Inhibition of Phosphoinositide 3-Kinase Ameliorates Dextran Sodium Sulfate-Induced Colitis in Mice

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ABSTRACT

The critical role of phosphoinositide 3-kinase γ (PI3Kγ) in inflammatory cell activation and recruitment makes it an attractive target for immunomodulatory therapy. 5-Quinoxilin-6-methylene-1,3-thiazolidine-2,4-dione (AS605240), a potent PI3Kγ inhibitor, has been reported to ameliorate chronic inflammatory disorders including rheumatoid arthritis, systemic lupus erythematosus, and atherosclerosis. However, its in vivo effect on intestinal inflammation remains unknown. Here we evaluated the protective and therapeutic potentials of AS605240 in mice with dextran sodium sulfate (DSS)-induced acute and chronic colitis. Our results showed that AS605240 improved survival rate, disease activity index, and histological damage score in mice administered DSS in both preventative and therapeutic studies. AS605240 treatment also significantly increased matrix metalloproteinase levels, macrophage infiltration, and CD4+ T-cell number in the colon of DSS-fed mice. The DSS-induced overproduction of colonic proinflammatory cytokines including interleukin (IL)-1β, tumor necrosis factor-α, and interferon-γ was significantly suppressed in mice undergoing AS605240 therapy, whereas colonic anti-inflammatory cytokines such as IL-4 were up-regulated. The down-regulation of the phospho-Akt level in immunological cells from the inflamed colon tissue and spleen of AS605240-treated mice was detected both by immunohistochemical analysis and Western blotting. These findings demonstrate that AS605240 may represent a promising novel agent for the treatment of inflammatory bowel disease by suppressing leukocyte infiltration as well as by immunoregulating the imbalance between proinflammatory and anti-inflammatory cytokines.

The intestinal bowel diseases (IBDs), including ulcerative colitis and Crohn’s disease, are characterized by chronic relapsing inflammatory disorders of the gastrointestinal tract. Despite extensive efforts, the etiology and pathogenesis of IBDs remain unclear and effective therapies with limited side effects are still lacking (Bouma and Strober, 2003; Baert et al., 2004). Therefore, development of new effective and well tolerated drugs for IBD therapy is necessary.

Chemokines, as detected in the inflamed colon of humans and in murine IBD models, are responsible for recruitment of leukocytes into the lamina propria (LP) of the intestine, which in most cases results in focal crypt damage and epithelial ulceration, the markers in the pathogenesis of IBD (MacDermott et al., 1998; Danese and Gasbarrini, 2005). Many studies have convincingly demonstrated that antagonists targeted against chemokine or receptor function can effectively inhibit acute and chronic inflammation via prevention of leukocyte chemotaxis and activation in animal models of IBD (Onuffer and Horuk, 2002). Recent studies have illustrated the fact that PI3Kγ acts as a key downstream signaling component that relays chemokine receptor signals (Curnock et al., 2002; Rückel et al., 2006). In brief, chemokines recruit immune cells through their action on G-protein-coupled receptors. PI3Kγ, directly activated by G-protein-coupled receptors, leads to the formation of phospho-
tidylinositol-(3,4,5)-triphosphate and consequently phospho-Akt. The interaction of phospho-Akt with phosphatidylinositol-(3,4,5)-triphosphate at the cell membrane stimulates phosphorylation of downstream targets, which regulate several inflammatory and immune functions, including recruitment of macrophages, neutrophils, and T-cell activation (Hirsch et al., 2000; Sasaki et al., 2000; Hawkins and Stephens, 2007). Given the central role of PI3K in regulating chemokine-induced migration of leukocytes and the fact that its expression is restricted mainly to the hematopoietic system, inhibition of PI3K is expected to offer an innovative rationale-based therapeutic strategy for inflammatory diseases without generating severe side effects (Rückle et al., 2006). In recent years, AS605240, a potent PI3K inhibitor, has been demonstrated to prevent inflammatory diseases in several murine disease models, including rheumatoid arthritis, systemic lupus erythematosus, and atherosclerosis (Barber et al., 2005; Camps et al., 2005; Fougerat et al., 2008). The purpose of this study, therefore, was to evaluate the effect of AS605240 on experimental colitis models induced by DSS.

