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ORIGINAL ARTICLE

Amitriptyline prevents CPT-11-induced early-onset diarrhea and colonic apoptosis without reducing overall gastrointestinal damage in a rat model of mucositis

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Abstract

Purpose Gastrointestinal mucositis (GIM) is one of the most debilitating side effects of the chemotherapy agent, irinotecan hydrochloride (CPT-11). The toll-like receptor (TLR) pathway is a key mediator implicated in the pathophysiology underlying GIM. The tricyclic antidepressant amitriptyline has been shown to inhibit TLR2 and TLR4 activity in in vitro models. The aim of this study was therefore to investigate the effect of amitriptyline on the development of GIM following CPT-11.

Methods Male albino Wistar rats were treated with either CPT-11 (125 mg/kg, i.p., $n = 18$), amitriptyline (20 mg/kg, $n = 18$), both agents ($n = 18$), or vehicle control ($n = 18$) and killed at 6, 48, or 96 h. Differences between groups in measurements of gastrointestinal toxicity (diarrhea and weight loss), mucosal injury (apoptosis and histopathology score), colonic expression of TLRs, and pro-inflammatory cytokines were determined.

Results CPT-11-induced diarrhea and colonic apoptosis were inhibited by amitriptyline at 6 h. However, rats were not protected from weight loss or mucosal injury over the time course of CPT-11-induced GIM. Interleukin-1 beta transcript expression was significantly decreased with amitriptyline treatment at 6 h, although protein expression did not differ between groups. There was no change in TLR4 or TLR2 expression in any group.

Conclusions Prophylactic amitriptyline was able to inhibit early intestinal damage in this rat model of CPT-11-induced GIM, but exacerbated late-onset injury. These findings do not support use of amitriptyline as an approach for mitigation of GIM in this setting.

Keywords Mucositis · Toll-like receptors · Diarrhea · Apoptosis · CPT-11 · Amitriptyline

Introduction

The cancer chemotherapy drug irinotecan hydrochloride (CPT-11) causes gastrointestinal mucositis (GIM), which manifests as diarrhea, in up to 80% of patients [1]. CPT-11 causes an early-onset diarrhea that occurs within 24 h and involves an adverse cholinergic reaction which can be prevented by atropine, and late-onset diarrhea, which appears after 24 h and is due to gastrointestinal (GI) mucosal damage. CPT-11 is a pro-drug that is converted to the toxic metabolite 7-ethyl-10-hydroxy-camptothecin (SN-38) and then is detoxified to SN-38 glucuronide (SN-38G) by liver UDP-glucuronosyltransferase isoforms. SN-38G undergoes enterohepatic recirculation to SN-38 via the action of β-glucuronidase-producing bacteria, which is thought to be a major factor underpinning the unique GI toxicity of CPT-11 (for review, see [2]). CPT-11-induced GIM is a complex phenomenon that currently lacks an effective intervention clinically. Previous preclinical models of CPT-11-induced diarrhea have shown some benefit with the use of probiotics [3], antibiotics [4], and agents that reduce inflammatory cytokine production [5], suggesting that microbes and host immune responses are key aspects of pathogenesis.

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Our group has identified the occurrence and severity of CPT-11-induced GIM in rats are associated with increased apoptosis, reduced proliferation, histological damage [6], and changes in the gut microflora [7]. In addition, activation of nuclear factor kappa B (NF-κB) follows CPT-11 administration, which upregulates expression of inflammatory mediators involved in mucositis development, including pro-inflammatory cytokines tumor necrosis factor alpha (TNF), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) [8, 9]. Recent research has further highlighted the role of toll-like receptor (TLR) signaling pathways in GIM development, in particular TLR2 and TLR4 pathways. Studies have revealed genetic absence of TLR2 and TLR4 protects against CPT-11-induced GIM, with improvement in diarrhea and intestinal permeability, and less histopathological damage to the small intestine in mice [10, 11]. These findings provide evidence that host immune-microbe interactions regulate mucosal response to CPT-11. Therefore, both TLR2 and TLR4 appear to have key roles in mediating CPT-11-induced GIM, most likely due to modified inflammatory responses and bacterial translocation, and provide a rationale target for intervention. Despite this, few studies have investigated the impact of pharmacological TLR inhibition on CPT-11-induced GIM.

