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RESEARCH PAPER

Blockade of adenosine A_{2B} receptors ameliorates murine colitis

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Background and purpose: The adenosine 2B (A_{2B}) receptor is the predominant adenosine receptor expressed in the colon. Acting through the A_{2B} receptor, adenosine mediates chloride secretion, as well as fibronectin and interleukin (IL)-6 synthesis and secretion in intestinal epithelial cells. A_{2B} receptor mRNA and protein expression are increased during human and murine colitis. However, the effect of the A_{2B} receptor in the activation of the intestinal inflammatory response is not known. In this study, we examined the effect of A_{2B} receptor antagonism on murine colitis.
Experimental approach: Dextran sodium sulphate (DSS)-treated mice and piroxicam-treated IL-$10^{-/-}$ mice were used as animal models of colitis. The A_{2B} receptor-selective antagonist, ATL-801, was given in the diet.
Key results: Mice fed ATL-801 along with DSS showed a significantly lower extent and severity of colitis than mice treated with DSS alone, as shown by reduced clinical symptoms, histological scores, IL-6 levels and proliferation indices. The administration of ATL-801 prevented weight loss, suppressed the inflammatory infiltrate into colonic mucosa and decreased epithelial hyperplasia in piroxicam-treated IL-$10^{-/-}$ mice. IL-6 and keratinocyte-derived chemokine (KC) concentrations in the supernatants of colonic organ cultures from colitic mice were significantly reduced by ATL-801 administration.
Conclusions and implications: Taken together, these data demonstrate that the intestinal epithelial A_{2B} receptor is an important mediator of pro-inflammatory responses in the intestine and that A_{2B} receptor blockade may be an effective therapeutic strategy to treat inflammatory bowel disease.
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Keywords: A_{2B} receptor; colitis; IBD; diarrhoea

Abbreviations: A_{2B}, adenosine 2B; DSS, dextran sodium sulphate; IBD, inflammatory bowel disease; IL, interleukin; KC, keratinocyte derived cytokine; MPO, myeloperoxidase; TUNEL, terminal deoxyuridine nick-end labelling

Introduction

Adenosine is a purine nucleoside that plays a key role in nucleic acid, energy and protein metabolism. As an extracellular autocoid, generated by the action of 5′ nucleotidase on adenosine 5′ monophosphate, it is a powerful mediator of cellular responses. Its levels increase under conditions of cellular stress, hypoxia or inflammation. Adenosine exerts its effects through one of the four known cell-surface G-protein-coupled receptors: adenosine A_1 (A_1), adenosine A_{2A} (A_{2A}), adenosine A_{2B} (A_{2B}) and adenosine A_3 (A_3) receptors (Alexander *et al*., 2008). Of these four adenosine receptors, the A_{2B} receptor is the predominant adenosine receptor expressed by colonic mucosa (Strohmeier *et al*., 1995). Indeed, in human colonic epithelia it is the only adenosine receptor expressed. It positively couples to adenylyl cyclase and it signals through cAMP (Strohmeier *et al*., 1995). The A_{2B} receptor is expressed at both the apical and basolateral surfaces of colonic epithelial cells. We recently demonstrated that A_{2B} receptor mRNA and protein expression are induced in epithelial cells during human and murine colitis (Kolachala *et al*., 2005a).

In the intestine, the A_{2B} receptor mediates adenosine-induced vectorial chloride secretion (Barrett, 1991; Strohmeier *et al*., 1995) which, when upregulated, leads to secretory diarrhoea. In addition, the A_{2B} receptor mediates the synthesis and secretion of interleukin (IL)-6 and fibronectin through the activation of the cAMP/CREB (cAMP response element-binding protein) signalling pathway. Interestingly, both IL-6 and fibronectin secretion, mediated by

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A_{2B} receptor stimulation, are apically polarized (Sitaraman *et al*., 2001). Studies in our laboratory and others have demonstrated that adenosine-mediated IL-6 secretion activates neutrophils (Sitaraman *et al*., 2001) and aids in neutrophil-mediated bacterial killing (Nadeau *et al*., 2002). Fibronectin released in the apical compartment significantly enhances the adherence and invasion of *Salmonella typhimurium* into epithelial cells (Walia *et al*., 2004; Dorsey *et al*., 2005).

The biological effects of adenosine in the intestine suggest that the A_{2B} receptor may be pro-inflammatory; hence, we hypothesized that A_{2B} receptor antagonists may have therapeutic benefits in inflammatory diseases, such as inflammatory bowel disease (IBD). To test this hypothesis, we studied the effect of a newly developed, highly specific inhibitor of the A_{2B} receptor, ATL-801, (*N*-[5-(1-cyclopropyl-2,6-dioxo-3-propyl-2,3,6,7-tetrahydro-1*H*-purin-8-yl)-pyridin-2-yl]-*N*-ethyl-nicotinamide; Wang *et al*., 2007) in the development of colitis using two established models of murine colitis (Cooper *et al*., 1993; Berg *et al*., 2002).

Materials and methods

Adenosine receptor-binding assay

Binding affinities of ATL-801 to adenosine receptors were evaluated using receptor plasmids (A_1, A_{2A}, A_{2B} and A_3) generated by PCR from human or mouse tissues and cloned into pcDNA 3. 1. HEK293 cells were stably transfected using lipofectin with selection in 1 mg mL^{-1} G418. Clones expressing high numbers of receptors were carried in 0.5 mg mL^{-1} G418. For radioligand-binding assays, membranes were prepared from transfected cells and the affinity of ATL-801 was determined by competition for radioligand binding. Membranes were incubated at room temperature for 2 h with radioligands (A_1 and A_3: [^{125}I]N^6-4-amino-3-iodo-benzyladenosine (^{125}I-ABA); A_{2A}: ^{125}I4-(2-[7-amino-2-[2-furyl] [1,2,4] triazolo[2,3-*a*] [1,3,5]triazin-5-yl-amino]ethyl) phenol (^{125}I-ZM-241385); A_{2B}: ^{125}I3-(4-aminobenzyl)-8-phenyloxyacetate-1-propyl-xanthine (^{125}I-ABOPX) 7, concentrations of ATL-801 ranging from 10^{-10} to 10^{-5}M and 1 U mL^{-1} adenosine deaminase, filtered over glass fiber filters, and retained radioactivity counted in a γ counter. Non-specific binding was measured in the presence of 100 μM 5′-(*N*-ethylcarboxamido) adenosine (NECA). IC_{50} values and inhibition constants (K_i values) were determined as described by Linden (1982)

Experimental animals

The Animal Care Committee of the Emory University, Atlanta, GA, approved all procedures performed on animals. Age- and sex-matched 6-week-old C57BL/6 and IL-10$^{-/-}$ mice on C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME, USA). C57BL/6 mice were used for experiments with dextran sodium sulphate (DSS). Mice were maintained on a 12-h dark–light cycle and allowed free access to powdered or pelleted diet and tap water under conditions of controlled temperatures (25 ± 2 °C).

