Inflammopharmacol (2010) 18:73–85
DOI 10.1007/s10787-010-0032-x

Inflammopharmacology

RESEARCH ARTICLE

Efficacy of a novel sphingosine kinase inhibitor in experimental Crohn's disease

Lynn W. Maines · Leo R. Fitzpatrick · Cecelia L. Green · Yan Zhuang · Charles D. Smith

Received: 20 November 2009 / Accepted: 20 January 2010 / Published online: 12 February 2010

Abstract
Aim Activation of sphingosine kinase (SK) is a key response to many inflammatory processes. The present studies test the hypothesis that an orally available SK inhibitor, ABC294640, would be effective in rodent models of Crohn's disease.
Methods Trinitrobenzene sulfonic acid (TNBS) was administered rectally to mice and rats. Rats were treated with ABC294640 orally alone or in combination with olsalazine and disease progression was monitored.
Results For both rodent species, treatment with ABC294640 attenuated disease progression. Colon samples from the ABC294640-treated animals had improved histology and cytokine parameters when compared with vehicle-treated animals. The expression of SK was similarly increased in TNBS-treated animals and in human colon tissue specimens from inflammatory bowel disease patients relative to normal, control patients.
Conclusions Sphingosine kinase may be a critical mediator of colonic damage during intestinal inflammation, and pharmacologic inhibitors of this enzyme may prove useful in the treatment of Crohn's disease.

Keywords Sphingosine kinase inhibitor · Sphingosine kinase · Trinitrobenzene sulfonic acid · Crohn's disease · Inflammatory bowel disease · Rats · Mice

L. W. Maines (✉) · C. L. Green · Y. Zhuang · C. D. Smith
Apogee Biotechnology Corporation, Hershey Center for Applied Research, 1214 Research Blvd, Suite 1016, Hummelstown, PA 17036, USA
e-mail: LWMaines@apogee-biotech.com

L. R. Fitzpatrick
Department of Pharmacology, Penn State College of Medicine, Hershey, PA, USA

Introduction

The roles of sphingolipid metabolism in mediating inflammatory responses have been frequently reviewed (Yamanaka and Shegogue et al. 2004; Baumruker and Bornancin et al. 2005; Chalfant and Spiegel 2005; Kee et al. 2005; El Alwani et al. 2006; Leclercq and Pitson 2006; Taha et al. 2006). Briefly, ceramide is produced by the hydrolysis of sphingomyelin in response to several inflammatory stresses, including TNFα (Mathias et al. 1991; Xia et al. 1998). Ceramide can be further hydrolyzed by ceramidase to produce sphingosine, which is rapidly phosphorylated by sphingosine kinase (SK) to produce sphingosine 1-phosphate (S1P). Therefore, activation of ceramidase and SK by cytokines and growth factors leads to rapid increases in the levels of S1P and depletion of ceramide. These conditions result in increased inflammation and proliferation cascades and inhibit apoptosis. Deregulation of apoptosis in phagocytes is an important component of the chronic inflammatory state in inflammatory bowel diseases (IBDs), and S1P has been shown to protect neutrophils from apoptosis in response to Fas, TNFα and ceramide (Kostin et al. 2003). Similarly, apoptosis of macrophages is blocked by S1P (Rabano et al. 2003).

In addition to its role in regulating cell proliferation and apoptosis, S1P has been shown to have several other important effects on cells that mediate immune functions. Platelets, monocytes and mast cells secrete S1P upon activation, promoting inflammatory cascades at the site of tissue damage (Yatomi et al. 1995; Prieschl et al. 1999).

The activation of SK is essential for the signaling responses to TNFα, since its ability to induce adhesion molecule expression via activation of NFκB is mimicked by S1P, and is blocked by the SK inhibitor dimethylsphingosine (Xia et al. 1998). S1P is also a mediator of Ca^{2+} influx during neutrophil activation by TNFα and other stimuli, leading to the production of superoxide and other toxic radicals (MacKinnon et al. 2002; Itagaki and Hauser 2003).

When considering the accumulating evidence for a pivotal role of SK in the regulation of inflammatory processes, pharmacological inhibition of SK is a new potential means of preventing and/or treating IBDs. However, therapeutically useful SK inhibitors have been described only recently (French et al. 2003a, b, Maines et al. 2006, 2008). These compounds inhibit S1P formation in intact cells, and demonstrate a high degree of selectivity for SK versus other lipid and protein kinases. Our lead SK inhibitor, ABC294640, has excellent oral bioavailability and in vivo SK inhibitory activity in rodents and can be administered chronically to rats and mice in efficacious doses without limiting systemic toxicities (Smith and French 2008). ABC294640 has been shown in a contracted 20-kinase panel to be specific towards SK and our recent data indicates it is specific to SK-2 with a Ki of approximately 30 μM (unpublished data). Importantly, ABC294640 represents the first pharmacologic probe to evaluate the biological roles of this SK isozyme.

In our previous work (Maines et al. 2008), we demonstrated that ABC294640 reduces the inflammatory disease parameters in the acute and chronic dextran sulfate sodium (DSS) models of ulcerative colitis (UC) in mice. The pathogenesis of the DSS model is different from the trinitrobenzene sulfonic acid (TNBS) model. Specifically, TNBS-induced colitis is thought to involve Th-1 and Th-17-directed immune responses. This immunological-based colitis is more responsive to typical IBD-drugs like corticosteroids than is DSS-induced colitis. Therefore, we have now evaluated ABC294640 against TNBS-induced colitis in rats and mice, to gain efficacy data in two species for a subsequent Investigational New Drug Application for ABC294640 and to gain a better understanding of the global therapeutic potential of this compound for IBD.

Materials and methods

Reagents

Trinitrobenzene sulfonic acid and prednisolone were purchased from Sigma–Aldrich (St. Louis, MO). ABC294640·HCl [3-(4-chlorophenyl)adamantane-1-carboxylic acid (pyridin-4-ylmethyl)amide, hydrochloride salt] was synthesized as described by Maines et al. (2008), and is hereafter referred to as ABC294640. Mice were dosed with drug in solution and for studies with rats, 10.5 ± 0.5 mg quantities of ABC294640 were filled into size 9 porcine hard gelatin capsules (Torpac; Fairfield, NJ). Rabbit polyclonal antibodies were raised against a 19 amino acid peptide unique to SK1 (Genbank Accession Number: AF200328) by Biosynthesis Inc. (Lewisville, TX), and have been previously demonstrated to detect human SK by immunohistochemistry (Maines et al. 2008).

Mouse TNBS model

On experimental days 0 and 7, age matched (46–63 days) male C57/BL/6 mice were anaesthetized with intraperitoneal ketamine/xylazine (136 mg/kg and 9.6 mg/kg, respectively) and a stainless steel catheter was carefully inserted into the colon (4 cm proximal to the anus). A solution of TNBS in 50% ethanol (0.1 ml volume of 40 mg/kg TNBS) was slowly administered with the animals being kept in a head-down position until they regained consciousness. Two control groups comprised mice administered either 50% ethanol or PBS in an identical fashion. Starting on day 6 and proceeding through day 9, animals were treated twice daily by oral gavage with either PBS containing 0.375% Tween-80 (vehicle) or 50 mg of ABC294640/kg) in vehicle. ABC294640 was administered by oral gavage at 50 mg/kg twice daily, which has been determined to be an effective dose in other inflammation models (French et al. 2006; Maines et al. 2008) with no related toxicities (unpublished data). On day 10, animals were killed by CO_2 asphyxiation and cervical dislocation per institutional IACUC requirements and the colons were removed. The colons were measured, weighed and scored for macroscopic inflammation as indicated below. The distal 3 cm was then transected for histology and biochemical analyses, as this was where disease manifestations were primarily observed.

