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

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Running Title Page

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d) Abbreviations: DSS, dextran sodium sulfate; DAI, disease activity index; GPCRs, G-protein–coupled receptors; LP, lamina propria; MPO, myeloperoxidase; PI3K, phosphoinositide 3-kinase; PIP3, Phosphatidylinositol-(3, 4, 5)-triphosphate.

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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. 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 on mice with DSS-induced acute and chronic colitis. Our results showed that AS605240 improved survival rate, disease activity index (DAI) and histological damage score in mice administered DSS in both the preventive and therapeutic studies. AS605240 treatment also significantly inhibited the increase of myeloperoxidase (MPO) levels, macrophage infiltration and CD4+ T cell number in the colon of DSS-fed mice. The DSS-induced overproductions of colonic proinflammatory cytokines including interleukin-1β (IL-1β), tumour necrosis factor-α (TNFα) and interferon-γ (IFNγ) were significantly suppressed in mice undergoing AS605240 therapy, while colonic anti-inflammatory cytokines such as interleukin-4 (IL-4) were up-regulated. The down-regulations of phospho-Akt level in immunological cells from the inflamed colon tissue and spleen of AS605240-treated mice were detected both by immunohistochemistry 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 unbalance between proinflammatory and anti-inflammatory cytokines.
**Introduction**

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

Chemokines, already detected in the inflamed colon of humans and murine IBD models, are responsible for recruitment of leukocytes into 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). A good deal of studies convincingly demonstrate that antagonists targeting against chemokine or receptor function can effectively inhibit acute and chronic inflammation via preventing leukocyte chemotaxis and activation in animal models of IBD (Onuffer and Horuk, 2002).

Recent studies have illustrated that PI3Kγ acts as a key downstream signaling component that relays chemokine-receptor signals (Curnock et al., 2002; Rückle et al., 2006). Briefly, chemokines recruit immune cells through their action on the G-protein–coupled receptors (GPCRs). PI3Kγ, directly activated by GPCRs, leads to the formation of phosphatidylinositol-(3, 4, 5)-triphosphate (PIP3) and
consequent phospho-Akt. The interaction of phospho-Akt with PIP3 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 PI3Kγ on regulating chemokine-induced migration of leukocyte and that its expression is mainly restricted to the haematopoietic system, inhibition of PI3Kγ 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 PI3Kγ 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.

Methods

Reagents and mice

AS605240 (Fig.1A), 5-quinoxilin-6-methylene-1,3-thiazolidine-2,4-dione, was
synthesized by our laboratory according to the patent (PCT/EP2003/050302), and dissolved in a vehicle (0.5% carboxymethylcellulose/0.25% Tween-20 (Sigma)). The structure and purity were identified by high performance liquid chromatography, mass spectrometry and nuclear magnetic resonance. Male C57BL/6 mice (6-8 week old, 18-20g body weight) were purchased from Sichuan University Animal Center (Chengdu, Sichuan, China) and kept under 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 were shown in Supplemental Figure 1-4 online. 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, MW 36000-50000, MP Biomedicals, LLC, Eschwege, Germany) for 5 or 7 days (Supplemental Figure 1 and 3). The DSS solutions were dissolved in sterile, distilled water and prepared fresh every other day. For chronic colitis induction, mice were induced by three cycles of administration of 2% DSS in drinking water for 5 days, alternating with 5-day periods of recovery (Supplemental Figure 4). To examine the survival rate (Supplemental Figure 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 of acute colitis (Supplemental Figure 1), mice were administered orally with 50mg/kg AS605240 twice daily, starting at the same day as DSS administration. Control group mice were given tap water and treated equally with vehicle on the experimental days 0-7. For therapeutic treatment of the recovery phase of acute colitis (acute established colitis, see Supplemental Figure 3), 2.5% DSS was given in drinking water for 5 days and then AS605240 (50mg/kg, orally by gavage twice daily) was given for 7 days (a total of 12 days for the experimental period). For therapeutic treatment of chronic colitis (Supplemental Figure 4), AS605240 (50mg/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 (DAI)**