Induction of DSS colitis

Colitis was induced in male C57BL/6 mice by oral administration of DSS (molecular wt. 50 000; ICN Biomedicals, Aurora, OH, USA) at 3% (wt/vol) in tap water *ad libitum* for 7 days. Age-matched male wild-type C57BL/6 mice receiving tap water served as controls. Mice were observed daily and evaluated for changes in body weight and development of clinical symptoms. Mice were given ATL-801 10 mg kg^{-1} diet (approximately 20 μg day^{-1} per mouse) during the administration of DSS.

Piroxicam-induced colitis

IL-10$^{-/-}$ mice spontaneously develop a chronic, T-cell-mediated, transmural colitis that shares many features with human Crohn's disease. On the basis of this model, there have been several clinical trials using IL-10 treatment for IBD in human patients (Schreiber *et al*., 2000). However, due to the inconsistency in the development of spontaneous colitis in IL-10$^{-/-}$ mice, Berg *et al*. (2002) have described rapid development of colitis in IL-10$^{-/-}$ mice treated with piroxicam, a non-steroidal anti-inflammatory drug. Accordingly, these mice were treated with piroxicam, 200 mg kg^{-1} diet, for 2 weeks to induce colitis. The diet containing piroxicam was prepared fresh every 2 days as described (Berg *et al*., 2002). Mice were given ATL-801 for a week prior to treatment with piroxicam, and ATL-801 treatment was continued during piroxicam administration. At 2 weeks after the induction of colitis, mice were killed and colonic tissue was removed for analysis.

Clinical score and histological scoring

Assessment of body weights, stool consistency and the presence of occult/gross blood by a guaic test (Hemoccult Sensa; Beckman Coulter, Fullerton, CA, USA) was performed daily for each mouse. Colitis was quantified with a clinical and histological score, as described by Cooper *et al*. (1993). Clinical score was based on weight loss, stool consistency and fecal blood (score range 0–12). Histological scoring was performed based on three variables, extent of inflammatory infiltrate, mucosal ulcers and severity of crypt damage (score range 0–11) (Cooper *et al*., 1993). IL-10$^{-/-}$ mouse colon histological scoring (ranging from 0 to 4) was performed as described by Berg *et al*. (2002) to assess intestinal lesions and their severity.

Cytokine measurements

In the DSS model, pro-inflammatory cytokines were measured by real-time PCR. Total RNA was extracted from DSS-treated, DSS + ATL-801 and control colonic tissue using the TRIzol reagent (Molecular Research Center Inc., Cincinnati, OH, USA). After quantification, a reverse transcription reaction was performed with 2 μg of each sample and oligo-dT primer, using the SuperScript First strand synthesis system for reverse transcription-PCR (Invitrogen, Carlsbad, CA, USA). The real-time iCycler sequence detection system (Bio-Rad, Hercules, CA, USA) was used for the real-time reverse transcription-PCR. Briefly, 3 ng of reverse-transcribed

cDNA, 500 nM of gene-specific primers and the iQ SYBR Green Supermix (Bio-Rad) were amplified at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The 36B4 expression levels were used as a reference, and fold induction was calculated using a standard curve. For graphical representation of quantitative PCR data, raw cycle threshold values (C_t values) obtained for treated mice were deducted from the C_t value obtained for internal 36B4 transcript levels, using the C_t method as follows: $\Delta\Delta C_T = (C_t \text{ target} - C_t \text{ 36B4})$ treatment$-(C_t \text{ target} - C_t$ 36B4) no treatment, and the final data were derived from $2^{-\Delta\Delta C_T}$. Primers used were: IL-6 sense 5′-ACAAGTCG GAGGCTTAATTACACAT-3′, antisense 5′-TTGCCATTGCAC AACTCTTTTC-3′; macrophage inhibitory protein (MIP)-2 sense 5′-AGTGAACTGCGCTGTCAATGC-3′, antisense 5′-AG GCAAACTTTTTGACCGCC-3′.

In the IL-$10^{-/-}$ model, pro-inflammatory cytokines were measured by ELISA in organ cultures. Colon organ cultures were prepared from IL-$10^{-/-}$ mice with and without ATL-801 along with piroxicam. Colons were dissected from mice and flushed with cold phosphate-buffered saline to remove fecal matter. Each colon was cut into 1 cm length and washed in Hanks' balanced salt solution with penicillin/streptomycin and cultured in serum-free RPMI-1640 supplemented with penicillin and streptomycin. Cultures were incubated at 37 °C in 5% CO_2. Supernatants were harvested after 24 h, centrifuged and stored at −80 °C before IL-6 and keratinocyte-derived chemokine (KC) levels were measured by ELISA (Gewirtz *et al*., 2001). T84 cells were plated on plastic. After reaching confluency, cells were treated with or without ATL-801 (100 μM) for 30 min and then treated with flagellin (100 ng mL^{-1}) for 5 h. IL-8 was measured in the supernatants by ELISA (Gewirtz *et al*., 2001).

Myeloperoxidase assay

Neutrophil infiltration into colon was quantified by measuring myeloperoxidase (MPO) activity as described previously (Castaneda *et al*., 2005). One unit of MPO activity was defined as the amount degrading 1 μmol of peroxide in 1 minute at 25 °C. The results were expressed as absorbance (per milligram protein).

Apoptosis and proliferation. Apoptotic cells were identified by fluorescent double immunofluorescent staining using caspase-3 and terminal deoxyuridine nick-end labelling (TUNEL). After deparaffinization and hydration, paraffin sections of colon were retrieved for antigens in a pressure cooker with sodium citrate (pH 6.0, 10 mM) for 10 min. After cooling, the sections were quenched in 3% H_2O_2 in methanol and then blocked with normal goat serum. Caspase-3 was detected with rabbit anti-cleaved caspase-3 IgG overnight at 4 °C followed by a labelled streptavidin–biotin staining method consisting of successive application of secondary antibody streptavidin, biotin horseradish peroxidase and cyanine-3 tyramide (Srinivasan *et al*., 2005). We performed TUNEL staining using the *in situ* cell death detection kit as described by the manufacturer (Roche Applied Science, Indianapolis, IN, USA). Nuclei were stained with Hoechst 33258 to count total crypt cell number. The apoptotic index was defined as number of caspase-3 and TUNEL-positive cells per crypt. Immunohistochemical staining for Ki67 was carried out using Vectastain ABC kit (Vector Laboratories Inc., Burlingame, CA, USA) according to the manufacturer's protocol. To perform the standard staining procedure, tissue sections were deparaffinized and rehydrated. Paraffin sections of colon were retrieved for antigens in a pressure cooker with sodium citrate (pH 6.0, 10 mM) for 10 min before the application of primary Ki67 antibody (1:500 dilutions, incubated 4°C overnight). Enzyme-conjugated secondary antibodies were applied and the specific staining was visualized after the addition of the enzyme-specific substrate. These tissues were counterstained by haematoxylin. Proliferation index was defined as number of Ki67-positive cells per crypt.

cAMP measurement

T84 cells were stimulated with adenosine (100 μM) or forskolin (10 μM) for 7 min, with or without ATL-801 (100 μM). Total cell lysates were processed in the presence of isobutylmethyl xanthine (1 mM) to inhibit phosphodiesterases. cAMP levels were measured in whole-cell lysates using a competitive cAMP immunoassay kit (Applied Biosystems, Bedford, MA, USA) according to the manufacturer's instructions. Luminescence was read with a Luminoscan Ascent plate reader (Thermo Labsystems, Needham Heights, MA, USA).

