A study linking toll-like receptors and irinotecan-induced gastrointestinal mucositis

Khloud G. Fakiha

Thesis submitted for degree of Doctor of Philosophy

Discipline of Medicine

The University of Adelaide

Australia

January 2015
Declaration

“I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other 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. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.”

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

I also give permission for the digital version of my thesis.

Khloud G. Fakiha

January 2015
Contents

Declaration .............................................................................................................. II

List of figures: ...................................................................................................... VII

List of tables: ...................................................................................................... X

List of abbreviations: ........................................................................................ XI

Abstract............................................................................................................. XII

Acknowledgment.............................................................................................. XV

Thesis explanation ............................................................................................ XVI

Chapter One ...................................................................................................... 1

1.0 Literature review ......................................................................................... 2

1.1 Introduction ................................................................................................... 2

1.2 Chemotherapy-induced gastrointestinal mucositis (GIM) ................................... 4

1.2.1 Irinotecan.................................................................................................. 4

1.2.2 Irinotecan metabolism ........................................................................... 4

1.2.3 Irinotecan-induced apoptosis ................................................................ 6

1.2.4 Irinotecan-induced diarrhoea .................................................................. 8

1.2.5 The role of intestinal microbiome in irinotecan-induced GIM ..................... 9

1.2.6 Irinotecan-induced morphological and histological damage ..................... 10

1.3 TLRs ............................................................................................................. 10

1.3.1 TLR2 and TLR4 ...................................................................................... 12

1.3.2 TLR2 and TLR4 involvement in intestinal inflammation ......................... 18

1.3.3 TLR2 and TLR4 involvement in cancer therapy-induced GIM ................. 20

1.3.4 Pro-inflammatory cytokine involvement in irinotecan-induced GIM .......... 21

1.3.5 Effects of TLR2 and TLR4 inhibition on mucosal injury ........................... 22

1.3.6 Amitriptyline (AMI) ............................................................................. 22

1.3.7 Amitriptyline metabolism ...................................................................... 23

1.3.8 Can AMI be used to prevent GIM development? ..................................... 25

1.4 Conclusion .................................................................................................. 26
Chapter Two .................................................................................................................. 29

2.0 General methods ........................................................................................................... 29

2.1 Histological analysis ...................................................................................................... 29

2.2 RNA extraction and reverse transcription ................................................................... 29

2.3 Real time-PCR .............................................................................................................. 30

2.4 Immunohistochemistry for TLR2, TLR4 and pro-inflammatory cytokine protein expression ..... 32

Chapter Three ................................................................................................................. 34

3.0 Toll-like receptors 2 and TLR4 and pro-inflammatory cytokines are ..................... 35

3.1 Introduction .................................................................................................................... 35

3.2 Materials and Methods ............................................................................................... 38

3.2.1 Experimental plan .................................................................................................... 38

3.2.2 Diarrhoea assessment ............................................................................................. 39

3.2.3 Toxicity classification: ............................................................................................ 39

3.2.4 Histological analysis ............................................................................................... 39

3.2.5 RNA extraction and reverse transcription ................................................................. 39

3.2.6 Real time-PCR ......................................................................................................... 40

3.2.7 Immunohistochemistry for TLR2, TLR4 and pro-inflammatory cytokine protein expression ..... 40

3.2.8 Statistical Analysis .................................................................................................. 40

3.3 Results .......................................................................................................................... 40

3.3.1 Response to treatment and histological analysis ..................................................... 40

3.3.2 TLR2 and TLR4 and pro-inflammatory cytokine mRNA expression ...................... 44

3.3.3 TLR2, TLR4 and pro-inflammatory cytokine protein expression following irinotecan treatment 47

3.3.4 The correlation between mRNA and protein expression: ........................................ 54

3.4 Discussion ..................................................................................................................... 58

Chapter Four ..................................................................................................................... 62