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* (Cooper et al., 1993). Weight loss was scored as
follows: 0, none; 1, 1-5%; 2, 5-10%; 3, 10-20%; 4, over 20%. Stool consistency was scored as follows: 0, well formed pellets; 2, loose stools; 4, diarrhea. Presence or absence of fecal blood was also scored as follows: 0, negative hemoccult test; 2, positive hemoccult test; 4, gross bleeding. 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 caecum 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 4um 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; 3, severe), depth of injury (0, none; 1, mucosal; 2, mucosal and submucosal; 3, transmural) and crypt damage (0, none; 1, basal 1/3 damaged; 2, basal 2/3 damaged; 3, only surface epithelium intact; 4, entire crypt and epithelium lost). The score of each parameter was multiplied by a factor reflecting the percentage of tissue involvement (1: 0-25%; 2: 26-50%; 3: 51-75%; 4: 76-100%) and summed to obtain a histological injury
score. Maximum possible score was 40.

**Assessment of colonic MPO activity**

MPO activity was measured according to the instruction of Myeloperoxidase Detection Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and the previous study (Krawisz et al., 1984).

**ELISA**

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

**RT-PCR**

Total RNA from the distal colon was isolated using TRIzol reagent (Invitrogen Corp) 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 shown as follows: TNFα(308bp), Forward: 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3' and Reverse: 5'-ACA TTC GAG GCT CCA GTG AA T TCG G-3'; IL-1β(382bp), Forward:
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5'-GCA ACT GTT CCT GAA CTC A-3' and Reverse: 5'-CTC GGA GCC TGT AGT GCA G-3'; IL-4(404bp), Forward: 5'-TAG TTG TCA TCC TGC TCT T-3' and Reverse: 5'-CTA CGA GTA ATC CAT TTG C-3'; and Beta actin(517bp), Forward: 5'-ATA TCG CTG CGC TGG TCG TC-3' and Reverse: 5'-AGG ATG GCG TGA GGG AGA GC-3'. The primer sequences of TNFα, IL-1β and IL-4 were obtained from the previous report (Kawakami et al., 1997). PCR amplification was carried out for 35 cycles as follows: denaturation at 94°C for 1 minute, primer annealing for 1 minute (The annealing temperatures were 57°C, 52°C, 50°C, and 59°C for TNFα, IL-1β, IL-4 and Beta actin, respectively), extension at 72°C for 30 seconds. The relative mRNA expression to Beta actin was performed using Quantity one software (4.6.1) and averaged from five mice in each group.

**Immunohistochemistry**

Colons were fixed in 10% formaldehyde, dehydrated, embedded in paraffin and sectioned (4μm). Sections were deparaffinized, rehydrated, and treated with 3% H₂O₂ in phosphate buffered saline and incubated overnight at 4°C with phospho-Akt (dilution 1:100, 587F11, Cell Signaling) or CD4 (dilution 1:600, GK1.5, Biolegend) or IFNγ (dilution 1:800, XMG1.2, Biolegend) monoclonal antibodies. Binding of primary antibody was detected with biotin-labeled goat
anti-rat or anti-rabbit IgG antibodies (dilution 1:200; KIT-0105M, Maixin Biotechnology, China), followed by streptavidin-horseradish peroxidase reaction and visualization with diaminobenzidine (Sigma) and counterstaining with hematoxylin. Positive cells were enumerated on 5 randomly chosen visual fields at ×400 magnification.

**Immunofluorescence**

The frozen sections of the distal colon were prepared to detect macrophage infiltration by immunofluorescence using FITC–conjugated rat anti-mouse F4/80 monoclonal antibody (dilution 1:50, BM8, Abcam) according to the instruction and the previous study (Buttler et al., 2008). Finally the sections were mounted with Vectashield mounting medium with diamidino phenyl indole (DAPI) (H-1500, Vector Laboratories, Burlingame, CA, USA). The numbers of F4/80-positive areas were measured on 5 randomly chosen visual fields at ×200 magnification with the aid of Adobe Photoshop software.