Electrophysiological studies

Electrophysiological studies were performed as described previously (Sugi *et al*., 2001; Kolachala *et al*., 2005b). Mice were killed by CO_2/hypothermia. The colon was removed and opened along the mesenteric border. The colon was then stripped of its external muscle by blunt dissection. A segment of mucosa from the distal colon was used for Ussing chamber studies. After attaining a sustained baseline I_{sc}, mucosal layers were stimulated with adenosine (100 μM) and forskolin (10 μM). Antagonist was added to both bathing solutions before stimulating with adenosine.

Statistical analysis

The data are presented as mean ± s.e. Statistical analysis was conducted using Student's *t*-test where $P<0.05$ was considered significant. In experiments wherein multiple group comparison was involved, we used ANOVA.

Reagents

Adenosine (Research Biochemicals Int., Natick, MA, USA), 5′-(*N*-ethylcarboxamido) adenosine and piroxicam (Sigma Aldrich, St Louis, MO, USA), isobutylmethyl xanthine (Biomol Research Laboratories Inc., Plymouth Meeting, PA), cAMP Screen Kit (Applied Biosystems), DSS (MP Biomedicals Inc., Aurora, OH, USA), IL-6 and KC Duoset ELISA kit (R&D Systems Inc., Minneapolis, MN, USA); ATL-801, the A_{2B} receptor-specific antagonist was a gift from Adenosine Therapeutics LLC. The radioiodinated radioligands were synthesized from non-radioactive precursors and carrier-free

[125]I and purified by HPLC in our laboratory. The diet containing the ATL-801 inhibitor was obtained from Harlan Tekad Research Diets (Madison, WI, USA). Antibodies Ki67 (Novocastra, Newcastle upon Tyne, UK), cleaved caspase-3 (Cell Signaling, Denvers, MA, USA), Vectastain immunohistochemistry kit (Vector Laboratories Inc.), TUNEL assay kit (Roche Applied Science). T84 cells are a colonic epithelial cell line, maintained in our laboratory.

Results

ATL-801 binds to the adenosine receptor

ATL-801 displayed high selectivity for the murine A_{2B} receptor ($K_i = 187 \pm 96.3$ nM, $n = 3$) and human A_{2B} receptor ($K_i = 19.32 \pm 2.2$ nM, $n = 4$) compared with its affinity to the murine A_1 receptor ($K_i = 5162 \pm 610$ nM, $n = 4$) and the A_{2A} receptor ($K_i = 3506 \pm 869$ nM 15% inhibition at 10 μm, $n = 3$). Radioligand binding to the mouse A_3 receptor was inhibited by only 10% at 10 μM (Table 1). Concentration–response curves of competitive binding assays, plotted as percent bound (B/B_0) against the log concentration of ATL-801, demonstrated that ATL-801 has high affinity and selectivity for the A_{2B} receptor (Figure 1).

A_{2B} receptor antagonism attenuates DSS-induced colitis

To investigate whether the A_{2B} receptor plays a role in the pathogenesis of intestinal inflammation, we used the DSS model of colitis. The mice were divided into four groups: (i) DSS alone, (ii) ATL-801 (10 mg kg^{-1}) + DSS, (iii) water alone and (iv) ATL-801 + water. The latter two groups served as control groups. The mice were assessed for the clinical signs of colitis (weight change, stool consistency and occult blood) (Cooper *et al*., 1993). All of the mice exposed to DSS alone developed clinical signs of colitis between days 5 and 6. As shown in Figure 2a, the mice receiving DSS alone had a clinical disease activity score of 9.4 ± 1.1. These mice had significant weight loss (5.9 ± 0.6%), frank blood in their stool (clinical score of 4) and diarrhoea. In contrast, mice given DSS and ATL-801 showed a significantly lower clinical score ($P < 0.003$; Figure 2a); some mice that received DSS + ATL-801 demonstrated occult blood loss, whereas others had no blood in their stool (clinical score of 0–1). Notably, mice given ATL-801 with DSS had solid stools, in contrast to DSS-fed mice, which had watery diarrhoea. The colons of mice fed ATL-801 and DSS showed solid stool pellets, which is reflected in their colon weight (0.48 ± 0.03 g), whereas the DSS-fed mice had no stool in their colons (0.26 ± 0.03 g, $P < 0.03$, $n = 5$).

In the next set of experiments, we tested the therapeutic efficacy of ATL-801 in the recovery (healing) phase of DSS-induced colitis. Colitis was induced by the administration of DSS in the drinking water for 7 days (colitic phase), after which the mice were switched to plain drinking water for 7 additional days (recovery phase). One group of mice was given a diet containing ATL-801 that was started during the recovery phase, whereas another group of mice continued on the diet without ATL-801. As shown in Figure 2b, mice given DSS and not receiving ATL-801 continued to lose weight during the recovery phase. In contrast, mice that received ATL-801 did not lose body weight and showed a trend towards recovery of their body weight. Taken together, these data demonstrate that mice were not only protected from DSS-induced colitis but also recovered faster when A_{2B} receptor signalling and function was inhibited.

As demonstrated by Okayasu *et al*. (1990), reduction of the colon length, which is used as a parameter of inflammation, correlated with the clinical data. Mice that received DSS alone had a 30% shorter colon length at the end of the experimental period (7 days of DSS) (4.5 ± 0.26 cm) compared with mice given water (6.5 ± 0.18 cm, $P < 0.0001$). ATL-801 prevented the reduction in colon length induced by DSS (6.04 ± 0.16 cm DSS + ATL-801, $P < 0.001$ compared with DSS alone).

DSS-induced colitis is characterized by the presence of inflammation of the colon, which is manifested by crypt destruction, mucosal damage, epithelial erosions and infiltration of inflammatory cells into the mucosal tissue. Tissues collected from mice exposed to DSS were examined histologically and compared with those that received DSS + ATL-801. Histological scoring was performed without the

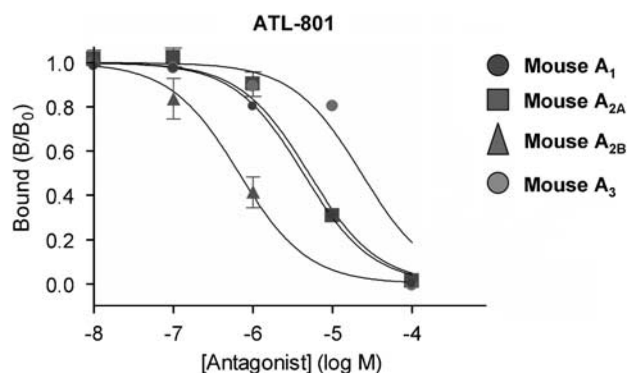

Figure 1 ATL-801 has high affinity and selectivity for the A_{2B} receptor. HEK293 cells were stably transfected with A_1, A_{2A}, A_{2B} and A_3 plasmids. Radioligand-binding assays were performed using ATL-801, as described in the Materials and methods section. Data are presented as percent bound (B/B_0) against the log concentration of adenosine receptor antagonist.

