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

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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 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.
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Thesis explanation

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.
Chapter One
1.0 Literature review

1.1 Introduction

One of the most common dose-limiting side effects associated with chemotherapy and radiation is damage to the alimentary tract mucosa, known clinically as gastrointestinal mucositis (GIM). GIM is ulceration and inflammation of the mucosal membranes of the mouth and/or the gastrointestinal tract due to chemical irritation in response to cytotoxic agents used during cancer treatment. Clinical symptoms include mouth and throat ulcers, abdominal pain, nausea, vomiting, rectal bleeding and diarrhoea (Keefe et al., 2007). After treatment with standard dose chemotherapy, approximately 40% of all patients and up to 100% of patients undergoing high-dose chemotherapy with bone marrow or stem cell transplantation reportedly develop GIM (Keefe et al., 1997; Keefe et al., 2000). When chemotherapeutic drugs damage the GI mucosa, malabsorption and malnutrition can occur, thus increasing the use of healthcare resources, such as fluid replacement, liquid diet, and total parenteral nutrition (Keefe et al., 2004). These patients are at risk of treatment reduction or discontinuation which may result in compromised survival outcomes. GIM adds substantial burden on the medical care required for cancer patients, increases healthcare costs and reduces patient quality of life (Elting et al., 2007).

Many drugs have been reported to cause GIM, including 5-fluorouracil, methotrexate, capecitabine and irinotecan hydrochloride (Rask et al., 1998; Cheng and Chang, 2003). Irinotecan hydrochloride (camptothecin-11, CPT-11) is among the chemotherapeutic drugs that are well known to cause GIM. Treatment with this drug has been demonstrated to increase the risk of developing GIM up to 60%, depending on the treatment regimen (Borner et al., 2005). Accordingly, diarrhoea is an increasingly recognised side effect associated with this drug. Treatment options for patients with GIM are limited, and prevention requires an understanding of the pathophysiological mechanisms underlying GIM development.

In the last decade, significant progress has been made in understanding the pathobiology of GIM. Currently, the pathobiology of GIM comprises five overlapping phases and in each phase, several signalling pathways are activated (Bowen et al., 2007b). Nuclear factor kappa B (NF-κB) and toll-like receptor (TLR) signalling are among the activated pathways after irinotecan administration.
and may be targetable by pharmacological inhibitors. It has been demonstrated, in our laboratory, that NF-κB, pro-inflammatory cytokines (Logan et al., 2008a; Logan et al., 2008b), apoptosis (Bowen et al., 2007a), pathogenic bacteria (Stringer et al., 2009a), inflammation (Al-Dasooqi et al., 2010) and loss of mucosal barrier integrity (Al-Dasooqi et al., 2011a) are among the main drivers of irinotecan-induced GIM. Activation of TLR signalling can lead to NF-κB activation, pro-inflammatory cytokine production, apoptosis and increased inflammation, all known to be involved in GIM, thus indicating potential TLR involvement in irinotecan-induced GIM.

TLRs belong to a family of pattern recognition receptors that play a role in innate and adaptive immunity and maintain gut homeostasis. Of specific interest, TLR2 and TLR4 have been shown to control gut homeostasis and to be involved in intestinal inflammation, proliferation, and inflammatory diseases (Fukata et al., 2006; Cario et al., 2007; Fukata and Abreu, 2007; Cario, 2008). TLR2 and TLR4 recognise invading pathogens and endogenous danger signals, resulting in the activation of their downstream signalling pathways. It has been proposed that TLR activation in response to danger molecules released following chemotherapy, such as high-mobility group box 1 (HMGB1) and heat shock proteins (HSPs), and products of gut pathogenic bacteria, such as lipopolysaccharide (LPS), results in the up-regulation of pro-inflammatory cytokines involved in GIM development. Recent evidence has highlighted the involvement of TLR2, TLR4 and TLR9 in chemotherapy-induced GIM (Ferreira et al., 2012; Kaczmarek et al., 2012; Hamada et al., 2013). However, to date, the role of TLRs in irinotecan-induced GIM has not been investigated specifically.

This review covers the mechanisms underlying irinotecan-induced GIM and the possible involvement of TLR2 and TLR4. Moreover, this review discusses the effects of TLR2 and TLR4 inhibition using a novel approach in irinotecan-induced GIM.
1.2 Chemotherapy-induced gastrointestinal mucositis (GIM)

1.2.1 Irinotecan

Irinotecan is classified as a plant alkaloid and topoisomerase I inhibitor. It is the hydrochloride salt of a semisynthetic derivative of camptothecin, a cytotoxic, quinoline-based alkaloid extracted from the Asian tree *Camptotheca acuminata*. It was licensed to Pfizer under the trade names Camptosar®, Camptothecin-11 and Campto®. It first received the United States Food and Drug Administration approval in 1996 for treatment of metastatic colorectal carcinoma (Berg, 1998). It is a prodrug that is converted to the active metabolite 7-Ethyl-10-hydroxycamptothecin (SN-38). SN-38 inhibits topoisomerase I activity by stabilising the cleavable complex between topoisomerase I and DNA, resulting in DNA breaks that inhibit DNA replication and trigger apoptotic cell death. As such, irinotecan, potently inhibits DNA replication, activating DNA damage responses and transient S-phase arrest (Pommier et al., 1998). Irinotecan is approved for the treatment of colorectal, lung, cervical and ovarian cancers (Rosen, 1998; Prados et al., 2004). The main side effects are neutropenia and diarrhoea. Approximately 60 to 80% of patients develop diarrhoea after treatment with this drug, depending on the treatment regimen (Borner et al., 2005). Hence, it is crucial to understand the mechanisms underlying irinotecan-induced GIM to help develop a better therapeutic plan. The following sections cover the metabolism of the drug, the known mechanisms of irinotecan-induced apoptosis, and the published research on how it can cause diarrhoea and mucosal injury.

1.2.2 Irinotecan metabolism

The metabolism and the pharmacokinetics of irinotecan are well characterised. Hepatic metabolism and biliary secretion are major pathways in irinotecan elimination and involve various enzymes, including esterases, UDP-glucuronosyltransferase (UGT1A1, UGT1A7 and UGT1A9) (Tallman et al., 2005), cytochrome P450 (CYP3A4 and CYP3A5) and β-glucuronidase. Irinotecan is converted by carboxylesterase (CES1, CES2 and CES3) to the active metabolite SN-38 (Santos et al., 2000; Mathijssen et al., 2001). Previous studies have demonstrated no significant differences in the activity of carboxylesterase enzyme between liver and colon tissues and that SN-38 itself is converted in the liver and intestine to its inactive form SN-38 glucuronide (SN-38G) by UGT1A1 (Guichard et al., 1999; Chen et al., 2013). UGT1A1 is one of the most important enzymes involved
in irinotecan metabolism, although other isoforms such as UGT1A7, UGT1A6, and UGT1A9 have been found to be important as well (Ciotti et al., 1999; Hanioka et al., 2001). The liver is the major site of glucuronidation. However, UGT1A1 is also expressed in extrahepatic tissues, including the gastrointestinal tract (Chen et al., 2013). Deletion of the UGT1 locus in the intestine has been found to increase susceptibility to irinotecan-induced diarrhoea and mortality, as a result of increased apoptosis and inflammation in the intestinal tract (Chen et al., 2013). Therefore, intestinal expression of UGT1A in the intestinal tract appears to be crucial for irinotecan detoxification.

Other important metabolites of irinotecan that are converted by CYP3A4 include 7-ethyl-10-[4-N(5-aminopentanoic acid)-1-piperidino]-carbonyloxy-camptothecin (APC) and 7-ethyl-10-(4-amino-1-piperidino)-carbonyloxy-camptothecin (NPC) (Santos et al., 2000). NPC can in turn be hydrolysed to SN-38 (Dodds et al., 1998). When SN-38G is excreted into the bile and subsequently enters the intestinal lumen, it can be converted back to SN-38 by β-glucuronidase produced by intestinal bacteria, such as *Escherichia coli*, *Bacteroides* species and *Clostridium perfringens* (Stringer et al., 2009a; Takakura et al., 2012). Recent findings using bacterial β-glucuronidase inhibitors suggested that this enzyme and the enterohepatic recirculation play an important role in late diarrhoea occurrence after treatment with irinotecan (Takasuna et al., 2006; Wallace et al., 2010; Roberts et al., 2013). A strong correlation has been reported between histological damage, diarrhoea and β-glucuronidase activity in the intestinal lumen whereas the correlation with intestinal tissue carboxylesterase activity was weaker in rat (Takasuna et al., 1996).

Biliary secretion is the most important elimination pathway for irinotecan and SN-38. ABCB1 and ABCG2 are two of the several ATP-binding cassette efflux transporters involved in irinotecan and SN-38 excretion (Iyer et al., 2002; Bansal et al., 2008; Shishido et al., 2013). Through enterohepatic recirculation, the gallbladder periodically releases SN-38 and/or SN-38G into the intestine where it is reabsorbed into the plasma thus leading to increased toxicity (Takakura et al., 2012). Irinotecan can be excreted in faeces and urine; SN-38 and NPC are mainly secreted in the faeces. In urine, SN-38G and APC are the main compounds detected 24 hours after drug administration (Sparreboom et al., 1998). The predominant excretion route of irinotecan is through faeces (64%) then followed by urine (32%) (Smith et al., 2006) which shows that the major toxic effect of irinotecan is in the gastrointestinal tract.
1.2.3 Irinotecan-induced apoptosis

Induction of apoptosis in malignant cells is a crucial pathway activated by chemotherapy treatment (Holden, 2001; Okada and Mak, 2004). However, normal epithelial cell apoptosis has long been recognised as one of the hallmarks of chemotherapy treatment and the development of GIM (Ijiri and Potten, 1983). Irinotecan induces apoptosis in a dose-dependent manner in both tumour and normal cells (Bowen et al., 2007a). Even though apoptosis in cancer cells has been reported following irinotecan treatment, the mechanism underlying apoptosis in normal tissues has not yet been fully elucidated. In response to DNA damage, many signalling pathways are activated. One of the sensor proteins recruited to the site of DNA damage is ataxia telangiectasia mutated (ATM) (Rudolf et al., 2012; Origanti et al., 2013). This subsequently results in activation of other signalling molecules, such as checkpoint kinase 1 (Chk1) and Chk2, which arrest cell cycle progression to allow for repair or to prevent the transmission of damaged chromosomes. ATM phosphorylation leads to rapid Chk1 phosphorylation. The latter leads to p53 phosphorylation that may result in either apoptosis, through pro-apoptotic proteins of the Bcl-2 family (including Bax, Bak, Noxa and Puma) or permanent cell cycle arrest, through p21 and 14-3-3σ. Rudolf et al. (2012) reported that irinotecan induces apoptosis and premature senescence depending on the cell type and varying activity of the stress kinases, including p38 kinase, and the contribution of selected microRNAs (Rudolf et al., 2012). In normal colonic epithelial cells, apoptosis occurs as a result of up-regulation of p53, which elevates p38 mitogen-activated protein kinases (p38MAPK), consequently initiating mitochondrial caspase-dependent apoptosis (Rudolf et al., 2012). In colonic fibroblast cells, however, premature senescence occurs after increased levels of p16 and lower activity of p38. Moreover, treatment of rat intestinal epithelial cells (IEC-6) with irinotecan has been demonstrated to cause a marked increase in the pro-apoptotic proteins Bax and Bak and to reduce the expression of the anti-apoptotic protein myeloid cell leukaemia sequence 1 (Mcl-1) (Bowen et al., 2007a) (Figure 1.1).
Figure 1.1: Irinotecan-induced apoptosis. The proposed cell injury pathways activated by irinotecan in normal colonic epithelial cells. DNA damage induced by irinotecan is sensed by ATM, which is phosphorylated and activates Chk1. Consequently, p53 will be phosphorylated and activate p38 MAPK mediating mitochondrial pathway caspase-dependent apoptosis.
1.2.4 Irinotecan-induced diarrhoea

Diarrhoea is one of the major complications associated with irinotecan treatment. Irinotecan induces two types of diarrhoea; early-onset diarrhoea, which is a cholinergic reaction to the drug that includes lacrimation, increased salivation, flushing, rhinitis and intestinal hyperperistalsis and can be prevented by atropine (Hyatt et al., 2005). Late onset diarrhoea occurs 24 h after drug administration (Gibson et al., 2003; Stein et al., 2010). It occurs in up to 80% of cancer patients, and is severe (National Cancer Institute (NCI) grade 3 and 4) in approximately 30 - 40% of patients (Rougier et al., 1997; Glimelius, 2005). Up to date, there are no preventative interventions that effectively reduce late onset diarrhoea. Different prophylactic and curative agents have been investigated in animal models and clinical trials. Investigated therapies include anti-diarrhoeal agents, such as loperamide, octreotide, acetorphan and budesonide (Saliba et al., 1998; Prados et al., 2004; Karthaus et al., 2005; Hoff et al., 2014), transporter enzyme inhibition, such as cyclosporine (Chester et al., 2003), alteration of intestinal bacteria using probiotics (Bowen et al., 2007c; Ooi et al., 2008) or antibiotics (Flieger et al., 2007), activated charcoal (Sergio et al., 2008), cytokines inhibitors, such as thalidomide and pentoxifylline (Melo et al., 2008) and other miscellaneous agents such as glutamine (Pan et al., 2005; Xue et al., 2008). However, results were not satisfactory for any agent, each with their own limitations. The current management guidelines recommended by the Multinational Association of Supportive Care in Cancer and International Society of Oral Oncology (MASCC/ISOO) for diarrhoea induced by standard or high dose chemotherapy is using octreotide as a second line if loperamide is ineffective (Lalla et al., 2014). The lack of effective interventions for diarrhoea induced by irinotecan could be a result of the complexity of irinotecan metabolism and the mechanisms behind diarrhoea occurrence. Several studies aimed at determining the mechanisms behind late diarrhoea occurrence have been conducted in the rat model of irinotecan-induced GIM (Gibson et al., 2003; Stringer et al., 2007b; Logan et al., 2008a; Stringer et al., 2009b; Al-Dasoqui et al., 2011a; Nakao et al., 2012). Direct intestinal damage is induced by irinotecan/SN-38, including increased apoptosis, accelerated levels of mucous secretion, changes in extracellular matrix components, and reductions in tight junction proteins, such as claudin-1 and occludin, which all result in loss of mucosal barrier integrity and is accompanied with water and electrolyte malabsorption.
1.2.5 The role of intestinal microbiome in irinotecan-induced GIM

Intestinal microflora also plays an important role in late diarrhoea occurrence (Brandi et al., 2006). Following irinotecan treatment, the proportion of intestinal bacteria is altered with increases in β-glucuronidase producing bacteria (Stringer et al., 2009a; Takakura et al., 2012). Evidence suggests that bacterial β-glucuronidase deconjugates SN-38G to SN-38, thereby exacerbating intestinal damage and diarrhoea severity (Takasuna et al., 1998; Fittkau et al., 2004; Stringer et al., 2009a). Alteration in intestinal bacteria has been reported to be associated with changes in goblet cells, excessive mucous secretion, and decreases in mucin gene expression (Muc2 and Muc4), all which may contribute to diarrhoea development (Stringer et al., 2009b). Bacterial β-glucuronidase plays an important role in diarrhoea occurrence following irinotecan treatment (Stringer et al., 2007a; Stringer et al., 2007b). This is supported by research showing that germ-free mice tend to be more resistant to irinotecan-induced GIM compared to conventional mice (Brandi et al., 2006). Moreover, inhibition of β-glucuronidase activity by antibiotics (penicillin and streptomycin) has been shown to be protective against irinotecan-induced diarrhoea (Takasuna et al., 1996). In the intestine, β-glucuronidase is produced primarily by Enterobacteriaceae, such as Escherichia coli (E. coli), Salmonella spp., Shigella spp. and Yersinia spp (Stringer et al., 2007a; Stringer et al., 2007b). Modification in the intestinal flora, especially E. coli, following treatment with irinotecan was found to be associated with increased expression of β-glucuronidase and diarrhoea in female Dark Agouti (DA) rats (Stringer et al., 2009a). After irinotecan treatment, the activity of β-glucuronidase in the jejunum was lower than the colon, likely related to the differences in the microflora between the two regions (Takasuna et al., 1996). Moreover, the relatively low level of damage in the small intestine compared to that in the colon could be associated with the lower exposure of the tissues to SN-38 or to the lower amount of β-glucuronidase in the lumen of the jejunum. These results strongly suggest that intestinal microflora play a role in the pathobiology of GIM. Given that one of the fundamental roles of TLRs is in microbial sensing (Brown et al., 2011), it is likely that altered microbial populations would have an impact on TLR signalling and therefore GIM development.
1.2.6 Irinotecan-induced morphological and histological damage

In addition to apoptosis and diarrhoea associated with irinotecan, other morphological and histological changes in the intestinal mucosa have been characterised using the same rat model of irinotecan-induced mucositis (Gibson et al., 2003; Logan et al., 2008a). In DA rats, irinotecan treatment was associated with villus blunting, epithelial atrophy and increased intensity of inflammation in jejunal mucosa. Damage in the colon peaked at 96 h and was characterised by complete crypt ablation, attenuated epithelium and polymorphonuclear cell infiltrate in the lamina propria. Moreover, crypt hyperplasia and increased mitotic figures were evident at later time points. Reduction in goblet cells and increases in mucous secretion were noted early following treatment (Stringer et al., 2009a). In this model, tissue restitution was initiated at 120 h following treatment. In addition, in mice treated with irinotecan, intestinal mucosal damage was associated with increased protein expression of TNF, IL-1β, IL-18, neutrophil infiltration measured by myeloperoxidase (MPO) activity, and increased inducible nitric oxide (iNOS) activity (Lima-Junior et al., 2012; Lima-Junior et al., 2014). The inflammatory reaction induced following irinotecan treatment modestly increased intestinal muscle contractility in response to acetylcholine in vitro even though the exact mechanism is still unclear (Lima-Junior et al., 2014). This suggests that inflammation induced by irinotecan plays a role in intestinal hyper-contractility and diarrhoea even though the exact mechanism needs to be investigated. Moreover, histological damage was found to be associated with tight junction defects, evident by deceased mRNA expression of occludin-1, claudin-1 and zona occludin-1 (ZO-1) which indicates an association between barrier injury and diarrhoea occurrence (Nakao et al., 2012; Wardill et al., 2013). Altering the barrier permeability increases the risk of bacterial translocation which stimulates the innate immune system, namely TLRs.

1.3 TLRs

The TLR family is a major class of transmembrane pattern recognition receptors capable of detecting pathogen-associated molecular patterns (PAMPs), including bacterial and viral products. TLRs also respond to damage-associated molecular patterns (DAMPs), including endogenous intracellular molecules released by activated or necrotic cells and extracellular matrix components (Brown et al., 2011). Each TLR shows a different expression pattern, extra- or intra-cellular localisation and signalling pathway, which is involved in the pathogenesis of different diseases.
Upon recognition, TLRs can activate inflammatory cytokines, co-stimulatory molecules, chemokines and type I interferons. They are expressed by a number of cell types throughout the gastrointestinal tract, including immune cells, such as polymorphonuclear neutrophils, monocytes, and dendritic cells, and non-immune cells, such as fibroblasts, endothelial, and epithelial cells (Abreu et al., 2002). Therefore, they are important for microbial recognition, innate and adaptive immunity, gut homeostasis, inflammation, inflammatory diseases and other cell responses. Up to date, 13 TLR members have been identified in human and 9 in rat (Kawai and Akira, 2010). Among the known TLR members, TLR1 to TLR9 have been identified in human intestinal epithelial cells (Otte et al., 2004).

TLRs are differentially distributed along the alimentary tract. TLR2 has been detected in the brush border of villus epithelial cells in the jejunum (Mantani et al., 2011), whereas TLR4 and TLR9 have been reported in the duodenum and all TLRs can be found in the intestinal crypts through the small intestine. Moreover, differential expression of TLR2 and TLR4 has been reported in the colon, and found to be associated with distributional differences in microbial population. In specific-pathogen free mice, but not in germ free mice, TLR2 was reportedly more highly expressed in the proximal colon, with its expression decreased gradually toward the distal colon, whereas TLR4 is conversely expressed highly in the distal colon and shows gradually decreased expression toward the proximal colon (Wang et al., 2010). The same study also reported that mucosa-associated microbiota was found to be significantly different between proximal and distal colon, which supports the role of microbiota in regional specific expression of TLR2 and TLR4 (Wang et al., 2010). Hence, even though TLRs are expressed in the intestine, their expression varies along the alimentary tract, which may be in response to differences in mucosa-associated microbiota along the alimentary tract.

In healthy intestine, epithelial cells coexist with commensal bacteria without initiation of inflammatory responses. However, the exact mechanism of tolerance has not been fully understood. Under stress conditions, enterocytes respond to TLR stimulation which leads to pro-inflammatory cytokine release (Gribar et al., 2008). This results in enterocyte apoptosis, reduced healing and reduced proliferation, thus promoting bacterial translocation and development of intestinal inflammation (Gribar et al., 2008). Since TLR2 and TLR4 have been shown to be involved in apoptosis, homeostasis, intestinal inflammation, and inflammatory bowel diseases, they are the focus of this review. Both of these TLRs have been previously shown to be involved...
in chemotherapy-induced GIM (Ferreira et al., 2012; Kaczmarek et al., 2012; Hamada et al., 2013), suggesting that they may be involved in irinotecan-induced GIM as well.

### 1.3.1 TLR2 and TLR4

Both TLR2 and TLR4 are extracellular receptors and recognise PAMPs and DAMPs. TLR2 senses exogenous ligands such as lipoproteins, peptidoglycans, and lipoteichoic acids. TLR4 agonists include LPS (Gay and Gangloff, 2007). Both TLR2 and TLR4 also respond to endogenous ligands, such as HSP70 and HMGB1 (Gay and Gangloff, 2007).

Upon stimulation, TLR2 forms a heterodimer with either TLR1 or TLR6, which act as co-receptors for recognising the molecular structure of the ligands and which are essential for downstream signalling (Brown et al., 2011). TLR2 activation can lead to two different signalling pathways, a myeloid differentiation primary response gene 88 (MyD88)-dependent pathway and a phosphatidylinositol 3-kinase (PI3K)-dependent pathway. TLR2 heterodimers bind to MyD88 and MyD88 adapter-like (Mal or TIRAP). This results in the formation of a complex of interleukin-1 receptor-associated kinases (IRAKs) and TNF receptor associated factor (TRAF6). TRAF6 ubiquitination activates a complex of transforming growth factor β activated kinase-1 (TAK1), TAK1 binding protein-1 (TAB1), and TAB2/3, resulting in the activation of the IκB kinase (IKK) complex. Subsequently, NF-κB translocates to the nucleus where it drives the transcription of cytokine genes (Gay and Gangloff, 2007; Takeuchi and Akira, 2010; Brown et al., 2011). The TAK1/IKK complex can also activate MAP kinases, Jun N-terminal kinase (JNK) and p38MAPK, leading to c-Jun and c-Fos activation to trigger inflammatory cytokine gene transcription (Figure 1.2).