Rat TNBS model

On day 0, female Sprague–Dawley rats (approximately 210 g) were anaesthetized (i.p.; 79.92 mg/kg ketamine plus 3.96 mg/kg xylazine). A stainless steel catheter was carefully inserted into the colon (8 cm proximal to the anus). Then, 1.0 ml total volume of TNBS solution (30 mg TNBS in 20% ethanol in PBS) was slowly delivered with animals being kept for 15-min intervals in a head-down position, level and head-up position. Treatment groups received oral doses of vehicle (0.375% Tween in PBS), prednisolone (5 mg/kg) in vehicle, osalazine in gelcaps, or ABC294640 in gelcaps. Animals were killed on day 6 by CO_2 asphyxiation and cervical dislocation per institutional IACUC requirements and colons removed. The colons were measured,

weighed and scored for macroscopic inflammation as indicated below. The distal 6 cm were then transected for histology and biochemical analyses. Colonic TNFα and IL-1β levels were determined as described previously by our laboratory (Fitzpatrick et al. 2000; Maines et al. 2008), using commercially available kits from Pierce Endogen (Rockford, IL).

Macroscopic score

Macroscopic inflammation within the distal 3 cm of each colon was scored using the scoring system as described by other investigators (Morris et al. 1989; McCafferty et al. 1999).

Histology score

The colon tissues were fixed with formalin overnight, followed by embedding in paraffin, sectioning and staining with haematoxylin–eosin. The sections were microscopically examined for histopathologic changes using the following scoring system. The histology score was determined by multiplying the percent involvement for each of the three following histological features by the percent area of involvement (Williams et al. 2001; Maines et al. 2008). Therefore, the minimal score is 0, and the maximal score is 40.

Myeloperoxidase assay

Mucosal myeloperoxidase (MPO) activity (Fitzpatrick et al. 2000) was determined as a measure of granulocyte infiltration into the colon.

Human tissue samples

Colon specimens were harvested from patients undergoing surgery at the Hershey Medical Center for a diagnosis of intestinal disease. Informed consent was obtained before surgery, as stipulated in the IRB-approved protocol # 19084. Two groups of patients were included in this study as described previously (Fitzpatrick et al. 2007). After bowel resection by a surgeon from the Department of Colon and Rectal Surgery, the intestinal tissue was sent to the Department of Anatomic Pathology Laboratory for Confirmation of the Diagnosis, and was banked within 30 min. For our studies, paraffin sections were obtained from macroscopically normal areas of the intestine from the non-IBD patients, and from areas of the intestine with obvious disease from the IBD patients, and stained for SK expression as described below.

Immunohistochemical analyses of SK expression

The expression of SK in colon specimens from the human patients, as well as from the rodent TNBS models was examined by immunohistochemistry. Briefly, sections were de-paraffinized in xylene and rehydrated in a series of ethanol dilutions. Sections were permeabilized with 0.5% Triton X-100 in PBS, and boiled for 10 min in 10 mM sodium citrate buffer for antigen retrieval. Slides were then incubated 10 min in 3% hydrogen peroxide to quench endogenous peroxidase. After washing in PBS, sections were blocked for 1 h with reagents from the VECTASTAIN ABC Kit (Vector Laboratories Inc.), and then incubated for 30 min at room temperature with the primary SK antibody. The samples were then washed with PBS, and incubated with biotinylated anti-rabbit IgG antibody for 30 min, followed by 30 min of incubation with DAB reagent for color development. Sections were lightly counterstained with hematoxylin, rehydrated, and mounted for analysis by bright-field microscopy. The percent area of SK expression was measured with a 25-mm ocular grid attached to an Olympus CH light microscope at a 400× magnification. Six areas of colonic tissue were evaluated per slide.

Statistics

Statistical analyses were performed using InStat (Version 3.01, GraphPad Software, Inc.). A one-way analysis of variance was performed with Tukey–Kramer multiple comparisons tests for evaluation of differences between groups.

Ethical considerations

All animal experimentation was conducted with procedures approved and monitored by the Penn State College of Medicine Institutional Animal Care and Use Committee (Office of Laboratory Animal Welfare Assurance Number A3045-01).

Results

Mouse TNBS model

To extend our previous findings that the SK inhibitor ABC294640 attenuates the development of DSS-induced UC in mice (Maines et al. 2008), we have studied this compound in the TNBS model of IBD, which models CD (Murthy and Flanigan 1999).

Change in body weight was the most global parameter of the IBD phenotype measured. As expected, non-TNBS-

treated mice gained weight while those receiving TNBS lost weight (Fig. 1a). Administration of ABC294640 nearly normalized the growth of the animals and this was associated with a general improvement in the appearance of the animals. Notably, the positive control drug prednisolone increased the loss of body weight (Fig. 1a), consistent with its known toxicity at therapeutic doses. The macroscopic scores for animals that received TNBS were consistently elevated in comparison with the ethanol alone controls, and this was largely normalized in the group that also received ABC294640. The treatment of the mice with prednisolone significantly attenuated macroscopic damage to a similar level found in PBS-treated mice.

As an objective measurement of inflammation-mediated oedema, hypertrophy and fibrosis, the weight of the distal 3 cm of each colon was determined (Fig. 1c). As with the macroscopic score, there was a substantial increase in colon weight of mice treated with ethanol alone compared with PBS. The addition of TNBS to the treatment protocol produced a further significant increase in colon weight, and this increase was significantly blocked by treatment of the mice with ABC294640. A comparable reduction was seen with prednisolone.

Granulocyte infiltration is frequently associated with IBD, and is markedly increased in rodent models of IBD (McCafferty et al. 1999; Williams et al. 2001; Maines et al. 2008). Therefore, MPO activity was assayed in the colon samples from the mice. As indicated in Fig. 1d, colonic MPO activity was substantially elevated in the TNBS-treated animals, when compared with control animals. The TNBS-induced increase in MPO activity was reduced by approximately 60% in mice receiving daily doses of ABC294640. As anticipated from the literature (Videla et al. 2006), prednisolone also suppressed neutrophil accumulation in the colon. Variability did not allow any changes to reach statistical significance.

Colons from mice with histology scores that correlated closely to the mean of their group were sectioned and stained and are shown in Fig. 2. Histological results of colon sections from the various treatment groups were consistent with the macroscopic scores and colon weights, revealing inflammation and damage in the TNBS-vehicle group (Fig. 2b, c) that was reduced or negated in the ABC294640-treated animals (Fig. 2d, e). It was also apparent that inflammatory cell influx was more substantial in TNBS-treated animals not receiving ABC294640 compared with those receiving the compound. The histology evaluations also suggested that the size of the muscularis layer, as well as oedema of the submucosa, was lessened in ABC294640-treated animals as compared to vehicle-treated animals (Fig. 2b, e). These observations are likely an explanation for the significant reductions in colon weight that were observed in the ABC294640-treated mice.

As a quantifiable measure of histological damage (Fig. 1e), animals receiving TNBS and oral vehicle typically had higher histology scores of 15–20 (representing moderate colitis) than non-TNBS controls (mean score <10). As with the other assays, the histology scores of TNBS mice given oral ABC294640 were consistently lower than the TNBS-vehicle animals, although some animals were only partially protected.

We have previously demonstrated that SK mRNA levels are elevated in human tumors (French et al. 2003a, b), and SK protein expression is increased in the colons of mice in the DSS model of UC (Maines et al. 2008). A similar increase in SK expression was observed in the colons from TNBS-induced colitis mice, when compared with PBS control animals (Fig. 3). SK staining was prominent in the mucosal epithelial cells, but was also seen in infiltrating leukocytes of TNBS-treated mice.

Rat TNBS model–dose response study

The rat TNBS model was used to consistently produce moderately severe colonic pathology. As shown in Fig. 4a, a discrete area of ulceration was typically produced in the rat distal colon on day 6 after TNBS administration. As shown in picture form (Fig. 4b) and by semi-quantitative analysis (Fig. 5b), colonic ulceration was less severe in animals treated with ABC294640. The mean body weight change was measured from days 0 to 6. As shown in Fig. 5, rats treated with either vehicle or ABC294640 had modest weight gains and animals that received prednisolone lost significant weight. On day 6, colons were harvested and evaluated for macroscopic damage. The vehicle group exhibited a macroscopic damage score relating to moderate colitis (5.57). All treatment groups had reduced scores (Fig. 5b).

