Cancer Chemother Pharmacol (2008) 61:775–784
DOI 10.1007/s00280-007-0534-4

ORIGINAL ARTICLE

Role of cytokines (TNF-α, IL-1β and KC) in the pathogenesis of CPT-11-induced intestinal mucositis in mice: effect of pentoxifylline and thalidomide

Maria Luisa P. Melo · Gerly A. C. Brito · Rudy C. Soares · Sarah B. L. M. Carvalho · Johan V. Silva · Pedro M. G. Soares · Mariana L. Vale · Marcellus H. L. P. Souza · Fernando Q. Cunha · Ronaldo A. Ribeiro

Received: 14 March 2007 / Accepted: 11 May 2007 / Published online: 12 July 2007

Abstract
Introduction Irinotecan (CPT-11) is an inhibitor of DNA topoisomerase I and is clinically effective against several cancers. A major toxic effect of CPT-11 is delayed diarrhea; however, the exact mechanism by which the drug induces diarrhea has not been established.
Purpose Elucidate the mechanisms of induction of delayed diarrhea and determine the effects of the cytokine production inhibitor pentoxifylline (PTX) and thalidomide (TLD) in the experimental model of intestinal mucositis, induced by CPT-11.
Materials and methods Intestinal mucositis was induced in male *Swiss* mice by intraperitoneal administration of CPT-11 (75 mg/kg) daily for 4 days. Animals received subcutaneous PTX (1.7, 5 and 15 mg/kg) or TLD (15, 30, 60 mg/kg) or 0.5 ml of saline daily for 5 and 7 days, starting 1 day before the first CPT-11 injection. The incidence of delayed diarrhea was monitored by scores and the animals were sacrificed on the 5th and 7th experimental day for histological analysis, immunohistochemistry for TNF-α and assay of myeloperoxidase (MPO) activity, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and KC ELISA.
Results CPT-11 caused significant diarrhea, histopathological alterations (inflammatory cell infiltration, loss of crypt architecture and villus shortening) and increased intestinal tissue MPO activity, TNF-α, IL-1β and KC level and TNF-α immuno-staining. PTX inhibited delayed diarrhea of mice submitted to intestinal mucositis and reduced histopathological damage, intestinal MPO activity, tissue level of TNF-α, IL-1β and KC and TNF-α immuno-staining. TLD significantly reduced the lesions induced by CPT-11 in intestinal mucosa, decreased MPO activity, TNF-α tissue level and TNF-α immuno-staining, but did not reduce the severity of diarrhea.
Conclusion These results suggest an important role of TNF-α, IL-1β and KC in the pathogenesis of intestinal mucositis induced by CPT-11.

Keywords CPT-11 · Mucositis · Intestine · Pentoxifylline · Thalidomide · Cytokine

M. L. P. Melo · R. C. Soares · S. B. L. M. Carvalho · J. V. Silva · P. M. G. Soares · M. L. Vale · M. H. L. P. Souza · R. A. Ribeiro (✉)
Departments of Physiology and Pharmacology, Faculty of Medicine, Federal University of Ceará, Rua Cel. Nunes de Melo, 1127, CEP 60.430-270 Fortaleza, CE, Brazil
e-mail: ribeiror@ufc.br

G. A. C. Brito
Departments of Morphology, Faculty of Medicine, Federal University of Ceará, Fortaleza, Brazil

M. L. P. Melo
Department of Nutrition, Health Sciences Centre, Statue University of Ceará, Fortaleza, Brazil

F. Q. Cunha
Department of Pharmacology, Faculty of Medicine, University of São Paulo, Ribeirão Preto, Brazil

Introduction

Irinotecan (CPT-11, 7-ethyl-10[4-[1-piperidino-1-piperidino]carbonyloxycampto-thecin), a potent DNA topoisomerase I inhibitor, is used clinically to treat colorectal, gastric, lung, breast, ovarian cancers, pancreatic and malignant lymphoma [7, 21, 27, 30]. CPT-11 is a prodrug which is activated by carboxylesterases to a 100- to 1000-fold more

cytotoxic metabolite SN-38 (7-ethyl-10-hydroxycampothecin). SN-38 is further converted to its glucuronide (SN-38G) by uridine diphosphate glucuronosyltransferase 1A isoforms, which can be converted back to SN-38 by intestinal microbial β-glicuronidase and undergo enterohepatic recycling. SN-38 is considered to be the main cause of diarrhea [7, 36, 40, 42]. A second less important metabolic pathway of CPT-11 is cytochrome P450. Biliary excretion is the major elimination route for CPT-11 and its major metabolites, with the urinary excretion being a less important pathway [15, 40].

The main clinically important toxic effects or dose-limiting factors of CPT-11 are severe delayed diarrhea and leukopenia [7, 18]. CPT-11 causes two types of diarrhea, first an early secretory diarrhea which is cholinergic in nature and can be prevented by the administration of atropine and second, a delayed diarrhea which has high incidence, and when it is serious, may limit the effectiveness of the treatment, since it is usually necessary to reduce or even interrupt its administration [12, 31].

Previous studies have demonstrated that thalidomide (TLD) significantly reduces CPT-11-induced intestinal toxicity and showed that TLD interferes with the pharmacokinetics and pharmacodynamics of CPT-11, decreasing the systemic exposure of SN-38, reducing half-life of this metabolite and diminishing the billiary excretion and cecal exposure of CPT-11, SN-38 and SN-38G. These actions could, at least partially, explain TLD preventive effect on CPT-11-induced intestinal lesions [41, 42]. Additionally, the same authors demonstrated that TLD inhibits CPT-11-induced production of cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-1β((IL-1β) and interferon-γ (IFN-γ) [41].

Thus, drugs that modulate cytokine synthesis may serve as potential therapeutic tools. Pentoxifylline (PTX) is a methylxanthine derivative which reduces the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-8 [16, 26, 37]. TLD, an α-N-phthalimidoglutarimide, is a synthetic glutamic acid derivative which inhibits TNF-α production by enhancing the degradation of its messenger RNA [24, 33] and alters plasma pharmacokinetics of CPT-11 and SN-38 [41, 42]. Based on this background, the aim of the present study was to investigate the mechanisms underlying CPT-11-induced delayed diarrhea and determine the effects of the cytokine inhibitors PTX and TLD on the mouse experimental model of CPT-11-induced intestinal mucositis.

Material and methods

Animals

Male *Swiss* mice, weighing 25–35 g, from the Federal University of Ceará, were used in the present study. The animals had free access to drinking water and food. All procedures and animal treatment were conducted in accordance with guidelines set forth by the US Department of Health, Education and Welfare [25] and were approved by our local ethics committee for experimental animal use.

Drugs

The following drugs were used: irinotecan hydrochloride (CPT-11, Camptosar®, Pharmacia and Upjohn Co, Kalamazoo, EUA, 100 mg ampoule); pentoxifylline (Trental®, Hoechst, São Paulo, Brazil, 100 mg ampoule); thalidomide (Talidomida®, CEME, Minas Gerais, Brazil, 100 mg tablet).