TLR2 stimulation can also lead to activation of the PI3K pathway. After TLR2 activation, PI3K is recruited to convert phosphatidylinositol 4,5-bisphosphate (PIP2) to PIP3, which leads to Akt activation. Activated Akt results in the inactivation of glycogen synthase kinase-3 (GSK-3), which differentially controls the production of pro-inflammatory cytokines (Brown et al., 2011). It was reported that the inhibition of the PI3K pathway enhanced LPS-induced TNF, NF-κB and JNK activation (Guha and Mackman, 2002). Through the PI3K pathway, TLR2 can enhance intestinal
tight junction barrier integrity. Stimulation of the TLR2/PI3K/Akt pathway was found to be able to modulate intestinal permeability in Caco-2 cells via intestinal trefoil factor 3 (TFF3) (Lin et al., 2013). TFF3 is a peptide that is involved in wound healing and mucosal protection. It plays a role in stabilising tight junctions by increasing claudin-1 and promoting the distribution of ZO-1 (Lin et al., 2013). TLR2 activation by TFF3 has been demonstrated to reduce IL-1β-induced increase in permeability and release of pro-inflammatory cytokines (Lin et al., 2013). The TLR2 agonist, Pam3CysSK4, has been shown to increase occludin in tight junctions in vitro (Karczewski et al., 2010). Therefore, depending on the activated pathway, TLR2 can have either protective or injurious effects. Recent evidence has shown that TLR4 activation involves the PI3K pathway as well (Kim et al., 2012).
Figure 1.2: TLR2 signalling pathways (MyD88-dependant and PI3K-dependent). TLR2 forms a heterodimer with either TLR1 or TLR6. TLR2 activation can lead to MyD88-dependent or PI3K-dependent pathways. In the MyD88-dependent pathway, MyD88 binds to MAL/TIRAP. This is followed by IRK1/IRAK4/TRAF6 activation and results in TAK1/TAB1/2/3/IKK activation complex. Consequently, NF-κB translocates to the nucleus and activates pro-inflammatory cytokines. In the PI3K-dependent pathway, PI3K can generate PIP3, which leads to Akt activation. Akt inhibits GSK3, resulting in increased expression of IL-10 and decrease in pro-inflammatory cytokines. Through FADD pathway, TLR2 can induce apoptosis.
TLR4 signalling activates two pathways: the MyD88-dependent pathway, which is similar to TLR2 MyD88-dependent pathway described above and the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent pathway. In the TRIF-dependant pathway (also called MyD88-independent pathway), TLR4 recruits TRAM to activate TRIF, resulting in TRIF being able to bind to TRAF3 and TRAF6 or with receptor (TNFRSF)-interacting serine-threonine kinase 1 (RIP), subsequently leading to activation of NF-κB. TRIF leads to interferon regulatory transcription factor 3 (IRF3) activation by IKK and TANK-binding kinase 1 (TBK1) stimulation, which leads to the production of type 1 interferons (IFN) and the anti-inflammatory cytokine IL-10 (Brown et al., 2011). It has been suggested that TRAF3 differentially controls the MyD88- and TRIF-dependent pathways (Tseng et al., 2010). Although the degradation of TRAF3 is required for the production of MAPK and inflammatory cytokines in the MyD88-dependent pathway, it is not required for type 1 IFN activation in the TRIF-dependent pathway (Figure 1.3).

TLR4 stimulation in response to endogenous or exogenous molecules has been implicated in intestinal injury. Robust evidence exists to show a link between TLR4 stimulation by intestinal microbiota and mucosal damage. LPS (also known as endotoxin), a gram negative bacterial product, is the most studied TLR4 ligand (Kessel et al., 2008; Li et al., 2011; Richter et al., 2012). LPS activation of TLR4 can impair gut integrity and leads to bacterial and endotoxin translocation across the intestinal mucosa (Kessel et al., 2008). LPS-induced endotoxaemia has been found to cause reduction in the mucosal weight, enterocyte proliferation and increased apoptosis in male Sprague-Dawley rats treated with LPS (Kessel et al., 2008). This was associated with increased mRNA expression of TLR4, MyD88 and TRAF6 in the mucosal ileum. Previous evidence has shown that inhibiting LPS-induced TLR4 stimulation can attenuate intestinal injury in animal models. Reduction in TLR4, MyD88 and TRAF6 protein expression, by glutamine treatment, was associated with a significant reduction in irinotecan-induced diarrhoea occurrence and severity, β-glucuronidase activity and increased intestinal weight (Xue et al., 2008). In another study using glutamine, reduction in TLR4, TRAF6 and MyD88 expression and mucosal injury was observed in rats treated with LPS (Kessel et al., 2008). Inhibition of the TLR4/NF-κB/macrophage inflammatory protein-2 (MIP-2) pathway, by berberine, shows a protective effect from LPS-induced intestinal injury in mice, likely by reducing enterocyte apoptosis and neutrophil infiltration (Li et al., 2011). LPS administration to IEC-6 cells has been found to impair wound healing in a dose-dependent manner (Richter et al., 2012). Accordingly, reductions in TLR4
mRNA expression, achieved by adding LPS binding protein (LBP), were found to improve cell restitution despite exposure to LPS. Therefore, it can be concluded that TLR4 stimulation by LPS results in increased inflammation, and apoptosis and reduced proliferation and healing, which are all important features of GIM.

In seeking to identify the DAMPs involved in TLR2- and TLR4-mediated intestinal injury, HSP70 and HMGB1 were among the ligands found to stimulate both TLRs and can be activated in response to irinotecan treatment (Piccinini and Midwood, 2010). HSP70 and HMGB1 were found to be up-regulated following irinotecan administration in colorectal tumour cells (Frey et al., 2012). It is possible that they can be increased in normal cells as well. HSP70 has been shown to activate TLR2 and TLR4 and to induce MyD88-dependent signalling (Asea et al., 2002; Zhou et al., 2005). This suggests that TLR2 and TLR4 might be associated with irinotecan-induced GIM via HMGB1 and HSP70 stimulation.
Figure 1.3: TLR4 signalling pathways (MyD88-dependant and –independent pathways). In the MyD88-dependent pathway, MyD88 binds to MAL/TIRAP. This is followed by IRK1/IRAK4/TRAF6 activation and results in TAK1/TAB1/2/3/IKK activation complex. Consequently, NF-κB translocates to the nucleus and activates pro-inflammatory cytokines. In the TRIF-dependent pathway, TRAM binds to TRIF which leads to TRAF3/TRAF6/RIP1 recruitment. Subsequently, NF-κB will be activated and pro-inflammatory cytokines will be upregulated. Through TRAF3/IRF3, type I INF can be activated.
1.3.2 TLR2 and TLR4 involvement in intestinal inflammation

Recognition of PAMPs and DAMPs by TLRs expressed by intestinal cells is an important component of immunity and is responsible for host-microbial interactions in the mucosa. Any dysregulation of this interaction may result in inflammation within the bowel (Fukata and Arditi, 2013). Substantial evidence indicates the involvement of TLR2 and TLR4 in inflammatory diseases, including Crohn’s disease (CD), irritable bowel syndrome (IBS) and ulcerative colitis (UC) (Cario and Podolsky, 2000; Sanchez-Munoz et al., 2011; Belmonte et al., 2012; Candia et al., 2012). Recently, it was reported that TLR2 and TLR4 mRNA and protein expression is increased significantly in the colonic mucosa of patients with active UC and CD (Brown et al., 2014). In another study, TLR2 and TLR4 mRNA and protein expression were reported to be significantly increased in the colonic mucosa of patients with irritable bowel syndrome (IBS), compared to healthy controls (Belmonte et al., 2012). In addition, several studies have investigated the involvement of TLR2 and TLR4 in animal models of acute colitis. Dong et al (2012) found increased colonic TLR2 and TLR4 expression following dextran sulfate sodium (DSS)-induced colitis and reported that it was associated with an increase in potentially pathogenic *E. coli* and a decrease in beneficial *Lactobacillus spp.* and *Bifidobacterium spp.* (Dong et al., 2012). DSS colitis-induced increase in *E. coli* count was decreased significantly in genetic knock-out mice deficient for TLR2 and/or TLR4 (Heimesaat et al., 2007). In addition, TLR deficiency was associated with a reduced disease activity index, reduced histopathological scoring and an increased count of *Lactobacillus spp.* and *Bifidobacterium spp.* (Dong et al., 2012). Recent research has shown that targeted inhibition of TLR2 and TLR4 has a protective effect from DSS-induced colitis. Inhibiting TLR4, using paeoniflorin, attenuated DSS-induced colitis through a mechanism involving the inhibition of NF-κBp65, JNK and p38MAPK (Zhang et al., 2014). In another study, TLR2 inhibition, using chloroquine, reduced colitis induced by DSS clinically and histologically (Nagar et al., 2014). When using TLR2 and TLR4 monoclonal antibodies, significant reductions in colitis index and histological scoring was reported (Dong et al., 2012). However, other investigators have reported that TLR2, TLR4 and MyD88 deficiency was not protective from clinical symptoms and histological injury in chronic inflammation despite the observed reduction in acute inflammation (Rakoff-Nahoum et al., 2004; Fukata et al., 2005). This suggests that TLR2 and TLR4 play different roles in regard to acute and chronic inflammation. Inhibition of both TLR2 and TLR4 could be protective from acute inflammation but not chronic inflammation.
On the other hand, TLR2 and TLR4 activation could have important protective effects for the intestine. Since TLR2 plays an important role in maintaining intestinal barrier integrity by preserving ZO-1 through a PI3K/Akt pathway (Cario et al., 2004; Lin et al., 2013), disruption of this pathway could possibly increase barrier permeability. Data from both in vitro and in vivo studies showed that TLR2 has protective effects against inflammatory stress-induced damage via TFF3 (Podolsky et al., 2009). In a model of chronic colitis, mice deficient in TLR2 or MyD88 showed severe signs of morbidity and increased mortality, whereas administration of a TLR2 ligand Pam3CysSK4 was found to significantly suppress mucosal inflammation and apoptosis thus restoring tight junction-associated integrity (Cario et al., 2007). This suggests that the entire loss of TLR2 could be harmful and impair commensal-mediated tight junction barrier integrity.

In response to endotoxin, TLR4 up-regulation showed protective effects in response to ethanol-induced gastric injury through cyclooxygenase-2 (COX-2) and prostaglandin E2 (PGE2) (Zhang et al., 2010). Furthermore, TLR4 activation showed protective effects against radiation-induced intestinal injury in rats (Riehl et al., 2012; Lacave-Lapalun et al., 2013). Administration of LPS after low dose colorectal radiation (20-Gy) showed a protective effect, although the effect was not seen following high dose radiation (72-Gy) (Lacave-Lapalun et al., 2013). Similarly, administration of hyaluronic acid, which is a ligand capable of binding to both TLR2 and TLR4, prior to low dose radiation reduced apoptosis and increased crypt survival by increasing COX-2 expression (Riehl et al., 2012). It was suggested that tolerance could be developed in moderate tissue damage by TLR4 activation but not after severe damage (Lacave-Lapalun et al., 2013). Recently, it was reported that both excessive and deficient expression of TLR4 can have injurious effects following ethanol-induced gastric mucosal injury (Ye et al., 2013a). HMGB1 was found to activate a TLR4/MyD88/NF-κB pathway after ethanol treatment. Subsequently, activated NF-κB can enhance the release of HMGB1 where it reactivates TLR4 signalling. It was proposed that TLR4 could play a protective role under homeostatic conditions where it could act to control pathogens that may be toxic to the host. However, under pathogenic conditions, TLR4 activation might lead to detrimental alteration in mucosal tissues (Ye et al., 2013a). Together, these findings suggest that differential cellular responses following TLR activation are disease context and stimuli specific.
1.3.3 TLR2 and TLR4 involvement in cancer therapy-induced GIM

Since diarrhoea is one of the main features of irinotecan-induced GIM, preventative therapy options depend on inhibiting the mechanism behind diarrhoea development. Administration of probiotics is increasingly gaining importance in the treatment of diarrhoea and inflammatory bowel diseases (Dai et al., 2013; Shadnoush et al., 2013). They can be useful for controlling microflora during cancer therapy as well (Bowen et al., 2007c; Ooi et al., 2008; Sezer et al., 2009; Ciorba et al., 2012). The use of probiotics has shown potential as a radioprotective factor in the intestine (Ciorba et al., 2012). The administration of Lactobacillus probiotic prior to radiation significantly improved survival and reduced epithelial apoptosis at the crypt base in C57BL/6 wild-type mice (Ciorba et al., 2012). This was demonstrated to be mediated through aTLR2/MyD88/COX-2 pathway. Treatment with VSL#3 probiotic was found to be effective in preventing irinotecan-induced diarrhoea, reducing weight loss and intestinal apoptosis in DA rats (Bowen et al., 2007c). This suggests that beneficial bacteria may protect the gastrointestinal tract from inflammation by down-regulating TLR expression and activity. As such, interventions targeted at down-regulating TLR4 and TLR2 may be effective in prevention of irinotecan-induced GIM.

In the context of chemotherapy-induced GIM, few studies have investigated the involvement of TLRs directly. Kaczmarek and colleagues (2011) reported that TLR2 deficiency significantly decreased histological damage, leukocytes in the lamina propria and apoptosis in ileal tissue in a model of doxorubicin-induced GIM. They suggested that the protective effects of TLR2 deficiency were mediated through GSK-3β inhibition (Kaczmarek et al., 2012). Ferreira et al (2012) demonstrated that TLR4 mRNA expression increased significantly 72 h after 5-fluorouracil administration in the ileum of mice compared to controls (Ferreira et al., 2012). Hamada and colleagues (2013) reported that mRNA expression of TLR4 and pro-inflammatory cytokines increased in the jejunum of Wistar rats following methotrexate administration (Hamada et al., 2013). Therefore, both TLRs have been shown to be involved in chemotherapy-induced GIM.

In summary, both TLR2 and TLR4 are expressed in the intestinal mucosa, respond to PAMPs and DAMPs, and play roles in innate and adaptive immunity. Both TLRs have been shown to be involved in intestinal inflammation and inflammatory bowel disease. Their inhibition was
protective from intestinal mucosal damage in acute inflammation and was associated with reductions in pathogenic bacteria. Both TLRs have been shown to be involved in chemotherapy-induced GIM. However, what is yet to be determined is TLR2 and TLR4’s relationship to irinotecan-induced GIM.

1.3.4 Pro-inflammatory cytokine involvement in irinotecan-induced GIM

There is substantial evidence that pro-inflammatory cytokines are up-regulated and related to mucosal injury severity in models of GIM (Logan et al., 2008a). However, the upstream regulation of these cytokines has yet to be elucidated. The following section provides linkages between TLRs and pro-inflammatory cytokines to support their critical involvement in GIM.

The activation of TLR2 and TLR4 can lead to NF-κB nuclear translocation, which culminates in up-regulation of genes involved in mucosal injury, including proinflammatory cytokines. Previous research has shown that intestinal NF-κB mRNA and protein expression is increased early following irinotecan treatment in a rat model of GIM (Bowen et al., 2007b; Logan et al., 2008a). This is associated with increased IL-1β, TNF, and IL-6 protein levels in the intestine (Logan et al., 2008a). When these proteins are up-regulated, they can initiate positive feedback loops resulting in NF-κB amplification and increased intestinal damage. Interestingly, these changes coincided with early histological damage and the initial occurrence of diarrhoea. In a separate study, inhibition of TNF and IL-1β tissue expression, using thalidomide, attenuated histological damage and inflammation but not delayed diarrhoea in irinotecan-treated mice (Melo et al., 2008). Conversely, administration of pentoxifylline, another inhibitor of TNF and IL-1β, was able to inhibit delayed diarrhoea as well as histopathological changes following treatment (Melo et al., 2008). This indicates that pro-inflammatory cytokines are essential markers of the pathogenesis of irinotecan-induced GIM.
1.3.5 Effects of TLR2 and TLR4 inhibition on mucosal injury

Several potential inhibitors of GIM development have been investigated to date. These inhibitors target single or multiple pathways associated with the pathogenesis of GIM. Inhibitors that target multiple pathways have been shown to be effective in ameliorating tissue damage. As such, palifermin was recently approved by the United States Food and Drug Administration for the prevention of oral mucositis (Keefe et al., 2006; Niscola et al., 2009). Palifermin has multiple biological activities that result in the modulation of signalling pathways, including those of pro-inflammatory cytokines, the Bcl-2 family, p53, Akt and p21 activated protein kinase 4 (Wildhaber et al., 2003; Bao et al., 2005). The effect of NF-κB inhibition on GIM development has also been investigated. Previous research has shown that the NF-κB inhibitor, 5-aminosalicylic acid, improved mucosal damage following 5-fluorouracil treatment in mice (Chang et al., 2012a). However, 5-aminosalicylic acid is not recommended as an intervention for patients since it can increase diarrhoea severity after radiotherapy (Gibson et al., 2013). Another inhibitor that was investigated was apolipoprotein E, which can reduce NF-κB and pro-inflammatory cytokines. This inhibitor was found to ameliorate intestinal inflammation, IL-1β and TNF expression, and apoptosis, but not weight loss, in a model of GIM (Azevedo et al., 2012). Considering that TLRs are upstream mediators of NF-κB and pro-inflammatory cytokines, they can be investigated as a target for treating GIM. Previous research has shown that using a TLR9 antagonist, ODN208, reduced doxorubicin-induced morphological and histological damage in the small intestine of mice (Kaczmarek et al., 2012). However, to date, no studies have investigated the effects of TLR2 and TLR4 inhibition on irinotecan-induced GIM.

1.3.6 Amitriptyline (AMI)

AMI hydrochloride is 3-(10,11-dihydro-5H-dibenzo[a,d]cycloheptene-5-ylidene)-N,N-dimethyl-1-propanamine hydrochloride (also known as Elavil, Endep, Levate and many others). It was approved as a tricyclic antidepressant drug by the United States of Food and Drug Administration in 1961 (Leucht et al., 2012). Its main mode of action is inhibiting the reuptake of serotonin and noradrenaline into the presynaptic terminal. It also blocks cholinergic, histaminergic and serotonergic receptors (Australian Medicines Handbook 2014). It is widely used for the management of psychological disorders and various types of pain, including neuropathic pain (Banerjee et al., 2013). It is effective in relieving cancer-related neuropathic pain, although is
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associated with adverse effects including, drowsiness, dizziness, mouth dryness, nausea, and constipation (Mishra et al., 2012). There is potential for AMI to be used to control cancer therapy-induced side effects.

1.3.7 Amitriptyline metabolism

AMI is well absorbed in the gastrointestinal tract. The peak plasma concentration occurs approximately 12 h after oral administration and the half-life varies between 1 - 50 h with an average of 15 h (Burch et al., 1984). In rats, it is rapidly metabolised with a half-life around 2.5 h in plasma after acute administration (Coudore et al., 1996). AMI is mainly metabolised by the hepatic microsomal enzymes CYP2D6, CYP2C19 and CYP3A4, respectively (Olesen and Linnet, 1997; Venkatakrishnan et al., 2001). It is biotransformed via N-demethylation to the active metabolite nortriptyline (accounting for 60% of total clearance and for 60-70% of urinary recovery of amitriptyline metabolites). It can also be metabolised by E-10 hydroxylation to 10-hydroxy amitriptyline (accounting for 15-20% of total urinary recovery) (Venkatakrishnan et al., 2001).

Recently, it was reported that the clearance of AMI by N-glucuronidation by UGT2B10 is higher than UGT1A4 and UGT1A3 (Kato et al., 2013). However, nortriptyline does not undergo glucuronidation in the human body (Kato et al., 2013). Others have reported that AMI is a non-selective UGT inhibitor at high concentrations in HEK293 cells (Uchaipichat et al., 2006). AMI was found to inhibit UGT2B7 but has minor inhibitory effect to other UGTs, including UGT1A1 and UGT1A9 (Donato et al., 2010) (Figure 1.4).

There are potentially important interactions between AMI and irinotecan (Santos et al., 2000; Tallman et al., 2005; Kato et al., 2013; Australian Medicines Handbook ). Both irinotecan and AMI are substrates for CYP3A4. In addition, AMI shows minor inhibitory effects on UGT1A1 and UGT1A9, which are involved in irinotecan metabolism. Therefore, there is a possibility of drug interaction between both drugs through inhibiting UGT1A enzymes. UGT1A enzymes are important for irinotecan detoxification. Therefore, inhibiting these enzymes could result in increasing SN-38 exposure.
Figure 1.4: AMI metabolism. In the liver, AMI undergoes demethylation mainly by CYP2C19 to its active metabolite nortriptyline which is excreted via bile and absorbed in the gastrointestinal tract. CYP2D6 mediates AMI hydroxylation to 10-hydroxy amitriptyline while UGT2B10 and UGT1A4 mediate N-glucuronidation.
1.3.8 Can AMI be used to prevent GIM development?

Theoretically, AMI can be used for treating GIM for the following reasons:

- It has anti-histaminic and anticholinergic effects.

AMI is among the antidepressant drugs that are used for treating IBS symptoms. It acts as an antihistamine and anticholinergic agent (Ohta et al., 1999; Brecht et al., 2007). Its weak anticholinergic affect could help in reducing colonic motility which may be relevant to its efficacy in treating IBS-diarrhoea (Bahar et al., 2008). It could also be the reason behind side effects including dry mouth, nausea and constipation. The antihistaminic effect of AMI could cause the sedative effect and may help in reducing the stress-induced diarrhoea in IBS (Spiller, 2006; Enginar et al., 2010). Therefore, AMI has a potential to reduce early diarrhoea occurrence following irinotecan.

- It has anti-inflammatory and TLR2 and TLR4 inhibitory effects.

AMI has anti-inflammatory effects in experimental models of acute inflammation (Sadeghi et al., 2011; Vismari et al., 2012; Gurgel et al., 2013). AMI (40-80 mg/kg) significantly reduced the migration of mononuclear leucocytes to the site of the inflammation and reduced IL-1β and TNF in the rat paw treated with carrageenan (Sadeghi et al., 2011; Vismari et al., 2012). AMI significantly inhibited TLR2 activity in HEK293 human cells stimulated by PAM3CSK4 and TLR4 activity in HEK293 cell-over-expressing human TLR4 incubated with LPS (Hutchinson et al., 2010b). These data suggest the possible inhibitory effect of AMI on TLR2, TLR4 and pro-inflammatory cytokines thus modulating irinotecan-induced GIM.

- It has anti-apoptotic effects.

AMI has been shown to affect cell death, depending on the treatment and the cell type. Pre-treatment with AMI (100 µM) for 24 h reduced hydrogen peroxide-induced apoptosis by up-regulating superoxide dismutase activity in PC12 cells (Kolla et al., 2005). AMI (1-25 µM) attenuated 1-methyl-4-phenylpyridinium-induced apoptosis in PC12 cells by suppressing altered mitochondrial membrane permeability that leads to cytochrome c release and caspase-3 activation (Han and Lee, 2009). These previous studies provide evidence for the ability of AMI to
reduce apoptosis which is an important feature of irinotecan-induced GIM. However, AMI has shown to be cytotoxic to cancer cells. It was reported that AMI had apoptotic effects on cancer cells, such as colon carcinoma, multiple myeloma and uterine leiomyosarcoma, \textit{in vitro} (Arimochi and Morita, 2006a; Mao et al., 2011b; Pula et al., 2013). This suggests that AMI would be protective in the intestine but should not affect the efficacy of chemotherapy.

- It has anti-diarrhoeal effects.