Materials and Methods

Reagents and Mice. AS605240 (Fig. 1A), was synthesized by our laboratory according to the patent (PCT/EP2003/050302; Rückle et

![AS605240](image)

**Fig. 1.** AS605240 prevents DSS-induced acute colitis in C57BL/6 mice. A, chemical structure of AS605240. B to E, clinical and histopathological indices. Mice were treated with either vehicle (control group), 2.5% DSS plus vehicle (vehicle-treated DSS group), or 2.5% DSS plus AS605240 (AS605240-treated DSS group) for 7 days (n = 8 per group). An averaged DAI (B) was assessed daily as described under Materials and Methods. On day 7, the mice were sacrificed. The colon was dissected for histological analysis with H&E staining (D) and the histological injury scores were obtained (E). The entire colon length (C) of each group was measured. F, changes in survival rate on days 0 to 14. AS605240 treatment markedly improves survival of mice fed with 3.5% DSS for 7 days (n = 10 per group). Data in B, C, E, and F are means ± S.D. #, p < 0.05 versus control; *, p < 0.05 versus DSS plus vehicle. Original magnification, 100× in D.
al., 2004) and dissolved in a vehicle (0.5% carboxymethylcellulose-0.25% Tween 20; Sigma-Aldrich, St. Louis, MO). The structure and purity were identified by high-performance liquid chromatography, mass spectrometry, and nuclear magnetic resonance. Male C57BL/6 mice (6–8 weeks old, 18–20 g b.wt.) were purchased from Sichuan University Animal Center (Chengdu, Sichuan, China) and kept under a specific pathogen-free environment. All studies involving mice were approved by the Institutional Animal Care and Use Committee of West China Center of Medical Sciences of Sichuan University.

**Induction of Colitis and AS605240 Treatment.** The experimental protocols are shown in Supplemental Figs. 1 to 4. Male C57BL/6 mice were weighed and randomized into treatment groups of 8 to 10 animals. For acute colitis induction, mice were fed 2.5% DSS (weight/volume, mol. wt. 36,000–50,000; MP Biomedicals, LLC, Eschwege, Germany) for 5 or 7 days (Supplemental Figs. 1 and 3). The DSS solutions were dissolved in sterile, distilled water and prepared fresh every other day. Chronic colitis was induced in mice by three cycles of administration of 2% DSS in drinking water for 5 days, alternating with 5-day periods of recovery (Supplemental Fig. 4). To examine the survival rate (Supplemental Fig. 2), mice were induced with 3.5% DSS in drinking water and the number of surviving mice was counted every day.

**AS605240 was used as described previously (Camps et al., 2005). For protective treatment against acute colitis (Supplemental Fig. 1), mice were administered 50 mg/kg AS605240 orally twice daily, starting on the same day as DSS administration. Control group mice were given tap water and treated in the same way with vehicle on experimental days 0 to 7. For therapeutically treatment of the recovery phase of acute colitis (acute established colitis, see Supplemental Fig. 3), 2.5% DSS was given in drinking water for 5 days and then AS605240 (50 mg/kg orally by gavage twice daily) was given for 7 days (a total of 12 days for the experimental period). For therapeutically treatment of chronic colitis (Supplemental Fig. 4), AS605240 (50 mg/kg orally by gavage twice daily) was initiated at day 11 after the third DSS administration and continued for 14 days.

**Determination of Disease Activity Index.** Animal body weight was recorded daily as well as stool consistency and the presence of occult or gross blood per rectum. These parameters were, respectively, scored by one trained observer blinded to the protocol as described previously by Cooper et al. (1993). Weight loss was scored as follows: 0, none; 1, to 5%; 2, 5 to 10%; 3, 10 to 20%; and 4, more than 20%. Stool consistency was scored as follows: 0, well formed pellets; 1, loose stools; and 4, diarrhea. The presence or absence of fecal blood was also scored as follows: 0, negative Hemoccult test; 2, positive Hemoccult test; and 4, gross bleeding. The clinical DAI, ranging from 0 to 4, was the sum of scores for these parameters divided by three.

**Histological Scoring and Colon Length.** After mice were sacrificed, the entire colon was removed from the cecum to the anus, and the colon length was measured as an indirect marker of inflammation. The distal colon was fixed in 10% buffered formalin for histological analysis. Sections (4-μm thick) were prepared and subjected to staining with hematoxylin and eosin (H&E). Slides were then examined and scored in a blinded fashion using a previously published grading system (Dieleman et al., 1998). Three independent parameters were measured: severity of inflammation (0, none; 1, slight; 2, moderate; and 3, severe), depth of injury (0, none; 1, mucosal; 2, mucosal and submucosal; and 3, transmural), and crypt damage (0, none; 1, basal one-third damaged; 2, basal two-thirds damaged; 3, only surface epithelium intact; and 4, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (1, 0 to 25%; 2, 25 to 50%; 3, 50 to 75%; and 4, 75 to 100%) and summed to obtain a histological injury score. The maximum possible score was 40.