Table 1 Affinities of ATL-801 for a range of human and mouse adenosine receptors

hA_1 K*i* (nM)	*hA_{2A} low* K*i* (nM)	*hA_{2B}* K*i* (nM)	*hA_3* K*i* (nM)	*Mouse A_1* K*i* (nM)	*Mouse A_{2A}* K*i* (nM)	*Mouse A_{2B}* K*i* (nM)	*Mouse A_3* K*i* (nM)
4983 ± 2661 $n = 7$	662.7 ± 90.9 $n = 6$	19.32 ± 2.2 $n = 4$	6314 ± 1828 $n = 6$	5162 ± 610 $n = 4$	15% (3506 ± 869) inhibition at 10 μm $n = 3$	187 ± 96.3 $n = 3$	10% inhibition at 10 μm $n = 3$

Affinity values (as K_i) were obtained using HEK293 cells, stably transfected with the receptors shown (see Materials and methods for details).

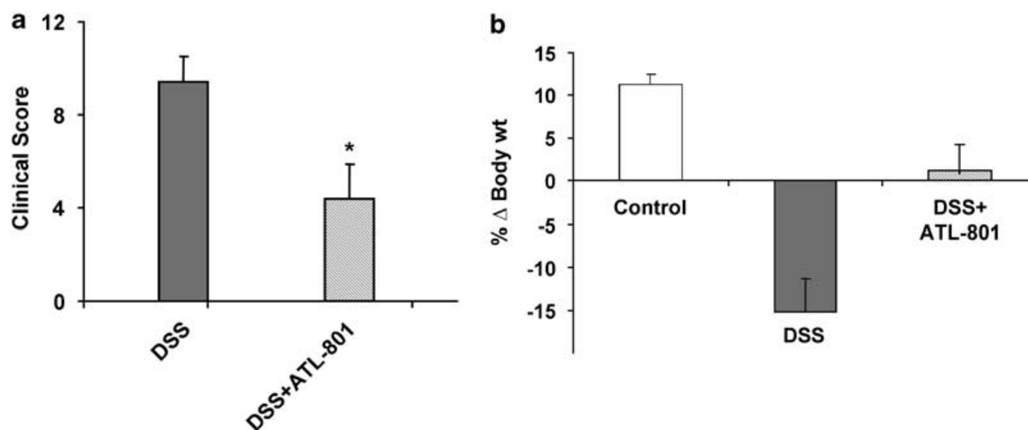

Figure 2 A_{2B} receptor antagonism attenuates dextran sodium sulphate (DSS)-induced colitis. Mice were weighed and randomized into four groups: (i) DSS alone (3%), (ii) DSS + ATL-801, (iii) water and (iv) ATL-801 alone (groups three and four served as controls; data from group three are shown here, which was similar to ATL-801 alone). Mice were killed after 7 days of ingesting DSS in drinking water. (**a**) Disease severity was assessed as described in the Materials and methods section. The data are presented as clinical scores. (**b**) Percent change in body weight in mice given DSS or DSS + ATL-801. Results are expressed as mean ± s.e., $n = 5$. *$P < 0.003$.

knowledge of the treatments, as described previously (Cooper *et al*., 1993). The histological data corroborated the clinical score and confirmed the protective role of ATL-801 against the development of colitis. As shown in Table 2 and Figure 3a, DSS-treated mice exhibited obvious signs of colon inflammation and tissue destruction. These mice had extensive crypt damage, epithelial erosion/ulceration, crypt abscess formation and infiltration of inflammatory cells into the lamina propria and muscularis mucosa of colonic sections (Table 2 and Figure 3a). In contrast, histological analysis of the sections from mice fed ATL-801 and DSS revealed significantly reduced histological inflammation, and these mice appeared to be protected from DSS-associated mucosal injury, having fewer inflammatory infiltrates and ulcerations (Figure 3a). Overall, a lower histological score was observed in mice given DSS + ATL-801, compared with the score in the DSS group ($P < 0.05$; Table 2). Histological signs of inflammation were not detected in the control groups that received water or water + ATL-801.

To confirm the histological findings with respect to granulocyte accumulation, we measured the activity of MPO in the colonic tissue. MPO is an enzyme specific to granulocyte lysosomes, and, therefore, it directly correlates with the number of neutrophils. Mice fed with DSS alone had significantly increased MPO activity (twofold, $P < 0.03$) compared with mice given DSS + ATL-801 (Figure 3b).

A_{2B} receptor antagonism suppresses pro-inflammatory cytokines associated with DSS-induced colitis

As the DSS-induced inflammatory response is associated with the production of pro-inflammatory cytokines, such as IL-6, we measured IL-6 mRNA levels in the colonic mucosal tissue of DSS-treated mice and compared them with mice that received DSS along with the antagonist. As shown in Figure 4a, mice treated with ATL-801 showed significantly lower levels of the mRNA for IL-6, compared with mice that received DSS alone ($P < 0.01$). Similarly, as shown in Figure 4b, levels of the mRNA for MIP-2 (a chemokine

Table 2 Histological assessment of colitis in mice treated with or without ATL-801 after ingestion of 3% DSS in drinking water for 6 days

Parameters	*Water*	*DSS*	*DSS + ATL 10 mg kg^{-1}*
Crypt damage	0	2.8 ± 1.5	1.9 ± 0.6
Inflammation	0.4 ± 0.2	3.7 ± 0.2	1.2 ± 0.2
Ulceration	0	2.6 ± 0.6	1.0 ± 1.0
Total lesion score	0.4 ± 0.2	9.1 ± 2.3	4.1 ± 1.8*

Abbreviation: DSS, dextran sodium sulphate. *$P < 0.05$.

secreted by macrophages and epithelial cells) were significantly lower in the ATL-801-treated group ($P < 0.02$) compared with mice that received DSS alone. Taken together, these data demonstrate that ATL-801 attenuated not only the clinical characteristics but also the histological features and inflammatory markers associated with DSS-induced colitis.

A_{2B} receptor antagonism reverses the effect of DSS colitis-induced changes in epithelial cell proliferation and apoptosis

Next, we examined whether blockade of the A_{2B} receptor affected epithelial cell survival in colitic mice. Epithelial cell proliferation was assessed by determining the number of cells that were positive for Ki67, and apoptosis was assessed with caspase-3 and TUNEL staining. As shown in Figure 5a, the proliferation index represented by the percent of Ki67-positive cells per crypt was significantly decreased in DSS-treated mice compared with DSS + ATL-801-treated mice (control vs DSS $P < 0.001$, DSS vs DSS + ATL-801, $P < 0.001$, $n = 5$). In addition, ATL-801 significantly inhibited the apoptosis associated with DSS-induced colitis. As shown in Figure 5b, mice treated with DSS showed an increase in apoptotic cells per crypt ($P < 0.01$).

A_{2B} receptor antagonism inhibits colitis in IL-$10^{-/-}$ mice

We next determined the effects of ATL-801 in the development of colitis in the IL-$10^{-/-}$ piroxicam model of colitis.

