Comparison of Prostaglandin E₂ Receptor Subtype 4 Agonist and Sulfasalazine in Mouse Colitis Prevention and Treatment

Guang-Liang Jiang, Wha Bin Im, Yariv Donde, and Larry A. Wheeler

Research and Development, Allergan Pharmaceuticals, Inc., Irvine, California

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ABSTRACT

Prodrugs of 5-aminosalicylic acid (5-ASA), such as sulfasalazine, have been the mainstay for the treatment and maintenance of inflammatory bowel disease (IBD) for decades, which is attributable to their antiadapative immune activity. However, 5-ASA compromises regeneration of intestinal epithelia and induces apoptosis. The majority of patients eventually undergo colectomy. Agonists for the prostaglandin E₂ subtype 4 (EP4) receptor have been shown to protect epithelial barrier against colitis-inducing agents and could be valuable alternatives for sulfasalazine. Here, we compared sulfasalazine and a novel EP4 agonist for their abilities to prevent colitis induction and relieve symptoms of established colitis in a dextran sulfate sodium–indomethacin mouse model. The EP4 agonist dose-dependently alleviated weight loss in colitis mice. Compared with sulfasalazine at 100 mg/kg on the colitis induction model, the EP4 agonist at 0.2 mg/kg was superior in reducing colitis symptoms, preventing increase of innate immune cells, and ameliorating inflammation in colon. In mice with established colitis, sulfasalazine quickly reversed weight loss but with fading efficacy. The EP4 agonist, in contrast, had slow but sustained effects on body weight gain and was more efficacious in epithelial regeneration. Such temporal differences between sulfasalazine and the EP4 agonist actions seemingly led to no additive effect in combination therapy. In conclusion, the EP4 agonist would be more efficacious in the maintenance of remission because of both anti-innate immune responses and epithelial regeneration activity, whereas sulfasalazine would be more suitable for induction of remission because of its rapid onset of antiadapative inflammation action.

Introduction

In inflammatory bowel disease (IBD) a compromised colonic mucosal barrier may greatly contribute to the entry of colonic antigens to the submucosal layer, where they initiate innate immune responses, trigger cytotoxic cytokine production, and further amplify inflammation by eliciting adaptive immune responses in the end. Typical phenotypic symptoms for IBD include body weight loss, diarrhea, fecal blood, and pain. Such debilitating symptoms could last for decades, requiring chronic drug treatments for the induction and maintenance of symptomatic remission. Current treatments are not highly successful because up to 60% of patients with IBD eventually have to undergo colectomy (Bernstein and Nabalamba, 2006; Kucharzik et al., 2006; Bewtra et al., 2007; Jiang et al., 2007).

For more than 50 years, the mainstay for treating IBD has been prodrugs of 5-ASA, such as sulfasalazine, despite various adverse effects and refractory cases (Bernstein and Nabalamba, 2006; Kucharzik et al., 2006; Bewtra et al., 2007). Sulfasalazine consists of 5-ASA covalently linked to sulfapyridine by an azo bond that is rapidly cleaved by bacteria in the terminal ileum and colon, thus releasing the active anti-inflammatory component, 5-ASA, which works topically in the colon (McQuaid, 2004). Anti-inflammatory actions of 5-ASA have been attributed to its abilities to induce T lymphocyte apoptosis, modulate inflammatory mediators from both cyclooxygenase/lipoxygenase pathways and nuclear factor-κB signals, an important transcription factor for proinflammatory cytokines, and activate peroxisome proliferator-activated receptor-γ (Wahl et al., 1998; Cavallini et al., 2001; Liptay et al., 2002; Doering et al., 2004; McQuaid, 2004; Rousseaux et al., 2005). Also noteworthy are the observations that 5-ASA inhibits regeneration and induces apoptosis of intestinal epithelia in colitis mucosa (Reinacher-Schick et al., 2000; Brown et al., 2010).