As an objective measurement of inflammation-mediated edema, hypertrophy and fibrosis, the weight of the distal 6 cm of each colon was determined (Fig. 5c). As with the macroscopic score, the vehicle group had the largest colonic weight. Improvements were seen in all drug treatment groups.

Histological examination of colon sections (Fig. 5d) yielded histology scores that were generally consistent with the macroscopic scores and colon weights. Inflammation and damage were evident in the vehicle group that was reduced in the drug-treated animals. Previous studies have shown that the K_i of SK2 for ABC294640 is 10 μM and circulating levels of ABC294640 in plasma reach levels in this range at a dose of 25 mg/kg (Fitzpatrick et al. 2010) and putatively explains the comparable results in the 25 and 50 mg/kg treatment groups. Representative colon sections from the vehicle-, prednisolone- and ABC294640 (25 mg/kg) treatment groups are shown in Fig. 6. Marked

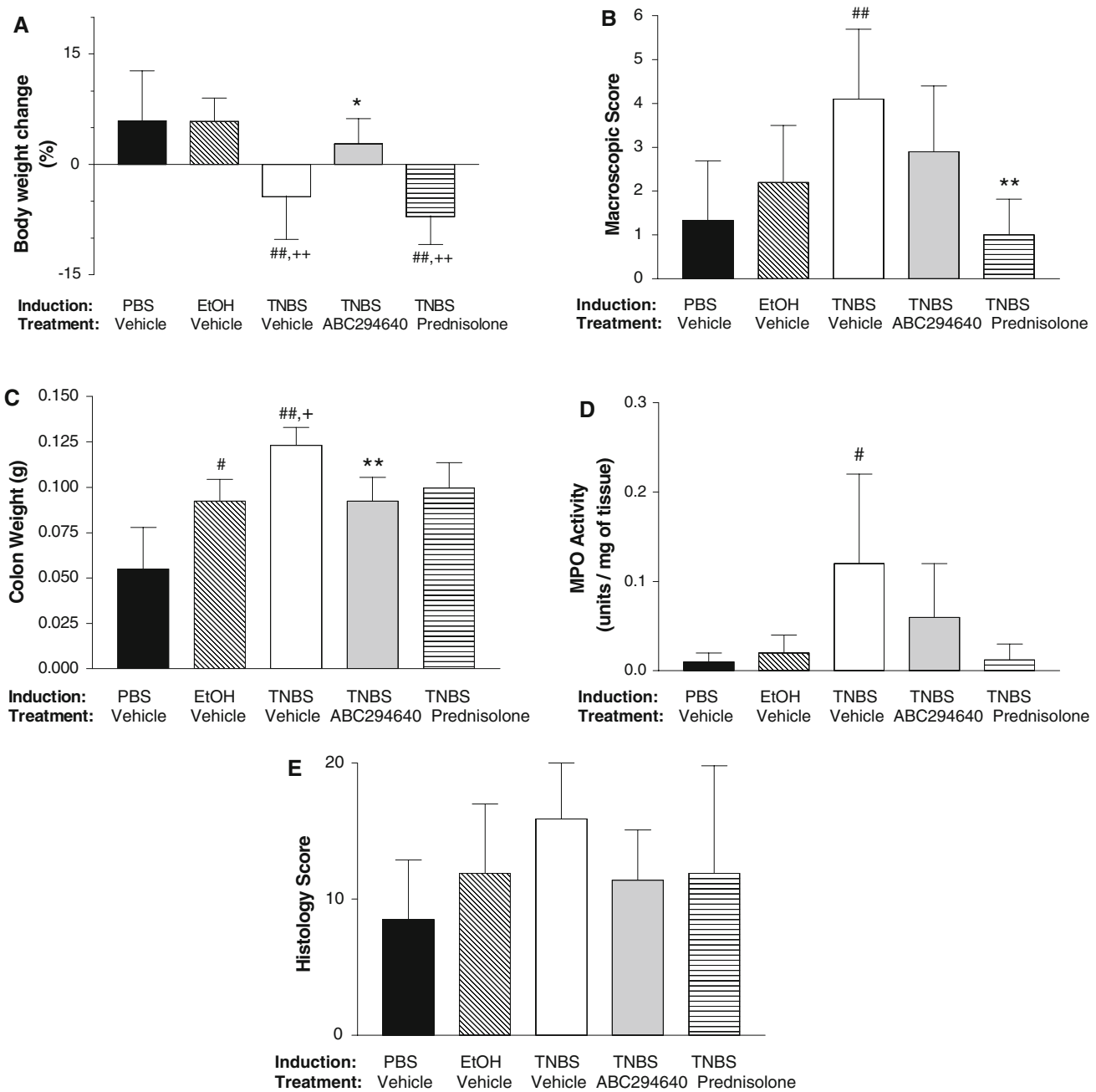

Fig. 1 Effects of ABC294640 on disease parameters in the mouse TNBS-colitis model. C57BL/6 mice were treated as follows: rectal PBS and oral vehicle (*black bars*); rectal 50% ethanol and oral vehicle (*diagonal hatched bars*); rectal TNBS and oral vehicle (*open bars*); rectal TNBS and oral ABC294640 (*gray bars* 50 mg/kg b.i.d.); or rectal TNBS and oral prednisolone (*horizontal hatched bars* 5 mg/kg b.i.d). At the time of killing (day 10), the following parameters were measured as described in "Materials and methods". **a** Body weight change, **b** macroscopic score, **c** colon weight, **d** myeloperoxidase activity and **e** histology score. *Values* represent the mean ± SEM for 4–5 mice per group. $^{*}P < 0.05$ or $^{**}P < 0.01$ versus vehicle/TNBS treatment, $^{\#}P < 0.05$ or $^{\#\#}P < 0.01$ or $^{\#\#\#}P < 0.001$ versus PBS/vehicle group, $^{+}P<0.05$ or $^{++}P < 0.01$ versus EtOH/vehicle group

inflammation and damage in the TNBS-vehicle group (Fig. 6a) was significantly reduced in both the prednisolone and ABC294640-treated animals (Figs. 6b, c, respectively). As in the mouse model, it was also apparent that leukocytes were present in much higher levels in vehicle-treated animals not receiving prednisolone or ABC294640. These histology studies also revealed reduced edema of the submucosa in prednisolone and ABC294640-treated animals as compared to vehicle animals (Fig. 6), as well as some evidence of attenuated colonic hypertrophy of the

