
<td>TLR4-dependent mechanisms for tight junction disruption</td>
<td>164</td>
</tr>
<tr>
<td>7.5.2</td>
<td>Clinical implications of barrier dysfunction</td>
<td>165</td>
</tr>
<tr>
<td>7.5.3</td>
<td>Contribution to diarrhoea</td>
<td>166</td>
</tr>
<tr>
<td>7.6</td>
<td>Conclusion</td>
<td>169</td>
</tr>
</tbody>
</table>
Chapter 8 A novel in vitro platform for the study of SN-38-induced mucosal damage and the development of TLR4-targeted therapeutic options............................................. 172

8.1 Abstract .................................................................................................................. 172
8.2 Introduction .............................................................................................................. 173
8.3 Materials and Methods .......................................................................................... 176
  8.3.1 Cell culture ....................................................................................................... 176
  8.3.2 Transmission electron microscopy ................................................................ 179
  8.3.3 Immunofluorescence for tight junction proteins .............................................. 179
  8.3.4 Liquid chromatography-mass spectrometry ..................................................... 180
  8.3.5 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of TLR4 expression ........................................................................................................ 182
  8.3.6 Statistical analysis ............................................................................................ 183
8.4 Results .................................................................................................................... 184
  8.4.1 Polyester membrane inserts support a polarised T84 phenotype with functional tight junctions in vitro ................................................................................ 184
  8.4.2 SN-38 stability in the transwell support system .............................................. 188
  8.4.3 T84 cells express TLR4 .................................................................................... 190
8.5 Discussion .............................................................................................................. 192
8.6 Conclusions ........................................................................................................... 195

Chapter 9 Addendum: Characterisation of SN-38 epithelial injury using novel in vitro model ............................................................................................................... 196

9.1 Rationale ................................................................................................................. 196
9.2 Materials and Methods .......................................................................................... 197
  9.2.1 Cell culture ....................................................................................................... 197
  9.2.2 SN-38 dose finding study ................................................................................. 197
  9.2.3 Characterisation of SN-38 induced barrier dysfunction .................................... 201
9.3 Results .................................................................................................................... 202
  9.3.1 SN-38 causes dose- and time-dependent decreases in TEER .................... 202
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.3.2 SN-38 induces barrier dysfunction in T84 monolayers</td>
<td>207</td>
</tr>
<tr>
<td>9.3.3 SN-38 causes electrophysiological changes in T84 monolayers</td>
<td>211</td>
</tr>
<tr>
<td>9.4 Discussion and Conclusions</td>
<td>213</td>
</tr>
<tr>
<td><strong>Chapter 10 General discussion</strong></td>
<td>215</td>
</tr>
<tr>
<td>10.1 Introduction</td>
<td>215</td>
</tr>
<tr>
<td>10.2 Tight junction disruption: a common trait of alimentary toxicity</td>
<td>216</td>
</tr>
<tr>
<td>10.3 TLR4-dependent mechanisms regulate irinotecan-induced barrier dysfunction and exacerbate clinical manifestations of gut toxicity</td>
<td>217</td>
</tr>
<tr>
<td>10.4 TLR4-dependent barrier dysfunction permits chloride-driven water fluxes, contributing to diarrhoea development</td>
<td>221</td>
</tr>
<tr>
<td>10.5 Translating <em>in vivo</em> mechanisms to novel <em>in vitro</em> model</td>
<td>222</td>
</tr>
<tr>
<td>10.6 Central barrier disruption permits TLR4-dependent pain pathways</td>
<td>222</td>
</tr>
<tr>
<td>10.7 Practical considerations for TLR4-targeted therapeutic approaches</td>
<td>225</td>
</tr>
<tr>
<td>10.8 Conclusions and future directions</td>
<td>226</td>
</tr>
<tr>
<td><strong>Chapter 11 References</strong></td>
<td>227</td>
</tr>
</tbody>
</table>
Acknowledgements

I extend my thanks and gratitude to my three supervisors, Professor Rachel Gibson, Dr Joanne Bowen and Professor Richard Logan for giving me the opportunity to undertake this PhD, reading many drafts and always providing encouragement while completing my studies. I would also like to thank Dr Janet Coller for her continued mentorship.

In addition, I would like to thank Ms Ysabella Van Sebille for her friendship and support throughout my candidature, Mr Kent Algate for his comedic relief and Dr Danijela Menicanin for her guidance and advice. You are my second family, and I would not have had as much fun as I did without you.

Thank you to members of the Cancer Treatment Toxicities Group for fostering such a positive research environment, it has been great to see the group grow during the past few years.

I would like to thank members of the Stem Cell Research Group, at the South Australian Health and Medical Research Institute. In particular, thank you Professor Andrew Zannettino and Dr Jacqueline Noll for guiding me through the CRISPR/Cas9 technique. I would also like to thank the following people for their technical support:

- Dr Lachlan Jolly: Neurogenetics Research Group
- Professor Mark Hutchinson: Neuroimmunopharmacology Research Group
- Dr Lyndsey Collins-Praino: Neurological Research Group
- Mr Jim Manavis: Neurological Research Group

I would also like to the The Florey Medical Research Foundation Project Grant in Cancer Research and the The Doctor Chun Chung Wong and Madam So Lau Lam Memorial Postgraduate Scholarship for their support. Thank you also to the Ray and Shirl Norman Cancer Research Trust and Australian Dental Research Foundation for providing funds to carry out my research projects. In addition, I would like to thank the Channel 9 Young Achiever Award committee for their continued support, the Australian Society for Medical Research and the Multinational Association of Supportive Care in Cancer for recognising my research.
Finally, thank you also to my family and friends for the support, encouragement and belief in me over the years. I would like to particularly thank my supportive partner, Nick, for his patience and encouragement throughout my entire candidature, and my mum and dad for their unwavering support.
Publications arising from this thesis


* denotes invited review
Contributions made by co-authors

Professor Rachel J Gibson


Professor Rachel J Gibson was my co-principal supervisor (with Dr Joanne M Bowen) and has therefore been listed on all publications arising from this thesis. Rachel helped design and interpret results as well as being responsible for obtaining funding for this project. She was also involved in drafting all manuscripts in preparation for publication.
Dr Joanne M Bowen


Contributions made by co-authors


Dr Joanne M Bowen was my co-principal supervisor (with Professor Rachel J Gibson) and has therefore been listed on all publications arising from this thesis. Joanne provided significant technical advice for much of the experimental work and also helped design and interpret results. She was also responsible for obtaining funding for this project and drafting all manuscripts in preparation for publication.
Contributions made by co-authors

Professor Richard M Logan


Professor Richard M Logan is my third supervisor. He has been listed as co-author on all publications from this thesis. Richard was involved in the original clinical study from which archival tissue samples were obtained and used for one of my studies (Chapter 3). He has also revised many drafts and provided assistance in gaining independent funding to support my research.
Ms Ysabella ZA Van Sebille


Ms Ysabella ZA Van Sebille is a member of the Cancer Treatment Toxicities group. Ysabella contributed to several publications arising from this thesis by assisting with laboratory and animal work, as well as reading draft manuscripts.
Contributions made by co-authors

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Ms Mander is part of the Adelaide Centre for Neuroscience Research. She has a keen interest in implications for blood brain barrier disruption in various disease states. Kim was involved in two reviews that were completed during my candidature, reading drafts and providing information about blood brain barrier regulation.
Ms Kate R Secombe


Ms Kate R Secombe was an honours student and research assistant in the Cancer Treatment Toxicities group during my candidature. During her honours degree, Kate helped with animal and laboratory work and has therefore been listed on the two publications arising from this study. During her time as a research assistant, she provided assistance in maintaining cell culture lines and stocks.
Contributions made by co-authors

Dr Janet K Coller


Dr Janet K Coller was responsible for obtaining funding to conduct my animal project. She also contributed significant time in reading draft manuscripts in preparation for publication.
Ms Imogen E Ball (nee White)


Ms Imogen E Ball is a research assistant in the Cancer Treatment Toxicities Group. Imogen was listed as co-author on the two publications based on her contribution to the animal study.
Professor Mark R Hutchinson


Professor Mark R Hutchinson was listed as co-author on the first publication arising from my animal study as he sourced and provided the BALB/c-*Tlr4*^+/-^ mice. Mark is highly specialised in the area of neuroimmunology, and he therefore provided advice regarding my analysis of neuroinflammation. He was also involved in preparing this manuscript for publication, reading several drafts.
Ms Vicky Staikopoulos


Ms Vicky Staikopoulos was a research officer in the Neuroimmunopharmacology Laboratory during my candidature. She was involved in training me on many techniques required for my animal study. These techniques were not available within the Cancer Treatment Toxicities Group. Vicky also assisted in analysis of glial immunohistochemistry and read several draft manuscripts.
Mr Jim Manavis


Mr Jim Manavis is head of histology in the School of Medicine, University of Adelaide. He has experience in immunohistochemical analysis of immune cells within the central nervous system as well as detecting changes in the blood brain barrier. Jim provided substantial advice regarding my analysis of central nervous system pathologies, an area my laboratory has little experience with. He provided technical assistance and read drafts of the manuscript.
Ms Romany Stansborough


Ms Romany Stansborough was an undergraduate during my candidature. Romany was involved in conducting parts of the laboratory work that contributed to the publication listed. She is now a member of the Cancer Treatment Toxicities Group.
Ms Joseph Shirren


Ms Joseph Shirren was an undergraduate during my candidature. Joseph was involved in conducting parts of the laboratory work that contributed to the publication listed. He is now a member of the Cancer Treatment Toxicities Group.
Dr Emma Bateman


Dr Emma Bateman is the laboratory manager of the Mucositis Research Group. Emma was involved in conducting the original animal study from which archival tissue samples were sought for the study listed. She also read several drafts of the manuscript and has therefore been listed as a co-author.
Ms Masooma Sultani


Ms Masooma Sultani was a member of the Mucositis and Gut Microbiome Research Groups during my candidature. She was involved in conducting the original animal study from which archival tissue samples were sought for the study listed.
Additional studies and publications

During my honours degree and PhD candidature, I published several first author reviews/primary research papers that are not presented in my thesis. These publications are listed below:


* denotes invited review
Co-authored publications

During my candidature, I was also involved in several other studies investigating intestinal toxicity. This involvement resulted in co-authorship of several manuscripts. These publications are not presented in my thesis, and are listed below:


Van Sebille YZA, Gibson RJ, Wardill HR and Bowen JM, ErbB small molecule tyrosine kinase inhibitor (TKI) induced diarrhoea: chloride secretion as a mechanistic hypothesis. *Cancer Treatment Reviews*. 41(7): 646-52.


* denotes invited review
Thesis explanation

The format of my thesis is as follows: a general introduction, a literature review, two research chapters, a second literature reviews, three research chapters, a general discussion and references. During my candidature, I made significant effort to publish my research findings. Each research chapter is presented in its original publication format. This may result in slight repetition between chapters arising from the same study.

My thesis has three distinct themes relating to the pathobiology of chemotherapy-induced gut toxicity. The first aims to characterise the extent of tight junction disruption in the alimentary tract following chemotherapy treatment (clinically and preclinically), giving rise to the first two research chapters (Chapter 2 and 3). The first publication (Chapter 2) was completed early in my candidature (2013). The second publication (Chapter 3) arose from independent research funding I obtained from the Australian Dental Research Foundation. Together, these chapters formed the scope and theme for my PhD, and are therefore followed by two literature reviews and the remaining four research chapters. The second theme relates to involvement of innate immune regulation in the development of chemotherapy-induced gut toxicity and barrier dysfunction, giving rise to an additional two primary research chapters (Chapter 6 and 7). The third aim of this thesis was to develop a high throughput in vitro model for the study of chemotherapy-induced mucosal injury and targeted therapeutic approaches. This is summarised in Chapters 8 and 9.

During my candidature, I had the opportunity to work with Professor Stephen Sonis from Dana-Farber Harvard Cancer Centre, Harvard University, Boston. After presenting my work at the Multinational Association for Supportive Care in Cancer in 2014 (Miami, USA), Professor Sonis and I developed the hypothesis that gut-derived inflammation affects central neurological functions. This formed the basis for my secondary literature review (Chapter 5) as well as an additional literature review on cytokine-mediated blood brain barrier permeability and its involvement in chemotherapy-induced cognitive decline. The latter literature review is not included as a chapter in this thesis, but as an appendix in its original publication format (PDF).
Nomenclature

This thesis contains variations in terminology relating to chemotherapy-induced gastrointestinal toxicity. This reflects failure in the field to accurately define this pathology. Inconsistencies within this thesis are due to requests made by reviewers during peer-review of each publication. I did not change the terminology from what was used in the original publications to avoid altering their content.

Please review the following nomenclature.

*Alimentary tract*: any region from mouth to anus

*Alimentary toxicity/mucositis*: ulceration/inflammation of the mouth or gastrointestinal tract

*Oral toxicity/mucositis*: ulceration and inflammation of the mouth

*Gastrointestinal/gut toxicity/mucositis*: ulceration/inflammation of the small or large intestine, rectum and anus

In addition, publications from within this thesis conform to standard nomenclature for naming genetically modified mice as per the guidelines outlined in International Committee on Standardised Genetic Nomenclature for Mice. Wild-type mice on a BALB/c background will be referred to as wild-type (WT). Toll-like receptor 4 knockout mice on a BALB/c background will be referred to as BALB/c-*Tlr4*<sup>-/-</sup>billy. Reference to the process of deleting a gene will be preferred to as knockout or *-/-*, e.g. *Tlr4*<sup>-/-</sup>.
Chapter 1 General introduction

This thesis investigates mucosal injury of the oral cavity and gastrointestinal tract following chemotherapy. The cytotoxic agents used during chemotherapy treatment are non-selective and indiscriminately target healthy cells. Mucosal linings, such as those found within the alimentary tract (mouth to anus), are particularly vulnerable due to their highly proliferative nature (Gutheil and Kearns, 1997, Al-Dasooqi et al., 2013a, Al-Dasooqi et al., 2013). Damage which occurs to these mucosal linings is broadly termed mucositis. However it is becoming increasingly recognised that this damage stems from a complex cascade of cellular events arising from the both mucosal and non-mucosal tissues (Sonis, 2004c). Therefore, for the purpose of this thesis, the term chemotherapy-induced alimentary toxicity has been used to describe damage throughout the entire alimentary tract, including both oral and gastrointestinal manifestations. The terms chemotherapy-induced oral toxicity (CIOT) and chemotherapy-induced gut toxicity (CIGT) will be used to describe experiments investigating manifestations of a specific region.

The purpose of this general introduction is to provide relevant background information regarding the current state of knowledge for chemotherapy-induced alimentary toxicity, with significant focus on the underlying mechanisms of CIGT and its associated diarrhoea. This chapter also aims to provide an overview of the thesis in regards to the main themes explored throughout the experimental chapters.

1.1 Chemistry-induced alimentary toxicity

Alimentary toxicity has become an increasingly recognised and better characterised side effect of chemotherapy (Keefe et al., 2007). The condition is characterised by breakdown of the mucosal barrier of the oral and gastrointestinal tract, resulting in (a) severe ulceration, (b) bacterial translocation and (c) an imbalance between secretion and absorption, manifesting as diarrhoea.

Alimentary toxicity is common, occurring in 40% of patients receiving standard dose chemotherapy and 100% of patients undergoing high dose chemotherapy or stem cell/bone marrow transplantation (Gibson and Stringer, 2009a, Wardill et al., 2014b). Whilst it has been documented that alimentary
toxicity significantly impacts on patient quality of life, this impact is difficult to quantify due to under-reporting of symptoms (Cirillo et al., 2009), the selective reporting of only severe forms of the condition (grades 3 and 4) and a failure to recognise either its frequency or its impact on patients (Sonis et al., 2015).

Chemotherapy-induced oral toxicity typically presents as ulcerative lesions impacting on the ability to eat, drink and speak (Al-Dasooqi et al., 2013). Similarly, gut toxicity, which manifests as clinically-diagnosed diarrhoea, ulceration, inflammation, pain and bleeding, renders patients unable to perform daily tasks (Sonis, 2004c). In both cases, patients are at a significantly greater risk of infection and require additional supportive care measures such as total parenteral nutrition, oral opioids, antibiotics/fungals and hospitalisation (Elting et al., 2003, Wardill et al., 2013). The provision of these supportive measures is also associated with significant economic impact. Several studies from both the US and Canada have made efforts to quantify this burden, well summarised by Carlotto et al., 2013. Although variations exist in the final estimates attributed to both oral and gut toxicity, most recent studies indicate incremental costs of 21,766 USD for severe forms of oral toxicity (Nonzee et al., 2008). The most recent study to assess diarrhoea indicated an additional cost of 2,717.90 USD per chemotherapy cycle (Mittmann et al., 2010), although these are considerably lower than those reported by Elting et al., (2003) suggesting severe diarrhoea resulted in an additional 8,443.55 USD per chemotherapy cycle (Elting et al., 2003). In addition to the substantial economic burden with which it is associated, chemotherapy-induced alimentary toxicity can also become acutely life threatening, requiring dose-reductions or complete treatment cessation, impacting on the provision of optimal cancer treatment (Al-Dasooqi et al., 2013a, Al-Dasooqi et al., 2013, Bowen et al., 2013a, Gibson et al., 2013). Chemotherapy-induced alimentary toxicity is therefore a major oncological problem, considered one of the most significant dose-limiting toxicities with no universally accepted preventative treatments.
1.2 Pathobiology

1.2.1 Common mechanisms underpin oral and gut toxicity

All cytotoxic agents cause alimentary toxicity of varying severities based on individual risk factors, tumour characteristics and treatment regimen (Lalla et al., 2014). Classically, oral and gut toxicity had been viewed independently of one another, thought to arise from distinct pathobiological mechanisms. However, the term alimentary toxicity was developed in 2004 to describe toxicity affecting both regions (Keefe, 2004a). This is based on the understanding that the alimentary tract is a single structure embryonically, from mouth to anus, coupled with the body having limited ways in which to respond to damage. It is therefore likely the mechanisms involved in toxicity are similar throughout the entire alimentary tract, with regional differences in the toxicity profile a consequence of local structure and differences in the mucosae (Keefe, 2004a, Keefe et al., 2004). This holistic approach to toxicity has seen the 5-phase model, originally developed to explain oral toxicity, applied to the development of gut toxicity.

1.2.2 The 5-phase model of alimentary toxicity

It is now universally accepted that the development of alimentary toxicity is the consequence of both direct cytotoxicity by chemotherapy and radiotherapy, characterised by irreversible DNA damage, as well as indirect tissue injury, primarily driven by proinflammatory signalling pathways. The broadly accepted pathobiology of chemotherapy-induced alimentary toxicity comprises five continuous and overlapping phases described by Sonis (2004) (Sonis, 2004c). The phases are: 1) initiation, 2) upregulation and message generation, 3) signalling and amplification, 4) ulceration and 5) healing. Briefly, direct cytotoxicity initiates the production of reactive oxygen species (ROS) which directly damage blood vessels, epithelial cells and submucosal tissue. ROS production also leads to an intense inflammation response driven primarily through nuclear factor kappa B (NFκB) signalling. When activated, this transcription factor causes significant upregulation of interleukin(IL)-1β, IL-6 and tumour necrosis factor (TNF) which all enhance NFκB expression through positive feedback mechanisms. NFκB activation also increases the synthesis and activation of adhesion molecules and
Chapter 1 General introduction

cyclooxygenase-2, leading to enhanced injury and apoptosis. This cascade of biological events eventually culminates to form the ulcerative stage of toxicity, characterised by clear breaches in the alimentary mucosa, bacterial colonisation and translocation, inflammation and pain. Finally, healing occurs through renewal of proliferation and differentiation of the epithelium, as well as local microbial flora. Although this model can be applied to most chemotherapeutic agents, as well as radiotherapy, each treatment modality has unique pathological features often based on the metabolism and pharmacokinetics of each anticancer drug (Wardill et al., 2016a). This thesis focuses predominantly on the gastrointestinal side effects associated with the chemotherapeutic drug, irinotecan hydrochloride (CPT-11, Pfizer). The major dose-limiting side effect of irinotecan is frequent and severe gut dysfunction, manifesting as diarrhoea (Li et al., 2015).

1.3 Irinotecan hydrochloride

Irinotecan hydrochloride is a chemotherapeutic drug used to treat colorectal carcinoma and a variety of other solid tumours (Ikuno et al., 1995, Takasuna et al., 1996, Tsunoda et al., 2010, Pedroso et al., 2015). Irinotecan serves as the water-soluble precursor of its lipophilic metabolite, SN-38, which is formed by carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and the dipiperdino side chain (Chabot, 1997). SN-38 is approximately 1000 times as potent as irinotecan as an inhibitor of the DNA enzyme topoisomerase I and its unique hepatobiliary metabolism is responsible for the high levels of intestinal toxicity with which it is associated (Araki et al., 1993). SN-38 is glucuronidated to SN-38-glucuronide (SN-38G) and detoxified in the liver via conjugation by the UDP glucurononosyltransferase 1A (UGT1A) family, which releases SN-38G into the intestines for elimination. However, in the intestinal lumen, bacterial β-glucoronidases are able to regenerate SN-38 from SN-38G. This second pass metabolism is key to the dose-limiting, and clinically diagnosed, diarrhoea associated with irinotecan treatment (Dodds et al., 2000, Di Paolo et al., 2006). The impact of this toxic metabolic process has also been demonstrated though a number of genetic trials, linking UGT1A single nucleotide polymorphisms (SNPs) with the incidence of irinotecan-induced diarrhoea. For example, a recent meta-analysis revealed that the
The **UGT1A1*28/*28** genotype was not only associated with worsened diarrhoea, but was also able to predict the relative risk of diarrhoea following medium (RR, 1.79; 95% CI, 1.08–2.97) and high (RR, 2.32; 95% CI, 1.25–4.28) dose irinotecan (Hu et al., 2010). It has also been shown that the presence of seven TA repeats (TA7), rather than six (TA6), in the TATA box of the **UGT1A1** promoter reduces enzyme expression and leads to impaired SN-38 conjugation and an increase in the severity of irinotecan-induced diarrhoea (Massacesi et al., 2006). Despite these findings, conclusions from pharmacogenetics studies within this field are not uniform and genetic testing remains reliant on clinical judgment.

### 1.3.1 Irinotecan-induced diarrhoea

Diarrhoea is considered the most severe, dose-limiting side effect of irinotecan treatment (Lalla et al., 2014). Irinotecan-induced diarrhoea presents bimodally, with an early secretory diarrhoea followed by a more severe, late-onset diarrhoea (Tsavaris et al., 2003, Gibson et al., 2007). A number of preclinical models have aimed to determine the underlying mechanisms responsible for this late-onset diarrhoea, with gastrointestinal atrophy, erythema and decreases in intestinal wet weights consistently reported (Logan et al., 2008a, Logan et al., 2009). Histopathologically, epithelial vacuolation, blood vessel dilation, goblet cell metaplasia and infiltration of polymorphic cells often accompany villous atrophy and crypt ablation (Logan et al., 2008a). Based on these changes, it is hypothesised that irinotecan-induced diarrhoea is characterised by malabsorption and mucin hypersecretion. In reality, it is likely that a combination of mechanisms contribute to diarrhoea development through disruption and imbalance of normal gut function resulting in secretory-, exudative- and osmotic-driven diarrhoea (Al-Dasooqi et al., 2013). More recently, it has been suggested that intestinal barrier function is critically important in the regulation of diarrhoea through its control of paracellular water movement and regulation of mucosal immunity (Wardill et al., 2012, Wardill and Bowen, 2013). The impact of chemotherapy, in particular irinotecan treatment, on intestinal barrier function and tight junction integrity forms the basis of this thesis.
1.3.2 Intestinal barrier dysfunction

The intestinal epithelium has two important and distinctly different roles within the gastrointestinal tract. It mediates the complex absorption of nutrients from the intestinal lumen, and simultaneously represents a barrier separating the internal milieu from the outside environment in both an immunologic and metabolic sense (Schulzke et al., 2009, Castoldi et al., 2015). In the gut, the luminal surface comes into direct contact with the highest concentrations of bacteria, antigens and a host of potentially toxic compounds (Castoldi et al., 2015). Consequently, the paradoxical functions of the intestinal barrier are critical in maintaining gastrointestinal health and homeostasis.

The intestinal barrier is formed and maintained predominantly through the actions of tight junctions; highly plastic structures able to undergo significant modulation (Gonzalez-Mariscal et al., 2008). Tight junctions are multi-protein complexes that form a selectively permeable seal between adjacent epithelial cells and demarcate the boundary between apical and basolateral membrane domains (Figure 1.1). The modification of tight junctions and paracellular permeability is dynamically regulated by various extracellular stimuli and is critical in maintaining the balance between health and disease susceptibility (Krug et al., 2014). Evidence from preclinical and clinical studies indicate intestinal tight junctions and barrier function play critical roles in the pathogenesis of intestinal and systemic diseases, with defects in their integrity and function reported in several gut disorders such as inflammatory bowel disease (Bertiaux-Vandaele et al., 2011, Rao et al., 2012, Landy et al., 2016).

There is significant anecdotal evidence implicating tight junctions in the development of CIGT. Preclinical studies have shown changes in the expression and localisation of key tight junction proteins following a number of chemotherapeutic agents (Hamada et al., 2010), including irinotecan (Nakao et al., 2012). However, conclusions are difficult to draw from these studies due to small sample sizes, varying treatment regimens and inconsistent findings. Clinically, the molecular changes in tight junctions have not been assessed, however changes in functional barrier indices
have been identified suggesting altered tight junction integrity (Keefe et al., 1997, Fazeney-Dorner et al., 2002, Blijlevens et al., 2005b, Melichar et al., 2008).

It is also becoming increasingly clear that many of the documented mediators of tight junction proteins constitute key steps in the pathobiology of CIGT (Wardill et al., 2012). For example, many proinflammatory cytokines have been extensively shown to induce proteolysis and internalisation of key tight junction proteins (Fish et al., 1999, Ma et al., 2004, Edelblum and Turner, 2009, Kimura et al., 2009, Beutheu Youmba et al., 2012). This supports the finding of barrier dysfunction in many gastrointestinal diseases characterised by inflammation (Li et al., 2013, Gong et al., 2016). In addition, extracellular matrix signalling, in particular the actions of matrix metalloproteinases (MMPs), have been recognised for their ability to disrupt intestinal barrier function (Al-Dasooqi et al., 2014). More recently, the impact of the gut microbiome on intestinal barrier function has received significant attention (Sheth et al., 2007, Nassour and Dubreuil, 2014, Sumitomo et al., 2016, Thaiss et al., 2016). This is of particular interest as gut dysbiosis emerging as a driver of intestinal toxicity (Wardill et al., 2013).
Figure 1.1 The intestinal tight junction is located at the apico-lateral border of epithelial cells.

Under healthy circumstances, intestinal tight junctions regulate the delicate balance between absorption and protection, providing a semi-permeable barrier throughout the alimentary tract. Proinflammatory cytokines, matrix metalloproteinase signalling and changes in the composition of the gut microbiota are all recognised for their detrimental effects on tight junction proteins and barrier function. *Adapted from Wardill et al., (2012).*
1.4 Gut dysbiosis: the catalyst for barrier dysfunction and innate immune activation

The microbiota of the gastrointestinal tract is a complex ecosystem, with crosstalk between the microbiota and its host critical for the preservation of tissue homeostasis (Thaiss et al., 2016). The crosstalk that exists between the microbiota and the innate mucosal immune system is of particular importance, given the unique ability of innate immune cells to initiate intense inflammatory responses whilst also maintaining tolerance to harmless pathogens (Scott et al., 2011). It is unsurprising therefore that perturbed interactions between the microbiome and the immune system have emerged as a pivotal driver of various gastrointestinal disease.

It is now recognised that gut dysbiosis plays a central role in the pathophysiology of CIGT, with alterations in the composition of the gut microbiota reported following treatment with a number of chemotherapeutic agents (Stringer et al., 2013, Montassier et al., 2015). Clinically, decreases in faecal Lactobacillus spp., Bifidobacterium spp., Bacteroides spp. and Enterococcus spp. were identified (n=26) following treatment with capecitabine, cisplatin/5-FU, FOLFOX, 5-FU/folinic acid, COFF and paclitaxel, carboplatin and gemcitabine (Stringer et al., 2013). Paralleled increases in Escherichia coli (E.coli) and Staphylococcus spp. were also identified. Unfortunately, the sample size was not large enough to perform agent- and symptom-specific analyses. In the setting of irinotecan-induced gut toxicity, comparable changes have been identified in vivo (Stringer et al., 2008). Importantly, increased β-glucuronidase activity was also seen following irinotecan (Stringer et al., 2008), likely exacerbating luminal activation of SN-38G and worsening toxicity.

The changes in the composition of the gut microbiota following chemotherapy are now well established, characterised by a gram-negative lipopolysaccharide (LPS) producing bacterial phenotype (Stringer et al., 2009c, Stringer et al., 2013). However, the mechanisms by which this altered gut microbiota contributes to the initiation and progression of symptoms are unclear. It has been suggested luminal changes in the gut microbiota drive dysfunction of the intestinal barrier, enhancing activation of the underlying innate immune system (Castoldi et al., 2015, Thaiss et al., 2016). This interaction has been suggested to underpin chronic inflammatory states such as colitis,
where an aberrant immune response is suggested to act as an adjunct to specific microbial alteration and barrier dysfunction (Thaiss et al., 2016). This hypothesis is also supported in the case of CIGT with studies showing eradication of the gut microbiota with administration of oral antibiotics is effective in reducing irinotecan-induced diarrhoea (Takasuna et al., 1996, Kurita et al., 2011). Similarly, it has been shown that irinotecan-induced gut toxicity is attenuated in germ-free mice (Pedroso et al., 2015). Germ-free mice were also protected from developing irinotecan-induced barrier dysfunction and subsequent inflammation. These findings suggest barrier dysfunction is driven through microbiota-dependent mechanisms which then initiate an aberrant innate immune response, likely to be regulated through the actions of Toll-like receptors (TLRs).

1.5 Toll-like receptors

TLRs are type I membrane proteins characterised by an ectodomain composed of leucine rich repeats, responsible for the recognition of pathogen-, microbial- and danger-associated molecular material (PAMPs, MAMPs and DAMPs) as well as a cytoplasmic domain homologous to the cytoplasmic region of the IL-1 receptor, required for downstream signalling (Kawai and Akira, 2006, Kawai and Akira, 2007). To date, 11 human TLRs have been identified, each of which respond to distinct pathogenic stimuli (Thaiss et al., 2016). Epithelial TLRs of the gastrointestinal tract are critical in the maintenance of homeostasis, including regulation of the microbiota composition (Vindigni et al., 2016). Activation of pattern recognition receptors, like TLRs, is directly coupled to the production of antimicrobial peptides and mucous, enhancing microbial balance and highlighting the bidirectional communication at the host-microbial interface (De Nardo, 2015, Thaiss et al., 2016).

Of the many TLR subtypes, TLR4 is well characterised as it recognises LPS from gram-negative bacteria, activating similar downstream intracellular signalling pathways to those previously documented for IL-1, binding to its co-receptor, activating NFκB and resulting in a powerful proinflammatory cascade (Gribar et al., 2008, Zhang et al., 2016). Based on its ability to detect
gram-negative bacterial products and its role in barrier modulation, this thesis focuses on only the impact of TLR4 signalling in the setting of irinotecan-induced gut toxicity.

TLR4 is typically expressed on antigen presenting cells such as dendritic cells and macrophages, however recent research has determined that enterocytes and T helper cells also express TLR4 and that its upregulation during periods of stress has important impacts on enterocyte function (Jilling et al., 2006, Leaphart et al., 2007, Reynolds et al., 2012). The interaction of TLR4 and its co-receptors CD14 and MD-2 with LPS results in recruitment of the adapter molecule myeloid differentiation marker 88 (MyD88), phosphorylation of IL-1 receptor associated kinase (IRAK) and recruitment of the adapter molecule tumour necrosis factor receptor-associated factor (TRAF)-6 (Medzhitov et al., 1998, Fukata et al., 2005, Zhang et al., 2010). Recent research has attempted to delineate the mechanisms by which TLR4 contributes to gastrointestinal immunity by altering intestinal microbes and MyD88 expression (Lai and Egan, 2013). Using a preclinical model, Lai and colleagues (2013) administered a broad-spectrum oral antibiotic to animals treated with 14Gy of whole body irradiation. Antibiotic administration attenuated weight loss, and increased food and water intake. Further, colonic crypt survival was increased, cellular proliferation enhanced and apoptosis reduced in mice treated with oral antibiotics. These findings indicate exposure to a regimen of oral antibiotics significantly altering the intestinal microbiota, affects sensitivity of colonic epithelial cells to radiation-induced damage, likely through reduced TLR4 activation.

These findings draw significant parallels with other models of gastrointestinal inflammation. For example, TLR4 deficiency (-/-) has been shown to significantly reduce acute inflammation, NFkB signalling, COX-2 expression and prostaglandin production in a preclinical model of dextran sulfate sodium (DSS)-induced colitis (Fukata et al., 2005). This supports findings showing elevated levels of TLR4 correlating with disease severity and tissue damage in a number of benign inflammatory bowel disorders (Fukata et al., 2005, Gribar et al., 2009, Kaczmarek et al., 2012, Hamada et al., 2013). Of particular interest is the effect of aberrant TLR4 expression on barrier function, with data now suggesting activation of TLR4 by LPS leads to breakdown of the tight junction complex and
increased paracellular permeability (Sheth et al., 2007, Gao et al., 2015, Guo et al., 2015).

Irinotecan-induced gut toxicity is characterised by altered barrier function and overexpression of intestinal TLR4. Hence, this thesis aims to investigate the impact of TLR4-dependent signalling in the development of irinotecan-induced gut toxicity and barrier dysfunction.

1.5.1 TLR4-mediated glial activation and pain

In addition to its roles in gastrointestinal homeostasis, barrier function and innate mucosal immunity, TLR4 has recently been recognised as a driver of pain signalling through its interaction with systemic LPS (Lewis et al., 2012, Wardill et al., 2015b). TLR4 resides on glial cells (astrocytes and macrophages) within the CNS and is able to regulate their immunological activity. It is well established glia have two distinct states; a quiescent basal state and an activated state (Garrison et al., 1991). When activated, glia produce a number of neuroexcitatory mediators which enhance neuronal excitability and theoretically increase pain signaling (Watkins et al., 2009). These neuroexcitatory mediators such as ROS, proinflammatory cytokines and chemokines enhance neuronal excitability by increasing pain associated neurotransmitter release from sensory afferents (Watkins et al., 2007), upregulation of the number and conductance of calcium permeable \( \alpha \)-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptor (Zhang et al., 2008) and downregulated expression of glial glutamate transporters (Kleibeuker et al., 2008). Indirect neuromodulation via the actions of glia is now considered a key driver of neuropathic-type pain in which peripheral stimuli drive central pain signalling through their actions on TLR4 (Garrison et al., 1994). Given CIGT is associated with significant serum LPS (Wardill et al., 2016a), my laboratory developed the hypothesis that centrally-located TLR4 and glia may be well positioned to enhance pain resulting from the chemotherapy-induced stimuli generated in the gut. This hypothesis parallels findings showing both microgliosis and astrocytosis administration of vincristine, paclitaxel, bortezomib and oxaliplatin (Di Cesare Mannelli et al., 2013, Ji et al., 2013, Di Cesare Mannelli et al., 2014, Li et al., 2014b, Robinson et al., 2014), whilst also challenging the way in which we approach toxicities, suggesting that some may have common underlying mechanisms. This reiterates
key themes from the recent MASCC/ISOO Symposium for Supportive Care in Cancer (2016) in which a holistic approach to chemotherapy-induced toxicity was encouraged to streamline patient management and reduce polypharmacy.

1.6 Hypotheses and aims

This thesis has three distinct themes relating to the pathobiology and treatment of chemotherapy-induced gut toxicity:

1. Characterisation of oral and intestinal tight junction integrity in response to irinotecan treatment

2. The impact of Toll-like receptor 4 (TLR4) signalling on the development and severity of irinotecan-induced gut toxicity and barrier dysfunction

3. Development of an in vitro model to (a) investigate the molecular mechanisms of irinotecan-induced mucosal damage and tight junction disruption, and (b) test the therapeutic efficacy of genetic and pharmacological TLR4 inhibition

In addition to gut toxicity, this thesis also explores the impact of TLR4-dependent signalling in the development of chemotherapy-induced pain, giving rise to the fourth theme:

4. Assessment of central glial activity in wild-type and BALB/c-\textit{Tlr4}\textsuperscript{-/-} mice treated with chemotherapy and correlate with clinical markers of pain

\textbf{Theme 1} Tight junctions are highly dynamic and plastic structures responsible for regulating and maintaining intestinal permeability and mucosal homeostasis. They are comprised of three protein families: claudin, occludin and ZO. It is widely recognised that the integrity of these proteins represents the functional capacity and structural morphology of the tight junction unit, particularly within the gastrointestinal tract. In addition, it is well described under pathological conditions (e.g. gut dysbiosis, inflammation), tight junction proteins are subject to intense modification resulting in altered tight junction integrity and breaches in the mucosal barrier.
Based on the highly plastic nature of intestinal tight junctions I aimed to characterise their molecular
integrity in a preclinical model of irinotecan-induced gut toxicity. The secondary aim of Theme 1
was to determine if comparable tight junction defects were seen in the oral cavity of patients
undergoing chemotherapy. This would confirm if tight junction defects (1) occur in response to
different treatment modalities, (2) are found throughout the entire alimentary tract and that they (3)
translate from preclinical to clinical models of toxicity.

**Theme 2** The finding of intestinal dysfunction seen in patients undergoing chemotherapy is
biologically and clinically significant providing insight into the mechanisms involved in CIGT. The
mechanisms responsible for the development of a ‘leaky’ gut however remained unclear. Gut
dysbiosis following chemotherapy is characterised by increases in LPS-producing, gram-negative
bacteria recognised for their ability to degrade the intestinal barrier. Literature suggests this is
predominantly via the molecular interaction between bacterial LPS and its receptor TLR4. Theme 2
therefore aimed to determine the impact of TLR4 signalling on irinotecan-induced intestinal barrier
dysfunction using a preclinical BALB/c-Tlr4−/− mouse model. It is well documented that barrier
dysfunction enhances activation of the innate immune system, exacerbating the severity of
gastrointestinal damage. The secondary aim of Theme 2 was to therefore characterise clinical,
histopathological and inflammatory markers of irinotecan-induced gut toxicity in a preclinical
BALB/c-Tlr4−/− mouse model.

**Theme 3** Assessing the molecular mechanisms involved in the development of CIGT and diarrhoea
are limited given the inherent difficulties in accessing the gastrointestinal tract. Comparably,
analysis of therapeutic interventions is typically approached using *in vivo* models which are time and
resource consuming. I therefore aimed to develop a new *in vitro* model for the study of
chemotherapy-induced mucosal injury and the high throughput analysis of therapeutic interventions.
This model aimed to recapitulate the complex pathological features of irinotecan-induced gut
toxicity *in vivo* including apoptosis, barrier dysfunction and tight junction augmentation, allowing
for paralleled structural and functional analyses.
**Theme 4** Heightened pain sensitivity is reported with varying incidence following chemotherapy treatment. Importantly, chemotherapy-induced pain and gut toxicity are commonly reported in the same subset of patients, suggesting common underlying mechanisms. There is a growing body of evidence linking TLR4 with altered pain signalling, thus the hypothesis emerged that TLR4 may be uniquely positioned to mediate both irinotecan-induced gut toxicity and pain. Theme 4 aimed to assess pain following induction of gut toxicity using irinotecan. Pain was assessed using the facial grimace criteria and central markers of glial activation in the lumbar spinal cord (L3/L4 site of colonic visceral afferents). A secondary aim was to determine if genetic knockout of TLR4 (Tlr4<sup>−/−</sup>) attenuated markers of pain signalling using a BALB/c-Tlr4<sup>−/−</sup>bally mouse model of toxicity.
# Statement of Authorship

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## Principal Author

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| Overall percentage (%) | 90% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
| Signature | Date |

## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate’s stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.

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| Contribution to the Paper | Senior author, supervisor. Provided guidance and editing of final draft. |
| Signature | Date |
Chapter 2 Chemotherapy-induced mucosal barrier dysfunction: an updated review on the role of tight junctions

[Wardill HR et al., (2013) Current Opinion in Supportive and Palliative Care. 7(2):155-161]

2.1 Abstract

Purpose of review: Gut toxicity, or mucositis, is a major dose-limiting side effect of chemotherapy that until recently received very little attention. Despite significant research, the mechanisms that underpin chemotherapy-induced gut toxicity remain unclear. Recently however there has been renewed interest in the role tight junctions play in the pathogenesis of chemotherapy-induced gut toxicity and associated diarrhoea. Thus, this review will cover the role of tight junctions in maintaining gastrointestinal homeostasis and touch on recently proposed mechanisms of how their disruption may contribute to the development of chemotherapy-induced diarrhoea.

Recent findings: There is a wealth of anecdotal evidence regarding the role of tight junctions in the pathogenesis of gut toxicity. Clinical evidence highlights altered permeability indices, however, few studies have quantified the molecular changes in tight junctions in response to chemotherapy. Further, many documented mediators of tight junction disruption have documented roles in the development of gut toxicity. This review will discuss the potential mechanisms by which tight junction disruption and mucosal barrier dysfunction occur, how they contribute to diarrhoea.

Summary: The significant clinical and economic impact associated with chemotherapy-induced gut toxicity and diarrhoea has only recently been appreciated. This has prompted significant research efforts in an attempt to reveal the pathophysiology of this debilitating complication. Renewed interest has been shown regarding the role of tight junctions in not only maintaining gastrointestinal health, but also contributing to mucosal barrier injury and diarrhoea development. More detailed research into the effect chemotherapy has on the molecular characteristics of tight junctions will lead to a better understanding of the pathophysiology of chemotherapy-induced gut toxicity and may uncover the therapeutic potential of tight junctions in treating diarrhoea.


**Chapter 2 Chemotherapy-induced mucosal barrier dysfunction: an updated review**

### 2.2 Introduction

Chemotherapy is a highly effective cytotoxic therapy used to treat a number of common malignancies (Gutheil and Kearns, 1997). Despite the advantages of this treatment option, chemotherapeutic agents are non-selective in nature and are associated with widespread cytotoxicity (Elting *et al.*, 2003). The alimentary mucosa is particularly susceptible to regimen-related damage and toxicity due to its high cellular turnover (Gibson and Bowen, 2011). The term alimentary toxicity is used to describe damage to the mucous membranes of the alimentary tract (mouth to anus) that occur following cytotoxic treatment (Keefe *et al.*, 2000). Regional forms of alimentary toxicity include oral toxicity (oral ulceration, inflammation and infection) and gut toxicity (diarrhoea). Both forms of toxicity manifest as severe mucosal inflammation and damage (Logan *et al.*, 2008a), which often lead to symptoms such as dysphagia, vomiting, diarrhoea, weight loss, rectal bleeding and infection (Elting *et al.*, 2003, Kang *et al.*, 2004, Sonis, 2004a, Sonis, 2004b). A wealth of research literature has been generated regarding potential therapeutic avenues for chemotherapy-induced alimentary toxicity (Beaven and Shea, 2007, Buchsel, 2008), however, improvements are often limited to toxicity affecting the oral mucosa due to the ease at which the it is accessed (Tooley *et al.*, 2009).

Despite being subject to extensive investigation, the molecular mechanisms that underpin chemotherapy-induced gut toxicity (CIGT) are unclear; thus development of preventative interventions remains a challenge to oncology practice. Consequently, gut toxicity following chemotherapy is associated with debilitating clinical symptoms, increased infection rates and severe dose-limitations; all of which compromise patient outcomes and add to the growing economic burden associated with cancer care (Elting *et al.*, 2003). As such, this review will focus on the pathophysiology of CIGT, investigating the mechanisms and clinical implications of tight junction disruption.
2.3 Mucosal barrier function

The development of a ‘leaky’ gut is a hallmark trait of compromised mucosal barrier function (Hollander, 1999, Melichar et al., 2001). Intestinal homeostasis and tight junction integrity are traditionally measured using non-invasive sugar permeability tests, which utilise a ratio of monosaccharide and disaccharide sugar probes (Hollander, 1999, Blijlevens et al., 2004). By using the ratio of two sugars, most commonly rhamnose and lactulose, variables such as gastrointestinal transit time and renal impairment are eliminated (Keefe et al., 1997). Recent clinical research has demonstrated severe intestinal dysfunction in patients with CIGT that extends far beyond direct cytotoxic insult (Keefe et al., 1997, Blijlevens et al., 2000, Keefe et al., 2000). Additionally, both the absorptive capacity of the small intestine and mucosal barrier function have been shown to be severely compromised following a host of cytotoxic therapies (Keefe et al., 1997, Keefe et al., 2000, Blijlevens et al., 2005a, Blijlevens et al., 2005b).

Tight junctions are key determinants of mucosal barrier function and intestinal permeability, regulating the passage of solutes across the intestinal epithelium via the paracellular pathway (Gonzalez-Mariscal et al., 2008). As such, mucosal barrier dysfunction is highly suggestive of tight junction disruption within the small bowel (Blijlevens et al., 2000, Blijlevens et al., 2005a) and dynamic regulation of tight junctions is therefore fundamental to gastrointestinal homeostasis (Cummins, 2012).

2.3.1 Molecular structure of tight junctions

The molecular architecture of the tight junction exhibits a complex arrangement of interacting cytoplasmic and transmembrane proteins (Gonzalez-Mariscal et al., 2008), which form continuous adhesive strands that circumscribe the apical-lateral margin of polarised epithelia (Anderson et al., 1993, Fanning and Anderson, 2009). Zonular occludens proteins (ZO-1, -2, -3) are cytosolic scaffolds that anchor peripherally located transmembrane proteins (occludin and claudins) to the actin cytoskeleton. Genetic knockout studies have demonstrated that the absence of ZO-1 (-/-) completely abrogates tight junction assembly and paracellular regulation (Umeda et al., 2006,
Tsukita et al., 2009) suggesting ZO-1 is integral to the barrier integrity and tight junction formation. Claudin proteins are the major component of tight junction strands, as shown by freeze fracture microscopy (Morita et al., 1999). Claudins constitute a family of over 20 proteins, which display variable extracellular loop distribution and therefore a wide range of functions (Gonzalez-Mariscal et al., 2008). Accordingly, claudin subtypes are responsible for different paracellular roles within the tight junction (Amasheh et al., 2002, Milatz et al., 2010). Several claudin subtypes, particularly claudin-1, have been implicated in a number of benign inflammatory disorders of the bowel characterised by mucosal barrier dysfunction (Schulzke et al., 2009, Bertiaux-Vandaele et al., 2011) and claudin proteins are therefore considered crucial to tight junction integrity and gastrointestinal barrier function. In addition, recent research points toward a relationship between claudin-1 and apoptosis, suggesting this tight junction protein may act as a potent anti-apoptotic mediator with proteolysis of claudin-1 resulting in increased rates of apoptosis (Liu et al., 2012). This is highly relevant to the setting of CIGT, given the large apoptotic phase involved in its pathogenesis.

Occludin is a tight junction protein with controversial roles in tight junction assembly and barrier function. Despite being one of the earliest identified proteins in the tight junction complex, the importance of occludin to tight junction integrity remains unclear. For example, occludin knockout (-/-) mice exhibit morphologically intact tight junctions with no detectable changes in barrier integrity or anion fluxes (Schulzke et al., 2005). Paradoxically, overexpression of occludin results in increased transepithelial resistance (Balda et al., 1996). In contrast, substantial data exists demonstrating alterations in the expression, localisation and phosphorylative state of occludin in pathologies associated with poor barrier function (Bertiaux-Vandaele et al., 2011, Hamada et al., 2013, Wardill et al., 2013, Wardill et al., 2014a).

### 2.4 Are alterations in intestinal tight junctions pivotal to CIGT development?

Currently, the molecular mechanisms that underpin the development of gut toxicity following chemotherapy are poorly understood. Recent research has highlighted roles for apoptosis (Keefe et al., 2000), matrix metalloproteinases (MMPs) (Al-Dasooqi et al., 2010, Al-Dasooqi et al., 2011a)
and the gut microbiome (Stringer et al., 2007, Stringer et al., 2009a, Stringer et al., 2009b) in the development of CIGT. More recently however, there has been a renewed interest regarding the role of tight junctions in the pathophysiology of gut toxicity (Wardill et al., 2012).

2.4.1 Clinical studies

Tight junctions were first hypothesised as a potential pathomechanism for CIGT in 1997, with Keefe et al. [14] demonstrating marked increases in intestinal permeability in patients receiving various chemotherapeutic agents, largely administered in combination. Intestinal permeability, measured by rhamnose/lactulose permeation, was perturbed in all patients, peaking 7 days following treatment and corresponding with the onset and duration of gastrointestinal symptoms [14,35]. Comparable clinical studies soon emerged [12,36-40], therefore, validating the efficacy of sugar permeability tests as simple, well tolerated and reliable test, while emphasising the profound effects of chemotherapeutic agents on mucosal barrier integrity. In line with these findings, Blijlevens et al. [15] identified significant abnormalities in intestinal permeability in haematopoietic stem cell transplant recipients treated with various myeloablatative treatments including melphalan, cyclophosphamide, idarubicin, etoposide, busulphan and total body irradiation. All treatment regimens caused significant mucosal dysfunction; however, mucosal injury was more pronounced and prolonged in patients treated with idarubicin. Together these studies suggest that regimen-related mucosal barrier dysfunction is not limited to a single chemotherapeutic agent, but a common phenomenon with cytotoxic treatment.

Morphological defects in tight junctions have also been identified following cytotoxic therapy. In 2000, Keefe et al [4] demonstrated a significant increase in the amount of open tight junctions within the small intestine of patients receiving combined chemotherapeutic regimens for a range of solid tumours. Importantly, peak tight junction disruption coincided with maximal intestinal permeability of patients receiving polydrug-chemotherapy reported by Fazeny-Dorner et al. [35]. Most recently, Wardill et al. (2015) showed clear changes in oral epithelial tight junctions in patients receiving standard dose chemotherapy. This study was the first to show that chemotherapy disrupts
key tight junction proteins of the oral cavity and therefore provides further evidence for a common pathway for alimentary mucositis

2.4.2 Preclinical studies

Despite substantial anecdotal evidence suggestive of tight junction disruption following chemotherapy, very few studies have investigated the molecular changes in tight junctions in response to cytotoxic treatment. Given that disruption to the expression and distribution of specific tight junction proteins drastically alters tight junction integrity and gastrointestinal homeostasis (Fanning and Anderson, 2009), this is an area of much needed research. Hamada and colleagues (Hamada et al., 2010) were the first to characterise alterations in tight junctions following the administration of methotrexate (MTX), a commonly used chemotherapeutic agent. Tumour-naïve rats were treated with 15 mg/kg of intravenous MTX once daily for 3-5 consecutive days and were killed 24 h following their final treatment. Mucosal barrier function, determined by fluorescein isothiocyanate-dextran (FITC-dextran) permeability, was significantly increased in MTX-treated rats indicating poor barrier integrity. This preclinical study highlighted significant changes in the expression, distribution and phosphorylation of ZO-1, however investigators were only limited to this single tight junction protein. The authors expanded upon these results in 2013, again showing decreases in ZO-1 expression and its phosphorylative state following MTX, as well as detachment from its cytoplasmic protein claudin-4 (Hamada et al., 2013). Reversible phosphorylation and detachment of tight junction proteins has been established as a vital aspect of tight junction integrity and barrier function (Sakakibara et al., 1997), thus the changes in ZO-1 identified by Hamada and colleagues highlight a potential mechanism of chemotherapy-induced mucosal barrier dysfunction.

More recently, marked decreases in small intestinal occludin and claudin-1 expression and distribution were demonstrated in MTX-treated Wistar rats (Beutheu Youmba et al., 2012). Comparable changes in these major tight junction proteins have also been identified in response to irinotecan treatment (Nakao et al., 2012) reiterating clinical suggestions that tight junction disruption is a common pathological finding following many cytotoxic agents.
evidence for tight junction involvement, this evidence is often conflicting within recent literature. For example, Nakao et al., (Nakao et al., 2012) identified decreased small intestinal permeability in irinotecan-treated rats, which contrasts with evidence of permeability increases in previously published literature (Table 2.1). Standardised methods of assessing intestinal permeability in both the clinical and in vivo setting are therefore required to ensure consistency across research. Further inconsistencies exist in the quantification of tight junction proteins (Table 2.1). For example, there are currently no universally accepted methods for assessing and quantifying tight junction protein distribution or expression. This is particularly evident in recent preclinical studies (Beuthe Youmba et al., 2012, Nakao et al., 2012) which present conflicting evidence regarding intestinal occludin and claudin-1 protein and mRNA expression. However, it is important to note that these studies investigate different cytotoxic agents and conflicting results may therefore reflect the differing mechanisms of action of these agents. Results from these studies must also be interpreted with caution due to small sample sizes and chemotherapeutic regimens that do not reflect clinical practice. Further preclinical work is therefore required to clarify chemotherapy-induced tight junction disruption, placing emphasis on the effect of chemotherapy on all tight junctions proteins and establishing a time-course for these changes. This would allow for correlation between tight junction disruption and documented events in the pathophysiology of CIGT such as peak histological damage and the onset of clinical symptoms (Sonis, 2004c). Validation of these changes in patient groups would then be warranted.
<table>
<thead>
<tr>
<th>Study</th>
<th>Experimental Design</th>
<th>Cytotoxic Agents</th>
<th>Permeability Assay</th>
<th>Mucosal Barrier Function</th>
<th>Tight Junction Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keefe 1997</td>
<td>Clinical (n=35)</td>
<td>Cyclophosphamide, carboplatin, melphalan, etoposide, busulfan, methotrexate, epirubicin</td>
<td>Urinary lactulose and mannitol excretion</td>
<td>Significantly increased (p&lt;0.05) intestinal permeability; peaked day 7 post-treatment</td>
<td>Data not provided</td>
</tr>
<tr>
<td>Melichar 2001</td>
<td>Clinical (n=10)</td>
<td>Cisplatin, folinic acid, 5-FU, tegafur, gemcitabine, paclitaxel, docorubicin</td>
<td>Urinary lactulose, D-xylene and mannitol excretion</td>
<td>Intestinal permeability to lactulose was significantly increased after treatment (p&lt;0.0001)</td>
<td>Data not provided</td>
</tr>
<tr>
<td>Fazeny-Dörner 2002</td>
<td>Clinical (n=14)</td>
<td>Polydrug-chemotherapy IFADI (ifosfamide, adriamycin, dacarbazine)</td>
<td>Urinary lactulose and mannitol excretion</td>
<td>Permeability index was significantly higher (p&lt;0.01) in patients receiving poly-drug chemotherapy; peaks coincided with maximal nausea symptoms.</td>
<td>Data not provided</td>
</tr>
<tr>
<td>Sukkar 2004</td>
<td>Clinical (n=12)</td>
<td>Adjuvent 5-FU and levamisole</td>
<td>Urinary lactulose and mannitol excretion</td>
<td>5-FU/LV treatment induced significant increase in intestinal permeability (p&lt;0.001).</td>
<td>Tight junction involvement speculated</td>
</tr>
<tr>
<td>Blijlevens 2005</td>
<td>Clinical (n=159)</td>
<td>BCNU, etoposide, cytarabine, melphalan, cyclophosphamide, total body irradiation, idarubicin, busulfan</td>
<td>Urinary lactulose, L-rhamnose, D-xylene and 3-O-methylglucose excretion</td>
<td>Significantly increase in intestinal permeability (p=0.003).</td>
<td>Tight junction involvement speculated</td>
</tr>
<tr>
<td>Choi 2007</td>
<td>Clinical (n=51)</td>
<td>Adjuvent 5-FU and levamisole</td>
<td>51Cr-EDTA urinary excretion</td>
<td>5-FU/LV treatment resulted in significantly increased intestinal permeability (p&lt;0.001); correlated with mucositis/toxicity severity (p&lt;0.001, r=0.898).</td>
<td>Authors hypothesised tight junction dysfunction; no follow up study</td>
</tr>
<tr>
<td>Hamada 2010</td>
<td>Rat In Vivo (no sample size provided)</td>
<td>Methotrexate (15 mg/kg; 3-5 days)</td>
<td>FD-4 permeability</td>
<td>Methotrexate administration caused increased FD-4 permeability (p&lt;0.01)</td>
<td>Cytoplasmic redistribution, altered expression and decreased tyrosine phosphorylation (p&lt;0.05) of jejunal ZO-1</td>
</tr>
<tr>
<td>Younba 2012</td>
<td>Rat In Vivo (n=8)</td>
<td>Methotrexate (2.5 mg/kg; 3 days)</td>
<td>Transepithelial resistance (Ω·cm²) was used as a measure of permeability; assessed using an epithelial volt-ohm-meter.</td>
<td>Methotrexate significantly increased permeability (p&lt;0.001)</td>
<td>Decreased occludin and claudin-1 expression in the jejunum (p&lt;0.05). No change in ZO-1 identified (p=ns).</td>
</tr>
<tr>
<td>Nakao 2012</td>
<td>Rat In Vivo (n=10)</td>
<td>Irinotecan (250 mg/kg)</td>
<td>Transepithelial resistance (Ω·cm²) was used as a measure of permeability; assessed in Ussing chambers</td>
<td>Irinotecan caused significantly increased permeability in the colon (p&lt;0.05)</td>
<td>Decreased occludin and claudin-1 protein and mRNA expression (p&lt;0.05) in the jejunum/colon. No change in ZO-1 expression (p=ns).</td>
</tr>
<tr>
<td>Hamada 2014</td>
<td>Rat In Vivo (n=8)</td>
<td>Methotrexate (15 mg/kg; 3-5 days)</td>
<td>Immunofluorescence, immunoprecipitation, RT-PCR</td>
<td>No functional analyses conducted</td>
<td>Increased expression (mRNA/protein) of the pore-forming tight junction proteins, claudin-2 and -4 Decreased occludin expression (mRNA/protein)</td>
</tr>
</tbody>
</table>
2.5 Regulators of tight junctions constitute key steps in the pathophysiology of gut toxicity

Tight junctions undergo a broad variety of changes in response to a number of physiological and pathological cues, thereby enhancing the functional plasticity of these highly dynamic structures. This regulatory feature of tight junctions enables them to control permeability and organise a number of diverse cellular processes including cell polarity, morphogenesis, cell proliferation and differentiation (Forster, 2008). Although tight junction modulation and regulation is pivotal for many physiological processes, various mediators of tissue injury are able modulate tight junction proteins (Gonzalez-Mariscal et al., 2008, Wardill et al., 2013) and increased intestinal permeability is a hallmark trait of many pathological states (Cummins, 2012). The involvement of tight junctions in the pathophysiology of CIGT is compelling given the wealth of in vitro, preclinical and clinical data indicating that many mediators of CIGT are potent regulators of tight junction integrity. In particular, the molecular cross talk between mediators of inflammation (e.g. IL-1β, TNF, IL-6, matrix metalloproteinases) and tight junctions makes these intercellular complexes an excellent target for research.

2.5.1 Proinflammatory cytokines disrupt barrier function

The effects of proinflammatory cytokines on barrier integrity is well described, explaining the finding of tight junction disruption in a number of inflammatory gut disorders. Inflammatory bowel diseases (IBDs) like Crohn’s disease are characterised by chronic and relapsing diarrhoea (Burgel et al., 2002). Research has shown downregulation of occludin, claudin-1 and claudin-8 in patients with Crohn’s disease, with paralleled increases in the cation channel-former claudin-2 (Schmitz et al., 1999, Schulzke et al., 2009). Intestinal sections from patients with Crohn’s disease and ulcerative colitis are also characterised by reduced tight junction strand formation and strand breaks (Schmitz et al., 1999). Importantly, these changes have been shown to correlate with the onset and duration of symptoms, such as abdominal discomfort, diarrhoea and pain, experienced by patients suggesting that tight junction disruption may contribute to the development of diarrhoea.
Recent *in vitro* research has solidified the modulatory roles of proinflammatory cytokines on tight junction integrity, indicating that interleukin-1β (IL-1β), tumour necrosis factor (TNF) and interferon gamma (IFNγ) are able to disrupt tight junction integrity (Ma *et al.*, 2004, Ma *et al.*, 2005, Al-Sadi *et al.*, 2008). Further, tight junction protein disruption was exacerbated following TNF exposure in conjunction with IFNγ, highlighting the synergistic effects of proinflammatory cytokines (Fish *et al.*, 1999, Wang *et al.*, 2005).

### 2.5.2 The extracellular matrix maintains mucosal homeostasis

Matrix metalloproteinases (MMPs), of the extracellular matrix, affect numerous biological phenomena and have recently been recognised for their proteolytic functions (Cummins, 2012). In fact, substantial *in vitro* evidence exists to support a role for MMP-mediated tight junction protein proteolysis, particularly in the case of occludin (Beauchesne *et al.*, 2009, Casas *et al.*, 2010). This is of particular importance as MMPs have recently been identified as key mediators in the development of chemotherapy-induced gut toxicity (CIGT), with significantly increased serum and tissue levels present during development of mucosal injury (Al-Dasooqi *et al.*, 2010). Although far more common in endothelial cells, MMP-mediated occludin proteolysis has been reported in epithelial tight junctions. Epithelial MMP-tight junction interactions were first established in the seminiferous epithelium of Sprague-Dawley rats. The seminiferous epithelium was isolated from 20-day-old rats and cultured with recombinant human TNFα (Siu *et al.*, 2003). Following TNF application, the authors reported correlating elevations in MMP-2/-9 subtypes, tight junction disruption and epithelial hyper-permeability. Elevated MMP-7 has also been identified in response to oestrogen-treatment in normal human vaginal epithelial cells (hEVECs) leading to occludin proteolysis and poor epithelial barrier function (Gorodeski, 2007). Importantly, inhibition of MMP-7 through oestrogen removal or pharmacological intervention prevented occludin degradation, and enhanced mucosal barrier function. More recently, MMP-tight junction interactions have been demonstrated using human airway epithelial models (Vermeer *et al.*, 2009) and human embryonic kidney cell lines (Jeong *et al.*, 2012). In both cases, MMP-9 activation caused altered expression and
localisation of occludin, claudin-1 and ZO-1, tight junction strand breaks and epithelial apoptosis, thus highlighting a clear role of MMPs in the regulation of tight junctions and barrier function.