Amitriptyline (AMI), a tricyclic antidepressant drug, is widely used in the management of psychological disorders, various types of pain [12], and irritable bowel disease [13], due to its anti-inflammatory effects. In vitro AMI and its metabolite, nortriptyline, decreased LPS-induced TNF and IL-1β release in glial cells [14], and AMI was shown to block agonist-induced TLR2 and TLR4 activation and IL-1β release [15]. These findings suggest a possible inhibitory effect of AMI on intestinal TLR2 and TLR4 signaling, which would modulate CPT-11-induced GIM by depressing inflammatory responses. Consequently, we hypothesized that TLR2 and TLR4 inhibition by AMI would attenuate CPT-11-induced GIM. The primary aim was to determine if AMI attenuates GIM following CPT-11 treatment.

Methods

Chemicals

AMI (Sigma-Aldrich, Castle Hill, Australia) was diluted in sterile distilled water. CPT-11 (kindly supplied by Pharmacia/Pfizer, NY, USA) was prepared in a sorbitol/lactic acid buffer (also used as vehicle) as described previously [16] and as required for the activation of the drug. Atropine was diluted to 0.01 mg/kg (Pfizer, NY, USA) in sterile distilled water and administered as a subcutaneous injection immediately prior to CPT-11.

Animals and ethics

The study was approved by the Animal Ethics Committees of The University of Adelaide (approval number: M-2012-026) and complied with the 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 and 8 weeks and weighing 184–300 g sourced from the Australian Research Centre (Perth). Rats were housed in Perspex cages at a temperature of 21 ± 1°C and subject to 12-h light/dark cycle with ad libitum access to standard chow and water.

Experimental design

After 1 week of acclimatization, 72 rats were randomly assigned into four groups ($n = 18$): (1) CPT-11; (2) AMI; (3) CPT-11 + AMI; and (4) vehicle control. All treatments were administered as intraperitoneal (i.p.) injections and all rats received the same number of injections to control for stresses. Timing of treatments is all relative to CPT-11/vehicle injection designated as time 0 h. Group 1 received water injection the morning (−24 h) and afternoon (−16 h) preceding 125 mg/kg CPT-11 (0 h). This was followed by a third water injection immediately after CPT-11 (0 h). Group 2 received 20 mg/kg AMI at −24, −16, and 0 h and vehicle (0 h). Group 3 received 20 mg/kg AMI at −24, −16, and 0 h and CPT-11 (0 h). Group 4 received water injection at −24, −16, and 0 h and vehicle (0 h). All rats received atropine to dampen cholinergic responses. The CPT-11 dose (125 mg/kg) used in this study has been shown previously in our laboratory to cause diarrhea in male albino Wistar rats (unpublished results). Repeated dosing of AMI was conducted to establish steady-state plasma levels and theoretically ensure the inhibition of TLR2 and TLR4 prior to CPT-11 injection (personal communication Prof. Mark Hutchinson).

Groups of rats ($n = 6$) were killed at 6, 48, and 96 h by cardiac puncture and cervical dislocation under 2% isofluorane in 100% O_2 anesthesia. The jejunum and colon were dissected and prepared for histological, immunochemistry, and molecular analyses as previously described [6, 17, 18]. 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 scrapings were stored in RNA later solution (Ambion, Life Technologies, Australia) at −70°C.

GI toxicity assessment

Animals were monitored daily to record diarrhea incidence and severity (graded as described previously [6]). Briefly, this was graded as 0, no diarrhea; 1, mild diarrhea (staining of

anus); 2, moderate diarrhea (staining over top of the legs and lower abdomen); and 3, severe diarrhea (staining over legs and higher abdomen, often associated with continual oozing). Body weight change, as a marker of GI toxicity, was also collected daily.