**Assessment of Colonic MPO Activity.** MPO activity was measured according to the instructions for the Myeloperoxidase Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and the previous study (Krawisz et al., 1984).

**ELISA.** Cytokine levels in frozen distal colon biopsies were measured with a commercially available ELISA kit (Biosources, San Jose, CA) according to the manufacturer’s instructions and then were expressed as picograms per milligram of total proteins.

**RT-PCR.** Total RNA from the distal colon was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA was generated using total RNA with the Reverse Transcriptase kit (TaKaRa Biotechnology Group, Dalian, China). All primer sequences were as follows: TNF-α (308 bp), forward 5’-GGC AGG TCT ACG TGG GCA TTC C-3’ and reverse 5’-ACG GTC CAG CTA GTG AAT TCG G-3’; IL-1β (382 bp), forward 5’-GCA ACT GTT CAA GCT A-3’ and reverse 5’-CTC GGA GCC TGT AGT GCA G-3’; IL-4 (404 bp), forward 5’-TAG TGG TCA TCG TGC TCT T-3’ and reverse 5’-CTA CGA GTC CAT TTG C-3’; and β-actin (517 bp), forward 5’-ATA TCG CTG CTC TGG TCG TC-3’ and reverse 5’-AGG ATG GCG TGA GGA AGC GC-3’. The primer sequences of TNF-α, IL-1β, and IL-4 were obtained from a previous report (Kawakami et al., 1997). PCR amplification was performed for 35 cycles as follows: denaturation at 94°C for 1 min, primer annealing for 1 min (the annealing temperatures were 57, 52, and 59°C for TNF-α, IL-1β, IL-4, and β-actin, respectively), and extension at 72°C for 30 s. The relative mRNA expression by β-actin was determined using Quantity One software (version 4.6.1) and averaged from five mice in each group.

**Immunohistochemistry.** Colonies were fixed in 10% formaldehyde, dehydrated, embedded in paraffin, and sectioned (4 μm). Sections were deparaffinized, rehydrated, and treated with 3% H2O2 in phosphate-buffered saline and incubated overnight at 4°C with phospho-Akt (587F11; dilution 1:100; Cell Signaling Technology Inc., Danvers, MA) or CD4 (GK1.5, dilution 1:600; BioLegend, San Diego, CA) or IFN-γ (XMG12, dilution 1:800; BioLegend) monoclonal antibodies. Binding of primary antibody was detected with biotin-labeled goat anti-rat or anti-rabbit IgG antibodies (KIT-0105M, dilution 1:200; Maixin Biotechnology, Fuzhou, China), followed by streptavidin-horseradish peroxidase reaction and visualization with diamino-benzidine (Sigma-Aldrich) and counterstaining with hematoxylin. Positive cells were enumerated on five randomly chosen visual fields at 400× magnification.

**Immunofluorescence.** The frozen sections of the distal colon were prepared to detect macrophage infiltration by immunofluorescence using fluorescein isothiocyanate-conjugated rat anti-mouse F4/80 monoclonal antibody (BM5, dilution 1:50; Abcam Inc., Cambridge, MA) according to the instructions and a previous study (Buttler et al., 2008). Finally, the sections were mounted with VECTASHIELD mounting medium with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). The numbers of F4/80-positive cells were measured on five randomly chosen visual fields at 200× magnification with the aid of Adobe Photoshop software.

**Western Blotting.** Spleen cell suspension and whole-cell protein extraction were performed as described previously (Mesel-Lemoine et al., 2006). The protein concentrations were determined by comparison with a known concentration of bovine serum albumin. The primary antibodies were a 1:750 dilution of the antibodies against Akt (Cell Signaling Technology, Inc.) and phospho-Akt (587F11; Cell Signaling Technology, Inc.), and a 1:8000 dilution of the antibody against β-actin (Sigma-Aldrich). Western blots were performed with horseradish peroxidase-conjugated IgG with the use of enhanced chemiluminescence detection reagents (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The film was scanned with a GS-700 imaging densitometer (Bio-Rad Laboratories, Hercules, CA). The relative phospho-Akt signal from each mouse was normalized relative to its Akt level using Quantity One software (version 4.6.1) and averaged from four mice in each group.

**Cell Preparation and Flow Cytometric Analysis.** LP cells in the colon were also prepared using a method described previously (Arstila et al., 2000). Flow cytometric analysis was performed following routine procedures by using 1 × 106 cells per sample. To measure
the expression of F4/80 (dilution 1:50; Abcam), cells were labeled with either a fluorescein isothiocyanate- or a phycoerythrin-labeled antibody (BD Biosciences Pharmingen, San Diego, CA). Flow cytometric analysis was conducted on a FACSCalibur flow cytometer (BD Biosciences Pharmingen) and analyzed by using Cell Quest software (BD Biosciences, San Jose, CA).