**Western blotting**

Spleen cells suspension and whole-cell protein extraction were performed as previously described (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) and phospho-Akt (587F11, Cell Signaling), and a 1:8000 dilution of the antibody against β-actin (Sigma-Aldrich). Western blots were performed with horseradish peroxidase–conjugated immunoglobulin G with the use of enhanced chemiluminescence detection reagents (Amersham International). The film was scanned with a GS-700 imaging densitometer (Bio-Rad). The relative phospho-Akt signal from each mouse was normalized relative to its Akt level using Quantity one software (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 previously described method (Arstila et al., 2000). Flow cytometry followed routine procedures by using 1×10^6 cells per sample. To measure the expression of F4/80 (dilution 1:50, Abcam), cells were labeled with either a FITC- or a phycoerythrin-labeled antibody (Pharmingen). Flow cytometric analysis was conducted on a FACS Calibur flow cytometer (Pharmingen) and analyzed by using the Cell Quest software (BD Biosciences).

Statistical analysis
Statistical analysis was performed with the SPSS software system (SPSS for Windows, version 13.0; SPSS Inc, Chicago, IL). Parametric data were statistically analyzed by the Student’s t test or one-way ANOVA followed by post hoc tests when appropriate. Differences in Non-parametric data were evaluated by the Mann-Whitney U test. Survival curves were statistically analyzed using Kaplan-Meier test. Data were expressed as means ± SD. 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 appeared 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 DSS-fed mice given vehicle on days 4-7 ($p < 0.05$; **Fig. 1B**). Furthermore, the 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 histopathologic event including marked crypt damage, ulceration, and infiltration of inflammatory cells (Fig. 1D). In contrast, the colons from AS605240-treated DSS mice showed relatively normal, exhibiting only mild evidence of inflammatory cell infiltration and mucosal injury (Fig. 1D). Furthermore, AS605240 also prevented 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 seven days exhibited 100% mortality rate by 8 days (Fig. 1F). Inversely, AS605240-treated DSS mice showed a marked increase in survival with 70% of mice alive on day 8. There were still 40% of mice alive even on day 14 of the end of treatment ($n = 10$, $p = 0.001$; Fig. 1F).

Effect of AS605240 on two established murine colitis induced by DSS
Acute established colitis in C57BL/6 mice after 5-day DSS administration persists for several weeks even if 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 three 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 two established colitis models showed marked crypts loss, erosions and inflammatory cell infiltrations including lymphocytes, macrophages and occasional neutrophils (**Fig. 2B 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. 2B and E**). We also employed 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 received a significantly improved histological score compared with vehicle-treated DSS mice ($p = 0.013$; **Fig. 2C**). In chronic established colitis, vehicle-treated DSS mice had a sharp elevated histological score compared with 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 obviously accompanied with decrease of inflammatory infiltration. Furthermore, the expression of total Akt in the lesion site did not be influenced by AS605240 treatment (Fig. 3A 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 detecting the protein expression of phospho-Akt in splenocyte
extracts by western blot analysis. A significant reduction of protein expression of phospho-Akt in splenocyte extracts was observed in AS605240-treated DSS group compared with that in vehicle-treated DSS group ($p < 0.01$, **Fig. 3C 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 different treatment groups of acute DSS colitis. By immunofluorescence, we observed a large number of macrophage infiltration in colonic samples from vehicle-treated DSS mice, which was mainly located in the mucosa of the lesion site. In sharp contrast, few infiltrating macrophage were detected both in AS605240 treated and untreated colonic samples (**Fig. 4A and B**). These results were further supported by the determination of the expression of F4/80 in colon LP cells of vehicle-treated and AS605240-treated DSS mice via flow cytometric analysis (**Fig. 4C and D**). Indeed, we observed that F4/80 positive cell number in the colon LP cell extracts was significantly lower in AS605240-treated DSS group than in 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 following 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 levels 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 difference between MPO activity in AS605240-treated DSS mice and control healthy mice ($p > 0.05$; Fig. 4E). Therefore, our results strongly suggest inhibition of neutrophil recruitment as a potential mechanism for the protective effect of AS605240.