Histological examination

Jejunum and colon tissue sections were stained with hematoxylin and eosin as previously described [6], and scanned using a NanoZoomer 2.0-HT Slide scanner (Hamamatsu, Japan). The occurrence of eight (jejunum) or six (colon) histological criteria was examined using established criteria [19]: 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 edema. Each parameter was scored as present = 1 or absent = 0, with the average total score plotted for each time point across groups.

Epithelial apoptosis and proliferation

Cleaved caspase-3 (apoptosis marker) and Ki-67 (proliferation marker) were detected using immunohistochemistry as previously described [17]. Primary antibodies against caspase-3 (ab4051, Abcam, Cambridge, UK) and Ki-67 (ab16667, Abcam, Cambridge, UK) were diluted to 1:100 with 5% normal goat serum. Caspase-3 staining was assessed by counting positive cells in 15 fields (×200 magnification) and calculation of the mean number of positive cells/crypt. Ki-67 staining was assessed by counting stained cells in 20 half crypts (×400 magnification) and calculation of the mean number of positive cells/half crypt.

Transcript expression of innate and adaptive immunity in colon

RNA was extracted from 20 mg of colonic mucosal scrapings (6 h kill point) by homogenizing tissues in 500 μl TRIzo1®Reagent (Invitrogen Life Technologies, Australia). RNA was isolated using a Nucleospin® RNA II kit (Nucleospin, Australia) following the manufacturer's instructions. RNA concentration and purity was measured using NanoDrop® Spectrophotometer and determined to be high purity, protein-free, phenol-free, and ethanol-free RNA samples. Differential gene expression between CPT-11- and AMI + CPT-11-treated rats was determined with a rat innate and adaptive immunity array (QIAGEN, Doncaster, Australia). All genes were normalized to the housekeeping gene lactate dehydrogenase A (Ldha) as this transcript had the most stable expression across groups as determined by $2^{-\Delta\Delta CT}$ analysis.

TLR2, TLR4, and IL-1β expression

TLR2, TLR4, and IL-1β were detected with immunohistochemistry using methodology described previously [17]. In brief, non-specific antibody binding was prevented with blocking solution (Covance, USA) for 20 min at room temperature. Then, avidin and biotin were blocked (following the manufacturer's instructions; Vector Laboratories, Inc., USA). Sections were incubated overnight at 4°C with primary antibodies diluted in blocking solution in a humidifying chamber, TLR2 (Novus Biologicals, USA), TLR4 (Abcam, UK), and IL-1β (Santa Cruz Biotechnology, USA). Sections were washed and then incubated with linking reagent and labeling reagent, and finally antibody binding was visualized using DAB according to the manufacturer's instructions (Ultra Streptavidin (USA) HRP Detection Kit, BioLegend, USA). All sections were scanned using a NanoZoomer 2.0-HT Slide scanner (Hamamatsu, Japan) and scored for relative abundance of positive staining in the epithelium using a 0–4 scale.

Statistical analysis

Group medians were compared by the Kruskal-Wallis test for diarrhea and histopathology scores. Average body weight changes from baseline and caspase-3 and Ki-67 detection were compared between groups using two-way ANOVA with Bonferroni post hoc test, and protein expression (TLR2, TLR4, and IL-1β) was compared between groups using one-way ANOVA with Tukey's post hoc test. Results were statistically significant at $P < 0.05$.

Results

GI toxicity

Weight loss Body weight of vehicle control rats increased 16.3 ± 1.0% over the experiment time course from baseline. CPT-11-alone rats lost 2.9 ± 1.3% body weight in the first 24 h after CPT-11, accompanied with acute-onset diarrhea. Rats then slowly regained weight, returning to baseline by the end of the study, although they remained significantly lighter than vehicle controls at 96 h ($P < 0.0001$). Rats treated with AMI alone lost 2.4 ± 0.4% body weight between − 24 and 0 h of the experiment that was significantly more compared to vehicle controls ($P = 0.004$). By 24 h, rats returned to baseline and then continued to gain weight until the end of the study, although they remained significantly lighter than vehicle controls ($P < 0.0001$). Rats treated with AMI + CPT-11 lost weight every day of the study up to 10.4 ± 1.1% at 96 h, with significantly greater weight loss compared to all other groups ($P < 0.0001$) (Fig. 1).