Emerging from IBD research is an alternative target, EP4, a subtype of the PGE₂ receptor family. EP4 receptors are constitutively expressed in the colonic epithelium and upregulated during IBD (Takafuji et al., 2000; Nitta et al., 2002). EP4 knockout mice are most susceptible to IBD induction (Kahashima et al., 2002). Moreover, agonists for EP4...
have been shown to inhibit the production of chemokines and cytotoxic cytokines from immune cells (Yamane et al., 2000; Nitta et al., 2002; Takayama et al., 2002; Ratcliffe et al., 2007), suppress inflammation at lesions of the gastrointestinal tract (Kabashima et al., 2002; Jiang et al., 2009), and promote epithelial cell survival and growth by activating antiapoptotic and proliferative cellular signaling pathways (Fujino et al., 2003; Hoshino et al., 2003; Joseph et al., 2005; Jiang et al., 2007). In live animals, EP4 agonists from several distinct chemical templates have been shown to prevent and improve IBD symptoms (Kabashima et al., 2002; Nitta et al., 2002; Jiang et al., 2007). In addition, a clinical trial has shown promising therapeutic effects of an EP4 agonist on 5-ASA refractory cases (Nakase et al., 2010).

However, direct comparison of EP4 agonists with prodrugs of 5-ASA, such as sulfasalazine, in IBD has not been studied. Such comparison would disclose the similarities and/or differences of these two agents in functionality and mechanisms and may facilitate strategic planning of colitis management.

In this study we compared sulfasalazine and a novel EP4-selective agonist (Fig. 1) bound to a cell membrane. The EC50 of 0.25 nM showed no detectable fluorometric imaging plate reader signal (hEP4) with an EC50 of 0.25 ± 0.03 and 11.1 ± 0.05 nM, respectively. On the other hand, the compound at 10 μM showed no detectable fluorometric imaging plate reader signals in human embryonic kidney 293 cells heterologously expressing hEP1, human progestadin F3 receptor, human progestadin I2 receptor, and human thromboxane A2 receptor and also in hEP2, hEP3, hGq, and human progestadin D2 receptor (Gq) where a chimeric G protein (in parentheses) was coexpressed to induce Ca2+ signal from Gs- and Gi-coupled receptors. Hence this compound was designated as an EP4-selective agonist. This agonist has more than 10 times higher affinity with EP4 receptor than the EP4 agonist we reported previously (Jiang et al., 2007). Most importantly, not like other EP4 agonists, it would have a minimal systemic exposure when targeted to colon delivery, because of its extreme susceptibility to hepatic metabolism (data not shown).

**Induction of Colitis and Treatments.** Mice were caged individually and provided with drinking water containing 5% DSS (average molecular weight of 8000) with indomethacin (4 mg/kg/day) to induce colitis (Kabashima et al., 2002; Jiang et al., 2007; Okayama et al., 2007). Indomethacin was first dissolved in dimethyl sulfoxide, then added to 5% DSS drinking water with a final dimethyl sulfoxide concentration of 0.1%. A few drops of Tween 80 were added to keep indomethacin in solution. The intake of indomethacin was calculated from daily water consumption. Drugs or vehicle was administered by intracolonic delivery. In brief, animals were kept under anesthesia with isofluorane. A sterile 3.5 French polyvinyl chloride tube (Tyco Healthcare, Mansfield, MA) was first lubricated with glycerol and then intrarectally advanced 8 cm into the cecum region by an experienced operator. This tube has a round tip, and its flexibility and rigidity allow a smooth insertion without damaging the colon. Drugs or vehicle (4% dimethyl sulfoxide in 0.9% normal saline) were given at 100 mg/kg/day and 5% DSS drinking water with a final dimethyl sulfoxide concentration of 0.1%.