Statistical Analysis. Statistical analysis was performed with the SPSS software program (SPSS for Windows, version 13.0; SPSS Inc., Chicago, IL). Parametric data were analyzed statistically with Student’s t-test or one-way analysis of variance followed by post hoc tests when appropriate. Differences in nonparametric data were evaluated by the Mann-Whitney U test. Survival curves were analyzed statistically using a Kaplan-Meier test. Data are expressed as mean ± S.D. A significant difference was defined as p < 0.05.

Results

AS605240 Treatment Protects against Acute DSS-Induced Colitis. Mice fed with DSS developed clinical, gross, and histological signs of colitis after 7 days of DSS administration. Vehicle-treated DSS mice showed body weight loss, diarrhea, and bleeding in feces, which ultimately resulted in a sharp increase of DAI, compared with control healthy mice. In contrast, DSS-fed mice receiving AS605240 treatment exhibited a markedly reduced DAI compared with that for DSS-fed mice given vehicle on days 4 to 7 (p < 0.05) (Fig. 1B). Furthermore, colon length in AS605240-treated DSS mice was remarkably longer than that in vehicle-treated DSS mice (p = 0.001) (Fig. 1C).

On H&E staining of colonic tissue sections, control animals showed no signs of inflammation, but the colons of vehicle-treated DSS mice developed a well established histopathological event including marked crypt damage, ulceration, and infiltration of inflammatory cells (Fig. 1D). In contrast, the colons from AS605240-treated DSS mice were relatively normal, exhibiting only mild evidence of inflammatory cell infiltration and mucosal injury (Fig. 1D). Furthermore, AS605240 also prevented a significantly DSS-induced increase in histological damage score (p = 0.006) (Fig. 1E).

AS605240 Treatment Extends Lifespan in a Mouse Model of DSS-Induced Colitis. In survival experiments, mice treated with 3.5% DSS plus vehicle for 7 days exhibited a 100% mortality rate by 8 days (Fig. 1F). Conversely, AS605240-treated DSS mice showed a marked increase in survival with 70% of mice alive on day 8, and 40% of mice still alive on day 14 at the end of treatment (n = 10, p = 0.001) (Fig. 1F).

Effect of AS605240 on Two Established Murine Colitis Models Induced by DSS. Acute established colitis in C57Bl/6 mice after 5 days of DSS administration persists for several weeks even after discontinuation of the administration (Cooper et al., 1993; Dieleman et al., 1998). Chronic colitis induced by multiple cycles of DSS can typically continue for at least 3 months (Steidler et al., 2000). Therefore, we evaluated the therapeutic effects of AS605240 on acute and chronic established murine colitis models.

As shown in Fig. 2, A and D, AS605240 treatment significantly reduced elevated DAI levels induced by DSS in both acute and chronic established murine colitis. In subsequent histological examination, the distal colons from mice of these two established colitis models showed marked crypt loss, erosions, and inflammatory cell infiltrations including lymphocytes, macrophages, and occasional neutrophils (Fig. 2, B and E). Conversely, the colon architecture of AS605240-treated DSS mice appeared relatively normal, displaying a restoration in the crypt architecture with goblet cell replenishment mucin and a significant reduction of inflammatory cell infiltration (Fig. 2, B and E). We also used a previously published histological injury scoring system to quantify the severity of inflammation (Dieleman et al., 1998). In acute established DSS colitis, AS605240-treated DSS mice had a significantly improved histological score compared with that for vehicle-treated DSS mice (p = 0.013) (Fig. 2C). In chronic established colitis, vehicle-treated DSS mice had a sharply elevated histological score compared with that for control healthy mice (mean score: 9.4). However, DSS-fed mice receiving AS605240 treatment had an average histological score of 3.3, which represented a nearly 65% decrease (p = 0.002) (Fig. 2F) in pathological symptoms. These results suggest that AS605240 influences the development of colitis in mice.

