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

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AMI: amitriptyline, ATM: ataxia telangiectasia mutated, B2M: Beta-2-microglobulin, CD: crohn's disease, CPT-11: irinotecan, COX-2: cyclooxygenase-2, CCL-2: Chemokine (C-C motif) ligand 2, Cas-3: caspase-3, DAMP: damage-associated molecular pattern, DA: Dark Agouti rat, DAB: diaminobenzidine, DMSO: dimethyl sulfoxide, DSS: dextran sulfate sodium, *E. coli: Escherichia coli*, FCS: foetal calf serum, FADD: Fas-Associated protein with Death Domain, GIM: gastrointestinal mucositis, GST: glutathione S-transferase, GSK-3β: glycogen synthase kinase 3, HMGB1: high-mobility group box 1, HSP: heat shock proteins, h: hour, H&E: haematoxylin and eosin, HCl: hydrogen chloride, H_2O_2: hydrogen Peroxide, IL-1β: interleukin-1 beta, IBD: inflammatory bowel diseases, IBS: irritable bowel syndrome, IEC-6: rat intestinal epithelial cell line, INF: interferon, i.p: intraperitoneal injection, i.v: intravenous injection, LPS: lipopolysaccharide, Ldha: Lactate dehydrogenase A, MMP: Matrix metalloproteinases, MD-2: Myeloid differentiation factor-2, MCP-1: monocyte chemoattractant protein-1, MPO: Myeloperoxidase, MyD88: myeloid differentiation primary response gene 88, NF-κB: nuclear factor kappa B, PAMP: pathogen-associated molecular patterns, PBS: phosphate buffered saline, PGE2: prostaglandin E2, P53: tumour suppressor protein, PI3K: phosphatidylinositide 3-kinase, RT-PCR: real time- polymerase chain reaction, ROS: reactive oxygen species, rhIL-1Ra: recombinant human interleukin-1 receptor antagonist, sc: subcutaneous injection, SN-38: 7-Ethyl-10-hydroxycamptothecin, SN-38G: SN-38 glucuronide, TLR: toll-like receptor, TNF: tumor necrosis factor, TRAF3: tumor necrosis factor receptor associated factor 3, Tj: tight junction, TFF3: trefoil factor 3, UBC: Ubiquitin C, UC: ulcerative colitis, UGT: UDP-glucuronosyltransferase and XTT: (2,3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide).

Abstract

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

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

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

The first study of this research project investigated the involvement of TLR2, TLR4 and pro-inflammatory cytokines in irinotecan-induced diarrhoea. TLR2, TLR4, interlukin-1 beta (IL-1β),

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

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

The third study investigated the ability of AMI to inhibit apoptosis induced by SN-38, the active metabolite of irinotecan, in a rat intestinal epithelial cell line, IEC-6. Cells were treated for 24 h with SN-38 (IC_{50} of apoptosis = 8.7 μM) alone or in combination with AMI (1 μM). At this dose, AMI inhibits TLR2 and TLR4 and is within the therapeutic range. Apoptosis and mRNA

expression of TLR2, TLR4 and pro-inflammatory cytokines were investigated. Results showed that treatment with SN-38 was associated with increased mRNA expression of TLR2, TLR4 and pro-inflammatory cytokines compared to AMI treated cells. It also showed that treatment with AMI attenuated apoptosis when administered with SN-38. Treatment with AMI and SN-38 was associated with significant reduction in mRNA expression of TLR2, TLR4 and IL-1β compared to SN-38 treated cells. However, TNF expression increased after treatment with both drugs compared to SN-38 treated cells, suggesting and that an alternative pathway to TLRs is activated which leads to TNF upregulation in IEC-6 cells.

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

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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.