### 2.6 Potential involvement of tight junctions in chemotherapy-induced diarrhoea

Chemotherapy-induced diarrhoea is a major clinical manifestation of CIGT that significantly effects the provision of optimal cancer care. Between 20% and 40% of patients receiving chemotherapy will experience dose-limiting diarrhoea, depending on the regimen and other treatment co-factors (Savarese et al., 1997, Gibson and Keefe, 2006). Although the prevalence of both gut and oral toxicity have been investigated in great detail, the mechanisms responsible for the development of chemotherapy-induced diarrhoea remain unclear (Gibson and Keefe, 2006). Much of the current literature is based on clinical observations, thus robust scientific investigation is lacking. It is thought that chemotherapy-induced diarrhoea development is a multifactorial process and may be caused by altered gut motility/decreased transit time associated with malabsorption and impaired absorptive ability, elevations in proinflammatory cytokines, alterations in the luminal microflora and transient lactulose intolerance (Gibson and Keefe, 2006, Gibson and Stringer, 2009b). Detailed research conducted to determine the mechanism of chemotherapy-induced diarrhoea has largely focused on irinotecan, which causes a biphasic diarrhoea response characterised by a severe, late-onset diarrhoea phenotype (Gibson and Stringer, 2009b). It has been hypothesised deglucuronidation of SN-38 by gut microflora, the active metabolite of irinotecan, may amplify toxicity causing severe intestinal damage and resulting in diarrhoea (Stringer et al., 2007). Further links have been established between SN-38, cyclooxygenase (COX)-2-mediated inflammation and prostaglandin release however, to date the literature has been unable to identify which biological cause is most pivotal. Recent research investigating the pathogenesis and symptomology of certain inflammatory bowel diseases (IBDs) has indicated that mucosal barrier dysfunction and tight junction defects contribute to diarrhoea development through leak-flux mechanisms which allow passive water movement toward the lumen (Amasheh et al., 2002). IBDs present with very similar clinical manifestations to those seen in patients with severe gut toxicity induced by chemotherapy, as such,
alterations in tight junctions and mucosal barrier integrity have been hypothesised as potential mechanisms of chemotherapy-induced diarrhoea (Figure 2.1). A recent preclinical study further supports this hypothesis by identifying marked derangement of intestinal electrolyte and water fluxes which strongly correlated with overt diarrhoea in MTX-treated rats (Carneiro-Filho et al., 2004). However, it is important to note that despite having similar clinical manifestations, IBDs and CIGT have vastly different pathogeneses and inflammatory characteristics. As such, any extrapolation of potential mechanisms from IBDs to CIGT must be interpreted with caution. Further, chemotherapy-induced diarrhoea is multifactorial and likely to be the result of confounding pathologies. Thus, tight junction disruption may play only a small role in its pathophysiology.

In addition to its hypothesised roles in paracellular fluid regulation and diarrhoea, mucosal barrier dysfunction also leads to dysregulated transit across the intestinal epithelium allowing penetration of potentially noxious agents and pathogens into the underlying tissue (Hering et al., 2012). This phenomenon was recently demonstrated, with pathogenic bacteria detected on the basolateral surface of the intestinal epithelium, in mesenteric lymph nodes and the spleen of irinotecan-treated rats (Nakao et al., 2012). Detailed investigations highlighted that this bacterial translocation exacerbated sepsis and worsened diarrhoea severity. As such, it is possible that mucosal barrier dysfunction may exert several detrimental effects, promoting diarrhoea development through altered leak-flux mechanisms, as well as increasing the risk of bacterial translocation, infection and sepsis. Clinically, the development of mucosal barrier dysfunction has been identified as a major risk factor for severe complications after cytotoxic therapy, placing patients at risk of developing graft versus host disease, veno-occlusive disease and systemic infections (Blijlevens et al., 2000, Blijlevens, 2005).
Figure 2.1 Proposed involvement of cytokine and MMP-dependent tight junction disruption in the development of chemotherapy-induced diarrhoea. Following chemotherapy, proinflammatory cytokines and MMPs are upregulated; both of which have been shown to alter tight junction integrity. Altered intestinal barrier function enables bacterial translocation and altered solute fluxes. Thus, the prevailing hypothesis elevated MMP and proinflammatory cytokine levels contribute, in part, to chemotherapy-induced diarrhea through tight junction disruption.
2.7 Where to now?

Tight junctions are highly plastic structures, able to undergo numerous modifications in response to various pathological cues. There is substantial anecdotal evidence suggesting that tight junctions play pivotal roles in the pathophysiology of gut toxicity, however, there has been minimal research investigating specific molecular changes in these highly plastic intercellular structures. Alterations in the expression and distribution of key tight junction proteins, occludin, claudin-1 and ZO-1, have been identified following cytotoxic treatment, thus substantiating preliminary evidence. Despite these advances, further investigation is now required to delineate the time-course of tight junction changes and the underlying mechanisms. Cytokine- and MMP-mediated tight junction proteolysis is a well-documented phenomenon in both endothelial and epithelial cell populations. Given that both proinflammatory cytokines and MMPs are intimately involved in the pathogenesis of CIGT, cytokine- and MMP-dependent epithelial tight junction alteration should be a focus for future research. Further research is now warranted to determine the role tight junctions play in its development.
### Statement of Authorship

<table>
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<tr>
<th>Title of Paper</th>
<th>Irinotecan disrupts tight junction proteins within the gut: implications for chemotherapy-induced gut toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Publication Status</td>
<td>□ Published □ Accepted for Publication □ Submitted for Publication □ Unpublished and Unsubmitted work written in manuscript style</td>
</tr>
</tbody>
</table>

### Principal Author

| Name of Principal Author (Candidate) | Hannah Wardill |
| Contribution to the Paper | I was responsible for all experimental work using archival tissue samples obtained from the original animal study (JARIII). This included analysis of clinical data, histopathology, immunohistochemistry and RT-PCR. |
| Overall percentage (%) | 75% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
| Signature | Date |

### Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| Name of Co-Author | Joanne Bowen |
| Contribution to the Paper | Joanne was involved in the original animal study as well as reviewing drafts of the manuscript. |
| Signature | Date |

| Name of Co-Author | Noor Al-Desoqui |
| Contribution to the Paper | Noor was a PhD student in the laboratory at the time and assisted with RT-PCR (primer design and data analysis). |
| Signature | Date |
## Chapter 3 Irinotecan causes tight junction disruption in the gut

<table>
<thead>
<tr>
<th>Name of Co-Author</th>
<th>Contribution to the Paper</th>
<th>Signature</th>
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<tbody>
<tr>
<td>Moeona Sultani</td>
<td>Moeona was responsible for running the original animal study.</td>
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<tr>
<td>Emma Bateman</td>
<td>Emma was responsible for helping with the original animal study.</td>
<td></td>
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</tr>
<tr>
<td>Romany B尔斯borough</td>
<td>Romany helped with Immunohistochemical analysis during an undergraduate research programme.</td>
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</tr>
<tr>
<td>Joseph Shirren</td>
<td>Joseph helped with some of the Immunohistochemical analysis during an undergraduate research programme.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rachel Gibson</td>
<td>Rachel was the primary supervisor for the animal study and downstream analyses, responsible for experimental design and manuscript production.</td>
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</table>
Chapter 3 Irinotecan causes tight junction disruption in the gut

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Chapter 3 Irinotecan disruptions tight junction proteins within the gut: implications for chemotherapy-induced gut toxicity


3.1 Abstract

Chemotherapy for cancer causes significant gut toxicity, leading to severe clinical manifestations and an increased economic burden. Despite much research, many of the underlying mechanisms remain poorly understood hindering effective treatment options. Recently there has been renewed interest in the role tight junctions play in the pathogenesis of chemotherapy-induced gut toxicity. To determine the underlying mechanisms of chemotherapy-induced gut toxicity, this study aimed to quantify the molecular changes in a key tight junction proteins, ZO-1, claudin-1 and occludin, using a well-established preclinical model of gut toxicity. Female tumour-bearing dark agouti rats received irinotecan or vehicle control and were assessed for validated parameters of gut toxicity including diarrhoea and weight loss. Rats were killed at 6 h, 24 h, 48 h, 72 h, 96 h and 120 h post-chemotherapy. Tight junction protein and mRNA expression in the jejunum and colon were assessed using semi-quantitative immunohistochemistry and RT-PCR. Significant changes in protein expression of tight junction proteins were seen in both the jejunum and colon, correlating with key histological changes and clinical features. mRNA levels of claudin-1 were significantly decreased 6 h after irinotecan in the small and large intestines. ZO-1 and occludin mRNA levels remained stable across the time-course of gut toxicity. Findings strongly suggest irinotecan causes tight junction defects, which lead to mucosal barrier dysfunction and the development of diarrhoea. Detailed research is now warranted to investigate regulation of tight junction proteins to delineate the underlying pathophysiology of gut toxicity and identify future therapeutic targets.
Chapter 3 Irinotecan causes tight junction disruption in the gut

3.2 Introduction

Chemotherapy for cancer can cause significant gut toxicity characterised by severe clinical manifestations affecting the entirety of the gastrointestinal tract (GIT) (Keefe et al., 2000). Symptoms such as pain, ulceration, vomiting and diarrhoea are a significant burden on patients’ quality of life, requiring greater resource utilisation and resulting in significant economic burden (Elting et al., 2003). Chemotherapy-induced gut toxicity (CIGT) is therefore a clinical and economic challenge to oncology practice (Keefe et al., 2007).

CIGT development is a multifactorial process characterised by dynamic biochemical interactions between chemotherapeutic agents and cellular constituents of the mucosa (Kang et al., 2004, Sonis, 2004a, Sonis, 2004c). Recent research has focused on the molecular mechanisms that underpin CIGT, highlighting roles for apoptosis (Keefe et al., 2000), the immune system (Logan et al., 2008a), the gut microbiome (Stringer et al., 2008) and matrix metalloproteinases (MMPs)(Al-Dasooqi et al., 2010). More recently, intestinal tight junctions were proposed to play important roles in the pathophysiology of CIGT (Wardill et al., 2012).

Dynamic regulation of tight junctions is fundamental to many physiological processes as disruption drastically alters mucosal barrier function and intestinal permeability, making these traits a hallmark of many pathological states (Bjarnason et al., 1995, Cummins, 2012). The molecular architecture of the tight junction exhibits a complex arrangement of interacting cytoplasmic and transmembrane proteins. Briefly, peripherally located zonular occludens (ZO) proteins interact to anchor tight junction membrane proteins to the cytoskeleton (Shin and Margolis, 2006, Gonzalez-Mariscal et al., 2008). These scaffolding proteins play important roles in tight junction formation and are crucial in barrier integrity (Gonzalez-Mariscal et al., 2008). Claudins are essential components of the intercellular tight junction and major determinants of paracellular solute fluxes (Will et al., 2008). The structural organisation of claudin proteins varies and different claudin subtypes are responsible for different roles within the tight junction (Gonzalez-Mariscal et al., 2008). Claudin-1 is of particular interest with regards to gastrointestinal inflammation and has been implicated in the...
pathophysiology of a number of inflammatory bowel disorders (IBDs) (Bertiaux-Vandaele et al., 2011). Recent research has also identified roles for claudin-1 in apoptosis (Schulzke et al., 2009) and cellular regeneration (Turner, 2009); both of which are key events in CIGT. Occludin is a key transmembrane protein integral to tight junction integrity and their functional capacity (McCarthy et al., 1996, Gonzalez-Mariscal et al., 2008, Ulluwishewa et al., 2011). For example, knockout (occludin−/−) mice exhibit morphologically intact tight junctions (Schulzke et al., 2005), however, poor tight junction integrity and mucosal barrier dysfunction follow. These results indicate likely roles for occludin in tight junction stability and barrier function as opposed to tight junction assembly (Saitou et al., 2000).

There is substantial anecdotal evidence to suggest that tight junctions contribute to CIGT (Wardill et al., 2012). In 1997, Keefe et al., identified transient abnormalities in intestinal permeability in patients receiving high-dose chemotherapy (Keefe et al., 1997). Comparable changes in intestinal permeability have also been shown in patients receiving various myeloablative treatments (Blijlevens et al., 2005a) indicating that intestinal function is compromised by a variety cytotoxic regimens. Furthermore, morphological defects in tight junctions have been identified following cytotoxic treatment, with Keefe et al. (2000) reporting significant increases in the number of open intestinal tight junctions in patients receiving high-dose chemotherapy. It is also important to consider biological regulators of tight junction integrity. It is well documented that both proinflammatory cytokines and pathogenic bacteria exhibit modulatory effects on tight junction proteins. Importantly, these are hallmark traits and key aspects of the pathobiology of CIGT and therefore suggest that changes in inflammatory signalling combined with gut dysbiosis are key drivers of tight junction breakdown in the setting of CIGT. This study therefore aimed to characterise the integrity of intestinal tight junctions in an in vivo model of irinotecan-induced gut toxicity.
3.3 Materials and Methods

3.3.1 Animals and ethics

Female Dark Agouti (DA) rats, weighing between 150 and 170 g were used for this study. Rats were housed in Perspex cages at a temperature of 22 ± 1 °C and subject to a 14 h light/10 h dark cycle. Animals had ad libitum access to autoclaved chow and water. Experimental design was approved by the Animal Ethics Committees of the Institute of Medical and Veterinary Science (IMVS), and The University of Adelaide, and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching (Council, 2004).

3.3.2 Experimental design

The dark agouti mammary adenocarcinoma (DAMA) rat model of irinotecan-induced gut toxicity was used to conduct this study. DA rats used in this study were tumour-bearing based on previous research has showing that the response to irinotecan is more pronounced in tumour-bearing rats compared to naïve rats (Gibson et al., 2007). Forty rats were randomly assigned to receive either irinotecan (n=5-9 per time point) or vehicle control (n=6). All rats received breast cancer innoculum as described previously (Gibson et al., 2002). Briefly, mammary adenocarcinoma tumours syngeneic with the DA rat were diced, homogenised and filtered through sterile gauze. A viable cell count was conducted using 0.4% w/v trypan blue before 150 µl of cells were implanted subcutaneously into both right and left flanks of the rat at a concentration of 2.0x10^7 cells/ml. Tumours were allowed to grow for one week prior to administration of chemotherapy and did not exceed 15% of their total body weight at study end point. All rats received 0.01 mg/kg subcutaneous atropine immediately prior to administration of either 175 mg/kg irinotecan (intraperitoneal) (kindly supplied by Pfizer, Kalamazoo, USA, administered in a sorbitol/lactic acid buffer: 45mg/mL sorbitol/0.9mg/mL lactic acid, pH 3.4), or vehicle control (sorbitol/lactic acid buffer). This buffer has previously been shown to have no gut toxicity effects (Gibson et al., 2003). Groups of rats were killed by exsanguination and cervical dislocation while under 3% isoflourane in 100% O_2 anesthesia at times 6, 24, 48, 72, 96 and 120 h post-irinotecan treatment.
3.3.3 Clinical assessment of gut toxicity

All rats were monitored four times daily for the presence of diarrhoea and other clinical parameters. Diarrhoea was quantified (by two independent assessors; MS/RJG) using a validated grading system\(^{48,51}\) where 0 = no diarrhoea, 1 = mild perianal staining, 2 = moderate staining covering hind legs, and 3 = severe staining covering hind legs and abdomen with continual anal leakage (Gibson et al., 2007). Rats were weighed daily to track weight loss/gain. Rats were killed if they displayed $\geq$15% weight loss or significant distress and clinical deterioration, in compliance with animal ethical requirements. All gut toxicity assessments were conducted in a blinded fashion.

3.3.4 Tissue preparation

The entire gastrointestinal tract from pyloric sphincter to rectum was dissected and flushed with chilled 1 X PBS (pH 7.4) to remove intestinal contents. Both the small and large intestines were weighed immediately after resection. Samples of small (jejunum) and large (colon) intestine were collected, snap frozen in liquid nitrogen or stored in RNAlater® (Sigma Aldrich, NSW, Australia; #R0901) at -20°C for molecular analyses. Additional samples (1cm in length) of jejunum and colon were fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax for histological and immunohistochemical analyses.

3.3.4.1 Immunohistochemical analysis of tight junction proteins

Immunohistochemical analysis was carried out using the Level 2 USA™ Ultra Streptavidin Detection System kit (Signet Laboratories, Dedham, USA; #929901) as per manufacturer’s instructions. Antigen retrieval was conducted using either a 10 mM/l citrate buffer (occludin and claudin-1) or protease pretreatment (Sigma Aldrich, NSW, Australia; #P5147). To reduce nonspecific staining sections were blocked in 3% hydrogen peroxidase ($\text{H}_2\text{O}_2$)/methanol solution and 5% normal blocking serum in 0.01M of PBS containing 0.1% sodium azide (NaN3). Sections were incubated with avidin/biotin blocking solution (Signet Laboratories, Dedham, USA; #31126) for 15 min, before the primary antibody was applied for overnight a 4°C (Table 3.1). Negative control sections had the primary antibody omitted and liver was used as a positive control in all
Chapter 3 Irinotecan causes tight junction disruption in the gut

experiments. Both linking and labeling reagents were applied (Signet Laboratories, Dedham, USA) followed by diaminobenzidine (DAB) chromogen in 0.03% hydrogen peroxidase. Slides were counterstained in Harris Haematoxylin, before being dehydrated, cleared and coverslipped. Slides were scanned using the NanoZoomer™ (Hamamatsu Photonics, Japan) and assessed with NanoZoomer™ Digital Pathology software v.2 (Histalim, Montpellier, France) and analysed using a validated semi-quantitative grading system (Al-Dasooqi et al., 2010); 0=no staining, 1=weak, 2=mild, 3=moderate and 4=intense. All samples were analysed in a blinded fashion by two investigators (HRW/RJG). As the focus of tight junction staining is epithelial, this was the only tissue region analysed.

<table>
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<td>Occludin Mouse monoclonal</td>
<td>Invitrogen 33-1500</td>
<td>2.5 µg/ml</td>
<td>1:200</td>
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<tr>
<td>Claudin-1 Rabbit polyclonal</td>
<td>Abcam ab15908</td>
<td>10 µg/ml</td>
<td>1:100</td>
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<td>ZO-1 Rabbit polyclonal</td>
<td>Invitrogen 61-7300</td>
<td>10 µg/ml</td>
<td>1:100</td>
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3.3.5 Real-time polymerase chain reaction (RT-PCR)

3.3.5.1 RNA extraction

RNA extraction was performed on jejunum and colon samples as per manufacturer’s instructions (NucleoSpin RNA Isolation Kit, Macherey-Nagel, Düren, Germany; #740933.250). Briefly, tissue samples were homogenized in 250 µl and 500 µl of TRIzol® Reagent (Invitrogen, Vic, Australia; #15596018), respectively. Samples were centrifuged for 15 minutes at 4°C and the upper aqueous layer removed. Following a sequence of filtration steps, RNA-binding conditions were adjusted and DNA digestion performed. A series of washing steps was carried out before highly pure mRNA was eluted in RNase-free water. Once eluted, RNA was stored at -80°C.
3.3.5.2 Assessment of RNA quantity and quality

RNA was quantified for yield (ng/µl) and purity using the Thermo Scientific Nanodrop 1000 spectrophotometer. RNA integrity was assessed at the Adelaide Microarray Facility (SA Pathology) using the Agilent 2100 Bioanalyser RNA 6000 Nano Chip (Series II) kit.

3.3.5.3 Reverse transcription and RT-PCR

1 µg of RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad, NSW, Australia; #1708890) as per manufacturer’s instructions. RT-PCR was performed using the Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Amplification mixes contained 1 µl of cDNA sample (100 ng/µl), 5 µl of SYBR green fluorescence dye, 3 µl of RNase-free water and 0.5 µl of each forward and reverse primer (prediluted to 50 pmol/µl) to make a total volume of 10 µl. Thermal cycling conditions included a denaturing step at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 56°C for 15 s and extension at 72°C for 20 s. All samples were run in triplicate. Primer efficiency was evaluated using standard curves and cycle threshold (Ct) values were calculated by Rotor Gene 6 analysis software. Ct values were used to quantify occludin gene expression, relative to untreated control calibrator and a validated housekeeping gene using the Pfaffl method of relative quantification (Pfaffl, 2001) Ubiquitin C (UBC), a validated housekeeping gene (Al-Dasooqi et al., 2011b), was used as a reference gene in all RT-PCR runs. Housekeeping gene stability was validated using the 2^ΔCT method (Livak and Schmittgen, 2001).

Table 3.2 RT-PCR primer specifications

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<tr>
<th>Target</th>
<th>Sequence 5'-3'</th>
<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>Reference</th>
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<tr>
<td>Rat Occludin</td>
<td>F: CACGTTGACCAATGC</td>
<td>118</td>
<td>54; 56</td>
<td>*</td>
</tr>
<tr>
<td>NM_031329.2</td>
<td>R: CCCGTTCCATGGCTC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat ZO-1</td>
<td>F: GATTCGATTGTGTTGTCC</td>
<td>106</td>
<td>56; 56</td>
<td>*</td>
</tr>
<tr>
<td>NM_001106266.1</td>
<td>R: TCATTGTAGCACCACCCGCC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rat Claudin-1</td>
<td>F: AGGTCTGGCGACATTGTTG</td>
<td>91</td>
<td>56; 54</td>
<td>*</td>
</tr>
<tr>
<td>NM_031699.2</td>
<td>R: TGGTGTTGGTGGTGAGGTGG</td>
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<td></td>
<td></td>
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<tr>
<td>Rat UBC</td>
<td>F: TCGTACCTTCTACCCACAGTACCTAG</td>
<td>82</td>
<td>58; 56</td>
<td>(Al-Dasooqi et al., 2011b)</td>
</tr>
<tr>
<td>NM_017313.1</td>
<td>R: GAAAATAGACACACCTCCCCCATCA</td>
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</table>

* Designed by PrimerBlast, analysed using NetPrimer software and synthesised by Geneworks Ltd.
Chapter 3 Irinotecan causes tight junction disruption in the gut

3.3.6 Statistical analysis

Data were compared using Prism version 6.0 (GraphPad® Software, San Diego, USA). A Kolmogorov-Smirnov test was used to assess normality and a Bartlett’s test was employed to assess equal variance. When normality and equal variance were confirmed, a one-way analysis of variance with a Tukey’s post hoc test was used to identify statistical significance between groups. In other cases, a Kruskal-Wallis with a Dunn’s multiple comparison was used to identify statistical significance. A p-value <0.05 was considered statistically significant.
3.4 Results

3.4.1 Irinotecan causes severe gut toxicity characterised by diarrhoea and weight loss

All rats receiving irinotecan developed gut toxicity represented by diarrhoea and significant weight loss (Figure 3.1). Diarrhoea occurred in a bi-phasic response, with symptoms first appearing at 6 h after irinotecan administration. There was an initial resolution of diarrhoea before a second, more severe diarrhoea appeared, with maximal symptoms seen 72 h post-irinotecan (**p<0.0001). Rats receiving vehicle control did not develop diarrhoea at any time point.

Rats receiving irinotecan had a peak weight loss compared to baseline at 72 h after chemotherapy (-11.1±6.6%; **p<0.0001) before recovery at 120 h (-0.25±6.7%; p>0.05). Rats receiving vehicle control continued to gain weight over the course of the experiment.

3.4.2 Irinotecan causes severe histopathological damage in the small and large intestine

Marked histological evidence of gut toxicity was observed in the jejunum and colon of irinotecan-treated rats (Figure 3.2). Characteristic apoptotic bodies were observed at 6 h post-irinotecan in the crypt epithelium of both regions of the gut. Gross architectural disturbances, including villous blunting and crypt degeneration throughout the mucosa, were particularly evident 48 h and 72 h after irinotecan administration (Figure 3.2). Restoration of the mucosa was evident by 120 h, indicated by the return of architectural integrity and the presence of mitotically active cells.
Figure 3.1 Measures of clinical toxicity. Percentage of rats with grade 0, 1, 2 and 3 diarrhoea between 6 and 120 h following vehicle (A; sorbitol/lactic acid buffer: 45 mg/mL sorbitol/0.9 mg/mL lactic acid, pH 3.4) or 175 mg/ml irinotecan (B). Peak diarrhoea was seen at 72 h following irinotecan treatment (p<0.0001). Data presented as relative percentage of total rats. A Chi Squared test was performed to determine statistical significance. (C) Percentage change in weight in vehicle and irinotecan-treated rats. *p<0.01 versus 120 h, **p<0.0001 versus 24 h and 120 h. Data presented as mean percentage relative to baseline body weight. A one-way ANOVA with Tukey’s post hoc was performed to determine significance (n=39).
Figure 3.2 Irinotecan administration causes severe histological damage in the jejunum and colon of DA rats. Characteristic apoptotic bodies are visible at 6 h and 96 h post-irinotecan. Gross architectural changes (villous blunting and crypt degeneration) are evident at 24 h, but are most severe at 48 h and 72 h hours. Restoration of the epithelium is evident at 120 h, indicated by mitotically active cells. Original magnification 20 X (villus) and 40 X (crypt). Scale bars represent 20 µm.
3.4.3 Irinotecan causes molecular defects in intestinal tight junction proteins

There were no significant changes in protein expression of ZO-1 in the jejunum at any time point investigated (p>0.05). In contrast, there was a significant decrease in ZO-1 protein expression 96 h following administration of irinotecan in the colonic crypts (apical crypt, #p=0.027; basal crypt ^p=0.005) (Figure 3.3). ZO-1 mRNA expression remained stable across the time course of gut toxicity (p>0.05) in the small and large intestine.

Claudin-1 protein expression was significantly decreased 6 h after irinotecan administration in both the apical and basal villus regions of the jejunum (#p=0.034, ^p=0.021; Figure 3.3). Expression returned to baseline at 72 h. No significance difference was observed in jejunal crypts (p>0.05). There was a significant decrease in claudin-1 protein expression at 24 h and 96 h following irinotecan in the apical and basal colonic crypts, respectively (#p=0.031, ^p=0.001; Figure 3.3). Claudin-1 mRNA expression was significantly downregulated 6 h following chemotherapy in both the jejunum (-8.3 fold change; *p<0.0001) and colon (-1.8 fold change; *p=0.001) of DA rats. Levels remained significantly low at 24 h in the colon only (-2.5 fold change; *p=0.002). A 9.8 fold increase in claudin-1 mRNA expression was observed in the jejunum at 72 h following irinotecan (*p<0.0001; Figure 3.4).

Occludin protein expression was significantly downregulated in the jejunal crypts 48 h following irinotecan administration (#p=0.048, ^p=0.049; Figure 3.3). This pattern of expression was mirrored in the colon, where occludin protein expression was significantly downregulated in both apical and basal crypt regions at 24 and 48 h following irinotecan treatment (24 h #p=0.033, ^p=0.017; 48 h #p=0.031, ^p=0.049; Figure 3.4). There was no change in occludin mRNA expression in the jejunum or colon of DA rats (p>0.05) at any time point under investigation.
Chapter 3 Irinotecan causes tight junction disruption in the gut

Figure 3.3 Irinotecan causes molecular defects in intestinal tight junctions of the DA rat.

**Jejunum** Claudin-1 and occludin were significantly downregulated in the jejunum at 6 h (claudin-1 apical villus \( p=0.034 \), basal villus \( ^p=0.021 \)) and 48 h (occludin basal villus \( p=0.048 \), crypt \( ^p=0.049 \)), respectively. There was no statistically significant change in ZO-1 protein expression in the jejunum. In the colon, ZO-1 showed significant protein downregulation at 96 h (apical crypt \( #p=0.027 \), basal crypt \( ^p=0.005 \)). **Colon** Claudin-1 protein expression was decreased at 24 h (apical crypt \( #p=0.031 \)) and 96 h (basal crypt \( ^p=0.001 \)) in the colon. Occludin protein expression was also decreased at 24 h (apical crypt \( #p=0.033 \); basal crypt \( ^p=0.017 \)) and 48 h (apical crypt \( #p=0.031 \); basal crypt \( ^p=0.049 \)) in the colon. Staining intensity was analysed in a blinded fashion using a validated semi-quantitative grading system (Bowen et al., 2005). A Kruskal-Wallis with a Dunn’s multiple comparison was performed to determine significance. Data presented as median values (n=39). For jejunal staining, * denotes significance in apical villous staining relative to vehicle controls, # denotes significance in basal villous staining relative to vehicle controls, ^ denotes significance in crypt staining relative to vehicle controls, where \( p<0.05 \). For colonic staining, # denotes significance in apical crypt staining relative to vehicle controls, ^ denotes significance in basal crypt staining relative to vehicle controls (\( p<0.05 \)).
Figure 3.4 Representative images of tight junction protein immunostaining in the colon of vehicle- and irinotecan treated rats. Original magnification 40 X.
Figure 3.5 RT-PCR analysis of ZO-1, claudin-1 and occludin in the jejunum and colon of rats treated with irinotecan or vehicle control. Irinotecan caused an 8.3 fold decrease in claudin-1 mRNA expression (*p<0.0001), relative to an internal housekeeper and untreated controls, in the jejunum 6 h following irinotecan followed by a 9.8 fold increase at 72 h (*p<0.0001). A -1.8 and -2.5 fold change in claudin-1 mRNA expression was observed in the colon of irinotecan treated DA rats at 6 h (*p=0.001) and 24 h (*p=0.002), respectively. The fold change in mRNA expression values were used to create the graphs. All data is relative to internal controls (untreated animals) and a validated housekeeping gene (UBC). Relative mRNA expression was calculated using the Pfaffl method of relative quantification, where y=1 reflects no change in mRNA expression. Data presented as individual points with median (n=39). A two-way ANOVA was performed to identify statistical significance (p<0.05).
3.4.4 RT-PCR efficiency and housekeeping gene stability (UBC)

The amplification efficiencies for each set of primers were measured using serial dilutions of cDNA in triplicate. PCR efficiency and a standard curve were calculated using Rotor Gene 6 software. Variations in cycle times and annealing temperatures did not yield comparable PCR efficiencies (ZO-1=1.01; claudin-1=1.1; occludin=1.21; UBC=1.31), therefore the Pfaffl method (Pfaffl, 2001) of relative quantification was employed.

The fold change in UBC expression was calculated using the $2^{ΔCt}$ method where control rats were used as a baseline. UBC showed no differential mRNA expression in the jejunum or colon of DA rats across the time-course of irinotecan-induced gut toxicity, indicating high stability as outlined by Al-Dasooqi et al., (2010).
3.5 Discussion

Chemotherapy-induced gut toxicity (CIGT) has become increasingly important as a dose-limiting factor of anti-cancer therapies, particularly with the advent of more aggressive chemotherapy regimens (Elting et al., 2003). Evidence exists for the involvement of tight junctions in the pathophysiology of CIGT (Wardill et al., 2012), however, to date few studies have conducted detailed investigations of intestinal tight junctions following chemotherapy. The present study therefore aimed to characterise the expression of three key tight junctional proteins, ZO-1, claudin-1 and occludin, in both the small and large intestine using a well-established pre-clinical model of gut toxicity. Key findings from this study indicate tight junction defects coincide with validated histopathological and clinical markers of gut toxicity (diarrhoea and weight loss), strongly implicating tight junctions in the development of CIGT.

Late-onset diarrhoea is a key dose-limiting factor for many chemotherapy patients (Gibson and Keefe, 2006), however to date there are no clear mechanisms underpinning its development. The clinical and economic impact associated with chemotherapy-induced diarrhoea has only recently been appreciated, prompting research efforts in an attempt to reveal the pathophysiology of this complication (Bowen et al., 2013a). Despite having vastly different pathogeneses, inflammatory bowel disorders (IBDs) are often used to identify potential mechanisms of CIGT due to highly comparable clinical manifestations (Schulzke et al., 2009). Recent research investigating the pathophysiology and symptomology of IBDs has shown that tight junction defects and barrier disturbances contribute to diarrhoea development through leak-flux mechanisms (Amasheh et al., 2002, Burgel et al., 2002, Amasheh et al., 2009). In line with this research, the present study identified tight junction defects which coincided with key events in the pathophysiology of CIGT and preceded the onset of clinical symptoms. Of particular importance was the significant decrease in ZO-1 protein expression in the colonic crypts at 96 h following insult, coinciding with the presence of severe late-onset diarrhoea observed in this animal model. It is likely that loss of tight
junction apposition increases intestinal electrolyte and water fluxes, which therefore contribute to diarrhoea development.

In addition to ZO-1 disruption, the current study identified significant changes in claudin-1 at both the protein and gene level. Previous research has identified emerging roles for claudin-1 in mediating apoptosis and aiding tissue regeneration (Turner, 2009, Liu et al., 2012). Liu and colleagues recently demonstrated this concept, reporting significantly increased levels of claudin-1 in human breast cancer MCF-7 cells - a cell line recognised for their low levels of apoptosis. In line with these findings, downregulation of claudin-1 by siRNA knockdown resulted in a significant increase in apoptosis markers such as caspase-8. Importantly, loss of claudin-1 was shown to increase the susceptibility of MCF-7 cells to TNF-induced apoptosis. In the current study, significantly reduced claudin-1 protein and mRNA expression was observed 6 h following irinotecan insult, coinciding with peak apoptosis levels (Keefe et al., 2000) thus supporting this novel role of claudin-1 as a potent anti-apoptotic mediator. Claudin-1 downregulation was also identified 24 h following chemotherapy, and this is likely due to the slower proliferative rate of the colon relative to the jejunum. In contrast to its anti-apoptosis mechanisms, it has also been suggested that claudin-1 contributes to tissue repair and regeneration (Kang et al., 2004, Sonis, 2004a, Sonis, 2004c). Our data show overexpression of claudin-1 at 72 h post-irinotecan, the established point of cellular regeneration and mitotic activity in this model of CIGT. Unexpectedly however, this was accompanied by a decrease in protein expression in the colonic crypts and which may be attributable to the second wave of apoptosis, as the body resets homeostasis (Keefe et al., 2000).

There is conflicting evidence regarding mRNA analysis of tight junction proteins following chemotherapy. Most recently, a preclinical study conducted by Nakao et al. (2012) investigated tight junction proteins in tumour-naïve rats and found tight junction changes were associated with gut barrier dysfunction. Specifically, a significant decrease in both occludin and claudin-1 mRNA expression was identified (Nakao et al., 2012), however given their small sample size (n=10) and single time-point evaluation, these conclusions should be interpreted with caution. In contrast,
several other preclinical studies found no change in occludin and ZO-1 mRNA expression following administration of methotrexate (MTX), but demonstrated decreased claudin-1 mRNA expression (Hamada et al., 2010, Beutheu Youmba et al., 2012). In accordance with these latter findings, the present study did not identify any significant change in occludin or ZO-1 mRNA expression following irinotecan administration. These inconsistencies highlight the need for further research to clarify the effect of various cytotoxic agents on tight junction protein mRNA levels at time points relevant to clinical symptoms.

The disparate protein and mRNA expression identified in the present study suggest that irinotecan indirectly modulates both occludin and ZO-1 through post-translational regulation. Post-translational modification of occludin and ZO-1, in particular proteolytic degradation, is a well-documented phenomenon (Cummins, 2012). Recent research has indicated proinflammatory cytokines are able to modulate tight junction proteins (Fish et al., 1999, Al-Sadi et al., 2008) and it is therefore hypothesised that cytokine-mediated tight junction modulation may play a key role in the development of CIGT (Wardill et al., 2012). In vitro application of tumour-necrosis factor (TNF) has been shown to induce actin and tight junction rearrangement, resulting in a time-dependent decrease in tight junction protein expression and a parallel increase in intestinal permeability (Mullin and Snock, 1990, Fish et al., 1999, Wang et al., 2005). Previous research has shown that elevated serum TNF and interleukin-1 are hallmarks of CIGT pathophysiology, with peaks occurring at 24-48 h post-chemotherapy (Logan et al., 2008b). The present study has shown occludin downregulation also occurs at these time-points, implying a correlation between the two. It is therefore suggested proinflammatory cytokines not only contribute to histological damage in the gut, but also mediate mucosal barrier function through tight junction modulation.

Matrix metalloproteinases (MMPs) are extracellular matrix signalling molecules recently recognised for their proteolytic functions (Cummins, 2012). Substantial in vivo evidence supports a role for MMP-mediated occludin proteolysis and membrane cleavage (Beauchesne et al., 2009, Casas et al., 2010). This is of particular importance as MMPs have recently been identified as key mediators of
intestinal toxicity induced by irinotecan (Al-Dasooqi et al., 2010). Al-Dasooqi and colleagues (2010) reported significantly increased small intestinal and serum levels of MMP-9 and MMP-12 following irinotecan. Given the proteolytic functions of MMPs this suggests MMP-mediated tight junction damage may occur. This correlation has previously been shown in the central nervous system, with MMP-9 levels in brain tissue correlating with reductions in occludin and ZO-1 protein levels (Beauchesne et al., 2009, Lischper et al., 2010). Further, MMP inhibition has been shown to ameliorate hypoxic-induced occludin proteolysis (Bauer et al., 2010). Further studies are now warranted in order to establish the molecular mechanisms of these post-translational regulations.

In addition to post-translational modulation, reversible phosphorylation of tight junction proteins has recently been established as a vital aspect of tight junction integrity and barrier regulation (Sakakibara et al., 1997, Cummins, 2012). Despite the importance of tight junction phosphorylation for optimal tight junction assembly and function (Sakakibara et al., 1997, Cummins, 2012), few studies have investigated post-translational phosphorylation of tight junction proteins in the gut. Hamada and colleagues (2010) reported decreased tyrosine phosphorylation of ZO-1 despite observing no change in ZO-1 protein and mRNA expression. Although not addressed in this study, dephosphorylation may be responsible for chemotherapy-induced intestinal barrier dysfunction and future investigations are now necessary.
3.6 Conclusion

Tight junction proteins can undergo a broad array of regulatory modification in response to various pathological cues, highlighting the plasticity of these dynamic signalling complexes. These molecular events are likely to contribute to mucosal barrier dysfunction, increased intestinal permeability and the development of diarrhoea; all characteristic of CIGT. It is likely that ZO-1 primarily is a late-onset, symptom-associated protein, whereas claudin-1 and occludin have more potential as prognostic markers as their modulation precedes barrier dysfunction and symptomology. Detailed research is now required to investigate post-translational modulation of tight junction proteins to elucidate the molecular mechanisms that underpin the development of gut toxicity. This may lead to a greater understanding of how tight junction modifications affect gastrointestinal homeostasis, thus revealing their therapeutic potential in chemotherapy-induced gut toxicity and other diseases characterised by barrier dysfunction.
# Chapter 4 Chemotherapy-induced oral tight junction disruption

## Statement of Authorship

<table>
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<th>Title of Paper</th>
<th>Tight junction defects are seen in the buccal mucosa of patients receiving standard dose chemotherapy for cancer</th>
</tr>
</thead>
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<tr>
<td>Publication Status</td>
<td>Published ☑  Accepted for Publication ☐ Submitted for Publication ☐  Unpublished and Unsubmitted work written in manuscript style ☐</td>
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### Principal Author

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<tr>
<th>Name of Principal Author (Candidate)</th>
<th>Hannah Wardill</th>
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</thead>
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<tr>
<td>Contribution to the Paper</td>
<td>I was responsible for all experimental work using archival tissue samples obtained in the original study (Gibson et al., 2006, Asia Pacific Journal of Clinical Oncology, 21(1):39-49). I performed all data analysis and prepared the manuscript for publication.</td>
</tr>
<tr>
<td>Overall percentage (%)</td>
<td>80%</td>
</tr>
<tr>
<td>Certification:</td>
<td>This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.</td>
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### Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate’s stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate’s stated contribution.

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<th>Richard Logan</th>
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<tr>
<td>Contribution to the Paper</td>
<td>Richard was responsible for obtaining the patient tissue samples for the original study. Richard is also my third supervisor, and therefore reviewed the manuscript prior to publication.</td>
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**Chapter 4 Chemotherapy-induced oral tight junction disruption**

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<th>Name of Co-Author</th>
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<tr>
<td>Contribution to the Paper</td>
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<td>Contribution to the Paper</td>
<td>Rachel was responsible for the original study and therefore obtained many of the patient buccal biopsies. She is also my co-primary supervisor and helped with preparation of the manuscript.</td>
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Chapter 4 Tight junction defects are seen in the buccal mucosa of patients receiving standard dose chemotherapy for cancer


4.1 Abstract

**Purpose** Oral toxicity (mucositis) is one of the most common and debilitating side effects of chemotherapy treatment. Patients are often unable to eat and drink, which can lead to poor clinical outcomes and extensive resource utilisation. The primary aim of this study was to determine the molecular integrity of oral epithelial tight junctions in patients undergoing chemotherapy. The secondary aim was to correlate these changes with proinflammatory cytokines and matrix metalloproteinase profiles. **Methods** Patients (n=23) were recruited from the Royal Adelaide Hospital between 2000-03. Each patient underwent two oral buccal mucosa biopsies (4mm): one prior to chemotherapy treatment and a second one after chemotherapy treatment. Oral buccal mucosa biopsies were also taken from 7 healthy volunteers with no history of cancer, chemo- or radiotherapy treatment or inflammatory disorders. Routine haematoxylin and eosin staining was performed to determine epithelial thickness. Immunohistochemical staining was performed for claudin-1, zonular occludens-1, occludin, interleukin-1β, tumour necrosis factor, interleukin-6, matrix metalloproteinase-2 and -9. **Results** Patients receiving standard dose chemotherapy had significant epithelial atrophy. Elevations in all cytokines and matrix metalloproteinases were seen, with significant lamina propria staining for interleukin-6 and tumour necrosis factor. Matrix metalloproteinase-2 appeared most upregulated within the oral epithelium. These changes coincided with altered tight junction staining properties. Changes in the staining intensity and localisation were both noted, with clear cytoplasmic staining for zonular occludens-1 and claudin-1 in patients treated with chemotherapy. **Conclusions** Chemotherapy causes defects in oral tight junctions, coupled with altered cytokine and matrix metalloproteinase profiles. Tight junction disruption in the epithelium may contribute to ulcer development or lead to poor tissue integrity and the timing of these events may be a target for preventative treatment.
4.2 Introduction

Chemotherapy treatment is associated with a host of debilitating side effects with varying effects on patient quality of life, resource utilisation and treatment efficacy. Over the past decade, there has been an appreciation gained for the impact of chemotherapy-induced alimentary toxicity on patient quality of life, leading to vast improvements in our understanding of its pathobiology (Sonis, 2004c, Carlotto et al., 2013). Alimentary toxicity is characterised by severe ulceration along the entire alimentary tract (Logan et al., 2008a), however, oral lesions are most easily accessed and therefore diagnosed. In fact, oral toxicity is frequently described as the most common dose-limiting factor for patients undergoing chemotherapy treatment, affecting 80-100% of those receiving high dose treatment (Keefe et al., 2000, Keefe, 2007). The development of oral toxicity in patients during cancer treatment places a significant clinical and economic burden on the provision of care. Additionally, oral toxicity can compromise treatment outcomes and, in itself, increase mortality through heightened infection risk. Despite its prevalence and clinical impact, there is limited data on the molecular mechanisms that underpin or initiate this toxicity.

It is currently accepted that the pathobiology of alimentary toxicity, in which oral toxicity is included, can be described using a continuous and overlapping 5-phase model proposed by Sonis in 2004 (Sonis, 2004a, Sonis, 2004c). This model was the first to recognise that alimentary toxicity is not purely an epithelial phenomenon, highlighting the dynamic interactions that occur between the epithelium, extra cellular matrix (ECM), submucosa and the chemotherapeutic agent itself. Consequently, the pathobiology is defined as the collective consequences of direct cytotoxicity, induced by the chemotherapeutic agent, as well as inflammatory-driven indirect cytotoxicity primarily controlled through nuclear factor kappa B (NFκB). Although this model of alimentary mucositis remains universally accepted, recent advances in our understanding have identified complimentary molecular mediators of toxicity. One such example is the emerging role of tight junctions (Al-Dasooqi et al., 2013) in regulating barrier dysfunction commonly observed following cytotoxic treatment.
Tight junctions are highly dynamic signalling complexes vital to epithelial homeostasis. Located at the apico-lateral boundary of adjacent epithelial cells, tight junctions are integral in maintaining epithelial adhesion as well as regulating paracellular permeability (Gonzalez-Mariscal et al., 2008). Tight junctions are primarily formed of four protein groups; claudins, zonular occludens (ZO), junctional adhesion molecules (JAMs) and occludin. Importantly, the molecular interactions of these proteins cause tight junctions to be highly malleable and plastic structure that assemble, grow, recognise and disassemble in response to various physiological and pathological cues. Based on their highly plastic nature, particularly in response to inflammatory mediators, tight junctions have gained significant attention in a number of inflammatory-based pathologies, including chemotherapy-induced gut toxicity (Wardill et al., 2012, Wardill et al., 2013, Wardill et al., 2014a). Tight junctions were first identified to be involved in the pathobiology of gut toxicity in 1997, with Keefe and colleagues (Keefe et al., 1997) showing increased and uncontrolled intestinal permeability in patients receiving high dose chemotherapy. In 2000, ultrastructural changes in small intestinal tight junctions were identified in patients receiving various chemotherapeutic treatment regimens (Keefe et al., 2000). Since the early 2000’s, several studies have identified molecular defects in intestinal tight junctions following chemotherapy treatment, with downregulation, redistributing and phosphorylation of occludin, ZO-1 and claudin-1 consistently reported (Hamada et al., 2010, Beutheu Youmba et al., 2012, Nakao et al., 2012, Hamada et al., 2013, Wardill et al., 2013, Wardill et al., 2014a). Tight junction disruption is therefore emerging as a key player in the pathobiology of alimentary toxicity.

Modification of tight junction proteins, particularly post-translationally, is a well-documented phenomenon and forms the basis of many inflammatory pathologies (Edelblum and Turner, 2009, Schulzke et al., 2009, Rebuffat et al., 2013). In the setting of both oral and gut toxicity, the interaction between proinflammatory cytokines, matrix metalloproteinases (MMPs) and tight junctions is compelling given the strong inflammatory component of mucositis (Logan et al., 2008b) and documented changes in MMP profiles (Al-Dasooqi et al., 2011a). The ability of proinflammatory cytokines and MMPs to degrade tight junctions is well-established (Cummins,
2012, Wardill and Bowen, 2013), highlighting a potential interaction between mediators of mucositis and tight junction disruption. Importantly, these mediators are not only found at elevated levels in the gut but also the oral cavity (Logan et al., 2008a) and circulating serum (Logan et al., 2008b) therefore suggesting tight junction disruption may also play a role in the pathobiology of oral toxicity. This study therefore aims to determine the phenotype of oral epithelial tight junctions in patients receiving chemotherapy and correlate with established changes in proinflammatory cytokines (Interleukin(IL)-1β, IL-6, tumour necrosis factor (TNF)) and MMP profiles (MMP-2, -9). Results from this study will determine if tight junction disruption is a common mechanism of alimentary toxicity, and may shed light on the underlying mechanisms responsible for barrier dysfunction.
4.3 Materials and Methods

4.3.1 Patients

Tissue samples were sourced from a previously conducted study (Gibson et al., 2006) published by Gibson et al., 2006. This previous study was approved Royal Adelaide Hospital Human Ethics Committee. Briefly, patients were recruited from the Department of Medical Oncology at the Royal Adelaide Hospital between 2000 and 2003 (n=23). The study included 7 male and 16 female patients with a median age of 52.4 years (32-86 years) (Gibson et al., 2006). Patients were excluded if they were undergoing concurrent radiotherapy to the head and neck, or if they had pre-existing mucosal damage. Tumour type was heterogeneous amongst patients and included breast, non-Hodgkin’s lymphoma, Hodgkin’s lymphoma, colorectal, lung and neuroendocrine pancreatic. Standard dose chemotherapy was used in all patients, administered over 1-4 hours (Gibson et al., 2006). Treatments included ABVD, AC, CMF, DOX, Docetaxel, CHOP, 5-FU/Folinic Acid, CAV and Streptozocin. For tabular breakdown of patient demographics and treatment regimens, please refer to Table 4.1.

Patients had a single oral buccal mucosa biopsy prior to the commencement of their first chemotherapy cycle and a second after cessation of their treatment (mean 4.8 days; range 3-11 days). Seven healthy volunteers (3M:4F), with no history of cancer, chemotherapy treatment and pre-existing mucosal damage were also recruited for the study. All biopsies were performed by a single operator. Pre-chemotherapy biopsies were taken on one side of the mouth and post-chemotherapy biopsies were taken on the opposite side. The surrounding buccal mucosa was injected with local anesthetic, and a small (4 mm) punch biopsy was taken. A single stitch was placed at the site of the biopsy if necessary. The number of previous chemotherapy cycles undergone by each patient was recorded at recruitment to determine if these contributed to histological or molecular changes in the oral cavity.
4.3.2 Clinical assessment of oral toxicity

Case note reviews were used to identify the presence/absence of toxicity in this patient cohort at the time of sample collection. Institutional reporting guidelines did not require mandatory reporting of oral symptoms in patient case notes, and therefore oral toxicities were not as comprehensively reported in this archival patient group as would be required today. Gibson et al., (2006) reported that greater than 50% of patients had mucositis symptoms of WHO grades 1-2 (relatively mild) ranging from mouth ulcers, loss of taste, mouth dryness, ‘thick’ feeling over the tongue and cheek area and fissured tongues (Gibson et al., 2006). For full tabular breakdown, please see Table 4.1.
# Table 4.1 Characteristics of patients that participated in the study

<table>
<thead>
<tr>
<th></th>
<th>Treated cohort</th>
<th>Healthy controls</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex (% of total patients)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M:</td>
<td>17.39</td>
<td>M: 42.85</td>
</tr>
<tr>
<td>F:</td>
<td>82.61</td>
<td>F: 57.15</td>
</tr>
<tr>
<td><strong>Mean age (years)</strong></td>
<td>52.42±10.3</td>
<td>31.71±8.2</td>
</tr>
<tr>
<td><strong>Primary tumour (% of total patients)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breast</td>
<td>43.48</td>
<td></td>
</tr>
<tr>
<td>Non-Hodgkin’s Lymphoma</td>
<td>8.70</td>
<td></td>
</tr>
<tr>
<td>Hodgkin’s Lymphoma</td>
<td>8.70</td>
<td></td>
</tr>
<tr>
<td>Colorectal</td>
<td>17.39</td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>8.70</td>
<td></td>
</tr>
<tr>
<td>Neuroendocrine Pancreatic</td>
<td>13.04</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment regimen (% of total patients)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AC+CMF</td>
<td>13.04</td>
<td></td>
</tr>
<tr>
<td>DOX+Docetaxel+CMF</td>
<td>4.35</td>
<td></td>
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<tr>
<td>CMF</td>
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<tr>
<td>AC</td>
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<tr>
<td>Docetaxel</td>
<td>4.35</td>
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<tr>
<td>AC+CMF</td>
<td>8.70</td>
<td></td>
</tr>
<tr>
<td>CHOP</td>
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<td></td>
</tr>
<tr>
<td>ABVD</td>
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</tr>
<tr>
<td>5-FU/Folinic Acid</td>
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<tr>
<td>5-FU/Leucovorin</td>
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<td></td>
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<tr>
<td>CAV</td>
<td>4.35</td>
<td></td>
</tr>
<tr>
<td>Streptozocin/5-FU</td>
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<td></td>
</tr>
<tr>
<td><strong>Toxic events (% of total patients)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>43.49</td>
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</tr>
<tr>
<td>Oral toxicity (total)</td>
<td>56.52</td>
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</tr>
<tr>
<td><em>Mouth ulcers</em></td>
<td>17.39</td>
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<tr>
<td><em>Lip ulcers</em></td>
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<tr>
<td><em>Erythema</em></td>
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<tr>
<td><em>White spots</em></td>
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<td></td>
</tr>
<tr>
<td><em>Tongue fissures</em></td>
<td>8.69</td>
<td></td>
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<tr>
<td><em>Pain</em></td>
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<td></td>
</tr>
<tr>
<td>Intestinal toxicity</td>
<td>4.35</td>
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</table>

ABVD, Adriamycin/Bleomycin/Vincristine/Dacarbazine; AC, Doxorubicin/Cyclophosphamide; CAV, Cyclophosphamide/Doxorubicin/Vincristine/Dacarbazine; CHOP, Cyclophosphamide/Doxorubicin/Vincristine/Prednisolone; CMF, Cyclophosphamide/Methotrexate/5-Fluorouracil; DOX, Doxorubicin; 5-FU, 5-Fluorouracil
4.3.3 Histopathological analysis of the oral epithelium

Oral buccal mucosa biopsies were cut at 5\(\mu\)m using the Leica Microtome and mounted onto glass microscope slides. Routine haematoxylin and eosin staining was conducted on all buccal mucosa biopsy samples. Briefly, sections were dewaxed and rehydrated through graded ethanols. Sections were placed in Harris Haematoxylin for 2 mins before being placed in 0.5% ammonia for 1 min. Sections were washed and placed in eosin for 2 mins before being dehydrated, cleared and coverslipped. Slides were scanned using a NanoZoomer (Hamamatsu Photonics, Japan) and analysed using NanoZoomer Digital Pathology software (Histalim, Montpelier, France). Epithelial thickness was measured ten times across the width of the tissue section and an average was determined (Al-Azri et al., 2014). All analysis was conducted in a blinded fashion.

4.3.4 Immunohistochemical analysis of tight junctions and inflammatory markers

Immunohistochemistry (IHC) was carried out on 4 \(\mu\)m sections of oral buccal mucosal cut on a rotary microtome and mounted onto FLEX IHC microscope slides (Flex Plus Detection System, Dako, Denmark; #K8020). Immunohistochemical analysis was performed for three tight junction proteins (claudin-1, ZO-1 and occludin), proinflammatory cytokines (IL-1\(\beta\), IL-6, TNF) as well as MMP-2 and MMP-9 (Table 4.2). Immunohistochemical analysis was performed using Dako reagents on an automated machine (AutostainerPlus, Dako, Denmark) following standard protocols supplied by the manufacturer. Briefly, sections were deparaffinised in histolene and rehydrated through graded ethanols before undergoing heat mediated antigen retrieval using an EDTA/Tris buffer (0.37g/L EDTA, 1.21g/L Tris; pH 9.0). Retrieval buffer was preheated to 65°C using the Dako PT LINK (pre-treatment module). Slides were immersed in the buffer and the temperature raised to 97°C for 20 min. After returning to 65°C, slides were removed and placed in the Dako AutostainerPlus and stained following manufacturer’s guidelines. Briefly, endogenous peroxidase was blocked using the FLEX peroxidase block followed by a serum-free protein block (Dako, Denmark; #X0909). Primary antibodies were suspended in the EnVision™ FLEX Antibody Diluent (Dako, Denmark; #K8006) and applied for 60. Negative controls had the primary antibody omitted.
The EnVision™ FLEX+ Rabbit/Mouse LINKER (Dako, Denmark; #K8019) was then applied for 30-60 min before DAB was used to visualise the target protein. Slides were removed from the automated stainer, counterstained in Harris Haematoxylin, dehydrated and coverslipped. Slides were scanned using the NanoZoomer (Hamamatsu Photonics, Japan) and assessed with NanoZoomer Digital Pathology software (Histalim, Montpellier, France). Healthy control samples were used as an internal positive control for tight junction proteins. Human tonsil was used as a positive control for IL-1β, IL-6, TNF, MMP-2 and MMP-9.

Slides were scanned using a NanoZoomer (Hamamatsu, Japan) and analysed using NanoZoomer Digital Pathology software (Histalim, Montpellier, France). Tight junction staining was analysed in the superficial/intermediate, prickle cell and basal epithelium as well as the endothelium of the lamina propria (Figure 4.1), whilst IL-1β, IL-6, TNF, MMP-2 and MMP-9 staining was analysed in the whole oral epithelium and lamina propria. Staining intensity was analysed using a validated semi-quantitative grading system (Al-Azri et al., 2014) from 0-3; where 0 = no staining, 1 = mild staining, 2 = moderate staining and 3 = intense staining (Figure 4.2) and was conducted in a blinded fashion (Al-Azri et al., 2014). In addition, the characteristics of tight junction staining, including membrane specificity and location, were assessed qualitatively.
## Table 4.2 Antibody specification and application.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Distributor</th>
<th>Catalogue #</th>
<th>Concentration</th>
<th>Polymer Type</th>
<th>Incubation period</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occludin</td>
<td>Invitrogen</td>
<td>33-1500</td>
<td>5 µg/ml</td>
<td>EnVision™ FLEX+</td>
<td>60 min</td>
</tr>
<tr>
<td>Mouse monoclonal</td>
<td></td>
<td></td>
<td></td>
<td>Rabbit LINKER</td>
<td></td>
</tr>
<tr>
<td>Claudin-1</td>
<td>Abcam</td>
<td>ab15908</td>
<td>2 µg/ml</td>
<td>EnVision™ FLEX+</td>
<td>60 min</td>
</tr>
<tr>
<td>Rabbit polyclonal</td>
<td></td>
<td></td>
<td></td>
<td>Rabbit LINKER</td>
<td></td>
</tr>
<tr>
<td>ZO-1</td>
<td>Invitrogen</td>
<td>61-7300</td>
<td>2.5 µg/ml</td>
<td>EnVision™ FLEX+</td>
<td>60 min</td>
</tr>
<tr>
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<td></td>
<td></td>
<td>Rabbit LINKER</td>
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<tr>
<td>TNF</td>
<td>Abcam</td>
<td>ab6671</td>
<td>10 µg/ml</td>
<td>EnVision™ FLEX+</td>
<td>30 min</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>Rabbit LINKER</td>
<td></td>
</tr>
<tr>
<td>IL-1β</td>
<td>Abcam</td>
<td>ab9787</td>
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<td>EnVision™ FLEX+</td>
<td>30 min</td>
</tr>
<tr>
<td>Rabbit polyclonal</td>
<td></td>
<td></td>
<td></td>
<td>Rabbit LINKER</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
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<td>ab6672</td>
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<td>EnVision™ FLEX+</td>
<td>30 min</td>
</tr>
<tr>
<td>Rabbit polyclonal</td>
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<td></td>
<td></td>
<td>Rabbit LINKER</td>
<td></td>
</tr>
<tr>
<td>MMP-2</td>
<td>Abcam</td>
<td>ab58803</td>
<td>1.25 µg/ml</td>
<td>EnVision™ FLEX+</td>
<td>30 min</td>
</tr>
<tr>
<td>Rabbit polyclonal</td>
<td></td>
<td></td>
<td></td>
<td>Rabbit LINKER</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td>Abcam</td>
<td>ab37150</td>
<td>1.25 µg/ml</td>
<td>EnVision™ FLEX+</td>
<td>30 min</td>
</tr>
<tr>
<td>Mouse monoclonal</td>
<td></td>
<td></td>
<td></td>
<td>Mouse LINKER</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.1 Histology of the human buccal mucosa. A photomicrograph of the buccal mucosa stained with haematoxylin and eosin (original magnification 40 X; scale bar shows 100 µm). The stratified squamous epithelium of the human buccal mucosa is non-keratinised, displaying four distinct layers: basal cells (B), prickle cell layer, the intermediated layer and the superficial epithelium. Mitotically active basal cells reside on the basement membrane, separating the epithelium from the underlying lamina propria. The lamina propria consists of loose connective tissue with rich vasculature and immune capabilities.
Figure 4.2 Semi-quantitative grading system representative images. Representative photomicrographs of the oral epithelium showing immunohistochemical staining of varying intensities. A-D indicate epithelial staining intensities, while lamina propria staining is shown in images E-H. Staining is graded on a scale of 0-3, where 0 = no staining, 1 = mild staining, 2 = moderate staining and 3 = intense staining.
4.3.5 Statistical analysis

Epithelial thickness and immunohistochemical staining were compared between healthy control samples, pre-chemotherapy samples and post-chemotherapy samples using GraphPad Prism 7.0. Data was assessed for normality using the D'Agostino-Pearson omnibus test. When normality was confirmed, a two-way analysis of variance (ANOVA) was performed with a Tukey’s post hoc. If normality was not achieved, a Kruskal-Wallis with a Dunn’s multiple comparison was performed. To determine the relationship between previous chemotherapy cycles and epithelial thickness, a linear regression model was applied and the coefficient of determination ($r^2$) was determined. A $p$-value $<0.05$ was considered significant.
4.4 Results

4.4.1 Chemotherapy causes significant epithelial atrophy consistent with oral toxicity

Epithelial atrophy was observed both before (\(**p=0.0008\)) and following chemotherapy cycles (\(***p<0.0001\); Figure 4.3A and 4.4). Given that patients were not naïve to chemotherapy treatment, it is likely that the atrophy observed prior to treatment was due to the previous cycles patients underwent. This was confirmed by a strong correlation between epithelial thickness and the number of previous chemotherapy cycles patients had undergone (\(r^2=0.66; ***p<0.0001\); Figure 4.3B).
Figure 4.3 (A) Epithelial atrophy was observed in cancer patients prior to chemotherapy (**p=0.0008) and following chemotherapy (***/p<0.0001). Epithelial atrophy was significantly more severe in patients following chemotherapy compared with those prior to the onset of treatment (*p=0.0042). (B) Correlation between epithelial thickness and previous cycles of chemotherapy. Data presented as mean+/-SEM (A) or individual points (B) with a linear regression model.
Figure 4.4 Representative histological images showing epithelial thickness for healthy controls (A) and patients following chemotherapy treatment (B). Original magnification 40 X, scale bars show 50 µm.
4.4.2 Chemotherapy increases proinflammatory cytokines and alters MMP profiles

Increases were seen in all proinflammatory cytokines and MMPs subtypes following chemotherapy (Figure 4.5 and 4.6). IL-1β and IL-6 showed increased expression in the epithelium of patients treated with chemotherapy (***p=0.0017, *p=0.0167, respectively). Although no significance change was seen in the epithelial expression of TNF across all groups (p>0.05; Figure 4.5 and 4.6), there was a significant increase in the lamina propria following chemotherapy treatment (***p<0.0001; Figure 4.5 and 4.6). This was consistent with the changes seen in IL-6, with significant increases in patients treated with chemotherapy (***p<0.0001; Figure 4.5 and 4.6). Both IL-6 and TNF appeared most prominent in the fibrous material and amorphous ground substance of the lamina propria (Figure 4.5 and 4.6). MMP-9 staining remained showed mild increases in staining expression in both the epithelium (**p=0.0039) and lamina propria (*p=0.0409) of patients treated with chemotherapy (Figure 4.5 and 4.6). MMP-2 staining was most significant in the epithelium of patients treated with chemotherapy (**p=0.001), with clear cytoplasmic staining in the prickle layer indicating active secretion (Figure 4.5 and 4.6). The vasculature and fibroblasts in the lamina propria also showed positive MMP-2 staining in patients treated with chemotherapy. Residual inflammatory signalling was evident in the oral cavity of patients exposed to previous chemotherapy treatment, with pre-chemotherapy biopsies displaying increased TNF in the lamina propria (***p<0.0001).