AS605240 Remarkably Reduces Expression of Akt Phosphorylation of Immunological Cells in DSS-Induced Acute Colitis. To elucidate the effect of AS605240 on the activation of Akt in DSS-induced acute colitis, we tested phospho-Akt expression in the colon tissues or splenocyte extracts from different treatment groups. Immunohistochemical staining for phospho-Akt was performed on colon tissue specimens. As shown in Fig. 3, A and B, the immunohistochemical analysis showed that DSS induced an enhanced phospho-Akt in mucosa and submucosa of the inflamed site of colonic tissue where inflammatory cells accumulated. In sharp contrast, up-regulated expression of phospho-Akt induced by DSS administration was remarkably decreased by AS605240 treatment (p = 0.001), which was obviously accompanied by a decrease in inflammatory infiltration. Furthermore, the expression of total Akt in the lesion site was not influenced by AS605240 treatment (Fig. 3, A and B). Therefore, this result reveals that AS605240 can significantly inhibit the phosphorylation of Akt in immunological cells from the inflamed colon tissue of DSS-fed mice. Furthermore, the result was further confirmed by detection of the protein expression of phospho-Akt in splenocyte extracts by Western blot analysis. A significant reduction in expression of phospho-Akt in splenocyte extracts was observed in the AS605240-treated DSS group compared with that in the vehicle-treated DSS group (p < 0.01) (Fig. 3, C and D). Taken together, the above results indicate that inhibition of Akt phosphorylation in immunocytes is an important signaling event in DSS-induced colitis.

Influence of AS605240 on Macrophage Infiltration in DSS-Induced Acute Colitis. Macrophage infiltration was measured in the colon tissue from groups with acute DSS colitis receiving different treatments. By immunofluorescence, we observed infiltration of a large number of macrophages in colonic samples from vehicle-treated DSS mice, which was mainly located in the mucosa of the lesion site. In sharp contrast, few infiltrating macrophages were detected in either AS605240-treated or untreated colonic samples (Fig. 4, A and B). These results were further supported by determination of the expression of F4/80 in colon LP cells of vehicle-treated and AS605240-treated DSS mice via flow cytometric analysis (Fig. 4, C and D). Indeed, we observed that...
the number of F4/80-positive cells in the colon LP cell extracts was significantly lower in the AS605240-treated DSS group than in the vehicle-treated DSS group \((p = 0.019)\) (Fig. 4D). Taken together, our results demonstrate that AS605240 can markedly reduce macrophage infiltration in the colon of DSS-fed mice.

**Effect of AS605240 on MPO Activity of Acute DSS-Induced Colitis.** MPO activity is a useful index for evaluating neutrophil infiltration in colonic tissues after induction of colitis (Takizawa et al., 1995). Using a standard enzymatic procedure, we observed that MPO levels in the colon tissue of vehicle-treated DSS mice were 3.3-fold greater than those in the colon tissue of control healthy mice \((p < 0.001)\) (Fig. 4E). In contrast, colonic MPO in AS605240-treated DSS mice reached levels 58% lower than those in vehicle-treated DSS mice \((p = 0.006)\) (Fig. 4E). Furthermore, there was no statistically significant difference between MPO activity in AS605240-treated DSS mice and control healthy mice \((p > 0.05)\) (Fig. 4E). Therefore, our results strongly support inhibition of neutrophil recruitment as a potential mechanism for the protective effect of AS605240.

**Effect of AS605240 on Infiltration of CD4\(^+\) T Cells in Two Established Murine Colitis Models Induced by DSS.** CD4\(^+\) T cells play an important role in worsening chronic colitis induced by multiple cycles of DSS and in the recovery phase of exaggerated colitis induced by DSS (Dielemann et al., 1998; Shintani et al., 1998). Thus, we measured the expression of CD4\(^+\) T cells in the colon tissue of all treatment groups from the two established murine colitis models by immunohistochemistry. A pronounced infiltration of CD4\(^+\) T cells was seen in the colon of mice with acute established colitis, of which the majority were inflammatory cells. In contrast, few CD4\(^+\) T cells were detected in the colon tissue of AS605240-treated DSS mice or control healthy mice.
We also observed similar results in DSS-induced chronic colitis (Fig. 5, C and D). Thus, our results confirmed that AS605240 can effectually inhibit DSS-induced colitis through the reduction of infiltration of CD4^+ T cells.

**Influence of AS605240 on the Production of Cytokines in the Colon of DSS-Treated Mice.** To investigate the influence of AS605240 on cytokine production, we measured the levels of proinflammatory (TNF-α, IL-1β, and IFN-γ) and anti-inflammatory cytokines (IL-4) in colonic tissue from different treatment groups. In the induction phase of acute DSS colitis, we observed elevated TNF-α and IL-1β mRNA expression on day 7 after DSS administration by RT-PCR. Significant reductions in TNF-α and IL-1β mRNA levels were also detected in the colon of DSS-exposed mice undergoing AS605240 therapy (p < 0.05) (Fig. 6, A and B). To further confirm these phenomena, protein levels of TNF-α and IL-1β in the colon tissue were also measured by ELISA. As depicted in Fig. 6, C and D, AS605240 treatment markedly inhibited the increases in TNF-α and IL-1β protein levels induced by DSS administration (TNF-α: 50% decrease, p = 0.017; IL-1β: 52% reduction, p = 0.001). In contrast, IL-4 mRNA expression in vehicle-treated DSS mice was significantly down-regulated compared with that in healthy control mice, whereas the down-regulation was prevented by AS605240 therapy (Fig. 6, A and B).