Induction of experimental intestinal mucositis

Experimental intestinal mucositis in mice was based on a model previously described by Ikuno et al. [18] and modified for our experimental conditions. Saline or CPT-11 (75 mg/kg) was administered intraperitonally (i.p.), for four consecutive days. On days 5 and 7, the mice were killed by cervical dislocation.

Experimental design

Mouse groups with intestinal mucositis were treated subcutaneously (s.c.) with PTX (1.7, 5 and 15 mg/kg or saline—0.5 ml) or TLD (15, 30 and 60 mg/kg or saline-DMSO 2%—0.5 ml) daily for 7 days, starting 1 day before the first CPT-11 injection.

Diarrhea assessment

The severity of diarrhea was monitored throughout the experimental period. Diarrhea observed after the final administration was considered to be delayed-onset diarrhea. The severity of the diarrhea was scored as described by Kurita et al. [20] as follows: 0—normal, normal stool or absent; 1—slight, slightly wet and soft stool; 2—moderate, wet and unformed stool with moderate perianal staining of the coat; and 3—severe, watery stool with severe perianal staining of the coat.

Histopathological analysis

On day 7, after killing, the intestines (duodenum, jejunum and ileum) were dissected. In each experiment, samples were removed for histopathological analysis. The specimens were fixed in 10% (v/v) neutral-buffered formalin, dehydrated and embedded in paraffin. Sections were cut and stained with haematoxylin and eosin (H&E) and examined by light microscopy (photomicrographs at $\times 100$ and $\times 400$ magnification). Blind method was used to avoid observer

bias. The severity of mucositis was graded using the following criteria previously described: grade 0, no lesion; grade 1, <10% crypts contain individual necrotic cells, grade 2, >10% crypts contain necrotic cells but the crypt architecture is intact; grade 3, >10% crypts contain necrotic cells showing focal loss of crypt architecture (<20%), villi are shortened, and variable hypertrophy/hiperbasophilia apparent in the remaining crypt cells; and grade 4, same as grade 3 except that the loss of crypt architecture and villous shortening are more extensive [39].

Intestinal morphometry

In the morphometric analysis a Nikon microscope with 10× objective lenses and micrometric 10× Leitz Wetzlar ocular lenses were used. The measures were made by the NIH image software for analysis of the histological sections. An average of 5 to 10 different linear measurements of crypt depth and villus height was considered. Height of the villus was considered from the top to the bottom, which corresponds to the junction of the crypt/villus; and the depth of the crypts, defined as invagination between adjacent villus.

Myeloperoxidase (MPO) assay

MPO activity, a marker for neutrophils in inflamed tissue, was measured in mice intestine, using a modified version from Bradley et al. [5]. The animals had a sample of intestine removed on day 7 for analysis of MPO activity. The specimen was stored at −70°C until required for assay. The mucosa was weighed and triturated using a Polytron Ultraturrax in ice-cold buffer solution (0.1 M NaCl, 20 mM $NaPO_4$, 15 mM NaEDTA), and the homogenate was centrifuged at 4°C for 10 min (4,200 rpm). The supernatant was collected for analyses in the enzyme-linked immunosorbance assay (ELISA) and values were expressed as units of MPO per mg of tissue.

Detection of cytokines (TNF-α, IL-1β, KC) in duodenum tissue

The animals had a sample of their intestine removed on day 5 and day 7 for analysis of cytokines. The specimen was stored at -70°C until required for assay. The tissue collected was homogenized and processed as described by Safieh-Garabedian et al. [32]. The detection of TNF-α, IL-1β and KC concentrations was determined by ELISA, as described previously [8]. Briefly, microtiter plates were coated overnight at 4°C with antibody against mice TNF-α, IL-1β and KC (2 μg/ml). After blocking the plates, the samples and standard at various dilutions were added in duplicate and incubated at 4°C for 24 h. The plates were washed three times with buffer. After washing the plates, biotinylated sheep polyclonal anti-TNF-α or anti-IL-1β or anti-KC (diluted 1:1000 with assay buffer 1% BSA), was added to the wells. After further incubation at room temperature for 1 h, the plates were washed and 50 μl of avidin-HRP diluted 1:5000 were added. The color reagent *o*-phenylenediamine (OPD; 50 μl) was added 15 min later and the plates were incubated in the dark at 37°C for 15–20 min. The enzyme reaction was stopped with H_2SO_4 and absorbance was measured at 490 nm. Values were expresses as picograms/milliliter (pg/ml).

Expression of TNF-α in duodenum tissue

Immunohistochemistry for TNF-α was performed using the streptavidin-biotin-peroxidase method [17] in formalin-fixed, paraffin-embedded tissue sections (4 μm thick), mounted on poly-L-lysine-coated microscope slides. The sections were deparaffinized and rehydrated through xylene and graded alcohols. After antigen retrieval, endogenous peroxidase was blocked (15 min) with 3% (v/v) hydrogen peroxide and washed in phosphate-buffered saline (PBS). Sections were incubated overnight (4°C) with primary anti-TNF-α antibody (polyclonal goat anti-mouse) diluted 1:200 in PBS plus bovine serum albumin (PBS-BSA). The slides were then incubated with biotinylated goat anti-IgG, diluted 1:200 in PBS-BSA. After washing, the slides were incubated with avidin-biotin-horseradish peroxidase conjugate (Strep ABC complex by Vectastain® ABC reagent and peroxidase substrate solution) for 30 min, according to the Vectastain protocol. TNF-α was visualized with the chromogen 3,3′diaminobenzidine (DAB). Negative control sections were processed simultaneously as described above but with the first antibody being replaced by PBS-BSA 5%. None of the negative controls showed TNF-α immunoreactivity. Slides were counterstained with Harry's haematoxylin, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped.