4.4.3 Tight junction defects are seen in the buccal mucosa following chemotherapy

Claudin-1 and ZO-1 protein expression decreased most notably in the basal (claudin-1: *p=0.0130, ZO-1: ***p<0.0001; Figure 4.7) and prickle cell layers (claudin-1: **p=0.0078, ZO-1: ***p<0.0001; Figure 4.7). Despite only modest changes in the overall staining intensity of tight junction proteins, clear changes in their localisation were evident (Figure 4.8). In healthy controls, ZO-1 and claudin-1 displayed strong specificity for the membrane, with epithelial staining showing the typical ‘cobblestone’ appearance. In patients treated with chemotherapy, claudin-1 expression appeared disrupted, particularly in the basal epithelium, and less specific for the membrane.
Membrane specificity was not evident until more superficial epithelial layers. This redistribution was also clear in ZO-1 staining characteristics, with clear cytoplasmic staining evident.
Figure 4.5 Representative staining for IL-6, TNF and MMP-2 in the oral mucosa of healthy controls and patient samples prior to and following chemotherapy. Staining intensity for IL-6 and TNF was most apparent in the lamina propria, staining fibrous material and amorphous ground substance. Low grade epithelial staining was evident in patients treated with chemotherapy. MMP-2 staining was most significant in the epithelium, with clear cytoplasmic staining in the prickle layer indicating active secretion. The vasculature as fibroblasts in the lamina propria showed positive MMP-2 staining in patients treated with chemotherapy. Original magnification 40 X; scale bars on images A-C show 100 µm.
Figure 4.6 Immunohistochemical analysis of proinflammatory cytokines and MMPs expression. All staining was analysed using a validated, semi-quantitative grading system (0-3). Data expressed as mean+/SEM. A Kruskal-Wallis with Bonferroni correction was performed to identify statistical significance, where; *p<0.05, **p<0.002, ***p=0.0001 ****p<0.0001.
Figure 4.7 Immunohistochemical analysis of tight junction protein expression. All staining was analysed using a validated, semi-quantitative grading system (0-3). Data expressed as mean+/SEM. A Kruskal-Wallis with Bonferroni correction was performed to identify statistical significance, where;

\*p<0.05, \**p<0.002, \***p=0.0001 \****p<0.0001.
Figure 4.8 Representative staining for claudin-1 (A-C) and ZO-1 (D-E) in the oral epithelium of healthy controls and patients treated with chemotherapy. The membrane specificity of claudin-1 staining is evident in the deep layers of the basal epithelium in health control patients (A, B: arrow head). In treated patients, staining is disrupted and less specific for the membrane, showing redistribution to the cytoplasm. Membrane specificity is not evident until more superficial epithelial layers (C: arrow head). ZO-1 staining displays typical cobblestone appearance in health controls (D). Following chemotherapy, internalization of ZO-1 is evident showing clear cytoplasmic staining (E: arrows). Original magnification 40x; scale bars on images A-C show 100 µm, scale bars on images D-E show 10 µm.
Chapter 4 Chemotherapy-induced oral tight junction disruption

4.5 Discussion

Recent clinical practice guidelines (Al-Dasooqi et al., 2013) and preclinical research outcomes (Wardill et al., 2013) have highlighted growing evidence indicating the impact of tight junction disruption in the development of chemotherapy-induced mucositis. In light of this new research avenue, the current study utilised archival tissue samples obtained from patients undergoing standard chemotherapy, with the aim of determining oral epithelial tight junction integrity and correlating with established changes in proinflammatory cytokine and MMP profiles.

An unexpected finding from the current study was significant epithelial atrophy seen in the buccal mucosa biopsies taken prior to chemotherapy treatment. Importantly, all patients recruited for the original study had received previous cycles of cytotoxic treatment indicating that treatment causes persistent, long-term changes in the oral cavity. Epithelial thickness strongly correlated with the number of previous treatments patients underwent. These results support the idea that affected tissue exhibits long-term ultrastructural changes. These changes in epithelial thickness were also accompanied by residual inflammation and extra cellular matrix signalling, with elevated staining intensity compared to healthy controls. Unfortunately, we were unable to access information regarding the timing of previous cytotoxic treatment and correlations could not be drawn.

This study is the first to identify chemotherapy-induced oral epithelial tight junction disruption in patients receiving chemotherapy. In fact, it is one of only a few clinical studies that have documented changes in tight junctions from clinical patient samples. Keefe and colleagues (2000) showed altered tight junction integrity in the duodenum of patients undergoing chemotherapy (Keefe et al., 2000). These changes, detected by transmission electron microscopy, were the first to suggest tight junction disruption may contribute to ulceration, loss of tissue integrity and diarrhoea development in patients undergoing chemotherapy. Consequently, chemotherapy-induced tight junction disruption may indeed be a critical aspect of oral ulceration – a major clinical aspect of mucositis. More importantly however, tight junctions provide an important paracellular barrier to potential pathogens and thus disruption may promote bacterial translocation and increase the risk of
local, or systemic, infection in already immunocompromised patients. This is a well-documented risk associated with tight junction disruption in the gastrointestinal tract, with chemotherapy-treated rats showing increased bacterial translocation to the mesenteric lymph nodes and spleen (Nakao et al., 2012) coupled with severe tight junction impairment. Implications for oral epithelial tight junction disruption may therefore not only promote mucosal breaches, but have detrimental effects on patients’ clinical health outcomes.

Tight junctions are highly plastic complexes, with the ability to change in response to a wide variety of physiological and pathological cues. Although reduced expression of key tight junction proteins is most widely documented, cytoplasmic redistribution of these proteins has also been shown to drastically affect their function. For example, Nassour et al., (2014) showed application of STb, a low molecular weight heat-resistance toxin produced by enterotoxigenic Escherichia coli, caused significant translocation of claudin-1 to the cytoplasm of T84 cells (Nassour and Dubreuil, 2014). This was accompanied by increased permeability of T84 monolayers and poor transepithelial resistance. In similar studies, redistribution of claudin-1 from the membrane to a more soluble form was associated with marked alterations in F-actin stress fibres (Ngendahayo Mukiza and Dubreuil, 2013). F-actin filament dissolution and condensation were also accompanied by redistribution and fragmentation of ZO-1 and occludin. This relationship has also been demonstrated in response to IL-1β treatment, with altered subcellular localisation of claudin-1 and ZO-1 shown in both thyroid cells (Rebuffat et al., 2013) and cultured human corneal epithelial (HCE) cells (Kimura et al., 2009). In the setting of chemotherapy-induced tight junction disruption, it has also been shown that downregulation and redistribution of ZO-1 drastically affects the function of intestinal tight junctions. For example, Hamada and colleagues showed that methotrexate-induced diarrhoea resulted in significantly increased permeability to fluorescein isothiocyanate (FITC)-dextran coupled with internalisation of ZO-1 in colonic epithelial cells (Hamada et al., 2010, Hamada et al., 2013). Although shown in a variety of cell types and in response to varying cues, these studies emphasise the significance of cytoplasmic redistribution of tight junction proteins and may offer mechanistic avenues to explore.
The current study has shown clear increases in several proinflammatory cytokines and MMP subtypes. This change comes as no surprise given the vast amount of research showing a strong inflammatory component to alimentary toxicity (Logan et al., 2008a, Logan et al., 2008b, Al-Dasooqi et al., 2010, Al-Azri et al., 2014). However, few studies have assessed cytokine and MMP expression in the oral epithelium of patients receiving chemotherapy, with most research coming from preclinical animal models. For example, our laboratory has previously shown elevations in IL-1β, TNF and IL-6 in the oral mucosa of tumour-bearing rats receiving chemotherapy (Logan et al., 2008a), paralleling the clinical changes observed in the current study. These results compliment earlier clinical findings showing increased NFκB and cyclooxygenase-2 expression in the oral cavity of patients following cytotoxic chemotherapy (Logan et al., 2007). Recent research has also shown elevated MMP-9 expression in the ventral surface of the tongue of tumour-bearing rats treated with chemotherapy (Al-Azri et al., 2014). These parallel earlier research showing a time dependent increase in both MMP-2 and MMP-9 in the jejunum following irinotecan administration (Al-Dasooqi et al., 2010). Although more substantial changes were seen preclinically, particularly for MMP-9, results again reflected the changes observed clinically. Importantly, the changes in proinflammatory cytokine and MMP profiles observed in our present study were clearly coupled with changes in tight junction integrity.

The idea that both proinflammatory cytokines and MMPs regulate tight junctions is not a new phenomenon, with strong supportive in vitro and in vivo evidence. The earliest evidence for proinflammatory-cytokine dependent tight junction disruption was seen in the setting of inflammatory bowel disorders, with clear changes in claudin-1, ZO-1 and occludin coinciding with peak relapse and remission phases (Bertiaux-Vandaele et al., 2011). Recent in vitro research has solidified the modulatory roles of proinflammatory cytokines on tight junction integrity, showing that IL-1β and TNF are able to disrupt tight junction integrity (Ma et al., 2004, Ma et al., 2005, Al-Sadi et al., 2008). Comparable effects have also been documented following exposure to MMPs (Al-Dasooqi et al., 2014), although much of the research to date has only focused on their effects on
endothelial tight junctions. Importantly however, interactions between proinflammatory cytokine signalling, MMP activity and epithelial tight junction integrity have been documented. In fact, treatment with TNF has been reported to activate both MMP-2 and MMP-9 resulting in tight junction disruption and epithelial hyper-permeability (Vermeer et al., 2009).

More recently, MMP-tight junction interactions have been demonstrated using human airway epithelial models (Vermeer et al., 2009) and human embryonic kidney cell lines (Jeong et al., 2012). In both cases, MMP-9 activation caused altered expression and localisation of occludin, claudin-1 and ZO-1, tight junction strand breaks and epithelial apoptosis, thus highlighting a clear role of MMPs in the regulation of tight junctions and barrier function. Given the wealth of supportive literature showing cytokine- and MMP-mediated tight junction disruption, the idea that these interactions underpin chemotherapy-induced oral toxicity is compelling. Given that these interactions have also been reported to contribute to chemotherapy-induced gut toxicity and associated diarrhoea, this study therefore indicates that tight junction defects occur throughout the entirety of the alimentary tract, regardless of anatomic site. This provides further evidence for a common pathway for mucositis development, which is modified as a consequence of local structural differences in the mucosae. These differences are overwhelming when comparing the oral mucosa to the gastrointestinal tract, however these structural differences may have implications for the resilience that different mucosae may exhibit in response to the effects of chemotherapeutic drugs.
4.6 Conclusion

Chemotherapy causes defects in key tight junction proteins of the oral cavity, characterised by decreased expression and cytoplasmic redistribution. This is the first study to identify changes in oral epithelial tight junctions of patients undergoing chemotherapy. This provides further evidence for a common pathway for alimentary mucositis, with regional differences the result of structural variations in the alimentary mucosae. Changes in oral epithelial tight junctions were coupled with altered cytokine and MMP profiles and the timing of these events may be a target for preventative treatment. It is therefore critical that these results be assessed in a more controlled manner to assess if tight junction disruption is in fact the cause of oral mucositis, or purely an effect. It must also be acknowledged that not all patients undergoing chemotherapy treatment developed clinical mucositis. Despite this, subclinical evidence of mucositis was apparent in the form of apoptosis (Gibson et al., 2006), inflammation, atrophy and perhaps tight junction defects. For a stronger understanding of the temporal relationship between mediators of inflammation, tight junctions and mucositis development to be establish, these investigations should now be extended into controlled animal studies as well as into larger patient cohorts with heterogeneous diagnoses and more detailed reporting of mucositis onset, severity and duration.
Chapter 5 TLR4: a common mechanism of regimen-related gut and neurotoxicity

Statement of Authorship

Title of Paper: Toll-like receptor 4 signalling: a common biological mechanism of regimen-related toxicities – an emerging hypothesis for neuropathy and gastrointestinal toxicity.

Publication Status: Published


Principal Author

Name of Principal Author (Candidate): Hannah Wardill

Contribution to the Paper: I was responsible for generation of the hypothesis and preparation of the manuscript.

Overall percentage (%): 80%

Certification: This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

Signature

Date

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate in include the publication in the thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author: Yasabella Van Sebille

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Contribution to the Paper: Kimberley provided expertise in neuropathology.

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## Chapter 5 TLR4: a common mechanism of regimen-related gut and neurotoxicity

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Chapter 5 Toll-like receptor 4 signalling: a common biological mechanism of regimen-related toxicities – an emerging hypothesis for neuropathy and gastrointestinal toxicity.


5.1 Abstract

Regimen-related toxicities remain a priority concern within the field of supportive care in cancer. Despite this, many forms of toxicity are under reported and consequently poorly characterised. Although there have been significant improvements in our understanding of regimen-related toxicities, symptom management continues to occur independently raising concerns such as drug interactions and the tendency to emphasise management of a single symptom at the expense of others. This review focuses on two important toxicities induced by chemotherapy; neuropathy/pain and gastrointestinal toxicity, introducing the Toll-like receptor (TLR) 4 pathway as a common component of their pathobiology. Given the global observation of toxicity clusters, identification of a common initiating factor provides an excellent opportunity to simultaneously target multiple side effects of anticancer treatment. Furthermore, identification of common biological underpinnings could perhaps reduce polypharmacy and have pharmacoeconomic benefits
5.2 Introduction

Regimen-related toxicities are universally underappreciated and often seen as the trade-off for remission (Muls, 2014). Studies suggest this is due to oncology follow-up clinics focusing on disease recurrence whilst rarely addressing symptom management and referral pathways (Muls, 2014). While research efforts into supportive care in cancer have seen significant improvement, regimen-related toxicities are viewed as biologically independent, but simultaneous events, perpetuating the silo mentality that typically exists within the supportive care domain. Individual, symptom-oriented therapeutic strategies also raise some important concerns, such as polypharmacy and drug side effects, and the tendency to emphasise management of a single symptom at the expense of others. Furthermore, this approach ignores global observations that regimen-related toxicities occur in symptom clusters (Aprile et al., 2009) which point to commonalities in their underlying biology, or at the least, overlapping mechanisms. In fact, in a retrospective review of 1000 cancer patients admitted for palliative care, each patient was reported to have greater than 10 symptoms (Homsi et al., 2002, Walsh and Rybicki, 2006). Based on these observations, we suggest a paradigm shift, moving towards the idea that toxicities should be approached more holistically (Cleeland et al., 2003), combining efforts of neurologists, gastroenterologists, oncologists and other leading experts to identify common mechanisms between these pathologies. This critical review will focus on two important regimen-related toxicities, neurotoxicity (pain) and gastrointestinal toxicity, introducing the Toll-like receptor (TLR) 4 pathway as a common component of their pathobiology.

Neurotoxicity is a poorly characterised, dose limiting side effect of chemotherapy treatment (Holmes, 2013) with symptoms typically falling under three broad categories, cognitive dysfunction, fatigue and neuropathy. Most commonly associated with platinum compounds (cisplatin and oxaliplatin), spindle poisons/antitubulins (vincristine and paclitaxel) and the newer targeted agents such as the proteasome inhibitors (bortezomib, ixabepilone, thalidomide) (Goel et al., 2008, Hoy, 2013), heightened pain perception (hyperalgesia) and allodynia remain under reported and ill-defined side effects of chemotherapy. Given the profound personal impact of these neurological
symptoms, chemotherapy-induced neurotoxicity is now considered a priority concern within the oncology arena, bringing together oncologists and neurologists to shed light on the mechanisms that underlie this pathology. Recent neuroimaging techniques suggest performance changes in neurological function occur in a subset of cancer patients, and that these changes may be associated with structural and functional alterations in the brain (Komaki et al., 1995). However, the molecular mechanism(s) involved in chemotherapy-induced neurotoxicity, specifically heightened pain perception, remain unclear and poorly studied. Recent speculation has led to several candidate mechanisms for neurotoxicity including oxidative stress, inflammation and DNA damage (Ahles and Saykin, 2007, Wigmore, 2013). It has also been proposed that some cytotoxic agents may damage neurons through binding to axonal microtubules to subsequently alter axonal transport (Tanner et al., 1998). This is however contradicted by a wealth of evidence showing no morphological changes in centrally-located neurons following various cytotoxic insults (Ginos et al., 1987, Gangloff et al., 2005). The lack of pathological changes observed in these neurons suggests that direct cytotoxicity is not sufficient to fully account for the range and severity of neurological symptoms experienced by patients, and more complex mechanisms are likely to be involved.

It has also been suggested that systemic proinflammatory and immune factors released following chemotherapy (Sonis, 2004c, Logan et al., 2008a) cause localised glial activation to further exacerbate neuronal responses and potentiate pain (Watkins et al., 2009). Glia have long been overlooked for their role in pain signalling, viewed only as structural supports of neurons of the CNS. It was not until the early 1990’s when the actions of glia in varying pain states were appreciated and it is now a well-documented component of neuropathic pain (Hutchinson et al., 2007b, Watkins et al., 2009). The most recent advent in the area of glia-mediated nociception is the role of the TLR family, specifically TLR4. TLRs are a family of transmembrane protein receptors able to recognise a diverse range of signals on exogenous and endogenous substances considered to be danger signals, and hence warrant activation of the innate immune system for the survival of the host (Fukata et al., 2005). TLR4 has been most extensively characterised as it recognises lipopolysaccharide (LPS) from gram-negative bacteria. TLR4 agonists activate similar downstream
Intracellular signalling pathways to those previously documented for interleukin(IL)-1, binding to its co-receptor, activating NFκB and resulting in a powerful proinflammatory cascade (Gribar et al., 2008).

In addition to severe neurotoxicity, chemotherapy is also recognised for causing severe gastrointestinal side effects. Gut toxicity is often a dose-limiting manifestation of chemotherapy treatment that affects a large proportion of patients, dependent on the dose of chemotherapy administered (Keefe, 2004b). Clinically, chemotherapy-induced gut toxicity (CIGT) is associated with severe gastrointestinal symptoms such as diarrhoea, infection and rectal bleeding (Muls, 2014). Characterised by severe ulceration, inflammation and pain, CIGT has recently been implicated with glial activation (Weng et al., 2003), elevated proinflammatory cytokines (IL-1β, IL-6, TNF) (Logan et al., 2008a) and, importantly, excessive TLR4 activation (Hamada et al., 2013). Like the CNS, the enteric nervous system is comprised of neurons and glia (Ruhl, 2005). The traditional role of glia has also been challenged in the enteric nervous system with research suggesting that enteric glia are capable of regulating gastrointestinal homeostasis, and critically, transmission of sensory information from the gut to the CNS (Toumi et al., 2003, Van Landeghem et al., 2011, Watson and Hughes, 2012). It is therefore tangible to suggest that peripheral toxicity, such as CIGT, may drive glial activation and thus exacerbate neuronal damage and pain perception.

5.3 Indirect neuromodulation through glial activation

5.3.1 The emerging role of glia in neuropathic pain

Glia is the collective term used to describe both astrocytes and microglia, the key supportive cells of the CNS. Traditionally, glia were viewed as structural supports for neurons, providing typically homeostatic roles including immune surveillance, clearance of debris, regulation of the ionic and chemical composition of the extracellular matrix and maintenance of blood brain barrier (BBB) integrity; glia are therefore considered pivotal to not only CNS homeostasis but also the survival of the host (Hutchinson et al., 2007a). It was not until the early 1990’s where these static, neurosupportive roles of glia were challenged and their roles under varying pain states
Acknowledged (Garrison et al., 1991). This paradigm shift in our understanding of glia followed early evidence showing an associative link between astrocyte activation and neuropathic pain (Garrison et al., 1991). The earliest evidence came from Garrison et al. (1991) where significantly elevated glial fibrillary acidic protein (GFAP) staining in the lumbar spinal cord was noted following sciatic nerve constriction. Garrison and colleagues furthered this work in 1994, showing activated glia in neuropathic animals (Garrison et al., 1994). Importantly, when an N-methyl-D-aspartate (NMDA) receptor inhibitor – MK-801 – was applied, both glial activation and neuropathic pain were improved. Several studies also report comparable changes in glial activity in various preclinical models of neuropathic pain (Garrison et al., 1991, Jiang et al., 2009, Mika et al., 2009) and subsequently glia are now considered a vital step in its pathobiology.

It is now well established that glia have two distinct states; a quiescent basal state and an activated state. Microglia have a classic quiescent phenotype under normal pain responses, responsible for surveying the extracellular space in search of potential danger, but producing no neuroexcitatory substances (Davalos et al., 2005). In contrast, astrocytes are active players in synaptic signalling even under basal conditions. They maintain house-keeping functions, providing energy sources and neurotransmitter precursors to neurons, cleaning debris and resorbing excess neurotransmitters. Upon activation, these glia shift from their basal state, to an activated state characterised by a reactive, proinflammatory response profile (Hutchinson et al., 2009). A variety of glial activation signals have been identified, some of which are very well characterised including neuronally released fractylkine and traditional neuronal nociceptive modulators and transmitters, such as reactive oxygen species (ROS), nitric oxide, prostaglandins, excitatory amino acids, substance P and proinflammatory cytokines (Liu et al., 2007). Upon activation, glia release substances (ROS, nitric oxide, prostaglandins, proinflammatory cytokines) that increase neuronal excitability, leading to pain enhancement. These neuroexcitatory mediators directly enhance neuronal excitability (Liu et al., 2007, Binshtok et al., 2008), increase pain associated neurotransmitter release from sensory afferents (Watkins et al., 2007), upregulate the number and conductance of calcium permeable α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and NMDA receptor (Zhang et al.,
Chapter 5 TLR4: a common mechanism of regimen-related gut and neurotoxicity

2008) and downregulate expression of glial glutamate transporters; all of which potentiate pain (Kleibeuker et al., 2008).

Although this mechanism of pain potentiation is well described in the setting of peripheral nerve damage, limited data exists regarding its role in chemotherapy-induced pain. Of the limited data, both microgliosis and astrocytosis are reported following administration of vincristine, paclitaxel, bortezomib and oxaliplatin (Di Cesare Mannelli et al., 2013, Ji et al., 2013, Di Cesare Mannelli et al., 2014, Li et al., 2014b, Robinson et al., 2014). Robinson et al. (2014) characterised patterns of glial activation in response to chemotherapy and typical spinal nerve ligation (Robinson et al., 2014). Consistent with previous peripheral nerve injury models, microglia activation was evident following spinal nerve ligation, but not chemotherapy administration. In contrast, astrocytes were activated following both oxaliplatin and bortezomib treatment in a manner that paralleled chemotherapy-evoked behavioral changes. Despite this disparity, the behavioral phenotype and activation of astrocytes were prevented by co-administration of minocycline hydrochloride – a microglial inhibitor – in both models, suggesting a common mechanism between both neuropathies. Similarly, Ji et al. (2013) reported significant astrocytic hypertrophy and activation, demonstrated by increased glial GFAP expression in the dorsal horn of vincristine-treated rats with mechanical allodynia (Ji et al., 2013). This was coupled by increased astrocytic expression of IL-1β and phosphorylation of the NMDA receptor in spinal dorsal horn neurons. Importantly, treatment with pentoxifylline, an anti-inflammatory agent, and an IL-1R antagonist attenuated phosphorylation of NMDA receptors and mechanical allodynia. Most recently, oxaliplatin treatment was also associated with microglia activation, however this activation was only transient (Di Cesare Mannelli et al., 2014). Microglia displayed a highly ramified phenotype, similar to that of vehicle-treated animals. The number of GFAP-expressing cells in the dorsal horn superficial laminae was significantly increased in oxaliplatin treated animals at 1, 2 and 3 weeks following treatment, correlating with the pain-profile. Importantly, although application of minocycline attenuated pain and glial activation, the efficacy of fluorocitrate – an astrocyte inhibitor – was significantly greater. Together, these studies highlight that glial activation, specifically astrocyte activation, is an important component of
chemoneuropathy and associated pain. Despite these promising findings, the initiating factor for glial activation following chemotherapy remains unclear. One potential candidate is the release of endogenous danger signals. Several neurological conditions such as peripheral nerve damage have been shown to elicit the release of these endogenous danger signals (Bianchi, 2007) which communicate cellular/tissue damage and/or stress independent of the release of classic neurotransmitters or neuromodulators (Watkins et al., 2009). On release of these danger signals, the innate immune pattern recognition receptor, TLR4, causes activation of TLR4-expressing cells including both microglia and astrocytes (Watkins et al., 2009). Given the extensive peripheral tissue damage observed following cytotoxic treatment, TLR4-mediated glial activation therefore presents as a novel pathway in the pathobiology of chemotherapy-induced pain.

### 5.3.2 TLR4-mediated glial activation

TLRs are a family of approximately ten single transmembrane receptors that recognise a diverse range of moieties or ‘patterns’ on exogenous and endogenous substances considered to be danger signals, and hence warrant activation of the innate immune system (Hutchinson et al., 2007b). Of the many TLR subtypes, TLR4 has been most extensively characterised with established roles in the host immune response. When activated, typically by lipopolysaccharide (LPS), TLR4 recruits adaptor molecules and kinases, initiating a downstream signalling cascade that culminates in the secretion of proinflammatory cytokines and chemokines (Takeuchi and Akira, 2002, Li et al., 2006, Trotta et al., 2014). This signalling cascade can be MyD88-dependent or -independent, with the MyD88-dependent pathway most commonly associated with translocation of NFκB and proinflammatory cytokine secretion. MyD88-dependent signalling typically requires the adaptor proteins TIRAP (TIR domain containing adaptor protein) and MyD88 to initiate the rapid production of proinflammatory cytokines, chemokines and their receptors TNF, IL-1α, IL-1β, IL-1ra, IL-6, IL-8, IL-10, IL-12, IL-23, and macrophage inflammatory protein (MIP)-1α, and MIP-1β (Lee and Kim, 2007). These factors facilitate the inflammatory response by increasing vascular permeability, directing dendritic cells and initiating macrophage migration from the periphery (O'Neill and Bowie,
Chapter 5 TLR4: a common mechanism of regimen-related gut and neurotoxicity

2007). In contrast, the independent signalling pathway is reliant on Toll-like receptor adaptor molecule (TICAM)-1, -2, the TIR-domain-containing adaptor inducing interferon-β (TRIF) or TRIF-related adaptor molecule (TRAM) resulting in the production of interferon-β and chemokines.

In addition to the well-documented roles of TLR4 signalling in the host immune response, recent evidence has also linked this immune receptor to a number of neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease (Trotta et al., 2014). TLR4 expression in the CNS was, until recently, limited to microglia, astrocytes and oligodendrocytes. Recent evidence has now shown that TLR4 is expressed on CNS structures exposed to the blood stream such as the choroid plexus, circumventricular organs and leptomeninges. This newly emerging distribution of TLR4 expression may therefore explain the innate immune response observed in the brain, which originates from areas devoid of a blood-brain barrier (Laflamme and Rivest, 2001). Furthermore, recent evidence has shown altered neuronal TLR4 expression in response to ischemia/reperfusion (Tang et al., 2007a). This is further supported by knockout studies, where the extent of energy deprivation-induced cell death and associated neurological deficit were significantly reduced in TLR4 deficient mice compared to wild-type (Tang et al., 2007b).

5.3.3 TLR4 in the central nervous system

There is accumulating evidence suggesting TLR4 contributes to neuronal death, BBB damage and inflammatory responses in the brain (Caso et al., 2007, Hua et al., 2007). Consequently, TLR4 has been implicated with several CNS pathologies, particularly those characterised by neuroinflammation and subsequent degeneration. It has been postulated that TLR4-mediated NFκB signalling plays a critical role in the development of neuroinflammation, leading to the secretion of proinflammatory cytokines, chemokines and enzymes such as cyclooxygenase (COX)-2 and matrix metalloproteinases (MMPs) (Wang et al., 2000, Lucas et al., 2006). Furthermore, it is suggested that these neuroinflammatory mediators are able to activate microglia leading to neuronal excitation or neuronal loss (Allan and Rothwell, 2001, Morganti-Kossmann et al., 2002). In fact, this phenomenon was recently demonstrated in the setting of Alzheimer’s disease; a neurodegenerative
disease characterised by microgliosis. Importantly, activated microglia have been identified surrounding senile plaque in the brains of Alzheimer’s disease patients and have been shown to express increased levels of TLR4 (Amasheh et al., 2009). Additionally, treatment of microglia with senile plaque material was shown to induce sharp peaks in the mRNA expression of many TLR subtypes, including TLR4, when compared with age-matched plaque-free tissue (Hanke and Kielian, 2011). It is therefore suspected that TLR4-mediated glial activation results in the production of nitric oxide, oxygen derived free radicals, proteases, adhesion molecules and proinflammatory cytokines which, when produced in excess, have detrimental effects on neuronal homeostasis and contribute to the development of neurodegenerative conditions such as Alzheimer’s disease (Carty and Bowie, 2011).

In addition to Alzheimer’s disease, TLR4 signalling has gained momentum regarding the pathobiology of Parkinson’s disease; a chronic, neurodegenerative condition characterised by loss of dopaminergic neurons in the substantia nigra pars compacta and the striatum of the basal ganglia (Dutta et al., 2008). Although the mechanisms responsible for Parkinson’s disease remain unclear, emerging evidence suggests a neuroinflammatory component to the condition (Tufekci et al., 2011). The presence of cytoplasmic alpha-synuclein (AS), or Lewy bodies, is the hallmark trait of Parkinson’s disease and the subject of significant molecular research. Stefanova and colleagues (2007) were the first to show elevated levels of TLR4 in AS cytoplasmic inclusions (Stefanova et al., 2007). These findings have since been extended with research now showing that TLR4 is essential for the AS-dependent activation of microglia, leading to the production of proinflammatory cytokines and ROS (Fellner et al., 2013). Importantly, this mechanism is unique to microglia, with astrocytic uptake of AS shown to be TLR4-independent. In contrast, the role for TLR4 in Parkinson’s disease is confounded by evidence showing that genetic TLR4 deletion results in reduced phagocytic activity of microglia, leading to heightened AS accumulation and exacerbated neurodegeneration (Zhang et al., 2005, Cookson, 2009). These results suggest that despite initiation of an inflammatory response, TLR4-mediated glial activation may be important in the clearance of AS, thus exerting a protective effect in Parkinson’s disease.
5.4 TLR4 and neuropathic pain

TLR4 has received significant attention for its roles in several neuroinflammatory disorders characterised by neurodegeneration (Grace et al., 2014). In addition to these emerging roles, TLR4 has also gained momentum for its role(s) in modulating neuropathic pain (Hutchinson et al., 2010, Lewis et al., 2012). Within the CNS, TLR4 is predominantly expressed by microglia, but expression may be upregulated on astrocytes under neuroinflammatory settings (Hutchinson et al., 2008). TLR4 appears to be directly relevant to the pathobiology of neuropathic pain, as it recognises and responds to endogenous danger signals, and thus has the ability to modulate pain signalling. TLR4 knockout and knockdown studies have demonstrated this emerging role for TLR4, with knockout/knockdowns suppressing the development and/or maintenance of nerve injury-induced allodynia (Tanga et al., 2005, Kim et al., 2007, Obata et al., 2008, Laird et al., 2009). Additionally, administration of a selective TLR4 antagonist has been shown to suppress well-established neuropathic pain induced by chronic constriction injury (Hutchinson et al., 2007b). In the setting of chemoneuropathy, recent research has shown that paclitaxel treatment is associated with elevated TLR4 expression and glial activation in the dorsal root ganglion. Additionally, application of both TLR4 and MyD88 antagonists significantly reduced peripheral neuropathy and associated pain (Li et al., 2014b). Taken together, these studies suggest that ongoing TLR4 activation and peripheral endogenous danger signalling is at the core of neuropathic pain, and may therefore contribute to the development of chemotherapy-induced pain and its associated clinical features.

5.4.1 Peripheral tissue damage activates central TLR4

Although the development of neuropathic pain through TLR4 activation is most extensively characterised in the setting of peripheral nerve injury, the production of these endogenous danger signals and other TLR4 ligands is not unique to this form of tissue damage. In fact, it is well established that chemotherapy treatment causes significant gut toxicity, which is characterised by excessive production of endogenous danger signals (pathological- and danger-associated molecular patterns; PAMPs/DAMPs) (Bianchi, 2007). In addition to this, recent research has shown
entocyte-expressed TLR4 is intimately involved in the initiation of gut toxicity following chemotherapy treatment, activating NFκB and mounting an immune response (Hamada et al., 2013). Given that TLR4 is activated by endogenous danger signals, centrally-located TLR4 and glia are well positioned to enhance pain resulting from inflammation in the periphery such as gut toxicity following chemotherapy. We therefore hypothesise that the molecular signals derived from gastrointestinal toxicity drive glial activation and subsequent neuropathy in a TLR4-dependent manner (Figure 5.1). This pathway appears to be initiated by damage that originates in the periphery, and thus the pathobiology of chemotherapy-induced pain may point to the existence of a gut-CNS axis.

The existence of a gut-CNS axis is not a new phenomenon (Tillisch, 2014). Based on paralleled comorbidities of gastrointestinal and neurological origin, there has been an appreciation gained for the existence of a gut-CNS axis and the roles it may play in governing neurological function (Rhee et al., 2009, Foster and McVey Neufeld, 2013). While candidate mechanisms of the gut-CNS axis include neural, endocrinal and immune pathways, the gut microbiota has emerged as a predominant player, although the mechanisms underpinning the gut-CNS axis remains unclear (Collins et al., 2012). Although a wealth of data exists supporting a role for the gut microbiota in modulating neurological function, there is evidence to suggest that immune cells produced within the gut may also exhibit neuromodulatory effects (Grenham et al., 2011). Disruption of the homeostatic state between the microbiota and the innate mucosal immune system of the host has been shown to result in activation of TLRs and consequent alteration of cytokine profiles, leading to impaired neurological function. It is suggested that these immune cells disrupt the BBB and upon crossing, are subsequently reactivated within the CNS. This phenomenon was recently demonstrated in mice receiving peripheral surgery, displaying BBB disruption and elevated TNF signalling which facilitated macrophage migration into the hippocampus and subsequent neurological decline (Degos et al., 2013). Additionally, administration of proinflammatory cytokines in rodents has been reported to induce depressive like symptoms, disrupted circadian rhythm and reduced appetite (Benzing et al., 1999, Ryan et al., 2007). Although a gut-CNS axis has not been applied to the setting of
Chapter 5 TLR4: a common mechanism of regimen-related gut and neurotoxicity

Chemotherapy-induced pain, these results support the hypothesis that CIGT is able to modulate CNS homeostasis, and may contribute to the development of chemoneuropathy. Furthermore, TLR4 may be uniquely positioned to modulate inflammatory responses in both the gut and CNS thus contributing to both toxicities. Given the potential role of TLR4 as a common initiating factor in both gut and CNS toxicity, it presents as an attractive therapeutic target.

5.5 Blood brain barrier disruption permits central pathology

It is well described that a proinflammatory state is associated with poor tight junction integrity and the induction of a ‘leaky’ barrier. This has been shown extensively in the gastrointestinal tract in response to chemotherapy (Schmitz et al., 1999, Chiba et al., 2006, Kimura et al., 2009), however newly emerging data suggest that tight junctions of the blood brain barrier may also be subject to such modification (Coelho-Santos et al., 2015, Gao et al., 2015). The finding of detectable levels of systemically administered chemotherapeutic agents within the central nervous system (CNS) (Bourke et al., 1973) supports this presumption and implies a level of BBB permeability that has not been previously appreciated. Increased levels of BBB permeability suggest that some chemotherapeutic agents are capable of disrupting its integrity, either directly or indirectly. Consequently, it has been suggested that systemic inflammation, derived from the gut, drives central blood brain barrier disruption leading to enhanced communication between the peripheral environment and CNS and, blood brain barrier disruption is therefore an important component to consider in our hypothesis. In support of this, localised breaches in blood brain barrier integrity and subsequent neuroinflammation have been identified in preclinical models of colitis (Natah et al., 2005). Increased microglial and astrocytic reactivity have also been shown to occur in similar models (Riazi et al., 2008, Wang et al., 2010, Nyuyki and Pittman, 2015). These findings clearly support a role for gut-derived inflammation in neuropathology, and it is likely that cytokine-mediated blood brain barrier breakdown facilities communication between the gut and CNS (Figure 5.1). For further discussion of cytokine-mediated blood brain barrier breakdown, please review by Wardill et al., (2016) included in Chapter 12.
Figure 5.1 Peripheral inflammation modulates central pain signalling through TLR4. Here we proposed that inflammatory mediators, released from the gastrointestinal tract upon chemotherapy-induced damage, are able to cross the blood brain barrier and enter the CNS. These mediators are potent ligands for TLR4, located on glial cells through the CNS, causing extensive glial activation. Upon activation, glia release substances that increase neuronal excitability, leading to pain enhancement. These neuroexcitatory mediators directly enhance neuronal excitability, increase pain associated neurotransmitter release from sensory afferents, upregulate the number and conductance of calcium permeable AMPA and NMDA receptor and downregulate expression of glial glutamate transporters; all of which potentiate pain. Adapted from Wardill et al., 2015 (Cancer Treatment Reviews).
5.6 Clinical translation

There is strong evidence suggesting that peripheral toxicity drives glial activation through TLR4 signalling; this review has highlighted evidence using the examples of CIGT and pain. Where the complexity lies is the sequence of these toxicities. Identifying whether these toxicities occur in unison or sequentially will shed light on the role of TLR4 as a common underlying mechanism. It is likely that TLR4-mediated pain is agent specific, and may be a case where one size, on a theoretical basis, does not fit all. For example, the clinical observation of neuropathy is rare amongst patients being treated with agents typically associated with high rates of gut toxicity (irinotecan, 5-fluorouracil, methotrexate), indicating potential independent mechanisms (Figure 5.2). However, the hypothesis of dual-toxicities of common biology is compelling with regards to the clinical use of proteasome inhibitors (bortezomib, thalidomide, ixabepilone, ixazomib) (Deeken et al., 2014, Huang et al., 2014a, Kumar et al., 2014) and taxanes (paclitaxel, docetaxel) (Luo et al., 2013, Ahn et al., 2014, Amiri-Kordestani et al., 2014), which commonly induce toxicities of both gastrointestinal and neurological origin, raising the question of equivalent risk.
Figure 5.2 Independent and common mechanisms of chemotherapy-induced gut toxicity and neurotoxicity. Wardill et al., 2015 (Cancer Treatment Reviews).
5.7 Conclusions and future directions

Regimen-related toxicities remain a priority concern within the field of supportive care in cancer. Despite this, many forms of toxicity are under reported and consequently poorly characterised. This holds particularly true for chemotherapy-induced gastrointestinal toxicity and neurotoxicity, specifically the symptom of pain. This review has highlighted TLR4 signalling as a common underlying pathway of both toxicities. Given the global observation of toxicity clusters, identification of a common initiating factor would provide an excellent opportunity to simultaneously target multiple side effects of anticancer treatments. Despite strong epidemiological evidence highlighting toxicity clusters, it remains unclear why some patients are more susceptible to severe toxicity. Evidence has shown TLR4 gene mutations (Thr399Ile) contribute to the severity of acute graft versus host disease, influencing the risk in patients undergoing allogenic transplantation (Elmaagcli et al., 2006). The TLR4 Asp299Gly polymorphism has also been identified as a risk factor for Crohn’s disease potentially contributing to disease phenotype (Brand et al., 2005). This emerging hypothesis for TLR4-mediated toxicities could therefore have the potential to drive biomarker development and risk evaluation techniques, presenting an attractive avenue for future research. Furthermore, identification of common biological underpinnings could perhaps reduce polypharmacy, lessen drug side effects, and have pharmacoeconomic benefits.

The ubiquitous involvement of the innate immune system in regimen-related toxicity makes TLR4 an overlooked candidate in the pathophysiology of other dose-limiting side effects of chemotherapy. For example, recent speculation suggests that proinflammatory cytokines are able to disrupt the hypothalamic-pituitary-adrenal (HPA) axis, to alter circadian rhythm and thus induce fatigue – an established side effect of chemotherapy (Morrow et al., 2002). In fact, decreased circulating levels of serum cortisol have been reported immediately following treatment with the platinum compounds cisplatin and carboplatin (Morrow et al., 2002), indicating impaired HPA axis function (Fredrikson et al., 1992, Hursti et al., 1993). Given the role of TLR4 in neuroinflammation, it is conceivable that activation of enterocyte-expressed TLR4 initiates the induction of a ‘cytokine storm’ which is able
to modulate the CNS and thus impact on the function of the HPA axis. Activation of TLR4 may therefore be the missing link in the initiation of this cascade where circulating proinflammatory cytokines can access the CNS and exert profound effects on behaviour and cognitive function. The induction of this ‘cytokine storm’ appears to be predominantly initiated by the activation of TLR4 in the gut – the largest immunological organ – and must therefore be adequately acknowledged if we are to adopt a holistic approach to toxicity.
# Statement of Authorship

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<th>Irinotecan-induced gastrointestinal dysfunction and pain are mediated by common TLR4-dependent mechanisms.</th>
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<td>Publication Status</td>
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## Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

1. the candidate’s stated contribution to the publication is accurate (as detailed above);
2. permission is granted for the candidate to include the publication in the thesis; and
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Chapter 6 Irinotecan-induced gastrointestinal dysfunction and pain are mediated by common TLR4-dependent mechanisms


6.1 Abstract

Strong epidemiological data indicate chemotherapy-induced gut toxicity and pain occur in parallel, indicating common underlying mechanisms. We have recently outlined evidence suggesting that Toll-like receptor 4 (TLR4) signalling may contribute to both side effects. We therefore aimed to determine if genetic deletion of TLR4 improves chemotherapy-induced gut toxicity and pain. Forty-two female wild-type (WT) and 42 Tlr4 null (−/−) BALB/c mice weighing between 18-25 g (10-13 weeks) received a single 270 mg/kg (i.p.) dose of irinotecan hydrochloride or vehicle control and were killed at 6, 24, 48, 72 and 96 h. Bacterial sequencing was conducted on caecal samples of control animals to determine gut microbiome profile. Gut toxicity was assessed using validated clinical and histopathological markers, permeability assays and inflammatory markers. Chemotherapy-induced pain was assessed using the validated rodent facial grimace criteria, as well as immunological markers of glial activation in the lumbar spinal cord. TLR4 deletion attenuated irinotecan-induced gut toxicity, with improvements in weight loss (p=0.0003) and diarrhoea (p<0.0001). Crypt apoptosis was significantly decreased in BALB/c-Tlr4−/−billy mice (p<0.0001) correlating with lower mucosal injury scores (p<0.005). Intestinal permeability to FITC-dextran (4kDa) and LPS translocation were greater in WT mice compared to BALB/c-Tlr4−/−billy (p=0.01 and p<0.0001, respectively). GFAP staining in the lumbar spinal cord, indicative of astrocytic activation, was increased at 6 and 72 h in WT mice compared to BALB/c-Tlr4−/−billy mice (p=0.008, p=0.01). These data indicate that TLR4 is uniquely positioned to mediate irinotecan-induced gut toxicity and pain, highlighting the possibility of a targetable gut/CNS axis for improved toxicity outcomes.
6.2 Introduction

Irinotecan-induced gut toxicity remains a priority concern within the field of supportive care in cancer. Typically used to treat a variety of solid tumours, irinotecan can cause severe diarrhoea, rectal bleeding and infection in patients, often resulting in dose reductions and treatment delays (Lalla et al., 2014). Irinotecan-induced diarrhoea is clinically significant as fluid/electrolyte imbalances can lead to renal insufficiency, malnutrition, and extreme dehydration. More importantly, these side effects have severe psychological impacts for patients and significantly the provision of optimal cancer care (Carlotto et al., 2013). Despite both its prevalence and clinical significance, the precise mechanisms that underpin gut toxicity remain unclear and therapeutic options for patients are limited (Lalla et al., 2014).

The broadly accepted pathophysiology of chemotherapy-induced gut toxicity (CIGT) comprises five continuous and overlapping phases described by Sonis (Sonis, 2004c). Although this model can be applied to most chemotherapeutic agents, each treatment modality has unique pathological features due to differences in the metabolism and pharmacokinetics of each anticancer drug. In the case of irinotecan, its unique enterohepatic recirculation is thought to be responsible for the high levels of intestinal toxicity. Irinotecan serves as the water-soluble precursor of the lipophilic metabolite SN-38, which is formed by carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and the dipiperdino side chain (Chabot, 1997). SN-38 is glucuronidated to the non-toxic SN-38 glucuronide (SN-38G) in the liver via the uridine-diphospho-glucuronosyl transferase (UGT1A) enzyme family, which then releases SN-38G into the intestine for elimination (Chabot, 1997). However, in the intestinal lumen, bacterial β-glucuronidases regenerate SN-38 from SN-38G (Stringer et al., 2008). This unique metabolic pathway not only results in high levels of intestinal toxicity, but also highlights the key relationship between toxicity and the gut microbiome (Stringer et al., 2008).

The gut microbiome has received significant attention for its role in the development of gut toxicity following chemotherapy, with documented changes in the balance of commensal and pathogen
bacteria following numerous chemotherapeutic agents (Stringer et al., 2009a, Stringer et al., 2009b, Stringer et al., 2009c). In light of these findings, the interaction between the gut microbiome and innate mucosal immune system has also gained interest, with particular emphasis on the impact of Toll-like receptor (TLR) signalling (Al-Dasooqi et al., 2013, Wardill et al., 2014c, Wardill et al., 2015b). TLRs are a family of transmembrane protein receptors recognising a diverse range of signals on exogenous and endogenous substances considered to be ‘dangerous’, and hence warranting activation of the innate immune system for host survival (Fukata et al., 2005, Jacobsen et al., 2014, De Nardo, 2015). TLR4 has been most extensively characterised as it recognisees, and responds to, lipopolysaccharide (LPS) from gram-negative bacteria. We have shown that TLR4 is overexpressed in the gut during peak injury (Bowen et al., 2012) and is undetectable at later time points associated with healing (Gibson et al., 2015). It has been hypothesised that TLR4 not only induces an exacerbated innate immune response resulting in a heightened toxicity profile but also regulates intestinal barrier integrity. This mechanism is particularly relevant in the setting of irinotecan-induced gut toxicity, as our preliminary in silico docking data indicate that SN-38 has the potential to act as a ligand for the TLR4/MD-2 complex (Figure 6.1).

TLR4 has also been hypothesised to mediate chemotherapy-induced pain through central glial activation (Wardill et al., 2015b), with strong clinical evidence showing chemotherapy-induced gut toxicity is often paralleled by the symptom of pain (Aprile et al., 2008, Gibson et al., 2015). This is suggestive of common underlying mechanisms. The ubiquitous involvement of the innate immune system in both chemotherapy-induced pain and gut toxicity therefore makes TLR4 a potentially overlooked candidate in the pathophysiology of these toxicities. We therefore hypothesise that TLR4-mediated signalling plays a central role in the development of irinotecan-induced gut toxicity and pain.
Figure 6.1 In silico docking of SN-38 with MD-2, the TLR4 accessory protein. These data provide evidence for the possible “off-target” action of SN-38 and supports a role for TLR4-mediated mechanisms in the development of irinotecan-induced toxicity. Image obtained from 2015 grant application by Bowen et al. (2015) National Health and Medical Research Committee. Image courtesy of Professor Mark Hutchinson.
Chapter 6 Irinotecan-induced gut toxicity and pain

6.3 Materials and Methods

6.3.1 Animal model and ethics

The study was approved by the Animal Ethics Committee of the University of Adelaide and complied with National Health and Research Council (Australia) Code of Practice for Animal Care in Research and Training (2014). Mice were group housed in ventilated cages with three to five animals per cage. They were housed in approved conditions on a 12 h light/dark cycle. Food and water were provided *ad libitum*.

6.3.2 Experimental design

All mice were on a BALB/c background. Forty-two female BALB/c-wild-type (WT) and BALB/c-*Tlr4*<sup>−/−</sup> mice (n<sub>total</sub>=84) weighing between 18-25 g (10-13 weeks) were used. WT BALB/c mice were obtained from the University of Adelaide Laboratory Animal Service (SA, Australia), and BALB/c-*Tlr4*<sup>−/−</sup> mice, back-crossed onto BALB/c for more than 10 generations, were kindly sourced from Professor Paul Foster from the University of Newcastle (NSW, Australia) and were originally sourced from Osaka, Japan (Phipps et al., 2009). All BALB/c-*Tlr4*<sup>−/−</sup> mice were homozygous null mutants and hence expressed no detectable TLR4 mRNA or protein (personal communication MRH). Mice were treated with a single 270 mg/kg intraperitoneal (i.p.) dose of irinotecan hydrochloride (kindly provided by Pharmacia/Pfizer, Mich, USA) prepared in a sorbitol/lactic acid buffer (45mg/ml sorbitol/0.9mg/ml lactic acid; pH 3.4; Sigma-Aldrich, NSW, Australia; D-sorbitol #S1876, lactic acid #252476), which was shown in pilot work to cause reproducible diarrhoea with no mortality. Control mice received the sorbitol/lactic acid buffer only.

All mice received 0.03 mg/kg of atropine subcutaneously (s.c.) immediately prior to treatment to reduce the cholinergic response to irinotecan. Mice were randomly assigned to treatment groups and killed at 6 h, 24 h, 48 h, 72 h and 96 h. Mice were anaesthetised using 200 mg/kg i.p. lilium sodium pentobarbital (60 mg/ml) and blood was collected from the facial vein. They were then killed via transcardial perfusion with cold, sterile 1 X PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4).
6.3.3 Clinical assessment of gut toxicity

All mice were monitored four times daily for the presence of diarrhoea and other clinical parameters. Diarrhoea was quantified (by two independent assessors) using a validated grading system where 0 = no diarrhoea, 1 = mild perianal staining, 2 = moderate staining covering hind legs, and 3 = severe staining covering hind legs and abdomen with continual anal leakage (Gibson et al., 2007). Mice were weighed daily to track weight loss/gain. Mice were killed if they displayed ≥15% weight loss or significant distress and clinical deterioration, in compliance with animal ethical requirements.

6.3.4 Facial grimace criteria

Chemotherapy-induced pain was measured by two independent assessors, in a blinded manner, 4 times daily in all mice using the validated rodent facial grimace criteria (Sotocinal et al., 2011), as previously published by my group (Gibson et al., 2015). Briefly, the scoring method consists of five distinct criteria: orbital tightening, cheek bulge, nose bulge, ear position and whisker position. Each criterion was scored as 0 = absent, 1 = moderate and 2 = severe. Please refer to Table 6.1 for detailed breakdown of scoring criteria.

| Table 6.1 Facial grimace criteria scoring aid as outlined by Sotocinal et al., 2011. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| **Orbital tightening**          | **0 (absent)**                  | **1 (moderate)**                | **2 (severe)**                  |
| Rounded eyes                    | Narrowing of orbital area       | Tightly closed eyelid or an eye squeeze (denoted by wrinkle around eye) |
| **Nose bulge**                  | Skin on bridge of nose should run flat with whisker region | Slight rounding of the skin on the nose | Clear rounded extension of skin visible on bridge of nose with characteristic wrinkles |
| **Cheek bulge**                 | Cheek muscles should not be obviously identified | Convex appearance of the cheek muscle from baseline potion | Clear demarcation of cheek muscle between eye and whiskers |
| **Eye position**                | Naturally forward facing, receptive ear position | Pulled apart and back from their baseline position | Ears drawn back featuring vertical ridges |
| **Whisker position**            | Baseline position               | Backward pointing whiskers      | Whiskers become flat against the face, may face forward or clump together |
6.3.5 Tissue preparation

**Gastrointestinal Tract** Following anesthesia with sodium pentobarbital, mice with perfused with chilled, sterile 1 X PBS (pH 7.4). The entire gastrointestinal tract from pyloric sphincter to rectum was dissected prior to perfusion with 4% PFA and flushed with chilled 1 X PBS (pH 7.4) to remove intestinal contents. Both the small and large intestines were weighed immediately after resection. Samples (1 cm in length) of jejunum, ileum and colon were collected and (1) drop-fixed using 10% neutral buffered saline for processing and embedding into paraffin wax, or (2) stored in RNAlater® (Sigma Aldrich, NSW, Australia; #R0901) at -20°C for molecular analyses.

**Central Nervous System** Mice were perfused with 4% PFA and the vertebral column dissected. Vertebral bodies were removed to expose the entire spinal cord. The entire spinal cord from cervical to lumbar regions was removed and the lumbar region prepared for further analysis (L3/L4). The mice were decapitated and brains extracted. All tissue stored in 4% PFA overnight for processing and embedding in paraffin wax.

6.3.6 Bacterial diversity profiling

It is well established that the gut microbiome is involved in the metabolism of irinotecan (Mathijssen et al., 2001). To confirm both WT and BALB/c-Tlr4-/-billy contain similar bacterial profiles, the caecal contents of 12 control animals (WT n=6, BALB/c-Tlr4-/-billy n=6) were aseptically collected and sent for genetic sequencing at the Australian Genomics Research Facility (Brisbane, Australia).

The sequencing details are as follows: Target: 341F-806R, Forward primer (341F): 5’-CCTAYGGRBGCASCAG-3’; Reverse primer (806R): 5’-GGACTACNNGGGTATCTAAT-3’; Read Length: 300bp.

6.3.6.1 Bioinformatics method

Paired-ends reads were assembled by aligning the forward and reverse reads using PEAR (Zhang et al., 2014) (version 0.9.5). Primers were trimmed using Seqtk (version 1.0). Trimmed sequences
were processed using Quantitative Insights into Microbial Ecology (QIIME 1.8) (Caporaso et al., 2010) USEARCH (Edgar, 2010, Edgar et al., 2011) (version 8.0.1623) and UPARSE software.

Using USEARCH tools, sequences were quality filtered, full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data were discarded. Sequences were clustered followed by chimera filtering using the “rdp_gold” database as a reference. To obtain number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%.

Using QIIME, taxonomy was assigned using Greengenes database (version 13_8, Aug 2013).

PEAR assembly read statistics were as follows: WT BALB/c control 59892 / 67175 (89.16%); BALB/c-Tlr4<sup>-/-</sup>billy control 57982 / 65950 (87.92%).

Data was assessed using unpaired, two-tailed t-tests with Welch’s corrections to determine statistical significance between the proportion of bacterial phylogenies in WT and BALB/c-Tlr4<sup>-/-</sup>billy mice. The Shannon’s diversity index was also determined.

### 6.3.7 Histopathological and immunohistochemical analysis

Haematoxylin and eosin (H&E) staining was performed on 5 μm sections of jejunum, ileum and colon cut on a rotary microtome and mounted onto glass Superfrost® microscope slides (Menzel-Gläser, Braunschweig, Germany). Slides were scanned using the NanoZoomer™ (Hamamatsu Photonics, Japan) and assessed with NanoZoomer™ Digital Pathology software view.2 (Histalim, Montpellier, France). The occurrence of eight histological criteria in the jejunum and ileum were examined to generate a total tissue injury score (Howarth et al., 1996). These criteria were villous fusion, villous atrophy, disruption of brush border and surface enterocytes, crypt loss/architectural disruption, disruption of crypt cells, infiltration of polymorphonuclear cells and lymphocytes, dilation of lymphatics and capillaries, and oedema. In the colon, the latter six criteria were examined. Each parameter was scored as present = 1 or absent = 0 in a blinded fashion by two independent assessors.
6.3.7.1 Immunohistochemical assessment of cellular markers of apoptosis and proliferation

Immunohistochemistry (IHC) was carried out on 5 µm sections of jejunum, ileum and colon, cut on a rotary microtome and mounted onto FLEX IHC microscope slides (Flex Plus Detection System, Dako, Denmark; #K8020). Immunohistochemical analysis was performed for caspase 3 (Abcam, Vic, Australia; #ab4051), a marker of apoptosis, and Ki67 (Abcam, Vic, Australia; #ab16667), a marker of proliferation. Changes in both parameters are validated markers for altered tissue kinetics and an excellent way to assess the subclinical severity of gut toxicity (Keefe et al., 2000).

Immunohistochemical analysis was performed using Dako reagents on an automated machine (AutostainerPlus™, Dako, Denmark; #AS480) following standard protocols supplied by the manufacturer. Briefly, sections were deparaffinised in histolene and rehydrated through graded ethanols before undergoing heat mediated antigen retrieval using an EDTA/Tris buffer (0.37 g/L EDTA, 1.21 g/L Tris; pH 9.0). Retrieval buffer was preheated to 65°C using the Dako PT LINK™ (pre-treatment module; Dako, Denmark; #PT101). Slides were immersed in the buffer and the temperature raised to 97°C for 20 min. After returning to 65°C, slides were removed and placed in the Dako AutostainerPlus™ (Dako, Denmark; #AS480) and stained following manufacturer’s guidelines. Negative controls had the primary antibody omitted. Slides were scanned using the NanoZoomer™ (Hamamatsu Photonics, Japan) and assessed with NanoZoomer™ Digital Pathology software view.2 (Histalim, Montpellier, France). Apoptosis was quantified by counting the number of positively stained cells for 15 crypts. Data were presented as average positively stained cells per crypt. Ki67 data was represented as the percentage of positively staining cells relative to total cells in the intestinal crypts. Only well oriented, non-oblique crypts were included for analysis. All staining was analysed by two independent assessors.

6.3.7.2 Immunohistochemical assessment of microglia and astrocyte reactivity expression markers

Immunostaining was conducted on 5 µm sections of lumbar spinal cord (L3/4), cut on a rotary microtome and mounted onto Superfrost® microscope slides (Menzel-Gläser, Braunschweig, Germany). Immunohistochemical analysis was performed for astrocytic Glial Fibrillary Acidic
Protein (GFAP), Clone 6F2, (DakoCytomation, Dako, Denmark; #M0761) and microglial Iba-1 (Wako, Virginia, USA; #019-19741). Briefly, sections were dewaxed on a hot air blower and in xylene, then dehydrated in 100% ethanol before being quenched for endogenous peroxidase activity with 0.5% hydrogen peroxide in methanol for 30 min. Slides were then washed in 0.1 M PBS (pH 7.4, 2 x 3 min) before being subjected to heat-mediated antigen retrieval using 0.1 M citrate buffer (pH 6.0). Non-specific binding was blocked by 3% normal horse serum (NHS; Sigma-Aldrich, NSW, Australia) for 30 min at room temperature. Primary antibodies were applied, using 3% NHS as the diluent, overnight at room temperature in a humid chamber (GFAP 2 µg/ml; Iba-1 0.1 µg/ml). Following incubation with the primary antibody, a secondary goat biotinylated anti-mouse/rabbit IgG (6 µg/ml) was applied to sections for 30 min at room temperature (Vector Laboratories, California, USA; anti-mouse #BA-9200; anti-rabbit #BA-1000). After a further PBS wash (2 x 3 min), slides were incubated with Pierce streptavidin peroxidase conjugate at 2 µg/ml (ThermoFisher Scientific, Vic, Australia; #21130) for 30 min at room temperature followed by another rinse with 0.1 M PBS. The immunocomplex was then visualised with precipitation of DAB (Sigma-Aldrich, NSW Australia; #D-5637) in the presence of hydrogen peroxide (3%). Slides were washed to remove excess DAB and lightly counterstained with haematoxylin, dehydrated and mounted with DePeX from histolene. Slides were scanned using the NanoZoomer™ (Hamamatsu Photonics, Japan) and assessed with NanoZoomer™ Digital Pathology software (Histalim, Montpellier, France). Staining was assessed in the dorsal column of the lumbar spinal cord using ImageJ 1.49 software and the previously validated colour deconvolution method (Figure 6.2) (Helps et al., 2012).
**Figure 6.2 Graphical representation of colour deconvolution analysis of DAB staining.** Colour deconvolution allows for the area and intensity of DAB staining to be assessed in a selected area. The area of interest is selected and the analysis applied. DAB staining is identified (red) and adjustedDAB% determined. This method of detection identifies proliferative and hypertrophic changes in glia, both of which are indicative of an activated state.
6.3.7.3 Immunohistochemical assessment of blood brain barrier permeability

Immunohistochemical analysis was also performed on 5 µm sections of brain, cut (mid-sagitally) on a rotary microtome and mounted onto Superfrost® microscope slides (Menzel-Gläser, Braunschweig, Germany). Immunostaining was performed using a rabbit polyclonal anti-human albumin antibody (Dako, Denmark; #A0001) as per the method described in 5.2.7.2. No antigen retrieval was required. Staining was assessed using a semi-quantitative grading system where 0 = no staining, 1 = mild staining with leakage localised to one region, 2 = moderate staining with two unrelated sites of leakage and 3 = intestine staining with ≥3 unrelated sites or global leakage. Staining was assessed in a blinded fashion by two independent assessors.

6.3.8 Assessment of in vivo intestinal permeability

6.3.8.1 4 kDa FITC-dextran assay

Three hours prior to kill time points, mice received a 500 mg/kg dose (75 mg/ml) of 4 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma-Aldrich, NSW, Australia; #46944) via oral gavage. Blood was collected from the facial vein into Multivette® 600 Serum-Gel with Clotting Activator capillary tubes (Sarstedt, Numbrecht, Germany; #15.1670.100) and stored on ice for 30 min. Samples were centrifuged at 11,000 x g for 5 min at room temperature and the serum isolated. Serum samples were diluted 1:3 with 1 X PBS and quantified using the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA) and Gen5 version 2.00.18 software relative to a standard curve (range 0.0001-10 µg/ml).

6.3.8.2 Serum Limulus Amebocyte Lysate (LAL) endotoxin assay

The Limulus Amebocyte Lysate (LAL) endotoxin assay was run on serum samples isolated from blood collected from the facial vein into Multivette® 600 Serum-Gel with Clotting Activator capillary tubes (Sarstedt, Numbrecht, Germany; #15.1670.100). The LAL QCL-1000™ endotoxin detection kit (Lonza, Basel, Switzerland; #50-647U, 50-648U) was then used to quantify serum
endotoxin, as per manufacturer’s guidelines. Endotoxin concentration was determined relative to a linear standard curve (range 0.1-1 EU/ml).

6.3.9 **Tissue cytokine protein quantification using the Luminex multiplex platform**

Irinotecan causes global gastrointestinal damage, however damage is typically most severe in the ileum and colon (Ijiri and Potten, 1983). Proinflammatory cytokine expression was therefore assessed using 30 mg of ileal and colonic tissue samples. Intestinal tissue samples were homogenised at room temperature using the QIAGEN TissueLyser LT (QIAGEN, NSW, Australia) for 5 min at 50 Hz in 500 µl of Radio-Immunoprecipitation Assay (RIPA) buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) (Sigma Aldrich, NSW, Australia; #R0278) supplemented with Roche protease inhibitor cocktail (Sigma Aldrich, NSW, Australia; #04693116001). Homogenates were centrifuged at 11,000 x g for 15 min at 4°C and the supernatant isolated, aliquoted and stored at -80°C. Total protein concentration was quantified using the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, VIC, Australia; #23225). A working concentration of 1 mg/ml was used for cytokine analysis.