In the recovery phase of acute DSS colitis, we found that colonic IFN-γ expression was notably lower in the AS605240-treated DSS group than that in vehicle-treated DSS group by immunohistochemical analysis (p = 0.016) (Fig. 5, A and B). We also observed that AS605240 treatment significantly reduced elevated colonic IFN-γ expression induced by DSS in chronic established colitis (p = 0.003) (Fig. 5, C and D). Taken together, our findings show that AS605240 prevented colonic inflammation caused by DSS through down-regulating the production of proinflammatory cytokines or/and up-regulating the production of anti-inflammatory cytokines.

**Discussion**

In the present study, we demonstrated that AS605240 had protective and therapeutic potentials in acute and chronic DSS colitis in vivo. In the preventative protocol, AS605240 significantly lessened the clinical and histopathological...
symptoms of DSS-fed mice and increased survival in an experimental murine model of acute colitis. In the therapeutic protocol, AS605240 treatment also diminished the loss of crypts, the infiltration of inflammatory cells, and the decreased histological score compared with vehicle-treated DSS-fed mice. These results indicate that AS605240 not only attenuates the induction of colitis but also has an inhibitory effect on established colitis, suggesting its clinical application potential for treatment of IBDs.

Immune cell recruitment triggered by chemokines requires intracellular signaling through the lipid kinase PI3Kα and the PI3K-dependent protein serine/threonine kinase Akt (the main downstream target of PI3K). The activation of PI3Kα-Akt signaling plays a pivotal role in various inflammatory, autoimmune, and allergic processes (Hirsch et al., 2000; Rückle et al., 2006; Hawkins and Stephens, 2007). AS605240, a potent PI3Kα inhibitor, has exhibited its favorable anti-inflammatory effects in murine models of several inflammatory diseases through effectively inhibiting the activation of the PI3Kα-Akt pathway in different immune cell types, which is reflected in the reduction of Akt phosphorylation induced by chemokines in immunological cells after AS605240 treatment (Barber et al., 2005; Camps et al., 2005; Fougerat et al., 2008). Our present study confirmed that AS605240 can also significantly block the induction and development of DSS-induced colitis accompanied by a decrease in the phospho-Akt level in immunological cells from both inflamed colon

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**Fig. 4.** AS605240 ameliorates the expression of F4/80 and MPO in the colon tissue of mice from DSS-induced acute colitis. A and B, immunofluorescence detection of F4/80-expressing cells. Colon tissues were obtained from different treatment groups and were processed for immunofluorescence analysis using F4/80 monoclonal antibody, and representative results from four independent animals are shown in A. The numbers of F4/80-expressing cells were determined as described under Materials and Methods and are shown in B. C and D, flow cytometry analysis was performed in colon LP cell extracts from different treatment groups with F4/80 monoclonal antibody. Representative results from six independent animals are shown in C. The numbers of F4/80-expressing cells were determined in six independent animals as described under Materials and Methods and are shown in D. E, MPO activity in the colonic tissue from mice receiving vehicle (control), DSS plus vehicle or DSS plus AS605240 was measured as described under Materials and Methods (n = 5/experimental group). Data in B, D, and E are means ± S.D. #, p < 0.05 versus control; *, p < 0.05 versus DSS plus vehicle. Arrows in A indicate the positive stained cells. Original magnification, 200× in A.
tissue and spleen of DSS-fed mice. Thus, we can conclude that inhibition of Akt phosphorylation in immunocytes is a key signaling event induced by AS605240 in DSS-induced colitis.

