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The effect of pentoxifylline and its metabolite-1 on inflammation and fibrosis in the TNBS model of colitis ☆

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ABSTRACT

TNBS-induced colitis has characteristics resembling human Crohn's disease including transmural inflammation, ulceration, and fibrosis. Current treatments target acute symptoms but do not necessarily prevent fibrotic complications of the disease. The aim of this study was to determine the effect of pentoxifylline and its primary metabolite (M-1) on fibrosis in the TNBS-induced colitis model. Myeloperoxidase activity and interleukin-18 are indicators of inflammation and were elevated in the TNBS model. The morphology damage score assesses colon damage and was also elevated in the TNBS model. Collagen as the indicator of fibrosis was quantified and visualized by the Sirius Red/Fast Green staining technique and collagen type I was assessed by Western analysis. Collagen was elevated in the TNBS-induced model. Pentoxifylline and M-1 treatment significantly attenuated colon damage and inflammation in TNBS-colitis ($P<0.05$). M-1 treatment significantly reduced the TNBS-induced increase in colon weight, colon thickness and total collagen content ($P<0.05$). Results suggest that pentoxifylline and M-1 inhibit intestinal fibrosis in this experimental model and may prove beneficial in the treatment of intestinal fibrosis associated with human Crohn's disease with the added benefit of inhibiting inflammation and ulceration. This is the first study to examine the effects of racemic M-1 *in vivo* and one of the few studies to examine the effect of drugs on both inflammation and fibrosis in an experimental model of colitis.

1. Introduction

The treatment of patients who suffer from inflammatory bowel disease can be difficult. There is a paucity of therapeutic options for the inflammatory bowel disease patient largely because of its complexity. Crohn's disease is a chronic relapsing form of inflammatory bowel disease of unknown origin, characterized by transmural inflammation, ulceration, bowel wall thickening, and fibrosis (Cotran et al., 1999; Fiocci, 1998; Pucilowska et al., 2000). We assess colonic damage and inflammation in the TNBS rat model, an experimental model of colitis that is characterized by transmural inflammation, ulceration, and fibrosis resembling Crohn's disease. In response to an unidentified antigen, inappropriate activation of the intestinal immune system results in a prolonged inflammatory process with the production of inflammatory mediators (Rogler and Andus, 1998). The cytokine interleukin-18 (IL-18) is elevated in serum of patients with inflammatory bowel disease and correlates with inflammatory markers (Haas et al., 2009). Fibrosis is a complication of chronic inflammation that leads to loss of tissue function. Fibrosis in Crohn's disease is observed when excessive collagen is deposited in all layers of the intestine (Pucilowska et al., 2000). The cells responsible for collagen deposition in the inflamed intestine are smooth muscle cells, fibroblasts and myofibroblasts (Lawrance et al., 2001). Fibrosis contributes to intestinal stricture formation, a serious complication of Crohn's disease, often requiring surgical resection (Farmer et al., 1985; Harper et al., 1987). Therapeutic intervention for Crohn's disease includes the use of immunomodulating and anti-inflammatory agents that target acute symptoms but do not necessarily alter the natural course of the disease or prevent fibrotic complications such as stricture formation (Brooks and Green, 2004). Anti-tumor necrosis factor-α has shown considerable promise in Crohn's disease patients refractory to standard treatment (D'Haens et al., 2001; Feagan, 2003; Hanauer et al., 2002; Stein and Hanauer, 2000) but the high cost, risk of side effects, and concern of long-term effects can limit its use in the general Crohn's disease population. Pentoxifylline is a well-known methylxanthine derivative (Ward and Clissold, 1987) which has anti-inflammatory effects including inhibition of TNFα and other cytokines involved in inflammatory bowel disease (Reimund et al., 1997). Pentoxifylline treatment attenuated intestinal inflammation and damage in experimental colitis (Murthy et al., 1999; Peterson and Davey, 1997). Our lab and others have shown that

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pentoxifylline is a potent anti-fibrogenic compound both *in vitro*, inhibiting proliferation and collagen synthesis by cultured fibroblasts and myofibroblasts (Isbrucker and Peterson, 1998; Peterson et al., 1994; Pinzani, 1995; Preaux et al., 1997), and *in vivo*, preventing hepatic fibrosis in an animal model (Peterson, 1993). We have also been investigating the *in vitro* effects of 1-(5-hydroxyhexyl)-3, 7-dimethylxanthine, the metabolite-1 (M-1) of pentoxifylline and have reported that M-1 inhibits fibroblast proliferation and collagen synthesis (Isbrucker and Peterson, 1998) suggesting that it too may have an antifibrotic potential. This study was designed to assess the *in vivo* effects of pentoxifylline and M-1 on intestinal inflammation and fibrosis in the well-established 2,4,6-trinitrobenzenesulfonic acid (TNBS) model of colitis in rats. TNBS-colitis has many characteristics resembling human Crohn's disease, including transmural inflammation and fibrosis, skip-segment ulceration, lymphoid infiltration and intestinal crypt distortion (Morris et al., 1989). In this study, we assessed the ability of pentoxifylline and M-1 to attenuate colon damage and inflammation associated with TNBS-colitis. Type I collagen was elevated in TNBS-treated colon tissue contributing to the fibrosis observed. Treatment with M-1 prevented TNBS-induced intestinal fibrosis suggesting that M-1 may target both inflammation and fibrosis which could have significant implications for the treatment of human Crohn's disease.