Table 1 Delayed diarrhea after administration of CPT-11 of mice treated with pentoxifylline (PTX) and thalidomide (TLD)

Day	Normal	CPT-11 (75 mg/kg)						
		Saline	PTX (mg/kg)			TLD (mg/kg)		
			1.7	5	15	15	30	60
5	0 (0–0)	0(0–3)	0(0–3)	0(0–3)	1(0–3)	0(0–3)	0(0–3)	0(0–3)
6	0 (0–0)	3(0–3)#	1(0–3)	1(0–2)	2(0–3)	1(0–3)	1(0–3)	1(0–3)
7	0 (0–0)	3(3–3)#	0(0–3)*	0(0–3)*	2(0–3)	1(0–3)	1(0–3)	1(0–3)

PTX (1.7 and 5 mg/kg) significantly inhibit delayed diarrhea CPT-11-induced. Data represent median values of scores and were analyzed by using Kruskal–Wallis and Dunn's test ($n = 8$). #$P < 0.05$ statistical differences compared to normal mice. *$P < 0.05$ statistical differences compared to mice submitted to intestinal mucositis and treated with saline

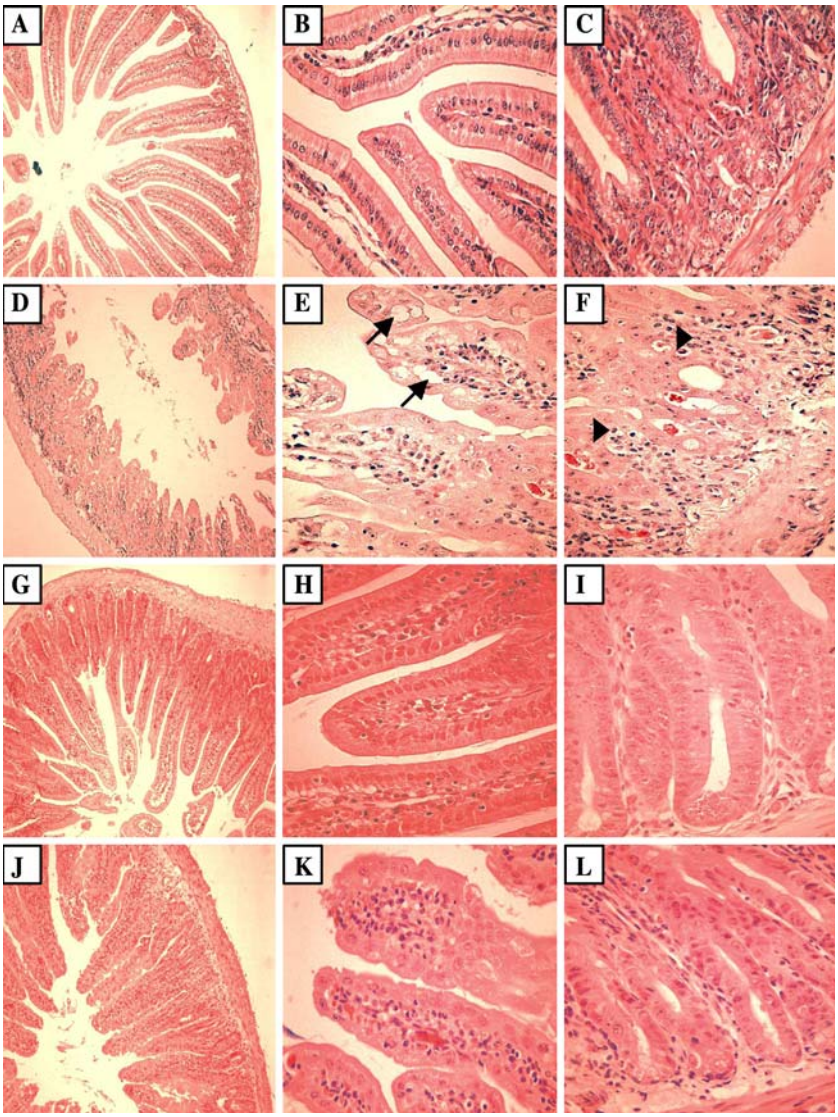

Fig. 1 Photomicrographs of duodenum. Normal mice, showing the preservation of villi (**a**, **b**) and crypts (**c**). Mice submitted to intestinal mucositis by CPT-11, showing villi shortened recovered with flattened and vacuolated cells (**d**, **e**; *arrow*), loss of crypt architecture and infiltration of inflammatory cells in the lamina propria (**f**; *arrowhead*). Mice submitted to intestinal mucositis by CPT-11 and treated with pentoxifylline (1.7 mg/kg), showing preservation of the villi and crypts (**g**, **h**, **i**). Mice submitted to intestinal mucositis by CPT-11 and treated with thalidomide (60 mg/kg), showing a smaller shortening of the villi (**j**, **k**) and preservation of the crypts (**l**). H&E staining (×100 and ×400 magnification)

Statistical analysis

The data are presented as means ± standard error of the mean (SEM) or medians, where appropriate. Analysis of Variance (ANOVA), followed by Bonferroni's test, was used to calculate the means, and Kruskal–Wallis followed by Dunn's test was used to compare medians. A P-value of <0.05 was considered as indicating significant differences.

Results

PTX, but not TLD reduced delayed diarrhea induced by CPT-11

CPT-11 (75 mg/kg) caused significant diarrhea ($P < 0.05$) on the 6th and 7th day after its first administration. PTX (1.7 and 5 mg/kg) significantly reduced the severity of the delayed diarrhea induced by CPT-11 in the 7th experimental day. TLD failed to prevent CPT-11-related delayed diarrheal symptoms (Table 1).

PTX and TLD reduced the histopathological alterations induced by CPT-11 on intestinal mucosa

CPT-11 induced shortened of the villi, loss of architecture the crypt and infiltration of inflammatory cells in the lamina propria (Fig. 1d, e, f), characterized mucositis grade 4. PTX (Fig. 1g, h, i) and TLD (Fig. 1j, k, l) significantly reduced the histopathological alterations observed in intestinal mucositis induced by CPT-11. The treatment with PTX in the 1.7 and 5 mg/kg and TLD in the 60 mg/kg, during 7 days, starting 1 day before of the mucositis induction, reduced the shortening of the villi, the infiltration of inflammatory cells and the alteration of crypt size and architecture. When the histological score was used it was observed