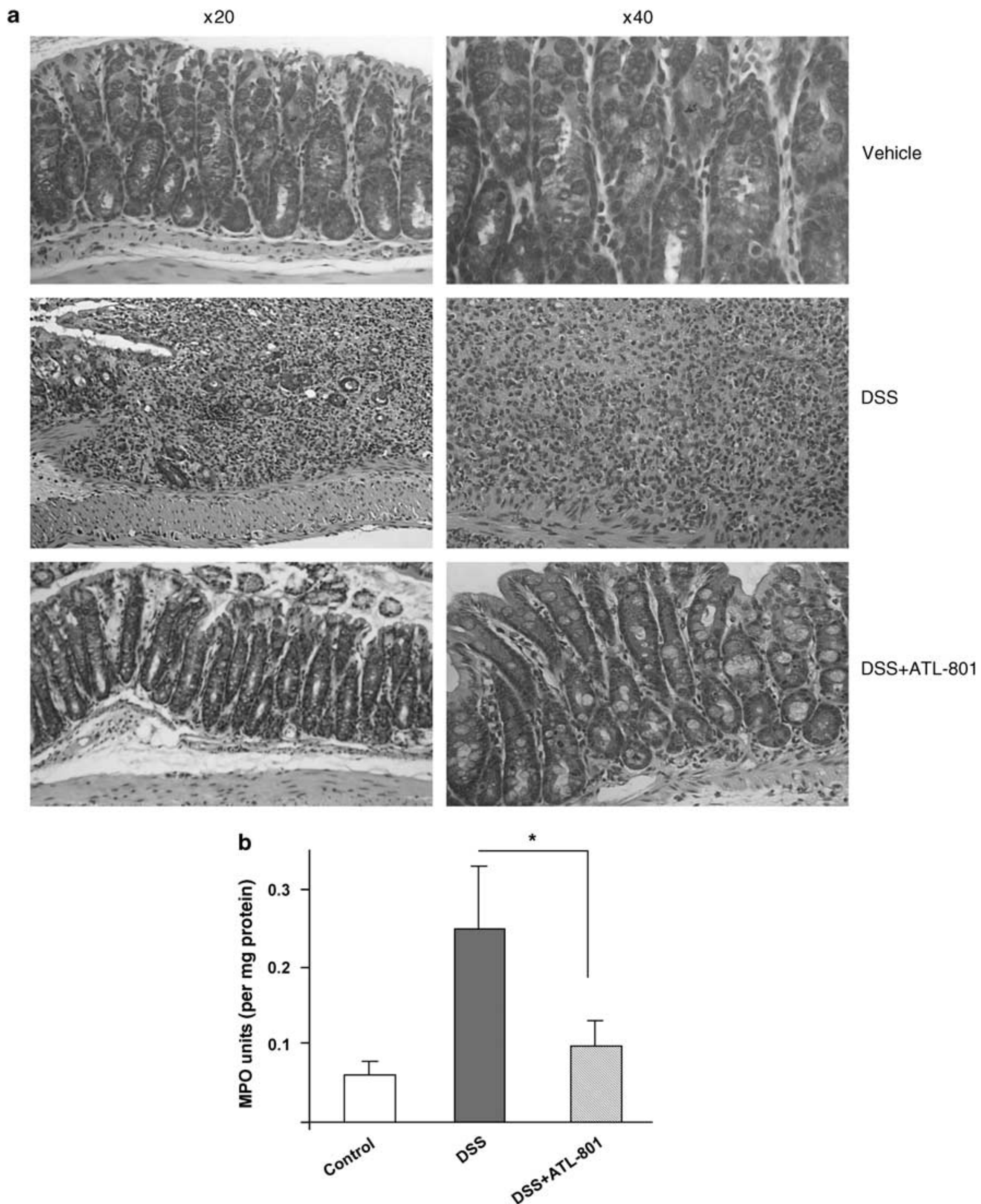

Figure 3 A_{2B} receptor antagonism inhibits inflammation associated with dextran sodium sulphate (DSS) ingestion. Mice were weighed and randomized into four groups: (i) DSS alone (3%), (ii) DSS + ATL-801, (iii) water and (iv) ATL-801 alone (groups three and four served as controls; data from group three are shown here, which was similar to ATL-801 alone). Mice were killed after 7 days of ingesting DSS in drinking water. (**a**) Colons were fixed in formalin, paraffin-embedded, sectioned and stained with haematoxylin and eosin. Representative sections of colons are shown. Control (upper panels), DSS alone (middle panels) and DSS + ATL-801 (bottom panels), $n = 5$. (**b**) Colons were snap frozen in liquid nitrogen, and myeloperoxidase was measured as an index of neutrophil infiltration into the injured tissue, as described in the Materials and methods section. Each bar represents mean ± s.e. $n = 5$ animals per group, $^*P < 0.038$.

IL-$10^{-/-}$ mice were treated with piroxicam ± ATL-801 in their diet, as described in the Materials and methods section. The mice were killed at the end of 4 weeks, colonic tissues were analysed and histological scoring (ranging from 0 to 4) was performed to assess intestinal lesions and their severity (Berg *et al.*, 2002). The IL-$10^{-/-}$ mice receiving piroxicam showed significant weight loss, whereas IL-$10^{-/-}$ mice that received piroxicam + ATL-801 maintained their body weight (Figure 6a). Furthermore, marked immune cell infiltrates were found in the mucosa and submucosa. Epithelial

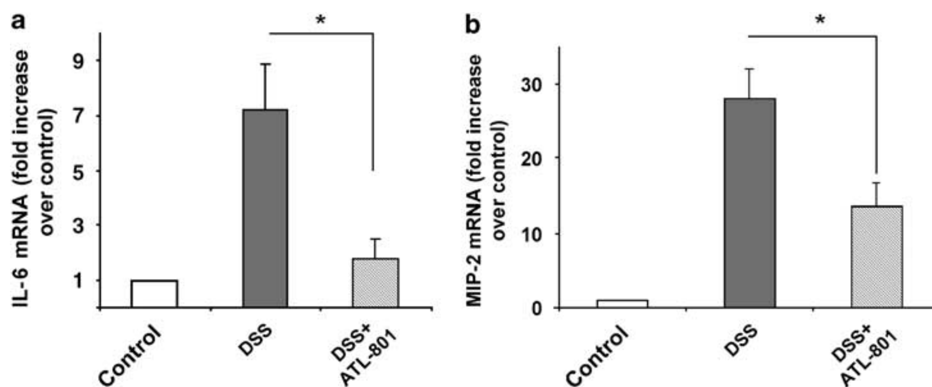

Figure 4 A_{2B} receptor antagonism decreased levels of mRNA for cytokines in colitis. Total RNA from colonic mucosal strips was isolated, reverse transcribed and subjected to real-time PCR analysis for IL-6 or macrophage inhibitory protein (MIP)-2 mRNA, as described in the Materials and methods section. (**a**) Normalized IL-6 mRNA is represented as fold increase over control mice, $n=5$, $P<0.01$. (**b**) Normalized MIP-2 mRNA is represented as fold increase over control mice, $n=5$, $^*P<0.002$.