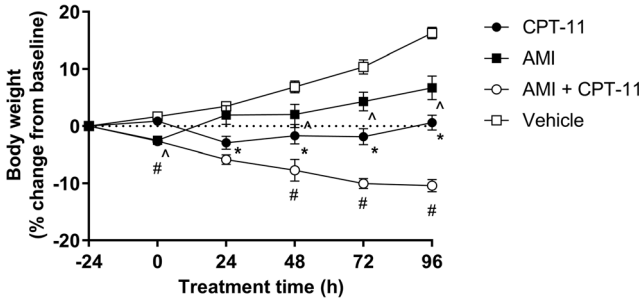

Fig. 1 Body weight. Change in body weight was measured over time and expressed as a percentage relative to baseline. Data shown as mean ± SEM. Significant difference between groups; vehicle and CPT-11 (*), vehicle and AMI (^), AMI + CPT-11 and CPT-11 (#), tested by 2-way ANOVA with Bonferroni multiple comparison test

Diarrhea Sixty-one percent and 6% of rats treated with CPT-11 alone showed mild or moderate diarrhea, respectively, at 6 h. Mild diarrhea reduced at 24 h to 25% of rats and was not observed at later time points. Rats treated with AMI alone showed no diarrhea at any time point. Sixteen percent of rats treated with AMI + CPT-11 exhibited mild diarrhea at 24 h, and 16% had moderate diarrhea at 96 h. Sixteen percent of rats treated with vehicle controls had mild diarrhea at 96 h. Diarrhea incidence and severity was significantly higher in CPT-11-treated rats compared to all other treatments at 6 h ($P = 0.005$).

Mucosal injury: histological analysis

In the jejunum, significantly increased histological damage was observed in the CPT-11 group compared to the vehicle controls at 48 h ($P = 0.005$) with villous atrophy and blunting, and disruption of crypt cells, while at 96 h, crypt regeneration and hyperplasia was observed (Fig. 2a).

In the colon, increased edema was observed in rats treated with AMI alone along with disruption in crypt cells. Complete crypt ablation and edema was observed in rats treated with AMI + CPT-11, with significantly increased histopathological scores compared to vehicle controls at 6 and 48 h ($P = 0.01$ and $P = 0.03$, respectively) (Fig. 2b). Crypt length did not differ significantly between any groups at any time point.

Apoptosis

In the jejunum, there was a significant increase in the number of caspase-3-positive apoptotic cells per crypt following CPT-11 alone (mean ± SEM = 2.5 ± 0.25 cells/crypt) and AMI + CPT-11 (2.2 ± 0.2 cells/crypt) compared to vehicle controls (0.03 ± 0.01 cells/crypt) and AMI alone (0.04 ± 0.02 cells/crypt) groups at 6 h ($P < 0.0001$). There were no other significant differences between groups at any other time point (Fig. 3a).

In the colon at 6 h, there was a significant increase in crypt apoptosis in rats treated with CPT-11 (1.8 ± 0.28 cells/crypt) compared to AMI + CPT-11 (0.6 ± 0.25 cells/crypt, $P < 0.0001$), AMI (0.2 ± 0.05 cells/crypt, $P < 0.0001$), and vehicle controls (0.1 ± 0.02 cells/crypt, $P < 0.0001$). In addition, a less pronounced increase in apoptosis in rats treated with AMI + CPT-11 compared to vehicle controls was noted at 6 h ($P = 0.04$). There were no other significant differences between groups at any time points (Fig. 3b, c).

Proliferation

Jejunum At 48 h, proliferation was significantly decreased with CPT-11 (20.2 ± 1.9 cells/half crypt) and AMI + CPT-11 (17.4 ± 1.4 cells/half crypt) compared to vehicle controls (29.6 ± 0.85 cells/half crypt, $P < 0.003$). At 96 h, this was reversed with a significant increase in proliferation with CPT-11 (45.2 ± 1.5 cells/half crypt) and AMI + CPT-11 (39.1 ± 3.6 cells/half crypt) compared to vehicle controls (29.6 ± 1.75, $P < 0.002$). There were no significant differences in proliferation between rats treated with CPT-11 and AMI + CPT-11 at any time point (Fig. 4a).