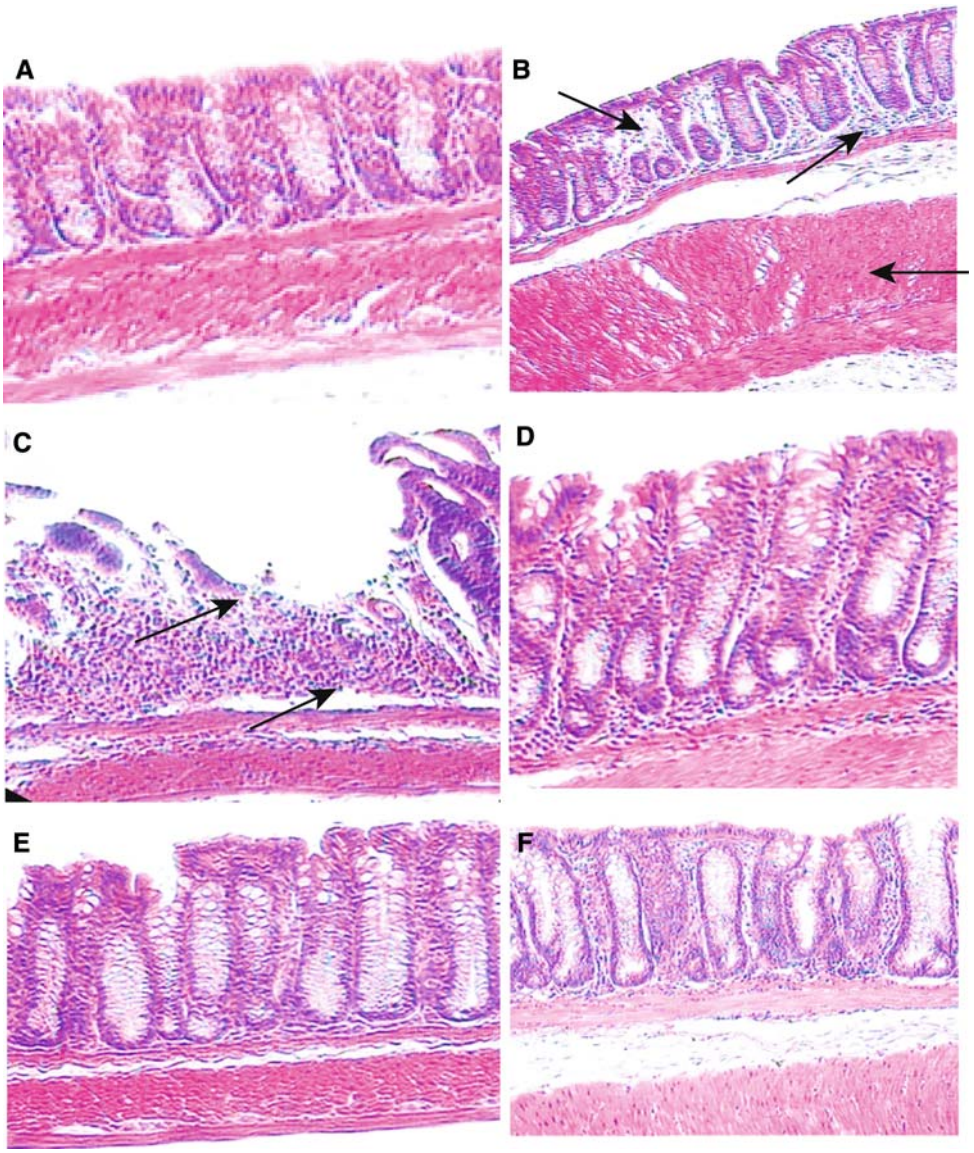

Fig. 2 Effects of ABC294640 on colon histology in the mouse TNBS-colitis model. Sections of colons from the animals described in Fig. 1 were stained with H&E and examined for pathologic changes. **a** Rectal PBS, oral vehicle-treated control animal representative of a colon with no disease morphology. **b** Rectal TNBS, oral vehicle-treated animal; *top arrow* shows moderate crypt disruption, *middle arrow* indicates leukocyte infiltration and *bottom arrow* points out severe thickening of the muscularis propria layer, **c** as in **b**, rectal TNBS and oral vehicle; *top arrow* shows an area of severe crypt damage and *bottom arrow* indicates substantial mucosal leukocyte infiltration. **d** A representative rectal TNBS, oral ABC294640-treated mouse with average disease intensity; *left arrow* shows that leukocytes are present at the bottom of the crypts, while the *right arrow* shows infiltrating leukocytes in the submucosa. **e** A rectal TNBS, oral ABC294640-treated complete responder showing normal crypt morphology similar to that of the rectal PBS-treated animals. **f** A rectal TNBS, oral prednisolone-treated mouse with some leukocyte infiltration in the lamina propria (*top arrow*) as well as at the base of the crypts near the muscularis mucosa (*bottom arrow*). The magnification was 150× for all panels

muscularis propria layer. These observations again are a likely explanation for the significant reductions in colon weight that were observed in the prednisolone- and ABC294640-treated mice.

Rat TNBS model—combination therapy study

The previous studies demonstrate that ABC294640 protects against colonic damage similar to the steroid prednisolone. Because olsalazine (Dipentum), a 5-aminosalicylic acid prodrug, is frequently used for maintenance therapy for UC, the ability of ABC294640 to be combined with this drug was examined in the rat TNBS model.

Disease parameters were measured in rats treated with ABC294640 or olsalazine alone or in combination. As shown in Fig. 7a, rats treated with vehicle or olsalazine lost weight between days 0 and 6; whereas, animals treated

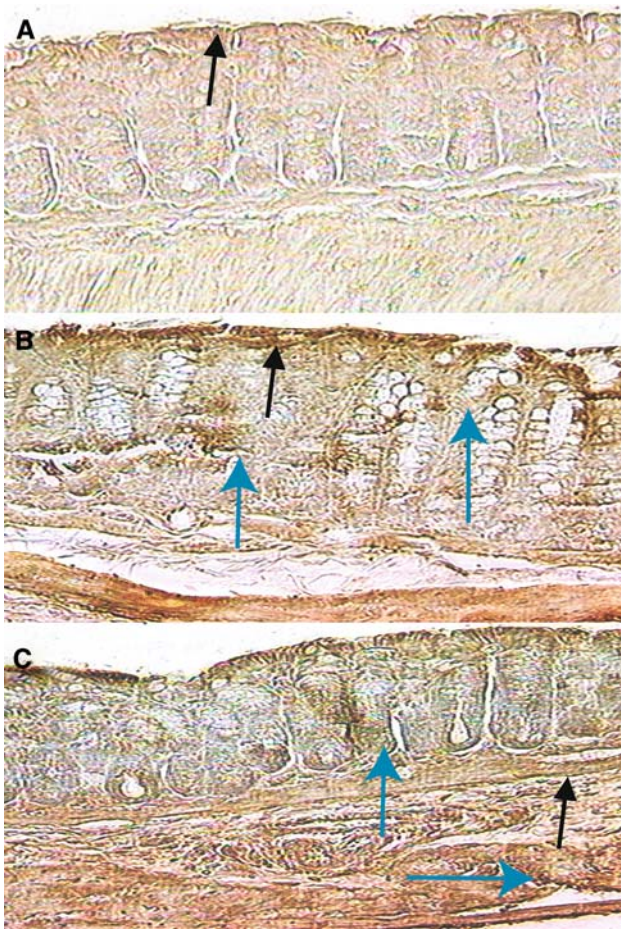

Fig. 3 SK expression in the mouse TNBS-colitis model. **a** A representative example of the mild SK expression pattern in the colon of a control, i.e. non-TNBS-treated, mouse. The *black arrow* shows staining of a surface epithelial cell. **b** A representative section of a TNBS-treated animal and reveals a general increase in SK staining with the most pronounced increase in the remnant surface colonic epithelial cells (*black arrow*), as well as in patches of infiltrating lamina propria leukocytes (*blue arrows*). **c** A representative section from another TNBS-treated animal, and elevated SK expression is evident in crypt colonic epithelial cells (*black arrow*), as well as in infiltrating leukocytes within the lamina propria and submucosa (*blue arrows*)

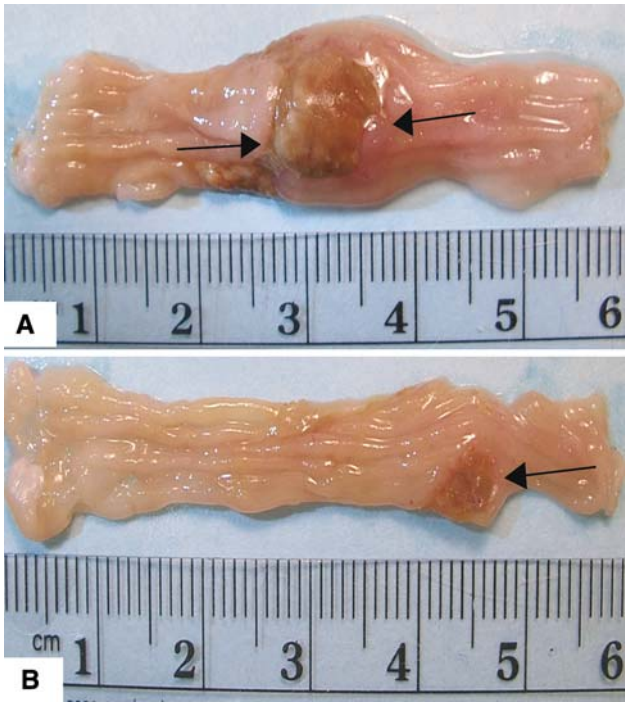

Fig. 4 Effect of ABC294640 on macroscopic damage to the rat colon in the TNBS-colitis model. Colons were harvested from TNBS-exposed rats treated with vehicle (**a**) or ABC249640 at 50 mg/kg, b.i.d. (**b**). The *double arrows* demarcate a large ulcer within the distal 4 cm segment of the rat colon. Colonic thickening is clearly evident in the adjacent ulcer area. There is a much smaller ulcer (*arrow*) at the 4.5-cm mark of the distal colon of the ABC294640-treated animal

with ABC294640 alone or in combination with olsalazine gained weight.