2. Materials and methods

2.1. Synthesis of metabolite-1 from pentoxifylline

Metabolite-1 [(1-(5-hydroxyhexyl)-3,7-dimethylxanthine] is a chiral molecule derived from pentoxifylline by the reduction of a single ketone group to a corresponding hydroxyl group (Fig. 1). A racemic mixture of metabolite-1 was synthesized from pentoxifylline in a non-selective chemical reduction reaction using sodium borohydride ($NaBH_4$) as the reducing agent. Pentoxifylline (10 g) was dissolved in methanol (500 ml) and 1.8 g $NaBH_4$ was slowly added. Solvent was evaporated off after 1 h of mixing. The crude product was dissolved in water and extracted with dichloromethane. The organic layer was dried and filtered, and the solvent was evaporated off leaving a powdered product (M-1). The product was recrystallized from 2-propanol at 4 °C, collected by filtration and dried before use (yield>80%). The metabolite was identified by proton nuclear magnetic resonance spectroscopy (^{1}H-NMR).

2.2. Animals

Virgin female Sprague–Dawley rats weighing 125–150 g upon arrival from Charles River Laboratories (Quebec, Canada) were maintained in the Carlton Animal Care Facility at Dalhousie University. Animals were housed 2–3 per cage with a 12-h light/dark cycle and *ad libitum* access to water and standard laboratory rat chow. Animals were given 1 week to acclimatize to the facility before experimentation began. The experimental protocol was approved by the University Committee on the use of Laboratory Animals at Dalhousie University.

2.3. TNBS-induced colitis

Colitis was induced on day 0 by intracolonic administration of 90 mg/kg TNBS in 50% ethanol (250 µl/rat). This dose was chosen because it reproducibly produced significant colitis as assessed by the macroscopic damage score. A catheter was inserted 6 cm into the lumen of the colon where the TNBS solution was ejected and then the animal was maintained in the Trendelenburg position for 5 min to ensure contact with the intestinal mucosa. Control animals received 250 µl of sterile saline. Animals were treated with pentoxifylline or M-1 at 72 h post-TNBS, to prevent a washout effect from this drug treatment.

2.4. Modulation of TNBS-induced colitis

Rats were randomly assigned to 6 treatment groups (n = 4) on day 3. Pentoxifylline (64 mg/kg) and M-1 (64 mg/kg) were administered by enema in a 250 µl volume twice-daily from days 3 to 14. Controls received 250 µl vehicle (saline). Additional control groups assessing the effects of pentoxifylline and M-1 in non-colitic rats were included. Animals were euthanized at day 14 and the distal 6 cm of colon was carefully excised and opened longitudinally and the colon was weighed and scored for macroscopic damage (described below). Longitudinal strips of tissue were frozen in liquid nitrogen for myeloperoxidase (MPO) assay and Western analysis. The remainder of the colon was rolled longitudinally, secured with suture thread and fixed in 4% paraformaldehyde for histological processing.

2.5. Macroscopic scoring of colon damage

The mucosal surface of the distal 6 cm of rat colon was scored for macroscopic damage using a well-established scale (Wallace et al.,

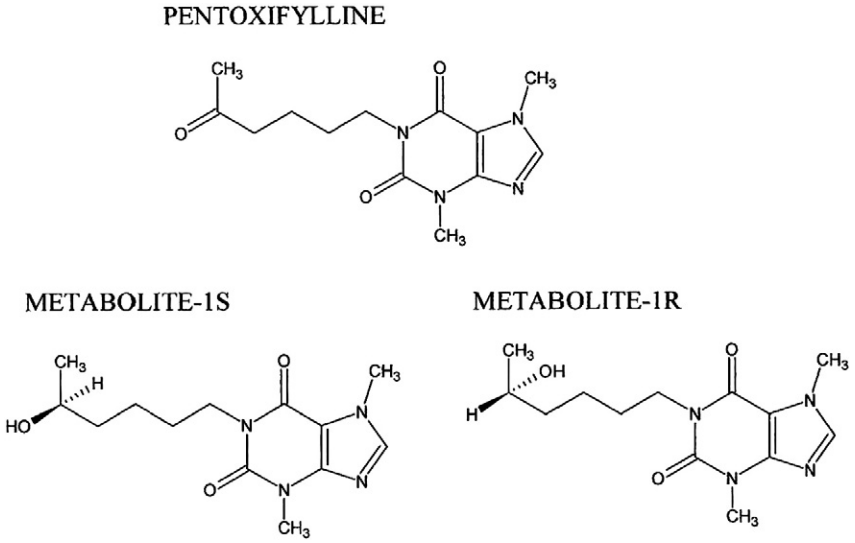

Fig. 1. Structures of pentoxifylline and its major metabolite (M-1). M-1 is formed from the reduction of a carboxyl group (C=O) on the pentoxifylline molecule (arrow) to a corresponding hydroxyl group (-OH) on M-1 forming a chiral center in the metabolite. The two spatial orientations of the hydroxyl group around the chiral carbon atom give rise to two enantiomers of the M-1 (M-1S and M-1R). [dark wedge = out of the plane of the page; dashed wedge = into the plane of the page].

1989) that takes into account the presence or absence of hyperemia, ulceration, adhesions, and diarrhea.