Cytokine concentration was measured in individual ileal and colonic homogenates using Luminex xMAP technology (Milliplex Mouse Cytokine Kit, Merck Millipore, Darmstadt, Germany; #MPMCYTOMAG70K08) as per manufacturer’s instructions. The cytokines analysed were: IL-1β, IL-6, TNFα, IL-10, IFN-γ, IL-2, IL-17α and MCP-1. Each 96-well plate included a 6-point standard curve and two quality controls provided by Merck Millipore.

6.3.10 **Statistical analysis**

Data were compared using Prism version 7.0 (GraphPad® Software, San Diego, USA). A D’Agostino-Pearson omnibus test was used to assess normality. When normality was confirmed, a two-way analysis of variance (ANOVA) with appropriate post hoc testing were performed to identify statistical significance between groups. In other cases, a Kruskal-Wallis test with Dunn’s multiple comparisons test and Bonferroni correction was performed. Diarrhoea data was assessed using a Chi-square test (Bland, 2005). A p-value of <0.05 was considered significant.
6.4 Results

6.4.1 Bacterial diversity profiling

Bacterial profiling showed comparable abundance of bacterial phyla in both WT and BALB/c-Tlr4<sup>−/−</sup> billy mice (Table 6.2; Figure 6.3). The majority of the microbiome comprised of Firmicutes (WT 76.66 ± 5.98%; BALB/c-Tlr4<sup>−/−</sup>billy 71.33 ± 2.66%) and Bacteroidetes (WT 22.46 ± 6.01%; BALB/c-Tlr4<sup>−/−</sup>billy 24.34 ± 3.00%) phyla. A two-tailed, unpaired t-test with Welch’s correction showed increased expression of the Proteobacteria and TM7 phyla in BALB/c-Tlr4<sup>−/−</sup>billy mice (Proteobacteria: WT 0.44 ± 0.12%; BALB/c-Tlr4<sup>−/−</sup>billy 1.93 ± 0.61%, p=0.046; TM7: WT 0.10±0.06%; BALB/c-Tlr4<sup>−/−</sup>billy 0.55 ± 0.16%, p=0.028).

The Shannon’s Diversity index was used to characterize species diversity between WT and BALB/c-Tlr4<sup>−/−</sup>billy mice. BALB/c-Tlr4<sup>−/−</sup>billy mice showed increased diversity at baseline (Figure 6.4; *p=0.078) which may account for differences seen in toxicity endpoints.
<table>
<thead>
<tr>
<th>Phylum</th>
<th>WT</th>
<th>BALB/c-Tlr4&lt;sup&gt;−/−&lt;/sup&gt;</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacteroidetes</td>
<td>22.45±6.00</td>
<td>24.34±3.01</td>
<td>0.79</td>
</tr>
<tr>
<td>Firmicutes</td>
<td>76.65±5.98</td>
<td>71.33±2.66</td>
<td>0.46</td>
</tr>
<tr>
<td>Proteobacteria</td>
<td>0.43±0.12</td>
<td>1.93±0.61</td>
<td>*0.05</td>
</tr>
<tr>
<td>TM7</td>
<td>0.10±0.06</td>
<td>0.56±0.16</td>
<td>*0.03</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>0.25±0.09</td>
<td>0.65±0.19</td>
<td>0.09</td>
</tr>
<tr>
<td>Cyanobacteria</td>
<td>0.02±0.01</td>
<td>0.10±0.04</td>
<td>0.08</td>
</tr>
<tr>
<td>Acidobacteria</td>
<td>0.002±0.001</td>
<td>0.0004±0.0003</td>
<td>0.26</td>
</tr>
<tr>
<td>Verrucomicrobia</td>
<td>0.0005±0.0005</td>
<td>0.13±0.11</td>
<td>0.27</td>
</tr>
<tr>
<td>Deferribacteres</td>
<td>0.005±0.003</td>
<td>0.0006±0.0003</td>
<td>0.23</td>
</tr>
<tr>
<td>Tenericutes</td>
<td>0.02±0.02</td>
<td>0.92±0.53</td>
<td>0.13</td>
</tr>
<tr>
<td>Chloroflexi</td>
<td>0.02±0.005</td>
<td>0.01±0.01</td>
<td>0.58</td>
</tr>
<tr>
<td>Nitrospirae</td>
<td>0.003±0.001</td>
<td>0.003±0.002</td>
<td>0.70</td>
</tr>
</tbody>
</table>
Figure 6.3 Relative abundance of bacterial phyla, class, order, family, genus and species in WT and BALB/c-Tlr4−/−billy mice treated with vehicle-control. Caecal contents were obtained from the caecum of vehicle-treated mice and sent for genetic sequencing at the Australian Genomics Research Facility (Brisbane, Australia). Data are presented as relative abundance (%).
Figure 6.4 Shannon’s diversity index for bacterial species in WT and BALB/c-Tlr4−/−billy mice treated with vehicle control. BALB/c-Tlr4−/−billy mice has a higher diversity index compared to WT mice, *p=0.078. Data presented as individual points with mean±SEM. A two-tailed, unpaired t-test with Welch’s correction was performed to identify statistical significance where p<0.05.
6.4.2 BALB/c-Tlr4^−/−billy mice have attenuated clinical manifestations of irinotecan-induced gut toxicity

Irinotecan caused diarrhoea in all mice from as early as 6 h (Figure 6.5A/B). Diarrhoea severity was significantly improved in BALB/c-Tlr4^−/−billy mice compared to WT (*p<0.0001). No diarrhoea was seen in any vehicle control animals (Figure 6.5C/D). Weight loss following irinotecan treatment was most severe at 72 h in WT (-9.96 ± 0.98% from baseline) and BALB/c-Tlr4^−/−billy mice (-5.68 ± 0.64% from baseline), the weight loss in BALB/c-Tlr4^−/−billy mice was significantly less than that seen in WT (*p<0.0001) (Figure 6.5E).

6.4.3 BALB/c-Tlr4^−/−billy mice have improved histological architecture in the small intestine

BALB/c-Tlr4^−/−billy mice treated were protected against irinotecan-induced mucosal tissue injury most effectively in the jejunum (Figure 6.6A), with improvements seen in BALB/c-Tlr4^−/−billy mice compared to WT at 48 (*p=0.003) and 72 h (*p=0.023). Despite improvements in diarrhoea, architectural tissue injury remained evident at 96 h in the jejunum (Figure 6.6A WT #p<0.0001; BALB/c-Tlr4^−/−billy ^p=0.003) and ileum (Figure 6.6B WT #p<0.0001; BALB/c-Tlr4^−/−billy ^p<0.0001). This late histopathology was not evident in the colon (Figure 6.6C p>0.05) suggesting colonic histopathology may be more indicative of diarrhoea severity. Representative images (Figure 6.7A-D) show villous blunting/fusion, crypt disruption and inflammatory infiltrate (subset panel, b).

Peak apoptosis was seen at 6 h in both WT and BALB/c-Tlr4^−/−billy mice (Figure 6.6D-F) with paralleled decreased in proliferation seen in the jejunum and ileum (Figure 6.6G/H). BALB/c-Tlr4^−/−billy mice had reduced apoptotic counts at 6 h in the jejunum (Figure 6.6D ***p<0.0001) and ileum (Figure 6.6E *p=0.002). Representative immunostaining (Figure 6.7E-H) shows crypt caspase 3 positive cells in the jejunal crypts. No change was seen in proliferation between WT and BALB/c-Tlr4^−/−billy mice in any region at any time point (p>0.05) (Figure 6.6G-I).
Figure 6.5 *Tlr4*<sup>−/−</sup> attenuates symptomatic parameters of gut toxicity: diarrhoea and weight loss. Diarrhoea profiles shown for WT BALB/c mice (A) and BALB/c-*Tlr4*<sup>−/−</sup> mice (B) treated with 270 mg/kg of irinotecan, as well as vehicle treated controls (C, D). Diarrhoea data is expressed as the percentage of total animals (per time point) with a particular grade of diarrhoea. Data was analysed using a Chi-Squared test. Diarrhoea was most significant at 24 h post-treatment in both treatment groups. Diarrhoea was significantly improved in BALB/c-*Tlr4*<sup>−/−</sup> mice compared to WT (***p<0.0001). Panel E shows weight loss over the 96 h time course. Data displayed as percentage weight change from baseline (0 h). A Kruskal-wallis with post hoc testing was performed to identify statistical significance. BALB/c-*Tlr4*<sup>−/−</sup> mice had significantly less body weight loss at 24 h (**p=0.003), 48 h, 72 h and 96 h (***p<0.0001).
Figure 6.6 Histopathological parameters in jejunum, ileum and colon of WT and BALB/c-\(Tlr4^{+/+}\) mice. There were significant increases in tissue injury scores in WT and BALB/c-\(Tlr4^{-/-}\) mice in the jejunum (A), ileum (B) and colon (C); # denotes a change from baseline in WT mice, where \(p<0.05\); ^ denotes a change from baseline in BALB/c-\(Tlr4^{-/-}\) mice, where \(p<0.05\). Data presented as interquartile range±min/max. TLR4 deletion reduced the severity of jejunal and ileal tissue damage (A *\(p=0.003\) 48 h, *\(p=0.023\) 72 h; B *\(p=0.005\) 96 h). Apoptosis was increased in the jejunum (D), ileum (E) and colon (F) in all treated animals (\(p<0.05\)). TLR4 deletion attenuated apoptosis in the jejunum and ileum (D ***\(p<0.0001\) 6 h; E *\(p=0.0023\) 6 h). Decreased small intestinal proliferation was noted at 6 h in all animals treated with irinotecan (G and H), however no differences were seen between WT and BALB/c-\(Tlr4^{-/-}\) mice. No change was seen in the colon.
Figure 6.7 Representative histological images showing morphological changes (A-D) and apoptosis (E-H) in the gut following irinotecan. Panel B shows clear jejunal tissue injury at 48 h characterised by crypt ablation, villous blunting/fusion, inflammatory infiltrate (subset panel, b). Crypt apoptosis (brown staining [arrow]; Panel F, H) was most significant at 6 h, preceding the onset of clinical symptoms and tissue injury. Scale bars show 30 μm. Original magnification 40 x.
6.4.4 TLR4-dependent signalling contributes to intestinal barrier disruption

Serum FITC-dextran was elevated in WT mice at 24 (#p<0.0001), 48 (#p=0.0043) and 72 h (#p=0.01) compared to vehicle controls (Figure 6.8A), indicating compromised intestinal barrier function. No statistically significant change was seen in BALB/c-Tlr4−/− mice at any time point (p>0.05). At 24 h post-irinotecan, WT mice had significantly greater serum FITC-dextran concentrations compared to BALB/c-Tlr4−/− mice (3209.59±1020.88 ng/ml vs. 1373.97±303.56 ng/ml; *p=0.01).

Serum endotoxin (LAL), a measure of LPS translocation, was elevated at all time points in WT mice treated with irinotecan (#p<0.005), with most significant peaks at 24 and 72 h (both #p<0.0001) (Figure 6.8B). Serum endotoxin was highest at 24 (^p=0.001), 48 (^p=0.003) and 96 h (^p=0.02) in BALB/c-Tlr4−/− mice treated with irinotecan compared to control. There was a significant difference in serum endotoxin between WT and BALB/c-Tlr4−/− mice at 72 h post irinotecan treatment (33.35 ± 2.19 EU/ml vs. 13.96±5.87 EU/ml; ***p<0.0001).
Figure 6.8 Ex vivo markers of barrier permeability, serum 4 kDa FITC-dextran (A) and serum endotoxin (B). FITC-dextran was administered as a 500 mg/kg dose, via oral gavage, 3 h prior to kill time point. Serum endotoxin was assessed using the Serum Limulus Amebocyte Lysate (LAL) endotoxin assay. Data is expressed as mean±SEM and was analysed using a two-way ANOVA with Tukey’s post hoc. Both markers of barrier disruption show elevations following treatment with irinotecan (# denotes a change from baseline in WT mice; ^ denotes a change from baseline in BALB/c-Tlr4-/-billy mice, where p<0.05). Maximum FITC-dextran permeability coincides with peak diarrhoea in WT animals (24 h). At this time point, TLR4 deletion decreased serum FITC-dextran levels (*p=0.0104) compared to WT. Serum endotoxin, indicative of LPS translocation, was reduced in BALB/c-Tlr4-/-billy mice at 72 h compared to WT mice treated with irinotecan (***p=0.0001).
6.4.5 BALB/c-Tlr4−/−billy mice exhibit a muted inflammatory response

BALB/c-Tlr4−/−billy mice treated with irinotecan showed no statistically significant increase in IL-1β, IL-6 TNFα, IFN, IL-2 or IL-17 expression in the ileum or colon when compared to vehicle controls (Figure 6.9 and 6.10). Reciprocally, they showed no decrease in the anti-inflammatory cytokine, IL-10. There were significant increases in the expression of IL-1β (#p=0.04 24 h; #p=0.004 48 h) and MCP1 (#p=0.005) in the ileum of WT mice treated with irinotecan (Figure 6.9). No statistically significant increase was seen in TLR4 deficient mice (Figure 6.9; p>0.05), although BALB/c-Tlr4−/−billy mice did show comparable increases in MCP1 at 48 h (^p=0.011). BALB/c-Tlr4−/−billy mice lacked the IL-6 response seen in WT animals at 6 h (#p=0.003, *p=00002 ileum; #p=0.0004, *p=0.0005 colon) (Figure 6.9 and 6.10). WT mice also showed increased ileal expression of TNFα (#p=0.01) 24 h after treatment with irinotecan and this was significantly elevated relative to treated BALB/c-Tlr4−/−billy mice (^p=0.02). There was a significant difference in IFN production at 24 and 72 h in WT mice compared to BALB/c-Tlr4−/−billy mice (^p=0.023 24 h, ^p=0.017 72 h). No change was seen in IL-2, IL-17 or IL-10 in WT or BALB/c-Tlr4−/−billy mice.
Figure 6.9 Cytokine expression in the ileum of vehicle-treated and irinotecan-treated WT and BALB/c-\textit{Tlr4}\textsuperscript{-/-}billy mice. Cytokine expression was assessed using Luminex xMAP technology. Data expression as mean±SEM (pg/ml). A two-way ANOVA with Tukey’s post hoc was performed to identify statistical significance. IL-1\textbeta expression increased in the ileum of WT animals treated with irinotecan compared to control (#p=0.0394 24 h; #p=0.0036 48 h; # denotes a change from baseline in WT mice; \textsuperscript{\^} denotes a change from baseline in BALB/c-\textit{Tlr4}\textsuperscript{-/-}billy mice, where p<0.05). BALB/c-\textit{Tlr4}\textsuperscript{-/-}billy mice lacked an IL-6 response at 6 h, with significantly lower expression compared to WT mice (**p=0.0002 ileum). TNF\textalpha expression peaked at 24 h in the ileum of WT mice treated with irinotecan (#p=0.0113). This was significantly elevated relative to BALB/c-\textit{Tlr4}\textsuperscript{-/-}billy mice (*p=0.0166), which showed no elevation in TNF\textalpha (p>0.05). No change was seen in the anti-inflammatory cytokine, IL-10.
Figure 6.10 Cytokine expression in the colon of vehicle-treated and irinotecan-treated WT and BALB/c-\textit{Tlr4}\textsuperscript{−/−} mice. Cytokine expression was assessed using Luminex xMAP technology. Data expression as mean±SEM (pg/ml). A two-way ANOVA with Tukey’s post hoc was performed to identify statistical significance. No change was seen in IL-1β expression in the colon of WT mice relative to vehicle-treated controls. Only a significant decrease was seen in BALB/c-\textit{Tlr4}\textsuperscript{−/−} mice (# denotes a change from baseline in WT mice; ^ denotes a change from baseline in BALB/c-\textit{Tlr4}\textsuperscript{−/−} mice, where p<0.05). Like the ileum, BALB/c-\textit{Tlr4}\textsuperscript{−/−} mice lacked an IL-6 response in the colon, with significantly lower expression compared to WT mice (**p=0.0005 colon). WT mice showed elevated IFN production at 24 and 72 h post-irinotecan, compared to vehicle-treated controls, however there was no difference compared to in BALB/c-mice. No change was seen in the anti-inflammatory cytokine, IL-10.
6.4.6 Irinotecan-induced pain is associated with TLR4-dependent astrocytic GFAP expression

Facial grimace criteria peaked at 6 h in both treated animal groups, reducing steadily for the remainder of the experimental time-course (Figure 6.11). From 6 to 72 h, BALB/c-mice had reduced facial grimace criteria compared to WT mice (**p<0.0001; Figure 6.11). Elevated GFAP staining, indicative of astrocyte activation, was seen at 6 h in WT animals compared to controls (#p=0.004) (Figure 6.11A). GFAP staining was significantly greater in WT compared to BALB/c-mice at 6 (*p=0.008) and 72 h (*p=0.01). No change was seen in Iba-1 staining in any animals (Figure 6.12B; p>0.05). Representative images (Figure 6.12C) support activation of astrocytes with obvious changes in phenotype 6 h after irinotecan in WT mice.

6.4.7 Irinotecan increases blood brain barrier permeability to albumin

Elevated albumin staining was seen in WT (#p=0.0001) and BALB/c-mice (^p=0.03) at 24 h, and in WT mice at 48 and 72 h (#p=0.006 and #p=0.03, respectively; Figure 6.13A/B), although there was no difference between WT and BALB/c-mice (p>0.05). Both parenchymal (Figure 6.13C) and perivascular (Figure 6.13D) albumin was evident in WT and BALB/c-mice treated with irinotecan, with minimal leakage in control animals (Figure 6.13B).
Figure 6.11 Facial grimace criteria following irinotecan treatment (A,B) or vehicle control (C,D). Facial grimace scores were assessed four times daily using the facial grimace criteria. Most significant facial pain was seen at 6 h following treatment with irinotecan. BALB/c-Tlr4−/− mice had lower facial pain scores at all time points (***p<0.0001 6-72 h; *p=0.0072 96 h). Data was analysed using a Kruskal-Wallis with Bonferroni correction, data presented as relative proportion of animals exhibiting varying severity of facial pain.
Figure 6.12 Glial activation, measured by GFAP and Iba-1 staining in the dorsal lumbar spinal cord of mice treated with irinotecan of vehicle control. GFAP and Iba-1 immunostaining was assessed in the dorsal column of the lumbar spinal cord. Increased astrocytic activation (GFAP) was seen in treated WT mice at 6 h compared to controls (A; #p=0.0041). This was not evident in BALB/c-Tlr4−/− mice (p>0.05). Irinotecan-treated WT mice showed increased GFAP staining compared to BALB/c-Tlr4−/− mice at 6 h (*p=0.008) and 72 h (*p=0.012). No change was seen in microglial activity (Iba-1) across the full time course in both WT and BALB/c-Tlr4−/− mice (B; p>0.05). Data presented as mean±SEM. Panel C shows representative images of GFAP staining in vehicle control WT mice and 6 h after irinotecan. WT mice treated with irinotecan displayed morphological changes in astrocyte phenotype (somatic hypertrophy, thickened and ramified processes) indicative of an activated state. Scale bars show 50 µm or 10 µm for representative images and subset panels, respectively. Original magnification 40 x.
Figure 6.13 Irinotecan causes blood brain barrier dysfunction. Staining was analysed using a semi-quantitative 0-3 grading system and represented as interquartile range±mix/max. A Kruskal-Wallis Bonferroni correction was performed to identify statistical significance in non-parametric data. Scale bars show 20 µm, 100 µm and 50 µm for panels B, C and D, respectively. Original magnification 40 x. Albumin staining, indicative of increased blood brain barrier transit, was elevated compared to untreated controls at 24 h (#p<0.0001), 48 h (#p=0.0063) and 72 h (#p=0.0325) in WT mice; this was only seen at 24 h (^p=0.0325) in BALB/c-Tlr4−/− mice. No differences were seen between WT and BALB/c-Tlr4−/− mice (p>0.05). Qualitative assessment showed that albumin leakage was not limited to a particular brain region, but affected the vasculature globally with both parenchymal (C) and perivascular (D) albumin staining noted. Minimal leakage was observed in control animals (B).
6.5 Discussion

Irinotecan is a commonly prescribed chemotherapeutic agent, however its therapeutic efficacy is often limited by its severe gastrointestinal side effects. Diarrhoea can significantly impact on patients' health outcomes, altering fluid/electrolyte balances and leading to renal insufficiency, malnutrition and extreme dehydration. In addition to severe gastrointestinal dysfunction, irinotecan causes central neuropathy and heightened pain sensitivity. Recent research suggests TLR4 may be uniquely positioned to regulate both of these toxicities associated with irinotecan treatment (Gibson et al., 2015, Wardill et al., 2015b). Results from the current study support this newly proposed hypothesis, highlighting significant improvements in symptomatic parameters and histopathological markers of gut toxicity in BALB/c-Tlr4<sup>-/-</sup> billy mice treated with irinotecan. This study is also the first to show paralleled improvements in in vivo pain markers and central glial reactivity following irinotecan.

The gut microbiota is critical in regulating the severity of gut toxicity, likely through its evolving crosstalk with innate immunity. It is well established that dysbiotic changes, predominantly the levels of LPS-producing, gram-negative bacteria, correlate with diarrhoea severity (Stringer et al., 2008, Stringer et al., 2013). Comparable levels of major phylogenies (fermicutes, bacteroidetes) were seen in vehicle treated WT and BALB/c-Tlr4<sup>-/-</sup> billy mice. However, small variations were seen in two relatively low abundance microbes and in overall species diversity. These differences seen in the composition of the gut microbiome in WT and BALB/c-Tlr4<sup>-/-</sup> billy mice are not surprising given the wealth of emerging evidence indicating both genetic and environment factors, such as breeding rooms/facilities, weigh significantly on the composition of the gut microbiome (Hufeldt et al., 2010).

At baseline, TLR4 knockout mice exhibited higher levels of the TM7 bacterial phyla. Little is known about this bacterial phylogeny, however it has been suggested to contribute to inflammatory pathologies within the gastrointestinal tract (Kuehbacher et al., 2008). More importantly, BALB/c-Tlr4<sup>-/-</sup> billy mice had elevated levels of β-glucuronidase-producing proteobacteria, likely increasing the
rate of SN-38 reactivation, and thus worsened gut toxicity. Despite this predisposition, BALB/c-\textit{Tlr4}\textsuperscript{-/-}mice showed improvements in both the duration and severity of symptoms compared to WT mice. This finding compliments recent research showing germ-free mice experienced less severe irinotecan-induced gut toxicity compared to conventional mice (Pedroso \textit{et al.}, 2015). Most importantly, the germ-free mice also had higher levels of unbound SN-38 and higher β-glucuronidase activity. This suggests, contrary to traditional beliefs, the second pass metabolism of irinotecan may not be the main factor in determining the severity of irinotecan-induced gut toxicity. Comparatively, depletion of the gut microbiome with oral antibiotics has shown to be effective in reducing irinotecan-induced diarrhoea (Kurita \textit{et al.}, 2011). It is now essential to determine if these improvements are the results of reduced microbial metabolism and SN-38 reactivation, or the result of reduced TLR4-mediated signalling and the intimate crosstalk between the microbiome and the innate immune system. Determination of which factor contributes more significantly to clinical outcomes would therefore better direct therapeutic research efforts.

Extensive literature exists showing the protective effect of TLR4 deletion in an inflammatory setting, however this appears to be limited to only acute insults, with TLR4 deficiency exacerbating chronic inflammatory diseases (Gibson \textit{et al.}, 2008). For example, significant improvements in acute inflammation have been shown in the absence of TLR4 and MyD88, a downstream signalling molecule of TLR4, following acute infection with \textit{Citrobacter rodentium} (Gibson \textit{et al.}, 2008). Similar results have also been demonstrated in methotrexate-induced gut toxicity, with MD-2 (a TLR4 accessory protein) deletion improving clinical and histological parameters of toxicity (Frank \textit{et al.}, 2015). Importantly, this study showed TLR4 and TLR2 appear to have opposing roles, with both genetic deletion and pharmacological inhibition of TLR2 worsening methotrexate-induced damage (Frank \textit{et al.}, 2015). It appears TLR2 has paradoxical roles in chemotherapy-induced gut toxicity, with improvements seen in \textit{Thr2}\textsuperscript{-/-}mice treated with irinotecan. This study also showed that \textit{Myd88}\textsuperscript{-/-}mice were also protected from developing severe irinotecan-induced gut toxicity, reiterating the importance of TLR4-dependent signalling (Frank \textit{et al.}, 2015). Despite support for TLR4 deletion providing protection against chemotherapy-induced gut toxicity, this does not appear
to be the case for acute and chronic colitis, with BALB/c-\(Tlr4^{-/-}\) mice more susceptible to ulceration and bleeding (Gibson et al., 2008). Although the unique mechanisms to each effect are not understood, these data do imply ambivalent roles for TLR4 in different inflammatory-based pathologies in the gastrointestinal tract.

It is well established that irinotecan-induced gut toxicity occurs through apoptosis of crypt epithelial cells through the gastrointestinal tract and consequently apoptosis is an established marker of toxicity severity (Keefe et al., 2000). Our results showed significantly decreased levels of apoptosis in the jejunum and ileum of irinotecan-treated BALB/c-\(Tlr4^{-/-}\) mice. This supports recent research suggesting that TLR4 signalling contributes to intestinal stem cell apoptosis through endoplasmic reticular stress-related mechanisms (Afrazi et al., 2014). We also saw levels of proliferation inversely parallel these changes in cellular dynamics. This is of great clinical significance as apoptosis is one of the initial steps in the cascade of biological events that results in the development of gut toxicity. If TLR4 deletion is able to profoundly impact such an early mediator of toxicity, it provides an excellent opportunity to intervene prior to architectural tissue damage, inflammation and bacterial translocation.

In this study, increased FITC-dextran permeability and endotoxin translocation were seen in irinotecan-treated WT mice, indicative of altered intestinal barrier function. Importantly, BALB/c-\(Tlr4^{-/-}\) mice maintained intestinal barrier function with no significant changes in FITC-dextran permeability and decreased LPS translocation. Surprisingly, BALB/c-\(Tlr4^{-/-}\) mice only showed mild improvements in serum endotoxin compared to WT mice and this did not appear to reflect the differences in intestinal damage. The failure to show differences at most time points in this study could be explained by evidence suggesting that TLR4 on hepatocytes is required for complete endotoxin clearance (Deng et al., 2013). Nonetheless, reducing permeability of the intestinal barrier has the potential to reduce bacterial translocation, thus reducing systemic toxicity and its associated biological and clinical implications. Highlighting this pathobiological mechanism, BALB/c-\(Tlr4^{-/-}\) mice displayed less severe inflammation compared to WT mice. The most significant difference was
seen for IL-6, in which BALB/c-Tlr4<sup>-/-</sup>bally mice showed no increase after treatment, supporting the idea that TLR4-dependent NFκB activation is required for the release of IL-6 from macrophages (Pathak et al., 2006). Stunted IL-6 production has also been seen in MyD88 deficient mice (Hayashi et al., 2001) and Tlr4<sup>-/-</sup> macrophages (Shoenfelt et al., 2009). Given the profound effect proinflammatory cytokines have on tight junction integrity, IL-6 is a likely candidate in the development of a leaky gut seen in WT mice treated with chemotherapy. It is also important to consider the reciprocal changes in anti-inflammatory cytokines as the dynamics between pro- and anti-inflammatory cytokines is becoming increasingly recognised in a number of gastrointestinal pathologies (Sultani et al., 2012). Of note is the marked decrease in IL-10 expression seen following irinotecan. Similar changes were also seen in IL-17α, a cytokine recognised for its paradoxical effects in inflammation. IL-17α has been implicated in a number of inflammatory based pathologies, such as inflammatory bowel disease (Harvey et al., 2014), however, recent research now suggests that the presence of IL-17α-producing γδT cells is critical for tissue repair, particularly in the lungs (Murdoch and Lloyd, 2010) and skin (Jameson et al., 2002). Importantly however, intra-epithelial γδT cells have also been shown to participate in tissue repair in the gut (Chen et al., 2002) suggesting that the lack of IL-17α expression seen in the current study may exacerbate toxicity.

The current study also identified, for the first time, disruption of the blood brain barrier in animals treated with irinotecan. Blood brain barrier disruption has been hypothesised to contribute to the development of ‘chemobrain’ and cognitive impairment seen following chemotherapy, allowing cytotoxic agents direct access to the CNS (Holmes, 2013). It has also been suggested uncontrolled blood brain barrier transit may potentiate the ability of peripheral inflammation to influence central pain signalling. It is becoming increasingly recognised that TLR4, expressed on centrally located glia, is able to recognise and respond to peripherally derived LPS and inflammatory mediators (Wardill et al., 2015b). We have shown translocation of LPS to systemic circulation following chemotherapy treatment, reflecting the swing towards a gram-negative, pathogenic gut microbiome profile following chemotherapy. Despite this, we saw no association in serum LPS, glial activation
and pain. Instead, astrocytic activation appeared to occur bimodally, with increases in GFAP staining seen at 6 and 72 h. This suggests that cellular events associated with apoptosis (which peaks at 6 h), or inflammation, may be more important in TLR4-mediated glial activation. This concept is particularly compelling when looking at recent research by Ji et al., (2013) who reported significant astrocytic hypertrophy and activation in the dorsal horn of vincristine-treated rats with mechanical allodynia (Ji et al., 2013). Treatment with pentoxifylline, an anti-inflammatory agent, attenuated astrocytic reactivity and mechanical allodynia. Astrocytic reactivity has also been identified in the lumbar spinal cord of rats receiving oxaliplatin treatment, providing evidence linking peripheral inflammation and central gliosis. It is now critical to determine if the irinotecan-induced gut toxicity and pain are independent, yet simultaneously occurring events that are both governed by TLR4, or if there is a true directional mechanism linking gut-derived inflammation and central pain signalling.

Data from the current study have clearly highlighted the involvement of TLR4 in the development of irinotecan-induced gut toxicity and pain, provide a unique opportunity to simultaneously treat irinotecan-induced toxicities. In all cases of TLR4-targeted therapeutic options, the effect on both the efficacy of the anti-cancer therapy and overall tumour kinetics are paramount. This is particularly the case when targeting TLR4, as recent evidence now suggests that it may play critical roles in chemotherapy efficacy, initiating an immune response critical for tumour regression. For example, an Apetoh et al. (2007) showed that TLR4 deficient animals had increased tumour growth under normal conditions and in response to doxorubicin (Apetoh et al., 2007). TLR4 inhibition has also been implicated in tumour regression, with several studies now showing that numerous cancer cell lines (e.g. SW260 [colon], CRC-526 [breast], PC3 [prostate]) overexpress TLR4 and that LPS stimulates their growth (Yang et al., 2010, Huang et al., 2014b, Jain et al., 2015). Although not assessed in the current study, future work for TLR4-target interventions will also need to clarify the role of TLR4 in normal gastrointestinal motility patterns given recent research implicating TLR4 in altered motility patterns following opioid treatment (Anitha et al., 2012, Farzi et al., 2015).
6.6 Conclusions

Given the ubiquitous involvement of the innate immune system, particularly TLR4, in gut homeostasis and central pain signalling, it presents as a potentially overlooked candidate in the treatment of chemotherapy-induced gut toxicity and pain. Our research has demonstrated that TLR4 is pivotal in the development of both toxicities. This research not only improves our understanding of the underlying mechanisms involved, but also reveals a promising opportunity to intervene in the complex pathophysiology of these dose-limiting side effects of chemotherapy. Research efforts must now be targeted at tailoring methods of inhibiting TLR4, keeping in mind the potential effects on tumour burden and gastrointestinal function.
# Chapter 7 Claudin-1 internalisation drives TLR4-dependent barrier dysfunction

## Statement of Authorship

<table>
<thead>
<tr>
<th>Title of Paper</th>
<th>TLR4-dependent claudin-1 internalisation contributes to irinotecan-induced diarrhoea through leak flux mechanisms and altered chloride secretion</th>
</tr>
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</table>
| Publication Status | Published  
Unpublished and Unsubmitted work written in manuscript style |
| Publication Details | Second research paper from the main animal study performed in my candidature. Submitted to Molecular Cancer Therapeutics. |

### Principal Author

| Name of Principal Author (Candidate) | Hannah Wardill |
| Contribution to the Paper | I was responsible for experimental design, organising the animal study, downstream tissue analyses, data acquisition, data analysis and manuscript preparation. |
| Overall percentage (%) | 90% |
| Certification | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
| Signature | Date |

### Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);

ii. permission is granted for the candidate to include the publication in their thesis; and

iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

| Name of Co-Author | Joanne Bowen |
| Contribution to the Paper | Joanne was responsible for obtaining research funding to support this study. She also helped with experimental design, data interpretation and preparation of the manuscript for publication. |
| Signature | Date |

| Name of Co-Author | Yasabella Van Sebille |
| Contribution to the Paper | Yasabella helped with the animal study. |
| Signature | Date |
Chapter 7 Claudin-1 internalisation drives TLR4-dependent barrier dysfunction

<table>
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<tr>
<td>Kate Seconbe</td>
<td>Kate is a research assistant in our laboratory and helped conduct the animal study.</td>
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<tr>
<td>Janet Cotter</td>
<td>Janet was responsible for obtaining research funding to support this study. She also helped with experimental design, data interpretation and preparation of the manuscript for publication.</td>
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<tr>
<td>Imogen Ball (nee White)</td>
<td>Imogen is a research assistant in our laboratory and helped conduct the animal study.</td>
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<td>Richard Logan</td>
<td>Richard was responsible for revising this manuscript for publication.</td>
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<tr>
<td>Rachel Gibson</td>
<td>Rachel was responsible for obtaining research funding to support this study. She also helped with experimental design, data interpretation and preparation of the manuscript for publication.</td>
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Chapter 7 TLR4-dependent claudin-1 internalisation and secretagogue-mediated chloride secretion regulate irinotecan-induced diarrhoea

[Wardill HR et al., (2016) Molecular Cancer Therapeutics. in press]

7.1 Abstract

We have previously shown increased intestinal permeability, to 4 kDa FITC-dextran, in BALB/c mice treated with irinotecan. Importantly, genetic deletion of Toll-like receptor 4 (TLR4; Tlr4<sup>−/−</sup>) protected against loss of barrier function indicating TLR4 is critical in tight junction regulation. The current study aimed to (1) determine the molecular characteristics of intestinal tight junctions in wild-type and Tlr4<sup>−/−</sup> BALB/c mice, and (2) characterise the secretory profile of the distal colon. Forty-two female wild-type (WT) and 42 Tlr4<sup>−/−</sup> (BALB/c-Tlr4<sup>−/−</sup>billy) BALB/c mice weighing between 18-25 g received a single 270 mg/kg (i.p.) dose of irinotecan hydrochloride or vehicle control and were killed at 6, 24, 48, 72 and 96 h. The secretory profile of the distal colon, following carbachol and forskolin, was assessed using Ussing chambers at all time points. Tight junction integrity was assessed at 24 h, when peak intestinal permeability and diarrhoea were reported, using immunofluorescence, western blotting and RT-PCR. Irinotecan caused internalisation of claudin-1 with focal lesions of ZO-1 and occludin proteolysis in the ileum and colon of WT mice. BALB/c-Tlr4<sup>−/−</sup>billy mice maintained phenotypically normal tight junctions. Baseline conductance, a measure of paracellular permeability, was increased in irinotecan-treated WT mice at 24 h (53.19±6.46 S/cm²; p=0.0008). No change was seen in BALB/c-Tlr4<sup>−/−</sup>billy mice. Increased carbachol-induced chloride secretion was seen in irinotecan-treated WT and BALB/c-Tlr4<sup>−/−</sup>billy mice at 24 h (WT 100.35±18.37 µA/cm²; p=0.022; BALB/c-Tlr4<sup>−/−</sup>billy 102.72±18.80 µA/cm²; p=0.023). Results suggest TLR4-dependent claudin-1 internalisation and secondary anion secretion contribute to irinotecan-induced diarrhoea.
7.2 Introduction

Irinotecan is a commonly prescribed chemotherapeutic agent used to treat a variety of solid tumours (Gibson et al., 2007, Yusof et al., 2016). Despite long-standing clinical efficacy, irinotecan is associated with a host of debilitating off target toxicities which severely impact its widespread implementation (Tsunoda et al., 2010). Of these side effects, gut toxicity presents as a major clinical obstacle in oncology practice with limited therapeutic avenues. Characterised by severe diarrhoea, rectal bleeding, pain and infection, irinotecan-induced gut toxicity is considered the most significant dose-limiting side effect of irinotecan, placing a substantial clinical and economic burden on the provision of optimal cancer care (Ikuno et al., 1995, Carlotto et al., 2013). The current understanding of the molecular mechanisms that drive gut toxicity has not yet led to any advances in its treatment and thus, a better understanding of the underlying biology is required. Recent research has outlined emerging evidence implicating intestinal barrier injury and tight junction disruption in the development of gut toxicity (Nakao et al., 2012, Wardill et al., 2013) however few studies have investigated their regulation and involvement in diarrhoea.

Tight junctions are highly dynamic signalling complexes critical to gastrointestinal homeostasis and the maintenance of barrier function (Wardill and Bowen, 2013). In the setting of chemotherapy-induced gut toxicity (CIGT), intestinal barrier dysfunction is most commonly associated with augmentation of key tight junction proteins claudin-1, zonular occludens(ZO)-1 and occludin; all of which are integral to maintenance of the tight junction unit (Cummins, 2012, Wardill et al., 2012). A large body of evidence demonstrates disruption of these proteins in a number of gastrointestinal pathologies, particularly those characterised by diarrhoea (Edelblum and Turner, 2009, Wardill and Bowen, 2013), with altered barrier function and tight junction integrity seen following a number of chemotherapeutic agents (Wardill and Bowen, 2013). Dual sugar permeability assays are typically used to non-invasively assess barrier function in patients receiving chemotherapy. Elevations in permeability indices have been identified in a number of patient cohorts and have been shown to correlate with the severity of gastrointestinal symptoms (Keefe et al., 1997, Melichar et al., 2001,
Fazeny-Dorner et al., 2002, Blijlevens et al., 2005a, Choi et al., 2007). Despite these findings, the molecular characteristics of tight junctions have been largely ignored clinically, assessed only in in vivo rat studies. These studies consistently show decreased expression, redistribution and phosphorylation of claudin-1, occludin and ZO-1 (Hamada et al., 2010, Beutheu Youmba et al., 2012, Nakao et al., 2012, Hamada et al., 2013), however few report these changes in combination with robust permeability data.

It is well documented that changes in tight junction integrity parallel the onset and severity of gastrointestinal symptoms (Bertiaux-Vandaele et al., 2011). In vivo studies have shown increased intestinal permeability, caused by irinotecan treatment, permits endotoxin and bacterial translocation, thus increasing the risk of secondary infection (Nakao et al., 2012). In patients with ulcerative colitis, loss of intestinal barrier function and tight junction integrity has been shown to contribute to diarrhoea development via passive back-flow of anions and water into the lumen (Schmitz et al., 1999). Intestinal barrier dysfunction has also been identified as a key risk factor for secondary complications in patients receiving multi-drug chemotherapy (van der Velden et al., 2010), increasing the risk of bacteremia, mucosal-related invasive fungal disease, typhlitis and sepsis (Blijlevens, 2005).

Despite strong clinical evidence indicating barrier dysfunction permits secondary toxicity and infectious consequences, the mechanisms by which barrier dysfunction and tight junction disruption contribute to irinotecan-induced diarrhoea remain unclear. We have recently demonstrated increased intestinal permeability to 4 kDa FITC-dextran in BALB/c mice treated with irinotecan, indicating poor tight junction integrity. Importantly, our findings demonstrated that genetic deletion of Toll-like receptor 4 (TLR4; Tlr4−/−) protected against the development of barrier dysfunction (Wardill et al., 2016a) and reduced the duration and severity of diarrhoea. We hypothesise that the ability of TLR4 to recruit powerful downstream inflammatory signals, recognised for their ability to disrupt tight junction proteins, makes it a potential driver of barrier dysfunction following chemotherapy. Research shows that TLR4, and its ligand lipopolysaccharide (LPS), are upregulated following
irinotecan (Bowen et al., 2012). This parallels other models of barrier dysfunction and supports the idea that TLR4 activation promotes barrier dysfunction (Kobayashi et al., 2013, Guo et al., 2015).

The current study therefore aims to investigate the morphology and molecular integrity of intestinal tight junctions in a model of irinotecan-induced diarrhoea, and gauge the importance of TLR4 in tight junction regulation. In addition, this study aims to assess the secretory profile of the intestine using Ussing chambers to determine the impact of altered anion secretion, secondary to barrier disruption, on diarrhoea development.
Chapter 7 Claudin-1 internalisation drives TLR4-dependent barrier dysfunction

7.3 Materials and Methods

7.3.1 Animal model and ethics

The study was approved by the Animal Ethics Committee of the University of Adelaide and complied with National Health and Research Council (Australia) Code of Practice for Animal Care in Research and Training (2014). Mice were group housed in ventilated cages with three to five animals per cage. They were housed in approved conditions on a 12 h light/dark cycle. Food and water were provided ad libitum.

7.3.2 Experimental design

All mice were on a BALB/c background. Forty-two female BALB/c-wild-type (WT) and BALB/c-\textit{Tlr4}\textsuperscript{-/-} mice (\(n_{\text{total}}=84\)) weighing between 18-25 g (10-13 weeks) were used. WT BALB/c mice were obtained from the University of Adelaide Laboratory Animal Service (SA, Australia), and BALB/c-\textit{Tlr4}\textsuperscript{-/-} mice, back-crossed onto BALB/c for more than 10 generations, were kindly provided by Professor Paul Foster from the University of Newcastle (NSW, Australia) and were originally sourced from Osaka, Japan (Phipps et al., 2009). All BALB/c-\textit{Tlr4}\textsuperscript{-/-} mice were homozygous null mutants and hence expressed no detectable TLR4 mRNA or protein (Wardill et al., 2016a). Mice were treated with a single 270 mg/kg intraperitoneal (i.p.) dose of irinotecan hydrochloride (kindly provided by Pharmacia/Pfizer, Michigan, USA) prepared in a sorbitol/lactic acid buffer (45 mg/ml sorbitol / 0.9 mg/ml lactic acid; pH 3.4; Sigma-Aldrich, NSW, Australia; D-sorbitol #S1876, lactic acid #252476), which was shown in our previous work to cause reproducible diarrhoea with no mortality (Wardill et al., 2016a). Control mice received the sorbitol/lactic acid buffer only. All mice received 0.03 mg/kg of atropine subcutaneously immediately prior to treatment to reduce the cholinergic response to irinotecan. Mice were randomly assigned to treatment groups and killed at 6, 24, 48, 72 and 96 h. Mice were anaesthetised using 200 mg/kg (intraperitoneal) ilium sodium pentobarbital (60 mg/ml) and blood was collected from the facial vein. They were killed via transcardial perfusion with cold, sterile 1 X PBS (pH 7.4) followed by 4% paraformaldehyde (PFA) in 0.1 M PBS (pH 7.4).
7.3.3 Clinical assessment of gut toxicity

Mice were assessed four times daily for response to irinotecan treatment as per Wardill et al., (2016). For clinical data please refer to Wardill et al., 2016 (Wardill et al., 2016a) (Chapter 6).

7.3.4 Tissue preparation

The entire gastrointestinal tract from the pyloric sphincter to the rectum was dissected prior to perfusion with 4% PFA and flushed with chilled 1 X PBS (pH 7.4) to remove intestinal contents. Both the small and large intestines were weighed immediately after resection. Samples (1 cm in length) of jejunum, ileum and colon were collected and (1) drop-fixed using 10% neutral buffered saline for processing and embedding into paraffin wax, or (2) stored in RNaLater® (Sigma Aldrich, NSW, Australia; #R0901) at -20°C for molecular analyses. Mucosal scrapings were also collected from the jejunum, ileum and colon, snap frozen and stored at -80°C.

7.3.5 Tight junction analysis

Tight junction analysis was performed on the jejunum, ileum and colon taken from WT and BALB/c-<i>Tlr4</i>−/−billy mice 24 h after irinotecan treatment as peak diarrhoea severity and serum FITC-dextran were seen at this time point. Tight junction analysis was also performed on six vehicle control mice from each genotype to ensure TLR4 deletion did not affect tight junction morphology.

7.3.5.1 Immunofluorescent analysis of tight junction proteins

Immunofluorescence (IF) was carried out on 4 µm sections of jejunum, ileum and colon, cut on a rotary microtome and mounted onto FLEX IHC microscope slides (Flex Plus Detection System, Dako, Denmark; #K8020). IF analysis was performed for key tight junction proteins: claudin-1, ZO-1 and occludin. IF was performed using Dako reagents on an automated machine (AutostainerPlus™, Dako, Denmark; #AS480) following standard protocols supplied by the manufacturer. Briefly, sections were deparaffinised in histolene and rehydrated through graded ethanols before undergoing heat-mediated antigen retrieval using an ethylenediaminetetraacetic acid-sodium bicarbonate (EDTA-NaOH) buffer (0.37 g/L EDTA, pH 8.0). Retrieval buffer was
preheated to 65°C using Dako PT LINK™ (pre-treatment module; Dako, Denmark; #PT101). Slides were immersed in the buffer and the temperature raised to 97°C for 20 min. After returning to 65°C, slides were placed in the Dako AutostainerPlus™ and tissue was blocked using 10% normal horse serum (NHS) in 1 X phosphate buffered saline (PBS). The primary antibodies were applied for 1 h using 5% NHS as a diluent. A fluorescently labeled secondary antibody (Donkey anti-rabbit or mouse IgG (H+L) Secondary Antibody, AlexaFluor® 568 or 488 conjugate, Invitrogen, Vic, Australia; #A10042) was applied at 0.8 µg/ml for a further 1 h, using 1 X PBS + 1% bovine serum albumin (BSA; Sigma-Aldrich, NSW, Australia; #A2058) and 2% foetal bovine serum (FBS; Sigma-Aldrich, NSW, Australia; #F2442) as a diluent. Slides were washed using 1 X PBS, counterstained using 1 µg/ml 4’,6-diamidino-2-phenylidole (DAPI; Life Sciences, Vic, Australia; #D1306) and coverslipped using an aqueous mounting medium (Fluorshield™, Sigma Aldrich, NSW, Australia; #F6182). Negative controls had the primary antibody omitted. Slides were visualised using the SP5 Spectral Scanning Confocal Microscope (Leica, Wetzlar, Germany). Immunofluorescence was assessed qualitatively for staining intensity and distribution in a blinded fashion.

IF antibody details are as follows: claudin-1 (Abcam ab15098; 2 µg/ml; 1:100; AlexaFluor® anti-rabbit 568 nm); ZO-1 (Invitrogen 61-7300; 2.5 µg/ml; 1:100; AlexaFluor® anti-rabbit 568 nm); occludin (Invitrogen 33-1500; 5 µg/ml; 1:100; AlexaFluor® anti-mouse 488 nm).

7.3.5.2 Western blotting for tight junction proteins

*Protein extraction and quantification*

Total protein was isolated from jejunal, ileal and colonic tissue samples. Tissue samples (30 mg) were immersed in 300 µl of Radio-Immunoprecipitation Assay (RIPA) buffer containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 0.1% protease inhibitor cocktail and 50 mM Tris, pH 8.0 (Sigma-Aldrich, NSW, Australia; #R0278). Samples were homogenised using the QIAGEN Tissue Lyser LT (Qiagen, NSW, Australia) at 50 Hz for 5 min. Homogenates were centrifuged at 11,000 g for 15 min at 4°C, and supernatant was collected, aliquoted and stored at -80°C. Total protein was quantified using the Pierce™ BCA Protein
Quantification Kit (ThermoFisher Scientific, Vic, Australia; #23225) relative to a 8-point standard curve (25 µg/ml – 2 mg/ml).

Western blot

Total protein lysates were quantified and 30 µg supplemented with 4 µl Bolt® reducing agent (ThermoFisher Scientific, Vic, Australia; #B0008) and 10 µl Bolt® lithium dodecyl sulfate (LDS) sample buffer (ThermoFisher Scientific, Vic, Australia; #B0007). The total volume was then adjusted to 40 µl with milliQ water. Samples were denatured for 10 min at 70°C before being loaded into precast Bolt® 4-12% Bis Tris Plus, SDS-PAGE Gels (12-well) (ThermoFisher Scientific, Vic, Australia; #NW04122BOX). Samples were separated using the Bolt® mini gel tank (ThermoFisher Scientific, Vic, Australia; #A25977) at 150 V for 45 min. Proteins were transferred to a polyvinylidene difluoride membrane using the iBlot® transfer stacks (ThermoFisher Scientific, Vic, Australia; #IB24002) and compatible iBlot® 2 gel transfer device (ThermoFisher Scientific, Vic, Australia; #IB21001). The membrane was washed with 1 X tris-buffered saline and Tween20 (TBST) and stained with Ponceau S red staining solution (Sigma-Aldrich, NSW, Australia; #09276) to confirm equal loading. The membrane was blocked and probed with primary and secondary antibodies using the iBind™ western device (ThermoFisher Scientific, Vic, Australia; #SLF1000) as per manufacturers guidelines. Western blots were assessed using ImageStudio® Lite, version 4.0. Signal intensity was determined relative to local background. Data were presented relative to loading control (GAPDH) and vehicle treated controls.

Antibody details are as follows: claudin-1 (Invitrogen 51-9000; 5 µg/ml; 1:50; IRDye® anti-rabbit 800 CW); ZO-1 (Invitrogen 61-7300; 6.25 µg/ml; 1:40; IRDye® anti-rabbit 800 CW); occludin (Invitrogen 71-1500; 1.25 µg/ml; 1:400; IRDye® anti-rabbit 800 CW); GAPDH (Abcam ab15822; 0.5 µg/ml; 1:2000; IRDye® anti-chicken 680 CW).
Table 7.1 Antibody specification and application.

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<td>2.5 µg/ml (1:200)</td>
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7.3.5.3 RT-PCR for tight junction proteins

RNA extraction and quantification

Total RNA was isolated from jejunal, ileal and colonic mucosal scrapings samples using the Macherey-Nagel NuceloSpin® RNA/Protein purification kit as per manufacturer’s instructions (Macherey-Nagel, Düren, Germany; #740933.250). Once eluted, RNA was stored at -80 °C. Total RNA was quantified using the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA), TAKE3 plate and Gen5 (version 2.00.18) software. RNA purity was also determined using the the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA), TAKE3 plate and Gen5 (version 2.00.18) software.

Reverse transcription and RT-PCR

500 ng of total RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad, NSW, Australia; #1708890) as per manufacturers instructions. RT-PCR was performed using the Rotor-Gene 3000 (Corbett Research, NSW, Australia). Amplification mixes contained 1-2 µl of cDNA sample (100 ng/µl), 5 µl of SYBR green fluorescence dye, 2-3 µl of RNase-free water and 0.5 µl of each forward and reverse primers, prediluted to 50 pmol/µl, to make a total volume of 10 µl.
Chapter 7 Claudin-1 internalisation drives TLR4-dependent barrier dysfunction

Primer details and RT-PCR conditions are as follows:

<table>
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<th>Target</th>
<th>Forward 5'-3'</th>
<th>Length (bp)</th>
<th>Tm (°C)</th>
<th>Reference</th>
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| Mouse Occludin NM_008756.2 | ACTGGGTCAGGGAATATCCA
                               | TCAGCAGCAGCCATGTACTC                  | 192        | 52; 54               | Nevado et al., 2014 |
| Mouse ZO-1 NM_001163574.1  | ACTCCCACTTCCCCCAAAAAC
                               | CACAGCTGAAGGACTCACA                    | 166        | 52; 54               | Nevado et al., 2014 |
| Mouse Claudin-1 NM_016674.4 | QIAGEN Mm_Cldn1_1_SG QuantiTect Primer Assay | -          | 55       | Cat No. QT00159278   |
| Mouse 18S rRNA NR_003278.3  | TCGGAACTGAGGCCATGATT
                               | TTTGCCTGTCGGTGTCCTTGG                  | 100        | 52; 54               | Sakai et al., 2014   |
| Mouse GAPDH NM_008084.2  | CCTCGTCCCCGTAAGACAAAAATG
                               | TCTCACCCTTTGCACCTGCAA                  | 100        | 54; 52               | Sakai et al., 2014   |

Thermal cycling conditions for ZO-1 and occludin included a denaturing step at 95 °C for 15 min, followed by 45 cycles of denaturation at 95°C for 10 s, annealing at 52 °C for 15 s and extension at 72 °C for 20 s. Claudin-1 cycling conditions were as follows: denaturing step at 95 °C for 15 min, followed by 45 cycles of denaturation at 94°C for 15s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s. All samples were run in triplicate with the inclusion of blank, negative controls.

Primer efficiency was evaluated using standard curves and experimental threshold (C_T) values were calculated by the Rotor Gene 6 programme. C_T values were used to quantify relative mRNA expression of each tight junction protein using the ΔC_T method, where relative expression = 2^(-[C_T(target)–C_T(housekeeper)]). Data are presented as relative mRNA expression to two housekeeping (reference) genes (18S, GAPDH). 18S and GAPDH were assessed for their suitability as housekeeping/reference genes using the ΔC_T method (Frank et al., 2015).

7.3.6 Electrophysiological analysis using Ussing chambers

Immediately following dissection, samples of distal colon were mounted into Ussing chambers (Physiologic Instruments, CA, USA; #EM-CSYS-8) for electrophysiological analyses. Briefly, the colon was cut longitudinally along the mesenteric attachment and placed onto a 0.1 cm² aperture slider (Physiologic Instruments, CA, USA; #P2303A). The tissue was mounted and continually bathed in an oxygenated, glucose-fortified Ringer’s solution at 37°C with the following composition

151
(composition in mM/L: NaCl 115.4; KCl 5; MgCl₂ 1.2; NaH₂PO₄ 0.6; NaHCO₃ 25; CaCl₂ 1.2 and glucose 10). Tissues were voltage-clamped to zero potential difference by the application of short-circuit current (Isc) and baseline was established. Tissues were allowed to equilibrate for 20 min and baseline Isc (µA/cm²) and conductance (S/cm²) were recorded. Tissues were pretreated with amiloride (20 µM) to inhibit the apical epithelial sodium channel before being treated with forskolin (adenosine 3’5’-cyclic monophosphate agonist; 10 µM) and carbachol (Ca²⁺ agonist; 100 µM), applied to the apical chamber. The Isc response was then measured, determined as the change in Isc following agonist administration (ΔµA/cm²), representing electrogenic chloride secretion.

7.3.7 Statistical analysis

Data were compared using Prism version 7.0 (GraphPad® Software, San Diego, USA). A D’Agostino-Pearson omnibus test was used to assess normality. When normality was confirmed, a two-way analysis of variance (ANOVA) with appropriate post hoc testing were performed to identify statistical significance between groups. In other cases, a Kruskal-Wallis test with Dunn’s multiple comparisons test and Bonferroni correction was performed. Diarrhoea data was assessed using a Chi-square test (Bland, 2005). A p-value of <0.05 was considered significant.
7.4 Results

7.4.1 Cytoplasmic redistribution of claudin-1 contributes to TLR4-dependent barrier disruption

7.4.1.1 Genetic deletion of TLR4 does not affect morphology of tight junction proteins

Qualitative analysis of IF for tight junction proteins showed membranous staining for all tight junction proteins in vehicle treated WT and BALB/c-Tlr4<sup>−/−</sup> mice. No apparent differences were noted in any tight junction protein between genotypes receiving vehicle (Figure 7.1-7.3; vehicle control panels).

7.4.1.2 Claudin-1 undergoes cytoplasmic translocation in response to irinotecan

Claudin-1 staining presented with sharp apical intensities (Figure 7.1B/H; arrow heads; red staining) and membranous staining down the apico-lateral border of the enterocyte. Marked claudin-1 internalization was evident at 24 h after irinotecan treatment in WT mice (Figure 7.1C/D, red staining; arrows). This was particularly evident in the ileum (Figure 7.1C/D), with complete loss of membranous staining specificity in some areas. Claudin-1 internalization was seen as uniform cytoplasmic staining. Evidence of claudin-1 internalization was also seen in the colon of WT animals following irinotecan treatment, characterised by cytoplasmic staining and loss of membrane specificity (Figure 7.1K/L). The degree of claudin-1 redistribution was comparatively less in BALB/c-Tlr4<sup>−/−</sup> mice treated with irinotecan in both gut regions. Membranous staining remained intact, although loss of apical staining intensity was evident (Figure 7.1G/H, O/P).

7.4.1.3 Irinotecan causes focal areas of ZO-1 and occludin proteolysis

IF staining for ZO-1 (Figure 7.2; red staining) showed focal areas of proteolysis in the ileum (Figure 7.2D; arrow heads) and colon (Figure 7.2L; arrow heads) of WT mice 24 h post-irinotecan. These focal areas of protein disruption were particularly evident in areas of epithelial injury (identified in our previous study (Wardill et al., 2016a)), often occurring alongside phenotypically normal tight junction staining (Figure 7.2D/L; arrows). Similar changes in occludin expression were also seen in
the ileum and colon of WT mice, with focal areas of proteolysis corresponding with frank epithelial damage (Figure 7.3D/L, arrow head; green staining). Staining appeared uniform for BALB/c-*Tlr4*<sup>−/−</sup> mice treated with irinotecan (Figure 7.2G/H, O/P; Figure 7.3G/H, O/P).

7.4.1.4 Western blotting shows no change in total protein expression of claudin-1, ZO-1 and occludin

Despite significant redistribution of claudin-1 and focal areas of ZO-1/occludin disruption following irinotecan treatment, western blot analysis revealed no quantitative changes in tight junction protein expression in any mouse group (Figure 7.4).

7.4.1.5 RT-PCR shows no change in the mRNA expression of tight junction proteins

Average RNA yield using the Macherey-Nagel NuceloSpin® Protein/RNA isolation kit was 464.44±276.47 ng/µl with an average $A_{260/280}$ of 2.07±0.06. cDNA conversion resulted in an average yield of 2077.69±347.44 ng/µl with an average $A_{260/280}$ of 1.76±0.11. Linear regression slope analysis revealed suitable primer efficiencies for ZO-1 (relative to 18S: 0.083; relative to GAPDH -0.080) and occludin (relative to 18S: -0.084; relative to GAPDH -0.080). Linear regression slope analysis for claudin-1 showed disparate primer efficiencies relative to each housekeeper. Housekeeping stability was analysed using the $\Delta C_{T}$ method, where relative expression = $2^{-[C_{T}(HK\ in \ controls) - C_{T}(HK\ in \ treated)]}$. 18S was eliminated as a suitable housekeeper based on differential expression in vehicle- and irinotecan-treated mice (data not shown).

Data showed no change in the relative mRNA expression of occludin or claudin-1 in WT or BALB/c-*Tlr4*<sup>−/−</sup> mice following irinotecan treatment (Figure 7.5). $\Delta C_{T}$ analysis revealed a significant decrease in the mRNA expression of ZO-1 (relative to GAPDH) in the colon 24 h after irinotecan treatment in WT mice (Figure 7.5H).
Figure 7.1 Representative images of claudin-1 immunofluorescence in the ileum and colon of vehicle- and irinotecan-treated WT and BALB/c-Tlr4<sup>−/−</sup> mice at 24 h. Claudin-1 staining was evident at the apico-lateral boundaries of epithelial cells, with distinct apical intensities (Panel B and F, arrow heads). This was seen in vehicle-treated WT and BALB/c-Tlr4<sup>−/−</sup> mice. No distinct changes in staining intensity were identified, although tight junction abnormalities typified by claudin-1 internalisation were seen in the ileum (Panel C/D; red staining) and colon (Panel K/L) of irinotecan treated WT. BALB/c-Tlr4<sup>−/−</sup> mice show only mild changes in staining following irinotecan, maintaining membrane specificity. Sections of ileum and colon were stained with a primary antibody to claudin-1 and visualised using an AlexaFluor anti-rabbit (680 nm, red). Blue counterstaining (DAPI, 405 nm) shows nuclei. GC = goblet cell. Original magnification 40 X.
Figure 7.2 Representative images of ZO-1 immunofluorescence in the ileum and colon of vehicle- and irinotecan-treated WT and BALB/c-Tlr4<sup>−/−</sup> mice at 24 h. Vehicle-treated mice showed phenotypically normal tight junctions, with apical ZO-1 staining (Panel A/B, E/F, I/J, M/N). No differences were seen between vehicle-treated WT and BALB/c-Tlr4<sup>−/−</sup> mice. Irinotecan-treated WT mice displayed focal areas of ZO-1 disruption, particularly in areas of epithelial injury (Panel C-D, K-L; arrow heads). These were found alongside areas of phenotypically normal ZO-1 staining (Panel K-L; arrows). Mild internalisation was also seen in WT mice (Panel D, arrow). BALB/c-Tlr4<sup>−/−</sup> mice showed no change in staining following irinotecan. Sections of ileum and colon were stained with a primary antibody to ZO-1 and visualised using an AlexaFluor anti-rabbit (680 nm, red). Blue counterstaining (DAPI, 405 nm) shows nuclei. Original magnification 40 X.
Figure 7.3 Representative images of occludin immunofluorescence in the ileum and colon of vehicle- and irinotecan-treated WT and BALB/c-Tlr4−/− mice at 24 h. Vehicle-treated mice showed phenotypically normal tight junctions, with apical occludin staining (Panel A-B, E-F, I-J, M-N). No differences were seen between vehicle-treated WT and BALB/c-Tlr4−/− mice. Like ZO-1, irinotecan-treated WT mice displayed focal areas of occludin disruption, particularly in areas of epithelial injury (Panel C-D, K-L; arrow heads). These were found alongside areas of phenotypically normal ZO-1 staining (Panel K-L; arrows). BALB/c-Tlr4−/− mice showed no change in staining intensity. Sections of ileum and colon were stained with a primary antibody to occludin and visualised using an AlexaFluor anti-mouse (800 nm, red). Blue counterstaining (DAPI, 405 nm) shows nuclei. PC = plasma cell; RBC = red blood cells. Original magnification 40 X.
Figure 7.4 Relative protein expression of claudin-1, ZO-1 and occludin in jejunal, ileal and colonic segments of vehicle- and irinotecan-treated BALB/c mice at 24 h. Protein expression was determined using ImageStudio® Lite software version 4.0 and represented as relative signal intensity (/GAPDH/vehicle controls). No changes were seen in occludin, ZO-1 or claudin-1 in any mouse groups following irinotecan treatment. Representative bands are shown from the ileum for occludin (Panel J), ZO-1 (Panel K) and claudin-1 (Panel L). Data are presented as mean±SEM and were analysed using a one-way ANOVA with Tukey’s post-hoc. A p-value of <0.05 was considered statistically significant.
Figure 7.5 Relative mRNA expression of claudin-1, ZO-1 and occludin in the jejunum (A-C), ileum (D-F) and colon (G-I) in vehicle- and irinotecan-treated BALB/c mice. mRNA expression presented relative to GAPDH, an internal housekeeping gene. No changes were seen in occludin or claudin-1 in any animals following irinotecan treatment. There was a decrease in ZO-1 mRNA expression (relative to GAPDH) in the colon of WT mice 24 h after irinotecan treatment (Panel H). Data are presented as median±IQR and were analysed using a one-way ANOVA with Tukey’s post-hoc. A p-value of <0.05 was considered statistically significant.
7.4.2 Irinotecan increases chloride secretion in the distal colon via TLR4-independent mechanisms

Ussing chamber studies indicated changes in baseline short-circuit current (Isc) and conductance in irinotecan-treated WT mice (Figure 7.6A-D). There was a significant difference in baseline Isc between WT and BALB/c-Tlr4−/−billy mice 24 h after irinotecan treatment (Figure 7.6A; WT 103.65±36.32 μA/cm²; BALB/c-Tlr4−/−billy 28.6727±10.03; *p=0.0168). Increased baseline conductance, a measure of paracellular permeability, was seen in WT mice at 24 h post-irinotecan treatment (Figure 7.6B/D; 53.19±6.46 S/cm², +105.62% relative to WT vehicle controls; #p=0.0008). There was no change seen in BALB/c-Tlr4−/−billy mice at any time after irinotecan compared to vehicle controls.