In the last few years, the use of antidepressants as anti-diarrhoeal agents in IBD patients has been investigated. As such, patients with irritable bowel syndrome (IBS) treated with AMI were more likely to experience reduction in IBS associated diarrhoea after 6 and 10 weeks of treatment than placebo controls (Bahar et al., 2008). AMI, at low doses, was found to be effective in relieving diarrhoea-predominant IBS (Vahedi et al., 2008). AMI when it was used with probiotics improved overall symptom of IBS, including diarrhoea, in about 66% of patients despite the side effects of this drug (Sohn et al., 2012). However, others reported that AMI was ineffective in treating IBD symptoms (Mikocka-Walus et al., 2006). Moreover, AMI as a psychological therapy can reduce stress associated with diarrhoea (Spiller, 2006).

- It can reduce gastric ulcers.

AMI has a protective effect from indomethacin and reserpine-induced gastric ulcer (Ji et al., 2012). Intraperitoneal injection of AMI (10 and 20 mg/kg) significantly reduced ulcer lesion and intraluminal bleeding in male Sprague-Daley rats. Since GIM is characterised as intestinal ulceration, AMI’s ability to reduce drug-induced gastric ulceration may translate to efficacy in the protection from GIM.

### 1.4 Conclusion

This review has provided evidence showing the potential relationship between TLR2 and TLR4 signalling and the development of GIM following irinotecan treatment. To date, the role of TLRs in GIM is ill defined. The proposed link between TLR2 and TLR4 and intestinal inflammation provides a new area for research to further understand the pathobiology of GIM. Few studies have
implicated the involvement of TLR2 and TLR4 in chemotherapy-induced GIM. Their inhibition may have protective effects from irinotecan-induced GIM and could present a potential clinical therapeutic opportunity in the treatment of GIM.

1.5 Aims of thesis

Given the literature summarised above, it was hypothesised that TLR2/TLR4 signalling is the primary driver of up-regulated pro-inflammatory cytokine expression involved in diarrhoea occurrence in rats treated with irinotecan. As such, TLR inhibition therefore could modulate irinotecan-induced GIM.

The first aim was to examine the expression patterns of TLR2, TLR4 and pro-inflammatory cytokines in the intestine of an established rat model of irinotecan-induced GIM and to correlate changes with diarrhoea occurrence.

The second aim was to investigate the effect of TLR2 and TLR4 inhibition, through AMI, on irinotecan-induced GIM symptoms in Wistar rats.

The last aim of this thesis was to investigate the effect of AMI on apoptosis and the expression of TLR2, TLR4 and pro-inflammatory cytokines after SN-38, the toxic metabolite of irinotecan, treatment to extend our knowledge about the effect of the drug on apoptosis.
Chapter Two
2.0 General methods

2.1 Histological analysis

Jejunum and colon samples were stained as previously described (Stringer et al., 2007b; Logan et al., 2008a). Briefly, paraffin-embedded tissue were cut to 4 µm sections and mounted onto glass slides. Sections were dewaxed, rehydrated and stained with Lillie-Mayer’s haematoxylin (Fronine Laboratory Supplier, Australia) for 10 minutes (min). Then, sections were differentiated in 1% acid alcohol and blued in Scott’s tap water (10 g magnesium sulphate, 1 g sodium hydrogen carbonate and 500 ml dH₂O). Sections were counterstained with eosin. Finally, they were dehydrated, cleared and mounted before histological examination. Sections were examined by light microscopy (Olympus BX51, USA) for histological analysis. Images were captured at 200x magnification.

2.2 RNA extraction and reverse transcription

Thirty mg of intestinal tissues were homogenised in 500 ml TRIzol®Reagent (Invitrogen Life Technologies, Mulgrave, Australia) then centrifuged to separate the RNA-containing aqueous phase. mRNA was purified using a Nucleospin® RNA II kit (Macherey-Nagel, Duren, Germany) following the manufacturer’s instructions. In brief, after RNA binding and adsorption on the silica membrane, DNA contamination, salt, metabolites and cellular components were removed by different washing buffers that were supplied with the kit. Total RNA was eluted in 40 µl RNase-free water (Nucleospin, Macherey-Nagel, Duren, Germany).

RNA concentration and purity was measured using NanoDrop® Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The mean (± SD) ratio of A260/280 was 2.09 ± 0.01, indicating high purity and protein-free RNA samples. The mean (± SD) ratio of A260/230 was 1.88 ± 0.05 to indicating phenol- and ethanol-free RNA.
RNA was converted to cDNA using the QuantiTect® reverse transcription kit (Qiagen, Doncaster, Australia). In each reaction, 1 µg of RNA was incubated in gDNA wipeout Buffer at 42 °C for 2 min to effectively remove contaminating genomic DNA. After Quantiscript reverse transcriptase, Quantiscript RT buffer, and RT primer mix addition, samples were incubated at 42 °C for 15 min followed by incubation at 95 °C for 3 min. Total cDNA concentration and purity were measured using the NanoDrop® Spectrophotometer then each sample was diluted to a working concentration of 100 ng/µl in RNase-free water (Qiagen, Doncaster, Australia). For cDNA purity, the mean (± SD) ratio of A260/280 was 1.8 ± 0.02 for all samples.

2.3 Real time-PCR

Primers: The primers that were used in this study are listed in Table 2.1. Some of the primers were used from previous published research (TLR2, IL-1β and TNF) and others were designed using Primer3 software (version 0.4.0) based on rattus norvegicus mRNA sequence for TLR4 (NM_019178.1) and IL-6 (NM_012589.2) in the GenBank database (NCBI, Bethesda, MD). Netprimer (Premier Biosoft, Palo Alto, Calif., USA) was used to check for the absence of primer dimers, potential hairpins, palindromes and secondary structures. Forward and reverse primers were synthesised by Geneworks Ltd. (Thebarton, S.A., Australia).

Real-time PCR (RT-PCR) was performed by using the Rotor Gene 3000 (Corbett Research, Australia). Amplification reactions contained 1 µl (100 ng) of cDNA sample, 5 µl of fluorescent dye SYBR green (QuantiTect SYBR green, Qiagen, Doncaster, Australia), 3 µl of nuclease-free water (Qiagen, Doncaster, Australia), and 0.5 µl of each forward and reverse primers (50 pmol) to make 10 µl as a final volume. Taq DNA polymerase activation at 95 °C for 15 min followed by 40 cycles of denaturation at 95 °C for 10 seconds, annealing at 53 °C (TLR4 and TNF) or 54 °C (TLR2, IL-1β and IL-6) and extension at 72 °C for 20 seconds. By using the Rotor-Gene software, the experimental cycle threshold (Ct) values were calculated. Amplification specificity of each product was confirmed by melt curve analysis. All samples were run in triplicate and delta delta Ct analysis was calculated using Rotor-Gene software and compared to baseline (control rat gene expression) in diarrhoea and no diarrhoea groups. Each gene was normalised with two housekeeping genes that were found to be the most stable genes in DA rat (Al-Dasooqi et al., 2011b), Ubiquitin C (UBC) and Beta-2-microglobulin (B2M), and the average was calculated. The
equivalent amplification efficiencies between housekeeping genes and genes of interest were confirmed using standard curves and linear regression analysis of delta C\textsubscript{t} results (representative figures shown in appendix).

Table 2.1: Primers sequences and characteristics

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence (5’-3’)</th>
<th>Size (bp)</th>
<th>Accession no.</th>
<th>Ref.</th>
</tr>
</thead>
</table>
| **IL-1B** | Forward: CTGGTACATCAGCACCTCTCAA  
Reverse: GAGACTGCCCATTCTCGACAA | 167 | NM_031512 | (Lacraz et al., 2009) |
| **TLR4** | Forward: CCATAAAAGCCGAAAGGTGA  
Reverse: CTGAGCAGGGTCTTCTCCAC | 159 | NM_138554.4 | Primer3.com |
| **TNF** | Forward: CTTCAAGGGACAAGGCTG  
Reverse: GAGGCTGACTTTTCTCCTG | 88 | NM_012675.3 | (Hutchinson et al., 2010a) |
| **IL-6** | Forward: CCACTGCCTTCCCTACTTCA  
Reverse: CAGAATTGCCATTGCACAAC | 159 | NM_012589.1 | Primer3.com |
| **TLR2** | Forward: CCTGGGCAGTCTTTGGAACATT  
Reverse: TTCCCCACTCTCAGGATTTGC | 179 | NM_003264.3 | (Moreira et al., 2012) |
| **B2M** | Forward: CGAGACCGATGTATATGCTTG  
Reverse: GTCCAGATGATTAGAGCTCCA | 114 | NM_012512.1 | (Al-Dasooqi et al., 2011b) |
| **UBC** | Forward: TCGTACCTTTCTCACACAGTATCTAG  
Reverse: AAAACTAAGACACCTCCCATCA | 82 | NM_017314.1 | (Al-Dasooqi et al., 2011b) |
2.4 Immunohistochemistry for TLR2, TLR4 and pro-inflammatory cytokine protein expression

Sections from the jejunum and colon of control and treated rats were cut at 4 µm thickness and mounted onto saline-coated slides. Sections were dewaxed in xylene and rehydrated in serial concentrations of ethanol (3x 100%, 90%, 70% and 50%). After washing sections with phosphate buffered saline (PBS) they were immersed in 10 mM citrate buffer (pH 6.0) and antigen retrieval was performed using a pressure cooker for 3 min on high pressure (The fast slow cooker-Breville, USA). Sections were allowed to cool for 20 min at room temperature then endogenous peroxidase was blocked using 3% H₂O₂ in methanol. Non-specific binding of antibodies was blocked by blocking solution (5% normal serum in 0.01M PBS containing 0.1% sodium azide) (Covance, USA) for 20 min at room temperature. Then avidin-biotin activity was blocked using avidin-biotin blocking kit (Vector Laboratories, Inc, Burlingame, USA) following manufacturer’s instructions. Sections were incubated overnight at 4 °C with primary antibody in humidifying chamber at 4 °C: TLR4 (Abcam, Cambridge, UK), TLR2 (Abcam, Cambridge, UK), IL-1β (H-153, Santa Cruz Biotechnology, Santa Cruz, CA; sc-7884), TNF (HP8001, Hycult Biotechnology b.v., Frontstraat, The Netherland) and IL-6 ((M-19)-R, Santa Cruz Biotechnology, Santa Cruz, CA; sc-1265-R) were diluted at 1:50, 1:200, 1:200, 1:75 and 1:1000, respectively. Then sections were incubated with linking reagent (Biotinylated Anti-Immunoglobulin Serum in 0.01M PBS containing 0.1% NaN₃, carrier protein and human serum) (Covance, New York, NY, USA) for 20 min at room temperature followed by the labelling reagent (Peroxidase Labelled ultra-Streptavidin in 0.01M PBS) (Covance, USA) for 20 min at room temperature. After washing with PBS for 3 min, antibody binding was visualised using diaminobenzidine (DAB) (Covance, USA) diluted in substrate buffer at 1:25 (Covance, USA). Subsequent to this, slides were washed with H₂O then counterstained with Lillie-Mayer’s haematoxylin, dehydrated and cleared in xylene before being coverslipped. Positive controls tissues used were rat brain for TLR2 and TLR4 and rat lung for pro-inflammatory cytokines as recommended by the manufacturer. Negative controls were processed similarly but the primary antibody was replaced with normal serum. They were used to ensure the specificity and accuracy of the staining.

Slides were scanned using NanoZoomer 2.0-HT Slide Scanner (Hamamatsu, Hamamatsu City, Japan). Qualitative analysis was performed using NDP software at 20x magnification. Staining intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong
staining; and 4, intense staining. This qualitative grading system has been published extensively (Bowen et al., 2005; Yeoh et al., 2005; Al-Dasooqi et al., 2010) and has been used routinely in our laboratory (Yeoh et al., 2005; Al-Dasooqi et al., 2010). All assessments were done in both jejunum (villi and crypt) and colon (crypt apical and basal region) and blinded to treatment. This assessment has been used routinely in our laboratory. The staining intensity was only considered in the epithelial layer for all regions since it is known that these cells express TLR2, TLR4 and pro-inflammatory cytokines.
Chapter Three
3.0 Toll-like receptors 2 and TLR4 and pro-inflammatory cytokines are possible mediators of irinotecan-induced GIM in Dark Agouti (DA) rats.

3.1 Introduction

Gastrointestinal mucositis (GIM) remains one of the most debilitating side effects of chemotherapy (Sonis et al., 2004). It occurs in approximately 40% of cancer patients undergoing standard dose chemotherapy and in 100% of cancer patients receiving high dose chemotherapy with bone marrow or stem cell transplantation (Keefe et al., 2000; Rubenstein et al., 2004). GIM manifests clinically as bloating, abdominal pain and diarrhoea (Sonis et al., 2004). In the last ten years, significant progress has been made in the understanding of the morphological changes and the underlying mechanisms behind chemotherapy-induced GIM (Gibson et al., 2003; Sonis, 2004; Sonis et al., 2004; Logan et al., 2009). With the advances in the understanding of GIM pathobiology, the need to design therapeutic strategies targeted to specific pathological pathways has become the focus of research recently.

Irinotecan, a topoisomerase-I inhibitor, is a well-known drug that causes GIM in cancer patients undergoing treatment protocols including this drug (Sonis et al., 2004). The use of irinotecan causes diarrhoea in about 60% of cancer patients, which limits treatment frequency, dosage and therefore reduces survival (Fittkau et al., 2004; Borner et al., 2005). Irinotecan alters gastrointestinal morphology in animal models of GIM, causing toxicity to both the jejunum and the colon (Gibson et al., 2003; Logan et al., 2008a; Stringer et al., 2009b). In the jejunum, irinotecan causes epithelial cell apoptosis, villous atrophy and crypt hypoplasia (Gibson et al., 2003; Logan et al., 2008a). In the colon, it causes decreased crypt length and increased crypt dilation and mucus secretion in addition to apoptosis (Gibson et al., 2003; Logan et al., 2008a). These morphological changes were associated with induction of inflammatory responses, significant alteration in cell kinetics, matrix metalloproteinases, mucus production, changes in the microflora and the activation of different cell regulatory pathways (Bowen et al., 2007b; Stringer et al., 2007a; Stringer et al., 2007b; Logan et al., 2008a; Al-Dasooqi et al., 2010; Al-Dasooqi et al., 2011a). One of the critical problems in GIM treatment is to distinguish the most critical factors behind the development of severe GIM, characterised by diarrhoea in animal model. In addition, patients (Sonis et al., 2004), and animals (Stringer et al., 2007b), respond differently to the same
treatment, which implies different levels of the activated signalling pathways that attribute to the occurrence of diarrhoea.

Previous research has shown that both oral mucositis and GIM result from complex biological events rather than simply clonogenic cell death (Sonis et al., 2007). GIM is associated with inflammatory infiltration, alterations in gut microbiome and activation of several signalling pathways implicated in the development of injury (Keefe et al., 2000; Sonis et al., 2007; Stringer et al., 2007b; Al-Dasooqi et al., 2010). Gene expression analysis of rat intestinal tissue following irinotecan treatment has helped to uncover the pathways involved (Bowen et al., 2007b). Toll-like receptor (TLR), nuclear factor kappa B (NF-κB), interleukin 6 (IL-6), and tumour necrosis factor (TNF)/stress signalling were among the signalling pathways suggested to be related to the development of GIM. Moreover, canonical pathway analysis on human peripheral blood monocyte-derived RNA has identified several pathways that are associated with oral mucositis in patients treated with chemo-radiation including carboplatin and paclitaxel (Sonis et al., 2007). Among the 14 pathways detected, TLR, NF-κB and IL-6 signalling pathways were significantly activated. TLR activation in cells leads to NF-κB up-regulation which results in pro-inflammatory cytokine secretion (Takeuchi and Akira, 2010). Despite ample evidence of the involvement of NF-κB and pro-inflammatory cytokines in GIM (Logan et al., 2008a; Logan et al., 2008b; Chang et al., 2012a), there is currently insufficient data in the literature for the role of TLRs in chemotherapy-induced GIM or diarrhoea. Kaczmarek et al (2012) has demonstrated the involvement of TLR2 and TLR9 in doxorubicin-induced small intestinal damage using genetic knockout TLR2−/− and TLR9−/− female C57BL/6 mice (Kaczmarek et al., 2012), although TLR expression was not compared between the jejunum and the colon or correlated with diarrhoea occurrence. Ferreira et al (2012) reported TLR4 mRNA up-regulation following 5-fluorouracil administration in female Swiss mice (Ferreira et al., 2012). Hamada et al (2013) reported an increase in mRNA expression of TLR4 in the jejunum of Wistar rats following methotrexate-induced GIM (Hamada et al., 2013). Other TLRs have not been investigated to date.

This study focused on the involvement of TLR2 and TLR4 in modulating pro-inflammatory cytokines expression after irinotecan-induced GIM for the following reasons:
TLR2 and TLR4 respond to pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) (Chang, 2010; Piccinini and Midwood, 2010). TLR2 recognises PAMPs derived from gram positive bacteria, viruses, fungi and parasites, including peptidoglycan and lipoteichoic acid, while TLR4 recognises ligands including lipopolysaccharide (LPS), paclitaxel and fibronectin (Gay and Gangloff, 2007; Chang, 2010; Piccinini and Midwood, 2010). Both TLRs respond to DAMPs, such as high-mobility group box 1 (HMGB1) and heat shock protein 70 (HSP70) (Piccinini and Midwood, 2010). It was proposed that DAMPs can be released from injured tissues in response to chemotherapy and PAMPs from colonising bacteria in the intestinal mucosa (Chang, 2010; Sonis, 2010). Therefore, both TLR4 and TLR2 could potentially play a major role in initiating GIM.

Moreover, TLR2 and TLR4 play a role in innate and adaptive immunity, inflammatory reactions, inflammatory diseases, and other cell responses (Chang, 2010). They are expressed at low levels in normal human colon tissues by intestinal epithelial cells (IECs) and predominantly in the crypt (Cario and Podolsky, 2000; Otte et al., 2004; Mantani et al., 2011). In the rat, TLR2 and TLR4 were detected in the undifferentiated epithelial cells and in the luminal substances of the intestinal crypt in the jejunum (Mantani et al., 2011). Even though TLRs are expressed in healthy intestine, their expression is increased during inflammation (Cario and Podolsky, 2000; Candia et al., 2012; Brown et al., 2014). Since GIM is characterised as inflammation along the alimentary tract, it is conceivable that TLRs are involved in the pathogenesis of GIM as well. During intestinal inflammation, TLR4 staining was found to be more intense in IEC, particularly in the colon and the ileum, of all patients with inflammatory bowel disease (IBD) compared to healthy controls (Cario and Podolsky, 2000). However, TLR2 expression remained unchanged in IBD patients. Moreover, pro-inflammatory cytokines have shown to modulate TLR expression in IEC (Abreu et al., 2002; Mueller et al., 2006). Pro-inflammatory cytokines, such as TNF and INF-γ, have been shown to induce the transcription of TLR4 (up to 1.4- and 1.2-fold, respectively) and its co-receptor MD2 (up to 1.4- and 2.5-fold, respectively) (Abreu et al., 2002). In contrast, IL-14 and IL-13, anti-inflammatory cytokines, suppressed IEC response to the TLR4 ligand, LPS, in mouse small intestinal cell lines (Lotz et al., 2007). Therefore, IECs have a potential to up-regulate TLR expression in response to cytokine signals or other stimuli. However, the correlation between GIM and TLR2, TLR4 and pro-inflammatory cytokine expressions has not yet been elucidated.
Since TLR2 and TLR4 have been shown to be involved in intestinal inflammation and mucosal injury, they could be involved in irinotecan-induced GIM. We hypothesised that TLR2 and TLR4 signalling is the primary driver of pro-inflammatory cytokine expression that leads to diarrhoea occurrence and severity in rats treated with irinotecan. This study aimed to use an established rat model of irinotecan-induced GIM to examine the expression patterns of TLR2, TLR4 and pro-inflammatory cytokines in the intestine and to correlate changes with diarrhoea occurrence.

3.2 Materials and Methods

3.2.1 Experimental plan

The study was approved by the Animal Ethics Committees of The Institute of Medical and Veterinary Sciences and of The University of Adelaide and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training. Archived samples of sixteen female Dark Agouti (DA) rats, aged between 6-8 weeks and weighing 150 - 170 g, were used in this study. Rats were divided into vehicle control (n = 6) and irinotecan-treated experimental groups (n = 10). All rats received 0.01 mg/kg subcutaneous (s.c) atropine (Pfizer, NY, U.S.A) directly prior to irinotecan treatment to reduce the cholinergic reaction to irinotecan (Hyatt et al., 2005). This was followed by a single intraperitoneal (i.p.) dose of 200 mg/kg of irinotecan, which has been shown to cause GIM in DA rats (Gibson et al., 2007). Irinotecan (kindly supplied by Pfizer, Kalamazoo, USA) was prepared in a sorbitol/lactic acid buffer (45 mg/ml sorbitol, 0.9 mg/ml lactic acid, pH 3.4), required for activation of the drug. The control group rats received subcutaneous atropine and an i.p. injection of the sorbitol/lactic acid buffer.

Rats were killed at 72 hours (h) (irinotecan, n = 4, control, n = 3) and 96 h (irinotecan, n = 6, control, n = 3) by cardiac puncture and cervical dislocation under 2% isofluorane in 100% O2 anaesthesia. These two time points have been shown previously to correspond with peak severity of irinotecan-induced diarrhoea (Stringer et al., 2009a). The jejunum and colon were dissected and flushed with chilled isotonic saline (0.9% w/v) to remove contents and 1 cm sections of the jejunum and colon were taken at 33% and 50% of the intestine length, respectively. Samples were fixed in 10% neutral buffered formalin before being processed and embedded in paraffin wax, or snap frozen in liquid nitrogen for molecular analysis.
3.2.2 Diarrhoea assessment

Animals were monitored four times daily for developing diarrhoea. Diarrhoea was graded as none, mild (staining of anus), moderate (staining top of legs and lower abdomen) and severe (staining over legs and higher abdomen as well as continual anal leakage) (Gibson et al., 2003; Stringer et al., 2007b; Logan et al., 2008a; Al-Dasooqi et al., 2010). This grading has been published previously and is used routinely in our lab (Gibson et al., 2003; Stringer et al., 2007b; Logan et al., 2008a; Al-Dasooqi et al., 2010).

3.2.3 Toxicity classification:

For the purpose of analysis, experimental rats were allocated to one of three subgroups based on response to irinotecan as follows: 1. Rats that developed diarrhoea at 72 h; 2. Rats that developed diarrhoea at 96 h; and 3. Rats that did not develop diarrhoea at any time following irinotecan treatment. Comparisons were made between these three experimental subgroups and the untreated controls.

3.2.4 Histological analysis

Jejunum and colon samples were stained as previously described in chapter two.

3.2.5 RNA extraction and reverse transcription

RNA was extracted and converted to cDNA as previously described in chapter two.
3.2.6 Real time-PCR

Primers: The primers that were used in this study are listed in chapter two (Table 2.1).

Real-time PCR (RT-PCR) was performed as described in chapter two.

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

Sections from the jejunum and colon of control and treated rats were cut and stained with TLR2, TLR4, IL-1β, TNF and IL-6 antibodies as previously described in chapter two.

3.2.8 Statistical Analysis

Results for mRNA expression (RT-PCR) and protein expression (immunohistochemistry) between groups were analysed using Kruskal-Wallis test with Dunn’s post-test. Correlations between RT-PCR and IHC results were investigated using Spearman rank correlations. Results were statistically significant at \( P < 0.05 \).