Colon There were no significant differences in proliferation in the colon between any treatments at any time point (Fig. 4b, c).

PCR array

Colonic mRNA expression of caspase-4, IL-1β, and interleukin-1 receptor 2 (IL-1r2) decreased (by 1.8-, 13-, and 4-fold, respectively) in rats treated with AMI + CPT-11 compared to CPT-11 at 6 h ($P = 0.005$, 0.038, and 0.01, respectively). Conversely, interferon gamma receptor 1 (INFγR1) mRNA expression increased by 1.4-fold in rats treated with AMI + CPT-11 compared to CPT-11 ($P = 0.02$). No other changes in mRNA expression were detected (Online Resource 1).

Protein expression of TLR2, TLR4, and IL-1β in the colon

There was no observable difference between groups for any target at 6 h in the colon when immunohistochemical staining was analyzed (Fig. 5).

Discussion

This study was the first to investigate the effect of AMI as a potential intervention for CPT-11-induced GIM in albino Wistar rats. We found that AMI exhibited anti-apoptotic properties by reducing early-onset diarrhea and activated caspase-3 levels 6 h after CPT-11 treatment. This was associated with decreased caspase-4, IL-1β, and IL-1r2 and increased

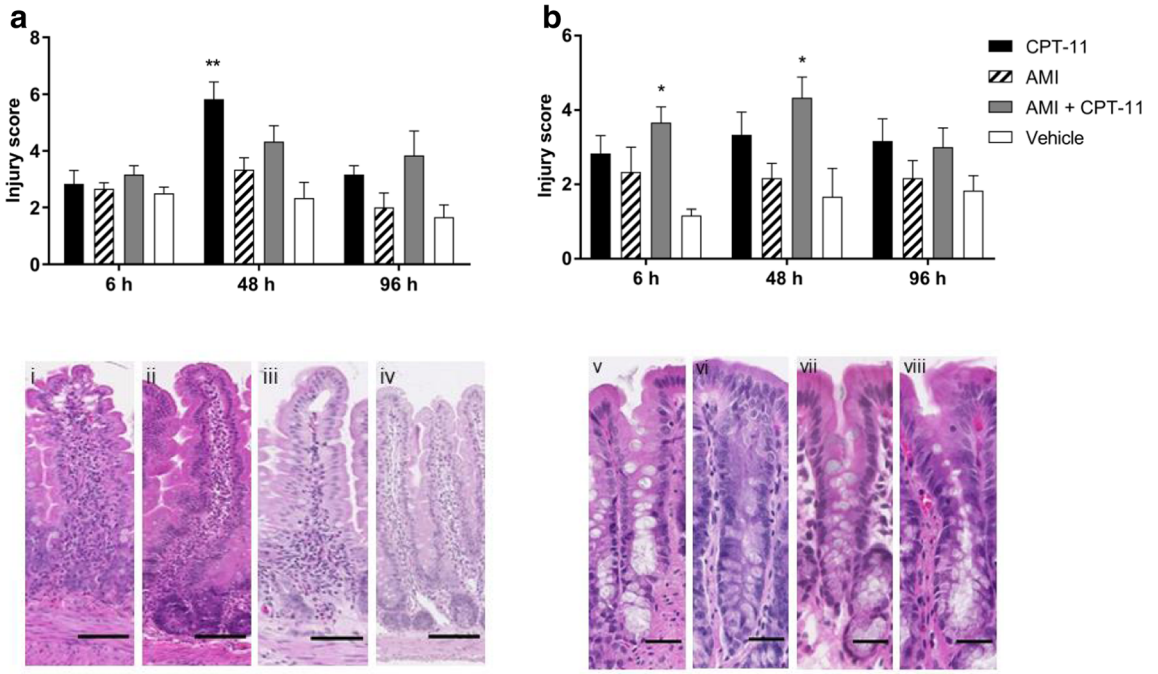

Fig. 2 Intestinal histopathology. Tissue injury in the jejunum (**a**) and colon (**b**) was scored against 8 and 6 criteria, respectively. Data shown as mean ± SEM, $*P < 0.05$ and $**P < 0.01$ compared to vehicle control, tested by 2-way ANOVA with Bonferroni multiple comparison test. Photomicrographs of representative tissue slides at 48 h in the jejunum: (i) CPT-11, (ii) AMI, (iii) AMI + CPT-11, and (iv) vehicle control; and colon: (v) CPT-11, (vi) AMI, (vii) AMI + CPT-11, and (viii) vehicle control. Scale bars are 50 μm