4.0 The effect of the TLR2 and TLR4 inhibitor, amitriptyline (AMI) on irinotecan-induced GIM63

4.1 Introduction: ................................................................................................................. 63

4.2 Methodology: ............................................................................................................... 66

IV
Chapter Five

5.0 The effect of Amitriptyline on SN-38-induced intestinal cell death and toll-like receptor expression

5.1 Introduction: ................................................................. 133

5.2 Methodology: .............................................................. 136

5.2.1 Cell line ..................................................................... 136

5.2.2 Cell culture ................................................................ 136

4.2.1 Drugs ........................................................................ 66

4.2.2 Experimental plan .......................................................... 66

4.2.3 GI toxicity and distress symptoms .................................. 69

4.2.4 Histological examination ................................................ 71

4.2.5 Tissue morphometry ...................................................... 71

4.2.6 Immunohistochemistry .................................................. 71

4.2.7 Caspase-3 and Ki-67 ..................................................... 71

4.2.8 TLR2, TLR4 and pro-inflammatory cytokines immunohistochemistry .......................................................................................... 72

4.2.8.1 MPO and MCP-1 immunohistochemistry .......................................................................................... 74

4.2.9 PCR array for innate and adaptive immunity in rat .... 75

4.2.9.1 RNA extraction and reverse transcription: .......................................................... 75

4.2.9.2 PCR array .................................................................. 75

4.2.10 Statistical analysis ......................................................... 75

4.3 Results ........................................................................... 76

4.3.1 Response to treatment and diarrhoea occurrence ............. 76

4.3.2 Histological analysis ...................................................... 82

4.3.3 Morphological changes in colon ..................................... 86

4.3.4 Apoptosis and proliferation ............................................ 88

4.3.4.1 Apoptosis ................................................................ 88

4.3.4.2 Proliferation ............................................................ 91

4.3.5 Protein expression of TLR2, TLR4 and pro-inflammatory cytokines in the jejunum and colon .......................................................... 94

4.3.6 Protein levels of MPO and MCP-1 in colon .................. 119

4.3.7 PCR array .................................................................. 122

4.4 Discussion: .................................................................... 126

Chapter Five ........................................................................ 132
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.2.3. Drug treatment</td>
<td>137</td>
</tr>
<tr>
<td>5.2.4. Cell proliferation assessment</td>
<td>137</td>
</tr>
<tr>
<td>5.2.5. Cell viability assessment</td>
<td>138</td>
</tr>
<tr>
<td>5.2.6. RNA extraction and reverse transcription</td>
<td>139</td>
</tr>
<tr>
<td>5.2.7. Primers</td>
<td>139</td>
</tr>
<tr>
<td>5.2.8. Real time PCR (RT-PCR)</td>
<td>139</td>
</tr>
<tr>
<td>5.2.9. Statistical analysis</td>
<td>140</td>
</tr>
<tr>
<td>5.3. Results</td>
<td>140</td>
</tr>
<tr>
<td>5.3.1. Cell proliferation and viability in response to SN-38</td>
<td>140</td>
</tr>
<tr>
<td>5.3.2. Effect of SN-38 and AMI on cell proliferation and viability</td>
<td>142</td>
</tr>
<tr>
<td>5.3.3. TLR2, TLR4 and pro-inflammatory cytokine mRNA expression after treatment with SN-38 and AMI</td>
<td>144</td>
</tr>
<tr>
<td>5.4. Discussion</td>
<td>148</td>
</tr>
</tbody>
</table>

Chapter Six .................................................................................. 153

6.0 General discussion .................................................................. 154

6.1. Introduction .......................................................................... 154

6.2. Are TLR2 and TLR4 involved in irinotecan-induced GIM? ............. 154

6.3. Does TLR2 and TLR4 inhibition modify irinotecan-induced GIM? ...... 157

Chapter Seven .............................................................................. 162

References .................................................................................... 163