**Materials and Methods.**

**Materials.** Female C57BL/6 mice at 5 months of age (22–25 g/mouse) were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Animal housing and handling procedures were performed according to the guidelines of Allergan’s animal care and use committee. DSS, IN, sulfasalazine, 5-ASA (cell culture grade), and 5-bromo-2-deoxyuridine (BrdU) were purchased from Sigma-Aldrich (St. Louis, MO). AlphaScreen cAMP assay kits were from PerkinElmer Life and Analytical Sciences (Waltham, MA). A guaiac paper test for hemoccult was purchased from Colostrogen Systems, Diagnostics Div., Tarrytown, NY) by persons masked to treatment. Indomethacin was chosen for the present study because of its reliable efficacy in decades of practice and it is least affected by local lumen environment compared with other 5-ASA prodrugs (McQuaid, 2004). Sulfasalazine was protected from light in all studies.

**Two sets of experiments were carried out.** In the first set, the EP4-selective agonist (0.01, 0.1, or 0.2 mg/kg/day) or sulfasalazine (100 mg/kg/day) was dosed 3 h before the initiation of DSS-IND drinking water and thereafter daily for 3 more days. Each group consisted of 11 mice. On day 5, the animals were sacrificed by decapitation under deep anesthesia with isofluorane. The colon and ileum were collected and fixed in 10% formalin for histopathological analysis. In the second set of study, mice were treated with the DSS-IND for the first 7 days, resulting in severe colitis in all mice. Then the mice were provided with normal drinking water, randomized into different groups and treated by vehicle, sulfasalazine (100 mg/kg/day), EP4-selective agonist (0.2 mg/kg/day), or a combination of sulfasalazine (100 mg/kg/day) and EP4 agonist (0.2 mg/kg/day) for the last 4 days. The animals were sacrificed on day 11.

**Assessment.** Food consumption, body weight, stool consistency, and occult blood in the stool were monitored daily. Diarrhea was scored as follows: 0, normal; 2, loose stool; 3, soft mud-like stool; 4, watery diarrhea. Hemoccult was assessed from the guaiac paper test and scored as follows: 0, normal; 2, trace positive; 3, strong positive; 4, gross bleeding (Kabashima et al., 2002; Jiang et al., 2007). Animals, including six age-matched healthy mice, were anesthetized by the end of the study. Blood was collected with BD Microtainer tubes (BD Biosciences, Franklin Lakes, NJ). Hematological profiles were analyzed with an automatic ADVIA120 Hematology System (Bayer Corp., Diagnostics Div., Tarrytown, NY) by persons masked to treatments. Then the mice were sacrificed, and colons were examined and fixed in 10% neutral formalin for histological analysis.

**For histology and immunological staining, colons were divided into proximal, middle, and distal portions and embedded with paraffin. Sections of 5 μm in thickness were prepared. To monitor cell proliferation, BrdU was injected intraperitoneally at 1.0 mg/0.1 ml of
normal saline 16 h before sacrifice. Paraffin-embedded sections were deparaffinized in xylene and rehydrated in ethanol. Antigen was retrieved with citrate buffer, pH 6.0, boiled for 5 min in a microwave, and slowly cooled down at room temperature. Immunofluorescence staining of BrdU was then performed following the manufacturer's instructions (Roche Applied Science, Penzberg, Germany). In brief, the sections were immerged with 65 l of anti-BrdU working solution (1:10 dilution) and incubated 24 h at 4°C in a humid atmosphere. Anti-mouse-Ig-fluorescein was applied for 30 min at room temperature afterward. The slides were costained with DAPI for 5 min and evaluated with a fluorescence microscope (Nikon, Tokyo, Japan).

Statistical Analysis. Data are presented as the means with S.E.M. and were analyzed by using two-tailed t test for any two-party comparison. A probability (P value) of less than 0.05 was considered significant.

Results

The EP4 Agonist Dose-Dependently Prevented Colitis Development. The EP4-selective agonist at 0.01, 0.1, and 0.2 mg/kg/day dose-dependently decreased the loss of body weight since day 2 and diarrhea and hemoccult scores since day 1 (data not shown), with the maximal effects at the dose of 0.2 mg/kg/day (Fig. 2).