INFγR1 transcript expression. However, AMI did not protect against CPT-11-induced weight loss or intestinal damage, rather exacerbating these measures of GI toxicity. Furthermore, expression of TLR2 and TLR4 was unchanged.

The occurrence of early-onset diarrhea in over 50% of rats treated with CPT-11 alone despite atropine administration is consistent with our previous work in Dark Agouti (DA) rats treated with 200 mg/kg [8]. However, CPT-11-induced late-onset diarrhea was not observed in this current study, with body weight recovering following an initial loss at 24 h. The dose of 125 mg/kg and the use of male rats make any comparison between this and previous work in female DA rats impractical. However, there was no significant colonic histopathology in CPT-11-treated rats, indicating that late-onset diarrhea and colonic, rather than jejunal, damage are closely related to diarrhea presentation.

Rats treated with AMI lost weight during the first 24 h of the study, possibly due to the sedative effect of this drug [20] indicated by their reluctance to move compared to other groups (data not shown). However, food and water intake and spontaneous locomotor activity were not quantified directly which is a limitation of the study. Further, the addition of AMI to CPT-11 treatment reduced acute diarrhea occurrence at 6 (0%) and 24 h (16%); however, this treatment was associated with late-onset diarrhea in 16% (1/6) of rats and continued weight loss up to 96 h. This reduction in early-onset diarrhea could be due to an anti-cholinergic action since AMI is known to have anti-cholinergic and anti-muscarinic effects [21]. Given that atropine treatment in rats treated with AMI + CPT-11 did not prevent early-onset diarrhea entirely, AMI may be having an additive effect, or may prevent diarrhea via a non-cholinergic mechanism yet to be determined.

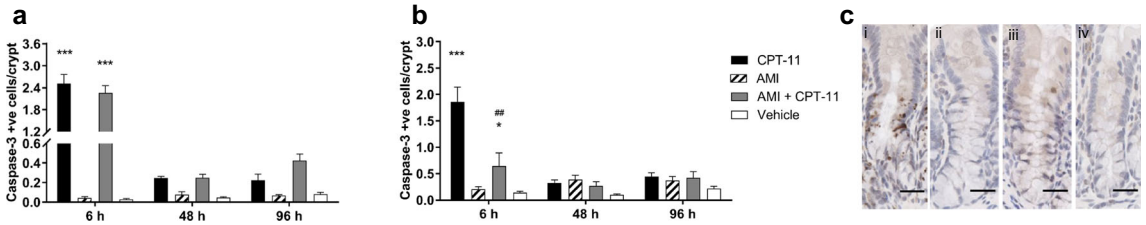

Fig. 3 Apoptosis quantification. Positive immunohistochemical detection of cleaved caspase-3 protein was counted in the **a** jejunum and **b** colon over time, with data presented as mean ± SEM cells/crypt, $***P < 0.0001$ and $*P < 0.05$ compared to vehicle control, $\#\#P < 0.01$ compared to CPT-11 alone, tested by 2-way ANOVA with Bonferroni multiple comparison test. **c** Representative photomicrographs of colon at 6 h: (i) CPT-11, (ii) AMI, (iii) AMI + CPT-11, (iv) vehicle control. Scale bars are 50 μm