3.3 Results

3.3.1 Response to treatment and histological analysis

Diarrhoea was observed in 6 of 10 (60%) rats that received irinotecan. At 72 h, 2 rats had mild diarrhoea. At 96 h, 2 rats had mild, 1 rat had moderate and 1 rat had severe diarrhoea (Figure 3.1). The 6 untreated control rats did not develop diarrhoea.

Marked histological damage was observed in both jejunum and colon in rats treated with irinotecan. In the jejunum, villus atrophy and blunting were observed in irinotecan treated rats with
diarrhoea at 72 and 96 h. Likewise, in the colon, changes included complete ablation of the crypts and oedema at 72 and 96 h (Figure 3.2).
Figure 3.1: Incidence of diarrhoea after irinotecan treatment. An increase in diarrhoea severity is evident at 96 h post treatment. At 72 h, 2 of 4 rats had mild diarrhoea. At 96 h, 2 of 6 rats had mild, 1 rat had moderate and 1 rat had severe diarrhoea.
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. Arrow showing villus atrophy at 72 h (A) and colonic crypt ablation at 96 h (B). Images were captured by light microscopy at 200 magnification. Scale bar is 100 µm.
3.3.2 TLR2 and TLR4 and pro-inflammatory cytokine mRNA expression

Jejunum:

The expression of TLR4, TLR2, IL-1β, TNF and IL-6 mRNA was not statistically different between groups (P = 0.09, 0.07, 0.9, 0.3 and 0.39, respectively) (Figure 3.3).

Colon:

The expression of TLR2, TLR4, IL-1β and TNF mRNA increased significantly in irinotecan treated rats who developed diarrhoea at 96 h up by approximately 7-, 6-, 9- and 3-fold change (median), respectively, compared to rats without diarrhoea (P = 0.008, P = 0.007, P = 0.032 and P = 0.039, respectively) (Figure 3.4). There were no significant changes in IL-6 expression between groups (P = 0.3).
Figure 3.3: Fold change in (A) TLR2, (B) TLR4, (C) IL-1β, (D) TNF and (E) IL-6 mRNA expression in jejunum compared to untreated controls (no diarrhoea n = 4; diarrhoea 72 h n = 2; and 96 h n = 4) after irinotecan treatment (200 mg/kg) in DA rats. The line represents median. Fold change compared to pooled untreated control group.
Figure 3.4: Fold change in (A) TLR2, (B) TLR4, (C) IL-1β, (D) TNF and (E) IL-6 mRNA expression in the colon compared to untreated controls (no diarrhoea, n = 4; diarrhoea 72h, n = 2; and 96 h, n = 4) after irinotecan treatment (200 mg/kg) in DA rats. The line represents median, * P < 0.05 and **P < 0.01, fold change compared to no diarrhoea and diarrhoea (72 h) groups.
3.3.3 TLR2, TLR4 and pro-inflammatory cytokine protein expression following irinotecan treatment

All slides were scored according to the staining intensity in the epithelial layer in all regions. The staining was cytoplasmic for all antibodies.

Jejunum:

Protein expression increased in the villi for TNF in irinotecan treated rats that developed diarrhoea at 96 h ($P < 0.0001$) compared to other groups. TLR4, TLR2, IL-1β and IL-6 protein expression was not significantly different between any groups (Figure 3.5, 3.6 and 3.7).

Colon:

TLR2, TLR4 and IL-1β protein expression increased significantly in the apical region of the crypt for irinotecan treated rats that developed diarrhoea at 96 h ($P = 0.04$, $P = 0.03$ and $P = 0.008$, respectively). In the basal region of the crypt, there were no significant differences in the staining for TLR2, TLR4, IL-1β, TNF and IL-6 between any groups (Figure 3.8, 3.9 and 3.10).
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. All control and experimental tissues were graded using a qualitative scale. The data are presented as median with range. ***P < 0.001 compared to other groups. Control (n = 6), irinotecan treated rats that did not develop diarrhoea (n = 4), developed diarrhoea at 72 h (n = 2), developed diarrhoea at 96 h (n = 4).
Figure 3.6: TLR2 immunostaining of the jejunum of DA rats. Photomicrographs are 200x original magnification. No diarrhoea: rats were treated with CPT-11 but did not develop diarrhoea; Diarrhoea (72 h): rats had diarrhoea 72 h after CPT-11 injection; Diarrhoea (96 h): rats had diarrhoea 96 h after CPT-11 injection; Control: rats had vehicle injections; and Negative control; primary antibody that was omitted from the immunostaining protocol to show specificity of secondary antibody. Scale bar is 50 µm.
Figure 3.7: TLR4 immunostaining of the jejunum of DA rats. Photomicrographs are 200x original magnification. No diarrhoea: rats were treated with CPT-11 but did not develop diarrhoea; Diarrhoea (72 h): rats had diarrhoea 72 h after CPT-11 injection; Diarrhoea (96 h): rats had diarrhoea 96 h after CPT-11 injection; Control: rats had vehicle injections; and Negative control; primary antibody that was omitted from the immunostaining protocol to show specificity of secondary antibody. Scale bar is 50 µm.
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. All control and experimental tissues were graded using a qualitative scale. The data are presented as median with range. *P < 0.05. Control (n = 6), irinotecan treated rats that did not develop diarrhoea (n = 4), developed diarrhoea at 72 h (n = 2), developed diarrhoea at 96 h (n = 4).
Figure 3.9: TLR2 immunostaining of the colon of DA rats. Photomicrographs are 200x original magnification. No diarrhoea: rats were treated with CPT-11 but did not develop diarrhoea; Diarrhoea (72 h): rats had diarrhoea 72 h after CPT-11 injection; Diarrhoea (96 h): rats had diarrhoea 96 h after CPT-11 injection; Control: rats had vehicle injections; and Negative control; primary antibody that was omitted from the immunostaining protocol to show specificity of secondary antibody. Scale bar is 50 µm.
Figure 3.10: TLR4 immunostaining of the colon of DA rats. Photomicrographs are 200x original magnification. No diarrhoea: rats were treated with CPT-11 but did not develop diarrhoea; Diarrhoea (72 h): rats had diarrhoea 72 h after CPT-11 injection; Diarrhoea (96 h): rats had diarrhoea 96 h after CPT-11 injection; Control: rats had vehicle injections; and Negative control; primary antibody that was omitted from the immunostaining protocol to show specificity of secondary antibody. Scale bar is 50 μm.
3.3.4 The correlation between mRNA and protein expression:

The correlation between mRNA and protein expression was calculated using Spearman test in all regions of the jejunum (crypt and villi) and colon (apical and basal regions of the crypt). Results are presented in table 3.1 (Figure 3.11 and 3.12).
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

<table>
<thead>
<tr>
<th>Marker</th>
<th>Correlation in jejunum</th>
<th>Correlation in colon</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Crypt P-value villi P-value Crypt (apical) P-value</td>
<td>Crypt (basal) P-value</td>
</tr>
<tr>
<td>TLR2</td>
<td>0.6 *0.007 0.5 0.06 0.5 *0.04</td>
<td>0.1 0.7</td>
</tr>
<tr>
<td>TLR4</td>
<td>0.5 *0.03 0.4 0.1 0.6 **0.009</td>
<td>0.5 *0.03</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.3 0.2 0.03 0.4 0.7 ***0.0005</td>
<td>0.7 ***0.0003</td>
</tr>
<tr>
<td>TNF</td>
<td>0.5 *0.03 0.3 0.2 0.4 0.07</td>
<td>0.2 0.3</td>
</tr>
<tr>
<td>IL-6</td>
<td>-0.06 0.2 -0.4 0.09 0.2 0.4</td>
<td>0.4 0.1</td>
</tr>
</tbody>
</table>
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.
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.
3.4 Discussion

This study showed that the expression of TLRs and pro-inflammatory cytokines were increased in rats that developed irinotecan-induced diarrhoea compared to rats that were resistant to developing diarrhoea, indicating a strong relationship between these inflammatory mediators and severity of mucosal injury. Previous studies have demonstrated conclusively that irinotecan causes diarrhoea in this model due to severe mucosal damage (Gibson et al., 2007; Stringer et al., 2009a). However, in these studies, rats receiving irinotecan did not always develop diarrhoea, mimicking the variable patient response seen clinically. As such, this implies that there is an association between diarrhoea occurrence and level of these inflammatory mediators.

TLR4 expression was strongly induced in the colon of rats with diarrhoea at 96 h. This finding was consistent at the mRNA and protein level. The effect was less pronounced in rats that developed diarrhoea at 72 h. However, a similar change in the jejunum was not seen, which indicates regional differences in the expression of these inflammatory mediators. TLR2, TLR4 and pro-inflammatory cytokine increased expression coincided with peak histological injury. At 96 h, the highest pathogen exposure, inflammation and intestinal pathogen microflora have been previously reported to occur (Logan et al., 2008a; Stringer et al., 2009a; Al-Dasooqi et al., 2010; Al-Dasooqi et al., 2011a). Previous work in our laboratory on the same samples showed that histological damage in the jejunum at 72 h included villous blunting, epithelial atrophy and increased inflammatory infiltration (Logan et al., 2008a; Al-Dasooqi et al., 2010). At 96 h, increased mitotic figures and occasional apoptotic bodies were observed (Stringer et al., 2009a). In the colon, complete crypt ablation was observed at 72 h following treatment (Al-Dasooqi et al., 2010). Extensive histological changes were evident in the colon at 96 h, with patchy crypt degeneration, dilated crypts with attenuated epithelium and few desquamated epithelial cells and polymorphonuclear cells in the lamina propria (Stringer et al., 2009a). Therefore, the increase in TLR2, TLR4 and pro-inflammatory cytokines is associated with increased histological damage in the colon at 96 h following irinotecan treatment. In these studies, irinotecan increases the histological damage. However, histological damage does not correlate with diarrhoea very well at 96 h. This suggests that the expression of TLRs and pro-inflammatory cytokines seems to correlate better with diarrhoea at this time point.
Our results showed lack of correlation between diarrhoea and gene expression at 72 h. This could due to the small sample size (n=2) at this time point or related to the differences in the features of the histopathology at 72 and 96 h as discussed previously.

The increased expression of TLRs and pro-inflammatory cytokines coincided with an increase in inflammation in the colon at the same time point in the same DA rat samples (Al-Dasoqi et al., 2010). Matrix metalloproteinases (MMPs), which play a role in inflammation and tissue injury, were up-regulated following irinotecan administration in DA rats (Al-Dasoqi et al., 2010). In the jejunum, MMP1 and MMP12 protein levels were increased at 96 h (P < 0.05) and were consistent with inflammatory cell infiltration during tissue primary damage while only MMP9 was up-regulated at 72 h (P < 0.05). In the colon, MMP1 was up-regulated at 72 h while MMP2, 9 and 12 levels were increased at 96 h (P < 0.05), which play a role in inflammation and maximal morphological damage. These findings support our results by showing an increase in inflammatory mediators in DA rats at 96 h. Moreover, NF-κB immunostaining, in the colon, has been shown to be increased significantly at 72 h following irinotecan treatment compared to control (P < 0.0001) in DA rats (Logan et al., 2008a). However, no study has investigated NF-κB, IL-1β, TNF and IL-6 protein expression at 96 h after irinotecan treatment in this model. A direct comparison between mRNA and protein levels of the inflammatory mediators had not previously been completed in this rat model. As such, it was surprising to find that there was lack of correlation between mRNA and protein in this study. The increase in infiltrating immune cells may explain the contradiction in our results between mRNA and protein levels. In mRNA expression, infiltrating immune cells could contribute to mRNA signal since RT-PCR analysis was conducted on whole tissue lysates. While in the immunostaining, epithelial cell protein expression was only evaluated. This approach excluded any positive staining of immune cells located in the lamina propria or submucosa which may have contributed to the overall expression.

Since TLRs respond to PAMPs derived from intestinal pathogenic microflora, it is conceivable that the increased expression of TLRs could be a result of altered colonic microflora. Previous research in our laboratory on the same samples has shown that the association between TLR and pro-inflammatory cytokines and diarrhoea occurrence at 96 h coincided with changes in the colon microflora, which play a role in TLR2 and TLR4 activation (Stringer et al., 2007a; Wang et al., 2010). It has been shown that intestinal microflora, especially E. coli, was increased in the colon at 96 h in DA rats following irinotecan administration which was associated with extensive
histological changes and diarrhoea severity (Stringer et al., 2009a). Similarly, at the same time point, an increase in β-glucuronidase, which is produced by E. coli, was observed in the colon while lower activity was detected in the jejunum (Takasuna et al., 1996; Stringer et al., 2009a). The increase in E. coli is likely to be a stimulator for TLR2 and TLR4 through LPS, which comprises part of the outer membrane of E. coli and is recognised by TLR2 and TLR4 (Beran et al., 2011). Therefore, the augmentation of E. coli in the colon may contribute to TLRs and pro-inflammatory cytokine up-regulation through this mechanism. Moreover, the increase in the TLRs and pro-inflammatory cytokines at 96 h coincided with diarrhoea severity, which could represent the ulceration phase of mucositis pathogenesis in this animal model, compared to 72 h.

Results from this study showed an increase in mRNA and protein levels of TLR2, TLR4 and IL-1β in the colon, while the jejunum showed an increase in TNF protein expression. The different results between jejunum and colon may due to several reasons, including differences in the structure and function, absorption rates, or differences in β-glucuronidase levels between both regions. Even though it has been proposed that the alimentary tract is formed from one structure embryologically, there are alterations in the histological structure between jejunum and colon to conform to the functional differences between both regions (Burkitt, 1994). Diarrhoea generally occurs as result of increased solute in the intestinal lumen which leads to osmotic movement of water into the lumen (Stringer et al., 2007a). Chemotherapy-induced diarrhoea is likely to be multifactorial, including alteration in gut motility resulting in less water absorption, colonic damage and changes in the microflora, which can all affect absorption and fluid transport (Stringer et al., 2007a). Under normal conditions, it is well known that the microflora is higher in the colon compared to the jejunum (Stringer et al., 2007a), suggesting that the microbe-epithelium interactions will be different in both regions. Moreover, previous studies have shown that irinotecan-induced diarrhoea is attributed to colonic damage and suggests that the microflora, especially E. coli, has significant effects on the development of diarrhoea through the hydrolysis of SN-38G, via β-glucuronidase, to SN-38 which may increase the level of toxicity (Takasuna et al., 1996; Stringer et al., 2009a). This enzymological effect is supported by the histological damage demonstrated in animal models. Therefore, the interaction between the microflora and the epithelium may differ in the jejunum and the colon because of the anatomical differences and β-glucuronidase levels. Another reason for these differences may be due to the increase in E. coli, as a LPS producer, in the colon compared to jejunum leading to the increase in TLRs and pro-inflammatory cytokines in the colon.
Our results showed an increase in TNF protein expression, but not mRNA, in the jejunum of rats that developed diarrhoea at 96 h. This suggests that these changes were post-translational in the jejunum. Similarly, Uddin, et al (2013) reported the lack of concordance between TLR2, TLR3 and TLR9 mRNA and protein levels between tissues from the gastrointestinal mucosa (Uddin et al., 2013). It was proposed that this could due to the effect of microRNA and epigenetics (O’Gorman et al., 2010; Uddin et al., 2013). Previous evidence has shown that LPS-induced up-regulation of pro-inflammatory cytokines, such as TNF and IL-6, can be inhibited at transcriptional levels through different mechanisms, such as preventing ubiquitination of NF-κB signalling (Chang et al., 2012b). TNF receptor-associated factor 3 (TRAF3) negatively regulates pro-inflammatory cytokines in both innate and adaptive immune signalling pathways. In the case of TLR4 stimulation, degradation of TRAF3 and ubiquitination promote the cytosolic translocation of MyD88-dependent signalling pathway and lead to the activation of NF-κB (Hildebrand et al., 2011). Hence, TRAF3 plays a role in the post-translational modification in TLR4 regulation. These could explain, in part, the post-translational activation of TNF in the jejunum. IL-1β is regulated by two steps: pro-IL-1β, which is synthesized in a TLR-dependent manner and IL-1β maturation that is activated independently of TLR signalling (Schroder and Tschopp, 2010). IL-6 has pro- and anti-inflammatory effects (Moller and Villiger, 2006). It is induced by IL-1 and TNF and can form a negative feedback loop inhibiting pro-inflammatory cytokines activation cascade.

This study investigated the role of TLR2, TLR4 and pro-inflammatory cytokines in irinotecan-induced-GIM. It showed an association between colonic TLRs and pro-inflammatory cytokines and diarrhoea occurrence and severity at 96 h. However, it did not investigate other TLRs, such as TLR9, and different time points, that present other phases of GIM. Global tissue expression can be investigated in addition to epithelial cells to help elucidate the effect of non-epithelial mediators of damage. Western blotting of downstream markers within activated pathways can also be investigated in the future to determine the factors that regulate signalling between ligand receptor (TLR) and injury effectors (cytokines). It should be acknowledged that the n values were low in this study and larger sample sizes could be investigated in the future to get more statistical differences. Finally, further studies are needed to explore TLRs correlations with GIM after treatment with different chemotherapy agents to test the robustness of these findings.
Chapter Four
4.0 The effect of the TLR2 and TLR4 inhibitor, amitriptyline (AMI) on irinotecan-induced GIM

4.1 Introduction:

Cancer treatment is associated with many complications, such as gastrointestinal mucositis (GIM), which is characterised by ulceration and inflammation along the lower alimentary tract. Cancer treatment carries a risk of GIM of up to 40% in cancer patients after standard dose chemotherapy and 100% after high dose chemotherapy (Keefe et al., 1997; Keefe et al., 2000). Patients with GIM suffer from abdominal pain, bloating, nausea, vomiting and diarrhoea (Keefe et al., 1997). They are often at increased risk of weight loss and infection and often need nutritional support (Elting et al., 2003). These symptoms lead to clinical consequences including, dose reduction or temporary discontinuation of the treatment until the patient recovers from the side effects (Elting et al., 2003; Elting et al., 2007). Currently there are limited effective interventions for GIM.

Irinotecan (CPT-11), a topoisomerase-I inhibitor, causes diarrhoea in approximately 60% of cancer patients (Borner et al., 2005). This limits treatment frequency, dosage and therefore may impact on survival (Sonis et al., 2004). Irinotecan is known to cause two distinct types of diarrhoea clinically, characterised as early and late onset. Early-onset diarrhoea occurs within 24 h and involves an adverse cholinergic reaction which can be prevented by atropine administration in patients (Stein et al., 2010). Late-onset diarrhoea appears after 24 h and is due to gastrointestinal mucosal damage. The exact mechanism of late-onset diarrhoea is still unclear. Irinotecan is a pro-drug that is converted by carboxylesterases to the active metabolite 7-Ethyl-10-hydroxy-camptothecin (SN-38). Detoxification of SN-38 occurs by hepatic and intestinal UDP-glucuronosyltransferase 1A1 (UGT1A1), UGT1A9 and UGT1A10. UGTs catalyse glucuronidation of SN-38 (SN-38G), which then undergoes biliary secretion. After its release into the gastrointestinal tract, SN-38G is subject to bacterial β-glucuronidase, which is able to convert it back to active SN-38 (Takasuna et al., 1996; 1998; Yamamoto et al., 2008). Inhibition of the bacterial enzyme β-glucuronidase protected mice from irinotecan-induced diarrhoea, implicating commensal bacteria in the aetiology of damage (Wallace et al., 2010). Recently, however, it was reported that intestinal expression of the UGT1A protein is crucial for SN-38 detoxification, as the absence of intestinal UGT1A1-mediated glucuronidation resulted in increased toxicity (Chen et al., 2013). As such, irinotecan-induced
toxicity is a complex phenomenon that can be modified at multiple regulatory steps during metabolism and excretion.

Research using animal models has led to considerable progress in understanding the pathobiology of irinotecan-induced GIM (Gibson et al., 2003; Stringer et al., 2007a; Stringer et al., 2007b; Logan et al., 2008a; Stringer et al., 2009a; Stringer et al., 2009b; Al-Dasooqi et al., 2010; Al-Dasooqi et al., 2011a). The occurrence and severity of irinotecan-induced diarrhoea, in dark agouti (DA) rats, was found to be associated with increased apoptosis and reduced proliferation, histological damage, increased inflammation, changes in the microflora, activation of multiple signalling pathways and up-regulation of nuclear factor kappa (NF-κB), which up-regulates many genes involved in mucositis development, including pro-inflammatory cytokines, tumour necrosis factor (TNF), interleukin-1 beta (IL-1β) and interleukin-6 (IL-6) in the intestines (Gibson et al., 2003; Stringer et al., 2007a; Logan et al., 2008a; Al-Dasooqi et al., 2010). Increased expression of inflammatory mediators is associated with histological damage, including crypt hypoplasia, villous atrophy and altered intestinal permeability (Logan et al., 2008a; Nakao et al., 2012), making it a key target for intervention.

The Toll-like Receptor (TLR) signalling pathway is among the pathways that have been identified as being activated in response to irinotecan treatment (Bowen et al., 2007b). Activation of TLRs either by invading pathogens or endogenous danger signals leads to NF-κB and pro-inflammatory cytokine up-regulation that is linked to GIM development (Brown et al., 2011). Mounting evidence has shown the involvement of TLR2 and TLR4 in epithelial barrier function and response to mucosal injury (Cario et al., 2007; Karczewski et al., 2010). TLR2 controls tight junction (TJ)-associated intestinal epithelial barrier integrity to balance mucosal homeostasis against inflammation (Cario et al., 2007), however, it has been shown that TLR2 deficiency has a protective effect against chemotherapy-induced mucosal injury by reducing apoptosis and severity of intestinal damage in a model of GIM (Kaczmarek et al., 2012). TLR4 also plays an important role in inflammation, proliferation and apoptosis in the intestinal response to epithelial injury (Fukata and Abreu, 2007), although TLR4 deficiency has yet to be investigated in models of GIM. Conversely, TLR4 and MyD88 signalling can have a protective effect from apoptosis and enhance proliferation in DSS-induced intestinal injury through activation of anti-inflammatory macrophages (M2) or cyclooxygenase (COX-2) and prostaglandin E2 (PGE2) (Fukata et al., 2006; Zheng et al., 2009). Therefore, both TLR2 and TLR4 have harmful and protective effects
depending on the stimuli, the cell type and the activated downstream pathways. Despite the improvement in the understanding of GIM pathology, few studies have investigated the role of TLR inhibition on chemotherapy-induced GIM. Previous research has shown 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 (Ferreira et al., 2012; Kaczmarek et al., 2012; Hamada et al., 2013). Research conducted in our laboratory has shown the association of TLR2, TLR4 and pro-inflammatory cytokine expression in the colon of DA rats with diarrhoea occurrence and severity at 96 h after irinotecan treatment (Chapter two results from this thesis). This implies that TLR2 and TLR4 play a role in mediating irinotecan-induced mucosal injury and provide a rationale target for intervention.