**Effect of AS605240 on CD4$^+$ T cells infiltration in two established murine colitis induced by DSS**

CD4$^+$ T cells play an important role in worsening the diseases in chronic colitis induced by multiple cycles of DSS or in the recovery phase of exaggerated
colitis induced by DSS (Dieleman 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 by immunohistochemistry. A pronounced infiltration of CD4+ T cells was seen in the colon of mice from acute established colitis, which accumulated the majority of inflammatory cells. In contrast, few CD4+ T cells were detected in the colon tissue of AS605240-treated DSS mice or control healthy mice (Fig. 5A and B). We also observed that similar results in DSS-induced chronic colitis (Fig. 5C and D). Thus, our results confirmed that AS605240 can effectually inhibit DSS-induced colitis through the reduction of CD4+ T cells infiltration.

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 (TNFα, IL-1β, IFNγ) and anti-inflammatory cytokines (IL-4) in the colonic tissue from different treatment groups.

In the induction phase of acute DSS colitis, we observed elevated TNFα and IL-1β mRNA expression on day 7 after DSS administration by RT-PCR. Significant reduction in TNFα and IL-1β mRNA levels were also detected in the
colon of DSS-exposed mice undergoing AS605240 therapy (p < 0.05, Fig. 6A and B). To further confirm these phenomena, protein levels of TNFα 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 increase of TNFα and IL-1β protein levels induced by DSS administration (TNFα: 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 while the down-regulation was prevented by AS605240 therapy (Fig. 6A and B).

In the recovery phase of acute DSS colitis, we found that colonic IFNγ expression was notably lower in AS605240-treated DSS group than that in vehicle-treated DSS group by immunohistochemistry (P =0.016, Fig. 5A and B). We also observed that AS605240 treatment significantly reduced elevated colonic IFNγ expression induced by DSS in chronic established colitis (P =0.003, Fig. 5C and D).

Taken together, our findings showed 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 therapeutical potentials of acute and chronic DSS colitis in vivo. In the preventative protocol, AS605240 significantly lessened the clinical and histopathologic symptoms of DSS-fed mice and increased survival in experimental murine model of acute colitis. In the therapeutic protocol, AS605240 treatment also diminished the loss of crypts, the infiltration of inflammatory cells and decreased histological score compared with vehicle-treated DSS-fed mice. These above results indicate that AS605240 not only attenuates the induction of colitis but also has an inhibitive effect on established colitis, suggesting its clinical application potential for IBD treatment.