2.6. Myeloperoxidase enzyme activity assay

MPO is an enzyme located in the intracellular primary granules of neutrophils. MPO activity is directly proportional to neutrophil number and can be used as a quantitative index of inflammation (Krawisz and Sharon, 1984). The assay used was a modification of previous methods (Bradley et al., 1982; Wallace, 1987). Briefly, strips of colon tissue 2 cm in length were cut from regions containing evidence of macroscopic damage. Tissue was homogenized in 0.5 ml HETAB buffer containing hexadecyltrimethylammonium bromide 0.5% (Sigma, Oakville, ON) in 50 mM phosphate buffer (pH 6.0). A second 0.5 ml HETAB buffer was used to wash the homogenizer probe and was added to the original tube (total volume 1 ml). Homogenates were sonicated and centrifuged for 10 min at 3100 rpm at 4 °C. Supernatants were then centrifuged for 5 min at 10,000 rpm. A 100 µl aliquot of supernatant was transferred to a cuvette containing 1.9 ml of *o*-dianisidine reagent consisting of 0.167 mg/ml *o*-dianisidine (Sigma, Oakville, ON) in 50 mM phosphate buffer (pH 6.0) with 0.0005% H_2O_2. The colorimetric reaction was measured by spectrophotometry (450 nm) at 0 min, 1 min, and 2 min to determine change in absorbance per minute. One unit of MPO activity is the amount needed to convert 1 µmol of H_2O_2 (change in absorbance of 1.13×10^{-2}). Samples were run in duplicate.

2.7. Preparation of colon tissue for H&E and Sirius Red/fast green staining

Paraformaldehye-fixed colon rolls were processed in buffered formalin prior to paraffin-embedding. Embedded tissues were cut into 8 µm sections, floated onto siliconized slides, and dried for 1 h at 60 °C. Sections were stained with hematoxylin and eosin (H&E) or Sirius Red/Fast Green (SR/FG) for visual analysis. Sections (15 µm) were cut and placed into glass test tubes for collagen quantitation by the SR/FG method described below.

2.8. Sirius Red/fast green method for quantitation of collagen

Collagen levels in colon tissue were assessed by a modification of the SR/FG method Lopez de Leon and Rojkind, 1985). Briefly, tissues were deparaffinized and incubated in 0.04% FG (BDH Inc., Toronto, ON) in saturated picric acid for 20 min. Tissues were then incubated in 0.1% FG/0.04% SR (BDH Inc., Toronto, ON) in saturated picric acid for 30 min and were washed well. Representative colon sections were also mounted on slides and kept for visualization of collagen staining. SR/FG stain of free-floating sections was eluted with 1 ml of 0.05 N NaOH in 50% methanol for collagen quantitation and read in a spectrophotometer at 540 nm and 604 nm, corresponding to peak absorbance values for SR and FG respectively. Collagen (µg) was calculated as a ratio of total protein (mg) in the colon section according to previously defined calculations (Lopez de Leon and Rojkind, 1985). Samples were assayed in quadruplicate and an average for each animal was obtained. To estimate the actual amount of collagen in a 6 cm colon segment, the collagen ratio was corrected for the protein concentration in the tissue and this was multiplied by the weight of the colon segment. The final calculation was as follows: µg collagen/mg protein × mg protein/g tissue × g tissue/6 cm colon segment.

2.9. Western analysis of colon lysates for collagen type I

Colon tissue lysates were prepared by pulverizing frozen tissue with a LN_2-cooled mortar and pestle. Ice-cold RIPA buffer (1% Igepal CA 630, 0.5% sodium deoxycholate, 0.1% SDS in PBS, pH 7.4) containing standard protease inhibitors (PMSF, aprotinin, sodium orthovanadate) was added at a ratio of 3 ul RIPA/1 mg tissue. Suspensions were homogenized followed by sonication and were incubated on ice for 30 min with additional PMSF (0.03 ul/mg tissue) before centrifugation (10 min, 10,000 rpm, 4 °C). Supernatants (lysates) were stored at −86 °C. A 1-h protein block was performed using 5% skim milk powder in Tris-buffered saline with 0.1% Tween (TBS-T) followed by overnight incubation at 4 °C with goat anti-collagen type I polyclonal (Santa-Cruz) 1:500 in TBS-T with 10% BSA. Membranes were incubated in blocking buffer for 1 h at RT with rabbit anti-goat IgG peroxidase conjugate (1:25,000; Sigma, Oakville, ON). Bands were visualized by enhanced chemiluminescence (ECL; Cell Signaling Technology) and equal loading was verified by amido black-staining of blots. Densitometry was analyzed by Scion Image software. Background density of each lane was subtracted from the corresponding band density. Measurements are reported in mean relative density units (RDU) ± S.E.M.

2.10. Interleukin-18 in colon tissues

IL-18 levels in colon lysates were analyzed by ELISA according to the manufacturer's instructions (Biosource, Toronto, ON). The level of detection of the Biosource Rat IL-18 kit was 4–1200 pg/ml.

2.11. Statistical analysis

Data were analyzed by an analysis of variance (ANOVA) and post-hoc analysis was carried out using the Student–Newman–Keuls method of multiple pairwise comparisons to analyze differences between groups, *i.e.*, when more than 2 variables were compared (Zar, 1974). SigmaStat version 2.0 software was used and the statistical significance was at $P<0.05$. Data are expressed as mean ± S.E.M.

3. Results

3.1. Changes in MPO activity and tissue architecture over time in TNBS-induced colitis

The change in MPO activity over time in this model corresponded with cellular infiltrate in the tissue visualized by H&E-staining of colon sections (Fig. 2). Fig. 2A shows that MPO activity in colon tissue was significantly elevated at 3, 7, and 14 days ($P<0.05$) compared to controls, with the highest elevation at 7 days post-TNBS. Fig. 2B–E compares cross-sections of colon from (B) a control rat and rats treated with 90 mg/kg TNBS at (C) 3 days, (D) 7 days and (E) 14 days post-TNBS. TNBS-treatment resulted in overall thickening of the colon tissue, especially in the submucosa and muscularis layers. Edema was present in the tissue at days 3 and 7. Cell density in the TNBS-treated tissue was much higher than control, visualized as increased nuclear (purple) staining. This was due to inflammatory infiltrate in the lamina propria as well as overgrowth of the muscularis. Mucosal crypt architecture was distorted, especially adjacent to sites of ulceration, and was completely absent in the ulcer.