Amitriptyline (AMI), a tricyclic antidepressant drug, is widely used in the management of psychological disorders and various types of pain, including neuropathic pain (Banerjee et al., 2013). AMI is mainly metabolised by the hepatic microsomal enzymes CYP2D6, CYP2C19 and CYP3A4 (Olesen and Linnet, 1997; Venkatakrishnan et al., 2001). However its long-term use is associated with adverse effects including, drowsiness, dizziness, mouth dryness, nausea and constipation (Mishra et al., 2012). Moreover, it is used in inflammatory bowel diseases and gastrointestinal disorders due to its anti-inflammatory effects (Bahar et al., 2008; Vahedi et al., 2008; Sohn et al., 2012). AMI reduces irritable bowel syndrome symptoms, including diarrhoea, in 66% of patients when given with probiotics (Sohn et al., 2012). AMI, and its metabolite nortriptyline, are able to decrease the release of nitric oxide, NF-κB, TNF, and IL-1β in glial cells (Obuchowicz et al., 2006; Vismari et al., 2012). In vitro, AMI blocked TLR4-related AKT activation in response to LPS, in a Myd88-dependent manner, resulting in pro-inflammatory cytokine inhibition (Hutchinson et al., 2010b). It was suggested that AMI possibly interacts with Myeloid differentiation factor-2 (MD-2) to inhibit TLR2 and TLR4 signalling (Hutchinson et al., 2010b). These findings suggest a possible inhibitory effect of AMI on intestinal TLR2 and TLR4 signalling, which would modulate irinotecan-induced GIM by depressing the response of pro-inflammatory cytokines. Despite the extensive use of AMI in clinical practice, there are limited data with regard to its effect on the gastrointestinal mucosa and particularly GIM. In this study, we hypothesised that TLR2 and TLR4 inhibition, via AMI, could modulate the effect of irinotecan-induced GIM. The primary aim of this study was to investigate the effect of TLR2 and TLR4 inhibition, through AMI, on irinotecan-induced GIM in Wistar rats. This study investigated the effect of AMI on GIM symptoms, histological damage, apoptosis and proliferation, inflammation, protein and mRNA expressions of TLRs and pro-inflammatory cytokines.
4.2 Methodology:

4.2.1 Drugs

AMI (Sigma-Aldrich, Castle Hill, Australia) was made up in distilled water. All rats received 0.01 mg/kg subcutaneous atropine (Pfizer, NY, U.S.A) immediately prior to irinotecan treatment to reduce the cholinergic reaction to irinotecan (Hyatt et al., 2005). Irinotecan (CPT-11), kindly supplied by Pfizer, Kalamazoo, USA, was prepared in a sorbitol/lactic acid buffer (45 mg/ml sorbitol, 0.9 mg/ml lactic acid, pH 3.4), required for the activation of the drug, hereafter referred to as vehicle.

4.2.2 Experimental plan

The study was approved by the Animal Ethics Committees of The University of Adelaide (approval number:M-2012-026) and complied with the 2014 National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training. Animals used in this study were male albino Wistar rats aged between 6-8 weeks and weighing 184-300 g. Rats were kept in room at 21 ± 1 ºC and subject to 14:10 h light-dark cycle. Seventy two rats were randomly divided into four groups: 1) CPT-11 (n = 18); 2) AMI (n = 18); 3) CPT-11+AMI (n = 18); and 4) control (n = 18). All rats received the same number of intraperitoneal (i.p) injections to control for stress, which were administered in the morning and in the afternoon, 8 h apart. Rats in group 1 received i.p water injection on the first day and i.p injections of irinotecan (125 mg/kg) and water the following day (designated 0 h). Group 2 received i.p injections of AMI (20 mg/kg) on the first day (-24 and -16 h) then vehicle and AMI injections on the second day (0 h). Group 3 received AMI injections in the first day (-24 and -16 h) then irinotecan and AMI injections on the second day (0 h). Group 4 received water injections in the first day (-24 and -16 h) and vehicle and water injections on the second day (0 h) (Figure 4.1). The irinotecan dose (125 mg/kg) used in this study has been shown previously in our laboratory to cause diarrhoea in male Wistar rats (Gibson, 2010). Repeated dosing of AMI injections were conducted to ensure the inhibition of TLRs and pro-inflammatory cytokines prior irinotecan injection, personal communication with Ass Prof. Mark Hutchinson, University of Adelaide. AMI dose (20 mg/kg) used in this study has been
shown previously to inhibit serum levels of TNF and IL-1β in male Wistar rats with acute inflammation (Sadeghi et al., 2011; Vismari et al., 2012).
Figure 4.1: Study design showing the four groups of the experiment. Irinotecan (CPT-11), amitriptyline (AMI), CPT-11+ AMI and vehicle. Red arrow (↑) shows CPT-11 injection times, purple arrow shows AMI injection times, blue arrow shows water injection times, green arrow shows vehicle injection times and X represents kill times.
Groups of rats (n = 6) were killed at 6, 48 and 96 h by cardiac puncture and cervical dislocation under 2% isofluorane in 100% O₂ anaesthesia. The jejunum and colon were dissected and flushed with chilled isotonic saline (0.9% w/v) to remove contents and 1 cm pieces of the jejunum and colon were taken at 33% and 50% of their length, respectively, for histological, immunohistochemistry and molecular analyses. For histological and immunohistochemistry analysis, all samples were fixed in 10% neutral buffered formalin before being processed and embedded in paraffin wax. For real-time PCR (RT-PCR) analysis, mucosal scraping of jejunum and colon tissues was stored in RNA later solution (Ambion, Life Technologies, Australia) at -70 °C. Microdissection samples were fixed in Clarke’s fixative (25% acetic acid in 70% ethanol) for 24 h then transferred to 70% ethanol at room temperature for storage.

### 4.2.3 GI toxicity and distress symptoms

Animals were monitored four times daily to record GI toxicity (diarrhoea and weight loss) and distress symptoms. Diarrhoea was graded as none, mild (staining of anus), moderate (staining of top of legs and lower abdomen) and severe (staining over legs and higher abdomen as well as continual anal leakage). This grading has been published previously and is used routinely in our lab (Gibson et al., 2003; Stringer et al., 2007b; Logan et al., 2008a; Al-Dasooqi et al., 2010). Rats were weighed once each day during the experiment.

Distress symptoms included a dull/ruffled coat, changes in temperament and reluctance to move. Each symptom was scored from 0-3. This grading, as defined by the Animal Ethics committee of University of Adelaide, was used in this study (Table 4.1). The total score for all criteria was averaged at each time point.
Table 4.1

Scoring of distress symptoms

<table>
<thead>
<tr>
<th>Symptom</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dull/Ruffled coat</td>
<td>Shiny coat</td>
<td>Coat raised around neck only</td>
<td>Coat raised around neck and belly</td>
<td>Coat raised over entire animal with or without loss of fur</td>
</tr>
<tr>
<td>Change in temperament</td>
<td>Normal disposition</td>
<td>Agitated</td>
<td>Stress marks on eyes/nose/paws</td>
<td>Stress marks with hunching</td>
</tr>
<tr>
<td>Reluctant to move</td>
<td>Movement as lift the lid</td>
<td>Movement once hand placed near/on rat</td>
<td>Movement only after picking up rat</td>
<td>No movement when handling rat</td>
</tr>
</tbody>
</table>
4.2.4 Histological examination

Jejunum and colon samples were stained as previously described in chapter two. The occurrence of eight histological criteria in the jejunum was examined. 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 last six criteria were examined. Each parameter was scored as present = 1 or absent = 0. The average total score was plotted for each time point. Criteria were modified from an established grading system previously published in models of GIM (Howarth et al., 1996).

4.2.5 Tissue morphometry

Clarke’s fixed colon tissues were stored in 70% ethanol. For microdissection, tissues were rehydrated in a series of ethanol and water. Then samples were incubated in 1M HCl at 60 °C for 7 min. After washing, samples were stained with Schiff’s reagent for 45 min at room temperature. Tissue was microdissected using a cataract knife and stereomicroscope (Olympus SZ60, USA) to visualise single rows of crypts. Microdissected tissues were placed onto microscope slide, fixed with 45% acetic acid and coverslipped. Crypt length was measured at 200x magnification by light microscopy (Olympus BX51, USA).

4.2.6 Immunohistochemistry

All slides were stained and scored as previously described in chapter 2.

4.2.7 Caspase-3 and Ki-67

Polyclonal rabbit anti-caspase-3 was used for apoptosis detection and monoclonal rabbit anti-Ki-67 antibody was used as a marker for proliferation in this study (Table 4.2).
Sections from the jejunum and colon of all rats were cut at 4 µm thickness and mounted onto silane-coated slides. Sections were dewaxed in xylene and rehydrated in serial concentrations of ethanol (3x 100%, 90%, 70% and 50%). After washing sections with phosphate buffered saline (PBS), they were immersed in 10 mM citrate buffer (pH 6.0) and antigen retrieval was performed using a pressure cooker for 3 min on high pressure (The fast slow cooker-Breville, USA). Sections were allowed to cool at room temperature for 20 min then endogenous peroxidase was blocked using 3% H₂O₂ in methanol. Non-specific binding of antibodies was blocked with 20% normal goat serum for 20 min at room temperature. Then, avidin and biotin activity was blocked using Avidin-Biotin Blocking kit (Vector Laboratories, Inc, Burlingame, USA) following manufacturer instructions. Sections were incubated for an hour at room temperature with the primary antibodies caspase-3 and Ki-67, diluted to 1:100 with 5% normal goat serum. Then, sections were incubated sequentially with the secondary antibody, goat anti-rabbit IgG (Vector laboratories, Burlingame, USA), diluted to 1:200 with 5% normal goat serum for 30 min at room temperature then ABC kit solution for 30 min (Vector laboratories, Burlingame, USA) at room temperature. Antibody binding was visualised by application of 3,3’-diaminobenzidine (DAB) (Invitrogen, Canada) for 2 min at room temperature. The slides were washed with distilled water and counterstained with Lillie-Mayer’s haematoxylin, dehydrated and cleared in xylene before being coverslipped.

Slides were scanned using NanoZoomer 2.0-HT Slide Scanner (Hamamatsu, Hamamatsu city, Japan). Using NDP software, caspase-3 staining was assessed by counting positive cells in 15 fields (20x magnification) and calculating the mean number of positive cells/crypt for each sample. Ki-67 staining was assessed by counting stained cells/20 half crypts (40x magnification) and calculating the mean number of positive cells/half crypt. Moreover, the percentage of stained cells/half crypt was calculated as well. All assessments were done blinded to treatment.

**4.2.8 TLR2, TLR4 and pro-inflammatory cytokines immunohistochemistry**

TLR2, TLR4 and pro-inflammatory cytokines antibodies were used to investigate changes in their expression after AMI and irinotecan treatment (Table 4.2). Immunostaining and scoring were performed as previously described in chapter 2.
Chapter four: The effect of TLR2 and TLR4 inhibition on irinotecan-induced GIM

Table 4.2: Antibodies used in this study

<table>
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<th>Clone</th>
<th>Concentration µg/ml</th>
<th>Secondary antibody</th>
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<tr>
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<td>Multispecies Linking reagent</td>
<td>Abcam Cat no: ab7202</td>
</tr>
</tbody>
</table>
4.2.8.1 MPO and MCP-1 immunohistochemistry

Myeloperoxidase (MPO) is a peroxidase enzyme that is expressed in neutrophils and can be used as a marker for inflamed tissues. The monocyte chemoattractant protein-1 (MCP-1 or CCL2) is expressed in monocytes/macrophages and was found to be up-regulated in response to peptidoglycan and lipopolysaccharide (LPS)-induced TLR2 and TLR4 activation in intestinal epithelial cells (Lan et al., 2005; Moue et al., 2008). Therefore, immunohistochemistry for both markers was performed to compare inflammation between all groups.

Sections from the colon of all rats were cut and processed as described previously (chapter 2). MPO and MCP-1 antibodies were used at dilution of 1:50 to stain positive cells.

Positive cells were counted in 10 fields at 40x magnification in the lamina propria using the digital scanner NanoZoomer (Hamamatsu, Hamamatsu city, Japan).
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4.2.9 PCR array for innate and adaptive immunity in rat

4.2.9.1 RNA extraction and reverse transcription:

RNA was extracted and converted to cDNA as described in chapter 2.

4.2.9.2 PCR array

Rat innate and adaptive immunity array (Qiagen, Doncaster, Australia) was used to evaluate the expression of 84 genes involved in the host response to invading pathogens and endogenous danger signals. The PCR array was performed for CPT-11 and CPT-11+ AMI groups to detect differential gene expressions between both groups at 6 h since this time point showed significant differences in apoptosis between these two groups. 100 ng of cDNA/reaction was prepared for each run. First strand cDNA was mixed with RT SYBR Green ROX FAST mastermix (Qiagen, Doncaster, Australia) then the mixture was added into 100-well RT² profiler PCR Array following manufacturer instructions. Relative expression was calculated using delta delta C_t analysis. All genes were normalised with the housekeeping gene Lactate dehydrogenase A (Ldha) since this gene had the most stable expression within our samples from the housekeeping genes in the array kit.

4.2.10 Statistical analysis

Results were statistically analysed by the Kruskal-Wallis test for diarrhoea, distress symptoms, injury scores and protein expression (TLR2 and TLR4, pro-inflammatory cytokines and MCP-1) to compare medians between all groups. Body and intestinal weight change, caspase-3, Ki-76 and MPO results were analysed using one-way ANOVA with Bonferroni post-hoc test to compare means between all groups. Results were statistically significant at P < 0.05.
4.3 Results

4.3.1 Response to treatment and diarrhoea occurrence

GI toxicity:

Weight loss: rats treated with AMI alone lost $2.4 \pm 0.4$ percentage of body weight between -24 and 0 h of the experiment. By 24 h, rats returned to baseline and then continued to gain weight until the end of the study. CPT-11 treated rats lost $2.9 \pm 1.3$ percentage of body weight in the first 24 h after CPT-11 injections, accompanied with diarrhoea occurrence. Rats slowly then regained weight, returning to baseline by the end of the study. Rats that were treated with both drugs lost weight every day of the study until the end of the experiment. Vehicle control group rats did not lose weight. Weight loss was significantly greater in the group that was treated with both drugs compared to vehicle controls ($P = 0.002$) and the AMI group ($P = 0.01$). Weight loss for rats treated with CPT-11 was significantly greater compared to vehicle controls ($P = 0.04$). Weight loss between rats treated with CPT-1 alone and rats treated with both drugs was not significantly different (Figure 4.2).

Diarrhoea: rats in the CPT-11 alone group showed mild (61%) and moderate (6%) diarrhoea within 6 h of treatment. At 24 h, only 25% of rats continued to have diarrhoea, which was mild. No diarrhoea was seen at later time points in this group. Rats treated with both drugs exhibited mild diarrhoea (16%) at 24 h and moderate diarrhoea (16%) at 96 h. AMI rats showed no diarrhoea at any time point. Vehicle control rats had mild diarrhoea (16%) at 96 h. Diarrhoea incidence and severity was significantly higher in CPT-11 group rats compared to all other groups at 6 h ($P = 0.005$) (Figure 4.3).
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Figure 4.2: Percentage weight change compared to the starting weight at each time point for all groups. CPT-11 dose was 125 mg/kg and AMI dose was 20 mg/kg repeated three times (-24, -16 and 0 h). Data shown is mean ± SD, n = 6, *P < 0.05 vs. control, ***P < 0.001 vs. control and AMI groups.
Figure 4.3: Incidence of diarrhoea at different time points. CPT-11 (rats treated with irinotecan), AMI (rats treated with AMI), CPT-11+ AMI (rats treated with both drugs) and control (rats injected with vehicle and water). *P < 0.0005 vs. other groups, Chi square test.
Distress symptoms:

Rats treated with CPT-11 showed significantly increased distress scores at 6 h and 48 h after treatment compared to controls (P = 0.009 and P = 0.01, respectively). Rats treated with AMI alone showed significantly higher distress symptoms from -24 to 24 h compared to control (P < 0.0001, P < 0.0001 and P = 0.0103, respectively) then recovered gradually until the end of the experiment. Total distress scores were significantly higher for rats treated with both drugs when compared to control at -24, 0, 24, 48 and 96 h (P < 0.0001, P < 00001, P = 0.02, P = 0.0002 and P = 0.04, respectively). Distress scores were higher in the group that was treated with both drugs compared to CPT-11 alone group (P < 0.0001) and controls (P < 0.0001) at -24 h. There were no significant differences between rats treated with CPT-11 alone and rats treated with both drugs at 0, 24, 48, 72 and 96 h (Figure 4.4).

Jejunum and colon weight:

A significant reduction in jejunum weight was observed for CPT-11 alone group rats compared to controls (mean ± SD = 2.6 ± 0.3 g, P < 0.0001) and AMI (mean ± SD = 2.8 ± 0.3 g, P < 0.0001) groups at 48 h. Similarly, the jejunum weight was significantly lower in the group that was treated with both drugs compared to control (mean ± SD = 1.9 ± 0.3 g, P = 0.0002) and AMI (mean ± SD = 2.1 ± 0.3 g, P < 0.0001) groups at the same time point (Figure 4.5). There were no significant differences in the jejunum weight between the rats treated with CPT-11 alone and those treated with both drugs (P = 0.57 and P > 0.9, respectively). There were no significant differences in the colon weight between any groups (P > 0.9).
Figure 4.4: Total score of distress symptoms (dull/ruffled coat, change in temperament, reluctant to move) for each group at different time points. Each criteria was scored from 0 - 3. Data shown is mean ± SD, n = 6 *P < 0.05, **P < 0.01, ***P < 0.001 vs. control and #P < 0.0001 vs. CPT-11.
Figure 4.5: A. Jejunum and B. colon weight for control and all treated groups. Data shown is mean ± SD, n = 6. ***P < 0.001 for CPT-11 and CPT-11+AMI vs. AMI and control groups.
4.3.2 Histological analysis

Marked histological changes were observed in jejunum for CPT-11 treated rats. In the jejunum, changes were evident as early as 6 h with increased apoptotic epithelial cells located in the crypts. Significantly increased histological damage (median = 5.5) was observed in this group compared to the control at 48 h (P = 0.005) with villous atrophy and blunting, and disruption of crypt cells. At 96 h, crypt hyperplasia was observed. There were no significant differences between groups treated with AMI or both drugs and control (Figure 4.6 and 4.7).

In the colon, the most pronounced histological damage was observed in the group that was treated with both drugs. At 6 h, disruption in crypt cells was evident, and at 48 h, complete crypt ablation with oedema was observed in this group. The histological damage was significantly higher in this group compared to control at 6 (median = 4) and 48 h (median = 4.5) (P = 0.01 and P = 0.03, respectively). It should be noted that increased oedema was also observed in the colon of rats treated with AMI alone. There were no significant differences between CPT-11, AMI and control groups (Figure 4.6 and 4.8).
Figure 4.6: Histological analysis in (A) the jejunum and (B) colon. In the jejunum, eight criteria were examined: 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 last six criteria were examined. Each parameter was scored as present = 1 or absent = 0. Data shown are medians with range, n = 6. *P < 0.05, **P < 0.01 vs. control.
Figure 4.7: Hematoxylin and eosin (H&E) staining illustrating histopathological damage in the jejunum at various time points for all groups. Photomicrographs are 200x original magnification. In CPT-11 group, arrow at 6 h shows apoptosis in the crypt, at 48 h shows crypt ablation and at 96 h shows crypt hyperplasia. In CPT-11+AMI group, arrow shows inflammatory infiltrate in the lamina propria at 48 h. Scale bar is 50 µm.
Figure 4.8: H&E staining illustrating histopathological damage in the colon at various time points for control and treatment groups. Arrow shows apoptotic bodies in CPT-11 and CPT-11+AMI groups at 6 h. Oedema observed in CPT-11+AMI group at 48 h as indicated by arrow. Scale bar is 50 µm.
4.3.3 Morphological changes in colon

Treatment with CPT-11 did not cause crypt shortening in this model. Furthermore, crypt length did not differ significantly between groups at any time point (Figure 4.9). At 48 h, median crypt length of CPT-11 treated rats was 234 µm, AMI 241 µm, both drugs 267 µm and control 230 µm ($P = 0.7$).
Figure 4.9: Crypt length in the colon for all experimental groups and control. Data are median with range, n = 6.
4.3.4 Apoptosis and proliferation

4.3.4.1 Apoptosis

The staining was nuclear for caspase-3 and Ki-67. Positively stained cells were counted as described in the methods.

In the jejunum, there was a significant increase in the number of caspase-3 positive apoptotic cells per crypt in CPT-11 group (mean ± SD = 2.5 ± 0.56 cells/crypt) and the group that was treated with both drugs (mean ± SD = 2.2 ± 0.49 cells/crypt) compared to control (mean ± SD = 0.03 ± 0.02) and AMI (mean ± SD = 0.04 ± 0.02 cells/crypt) groups at 6 h post treatment (P < 0.0001). At 48 h, caspase-3 positive apoptotic cells increased significantly in the group that was treated with CPT-11 (mean ± SD = 0.2 ± 0.03) compared to AMI (mean ± SD = 0.07 ± 0.06 cells/crypt, P = 0.003) and control (mean ± SD = 0.04 ± 0.01 cells/crypt, P = 0.0008). Similarly, apoptosis increased significantly in rats treated with both drugs (mean ± SD = 0.2 ± 0.09 cells/crypt) compared to AMI (P = 0.002) and control (P = 0.0004). Apoptosis was also increased in the group that was treated with both drugs (mean ± SD = 0.4 ± 0.1 cells/crypt) compared to AMI (mean ± SD = 0.06 ± 0.02, P = 0.0003) and control (mean ± SD = 0.09 ± 0.03, P = 0.002) at 96 h (Figure 4.10 A).

In the colon, at 6 h, there was a significant increase in apoptotic cells in the group that was treated with CPT-11 (mean ± SD = 1.8 ± 0.6 cells/crypt) compared to the groups that were treated with both drugs (mean ± SD = 0.6 ± 0.6 cells/crypt, P = 0.001), AMI (mean ± SD = 0.2 ± 0.1 cells/crypt, P < 0.0001) or control (mean ± SD = 0.1 ± 0.05 cells/crypt, P < 0.0001) (Figure 4.10 B and 4.11). Apoptosis increased in the group that was treated with AMI compared to control at 48 h (P = 0.04). There were no significant differences at 96 h between any experimental groups.
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. The data are mean number of stained cells/crypt ± SD, n = 6. *P < 0.05, ***P < 0.001 and ****P < 0.0001 compared to control, ##P < 0.01, ###P < 0.001 and ####P < 0.0001 compared to AMI group, ^^P < 0.01 compared to CPT-11+AMI group.
Figure 4.11: Caspase-3 immunohistochemistry in the colon at 6 h in all experimental groups. Negative control presents slides stained with secondary antibody only. Photomicrographs are at 200 x original magnification. Arrow is showing postively stained cell. Scale bar is 50 µm.
4.3.4.2 Proliferation

Jejunum:

Results were presented as the average of the actual number of Ki-67 positively stained cells/half crypt (Figure 4.12). Rats administered CPT-11 alone had significantly reduced proliferative crypt cells (Ki67-positive) at 6 h (mean ± SD = 24.5 ± 1.6 cells/half crypt) compared to AMI treated rats (mean ± SD = 32.2 ± 4.1 cells/half crypt, P = 0.01). At 48 h, proliferation was still significantly inhibited in CPT-11 rats (mean ± SD = 20.2 ± 4.1 cells/half crypt) compared to AMI treated rats (mean ± SD = 29 ± 1.8 cells/half crypt, P < 0.0001) and also compared to control rats (mean ± SD = 31±2.2 cells/half crypt, P = 0.0006). This was followed by significant increase in proliferation at 96 h (mean ± SD = 45.2 ± 3.3 cells/half crypt) compared to AMI (mean ± SD = 31.6 ± 2, P = 0.003) and control (mean ± SD = 29.6 ± 3.9, P = 0.0008). The group that was treated with both drugs did not show changes in proliferation compared to control at 6 h but showed a significant decrease in proliferation at 48 h (mean ± SD = 17.3 ± 3 cells/half crypt) compared to AMI (P < 0.0001) and control (P < 0.0001). A significant increase in proliferation was noted at 96 h compared to control (P = 0.04). There were no significant differences in Ki-67 protein expression between rats treated with CPT-11 and the rats treated with both drugs at any time point.