WT and BALB/c-Tlr4−/−billy mice exhibited increased ΔIsc after carbachol administration 24 h (Figure 7.7A; WT 100.35±18.37 μA/cm²; #p=0.022; BALB/c-Tlr4−/−billy 102.72±18.80 μA/cm²; ^p=0.023) and WT at 48 h (99.75±25.22 μA/cm²; #p=0.0244) after irinotecan. Irinotecan-treated WT mice also showed increased ΔIsc in response to forskolin at 72 h (82.18±16.54 μA/cm²; #p=0.025). No change was seen in irinotecan-treated BALB/c-Tlr4−/−billy mice in response to forskolin compared to vehicle controls (65.60±18.28 μA/cm²; p=0.999). Unstimulated (baseline) current and secretory responses to both carbachol and forskolin did not correlate with diarrhoea severity (Figure 7.7C/D; baseline r²=0.08, carbachol r²=0.04, forskolin r²=0.04). # denotes a change relative to untreated vehicle controls in WT mice; ^ denotes a change relative to untreated vehicle controls in BALB/c-Tlr4−/−billy mice; * denotes a significant difference between WT and BALB/c-Tlr4−/−billy mice, where p<0.05.
Figure 7.6 Baseline short-circuit current (Isc) and conductance of the distal colon at 24 h.

Segments of the distal colon were dissected and opened longitudinally, before being mounted into Ussing chambers. Increased baseline Isc was seen in WT mice compared to BALB/c-Tlr4−/− mice at 24 h (A; *p=0.0168), indicative of a pro-diarrhoea state. Increased conductance was also seen in WT mice 24 h after irinotecan treatment (B; #p=0.0008). # denotes a change relative to untreated vehicle controls in WT mice; # denotes a change relative to untreated vehicle controls in BALB/c mice; * denotes a significant difference between WT and BALB/c-Tlr4−/− mice, where p<0.05.

Data has been presented as absolute values (A/B) and baseline corrected (% relative to vehicle controls; C/D) to account for baseline differences between mouse groups. Data presented as mean±SEM. A two-way ANOVA with Tukey’s post-hoc was performed to identify statistical significance where p<0.05.
Figure 7.7 Change in short-circuit current (ΔIsc) in response to carbachol (A), a Ca\(^{2+}\) agonist, and forskolin (B), an adenosine 3’5’-cyclic monophosphate agonist. Irinotecan treatment elevated the response to carbachol in irinotecan-treated WT and BALB/c-\(\text{Tlr4}^{-/-}\) mice at 24 h (WT \#p=0.0222, BALB/c-\(\text{Tlr4}^{-/-}\) \(^{*}\)p=0.0229) and only WT at 48 h (#p=0.0244; Panel A) compared to their respective vehicle controls. The change in Isc following administration of forskolin was elevated in WT mice at 72 h post-treatment (#p=0.025; Panel B) compared to vehicle controls. No differences were seen between WT and BALB/c-\(\text{Tlr4}^{-/-}\) mice in response to either agonist at any time point. Data presented as mean±SEM. A two-way ANOVA with Tukey’s post-hoc was performed to identify statistical significance, where p<0.05. Correlation between stimulated current (Panel C) or unstimulated current (Panel D) and diarrhoea severity. A linear regression model was applied to the data sets and r\(^2\) values calculated.
7.5 Discussion

Irinotecan is a commonly prescribed chemotherapeutic agent, however its therapeutic efficacy is often limited by its severe gastrointestinal side effects, notably late-onset diarrhoea (Ikuno et al., 1995). Diarrhoea significantly impacts on patients’ clinical outcomes (Carlutto et al., 2013), however the underlying mechanisms remain unclear and therapeutic interventions limited (Bowen et al., 2013b). It is becoming increasingly clear that irinotecan causes significant intestinal barrier disruption, characterised by increased paracellular permeability and tight junction breakdown (Nakao et al., 2012, Wardill et al., 2016a). Our previous research has shown that BALB/c-\textit{Tlr4}^{-/-}\textit{billy} mice are protected from developing irinotecan-induced intestinal barrier disruption, supporting the idea that TLR4-dependent mechanisms are critical in tight junction disruption (Wardill et al., 2016a). Results from the current study support this hypothesis, showing improved tight junction integrity in irinotecan-treated BALB/c-\textit{Tlr4}^{-/-}\textit{billy} mice compared to their WT counterparts. This is the first study to compare functional in vivo permeability, ex vivo electrogenic measures of barrier function and morphological assessment of tight junction proteins in the setting of chemotherapy-induced barrier dysfunction.

Tight junctions are critical in maintaining gastrointestinal health and homeostasis. Despite this, they are highly plastic structures vulnerable to post-transcriptional and -translational modification by a variety of pathological cues (Gonzalez-Mariscal et al., 2008, Khan and Asif, 2015). Tight junction disruption has been identified following treatment with a number of chemotherapeutic agents, both preclinically (Nakao et al., 2012, Wardill et al., 2013) and clinically (Keefe et al., 1997, Blijlevens et al., 2005a, Wardill et al., 2016c), however the mechanisms that underpin their breakdown remain unclear. To date, many studies have shown architectural abnormalities (Keefe et al., 1997), functional alterations (Blijlevens et al., 2005a, Lutgens et al., 2005) and downregulation of key tight junction proteins such as claudin-1, ZO-1 and occludin (Hamada et al., 2010, Nakao et al., 2012). The present study did not identify any alterations in the protein expression of these tight junction units in any region of the gut. Instead, significant derangement of these proteins was identified,
Chapter 7 Claudin-1 internalisation drives TLR4-dependent barrier dysfunction

characterised by severe cytoplasmic redistribution and disassembly of the tight junction unit. Internalization of tight junction proteins is well recognised to contribute to poor barrier function and loss of tight junction apposition (Gonzalez-Mariscal et al., 2008). In the current study, cytoplasmic redistribution of claudin-1 was seen at 24 h; where peak barrier dysfunction and diarrhoea were detected.

7.5.1 TLR4-dependent mechanisms for tight junction disruption

In addition to demonstrating tight junction disruption in response to irinotecan, this study has also provided evidence implicating TLR4-dependent mechanisms in tight junction regulation. This is a growing area of research, with the interaction between TLR4 and its ligand, LPS, as well as its powerful downstream effects on inflammation providing a strong rationale for its involvement. Evidence suggests that LPS/TLR4-dependent tight junction disruption occurs via direct epithelial processes (Guo et al., 2015). This has been shown using in vitro models of LPS-induced barrier dysfunction, with LPS administration resulting in TLR4-dependent activation of focal adhesion kinase (FAK) and tight junction disruption in Caco-2 monolayers. Importantly, small interfering (si) RNA silencing of TLR4 prevented LPS-induced disruption. This was also confirmed with in vivo, intestinal epithelial-specific knockdown of TLR4.

Despite a growing body of evidence showing direct TLR4-mediated regulation of tight junctions, it is likely that this mechanism occurs in concert with cytokine-mediated tight junction disruption. A large body of evidence exists supporting a role for proinflammatory cytokine-mediated tight junction disruption (Gonzalez-Mariscal et al., 2008, Schulzke et al., 2009) explaining the observation of intestinal barrier disruption in diseases characterised by inflammation (Schmitz et al., 1999, Xu et al., 2016). Conversely, evidence also suggests that loss of anti-inflammatory cytokines can be detrimental in regulation of barrier function (Contreras-Ruiz et al., 2012, Foti Cuzzola et al., 2013). This molecular crosstalk between inflammatory cytokines and tight junctions is compelling with regards to irinotecan-induced gut toxicity as peak interleukin(IL)-1β, interferon (IFN)γ and tumour necrosis factor (TNF) levels coincide with intestinal barrier dysfunction and cytoplasmic
redistribution of claudin-1 in WT mice treated with irinotecan (Wardill et al., 2016a). Although associative links have been identified between a proinflammatory state and tight junction disruption, much of the research has been conducted in in vitro models as in vivo models can be complicated by cytokine-dependent immune cell recruitment and activation within the mucosa (Edelblum and Turner, 2009).

A particularly strong case for IL-1β-mediated tight junction disruption exists given TLR4 acts as its main upstream regulator (De Nardo, 2015). Addition of IL-1β to growth media has been shown to directly increase epithelial permeability and decreased expression of key tight junction proteins in Caco-2 cells (representative of the small intestinal epithelium) (Al-Sadi et al., 2008). Similarly, TNF has been shown to directly impair tight junction function through actin and protein rearrangement (Mullin and Snock, 1990). Treatment of Caco-2 cells with TNF has been shown to cause a time-dependent increase in permeability, paralleled by decreased expression and altered distribution of tight junction proteins (Ma et al., 2004). Importantly, Fish et al., (1999) suggest that TNF acts synergistically with IFNγ, both of which were shown to be upregulated following irinotecan treatment (Fish et al., 1999). IFNγ alone has also been recognised for its effects on tight junction proteins, causing disassembly and redistribution of occludin and claudin-1 through increased Rho-associated kinase and subsequent phosphorylation of myosin II regulatory light chain (MLC) (Utech et al., 2005). Given that BALB/c-Tlr4<sup>−/−</sup> mice showed no significant change in IL-1β, TNF and IFNγ (Wardill et al., 2016a), it is possible that proinflammatory cytokine production drives barrier dysfunction in a TLR4-dependent manner. Further, the barrier protective effects of anti-inflammatory cytokines, such as IL-10, also warrant further investigation as research suggests these cytokines are downregulated following chemotherapy (Sultani et al., 2012).

7.5.2 Clinical implications of barrier dysfunction

The clinical consequences of intestinal barrier dysfunction are becoming increasingly recognised in the setting of chemotherapy-induced gut toxicity. We have shown significant barrier dysfunction at 24 h which precedes serum detection of LPS (Wardill et al., 2016a), supporting Blijlevens et al.,
(2000) who describes that loss of barrier function is critical in preventing secondary, systemic toxicity. LPS translocation has also been suggested to underpin cancer-related sickness behaviour (Hines et al., 2013) and secondary toxicities such as chemotherapy-induced pain (Wardill et al., 2015b). In addition to permitting LPS translocation, loss of barrier integrity has also been shown to result in bacterial translocation and colonisation in mesenteric lymph nodes and the spleen following treatment with irinotecan, thus increasing the risk for infection, graft versus host disease (Blijlevens et al., 2000) and sepsis (van der Velden et al., 2010). It is also likely that loss of paracellular integrity allows greater exposure, and subsequent activation, of the innate mucosal immune system, driving a heightened inflammatory response following cytotoxic insult. Barrier dysfunction is therefore likely to exacerbate direct cytotoxic injury in the gut, thus worsening clinical outcomes for patients. By understanding the mechanisms that lead to barrier dysfunction, therapeutic interventions may be targeted to prevent local toxicity transitioning to systemic toxicity, reducing the associated risk such as infection, sepsis and pain.

7.5.3 Contribution to diarrhoea

Our previous research shows that intestinal permeability best reflects the clinical progression of diarrhoea following irinotecan treatment (Wardill et al., 2016a). This parallels clinical findings in patients with inflammatory bowel disease, with barrier function and tight junction integrity correlating with the onset, severity and duration of diarrhoea (Bertiaux-Vandaele et al., 2011). A wealth of studies show associative links between barrier dysfunction and diarrhoea, however the mechanisms by which barrier dysfunction contributes to diarrhoea remain unclear. It has been suggested that passive ‘leak flux’ mechanisms may be involved (Schmitz et al., 1999), where loss of barrier integrity allows passive leakage of solute and water into the lumen of the gut (Schmitz et al., 1999). Results from the current study also show, for the first time, that irinotecan-induced diarrhoea has a secretory component to its pathophysiology. Electrophysiological studies using Ussing chambers showed hyper-responsiveness to both carbachol and forskolin following irinotecan, indicating that irinotecan promotes active chloride secretion in a TLR4-independent manner. This
suggests the presence of a chloride-dependent osmotic drive toward the intestinal lumen. Coupled with the backflow of solutes and water through unregulated barrier function, it is possible that both passive and active secretory processes may underpin diarrhoea development in cases of tight junction disruption. This would explain the disparity between chloride secretion and diarrhoea severity seen in this study. Given the significant differences in barrier function between WT and BALB/c-\textit{Tlr4}^{-/-}\textit{billy} mice we suggest the osmotic drive, established by chloride secretion, is exaggerated in cases of barrier dysfunction and although increased osmotic drive may be present in both animal groups, its effect on diarrhoea severity is highly dependent on the integrity of the mucosal barrier. Further investigation is now required to assess this hypothesis and dissect net ion/water movement in all regions of the gut. Additionally, conclusions can not be drawn regarding paracellular sodium movement – a key driver of paracellular water, regulated by the pore-forming claudin-2 protein (Goswami \textit{et al.}, 2014). Future studies should therefore focus on the synergism between barrier dysfunction, active chloride secretion and paracellular sodium/water fluxes in chemotherapy-induced diarrhoea.
Figure 7.8 Graphical representation of proposed synergism of TLR4-dependent barrier dysfunction and altered chloride secretion on the development of irinotecan-induced diarrhoea. Data show distinctly difference diarrhoea profiles in WT and BALB/c-Tlr4^{--} mice, yet comparable secretory profiles (green CaCC and CFTR channels). Given the vast differences in barrier function between genotypes, regulated by tight junctions, it is hypothesised that although this osmotic drive is present in both genotypes, its effect on diarrhoea is highly dependent on the integrity of the mucosal barrier.
7.6 Conclusion

Tight junction disruption is a hallmark trait of many pathological states. A wealth of research now implicates poor tight junction integrity following treatment with various chemotherapeutic agents. We have shown that irinotecan treatment causes tight junction disruption, characterised by claudin-1 internalization, and barrier dysfunction via TLR4-dependent mechanisms. We have outlined three mechanisms by which TLR4 may regulate tight junction disruption; 1) through direct epithelial events mediated through luminal LPS, 2) through its downstream effects on inflammation and 3) through the production of zonulin. In reality, it is likely that these mechanisms occur in concert with one another given the highly multifactorial biology of irinotecan-induced gut toxicity. Nonetheless, TLR4 appears to be critical in the development of barrier dysfunction, with overarching effects on gut toxicity. In addition, this study suggests that tight junctions contribute to irinotecan-induced diarrhoea through passive, leak-flux mechanism and active secretory processes. This study is the first to show upregulated chloride secretion following irinotecan treatment and provides a novel avenue for the treatment of irinotecan-induced diarrhoea.
# Chapter 8 *In vitro* model of SN-38-induced mucosal injury

## Statement of Authorship

<table>
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<th>A novel <em>in vitro</em> platform for the study of SN-38-induced mucosal damage and toll-like receptor 4-targeted therapeutic options.</th>
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<td>Publication Status</td>
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| Contribution to the Paper | I was responsible for experimental design, generation of the *in vitro* model, cytological analyses, data acquisition, data analysis and manuscript preparation |
| Overall percentage (%) | 90% |
| Certification: | This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper. |
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### Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

i. the candidate's stated contribution to the publication is accurate (as detailed above);  
ii. permission is granted for the candidate to include the publication in the thesis; and  
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Chapter 8 A novel in vitro platform for the study of SN-38-induced mucosal damage and the development of TLR4-targeted therapeutic options


8.1 Abstract

Tight junction and epithelial barrier disruption are common traits of many gastrointestinal pathologies, including chemotherapy-induced gut toxicity. Currently, there are no validated in vitro models suitable for the study of chemotherapy-induced mucosal damage that allow paralleled functional and structural analyses of tight junction integrity. We therefore aimed to determine if a transparent, polyester membrane insert supports a polarised T84 monolayer with the phenotypically normal tight junctions. T84 cells (passage 5-15) were seeded into either 0.6 cm$^2$, 0.4 µm pore mixed cellulose transwell hanging inserts, or 1.12 cm$^2$ 0.4 µm pore polyester transwell inserts at varying densities. Transepithelial electrical resistance (TEER) was measured daily to assess barrier formation. Immunofluorescence for key tight junction proteins (occludin, ZO-1, claudin-1) and transmission electron microscopy were performed to assess tight junction integrity, organelle distribution and polarity. RT-PCR was performed to determine expression of TLR4. Liquid chromatography was also conducted to assess SN-38 degradation in this model. Polyester membrane inserts support a polarised T84 phenotype with functional tight junctions in vitro. Transmission electron microscopy indicated polarity, with apico-laterally located tight junctions. Immunofluorescence showed membranous staining for all tight junction proteins. No internalisation was evident. T84 cells expressed Toll-like receptor 4 (TLR4), although this was significantly lower than levels seen in HT29 cells (p=0.0377). SN-38 underwent more rapid degradation in the presence of cells (-76.04±1.86%) compared to blank membrane (-48.39±4.01%), indicating metabolic processes. Polyester membrane inserts provide a novel platform for paralleled functional and structural analysis of tight junction integrity in T84 monolayers. T84 cells exhibit the unique ability to metabolise SN-38 as well as expressing TLR4, making this an excellent platform to study clinically relevant therapeutic interventions for SN-38-induced mucosal damage by targeting TLR4.
8.2 Introduction

The intestinal epithelium has two important and distinctly different roles within the gastrointestinal tract. It mediates the complex absorption of nutrients from the intestinal lumen, and simultaneously represents a barrier separating the internal milieu from the outside environment in both an immunologic and metabolic sense (Melichar et al., 2008). In the gut, the luminal surface comes into direct contact with the highest concentrations of bacteria (Ley et al., 2006), antigens and a host of potentially toxic compounds (Hollander, 1999). Consequently, the paradoxical functions of the intestinal barrier are critical in maintaining gastrointestinal health and homeostasis (Donato et al., 2011).

Tight junctions provide a paracellular barrier that is selectively permeable to ions and macromolecules. The molecular characteristics and functional properties of tight junctions are subject to modification by a variety of cues, both physiological and pathological, highlighting the highly dynamic nature of these structures (Wardill et al., 2013). Consequently, tight junction disruption often leads to the development of a leaky gut (Schulzke et al., 2009); a hallmark feature of compromised mucosal barrier function and many pathological states (Cummins, 2012). A thorough understanding of tight junction regulation, signalling and modification is therefore critical to determine how they may contribute to disease progression. This holds particularly true for chemotherapy-induced gut toxicity, which is characterised clinically by increased intestinal permeability (Keefe et al., 1997) and tight junction defects (Wardill and Bowen, 2013, Wardill et al., 2016c). Despite molecular disruption to tight junctions being reported, the underlying mechanisms are unclear and difficult to identify in full physiological systems.

Chemotherapy treatment has long been recognised to induce a leaky gut, with recent research suggesting tight junction disruption may contribute to the development of clinically diagnosed diarrhoea through altered leak-flux mechanisms (Wardill et al., 2012). Irinotecan is a chemotherapeutic drug associated with exceptionally high levels of intestinal toxicity. It serves as the water-soluble precursor of the lipophilic metabolite, SN-38, which is formed by
carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin moiety and the dipiperdino side chain (Umezawa et al., 2000). SN-38 is approximately 1000 times as potent as irinotecan as an inhibitor of topoisomerase I and its unique hepatobiliary metabolism is responsible for the high levels of intestinal toxicity (Gibson et al., 2007). SN-38 is glucuronidated to SN-38 glucuronide (SN-38G) and detoxified in the liver via conjugation by the uridine-diphospho-glucuronosyl transferase (UGT1A) family, which releases SN-38G into the intestines for elimination (Araki et al., 1993). However, in the intestinal lumen, bacterial β-glucoronidases are able to regenerate SN-38 from SN-38G (Pedroso et al., 2015). This second pass metabolism is key to the dose-limiting, and clinically diagnosed, diarrhoea associated with irinotecan treatment.

Although our understanding of irinotecan and SN-38-induced gastrointestinal toxicity is improving, many studies’ mechanistic data are limited to due the difficulties in accessing the GIT. In vitro models therefore offer an appealing alternative of studying gastrointestinal-related pathologies. Epithelial cell lines derived from the intestine can be cultured as monolayers to mimic the intestinal epithelium and provide insight into the physiological characteristics of tight junctions and epithelial barrier function (Donato et al., 2011). The human colonic epithelial cell line, T84, derived from a colonic carcinoma, is widely used in vitro to assess intestinal barrier function and tight junction integrity (Cartwright et al., 1984, Dharmsathaphorn et al., 1984).

T84 cells are typically cultured in vitro using a mixed-cellulose membrane, semi-permeable transport system (Figure 8.1), which readily allows assessment of barrier function. However, these opaque mixed-cellulose membranes, although considered gold-standard, limit downstream imaging techniques such as light or confocal microscopy, critical for a thorough understanding of tight junction signalling and integrity. This study therefore aimed to determine if a transparent, polyester membrane insert supports a polarised T84 monolayer with phenotypically normal tight junctions. The long-term goal is for this model to be used for interrogation of complex gastrointestinal physiology under normal and challenged states. If successful, this model will be used to study mechanisms of chemotherapy (specifically irinotecan)-induced mucosal damage in a simple, high-
throughput manner. Additionally, this novel platform could be used to assess the efficacy of anti-mucotoxic agents for both preclinical and clinical translation.
8.3 Materials and Methods

8.3.1 Cell culture

Cryopreserved T84 cells (passage 5-15) derived from a human colorectal carcinoma were obtained from Culture Collections (Porton Down, UK; #88021101). HT29 cells (passage 5-15), derived from a human colorectal carcinoma with an epithelial phenotype, were kindly provided by Dr J Hardingham (Queen Elizabeth Hospital, South Australia). MCF-7 cells (passage 1-6), derived from a human breast carcinoma were also a kind gift from Professor M Brown (Royal Adelaide Hospital, South Australia). All cell lines retained their original morphology and growth characteristics over the range of passages used (data not shown).

Cells were thawed in a 37°C water bath and maintained in a 75 cm² or 150 cm² sterile cell culture flask (Corning Life Sciences, MA, USA) at 37°C with 5% CO₂. T84 and HT29 cell culture media was Dulbecco’s Modified Eagle Medium(DMEM)/Ham’s F-12 Nutrient Mixture containing 15 mM HEPES, L-glutamine and sodium bicarbonate (DMEM/F-12; Sigma-Aldrich, Castle Hill, NSW, Australia; #D8437) supplemented with 1% penicillin/gentamicin+fungizone, 10% foetal bovine serum and 2 mM L-glutamine (complete DMEM). MCF-7 cells were maintained in RMPI media supplemented with 2 mM L-glutamine and 10% FBS. Experimental cell cultures were grown in sterile, multi-well tissue culture plates under identical growth conditions. Cell lines were routinely passaged when culture monolayers reached approximately 80% confluence at subculture ratios between 1:3 and 1:6 in fresh growth medium. Cells were detached by aspirating growth medium, washing with 1X phosphate buffered saline (PBS; pH 7.4) and incubating with 3 ml of trypsin-EDTA for 10 min at 37°C (0.05% trypsin, 0.53 mM EDTA; Invitrogen, Mulgrave, VIC, Australia). The reaction was then quenched by the addition of growth medium. Cells were centrifuged at 300 g for 5 min, supernatant removed and cells resuspended in fresh, complete DMEM. Cell count were conducted using an automated cell counter (BioRad, NSA, Australia) and were seeded into either 0.6 cm², 0.4 µm pore mixed cellulose transwell hanging inserts (Invitrogen, VIC, Australia; #PIHA01250), or 1.12 cm² 0.4 µm pore polyester transwell inserts (Corning Life Sciences, MA,
USA; #CLS3801). T84 cells were seeded at the following densities: $5 \times 10^4$/cm$^2$; $1 \times 10^5$/cm$^2$; $2 \times 10^5$/cm$^2$ and $4 \times 10^5$/cm$^2$ into the apical chamber. Cell culture media in both the apical and basolateral chambers was changed every 48 h. Transepithelial electrical resistance (TEER) was measured daily using an EVOM2 epithelial volt-ohm-meter with chopstick electrodes (World Precision Instruments, Sarasota, FL, USA) for 1 week during the growth period and area adjusted for analysis using the following formula; $\text{TEER monolayer (}$ cm$^{-2}$) = [raw TEER (}$ \Omega$) – TEER blank (}$ \Omega$)/area of membrane (cm$^2$). All experiments were performed in triplicate and repeated twice.
Figure 8.1 (A) Permeable transport support system. This system consists of an inner chamber allowing the T84 monolayer to be suspended in a supportive media (DMEM/Ham’s/F12 + 1% penicillin/gentamicin + fungizone; 2 mM L-glutamine; 10% FBS). (B) Electric volt ohm-metre. The electrodes of an electric volt ohm-meter pass current and measure voltage to determine the transepithelial resistance of the monolayer (ohms). For reproducible results, the position and stability of the electrodes must remain consistent.
8.3.2 Transmission electron microscopy

After determining optimal cell density (1x10^5/cm²), cells were seeded into both 0.6 cm², 0.4 μm pore mixed cellulose transwell hanging inserts (Invitrogen, VIC, Australia; #PIHA01250), or 1.12 cm² 0.4 μm pore polyester transwell inserts (Corning Life Sciences, MA, USA; #CLS3801) at a density of 1x10^5/cm². Cell culture media was changed every 48 hours. TEER was measured daily from day 3 using an EVOM2 epithelial volt-ohm-meter. Once high, stable TEER was achieved monolayers were fixed overnight in 4% paraformaldehyde/1.25% glutaraldehyde (electron microscopy grade) in 1 X PBS (4% sucrose; pH 7.2). Monolayers were washed with 1x PBS + 4% sucrose (v/v) before being post-fixed in 2% osmium tetroxide (w/v) for 1 hour. Monolayers were dehydrated through graded ethanols, removed from the transwell support system and mounted in resin before being polymerised at 70°C for 24 hours. 80μm thick sections were cut on a Leica Ultracut S ultramicrotome using a diamond knife. Sections were picked up on 200mesh copper/palladium grids and stained with uranyl acetate and lead citrate. Grids were then visualised using the Philips CM200 transmission electron microscope (TEM). Monolayers were assessed for the presence of tight junctions, organelle distribution, polarity and monolayer formation. Monolayers with TEER values over 1000 Ω/cm² were used in all experiments.

8.3.3 Immunofluorescence for tight junction proteins

A secondary aim of this study was to determine if the polyester membrane transwell support systems support immunofluorescence and confocal imaging. T84 cells were seeded into 1.12 cm² 0.4 μm pore polyester transwell inserts at a density of 1x10^5/cm². Once T84 monolayers had developed stable TEER values >1000 Ω/cm² cell culture media was aspirated and cells washed with ice-cold 1X PBS pH 7.4. A fixing solution (1:1 v/v acetone/methanol stored at -20°C) was applied to the apical chamber (500 μl) for 15 min. Cells were rinsed with 1X PBS and permeabilised using 0.1% (v/v) Triton X-100/PBS for 3 min. After 2 x 5 min washes in 1X PBS, cells were blocked overnight in 3% (w/v) bovine serum albumin (BSA)/PBS at 4°C (Sigma-Aldrich, NSW, Australia; #9048-46-8). The blocking solution was aspirated and 200 μl of primary antibody was applied to the apical
Chapter 8 *In vitro* model of SN-38-induced mucosal injury

chamber (mouse mAb occludin, Invitrogen #33-1500, 5 µg/ml (1:100); rabbit pAb ZO-1, Invitrogen #61-7300, 2.5 µg/ml (1:100); rabbit pAb claudin-1, Abcam #ab15098, 2 µg/ml (1:100)). All primary antibodies were diluted in 1% (w/v) BSA/PBS and incubated for 1 h at room temperature. The primary antibody was then aspirated, and cells washed in 1X PBS+0.05% Tween (4 x 5 min). 200 µl of fluorescent-conjugated secondary antibody (anti-mouse 488; anti-rabbit 568 Alexa Fluor®; Invitrogen, VIC, Australia; #A28175, #A-11011) was added to the apical chamber at 100 µg/ml (1:200). All secondary antibodies were diluted in 1% (w/v) BSA/PBS and incubated for 1 hour at room temperature. For nuclear staining, cells were incubated with 1 µg/ml 4’,6-diamidino-2-phenylidole (DAPI) for 10 min at room temperature. Cells were washed in 1X PBS+0.05 Tween for 4 x 5 min before the membranes were removed from the inserts and mounted onto glass microscope slides using Fluoroshield™ (Sigma-Aldrich, NSW, Australia; #F6182). Cells were visualised using the SP5 Spectral Scanning Confocal Microscope (Leica, Wetzlar, Germany). Negative controls had the primary antibody omitted.

**8.3.4 Liquid chromatography-mass spectrometry**

The long-term goal is for this *in vitro* model to be used to assess the mechanisms involved in SN-38-induced mucosal damage to identify potential targets for the development of interventions. It is well recognised that SN-38, the active metabolite of irinotecan, is a basic compound and typically unstable in many physiological solutions. It is therefore important to characterise the degradation and potential metabolism of SN-38 in this model prior to implementing its usage. As per section 7.2.3, cells were seeded in triplicate into polyester membrane transwell support systems and TEER monitored daily. When a stable TEER >1000 Ω/cm² was achieved, cells were treated with 5 µM SN-38 in the apical and basolateral chambers. To determine the natural degradation of SN-38, transwell systems containing no T84 cells were filled with SN-38 supplemented cell culture media (5 µM). Each transwell system with T84 cells and without T84 cells (negative control) was subject to identical conditions. 25 µl from the apical and basolateral chambers was collected at 0 h, 1 h, 3 h, 6
h, 24 h and 48 h. Apical and basolateral samples were combined (50 µl) to produce a single sample per time point. All experiments were performed in triplicate and repeated.

8.3.4.1 Sample preparation

50 µl of sampled cell culture media was added to 150 µl of ice-cold acetonitrile with 0.1% formic acid (containing 20 ng/ml of IS). Samples were vortexed for 10 s and centrifuged at 13,300 rpm for 10 min at room temperature. A 180 µl aliquot of the supernatant was transferred to a clean microtube and 10 µl samples were analysed in triplicate using liquid chromatography–mass spectrometry (ABSCIEX TripleTOF™ 5600 LC/MS/MS).

8.3.4.2 Chromatographic conditions

The ABSCIEX TripleTOF™ 5600 LC/MS/MS was used to perform liquid chromatography–mass spectrometry analysis. Chromatographic separation was achieved by using a Kinetex C18 (2.6 µl, 50 mm x 3.0 mm) analytical column (Phenomenex, NSW, Australia; #00A-4633-AN). The mobile phase A was 5% acetonitrile, 95% water, 0.1% formic acid. Mobile phase B was 95% acetonitrile, 95% water, 0.1% formic acid with a 0.2 ml/min flow rate. The gradient system commenced with 90/10 mobile phase A/B for 30 s, whilst mobile phase A was gradually decreased to 0% by 2 min. This was maintained for 3 min before returning to initial conditions. The retention times for SN-38 and camptothecin (CPT; internal standard) were 2.1 min and 2.16 min, respectively.

8.3.4.3 Standard curve generation

A working standard solution of 1 µg/ml was diluted accordingly to achieve: 500 ng/ml, 375 ng/ml, 250 ng/ml, 125 ng/ml, 50 ng/ml, 25 ng/ml, 5 ng/ml and 2.5 ng/ml of SN-38. 10 µl of each solution were spiked into 40 µl of blank plasma. Final SN-38 concentrations were 100 ng/ml, 75 ng/ml, 25 ng/ml, 5 ng/ml, 1 ng/ml and 0.5 ng/ml. The lower limit of detection was 0.1 ng/ml and lower limit of quantification was 0.5 ng/ml. SN-38 concentration was determined by interpolation from the calibration curve and presented as molarity and percentage decrease from baseline.
8.3.5 Reverse transcription-polymerase chain reaction (RT-PCR) analysis of TLR4 expression

Toll-like receptor 4 (TLR4) is an emerging mediator of irinotecan-induced gut toxicity and a promising target for the development of potential interventions. In order for this platform to be used in the development of TLR4-targeted interventions, it is imperative that TLR4 expression be confirmed. HT29 and Caco-2 cells, known to express TLR4, were used as a positive control.

8.3.5.1 RNA isolation

To determine TLR4 mRNA expression, T84 (p7), Caco-2 (p46) and HT29 (p5) cells were grown to confluence in 90mm culture dishes. Total RNA extraction was performed on each cell line using the NucleoSpin RNA Isolation Kit as per manufacturer’s instructions (NucleoSpin RNA Isolation Kit, Macherey-Nagel, Düren, Germany; #740955). Once eluted, RNA was stored at -80 °C. Total RNA was quantified using the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA), TAKE3 plate and Gen5 (version 2.00.18) software. RNA purity was also determined using the the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA), TAKE3 plate and Gen5 (version 2.00.18) software. RNA integrity was assessed at the Adelaide Microarray Facility (South Australia Health and Medical Research Institute) using the Agilent 2100 Bioanalyser RNA 600 Nano Chip (Series II) kit.

8.3.5.2 Reverse transcription and RT-PCR

1 μg of total RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad, NSA, Australia; #1708890) as per manufacturers instructions. RT-PCR was performed using the Rotor-Gene 3000 (Corbett Research, NSW, Australia). Amplification mixes contained 2 µl of cDNA sample (100 ng/µl), 5 µl of SYBR green fluorescence dye, 2 µl of RNase-free water and 0.5 µl of each forward and reverse primers, prediluted to 50 pmol/µl, to make a total volume of 10 µl.

Thermal cycling conditions included a denaturing step at 95 °C for 15 min, followed by 40 cycles of denaturation at 95°C for 15s, annealing at 60 °C for 15s and extension at 72 °C for 15 s. All samples
were run in triplicate. Primer efficiency was evaluated using standard curves and experimental threshold (Ct) values were calculated by the Rotor Gene 6 programme. $C_T$ values were used to quantify relative mRNA expression of TLR4 in T84 cells and human colonic tissue using the $\Delta C_T$ method, relative where TLR4 expression = $2^{-\Delta C_T(target) - \Delta C_T(reference)}$. $\beta$-actin was used as an internal housekeeping (reference) gene. For primer details, see Table 8.1.

<table>
<thead>
<tr>
<th>Table 8.1 Primer sequences and characteristics</th>
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<tr>
<td><strong>Gene</strong></td>
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</tr>
<tr>
<td>Human TLR4</td>
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<td></td>
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<tr>
<td>Human $\beta$-actin</td>
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* Designed by Primer3, analysed using NetPrimer software and synthesised by Geneworks Ltd.

### 8.3.6 Statistical analysis

Data were compared using Prism version 7.0 (GraphPad® Software, San Diego, USA). A D’Agostino-Pearson omnibus test was used to assess normality. When normality was confirmed, a paired t-test or two-way analysis of variance with appropriate post hoc testing were performed to identify statistical significance. In other cases, a Kruskal-Wallis test with Dunn’s multiple comparisons test and Bonferroni correction was performed. A p-value of $<0.05$ was considered significant.
8.4 Results

8.4.1 Polyester membrane inserts support a polarised T84 phenotype with functional tight junctions in vitro

T84 cells seeded at all density ranges, in both transwell support systems, achieved TEER values greater than 1000 Ω/cm² within seven days (Figure 8.2A/B). T84 cells seeded at ≥2x10⁵ cells/cm² had an elevated trajectory, whilst a seeding density of 5x10⁴ cells/cm² displayed delayed maturation and resistance development. Monolayers seeded at ≤5x10⁴ cells/cm² displayed delayed maturation and resistance development. This trajectory is likely to facilitate optimal differentiation. When seeded at 1x10⁵/cm², T84 cells exhibited comparable resistance development characteristics in both transwell support systems, with no statistically significant differences observed (Figure 8.2C).

TEM analysis confirmed T84 monolayers grown on polyester membranes displayed polarity, with basally-located nuclei and apical microvilli (Figure 8.3A). TEM analysis also showed intercellular junctional complexes (tight junction, intermediate junction and desmosome) at the apico-lateral border of cells (Figure 8.3B/C). The presence of molecularly intact tight junctions in polyester membrane support system was confirmed by immunofluorescence for three major tight junction proteins: zonular occludens-1 (ZO-1), occludin and claudin-1 (Figure 8.4). No internalization or cytoplasmic redistribution was evident. These results also highlight the imaging potential of this support system, facilitating both light and confocal microscopy techniques. No fluorescence was detected on any membrane that had the primary antibody omitted (data not shown).
Figure 8.2 Transepithelial resistance (TEER) development over seven days in T84 cells in (A) mixed cellulose membrane support systems and, (B) polyester membrane support systems. All seeding densities achieved resistance in both membrane types within seven days. T84 cells seeded at ≥2x10⁵ cells/cm² had an elevated trajectory, whilst a seeding density of 5x10⁴ cells/cm² displayed delayed maturation and resistance development. (C) **Optimal seeding density appears to be 1x10⁵ cells/cm² with monolayers showing comparable resistance development in both support systems (p>0.05).** TEER was measured using an EVOM2 epithelial volt-ohm-meter (Physiologic Instruments). Data expressed as mean±SEM and analysed using a one-way analysis of variance with Tukey’s post hoc.
Figure 8.3 Transmission electron microscopy images of T84 cells grown on polyester membrane hanging inserts. Panel A (4200x) shows cellular polarity with apical microvilli (arrow) and a basally-located nucleus (N). Panel B (16500x) shows intercellular junction complexes at the apico-lateral boundary. Panel C (26500x) shows high power image of the junctional complex; TJ = tight junction, IJ = intermediate junction, D = desmosome.
Figure 8.4 Immunofluorescent staining of (A) ZO-1 (red), (B) occludin (green) and (C) claudin-1 (cyan) in T84 monolayers. The border of each cell can be distinguished by immunocytochemical circumferential staining of each tight junction protein. T84 monolayers were fixed in 1:1 (v/v) acetone/methanol, before being permeabilised with 0.1% TritonX-100. Cells were blocked with 3% BSA before the primary antibodies for ZO-1, occludin and claudin-1 were applied. Monolayers were then incubated with fluorescently-conjugated secondary anti-rabbit/mouse antibodies at 568 nm and 488 nm, respectively. Panel C has been pseudocoloured cyan for this figure. (D) XZ image showing occludin staining (arrow) at the cell periphery. The composite XZ stack shows apically located occludin staining which ceases toward the basal surface of the cell. The XZ composite was generated from 30 x 1 μm z-sections using the public domain Java image processing programme, Image J.
8.4.2 SN-38 stability in the transwell support system

SN-38 underwent rapid degradation in cell culture media alone (negative control; -T84 monolayer), with a 48.39±4.01% decrease from baseline by 1 h (Figure 8.6A/B). This decline was more pronounced in transwell systems with T84 monolayers (-76.04±1.86%) indicating metabolic processes (*p<0.0001; *p=0.0126). SN-38 concentration plateaued between 1 h and 24 h, although a small increase was observed at 48 h in the presence of T84 monolayers suggesting possible efflux mechanisms.
Figure 8.5 SN-38 concentration in transwell support system±T84 monolayers. Data presented as (A) concentration (nM) or, (B) relative to baseline (%). Transwell support systems±T84 monolayers were treated with 5 µM SN-38. The apical and basolateral chambers were sampled at 1 h, 3 h, 6 h, 24 h and 48 h. SN38 concentration was determined by liquid chromatography–mass spectrometry (ABSCIEX TripleTOFTM 5600 LC/MS/MS). SN-38 underwent rapid degradation in cell culture media, with a 48.39±4.01% decrease from baseline by 1 h. Degradation is more pronounced in transwell supports with T84 monolayers suggesting metabolic processes (***p<0.0001; *p=0.0126). Possible efflux of SN-38 can be seen at 48 h, with a spike in concentration. This is only observed in the presence of T84 monolayers.
8.4.3 T84 cells express TLR4

RNA integrity numbers (RIN) were assigned to each sample using the Agilent Bioanalyser. Caco-2 cell yielded the highest RIN score (RIN: 8.90). HT29 and T84 cells had RIN scores of 8.00 and 7.90 respectively. RNA A$_{260/280}$ ratios for Caco-2, HT29 and T84 cells were 2.098, 2.089 and 2.090, respectively, indicating pure, protein-free samples.

Under basal conditions, T84 cells expressed TLR4 (Figure 8.6), although this was significantly less than that of the HT29 cells (*p=0.037).
Chapter 8 *In vitro* model of SN-38-induced mucosal injury

Figure 8.6 TLR4 mRNA expression in T84 and HT29 cells relative to internal housekeeping gene β-actin. Relative expression was determined using the ΔCₜ method. Expression of TLR4 in Caco-2 cells was below detectable levels. Data expressed as mean±SEM. A one-way analysis of variance with Tukey’s post hoc was performed to determine statistical significance.
8.5 Discussion

This study has clearly highlighted the potential application for clear, polyester membrane transwell support systems in the investigation of gastrointestinal pathology. Comparable T84 epithelial cell growth patterns were shown in this model and the traditionally used, gold-standard mixed-cellulose membranes. T84 cells display normal morphological features when grown on polyester membranes, with a polarised phenotype complete with apical microvilli and apico-lateral tight junctions. It is intended for this model to be used for side-by-side structural and functional analysis of tight junctions and their contribution to the development of chemotherapy-induced gastrointestinal dysfunction. Correspondingly, we have characterised SN-38 metabolism in this model, highlighting the metabolic capabilities of T84 cells. This study also confirmed expression of TLR4, a key mediator of toxicity and promising target for therapeutic interventions.

Given the inherent challenges in accessing the gastrointestinal tract, a simple in vitro model for interrogation of complex gastrointestinal physiology is critical in unraveling the mechanisms of chemotherapy-induced diarrhoea. Characterisation of this model highlights its suitability for the study for SN-38-induced mucosal damage and the mechanisms and/or implications for tight junction disruption. Ultrastructurally, these monolayers displayed normal characteristics of the intestinal epithelium, with a microvillus-studded apical membrane, polarity and typical organelle distribution, supporting previous phenotype reports (Dharmsathaphorn et al., 1984). Most importantly, intercellular junction complexes were evident and apical tight junctions appeared phenotypically normal displaying apposing leaflets. Molecularly, tight junctions of polyester membrane T84 monolayers expressed the key architectural proteins, ZO-1, claudin-1 and occludin which exhibited typical ‘honeycomb’ or ‘cobblestone’ distribution.

It is well documented that tight junctions undergo an array of molecular changes in response to pathological cues (Gonzalez-Mariscal et al., 2008) with post-translational degradation (Cummins, 2012) and redistribution/internalisation of tight junction proteins reported in response to inflammatory mediators (Chiba et al., 2006, Edelblum and Turner, 2009, Gao et al., 2015), kinases
Chapter 8 *In vitro* model of SN-38-induced mucosal injury

(Jiang *et al.*, 2015) and microbiota changes (Nassour and Dubreuil, 2014, Staff, 2015). Internalisation is described most commonly for ZO-1, as it resides in the cytoplasm adjacent to the plasma membrane of the cell. It has been reported that under pathological states, particularly those with an inflammatory component, ZO-1 can detach from the junctional complex leading to altered tight junction integrity and barrier disruption (Hamada *et al.*, 2013). There was no evident internalisation of ZO-1, claudin-1 or occludin in T84 monolayers grown in polyester membrane support systems, indicating that this system supports molecularly intact, functional tight junctions that resemble that of the gastrointestinal tract. Furthermore, the transparent properties of the polyester membranes enable powerful confocal and light microscopy, enhancing the structural analysis of these complex intercellular structures and allowing paralleled functional and structural analyses. This feature is a significant advantage of this *in vitro* model, as structural tight junction analysis is typically achieved by growing T84 cells on coverslips. The polyester membrane transwell support system therefore provides a novel platform for interrogation of complex gastrointestinal physiology under normal or challenged situations and will enable investigation of SN-38-induced mucosal damage.

SN-38 is the semi-synthetic analogue of the naturally occurring anticancer alkaloid camptothecins and the active metabolite of irinotecan. The hydroxyl group at the C$_{10}$ position and ethyl group at the C$_{7}$ position both help to stabilise SN-38 in physiological environments and thus improve its potency (Bala *et al.*, 2013). Despite this, SN-38 is poorly solubilised and remains relatively unstable in physiological solutions. For example, the lactone ring of SN-38 is stable at pH $\leq 4.5$ but hydrolyses completely to its carboxylated form at pH $\geq 9$. At pH 6.7, both forms are in equilibrium. In the *in vitro* T84 transwell model used in this study, SN-38 underwent rapid degradation in physiologically stable cell culture media alone (negative control) highlighting not only the instability of SN-38, but also its relatively short half-life. In the presence of T84 cells, this degradation was more pronounced, with a further 27.65% reduction from baseline suggesting T84 cells express the enzymes required for SN-38 metabolism (e.g. UGT1A) and further highlights the suitability of this model for the study of SN-38-induced mucosal damage. In addition to metabolism, our results also showed evidence of
SN-38 efflux at 48 hours, indicating possible expression of efflux transporters that utilise SN-38 as a substrate in this T84 cell line. ATP binding cassette (ABC) transporters have been characterised in other human colorectal carcinoma derived cell lines, such as LS513 (Salphati et al., 2009), but their expression in T84 cells is unclear. The ABC transporter, multidrug resistance gene 1 (MDR1), as well as and cytochrome P450 isoform 3A4 (CYP3A4), have also been detected in LS180 cells, whereas only CYP3A4 was inducible in Caco-2 cells and TC-7 cells (Pfrunder et al., 2003).

Naruhashi and colleagues (2011) have recently shown that mRNA expression the ABC transporters MDR1, multidrug resistance-associated protein (MRP) 2 and MRP3 is comparable between Caco-2 lines and T84 monolayers (Naruhashi et al., 2011), with minimal differences their pattern of change in response to various endogenous compounds and xenobiotics. Despite similar expression, the intrinsic function of MDR1 was stronger in Caco-2 cells suggesting Caco-2 models are more suitable for the study of drug transport. Despite this, T84 cells were found to be more sensitive to stimulation by these endogenous compounds/xenobiotics presumably due to their relatively undifferentiated state relative to Caco-2 monolayers. T84 cells therefore present as a superior model for assessing the induction capacity of compounds and mechanisms of gastrointestinal pathology, whilst Caco-2 models are advantageous in the analysis of drug transport mechanisms and pro-drug development. We also showed that T84 cell lines express TLR4, whereas the Caco-2 cell line did not. Although this largely does not support the literature, it has been reported that some Caco-2 cells will only express TLR4 when stimulated with LPS (Hsu et al., 2011). In fact, TLR4 expression is comparatively lower in Caco-2 cell lines when compared with numerous other colonic cell lines such as HT29s and T84s (Hsu et al., 2011). This is also the case for TLR4 expression in T84 cells, which in our study was comparatively lower than that seen in HT29 cells. Nonetheless, the current study has definitively shown that T84 cells express TLR4, thus supporting the use of this novel in vitro platform for the study of TLR4-targeted interventions for chemotherapy-induced gut toxicity. Development of this in vitro model would therefore not only provide insight into the mechanisms of SN-38-induced mucosal damage, but would also provide an excellent platform in which to investigate pharmacological strategies critical in the development of effective treatment strategies.
8.6 Conclusions

Given the inherent challenges in accessing the gastrointestinal tract, the study of chemotherapy-induced gut toxicity remains difficult. A simple model for interrogation of complex gastrointestinal physiology is therefore critical in unraveling the mechanisms of symptoms such as diarrhoea. This study has successfully demonstrated the use of T84 cells, grown in transparent polyester transwell support systems, as a suitable model for the study of chemotherapy-induced mucosal damage. This platform supports a polarised T84 phenotype with functional tight junctions, allowing for in-depth permeability studies. Additionally, the transparent properties of these inserts allows for sophisticated downstream analyses such as live-cell fluorescent imaging or confocal microscopy. This study has also demonstrated that T84 cells exhibit the unique ability to metabolise SN-38 as well as expressing TLR4, making this an excellent platform for the study of clinically relevant therapeutic interventions for SN-38-induced mucosal damage by targeting TLR4.
Chapter 9 Addendum: Characterisation of SN-38 epithelial injury using novel *in vitro* model

9.1 Rationale

The study of irinotecan-induced gut toxicity and diarrhoea is limited due to the inherent challenges in accessing the gastrointestinal tract. Animal models are typically used to assess the mechanisms involved in symptom generation and the efficacy of anti-diarrhoeal therapeutics. This approach is both time and resource dependent. In Chapter 8 of this thesis, I developed a new *in vitro* model for the high throughput assessment of the molecular mechanisms of mucosal injury. Theoretically, this model can be applied to a number of gastrointestinal pathologies, allowing for paralleled functional and structural analyses of barrier function and epithelial transport mechanisms. For use in the setting of irinotecan-induced gut toxicity, further validation of this model is required to ensure translation of key pathological features from established *in vivo* models.
9.2 Materials and Methods

9.2.1 Cell culture

T84 cells, representative of the human colonic epithelium, were cultured as per methods outlined in Chapter 8 (section 8.3.1).

9.2.2 SN-38 dose finding study

T84 monolayers were grown to confluence and treated once a high, stable TEER was achieved (>1000 Ω/cm²). T84 monolayers were treated with apical/basolateral SN-38 at the following doses: 1, 2.5, 2.75, 3.0, and 10 µM, as well as a 0.01% DMSO vehicle control and 0.05% Triton X (TX)-100 positive control. TX-100 was chosen as a positive control based on its ability to disrupt barrier integrity through breakdown of the plasma membrane. TEER was measured at -30 min (baseline), immediately after treatment (0 h) and 3, 6, 24 and 48 h after treatment.

Immunofluorescence staining for caspase 3 was performed at 3, 6, 24 and 48 h to determine the extent of apoptosis. Briefly, cells were washed in cold 1 X PBS and fixed in 1:1 (v/v) methanol/acetone. Cells were permeabilised using 0.1% (v/v) TX-100/PBS for 3 min. After 2 x 5 min washes in 1X PBS, cells were blocked overnight in 3% (w/v) bovine serum albumin (BSA)/PBS at 4°C (Sigma-Aldrich, NSW, Australia; 9048-46-8). The blocking solution was aspirated and 200 µl of primary antibody was applied to the apical chamber (rabbit, polyclonal anti-caspase-3, 2 µg/ml, Abcam, Vic, Australia; #ab4051). The primary antibody was diluted in 1% (w/v) BSA/PBS and incubated for 1 h at room temperature. The primary antibody was then aspirated, and cells washed in 1X PBS+0.05% Tween (4 x 5 min). 200 µl of fluorescent-conjugated secondary antibody (anti-rabbit 568 AlexaFluor®; Invitrogen, VIC, Australia; #A11011) was added to the apical chamber at 100 µg/ml. The secondary antibody was diluted in 1% (w/v) BSA/PBS and incubated for 1 h at room temperature. For nuclear staining, cells were incubated with 1 µg/ml 4’,6-diamidino-2-phenylidole (DAPI) for 10 min at room temperature. Cells were washed in 1X PBS+0.05 Tween for 4 x 5 min before the membranes were removed from the inserts and mounted onto glass microscope
slides using Fluoroshield™ (Sigma-Aldrich, NSW, Australia; #F6182). Cells were visualised using the SP5 Spectral Scanning Confocal Microscope (Leica, Wetzlar, Germany).

Four areas from each membrane were imaged (40 X magnification), using the Leica SP5 Spectral Scanning Confocal Microscope (Wetzlar, Germany), providing a range of areas for analysis (Figure 9.1). Caspase-3 staining was quantified using ImageJ software. Briefly, channels were separated and RGB images converted to 8-bit. Automated thresholding was performed using the MaxEntropy algorithm (Sahoo et al., 1988, Mahmoudi and El Zaart, 2012). MaxEntropy was chosen based on qualitative assessment of its ability to identify apoptotic bodies and exclude non-specific staining (Figure 9.1C-F/9.2). Thresholding algorithms were deemed unsuitable if staining was not detected (Figure 9.1C), or non-specific staining was detected (Figure 9.1F). Total stained area and Integrated Density were calculated. All experiments were performed in triplicate and repeated.
Figure 9.1 Analysis of T84 caspase-3 staining. Four areas were visualized and captured using the Leica SP5 Spectral Scanning Confocal Microscope at 40x magnification (Panel A). Using ImageJ software, 568 nm (red) and 405 nm (blue) channels were separated and the RGB image was converted to an 8-bit image. Automated thresholding, using MaxEntropy, was conducted. Percentage stained area and Integrated Density were determined. All time points/dose schedules were conducted in triplicated and repeated. Thresholding was performed on 8-bit images derived from original RGB images (Panel C). MaxEntropy (Panel E) was chosen based on its ability to identify apoptotic bodies (red, Panel D). Thresholding algorithms were deemed unsuitable if staining was not detected (Panel B), or non-specific staining was identified (Panel F).
Figure 9.2 Montage of threshold algorithms provided by ImageJ software for detection of positive caspase-3 staining. ImageJ software provides a number of predetermined algorithms for the detection of positive staining. MaxEntropy (outlined in red) was chosen based on its ability to detect caspase positive cells, whilst excluding non-specific staining.
9.2.3 Characterisation of SN-38 induced barrier dysfunction

To determine the parallels between in vivo markers of irinotecan-induced barrier dysfunction and SN-38-induced changes in vitro, mucosal barrier function and tight junction integrity were assessed. Mucosal barrier function was assessed using 4 kDa fluorescein isothiocyanate (FITC)-dextran applied to the apical surface in Ussing chambers. Briefly, T84 monolayers were placed into 1.12 cm² aperture slider (Physiologic Instruments, CA, USA; #P2303A). The tissue was mounted and continually bathed in an oxygenated, glucose-fortified Ringer’s solution at 37°C with the following composition (composition in mM: NaCl 115.4; KCl 5; MgCl₂ 1.2; NaH₂PO₄ 0.6; NaHCO₃ 25; CaCl₂ 1.2 and glucose 10). Tissues were voltage-clamped to zero potential difference by the application of short-circuit current (Isc) and baseline was established. Tissues were allowed to equilibrate for 20 min and baseline Isc (µA/cm²) and conductance (S/cm²) were recorded. At T=0, 100 µl of 110 mg/ml FITC-dextran was added to the apical chamber. Samples were collected basolaterally at T=0, 15, 30, 60, 90 and 120 min. A membrane-only positive control was used in all runs. Samples were diluted 1:3 with 1 X PBS and quantified using the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA) and Gen5 version 2.00.18 software relative to a standard curve (range 0.0001-10 µg/ml). Tight junction integrity was also assessed using previously described immunofluorescence for claudin-1, ZO-1 and occludin (Wardill et al., 2016b).
9.3 Results

9.3.1 SN-38 causes dose- and time-dependent decreases in TEER

There was a dose-dependent decrease in TEER in T84 monolayers treated with SN-38. TX-100, the positive control, caused an immediate and persistent loss of TEER from 3 h after administration (-98.70±6.13% Table 9.1 and Figure 9.3). All remaining treatments, including the DMSO vehicle, caused a transient decrease in TEER at 3 and 6 h (Table 9.1 and Figure 9.3A). This decrease only persisted in SN-38 treated T84 cells. At 24 h, there was a dose-dependent decrease in TEER (Figure 9.3B). It was important to find a dose of SN-38 that caused a significant decrease in TEER, which mimicked that seen in vivo. It was also important to determine a dose which caused only a transient decrease in TEER, with TEER stabilising after peak injury (24 h). Based on these parameters, a dose of 2.75 µM was chosen for all remaining in vitro studies. This dose of SN-38 caused a 17.31±9.38% decrease in TEER at 24 h (Table 9.1, Figure 9.3).

Apoptotic studies revealed minimal cytotoxicity at this dose. To mimic in vivo pathology, it was important to induce a small degree of apoptosis, without causing severe damage to the monolayer. This allows analysis of the subtle, non-apoptotic mechanisms involved in barrier integrity and SN-38 induced mucosal damage. Although apoptotic bodies were evident (Figure 9.4A-E), 2.75 µM caused no significant increase in quantitative measures of caspase-3 staining (Figure 9.5A/B). Peak apoptosis occurred between 6 and 24 h, representing in vivo patterns of apoptosis. Peak fluorescence intensity for both 5 and 10 µM was seen at 6 h (5 µM: 455.65±63.43, 10 µM: 477.78±71.36; ***p<0.0001), indicating the presence of intensely stained caspase-3 positive cell bodies (Figure 9.5A). Total area stained (%) peaked at 24 h (5 µM: 1.71±0.17%, 10 µM: 2.46±0.26%; ***p<0.0001), suggesting that the apoptotic bodies were becoming more diffuse.
Figure 9.3 (A) Change in TEER, relative to baseline, for varying doses of SN-38 administered to T84 monolayers. Data show a transient decrease in TEER for all doses of SN-38 as well as vehicle control (DMSO) at 3 and 6 h. This change remains persistent in monolayers treated with >1 µM SN-38. Doses > 2.75 µM causes irreversible changes in TEER deeming the monolayers unsuitable for downstream analyses. (B) Dose response curve for change in TEER at 24 h. Data expressed as percentage decreased in TEER from baseline (mean ± SEM).
Table 9.1 Change in TEER (relative to baseline) following varying doses of SN-38, 0.05% TX-100 and 0.01% DMSO.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>1 µM SN-38</th>
<th>2.5 µM SN-38</th>
<th>2.75 µM SN-38</th>
<th>3 µM SN-38</th>
<th>3.5 µM SN-38</th>
<th>5 µM SN-38</th>
<th>10 µM SN-38</th>
<th>TX-100 (0.05%)</th>
<th>DMSO (0.01%)</th>
</tr>
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<tr>
<td>-0.5</td>
<td>-2.35</td>
<td>8.54</td>
<td>-1.26</td>
<td>5.58</td>
<td>-0.19</td>
<td>12.05</td>
<td>-0.28</td>
<td>10.36</td>
<td>0.03</td>
</tr>
<tr>
<td>0</td>
<td>2.35</td>
<td>6.12</td>
<td>1.26</td>
<td>5.49</td>
<td>0.19</td>
<td>11.94</td>
<td>0.28</td>
<td>10.39</td>
<td>-0.03</td>
</tr>
<tr>
<td>48</td>
<td>0.71</td>
<td>9.48</td>
<td>6.40</td>
<td>7.45</td>
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<td>9.33</td>
<td>-33.17</td>
<td>8.44</td>
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</table>
Figure 9.4 Representative images showing caspase-3 immunofluorescence in T84 monolayers treated with SN-38. DMSO, vehicle treated monolayers showed no significant apoptosis at any time point. Apoptotic bodies are evident from 3 h after SN-38 administration, peaking at 6-24 h. By 48 h, staining appears diffuse as the monolayers begin to regenerate. Red staining = caspase-3 positive cells/apoptotic bodies. Blue staining = 4′,6-diamidino-2-phenylidole (DAPI) to show T84 nuclei. Original magnification 40 X. Scale bars show 10 μM.
Figure 9.5 Quantitative analysis of apoptotic, caspase-3 positive staining using ImageJ software. Fluorescence intensity (A) peaked at 6 h post SN-38 treatment, indicating the presence of intensely stained caspase-3 positive cells. Percentage area stained (B) peaked later, at 24 h, indicating more diffuse staining. Caspase-3 staining was quantified using automated thresholding algorithm. A two-way analysis of variance with post hoc testing was performed to determine statistical significance. Data expressed as mean ± SEM where p<0.05 is considered statistically significant; * p<0.05, ** p<0.001, *** p<0.0001.
9.3.2 SN-38 induces barrier dysfunction in T84 monolayers

Basolateral translocation of 4 kDa FITC-dextran was detected in both vehicle-treated and SN-38 treated T84 monolayers (Table 9.2). This was more pronounced in SN-38 treated monolayers (Table 9.2, Figure 9.6 ** p<0.001, ***p<0.0001). Membrane-only, positive controls showed significant FITC-dextran permeability peaking at 90 min (data not shown 236.94±18.96 µg/ml). FITC-dextran permeability was also accompanied by claudin-1 internalisation seen in SN-38 treated T84 monolayers (Figure 9.7C). ZO-1 and occludin expression remain unaffected (Figure 9.7F and I).
Table 9.2 Basolateral FITC-dextran (% of apical dose) following treatment with DMSO control and SN-38.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>SN-38 (2.75 µM)</th>
<th>Mean (%)</th>
<th>SEM (%)</th>
<th>DMSO vehicle</th>
<th>Mean (%)</th>
<th>SEM (%)</th>
<th>Membran only control</th>
<th>Mean (%)</th>
<th>SEM (%)</th>
</tr>
</thead>
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<tr>
<td>0</td>
<td>0.02</td>
<td>0.02</td>
<td>0.00</td>
<td>0.00</td>
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<tr>
<td>15</td>
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<td>0.55</td>
<td>0.00</td>
<td>0.00</td>
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<td>1.34</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
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<td>1.34</td>
<td>0.77</td>
<td>0.34</td>
<td>47.10</td>
<td>2.97</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>8.59</td>
<td>1.32</td>
<td>2.21</td>
<td>0.84</td>
<td>73.90</td>
<td>4.41</td>
<td></td>
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<tr>
<td>90</td>
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<td>0.97</td>
<td>3.85</td>
<td>0.61</td>
<td>103.02</td>
<td>8.24</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>120</td>
<td>14.27</td>
<td>1.20</td>
<td>6.16</td>
<td>0.79</td>
<td>101.50</td>
<td>8.61</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 9.6 SN-38 induced barrier permeability to 4 kDa FITC-dextran. There was a natural movement of FITC-dextran across DMSO treated monolayers. This was significantly increased following 24 h treatment with 2.75 µM SN-38. Data presented as mean ± SEM, n = 6 data for DMSO vehicle and SN-38 treated monolayers. A two-way analysis of variance with Bonferroni correction was performed to identify statistical significance, where p<0.05 was considered significant; * p<0.05, ** p<0.001, *** p<0.0001.
Figure 9.7 Immunofluorescence for claudin-1 (red), ZO-1 (purple) and occludin (green) in DMSO- and SN-38-treated T84 monolayers. No changes were seen in the distribution or staining intensity for ZO-1 or occludin. Significant claudin-1 internalisation was evident at 24 h post SN-38 treatment. Following fixation and overnight blocking, monolayers were incubated with fluorescently-conjugated secondary anti-rabbit/mouse antibodies at 568 nm (claudin-1 and ZO-1) and 488 nm (occludin). ZO-1 staining has been pseudocoloured purple for this figure. Original magnification 40 X; scale bars show 50 µm and 5 µm.
9.3.3  SN-38 causes electrophysiological changes in T84 monolayers

*In vitro* SN-38 treatment decreased baseline resistance (Figure 9.8A; *p<0.006) and increased baseline conductance (Figure 9.8B; ***p<0.0001); both markers of paracellular permeability. No change in baseline short circuit current (Isc) was seen following SN-38 treatment (Figure 9.8C).
Figure 9.8 Baseline resistance (A), conductance (B) and short circuit current (C) in T84 monolayers 24 h after treatment with 0.01% DMSO or 2.75 µM SN-38. At 24 h, there was a significant decrease in transepithelial resistance (*p=0.006) and a complimentary increase in conductance (**p<0.0001). No change in baseline short circuit current was seen in T84 cells treated with SN-38 compared to DMSO controls. Data presented as mean ± SEM data. A two-tailed, unpaired t-test was performed to determine statistical significance, where p<0.05 was considered significant; * p<0.05, ** p<0.001, *** p<0.0001.
9.4 Discussion and Conclusions

This study aimed to characterise SN-38-induced epithelial injury in a novel *in vitro* model of mucosal toxicity. It showed translation of key pathological features from validated *in vivo* models, reinforcing its use in the study of irinotecan-induced gut toxicity in a high-throughput manner.