Comparison of EP4 Agonist with Sulfasalazine on Colitis Prevention. In a head-to-head test, the EP4-selective agonist at 0.2 mg/kg/day seemed to be superior to sulfasalazine (100 mg/kg/day) in ameliorating colitis symptoms (Fig. 3). The EP4 agonist and sulfasalazine reduced body weight loss by 90 and 75%, respectively, on day 2; 67 and 58%, respectively, on day 3; and 45 and 32%, respectively, on day 4 (P < 0.005 or P < 0.05; Fig. 3A). Moreover, the EP4 agonist decreased diarrhea and hemoccult scores more effectively than sulfasalazine on days 2, 3, and 4 (Fig. 3, C and D) and at the same time induced more food intake than sulfasalazine (Fig. 3B). Overall, the EP4 agonist treatment improved the general well-being of mice compared with those treated with vehicle or sulfasalazine.

Automated hematology analysis showed significant increases of inflammatory cells in the blood of colitis animals along with severe anemia (Fig. 4, A and B). However, sulfasalazine and the EP4 agonist prevented the increase of overall white blood cells by approximately 27% 4 days after colitis induction (Fig. 4A). Among the subsets of white blood cells, neutrophils and monocytes, which reflect the innate immune responses, however, were far less abundant in mice treated with the EP4 agonist than with sulfasalazine. Particularly, the number of monocytes in EP4 agonist-treated animals was not significantly different from that of normal mice (P > 0.05) (Fig. 4A). This may imply that colitis-inducing agents caused less mucosal damage in the presence of the EP4-selective agonist than sulfasalazine, because the infiltration of neutrophils and monocytes correlates with the extent of mucosal damage and disease severity (Gouni-Berthold et al., 1999; Konikoff and Denson, 2006). Also the EP4-selective agonist, not sulfasalazine, significantly prevented DSS-IND-

![Fig. 2. The dose-response effect of the EP4-selective agonist on accumulated body weight loss of mice subjected to colitis induction. **, P < 0.01 compared with vehicle group at each time point. n = 11/group.](image)

![Fig. 3. Effects of the EP4 agonist (0.2 mg/kg/day) and sulfasalazine (100 mg/kg/day) on preventing colitis development. The drugs or vehicle were administered 3 h before the supplement of colitis-inducing agents and daily for 3 more days to mice on colitis-inducing agents (n = 11/group). A, body weight loss was monitored daily and normalized against the initial weight. *, P = 0.02; **, P < 0.001–0.003 compared with vehicle at corresponding time point. B, average daily food consumption per mouse. C, diarrhea scores. *, P < 0.05; **, P < 0.01 compared with vehicle at corresponding time point. D, hemoccult scores. *, P < 0.05 versus EP4-selective agonist; **, P < 0.01 compared with vehicle at corresponding time point.](image)
induced decrease in red blood cells, hemoglobin, and hematocrit \( (P < 0.01; \text{Fig. 4B}) \), further supporting minimal mucosal damage in the EP4 agonist-treated group. On the other hand, sulfasalazine significantly prevented only the increase of lymphocytes, which are involved in the antigen-mediated adaptive immune response, consistent with its known anti-inflammatory role (Liptay et al., 2002; Doering et al., 2004).

The macroscopic observation on day 5 showed that chocolate-colored watery contents filled the colons from vehicle-treated animals. Colons from sulfasalazine treatment were filled with dark brown-colored loose stool. EP4-treated animals had stools of almost normal color and consistency in their colons. EP4 agonist prevented colon shortening compared with vehicle or sulfasalazine treatment and showed no difference from normal mice (Fig. 4C). On H&E-stained colon sections on day 5 vehicle-treated colon showed disruption of epithelial lining, infiltration of lamina propria and submucosa by a large quantity of inflammatory cells, and edema of submucosa; compared with the vehicle group, sulfasalazine treatment markedly reduced inflammatory cell infiltration at the lamina propria and submucosa with subtle lesions to the epithelial layer; the EP4-agonist treatment preserved the integrity of the epithelial layer, abolished edema, and showed sparse infiltration of inflammatory cells at submucosa (Fig. 4D).