It is well accepted that excessive leukocyte recruitment triggered by chemokines into the injured colonic tissue plays a key role in the pathogenesis of IBD (MacDermott et al., 1998; Danese and Gasbarrini, 2005). In this study, we observed that orally administered AS605240 significantly reduced macrophage and neutrophil infiltration in acute DSS colitis. This reduction could be explained by the important role of the chemokine-PI3K-Akt signaling pathway in macrophage and neutrophil recruitment at the damage site (Hirsch et al., 2000; Sasaki et al., 2000), and it may be an important mechanism of the AS605240 effect in colitis, because the induced phase of acute DSS colitis is highly lymphocyte-independent and principally mediated by neutrophils and macrophages (Dieleman et al., 1994). We also observed that in both established colitis models AS605240 decreased CD4^+ T-cell infiltration in the colonic tissue as well as the development of disease. This result is consistent with the results of Barber et al. (2005) in a murine model of systemic lupus erythematosus and further confirms the effect of AS605240 on CD4^+ T-cell infiltration. However, the exact underlying mechanism is still unclear. PI3K involvement in T-cell migration is controversial. In in vitro studies, p110γ-deficient T cells exhibit decreased chemotactic responses to the lymphoid chemokines such as CCL19, CCL21, and CXCL12 (Reif et al., 2004). More importantly, this recent finding illustrates that p110γ regulates chemokine-dependent migration of antigen-experienced effector CD4 T cells into inflammatory sites by influencing F-actin (downstream of chemokine receptors) polarization during adaptive immunological responses in vivo (Thomas et al., 2008). These results suggest the importance of PI3K in T-cell migration. However, there are also some opposing reports. Nombela-Arrieta et al. (2007) observed that PI3K deficiency has no significant effects on migration velocities of T cells using multiphoton intravital microscopy. In an in vivo study, CD4^+ T-cell invasion or lymphoproliferation in p65PI3K-transgenic mice was not influenced by the lack of PI3Kγ (Barber et al., 2006). Therefore, it would be interesting to elucidate the effects of PI3Kγ on T-cell migration in chronic inflammatory diseases. Furthermore, it was reported that PI3Kγ controls T-cell survival in a previous study (Swat et al., 2006), but there is also considerable doubt about the specific role of PI3Kγ in T-cell activation (Sasaki et al., 2000; Barber et al., 2005, 2006; Alcázar et al., 2007; Fougerat et al., 2008; Gar-

Fig. 5. AS605240 decreases CD4^+ T-cell infiltration and IFN-γ level in the colonic tissue of mice from acute and chronic established colitis. A and B, immunohistochemical analysis of CD4 and IFN-γ in the colon of different treatment groups in acute established colitis. Representative results from five independent animals are shown in A. The numbers of CD4- or IFN-γ-expressing cells were determined in five independent animals as described under Materials and Methods and are shown in B. C and D, immunohistochemical detection of CD4 and IFN-γ in the colon from different treatment groups in chronic established colitis. Representative results from five independent animals are shown in C. The numbers of CD4- or IFN-γ-positive cells were determined in five independent animals as described under Materials and Methods and are shown in D. Data in B and D are means ± S.D., * p < 0.05 versus DSS plus vehicle. Original magnification, 400× in A and C.
čon et al., 2008; Ji et al., 2008). AS605240 is a potent PI3K\(\gamma\) inhibitor (IC\(_{50}\) 8 nM), but also shows an inhibitory effect on PI3K IA isoforms (IC\(_{50}\) : PI3K\(\alpha\), 60 nM; PI3K\(\beta\), 270 nM; and PI3K\(\delta\), 300 nM) (Barber et al., 2005). Of the three PI3K IA isoforms, PI3K\(\alpha\) and PI3K\(\beta\) are mainly involved in normal growth and development of animals. PI3K\(\delta\), mainly restricted to the hematopoietic cells, has been reported to act as an important regulator of T-cell activation, proliferation, and differentiation in vitro or in vivo (Rückle et al., 2006; Rommel et al., 2007). Thus, it is possible that AS605240 can also partially inhibit the PI3K\(\delta\) isoform, resulting in the inhibition of T-cell activation, although PI3K\(\gamma\) involvement in T-cell activation is controversial. Given the dual inhibition of AS605240 on PI3K\(\gamma\) and other possible class IA PI3K isoforms (such as the \(\delta\) isoform), AS605240 may effectively control T-cell activation and survival. Hence, we speculate that AS605240 may significantly decrease pathogenic T-cell generation or increase T-cell apoptosis (especially CD4\(^+\) T cells), even though it has no effect on T-cell migration, thus significantly reducing pathogenic CD4\(^+\) T-cell infiltration in the colon tissue of mice with established DSS-induced colitis. This may be one of the important mechanisms concerning how AS605240 reverses acute and chronic established colitis as well as other chronic inflammatory diseases such as systemic lupus erythematosus and atherosclerosis (Barber et al., 2005; Fougerat et al., 2008).