3.2. Changes in colonic levels of interleukin-18 (IL-18) over time in the TNBS-induced colitis

The change in IL-18 levels in colonic lysates over time in the TNBS model is shown in Fig. 3 and demonstrates that the IL-18 levels peak at 72 h.

3.3. Effect of pentoxifylline and M-1 on colon damage in TNBS-induced colitis

Treatment with TNBS caused significant colitis with an average damage score of 6.9 ± 0.8 compared to control animals not exposed to TNBS (damage score = 1 ± 0; $P<0.001$) as shown in Table 1. Intracolonic treatment with pentoxifylline or M-1 (64 mg/kg i.c. bid) from days 3–14 significantly attenuated the damage induced by TNBS

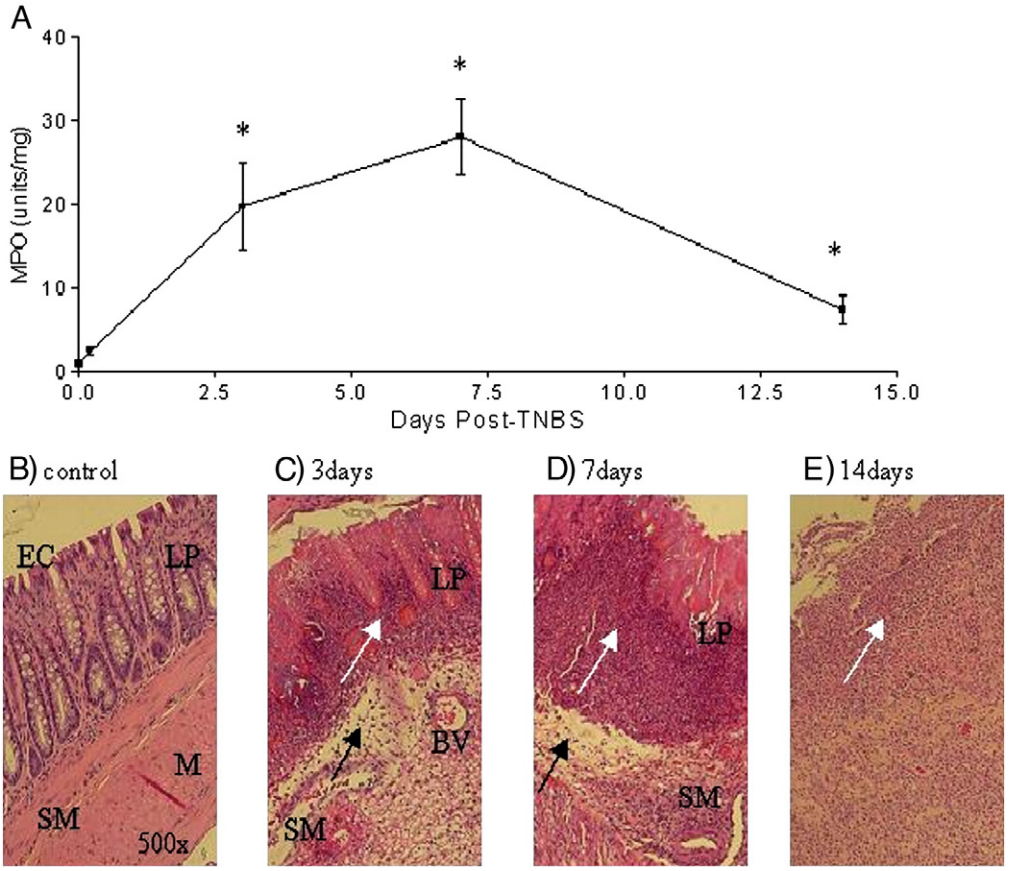

Fig. 2. Changes in MPO activity and inflammatory infiltrate over time in TNBS-colitis. A. MPO activity (units/mg tissue) in colon tissue was measured at 4 h, 3 days, 7 days, and 14 days post-TNBS. [* = significant difference from control, $P<0.05$] B. H&E-stained colon section taken from a control animal; note the intact epithelial cell layer (EC), normal crypt formation and basal level of inflammatory cells in the lamina propria (LP) visualized as dark purple nuclei. The submucosal layer (SM) is a narrow band between the LP and the muscularis (M). C. At day 3 post-TNBS, edema and inflammatory cells were present in the thickened submucosa (black arrows) and LP. Infiltration of inflammatory cells into the LP (white arrows) corresponded to a significant increase in MPO activity. D. At day 7 post-TNBS, there was continuing evidence of edema in the SM. The inflammatory infiltrate extending into the LP was thick and dense and corresponded to an elevated MPO activity. Note the abnormal appearance of the epithelial layer and crypt architecture. E. By day 14 post-TNBS, the epithelial layer and crypts were completely destroyed at sites of ulceration and a heavy inflammatory infiltrate was scattered throughout the surrounding tissue. This cellular infiltrate was less dense than that seen at days 3 and 7 (and this appears to be reflected in MPO activity) but was present in all layers of the colon.

(damage score $S=4.6\pm0.9$ and 4.2 ± 0.6 respectively, *vs.* 6.9 ± 0.8; $P<0.01$). Colons from control animal treated with pentoxifylline or M-1 were normal in appearance (damage score = 1, not shown). The results shown in Table 1 indicate that treatment with a lower dose of pentoxifylline (32 mg/kg) or M-1 (32 mg/kg) did not significantly alter the morphology damage score in TNBS treated animals.