**The EP4 Agonist and Sulfasalazine in the Treatment of Established Colitis.** We also compared the EP4-selective agonist and sulfasalazine for their ability to improve symptoms in mice with established colitis from the treatment of DSS-IND for 7 days. With established colitis, animals lost body weight by more than 15\% on day 7 and displayed severe diarrhea (Fig. 5, A and B) and gross colonic bleeding (data not shown). Sulfasalazine rapidly reversed weight loss with a gain of 4.7\% on the first day of treatment (day 8), whereas the vehicle group showed a loss of 2.3\%. On days 9, 10, and 11, the sulfasalazine group produced a weight gain of 3.1, 2.1, and 1.5\%, respectively, but not significantly different from those of the vehicle group. Apparently sulfasalazine was most effective on the first day of treatment, probably reflecting its early control of adaptive inflammation. EP4 agonist, in contrast, markedly increased animal weight on the third and fourth day of treatment (6.3 and 6.3\%, respectively), but not so much on the first and second day of treatment, indicating that the main contribution may result from its time-consuming regeneration of epithelial layer, as shown with BrdU incorporation (Fig. 5C).

EP4 agonist and sulfasalazine in combination changed animal weight by −2.8, 2.8, 6.8, and 6.0\% daily from days 8, 9, 10 and 11, respectively (Fig. 5A). The combination therapy thus seems to be not additive, because of the considerable temporal gap between the two drug activities. With respect to diarrhea, both drugs singly or in combination lowered scores significantly, but their effects were not additive (Fig. 5B).

Further histological observations with colonic sections revealed that DSS-IND treatment alone reduced BrdU-positive cells. At the end of the study, the EP4-selective agonist restored BrdU-positive epithelia to the normal level, whereas sulfasalazine failed to do so (Fig. 5C). This indicates that the EP4 agonist and sulfasalazine may play distinctive roles in epithelial regenerative repair.

**Discussion**

Animals treated with a low dose of low molecular weight DSS present with body weight gain and symptom-free and marginal pathological changes in colonic mucosa (Morteau et al., 2000; Kabashima et al., 2002; Jiang et al., 2007). In contrast, in cyclooxygenase 1 (COX-1) or COX-2 knockout
mice, DSS alone produced severe colitis (Morteau et al., 2000). Evidence shows that a low dose of DSS with a low dose of indomethacin induces severe pathological changes in colon resembling acute flares of human colitis with all of the classic symptoms seen in humans (Kabashima et al., 2002; Singh et al., 2004; Jiang et al., 2007; Okayama et al., 2007). This is a common reproducible colitis model for preclinical evaluation of new therapeutics. On the other hand, the addition of a low dose of indomethacin does not produce any damage on normal gastrointestinal mucosa (Takeuchi et al., 1986), but it eliminates the effects of COX activation induced by DSS treatment, such as elevation of endogenous PGE₂ (Morteau et al., 2000; Okayama et al., 2007) or weakening sulfasalazine effect partially derived from COX inhibition (McQuaid, 2004). So the current DSS-IND model is fairly balanced for comparing exogenous EP4 agonist with sulfasalazine.

We have shown here that an EP4-selective agonist prevented colitis more effectively than sulfasalazine, one of the most widely used drugs for IBD. EP4 agonist-treated mice had the least body weight loss, the lowest diarrhea and hematocrit scores, the least anemia and elevation of inflammatory cells, and the most food intake compared with mice treated with sulfasalazine or vehicle in a colitis-induction model.

