Therapeutic Effect of SHI-219, A Novel Water Soluble Prodrug of EG626 (Phtalazinol), on Mouse Dextran Sodium Sulfate -Induced Colitis

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Key Words: Inflammatory bowel disease (IBD), dextran sodium sulfate -induced colitis, Phtalazinol, Arachidonic Acid, COX-2, Inflammatory cytokine

ABSTRACT

Cytokines such as IL-1β, tumor necrosis factor (TNF)-α, IL-6 and IL-8 are increased, and leukotriene (LT)B4, thromboxane (TX)B2 and PGE2 participate in inflamed colonic mucosa after administration of mouse dextran sodium sulfate (DSS). EG626 (Phtalazinol), has been shown to inhibit cyclic AMP phosphodiesterase in arteries and platelets, has an effect of anti-inflammation. The effect of SHI-219, a novel water soluble prodrug of EG626, was examined in mouse DSS-induced colitis using drinking water containing 5% DSS. When SHI-219 was given everyday, the disease activity index (DAI) representing clinical symptoms improved and the histological score decreased; furthermore, IL-1β, IL-6, and TNF-α concentrations in rectal mucosa were lower compared with the Control group. Also TXB2 and LTB4 concentrations in rectal mucosa were lower, but PGE2 concentrations in rectal mucosa were not inhibited. And then Cyclooxygenase (COX)-2 expression also correlated with the degree of inflammation in the intestinal mucosa in the SHI-219 -treated group, indicating that SHI-219 did not inhibit COX-2 expression by immunohistochemical staining. These results suggest that administration of SHI-219 may be effective in ulcerative colitis.
Introduction

Inflammatory bowel disease (IBD), including Ulcerative colitis (UC) and Crohn's disease (CD) is an idiopathic, non-specific inflammatory disorder primarily involving the mucosa and submucosa of the colon. Although the pathophysiology of these disorders is not known with certainty, a growing body of experimental and clinical data suggest that this type of chronic gut inflammation might result from a maladjusted immune response to certain bacterial antigens [1)-3]. This uncontrolled activation of the immune system results in the release of various cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, IL-6, and IL-8, and other chemical mediators, such as leukotriene (LT) B⁴, thromboxane (TX) B₂, prostaglandin (PG) E₂ and cytokines, at the inflammatory site in patients with IBD [4)-6] that contributes to the regulation of the local inflammatory response. An abnormality of this network might be related to the development and persistence of mucosal inflammation in IBD patients [7]-[9].

Among the inflammatory mediators, LTB₄ is known as a potent mediator of chemotaxis and aggregation of neutrophils. In fact, LTB₄ accounts for most of the chemotactic activity detected in rectal dialysate from colitis patients [10]. TXB₂ is known as a proinflammatory mediator that can induce vasoconstriction and might result in damage to the gastrointestinal and colonic mucosa. In experimental animal models, TXA₂ promoted gastric mucosal ulceration and necrosis [11]. By contrast, PGE₂, exhibits many proinflammatory effects, contributing to vascular permeability, edema and intestinal fluid secretion, but treatment with cyclooxygenase inhibitors does not improve colonic lesions and causes relapse in patients with inflammatory bowel disease [12][13]. In previous investigations, exogenous administra- tion of PGE₂ has been shown to prevent colonic mucosal damage induced in experimental animals [14][15]. These results suggest that PGE₂ is important for cytoprotection in the colonic mucosa [16].

Clinical trials of EG626 (Phthalazinol), a platelet aggregation inhibitor, were conducted in patients with thalamic pain and arteriosclerosis in the 1980’s and its effectiveness was confirmed. It has also been reported in many studies that EG626 has been shown to inhibit cyclic AMP phosphodiesterase in arteries and platelets and eicosanoids, such as TXA₂, and thus exhibits an anti-inflammatory effect [17]-[18].

In the present study, we utilized a simple and reproducible, murine model of dextran sulfate sodium (DSS)-induced colitis, as an experimental model of colitis resembling human UC. We sought to elucidate the cytoprotective mechanisms of intraperitoneal SHI-219, a novel water soluble prodrug of EG626, in DSS-induced colitis by investigating the effects on lesion formation, as well as local expression and secretion of proinflammatory mediators, including proinflammatory cytokines and eicosanoids.

Materials and Methods

Animals

Six-week-old female BALB/c mice (SLC Co., Shizuoka, Japan) weighing about 20g were used in the study. Standard mouse chow pellets and water were provided ad libitum.

Experimental protocol

In preliminary investigations, we initially examined the clinical mediators and histological mucosal damage during the time course of DSS colitis.