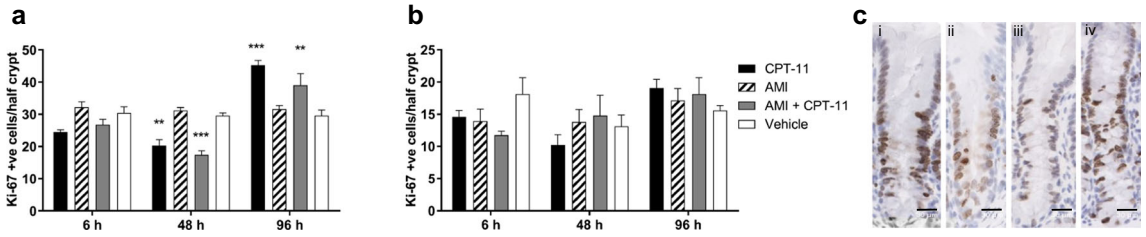

Fig. 4 Proliferation quantification. Positive immunohistochemical detection of Ki-67 protein was counted in the **a** jejunum and **b** colon over time, with data presented as mean ± SEM cells/half crypt, ***$P < 0.0001$ and **$P < 0.01$ compared to vehicle control, tested by 2-way ANOVA with Bonferroni multiple comparison test. **c** Representative photomicrographs of colon at 6 h: (i) CPT-11, (ii) AMI, (iii) AMI + CPT-11, and (iv) vehicle control. Scale bars are 30 μm

These results are in agreement with clinical studies demonstrating AMI effectiveness in irritable bowel syndrome with diarrhea [22, 23].

Regarding the late-onset diarrhea and weight loss, this indicates that AMI co-treatment was associated with an unidentified systemic toxicity, independent of early intestinal pathology and unlikely to be related to circulating levels after 24 h due to its short half-life of approximately 5 h in rats [24]. More likely, this exacerbation of GI toxicity could be a result of drug interactions altering SN-38 detoxification pathways. For example, AMI has been shown to be a non-selective inhibitor of UGTs [25] that would reduce SN-38 glucuronidation and increase intestinal exposure to toxic SN-38. Alternatively, AMI also inhibits glutathione S-transferase (GST-π and GST-α) enzyme activity [26], another pathway of cellular detoxification of CPT-11 [27]. These interactions could explain the observed 16% moderate diarrhea at 96 h in rats treated with both AMI and CPT-11. One rat (out of 6) in the vehicle control group had mild diarrhea at 96 h of unknown cause and even though overdose of sorbitol can cause GI symptoms, including diarrhea, in albino Wistar rats [28], the timing of the diarrhea onset makes it unlikely to be related to sorbitol. It is more likely that the mild and intermittent diarrhea was related to handling stress.

A significant increase in apoptosis at 6 h was observed in both the jejunum and colon of rats treated with CPT-11 compared to vehicle control, similar to previous studies in DA rats treated with CPT-11 [3]. The addition of AMI was able to significantly reduce CPT-11-induced apoptosis in colonic epithelium at 6 h, and for that reason, further assessment of AMI impact focused on this early time point. These observations are in agreement with a previous study in mice with cisplatin-induced acute kidney damage, where AMI inhibited ceramide production and protected against apoptosis [29], indicating that this may represent a mechanism for epithelium protection. In contrast, there is mixed evidence from previous in vitro research. For example, one in vitro study has shown that AMI has a protective effect from drug-induced apoptosis in rat PC-12 cells via various mechanisms including altered mitochrondrial permeability and subsequent measures of oxidative stress [30]. However, another study has shown that AMI at high concentration (100 μM) increases apoptosis in rat dorsal root ganglia [31].

We sought to elucidate the mechanism of apoptosis protection by AMI in our model using a PCR array of innate and adaptive immunity transcripts. Our results showed a significant decrease in mRNA expression of IL-1β, IL-1r2, and caspase-4 and increased expression of INFγR1 in rats treated with AMI + CPT-11 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 can be activated in a non-canonical pathway by caspase-4 [32]. Thus, reduction in caspase-4 should theoretically result in reduced activation of IL-1β and consequently inhibition of apoptosis. The available evidence seems to suggest that treatment with AMI + CPT-11 reduced apoptosis through a caspase-dependent