It is well known that the chronic inflammation in IBD may be caused by an imbalance in proinflammatory and anti-

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**Fig. 6.** Influence of AS605240 on IL-1\(\beta\), TNF-\(\alpha\), and IL-4 levels in the colon tissue of mice from DSS-induced acute colitis. A and B, RT-PCR analysis for IL-1\(\beta\), TNF-\(\alpha\), and IL-4 was performed on total RNAs extracted from the colons of different treatment groups as described under Materials and Methods. Representative results from five independent animals are shown in A. The levels of IL-1\(\beta\), TNF-\(\alpha\), and IL-4 mRNA were normalized to the levels of \(\beta\)-actin mRNA and showed in B. C and D, protein levels of TNF-\(\alpha\) and IL-1\(\beta\) in the colon from mice given vehicle (control), DSS plus vehicle, or DSS plus AS605240 were determined by ELISA. \(n = 5\) group. Data in B, C, and D are means ± S.D. #, \(p < 0.05\) versus control; *, \(p < 0.05\) versus DSS plus vehicle.
inflammatory cytokines to response to an initial event (Rogler and Andus, 1998). There are several indications showing proinflammatory cytokines (e.g., IL-1β, TNF-α, and IFN-γ) are involved in the pathogenesis of colitis. Elevated IL-1β levels are found in patients with IBD who have active disease as well as in experimental IBD models (Dieleman et al., 1996; Guimbard et al., 1998). We can also infer a pathogenic role of TNF-α in acute and chronic DSS colitis from the observed raised levels of TNF-α as well as from the beneficial effects obtained by the use of anti-TNF monoclonal antibody therapy alone or in combination with pentoxifylline, a TNF release inhibitor (Watkins et al., 1997; Murthy et al., 1999). Furthermore, recent studies in chronic DSS-induced colitis show that human IBD.

ment of AS605240 as a therapeutic agent for the prevention of AS605240 inhibits joint inflammation and damage in mouse models. We observed a reduction of TNF-α and IL-1β in mouse models and the disease activity index (DAI) was significantly decreased in DSS-induced colitis (Watkins et al., 1997; Murthy et al., 1999). Previous studies report that IL-4 has immunoregulatory and anti-inflammatory activities and that the production of IL-4 in inflammatory cells (a major resource of the three cytokines) in the colon tissue after AS605240 treatment. Previous studies report that IL-4 has immunoregulatory and anti-inflammatory activities and that the production of IL-4 in inflammatory bowel disease is remarkably lower than that in normal control group (West et al., 1996; Rogler and Andus, 1998). Consistent with previous results, our results showed that the IL-4 mRNA in the colonic tissue of mice was decreased by DSS intervention but restored to the basal level by AS605240 treatment. Taken together, these results indicate that AS605240 may inhibit colonic inflammation and immune responses through reducing the production of proinflammatory cytokines and up-regulating the levels of anti-inflammatory cytokines.

In conclusion, our study demonstrates for the first time that AS605240 can effectively protect and treat DSS-induced murine colitis by targeting of PI3K and/or other possible PI3K IA isoforms and consequently suppressing leukocyte infiltration as well as immunoregulating the imbalance between proinflammatory and anti-inflammatory cytokines. Our findings might be of great importance for the development of AS605240 as a therapeutic agent for the prevention and treatment of human IBD.

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References


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Supplemental Figure 1: Experimental design for evaluating the protective effect of AS605240 on acute DSS-induced colitis.

Group 1: Control healthy mice
Group 2: DSS + vehicle
Group 3: DSS + AS605240

Day

| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |

2.5% DSS

DAI

Sacrifice

Colon length
Histology
Mpo
Flow cytometric
Immunofluorescence
Western blotting

RT-PCR (TNF-α; IL-1β; IL-4)
ELISA (TNF-α; IL-1β)

Immunohistochemistry (Phospho-Akt)
Supplemental Figure 2: Experimental design for assessing the influence of AS605240 on survival rate of DSS-fed mice.

Group 1: DSS + vehicle

Group 2: DSS + AS605240
Supplemental Figure 3: AS605240 therapy protocol for the recovery phase of acute DSS-induced colitis (acute established colitis).

Group 1: Control healthy mice
Group 2: DSS + vehicle
Group 3: DSS + AS605240

Day 0 1 2 3 4 5 6 7 8 9 10 11 12

AS605240 or vehicle

2.5% DSS
Tap water

DAI

Sacrifice

Histology

Immunohistochemistry (CD4; IFN-γ)
Supplemental Figure 4: AS605240 treatment protocol for chronic murine colitis induced by three cycles of DSS (chronic established colitis).

- **Group 1**: Control healthy mice
- **Group 2**: DSS + vehicle
- **Group 3**: DSS + AS605240