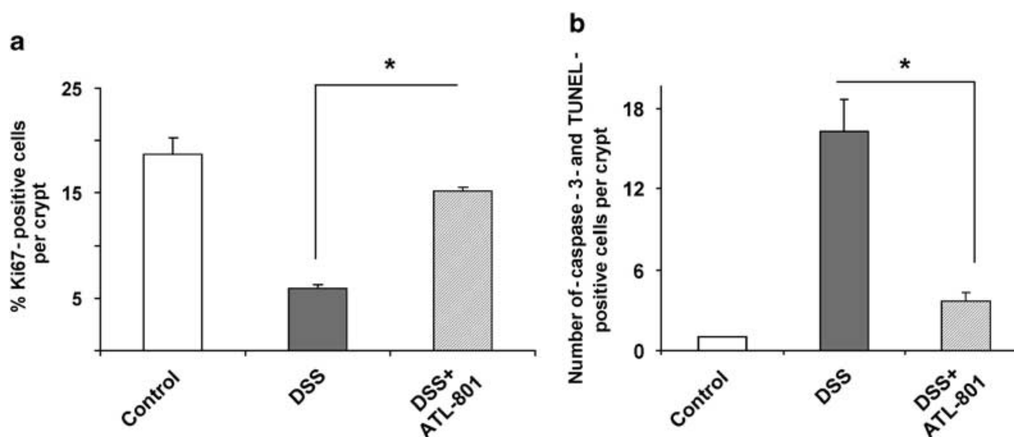

Figure 5 A_{2B} receptor antagonism reversed the effect of epithelial proliferation and apoptosis in colons from mice with DSS-induced colitis. Paraffin-embedded sections were stained for Ki67 or caspase-3 and TUNEL as described in the Materials and methods section. (**a**) Proliferation index (percent Ki67-positive cells per crypt). (**b**) Apoptotic index (number of caspase-3- and TUNEL-positive cells per crypt). Data represent averages of a total of 30 crypts per mouse, three mice per group, $^*P<0.001$.

hyperplasia was common in areas with inflammation (Figure 6b, upper panel). However, IL-$10^{-/-}$ mice treated with ATL-801 showed reduced inflammatory infiltrate as well as reduced epithelial hyperplasia (Figure 6b, lower panel). The mean histological score was significantly higher in IL-$10^{-/-}$ mice treated with piroxicam (2.8 ± 1.0) compared with the ATL-801-treated groups, which showed a histological score of 1.3 ± 0.3 ($P<0.009$). The IL-$10^{-/-}$ mice treated with ATL-801 alone showed normal histology (data not shown). Further, the IL-$10^{-/-}$ mice that received piroxicam showed significantly reduced colon length (7.0 ± 0.25 cm) compared with the IL-$10^{-/-}$ mice that received ATL-801 + piroxicam (8.4 ± 0.23 cm; $P<0.008$). Taken together, these data suggest that inhibition of the A_{2B} receptor reduced the extent and severity of colitis in the IL-$10^{-/-}$ mice/piroxicam model.

A_{2B} receptor antagonism suppresses the synthesis of pro-inflammatory cytokines induced by piroxicam in IL-$10^{-/-}$ mice

To determine the effect of A_{2B} receptor inhibition on pro-inflammatory cytokine synthesis, we obtained colonic tissue from IL-$10^{-/-}$ mice treated with piroxicam, with or without ATL-801. IL-6 and KC levels were measured in the colon culture supernatant, as described in the Materials and methods section. IL-6 levels were upregulated in colon cultures of IL-$10^{-/-}$ mice treated with piroxicam alone, compared with the piroxicam + ATL-801-treated mice ($P<0.006$) and untreated mice (Figure 7a). A similar inhibition of KC secretion was seen in tissue from mice treated with piroxicam + ATL-801 compared with tissue from mice given piroxicam alone (Figure 7b). ATL-801, alone, had no effect on IL-6 or KC levels (data not shown).

A_{2B} receptor antagonism inhibits proliferation and enhances apoptosis in IL-$10^{-/-}$ mice with piroxicam-induced colitis

We further examined the effect of ATL-801 on epithelial cell survival and apoptosis in IL-$10^{-/-}$ mice treated with piroxicam. Epithelial cell proliferation was assessed by determining the number of cells staining positive for Ki67, whereas apoptosis was assessed by caspase-3 and TUNEL staining. As shown in Figure 8a, the proliferation index, represented by the number of Ki67-positive cells

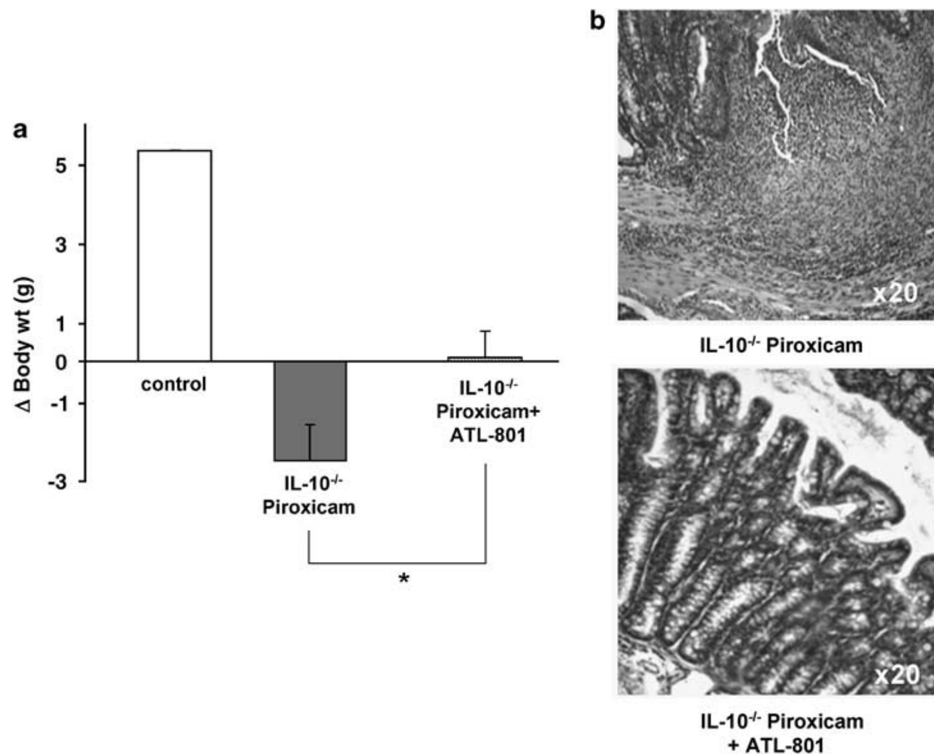

Figure 6 A_{2B} receptor antagonism inhibits colitis in IL-$10^{-/-}$ mice. IL-$10^{-/-}$ mice were weighed and randomized into four groups: untreated, IL-$10^{-/-}$ mice that received piroxicam alone (200 mg kg^{-1} diet), IL-$10^{-/-}$ mice that received piroxicam + ATL-801 (10 mg kg^{-1}) and IL-$10^{-/-}$ mice that received ATL-801 alone ($n = 5$ per group). (**a**) Body weight change from baseline (grams), $^{*}P < 0.05$ (**b**) Colons were fixed in formalin, paraffin-embedded, sectioned and stained with haematoxylin and eosin. Representative sections of colons from IL-$10^{-/-}$ mice that received piroxicam alone (top panel) or piroxicam + ATL-801 (bottom panel) are shown (magnification $\times$ 20).