Colons were harvested on day 6 and evaluated for macroscopic damage. As indicated in Fig. 7b, the vehicle group exhibited a macroscopic damage score of 7.1, indicating more severe damage than in the previous dose–response study. All treatment groups showed reduced scores with the combination therapy having the lowest score.

The weight of the distal 6 cm of each colon was measured (Fig. 7c). The vehicle group had the largest colonic weight, indicative of the most damage, with improvements being seen in all treatment groups. In addition, the best result was seen in the combination therapy group.

To assess the extent of neutrophil infiltration, MPO activity was assayed in colon samples. As indicated in Fig. 7d, colonic MPO activity was significantly decreased in the combination therapy group when compared with the vehicle group.

Histological examination of colon sections was performed (Fig. 7e), and scores were consistent with the macroscopic scores and colon weights. Specifically, the results revealed reduced colonic damage and inflammation in all drug treatment groups, although statistical significance was not reached.

We also measured the levels of the proinflammatory cytokines TNFα and IL-1β. As expected from the relevant literature (Stasi et al. 2004; Zhang et al. 2006), colonic levels of both cytokines were markedly increased in the vehicle/TNBS group. The treatment of rats ABC294640 alone or in combination with olsalazine significantly reduced ($P < 0.05$) the colonic cytokine levels to control levels (Fig. 8). In contrast, olsalazine alone was ineffective at reducing TNFα and IL-1β levels.

Figure 9 shows the SK immunohistochemistry results from the rat colon. Generally, the colonic SK staining pattern was similar to that found with murine TNBS-induced colitis. Increased SK staining was found in areas

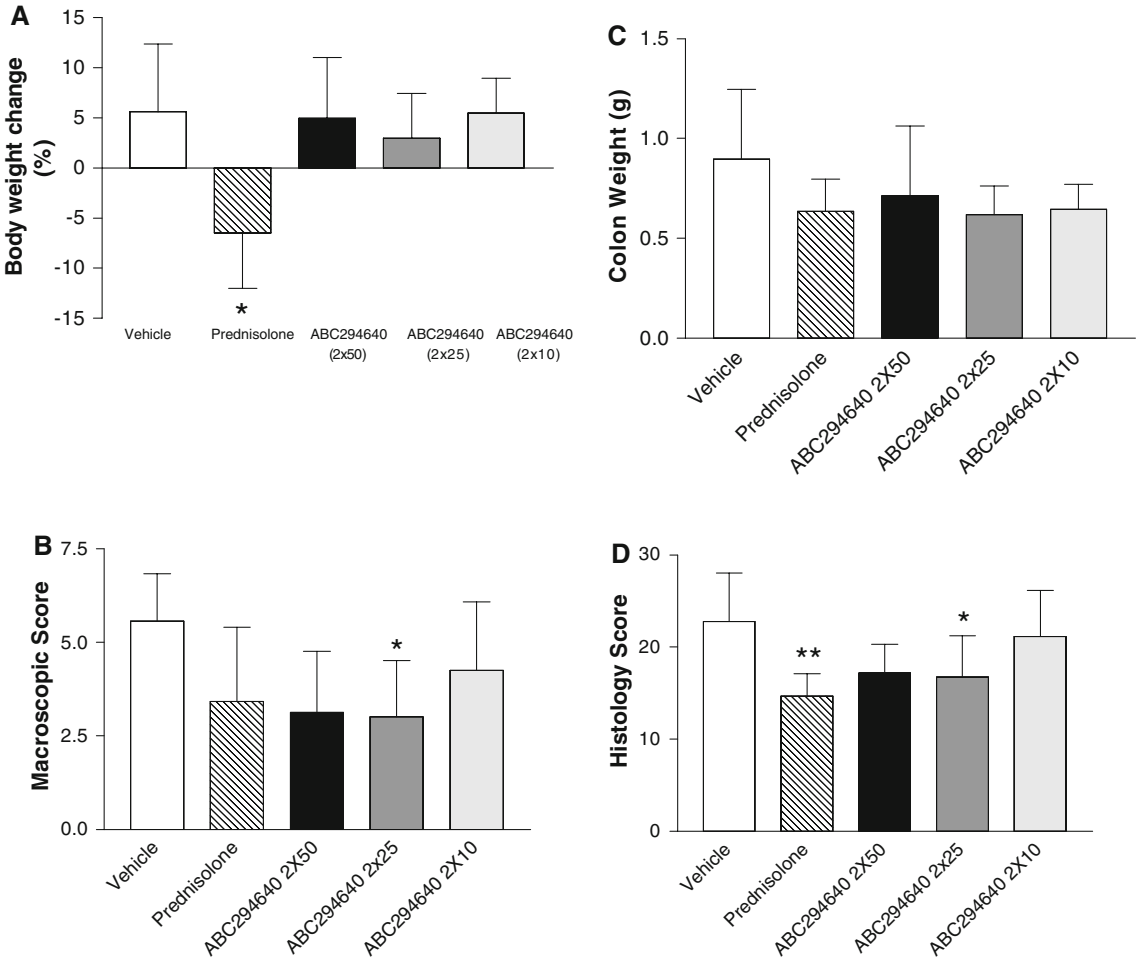

Fig. 5 Dose–response of ABC294640 effects on disease parameters in rat TNBS-colitis model. TNBS-exposed animals were treated twice a day as follows: oral vehicle (*white bars*); oral prednisolone (5 mg/kg *diagonal hatched bars*, via suspension in 0.375% Tween in PBS); oral ABC294640 (50, 25 and 10 mg/kg *black*, *dark gray* and *light gray bars*, respectively, via gelcap). At the time of killing (day 6), the following parameters were measured as described in "Materials and methods". **a** Body weight change, **b** macroscopic score, **c** colon weight, **d** histology score. Values represent the mean ± SD for 7–9 rats per group. $*P < 0.05$ or $**P < 0.01$ versus vehicle/TNBS treatment

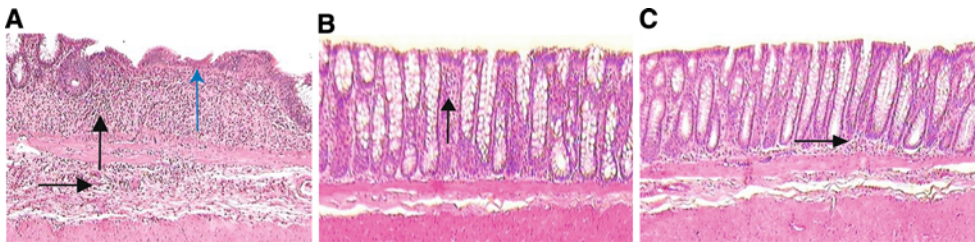

Fig. 6 Effects of ABC294640 on colon histology in the rat TNBS-colitis model. Sections of colons from the animals described in Fig. 5 were stained with H&E and examined for pathological changes. **a** Oral vehicle-treated animal showing an area of ulceration with the loss of crypts (*blue arrow*), severe leukocyte infiltration is apparent in the lamina propria and submucosa (*black arrows*). **b** Oral prednisolone group showing dramatic reduction in disease parameters when compared with **a**. Modest cellular infiltration is apparent in the lamina propria (*black arrow*). **c** A representative oral ABC294640-treated rat (25 mg/kg) with histological pathology similar to that of the prednisolone group in **b**, both when compared with the vehicle-treated group represented in **a**. Leukocyte infiltration (*arrow*) was apparent at the bottom of the crypts near the muscularis mucosa (magnification 100× for all panels)