Mice were randomized into four groups. The first group, designated as Normal (n=5), received tap water for 7 days. The second group, designated Control (n=5), received 5% DSS in 0.3 ml of distilled water (i.p.) daily for 7 days. The third group, designated SHI-219 (2mg) (n=5), received 5% DSS for 7 days and SHI-219 (2mg/mice) in 0.3ml of distilled water i.p. daily. The fourth group, designated SHI-219 (0.5 mg) (n=5), received SHI-219 (0.5mg/mice) in 0.3ml of distilled water i.p. daily. Preliminary investigations demonstrated that experimental colitis with DSS exhibited a high correlation between fecal blood by Hemoccult and visual observations by experienced veterinarians. Mice in this investigation study were sacrificed under anesthesia on day 7 of the DSS challenge.

Evaluation of colitis

Disease Activity Index (DAI) was determined by an investigator blinded to the protocol by scoring the extent of body weight loss, stool hemoccult positivity or gross bleeding, and stool consistency in accordance with the method described by Murthy et al. (Table 1) at the time of sacrifice[20][21].

Blood samples were collected directly from the heart immediately before sacrifice to determine hemoglobin concentration and white blood cell
SHI-219 inhibits colitis

The rectum was removed and opened longitudinally; half of the sample was utilized for histological examination and the remaining sample was used to measure mucosal cytokine and eicosanoid concentrations.

For Histological Damage Score (HDS), the rectum was fixed in 10% neutral buffered formalin; 4-mm specimens were subjected to hematoxylin and eosin (H.E.) staining. Randomly selected fields (n=15) magnified 100 times in each section were inspected and graded by a pathologist blinded to the treatment protocol (Fig. 1). The mean score was calculated for each section.

Table 1. Disease activity index

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss</th>
<th>Stool *)</th>
<th>Occult/gross bleeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>1-5%</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>5-10%</td>
<td>Loose stools</td>
<td>Hemoccult +</td>
</tr>
<tr>
<td>3</td>
<td>11-15%</td>
<td>Normal</td>
<td>Gross bleeding</td>
</tr>
<tr>
<td>4</td>
<td>&gt; 15%</td>
<td>Diarrhea</td>
<td></td>
</tr>
</tbody>
</table>

*Normal stools = well formed pellets; loose = pasty stools which do not stick to the anus; diarrhea = liquid stools that stick to the anus.

Fig. 1 Histological damage score

Grade 0 = Normal colonic mucosa
Grade 1 = Loss of one-third of the crypts
Grade 2 = Loss of two-thirds of the crypts
Grade 3 = The lamina propria is covered with a single layer of epithelium and mild inflammatory cell infiltration is present
Grade 4 = Erosions and marked inflammatory cell infiltration are present

15 randomly selected fields (magnified 100 times) in each section were inspected and graded as below by a pathologist in our hospital who was blinded to the treatment protocol. By scoring the grades in 15 fields, the mean in each section was calculated.
Myeloperoxidase (MPO) activation in colonic mucosa

MPO activity was assessed in colonic mucosa (n=5/group). After homogenization, tissue from the proximal large intestine of each mice was incubated at room temperature for 15 min with tetramethylbenzidine (TMBZ; Dojindo Laboratories, Kumamoto, Japan) and H2O2 and the absorbance was measured at 630 nm. Simultaneously, the protein content of the tissue homogenates was measured using a commercially available protein assay (Bio-Rad Laboratories, Hercules, CA, USA) and the resultant absorbance values (A) for the MPO assay were quantified relative to sample protein concentrations. At the initial measurement, human MPO (Sigma, St. Louis, MO, USA) was tested using the guaiacol method and used as a standard. Absorbance was measured and converted to MPO activity (mU/mg) according to the following formula: MPO activity (mU/mg) = [OD630/protein concentration (mg/ml)] × 6.24

Immunoassay of cytokines in colonic mucosa

IL-1β, IL-6, and TNF-α concentrations were measured in homogenized rectal tissue by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol (R&D Systems, Minneapolis, USA). Briefly, after biotinylated antibody reagent was added in 96-well plates, supernatants of homogenized rectal tissue were incubated at 37°C in 5% CO2 for 2 hours. After washing with PBS, streptavidin-peroxidase (HRP) solution was added and the plate was incubated for 30 minutes at room temperature. The absorbance was measured at 590 nm using a microplate reader.

The total protein concentration was determined by the Lowry method, and the absorbance was calculated per milligram of protein.