Appendix ......................................................................................... 185
List of figures:

Figure 1.1: Irinotecan-induced apoptosis................................................................. 7
Figure 1.2: TLR2 signalling pathways (MyD88-dependant and PI3K-dependent) .......... 14
Figure 1.3: TLR4 signalling pathways (MyD88-dependant and –independent pathways) .... 17
Figure 1.4: AMI metabolism.................................................................................. 24
Figure 3.1: Incidence of diarrhoea after irinotecan treatment................................. 42
Figure 3.2: Haematoxylin and eosin (H&E) stains comparing the histological damage: A) in the jejunum; and B) in the colon between rats with and without diarrhoea at 72 and 96 h........ 43
Figure 3.3: Fold change in TLR2, TLR4, IL-1β, TNF and IL-6 mRNA expression in jejunum compared to untreated controls................................................................. 45
Figure 3.4: Fold change in TLR2, TLR4, IL-1β, TNF and IL-6 mRNA expression in the colon compared to untreated controls................................................................. 46
Figure 3.5: TLR2, TLR4 and pro-inflammatory cytokine protein expression in all experimental groups and untreated control in the jejunum A) crypt and B) villi.................................................. 48
Figure 3.6: TLR2 immunostaining of the jejunum of all DA rat groups treated with CPT-11, control and negative control................................................................................ 50
Figure 3.7: TLR4 immunostaining of the jejunum of all DA rat groups treated with CPT-11, control and negative control................................................................................ 50
Figure 3.8: TLR2, TLR4 and pro-inflammatory cytokine protein expression in experimental groups and untreated control in the colon A) apical and B) basal region of the crypt............. 51
Figure 3.9: TLR2 immunostaining of the colon of all DA rat groups treated with CPT-11, control and negative control................................................................................ 52
Figure 3.10: TLR4 immunostaining of the colon of all DA rat groups treated with CPT-11, control and negative control................................................................................ 53
Figure 3.11: The correlation between mRNA (fold change compared to control) and protein expression (staining score) for TLR4, TLR2 and pro-inflammatory cytokines in the jejunum (crypt and villi) following irinotecan administration...................................................... 56
Figure 3.12: The correlation between mRNA (fold change from control) and protein (staining score) expression for TLR4, TLR2 and pro-inflammatory cytokines in the colon (apical and basal regions) following irinotecan administration...................................................... 57
Figure 4.1: Study design showing the four groups of the experiment. Irinotecan (CPT-11), amitriptyline (AMI), CPT-11+ AMI and vehicle............................................................. 68
Figure 4.2: Percentage weight change compared to the starting weight at each time point for all groups. .................................77
Figure 4.3: Incidence of diarrhoea in treated groups and control at different time points........78
Figure 4.4: Total score of distress symptoms (dull/ruffled coat, change in temperament, reluctant to move.) for each group at different time points..........................................................80
Figure 4.5: A. Jejunum and B. colon weight for control and all treated groups..................81
Figure 4.6: Histological analysis in (A) the jejunum and (B) colon..................................83
Figure 4.7: Hematoxylin and eosin (H&E) staining illustrating histopathological damage in the jejunum at various time points for all groups.................................................................84
Figure 4.8: H&E staining illustrating histopathological damage in the colon at various time points for control and treatment groups.................................................................85
Figure 4.9: Crypt length in the colon for all experimental groups and control.....................87
Figure 4.10: Changes in cell apoptosis as identified by caspase-3 immunohistochemistry in the A) jejunum and B) colon at 6, 48 and 96 h in all experimental groups......................................................89
Figure 4.11: Caspase-3 immunohistochemistry in the colon at 6 h in all experimental groups.....90
Figure 4.12: Changes in cell proliferation as indicated by Ki-67 immunostaining in A) jejunum and B) colon as a percentage of positive cells/half crypt..........................................................92
Figure 4.13: Ki-67 immunostaining in colon for control and treated groups (CPT-11, AMI, CPT-11+AMI) at 6 h showing positive proliferative cells (brown)..................................................93