3.4. Effect of pentoxifylline and M-1 on myeloperoxidase activity in TNBS-induced colitis

MPO activity in colon tissue from control animals was very low (0.36 ± 0.1 units/mg tissue) as illustrated in Table 1. Treatment of control animals with pentoxifylline or M-1 had no effect on basal MPO activity (not shown). Colonic tissue obtained from TNBS-treated animals had significantly elevated MPO levels at day 14 compared to controls (10.1 ± 3 *vs.* 0.36 ± 0.1 units/mg tissue; $P<0.001$). Intracolonic treatment with pentoxifylline or M-1 (64 mg/kg bid) from days 3–14 significantly attenuated the TNBS-induced increase in MPO activity (5.4 ± 2 and 3.7 ± 1 units/mg tissue respectively, *vs.* 10.1 ± 3 units/mg tissue; $P<0.05$). Tissue for analysis of MPO activity was taken from sites of colon damage. The results shown in Table 1 indicate that treatment with a lower dose of pentoxifylline (32 mg/kg) did not significantly alter the MPO activity in TNBS treated animals while treatment with the lower dose of M-1 (32 mg/kg) significantly decreased the MPO activity in TNBS treated animals. The results for the MPO corresponded to histology (not shown) in a similar manner as illustrated in Fig. 2.

3.5. Effect of M-1 on colon weight and thickness in TNBS-induced colitis

Colon wet weight and thickness are indirect measures of inflammation and/or fibrosis in the tissue. Induction of colitis with TNBS caused a significant increase in colon wet weight compared to saline treated controls (1.40 ± 0.2 *vs.* 0.54 ± 0.02 g/6 cm; $P<0.001$) as shown in Table 1. In colitic rats, treatment with M-1 (64 mg/kg bid) for 11 days significantly reduced colon weight compared to TNBS-treated animals (0.86 ± 0.06 *vs.* 1.40 ± 0.2 g/6 cm; $P<0.001$). There was no difference in colon weight between drug-treated controls and saline-treated controls indicating that pentoxifylline and M-1 had no effect on basal colon weight. The results shown in Table 1 also indicate that treatment with both doses of pentoxifylline (32 mg/kg and 64 mg/kg) or M-1 (32 mg/kg) did not significantly alter the colon weight in TNBS treated animals. Colon thickness (lumen to serosa) was measured at its maximum point using a light microscope (magnification 25×) with a scale embedded in the eyepiece. Scale units were converted to millimeters (mm). Data are expressed as mean maximum colon thickness (mm) ± S.E.M. (Fig. 4). TNBS treatment significantly increased maximum colon thickness compared to controls (2.1 ± 0.2 *vs.* 0.7 ± 0.05 mm; $P<0.05$). M-1 treatment significantly reduced maximum colon thickness in TNBS-treated rats (1.4 ± 0.1 *vs.* 2.1 ± 0.2 mm; $P<0.05$).

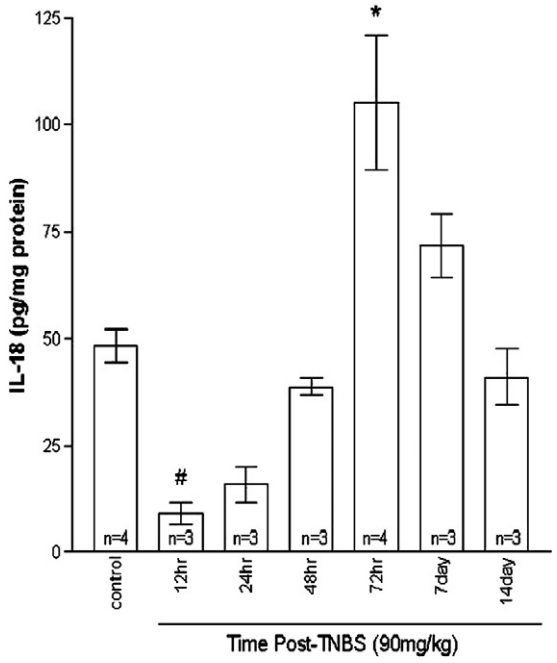

Fig. 3. Changes in IL-18 in colon tissue in TNBS-induced colitis. IL-18 levels in colon lysates were analyzed by ELISA. Results are expressed as mean ± S.E.M. * = significant difference from control, P<0.05.

3.6. Effect of M-1 and pentoxifylline on colonic collagen in TNBS-induced colitis

TNBS-treatment resulted in a visible elevation of tissue collagen in SR/FG-stained colon sections which was shown as a significant increase in collagen score in the TNBS group compared to controls (35 ± 5 *vs.* 15 ± 0.6; P<0.05) (Fig. 5A). While the effect of pentoxifylline alone did not reach significance, the effect of one of its metabolites, M-1 significantly reduced colonic collagen scores compared to TNBS-treated rats (19 ± 1.7 *vs.* 35 ± 5; P<0.05). The collagen score showed an excellent correlation with the damage score (Fig. 5B).

3.7. Western analysis of collagen type I in colon lysates

Colon lysates were analyzed by Western immunoblotting for collagen type I protein (Fig. 6A). Densitometric analysis of Western results (Fig. 6B) revealed a significant increase in collagen type I protein in TNBS-treated colon tissue (P<0.001). Collagen type I levels in drug-treated tissue samples were reduced compared to TNBS-treated tissue and appeared to relate to the level of colonic damage. Of interest, the association between collagen content and damage score was again observed here; three samples from each drug-treated group (lanes 7–9, 12–14) were from animals with low damage scores (damage score = 2–4). The lysates (lanes 10 and 11) are from each treatment group with a moderate level of colonic damage (damage score = 5 and 6, respectively), and there was an obvious greater amount of collagen type I detected by Western analysis.