In addition, we observed, for the first time, another unique characteristic for the EP4-selective agonist not shared with sulfasalazine: its block of neutrophil and monocyte increases, which reflects inflammatory innate immune responses. It has been shown that EP4 activation inhibits the release of chemokines and cytokines from neutrophils and monocytes (Yamane et al., 2000; Takayama et al., 2002; Ratcliffe et al., 2007) and thus abolishes the escalation of inflammation into adaptive immune responses mainly mediated by lymphocytes in the later phase (Nitta et al., 2002; Jiang et al., 2009). In addition, it has been observed that EP4 activation decreases neutrophil aggregation in vitro (Wise, 1998) and reduced neutrophil aggregation prevents tissue destruction and ameliorates colitis symptoms (Farooq et al., 2009; Sina et al., 2009). Such inhibition of innate immune responses by the EP4 agonist in the early phase of inflammation seems to be more effective in the prevention of colitis onset than the inhibition of adaptive immune responses by agents in the later phase, such as sulfasalazine, as seen in the present study. Furthermore, T lymphocytes express EP4 receptors (Cosme et al., 2000), and EP4 agonist suppresses the proliferation of isolated lymphocytes from colon lamina propria (Kabashima et al., 2002). So EP4 agonist may have direct dual roles in the inhibition of both innate and adaptive immune responses in colitis.

In addition to its ability to inhibit innate immune responses, the EP4 agonist inhibits epithelial apoptosis (Jiang et al., 2007) and promotes epithelial proliferation (Kabashima et al., 2002; Jiang et al., 2007, 2009) via activation of the AKT/extracellular signal-regulated kinase 1/2 pathway (Fujino et al., 2003; Joseph et al., 2005). These properties distinguish EP4 agonists from 5-ASA and its prodrugs. 5-ASA inhibits epithelial Akt activation in colitis, induces apoptosis, and decreases regeneration of intestinal epithelia in normal human and colitis animals (Reinacher-Schick et al., 2000; Brown et al., 2010). It is believed that IBD is initiated by the insult at the level of epithelial cells, and local inflammation could be secondary (Cooper et al., 1993; Poritz et al., 2007). So agents suppressing secondary inflammation are necessary but not sufficient for preventing relapse during the maintenance phase.

Anti-inflammation agents, such as 5-ASA prodrugs, are
currently used at a reduced dose to maintain IBD remission, a phase without obvious inflammation, although with a high ratio of relapse (Sandborn et al., 2010). It would be more reasonable to replace it with agents of both quenching inflammation in early phases and strengthening epithelial barrier function, such as the EP4 template tested in the current study. In addition, patients refractory to 5-ASA (not 5-ASA-sensitive cases) often are associated with depressed COX-2 expression (Brown et al., 2010) and responded favorably to EP4 agonist treatment (Nakase et al., 2010).

We also observed that in the established colitis model sulfasalazine readily reversed body weight loss on the first day of treatment, whereas vehicle or EP4-treated animals continued losing weight. These results indicate that sulfasalazine is a more potent agent than the EP4 agonist against severe inflammation dominated by large amounts of adaptive immune cells and cytotoxic cytokines. This makes sulfasalazine a better drug of choice than EP4 agonist in treating acute flares. However, in this established colitis model, the EP4-selective agonist showed more sustained and sustained gains at later time points than sulfasalazine, and more importantly it restored epithelial regeneration. So EP4 agonist monotherapy would be desirable for the improvement of epithelial barrier during the late phase of remission induction. In fact, complete healing of mucosa is the only factor significantly associated with sustained clinical remission (Baert et al., 2010). It is also noteworthy that sulfasalazine and EP4 agonist's effects on weight recovery were temporally separated. Therefore, it is not surprising to observe that the combination regimen did not produce many additive effects on weight gain or diarrhea change.

Systemic exposure of an EP4 agonist may cause some limited side effects, such as hot flashes, constipation, and elevated eosinophilia, which have been reported to be mild and transient (Nakase et al., 2010). Moreover, targeted colonic delivery would further minimize potential side effects.

In conclusion, an EP4-selective agonist showed better profiles in preventing the onset of colitis than sulfasalazine and slower recovery with enhanced epithelial regeneration in treating established colitis. Therefore, it may be a therapeutics valuable for chronic remission maintenance and induction of remission. We hope this study encourages future clinical trials for EP4 agonists on colitis, particularly patients refractory to produgs of 5-ASA.

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Address correspondence to: Dr. Guang-Liang Jiang, Research and Development, Allergan Pharmaceuticals, Inc., 2525 Dupont Dr., Irvine, CA 92612. E-mail: jiang_guang-liang@allergan.com.