Measurement of PGE2, TXB2, and LTB4 levels in colonic mucosa

Tissue samples from the distal large intestine of each mouse were homogenized in ethyl alcohol and the supernatant was collected. PGE2, TXB2, and LTB4 levels in the supernatant were measured using specific ELISA Kits (Cayman Chemical, Michigan, USA) according to the manufacturer’s protocols and were expressed per milligram of tissue.

Detection of COX-2 expression in colonic mucosa

Mice were sacrificed on day 7, and rectal specimens were stained with anti-mouse COX-2 polyclonal antibody (TAKARA BIO INC., Shiga, Japan). Briefly, the specimens were fixed in 3.3% formalin in phosphate-buffered saline (PBS) at 4°C overnight and embedded in OCT compound (Tissue Tek). Embedded samples were quickly frozen in liquid nitrogen, and 4-µm serial sections were prepared. Air-dried samples were fixed with acetone for 15 minutes, and endogenous peroxidase was blocked with 0.3% H2O2 in 50% methanol for 15 min at room temperature. Nonspecific binding was blocked with 2% bovine serum albumin and the tissue was incubated with 20 µg/ml biotinylated anti-mouse COX-2 monoclonal antibody overnight at 4°C, followed by incubation with avidin-biotin peroxidase complex at room temperature for 20 min. After washing with PBS, the tissues were incubated with 0.03% 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich Co., St. Louis, USA) containing 0.003% hydrogen peroxide. All sections were counterstained with Mayer’s hematoxylin 25).

Statistical analysis

All numerical values are expressed as mean 7 SEM. Data sets were analyzed by Fischer’s protected least significant difference (PLSD) comparison tests for post hoc t test. Differences of P<0.01 and P<0.05 were considered statistically significant.

Results

Time course of the development of damage in DSS colitis

In a preliminary investigation, the changes in DSS colitis over time were investigated up to 10 days. The DAI for evaluation of clinical findings began to increase on Day 3 with a gradual increase (the main symptom was diarrhea). Similarly, HDS began to increase on Day 3 and inflammation was aggravated with administration. During the study period, severe inflammation including death was observed on Day 10. Therefore, it was considered that evaluation on Day 7 was appropriate. Pro-inflammatory cytokines were also determined simultaneously and it was confirmed that IL-1β, IL-6, and TNF-α levels increased gradually (data not shown). (Fig. 2).
SHI-219 inhibits colitis

In the Control and SHI-219-treated groups, pasty to liquid, grossly bloody stools, weight loss, and severe anemia were observed in all mice. The colons were shortened, but gross ulcerations were not evident. When SHI-219 was administered, bloody stools were not evident, stools were better-formed, and the weight loss was attenuated. Furthermore, DAI s were significantly lower in mice treated with SHI-219 (2mg/mice) than those in the Control group (P<0.01). Histologically, specimens obtained from the Control group exhibited sporadic erosions with marked inflammatory cell infiltration in the lamina propria. In contrast, treatment with SHI-219 resulted in fewer erosions, and inflammatory cell infiltration was nearly absent in the rectum. Histological damage scores of the SHI-219-treated mice were significantly lower than in the Control group (P<0.01). (Table 2)

Table 2. The effect of SHI-219 on clinical indices and histological damage score
The Normal, Control, and SHI-219 (2mg/mice, 0.5mg/mice) groups were evaluated on day 7. SHI-219 was administered intraperitoneally each day. Results are expressed as the mean±S.E.M. (n= 5). *Significantly different from Control group at *P<0.01and **P<0.05. Hb: hemoglobin; WBC: white blood cell.
MPO activity in the colonic mucosa

MPO activity in the colonic mucosa was 0.671 ± 0.089 mU/mg protein in the Control group, 0.406 ± 0.026 mU/mg protein in the Normal group, 0.352 ± 0.048 mU/mg protein in SHI-219 (2mg) treated group (P<0.05 to Control) and 0.301 ± 0.017 mU/mg protein in SHI-219 (0.5 mg) treated group (P<0.01 to Control). MPO activity was significantly higher in the Control group compared with the Normal and SHI-219 treated groups. (Fig. 3)

The effect of SHI-219 on local cytokine concentrations

As shown in Table 3, the mucosal concentrations of IL-1β, IL-6, and TNF-α were significantly higher in the DSS group compared with the normal group. Treatment with SHI-219 significantly reduced mucosal cytokine concentrations (P<0.01). (Table 3)

Arachidonic acid metabolites in the colonic mucosa

As shown in Figure 4a, the mucosal concentrations of TXB2 and LTB4 were significantly higher in the DSS group compared with the Normal group. Treatment with SHI-219 significantly reduced these concentrations (P<0.01). On the other hand, mucosal concentrations of PGE2 correlated with the degree of inflammation in DSS colitis and SHI-219-treated groups, as shown in Figure 4b.