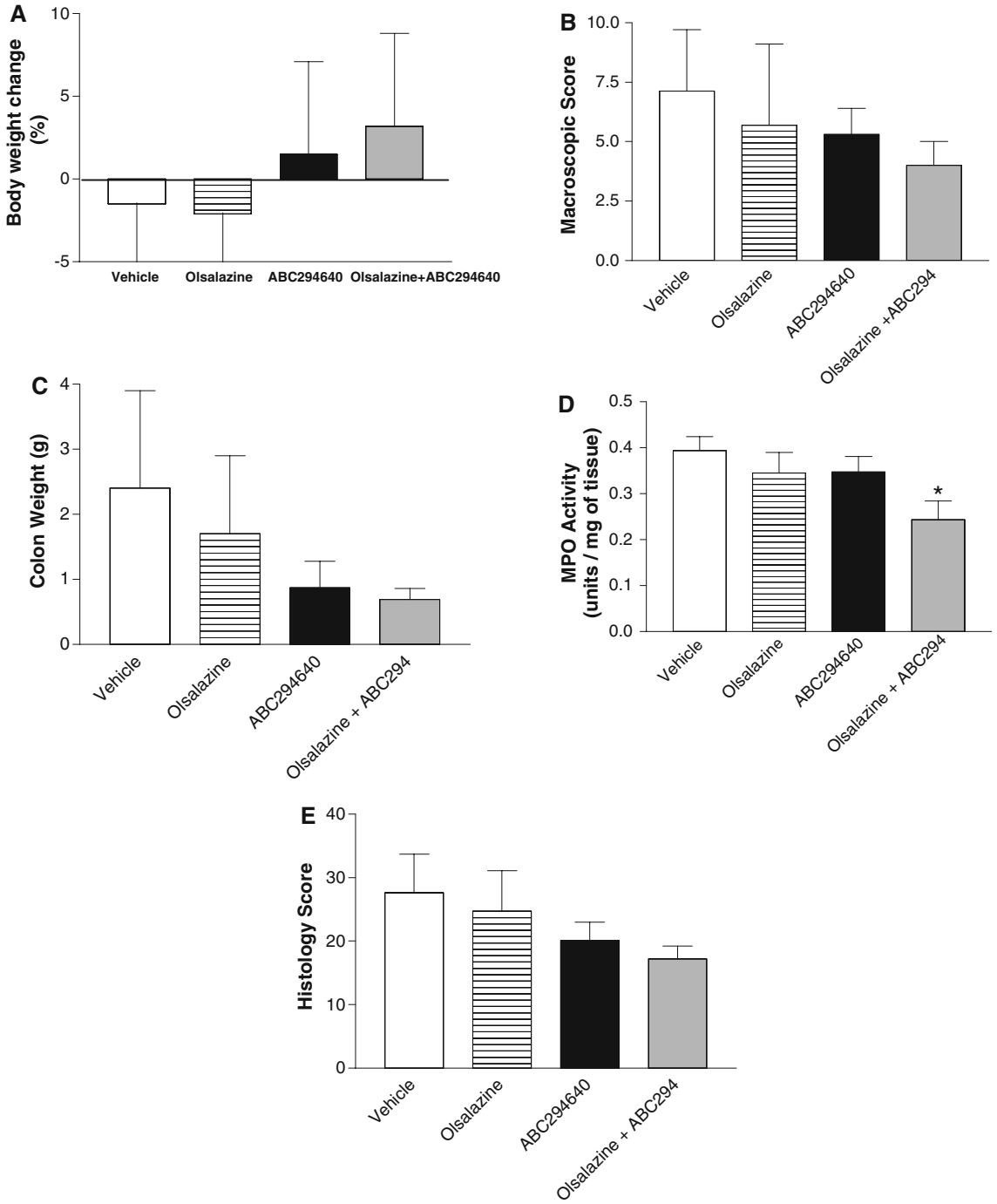

Fig. 7 Effects of ABC294640 in combination with olsalazine on disease parameters in the rat TNBS-colitis model. Animals were treated twice a day as follows: oral sham gavage (*white bars*); oral olsalazine (50 mg/kg *horizontal hatched bars*, via gel cap); ABC294640 (25 mg/kg *black bars*, via gelcap); olsalazine and ABC294640 (50 and 25 mg/kg, respectively, *gray bars*, via gel cap). At the time of killing (day 6), the following parameters were measured as described in "Materials and methods". **a** Body weight change, **b** macroscopic score, **c** colon weight, **d** myeloperoxidase activity, **e** histology score. Values represent the mean ± SD for 5–8 rats per group

of TNBS-induced mucosal damage (black arrows in Fig. 9b). Moreover, SK staining was apparent in both colonic epithelial cells and infiltrating leukocytes, following the intracolonic administration of TNBS to rats (Fig. 9c).

Human IBD

Although the rodent models have proven to be very useful in drug evaluation, it is critical to confirm that molecular alterations in these models mimic changes in human IBD.

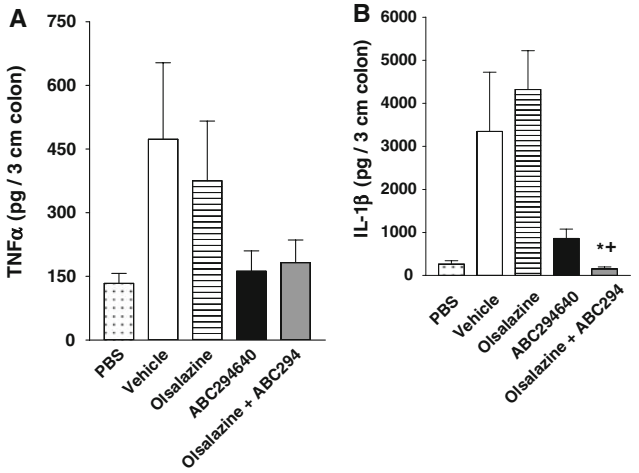

Fig. 8 Effects of ABC294640 in combination with olsalazine on colonic cytokines in the rat TNBS-colitis model. Colons from animals described if Fig. 7 were evaluated for inflammatory cytokines (PBS denotes animals that received rectal PBS and oral vehicle, all other groups are denoted by their oral treatment following rectal TNBS treatment. **a** Colonic TNFα content (pg per 3-cm colon). **b** Colonic IL-1β content (pg per 3-cm colon). Values represent the mean ± SEM for 4–8 rats per group. $^{*}P < 0.05$ versus vehicle, $^{+}P < 0.05$ versus olsalazine

Therefore, we conducted a pilot study of SK expression in colon specimens collected from human IBD patients. SK expression was found by immunohistochemistry in all of the colon samples. In the colon of a non-IBD control patient (colon cancer), SK expression was predominantly in the surface epithelial cells (black arrow, Fig. 10a). Interestingly, there were marked increases in SK expression within the colon of patients with active IBD. Specifically, as shown in Fig. 10b (from a CD patient), SK expression predominated in the surface epithelial cells (top black arrow), remnant crypts (bottom black arrow), and in lamina propria leukocytes (red arrow). A semi-quantitative analysis revealed a near doubling of the SK expression between IBD and non-IBD patients (Fig. 10c). The human colon samples were also evaluated for a total histology score and revealed that much higher histology scores were observed in the IBD samples than in control samples (Fig. 10d). Although perhaps expected, these results confirmed the presence of substantial inflammation and pathology in these colonic samples. As also shown in Fig. 10d, a correlation analysis was performed, and showed a significant association ($r = 0.762$, $P = 0.017$) between the colonic SK expression and histology score for this cohort of nine patients.

Discussion

Recognizing that a number of animal models for IBD have been described in the literature, all providing incomplete recapitulation of the diseases in humans, we have now utilized the TNBS model in mice to follow-up on the positive effects of the SK inhibitors in the DSS model of UC (Maines et al. 2008). As discussed by Murthy and Flanigan (1999), the TNBS model provides a rapid, reliable and reproducible IBD model. Covalent binding of TNBS to luminal cell surface proteins induces a CD^{4+} T cell response and cytokine production. Specifically, application of the hapten TNBS to the colon in the presence of ethanol results in transmural infiltrative disease that is limited to the colon and typically appears to be an IL-12-driven, Th1-mediated immunologic response. The role of TNFα in the development of the disease has been well documented, because the inflammatory response does not occur in TNFα-deficient animals and is markedly potentiated in mice that over-express this cytokine (Neurath et al. 1997).