Colon:

Following CPT-11 treatment, proliferation decreased at 6 h as indicated by Ki-67 positively stained cells (mean ± SD = 14.5 ± 2.3 cells/half crypt) and 48 h (mean ± SD = 10.2 ± 3.9 cells/half crypt) then increased at 96 h (mean ± SD = 19 ± 2.9 cells/half crypt). Similarly, rats treated with both drugs showed a decrease in proliferation at 6 h (mean ± SD = 11.7 ± 1.5 cells/half crypt) followed by slight increase at 48 h (mean ± SD = 14.8 ± 7 cells/half crypt) and 96 h (mean ± SD = 18.1 ± 6.3 cells/half crypt). However, there were no significant differences between any groups at any time point (Figure 4.12 and 4.13).
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. Data are mean ± SD, n = 6. #P < 0.05, ##P < 0.01 and ####P < 0.0001 compared to AMI group. *P < 0.05 and ***P < 0.001 compared to control.
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). Arrow shows positively stained cell. Photomicrographs are 200 x original magnification. Scale bar is 50 µm.
4.3.5 Protein expression of TLR2, TLR4 and pro-inflammatory cytokines in the jejunum and colon

The staining was cytoplasmic for TLRs and pro-inflammatory cytokines and was assessed in the epithelial layer only.

TLR2:

There were no significant differences in the staining intensity between groups in the crypt and villi regions of the jejunum. TLR2 protein expression did not differ in the crypt between any group at 6, 48 and 96 h (P = 0.1, 0.8 and 0.3, respectively). Similarly, TLR2 staining in the villi did not show significant differences between any group at 6, 48 and 96 h (P = 0.6, 0.7 and 0.8, respectively) (Figure 4.14 and 4.15).

In the colon, TLR2 staining in the apical layers was not significantly different between any group at 6 and 96 h (P = 0.1 and 0.9, respectively). TLR2 staining intensity showed an increase in the apical region of the crypt in the AMI group compared to controls at 48 h (P = 0.04). In the basal region of the crypt, there were no significant differences in TLR2 staining at 6, 48 and 96 h (P = 0.4, 0.9 and 0.9, respectively) (Figure 4.16 and 4.17).
Figure 4.14: Changes in TLR2 staining in the jejunum (A) crypt and (B) villi regions at 6, 48 and 96 h. All samples were graded by a qualitative scale. Data shown with median indicated by line, n = 6.
Figure 4.15: TLR2 immunostaining in the jejunum for all groups. Photomicrographs are 200x original magnification. Scale bar is 50 µm.
Figure 4.16: Changes in TLR2 immunostaining in the colon (A) apical and (B) basal regions at 6, 48 and 96 h. All samples were graded by a qualitative scale. Data shown with median indicated by line, n = 6, *P < 0.05.
Figure 4.17: TLR2 immunostaining in the colon for all groups. Photomicrographs are 400x original magnification. Scale bar is 50 µm.
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TLR4: No significant differences in the staining intensity were observed at any time points in the crypt (P = 0.1, 0.5 and 0.2, respectively) and villi regions of the jejunum (P = 0.3, 0.8 and 0.09, respectively) between groups (Figure 4.18 and 4.19).

In the colon, TLR4 levels in the apical layer did not differ significantly between any group at 6, 48 and 96 h (P = 0.1, 0.6 and 0.8, respectively). Similarly, in the basal layer, TLR4 protein expression was not statistically significant between any groups at 6, 48 and 96 h (P = 0.4, 0.9 and 0.9, respectively) (Figure 4.20 and 4.21).
Figure 4.18: Changes in TLR4 staining in the jejunum (A) crypt and (B) villi regions at 6, 48 and 96 h. All samples were graded by qualitative scale. Data shown with median indicated by line, n = 6.
Figure 4.19: TLR4 immunostaining in the jejunum of all groups. Photomicrographs are 200x original magnification. Scale bar is 50 µm.
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. All samples were graded by qualitative scale Data shown with median indicated by line, n = 6.
Figure 4.21: Tissue immunostaining of TLR4 in the colon for all groups at different time points. Photomicrographs are 400x original magnification. Scale bar is 50 µm.
IL-1β:

In the jejunum, protein expression of IL-1β did not differ significantly between any group in the villi (P > 0.05) and crypts (P > 0.05) (Figure 4.22 and 4.23).

In the colon, there were no significant differences in IL-1β protein levels between any group in both apical (P > 0.05) and basal regions of the crypt (P > 0.05) (Figure 4.24 and 4.25).
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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. All samples were graded by qualitative scale. Data shown with median indicated by line, n = 6.
Figure 4.23: IL-1β immunostaining in the jejunum of all groups. Photomicrographs are 200x original magnification. Scale bar is 50 µm.
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. All samples were graded by qualitative scale. Data shown with median indicated by line, n = 6.
Figure 4.25: IL-1β immunostaining in the colon for all groups. Photomicrographs are 400x original magnification. Scale bar is 50 µm.
TNF:

In the jejunum, no significant differences in TNF protein expression were observed between any group at 6, 48 and 96 h (P = 0.1, 0.1 and 0.8, respectively). TNF expression, similarly, was not statistically different between groups at all-time points (P = 0.1, 01 and 0.6, respectively) (Figure 4.26 and 4.27).

In the colon, TNF staining in the apical region of the crypt was more intense in the irinotecan group compared to the control at 6 (P = 0.04) and 48 h (P = 0.01). No significant differences were observed between any groups in basal crypt TNF staining (Figure 4.28 and 4.29).
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. All samples were graded by qualitative scale. Data shown with median indicated by line, n = 6.
Figure 4.27: TNF immunostaining in the jejunum of all groups. Photomicrographs are 200x original magnification. Scale bar is 50 µm.
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. All samples were graded by qualitative scale. Data shown with median indicated by line, n = 6. *P < 0.05 vs. control.
Figure 4.29: TNF immunostaining in the colon for all groups. Photomicrographs are 400x original magnification. Scale bar is 50 µm.
IL-6:

IL-6 protein expression was not statistically significant between any groups in the crypt (P > 0.05) and villi of the jejunum (P > 0.05) (Figure 4.30 and 4.31).

In the colon, similarly, there were no statistically significant differences in IL-6 protein expression between any groups (P > 0.05) (Figure 4.32 and 4.33).
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. All samples were graded by qualitative scale. Data shown with median indicated by line, n = 6.
Figure 4.31: IL-6 immunostaining in the jejunum of all groups. Photomicrographs are 200x original magnification. Scale bar is 50 µm.
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. All samples were graded by qualitative scale. Data shown with median indicated by line, n = 6.
Figure 4.33: IL-6 immunostaining in the colon for all groups. Photomicrographs are 400x original magnification. Scale bar is 50 µm.
4.3.6 Protein levels of MPO and MCP-1 in colon

Since AMI was effective at reducing irinotecan-induced apoptosis in the colon at 6 h, MPO and MCP-1 staining was conducted in this region to investigate treatment effect on neutrophils and macrophages and thus inflammation. The staining was scored in the lamina propria (representative images shown in appendix). There were no significant differences in MPO immunostaining between all groups at 6, 48 and 96 h (P = 0.8, 0.6 and 0.3 respectively). MCP-1 protein expression was not statistically different between groups at 6, 48 and 96 h (P = 0.8, 0.9 and 0.9, respectively) (Figure 4.34 and 4.35). The MPO cell count in the colon of rats treated with CPT-11 was 18 ± 9.8 +ve cells/field of view at 6 h then decreased to 12 ± 6.4 and 12 ± 7.2 +ve cells/field of view at 48 and 96 h, respectively. In rats treated with both drugs, MPO staining at 6 h was 12 ± 7 +ve cells/field of view then increased at 48 h to 14 ± 6.9 +ve cells/field of view but decreased to 9 ± 5.7 +ve cells/field of view at 96 h. Similarly, MCP-1 counting for rats treated with both drugs increased from 35 ± 16 +ve cells/field of view at 6 h to 43 ± 12 +ve cells/field of view at 48 h then decreased at 96 h to 25.5 ± 11 +ve cells/field of view.
Figure 4.34: MPO immunostaining in the colon at 6, 48 and 96 h. Data are mean ± SD. Field of view is defined as the average of the cells counted in 10 fields at 400x magnification in the lamina propria using the digital scanner for each sample.
Figure 4.35: MCP-1 immunostaining in the colon at 6, 48 and 96 h. Data are medians with range. Field of view is defined as the average of the +ve cells count in 10 fields at 400x magnification in the lamina propria using the digital scanner for each sample.
4.3.7 PCR array

PCR array results showed that the colonic mRNA expression of caspase-4, IL-1β and interleukin-1 receptor 2 (IL-1r2) decreased (1.8-, 13- and 4-fold, respectively) in rats treated with both drugs compared to rats treated with CPT-11 only at 6 h (P = 0.005, 0.038 and 0.01, respectively). However, interferon gamma receptor 1 (INFγR1) mRNA expression increased in rats treated with both drugs up to 1.4-fold change compared to CPT-11 group (P = 0.02). No changes in the mRNA expression of TLRs and pro-inflammatory cytokines were detected (Table 4.3).
Table 4.3

PCR array results comparing CPT-11+AMI group to CPT-11 group

<table>
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<th>Sig.</th>
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### Chapter four: The effect of TLR2 and TLR4 inhibition on irinotecan-induced GIM

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PCR: polymerase chain reaction.
4.4 Discussion:

This study was conducted to investigate the effect of AMI as an inhibitor of TLR2 and TLR4 and pro-inflammatory cytokines on CPT-11-induced GIM in Wistar rats. AMI is a prescribed antidepressant drug for cancer patients (Banerjee et al., 2013). Since it has anti-inflammatory effects, it is also prescribed for inflammatory bowel diseases (Bahar et al., 2008; Vahedi et al., 2008; Sohn et al., 2012). Moreover, AMI has been shown previously to inhibit TLR2 and TLR4 and pro-inflammatory cytokines (Hutchinson et al., 2010b). We hypothesised that AMI could modulate the gastrointestinal toxic effects of CPT-11 through the inhibition of TLR2 and TLR4 and pro-inflammatory cytokines in the intestines. Up to date no study has investigated the TLR inhibitory effect of AMI on CPT-11-induced GIM.

Key findings from this study indicated the ability of AMI to reduce early-onset diarrhoea occurrence and apoptosis in colonic epithelium 6 h after treatment with CPT-11. However, it was not protective from weight loss and distress symptoms. Intestinal protein and mRNA expression of TLRs and pro-inflammatory cytokines did not differ between rats treated with CPT-11 and rats treated with both drugs.

In this study, early-onset diarrhoea (within 24 h) was observed in over 50% of rats treated with CPT-11 alone. Previous evidence supports our findings of early-onset diarrhoea occurrence after treatment with CPT-11 despite pre-treatment with atropine which is designed to minimise the cholinergic response (Logan et al., 2008a). Diarrhoea was observed in approximately 50% of rats 24 h after treatment in a previous study using DA rats treated with 200mg/kg CPT-11 despite the administration of atropine (Logan et al., 2008a). However, late-onset diarrhoea was not observed in rats treated with CPT-11 in this study, which is the predominant form of diarrhoea expressed in the DA model. This could be due to the inherent differences between the two strains of rat and differences in the administered doses. The colonic damage observed in DA rats at 96 h following CPT-11 administration (200 mg/kg) was considerably higher than the damage observed in Wistar rats treated with CPT-11 (125 mg/kg) at the same time point. In DA rats, patchy crypt degeneration, dilated crypts with attenuated epithelium, polymorphonuclear cells in the lamina propria and increased crypt hyperplasia and mitotic figures were observed at 96 h (Stringer et al., 2009a). However, in Wistar rats from this study, there was no significant colonic damage in CPT-
11 treated rats at the same time point, indicating that late-onset diarrhoea and tissue damage are closely related. Interestingly, an i.p. dose of 200 mg/kg CPT-11 is fatal in male Wistar rats (Gibson, 2010), highlighting a substantial strain difference in sensitivity to irinotecan. It should be noted that one rat (out of 6) in the vehicle control group had diarrhoea. It is well known that an over dose of sorbitol can cause GI symptoms, including diarrhoea, in human and rat (Islam and Sakaguchi, 2006). However, injection of sorbitol/lactic acid buffer did not cause diarrhoea in DA rats previously (Logan et al., 2008a; Stringer et al., 2009a) and the timing of the diarrhoea onset makes it unlikely to be related to sorbitol.

The addition of AMI to CPT-11 treatment reduced acute diarrhoea occurrence at 6 h (0%) and 24 h (16%). The reduction in acute diarrhoea after treatment with both drugs could be due to the anticholinergic effect of AMI. AMI is known to have anticholinergic effects in vitro and in vivo (Richelson and Nelson, 1984; Warrington et al., 1989; Penttila et al., 2001). It reduces whole gut motility and increases colonic transit time (Santoro et al., 2000), yet it has only 2% of the anticholinergic activity of atropine (Chew et al., 2006). Given that atropine treatment in these rats was not sufficient to prevent early-onset diarrhoea entirely, AMI may be having an additive effect, or may prevent diarrhoea via a non-cholinergic mechanism. AMI has been shown previously to be effective in inflammatory bowel diseases with diarrhoea even though the exact mechanism behind it has not been fully investigated (Bahar et al., 2008; Vahedi et al., 2008). In this study, however, treatment with AMI+CPT-11 was associated with late-onset diarrhoea in 16% (1/6) of rats at 96 h, whilst rats treated with CPT-11 alone had no late-onset diarrhoea. In addition, animals treated with both drugs continued to lose weight up to 96 h, whereas CPT-11 alone rats had returned to baseline weight, indicating that AMI co-treatment was associated with an unidentified systemic toxicity manifesting as weight loss in these animals. As such, these findings do not support a role for AMI in prevention of CPT-11-induced late-onset diarrhoea.

A significant increase in apoptosis at 6 h was observed in both jejunum and colon of rats treated with CPT-11 compared to control. Our results are supported with previous studies showing that CPT-11 increases apoptosis at 6 h in the jejunum and colon of DA rats (Bowen et al., 2007a). It is well known that CPT-11, as a topoisomerase I inhibitor, arrests the replication of DNA by stabilising the cleavage complex of topoisomerase I and DNA. Consequently, double-strand DNA breaks accumulate and pathways in response to this stress will be activated. DNA damage is highly toxic to the cells and leads to cell death. The apoptotic pathway induced by CPT-11 in normal
intestinal mucosa is not fully understood (Merritt et al., 1997; Bowen et al., 2007a; Rudolf et al., 2012). In CPT-11 treated DA rats, apoptosis was associated with increases in p53, Bax and Bak expression in crypt epithelial cells (Bowen et al., 2007a). Mounting evidence has suggested that CPT-11-induced apoptosis can involve p53-independent and -dependant pathways (Merritt et al., 1997; Bowen et al., 2007a). In normal colonic epithelial cells, treatment with CPT-11 is sensed by ataxia telangietasia mutated (ATM) and Chk1 phosphorylation, which leads to p53 phosphorylation. Up-regulated expression of p53 activates p38 kinase and initiates caspase-dependant apoptosis (Rudolf et al., 2012). This was represented by the increase in crypt epithelial cells expressing caspase-3 6 h following CPT-11 administration.

The addition of AMI was able to reduce CPT-11-induced apoptosis in colonic epithelium. Previous in vitro research has shown that AMI has a protective effect from dexamethasone-induced apoptosis in human neuroblastoma cells (Leskiewicz et al., 2013). Moreover, AMI treatment can reduce 1-methyl-4-phenylpyridinium MPP+-induced apoptosis in neuronal PC12 cells by changing mitochondrial membrane permeability, reducing cytochrome c release, Bax and subsequent caspase-3 activation (Han and Lee, 2009). It also increases the expression of the anti-apoptotic protein Bcl-2. Overexpression of Bcl-2 is associated with increases in glutathione levels which increase resistance to SN-38-induced apoptosis in leukemia 697 cells (Yoshida et al., 2006). However, other studies have shown that AMI at high concentration (100 µM) increases apoptosis in rat dorsal root ganglia (Lirk et al., 2006a; Lirk et al., 2006b). This indicates that AMI may potentially reduce CPT-11-induced apoptosis by up-regulating anti-apoptotic proteins and inhibiting caspase-3 activation in the rat colon. However, the exact mechanism has not been studied.

The anti-apoptotic effects of AMI were confined to the colon. The lack of apoptosis protection in the jejunum might be related to differences in structure and function, stem cell sensitivity to cytotoxic insult (Bach et al., 2006), and the kinetics of proliferation between both regions (Al-Dasooqi et al., 2011a). Furthermore, it could be related to differences in the expression of TLR2 and TLR4 in various sections of the intestine. When TLR2 and TLR4 mRNA expression was investigated in Wistar rats, their expression was higher in the colon compared to the jejunum which was proposed to be caused by varied distribution of commensal bacterial species at different parts of the intestine (Kosik-Bogacka et al., 2012). As such, relative inhibition of TLRs by AMI in the colon would theoretically be greater than in the small intestine, and perhaps explain why
The expression of TLR2, TLR4 and pro-inflammatory cytokines were not significantly increased in our model, indicating that irinotecan did not induce a robust inflammatory response. In addition, the CPT-11 dose used did not induce late-onset diarrhoea or profound histological damage. Previous research has shown that the increase in TLR2, TLR4, TLR9 and pro-inflammatory cytokines are associated with late-onset diarrhoea (48 - 96 h) after chemotherapeutic treatment (Logan et al., 2008a; Ferreira et al., 2012; Kaczmarek et al., 2012). As such, it seems that TLR and
pro-inflammatory cytokines are involved in severe GIM, but play a smaller role in more modest tissue injury models of GIM such as that created in our study. Research conducted in our laboratory (chapter two results from this thesis) has shown an association between colonic TLR2 and TLR4 mRNA expression at 96 h and diarrhoea occurrence and severity after CPT-11-induced GIM. Hence, our results indicate that TLRs are only elevated in response to intestinal injury, which is sufficiently severe to cause late-onset diarrhoea. This is a fundamental difference between the DA model and the Wistar model. Moreover, mRNA was isolated from the whole intestinal tissues in DA rats while mRNA was isolated from mucosal layer only in Wistar rat. This suggests that other cell types, such as infiltrating immune cells, in the submucosa play a role in the expression of these mediators. TLR and pro-inflammatory cytokine expression were examined only in the epithelial layer. However, other cell types express these mediators and can be examined in the future.

Since AMI was effective at reducing CPT-11-induced apoptosis in the colon at 6 h, we conducted a PCR array analysis to determine differential gene expression profiles responsible for the protection. Our results showed a significant decrease in mRNA expression of IL-1β, IL-1r2 and caspase-4 but increased expression of INFγR1 in rats treated with CPT-11+AMI compared to CPT-11 alone. IL-1β plays an important role in host immune and pro-inflammatory responses, which is crucial for GIM. Pro-IL-1β requires the converting enzyme, caspase-1, for the release of the mature and active IL-1β protein (Friedlander et al., 1996; Siegmund, 2002). Caspase-1 activation requires caspase-4 (Sollberger et al., 2012). Thus reduction in caspase-4 will result in reduced activation of IL-1β and consequently inhibition of apoptosis. The available evidence seems to suggest that treatment with CPT-11+AMI reduced apoptosis through IL1β inhibition, although protein staining results suggest that the signal is derived from non-epithelial cells. In contrast, our results expectantly showed an increase in the mRNA expression of INFγR1 in combination-treated rat colons. Previous research has shown that treatment with AMI and its active metabolite nortriptyline have shown to significantly decrease blood INFγ in human (P = 0.004 and P = 0.037, respectively) (Himmerich et al., 2010). AMI may have protected cells expressing this receptor against apoptosis, thereby contributing to the relatively increased signal compared to CPT-11 alone.

In summary, this study provides an overview of the effect of AMI on CPT-11-induced intestinal injury. AMI is prescribed for cancer patients and so is taken in combination with chemotherapeutic
drugs (Pezzella et al., 2001; Kautio et al., 2008; Kautio et al., 2009; Banerjee et al., 2013; Toftegard et al., 2013). However, drug interactions should be considered for chemotherapeutic treatment safety and efficacy. Furthermore, TLR2 and TLR4 inhibition should be investigated by specific inhibitors that avoid the off target effects of AMI for effective prevention of GIM.
Chapter Five
5.0 The effect of Amitriptyline on SN-38-induced intestinal cell death and toll-like receptor expression.

5.1 Introduction:

Gastrointestinal mucositis (GIM) is a common side effect of chemotherapy that results in dose reduction or temporary discontinuation and thus impairs treatment efficacy and increases costs (Sonis, 2004). The main histological features of GIM are apoptosis, crypt hypoplasia, reduction in villous area, and inflammation (Keefe et al., 2000; Logan et al., 2008a). The incidence and severity of GIM depend on multiple factors including the use of single or multimodality therapy and the dose intensity of the regimen (Sonis et al., 2004). Despite advances in understanding the pathobiology of GIM, there is no clinically effective intervention for preventing GIM development. Preventative therapies should be designed based on the increased understanding of the pathology of GIM.

Irinotecan is a commonly used drug for treating gastric and colorectal cancers (Rothenberg, 2001; Yu et al., 2005). Despite the effectiveness of this drug against tumours, its efficacy and safety is compromised because of GIM occurrence in a large proportion of patients, mostly manifesting as diarrhoea. Up to 80% of cancer patients develop diarrhoea after treatment with irinotecan depending on the regimen (Glimelius, 2005). Irinotecan induces early diarrhoea, which is a result of increased cholinergic activity, and late-onset diarrhoea, due to exposure to the toxic metabolite, 7-Ethyl-10-hydroxy-camptothecin (SN-38). Irinotecan is a prodrug that is converted to SN-38 by carboxylesterases in the liver and intestine. Detoxification of SN-38 occurs via hepatic and intestinal UDP glucuronosyltransferase 1A1- (UGT1A1), UGT1A9- and UGT1A10-dependent glucuronidation, converting SN-38 to SN-38 glucuronide (SN-38G) (Chen et al., 2013). Following enterohepatic recirculation, SN-38 is reactivated within the intestine by β-glucuronidase, expressed by resident bacteria, which cleaves the glucuronide group from SN-38G (Takakura et al., 2012). SN-38 produces characteristic mucosal changes in the intestine by inducing apoptosis. From animal model studies, it is well known that irinotecan induces apoptosis in small and large intestinal epithelial cells, followed by morphological and histological changes with alterations in multiple signalling pathways, including toll-like receptors (TLR) (Bowen et al., 2007b; Logan et al., 2008a; Al-Dasooqi et al., 2010; Al-Dasooqi et al., 2011a). The mucosal damage reduces water absorption from the gastrointestinal tract and results in diarrhoea occurrence.
As a topoisomerase I inhibitor, SN-38 blocks the DNA ligation of topoisomerase I cleavage complex leading to DNA strand breaks (Pommier et al., 1998). Many signalling pathways are activated in response to this DNA damage, which arrest cell cycle progression to allow for repair or to prevent transmission of damaged chromosomes. SN-38-induced DNA damage could lead to p53 phosphorylation, which results in either apoptosis, mediated by pro-apoptotic proteins of the Bcl-2 family (Bax, Bak, Noxa, Puma) or permanent cell cycle arrest orchestrated through p21 and 14-3-3σ depending on the type of the exposed cell (Rudolf et al., 2012; Origanti et al., 2013). In normal colonic epithelial cells, up-regulated expression of p53 activates p38 kinase and initiates caspase-dependant apoptosis (Rudolf et al., 2012). It has been shown that treatment of rat small intestinal epithelial cells (IEC-6) with irinotecan causes a marked increase in the pro-apoptotic proteins Bax and Bak expression (Bowen et al., 2007a).