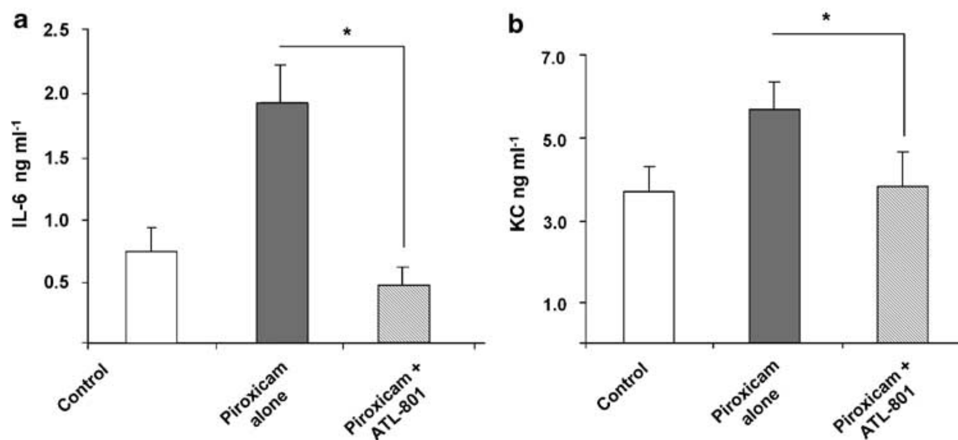

Figure 7 The A_{2B} receptor antagonist suppressed the production of pro-inflammatory cytokines. Colonic tissue culture supernatants, obtained from IL-$10^{-/-}$ mice treated with piroxicam, with or without ATL-801 ($n = 5$ per group), were processed for IL-6 (**a**) and KC (**b**) secretion, as described in the Materials and methods section, $^{*}P < 0.006$.

per crypt, showed a significant increase in the IL-$10^{-/-}$ mice that received piroxicam compared with the piroxicam + ATL-801-treated mice ($P < 0.001$, $n = 4$). In addition, the IL-$10^{-/-}$ mice treated with piroxicam + ATL-801 showed an increased apoptotic index (number of TUNEL- and caspase-3-positive cells per crypt) compared with the IL-$10^{-/-}$ mice treated with piroxicam alone (Figure 8b). These data are consistent with the inhibition of hyperplasia that is characteristic of colitis associated with piroxicam-treated IL-$10^{-/-}$ mice.

The A_{2B} receptor antagonist, ATL-801, inhibits adenosine-mediated cAMP levels and short circuit current (I_{sc})

To see the functional aspects of A_{2B} receptor inhibition, T84 colonic epithelial cells were treated with adenosine

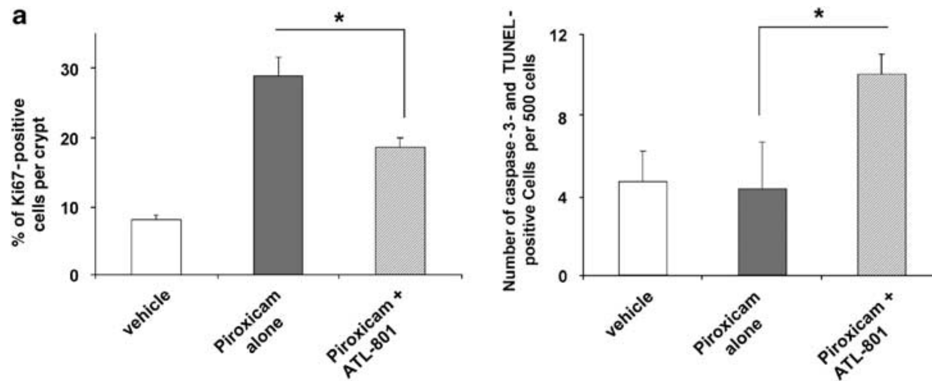

Figure 8 The A_{2B} receptor antagonist suppressed epithelial proliferation and induced apoptosis in piroxicam-induced colitis. Paraffin-embedded colonic sections from IL-$10^{-/-}$ mice treated with piroxicam, with or without ATL-801, were processed for Ki67 analysis (**a**) or caspase-3 and TUNEL staining (**b**) as described in the Materials and methods section. Data represent average proliferation and apoptotic indices, respectively, from a total of 30 crypts per section, from three independent mice, *$P<0.001$.

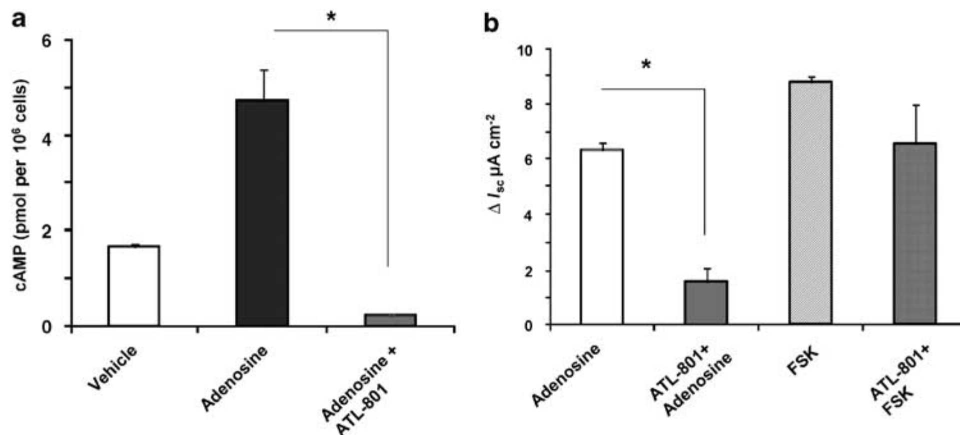

Figure 9 The A_{2B} receptor antagonist, ATL-801, inhibited adenosine-induced cAMP levels and short circuit current (I_{sc}). (**a**) Monolayers of T84 cells were grown on snap wells and mounted in an Ussing chamber. The cells were stimulated with adenosine (100 mM) after pretreatment with or without ATL-801. cAMP levels were measured as described in the Materials and methods section. Data are represented as fold increase over control, ATL-801 + adenosine and adenosine alone $P<0.0025$, $n=3$. (**b**) Colonic mucosal strips from the distal colon were mounted in an Ussing chamber. The increase in I_{sc} was determined as described in the Materials and methods section. After obtaining a baseline I_{sc}, the mucosa was stimulated with adenosine (100 mM) or forskolin (FSK; 10 μM). In addition, colonic mucosal strips were pretreated with ATL-801 (100 mM), 5 min before stimulating with adenosine or FSK (10 mM). Data represent ΔI_{sc} (ATL-801 + adenosine) vs adenosine alone, *$P<0.004$, $n=3$.