Immune cells recruitment triggered by chemokines requires intracellular signaling through the lipid kinase PI3Kγ and the PI3-kinase-dependent protein serine/threonine kinase Akt (the main downstream target of PI3K). The activation of PI3Kγ-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 PI3Kγ inhibitor, has exhibited its favorable anti-inflammatory effects on murine models of several
inflammatory diseases through effectively inhibiting the activation of PI3Kγ-Akt pathway in different immune cell types, which reflecting 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 with the decrease of phospho-Akt level in immunological cells from both inflamed colon tissue and spleen of DSS-fed mice. So, we can think 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 chemokine-PI3Kγ-Akt signaling pathway for macrophage and neutrophil recruitment at the damage site (Hirsch et al., 2000; Sasaki et al., 2000) and it may be an important mechanism of AS605240 effect in colitis, because the induced phase of acute DSS colitis is highly lymphocyte independent and principally mediated by neutrophil and
macrophage (Dieleman et al., 1994). We also observed that in both established colitis AS605240 decreased CD4+ T-cell infiltration in the colonic tissue as well as the development of disease in both established colitis. This result is consistent with Barber DF et al reports in a murine model of systemic lupus erythematosus (Barber et al., 2005), and further confirms AS605240 effect on CD4+ T-cell infiltration. However, the exact underlying mechanism is still unclear. PI3Kγ involvement in T-cell migration is controversial. 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, the 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 immunologic responses in vivo (Thomas et al., 2008). These results suggest the importance of PI3Kγ in T-cell migration. However, there are also some adverse reports. Nombela-Arrieta C et al observe that PI3Kγ deficiency has no significant effects on migration velocities of T cells using multiphoton intravital microscopy (Nombela-Arrieta et al., 2007). In vivo study, CD4+ T-cell invasion or lymphoproliferation in p65PI3K-transgenic mice is not influenced by the lack of PI3Kγ (Barber et al., 2006). Therefore, it would be interesting to elucidate PI3Kγ effects on T-cell migration in chronic
inflammatory diseases. Furthermore, it is reported that PI3Kγ controls T-cell survival in the previous study (Swat et al., 2006), but there is also lots of 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; Ji et al 2008; Garçon et al 2008). AS605240 is a potent PI3Kγ inhibitor (IC50 = 8 nM), but shows also a certain degree of the inhibitory effect on PI3K IA isoforms (IC50: PI3Kα = 60 nM, PI3Kβ = 270 nM, PI3Kδ = 300 nM) (Barber et al., 2005). In three PI3K IA isoforms, PI3Kα and PI3Kβ are mainly involved in normal growth and development of animals. PI3Kδ, mainly restricted to the hematopoietic cells, has been reported to act as an important regulator on 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 PI3Kδ isoform, resulting in the inhibition of T-cell activation although PI3Kγ involvement in T-cell activation is controversial. Given that the dual inhibition of AS605240 on PI3Kγ and other possible class IA PI3K (such as δ isoform), AS605240 may effectively control T cell activation and survival. Hence, we speculate that AS605240 may significantly decrease pathogenic T-cell generation or/and increase T-cell apoptosis (especially CD4+ T cells), even though having no intervention with T-cell migration, thus significantly reducing pathogenic CD4+ T cells infiltration in the colon tissue of mice from
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 has been well known that the chronic inflammation in IBD may be on account of an imbalance of proinflammatory and anti-inflammatory cytokines in response to an initial event (Rogler and Andus, 1998). There are a number of evidences showing that proinflammatory cytokines (e.g. IL-1β, TNFα and IFNγ) are involved in the pathogenesis of colitis. Elevated IL-1β levels are measured in IBD patients with active disease as well as in experimental IBD models (Dieleman et al., 1996; Guimbaud et al., 1998). We can also infer a pathogenic role of TNFα in acute and chronic DSS colitis from the observed raised levels of TNFα as well as from the beneficial effects obtained by the use of anti-TNF mAb therapy alone or combining with pentoxifylline, a TNF release inhibitor (Watkins et al., 1997; Murthy et al., 1999). Furthermore, recent studies in chronic DSS-induced colitis have showed that raised IFNγ expression and increased numbers of IFNγ secreting cells (mainly CD4+ T cells) are detected with development of lesions and that the utilization of neutralizing antibodies to IFNγ markedly diminishes histological scores (Obermeier et al., 1999). These results show that IL-1β, TNFα and IFNγ played important roles in initiation
and/or perpetuation of colitis. In our study, expression levels of TNFα, IL-1β and IFNγ in the colon tissue were enhanced by DSS and significantly suppressed by AS605240 treatment, which may be owing to the decrease of activated macrophages, neutrophils or CD4+ T cells (a major resource of the three cytokines) infiltration in the colon tissue after AS605240 treatment. Previous studies report that IL-4 has immunoregulatory and anti-inflammatory activities and 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). In consistence with previous reports, 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/or 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 PI3Kγ and/or other possible PI3K IA isoforms and consequently suppressing leukocyte infiltration as well as immunoregulating the unbalance between proinflammatory and anti-inflammatory cytokines. Our findings might be of great importance to develop AS605240 as a therapeutic agent to the prevention
and treatment of human IBD.
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Footnotes

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Legends for Figures

Figure 1. AS605240 prevents DSS-induced acute colitis in C57BL/6 mice.

(A) Chemical structure of AS605240. (B-E) The clinical and histopathologic 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 in 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 0-14. 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 ± SD. #, P < 0.05 vs. Control; *, P < 0.05 vs. DSS plus vehicle. Original magnification, ×100 in D.