Figure 4.14: Changes in TLR2 staining in the jejunum (A) crypt and (B) villi regions at 6, 48 and 96 h......................................................................................................................95
Figure 4.15: TLR2 immunostaining in the jejunum for all groups.......................................96
Figure 4.16: Changes in TLR2 immunostaining in the colon (A) apical and (B) basal regions at 6, 48 and 96 h.........................................................................................................97
Figure 4.17: TLR2 immunostaining in the colon for all groups...........................................98
Figure 4.18: Changes in TLR4 staining in the jejunum (A) crypt and (B) villi regions at 6, 48 and 96 h.................................................................................................................100
Figure 4.19: TLR4 immunostaining in the jejunum of all groups.......................................101
Figure 4.20: Changes in TLR4 immunostaining in the colon (A) apical and (B) basal regions at (6, 48 and 96 h) in control and experimental groups.........................................................102
Figure 4.21: Tissue immunostaining of TLR4 in the colon for all groups.........................103
Figure 4.22: Changes in IL-1β staining in the jejunum (A) crypt and (B) villi regions at (6, 48 and 96 h) in control and experimental groups. .................................................................105
Figure 4.23: IL-1β immunostaining in the jejunum of all groups.......................................106
Figure 4.24: Changes in IL-1β immunostaining in the colon (A) apical and (B) basal regions at (6, 48 and 96 h) in control and experimental groups.................................................................107
Figure 4.25: IL-1β immunostaining in the colon for all groups..................................................108
Figure 4.26: Changes in TNF staining in the jejunum (A) crypt and (B) villi regions at (6, 48 and 96 h) in control and experimental groups........................................................................110
Figure 4.27: TNF immunostaining in the jejunum of all groups..................................................111
Figure 4.28: Changes in TNF immunostaining in the colon (A) apical and (B) basal regions at (6, 48 and 96 h) in control and experimental groups........................................................................112
Figure 4.29: TNF immunostaining in the colon for all groups..................................................113
Figure 4.30: Changes in IL-6 staining in the jejunum (A) crypt and (B) villi regions at (6, 48 and 96 h) in control and experimental groups.............................................................................115
Figure 4.31: IL-6 immunostaining in the jejunum of all groups..................................................116
Figure 4.32: Changes in IL-6 immunostaining in the colon (A) apical and (B) basal regions at (6, 48 and 96 h) in control and experimental groups........................................................................117
Figure 4.33: IL-6 immunostaining in the colon for all groups..................................................118
Figure 4.34: MPO immunostaining in the colon at 6, 48 and 96 h............................................120
Figure 4.35: MCP-1 immunostaining in the colon at 6, 48 and 96 h........................................121
Figure 5.1: IEC-6 treated with different concentrations of SN-38 to detect 50% of cell inhibition by XTT assay.........................................................................................................................141
Figure 5.2: IEC-6 treated with different concentrations of SN-38 to detect 50% of cell inhibition by trypan blue method............................................................................................141
Figure 5.3: Effect of AMI on cell viability following SN-38 treatment (24 h) in the IEC-6 cell line by the XTT assay..............................................................................................................143
Figure 5.4: Effect of AMI on cell viability following SN-38 treatment (24 h) in the IEC-6 cell line by the trypan blue method................................................................................................143
Figure 5.5: TLR4 mRNA expression in IEC cells after treatment with AMI (24 h) at different concentrations..................................................................................................................145
Figure 5.6: TLR2 mRNA expression in IEC cells after treatment with AMI (24 h) at different concentrations........................................................................................................................145
Figure 5.7: mRNA expression of (A) TLR2 and (B) TLR4 in all treated groups.......................146
Figure 5.8: mRNA expression of (A) IL-1β, (B) TNF and (C) IL-6 in all treated groups...........147
List of tables:

Table 2.1: Primers sequences and characteristics.................................................................31
Table 3.1: Correlation between mRNA and protein expression using Spearman rank test in the jejunum and colon of DA rats treated with irinotecan-induced GIM.................................55
Table 4.1: Scoring of distress symptoms.............................................................................70
Table 4.2: Antibodies used in this study.............................................................................73
Table 4.3: PCR array results comparing CPT-11+AMI group to CPT-11 group.....................123
List of abbreviations:

Abstract

Gastrointestinal mucositis (GIM) has become increasingly recognised as a major toxicity of cancer treatment. The efficacy and safe use of irinotecan (a topoisomerase I inhibitor chemotherapeutic drug) is compromised because of GIM. Severe GIM often necessitates dose reduction or treatment discontinuation thus compromising patient survival. In rat studies, irinotecan has been shown to cause apoptosis, histological damage, inflammation and activation of signalling pathways. These signalling pathways include nuclear factor kappa B (NF-κB), tumour necrosis factor (TNF)/stress and toll-like receptors (TLR). The stimulation of TLRs can lead to early or late NF-κB activation, which up-regulates many genes involved in the development of GIM, including pro-inflammatory cytokines. Despite extensive research in this area, there is currently no clinically therapeutic intervention to prevent GIM development following irinotecan administration. Therefore, research focusing on designing therapeutic strategies targeted to specific pathological pathways is greatly needed.

In the light of newly emerging roles in inflammatory diseases for TLR2 and TLR4, these receptors have gained significant attention in the development of GIM. TLRs play a role in NF-κB regulation, pro-inflammatory cytokine activation, intestinal inflammation, and regulation of proliferation and apoptosis. Given that these cellular events are key characteristics of GIM, it is suggested that they may be central mediators of the injury process. Recently, it was reported that TLR2 and TLR9 are involved in doxorubicin-induced GIM, and TLR4 is involved in 5-fluorouracil- and methotrexate-induced GIM, providing further evidence as targets for intervention.

As such, the overarching objective of this PhD project was to investigate the involvement of TLR2, TLR4 and pro-inflammatory cytokines in irinotecan-induced GIM and the effect of their inhibition, by the antidepressant drug amitriptyline (AMI), on the development of GIM.

The first study of this research project investigated the involvement of TLR2, TLR4 and pro-inflammatory cytokines in irinotecan-induced diarrhoea. TLR2, TLR4, interlukin-1 beta (IL-1β),
TNF and interleukin-6 (IL-6) mRNA and protein expression was investigated in the colon and jejunum of Dark Agouti rats treated with irinotecan (200 mg/kg intraperitoneally). The expression of each marker (at 72 and 96 h) was compared between rats that developed diarrhoea and rats that did not develop diarrhoea following treatment. These two time points have shown to present maximum damage severity and diarrhoea occurrence, respectively, following irinotecan administration in our rat model. Results showed that mRNA expression of TLR2, TLR4, IL-1β and TNF increased significantly in the colon of rats that developed diarrhoea at 96 h compared to rats that did not develop diarrhoea. TLR2, TLR4 and IL-1β protein expression significantly increased in the apical region of the colonic crypts of the same group compared to the control. This indicated a strong relationship between these inflammatory mediators and severity of mucosal injury.