(100 mM), with or without ATL-801 (100 mM). As shown in Figure 9a, ATL-801 significantly inhibited adenosine-mediated changes in cAMP levels ($P<0.002$, $n=6$; Figure 9a). To determine the effect of ATL-801 on adenosine-induced short circuit currents (I_{sc}) *in vivo*, mucosal strips from murine colons were placed in Ussing chambers and the baseline I_{sc} was measured. As shown in Figure 9b, stimulation of the mouse colonic mucosa with adenosine (100 mM) showed a maximum ΔI_{sc} and addition of ATL-801 (100 μM) virtually abolished this adenosine-induced I_{sc} ($P<0.004$, $n=3$). However, as shown in Figure 9b, ATL-801 did not affect forskolin (a direct activator of adenylyl cyclase)-mediated I_{sc}. Taken together, these data demonstrate that ATL-801 is a potent inhibitor of adenosine-mediated cAMP response and I_{sc}. Furthermore, these data demonstrate that the ATL-801 effect is specific to adenosine-induced I_{sc} and does not affect other cAMP-dependent I_{sc} responses.

Discussion

In this study, we report that a selective A_{2B} receptor antagonist, ATL-801, ameliorates experimental colitis in two mouse models that represent both acute and chronic forms of gut inflammation. In the first model, colitis was induced by the oral administration of DSS. ATL-801 protected these mice from DSS-induced colitis and reduced inflammatory cell infiltration, focal crypt damage, epithelial injury and ulceration. One of the findings was that mice with DSS-induced colitis and treated with ATL-8801 showed a marked reduction in diarrhoea, as demonstrated by solid stool pellets and increased stool weight, compared with mice ingesting with DSS alone. This result is consistent with a known effect of adenosine in mediating cAMP-dependent chloride secretion and secretory diarrhoea (Li *et al*., 2005). Although the A_{2B} receptor has been shown to mediate

chloride secretion in epithelial cell lines *in vitro*, this is the first demonstration of an effect of the A_{2B} receptor on diarrhoea *in vivo*. diarrhoea associated with intestinal inflammation is multifactorial. The inhibition of adenosine-induced chloride secretion by ATL-801 in colonic mucosal strips *ex vivo*, in conjunction with the inhibition of colitis-associated diarrhoea by ATL-801, suggests that inhibition of chloride secretion is a possible mechanism by which ATL-801 reduced diarrhoea in the DSS-induced colitis model.

In addition to the effect of ATL-801 on diarrhoea, inflammatory infiltration was reduced in both DSS and IL-$10^{-/-}$ mouse models of colitis. In the DSS model, which is characterized by neutrophil infiltration, crypt injury and ulcers, ATL-801 reduced the extent of neutrophil infiltration, as assessed by histology and the MPO assay. There was significantly less ulceration and crypt damage after ATL-801 treatment, and this effect was dose dependent. Similarly, ATL-801 protected animals against the chronic inflammatory infiltrate and epithelial hyperplasia that characterized colitis in IL-$10^{-/-}$ mice. Further, ATL-801 reversed changes in epithelial cell survival associated with DSS-induced or piroxicam/IL-$10^{-/-}$-associated colitis. DSS-induced colitis has been reported to be associated with an inhibition of cell proliferation and increased apoptosis (Vetuschi *et al*., 2002), whereas piroxicam-induced colitis in IL-$10^{-/-}$ mice has been shown to be associated with epithelial hyperplasia. Finally, the protective effect of ATL-801 on colitis was reflected by reduced pro-inflammatory cytokine secretion from colons of colitic mice.

The A_{2B} receptor mediates biological responses in several tissues and, depending on the cell type or tissue, the A_{2B} receptor mediates pro- or anti-inflammatory effects. For example, in the lung, the A_{2B} receptor mediates bronchoconstriction in asthma by mediating pro-inflammatory and pro-fibrogenic effects. A_{2B} receptor antagonists are highly effective at reducing pulmonary injury and inflammation (Sun *et al*., 2006). In contrast, the A_{2B} receptor has been shown to mediate a protective effect against endotoxin-induced sepsis, by regulating inflammation and vascular adhesion (Yang *et al*., 2006) and against cardiac fibrosis, by modulating cardiac fibroblastic proliferation (Chen *et al*., 2004). In fibroblasts and endothelial cells, the A_{2B} receptor has been shown to inhibit tumour necrosis factor alpha (TNF-α) synthesis and signalling (Zhang *et al*., 2005; Kreckler *et al*., 2006). In addition, the A_{2B} receptor can act in an anti-inflammatory manner; it modulates macrophage function by inhibiting the production of TNF-α and IL-1β, whereas stimulating IL-10 and inhibiting cell proliferation (Xaus *et al*., 1999; Nemeth *et al*., 2005; Sipka *et al*., 2005; Kreckler *et al*., 2006). Our results suggest that during colitis, inhibition of the A_{2B} receptor may offer a regulatory signal that suppresses the pro-inflammatory effects, thereby ameliorating tissue damage.

Both immune cells and colonic epithelial cells express A_{2B} receptors. In colonic epithelial cells, the A_{2B} receptor is the only adenosine receptor expressed, whereas immune cells express multiple adenosine receptor subtypes (Cronstein *et al*., 1990; Salmon and Cronstein, 1990). With the combination of pharmacological data using selective ligands as well as gene knockout mice, important advances have been made towards an explanation of the role of adenosine receptors in IBD. Recent studies suggest a protective role for the A_{2A} receptor subtype (Odashima *et al*., 2005; Naganuma *et al*., 2006) in some models of murine colitis. The protective effect of the A_{2A} receptor on the development of colitis has been shown to be mediated by A_{2A} receptors expressed in T cells (Naganuma *et al*., 2006). A_3 receptors have been demonstrated to mediate a pro-inflammatory effect in two models of colitis, similar to our observations with A_{2B} receptor antagonism (Mabley *et al*., 2003). Whether or not the protective effect of A_{2B} receptor antagonism is mediated by epithelial cells, immune cells or both is unclear at this time. We have previously demonstrated that A_{2B} receptor expression is upregulated in epithelial cells in animal models (DSS) as well as in human IBD (Kolachala *et al*., 2005a). We further demonstrated that the increase in A_{2B} receptor expression is mediated by TNF-α (Kolachala *et al*., 2005a). Moreover, adenosine levels are highly upregulated in luminal fluid during active flare-ups of IBD, and adenosine induces recruitment of the A_{2B} receptor, preferentially, to the apical membrane. In addition, ATL-801 is a highly polar compound whose systemic bioavailability is restricted by its limited absorption through the gastrointestinal tract (J Linden, unpublished data). Given these effects of adenosine and the limited absorption of ATL-801, it is likely that the inflammatory response and its inhibition by ATL-801 are, at least in part, mediated by the colonic epithelial A_{2B} receptor. However, further studies are required to delineate the role of mucosal vs immune cell A_{2B} receptors in the development of colitis.

In conclusion, the data presented in this study demonstrate important anti-inflammatory effects of the selective A_{2B} receptor antagonist, ATL-801. Thus, modulation of A_{2B} receptor-mediated signalling through selective A_{2B} receptor antagonism could provide a firm basis for developing adenosine A_{2B} receptor antagonists as a new therapeutic approach for patients with IBD.

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