4. Discussion

TNBS-induced fibrosis has been studied in animal models of pulmonary interstitial fibrosis (Kimura et al., 1992; Zhang-Hoover et al., 2000), pancreatic fibrosis (Haber et al., 1999; Puig-Divi et al., 1996), and chronic cholangitis (Mourelle et al., 1995) but few studies have examined intestinal fibrosis in TNBS-induced colitis. The goal of the present study was to determine the effect of pentoxifylline and racemic M-1 on inflammation and fibrosis in TNBS-colitis. We have developed a simple, reproducible, and inexpensive method to synthesize racemic M-1 from pentoxifylline using a non-selective chemical reduction reaction (structures shown in Fig. 1).

In this study, rats received 90 mg/kg TNBS intracolonically at the onset of the experiment and drug treatments were not initiated for 72-h to prevent a washout effect and to ensure development of a good response to the hapten. A preliminary experiment had revealed that the effects of TNBS were severely dampened when intracolonic treatments were initiated 1-h after TNBS (data not shown) suggesting a "wash-out" effect, therefore it was important to initiate intracolonic treatment after establishment of colitis and hence 72 h post TNBS treatment was chosen as the optimum time for drug intervention. Evaluation of H&E-stained colon sections revealed that intestinal crypt distortion, edema, and inflammatory infiltrate, hallmarks of active Crohn's disease (Cotran et al., 1999), were evident by day 3 post-TNBS (Fig. 2C). A corresponding peak in colonic IL-18 was also observed at 72 h post TNBS. The visible increase in density of inflammatory infiltrate in response to TNBS (Fig. 2C–E) corresponded with increased values obtained for colonic MPO activity (Fig. 2A) with the highest elevation seen at 7 days post-TNBS. Ulceration featuring total loss of intestinal crypt architecture was evident at day 14 (Fig. 2E). Colon thickness and cell density were also visibly increased at this timepoint. The increased cellularity was due to invading inflammatory cells as well as hyperproliferation of resident cells, including smooth muscle cells and fibroblasts involved in fibrosis (Lawrance et al., 2001; Pucilowska et al., 2000). MPO activity remained significantly elevated at day 14 (Fig. 2E) indicating that neutrophils were a major component of the inflammatory infiltrate at this timepoint. Morphology damage score was elevated 7-fold and MPO activity was elevated 25-fold in TNBS treated animals compared to controls (Table 1). Regression analysis indicated that MPO activity demonstrated a positive correlation with morphology damage score ($r^2 = 0.923$, P = 0.001) in the TNBS-induced colitis model.

Our results also indicate that the IL-18 levels in colon lysates peak at 72 h post TNBS treatment (Fig. 3), while Videla et al., 1998 reported that peak levels of TNF alpha in colonic tissue were reached on days 4

Table 1
Effect of pentoxifylline or M-1 on morphology damage score (MDS), myeloperoxidase (MPO) activity and colon weight in TNBS treated animals.

	Morphology damage score	Myeloperoxidase activity	Colon weight
Control	1.0 ± 0	0.4 ± 0.1	0.6 ± 0.02
TNBS	6.9 ± 0.8[a]	10.1 ± 3.0[a]	1.3 ± 0.1[a]
TNBS + Pentoxifylline (32 mg/kg)	7.0 ± 1.0[a]	7.4 ± 1.4[a]	1.3 ± 0.1[a]
TNBS + Pentoxifylline (64 mg/kg)	4.6 ± 0.9[a,b]	5.4 ± 2.0[a,b]	1.2 ± 0.01[a]
TNBS + M-1 (32 mg/kg)	4.9 ± 1.0[a]	5.2 ± 1.5[a,b]	1.1 ± 0.1[a]
TNBS + M-1 (64 mg/kg)	4.2 ± 0.6[a,b]	3.7 ± 1.0[a,b]	0.9 ± 0.1[a,b]

Results are mean ± S.E.M. (n = 8) and are pooled data from two separate experiments with similar results.
[a] P<0.05 compared to control.
[b] P<0.05 compared to TNBS.

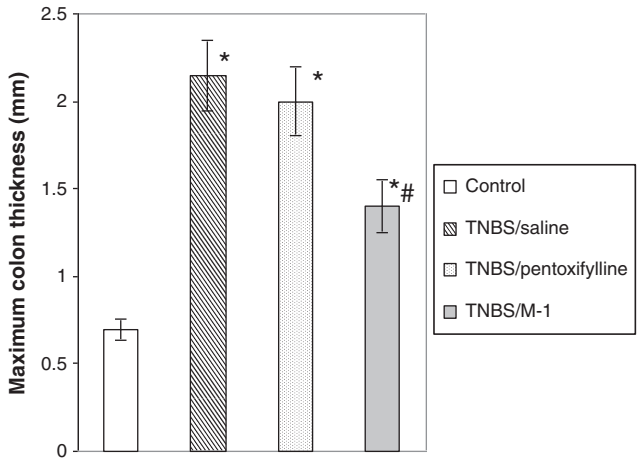

Fig. 4. Effect of M-1 and Pentoxifylline on colon thickness in TNBS-Colitis. Rat colons (distal 6 cm) were removed at day 14 post-TNBS and colon thickness (mm) was measured at its maximum point by microscopic examination of H&E-stained sections. Rats were treated with M-1 or pentoxifylline (64 mg/kg i.c. bid) from days 3 to 14 following exposure to TNBS (90 mg/kg) or saline. Results are mean ± S.E.M. (n = 8) and are pooled data from two separate experiments with similar results. * = significant difference from Sal/Sal; $P<0.05$., # = significant difference from TNBS/Sal, $P<0.05$.

and 10 post TNBS. Others have also reported that TNF alpha is increased in the TNBS model (Dutra et al., 2011; Isik et al., 2011; Miroliaee et al., 2011; Yin et al., 2011) and that TNF alpha, TNFRI, and TNFRII mRNA expression was increased significantly in the colon of TNBS experimental animals compared to controls (Rojas-Cartagena et al., 2005). Our regression analysis indicated that colonic IL-18 levels demonstrated a positive correlation with MPO activity ($r^2 = 0.757$, $P = 0.01$) and a positive correlation with morphology damage score ($r^2 = 0.648$, $P<0.05$) in the TNBS-induced colitis model. Recently, Carvalho et al. (2007) reported that TNF alpha levels also correlated with damage score in the TNBS model.