The second study of this project investigated the effect of AMI, a TLR2 and TLR4 inhibitor, in irinotecan-induced GIM. Clinical markers, histological changes, gene expression, and inflammation were compared between Wistar rats treated with irinotecan (125 mg/kg intraperitoneally, administered at 0 h), and combination of irinotecan and AMI (20 mg/kg intraperitoneally; administered at -24, -16 h and 0 h). Rats were then killed at 6, 48 and 96 h post treatment. Results showed that AMI reduced early-onset diarrhoea and colonic epithelial apoptosis in the colon at 6 h post treatment. However, rats were not protected against weight loss, histological damage, distress symptoms, inflammation and late-onset diarrhoea. PCR array analysis showed a significant decrease in caspase-4, IL-1β and IL-1 receptor 2 and increase in interferon γ receptor 1 (INFγ) mRNA expression in rats treated with irinotecan and AMI compared to rats treated with irinotecan alone. AMI was not able to protect rats against GIM but had protective effect from early-onset diarrhoea and anti-apoptotic effects. The study limitation was the increase in toxic symptoms after treatment with both drugs compared to irinotecan alone. This could be related to the ability of AMI to alter irinotecan metabolism through inhibiting the detoxification of the active metabolite SN-38 thus increasing the cytotoxicity of irinotecan. This could explain partially the histological damage, distress symptoms, inflammation and late diarrhoea occurrence after treatment with both drugs.

The third study investigated the ability of AMI to inhibit apoptosis induced by SN-38, the active metabolite of irinotecan, in a rat intestinal epithelial cell line, IEC-6. Cells were treated for 24 h with SN-38 (IC_{50} of apoptosis = 8.7 μM) alone or in combination with AMI (1 μM). At this dose, AMI inhibits TLR2 and TLR4 and is within the therapeutic range. Apoptosis and mRNA
expression of TLR2, TLR4 and pro-inflammatory cytokines were investigated. Results showed that treatment with SN-38 was associated with increased mRNA expression of TLR2, TLR4 and pro-inflammatory cytokines compared to AMI treated cells. It also showed that treatment with AMI attenuated apoptosis when administered with SN-38. Treatment with AMI and SN-38 was associated with significant reduction in mRNA expression of TLR2, TLR4 and IL-1β compared to SN-38 treated cells. However, TNF expression increased after treatment with both drugs compared to SN-38 treated cells, suggesting and that an alternative pathway to TLRs is activated which leads to TNF upregulation in IEC-6 cells.

In conclusion, this thesis provides an overview of the involvement of TLR2 and TLR4 in irinotecan-induced GIM and the effect of AMI on intestinal injury. Despite the use of AMI for neuropathic and cancer pain, drug interactions should be considered for chemotherapeutic treatment safety and efficacy. Furthermore, the role of TLR2 and TLR4 should be investigated by other specific inhibitors to avoid the “off target” effects of AMI. Findings may then be translated for effective prevention of GIM occurrence clinically.
Acknowledgment

My utter gratitude is to Allah almighty for the blessing and guidance during difficult times.

Limitless thanks go to my principal supervisor, Dr. Joanne Bowen who did not reserve any capacity to help, guide, encourage and support me to complete this work.

I extend my thanks to my co-supervisors, Prof. Richard Logan and Dr. Janet Coller for giving me the opportunity to undertake my PhD, providing constructive feedback and reading my drafts.

Thanks to Dr. Andrea Stringer for her help with laboratory techniques, guidance, support and encouragement to complete this work.

In addition, I thank all members of the Mucositis Research Group, Rachel Gibson, Emma Bateman, Erin Plews, Bronwen Mayo, Imogen White and Wan Nor I’zzah for their help over the years.

I must also acknowledge the support that I received from Ministry of Higher Education in Saudi Arabia for sponsoring my study.

Special thanks to my parents for their prayers, encouragement, support, belief and allowing me to pursue my dream as long as I can.

Thanks must go to all my family and friends, Ruba, Nameen, Dur and Nour, for their support and encouragement.

Finally, special thanks to my supportive lovely husband Akram Qutob. Without his help, support, and encouragement, I couldn’t have done this work. My daughters, Maryam, Malak and Talah, without their love, smiles, I would not have the determination to go through my postgraduate study in Australia.
This thesis is composed of seven chapters as follows: literature review, general methods, three distinct research chapters, general discussion, references and appendix. Each research chapter was written with introduction, material and methods, results and discussion, all references are included in the final chapter.