In addition to the apoptotic effect of irinotecan, it leads to up-regulation of multiple pro-inflammatory cytokine receptors, including members of the tumour necrosis factor (TNF) receptor family in rat intestine at 6 h following treatment (Bowen et al., 2007b). It has also been shown to increase NF-κB and pro-inflammatory cytokines in rat intestine between 2 and 12 h (Logan et al., 2008a). Moreover, the TLR signalling pathway has been demonstrated to be among the activated pathways after irinotecan treatment in microarray experiments using the same animal model (Bowen et al., 2007b). TLR2 and TLR4 activation utilises two signalling pathways: myeloid differentiation primary response gene 88 (MyD88)-dependent or -independent signalling pathways (Brown et al., 2011). Myd88-dependent signalling results in the activation of NF-κB and the release of pro-inflammatory cytokines, while MyD88-independent signalling results in the induction of interferon (IFN) type I and late activation of NF-κB (Akira and Takeda, 2004; McGettrick and O'Neill, 2010). Since TLR activation leads to NF-κB translocation and pro-inflammatory cytokine release, it has been proposed to be involved in irinotecan-induced GIM. Recently, a few studies have investigated the involvement of TLRs in chemotherapy-induced GIM. Kaczmarek, et al (2012) demonstrated the role of TLR2 and TLR9 signalling in doxorubicin-induced GIM (Kaczmarek et al., 2012), where TLR2 deficient mice had a significant decrease in apoptosis and histological damage compared to wild-type mice after treatment with doxorubicin; Ferreira et al (2012) reported significant increase in TLR4 expression 72 h after 5-fluorouracil-induced GIM (Ferreira et al., 2012); Hamada et al (2013) reported a significant increase in TLR4 mRNA and protein expression following methotrexate administration in the small intestine of Wistar rats when compared to control (Hamada et al., 2013). However, no published study has
investigated the involvement of TLR2 and TLR4 in irinotecan-induced GIM in a cultured epithelial model.

The role of TLR2 and TLR4 in irinotecan-induced GIM can be examined through pharmacological targeting with specific inhibitors. Amitriptyline (AMI), a tricyclic antidepressant drug, has been shown previously to inhibit the activity of TLR2, TLR4 and pro-inflammatory cytokines in HEK293 cells over-expressing TLR2 and TLR4 (Hutchinson et al., 2010b). It was also found to be a non-selective inhibitor of human UGTs in HEK293 cells (Uchaipichat et al., 2006). It is widely used in inflammatory bowel diseases and gastrointestinal disorders due to its anti-inflammatory effect (Bahar et al., 2008; Vahedi et al., 2008; Sohn et al., 2012). AMI reduced irritable bowel syndrome (IBS) symptoms, including diarrhoea, in 66% of patients in a recent clinical trial of IBS-diarrhoea treated with AMI in combination with probiotics (Sohn et al., 2012). AMI, and its metabolite nortriptyline, are able to decrease nitric oxide production, NF-κB, TNF, and IL-1β production, which has shown to be involved in TLR signalling pathway and GIM development (Obuchowicz et al., 2006; Vismari et al., 2012). Furthermore, AMI has been shown to affect cell death, depending on the treatment and the cell type; Pre-treatment with AMI for 24 h (hours) (100 µM/L) reduced hydrogen peroxide-induced apoptosis by up-regulating superoxide dismutase activity in PC12 cells (Kolla et al., 2005). AMI attenuated 1-methyl-4-phenylpyridinium-induced apoptosis in PC12 cells by suppressing altered mitochondrial membrane permeability that leads to cytochrome c release and caspase-3 activation (Han and Lee, 2009). These previous studies provide evidence for the inhibitory effect of AMI on TLR2 and TLR4 and apoptosis, thus highlighting the potential for AMI modulation of irinotecan-induced GIM.

Our previous work has shown that irinotecan-induced apoptosis was significantly inhibited (3.3-fold) with AMI (6 h) in the colon of Wistar rats (chapter three results of this thesis). The current study was conducted to further examine the ability of AMI to inhibit apoptosis and to determine the role of TLR activation using the rat intestinal epithelial cell line (IEC-6). IEC-6 are rat small intestine epithelial cells that express TLRs, produce pro-inflammatory cytokines and respond to LPS stimulation (Cario et al., 2007; Alcamo et al., 2012). Previous research found that in response to irinotecan treatment, IEC-6 cell number decreased in a dose-dependent manner with marked increases in the pro-apoptotic proteins Bax and Bak and reduction in the anti-apoptotic protein induced myeloid leukemia cell differentiation protein (Mcl-1) (Bowen et al., 2007a). As an extension of the previous work using irinotecan, we investigated the toxic metabolite SN-38 to
elucidate the apoptotic mechanism in IEC-6 cells. Up to date, no study has investigated SN-38 effects on IEC-6.

In this study, we hypothesised that AMI can attenuate irinotecan-induced apoptosis through TLR inhibition. The primary aim of this study was to investigate the effect of AMI on apoptosis and the expression of TLR2, TLR4 and pro-inflammatory cytokines after SN-38 treatment to extend our knowledge about the effect of the drug on apoptosis. IEC-6 cells were used in this study to investigate epithelial-specific responses to TLR inhibition.

5.2 Methodology:

5.2.1 Cell line

IEC-6, an epithelial cell line was obtained from the American Type Culture Collection (Rockville, MD, USA). IEC-6 is derived from adult rat jejunum and displays characteristics of an undifferentiated crypt cell type. Assays using this cell line were carried out between passage 3 and 11 and cells retained their original morphology and growth characteristics over the range of passages used.

5.2.2 Cell culture

IEC-6 cells were maintained in Dulbecco’s modified Eagle’s media supplemented to contain 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 50 U/ml penicillin, 50 µg/ml gentamycin, 1 µg/ml fungizone, 4 mM L-glutamine, 10 µg/ml bovine insulin and 10% foetal calf serum (FCS) (Bovogen Biological Pty Ltd, East Keilor, VIC, Australia). Experimental cell cultures were grown in sterile multi-well tissue culture plates (Falcon, BD, North Ryde, NSW, Australia) in identical growth condition. Media and supplements were purchased from Gibco (Breda, The Netherlands).

Cells were routinely passaged when culture monolayers reached approximately 80% confluence. Cells were subcultured in ratio of 1:3 in fresh growth medium. Monolayers were detached by using
trypsin-EDTA solution (0.05% trypsin, 0.53 mM EDTA). Cells were incubated at 37 °C for 10 min (minutes) until cells detached from the plate surface. The reaction was neutralised by adding fresh growth medium and cells were subcultured for experimentation or to start new passage. For experiments, cells were centrifuged at 250xg for 5 min at 21 °C and supernatant was removed. Fresh medium (1 ml) was added to the cells and resuspended to perform cell counting. Assessment of cell number was carried out by adding equal amounts of trypan blue (0.4%) and cell suspension (1:1) and counted on a haematocytometer. The remaining cells were centrifuged again and resuspended in fresh medium to obtain a cell density of 1x10^5 cells/ml.

5.2.3 Drug treatment

SN-38 was purchased from Tocris Bioscience (Bristol, UK) and kept at concentration of 10 µM in 100% dimethyl sulfoxide (DMSO) and used at final concentration of 0.1% DMSO in culture medium. The drug was stored at 4 °C in a light-proof container under sterile conditions. All further dilutions of SN-38 were in DMEM media and carried out immediately prior to experimental use. Amitriptyline hydrochloride (AMI) was purchased from Sigma-Aldrich (NSW, Australia) and was diluted in MilliQ water immediately prior to addition to cells. Control cells were grown in DMEM media.

5.2.4 Cell proliferation assessment

XTT:

To measure the proportion of proliferating cells following treatment with SN-38 and AMI, XTT assays (Roche Diagnostic, Castle Hill, Australia) were carried out in multi-well reactions. Cells were subcultured into 96-well plastic tissue culture plates (Falcon, BD, North Ryde, NSW, Australia) at density of 10,000 cells in 100 µl of growth medium. After initial 48 h incubation, SN-38 (+/- AMI) was added to all wells at differing specifically determined concentrations then incubated for 24 h. AMI concentrations tested (0.1, 0.5 and 1µM) were within the therapeutic range of the drug, converted from human to rat (Jorgensen, 1977; Chan et al., 1980; Kuss et al., 1985; Warrington et al., 1986; Wong et al., 1996; Rosenzweig et al., 1998; Kukes et al., 2009). SN-38 concentrations tested were from 0.01 to 100 µM to determine the concentration that kills
50% of the cells (IC$_{50}$). At the end of the treatment period, the media was aspirated and replaced with 100 µl fresh medium. Each well received 50 µl of freshly prepared XTT labelling mixture (tetrazolium salt XTT and electron coupling reagent). Metabolically active cells cleave XTT to soluble formazan dye which was quantitated after 6 h using a scanning multi-well Spectrophotometer (Synergy MX, Biotek instruments, Inc, USA) at absorbance wavelengths of 490 and 690 nm. The absorbance was calculated as (A$_{490}$ value - A$_{690}$ value). Cell proliferation was calculated as:

\[
\text{Cell proliferation (\%)} = \frac{\text{absorbance value for treated cells}}{\text{absorbance value for control}} \times 100
\]

Treatment was carried out in triplicate and all experiments were repeated three times and averaged to obtain meaningful data.

### 5.2.5 Cell viability assessment

To assess response of IEC-6 to differing doses of SN-38 and to confirm XTT results, a trypan blue assay was employed. Cell culture suspension was seeded into 6-well plastic tissue culture plates (Nunc, Denmark) at density of 2 x 10$^5$ cells/well in 500 µl of growth medium. Cells were incubated for 48 h then SN-38 (+/- AMI) at different concentrations was added followed by incubation for 24 h. Control was cells incubated in fresh growth medium. Cell monolayers were detached with trypsin/EDTA solution and added to 10 ml sterile tubes (Falcon, BD, North Ryde, NSW, Australia). Following centrifugation at 250xg for 5 min at 21 °C, cells were resuspended in 1 ml of fresh media. Viable cell counts were performed with addition of 0.4% trypan blue at a 1:1 ratio of cell suspension, visualised with a haematocytometer and shown as percentage of cells capable of excluding the dye. Cells which take up the dye are believed to be undergoing late stage cell death. Counts of treated cells in each group were compared to untreated controls. Each experiment was repeated three times. Then the half maximal inhibitory concentration (IC$_{50}$) for SN-38 was derived from a nonlinear regression model (curve fit) based on sigmoidal dose response curve (variable) and computed using GraphPad Prism 6 software. After detecting the SN-38 (IC$_{50}$) and AMI (TLR inhibitory) concentrations, as described below, cells count after treatment with both drugs was performed to confirm XTT results.
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5.2.6 RNA extraction and reverse transcription

After treating the cells with drugs as explained above, cells were detached with trypsin/EDTA solution and transferred to 10 ml sterile tubes (Falcon, BD, North Ryde, NSW, Australia). Following centrifugation at 250xg for 5 min at 21 ºC, cells were resuspended in 1 ml of sterile phosphate-buffered Saline (PBS) to remove residual medium. After a further spin and removing the supernatant, 350 µl TRIzol® Reagent (Invitrogen Life Technologies, Mulgrave, Australia) was added for 5 min then followed by the addition of 100 µl chloroform for 3 min. Cells were centrifuged for 15 min at 13400 xg at 4 ºC. After washing with ethanol (70%), RNA was isolated and converted to cDNA as described in chapter 2.

5.2.7 Primers

The primers that were used in this study are listed in chapter 2.

5.2.8 Real time PCR (RT-PCR)

RT-PCR was performed as described in chapter 2.
5.2.9 Statistical analysis

Statistical analysis was carried out using GraphPad Prism 6 and results were analysed by the Kruskal-Wallis test followed by Dunn’s multiple comparison test to identify differences between groups. P-values < 0.05 were considered statistically significant.

5.3 Results

5.3.1 Cell proliferation and viability in response to SN-38

The cytotoxicity of SN-38 was determined using XTT and trypan blue assays. Results showed that SN-38 caused a dose-dependent decrease in cell viability. XTT results showed that after 24 h SN-38 at concentration of 8.7 µM killed 50% of the cells (mean IC\textsubscript{50} ± SD = 8.7 ± 0.02, 95% CI = 5.06 to 12.52) (Figure 5.1). Trypan blue assay confirmed our results (mean IC\textsubscript{50} ± SD = 8.1 ± 0.02, 95% CI = 5.68 to 10.6) (Figure 5.2).
Figure 5.1: IEC-6 treated with different concentrations of SN-38 to detect 50% of cell inhibition by XTT assay. Each experiment was repeated three times with three replicates in each. Data shown are mean ± SEM. Red line shows the sigmoidal curve that was used to fit the line. Grey line connects the mean of actual data between all concentrations.

Figure 5.2: IEC-6 treated with different concentrations of SN-38 to detect 50% of cell inhibition by trypan blue method. Each experiment was repeated three times. Data shown are mean ± SEM. Red line shows the sigmoidal curve that was used to fit the line. Grey line connects the mean of actual data between all concentrations.
5.3.2 Effect of SN-38 and AMI on cell proliferation and viability

Treatment with SN-38 (8.7 µM) decreased IEC-6 cell proliferation significantly when compared to cells treated with AMI (1 µM). AMI alone had no significant effect on cell viability. SN-38 decreased viable cell count significantly (43.8%) compared to AMI (85.3%) ($P = 0.0072$). Treatment with both drugs (SN-38 + AMI) increased cell proliferation compared to treatment with SN-38 alone (57.3%) even though it was not statistically significant ($P = 0.07$). Results were confirmed via trypan blue assay (Figure 5.3 and 5.4).
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. Data is relative to 0.1% DMSO control value. Each experiment was carried out on triplicate and repeated three times. Data are medians with range. **P < 0.01.

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. Data is relative to 0.1% DMSO control value. Each experiment was repeated three times. Data shown are medians with range. **P < 0.01.
5.3.3 TLR2, TLR4 and pro-inflammatory cytokine mRNA expression after treatment with SN-38 and AMI

Characterisation of the impact of AMI alone on TLR expression was evaluated using different concentrations of the drug compared to untreated cells. The range of AMI concentrations that were used in this study were 0.1-1 µM. There were no significant differences in TLR2 and TLR4 expression at any concentration (P = 0.6 and 0.7, respectively). Therefore, the concentration of (1 µM) was chosen because it represents the mean plasma concentration with the therapeutic doses of AMI.

Treatment with SN-38 (8.7 µM for 24 h) increased TLR2 and TLR4 mRNA expression significantly up to 2.4- and 3-fold change (median), respectively, compared to AMI treated cells (P = 0.0002 and P = 0.0005, respectively). Treatment with SN-38 and AMI decreased TLR2 mRNA expression (median = -0.49-fold change) significantly compared to SN-38 treated cells (median = -2.9-fold change) (P = 0.0014). TLR4 expression following AMI and SN-38 was decreased significantly (median = -2.6-fold change) when compared to cells treated with SN-38 alone (median = -3.3-fold change) (P = 0.04). Therefore, AMI was able to reduce TLR expression induced by SN-38 (Figure 5.7).

Treatment with SN-38 similarly, increased IL-1β, TNF and IL-6 mRNA expression by 4.9-, 4.1- and 20.1-fold changes (median), respectively, compared to untreated control. Treatment with AMI and SN-38 decreased IL-1β expression significantly (0.2-fold change median) compared with SN-38 alone (4.9-fold change median, P = 0.002). However, TNF expression increased significantly (15.1-fold change median) in cells treated with both drugs compared to cells treated with SN-38 alone (4.1-fold change median, P = 0.01). IL-6 expression increased in combination treated cell (44.1-fold change median) compared to in SN-38 treated cells (20-fold change median) but was not statistically significant (P = 0.8). Therefore, treatment with AMI inhibited SN-38-induced IL-1β expression but was not able to prevent IL-6 up-regulation. Combination treatment led to an amplified increase in TNF expression (Figure 5.8).
Figure 5.5: TLR4 mRNA expression in IEC cells after treatment with AMI (24 h) at different concentrations. Controls administered vehicle (0.1% DMSO). Expression is normalised to UBC and B2M. Each experiment was repeated three times. Line represents median.

Figure 5.6: TLR2 mRNA expression in IEC cells after treatment with AMI (24 h) at different concentrations. Controls administered vehicle (0.1% DMSO). Expression is normalised to UBC and B2M. Each experiment was repeated three times. Line represents median.
Figure 5.7: mRNA expression of (A) TLR2 and (B) TLR4 in all treated groups. IEC-6 cells were treated with drugs for 24 h then RNA was isolated to perform RT-PCR. AMI concentration used was 1 µM and SN-38 concentration was 8.7 µM. Controls administered vehicle (0.1% DMSO). Expression is normalised to UBC and B2M. Each experiment was repeated three times. Line represents median. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 5.8: mRNA expression of (A) IL-1β, (B) TNF and (C) IL-6 in all treated groups. IEC-6 cells were treated with drugs for 24 h then RNA was isolated to perform RT-PCR. AMI concentration used was 1 µM and SN-38 concentration was 8.7 µM. Controls administered vehicle (0.1% DMSO). Expression is normalised to UBC and B2M. Each experiment was repeated three times. Line represents median. *P < 0.05, **P < 0.01, ***P < 0.001.
5.4 Discussion:

As discussed in Chapter 3 of this thesis, I have previously shown the ability of AMI to reduce early apoptosis in the rat colon after treatment with AMI and irinotecan, a prodrug of SN-38. As such, this study was conducted to investigate the inhibitory effect of AMI on apoptosis in normal intestinal epithelial cells and its effect on TLR2 and TLR4 in this process. The current study showed that SN-38-induced apoptosis was associated with increased expression of TLR2, TLR4 and pro-inflammatory cytokines. It was also found that AMI attenuated cell death after SN-38 treatment, and this was associated with significant reduction in mRNA expression of TLR2, TLR4 and IL-1β. Conversely, TNF expression was increased after cotreatment with AMI and SN-38.

This study investigated the effect of SN-38 on cell proliferation and showed dose-dependent decreases in proliferation. Our results are supported by previous research showing that irinotecan caused apoptosis in a dose dependant manner in IEC-6 cells (Bowen et al., 2007a). It was suggested that irinotecan induces apoptosis as a result of the generation of reactive oxygen species (ROS) and direct DNA damage (Bowen et al., 2007a).

This study showed that AMI modestly attenuated SN-38-induced apoptosis in IEC-6 cells. Our results are supported by previous research that showed that incubation with AMI at identical doses for 24 h prevented dexamethasone-induced apoptosis in SH-SY5Y human neuroblastoma cells (P < 0.05) (Leskiewicz et al., 2013). Other studies showed that pre-treatment with AMI at higher doses (1-10 μM) had a protective effect from 1-methyl-4-phenylpyridinium (MPP+)-induced neuronal cell death and beyond this concentration the effect declined (Han and Lee, 2009). It was suggested that AMI at therapeutic concentrations protects from neuronal cell death due to toxic insults but higher concentrations diminish the protection (Han and Lee, 2009). However, it was reported that treatment with higher concentrations of AMI (50 and 100 μM/L) has a cytoprotective effect from hydrogen peroxide H₂O₂-induced cell death (P < 0.001) (Kolla et al., 2005). Other studies showed that AMI can cause apoptosis in tumour cells most probably by enhancing caspase-3 and reducing Bcl-2 expression (Arimochi and Morita, 2006b; Mao et al., 2011a; Pula et al., 2013). From all of these studies, it is clear that the anti-apoptotic effect of AMI depends on the dose, cell type and culture conditions (Drzyzga et al., 2009); it is reasonable to assume that AMI at similarly therapeutic concentrations could possess anti-apoptotic properties in IEC-6 cells.
Apoptosis induced by SN-38 was associated with enhanced expression of TLRs. This study showed a significant increase in mRNA expression of TLR2 and TLR4 after 24 h from the administration of SN-38 compared to AMI in IEC-6 cells. The increased mRNA expression of TLR2 and TLR4 was associated with increased expression of pro-inflammatory cytokines which is consistent with other studies demonstrating the up-regulation of pro-inflammatory cytokines after treatment with irinotecan in an animal model (Logan et al., 2008a). TLR2 and TLR4 respond to damage-associated molecule patterns that can be a result of the generation of reactive oxygen species (ROS) after cancer treatment. Recently, it was reported that irinotecan increased the release of the high-mobility group box 1 (HMGB1) and the heat shock protein 70 (HSP70) in SW480 colorectal tumour cells 24 h after treatment, which is more likely to be the reason behind TLR4 and TLR2 up-regulation in this cell line (Frey et al., 2012).

Up-regulation of TLR2 and TLR4 in response to mucosal injury can lead to the activation of pro-inflammatory cytokines and apoptosis (Fukata et al., 2005; Fukata et al., 2006; Salaun et al., 2007). It was reported that ethanol-induced gastric injury is associated with HMGB1-mediated TLR4/MyD88/NF-κB signalling pathway that leads to IL-1B and TNF up-regulation in mice (Ye et al., 2013a; Ye et al., 2013b). This was associated with increased apoptosis, which was indicated by an increase in the Bax/Bcl-2 ratio. In IEC-6 cells, TLR4 protein expression up-regulation by LPS stimulation caused increased apoptosis and significant reduction in proliferation and cell migration (Leaphart et al., 2007). Additionally, TLR2 activation by bacterial lipoprotein was shown to cause apoptosis through MyD88/FADD pathway, thus activation of caspases, in human kidney epithelial 293 cells (Aliprantis et al., 2000). Previous research has shown elevated expression of TLR2 after doxorubicin-induced GIM that was associated with apoptosis in an animal model (Kaczmarek et al., 2012). Inhibition of TLR2 has shown to reduce GIM severity. TLR2 deficiency has been shown to activate glycogen synthase kinase 3 (GSK-3β), which resulted in reduced apoptosis and damage severity (Kaczmarek et al., 2012). However, TLR activation also has a protective role from mucosal damage, under certain conditions (Zhang et al., 2010; Lacave-Lapalun et al., 2013).

TLR2 and TLR4 can have an apoptotic or anti-apoptotic effect depending on the activated pathways. TLR4 deficiency was associated with increased apoptosis and reduced proliferation following dextran sulfate sodium (DSS)-induced intestinal mucosal injury (Fukata et al., 2006). TLR4 signalling through NF-κB/MAPK/COX-2 pathway is required for optimal proliferation and
protection from apoptosis (Fukata et al., 2006). LPS-induced TLR4 activation promoted repair and proliferation after a higher irradiation dose (Lacave-Lapalun et al., 2013). Activation of TLR2 through its ligand PCSK significantly suppressed inflammation and apoptosis in an animal model of colitis via MyD88/PI3K/Akt-mediated cell survival and limited pro-inflammatory cytokines through the MAPK-NF-κB pathway (Cario et al., 2007; Cario, 2008). Stimulation of IEC-6 cells with TLR2 ligand, synthetic PCSK, resulted in the activation of PI3K/Akt pathway which promotes cell survival (Cario et al., 2007). Moreover, TLR2 activation with PCSK mediated the induction of TFF3, in goblet cells, and conferred anti-apoptotic protection of intestinal mucosa against DSS-induced colitis (Podolsky et al., 2009). These findings imply that TLR2 and TLR4 exert mucosal protection properties in different epithelial cell types via different activated pathways. In the context of chemotherapy-induced GIM, TLR2 and TLR4 were up-regulated after treatment with SN-38 in intestinal epithelial cells, IEC-6, and were associated with increased apoptosis.