Intracolonic treatment with pentoxifylline or M-1 (64 mg/kg bid) from days 3 to 14 significantly attenuated colon damage and inflammation associated with TNBS-colitis (Table 1). There was no significant difference between the effects of pentoxifylline and M-1 on colon damage score or MPO activity, suggesting that these two drugs have a similar anti-inflammatory effect in TNBS-colitis even though their mechanisms of action may not be the same (Bursten et al., 1994; Coon et al., 1999; Jimenez et al., 2001; Sturm et al., 2002). Pentoxifylline in a dose-dependent manner inhibited the production of IL-18 (Aricha et al., 2006; Samardzic et al., 2001; Vukanić et al., 2007) and also dose dependently decreased TNF alpha (Appleyard et al., 1996; Aricha et al., 2006; Vukanić et al., 2007), and produced lower levels of IL-12 (Aricha et al., 2006; Vukanić et al., 2007) and higher levels of IL-10 (Vukanić et al., 2007).

M-1, in contrast to pentoxifylline, significantly reduced colon weight and thickness in colitic animals, suggesting that M-1 was having an additional effect compared to pentoxifylline (Table 1 and Fig. 4). Colon weight and thickness likely reflect a combination of edema, inflammation, and fibrosis induced by TNBS. Analysis of H&E-stained colon tissue at day 14 in TNBS treated animals compared to control (Fig. 2E and A) indicated that edema in the tissue had subsided by day 14. There was no obvious difference in inflammatory infiltrate between pentoxifylline and M-1 groups (not shown), which was also supported by the similarity in effect of pentoxifylline and M-1 on MPO activity (Table 1). The different effects of M-1 and pentoxifylline on colon weight and thickness may relate to fibrosis in the tissue.

This is one of the few studies to examine the effects of pharmacological agents on inflammation-induced fibrosis in TNBS-colitis. Collagen deposition in the colon was measured and visualized using a modification of the Sirius Red/Fast Green method of tissue collagen quantitation. This modified collagen score revealed that collagen was

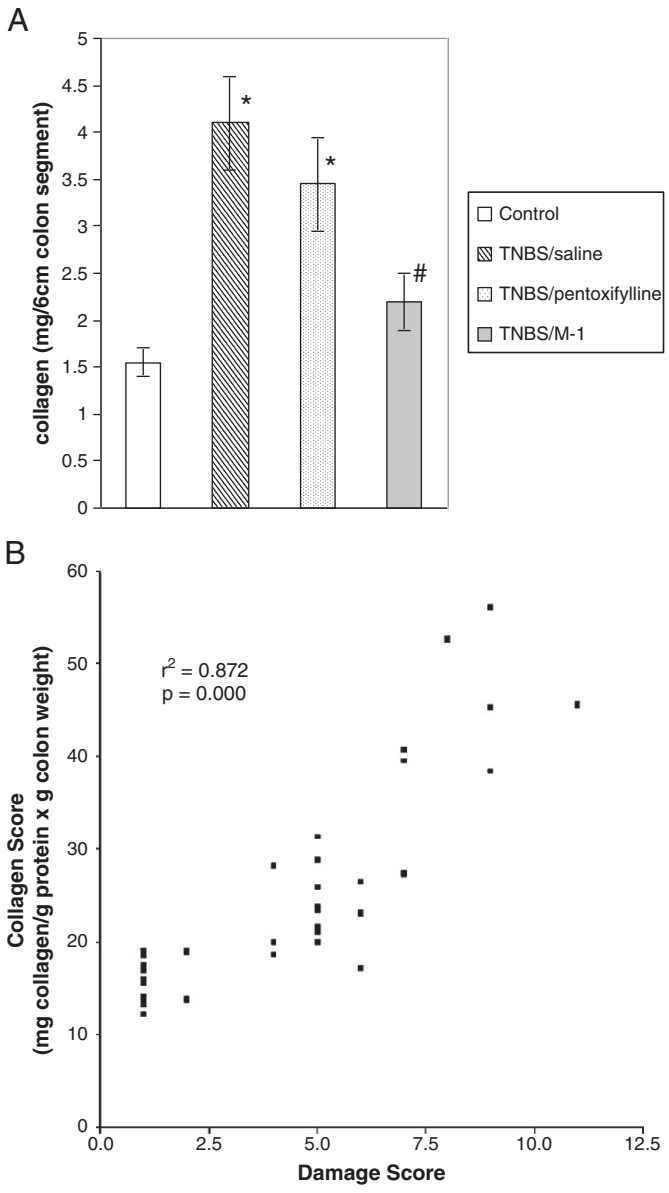

Fig. 5. Collagen quantitation in colon tissue using the SR/FG method. A. Collagen ratio modified to account for variability in colon weight (μg collagen/mg protein × g tissue/6 cm colon segment). B. Correlation of collagen score to damage score. Rats were treated with M-1 or pentoxifylline (64 mg/kg i.c. bid) from days 3 to 14 following exposure to TNBS (90 mg/kg). Results are mean ± S.E.M. (n = 8) and are pooled data from two separate experiments with similar results. * = significant difference from Sal/Sal; $P<0.05$; # = significant difference from TNBS/Sal, $P<0.05$.

significantly increased in the TNBS group compared to controls (Fig. 5A). This modified collagen score showed a significant positive correlation with damage score, expected in a model of inflammation-induced fibrosis (Fig. 5B).