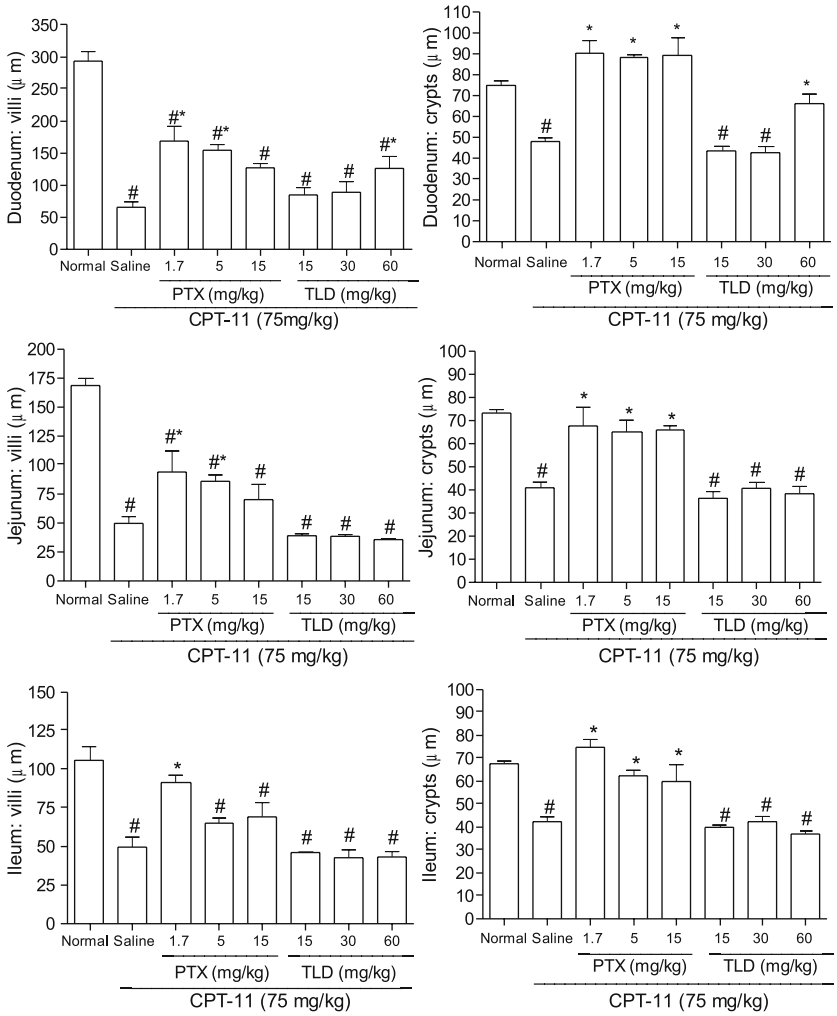

Fig. 2 Pentoxyfilline (PTX 1.7 and 5 mg/kg) and thalidomide (TLD 60 mg/kg) reduced the shortening of the villi and increase the depth of the duodenum crypts in mice submitted to intestinal mucositis. *Bars* represent the mean ± standard error of the mean (SEM). #$P < 0.05$ statistical difference compared to normal mice. *$P < 0.05$ statistical difference compared to mice submitted to intestinal mucositis treated with saline. The number of animals in each group was a least six. ANOVA and Bonferroni's test

Table 2 Histological grading of mucositis for intestine of mice treated with pentoxifylline (PTX) and thalidomide (TLD) on day 7 after CPT-11 administration

Day	Normal	CPT-11 (75 mg/kg)						
		Saline	PTX (mg/kg)			TLD (mg/kg)		
			1.7	5	15	15	30	60
Duodenum	0 (0–0)	4(3–4)#	1(0–4)*	2(0–3)*	3(1–4)	2(1–4)#	2(1–4)	1(1–4)*
Jejunum	0 (0–0)	4(4–4)#	2(0–4)*	2(0–4)*	4(0–4)#	4(1–4)	3(1–4)#	3(1–4)#
Ileum	0 (0–0)	4(4–4)#	3(0–4)*	4(3–4)#	4(3–4)#	2(1–4)	4(1–4)#	4(1–4)#

PTX (1.7 and 5 mg/kg) and TLD (60 mg/kg) significantly reduced grade of CPT-11-induced intestinal mucositis. Data represent median values of scores and were analyzed by using Kruskal–Wallis and Dunn's test ($n = 8$). #$P < 0.05$ statistical differences compared to normal mice. *$P < 0.05$ statistical differences compared to mice submitted to intestinal mucositis and treated with saline

that PTX in the 1.7 mg/kg dose reduced grade of CPT-11-induced mucositis in the duodenum, jejunum, ileum (grades 1, 2 and 3, respectively). On the other hand, the dose of 5 mg/kg had a better effect at duodenum and jejunum than in ileum. TLD (60 mg/kg) significantly reduced the histological alterations observed in duodenum (grade 1) (Table 2).

The results of the morphometric analysis of the animals with CPT-11-induced intestinal mucositis treated or not treated with PTX or TLD as observed in Fig. 2. CPT-11 significantly ($P < 0.05$) shortened both the villus height and crypt dept in all intestinal segments. PTX treatment significantly ($P < 0.05$) enlarged the depth of the intestinal crypts in the segments of the duodenum, jejunum and ileum. It

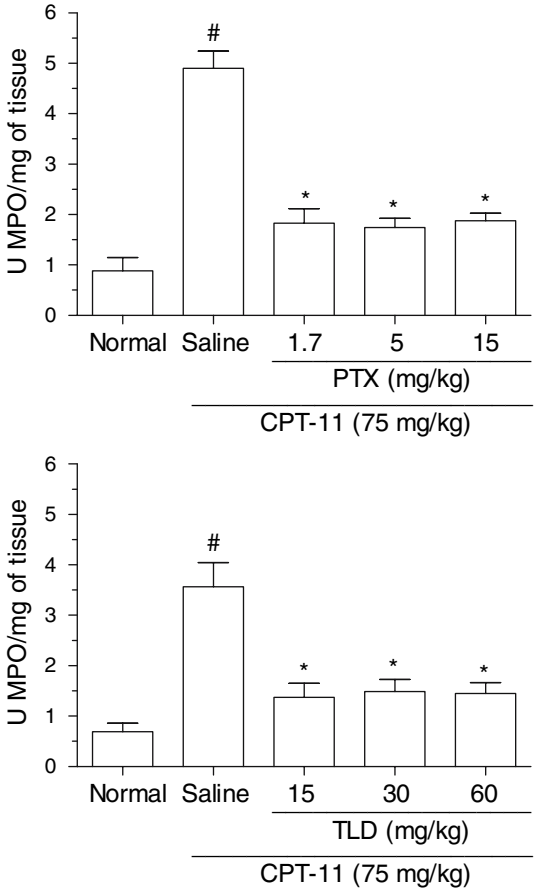

Fig. 3 Pentoxyfilline (PTX 1.7, 5 and 15 mg/kg) and thalidomide (TLD 15, 30 and 60 mg/kg) inhibit myeloperoxidase (MPO) activity in intestine of mice submitted to intestinal mucositis. *Bars* represent the mean ± standard error of the mean (SEM) expressed as units of MPO per mg of tissue. #$P < 0.05$ statistical difference compared to normal mice. *$P < 0.05$ statistical difference compared to mice submitted to intestinal mucositis treated with saline. The number of animals in each group was a least six. ANOVA and Bonferroni's test