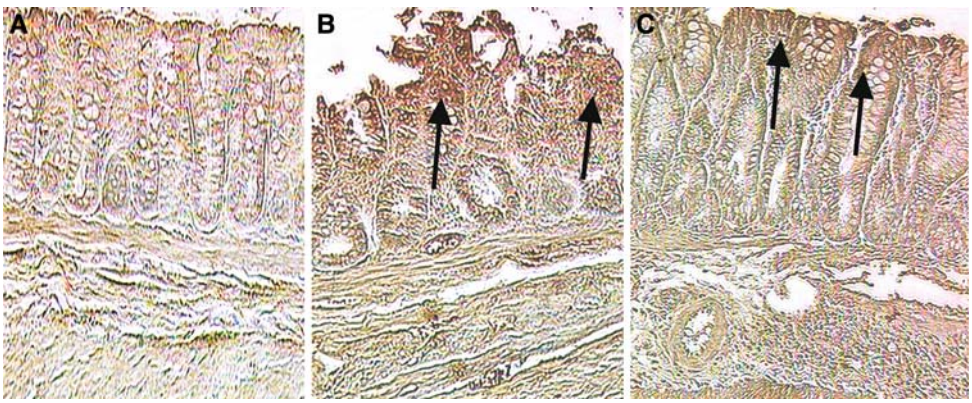

Fig. 9 SK expression in the rat TNBS-colitis model. **a** A representative example of SK expression in the colon of a control, i.e. non-TNBS-exposed, rat. The *black arrow* shows staining of a surface epithelial cell. **b** A representative section of a TNBS-exposed animal and reveals a general increase in SK staining with the most pronounced increase in the areas of mucosal damage (*black arrows*). **c** A representative section from another TNBS-exposed animal. SK staining is present in crypt colonic epithelial cells (*black arrows*), as well as in infiltrating leukocytes within the submucosa (*red arrow*)

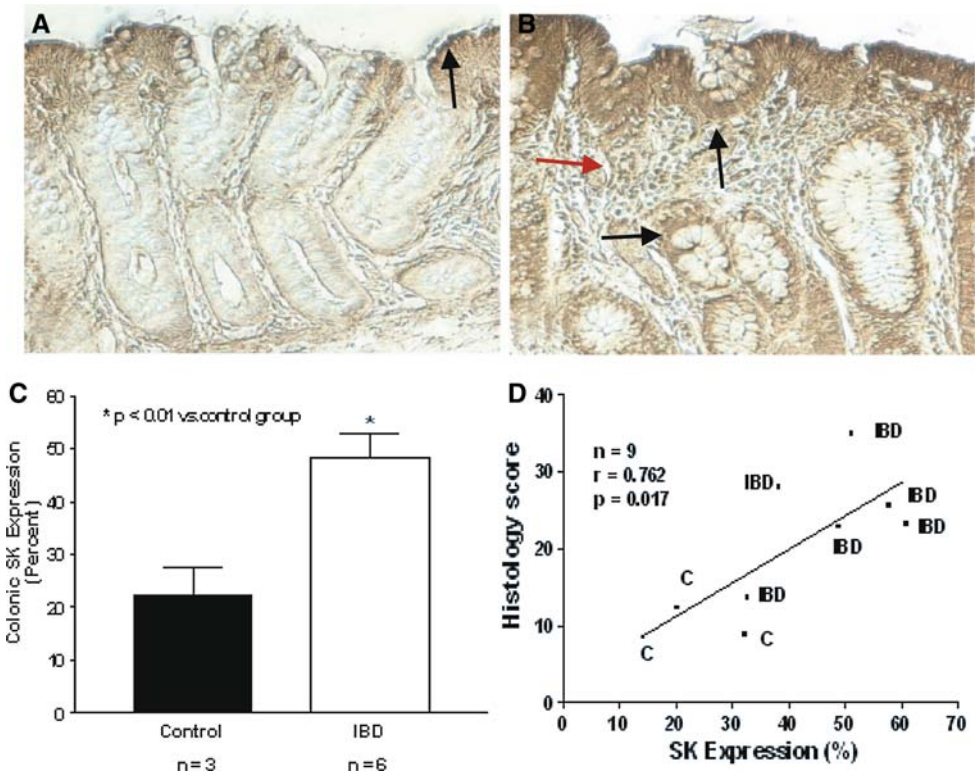

Fig. 10 Immunohistochemical staining of SK in the colons of human patients. **a** A representative result of SK expression (*arrow*) in a control patient sample, i.e. non-IBD. **b** A marked increase in SK staining in the epithelial cells (*black arrows*) as well as in the lamina propria (*red arrow*) from an IBD patient tissue sample. **c** A quantitative analysis of the percent SK expression results from all human samples tested, and **d** a correlation between SK expression levels and disease intensity in human control (**c**) and IBD tissue samples

In addition, antibodies against TNFα and pentoxifylline strongly reduced colonic and systemic inflammation in the TNBS model (Armstrong et al. 2001). The end result of the inflammatory cascade is the production of colonic ulceration and transmural inflammation (Murthy and Flanigan 1999). Indeed, in this study, we confirmed that TNFα (as well as IL-1β) was increased in the rat colon following TNBS exposure.

Previously, we showed that both colonic SK expression and SIP levels were increased following the administration of DSS to C57 BL/6 mice (Maines et al. 2008). Similarly, the current data suggest increased SK expression in the colons of mice and rats with TNBS-induced colitis, as well as in the colons of IBD patients. This target validation in the human is important evidence that the SK inhibitors may be effective in the clinical setting. As a whole, our results indicate that the TNBS model replicates the increased SK expression with intestinal inflammation, as observed in human IBD. Consequently, this data validate the use of the TNBS-colitis model for studying the effects of SK inhibitors in IBD.

Corticosteroids, such as dexamethasone can inhibit the inflammatory process in the TNBS model, suggesting this colitis is sensitive to the pharmacological action of these drugs (Nakase et al. 2001). With the goal of advancing a lead SK inhibitor into the clinic, we chose prednisolone as a comparator drug in our IBD studies. Conventional corticosteroids have demonstrated efficacy in active CD, but are used as sparingly due to deleterious side effects including immune suppression and osteoporosis (Sandborn et al. 2007). In pediatric IBD, steroids have even been shown to reduce bone density and formation (Canalis et al. 2002). In our studies, prednisolone-treated rats exhibited significant weight loss. Current medication strategies seek to eliminate a reliance on prednisone as corticosteroids are not an ideal first-line therapy for CD (Sandborn et al. 2007).

The search for a drug with similar efficacy to steroids without the side effects is thus of the utmost importance for the clinical management of IBD. As one option, 5-ASA drugs are considered marginally effective in the management of CD (Sandborn et al. 2007). Our combined data suggest that the novel SK-2 inhibitor ABC294640 performs comparably to prednisolone over multiple parameters, without the undesired weight loss. In addition, the combination of ABC294640 with olsalazine was tested as a possible scenario in which the SK inhibitor would be used as an add-on therapy to 5-ASA. The results from these studies are encouraging, with an apparent further improvement with the combination as was anticipated due to the different mechanisms of action of the two compounds. Interestingly, combination treatment with olsalazine and ABC294640 resulted in near normalization of the colonic TNFα and IL-1β contents, while olsalazine was devoid of anti-cytokine activity. In this regard, activation of SK is essential for the signaling responses to

TNFα because its ability to induce adhesion molecule expression via activation of NFκB is mimicked by S1P, and is blocked by the SK inhibitor dimethylsphingosine (Xia et al. 1998). We have previously shown that ABC294640 blocks TNFα-induced VCAM expression (Maines et al. 2008). The clear role of SK in the mechanism of action of TNFα (Xia et al. 1999; Niwa et al. 2000; Osawa et al. 2001) suggests that SK inhibitors could have additive or synergistic effects with other therapeutic modalities, such as remicade.