Other investigators have assessed SR-stained collagen by visual histochemical analysis (Last et al., 2004) often accompanied by development of a semi-quantitative scoring system (Du et al., 2001), automated digital image analysis (Ejeil et al., 2003), computerized histomorphometry (Murphy and Nicholson, 2003), and Sirius Red-polarizing microscopy-morphometry which distinguishes between certain types of collagen by their unique fibril properties (Che and Huang, 1999). The method described in this study provides a simple means of quantitating collagen. Our modification of the collagen calculation may be useful in assessing collagen in other forms of inflamed tissue.

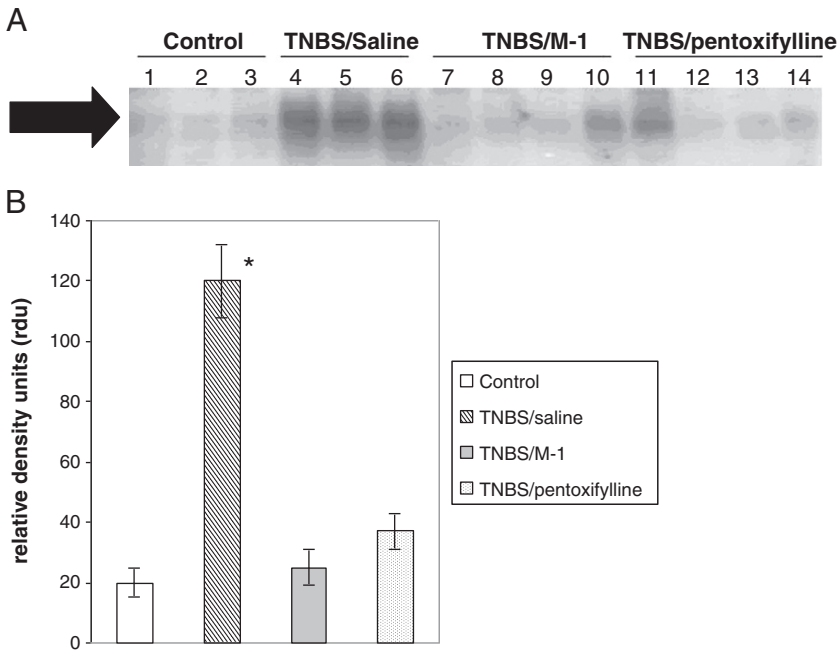

Fig. 6. Collagen type I in colon samples from TNBS-colitis experiments. Collagen type I was assessed in lysate samples by Western immunoblotting followed by densitometric analysis. A. Groups shown are controls (lanes 1–3), TNBS/Sal (lanes 4–6), TNBS/M-1 (lanes 7–10), and TNBS/pentoxifylline (lanes 11–14). B. Densitometric analysis of bands from the Western analysis. Background density was subtracted from the corresponding band density for each lane. Immunoblots were stained with amido black to verify equal loading of protein. * = significant difference from Sal/Sal; $P<0.05$.

In our study, colitic rats had significantly increased intestinal collagen levels (Fig. 5A) and treatment with M-1 significantly attenuated this fibrotic response. Western analysis indicated that collagen type I was significantly elevated in colonic tissue of the colitis model and that this effect was blocked by both pentoxifylline and M-1 (Fig. 6A and B). It should be noted that pentoxifylline has attenuated fibrosis in other disease models including glomerulonephritis (Lin et al., 2002), Peyronie's disease (Valente et al., 2003), and hepatic fibrosis (Peterson, 1993). We have recently reported that combination of ciprofloxacin and pentoxifylline greatly increased the levels of pentoxifylline and its M-1, via an effect on CYP1A2 and CYP2E1 (Peterson et al., 2004) and that combination of ciprofloxacin and M-1 resulted in an even higher sera level of the biologically active drug (Raoul et al., 2007). Our results also suggest that pentoxifylline inhibits two hallmarks of hepatic fibrosis (Verma et al., 2007).

We have recently shown that both pentoxifylline and IL-18 antibody blocked a key step in fibrosis (Khan et al., 2008). Our recent results indicate that IL-18 levels were significantly elevated in collagenous colitis, a form of inflammatory bowel disease that is characterized by collagen deposition and that the levels of IL-18 was subsequently decreased in these patients following treatment with pentoxifylline that also decreased collagen deposition in colonic tissue (Peterson et al. submitted to J Interferon Cytokine Res 2011). Our results suggest that for the first time that IL-18 may play a key role in the development of the inflammation and subsequent fibrosis in this TNBS model of colitis. Our results are supported by the recent suggestion that IL-18 may play a role in cardiac fibrosis (Fix et al., 2011).

In summary, both pentoxifylline and one of its metabolites M-1 significantly attenuated colon damage and inflammation associated with TNBS-colitis. Significant fibrosis was induced in colitic animals characterized by increased collagen deposition. Treatment with M-1 resulted in the reduction of TNBS-induced intestinal fibrosis. Though this metabolite is not currently available as a drug of choice, it is produced from pentoxifylline *in vivo* and its concentration can be effectively increased by combining pentoxifylline with other drugs including ciprofloxacin and this could have important implications for human Crohn's disease since there are currently no treatments that target both inflammation and fibrosis in this disease.

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