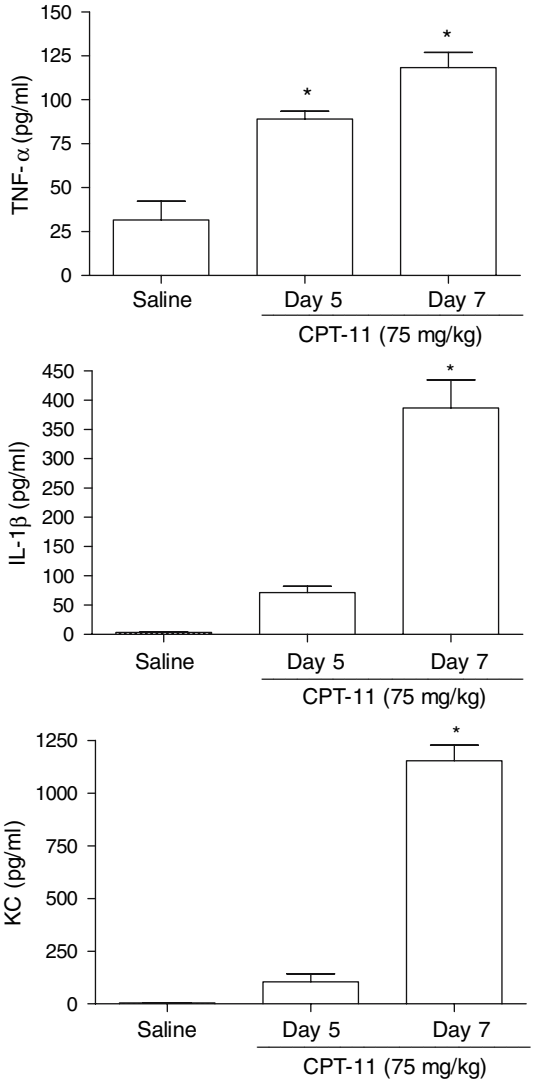

Fig. 4 CPT-11 increased the intestinal tissue level of TNF-α, IL-1β and KC in the duodenum of mice. *Bars* represent the mean ± standard error of the mean (SEM) of the concentration in pg/ml. #$P < 0.05$ statistical difference compared to normal mice. *$P < 0.05$ statistical difference compared to mice submitted to intestinal mucositis treated with saline. The number of animals in each group was a least five. ANOVA and Bonferroni's test

was also observed that PTX in the 1.7 and 5 mg/kg significantly ($P < 0.05$) reduced the shortening of the villi in the duodenum and jejunum. On the hand, in the ileum, only the smaller dose (1.7 mg/kg) significantly ($P < 0.05$) reduced the shortening of the villi. TLD (60 mg/kg) treatment significantly increase ($P < 0.05$) the depth of the intestinal crypts and the villi height in the duodenum.

MPO activity inhibition by PTX and TLD in CPT-11-induced intestinal mucositis

MPO activity was measured in intestinal tissue as an indicator of neutrophil infiltration. Our results showed that CPT-11 (75 mg/kg) induced a significant increase in MPO activity at day 7 after mucositis induction, in comparison to non-treated animals. PTX (1.7, 5, 15 mg/kg) and TLD (15, 30, 60 mg/kg) administered daily for 7 days, starting 1 day before CPT-11 treatment, significantly blocked ($P < 0.05$) this elevation in MPO activity (Fig. 3).

Inhibition of the pro-inflammatory cytokine production by PTX and TLD in CPT-11-induced intestinal mucositis

CPT-11 (75 mg/kg) significantly ($P < 0.05$) increased the intestinal tissue level of TNF-α on days 5 and 7. It was also observed that mice submitted to CPT-11-induced intestinal mucositis on day 7 increase levels of IL-1β and KC (Fig. 4). PTX (1.7 mg/kg) by 7 days reverted the raise of TNF-α, IL-1β and KC level induced by administration of

CPT-11 on day 7 ($P < 0.05$). TLD (60 mg/kg) inhibited TNF-α tissue level without affecting IL-1β and KC (Fig. 5).

Immunohistochemical reation for TNF-α

The duodenum of mice submitted to mucositis by CPT-11 showed marked immuno-staining for TNF-α. Areas of intense staining corresponded with areas of surface epithelium and lamina propria (Fig. 6c). Moderate TNF-α staining was seen in epithelial cells of the normal duodenum (Fig. 6b). PTX (1.7 mg/kg) and TLD (60 mg/kg) caused decrease of TNF-α immuno-staining in the duodenum tissue when compared with group of animals subjected to experimental mucositis that did not receive treatment (Fig. 6d and e).

Discussion

In this study, it was demonstrated that treating mice with CPT-11 caused significant intestinal mucositis presenting mucosa damage with small denuded areas. Most of the villi were flattened, the intestinal epithelial cells were vacuolated and the crypts were necrotic or shortened. The lamina propria presented intense infiltration of inflammatory cells. These data are in accordance with previous studies, showing similar aspects of intestinal mucositis induced by CPT-11 [11, 18, 20]. These authors have shown that CPT-11 treatment caused severe intestinal damage with villus and crypt hypoplasia and apoptosis in crypts of the small and large intestines [11].

The observation that CPT-11 treatment caused increase in MPO activity, in comparison with non-treated animals, demonstrated the combination of mucosal injury with neutrophil infiltration, supporting the hypothesis of the occurrence of an inflammatory phase in the mucositis pathological process [35]. Neutrophil migration represents a central component of the immune response, employing potent effect mechanism such as phagocytosis, production of reactive oxygen species and the release of inflammatory mediators [29].

We also demonstrated that delayed diarrhea symptoms caused by CPT-11 began on 6th to 7th experimental day. Consistently, pathological change in the gastrointestinal tract was most profound at this stage. These data were corroborated by other authors [18, 20]. Diarrhea may be caused by abnormalities of intestinal absorption or secretion due to a change in intestinal microflora, increased peristalsis or drug-induced epithelial damage [11, 18, 36, 40]. Furthermore, this model reproduces important signs and symptoms seen in chemotherapy-induced intestinal mucositis in patients, and has therefore been extensively used in the study of this condition. Despite considerable efforts to treat

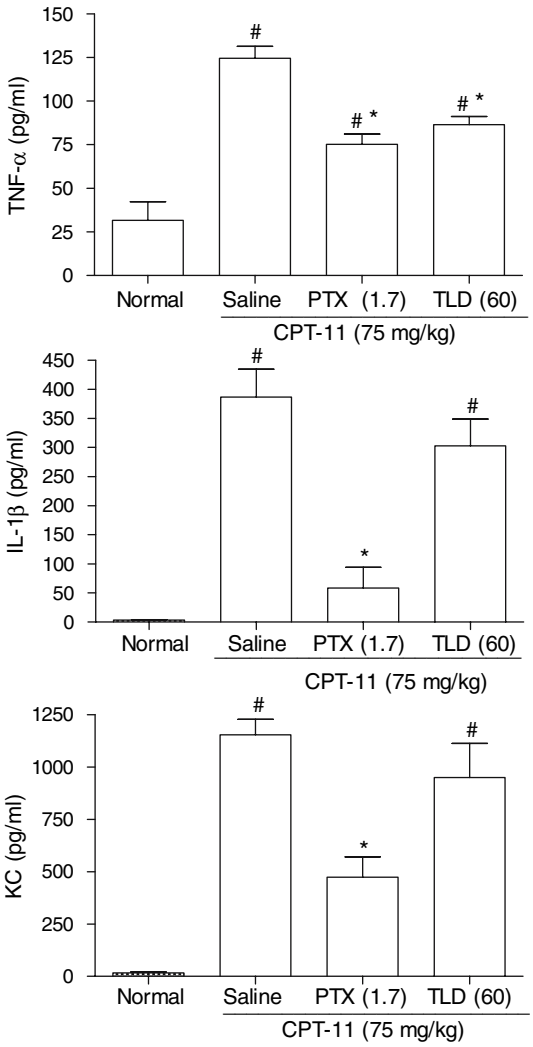

Fig. 5 Pentoxyfilline (PTX 1.7 mg/kg) and thalidomide (TLD 60 mg/kg) reduced TNF-α, and PTX also decrease IL-1β and KC intestinal tissue level in mice submitted to intestinal mucositis. *Bars* represent the mean ± standard error of the mean (SEM) of the concentration in pg/ml. #$P < 0.05$ statistical difference compared to normal mice. *$P < 0.05$ statistical difference compared to mice submitted to intestinal mucositis treated with saline. The number in each group was a least five. ANOVA and Bonferroni's test

chemotherapy-induced intestinal mucositis adequately, this pathological disorder continues to be an important dose-limiting complication cancer chemotherapy.