This study showed the ability of AMI to decrease TLRs and pro-inflammatory cytokine expression when it was compared to SN-38 treated cells. At all AMI concentrations examined, there was a decrease in TLR4 expression. AMI has shown previously to inhibit TLR2, TLR4 and pro-inflammatory cytokines in neural cells (Obuchowicz et al., 2006; Hutchinson et al., 2010b). It was also reported that the anti-inflammatory effect of AMI is due to inhibiting pro-inflammatory cytokines and nitric oxide production in the acute inflammatory response in rats (Obuchowicz et al., 2006; Sadeghi et al., 2011; Vismari et al., 2012). The inhibition of TLR expression by AMI could be via inhibiting the activation at MD-2 as suggested by Hutchinson, et al (2010) (Hutchinson et al., 2010b).

In addition to the effect of AMI on apoptosis through the inhibition of TLR2 and TLR4, AMI could have a cytoprotective effect against SN-38-induced apoptosis through increasing the expression of Bcl-2 and glutathione (GSH). In previous studies, AMI was able to inhibit apoptosis through suppressing ROS and Bax and increasing Bcl-2 and GSH levels, which are required for resistance to oxidative stress in differentiated PC12 cells (Yoshida et al., 2006; Han and Lee, 2009). It has been reported that overexpression of Bcl-2 in human leukemia 697 cells is associated with increased intracellular content of GSH which mediates resistance to cell death after treatment with SN-38 (Yoshida et al., 2006). However, the effect of AMI on Bcl-2 may be dose-dependent since a lower dose (5 mg/kg), but not a higher dose (10 mg/kg), increased Bcl-2 in hippocampal
mossy fibers, (Xu et al., 2003). In vitro, treatment with AMI (100 µM/L) for 24 h increased cell viability after H2O2-induced apoptosis by the up-regulation of superoxide dismutase in PC12 cells (Kolla et al., 2005). The cytoprotective effect of AMI was shown to be due to the suppression of the formation of reactive oxygen species (ROS) and inhibition of (MPP+)-induced depletion of glutathione GSH levels (Han and Lee, 2009). This may contribute to AMI effect on cell viability after treatment with both drugs.

The current study also showed that treatment with SN-38 in combination with AMI decreased TLR and IL-1β mRNA expression compared to SN-38 alone but increased the expression of TNF. IL-1R (IL-1 receptor) is required for IL1α and IL-1β activation. It is also required for the activation of TLR2 and TLR4 and leads to MyD88-dependent pathway but cannot activate the MyD88-independent pathway (Virtue et al., 2012). Apoptosis triggered by TLR2 and TLR4, in endothelial cells, appears to depend on MyD88 association with FADD (Virtue et al., 2012). Therefore, the inhibition of IL-1β expression can lead to inhibition of the TLR2 and TLR4/MyD88-dependent pathway which could result in the inhibition of apoptosis. This could partially explain the ability of AMI to reduce cell death when it is administered with SN-38.

The increased expression of TNF can have pro- or anti-apoptotic effects (Kubota et al., 2001). TNF is able to induce converse effects, depending on the second messenger system that conveys its signal within the cell. For example, the induction of caspases by TNF can lead to apoptosis, whereas the activation of NF-κB and Bcl-2 can lead to cell growth and proliferation (Kubota et al., 2001). Since AMI has been shown to increase Bcl-2 (Xu et al., 2003), it is suggested that TNF up-regulation has an anti-apoptotic effect through Bcl-2 expression. The autocrine activation of TNF can inhibit apoptosis in dendritic cells by inhibiting BAK and increasing Bcl-2 expression (Lehner et al., 2012). Moreover, it was reported that TNF stimulation induces IEC-6 proliferation in a dose dependant manner and requires protein tyrosine phosphorylation and MAPK activation (Dionne et al., 1998). However, under some conditions, the excessive secretion of inflammatory factors such as TNF or FasL may contribute to TLR2-triggered apoptosis (Salaun et al., 2007).

The major detoxification of SN-38 is through UGTs, which is highly expressed in the liver and intestine tissues (Chen et al., 2013). However, no study has investigated UGT expression specifically in IEC-6. Previous evidence has investigated UGT levels in liver and intestinal
microsomes isolated from human and rat (Tallman et al., 2005). The rank order of SN-38 glucuronidation in rat was UGT1A7 > 1A8 > 1A1. When hepatic, lung and intestinal cancer cell lines were treated with SN-38, the IC$_{50}$ value of SN-38 were much lower than our study (Ulukan et al., 2001; Takahata et al., 2008). The discrepancy in IC$_{50}$ values may be due to the different cell lines (normal/ tumour) and incubation times (24 h/ 120 h). However, it could be related to differences in UGT levels between normal and cancer cells. UGTs were found to be highly expressed in normal colonic mucosa compared to colon cancer tissues (Giuliani et al., 2005). UGT1A1 gene expression was found to be lower in mice tumour tissues compared to the surrounding normal colonic tissues (Chen et al., 2013). Therefore, there is a possibility that our cells express UGT, thus a possibility of SN-38 deactivation. Future studies are needed to quantify UGT expression in intestinal cell lines to elucidate the influence of the detoxification on cell response to SN-38.

In conclusion, results from this study showed the involvement of TLR2 and TLR4 in intestinal epithelial apoptosis induced by SN-38. The effect of AMI in attenuating apoptosis following SN-38 administration could be through TLR inhibition when AMI was administered within the therapeutic range of this drug.
Chapter Six
6.0 General discussion

6.1 Introduction

The primary focus of the studies contained in this thesis was to increase the understanding of the role of TLRs in irinotecan-induced GIM and to assess TLRs as a therapeutic target for the prevention of GIM. Key findings from this thesis showed the association of TLR2, TLR4 and pro-inflammatory cytokines and diarrhoea occurrence and severity following irinotecan administration in Da rats. The administration of AMI, TLR2 and TLR4 inhibitor, reduced diarrhoea occurrence and apoptosis induced by irinotecan but was not protective from histological damage and distress symptoms in Wistar rats. In vitro, AMI attenuated apoptosis after SN-38 treatment, and this was associated with significant reduction in mRNA expression of TLR2, TLR4 and IL-1β while TNF expression was increased after co-treatment with AMI and SN-38.

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

TLR2 and TLR4 have been shown to play a role in NF-κB regulation (Brown et al., 2011), pro-inflammatory cytokine activation (Takeuchi and Akira, 2010), intestinal inflammation (Gribar et al., 2008) and regulation of proliferation and apoptosis (Fukata et al., 2006; Cario et al., 2007), which are the main characteristics of GIM. Hence, it is conceivable that they are involved in GIM development, although to date, there has been very little research directly linking TLR expression and GIM. Data from this thesis provided evidence that both TLR2 and TLR4 are associated with GIM development following irinotecan (in vivo) and its metabolite SN-38 (in vitro). In vivo data demonstrated the association between colonic expression of these inflammatory mediators and diarrhoea occurrence in the colon after a single dose of irinotecan (200 mg/kg) in Dark Agouti (DA) rats. Previous research in our laboratory has indicated an increase in the pathogenic (gram negative) bacteria, E. coli, following irinotecan treatment in the same rat model (Stringer et al., 2009a). However, the increase in E. coli was not compared between rats with and without diarrhoea in treated rats in this study. Increased colonisation by β-glucuronidase-producing bacteria plays an important role in GIM development; first, it increases the reactivation of SN-38, thus increasing mucosal toxicity and diarrhoea severity; and second, bacterial products, such as lipopolysaccharide (LPS), and other pathogen-associated molecular patterns released from these bacteria can lead to TLR activation. Therefore, this could explain partially the increase of TLRs in...
irinotecan treated rats. However, quantification of β-glucuronidase and LPS between rats with and without diarrhoea is needed to elucidate the exact mechanism behind TLR up-regulation and diarrhoea induced by irinotecan.

In addition, the utilisation of an in vitro intestinal epithelial cell line, IEC-6, in this thesis has provided evidence of the involvement of TLRs in GIM development following the administration of the active metabolite of irinotecan, SN-38. In vitro data showed that TLR2 and TLR4 mRNA are up-regulated at 24 h which corresponds to the early stages of GIM development and this up-regulation was associated with apoptosis. Early increased mRNA expression of TLR could be considered a response to damage associated molecular patterns (DAMPs), which are generated by injured cells after SN-38 treatment. DAMPS which act as ligands for TLR2 and TLR4 include high-mobility group protein B1 (HMGB1) and heat-shock proteins (HSPs) (Chang, 2010). Both HMGB1 and HSP70 were found to be increased following irinotecan treatment (Frey et al., 2012). Our results are in agreement with previous findings by showing that TLR4 mRNA and protein expression increase significantly 24 h following methotrexate administration in the jejunum of Wistar rats (Hamada et al., 2013). Whilst in knockout mice, TLR2 deficiency protects against doxorubicin-induced apoptosis (Kaczmarek et al., 2012). As such, TLR2 and TLR4 clearly play a role in mucosal responses to insults from chemotherapy treatment.

The increase of TLR mRNA and protein expression in rats with diarrhoea was evident in the colon. The study described in chapter 2 showed differences in the expression of TLRs and pro-inflammatory cytokines between both regions in DA rats with diarrhoea. Regional differences could be related to either differences in the structure and function of jejunum and colon, water absorption rates or differences in β-glucuronidase levels by intestinal microflora (Stringer et al., 2007a; Stringer et al., 2009a). In addition, TLR expression, in healthy intestine, differs along the alimentary tract. It was reported that TLR2 and TLR4 mRNA expression were higher in the colon compared to the jejunum in Wistar rats (Kosik-Bogacka et al., 2012). TLR2 is highly expressed in the proximal colon while TLR4 is more highly expressed in the distal colon suggesting an effect of microflora in this variation in mice (Wang et al., 2010). Even though TLR2 and TLR4 expression has been identified in different animal models (mice) and strains (Wistars), it is conceivable that TLRs expression is distributed differentially along the alimentary tract with the contribution of microflora. Therefore, TLR2 and TLR4 involvement in irinotecan-induced GIM is likely to be associated with diarrhoea severity which is linked to colonic damage. However, this finding may
be specific to irinotecan since different models of GIM using different chemotherapeutic drugs have shown increased expression of TLRs in the jejunum (Ferreira et al., 2012; Kaczmarek et al., 2012; Hamada et al., 2013). It was also reported that TLR protein expression in the colon of tumour-bearing rat model of GIM treated with irinotecan was unchanged (Bowen et al., 2012). Currently the reason behind this seemingly contradictory difference in transcript and protein levels of TLRs in response to irinotecan is unclear. More research is required to investigate TLR expression at different time points following irinotecan treatment along the alimentary tract and the relationship with GIM severity.

Differences in our results compared to others could be related to the differences in the administration route of irinotecan and strain differences. In our model, rats had one intraperitoneal (i.p) injection of irinotecan (250 mg/kg) to allow for early damage investigation while other models had multiple intravenous (i.v) injections of irinotecan (50 mg/kg X 3 days) to allow investigation of late damage (Boushey et al., 2001; Javle et al., 2007). The use of tumour bearing model should be considered as the pathophysiology may differ between healthy and tumour models. In addition the metabolism of irinotecan differs between i.p. and i.v, which should be considered for pharmacokinetics and efficacy of irinotecan and when translated to the level of clinical use (Guichard et al., 1998).

In addition, it is well known that TLRs and pro-inflammatory cytokines are expressed in many cell types in the intestinal tract, such as infiltrating immune cells, and differentially along the alimentary tract. In this project, these mediators were only investigated in the epithelial layer at different regions of the jejunum (villi versus crypt) and the colon (apical and basal regions of the crypt). Future research could investigate their expression at different cells types and in different regions along the alimentary tract.

In summary, the current project has provided additional data that contributes to the understanding of GIM pathophysiology. This was achieved by showing that the mRNA and protein expression of TLR2, TLR4 and pro-inflammatory cytokines was increased in DA rats that developed diarrhoea following irinotecan treatment compared to rats that were resistant to developing diarrhoea. This indicated a strong relationship between these inflammatory mediators and severity of mucosal injury. In addition, increased mRNA and protein expression of TLR2 and TLR4 was associated
with apoptosis of epithelial cells. Previous studies have demonstrated conclusively that irinotecan induces intestinal apoptosis and causes diarrhoea in this model due to severe mucosal damage (Gibson et al., 2007; Stringer et al., 2009a). However, in these studies, rats receiving irinotecan did not always develop diarrhoea, mimicking the variable patient response seen clinically. As such, this implies that there is an association between diarrhoea occurrence, its severity and these inflammatory mediators, which provides a rationale for targeted intervention.

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

Previous evidence has shown that TLR2 and TLR4 are involved in chemotherapy-induced GIM (Bowen et al., 2012; Ferreira et al., 2012; Kaczmarek et al., 2012; Hamada et al., 2013). In the light of the preliminary data from the first study of this project (chapter two), it was important to investigate the effect of TLR2 and TLR4 inhibition on GIM development following irinotecan treatment. This can be achieved by different methods; gene knockout or TLR antagonism by pharmacological inhibition. TLR2 knockout mice have been used previously to investigate its involvement in doxorubicin-induced GIM and showed positive outcomes such as reduction in histological damage, apoptosis and acute inflammation (Krysko et al., 2011; Kaczmarek et al., 2012). However, there is a need to investigate inhibitors that can be used clinically. Using antagonist (antibodies) has shown positive results with irinotecan-induced GIM in animal models. Recently, it was reported that pre-treatment with recombinant human interleukin-1 receptor antagonist (rhIL-1Ra) reduces incidence and severity of diarrhoea following irinotecan treatment (i.p. 100 mg/kg x 3 days) in tumour bearing mice (Wang et al., 2014). Pharmacological drugs that have TLR inhibitory effects could be used to effectively investigate the role of TLRs in GIM development and such results could be translated into clinical practice.

Significant effort has been made to evaluate palliative and therapeutic strategies to treat chemotherapy-induced GIM (Gibson et al., 2013). Prophylactic medications that were recommended for GIM treatment include loperamide, tincture of opium and octreotide (Gibson et al., 2013). They work by slowing gut motility and increasing fluid re-absorption. By knowing the markers involved in GIM development, prophylactic medications can prevent the development of GIM. Hence, studies included in this thesis investigated a drug, amitriptyline (AMI), which can inhibit TLR2 and TLR4 and has weak anti-cholinergic activity.
AMI, a tricyclic anti-depressant, was investigated for several reasons. It is prescribed for cancer patients for relieving cancer related neuropathic pain (Mishra et al., 2012; Banerjee et al., 2013). However it’s long term use is associated with adverse effects including, drowsiness, dizziness, mouth dryness, nausea and constipation (Mishra et al., 2012). Moreover, it is widely used in inflammatory bowel diseases and gastrointestinal disorders due to its anti-inflammatory effects (Bahar et al., 2008; Vahedi et al., 2008; Sohn et al., 2012). AMI has anti-inflammatory effect including inhibition of TLR2 and TLR4 activity, NF-κB, IL-1β and TNF serum and protein levels (Hutchinson et al., 2010b; Sadeghi et al., 2011; Vismari et al., 2012). It has anti-apoptotic effects depending on the dose and the cell type (Kolla et al., 2005; Arimochi and Morita, 2006b; Han and Lee, 2009). It has anti-apoptotic effect at moderate dose (1-25 µM) in PC12 cells (Han and Lee, 2009). It has anti-diarrhoeal effect due to its anticholinergic properties which reduces colonic motility (Brecht et al., 2007). This evidence suggests a possible inhibitory effect of AMI on intestinal TLR2 and TLR4 expression, which could modulate irinotecan-induced GIM. Despite the extensive use of this drug in clinical practice (Vahedi et al., 2008; Sohn et al., 2012; Banerjee et al., 2013), there are limited data with regard to its effect on the gastrointestinal mucosa and particularly GIM.

Our results showed the ability of AMI to reduce acute diarrhoea occurrence and colonic apoptosis after irinotecan treatment in Wistar rats. However, rats were not protected against weight loss, distress symptoms, and histological damage in the intestines. To determine the transcriptional responses that may have been responsible for apoptosis protection by AMI, a PCR array of innate immunity transcripts was conducted. Expression of Interferon gamma receptor 1 (INFγr1) was increased while interlukin-1 beta (IL-1β), interleukin-1 receptor 2 (IL-1r2) and caspase-4 were decreased in the colon of rats treated with both AMI and irinotecan compared to irinotecan alone. The reduction in IL-1r2, caspase-4 and IL-1β expression suggests that the ability to reduce apoptosis is mediated through IL-1β inhibition (Antonopoulos et al., 2013), and our in vitro studies in IEC-6 cells confirmed that AMI reduced IL-1β mRNA expression following SN-38 treatment. Reduction in IL-1r2 and caspase-4 are essential for IL-1β inhibition (Friedlander et al., 1996; Siegmund, 2002). INFγ-mediated effects requires the activation of both INFγr1 and INFγr2 (Plataniyas, 2005). Even though treatment with AMI increased INFγr1, it did not translate to increased apoptosis. Therefore, these results strongly suggest that inhibition of apoptosis was through IL-1β inhibition.
Protein expression of TLRs did not differ between rats treated with irinotecan alone and rats treated with AMI and irinotecan in both regions. In fact, TLR2, TLR4, IL-1β, TNF and IL-6 were not significantly increased in our Wistar model compared to untreated controls, indicating that irinotecan did not induce a robust inflammatory response. In addition, in our current model, late onset diarrhoea was not observed. These findings were in direct contrast to results in the first study of the thesis (chapter 2) which examined DA rats. Differences in study design may account somewhat for the divergent results, including the use of different rat strains and sex in each study (female DA vs. male Wistar) and different dose of irinotecan used (200 vs. 125 mg/kg). Wistar rats were used in this study since previous work on our laboratory showed that 125 mg/kg irinotecan induced late-onset diarrhoea in this model (Gibson, 2010). However, in our study rats did not develop diarrhoea following the same dose. In addition, while 200 mg/kg was sufficient to induce late-onset diarrhoea in female DA rats, it was fatal for male Wistar rat (Gibson et al., 2007; Gibson, 2010), which is clearly showing strain differences between two models. In addition, there are differences in the levels of metabolising enzymes (CYPs) between both strains/sex, which could interfere with drug metabolism thus leading to different exposure to drug metabolites (Morita et al., 1998; Staack et al., 2004). This suggests that Wistars have a different profile of GIM compared to DA rats. Given the lack of late-onset diarrhoea in our study, it shows that the male Wistar rat is not an effective strain to model irinotecan-induced GIM and makes interpretation of the findings difficult.

Nonetheless, the lack of TLR up-regulation in this study supports our previous findings that TLR2 and TLR4 expression is associated with irinotecan-induced late onset diarrhoea and severity of mucosal injury. Previous research has shown that increases in TLR2, TLR4 and pro-inflammatory cytokines are associated with chemotherapy-induced late-onset diarrhoea. Increased TLR2 expression was associated with diarrhoea occurrence, apoptosis, histological damage, all of which were evident before bacterial translocation and intestinal ulceration in a doxorubicin-induced GIM model (Kaczmarek et al., 2012). TLR4 expression increased up to 72 h after 5-fluorouracil-induced GIM (Ferreira et al., 2012). Results from our study on DA rats have shown an association between colonic TLR2 and TLR4 mRNA expressions at 96 h and diarrhoea occurrence and severity after irinotecan-induced GIM. Hence, our results strongly indicate that TLRs are only elevated in vivo in response to intestinal mucosal injury that is sufficiently severe to cause diarrhoea. Hence, TLR inhibition has the potential to modify chemotherapy-induced diarrhoea. Although our results did not show an increase in tissue expression of TLRs and pro-inflammatory
cytokines at the protein level, secreted levels and activity of these mediators need to be investigated to rule out more subtle changes contributing to altered gut function.

Even though our results show that combined treatment of AMI and irinotecan has protective effect from apoptosis induced by irinotecan, it was not protective against weight loss and distress symptoms compared to irinotecan alone treated rats. This was an important observation since it could be a result of drug interactions that reduce safety of irinotecan. The interaction could be related to the effect of AMI on SN-38 metabolism. Previous evidence has shown that AMI is a weak inhibitor of UDP glucuronosyltransferases (UGTs), which are important for irinotecan detoxification (Uchaipichat et al., 2006; Donato et al., 2010). Thus, there is a possibility of reduction in SN-38 glucuronidation and thus prolonged toxicity. Even though AMI is prescribed for cancer patients and so is taken in combination with chemotherapeutic drugs (Pezzella et al., 2001; Kautio et al., 2008; Kautio et al., 2009; Banerjee et al., 2013; Toftegard et al., 2013), close management of patients’ symptoms should be considered since our results suggests that the intestinal mucosa is not protected from irinotecan toxicity when used with AMI.

In contrast to our results, recent research has shown that methotrexate-induced GIM is associated with a decrease in TLR4 mRNA and protein expression (Sukhotnik et al., 2014). Administration of glutamine attenuated methotrexate-induced GIM and showed an increase in TLR4 expression. Since methotrexate is well known to cause inflammatory responses, it was suggested that TLR4 down-regulation by methotrexate is compensatory, in that activated receptors mediate their own down-regulation to limit the response to stimulus. In that, TLRs are differentially expressed in intestinal leukocytes and epithelial cells and chemotherapy-induced GIM could modulate the interaction between leukocyte-epithelial cells. The exact mechanism of the protective effect of glutamine is still poorly understood. It includes regulation of occludin and claudin, through NF-κB which is involved in TLR4 signalling (Beutheu et al., 2014). This suggests a cross-talk between TLR, and NF-κB signalling. Therefore, TLR expression following injury should be investigated for each chemotherapeutic drug and the role of its inhibition by different inhibitors to understand their role in GIM.

In summary, this study provides an overview of the effect of AMI in irinotecan-induced apoptosis. Despite the extensive clinical use of AMI, drug interactions should be considered in
chemotherapeutic treatment safety and efficacy. Recently, a systematic review recommended that clinicians should not use AMI for cancer patients undergoing neurotoxic agents, such as oxaliplatin and paclitaxel (Hershman et al., 2014). Further investigations are required to investigate TLR2 and TLR4 through specific inhibitors to avoid the off target effects of AMI and which can be translated clinically for effective prevention of the occurrence of GIM. Moreover, results from this study confirmed the complexity and the multifactorial effects of GIM which should be considered for intervention in the future.
Chapter Seven
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Appendix
A. TLR4

B. TLR2
C. IL-1B

D. TNF
E. IL-6

Figure showing the melt curve of RT-PCR for A. TLR4, B. TLR2, C. IL-1B, D. TNF and E. IL-6.
Figure showing (A) MPO and (B) MCP-1 immunostaining. Arrows showing positively stained cells. Cells were counted in the lamina propria layer. Photomicrographs are 400x original magnification. Scale bar is 50 µm.