![Fig. 3 MPO activity](image)

The myeloperoxidase (MPO) activity was assessed in each group. Results are expressed as the mean ± SEM (n=5). MPO activity was significantly higher in the Control group than in the Normal and SHI-219 groups.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Control</th>
<th>SHI-219 (2mg/mice)</th>
<th>SHI-219 (0.5mg/mice)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>IL-1β</strong></td>
<td>8.2±1.3</td>
<td>152.3±21.2</td>
<td>56.3±17.2 **</td>
<td>116.5±24.6</td>
</tr>
<tr>
<td><strong>IL-6</strong></td>
<td>0.483±0.251</td>
<td>17.477±4.255</td>
<td>3.398±0.291 **</td>
<td>18.934±5.634</td>
</tr>
<tr>
<td><strong>TNF-α</strong></td>
<td>0.043±0.023</td>
<td>4.113±0.981</td>
<td>0.585±0.245 **</td>
<td>2.372±0.564</td>
</tr>
</tbody>
</table>

Table 3. The effect of SHI-219 on local cytokine concentrations
Results are expressed as the mean ± S.E.M. (n=5).
**Significantly different from Control group at P<0.05.
SHI-219 inhibits colitis

Fig. 4a TXB₂

Concentrations of mucosal TXB₂ in the Control group were significantly higher than in the Normal group. These values were significantly reduced in the SHI-219 groups.

Fig. 4b LTB₄

These values were significantly reduced in the SHI-219 groups.

Fig. 4c PGE₂

These values were significantly reduced in the SHI-219 groups.

**COX-2 staining**

No expression was observed in the Normal group. However, in the control group in which inflammatory cell infiltration (grade 3) was observed significantly at the base of crypts, COX-2 expression was observed around the inflammatory cells at the base of crypts (▼: black arrow head).

Furthermore, COX-2 expression was also evident in the SHI-219-treated group, which exhibited similar inflammation (grade 3) to the Control group. COX-2 expression also correlated with the degree of inflammation in the intestinal mucosa in the SHI-219-treated group, indicating that SHI-219 did not inhibit COX-2 expression. (Fig. 5)
Discussion

IBD, comprising Crohn's disease and ulcerative colitis, is a severe condition that is usually treated with aminosalicylates, glucocorticoids, and immunosuppressants. However, the in vitro inhibitory effects of sulfasalazine and 5-aminosalicylic acid on eicosanoid production are relatively weak.

Long-term use of glucocorticoids, while effective in suppressing active inflammation, has been associated with high rates of relapse and unacceptable toxicity. On the other hand, 6-mercaptopurine and its prodrug azathioprine are effective in inducing and maintaining remission. However, a significant number of patients are resistant or intolerant to thiopurines. Therefore, the search for new therapeutic strategies is warranted.

Many researchers have produced UC-like enteritis in animal models to investigate the pathophysiology and potential efficacy of experimental therapeutics. In the elucidation of the mechanism of UC, the data using clinical materials is somewhat difficult to analyze because of the influence of treatment and additional clinical courses. Therefore, investigation of the pathogenesis of UC is generally performed using models of experimental colitis. In 1969, Marcus & Watt first produced a UC-like model using oral administration of a sulphated polysaccharide, carrageenan, to guinea pigs and rabbits. Subsequently, Ohkusa induced enteritis in hamsters by oral exposure to another sulphated polysaccharide, DSS, with a molecular weight of 54,000, reporting that lesions exhibited erosion, ulceration, inflammatory cell infiltration, crypt abscesses and epithelioglandular hyperplasia, consistent with human UC. Koyama et al. concluded that this model more closely resembled UC than previous ones. We produced DSS-induced colitis according to this model to examine the expression and secretion of inflammatory cytokines in lesions of the large intestine.

Abnormal metabolism of arachidonic acid is another vital factor in the pathogenesis of IBD. As the crucial synthetase in the arachidonic acid metabolic pathway, cyclooxygenase-2 (COX-2) is shown to be a major arachidonic acid metabolite from platelets and myofibroblasts, respectively; i.e., 6-keto-PGF1α, TX metabolites and PDGF from platelets, and arachidonate metabolites from macrophages, contribute to bowel hyperemia, edema and even dysfunction. In addition, TXB2 could also induce platelet aggregation, vasoconstriction and microthrombosis, which would aggravate the inflammatory response. Neutrophils, which infiltrate the inflamed colonic mucosa substantially, are considered to metabolize arachidonic acid mainly via the 5-lipoxygenase pathway to produce LTB4, whereas platelets produce TXB2 exclusively. Administration of either COX-2 or thromboxane synthase inhibitors has been shown to be useful in the treatment of IBD.