SN-38 caused a time-dependent decrease in transepithelial resistance (TEER). A transient decrease at 3-6 h was seen in all treatment regimens, including 0.01% DMSO vehicle. This non-specific change in TEER most likely reflects the sensitivity of monolayers to physical stimuli, including replacing the culture media. This is supported by the lack of apoptosis and tight junction disruption seen following DMSO treatment. At 24 h, true cytotoxic changes in TEER were observed, with DMSO treated monolayers returning to baseline TEER. Doses of SN-38 above 1 µM caused a significant decrease in TEER at 24 h. This was coupled by caspase-3 positive apoptotic cells. Importantly, comparable changes in transcolonic resistance were seen *in vivo* (Chapter 7) with a 27.6% decrease at 24 h post-irinotecan (270 mg/kg i.p.).

A secondary aim of this study was to identify a suitable dose of SN-38 for future use in this model. SN-38 caused a dose-dependent decrease in TEER at 24 h. To replicate *in vivo* data (Chapter 7), I aimed to induce a transient 20% decrease in TEER at 24 h, with a return toward baseline or stabilisation at 48 h. It was also important to induce minimal apoptosis so more discrete cellular mechanisms could be assessed. Based on these parameters, a dose of 2.75 µM was therefore used for remaining experiments.

This model has been specifically designed for the assessment of barrier function and tight junction integrity, allowing for paralleled function and structural analyses. This is particularly important in the setting of irinotecan-induced gut toxicity as results from within this thesis have shown significant changes in barrier function and augmentation of key tight junction proteins. In this model, SN-38 caused changes in all key parameters of barrier integrity; decreasing transepithelial resistance, increasing FITC-dextran permeability and causing morphological changes in the distribution of claudin-1 expression. This parallels *in vivo* data showing elevated serum FITC-
dextran 24 h after irinotecan treatment (Chapter 6). Importantly, claudin-1 internalisation was a key histopathological finding \textit{in vivo} (Chapter 7) and mirrored here, hypothesised to initiate barrier dysfunction and contribute to diarrhoea through leak-flux mechanisms. It was also shown active, secretory processes contribute to irinotecan-induced diarrhoea in a TLR4-independent manner (Chapter 7). Electrophysiological analyses in the current study showed no increase in baseline short circuit current, suggesting that the secretory processes thought to underpin diarrhoea are the result of complex physiological processes, and are not mediated through direct epithelial mechanisms. This hypothesis is supported by a wealth of literature showing that mucosal immune responses and proinflammatory processes are critical in the induction of a pro-secretory state (Madsen et al., 1997, Zamuner et al., 2003).

In summary, this model exhibits clear parallels with established \textit{in vivo} markers of irinotecan-induced gut toxicity. This reinforces the use of T84 cells, grown in transparent polyester transwell support systems, as a suitable model for the study of chemotherapy-induced epithelial damage and high throughput selection of therapeutic interventions.
Chapter 10 General discussion

10.1 Introduction

Intestinal barrier dysfunction is a well described component of many inflammatory-based gut pathologies associated with diarrhoea (Balda et al., 1996, Blijlevens et al., 2000). Characterised by alterations in the molecular integrity of tight junctions, barrier dysfunction has been hypothesised to underpin the development of chemotherapy-induced gut toxicity (CIGT) (Wardill et al., 2012, Wardill et al., 2013, Wardill et al., 2014c). This hypothesis is based upon strong anecdotal evidence which highlights elevations in established regulators of tight junctions, including proinflammatory cytokines (Fish et al., 1999, Edelblum and Turner, 2009, Schulzke et al., 2009), matrix metalloproteinases (MMPs) (Vermeer et al., 2009), gut dysbiosis and lipopolysaccharide (LPS) (Sheth et al., 2007, Kobayashi et al., 2013, Guo et al., 2015, Qin et al., 2015), following treatment with chemotherapy. Despite this, the molecular changes in tight junctions are yet to be characterised and their impact on diarrhoea development is unclear.

This thesis firstly investigated the extent of tight junction disruption following irinotecan in a rat model of gut toxicity (Chapter 3). I also had the opportunity to extend upon these findings to characterise the integrity of tight junctions in the oral cavity of patients receiving standard-dose chemotherapy (Chapter 4). Data from these studies provide strong evidence implicating tight junction disruption throughout the entire alimentary tract, in both the preclinical and clinical setting. Remaining studies within this thesis investigated the mechanisms responsible for tight junction disruption, with emphasis on the innate mucosal immune receptor, Toll-like receptor 4 (TLR4). This work details the impact of TLR4 signalling through in vitro and in vivo genetic modulation and pharmacological inhibition. This thesis also investigated the role of TLR4 in the development of irinotecan-induced pain.
10.2 Tight junction disruption: a common trait of alimentary toxicity

Previous research has shown alterations in functional measures of intestinal barrier function and tight junction integrity following various cytotoxic treatments (outlined in Table 2.1), however the molecular characteristics of tight junctions and their regulation have been largely ignored. The study described in Chapter 3 showed tight junction defects throughout regions of the rat gastrointestinal tract in response to irinotecan. Importantly, these changes coincided with key events in the pathophysiology of gut toxicity including inflammation, architectural tissue damage and the development of late-onset diarrhoea; providing a clear time-course of tight junction regulation. In addition, results showed tight junction defects occurred as early at 6 h post-irinotecan, with decreases in claudin-1 protein and mRNA expression. This indicates that tight junction disruption precedes the onset of clinical symptoms therefore supporting the hypothesis that mucosal barrier injury is a key risk factor in the development of secondary complications of chemotherapy-induced gut toxicity (van der Velden et al., 2010).

In addition to intestinal tight junction disruption, results from this thesis (Chapter 4) clearly demonstrate tight junction disruption also occurs in the oral cavity of humans receiving chemotherapy. This study is one of only a few studies which have documented changes in tight junctions from clinical samples. Keefe et al., (2000) showed altered tight junction integrity, by transmission electron microscopy, in the duodenum of patients undergoing high-dose chemotherapy (Keefe et al., 2000). That study (Keefe et al., 2000) was the first to suggest tight junction disruption may contribute to mucosal ulceration and gastrointestinal symptoms, as opposed to being a consequence of them. It is therefore likely that changes in oral epithelial tight junctions contribute to oral ulceration and tissue breakdown, and may also increase the risk of infection. Importantly, work detailed in this thesis (Chapter 4) builds upon the work by Keefe et al., (2000), describing the molecular changes in key tight junction proteins as opposed to providing only qualitative description of tight junction alignment (open versus closed).
Together these studies (Chapter 3 and 4) show translation of a mechanism from a preclinical model to the clinic. Additionally, they highlight that tight junction disruption occurs throughout the entire alimentary tract following chemotherapy and precedes a number of validated features of mucosal injury. This provides further evidence for a common pathway for alimentary toxicity, with regional differences the result of structural variations in the alimentary mucosae.

10.3 TLR4-dependent mechanisms regulate irinotecan-induced barrier dysfunction and exacerbate clinical manifestations of gut toxicity

A growing body of evidence exists supporting a role for TLR4 in the regulation of barrier function. This predominantly stems from the molecular control that TLR4 exerts over many established regulators of tight junction proteins. For example, it has been shown that bacterial LPS, the main ligand of TLR4, is critical in regulating tight junction integrity (Sheth et al., 2007, Kobayashi et al., 2013, Li et al., 2013). This was first shown associatively, with increases in luminal LPS production coinciding with the induction of intestinal barrier dysfunction (Qin et al., 2015). However more recently, the cellular events leading to LPS-induced intestinal barrier dysfunction have been described in more detail, implicating focal adhesion kinase (FAK) and nuclear factor kappa B (NFκB) (Guo et al., 2015). This phenomenon has also been shown to underpin the development of blood brain barrier dysfunction, with circulating LPS causing TLR4-dependent endothelial tight junction breakdown (Gao et al., 2015, Qin et al., 2015). In addition, the downstream inflammatory signals of TLR4 have also been implicated in the control of tight junctions. Cytokine-mediated tight junction disruption is likely the best characterised mechanism leading to tight junction disruption and consequently barrier dysfunction (Blijlevens et al., 2005b, Chiba et al., 2006, Edelblum and Turner, 2009). More recently, a link between TLR4 and protein kinase C (PKC) has been suggested to underpin the development of barrier dysfunction (Wardill et al., 2014c). Taken together, these results indicate TLR4 may be uniquely positioned to regulate tight junctions via a number of distinct mechanisms and is therefore likely to be central to chemotherapy-induced barrier dysfunction.
Studies carried out during this thesis explored the phenomenon of TLR4-mediated barrier disruption using a novel BALB/c-Tlr4<sup>-/-</sup> billy mouse model of irinotecan-induced gut toxicity. In addition to its effect on barrier function, it was also hypothesised that TLR4 was involved in other areas of the pathophysiology of irinotecan-induced gut toxicity, and hence, genetic deletion would improve symptomatic manifestations and subclinical markers of toxicity. Previous studies from within my laboratory have shown that TLR4, and its downstream signalling molecules such as NFκB, are upregulated following chemotherapy (Logan <i>et al.</i>, 2007, Bowen <i>et al.</i>, 2012). In addition, preliminary <i>in silico</i> docking studies, conducted in my laboratory, have shown that the active metabolite of irinotecan (SN-38) acts as a ligand for the MD-2/TLR4 complex. This suggests TLR4 activation may be particularly important in the setting of irinotecan-induced gut toxicity.

10.3.1.1 Gut microbiome composition

Given the use of genetically modified animals in this study, it was important to determine the composition of the gut microbiome of each genotype (WT and BALB/c-Tlr4<sup>-/-</sup> billy), as evidence indicates both genotype and environmental factors (such as breeding facility) strongly influence the gastrointestinal bacterial community (Hufeldt <i>et al.</i>, 2010). Investigation of the gut microbiome from vehicle control WT and BALB/c-Tlr4<sup>-/-</sup> billy mice showed similar population of major bacterial phylogenies, bacteroides and fermicutes. Differences were however detected in low abundance microbes, proteobacteria and TM7. Proteobacteria is a β-glucuronidase producing bacterium which enhances reactivation of SN-38 in the intestinal lumen. Similarly, upregulated TM7 is often associated with a proinflammatory state within the gastrointestinal system (Kuehbacher <i>et al.</i>, 2008), and hence BALB/c-Tlr4<sup>-/-</sup> billy mice may have been predisposed to develop worsened toxicity based on their bacterial profile. These small differences in the microbial phenotype may reflect the differences seen in baseline permeability in WT and BALB/c-Tlr4<sup>-/-</sup> billy mice, with the BALB/c-Tlr4<sup>-/-</sup> billy mice having increased FITC-dextran permeability at baseline compared to WT mice. Underlying barrier dysfunction, as seen in BALB/c-Tlr4<sup>-/-</sup> billy mice, would allow for increased exposure of luminal contents to the underlying mucosal immune system, with CD103<sup>+</sup> dendritic cells of the
lamina propria able to relay information to mesenteric lymph nodes to promote tolerance. If this is the case, the BALB/c-Tlr4\textsuperscript{–/–}mice may have had a greater tolerance to insult. This is the first study to characterise the microbial composition of BALB/c-Tlr4\textsuperscript{–/–}mice and highlights the need to routinely characterise the composition of the gut microbiome in various animal populations.

10.3.1.2 TLR4 activation drives irinotecan-induced gut toxicity

Results from this thesis (Chapter 6) show improved clinical parameters of gut toxicity (diarrhoea and weight loss) in BALB/c-Tlr4\textsuperscript{–/–}mice treated with irinotecan, supporting the growing body of evidence suggesting dysregulated TLR4 signalling can be detrimental to gastrointestinal health (Shoenfelt et al., 2009, Deng et al., 2013, Huang et al., 2014b, Guo et al., 2015). These results compliment recent findings showing improved clinical manifestations of irinotecan-induced gut toxicity in germ-free mice compared to their conventional counterparts (Pedroso et al., 2015). Importantly, germ-free mice also had higher levels of unbound SN-38 and higher β-glucuronidases activity. This, coupled with the microbiome composition of the BALB/c-Tlr4\textsuperscript{–/–}mice, suggests the unique hepatobiliary metabolism of irinotecan and deglucuronidation of SN-38 may not be the main factor in determining the severity of irinotecan-induced gut toxicity, thus challenging traditional views on its pathophysiology.

Improvements were also seen in BALB/c-Tlr4\textsuperscript{–/–}mice in a number of validated histopathological markers of toxicity including apoptosis and tissue morphometry. The effect on apoptosis was particularly compelling as it is one of the earliest predictive markers of toxicity and predisposes to significant tissue injury (Gibson et al., 2006, Gibson and Shillitoe, 2006). Apoptosis is typically thought to be the result of direct cytotoxicity, induced by the chemotherapeutic agent (Sonis, 2004a), however results from this study (Chapter 6) clearly indicate that TLR4-dependent mechanisms are responsible for a degree of crypt apoptosis, highlighting a targetable aspect of the pathophysiology. It is important to note however that this thesis did not measure circulating SN-38 and lower exposure/re-activation to this toxic metabolite cannot be excluded as a potential cause for the improvements seen in BALB/c-Tlr4\textsuperscript{–/–}mice.
Through investigations carried out in this thesis, it is also evident that TLR4-dependent mechanisms contribute to irinotecan-induced barrier dysfunction, demonstrated by increased permeability to 4 kDa FITC-dextran and subsequent LPS translocation. This parallels work by Nakao et al., (2012) showing increased permeability in irinotecan-treated rats and increased colonisation of pathogenic bacteria into mesenteric lymph nodes and the spleen (Nakao et al., 2012). Further investigation demonstrated barrier dysfunction coincided with elevations in inflammation (interleukin (IL)-1β and tumour necrosis factor (TNF)) and paralleled the development of clinically-diagnosed diarrhoea (Chapter 6). Importantly, BALB/c-∗Tlr4−/−∗ mice showed no elevation in either of these cytokines, reflecting the molecular control of TLR4 on downstream inflammation, and were protected from changes in barrier function. This associatively highlights the ability of proinflammatory cytokines to disrupt intestinal barrier dysfunction.

Molecular investigations showed that, in my Tlr4−/− model of gut toxicity, barrier dysfunction was regulated by cytoplasmic redistribution of the tight junction protein, claudin-1. This was coupled with focal areas of occludin and ZO-1 loss, particularly in areas where epithelial injury was evident. Despite these focal lesions, no quantifiable changes in total protein or mRNA expression were identified suggesting that TLR4-dependent claudin-1 disassembly is sufficient to cause severe barrier dysfunction. Cytoplasmic redistribution of tight junction proteins has been shown to occur in response to some bacterial toxins, such as LPS (Nassour and Dubreuil, 2014), as well as to some proinflammatory cytokines and proteinases (Chiba et al., 2006, Vermeer et al., 2009). Strong evidence also indicates MyD88-dependent zonulin production regulates tight junction protein distribution, causing protein internalisation and breakdown of the tight junction unit (Fasano, 2011). Although zonulin antagonism has already been assessed for its safety and efficacy in coeliac patients (Paterson et al., 2007), augmentation of the upstream regulators, such as MyD88 or TLR4, has strong therapeutic potential given the multifactorial way in which TLR4 regulates tight junctions.
10.4 TLR4-dependent barrier dysfunction permits chloride-driven water fluxes, contributing to diarrhoea development

Studies conducted in this thesis have shown tight junction disruption is a common trait of both oral and gastrointestinal toxicity, occurring in a TLR4-dependent manner. It is clear from results within this thesis, and research from within the field, that loss of barrier function exacerbates toxicity, by promoting bacterial and LPS translocation and increasing the risk of secondary infection (Nakao et al., 2012). Despite positive findings of tight junction disruption in many pathologies characterised by diarrhoea, the mechanisms by which barrier dysfunction causes diarrhoea remain unclear. Based on results from this thesis, I hypothesise the interaction between barrier function, anion secretion and paracellular water movement is critical in the development of irinotecan-induced diarrhoea.

Electrophysiological investigations using Ussing chambers (Chapter 7) showed irinotecan treatment increased the colonic response to carbachol and forskolin, secretagogues that increase intracellular calcium and cyclic adenosine monophosphate (cAMP) and thus increase chloride secretion through the calcium activated chloride channel (CaCC) and the cystic fibrosis transmembrane conductance regulator (CFTR) channel, respectively. Importantly, there were no significant differences in the response between WT and BALB/c-Tlr4<sup>−/−</sup> mice. This shows, for the first time experimentally, that irinotecan-induced diarrhoea has a secretory component to its pathophysiology. It also demonstrates chloride secretion occurs in a TLR4-independent manner, with each animal genotype having comparable responses to each chloride channel agonist. Importantly, neither stimulated chloride secretion nor baseline current correlated with the severity of diarrhoea seen in each genotype suggesting secretory mechanisms alone are unlikely to be driving diarrhoea through their effect on paracellular water movement. It is therefore important to consider this mechanism in the context of barrier dysfunction. Increased luminal chloride secretion is likely to provide a strong osmotic drive toward the lumen for paracellular water movement. In the context of barrier dysfunction, it is difficult to dissect net ion/water movement, however it is plausible that the degree of barrier dysfunction promotes heightened paracellular water movement in the context of chloride-dependent osmotic drive. Paradoxically, augmentation of the epithelial barrier could allow for
luminal chloride to move passively down its concentration gradient, abolishing its effect on osmosis. Future research should therefore focus on the synergism between altered secretory processes and barrier dysfunction to determine their role in diarrhoea development.

10.5 Translating *in vivo* mechanisms to novel *in vitro* model

Given the inherent challenges in accessing the gastrointestinal tract, the study of CIGT remains difficult. A simple model for interrogation of complex gastrointestinal physiology is therefore critical in unraveling the mechanisms of symptoms such as diarrhoea. Studies from within this thesis (Chapter 8 and 9) have shown T84 monolayers, grown in transparent polyester transwell support systems, are a suitable model for the study of chemotherapy-induced epithelial damage. These cells exhibit polarity and phenotypically intact tight junction, with a microvillus-studded apical membrane. Importantly, I showed translation of key pathological findings from *in vivo* studies to my new *in vitro* model. This model allows for paralleled functional and structural analyses of tight junctions in response to SN-38 treatment. Consistent with *in vivo* findings, there was a dose- and time-dependent decrease in transepithelial electrical resistance and induction of apoptosis following SN-38. Treatment with SN-38 also caused disruption of colonic epithelial (T84) barrier function, increasing permeability of FITC-dextran and causing internalisation/disruption of claudin-1. This shows translation of a pathological finding from the *in vitro* setting to the clinic, thus strengthening support for barrier function as a key driver in the pathophysiology of gut toxicity.

10.6 Central barrier disruption permits TLR4-dependent pain pathways

It is becoming increasingly recognised that a number of chemotherapy-induced toxicities present in the same subset of patients, or in response to the same treatment regimens, suggesting common underlying mechanisms (Aprile *et al.*, 2008). A study by Aprile *et al.*, (2008) reported epidemiological links between the incidence of chemotherapy-induced gut toxicity and neurotoxicity (pain). This is not surprising as it is becoming increasingly evident that changes in gut health can profoundly affect neurological function, and vice versa (Low *et al.*, 2015, Emge *et al.*, 2016, Yarandi *et al.*, 2016). This phenomenon has been shown to underpin a number of neurological
conditions from Parkinson’s disease (Scheperjans et al., 2015) to autism (Li and Zhou, 2016) (Ghaisas et al., 2016). Additionally, studies suggest that peripheral stimuli, characterised by an inflammatory component, have the ability to alter central pain pathways through their effect on glia – the supportive cells of the CNS (Watkins et al., 2007, Riazi et al., 2008, Ji et al., 2013, Wardill et al., 2015b). Based on these findings, myself and Professor Stephen Sonis proposed a hypothesis suggesting that chemotherapy-induced gut toxicity and pain have common biological causes relating to TLR4 – the gatekeeper of glial activation (Wardill et al., 2015a, Wardill et al., 2015b). Research also suggests TLR4 is critical in the breakdown of the blood brain barrier (Gao et al., 2015), thus facilitating access of peripheral factors to the central nervous system. In the case of gut toxicity and pain, the interaction between LPS and centrally-located TLR4 is compelling given research showing that TLR4-dependent glial activation increases pain signalling through neuroinflammatory pathways (Hutchinson et al., 2007b, Watkins et al., 2009, Lewis et al., 2012). Hence, this thesis investigated the severity and duration of pain following irinotecan in my Tlr4−/− mouse model of irinotecan-induced gut toxicity to determine if peripheral inflammation and LPS production promoted pain and glial activation.

Through a number of investigations, it was shown that irinotecan caused blood brain barrier disruption, although this did not appear to be TLR4-dependent. This is the first time that blood brain barrier disruption has been shown to occur in a model of irinotecan-induced gut toxicity and suggests uncontrolled pathological communication between the periphery and CNS. It also supports research showing blood brain barrier dysfunction and central gliosis in patients with inflammatory bowel disease (Brown et al., 2002) and parasitic gut infections (Bercik et al., 2010). In addition to blood brain barrier dysfunction, it was also shown BALB/c-Tlr4+/− mice had significantly lower markers of central pain signalling and reduced astrocytic reactivity in the lumbar spinal cord, measured by the facial grimace criteria and GFAP staining, respectively. This compliments existing research showing astrocytic reactivity is critical in the development of chemotherapy-induced pain (Di Cesare Mannelli et al., 2014), however is the first to implicate stimuli originating in the periphery.
The idea that peripheral stimuli are able to modulate central functions is gaining significant attention within the literature. I hypothesised that, through its interaction with TLR4, LPS was the most likely candidate responsible for driving enhanced pain signalling. This supported research showing that TLR4-dependent astrocyte activation was critical in the development of paclitaxel-induced neuropathy (Li et al., 2014b). Despite this, my results show pain and serum LPS (endotoxin) peak at different time points across the time-course of gut injury, and no correlations could be drawn between LPS and pain \( (r^2=0.01; p=0.781; n=12) \) or LPS and glial activation in the lumbar spinal cord \( (r^2=0.21; p=0.180; n=12) \). This suggested that alternative peripheral stimuli may be responsible for central glial activation seen in WT mice treated with irinotecan. Research from Ji et al., (2013) shows administration of pentoxyfylline (an anti-inflammatory agent) prevented central astrocyte activation in a model of vincristine-induced neurotoxicity (Ji et al., 2013). This supports the moderate correlation seen between IL-6 and astrocyte reactivity \( (r^2=0.69; p=0.0007; n=12) \) in mice treated with irinotecan and instead suggests that gut derived inflammation, rather than LPS, may drive changes in central glial activation. Despite this, no correlations could be drawn between any of the downstream analyses and facial pain scores, thus suggesting that although glial activation may occur following irinotecan treatment, it may not be critical in the pathophysiology of pain. It is also important to consider the inherent limitations of the facial grimace criteria used to assess pain in this study. The facial grimace criteria is a validated marker of discomfort and pain, however is unable to discriminate between different forms of pain. This study would have benefited from inclusion of an additional measure of pain (e.g. Von Frey, paw pressure, thermal tests) designed to determine neuropathic-type pain. Nonetheless, these findings suggest that TLR4 is central to the development of irinotecan-induced gut toxicity and pain, providing a unique opportunity to simultaneously treat both toxicities. It is now critical to determine if the irinotecan-induced gut toxicity and pain are independent, yet simultaneously occurring events that are both governed by TLR4, or if there is a true directional mechanism linking one to the other highlighting the existence of a gut/CNS axis.
10.7 Practical considerations for TLR4-targeted therapeutic approaches

Results obtained from within this thesis demonstrate TLR4-dependent mechanisms not only underpin the development of irinotecan-induced gut toxicity but also intestinal barrier function. Furthermore, results also indicate TLR4 is central to both the development of irinotecan-induced gut toxicity and pain. This provides an exciting opportunity to simultaneously target both side effects, reducing polypharmacy and streamlining patient management. However, in all cases of TLR4-targeted therapeutic options, the effect on both the efficacy of the anti-cancer therapy and overall tumour kinetics are paramount. This is particularly the case when targeting TLR4, as recent evidence now suggests this innate immune receptor may play critical roles in tumour growth and chemotherapy efficacy. For example, Apetoh et al. (2007) showed TLR4 deficient animals had increased tumour growth under normal conditions and in response to doxorubicin (Apetoh et al., 2007). They concluded that dying tumour cells elicit a TLR4-dependent immune response that not only mediates tumour regression, but also determines the long-term survival of the animal or patient.

TLR4 inhibition has also been implicated in tumour regression, with several studies now showing that numerous cancer cell lines overexpress TLR4 and that LPS stimulates their growth (Huang et al., 2014b). For example, Huang et al., (2014) showed that the mRNA and protein expression of TLR4 and its downstream transcription factor NFκB were upregulated in human colon cancer specimens and stimulation with LPS caused SW620 cells to proliferate (Huang et al., 2014b). In addition, treatment with the TLR4 inhibitor, CRC-526, reduced overall tumour burden in mice. Similarly, TLR4 knockdowns in PC3 cells (prostate cancer cell line) exhibit reduced proliferation and migration tendencies. These findings have also been noted in models of breast cancer (Yang et al., 2010), as well as non-small cell lung cancer (Li et al., 2014a) and TLR4 inhibition may therefore present as a very positive advent in our approach to tackling chemotherapy-induced toxicity. Studies using tumour-bearing rodents are therefore necessary for future studies investigating the efficacy of TLR4 antagonism, thus highlighting a limitation of the model developed and used within this thesis. Another limitation of the BALB/c-Tlr4−/− mouse model used in this thesis is that it is a global
knockout model. Gut-, immune-, CNS- and tumour-specific knockout models or tamoxifen-inducible models would allow for controlled assessment of TLR4-dependent mechanisms and their contribution to symptom generation without confounding effects of maturation without an intact innate immune system.

10.8 Conclusions and future directions

The studies carried out in this thesis aimed to better characterise the impact that altered barrier function plays in the development of irinotecan-induced gut toxicity, and the mechanisms that control it. Through a number of investigations, this thesis has provided evidence that barrier disruption is critical in the progression of gastrointestinal symptoms and is a significant risk factor for secondary toxicity and worsened patient outcomes. It is likely that gut dysbiosis, seen following irinotecan treatment, acts as a catalyst for adjuvant intestinal barrier dysfunction and secondary TLR4-dependent innate immune activation.

This thesis has also demonstrated barrier disruption is not exclusive to the gastrointestinal tract, with increased permeability of the blood brain barrier seen following chemotherapy. This is the first time blood brain barrier disruption has been observed following chemotherapy, and supports the idea that gut-derived inflammation has significant systemic effects. This study also showed irinotecan treatment caused central astrocyte reactivity and heightened pain sensitivity; both of which appear to be TLR4-dependent. This is the first study to highlight that TLR4 is critical in the regulation of chemotherapy-induced barrier dysfunction, gut toxicity and pain. It can be concluded that barrier disruption, whether it be in the gastrointestinal tract or central nervous system, permits significant immune modulation that appears to be mediated by TLR4. This immune receptor therefore presents as a promising therapeutic target for the treatment of both chemotherapy-induced gut toxicity and pain, and findings from this thesis will direct new research in the field of chemotherapy-related side effects.
Chapter 11 References


Chapter 11 References


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Chapter 11 References


Chemotherapy-induced mucosal barrier dysfunction: an updated review on the role of intestinal tight junctions

Hannah R. Wardill and Joanne M. Bowen

Purpose of review
Gut toxicity, or mucositis, is a major dose-limiting side effect of chemotherapy that until recently received very little attention. Despite significant research, the mechanisms that underpin chemotherapy-induced gut toxicity (CIGT) remain unclear. Recently however, there has been renewed interest in the role tight junctions play in the pathogenesis of CIGT and associated diarrhea. Thus, this review will cover the role of tight junctions in maintaining gastrointestinal homeostasis and touch on recently proposed mechanisms of how tight junctions may contribute to the development of chemotherapy-induced diarrhea.

Recent findings
There is a wealth of anecdotal evidence regarding the role of tight junctions in the pathogenesis of gut toxicity. However, few studies have quantified or assessed the molecular changes in tight junctions in response to chemotherapy. This review will highlight the major findings of these studies and discuss the potential mechanisms by which tight junction disruption and mucosal barrier dysfunction may contribute to diarrhea.

Summary
The significant clinical and economic impact associated with CIGT and diarrhea has only recently been appreciated. This has prompted significant research efforts in an attempt to reveal the pathophysiology of this debilitating complication. Renewed interest has been shown regarding the role of tight junctions in not only maintaining gastrointestinal health, but also contributing to mucosal barrier injury and diarrhea development. More detailed research into the effect chemotherapy has on the molecular characteristics of tight junctions will lead to a better understanding of the pathophysiology of CIGT and may uncover the therapeutic potential of tight junctions in treating diarrhea.

Keywords
chemotherapy, diarrhea, mechanisms, mucosal barrier dysfunction, tight junctions, toxicity

INTRODUCTION
Chemotherapy is a highly effective cytotoxic therapy used to treat a number of common malignancies [1]. Despite the advantages of this treatment option, chemotherapeutic agents are nonselective in nature and are associated with widespread cytotoxicity [2]. The alimentary mucosa is particularly susceptible to regimen-related damage and toxicity due to its high cellular turnover [3]. Clinically, alimentary toxicity, or mucositis, is used to describe damage to the mucous membranes of the alimentary tract that occur following cytotoxic treatment [4]. Alimentary tract toxicity manifests as confluent ulcers affecting the entirety of the tract, which often lead to symptoms such as dysphagia, vomiting, diarrhea, weight loss, rectal bleeding and infection [2,5–7]. A wealth of research literature has been generated regarding potential therapeutic avenues for chemotherapy-induced toxicity [8,9]; however, improvements are often limited to toxicity affecting the oral mucosa owing to the ease at which the oral cavity is accessed [10]. Despite being subject to extensive investigation, the molecular mechanisms that underpin chemotherapy-induced gut toxicity

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Curr Opin Support Palliat Care 2013, 7:155–161
DOI:10.1097/SPC.0b013e32835f3e8c
Diarrhea is a major clinical manifestation of CIGT that significantly elevates the risk of dose-reduction, bleeding, infection and the duration and costs of hospitalization. 

Diarrhea development is thought to result from multiple factors; however, exact mechanisms are unclear. Tight junctions show potential involvement in the development of CID given their roles in maintaining gastrointestinal homeostasis and mucosal barrier integrity. Few research efforts have been made to elucidate the role of tight junctions in CID, although several studies have briefly quantified the molecular changes in tight junctions following chemotherapy. Detailed research will allow for a greater understanding of the pathogenesis of CID and may reveal the therapeutic potential of tight junctions. 

The development of a ‘leaky’ gut is a hallmark trait of compromised mucosal barrier function [11,12]. Intestinal homeostasis and tight junction integrity are traditionally measured using noninvasive sugar permeability tests, which utilize a ratio of monosaccharide and disaccharide sugar probes [11,13]. By using the ratio of two sugars, most commonly rhamnose and lactulose, variables such as gastrointestinal transit time and renal impairment are eliminated [14]. Recent clinical research has demonstrated severe intestinal dysfunction in patients with CIGT that extends far beyond direct cytotoxic insult [4,14,15]. Additionally, both the absorptive capacity of the small intestine and mucosal barrier function have been shown to be severely compromised following a host of cytotoxic therapies [14,15]. Tight junctions are key determinants of mucosal barrier function and intestinal permeability, regulating the passage of solutes across the intestinal epithelium via the paracellular pathway [16]. As such, mucosal barrier dysfunction is highly suggestive of tight junction disruption within the small bowel [15,17]. Dynamic regulation of tight junctions is therefore fundamental to gastrointestinal homeostasis [18].

**MOLECULAR STRUCTURE OF TIGHT JUNCTIONS**

The molecular architecture of the tight junction exhibits a complex arrangement of interacting cytoplasmic and transmembrane proteins [16], which form continuous adhesive strands that circumscribe the apical–lateral margin of polarized epithelia [19,20]. Zonular occludens proteins (ZO-1, ZO-2 and ZO-3) are cytosolic scaffolds that anchor peripherally located transmembrane proteins (occludin and claudins) to the actin cytoskeleton. Knockout studies (ZO-1 ) have demonstrated that the absence of ZO-1 completely abrogates tight junction assembly and paracellular regulation [21,22]. Zonular occludens proteins are therefore integral to the barrier integrity and tight junction formation. Claudin proteins are the major component of tight junction strands, as shown by freeze-fracture microscopy [23]. Claudins constitute a family of over 20 proteins, which display variable extracellular loop distribution and thus function [16]. Accordingly, claudin subtypes are responsible for different paracellular roles within the tight junction [24,25]. Several claudin subtypes, particularly claudin-1, have been implicated in a number of benign inflammatory disorders of the bowel characterized by mucosal barrier dysfunction [26,27]. Claudin proteins are, therefore, considered crucial to tight junction integrity and gastrointestinal homeostasis. Occludin is a well-characterized transmembrane protein of the tight junction. The importance of occludin to tight junction integrity has been extensively documented through numerous in vitro and in vivo investigations [16,28,29]. For example, knockout (occludin ) mice exhibit morphologically intact tight junctions [30], however, poor tight junction integrity and mucosal barrier dysfunction follow. These results indicate likely roles for occludin in tight junction stability and barrier function as opposed to tight junction assembly.

**ARE ALTERATIONS IN INTESTINAL TIGHT JUNCTIONS PIVOTAL TO CHEMOTHERAPY-INDUCED GUT TOXICITY DEVELOPMENT?**

Currently, the molecular mechanisms that underpin the development of gut toxicity following chemotherapy are poorly understood. Recent research has highlighted roles for apoptosis [4], matrix
metalloproteinases (MMPs) [31] and the gut microbiome [32,33] in the development of CIGT. More recently however, there has been a renewed interest regarding the role of tight junctions in the pathophysiology of gut toxicity [34].

Tight junctions were first hypothesized as a potential pathomechanism for CIGT in 1997, with Keefe et al. [14] demonstrating marked increases in intestinal permeability in patients receiving various chemotherapeutic agents, largely administered in combination. Intestinal permeability, measured by rhamnose/lactulose permeation, was perturbed in all patients, peaking 7 days following treatment and corresponding with the onset and duration of gastrointestinal symptoms [14,35]. Comparable clinical studies soon emerged [12,36–40], therefore validating the efficacy of sugar permeability tests as a simple, well tolerated and reliable test, while emphasizing the profound effects of chemotherapeutic agents on mucosal barrier integrity (Table 1). In line with these findings, Blijlevens et al. [15] identified significant abnormalities in intestinal permeability in haematopoietic stem cell transplant recipients treated with various myeloablative treatments including melphalan, cyclophosphamide, irinotecan, etoposide, busulphan and total body irradiation (Table 1). All treatment regimens caused significant mucosal dysfunction; however, damage was more pronounced and prolonged in patients treated with irinotecan. Together these studies suggest that regimen-related mucosal barrier dysfunction is not limited to a single chemotherapeutic agent, but common to numerous cytotoxic treatments.

Morphological defects in tight junctions have also been identified following cytotoxic therapy. In 2000, Keefe et al. [4] demonstrated a significant increase in the amount of open tight junctions within the small intestine of patients receiving selected combined chemotherapeutic regimens. Importantly, peak tight junction disruption coincided with maximal intestinal permeability of patients receiving polydrug-chemotherapy reported by Fazeny-Dorner et al. [35]. Given the importance of tight junction integrity to mucosal barrier function and gastrointestinal health, these studies suggest a key role for tight junctions in the pathophysiology of gut toxicity following chemotherapy [34].

Despite substantial anecdotal evidence suggestive of tight junction disruption following chemotherapy, very few studies have investigated the molecular changes in tight junctions in response to cytotoxic treatment. Given that disruption to the expression and distribution of specific tight junction proteins drastically alters tight junction integrity and gastrointestinal homeostasis [20], this is an area of much needed research. Hamada et al. [41] were the first to characterize alterations in tight junctions following the administration of methotrexate (MTX), a commonly used chemotherapeutic agent. Briefly, tumor-naive rats were treated with 15 mg/kg of MTX once daily for 3–5 consecutive days and were killed 24 h following their final treatment. Mucosal barrier function, determined by fluorescein isothiocyanate-dextran permeability, was significantly increased in MTX-treated rats indicating poor barrier integrity. Although investigations were limited to just a single tight junction protein, ZO-1, this preclinical study highlighted significant changes in its expression, distribution and phosphorylation. Reversible phosphorylation of tight junction proteins has been established as a vital aspect of tight junction integrity and barrier function [42], thus the changes in ZO-1 phosphorylation identified by Hamada et al. highlight a potential mechanism of chemotherapy-induced mucosal barrier dysfunction.

More recently, marked decreases in small intestinal occludin and claudin-1 expression and distribution were demonstrated in MTX-treated Wistar rats [43**]. Comparable changes in these major tight junction proteins have also been identified in response to irinotecan treatment [44], suggesting that tight junction disruption may be a common underlying mechanism of gut toxicity induced by various cytotoxic agents. Despite emerging evidence for tight junction involvement, this evidence is often conflicting within recent literature. For example, differences in permeability data exist, with Nakao et al. [44] identifying decreased small intestinal permeability in irinotecan-treated rats, a finding that contrasts with evidence of permeability increases reported in previously published literature (Table 1). Standard methods of assessing intestinal permeability in both clinical and in-vitro settings are, therefore, required to ensure consistency across research. Further inconsistencies exist in the quantification of tight junction proteins (Table 1). This situation is particularly evident in recent preclinical studies [43**,44] that present conflicting evidence regarding intestinal occludin and claudin-1 protein and mRNA expression. However, it is important to note that these studies investigate different cytotoxic agents (MTX and irinotecan) and conflicting results may, therefore, reflect the differing mechanisms of action of these agents. Results from these studies must also be interpreted with caution due to their small sample size and chemotherapeutic regimens that do not reflect clinical practice. Further preclinical work is, therefore, required to clarify chemotherapy-induced tight junction disruption, placing emphasis on the effect of chemotherapy.
### Table 1. Key studies highlighting the effect of various cytotoxic agents on mucosal barrier function, intestinal permeability and the molecular architecture of tight junctions

<table>
<thead>
<tr>
<th>References</th>
<th>Experimental design</th>
<th>Cytotoxic agents</th>
<th>Permeability assay</th>
<th>Mucosal barrier function</th>
<th>Tight junction characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Keefe [14]</td>
<td>Clinical (n = 35)</td>
<td>Cyclophosphamide, carboplatin, melphalan, etoposide, busulphan, methotrexate, epirubicin</td>
<td>Urinary lactulose and mannitol excretion</td>
<td>Significantly increased (P &lt; 0.05) intestinal permeability; peaked day 7 after treatment</td>
<td>Data not provided</td>
</tr>
<tr>
<td>Melichar [12]</td>
<td>Clinical (n = 10)</td>
<td>Cisplatin, folic acid, SFU, tegafur, gemcitabine, paclitaxel, doxorubicin</td>
<td>Urinary lactulose, D-xylene and mannitol excretion</td>
<td>Intestinal permeability to lactulose was significantly increased after treatment (P &lt; 0.0001)</td>
<td>Data not provided</td>
</tr>
<tr>
<td>Fazeny-Dörner [35]</td>
<td>Clinical (n = 14)</td>
<td>Polydrug-chemotherapy IFADIC (ifosfamide, Adriamycin, dacarbazine)</td>
<td>Urinary lactulose and mannitol excretion</td>
<td>Permeability index was significantly higher (P &lt; 0.01) in patients receiving polydrug chemotherapy; peaks coincided with maximal nausea symptoms</td>
<td>Data not provided</td>
</tr>
<tr>
<td>Sukkar [37]</td>
<td>Clinical (n = 12)</td>
<td>Adjuvant SFU and levamisole</td>
<td>Urinary lactulose and mannitol excretion</td>
<td>5-FU/LV treatment induced significant increase in intestinal permeability (P &lt; 0.0001)</td>
<td>Tight junction involvement speculated</td>
</tr>
<tr>
<td>Blijlevens [15]</td>
<td>Clinical (n = 159)</td>
<td>BCNU, etoposide, cytarabine, melphalan, cyclophosphamide, total body irradiation, idarubicin, busulphan</td>
<td>Urinary lactulose, urhamose, D-xylene and 3-O-methylglucose excretion</td>
<td>Significantly increase in intestinal permeability (P &lt; 0.003)</td>
<td>Tight junction involvement speculated</td>
</tr>
<tr>
<td>Choi [38]</td>
<td>Clinical (n = 51)</td>
<td>Adjuvant SFU and levamisole</td>
<td>$^{51}$Cr-EDTA urinary excretion</td>
<td>SFU/LV treatment resulted in significantly increased intestinal permeability (P &lt; 0.001); correlated with mucositis/toxicity severity (P &lt; 0.001, r = 0.898)</td>
<td>Authors hypothesized tight junction dysfunction; no follow up study</td>
</tr>
<tr>
<td>Hamada [41]</td>
<td>Preclinical in vivo (no sample size provided)</td>
<td>Methotrexate (1.5 mg/kg; 3–5 days)</td>
<td>FD-4 permeability</td>
<td>Methotrexate administration caused increased FD-4 permeability (P &lt; 0.01)</td>
<td>Cytoplasmic redistribution, altered expression and decreased tyrosine phosphorylation (P &lt; 0.05) of jejunal ZO-1</td>
</tr>
<tr>
<td>Youmba [43*]</td>
<td>Preclinical in vivo (n = 8)</td>
<td>In vivo methotrexate (2.5 mg/kg; 3 days)</td>
<td>Trans-epithelial resistance (Ω cm$^2$) was used as a measure of permeability; assessed using an epithelial volt-ohm-meter</td>
<td>Methotrexate significantly increased permeability (P &lt; 0.001)</td>
<td>Decreased occludin and claudin-1 expression in the jejunum (P &lt; 0.05)</td>
</tr>
<tr>
<td>Nakao [44]</td>
<td>Preclinical in vivo (n = 10)</td>
<td>Irinotecan (250 mg/kg)</td>
<td>Trans-epithelial resistance (Ω cm$^2$) was used as a measure of permeability; assessed in Ussing chambers</td>
<td>Irinotecan caused significantly increased permeability in the colon (P &lt; 0.05)</td>
<td>Decreased occludin and claudin-1 protein and mRNA expression (P &lt; 0.05) in the jejunum and colon</td>
</tr>
</tbody>
</table>

5-FU, 5-fluorouracil; BCNU, bis-chloroethylnitrosourea; EDTA, ethylenediaminetetraacetic acid; FD4, fluorescein isothiocyanate-labeled dextran; IFADIC, ifosfamide, Adriamycin, dacarbazine; LV, L-leucovorin; ZO, zonular occludens.
on all tight junctions proteins and establishing a time course for these changes. This approach would allow for correlation between tight junction disruption and documented events in the pathophysiology of CIGT such as peak histological damage and the onset of clinical symptoms [7]. Validation of these changes in patient groups would then be warranted.

**MATRIX METALLOPROTEINASES MEDIATE TIGHT JUNCTION PROTEINS**

Matrix metalloproteinases (MMPs) affect numerous biological phenomena and have recently been recognized for their proteolytic functions [18]. In fact, substantial in-vitro evidence exists to support a role for MMP-mediated tight junction protein proteolysis, particularly in the case of occludin [45,46]. This factor is of particular importance as MMPs have recently been identified as key mediators in the development of CIGT, with significantly increased serum and tissue levels present during development of mucosal injury [31]. Although far more common in endothelial cells, MMP-mediated occludin proteolysis has been reported in epithelial tight junctions. For example, estrogen-induced MMP-7 elevation in human ectocervical epithelial cells was shown to cause occludin downregulation and degradation [47]. Importantly, inhibition of MMP-7 ameliorated occludin proteolysis and restored barrier function. Similarly, a recent preclinical study indicated that MMP-2 and MMP-9, both of which are strongly implicated in the pathogenesis of gut toxicity, induced ZO-1 and occludin downregulation [48]. Given the emerging evidence for MMP-dependent tight junction disruption, the prevailing hypothesis is that elevated MMP levels contribute, in part, to mucosal barrier dysfunction following cytotoxic therapy through tight junction proteolysis (Fig. 1).

**POTENTIAL INVOLVEMENT OF TIGHT JUNCTIONS IN CHEMOTHERAPY-INDUCED DIARRHEA**

Chemotherapy-induced diarrhea (CID) is a major clinical manifestation of CIGT that has only been recently appreciated. Between 20 and 40% of patients receiving chemotherapy will experience significant diarrhea, depending on the regimen and other treatment cofactors [49]. CID is also a major dose-limiting factor [50]. In severe cases, cessation of treatment is required, which leads to compromised patient outcomes [51]. Although the mechanism of both gut and oral toxicity have been investigated in great detail, the mechanisms responsible for the development of CID remain unclear [49]. Much of the current literature is based on

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**FIGURE 1.** Proposed mechanisms for matrix metalloproteinase (MMP)-dependent tight junction disruption and subsequent mucosal barrier dysfunction. MMPs are significantly elevated following chemotherapy [31] and have been shown *in vitro* to induce tight junction proteolysis [47]. Altered mucosal barrier function enables bacterial translocation and altered solute fluxes. Thus, the prevailing hypothesis elevated MMP levels contribute, in part, to chemotherapy-induced diarrhea through tight junction proteolysis.
clinical observations, thus robust scientific investigation is lacking. It is thought that CID development is a multifactorial process and may be caused by altered gut motility, decreased transit time associated with malabsorption and impaired absorptive ability, elevations in proinflammatory cytokines and associated inflammation, alterations in the luminal microflora and transient lactulose intolerance [49,52]. Detailed research conducted to delineate the mechanism of CID has largely focused on irinotecan, which causes a biphasic diarrhea response [52]. It has been postulated that deglucuronidation of 7-ethyl-10-hydroxycamptothecin (SN-38), the active metabolite of irinotecan, may amplify toxicity causing severe intestinal damage and resulting in diarrhea [33]. Further links have been established between SN-38, cyclooxygenase-2-mediated inflammation and prostaglandin release, however, to date the literature has been unable to identify which biological cause is most pivotal. Recent research investigating the pathogenesis and symptomology of certain inflammatory bowel disorders (IBDs) has indicated that mucosal barrier dysfunction and tight junction defects contribute to diarrhea development through leak-flux mechanisms [24]. IBDs present with very similar clinical manifestations to those seen in patients with severe gut toxicity induced by chemotherapy. As such, alterations in tight junctions and mucosal barrier integrity have been hypothesized as potential mechanisms of CID. A recent preclinical study further supports this hypothesis by identifying marked derangement of intestinal electrolyte and water fluxes that strongly correlated with overt diarrhea in MTX-treated rats [53]. However, it is important to note that despite having similar clinical manifestations, IBDs and CIGT have vastly different pathogeneses and inflammatory characteristics. As such, any extrapolation of potential mechanisms from IBDs to CIGT must be interpreted with caution. Further, CID development is multifactorial and likely to be the result of confounding diseases. Thus, tight junction disruption may play only a small role in its pathophysiology. Mucosal barrier dysfunction also leads to dysregulated transit across the intestinal epithelium allowing penetration of potentially noxious agents and pathogens into the underlying tissue [54]. This phenomenon was recently reported, with pathogenic bacteria identified on the epithelial basolateral surface and in mesenteric lymph nodes of irinotecan-treated rats [44]. Detailed investigations highlighted that this bacterial translocation exacerbated sepsis and worsened diarrhea severity. As such, it is possible that mucosal barrier dysfunction may contribute to diarrhea development through leak-flux mechanisms, comparable with that seen in IBDs, and also bacterial translocation, thus reiterating the importance of the tight junction integrity in gastrointestinal health.

CONCLUSION
Tight junctions are highly dynamic structures, able to undergo numerous modifications in response to various pathological cues. There is substantial anecdotal evidence suggesting that tight junctions play pivotal roles in the pathophysiology of gut toxicity. However, there has been minimal research investigating specific molecular changes in these highly plastic intercellular structures. Alterations in the expression and distribution of key tight junction proteins, occludin, claudin-1 and ZO-1, have been identified, thus substantiating preliminary evidence. Despite these advances, further investigation is now required to delineate the time-course of tight junction changes and the underlying mechanisms. MMP-mediated tight junction proteolysis is a well-documented phenomenon in endothelial tight junctions, specifically in the blood–brain barrier. Given that MMPs are intimately involved in the pathogenesis of CIGT, MMP-dependent epithelial tight junction alteration should be a focus for future research. Additionally, mucosal barrier dysfunction has recently been linked with the development of diarrhea in other cases of intestinal inflammation. Given the profound clinical and economic burden associated with CID, further research is now warranted into the role tight junctions play in its development.

Acknowledgements
Ms Hannah R Wardill is the recipient of an Australian Post-Graduate Award.

Conflicts of interest
There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING
Papers of particular interest, published within the annual period of review, have been highlighted as:
- of special interest
- of outstanding interest
Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 236–237).

Chemotherapy-induced mucosal barrier dysfunction Wardill and Bowen

Chemotherapy for cancer can cause significant gut toxicity, leading to severe clinical manifestations and an increased economic burden. Despite much research, many of the underlying mechanisms remain poorly understood hindering effective treatment options. Recently there has been renewed interest in the role tight junctions play in the pathogenesis of chemotherapy-induced gut toxicity. To delineate the underlying mechanisms of chemotherapy-induced gut toxicity, this study aimed to quantify the molecular changes in key tight junction proteins, ZO-1, claudin-1, and occludin, using a well-established preclinical model of gut toxicity. Female tumor-bearing dark agouti rats received irinotecan or vehicle control and were assessed for validated parameters of gut toxicity including diarrhea and weight loss. Rats were killed at 6, 24, 48, 72, 96, and 120 h post-chemotherapy. Tight junction protein and mRNA expression in the small and large intestines were assessed using semi-quantitative immunohistochemistry and RT-PCR. Significant changes in protein expression of tight junction proteins were seen in both the jejunum and colon, correlating with key histological changes and clinical features. mRNA levels of claudin-1 were significantly decreased early after irinotecan in the small and large intestines. ZO-1 and occludin mRNA levels remained stable across the time-course of gut toxicity. Findings strongly suggest irinotecan causes tight junction defects which lead to mucosal barrier dysfunction and the development of diarrhea. Detailed research is now warranted to investigate posttranslational regulation of tight junction proteins to delineate the underlying pathophysiology of gut toxicity and identify future therapeutic targets.

**Introduction**

Chemotherapy for cancer can cause significant gut toxicity leading to severe clinical manifestations affecting the entirety of the gastrointestinal tract (GIT). Symptoms including but not limited to pain, ulceration, vomiting, and diarrhea, are a significant burden on patients’ quality of life, requiring greater resource utilization, and resulting in significant economic burden. Chemotherapy-induced gut toxicity (CIGT) is therefore a clinical and economic challenge to oncology practice. Currently there are limited treatment options for patients with CIGT and development of effective preventative therapies is hampered by a lack of understanding of the underlying pathobiological mechanisms.

CIGT development is a multifactorial process characterized by dynamic biochemical interactions between chemotherapeutic agents and cellular constituents of the mucosa. Recent research has focused on the molecular mechanisms that underpin CIGT, highlighting roles for apoptosis, the immune system, the gut microbiome, and matrix metalloproteinases (MMPs). More recently, intestinal tight junctions were proposed to play important roles in the pathophysiology of CIGT.

Dynamic regulation of tight junctions is fundamental to many physiological processes as disruption drastically alters mucosal barrier function and intestinal permeability, making these traits a hallmark of many pathological states. The molecular architecture of the tight junction exhibits a complex arrangement of interacting cytoplasmic and transmembrane proteins. Briefly, peripherally located zonular occludens (ZO) proteins interact to anchor tight junction membrane proteins to the cytoskeleton. These scaffolding proteins play important roles in tight junction formation and are crucial in barrier integrity. Claudins are essential components of the intercellular tight junction and major determinants of paracellular solute fluxes. The structural organization of claudin proteins varies and different claudin subtypes are responsible for different roles within the tight junction.

Caudin-1 is of particular interest with regards to gastrointestinal inflammation and has been implicated in the pathophysiology of a number of inflammatory bowel disorders (IBDs). Further, recent research has identified roles for claudin-1 in apoptosis and cellular regeneration, both of which are key events in CIGT. Occludin is a key transmembrane protein integral to tight junction integrity. The importance of occludin to tight junction...
function has been conclusively demonstrated through numerous investigations. For example, knockout (occludin−/−) mice exhibit morphologically intact tight junctions, however, poor tight junction integrity and mucosal barrier dysfunction follow. These results indicate likely roles for occludin in tight junction stability and barrier function as opposed to tight junction assembly.

There is substantial anecdotal evidence to suggest that tight junctions play key roles in the development of CIGT. In 1997, Keefe et al. identified a transient abnormality in intestinal permeability in patients receiving high-dose chemotherapy. Marked abnormalities in intestinal permeability have also been shown in patients receiving various myeloablative treatments indicating that intestinal function is compromised by various cytotoxic regimens. Further, morphological defects in tight junctions have been identified, with Keefe et al. (2000) demonstrating significant increases in the number of open intestinal tight junctions in patients receiving high-dose chemotherapy. Given the regulatory roles of tight junctions in maintaining mucosal barrier function, these data strongly suggest tight junction involvement in CIGT pathophysiology.

In addition to these clinical findings, recent in vivo research has identified elevated proinflammatory cytokines and pathogenic bacteria as hallmarks of CIGT. Importantly, proinflammatory cytokines and pathogenic bacteria exhibit modulatory effects on tight junction proteins and therefore may be responsible for changes in barrier integrity. Breakdown of the mucosal barrier enables noxious agents and pathogens to penetrate the epithelium. This phenomenon was recently reported, with bacteria found in the basolateral compartment and mesenteric lymph nodes of chemotherapy-treated rats. It is therefore hypothesized that bacteria- and proinflammatory cytokine-mediated tight junction disruption are pivotal in the development of gut toxicity.

**Results**

**Irinotecan causes significant gut toxicity**

All rats receiving irinotecan developed gut toxicity represented by diarrhea and significant weight loss. Diarrhea occurred in a biphasic response, with symptoms first appearing at 6 h after irinotecan administration. There was an initial resolution of diarrhea before a second more severe diarrhea appeared, with maximal symptoms seen 72 h post-irinotecan. Rats receiving vehicle control did not develop diarrhea at any time point (Fig. 1A).

Rats receiving irinotecan had a peak weight loss compared with baseline at 72 h after chemotherapy (mean ± SD = 11.1 ± 6.6%, P < 0.0001) before recovery at 120 h (mean ± SD = -0.25 ± 6.7%). Rats receiving vehicle control continued to gain weight over the course of the experiment (Fig. 1B).

**Irinotecan causes severe histological damage in the small and large intestines**

Marked histological evidence of gut toxicity was observed in the jejunum and colon of irinotecan-treated rats (Fig. 2). Characteristic apoptotic bodies were observed at 6 h post-irinotecan in the crypt epithelium of the both regions of the gut. Gross architectural disturbances, including villous blunting and crypt degeneration throughout the mucosa, were particularly evident 48 h and 72 h after irinotecan administration (Fig. 2). Restoration of the mucosa was evident by 120 h, indicated by the return of architectural integrity and the presence of mitotically active cells.

**Irinotecan causes molecular defects in intestinal tight junction proteins**

ZO-1

There were no significant changes in protein expression of ZO-1 in the jejunum at any time point investigated (P = ns) (Fig. 3). In contrast, there was a significant decrease in ZO-1 protein expression 96 h following administration of irinotecan in the colonic crypts (P < 0.05) (Figs. 4 and 5). ZO-1 mRNA expression remained stable across the time course of gut toxicity (P = ns) in the small and large intestine (data not shown).

Claudin-1

Claudin-1 protein expression was significantly decreased 6 h after irinotecan administration in both the apical and basal villus regions of the jejunum (P < 0.05) (Fig. 3). Expression returned to baseline at 72 h. No significance difference was observed in jejunal crypts (P = ns). There was a significant decrease in claudin-1 protein expression at 24 and 96 h following irinotecan in the...
apical and basal colonic crypts, respectively ($P < 0.05$) (Figs. 4 and 5). Claudin-1 mRNA expression was significantly downregulated ($P < 0.05$) 6 h following chemotherapy in both the jejenum (~8.3-fold change) and colon (~1.8-fold change) of DA rats. Levels remained significantly ($P < 0.05$) low at 24 h in the colon only (~2.5-fold change). A 9.8-fold increase in claudin-1 mRNA expression was observed in the jejunum at 72 h following irinotecan (Fig. 6).

**Occludin**

Occludin protein expression was significantly downregulated in the jejunal crypts 48 h following irinotecan administration ($P < 0.05$) (Fig. 3). This pattern of expression was mirrored in the colon, where occludin protein expression was significantly downregulated ($P < 0.05$) in both apical and basal crypt regions at 48 h following irinotecan treatment (Fig. 4 and 5). There was no change in occludin mRNA expression in the jejunum or colon of DA rats ($P = \text{ns}$) at any time point under investigation (data not shown).

**RT-PCR efficiency**

The amplification efficiencies for each set of primers were measured using serial dilutions of cDNA in triplicate. PCR efficiency and a standard curve were calculated by the Rotor Gene 6 program. Variations in cycle times and annealing temperatures did not yield comparable PCR efficiencies (ZO-1 = 1.01; claudin-1 = 1.1; occludin = 1.21; UBC = 1.31); therefore the Pfaffl method\(^{29}\) of relative quantification was employed.
Stability of housekeeping gene (UBC)

The fold change in UBC expression was calculated using the $2^{-\Delta\Delta C_{t}}$ method where control rats were used as a baseline. UBC showed no differential mRNA expression in the jejunum or colon of DA rats across the time-course of irinotecan-induced gut toxicity (data not shown), indicating high stability.

Discussion

Chemotherapy-induced gut toxicity (CIGT) has become increasingly important as a dose-limiting factor of anti-cancer therapies, particularly with the advent of more aggressive chemotherapy regimens. Evidence exists for the involvement of tight junctions in the pathophysiology of CIGT, however, to date few studies have conducted detailed investigations of intestinal tight junctions following chemotherapy. The present study therefore aimed to characterize the expression of three key tight junctional proteins, ZO-1, claudin-1, and occludin, in both the small and large intestine using a well-established pre-clinical model of gut toxicity.

Figure 4. Irinotecan causes tight junction defects in the colon of DA rats. ZO-1 protein expression was significantly decreased in the apical crypt epithelium 96 h following chemotherapy. Irinotecan caused significant downregulation in claudin-1 protein expression in the apical crypt epithelium 24 h following treatment, while a significant decrease was observed 96 h following irinotecan in the crypt crypt epithelium of the colon. Significant downregulation in occludin protein expression was observed at 24 and 48 h following irinotecan administration in the apical and basal crypt epithelium. ZO-1, claudin-1, and occludin protein expression was analyzed in the basal and apical crypt epithelium of the colon. Staining intensity was analyzed in a blinded fashion (HR Wardill and RJ Gibson) using a validated semi-quantitative grading system. A Kruskall–Wallis with a Dunn multiple comparison was performed to determine significance. Data presented as median values ($n = 39$); □ $P < 0.05$ vs. control.

Figure 5. Tight junction protein (ZO-1, claudin-1, and occludin) immunostaining in the colon at selected time points following irinotecan (175 mg/kg ip) administration. Photomicrographs taken at original magnification 400×.
Mechanisms of CIGT due to highly comparable clinical manifestations of IBDs are often used to identify potential mechanisms of intestinal electrolyte and water fluxes which correlate with overt treatment-induced diarrhea. Despite having vastly different pathogeneses, inflammatory processes of IBDs have shown that tight junction defects and gene levels strongly implicated tight junctions in the development of CIGT.