In the present investigation, it was demonstrated that PTX significantly reduced the CPT-11-induced lesions in the intestinal mucosa, improving the recovery of crypts and villi. The lowest doses of PTX (1.7 and 5 mg/kg) had a better protector effect in intestinal structures and reduced the severity of CPT-11-induced diarrhea. However, the highest dose (15 mg/kg) did not reduce diarrhea nor intestinal mucositis grade, determined by the histopathological score, despite the fact that this dose reduced the CPT-11-induced crypt morphometric alterations. So a protector effect of this

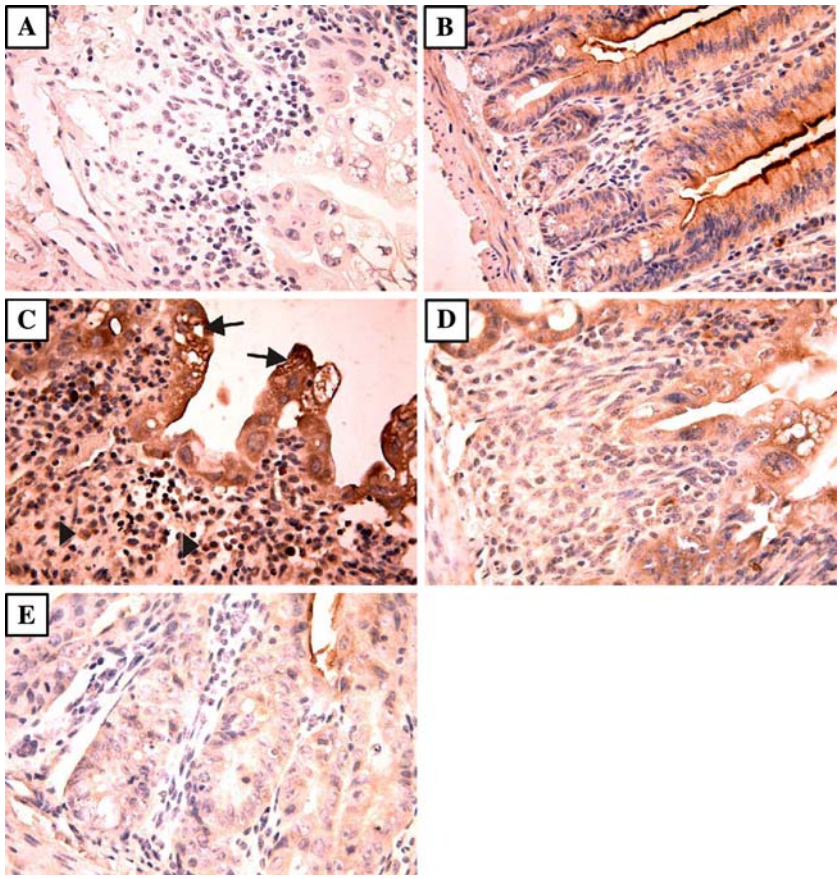

Fig. 6 Representative examples of TNF-α immunohistochemistry in the duodenum of mice (×400 magnification). **b** Moderate TNF-α staining was seen in epithelial cells of normal duodenum. **c** The duodenum of mice which received CPT-11 and saline, presented intense immunostaining for TNF-α on the surface epithelium (*arrow*) and on lamina propria cells (*arrow head*). **d**, **e** The treatment with pentoxifylline (1.7 mg/kg) and thalidomide (60 mg/kg) considerably reduced the immunostaining in the surface epithelium and lamina propria. **a** Negative control represents a sample of the duodenum where the first antibody was replaced by PBS-BSA 5% and no staining was detected

dose may be considered since improvement in morphometric alterations may precede the recovery of functional alterations in intestinal mucositis [35].

The microscopic effects were associated with reduced neutrophil infiltration detected by MPO activity. This effect of PTX is consistent with previous reports showing that PTX has inhibited the migration of neutrophils in the peritoneal cavity induced by *Clostridium difficile* toxin A [6] and in the synovial cavity induced by ovalbumin [4]. A number of investigators have also described anti-inflammatory effects of PTX. The treatment with a single dose with PTX significantly attenuates inflammatory response in experimental pancreatitis induced by cerulean [13]. The administration of PTX in patients submitted to transplant bone marrow reduced the severity of mucositis and reduced the need of total parenteral nutrition [3]. Later reports have not proven this clinic benefit [10]. Recently, it was shown that PTX and TLD protects hamsters from oral mucositis induced by 5-fluorouracil [22]. However, the effect of PTX on CPT-11-induced delayed diarrhea was not yet studied.

Here, the lowest doses of PTX had better anti-inflammatory effect. Accordingly Abdel-Salam et al. [1] demonstrated that administration of PTX in rats 30 min before injection of carrageenan reduces paw edema. However, higher doses of PTX, although causing marked early suppression of the inflammatory response, has its effects sustained for only two hours, with return of inflammation to control levels by the end of experimental period. This might suggest that the reduction in anti-inflammatory effects seen with the higher doses of the drug is a consequence of a marked vasodilator activity leading to increased functional lumen, intravascular pressure and microvascular permeability. In addition, release of prostacyclin or nitric oxide [28], following the higher doses of the drug could have induced inflammatory exudation [1]. Besides, PTX shows a regulator effect of IL-10, an anti-inflammatory cytokine which seems to be drug concentration dependent. In vitro and in vivo, higher concentrations of PTX inhibit IL-10, while lower concentrations induce higher synthesis of this cytokine [23].