However, in 1983 when Goldin et al. administered 15,15-methyl PGE2 (200mg/day) orally to UC patients to maintain disease remission, the treatment was unsuccessful due to diarrhoea. As a result, LTB4 has subsequently received more attention than PG. Nevertheless, possible cytoprotective effects of PG have been investigated in various models of experimental enteritis.

In other previous studies, PGE2 has been shown to be a major arachidonic acid metabolite synthesized by cyclooxygenase (COX) that contributes to immune regulation. PGE2 acts on macrophages and exhibits inhibitory effects on macrophage activation. Furthermore, subsequent studies demonstrated anti-inflammatory effects of PGE2, including suppression of T-cell induction and neutrophil function. Consequently, PGE2 has recently been considered to be primarily an inhibitor of inflammation. Our previous studies also suggested that rectal injection of PGE2 might prove to be a safe and effective way to treat ulcerative colitis.

Steroids also inhibit the production of PGE2 through inhibition of COX-2. Therefore, a desirable new treatment mechanism would inhibit the production of proinflammatory substances, such as TXB2 and LTB2, but have no effect on the useful COX-2 or PGE2. Different inflammatory mechanisms exist in leukocytes, macrophages, platelets and myofibroblasts, respectively; i.e., MPO activity in leukocytes, inflammatory cytokine and arachidonate metabolites from macrophages, PAF, TX metabolites and PDGF from platelets, and COX-2 and various growth factors from myofibroblasts. These chemical mediators were examined in the current investigation.

In the present study, we employed the UC model in mice to determine whether or not the ameliorative effects of SHI-219 on experimental colitis existed, as assessed by DAI, HDS and MPO.
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activities; parameters that are generally utilized to assess the severity of colonic inflammation in inflammatory bowel disease. Compared with the normal controls, these parameters were all significantly increased in the colonic mucosa of the DSS-induced animals (P<0.01). However, these elevated parameters were significantly ameliorated (P<0.05 or 0.01) as shown in Table 1 after the DSS-induced mice were treated with SHI-219 (0.5, 2.0 mg/mice) as described in the experimental protocol; in this model a therapeutic dose of SHI-219 (2.0mg) was as effective as SHI-219 (0.5mg).

Excessive expression of TXB₂ and LTB₄ in colonic mucosa was reduced to a greater extent in the SHI-219 (2mg) subgroup than in the SHI-219 (2.0mg) subgroup, but PGE₂ levels did not differ significantly between groups (Figure 4a,b). Furthermore, PGE₂ concentration in the colonic mucosa was related the degree of inflammation in each of the groups and COX-2, which synthesizes PGE₂, was also expressed in mice with inflammatory colitis. Based on these results, SHI-219, ameliorated the colonic lesion in experimental colitis, and inhibited the expression of TXB₂ and LTB₄.

A newly synthesized compound, EG626, has been shown to inhibit cyclic AMP phosphodiesterase in arteries and platelets. Moreover, this agent also possesses highly potent, reversible and competitive antagonistic effects on TXA₂, which is known to induce potent arterial contractions. Preliminary data also suggested that EG626 potentiated the anti-aggregating activity of PGI₂, suggesting that both effects might be partly due to the inhibition of cyclic AMP phosphodiesterase.

In the current study, SHI-219, a novel water soluble prodrug of EG626, exhibited an inhibitory effect on DSS-induced colitis, which likely occurred through inhibition of the production of proinflammatory substances, including TXA₂ and LTB₄. Furthermore, SHI-219 did not inhibit the cytoprotective effects of PGE₂ and COX-2, which suggested that SHI-219 inhibits anti-inflammatory effect due to inhibition of LOX and TXs, but not through COX-2 and mPGES-1 expression. Further studies should be conducted in the future to study the effects on other enzymes, including LOX, TXs, mPGES-1, etc., to expand the indications for the use of SHI-219.

In conclusion, the results of this study demonstrate that treatment with SHI-219 can ameliorate the pathological, and clinical damage of DSS-induced colitis. The compound inhibited both LTB₄ and TXB₂ production in DSS-induced mice, but did not inhibit COX-2 or PGE₂. The properties of SHI-219 may make it a potential new therapeutic drug for inflammatory bowel disease.

ACKNOWLEDGMENT

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