Late-onset diarrhea is a key dose-limiting factor for many chemotherapy patients, however to date there are no clear mechanisms underpinning its development. The clinical and economic impact associated with chemotherapy-induced diarrhea has only recently been appreciated, prompting research efforts in an attempt to reveal the pathophysiology of this complication. Despite having vastly different pathogeneses, inflammatory bowel disorders (IBDs) are often used to identify potential mechanisms of CIGT due to highly comparable clinical manifestations. Recent research investigating the pathophysiology and symptomology of IBDs has shown that tight junction defects and barrier disturbances contribute to diarrhea development through leak-flux mechanisms. In line with this research, the present study identified tight junction defects which coincided with key events in the pathophysiology of CIGT and preceded the onset of clinical symptoms. Of particular importance was the significant decrease in ZO-1 protein expression in the colonic crypts at 96 h following insult, coinciding with the presence of severe late-onset diarrhea observed in our animal model. Previous research has also shown that chemotherapy induces significant derangement of intestinal electrolyte and water fluxes which correlate with overt treatment-induced diarrhea. Given that tight junctions are key regulators of mucosal barrier function, it is feasible that disruption to tight junction proteins, particularly ZO-1, may contribute to the development of late-onset diarrhea through leak-flux mechanisms. These findings highlight tight junctions as a potential therapeutic target in the clinical setting and detailed investigations are now required.

In addition to ZO-1 disruption, the current study identified significant changes in claudin-1 at both the protein and gene level. Previous research has identified emerging roles for claudin-1 in mediating apoptosis and aiding tissue regeneration. Liu and colleagues demonstrated this concept, reporting significantly increased levels of claudin-1 in human breast cancer MCF-7 cells—a cell line recognized for their low levels of apoptosis. In line with these findings, downregulation of claudin-1 by siRNA knockdown resulted in a significant increase in apoptosis markers such as caspase-8. Further, loss of claudin-1 was shown to increase the susceptibility of MCF-7 cells to TNF-induced apoptosis. In the current study, significantly reduced claudin-1 protein and mRNA expression was observed 6 h following irinotecan insult, coinciding with peak apoptosis levels thus supporting this novel role of claudin-1 as a potent anti-apoptotic mediator. Claudin-1 downregulation was also identified 24 h following chemotherapy, and this is likely due to the slower proliferative rate of the colon relative to the jejunum. In addition to apoptosis, our results also support a role for claudin-1 in aiding tissue regeneration, a newly identified function of claudin-1, as its overexpression coincides with the known time point of cellular regeneration in the epithelium of the jejunum and colon (72 h). Unexpectedly however, this was accompanied by a decrease in protein expression in the colonic crypts and which may be attributable to the second wave of apoptosis, as the body resets homeostasis.

There is conflicting evidence regarding mRNA analysis of tight junction proteins following chemotherapy. Most recently, a preclinical study conducted by Nakao et al. (2012) investigated tight junction proteins in tumor-naive rats and found tight junction changes were associated with gut barrier dysfunction. Specifically, a significant decrease in both occludin and claudin-1 mRNA expression was identified; however given their small sample size and single time-point evaluation, these conclusions should be interpreted with caution. In contrast, several other preclinical studies found no change in occludin and claudin-1 mRNA expression following administration of methotrexate (MTX), but demonstrated decreased claudin-1 mRNA expression. In accordance with these latter findings, our study did not identify any significant change in occludin or ZO-1 mRNA expression following irinotecan administration. These inconsistencies highlight the need for further research to clarify the effect of various cytotoxic agents on tight junction protein mRNA levels at time points relevant to clinical symptoms.

The disparate protein and mRNA expression identified in the present study suggest that irinotecan indirectly modulates both occludin and ZO-1 through posttranslational regulation. Posttranslational modification of occludin and ZO-1, in particular proteolytic degradation, is a well-documented phenomenon. Recent research has indicated proinflammatory cytokines are
able to modulate tight junction proteins and it is therefore hypothesized that cytokine-mediated tight junction modulation may play a key role in the development of CIGT. In vitro application of tumor necrosis factor (TNF) has been shown to induce actin and tight junction rearrangement, resulting in a time-dependent decrease in tight junction protein expression and a parallel increase in intestinal permeability. Previous research has shown that elevated serum TNF and interleukin-1 are hallmarks of CIGT pathophysiology, with peaks occurring at 24–48 h post-chemotherapy. The present study has shown that occludin downregulation also occurs at these time-points, implying a correlation between the two. It is therefore suggested that proinflammatory cytokines not only contribute to histological damage in the gut, but also mediate mucosal barrier function through tight junction modulation.

Matrix metalloproteinases (MMPs) are extracellular matrix signaling molecules recently recognized for their proteolytic functions. Substantial in vivo evidence supports a role for MMP-mediated occludin proteolysis and membrane cleavage. This is of particular importance as MMPs have recently been identified as key mediators of intestinal toxicity induced by irinotecan. Al-Dasooqi and colleagues (2010) reported significantly increased small intestinal and serum levels of MMP-9 and MMP-12 following irinotecan. Given the proteolytic functions of MMPs this suggests MMP-mediated tight junction damage may occur. This correlation has previously been shown in the central nervous system, with MMP-9 levels in brain tissue correlating with reductions in occludin and ZO-1 protein levels. Further, MMP inhibition has been shown to ameliorate hypoxia-induced occludin proteolysis. Further studies are now warranted in order to establish the molecular mechanisms of these posttranslational regulations.

In addition to posttranslational modulation, reversible phosphorylation of tight junction proteins has recently been established as a vital aspect of tight junction integrity and barrier regulation. Despite the importance of tight junction phosphorylation for optimal tight junction assembly and function, few studies have investigated posttranslational phosphorylation of tight junction proteins in the gut. Hamada and colleagues reported decreased tyrosine phosphorylation of ZO-1 despite observing no change in ZO-1 protein and mRNA expression. Although not addressed in this study, dephosphorylation may be responsible for chemotheraphy-induced intestinal barrier dysfunction and future investigations are now necessary.

Conclusion

Tight junction proteins can undergo a broad array of regulatory modification in response to various pathological cues, highlighting the plasticity of these dynamic signaling complexes. These molecular events are likely to contribute to mucosal barrier dysfunction, increased intestinal permeability and the development of diarrhea; all characteristic of CIGT. It is likely that ZO-1 primarily is a late-onset and symptom-associated protein, whereas claudin-1 and occludin have more potential as prognostic markers as their modulation precedes barrier dysfunction and symptomology. Detailed research is now required to investigate posttranslational modulation of tight junction proteins to elucidate the molecular mechanisms that underpin the development of gut toxicity. This may lead to a greater understanding of how tight junction modifications affect gastrointestinal homeostasis, thus revealing their therapeutic potential in chemotherapy-induced gut toxicity and other diseases characterized by barrier dysfunction.

Methods

Animals and ethics

Female Dark Agouti (DA) rats, weighing between 150 and 170 g were used for this study. Rats were housed in Perspex cages at a temperature of 22 ± 1 °C and subject to a 14 h light/10 h dark cycle. Animals had ad libitum access to autoclaved chow and water. Experimental design was approved by the Animal Ethics Committee of the Institute of Medical and Veterinary Science (IMVS), and The University of Adelaide, and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Teaching.

Experimental design

The dark agouti mammary adenocarcinoma (DAMA) rat model of irinotecan-induced gut toxicity was used to conduct this study. DA rats are tumor-bearing as our previous research has shown that the response to irinotecan is more pronounced in tumor-bearing rats compared with naive rats. Forty rats were randomly assigned to receive either irinotecan (n = 5–9 per time point) or vehicle control (n = 6). All rats received breast cancer inoculum as described previously. Briefly, mammary adenocarcinoma tumors syngeneic with the DA rat were diced, homogenized and filtered through sterile gauze. A viable cell count was conducted using 0.4% w/v trypan blue before 150 μL of cells were implanted subcutaneously into both right and left flanks of the rat at a concentration of 2.0 × 10^6 cells/mL. Tumors were allowed to grow for one week prior to administration of chemotherapy and did not exceed 15% of their total body weight at study end point. All rats received 0.01 mg/kg subcutaneous atropine (to reduce the cholinergic reaction) immediately prior to administration of either 175 mg/kg irinotecan (intraperitoneal) (kindly supplied by Pfzer, administered in a sorbitol/lactic acid buffer: 45 mg/mL sorbitol/0.9 mg/mL lactic acid, pH 3.4), or vehicle control (sorbitol/lactic acid buffer). This buffer has previously been shown to have no gut toxicity effects. Groups of rats were killed by exsanguination and cervical dislocation while under 3% isoflourane in 100% O_2 anesthesia at times 6, 24, 48, 72, 96, and 120 h post-irinotecan treatment. The entire gastrointestinal tract (from the pyloric sphincter to the rectum) was dissected out and separated into the small intestine (pyloric sphincter to ileocecal sphincter) and colon (ascending colon to rectum). The small intestines and colons were flushed with chilled, sterile saline (Baxter Healthcare), and 1 cm samples dissected from 50% of the length of each, and fixed in 10% neutral buffered formalin, processed, and embedded in paraffin for immunohistological analyses.
Gut toxicity assessment

Gut toxicity assessment, including measures of weight loss and diarrhea, was recorded on a daily basis including baseline. Animals were weighed daily at the same time, and total weight loss/gain recorded. Further, diarrhea occurrence and severity was recorded 4x daily according to previous grading:

- 0, no diarrhea;
- 1, mild diarrhea (staining of anus);
- 2, moderate diarrhea (staining spreading over top of legs);
- 3, severe diarrhea (staining over legs and abdomen, often with continual anal leakage).

All gut toxicity assessments were conducted in a blinded fashion (RJ Gibson and M Sultani).

Tissue preparation

Samples of small (jejunum) and large (colon) intestine were collected, snap frozen in liquid nitrogen and stored in RNAlater (Ambion) for molecular analysis. All tissues were stored at −80 °C until required. Additional samples (1 cm in length) of jejunum and colon were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin wax for histological and immunohistochemical analyses.

Histological analysis

Sections of jejunum and colon were cut from paraffin blocks at 5 μm and mounted onto glass microscope slides. Slides were dewaxed in xylene, stained with Lilli-Mayer hematoxylin for 10 min and counterstained in eosin for 3 min. Slides were dehydrated through graded ethanol, cleared in xylene and coverslipped. Slides were scanned using the NanoZoomer (Hamamatsu Photonics) and assessed with NanoZoomer Digital Pathology software (Histalim) and coverslipped. Slides were scanned using the NanoZoomer Digital Pathology software (Histalim) using the Agilent 2100 Bioanalyser RNA 6000 Nano Chip (Series II) kit.

Immunohistochemistry

Immunohistochemical analysis was performed using the Level 2 USA™ Ultra Streptavidin Detection System kit (Signet Laboratories) as per manufacturer’s instructions. Antigen retrieval was conducted using either a 10 mM/l citrate buffer (occludin and claudin-1) or protease pretreatment (Sigma PS147). To reduce nonspecific staining sections were blocked in 3% hydrogen peroxidase (H₂O₂)/methanol solution and 5% normal blocking serum in 0.01 M of PBS containing 0.1% sodium azide (NaN₃). Sections were incubated with avidin/biotin blocking solution (Signet Laboratories) for 15 min, before the primary antibody was applied for overnight a 4 °C (rabbit polyclonal anti-claudin-1 (10 μg/μl) and anti-ZO-1 (10 μg/μl); mouse monoclonal anti-occludin (2.5 μg/μl)). Negative control sections had the primary antibody omitted and liver was used as a positive control in all experiments. Both linking and labeling reagents were applied (Signet Laboratories) followed by diaminobenzidine (DAB) chromogen in 0.03% hydrogen peroxidase. Slides were counterstained in Harris Haematoxylin, before being dehydrated, cleared, and coverslipped. Liver was used as a positive control in all immunohistochemistry runs. Slides were assessed using NanoZoomer Digital Pathology software (Histalim) and analyzed using a validated semi-quantitative grading system: 0 = no staining, 1 = weak, 2 = mild, 3 = moderate, and 4 = intense.

RNA extraction

RNA extraction was performed on jejunum and colon samples as per manufacturer’s instructions (NucleoSpin RNA Isolation Kit, Macherey-Nagel). Briefly, tissue samples were homogenized in 250 μl and 500 μl of TRIzol® Reagent (Invitrogen), respectively. Samples were centrifuged for 15 min at 4 °C and the upper aqueous layer removed. Following a sequence of filtration steps, RNA-binding conditions were adjusted and DNA digestion performed. A series of washing steps was performed before highly pure mRNA was eluted in RNase-free water. Once eluted, RNA was stored at −80 °C.

Assessment of RNA quantity and quality

RNA was quantified for yield (ng/μl) and purity using the Thermo Scientific Nanodrop 1000 spectrophotometer. RNA integrity was assessed at the Adelaide Microarray Facility (SA Pathology) using the Agilent 2100 Bioanalyzer RNA 6000 Nano Chip (Series II) kit.

Reversal transcription and RT-PCR

One microgram of RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad) as per manufacturer’s instructions. RT-PCR was performed using the Rotor-Gene 3000 (Corbett Research). Amplification mixes contained 1 μl of cDNA sample (100 ng/μl), 5 μl of SYBR green fluorescence dye, 3 μl of RNase-free water and 0.5 μl of each forward and reverse primer (prediluted to 50 pmol/μl) to make a total volume of 10 μl. Thermal cycling conditions included a denaturing step at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 10 s, annealing at 56 °C for 15 s, and extension at 72 °C for 20 s. All samples were run in triplicate. Primer efficiency was evaluated using standard curves and cycle threshold (Ct) values were calculated by Rotor Gene 6 analysis software. Ct values were used to quantify occludin gene expression, relative to untreated control calibrator and a validated housekeeping gene using the Pfaffl method of relative quantification. Ubiquitin C (UBC), a validated housekeeping gene, was used as a reference gene in all RT-PCR runs. Housekeeping gene stability was validated using the 2−ΔΔCt method.

Statistical analysis

Data were compared using Prism version 6.0 (GraphPad® Software). A Kolmogorov–Smirnov test was used to assess normality and a Bartlett test was employed to assess equal variance. When normality and equal variance were confirmed, a one-way analysis of variance with a Tukey post hoc test was used to identify statistical significance between groups. In other cases, a Kruskall–Wallis with a Dunn multiple comparison was used to identify statistical significance. A P value < 0.05 was considered statistically significant.

Disclosure of Potential Conflicts of Interest

No potential conflicts were disclosed.
Acknowledgments

The authors would like to thank Mrs Emily Schneider, Mrs Nadia Gagliardi, and Mr Chris Leigh from the School of Medical Sciences for technical assistance with immunohistochemistry staining and Mr Távikh Morgenstern for technical assistance with figure layouts. J.B. is the recipient of an NHMRC Post-Doctoral Training Fellowship, N.A.-D. is the recipient of a Clinical Centre of Research Excellence Post-Doctoral Training Fellowship. Funding for this project was provided by a Cure Cancer/Cancer Australia Research Grant and a South Australian Professional Development Scholarship awarded to R.J.G.


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Tight junction defects are seen in the buccal mucosa of patients receiving standard dose chemotherapy for cancer

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Received: 21 July 2015 / Accepted: 21 September 2015 / Published online: 6 October 2015 © Springer-Verlag Berlin Heidelberg 2015

Abstract

Purpose Oral mucositis is one of the most common and debilitating side effects of chemotherapy treatment. Patients are often unable to eat and drink, which can lead to poor clinical outcomes and extensive resource utilisation. The primary aim of this study was to determine the molecular integrity of oral epithelial tight junctions in patients undergoing chemotherapy. The secondary aim was to correlate these changes with proinflammatory cytokines and matrix metalloproteinase profiles.

Methods Patients (n = 23) were recruited from the Royal Adelaide Hospital between 2000 and 2003. Each patient underwent two oral buccal mucosa biopsies (4 mm): one prior to chemotherapy treatment and a second one after chemotherapy treatment. Oral buccal mucosa biopsies were also taken from seven healthy volunteers with no history of cancer, chemo- or radiotherapy treatment or inflammatory disorders. Routine haematoxylin and eosin staining was performed to determine epithelial thickness. Immunohistochemical staining was performed for claudin-1, zonular occludens-1, occludin, interleukin-1β, tumour necrosis factor, interleukin-6, matrix metalloproteinase-2 and metalloproteinase-9.

Results Patients receiving standard dose chemotherapy had significant epithelial atrophy. Elevations in all cytokines and matrix metalloproteinases were seen, with significant lamina propria staining for interleukin-6 and tumour necrosis factor. Matrix metalloproteinase-2 appeared most upregulated within the oral epithelium. These changes coincided with altered tight junction staining properties. Changes in the staining intensity and localisation were both noted, with clear cytoplasmic staining for zonular occludens-1 and claudin-1 in patients treated with chemotherapy.

Conclusions Chemotherapy causes defects in oral tight junctions, coupled with altered cytokine and matrix metalloproteinase profiles. Tight junction disruption in the epithelium may contribute to ulcer development or lead to poor tissue integrity, and the timing of these events may be a target for preventative treatment.

Keywords Mucositis · Chemotherapy · Oral toxicity · Tight junctions · Clinical · Oral mucosa

Introduction

Chemotherapy treatment is associated with a host of debilitating side effects with varying effects on patient quality of life, resource utilisation and treatment efficacy. Over the past decade, there has been an appreciation gained for the impact of chemotherapy-induced alimentary mucositis on patient quality of life, leading to vast improvements in our understanding of its pathobiology [1, 2]. Mucositis is characterised by severe
ulceration along the entire alimentary tract [3]; however, oral lesions are most easily accessed and therefore diagnosed. In fact, oral mucositis is frequently described as the most common dose-limiting factor for patients undergoing chemotherapy treatment, affecting 80–100 % of those receiving high-dose treatment [4, 5]. The development of oral mucositis in patients during cancer treatment places a significant clinical and economic burden on the provision of care. Additionally, oral mucositis can compromise treatment outcomes and, in itself, increases mortality through heightened infection risk. Despite its prevalence and clinical impact, there is limited data on the molecular mechanisms that underpin or initiate this toxicity.

It is currently accepted that the pathobiology of alimentary toxicity, in which oral mucositis is included, can be described using a continuous and overlapping 5-phase model proposed by Sonis in 2004 [6, 7]. This model was the first to recognise that alimentary toxicity is not purely an epithelial phenomenon, highlighting the dynamic interactions that occur between the epithelium, extra cellular matrix (ECM), submucosa and the chemotherapeutic agent itself. Consequently, the pathobiology is defined as the collective consequences of direct cytotoxicity, induced by the chemotherapeutic agent, as well as inflammatory-driven indirect cytotoxicity primarily controlled through nuclear factor kappa B (NF-κB). Although this model of alimentary mucositis remains universally accepted, recent advances in our understanding have identified complimentary molecular mediators of toxicity. One such example is the emerging role of tight junctions [8] in regulating barrier dysfunction commonly observed following cytotoxic treatment.

Tight junctions are highly dynamic signalling complexes vital to epithelial homeostasis. Located at the apico-lateral boundary of adjacent epithelial cells, tight junctions are integral in maintaining epithelial adhesion as well as regulating paracellular permeability [9]. Tight junctions are primarily formed of four protein groups; claudins, zonulin occludens (ZO), junctional adhesion molecules (JAMs) and occludin. Importantly, the molecular interactions of these proteins cause tight junctions to be highly malleable and plastic structure that assemble, grow, recognise and disassemble in response to various physiological and pathological cues. Based on their highly plastic nature, particularly in response to inflammatory mediators, tight junctions have gained significant attention in a number of inflammatory-based gastrointestinal pathologies, including mucositis [10, 11]. Tight junctions were first identified to be involved in the pathobiology of gastrointestinal (GI) mucositis in 1997, with Keefe and colleagues [12] showing increased and uncontrolled intestinal permeability in patients receiving high-dose chemotherapy. In 2000, ultrastructural changes in small intestinal tight junctions were identified in patients receiving various chemotherapeutic treatment regimens [5]. Since the early 2000s, several studies have identified molecular defects in intestinal tight junctions following chemotherapy treatment, with downregulation, redistributing and phosphorylation of occludin, ZO-1 and claudin-1 consistently reported [13–17]. Tight junction disruption is therefore emerging as a key player in the pathobiology of mucositis.

Modification of tight junction proteins, particularly post-translationally, is a well-documented phenomenon and forms the basis of many inflammatory pathologies [18–20]. In the setting of both oral and GI mucositis, the interaction between proinflammatory cytokines, matrix metalloproteinases (MMP) and tight junctions is compelling given the strong inflammatory component of mucositis [21] and documented changes in MMP profiles [22]. The ability of proinflammatory cytokines and MMPs to degrade tight junctions is well established [23, 24], highlighting a potential interaction between mediators of mucositis and tight junction disruption. Importantly, these mediators are not only found at elevated levels in the gut but also the oral cavity [3] and circulating serum [21] therefore suggesting that tight junction disruption may also play a role in the pathobiology of oral mucositis. This study therefore aims to determine the phenotype of oral epithelial tight junctions in patients receiving chemotherapy and correlate with established changes in proinflammatory cytokines (IL-1β, IL-6, TNF) and MMP profiles (MMP-2, MMP-9). Results from this study will determine if tight junction disruption is a common mechanism of oral and GI mucositis and may shed light on the underlying mechanisms responsible for barrier dysfunction.

Materials and methods

Patients

Tissue samples were sourced from a previously conducted study [25] published by Gibson et al. 2006. This previous study was approved by Royal Adelaide Hospital Human Ethics Committee. Briefly, patients were recruited from the Department of Medical Oncology at the Royal Adelaide Hospital between 2000 and 2003 (n = 23). The study included 7 male and 16 female patients with a median age of 52.4 years (32–86 years) [25]. Patients were excluded if they were undergoing concurrent radiotherapy to the head and neck or if they had pre-existing mucosal damage. Tumour type was heterogeneous amongst patients and included breast, non-Hodgkin’s lymphoma, Hodgkin’s lymphoma, colorectal, lung and neuroendocrine pancreatic. Standard dose chemotherapy was used in all patients, administered over 1–4 h [25]. Treatments included ABVD, AC, CMF, DOX, Docetaxel, CHOP, 5-FU/Folinic Acid, CAV and Streptozocin. For tabular breakdown of patient demographics and treatment regimens, please refer to Gibson et al. 2006.

Patients had a single oral buccal mucosa biopsy prior to the commencement of their first chemotherapy cycle and a second after cessation of their treatment (mean 4.8 days; range 3–11 days). Seven healthy volunteers (3 M:4F), with no history of cancer, chemotherapy treatment and pre-existing mucosal
damage, were also recruited for the study. All biopsies were performed by a single operator. Pre-chemotherapy biopsies were taken on one side of the mouth, and post-chemotherapy biopsies were taken on the opposite side. The surrounding buccal mucosa was injected with local anaesthetic, and a small (4 mm) punch biopsy was taken. A single stitch was placed at the site of the biopsy if necessary. The number of previous chemotherapy cycles undergone by each patient was recorded at recruitment to determine if these contributed to histological or molecular changes in the oral cavity.

Clinical assessment of oral mucositis

Case note reviews were used to identify the presence/absence of mucositis in this patient cohort at the time of sample collection. Institutional reporting guidelines did not require mandatory reporting of oral mucositis symptoms in patient case notes, and therefore, oral toxicities were not as comprehensively reported in this archival patient group as would be required today. Gibson et al. (2006) reported that 50 % of patients had mucositis symptoms of WHO grades 1–2 (relatively mild) ranging from mouth ulcers, loss of taste, mouth dryness, ‘thick’ feeling over the tongue and cheek area and fissured tongues [25]. For full tabular breakdown of mucositis severity and symptoms, please refer to Gibson et al. (2006) [25].

Histological analysis

Oral buccal mucosa biopsies were cut at 5 μm using the Leica Microtome and mounted onto glass microscope slides. Routine haematoxylin and eosin staining was conducted on all buccal mucosa biopsy samples. Briefly, sections were dewaxed and rehydrated through graded ethanol. Sections were placed in Harris Haematoxylin for 2 min before being placed in 0.5 % ammonia for 1 min. Sections were washed and placed in eosin for 2 min before being dehydrated, cleared and coverslipped. Slides were scanned using a NanoZoomer (Hamamatsu Photonics, Japan) and analysed using NanoZoomer Digital Pathology software (Histalim, Montpellier, France). Epithelial thickness was measured ten times across the width of the tissue section and an average determined [26]. All analyses were conducted in a blinded fashion.

Immunohistochemistry

Immunohistochemistry (IHC) was carried out on 4 μm sections of oral buccal mucosal cut on a rotary microtome and mounted onto FLEX IHC microscope slides (Flex Plus Detection System, Dako, Denmark; #K8020). Immunohistochemical analysis was performed for three tight junction proteins (claudin-1, ZO-1 and occludin), proinflammatory cytokines (IL-1β, IL-6, TNF) as well as MMP-2 and MMP-9 (Table 1). Immunohistochemical analysis was performed using Dako reagents on an automated machine (AutostainerPlus, Dako, Denmark) following standard protocols supplied by the manufacturer. Briefly, sections were deparaffinised in histolene and rehydrated through graded ethanols before undergoing heat mediated antigen retrieval using an EDTA/Tris buffer (0.37 g/L EDTA, 1.21 g/L Tris; pH 9.0). Retrieval buffer was preheated to 65 °C using the Dako PT LINK (pre-treatment module). Slides were immersed in the buffer and the temperature raised to 97 °C for 20 min. After returning to

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65 °C, slides were removed and placed in the Dako AutostainerPlus and stained following manufacturer’s guidelines. Briefly, endogenous peroxidase was blocked using the FLEX peroxidase block followed by a serum-free protein block (Dako, Denmark; #X0909). Primary antibodies were suspended in the EnVision™ FLEX Antibody Diluent (Dako, Denmark; #K8006) and applied for 60. Negative controls had the primary antibody omitted. The EnVision™ FLEX+ Rabbit/ Mouse LINKER (Dako, Denmark; #K8019) was then applied for 30–60 min before DAB was used to visualise the target protein. Slides were removed from the automated stainer, counterstained in Harris Haematoxylin, dehydrated and coverslipped. Slides were scanned using the NanoZoomer (Hamamatsu Photonics, Japan) and assessed with NanoZoomer Digital Pathology software (Histalim, Montpellier, France). Healthy control samples were used as an internal positive control for tight junction proteins. Human tonsil was used as a positive control for IL-1β, IL-6, TNF, MMP-2 and MMP-9.

Slides were scanned using a NanoZoomer (Hamamatsu, Japan) and analysed using NanoZoomer Digital Pathology software (Histalim, Montpellier, France). Tight junction staining was analysed in the superficial/intermediate, prickle cell and basal epithelium as well as the endothelium of the lamina propria (Fig. 1), whilst IL-1β, IL-6, TNF, MMP-2 and MMP-9 staining was analysed in the whole oral epithelium and lamina propria. Staining intensity was analysed using a validated semi-quantitative grading system [26] from 0 to 3; where 0 = no staining, 1 = mild staining, 2 = moderate staining and 3 = intense staining (Fig. 2) and was conducted in a blinded fashion [26]. In addition, the characteristics of tight junction staining, including membrane specificity and location, were assessed qualitatively.

Statistical analysis

Epithelial thickness and immunohistochemical staining were compared between healthy control samples, pre-chemotherapy samples and post-chemotherapy samples using GraphPad Prism 7.0. Data was assessed for normality using the D’Agostino-Pearson omnibus test. When normality was confirmed, a two-way analysis of variance (ANOVA) was performed with a Tukey’s post hoc. If normality was not achieved, a Kruskal-Wallis with a Dunn’s multiple comparison was performed. To determine the relationship between previous chemotherapy cycles and epithelial thickness, a linear regression model was applied and the coefficient of determination ($r^2$) was determined. A $p$ value < 0.05 was considered significant.

Results

Chemotherapy causes significant epithelial atrophy

Epithelial atrophy was observed both before ($p = 0.0008$) and following chemotherapy cycles ($p < 0.0001$; Fig. 3a, c). Given that patients were not naïve to chemotherapy treatment, it is likely that the atrophy observed prior to treatment was due to the previous cycles patients underwent. This was confirmed by a strong correlation between epithelial thickness and the

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Fig. 1 Histology of the human oral mucosa. A photomicrograph of the oral cavity stained with haematoxylin and eosin (original magnification 40×; scale bar shows 100 μm). The stratified squamous epithelium of the human oral cavity is non-keratinised, displaying four distinct layers: basal cells (B), prickle cell layer, the intermediated layer and the superficial epithelium. Mitotically active basal cells reside on the basement membrane, separating the epithelium from the underlying lamina propria. The lamina propria consists of loose connective tissue with rich vasculature and immune capabilities.
Fig. 2 Semi-quantitative grading system representative images. Representative photomicrographs of the oral epithelium showing immunohistochemical staining of varying intensities. a–d indicate epithelial staining intensities, whilst lamina propria staining is shown in images e–h. Staining is graded on a scale of 0–3, where 0 = no staining, 1 = mild staining, 2 = moderate staining and 3 = intense staining.

Fig. 3 a Epithelial atrophy was observed in cancer patients prior to chemotherapy (\(* *p = 0.0008\)) and following chemotherapy (\(* * * *p < 0.0001\)). Epithelial atrophy was significantly more severe in patients following chemotherapy compared with those prior to the onset of treatment (\(*p = 0.0042\)). b Correlation between epithelial thickness and previous cycles of chemotherapy. Data presented as mean ± SEM (b) or individual points (b) with a linear regression model. c Representative histological images showing epithelial thickness for healthy controls and patients following chemotherapy treatment. Original magnification 40×; scale bars show 50 μm.
Chemotherapy increases proinflammatory cytokines and alters MMP profiles

Increases were seen in all proinflammatory cytokines and MMPs subtypes following chemotherapy (Fig. 4). IL-1β and IL-6 showed increased expression in the epithelium of patients treated with chemotherapy ($p = 0.0017, p = 0.0167$, respectively). Although no significant change was seen in the epithelial expression of TNF across all groups ($p > 0.05$), there was a significant increase in the lamina propria following chemotherapy treatment ($p < 0.0001$). This was consistent with the changes seen in IL-6, with significant increases in patients treated with chemotherapy ($p < 0.0001$). Both IL-6 and TNF appeared most prominent in the fibrous material and amorphous ground substance of the lamina propria (Fig. 4b). MMP-9 staining remained showed mild increases in staining expression in both the epithelium ($p = 0.0039$) and lamina propria ($p = 0.0409$) of patients treated with chemotherapy. MMP-2 staining was most significant in the epithelium of patients treated with chemotherapy ($p = 0.001$), with clear cytoplasmic staining in the prickle layer indicating active secretion. The vasculature and fibroblasts in the lamina propria also showed positive MMP-2 staining in patients treated with chemotherapy.

Residual inflammatory signalling was evident in the oral cavity of patients exposed to previous chemotherapy treatment, with pre-chemotherapy biopsies displaying increased TNF in the lamina propria ($p < 0.0001$).

Tight junction defects are seen following chemotherapy

Claudin-1 and ZO-1 protein expression decreased most notably in the basal (claudin-1: $p = 0.0130$, ZO-1: $p < 0.0001$) and prickle cell layers (claudin-1: $p = 0.0078$, ZO-1: $p < 0.0001$). Despite only modest changes in the overall staining intensity of tight junction proteins, clear changes in their localisation were evident (Fig. 5). In healthy controls, ZO-1 and claudin-1 displayed strong specificity for the membrane, with epithelial staining showing the typical ‘cobblestone’ appearance. In patients treated with chemotherapy, claudin-1 expression appears disrupted, particularly in the basal epithelium, and less specific for the membrane. Membrane specificity is not evident until more superficial epithelial layers. This redistribution is also clear in ZO-1 staining characteristics, with clear cytoplasmic staining evident.

Discussion

Recent clinical practice guidelines [8] and preclinical research outcomes [10] have highlighted the growing evidence...
indicating the impact of tight junction disruption in the development of chemotherapy-induced mucositis. In light of this new research avenue, the current study utilised archival tissue samples obtained from patients undergoing standard chemotherapy, with the aim of determining oral epithelial tight junction integrity and correlating with established changes in pro-inflammatory cytokine and MMP profiles.

An unexpected finding from the current study was significant epithelial atrophy seen in the buccal mucosa biopsies taken prior to chemotherapy treatment. Importantly, all patients recruited for the original study had received previous cycles of cytotoxic treatment indicating that treatment causes persistent, long-term changes in the oral cavity. Epithelial thickness strongly correlated with the number of previous treatments patients underwent. These results support the idea that affected tissue exhibits long-term ultrastructural changes. These changes in epithelial thickness were also accompanied by residual inflammation and extra cellular matrix signalling, with elevated staining intensity compared to healthy controls. Unfortunately, we were unable to access information Regarding the timing of previous cytotoxic treatment and correlations could not be drawn.

This study is the first to identify chemotherapy-induced oral epithelial tight junction disruption in patients receiving chemotherapy. In fact, it is one of only a few clinical studies that have documented changes in tight junctions from clinical patient samples. Keefe and colleagues (2000) showed altered tight junction integrity in the duodenum of patients undergoing chemotherapy [5]. These changes, detected by transmission electron microscopy, were the first to suggest that tight junction disruption may contribute to ulceration, loss of tissue integrity and diarrhoea development in patients undergoing chemotherapy. Consequently, chemotherapy-induced tight junction disruption may indeed be a critical aspect of oral ulceration—a major clinical aspect of mucositis. More importantly, however, tight junctions provide an important paracellular barrier to potential pathogens and thus disruption may promote bacterial translocation and increase the risk of local, or systemic, infection in already immunocompromised patients. This is a well-documented risk associated with tight junction disruption in the gastrointestinal tract, with chemotherapy-treated rats showing increased bacterial translocation to the mesenteric lymph nodes and spleen [27] coupled with severe tight junction impairment. Implications for oral epithelial tight junction disruption may therefore not only promote mucosal breaches but have detrimental effects on patients’ clinical health outcomes.

Tight junctions are highly plastic complexes, with the ability to change in response to a wide variety of physiological and pathological cues. Although reduced expression of key tight junction proteins is most widely documented, cytoplasmic redistribution of these proteins has also been shown to drastically affect their function. For example, Nassour et al. (2014) showed that application of STb, a low molecular weight heat-resistance toxin produced by enterotoxigenic Escherichia coli, caused significant translocation of claudin-1 to the cytoplasm of T84 cells [28]. This was accompanied by increased permeability of T84 monolayers and poor transepithelial resistance. In similar studies, redistribution of claudin-1 from the membrane to a more soluble form was associated with marked alterations in F-actin

![Fig. 5 Representative staining for claudin-1 (a–c) and ZO-1 (d–e) in the oral epithelium of healthy controls and patients treated with chemotherapy. The membrane specificity of claudin-1 staining is evident in the deep layers of the basal epithelium in health control patients (a, b: arrow head). In treated patients, staining is disrupted and less specific for the membrane, showing redistribution to the cytoplasm. Membrane specificity is not evident until more superficial epithelial layers (c: arrow head). ZO-1 staining displays typical cobblestone appearance in health controls (d). Following chemotherapy, internalisation of ZO-1 is evident showing clear cytoplasmic staining (e: arrows). Original magnification 40×; scale bars on images a–e show 100 μm; scale bars on images d–e show 10 μm]
supportive roles of proinflammatory cytokines and MMPs in the regulation of tight junctions and barrier function. Importantly, however, interactions between proinflammatory cytokine signalling, MMP activity and epithelial tight junction integrity have been documented. In fact, treatment with TNF has been reported to activate both MMP-2 and MMP-9 resulting in tight junction disruption and epithelial hyper-permeability.

More recently, MMP-tight junction interactions have been demonstrated using human airway epithelial models and human embryonic kidney cell lines. In both cases, MMP-9 activation caused altered expression and localisation of occludin, claudin-1 and ZO-1, tight junction strand breaks and epithelial apoptosis, thus highlighting a clear role of MMPs in the regulation of tight junctions and barrier function. Given the wealth of supportive literature showing cytokine- and MMP-mediated tight junction disruption, the idea that these interactions underpin chemotherapy-induced oral toxicity is compelling. Given that these interactions have also been reported to contribute to chemotherapy-induced gut toxicity and associated diarrhoea, this study therefore indicates that tight junction defects occur throughout the entirety of the alimentary tract, regardless of anatomic site. This provides further evidence for a common pathway for mucositis development, which is modified as a consequence of local structural differences in the mucosa. These differences are overwhelming when comparing the oral mucosa to the gastrointestinal tract; however, these structural differences may have implications for the resilience that different mucosae may exhibit in response to the effects of chemotherapeutic drugs.

Conclusions
Chemotherapy causes defects in key tight junction proteins of the oral cavity, characterised by decreased expression and cytoplasmic redistribution. This is the first study to identify changes in oral epithelial tight junctions of patients undergoing chemotherapy. This provides further evidence for a common pathway for alimentary mucositis, with regional differences the result of structural variations in the alimentary mucosa. Changes in oral epithelial tight junctions were coupled with altered cytokine and MMP profiles, and the timing of these events may be a target for preventative treatment. It is therefore critical that these results be assessed in a more controlled manner to assess if tight junction disruption is in fact the cause of oral mucositis or purely an effect. It must also be acknowledged that not all patients undergoing chemotherapy treatment developed clinical mucositis. Despite this, subclinical evidence of mucositis was apparent in the form of apoptosis, inflammation, atrophy and perhaps tight junction defects. For a stronger understanding of the temporal relationship between mediators of inflammation, tight junctions and mucositis development to be established, these investigations should now be extended into controlled animal studies as well.

stress fibres. F-actin filament dissolution and condensation were also accompanied by redistribution and fragmentation of ZO-1 and occludin. This relationship has also been demonstrated in response to IL-1β treatment, with altered subcellular localisation of claudin-1 and ZO-1 shown in both thyroid cells and cultured human corneal epithelial (HCE) cells. In the setting of chemotherapy-induced tight junction disruption, it has also been shown that downregulation and redistribution of ZO-1 drastically affects the function of intestinal tight junctions. For example, Hamada and colleagues showed that methotrexate-induced diarrhoea resulted in significantly increased permeability to fluorescein isothiocyanate-dextran coupled with internalisation of ZO-1 in colonic epithelial cells. Although shown in a variety of cell types and in response to varying cues, these studies emphasise the significance of cytoplasmic redistribution of tight junction proteins and may offer mechanistic avenues to explore.

The current study has shown clear increases in several proinflammatory cytokines and MMP subtypes. This change comes as no surprise given the vast amount of research showing a strong inflammatory component to alimentary toxicity. However, few studies have assessed cytokine and MMP expression in the oral epithelium of patients receiving chemotherapy, with most research coming from preclinical animal models. For example, our laboratory has previously shown elevations in IL-1β, TNF and IL-6 in the oral mucosa of tumour-bearing rats receiving chemotherapy, paralleling the clinical changes observed in the current study. These results compliment earlier clinical findings showing increased NFκB and cyclooxygenase-2 expression in the oral cavity of patients following cytotoxic chemotherapy. Recent research has also shown elevated MMP-9 expression in the ventral surface of the tongue of tumour-bearing rats treated with chemotherapy. This parallels earlier research showing a time-dependent increase in both MMP-2 and MMP-9 in the jejunum following irinotecan administration. Although more substantial changes were seen preclinically, particularly for MMP-9, results again reflected the changes observed clinically. Importantly, the changes in proinflammatory cytokine and MMP profiles observed in our present study were clearly coupled with changes in tight junction integrity.

The idea that both proinflammatory cytokines and MMPs regulate tight junctions is not a new phenomenon, with strong supportive in vitro and in vivo evidence. The earliest evidence for proinflammatory cytokine-dependent tight junction disruption was seen in the setting of inflammatory bowel disorders, with clear changes in claudin-1, ZO-1 and occludin coinciding with peak relapse and remission phases. Recent in vitro research has solidified the modulatory roles of proinflammatory cytokines on tight junction integrity, showing that IL-1β and TNF are able to disrupt tight junction integrity. Comparable effects have also been documented following exposure to MMPs, although much of the research to date has only focused on their effects on endothelial tight junctions.
as into larger patient cohorts with heterogeneous diagnoses and more detailed reporting of mucositis onset, severity and duration.

Acknowledgments This current study was supported by funds awarded to Ms. Hannah Wardill by the Australian Dental Research Foundation (AGRF 2013-3). Ms. Hannah Wardill and Ms. Yasabella Van Sebille are recipients of the Australian Postgraduate Award. Ms. Hannah Wardill is also the recipient of the Florey Medical Research Foundation Doctor Chun Chung Wong and Madam So Sau Lam Memorial Postgraduate Cancer Research Top Up Scholarship.

Compliance with ethical standards Conflict of interest The authors declare that they have no competing interests.

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NOTE:
This publication is included on pages 294 - 303 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1002/ijc.28656](http://dx.doi.org/10.1002/ijc.28656)
Complications of Treatment

Toll-like receptor 4 signaling: A common biological mechanism of regimen-related toxicities
An emerging hypothesis for neuropathy and gastrointestinal toxicity

Hannah R. Wardill a, b, Ysabella Z.A. Van Sebille b, Kimberley A. Mander c, Rachel J. Gibson a, Richard M. Logan d, Joanne M. Bowen b, Stephen T. Sonis e

Abstract
Regimen-related toxicities remain a priority concern within the field of supportive care in cancer. Despite this, many forms of toxicity are under reported and consequently poorly characterised. Although there have been significant improvements in our understanding of regimen-related toxicities, symptom management continues to occur independently raising concerns such as drug interactions and the tendency to emphasise management of a single symptom at the expense of others. This review focuses on two important toxicities induced by chemotherapy; neuropathy/pain and gastrointestinal toxicity, introducing the Toll-like receptor (TLR) 4 pathway as a common component of their pathobiology. Given the global observation of toxicity clusters, identification of a common initiating factor provides an excellent opportunity to simultaneously target multiple side effects of anticancer treatment. Furthermore, identification of common biological underpinnings could perhaps reduce polypharmacy and have pharmacoeconomic benefits.

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Introduction
Regimen-related toxicities are universally underappreciated and often seen as the trade-off for remission [1]. Studies suggest this is due to oncology follow-up clinics focusing on disease recurrence whilst rarely addressing symptom management and referral pathways [1]. While research efforts into supportive care in cancer have seen significant improvement, regimen-related toxicities are viewed as biologically independent, but simultaneous events, perpetuating the silo mentality that typically exists within the supportive care domain. Individual, symptom-oriented therapeutic strategies also raise some important concerns, such as polypharmacy and drug side effects, and the tendency to emphasise management of a single symptom at the expense of others. Furthermore, this approach ignores global observations that regimen-related toxicities occur in symptom clusters [2] which point to commonalities in their underlying biology, or at the least, overlapping mechanisms. In fact, in a retrospective review of 1000 cancer patients admitted for palliative care, each patient was reported to have greater than 10 symptoms [3,4]. Based on these observations, we suggest a paradigm shift, moving towards the idea that toxicities should be approached more holistically [5], combining efforts of neurologists, gastroenterologists, oncologists and other leading experts to identify common mechanisms between these pathologies. This critical review will focus on two important regimen-related toxicities, neurotoxicity and gastrointestinal (GI) toxicity, introducing the Toll-like receptor (TLR) 4 pathway as a common component of their pathobiology.

Neurotoxicity is a poorly characterised, dose limiting side effect of chemotherapy treatment [6] with symptoms typically falling under three broad categories, cognitive dysfunction, fatigue and neuropathy. Most commonly associated with platinum compounds (cisplatin and oxaliplatin), spindle poisons/antitubulins...
(vincristine and paclitaxel) and the newer targeted agents such as the proteasome inhibitors (bortezomib, ixabepilone, thalidomide) [7,8], heightened pain perception (hyperalgesia) and allodynia remain under reported and ill-defined side effects of chemotherapy. Given the profound personal impact of these neurological symptoms, chemotherapy-induced neurotoxicity is now considered a priority concern within the oncology arena, bringing together oncologists and neurologists to shed light on the mechanisms that underlie this pathology. Recent neuroimaging techniques suggest performance changes in neurological function occur in a subset of cancer patients, and that these changes may be associated with structural and functional alterations in the brain [9]. However, the molecular mechanisms involved in chemotherapy-induced neurotoxicity, specifically heightened pain perception (chemoneuropathy), remain unclear and poorly studied. Recent speculation has led to several candidate mechanisms for neurotoxicity including oxidative stress, inflammation and DNA damage [10,11]. It has also been proposed that some cytotoxic agents may damage neurons through binding to axonal microtubules to subsequently alter axonal transport [12]. This is however contradicted by a wealth of evidence showing no morphological changes in centrally-located neurons following various cytotoxic insults [13,14]. The lack of pathological changes observed in these neurons suggests that direct cytotoxicity is not sufficient to fully account for the range and severity of neurological symptoms experienced by patients, and more complex mechanisms are likely to be involved.

It has been suggested that systemic proinflammatory and immune factors released following chemotherapy [15,16] cause localised glial activation to further exacerbate neuronal responses and potentiate pain [17]. Glia have long been overlooked for their role in pain signaling, viewed only as structural supports of neurons of the CNS. It was not until the early 1990’s when the actions of glia in varying pain states were appreciated and it is now a well-documented component of neuropathic pain [17,18]. The most recent advent in the area of glia-mediated nociception is the role of the Toll-like receptor (TLR) family, specifically TLR4. TLRs are a family of transmembrane protein receptors that recognise a diverse range of signals on exogenous and endogenous substances considered to be danger signals, and hence warrant activation of the innate immune system for the survival of the host [19]. TLR4 has been most extensively characterised as it recognises lipopolysaccharide (LPS) from gram-negative bacteria. TLR4 agonists activate similar downstream intracellular signaling pathways to those previously documented for interleukin (IL)-1, binding to its co-receptor, activating nuclear factor kappaB (NFkB) and resulting in a powerful proinflammatory cascade [20].

In addition to severe neurotoxicity, chemotherapy is also recognised for causing severe gastrointestinal side effects. Gut toxicity is often a dose-limiting manifestation of chemotherapy treatment that affects a large proportion of patients, dependent on the dose of chemotherapy administered [21]. Clinically, chemotherapy-induced gut toxicity (CIGT) is associated with severe gastrointestinal symptoms such as diarrhea, infection and rectal bleeding [1]. Characterised by severe ulceration, inflammation and pain, CIGT has recently been implicated with glial activation [22], elevated proinflammatory cytokines (IL-1β IL-6, TNF) [15] and, importantly, excessive TLR4 activation [23]. Like the CNS, the enteric nervous system is comprised of neurons and glia [24]. The traditional role of glia has also been challenged in the enteric nervous system with research suggesting that enteric glia are capable of regulating gastrointestinal homeostasis, and critically, transmission of sensory information from the gut to the CNS [25–27]. It is therefore tangible to suggest that peripheral toxicity, such as CIGT, may drive glial activation and thus exacerbate neuronal damage and pain perception.

**Indirect neuromodulation through glial activation**

**The emerging role of glia in neuropathic pain**

Glia is the collective term used to describe both astrocytes and microglia, the key supportive cells of the CNS. Traditionally, glia were viewed as structural supports for neurons, providing typically homeostatic roles including immune surveillance, clearance of debris, regulation of the ionic and chemical composition of the extracellular matrix and maintenance of blood brain barrier (BBB) integrity; glia are therefore considered pivotal to not only CNS homeostasis but also the survival of the host [18]. It was not until the early 1990’s where these static, neurosupportive roles of glia were challenged and their roles under varying pain states acknowledged [28]. This paradigm shift in our understanding of glia followed early evidence showing an associative link between astrocyte activation and neuropathic pain [28]. The earliest evidence came from Garrison et al. (1991) where significantly elevated glial fibrillary acidic protein (GFAP) staining in the lumbar spinal cord was noted following sciatic nerve constriction. Garrison and colleagues furthered this work in 1994, showing activated glia in neuropathic animals [29]. Importantly, when an N-methyl-D-aspartate (NMDA) receptor inhibitor – MK-801 – was applied, both glial activation and neuropathic pain were improved. Several studies also report comparable changes in glial activity in various preclinical models of neuropathic pain [28,30,31] and subsequently glia are considered a vital step in its pathobiology.

It is now well established that glia have two distinct states; a quiescent baseline state and an activated state. Microglia have a classic quiescent phenotype under normal pain responses, responsible for surveying the extracellular space in search of potential danger, but producing no neuroexcitatory substances [32]. In contrast, astrocytes are active players in synaptic signaling even under basal conditions. They maintain house-keeping functions, providing energy sources and neurotransmitter precursors to neurons, cleaning debris and resorbing excess neurotransmitters. Upon activation, these glia shift from their basal state, to an activated state characterised by a reactive, proinflammatory response profile [17]. A variety of glial activation signals have been identified, some of which are very well characterised including neuronally released fractalkine and traditional neuronal nociceptive modulators and transmitter, such as reactive oxygen species (ROS), nitric oxide, prostaglandins, excitatory amino acids, substance P and proinflammatory cytokines [33]. Upon activation, glia release substances (ROS, nitric oxide, prostaglandins, proinflammatory cytokines) that increase neuronal excitability, leading to pain enhancement. These neuroexcitatory mediators directly enhance neuronal excitability [33,34], increase pain associated neurotransmitter release from sensory afferents [35], upregulate the number and conductance of calcium permeable α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) and NMDA receptors [36] and downregulate expression of glial glutamate transporters; all of which potentiate pain [37].

Although this mechanism of pain potentiation is well described in the setting of peripheral nerve damage, limited data exists regarding its role in chemotherapy-induced pain. Of the limited data, both microgliosis and astrocytosis are reported following administration of vincristine, paclitaxel, bortezomib and oxaliplatin [38–42]. Robinson et al. (2014) characterised patterns of glial activation in response to chemotherapy and typical spinal nerve ligation [42]. Consistent with previous peripheral nerve injury models, microglia activation was evident following spinal nerve ligation, but not chemotherapy administration. In contrast, astrocytes were activated following both oxaliplatin and bortezomib treatment in a manner that paralleled chemotherapy-evoked behavioural changes. Despite this disparity, the behavioural
phenotype and activation of astrocytes were prevented by co-administration of minocycline hydrochloride – a microglial inhibitor – in both models, suggesting a common mechanism between both neuropathies. Similarly, Ji et al. (2013) reported significant astrocytic hypertrophy and activation, demonstrated by increased glial GFAP expression in the dorsal horn of vincristine-treated rats with mechanical allodynia [38]. This was coupled by increased astrocytic expression of IL-1β and phosphorylation of the NMDA receptor in spinal dorsal horn neurons. Importantly, treatment with pentoxifylline, an anti-inflammatory agent and an IL-1 antagonist, attenuated phosphorylation of NMDA receptors and mechanical allodynia. Most recently, oxaliplatin treatment was also associated with microglia activation, however this was only transient [40]. Microglia displayed a highly ramified phenotype, similar to that of vehicle-treated animals. The number of GFAP-expressing cells in the dorsal horn superficial laminae was significantly increased in oxaliplatin treated animals at 1, 2 and 3 weeks following treatment, correlating with the pain-profile. Importantly, although application of minocycline attenuated pain and glial activation, the efficacy of fluorocitrate – an astrocyte inhibitor – was significantly greater. Together, these studies highlight that glial activation, specifically astrocyte activation, is an important component of chemoneuropathy and associated pain. Despite these promising findings, the initiating factor for glial activation following chemotherapy remains unclear. One potential candidate is the release of endogenous danger signals. Several neurological conditions such as peripheral nerve damage have been shown to elicit the release of these endogenous danger signals [43] which communicate cellular/tissue damage and/or stress independent of the release of classic neurotransmitters or neuromodulators [17]. On release of these danger signals, the innate immune pattern recognition receptor, TLR4, causes activation of TLR4-expressing cells including both microglia and astrocytes [17]. Given the extensive peripheral tissue damage observed following cytotoxic treatment, TLR4-mediated glial activation therefore presents as a novel pathway in the pathobiology of chemoneuropathy.

**TLR4-mediated glial activation**

TLRs are a family of approximately ten single transmembrane receptors that recognise a diverse range of moieties or ‘patterns’ on exogenous and endogenous substances considered to be danger signals, and hence warrant activation of the innate immune system [18]. Of the many TLR subtypes, TLR4 has been most extensively characterised with established roles in the host immune response. When activated, typically by lipopolysaccharide (LPS), TLR4 recruits adaptor molecules and kinases, initiating a downstream signaling cascade that culminates in the secretion of proinflammatory cytokines and chemokines [44–46]. This signaling cascade can be MyD88-dependent or -independent, with the MyD88-dependent pathway most commonly associated with translocation of NFκB and proinflammatory cytokine secretion. MyD88-dependent signaling typically requires the adaptor proteins TIRAP (TIR domain containing adaptor protein) and MyD88 to initiate the rapid production of proinflammatory cytokines, chemokines and their receptors TNF, IL-1α, IL-1β, IL-1ra, IL-6, IL-8, IL-10, IL-12p40, IL-23, macrophage inflammatory protein (MIP)-1α, and MIP-1β [47]. These factors facilitate the inflammatory response by increasing vascular permeability, directing dendritic cells and initiating macrophage migration from the periphery [48]. In contrast, the independent signaling pathway is reliant on Toll-like receptor adaptor molecule (TICAM)–1, –2, the TIR-domain-containing adaptor inducing interferon-β (TRIF) or TRIF-related adaptor molecule (TRAM) resulting in the production of interferon-β and chemokines.

In addition to the well-documented roles of TLR4 signaling in the host immune response, recent evidence has also linked this immune receptor to a number of neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease [44]. TLR4 expression in the CNS was, until recently, limited to microglia, astrocytes and oligodendrocytes. Recent evidence has now shown that TLR4 is expressed on CNS structures exposed to the blood stream such as the choroid plexus, circumventricular organs and leptominings. This newly emerging distribution of TLR4 expression may therefore explain the innate immune response observed in the brain, which originates from areas devoid of a blood–brain barrier [49]. Furthermore, recent evidence has shown altered neuronal TLR4 expression in response to ischaemia/reperfusion [50]. This is further supported by knockout studies, where the extent of energy deprivation-induced cell death and associated neurological deficit were significantly reduced in TLR4 deficient mice compared to wild-type [51].

**TLR4 in the central nervous system**

There is accumulating evidence that TLR4 contributes to neuronal death, BBB damage and inflammatory responses in the brain [52,53]. Consequently, TLR4 has been implicated with several CNS pathologies, particularly those characterised by neuroinflammation and subsequent degeneration. It has been postulated that TLR4-mediated NFκB signaling plays a critical role in the development of neuroinflammation, leading to the secretion of proinflammatory cytokines, chemokines and enzymes such as cyclooxygenase (COX)-2 and matrix metalloproteinases (MMPs) [54,55]. Furthermore, it is suggested that these neuroinflammatory mediators are able to activate microglia leading to neuronal excitation or neuronal loss [56,57]. In fact, this phenomenon was recently demonstrated in the setting of Alzheimer’s disease; a neurodegenerative disease characterised by microgliosis. Importantly, activated microglia have been identified surrounding senile plaque in the brains of Alzheimer’s disease patients and have been shown to express increased levels of TLR4 [58]. Additionally, treatment of microglia with senile plaque material was shown to induce sharp peaks in the mRNA expression of many TLR subtypes, including TLR4, when compared with age-matched plaque-free tissue [59]. It is therefore suspected that TLR4-mediated glial activation results in the production of nitric oxide, oxygen derived free radicals, proteases, adhesion molecules and proinflammatory cytokines which, when produced in excess, have detrimental effects on neuronal homeostasis and contribute to the development of neurodegenerative conditions such as Alzheimer’s disease [60].

In addition to Alzheimer’s disease, TLR4 signaling has gained momentum regarding the pathobiology of Parkinson’s disease; a chronic, neurodegenerative condition characterised by loss of dopaminergic neurons in the substantia nigra pars compacta and the striatum of the basal ganglia [61]. Although the mechanisms responsible for Parkinson’s disease remain unclear, emerging evidence suggests a neuroinflammatory component to the condition [62]. The presence of cytoplasmic alpha-synuclein (AS), or Lewy bodies, is the hallmark trait of Parkinson’s disease and the subject of significant molecular research. Stefanova and colleagues (2007) were the first to show elevated levels of TLR4 in AS cytoplasmic inclusions [63]. These findings have since been extended with research now showing that TLR4 is essential for the AS-dependent activation of microglia, leading to the production of proinflammatory cytokines and ROS [64]. Importantly, this mechanism is unique to microglia, with astrocytic uptake of AS shown to be TLR4-independent. In contrast, the role for TLR4 in Parkinson’s disease is confounded by evidence showing that genetic TLR4 deletion results in reduced phagocytic activity of microglia, leading to heightened AS accumulation and exacerbated neurodegeneration [65,66]. These results suggest that despite initiation of an inflammatory response, TLR4-mediated glial activation may be important
TLR4 and neuropathic pain

TLR4 has received significant attention for its roles in several neuroinflammatory disorders characterised by neurodegeneration. In addition to these emerging roles, TLR4 has also gained momentum for its role(s) in modulating neuropathic pain. Within the CNS, TLR4 is predominantly expressed by microglia, but expression may be upregulated on astrocytes under neuroinflammatory settings [67]. TLR4 appears to be directly relevant to the pathobiology of neuropathic pain, as it recognises and responds to endogenous danger signals, and thus has the ability to modulate pain signaling. TLR4 knockout and knockdown studies have demonstrated this emerging role for TLR4, with knockout/knockdowns suppressing the development and/or maintenance of nerve injury-induced allodynia [68–71]. Additionally, administration of a selective TLR4 antagonist has been shown to suppress well-established neuropathic pain induced by chronic constriction injury [18]. In the setting of chemoneuropathy, recent research has shown that paclitaxel treatment is associated with elevated TLR4 expression and glial activation in the dorsal root ganglion. Additionally, application of both TLR4 and MyD88 antagonists significantly reduced peripheral neuropathy and associated pain [39]. Taken together, these studies suggest that ongoing TLR4 activation and peripheral endogenous danger signaling is at the core of neuropathic pain, and may therefore contribute to the development of chemoneuropathy and its associated clinical features.

Peripheral tissue damage activates central TLR4

Although the development of neuropathic pain through TLR4 activation is most extensively characterised in the setting of peripheral nerve injury, the production of these endogenous danger signals and other TLR4 ligands is not unique to this form of tissue damage. In fact, it is well established that chemotherapy treatment causes significant gut toxicity, which is characterised by excessive production of endogenous danger signals (pathological- and danger-associated molecular patterns; PAMPs/DAMPs) [43]. In addition to this, recent research has shown enterocyte-expressed TLR4 is intimately involved in the initiation of gut toxicity following chemotherapy treatment, activating NF-$kappa$B and mounting an immune response [72]. Given that TLR4 is activated by endogenous danger signals, centrally-located TLR4 and glia are well positioned to enhance pain resulting from inflammation in the periphery such as gut toxicity following chemotherapy. We therefore hypothesise that the molecular signals derived from gastrointestinal toxicity drive glial activation and subsequent neuropathy in a TLR4-dependent manner (Fig. 1). This pathway appears to be initiated by damage that originates in the periphery, and thus the pathobiology of chemoneuropathy may point to the existence of a gut-CNS axis.

The existence of a gut-CNS axis is not a new phenomenon [73]. Based on paralleled comorbidities of gastrointestinal and neurological origin, there has been an appreciation gained for the existence of a gut-CNS axis and the roles it may play in governing neurological function [74,75]. While candidate mechanisms of the gut-CNS axis include neural, endocrinal and immune pathways, the gut microbiota has emerged as a predominant player, although the mechanisms underpinning the gut-CNS axis remains unclear [76]. Although a wealth of data exists supporting a role for the gut microbiota in modulating neurological function, there is evidence to suggest that immune cells produced within the gut may also exhibit neuromodulatory effects [77]. Disruption of the homeostatic state between the microbiota and the innate mucosal...
immune system of the host has been shown to result in activation of TLRs and consequent alteration of cytokine profiles, leading to impaired neurological function. It is suggested that these immune cells disrupt the BBB and upon crossing, are subsequently reacti-
vated within the CNS. This phenomenon was recently demon-
strated in mice receiving peripheral surgery, displaying BBB
disruption and elevated TNF signaling which facilitated macro-
phage migration into the hippocampus and subsequent neurologi-
cal decline [78]. Additionally, administration of proinflammatory
cytokines in rodents has been reported to induce depressive like
symptoms, disrupted circadian rhythm and reduced appetite
[79,80]. Although a gut-CNS axis has not been applied to the
setting of chemotherapy-induced pain, these results support the
hypothesis that CIGT is able to modulate CNS homeostasis, and
may contribute to the development of chemoneuropathy. Furth-
ernore, TLR4 may be uniquely positioned to modulate inflam-
matory responses in both the gut and CNS thus contributing
to both toxicities and it therefore presents as an attractive
therapeutic target.

Clinical translation

There is strong evidence suggesting that peripheral toxicity
drives glial activation through TLR4 signaling; this review has
highlighted evidence using the examples of CIGT and pain. Where
the complexity lies is the sequence of these toxicities. Identifying
whether these toxicities occur in unison or sequentially will shed
light on the role of TLR4 as a common underlying mechanism. It
is likely that TLR4-mediated pain is agent specific, and may be a
case where one size, on a theoretical basis, does not fit all. For
example, the clinical observation of neuroopathy is rare amongst
patients being treated with agents typically associated with high
rates of gut toxicity (irinotecan, 5-fluorouracil, methotrexate), indi-
cating potential independent mechanisms (Fig. 2). However, the
hypothesis of dual-toxicities of common biology is compelling
with regards to the clinical use proteasome inhibitors (bortezomib,
thalidomide, ixabepilone, ixazomib) [81–83] and taxanes (pacli-
taxel, docetaxel) [84–86], which commonly induce toxicities of both
gastrointestinal and neurological origin, raising the question of
equivalent risk.

Conclusions and future directions [87]

Regimen-related toxicities remain a priority concern within the
field of supportive care in cancer. Despite this, many forms of tox-
icity are under reported and consequently poorly characterised.
This holds particularly true for chemotherapy-induced gastroin-
testinal toxicity and neurotoxicity, specifically the symptom of pain.
This review has highlighted TLR4 signaling as a common underly-
ing pathway of both toxicities. Given the global observation of tox-
icity clusters, identification of a common initiating factor would
provide an excellent opportunity to simultaneously target multiple
side effects of anticancer treatments. Despite strong epidemiolog-
evidence highlighting toxicity clusters, it remains unclear
why some patients are more susceptible to severe toxicity. Ev-
dence has shown TLR4 gene mutations (Thr399Ile) contribute to
the severity of acute Graph versus Host Disease, influencing the
risk in patients undergoing allogenic transplantation [88]. The
TLR4 Asp299Gly polymorphism has also been identified as a risk
factor for Crohn’s disease potentially contributing to disease
phenotype [87]. This emerging hypothesis for TLR4-mediated
toxicities could therefore have the potential to drive biomarker
development and risk evaluation techniques, presenting an
attractive avenue for future research. Furthermore, identification
of common biological underpinnings could perhaps reduce poly-
pharmacy, lessen drug side effects, and have pharmacoeconomic
benefits.