TLD, in a similar manner to PTX significantly reduced the lesions induced by CPT-11 in intestinal mucosa and neutrophil infiltration detected by MPO activity, but did not reduce the severity of diarrhea. The more effective inhibitory action of PTX compared with TLD may be in part explained by distinct effects other than inhibiting TNF-α. It has been demonstrated that PTX reduces production of cytokines, such as TNF-α, IL-1β and IL-8 [16, 26, 37]. TLD was shown to inhibit TNF-α production [24, 33] without affecting the production of either IL-1β [19, 24, 33] or

IL-8 [19]. In the present study, we demonstrated that CPT-11 increases TNF-α in 5th and 7th experimental day. CPT-11 increase IL-1β and KC level in intestinal tissue on day 7. Thus, the protective effect of PTX found in the present study could be explained by its capacity to inhibit the production of these three inflammatory cytokines (TNF-α, IL-1β and KC).

Clinical trial demonstrated that TLD with CPT-11 ameliorated the gastrointestinal toxicity and enhanced the antitumor activity of CPT-11 in colorectal cancer patients [14]. However, others studies did not confirm its anti-diarrhea activity [2]. In rats, TLD inhibited TNF-α production intestinal, epithelial apoptosis and reduced CPT-11-induced diarrhea [41]. Other studies showed that these preventive effects of TLD were associated with changes in CPT-11 pharmacokinetic and pharmacodynamic [41, 42].

In accordance to our data, it has been demonstrated that cytokines regulate and amplify the immune response, induce tissue injury and mediate complications such as diarrhea [34]. Williams [38] reported that inflammatory cytokines, such IL-1, IL-6, TNF-α, IFN-γ and IL-2 contribute to the severity and maintenance of injury in the intestinal mucositis. De Koning et al. [9] demonstrated that mucosal immune response has an important role during methotrexate-induced mucositis, TNF-α contributes to and IL-10 regulates mucosal damage by restricting excessive mucositis. It is also known that IL-1α, IL-1β and TNF-α stimulate secretion of others cytokines, metabolites of arachidonic acid and proteases by macrophages, neutrophils, smooth muscle cells, fibroblast and epithelial cells [34]. KC, the mouse ortholog of human IL-8, is an important chemokine in the intestinal inflammatory process, recognized as a powerful neutrophils chemotaxic factor [29].

In conclusion, TNF-α, IL-1β and KC are important mediators in pathogenesis of intestinal mucositis. PTX and TLD showed a protector effect in intestinal structures. However, only PTX, reduced the severity of CPT-11-induced diarrhea. This result may be explained by TLD more selective TNF-α inhibition. The possibility of using these drugs in the treatment of humans merits further investigation.

Acknowledgments The authors thank Maria Silvandira França Pinheiro, Department of Physiology and Pharmacology, and José Ivan Rodrigues de Sousa, Department of Morphology, Faculty of Medicine, Federal University of Ceará, Brazil. This work was supported by the Brazilian Agency for Scientific and Technological Development (CNPq).

References

1. Abdel-Salam OME, Baiuomy AR, El-Shenawy SM, Arbid MS (2003) The anti-inflammatory effects of the phosphodiesterase inhibitor pentoxifylline in the rat. Pharmacol Res 47:331–340
2. Allegrini G, Di Paolo A, Cerri E, Cupini S, Amatori F, Masi G, Danesi R, Marcucci L, Bocci G, Tacca MD, Falcone A (2006) Irinotecan in combination with thalidomide in patients with advanced solid tumors: a clinical study with pharmacodynamic and pharmacokinetic evaluation. Cancer Chemother Pharmacol 58:585–593
3. Bianco JA, Appelbaum FR, Nemunaitis J, Almgren J, Andrews F, Kettner P, Shields A, Singer JW (1991) Phase I–II trial of pentoxifylline for the prevention of transplant-related toxicities following bone marrow transplantion. Blood 78(5):1205–1211
4. Bombini G, Canetti C, Rocha FAC, Cunha FQ (2004) Tumour necrosis factor-α mediates neutrophil migration to the knee synovial cavity during immune inflammation. Eur J Pharmacol 496:197–204
5. Bradley PP, Christensen RD, Rothstein G (1982) Cellular and extracellular myeloperoxidase in pyogenic inflammation. Blood 60:618–22
6. Carneiro-Filho BA, Sousa MLP, Lima AAM, Ribeiro RA (2001) The effect of tumour necrosis factor (TNF) inhibitors in *Clostridium difficile* toxin-induced paw oedeme and neutrophil migration. Pharmacol Toxicol 88:313–318
7. Chester JD, Joel SP, Cheeseman SL, Hall GD, Braun MS, Perry J, Davis T, Button CJ, Seymour MT (2003) Phase I and pharmacokinetic study of intravenous irinotecan plus oral ciclosporin in patients with fluorouracil-refractory metastatic colon cancer. J Clin Oncol 21(6):1125–1132
8. Cunha FQ, Boukili MA, Motta JIB, Vargaftig BB, Ferreira SH (1993) Blockade by fenspiride of endotoxin-induced neutrophil migration in the rat. Eur J Pharmacol 238:47–52
9. De Koning BAE, van Dieren JM, Lindenbergh-Kortleve DJ, van der Sluis M, Matsumoto T, Yamaguchi K, Einerthand AW, Samsom JN, Pieters R, Nieuwenhuis EES (2006) Contributions of mucosal immune cells to methotrexate-induced mucositis. Int Immunol 24:1–9
10. Ferrà C, de Sanjosé S, Lastra CF, Martí F, Mariño EL, Sureda A, Brunet S, Gallardo D, Berlanga JJ, García J, Grañena A (1997) Pentoxifylline, ciprofloxacin and prednisone failed to prevent transplant-related toxicities in bone marrow transplant recipients and were associated with an increased incidence of infectious complications. Bone Marrow Transplant 20:1075–1080
11. Gibson RJ, Bowen JM, Inglis MRB, Cummins AG, Keefe DMK (2003) Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer. J Gastroenterol Hepatol 18:1095–1100
12. Gibson RJ Keefe DMK (2006) Cancer chemotherapy-induced diarrhea and constipation: mechanism of damage and prevention strategies. Support Care Cancer 14(9):890–900
13. Gómez-Cambronero L, Camps B, de la Asunción JG, Cerdá M, Pellín A, Pallardo FV, Calvete J, Sweiry JH, Mann GE, Viña J, Sastre J (2000) Pentoxifylline ameliorates cerulein-induced pancreatitis in rats: role of glutathione and nitric oxide. J Pharmacol Exp Ther 293(2):670–676
14. Govindarajan R, Heaton KM, Broadwater R, Zeltlin A, Lang NP, Hauer-Jensen M (2000) Effect of thalidomide on gastrointestinal toxic effects of irinotecan. Lancet 356:566–567
15. Gupta E, Wang X, Ramirez J, Ratain MJ (1997) Modulation of glucuronidation of SN-38, the active metabolite of irinotecan, by valproic acid and phenobarbital. Cancer Chemother Pharmacol 39:440–444
16. Gutierrez-Reyes G, Lopez-Ortal P, Sixtos S, Cruz S, Ramirez-Iglesias MT, Gutierrez-Ruiz MC, Sanchez-Avila F, Roldan E, Vargas-Voracková F, Kershenobich D (2006) Effect of pentoxifylline on levels of pro-inflammatory cytokines during chronic hepatitis C. Scand J Immunol 63(6):461–467
17. Hsu SM, Raine L (1981) Protein A, avidin, and biotin in immunohistochemistry. J Histochem Cytochem 29(11):1349–1353