The present work extends our previous results in the DSS colitis models (Maines et al. 2008) and demonstrates that a novel inhibitor of SK (ABC294640) provides protection against IBD in the TNBS-colitis model in an overall similar manner to prednisolone. Moreover, the side-effect profile of ABC294640 was superior to that of prednisolone. ABC294640 may find utility as a single therapeutic agent, or as part of a targeted combination therapy approach for the treatment of intestinal inflammation. Taken together, these animal and human data lend further evidence to warrant investigation of a first in class small molecule SK-2 inhibitor in a clinical setting for the treatment of IBDs.

Acknowledgments We thank Dr. Walter Koltun (Department of Surgery) of the Penn State College of Medicine for providing the human tissue samples.

References

Armstrong AM, Foulkes R et al (2001) Tumour necrosis factor inhibitors reduce the acute-phase response in hapten-induced colitis. Br J Surg 88(2):235–240

Baumruker T, Bornancin F et al (2005) The role of sphingosine and ceramide kinases in inflammatory responses. Immunol Lett 96(2):175–185

Canalis E, Pereira RC et al (2002) Effects of glucocorticoids on the skeleton. J Pediatr Endocrinol Metab 15(5):1341–1345

Chalfant CE, Spiegel S (2005) Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling. J Cell Sci 118(Pt 20):4605–4612

El Alwani M, Wu BX et al (2006) Bioactive sphingolipids in the modulation of the inflammatory response. Pharmacol Ther 112(1):171–183

Fitzpatrick LR, Wang J et al (2000) In vitro and in vivo effects of gliotoxin, a fungal metabolite: efficacy against dextran sodium sulfate-induced colitis in rats. Dig Dis Sci 45(12):2327–2336

Fitzpatrick LR, Small JS et al (2007) Enhanced intestinal expression of the proteasome subunit low molecular mass polypeptide 2 in patients with inflammatory bowel disease. Dis Colon Rectum 50(3):337–348

French K, Zhuang Y et al. (2010) Pharmacology and Antitumor Activity of ABC294640, a selective inhibitor of sphingosine kinase-2. JPET. doi:10.1124/JPET.109.163444 (in press)

French KJ, Schrecengost RS et al (2003a) Discovery and evaluation of inhibitors of human sphingosine kinase. Cancer Res 63(18):5962–5969

French KJ, Schrecengost RS et al (2003b) Discovery and evaluation of inhibitors of human sphingosine kinase. Cancer Res 63(18):5962–5969

French KJ, Upson JJ et al (2006) Antitumor activity of sphingosine kinase inhibitors. J Pharm Exp Ther 318(2):596–603

Itagaki K, Hauser CJ (2003) Sphingosine 1-phosphate, a diffusible calcium influx factor mediating store-operated calcium entry. J Biol Chem 278(30):27540–27547

Kee TH, Vit P et al (2005) Sphingosine kinase signalling in immune cells. Clin Exp Pharmacol Physiol 32(3):153–161

Kostin S, Pool L et al (2003) Myocytes die by multiple mechanisms in failing human hearts. Circ Res 92(7):715–724

Leclercq TM, Pitson SM (2006) Cellular signalling by sphingosine kinase and sphingosine 1-phosphate. IUBMB Life 58(8):467–472

MacKinnon AC, Buckley A et al (2002) Sphingosine kinase: a point of convergence in the action of diverse neutrophil priming agents. J Immunol 169(11):6394–6400

Maines LW, French KJ et al (2006) Pharmacologic manipulation of sphingosine kinase in retinal endothelial cells: implications for angiogenic ocular diseases. Invest Ophthalmol Vis Sci 47(11):5022–5031

Maines LW, Fitzpatrick LR et al (2008) Suppression of ulcerative colitis in mice by orally available inhibitors of sphingosine kinase. Dig Dis Sci 53(4):997–1012

Mathias S, Dressler KA et al (1991) Characterization of a ceramide-activated protein kinase: stimulation by tumor necrosis factor alpha. Proc Natl Acad Sci USA 88(22):10009–10013

McCafferty DM, Miampamba M et al (1999) Role of inducible nitric oxide synthase in trinitrobenzene sulphonic acid induced colitis in mice. Gut 45(6):864–873

Morris GP, Beck PL et al (1989) Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 96(3):795–803

Murthy S, Flanigan A (1999) Animal models of inflammatory bowel disease. Birkhauser Verlag, Basel

Nakase H, Okazaki K et al (2001) An oral drug delivery system targeting immune-regulating cells ameliorates mucosal injury in trinitrobenzene sulfonic acid-induced colitis. J Pharmacol Exp Ther 297(3):1122–1128

Neurath MF, Fuss I et al (1997) Predominant pathogenic role of tumor necrosis factor in experimental colitis in mice. Eur J Immunol 27(7):1743–1750

Niwa M, Kozawa O et al (2000) Tumor necrosis factor-alpha-mediated signal transduction in human neutrophils: involvement of sphingomyelin metabolites in the priming effect of TNF-alpha on the fMLP-stimulated superoxide production. Life Sci 66(3):245–256

Osawa Y, Banno Y et al (2001) TNF-alpha-induced sphingosine 1-phosphate inhibits apoptosis through a phosphatidylinositol 3-kinase/Akt pathway in human hepatocytes. J Immunol 167(1):173–180

Prieschl EE, Csonga R et al (1999) The balance between sphingosine and sphingosine-1-phosphate is decisive for mast cell activation after Fc epsilon receptor I triggering. J Exp Med 190(1):1–8

Rabano M, Pena A et al (2003) Sphingosine-1-phosphate stimulates cortisol secretion. FEBS Lett 535(1–3):101–105

Sandborn WJ, Feagan BG et al (2007) Medical management of mild to moderate Crohn's disease: evidence-based treatment algorithms for induction and maintenance of remission. Aliment Pharmacol Ther 26(7):987–1003

Smith CD, French KJ et al. (2008) Sphingosine kinase inhibitors. US, Apogee Biotechnology Corporation, US 7,338,961 B2

Stasi MA, Ruggiero V et al (2004) Ameliorating effects of the immunomodulator 3-(2-ethylphenyl)-5-(3-methoxyphenyl)-1H–1, 2, 4-triazole in an experimental model of colitis in the rat. Eur J Pharmacol 494(2–3):263–272

Taha TA, Hannun YA et al (2006) Sphingosine kinase: biochemical and cellular regulation and role in disease. J Biochem Mol Biol 39(2):113–131

Videla S, Vilaseca J et al (2006) Selective inhibition of phosphodiesterase-4 ameliorates chronic colitis and prevents intestinal fibrosis. J Pharmacol Exp Ther 316(2):940–945

Williams KL, Fuller CR et al (2001) Enhanced survival and mucosal repair after dextran sodium sulfate-induced colitis in transgenic mice that overexpress growth hormone. Gastroenterology 120(4):925–937

Xia P, Gamble JR et al (1998) Tumor necrosis factor-alpha induces adhesion molecule expression through the sphingosine kinase pathway. Proc Natl Acad Sci USA 95(24):14196–14201

Xia P, Wang L et al (1999) Activation of sphingosine kinase by tumor necrosis factor-alpha inhibits apoptosis in human endothelial cells. J Biol Chem 274(48):34499–34505

Yamanaka M, Shegogue D et al (2004) Sphingosine kinase 1 (SPHK1) is induced by transforming growth factor-beta and mediates TIMP-1 up-regulation. J Biol Chem 279(52):53994–54001

Yatomi Y, Ruan F et al (1995) Sphingosine-1-phosphate: a platelet-activating sphingolipid released from agonist-stimulated human platelets. Blood 86(1):193–202

Zhang M, Deng C et al (2006) Curcumin inhibits trinitrobenzene sulphonic acid-induced colitis in rats by activation of peroxisome proliferator-activated receptor gamma. Int Immunopharmacol 6(8):1233–1242