The ubiquitous involvement of the innate immune system in
regimen-related toxicity makes TLR4 an overlooked candidate in
the pathophysiology of other dose-limiting side effects of
chemotherapy. For example, recent speculation suggests that
proinflammatory cytokines are able to disrupt the hypothalamic–
piitary–adrenal (HPA) axis, to alter circadian rhythm and thus
induce fatigue – an established side effect of chemotherapy [89].
In fact, decreased circulating levels of serum cortisol have been
reported immediately following treatment with the platinum com-
pounds cisplatin and carboplatin [89], indicating impaired HPA
axis function [90,91]. Given the role of TLR4 in neuroinflammation,
it is conceivable that activation of enterocyte-expressed TLR4 initi-
ates the induction of a ‘cytokine storm’ which is able to modulate
the CNS and thus impact on the function of the HPA axis. Activation
of TLR4 may therefore be the missing link in the initiation of this

Fig. 2. Independent and common mechanisms of gastrointestinal and neurotoxicity.
cascade where circulating proinflammatory cytokines can access the CNS and exert profound effects on behaviour and cognitive function. The induction of this ‘cytokine storm’ appears to be predominantly initiated by the activation of TLR4 in the gut – the largest immunological organ – and must therefore be adequately acknowledged if we are to adopt a holistic approach to toxicity.

Conflict of interest statement

There are no conflicts of interest to declare on behalf of any authors.

Acknowledgement

Thank you Mr. Tavik Morgenstern for kindly providing the illustration for this manuscript.

References


NOTE:
This publication is included on pages 311 - 321 in the print copy of the thesis held in the University of Adelaide Library.

It is also available online to authorised users at:

[http://dx.doi.org/10.1158/1535-7163.MCT-15-0990](http://dx.doi.org/10.1158/1535-7163.MCT-15-0990)
A novel in vitro platform for the study of SN38-induced mucosal damage and the development of Toll-like receptor 4-targeted therapeutic options

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Abstract

Tight junction and epithelial barrier disruption is a common trait of many gastrointestinal pathologies, including chemotherapy-induced gut toxicity. Currently, there are no validated in vitro models suitable for the study of chemotherapy-induced mucosal damage that allow paralleled functional and structural analyses of tight junction integrity. We therefore aimed to determine if a transparent, polyester membrane insert supports a polarized T84 monolayer with the phenotypically normal tight junctions. T84 cells (passage 5–15) were seeded into either 0.6 cm², 0.4 μm pore mixed-cellulose transwell hanging inserts or 1.12 cm², 0.4 μm pore polyester transwell inserts at varying densities. Transepithelial electrical resistance was measured daily to assess barrier formation. Immunofluorescence for key tight junction proteins (occludin, zonular occludens-1, claudin-1) and transmission electron microscopy were performed to assess tight junction integrity, organelle distribution, and polarity. Reverse transcription-polymerase chain reaction was performed to determine expression of toll-like receptor 4 (TLR4). Liquid chromatography was also conducted to assess SN38 degradation in this model. Polyester membrane inserts support a polarized T84 phenotype with functional tight junctions in vitro. Transmission electron microscopy indicated polarity, with apico-laterally located tight junctions. Immunofluorescence showed membranous staining for all tight junction proteins. No internalization was evident. T84 cells expressed TLR4, although this was significantly lower than levels seen in HT29 cells (P = .0377). SN38 underwent more rapid degradation in the presence of cells (−76.04 ± 1.86%) compared to blank membrane (−48.39 ± 4.01%), indicating metabolic processes. Polyester membrane inserts provide a novel platform for paralleled functional and structural analysis of tight junction integrity in T84 monolayers. T84 cells exhibit the unique ability to metabolize SN38 as well as expressing TLR4, making this an excellent platform to study clinically relevant therapeutic interventions for SN38-induced mucosal damage by targeting TLR4.

Keywords: in vitro model, transwell support, barrier function, tight junctions, SN38, toll-like receptor 4

Introduction

The intestinal epithelium has two important and distinctly different roles within the gastrointestinal tract (GIT). It mediates the complex absorption of nutrients from the intestinal lumen and simultaneously represents a barrier separating the internal milieu from the outside environment in both an immunologic and metabolic sense. In the gut, the luminal surface comes into direct contact with the highest concentrations of bacteria, antigens, and a host of potentially toxic compounds. Consequently, the paradoxical functions of the intestinal barrier are critical in maintaining gastrointestinal health and homeostasis.

Tight junctions provide a paracellular barrier that is selectively permeable to ions and macromolecules. The molecular characteristics and functional properties of tight junctions are subject to modification by a variety of cues, both physiological and pathological, highlighting the highly dynamic nature of these structures. Consequently, tight junction disruption often leads to the development of a leaky gut, a hallmark feature of compromised mucosal barrier function and many pathological states. A thorough understanding of tight junction regulation, signaling, and modification is therefore critical to determine how they may contribute to disease progression. This holds particularly
true for chemotherapy-induced gut toxicity, which is characterized clinically by increased intestinal permeability and tight junction defects.\textsuperscript{9,10} Despite molecular disruption to tight junctions being reported, the underlying mechanisms are unclear and difficult to identify in full physiological systems.

Chemotherapy treatment has long been recognized to induce a leaky gut, with recent research suggesting tight junction disruption may contribute to the development of clinically diagnosed diarrhea through altered leak-flux mechanisms.\textsuperscript{11} Irinotecan is a chemotherapeutic drug associated with exceptionally high levels of intestinal toxicity. It serves as the water-soluble precursor of the lipophilic metabolite, SN38, which is formed by carboxylesterase-mediated cleavage of the carbamate bond between the camptothecin (CPT) moiety and the dipiperdino side chain.\textsuperscript{12} SN38 is approximately 1000 times as potent as irinotecan as an inhibitor of topoisomerase I and its unique hepatobiliary metabolism is responsible for the high levels of intestinal toxicity.\textsuperscript{13} SN38 is glucoronidated to SN38 glucuronide (SN38G) and detoxified in the liver via conjugation by the uridine-diphospho-glucuronosyl transferase (UGT1A) family, which releases SN38G into the intestines for elimination.\textsuperscript{14} However, in the intestinal lumen, bacterial β-glucuronidases are able to regenerate SN38 from SN38G.\textsuperscript{15} This second pass metabolism is key to the dose-limiting, and clinically diagnosed, diarrhea associated with irinotecan treatment.

Although our understanding of irinotecan and SN38-induced gastrointestinal toxicity is improving, many studies’ mechanistic data are limited to due the difficulties in accessing the GIT. \textit{In vitro} models therefore offer an appealing alternative of studying GI-related pathologies. Epithelial cell lines derived from the intestine can be cultured as monolayers to mimic the intestinal epithelium and provide insight into the physiological characteristics of tight junctions and epithelial barrier function.\textsuperscript{4} The human colonic epithelial cell line, T84, derived from a colonic carcinoma, is widely used \textit{in vitro} to assess intestinal barrier function and tight junction integrity.\textsuperscript{16,17} T84 cells are typically cultured \textit{in vitro} using a mixed-cellulose membrane, semi-permeable transport system (Figure 1), which readily allows assessment of barrier function. However, these opaque mixed-cellulose membranes, although considered gold-standard, limit downstream imaging techniques such as light or confocal microscopy, critical for a thorough understanding of tight junction signaling and integrity. We therefore aimed to determine if a transparent, polyester membrane insert supports a polarized T84 monolayer with phenotypically normal tight junctions. The long-term goal is for our model to be used for interrogation of complex gastrointestinal physiology under normal and challenged states. If successful, this model will be used to study mechanisms of chemotherapy (specifically irinotecan)-induced mucosal damage in a simple, high-throughput manner. Additionally, this novel platform could be used to assess the efficacy of antimucotoxic agents for both preclinical and clinical translation.

**Materials and methods**

**Cell culture**

Cryopreserved T84 cells (passage 5–15) derived from a human colorectal carcinoma were obtained from Culture Collections (Porton Down, UK; 88021101). HT29 cells (passage 5–15), derived from a human colorectal carcinoma with an epithelial phenotype, were kindly provided by Dr J Hardingham (Queen Elizabeth Hospital, South Australia). MCF-7 cells (passage 1–6) derived from a human breast carcinoma were also a kind gift from Professor M Brown (Royal Adelaide Hospital, South Australia). All cells lines retained their original morphology and growth characteristics over the range of passages used (data not shown).

Cells were thawed in a 37°C water bath and maintained in a 75 cm\textsuperscript{2} or 150 cm\textsuperscript{2} sterile cell culture flask (Corning Life Sciences, MA, USA) at 37°C with 5% CO\textsubscript{2}. T84 and HT29
cell culture media was Dulbecco’s Modified Eagle Medium (DMEM)/Ham’s F-12 Nutrient Mixture containing 15 mM HEPES, L-glutamine, and sodium bicarbonate (DMEM/F-12; Sigma-Aldrich, Castle Hill, NSW, Australia; D8437) supplemented with 1% penicillin/gentamicin + fungizone, 10% foetal bovine serum, and 1 mM L-glutamine (complete DMEM). MCF-7 cells were maintained in Roswell Park Memorial Institute (RPMI) media supplemented with 2 mM L-glutamine and 10% Foetal Bovine Serum (FBS). Experimental cell cultures were grown in sterile, multi-well tissue culture plates under identical growth conditions. Cell lines were routinely passaged when culture monolayers reached approximately 80% confluence at subculture ratios between 1:3 and 1:6 in fresh growth medium. Cells were detached by aspirating growth medium, washing with 1X phosphate buffered saline (PBS; pH 7.4) and incubating with 3 ml of trypsin-ethylenediaminetraacetic acid (EDTA) for 10 min at 37°C (0.05% trypsin, 0.53 mM EDTA; Invitrogen, Mulgrave, VIC, Australia). The reaction was then quenched by the addition of fresh medium. Cells were centrifuged at 300 g for 5 min, supernatant removed and cells resuspended in fresh, complete DMEM. Cell counts were conducted using an automated cell counter (BioRad, NSW, Australia) and were seeded into either 0.6 cm², 0.4 mm pore mixed-cellulose transwell hanging inserts (Invitrogen, VIC, Australia; PIHA01250) or 1.12 cm², 0.4 mm pore polyester transwell inserts (Corning, MA, USA; CLS3801). T84 cells were seeded at the following densities: 50,000 cells/cm²; 100,000 cells/cm²; 200,000 cells/cm²; and 400,000 cells/cm² into the apical chamber. Cell culture media in both the apical and basolateral chambers was changed every 48 h. Transepithelial electrical resistance (TEER) was measured daily using an EVOM2 epithelial volt-ohm-meter with chopstick electrodes (World Precision Instruments, Sarasota, FL, USA) for one week during the growth period and area adjusted for analysis using the following formula; TEER monolayer (Ω/cm²) = [raw TEER (Ω) - TEER blank (Ω)]/area of membrane (cm²). All experiments were performed in triplicate and repeated twice.

Transmission electron microscopy

After determining optimal cell density (100,000 cells/cm²), cells were seeded into both 0.6 cm², 0.4 mm pore mixed-cellulose transwell hanging inserts (Invitrogen, VIC, Australia; PIHA01250) and 1.12 cm², 0.4 mm pore polyester transwell inserts (Corning, MA, USA; CLS3801) at a density of 100,000 cells/cm². Cell culture media was changed every 48 h. TEER was measured daily from day 3 using an EVOM2 epithelial volt-ohm-meter. Once high, stable TEER was achieved, monolayers were fixed overnight in 4% paraformaldehyde/1.25% glutaraldehyde (electron microscopy grade) in 1X PBS (4% sucrose; pH 7.2). Monolayers were washed with 1X PBS + 4% sucrose (v/v) before being postfixed in 2% osmium tetroxide (w/v) for 1 h. Monolayers were dehydrated through graded ethanol, removed from the transwell support system and mounted in resin before being polymerized at 70°C for 24 h; 80 μm thick sections were cut on a Leica Ultracut S ultramicrotome using a diamond knife. Sections were picked up on 200 mesh copper/palladium grids and stained with uranyl acetate and Lead Citrate. Grids were then visualized using the Philips CM200 transmission electron microscope (TEM). Monolayers were assessed for the presence of tight junctions, organelle distribution, polarity, and monolayer formation. Monolayers with TEER values over 1000 Ω/cm² were used in all experiments.

Immunofluorescence

A secondary aim of this study was to determine if the polyester membrane transwell support systems support immunofluorescence and confocal imaging. T84 cells were seeded into 1.12 cm², 0.4 μm pore polyester transwell inserts at a density of 100,000 cells/cm². Once T84 monolayers had developed stable TEER values > 1000 Ω/cm², cell culture media was aspirated and cells washed with ice-cold 1X PBS pH 7.4. A fixing solution (1:1 v/v acetone/methanol stored at −20°C) was applied to the apical chamber (500 μl) for 15 min. Cells were rinsed with 1X PBS and permeabilized using 0.1% (v/v) Triton X-100/PBS for 3 min. After 2 × 5 min washes in 1X PBS, cells were blocked overnight in 3% (w/v) bovine serum albumin (BSA)/PBS at 4°C (Sigma-Aldrich, NSW, Australia; 9048-46-8). The blocking solution was aspirated and 200 μl of primary antibody was applied to the apical chamber (mouse mAb occladin, Invitrogen 33–1500, 5 μg/ml; rabbit pAb ZO-1, Invitrogen 61–7300, 2.5 μg/ml; rabbit pAb claudin-1, Abcam ab15098, 2 μg/ml). All primary antibodies were diluted in 1% (w/v) BSA/PBS and incubated for 1 h at room temperature. The primary antibody was then aspirated, and cells washed in 1X PBS + 0.05% Tween (4 × 5 min); 200 μl of fluorescent-conjugated secondary antibody (antimouse 488; antirabbit 568 Alexa Fluor®; Invitrogen, VIC, Australia) was added to the apical chamber at 100 μg/ml. All secondary antibodies were diluted in 1% (w/v) BSA/PBS and incubated for 1 h at room temperature. For nuclear staining, cells were incubated with 1 μg/ml 4’,6-diamidino-2-phenylindole for 10 min at room temperature. Cells were washed in 1X PBS + 0.05 Tween for 4 × 5 min before the membranes were removed from the inserts and mounted onto glass microscope slides using Fluoroshield™ (Sigma-Aldrich, NSW, Australia; #F6182). Cells were visualized using the SP5 Spectral Scanning Confocal Microscope (Leica, Wetzlar, Germany). Negative controls had the primary antibody omitted.

Liquid chromatography – Mass spectrometry

The long-term goal is for this in vitro model to be used to assess the mechanisms involved in SN38-induced mucosal damage to identify potential targets for the development of interventions. It is well recognized that SN38, the active metabolite of irinotecan, is a basic compound and typically unstable in many physiological solutions. It is therefore important to characterize the degradation and potential metabolism of SN38 in this model prior to implementing its usage. As per ‘Immunofluorescence’ section, cells were seeded in triplicate into polyester membrane transwell support systems and TEER monitored daily. When a stable TEER > 1000 Ω/cm² was achieved, cells were treated with...
5 μM SN38 in the apical and basolateral chambers. To determine the natural degradation of SN38, transwell systems containing no T84 cells were filled with SN38 supplemented cell culture media (5 μM). Each transwell system with T84 cells and without T84 cells (negative control) was subject to identical conditions; 25 μl from the apical and basolateral chambers was collected at 0 h, 1 h, 3 h, 6 h, 24 h, and 48 h. Apical and basolateral samples were combined (50 μl) to produce a single sample per time point. All experiments were performed in triplicate and repeated.

**Sample preparation.** Fifty μl of sampled cell culture media were added to 150 μl of ice-cold acetonitrile with 0.1% formic acid (containing 20 ng/ml of IS). Samples were vortexed for 10 s and centrifuged at 13,300 rpm for 10 min at room temperature. A 180 μl aliquot of the supernatant was transferred to a clean microtube and 10 μl samples were analyzed in triplicate using liquid chromatography–mass spectrometry (ABSCIEX TripleTOF™ 5600 LC/MS/MS).

**Chromatographic conditions.** The ABSCIEX TripleTOF™ 5600 LC/MS/MS was used to perform liquid chromatography–mass spectrometry analysis. Chromatographic separation was achieved by using a Kinetex C18 (2.6 μm, 50 mm x 3.0 mm) analytical column (Phenomenex, NSW, Australia; #00 A-4633-AN). The mobile phase A was 5% acetonitrile, 95% water, 0.1% formic acid. Mobile phase B was 95% acetonitrile, 95% water, 0.1% formic acid with a 0.2 ml/min flow rate. The gradient system commenced with 90/10 mobile phase A/B for 30 s, whilst mobile phase A was gradually decreased to 0% by 2 min. This was maintained for 3 min before returning to initial conditions. The retention times for SN38 and CPT (internal standard) were 2.1 min and 2.16 min, respectively.

**Standard curve generation.** A working standard solution of 1 μg/ml was diluted accordingly to achieve 500 ng/ml, 250 ng/ml, 125 ng/ml, 50 ng/ml, 25 ng/ml, 5 ng/ml, and 2.5 ng/ml of SN38; 10 μl of each solution were spiked into 40 μl of blank plasma. Final SN38 concentrations were 100 ng/ml, 75 ng/ml, 25 ng/ml, 5 ng/ml, 1 ng/ml, and 0.5 ng/ml. The lower limit of detection was 0.1 ng/ml and lower limit of quantification was 0.5 ng/ml. SN38 concentration was determined by interpolation from the calibration curve and presented as molarity and percentage decrease from baseline.

**Reverse transcription-polymerase chain reaction (RT-PCR) analysis of Toll-like receptor 4 (TLR4)**

TLR4 is an emerging mediator of irinotecan-induced gut toxicity and a promising target for the development of potential interventions. In order for this platform to be used in the development of TLR4-targeted interventions, it is imperative that TLR4 expression be confirmed. TLR4 expression in HT29 and Caco-2 cells was used as a positive control.

**Ribonucleic acid (RNA) isolation.** To determine TLR4 mRNA expression, T84 (p7), Caco-2 (p46), and HT29 (p5) cells were grown to confluence in 90 mm culture dishes. Total RNA extraction was performed on each cell line using the NucleoSpin RNA Isolation Kit as per manufacturer’s instructions (NucleoSpin RNA Isolation Kit, Macherey-Nagel, Düren, Germany; #740955). Once eluted, RNA was stored at −80°C. Total RNA was quantified using the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA), TAKE3 plate, and Gen5 (version 2.0.0.18) software. RNA purity was also determined using the the BioTek Synergy™ Mx Microplate Reader (BioTek, Vermont, USA), TAKE3 plate, and Gen5 (version 2.0.0.18) software. RNA integrity was assessed at the Adelaide Microarray Facility (South Australia Health and Medical Research Institute) using the Agilent 2100 Bioanalyzer RNA 600 Nano Chip (Series II) kit.

**Reverse transcription and RT-PCR.** One mcg of total RNA was reverse transcribed using the iScript™ cDNA Synthesis Kit (BioRad, NSA, Australia; #1708890) as per manufacturer’s instructions. RT-PCR was performed using the Rotor-Gene 3000 (Corbett Research, NSW, Australia). Amplification mixes contained 2 μl of cDNA sample (100 ng/μl), 5 μl of Synergy Brands (SYBR) green fluorescence dye, 2 μl of RNase-free water, and 0.5 μl of each forward and reverse primers, prediluted to 50 pmol/μl, to make a total volume of 10 μl. Thermal cycling conditions included a denaturing step at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s and extension at 72°C for 15 s. All samples were run in triplicate. Primer efficiency was evaluated using standard curves and experimental threshold (Ct) values were calculated by the Rotor Gene 6 programme. Ct values were used to quantify relative mRNA expression of TLR4 in T84 cells and human colonic tissue using the ΔCt method, relative where TLR4 expression = 2^(-ΔCt) (target) – C_{t} (reference)). β-actin was used as an internal housekeeping (reference) gene.

Primer details are as follows: TLR4 primer sequence 5'->3' F: TGA GCA GTC GTG CTG GTA TC (Tm=54°C), R: CAG GGC TTT TCT GAG TCG TC (Tm=54°C), NM_001101.3, 116 bp. Cyt values were used to calculate relative mRNA expression of TLR4 in T84 cells and human colonic tissue using the ΔCt method, relative where TLR4 expression = 2^(-ΔCt) (target) – C_{t} (reference)). β-actin was used as an internal housekeeping (reference) gene.

**Statistical analysis**

Data were compared using Prism version 7.0 (GraphPad® Software, San Diego, USA). A D’Agostino-Pearson omnibus test was used to assess normality. When normality was confirmed, a paired t-test or two-way analysis of variance with appropriate post hoc testing were performed to identify statistical significance. In other cases, a Kruskall-Wallis test with Dunn’s multiple comparisons test and Bonferroni correction was performed. A P-value of <.05 was considered significant.

**Results**

Polyester membrane inserts support a polarised T84 phenotype with functional tight junctions in vitro T84 cells seeded at all density ranges, in both transwell support systems, achieved TEER values greater than 1000 Ω/cm² within
seven days (Figure 2(a) and (b)). T84 cells seeded at ≥200,000 cells/cm² had an elevated trajectory, whilst a seeding density of 50,000 cells/cm² displayed delayed maturation and resistance development. Monolayers seeded at 100,000 cells/cm² displayed consistent and controlled development of resistance. This trajectory is likely to facilitate optimal differentiation. When seeded at 100,000 cells/cm², T84 cells exhibited comparable resistance development characteristics in both transwell support systems, with no statistically significant differences observed (Figure 2(c)).

TEM analysis confirmed T84 monolayers grown on polyester membranes displayed polarity, with basally located nuclei and apical microvilli (Figure 3(a)). TEM analysis also showed intercellular junctional complexes (tight junction, intermediate junction, and desmosome) at the apico-lateral border of cells (Figure 3(b) and (c)). The presence of molecularly intact tight junctions in polyester membrane support system was confirmed by immunofluorescence for three major tight junction proteins: zonular occludens-1 (ZO-1), occluding, and claudin-1 (Figure 4(a) to (c)). No internalization or cytoplasmic redistribution was evident. These results also highlight the imaging potential of this support system, facilitating both light and confocal microscopy techniques. No fluorescence was detected on any membrane that had the primary antibody omitted (data not shown).

SN38 stability in the transwell support system
SN38 underwent rapid degradation in cell culture media alone (negative control; −T84 monolayer), with a 48.39% ± 4.01% decrease from baseline by 1 h (Figure 5(a) and (b)). This decline was more pronounced in transwell systems with T84 monolayers (−76.04% ± 1.86%) indicating metabolic processes (*P < .0001; P = .0126). SN38 concentration plateaued between 1 h and 24 h, although a small increase was observed at 48 h in the presence of T84 monolayers suggesting possible efflux mechanisms.

T84 cells express TLR4
RNA integrity numbers (RIN) were assigned to each sample using the Agilent Bioanalyser. Caco-2 cell yielded the highest RIN score (RIN: 8.90). HT29 and T84 cells had RIN scores of 8.00 and 7.90, respectively. RNA A₂₆₀/A₂₈₀ ratios for Caco-2, HT29, and T84 cells were 2.098, 2.089, and 2.090, respectively, indicating pure, protein-free samples.

Under basal conditions, T84 cells expressed TLR4 (Figure 6), although this was significantly less than that of the HT29 cells (*P = .037).

Discussion
This study has clearly highlighted the potential application for clear, polyester membrane transwell support systems in the investigation of gastrointestinal pathology. We have shown comparable T84 epithelial cell growth patterns in this model and the traditionally used, gold-standard mixed-cellulose membranes. T84 cells display normal morphological features when grown on polyester membranes, with a polarized phenotype complete with apical microvilli.
Figure 3  Transmission electron microscopy images of T84 cells grown on polyester membrane hanging inserts. Panel A (4200×) shows cellular polarity with apical microvilli (arrow) and a basally located nucleus (N). Panel B (16500×) shows intercellular junction complexes at the apico-lateral boundary. Panel C (26500×) shows high power image of the junctional complex; TJ = tight junction, IJ = intermediate junction; D = desmosome.

Figure 4  Immunofluorescent staining of (a) ZO-1 (red), (b) occludin (green), and (c) claudin-1 (cyan) in T84 monolayers. The border of each cell can be distinguished by immunocytochemical circumferential staining of each tight junction protein. T84 monolayers were fixed in 1:1 (v/v) acetone/methanol, before being permeabilized with 0.1% TritonX-100. Cells were blocked with 3% BSA before the primary antibodies for ZO-1, occludin, and claudin-1 were applied. Monolayers were then incubated with fluorescently conjugated secondary antirabbit/mouse antibodies at 568 nm and 488 nm, respectively. Panel C has been pseudocolored cyan for this figure. (d) XZ image showing occludin staining (arrow) at the cell periphery. The composite XZ stack shows apically located occludin staining which ceases toward the basal surface of the cell. The XZ composite was generated from 30 μm x 1 μm z-sections using the public domain Java image processing programme, Image J. (A color version of this figure is available in the online journal.)

Figure 5  SN38 concentration in transwell support system ± T84 monolayers. Data presented as (a) concentration (nM) or (b) relative to baseline (%). Transwell support systems ± T84 monolayers were treated with 5 μM SN38. The apical and basolateral chambers were sampled at 1 h, 3 h, 6 h, 24 h, and 48 h. SN38 concentration was determined by liquid chromatography–mass spectrometry (ABSCIEX TripleTOF™ 5600 LC/MS/MS). SN38 underwent rapid degradation in cell culture media, with a 48.39% ± 4.01% decrease from baseline by 1 h. Degradation is more pronounced in transwell supports with T84 monolayers suggesting metabolic processes (*P < .0001; tP = .0126). Possible efflux of SN38 can be seen at 48 h, with a spike in concentration. This is only observed in the presence of T84 monolayers.
and apico-lateral tight junctions. We intend for this model to be used for side-by-side structural and functional analysis of tight junctions and their contribution to the development of chemotherapy-induced gastrointestinal dysfunction. Correspondingly, we have characterized SN38 metabolism in this model, highlighting the metabolic capabilities of T84 cells. We have also confirmed expression of TLR4, a key mediator of toxicity and promising target for therapeutic interventions.

Given the inherent challenges in accessing the GIT, a simple in vitro model for interrogation of complex gastrointestinal physiology is critical in unraveling the mechanisms of chemotherapy-induced diarrhea. Characterization of this model highlights its suitability for the study for SN38-induced mucosal damage and the mechanisms and/or implications for tight junction disruption. Ultrastructurally, these monolayers displayed normal characteristics of the intestinal epithelium, with a microvillus-studded apical membrane, polarity, and typical organelle distribution, supporting previous phenotype reports. 

Most importantly, intercellular junction complexes were evident and apical tight junctions appeared phenotypically normal displaying apposing leaflets. Molecularly, tight junctions of polyester membrane T84 monolayers expressed the key architectural proteins, ZO-1, claudin-1, and occludin which exhibited typical 'honeycomb' or 'cobblestone' distribution.

It is well documented that tight junctions undergo an array of molecular changes in response to pathological cues with post-translational degradation and redistribution/internalisation of tight junction proteins reported in response to inflammatory mediators, kinases, and microbiota changes. Internalization is described most commonly for ZO-1, as it resides in the cytoplasm adjacent to the plasma membrane of the cell. It has been reported that under pathological states, particularly those with an inflammatory component, ZO-1 can detach from the junctional complex leading to altered tight junction integrity and barrier disruption. There was no evident internalization of ZO-1, claudin-1, or occludin in T84 monolayers grown in polyester membrane support systems, indicating that this systems supports molecularly intact, functional tight junctions that resemble that of the GIT. Furthermore, the transparent properties of the polyester membranes enable powerful confocal and light microscopy, enhancing the structural analysis of these complex intercellular structures and allowing paralleled functional and structural analyses. This feature is a significant advantage of this in vitro model, as structural tight junction analysis is typically achieved by growing T84 cells on coverslips. The polyester membrane transwell support system therefore provides a novel platform for interrogation of complex gastrointestinal physiology under normal or challenged situations and will enable investigation of SN38-induced mucosal damage.

SN38 is the semi-synthetic analogue of the naturally occurring anticancer alkaloid CPTs and the active metabolite of irinotecan. The hydroxyl group at the C10 position and ethyl group at the C7 position both help to stabilize SN38 in physiological environments and thus improve its potency. Despite this, SN38 is poorly solubilized and remains relatively unstable in physiological solutions. For example, the lactone ring of SN38 is stable at pH ≤ 4.5 but hydrolyses completely to its carboxylated form at pH ≥ 9. At pH 6.7, both forms are in equilibrium. In our in vitro T84 transwell model, SN38 underwent rapid degradation in physiologically stable cell culture media alone (negative control) highlighting not only the instability of SN38 but also its relatively short half-life. In the presence of T84 cells, this degradation was more pronounced, with a further 27.65% reduction from baseline suggesting T84 cells express the enzymes required for SN38 metabolism (e.g. UGT1A) and further highlights the suitability of this model for the study of SN38-induced mucosal damage. In addition to metabolism, our results also showed evidence of SN38 efflux at 48 h, indicating possible expression of efflux transporters that utilize SN38 as a substrate in this T84 cell line. ATP binding cassette (ABC) transporters have been characterized in other human colorectal carcinoma derived cell lines, such as LS180, but their expression in T84 cells is unclear. The ABC transporter, multidrug resistance gene 1 (MDR1), as well as and cytochrome P450 isofrom 3A4 (CYP3A4), have also been detected in LS180 cells, whereas only CYP3A4 was inducible in Caco-2 cells and TC-7 cells. 

Naruhashi et al. have recently shown that mRNA expression the ABC transporters MDR1, MDR-associated protein (MRP) 2, and MRP3 is comparable between caco-2 lines and T84 monolayers, with minimal differences their pattern of change in response to various endogenous compounds and xenobiotics. Despite similar expression, the intrinsic function of MDR1 was stronger in caco-2 cells suggesting caco-2 cells express the enzymes required for SN38 metabolism (e.g. UGT1A) and further highlights the suitability of this model for the study of SN38-induced mucosal damage. In addition to metabolism, our results also showed evidence of SN38 efflux at 48 h, indicating possible expression of efflux transporters that utilize SN38 as a substrate in this T84 cell line. ATP binding cassette (ABC) transporters have been characterized in other human colorectal carcinoma derived cell lines, such as LS180, but their expression in T84 cells is unclear. The ABC transporter, multidrug resistance gene 1 (MDR1), as well as and cytochrome P450 isofrom 3A4 (CYP3A4), have also been detected in LS180 cells, whereas only CYP3A4 was inducible in Caco-2 cells and TC-7 cells. 

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Figure 6 TLR4 mRNA expression in T84 and HT29 cells relative to internal housekeeping gene β-actin. Relative expression was determined using the ΔΔCT method. Expression of TLR4 in Caco-2 cells was below detectable levels. Data expressed as mean ± SEM. A one-way analysis of variance with Tukey’s post hoc was performed to determine statistical significance.
stimulated with lipopolysaccharide (LPS).\textsuperscript{31} In fact, TLR4 expression is comparatively lower in caco-2 cell lines when compared with numerous other colonic cell lines such as HT29s and T84s.\textsuperscript{31} This is also the case for TLR4 expression in T84 cells, which in our study was comparatively lower than that seen in HT29 cells. Nonetheless, the current study has definitively shown that T84 cells express TLR4, thus supporting the use of this novel \textit{in vitro} platform for the study of TLR4-targeted interventions for chemotherapy-induced gut toxicity. Development of this \textit{in vitro} model would therefore not only provide insight into the mechanisms of SN38-induced mucosal damage but would also provide an excellent platform in which to investigate pharmacological strategies critical in the development of effective treatment strategies.

Given the inherent challenges in accessing the GIT, the study of chemotherapy-induced gut toxicity remains difficult. A simple model for interrogation of complex gastrointestinal physiology is therefore critical in unraveling the mechanisms of symptoms such as diarrhea. This study has successfully demonstrated the use of T84 cells, grown in transparent polyester transwell support systems, as a suitable model for the study of chemotherapy-induced mucosal damage. This platform supports a polarized T84 phenotype with functional tight junctions, allowing for in-depth permeability studies. Additionally, the transparent properties of these inserts allow for sophisticated downstream analyses such as live-cell fluorescent imaging or confocal microscopy. We also demonstrated that T84 cells exhibit the unique ability to metabolize SN38 as well as expressing TLR4, making this an excellent platform for the study of clinically relevant therapeutic interventions for SN38-induced mucosal damage by targeting TLR4.

**Author contributions:** All authors participated in the design, interpretation of the studies, and analysis of the data and review of the manuscript; HRW, KRS, YZAVS conducted the experiments; HRW, JMB, RJG, RML designed the model; HRW, YZAVS, RJG, JMB wrote the manuscript.

**ACKNOWLEDGEMENTS**

The current study was supported by funds from the Florey Medical Research Foundation Project Grant in Cancer Research for PhD students awarded to Ms Hannah Wardill. Ms Hannah Wardill is also the recipient of the Florey Medical Research Foundation Doctor Chun Chung Wong and Madam So Sau Lam Memorial Postgraduate Cancer Research Top Up Scholarship 2015 and an Australian Postgraduate Award. Ms Yasabella Van Sebille is also a recipient of an Australian Postgraduate Award. The authors would like to thank Dr Scott Smid for providing Caco-2 cells, Dr Jennifer Hardingham for providing HT29 cells, and Professor M Brown for providing MCF-7 cells.

**DECLARATION OF CONFLICTING INTERESTS**

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**ETHICAL APPROVAL**

This article does not contain any studies with human participants or animals performed by any of the authors.

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(Received October 27, 2015, Accepted February 19, 2016)
Cytokine-mediated blood brain barrier disruption as a conduit for cancer/chemotherapy-associated neurotoxicity and cognitive dysfunction

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Neurotoxicity is a common side effect of chemotherapy treatment, with unclear molecular mechanisms. Clinical studies suggest that the most frequent neurotoxic adverse events affect memory and learning, attention, concentration, processing speeds and executive function. Emerging preclinical research points toward direct cellular toxicity and induction of neuroinflammation as key drivers of neurotoxicity and subsequent cognitive impairment. Emerging data now show detectable levels of some chemotherapeutic agents within the CNS, indicating potential disruption of blood brain barrier integrity or transport mechanisms. Blood brain barrier disruption is a key aspect of many neurocognitive disorders, particularly those characterized by a proinflammatory state. Importantly, many proinflammatory mediators able to modulate the blood brain barrier are generated by tissues and organs that are targets for chemotherapy-associated toxicities. This review therefore aims to explore the hypothesis that peripherally derived inflammatory cytokines disrupt blood brain barrier permeability, thereby increasing direct access of chemotherapeutic agents into the CNS to facilitate neuroinflammation and central neurotoxicity.

Neurotoxicity and its associated cognitive manifestations are poorly characterized, dose-limiting side effects of chemotherapy treatment. Clinically, the impact of chemotherapy on cognition has been most extensively studied in breast cancer patients, however it is becoming increasingly recognized that cognitive symptoms affect a large portion of patients with varying malignancies and treatments. Despite its prevalence, cognitive dysfunction, often referred to as chemotherapy-induced neurotoxicity, remains an under-reported and ill-defined complication of anti-cancer treatment. Cognitive symptoms are vast, but are most commonly reported to affect memory and learning, attention, concentration, processing speeds and executive function. Importantly, unlike many acute chemotherapy-related toxicities, cognitive dysfunction presents both acutely and chronically, compromising quality of life for patients unable to return to prior levels of social and academic interaction.

Given its frequency, and its acute and chronic impact, the importance of better understanding chemotherapy-induced neurotoxicity has become a priority with an obvious goal of developing effective interventions. Like the overwhelming majority of regimen-related toxicities, changes in neurological function occur in a subset of cancer patients and curiously these changes may or may not be associated with structural and functional alterations in the brain. Compounding our ability to attribute cognitive changes directly to treatment has been the finding that impairment has been reported among cancer patients who are treatment naïve.

As yet, the molecular mechanism(s) involved in chemotherapy-induced neurotoxicity have not been clearly defined, however there is strong evidence implicating direct cytotoxicity and associated inflammatory mechanisms. Currently, the bulk of studies assessing the latter focus on neuroinflammatory pathways, however, it is important to consider the impact of cytokines derived from the tumor, as well as

Key words: neurotoxicity, cognitive dysfunction, blood brain barrier, inflammation, chemotherapy-induced gut toxicity

Abbreviations: BBB: blood brain barrier; BMECs: brain microvascular endothelial cells; CIGT: chemotherapy-induced gut toxicity; IL: interleukin; INF: interferon gamma; MMP: matrix metalloproteinase; MTX: methotrexate; Myd88: myeloid differentiation factor; TKI: tyrosine kinase inhibitors; TLR: toll-like receptor; TNF: tumour necrosis factor; VEGFR: vascular epidermal growth factor receptor; ZO: zonular occludens; 5-FU-: 5-fluorouracil

DOI: 10.1002/ijc.30252

History: Received 5 June 2016; Accepted 21 June 2016; Online 1 July 2016
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those elicited by the effect of chemotherapy on normal or tumour tissue. Most likely, direct cytotoxicity and neuroinflammation occur in concert with cytokine-mediated disruption of the blood brain barrier (BBB) serving to enhance drug penetration to augment local levels and result in amplification of cognitive symptoms.

The finding of detectable levels of systemically administered chemotherapeutic agents within the central nervous system (CNS) supports this presumption and implies a level of BBB permeability that has not been previously appreciated. Increased levels of BBB permeability suggest that some chemotherapeutic agents are capable of disrupting its integrity, either directly or indirectly. Convincing evidence also exists linking BBB dysfunction with a proinflammatory state, with BBB dysfunction reported in patients with chronic inflammatory diseases as well as being a consequence of many forms of regimen-related peripheral toxicities. Among these, chemotherapy-induced mucosal injury, especially of the gastrointestinal tract, provides a compelling example of how focal chemotherapy-induced tissue damage can serve as a conduit for central neurotoxicity. Of interest, chemotherapy-induced gut toxicity (CIGT) has recently been shown to increase central markers of pain and neuroinflammation highlighting the ability of peripherally derived inflammation to profoundly affect CNS function.

Structural and Neuroimaging Studies That Define the Scope of Chemotherapy-associated Changes in the Brain

The neural basis for neurological deficiencies in cancer patients has been investigated with both structural and functional neuroimaging. Voxel-based morphometry (VBM) and diffusion-tensor imaging (DTI) are structural imaging techniques able to detect changes in both white and gray matter, whilst, functional magnetic resonance imaging (fMRI) and positron emission tomography (PET) studies enable assessment of functional deficiencies when structural changes are not evident. While these technologies have not been applied broadly, limited data suggest that chemotherapy is consistently associated with, changes in white matter (WM) structures. WM hyperintensities and hippocampal lesions have been identified using basic neuroimaging techniques in breast cancer patients treated with various chemotherapy regimens.  15-17

T1-VBM studies, an automated and quantitative method of neuroimaging which theoretically provides an unbiased, comprehensive and highly reliable assessment sensitive to local changes, have demonstrated diffuse cortical and subcortical WM and bilateral neocortical gray matter (GM) volume reductions or deficiencies in the superior frontal gyrus, parahippocampal gyrus, cingulate gyrus and the precuneus gyrus.  20-22 Based on the spectrum of neurocognitive symptoms seen in cancer patients and the well-documented function of the hippocampus, a growing body of research now shows impaired neurogenesis and hippocampal function likely contribute to neurotoxicity.  23,24 In support of this, hippocampal alterations have been identified in response to a spectrum of chemotherapeutic agents in a number of patient cohorts.

Structural changes, indicative of direct neurotoxicity, are often seen in conjunction with neurocognitive functional deficiencies detected through DTI and digital symbol testing (DST); a measure of processing speed. Although studies are limited in size and number, results have indicated associations between the integrity of the corpus collosum and processing speeds of patients receiving adjuvant chemotherapy for breast cancer.  27 Further associations have been identified between processing speeds and frontal WM integrity.  28 The largest study to investigate this association was conducted by Deprez et al. in premenopausal women with breast cancer. Patients receiving adjuvant chemotherapy exhibited worsening attention, psychomotor speed, verbal learning and memory, as well as decreased microstructural integrity in widespread regions of the corona radiata and the corpus collosum, compared to matched controls, reinforcing that WM changes may be the source of cognitive deficits seen in chemotherapy-treated patients.  29,29

Results of neuroimaging studies have been informative relative to describing observed structural and functional deficiency relationships associated with cancer- and chemotherapy-associated cognitive dysfunction. However, they are unable to mechanistically define the pathogenesis of chemotherapy-associated neurological toxicities. And at this early stage, their interpretation is limited by heterogeneity in experimental methodology, and confounded by neurological comorbidities commonly seen in cancer patients such as depression and anxiety, which can produce similar structural manifestations.  1 Furthermore, the inclusion of predominantly elderly patients, a lack of pretreatment baseline controls and presence of structural deficits in treatment-naive patients clouds the ability to make definitive conclusions regarding the mechanisms of chemotherapy-induced neurotoxicity.

Blood Brain Barrier Dysfunction: An Accelerant for Neurotoxicity?

The presence of chemotherapeutic agents in the CNS after systemic administration indicates their ability to cross the BBB, either physiologically or pathologically.  30 Early research has demonstrated detectable levels of intravenous administered cisplatin, bis-chloroethyl nitrosourea (BCNU) and paclitaxel in the brains of rodents using PET.  30,31 This phenomenon has also been seen in higher order primates, with detectable levels of 5-fluorouracil in the cerebrospinal fluid after intravenous administration.  14 Although therapeutic drug levels effective for CNS malignancies were not seen, drug concentrations were sufficient to induce apoptosis and neuronal damage associated with neurological dysfunction.  32 In addition to these findings, it is well established that a number of proinflammatory cytokines have detrimental effects on tight junctions and thus the integrity of the BBB. This is critically important when considering BBB breakdown
in cancer patients, as there are a number of sources of proinflammatory cytokines, derived from the tumour and the effects of chemotherapy on normal tissue.

Mediators of inflammation disrupt blood brain barrier integrity

The BBB (Fig. 1) is highly plastic and can undergo significant modification in response to a raft of physiological and pathologies cues. Subsequently, impairment of the BBB has been implicated in a number of CNS pathologies, particularly those characterized by a proinflammatory state. For example, traumatic brain injury (TBI) is often accompanied by a large inflammatory response resulting in grossly abnormal BBB permeability, the influx of inflammatory cells and subsequent oedema. A similar mechanism is now also hypothesized to play a role in the pathogenesis of stroke in which a weakening of the BBB, associated with a transient breakdown of tight junction proteins, is thought to contribute to the haemorrhagic transformation manifested by a heightened inflammatory state and worsened prognosis.

In light of these clinical associations between inflammation and BBB breakdown, there is now a wealth of in vitro and in vivo data demonstrating the ability of proinflammatory cytokines to disrupt the BBB (Table 1). From these

Figure 1. Blood brain barrier transport mechanisms. Cerebral endothelial cells of the blood brain barrier (Panel A) have specialised circumferential tight junctions and intracellular caveolae, which regulate blood brain barrier transit (Panel B). Tight junctions are highly plastic, multi-protein structures traversing the intercellular junction (Panel C). Each tight junction comprises the cytoplasmic protein family zonular occludens (ZO1, ZO2, ZO3) and the transmembraneous protein families claudin, JAMs and occludin. Caveolae, comprised of Cav-1, Cav-2 and Cav-3, control transcellular permeability within the blood brain barrier (Panel D).
Table 1. The action of established mediators of gut toxicity on blood–brain barrier regulation and dysfunction

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Effect on tight junctions and paracellular permeability</th>
<th>Effect on caveolae-mediated transcytosis</th>
<th>Established role in gut toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proinflammatory cytokines</td>
<td>Il-1β, Il-1 and TNF exposure caused increased paracellular permeability and reduced ZO-1 expression in an in vitro model of the blood brain barrier (THBMEC). Consequently, many inflammatory CNS pathologies are characterised by increased blood brain barrier permeability.</td>
<td>IL-1β induces translocation of IL-1R1 and recruitment of signaling molecules to caveolin-enriched lipid rafts, preceeding caveolin-dependent endocytosis. Caveolae are involved in TNF endocytosis in BBB endothelia and eventual transport across the BBB from the luminal to abluminal side. TNF also induces translocation of its receptors, TNFR1 and TNFR2, to endothelial caveolae.</td>
<td>Well-documented mediators of toxicity; significantly elevated levels of Il-1β, Il-6 and TNF following cytotoxic insult. Initiate an inflammatory response, and amplify NFκB signaling.</td>
</tr>
<tr>
<td>MMPs</td>
<td>BMECs exposed to oxidative stress expressed significantly elevated MMP-9 activity paralleled by downregulation and redistribution of occludin. Elevated levels of MMP-2/-9 have also been report in several CNS pathologies such as cerebral ischemia, leading to increased barrier permeability and cerebral oedema. Inhibition of both MMP-2/-9 has been shown to attenuate oedema formation.</td>
<td>Membrane-type 1 (MT1) MMP is present at endothelial caveolae with caveolin-1 constituting a novel pathway for MT1-MMP internalization in human endothelial cells.</td>
<td>Altered tissue and serum levels of various MMPs and their inhibitors (TIMPs) have been reported following irinotecan. Hypothesized to contribute to development of gut toxicity through inflammatory pathways, altered extracellular matrix composition, adhesion molecules and tight junctions.</td>
</tr>
<tr>
<td>ROS</td>
<td>Increased presence of ROS correlates with cytoskeletal rearrangements, redistribution and disappearance of TJ proteins claudin-5 and occludin.</td>
<td>Peroxynitrite (ONOO⁻) exposure is related to the impaired expression of Cav-1 in endothelial membrane associated with vascular disturbances of diabetes. Specifically, regulation of eNOS within caveolae is an important physiological mechanism for control of vascular reactivity, and is thought to have a role in the suppression of inflammatory signaling pathway. During IBD the loss of Cav-1, and thus caveolae, results in reduced tissue pathology likely due to decreased inflammatory and angiogenic signaling; this protection is also lost with endothelial cell-specific restoration of Cav-1.</td>
<td>Upregulated ROS is a key step in the initiation of gut toxicity, occurring almost instantaneously after cytotoxic treatment. It is currently part of the universally accepted model of gut toxicity.</td>
</tr>
<tr>
<td>Substance P</td>
<td>Substance P (SP) shown to induce changes in ZO-1 and claudin-5 in HBMECs and correlate with increased permeability. SP is released early as part of a neurogenic inflammatory response. In so doing, it facilitates an increase in the permeability of the blood–brain barrier. At the cellular level, SP has been shown to directly result in neuronal cell death.</td>
<td>SP receptor the NK-1R is localized within endothelial caveolae though their role requires further elucidation.</td>
<td>Preclinical studies have shown elevated levels of SP, and NK-1R, in the small bowel following cytotoxic insult; 5-fluorouracil caused a 3-fold increase in the mRNA expression of NK-1R and significant elevations in SHT3 and NK-1R immunopositive cells. Regulated through NFκB, SP is thought to activate MMPs and initiate an inflammatory response.</td>
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</table>
Table 1. The action of established mediators of gut toxicity on blood–brain barrier regulation and dysfunction (Continued)

<table>
<thead>
<tr>
<th>Mediator</th>
<th>Effect on tight junctions and paracellular permeability</th>
<th>Established role in gut toxicity</th>
</tr>
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<tbody>
<tr>
<td>TLR4</td>
<td>Ischaemic stroke is associated with heightened TLR4 expression. Pharmacological inhibition or genetic deletion of TLR4 attenuated barrier and tight junction disruption.</td>
<td>The innate immune system is critically important in the development of gut toxicity following chemotherapy, and suggests that these off targets are potential facilitators of CNS pathology.</td>
</tr>
<tr>
<td>VEGF</td>
<td>VEGF disrupts tight junctions, and it has been shown to be involved in BBB breakdown and immune cell infiltration in vivo. VEGF significantly mediates BBB breakdown in response to inflammatory signals.</td>
<td>It is well documented that increased MMP activity correlates with elevated permeability of both endothelial and epithelial barriers (Table 1), strongly implying MMP-mediated tight junction disruption. Particularly robust evidence supports a role for MMP-mediated tight junction disruption in the BBB as endothelial cells, astrocytes and pericytes are all potent sources of these signaling proteins. Brain-derived microvascular endothelial cells (BMECs) exposed to oxidative stress expressed significantly elevated MMP-9 activity paralleled by downregulation of occludin. Numerous preclinical studies also support a role for MMP-mediated tight junction disruption. For example, MMP-2/9 levels have been shown to be significantly elevated following cerebral ischemia leading to tight junction protein degradation, increased BBB permeability and oedema. Furthermore, inhibition of MMP-2/9 has been shown to reduce vascular permeability and attenuate tight junction disruption. The impact of MMPs on caveolae-mediated transcytosis is now also being recognized for its potential role in CNS pathologies characterized by BBB disruption (Table 1).</td>
</tr>
<tr>
<td>MMP-2/-9</td>
<td>MMP-2/-9 is known to exacerbate chemotherapy-induced diarrhoea, implicating VEGF activity in cytotoxic therapy-induced gut toxicity.</td>
<td>The impact of MMP-2/-9 on tight junction disruption in the BBB has been shown to be generated by tissues and organs that are targets for chemotherapy-associated toxiciies. Among these, chemotherapy-induced mucosal injury, especially of the gastrointestinal tract, provides a compelling example of how focally induced chemotherapy tissue damage can serve as a conduit for central neurotoxicity. While suggested by Seigers and Fardell, the potential impact of peripheral inflammatory mediators as a driver of central toxicity has hardly been explored.</td>
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<tr>
<td>IL-1β</td>
<td>IL-1β is known to exacerbate chemotherapy-induced diarrhoea, implicating MMP activity in cytotoxic therapy-induced gut toxicity.</td>
<td>The development of CIGT is a dynamic process, characterized by observations, it is clear that some cytokines exclusively affect paracellular barriers (e.g., IL-1β), through breakdown and translocation of tight junction proteins, whilst others target transcellular processes mediated by caveoleae (e.g., TNFα). A number of vasogenic agents (histamine, substance P) and proteases associated with inflammation, have also been identified to promote BBB remodeling. Of interest is the impact of matrix metalloproteinases (MMP) on tight junction integrity (Table 1) given the high levels of circulating MMPs observed after chemotherapy.</td>
</tr>
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overlapping and simultaneous biological events. Logan et al.\textsuperscript{51} demonstrated that the administration of irinotecan, 5-fluorouracil and methotrexate induced significant elevations in TNF-\(\alpha\), IL-1\(\beta\) and IL-6 in tumour-bearing rats. Importantly, these proinflammatory cytokines not only damage surrounding tissue through pro-apoptotic signals (e.g., caspases 3 activation), but they are also highly efficient activators of NFkB thus amplifying the mucotoxic cascade. Furthermore, TNF-\(\alpha\) and IL-1\(\beta\) induce MMP-1, MMP-2 and MMP-3 activation, which is thought to contribute to development of gut toxicity through inflammatory pathways, altered extracellular matrix composition, adhesion molecules and tight junction disruption. Toll-like receptor (TLR)4-dependent mechanisms have also been linked to the development of gut toxicity, with increases in its expression seen following chemothera-
py\textsuperscript{81} as well as improvements in symptomatic parameters seen following genetic manipulation of its downstream signaling molecules (e.g., MyD88, MD-2)\textsuperscript{82,83}. This aspect of CIGT has significant implications for BBB maintenance, as it is becoming increasingly clear that TLR4-dependent signaling pathways are critical for tight junction integrity.\textsuperscript{84}

TLR4-mediated barrier modulation has been shown in both endothelial and epithelial models. For example, Gao et al. (2015)\textsuperscript{85} recently showed that traumatic brain injury was not only associated with traditional proinflammatory markers, but also elevated TLR4 signaling and uncontrolled BBB transit. Importantly, administration of a vascular endothelial growth inhibitor (VEGI) up-regulated the tight junction proteins (claudin-5, ZO-1, occludin) and attenuated TLR4 activation, NF-\(\kappa\)B signaling and the production of proinflammatory cyto-
kines, as well as improving markers of brain injury. Alcohol-
induced steatohepatitis is also well documented to present with acute intestinal barrier disruption, resulting from impaired tight junction protein expression.\textsuperscript{86} In this study, administration of a TLR4 monoclonal antibody attenuated both functional and molecular markers of barrier function, emphasizing the importance of TLR4-mediated tight junction disruption in an inflammatory setting.

TLR4-dependent tight junction disruption has also been shown to occur in response to irinotecan treatment in a TLR4 knockout (\(-/-\)) mouse model of gut toxicity.\textsuperscript{80} Following irinotecan, increased permeability of both the intestinal barrier and BBB were detected, both seen at 24 h post-treatment. Although TLR4 knockout animals only showed improvements in intestinal barrier disruption, this study is the first to demon-
strate central neurotoxic changes in a model of chemotherapy-
induced gut toxicity and reinforces the bidirectional communi-
cation that exists between the gastrointestinal system and CNS. It is likely this communication that underpins the preva-

cent comorbidities affecting these organ systems.

**Intestinal inflammation drives CNS changes**

A number of intestinal pathologies are associated with an increased risk of behavioral comorbidities as indicated by increased rates of depression, mood disorders and cognitive dysfunction in patients with inflammatory bowel disease (IBD).\textsuperscript{87} For example, elevated circulating proinflammatory cytokines, increased in intestinal permeability and the number of circulating monocytes are commonly reported in acute phases of trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice.\textsuperscript{88} Importantly, these are accompanied by localized breaches in the BBB\textsuperscript{89} leading to increased neuroinflamma-
tion\textsuperscript{90,91} and associated cognitive disturbance. These findings are consistent with those of Zonis et al.\textsuperscript{92} who, using a differ-
ent murine IBD model (dextran sodium sulfate), found increased microglial and astrocytic reactivity in the hippocampus of treated mice. These results also compliment data indicating altered neuronal function and increased anxiety-
like behavior in models of parasitic gut inflammation.\textsuperscript{93,94}

Other reports support the concept that patients with chronic inflammatory states initiated by autoimmune diseases, cancer or infections are at higher risk for central neurological pathology and that there is a high likelihood that such changes are mediated by proinflammatory cytokines directly impacting the brain.\textsuperscript{95}

**Neuroinflammation and Cognitive Dysfunction**

Increased systemic proinflammatory cytokine production has been previously suggested as a candidate mechanism for cog-
nitive dysfunction in cancer patients.\textsuperscript{96} It is therefore possible that proinflammatory cytokines may be involved in several aspects of neurotoxicity by; (i) increasing BBB transit, and (ii) permitting neuroinflammation and associated tissue manifestations.

Substantial data, from a spectrum of clinical settings, highlight links between peripheral inflammation and cogni-
tive symptoms. For example, peripheral activation of the immune system by a subseptic dose of lipopolysaccharide (LPS) has been shown to increase cytokine expression within the brain\textsuperscript{96–98} at levels associated with learning and memory disruption in both models of disease and health.\textsuperscript{99–101} In healthy volunteers, LPS leads to increased levels of IL-1, TNF-\(\alpha\) and IL-6 resulting in impaired working memory and cognitive dysfunction.\textsuperscript{102} Similarly, increased peripheral inflammation has also been associated with gradual cognitive decline and the development of dementia in the elderly population.\textsuperscript{103}

Interestingly, the use of IFN-\(\alpha\) and IL-2 (proinflammatory cytokines) as anti-cancer agents is highly linked to the devel-

optment of depression and other cognitive impairments.\textsuperscript{104,105} however, there is only limited clinical data from cancer patients in which correlations between circulating cytokines and cognitive function have been evaluated. Meyers et al.\textsuperscript{105} reported an association between elevated levels of circulating IL-6 and worsened executive function in patients with acute myeloid leukemia. In addition, elevated IL-6 and TNF-\(\alpha\) seen in chemotherapy-treated breast cancer survivors correlated with persistent hippocampal structural changes and reduced verbal memory performance\textsuperscript{107,108} well beyond the period during which patients received drug infusions.
When looking at other CNS pathologies characterized by neuroinflammation and cognitive impairment, Alzheimer’s disease (AD) provides additional insight. Patients with AD often have elevated levels of TNFα in the cerebrospinal fluid and parenchyma, as well as expressing TNFα-related polymorphisms. The pathological level of cytokines resulting in this chronic inflammatory state mimics that noted in cancer patients and perpetuates neuronal loss and cognitive decline. Importantly, treatment with anti-TNFα agents in patients with AD has been shown to favorably impact the development of cognitive dysfunction.

Of particular importance to our proposed hypothesis is that AD is often accompanied by increased BBB transit, leading to heightened inflammatory influx and worsened clinical outcomes.

Figure 2. Schematic highlighting multifactorial pathobiology of cognitive dysfunction. Many cognitive disorders are characterised by blood brain barrier disruption and neuroinflammation. The impact of peripheral inflammation on central neurovascular integrity and the subsequent development of neuroinflammation is now considered a key driver in many neurological disease characterised by systemic inflammation. Blood brain barrier disruption may therefore compliment what is currently understood about neurotoxicity, by enhancing exposure of the CNS to chemotherapeutic agents (direct neurotoxicity) and permitting neuroinflammation (indirect). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

When looking at other CNS pathologies characterized by neuroinflammation and cognitive impairment, Alzheimer’s disease (AD) provides additional insight. Patients with AD often have elevated levels of TNFα in the cerebrospinal fluid and parenchyma, as well as expressing TNFα-related polymorphisms. The pathological level of cytokines resulting in this chronic inflammatory state mimics that noted in cancer patients and perpetuates neuronal loss and cognitive decline. Importantly, treatment with anti-TNFα agents in patients with AD has been shown to favorably impact the development of cognitive dysfunction. Of particular importance to our proposed hypothesis is that AD is often accompanied by increased BBB transit, leading to heightened inflammatory influx and worsened clinical outcomes.

Tumour-dependent cytokine production
Further confounding our understanding of how peripheral inflammation, BBB disruption and neuroinflammation contribute to cognitive dysfunction is the fact that results from clinical studies are neither uniform nor concrete. Cognitive dysfunction has been reported in patients with breast cancer or colorectal cancer after diagnosis, but before the administration of any anti-cancer treatment. Similarly, in a recent study comparing patients with localized colorectal cancer (n = 289) who received or did not receive chemotherapy, patients with metastatic or recurrent colorectal cancer (CRC) (n = 73) and healthy controls, Vardy et al. reported significant differences in cognitive dysfunction prior to, and following, treatment between patients with CRC and healthy controls. Surprisingly, there was no difference in the degree of cognitive dysfunction between patients who received chemotherapy and those who did not. In addition, the extent of disease (local vs. metastatic) did not affect neurological function clouding understanding of tumour-driven effects on cognition. While levels of proinflammatory cytokine levels were elevated in the CRC cohorts vs. controls, there was no statistically significant relationship between them and cognitive dysfunction. Patel et al.’s study of 174 newly diagnosed patients with breast cancer also reported baseline levels of cognitive dysfunction and elevations in proinflammatory cytokine levels (TNF). However, elevations in TNF were no higher in cancer patients compared to a noncancer, demographically similar control group.

The Significance of Symptom Chronicity
Despite patient and study heterogeneity and methodological limitations, neurotoxicity is defined by the chronicity of symptoms which often persist long after treatment.

Int. J. Cancer: 00, 00–00 (2016) © 2016 UICC
cessation. It has been reported that patients treated with high-dose chemotherapy and those that receive autologous haemopoietic stem cell transplantation have significant impairments in cognitive function up to 1 year after cessation of their treatment. The longevity of cognitive symptoms has also been assessed in survivors of childhood acute lymphoblastic leukaemia 6–18 years after remission. Deficits in figural memory as well as reduced hippocampal volume were also noted. Similarly, in breast cancer patients, chemotherapy treatment resulted in reduced gray matter volume in the right parahippocampal gyrus compared to untreated cancer patients, which correlated with reduced memory performance at 1 but not 3 years post-treatment. These studies are somewhat complimented by two prospective studies that show hippocampal volume reductions at 1 month following chemotherapy treatment, which was lost at further time points (1 year). More persistent changes were observed in a recent investigation of 19 breast cancer patients, who showed right hippocampal gray matter volume reductions at both 1 month and 1 year after treatment completion.

Although variations exist in the time-course of these symptoms, chronicity is almost always reported and is a defining characteristic of neurotoxicity. Given the relatively short half-life of many chemotherapeutic agents, the chronicity of symptoms is biologically significant, and suggests that the impact of direct cytotoxicity would presumably be minimal; with chronic, reactive inflammatory processes, a more likely candidate. However, it is important to consider that neurotoxicity is unlikely to be attributable to a single mechanism, rather, various mechanisms may converge additively or synergistically to result in the heterogeneous symptoms seen in patients (Fig. 2). This is well described by Dietrich et al., who highlights key mechanistic drivers such as oxidative stress, direct cellular toxicity and inflammation that contribute to altered cellular kinetics in the hippocampus as well as neurovascular/BBB disruption. If a prolonged period of inflammation is present, either systemically or centrally, altered BBB transit could parallel the chronic, long-term changes seen in patients and may provide a better biological understanding.

**Conclusion**

Regimen-related toxicities are poorly characterized and often have significant effects on patient quality of life. We have previously highlighted the importance of symptom clusters, emphasizing the possibility of common underlying mechanisms, which could perhaps be simultaneously targeted. Cognitive impairment is particularly devastating to patients, and currently has no universally accepted mechanism. This review has proposed that, contrary to traditional beliefs, chemotherapeutic agents can in fact gain access to the CNS. Importantly, we suggest that this is likely due to upregulated and uncontrolled BBB transit. The BBB, like many interfaces within the body, is subject to intense modification highlighting the plasticity of tight junctions and transcytotic mediators. Based on symptom clustering and potential linkages between the gut and CNS, we suggest that peripherally derived inflammatory mediators are responsible for inducing BBB dysfunction, thus permitting central neurotoxicity. Importantly, neurotoxic changes may occur through the direct actions of the chemotherapeutic drug itself, or present as the behavioral manifestation of neuroinflammation. BBB disruption may therefore be the missing link in our understanding of how gut/CNS communication is involved in the development of two critically important regimen-related toxicities. Furthermore, it presents as an exciting opportunity to target peripheral inflammation and achieve wider reaching clinical outcomes.

**Acknowledgements**

Ms Hannah Wardill is the recipient of the Florey Medical Research Foundation Doctor Chun Chung Wong and Madam So Sau Lam Memorial Postgraduate Cancer Research Top Up Scholarship 2015. Ms Hannah Wardill, Ms Yasabella Van Sebille and Ms Kimberly Mander are recipients of an Australian Postgraduate Award.

**References**