18. Ikuno N, Soda H, Watanabe M, Oka M (1995) Irinotecan (CPT-11) and characteristic mucosal changes in the mouse ileum and cecum (reports). J Natl Cancer Inst 87(24):1876–1883
19. Kim YS, Kim JS, Jung HC, Song IS (2004) The effects of thalidomide on the stimulation of NF-κB activity and TNF-α production by lipopolysaccharide in a human colonic epithelial cell line. Mol Cells 17(2):210–216
20. Kurita A, Kado S, Kaneda N, Onoue M, Hashimoto S, Yokokura T (2000) Modified irinotecan hydrochloloride (CPT-11) administration schedule improves induction of delayed-onset diarrhea in rats. Cancer Chemother Pharmacol 46:211–220
21. Langer CJ (2004) Irinotecan in advanced lung cancer: focus on North American trials. Oncology 18(7):17–28
22. Lima V, Brito GAC, Cunha FQ, Rebouças CG, Falcão BAA, Augusto RF, Souza MLP, Leitão BT, Ribeiro RA (2005) Effects of the tumour necrosis factor-α inhibitors pentoxifylline and thalidomide in short-term experimental oral mucositis in hamsters. Eur J Oral Sci 113(3):210–217
23. Marcinkiewicz J, Grabowska A, Lauterbach R, Bobek M (2000) Differential effects of pentoxifylline, a non-specific phosphodiesterase inhibitor, on the production of IL-10, IL-12 p40 and p35 subunits by murine peritoneal macrophages. Immunopharmacology 49:335–343
24. Moreira AL, Sampaio EP, Zmuidzinas A, Frindt P, Smith KA, Kaplan G (1993) Thalidomide exerts its inhibitory action on tumor necrosis factor α by enhancing mRNA degradation. J Exp Med 177:1675–1680
25. National Research Council (1986) Guide for the care and use of laboratory animals. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council. National Academy Press, Washington, DC
26. Neuner P, Klosner E, Schauer E, Pourmojib M, Macheiner W, Grünwald C, Knobler R, Schwarz A, Luger TA, Schwarz T (1994) Pentoxifylline *in vivo* down-regulates the release of IL-1β, IL-6, IL-8 and tumour necrosis factor-α by human peripheral blood mononuclear cells. Immunology 83:262–267
27. Perez EA, Hillman DW, Mailliard JA, Ingle JN, Ryan JM, Fitch TR, Rowland KM, Kardinal CG, Krook JE, Kugler JW, Dakhil SR (2004) Randomized phase II study of two irinotecan schedules for patients with metastatic breast cancer refractory to an anthracycline, a taxane, or both. J Clin Oncol 22(14):2849–2855
28. Pohanka E, Sinzinger H (1986) Effect of a single pentoxifylline administration on platelet sensitivity, plasma factor activity, plasma-6-oxo-PGF_1 alpha and thromboxane B_2 in health volunteers. Prostaglandins Leukot Med 22:191–200
29. Reaves TA, Chin AC, Parkos CA (2005) Neutrophil transepithelial migration: role of toll-like receptors in mucosal inflammation. Mem Inst Oswaldo Cruz 100(suppl 1):191–198
30. Rocha-Lima CM, Green MR, Rotche R, Miller WH Jr, Jeffrey GM, Cisar LA, Morganti A, Orlando N, Gruia G, Miller LL (2004) Irinotecan plus gemcitabine results in no survival advantage compared with gemcitabine monotherapy in patients with locally advanced or metastatic pancreatic cancer despite increased tumor response rate. J Clin Oncol 22:18–21
31. Rubenstein EB, Peterson DE, Schubert M, Keefe D, McGuire D, Epstein J, Elting LS, Fox PC, Cooksley C, Sonis ST (2004) Clinical practice guidelines for the prevention and treatment of cancer therapy-induced oral and gastrointestinal mucositis. Cancer 100(suppl 9):2026–46
32. Safieh-Garabedian B, Poole S, Allchorne A, Winter J, Woolf CJ (1995) Contribution of interleukin-1 beta to the inflammation-induced increase in nerve growth factor levels and inflammatory hyperalgesia. Br J Pharmacol 115:1265–1275
33. Sampaio EP, Sarno EN, Galilly R, Cohn ZA, Kaplan G (1991) Thalidomide selectively inhibits tumor necrosis factor α production by stimulated human monocytes. J Exp Med 173:699–703
34. Sartor RB (1994) Cytokines in intestinal inflammation: pathophysiological and clinical considerations. Gastroenterology 106:533–539
35. Sonis ST (2004) The pathobiology of mucositis. Nat Rev Cancer 4(4):277–284
36. Takasuna K, Hagiwara T, Watanabe K, Onose S, Yoshida S, Kumazawa E, Nagai E, Kamataki T (2006) Optimal antidiarrhea treatment for antitumor agent irinotecan hydrochloride (CPT-11)-induced delayed diarrhea. Cancer Chemother Pharmacol 25:1–10
37. Van Furth AM, Verhard-Seijmonbergen EM, Van Furth R, Langermans JAM (1997) Effect of lisofylline and pentoxifylline on the bacterial-stimulated production of TNF-α, IL-1β and IL-10 by human leucocytes. Immunology 91:193–196
38. Williams DA (2001) inflammatory cytokines and mucosal injury. J Natl Cancer Inst Monogr 29:26–30
39. Woo PCY, Ng WF, Leung HCH, Tsoi HW, Yuen KY (2000) Clarithromycin attenuates cyclophosphamide-induced mucositis in mice. Pharmacol Res 41(5):526–32
40. Xie R, Mathijssen RHJ, Sparreboom A, Verweij J, Karlsson MO (2002) Clinical pharmacokinetics of irinotecan and its metabolites in relation with diarrhea. Clin Pharmacol Ther 72(3):265–275
41. Yang XX, Hu ZP, Xu AL, Duan W, Zhu YZ, Huang M, Sheu FS, Zhang Q, Bian JS, Chan E, Li X, Wang JC, Zhou SF (2006) A mechanistic study on reduced toxicity of irinotecan by coadministered thalidomide, a tumor necrosis factor-alpha inhibitor. J Pharmacol Exp Ther 319(1):82–104
42. Yang XX, Hu ZP, Chan SY, Duan W, Ho PC, Boelsterli UA, Ng KY, Chan E, Bian JS, Chen YZ, Huang M, Zhou SF (2006) Pharmacokinetic mechanisms for reduced toxicity of irinotecan by coadministered thalidomide. Curr Drug Metab 7(4):431–455