Figure 2. Effect of AS605240 on murine models of two established DSS colitis.

(A-C) The influence of AS605240 treatment on the recovery phase of acute DSS colitis. An averaged DAI (A) was assessed every other day as described in
Methods. On day 12 of the experimental period, paraffin embedded sections of the colon were stained with haematoxylin and eosin (B), and the histological scores were obtained (C). (D-F) The effect of AS605240 on chronic colitis model induced by three cycle DSS. An averaged DAI (D) was assessed every other day as described in Methods. On day 14 after AS605240 treatment, the colon was dissected for histological analysis with H&E staining (E) and the histological scores were gained (F). Data in A, C, D and F are means ± SD (n≥6 per group). #, P < 0.05 vs. Control; *, P < 0.05 vs. DSS plus vehicle. Original magnification, ×100 in B, E.

Figure 3. AS605240 reduces the levels of phospho-Akt in immunological cells from the inflamed colon tissue or spleen of mice fed with DSS.

(A and B) Immunohistochemical analysis with anti-phospho-Akt or anti-Akt antibody was performed on the colons from different treatment groups as described in Methods. Representative results from 5 independent animals are shown in A. The numbers of phospho-Akt- or Akt-expressing cells were determined as described in Methods and are shown in B (n = 5, per experimental group). (C and D) Splenocyte extracts from different treatment groups were assayed by western blot analysis using antibodies to phospho-Akt, Akt or Beta actin. Representative results from 4 independent experiments are
shown in C. Mean percentage of phospho-Akt signal relative to its Akt level from four mice in each group is demonstrated in D. Data in B and D are means ± SD, #, \( P < 0.05 \) vs. Control; * , \( P < 0.05 \) vs. DSS plus vehicle. Original magnification, ×400 in A.

Figure 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. Colons were obtained from different treatment groups and processed for immunofluorescence analysis using F4/80 monoclonal antibody and representative results from 4 independent animals are shown in A. The numbers of F4/80-expressing cells were determined as described in 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 6 independent animals are shown in C. The numbers of F4/80-expressing cells were determined on 6 independent animals as described in 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 in Methods (\( n = 5 \), per experimental group). Data in B, D and E are Means ± SD; #, \( P < 0.05 \) vs. Control; * , \( P <
0.05 vs. DSS plus vehicle. Arrows in A indicate the positive stained cells. Original magnification, ×200 in A.

Figure 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) The immunohistochemical analysis of CD4 and IFNγ in the colon of different treatment groups in acute established colitis. Representative results from 5 independent animals are shown in A. The numbers of CD4- or IFNγ-expressing cells were determined on 5 independent animals as described in Methods and are shown in B. (C and D) The immunohistochemical detection of CD4 and IFNγ in the colon from different treatment groups in chronic established colitis. Representative results from 5 independent animals are shown in C. The numbers of CD4- or IFNγ-positive cells were determined on 5 independent animals as described in Methods and are shown in D. Data in B and D are means ± SD; *, P < 0.05 vs. DSS plus vehicle. Original magnification, ×400 in A, C.

Figure 6. Influence of AS605240 on IL-1β, TNFα and IL-4 levels in the colon tissue of mice from DSS-induced acute colitis.

(A and B) RT-PCR analysis for IL-1β, TNFα, IL-4 was performed on total
RNAs extracted from the colons of different treatment groups as described in Methods. Representative results from 5 independent animals are shown in A. The levels of IL-1β, TNFα and IL-4 mRNA were normalized to the levels of Beta actin mRNA and showed in B. (C and D) Protein levels of TNFα and IL-1β in the colon from mice given vehicle (control), DSS plus vehicle or DSS plus AS605240 were determined by ELISA (n = 5 per group); Data in B, C and D are means ± SD; #, P < 0.05 vs. Control; * , P < 0.05 vs. DSS plus vehicle.
Figure 1
Figure 2
Figure 4
Figure 5
Figure 6