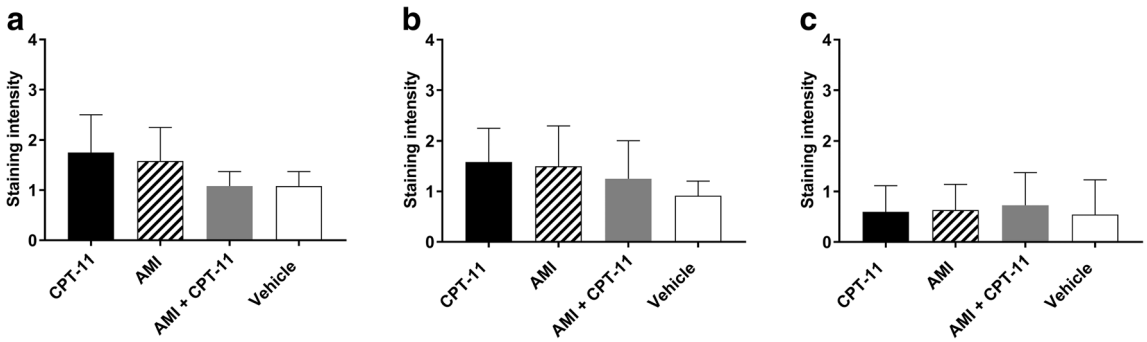

Fig. 5 Qualitative immunohistochemistry. Intensity of immunostaining in the colonic epithelium for **a** TLR2, **b** TLR4, and **c** IL-1β at 6 h. Data shown is mean ± SEM

pathway that involved IL-1β inhibition. In contrast, mRNA expression of INFγR1 increased and would support a pro-apoptotic and pro-inflammatory response [33].

Examination of protein expression levels revealed a lack of concordance between mRNA and protein results with no change in IL-1β immunostaining observed. This perhaps indicates that CPT-11 did not induce a robust inflammatory response nor are inflammatory factors likely to play a significant role in regulating early apoptosis. Previous research has shown that intestinal expression of TLR2, TLR4, TLR9, and pro-inflammatory cytokines is associated with chemotherapy-induced GIM [8, 11, 34, 35]. As such, it seems that TLRs and pro-inflammatory cytokines are involved in severe GIM but play an uncertain role in more modest tissue injury models of GIM such as that created in our current study. Research conducted in our laboratory has shown an association between colonic TLR2 and TLR4 mRNA expression at 96 h and diarrhea severity after CPT-11 [36]. Hence, our results indicate that TLRs are only elevated in response to intestinal injury that is sufficiently severe to cause increased permeability, bacterial translocation, and subsequent late-onset diarrhea. Nonetheless, the underlying cause of discordance between mRNA and protein expression requires further investigation. One explanation could be that in our current study, mRNA was isolated from the mucosal layer only, and immunostaining was quantified in the epithelium, with the possibility that cells occupying other tissue compartments likely contribute a relatively greater signal for TLRs as seen in other studies. As such, further work is required to dissect the role of individual targets on AMI-mediated apoptosis protection in the colon. Furthermore, the lack of specificity of AMI as a TLR2/4 antagonist complicates any interpretation. Other than its interaction with 5-HT receptors, it has receptor binding activity at muscarinic cholinergic and histamine H1 receptors [37], which have the potential to impact gastrointestinal function, and is a limitation of the current study. To date, the impact of AMI on intestinal activity of TLR2/4 has not been examined and should form the basis of future studies to complement the evidence generated from genetic knock out models [10, 11].

In summary, this study demonstrated that AMI was ineffective at preventing CPT-11-induced GIM, although showed some activity in prevention of early-onset diarrhea and colonic apoptosis. The contribution of TLR2 and 4 in this model appears negligible given that CPT-11 did not lead to an observable up-regulation of these targets. As such, further work needs to be conducted to identify if AMI has any ability to modulate intestinal TLR activity.

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Compliance with ethical standards

The study was approved by the Animal Ethics Committees of The University of Adelaide (approval number: M-2012-026) and complied with the National Health and Medical Research Council (Australia) Code of Practice for Animal Care in Research and Training.

Conflict of interest The authors declare that there are no conflicts of interest.

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