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The adenosine A_{2A} receptor agonist CGS 21680 fails to ameliorate the course of dextran sulphate-induced colitis in mice

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Abstract

Objective—In this study we investigated the effect of CGS 21680 (2-*p*-(2-Carboxyethyl) phenethylamino-5 -N-ethylcarboxamidoadenosine hydrochloride), an adenosine A_{2A} receptor agonist, in a model of dextran sulphate sodium (DSS)-induced colitis.

Methods—NMRI mice were fed 5 % (w/v) DSS, and were treated intraperitoneally with 0.5 mg/kg CGS 21680 or vehicle for 10 days. Changes of bodyweight, colon length, the incidence of rectal bleeding, levels of macrophage inflammatory protein (MIP)-1 α , MIP-2, interferon γ , interleukin (IL)-1 β , IL-12 and tumour necrosis factor- α from homogenates of colon biopsies, and the release of [³H]acetylcholine (ACh) from longitudinal muscle strip were determined.

Results—DSS significantly decreased bodyweight, colon length, and it increased the incidence of rectal bleeding and levels of MIP-1 α , MIP-2 and IL-1 β compared to DSS-untreated animals. CGS 21680 had no effect on these changes. No change could be observed in release of ACh in DSS-induced colitis with or without CGS 21680.

Conclusion—In summary, CGS 21680 is ineffective in ameliorating DSS-induced colitis in mice.

Keywords

Dextran sulphate; Colitis; Adenosine A_{2A} receptor; CGS 21680

Introduction

Crohn's disease and ulcerative colitis, collectively termed inflammatory bowel disease (IBD), are characterised by chronic inflammation of the gastrointestinal tract with unknown etiology. The recent establishment of animal models for IBD has provided evidence that immune dysregulation and altered cytokine secretion patterns may play an important role in the pathology of IBD [1]. Some of the most widely used animal models of colitis comprise the administration of either toxic chemicals or immune reactive substances [2–6]. One of these animal models of IBD is colitis induced by the oral administration of dextran sulphate sodium (DSS) [7]. Mice with colitis induced by DSS exhibit lymphoid hyperplasia, inflammatory cell infiltration, focal crypt damage as well as epithelial injury and ulceration [4,7,8]. Although the

murine DSS model of IBD differs from the human disease in some ways, it is a widely used preclinical model for testing the efficacy of treatments for IBDs [4,9,10].

It is well recognised that certain naturally occurring purines are effective modulators of the immune system. Among these purines adenosine is the best characterised, and has been shown to affect almost all aspects of an immune response [11–16]. Adenosine and its analogs can ameliorate the course of a variety of inflammatory diseases including endotoxin shock [17], rheumatoid arthritis [18], pleural inflammation [19], uveitis [20], but adenosine can lead to the exacerbation of inflammation and damage [21]. The beneficial effects of adenosine are partly mediated by the inhibition of deleterious immune-mediated processes, including the release of injurious proinflammatory cytokines and free radicals [22–28]. Adenosine, following its release from cells or after being formed extracellularly, diffuses to cell membrane of surrounding cells where it interacts with four receptors: A₁, A_{2A}, A_{2B} and A₃. [29,30]. All of these receptors are members of the G protein-coupled family of receptors [29], and are expressed on the surface of macrophages and lymphocytes [31–36]. The most potent anti-inflammatory and immuno-suppressive effects of adenosine are generally attributed to occupancy of A_{2A} receptors expressed on immune cells [16]. Because relatively little is known about the effect of A_{2A} receptor stimulation on the development of IBD, in this work we studied the therapeutic efficacy of A_{2A} receptor activation in DSS-induced colitis in mice.

Materials and methods

Materials

Reagents were obtained from the following sources: dextran sulphate sodium salt (DSS; MW 36,000–50,000) was from ICN Pharmaceuticals (Costa Mesa, CA); CGS 21680 (2-*p*-(2-Carboxyethyl)phenethylamino-5'-N-ethylcarboxamidoadenosine hydrochloride), dimethyl sulfoxide (DMSO), NaF, NaCl, Na-deoxycholate, Na₃VO₄, Tris-HCl, NP-40, phenylmethanesulfonyl fluoride (PMSF), aprotinin, leupeptin, pepstatin were from Sigma (St. Louis, MO); NMRI mice were from TOXICOOP (Budapest, Hungary); and specific cytokine ELISA kits were from R&D Systems (Minneapolis, MN).

Induction of colitis and treatment

Male NMRI mice, 8 wk of age, weighing 25–27 g were used for these studies. Mice were kept in individual cages in the Animal Unit for 7 days before starting the experiments. Animals received food and water *ad libitum*, and lighting was maintained on a 12-h cycle. All procedures were carried out in accordance with the European Community guidelines for the use of experimental animals and those of the institutional ethics committee.

Mice were fed 5 % (w/v) DSS (molecular mass 36–50 kDa), dissolved in the drinking water, throughout the experiment [37]. CGS 21680 was administered intraperitoneally at a dose of 0.5 mg/kg/day throughout the entire study. Control mice were treated with vehicle which was 10 % (v/v) DMSO in saline.

Evaluation of colitis severity and drug effects

Parameters recorded in the experiments were bodyweight, colon length, and bleeding from the rectum as determined by ocular inspection. Mice were weighed on every day and colitis-induced weight change was expressed as a percentage of the original weight. Mice were killed by cervical dislocation, and the colon resected between the ileocecal junction and the proximal rectum, close to its passage under the pelvisternum. The colon was placed on a non-absorbent surface and its length measured with a ruler.

Cytokine production

A colon biopsy was removed and snap frozen in liquid nitrogen. The samples were then homogenised (100 mg/ml) in modified RIPA buffer containing 50 mM Tris-HCl pH 7.4, 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na₃VO₄, 1 mM NaF, and protease inhibitors (1 mM PMSF, 1–1 µg/ml aprotinin, leupeptin and pepstatin). Samples were centrifuged at 15,000 × g for 15 min, and the supernatants frozen at –80 °C until assay. Cytokine levels were determined using ELISA.

[³H]acetylcholine (ACh) release

Experiments were performed at 37 °C in modified Krebs' solution containing (mM) 113 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, which was continuously saturated with 25 NaHCO₃, and 11.5 D-glucose, + 5 % carbogen gas (95 % O₂ CO₂). Longitudinal muscle strips were incubated for 45 min in 1 ml of modified and saturated Krebs' solution in the presence of 4 µCi/ml [³H]ACh (82.0 Ci/mmol; Amersham Pharmacia Bio-tech, Arlington Heights, IL). After incubation the strips were transferred to thermoregulated (37 °C) organ baths (internal volume, 0.2 ml). The tissue was perfused at a rate of 1 ml/min. The perfusing solution contained 10 µM hemicholinium-3 to inhibit reuptake of [³H]choline originating from hydrolysis of [³H]ACh. After 60 min of preperfusion, the outflow was collected in 10-ml (10-min) fractions for an additional 60 min. Tissues were stimulated at 30 V, 5 min, 2 Hz, 2 msec (600 pulses), then 30 V, 1 min, 10 Hz, 2 msec (600 pulses), via platinum electrodes using a Grass S88 stimulator during the 3rd and 6th collection periods (S₁ and S₂). At the end of the perfusion period, the tissue was removed from the organ bath and homogenised in 500 µl of 10 % trichloroacetic acid. To determine radioactivity released from the tissue, aliquots (0.5 ml) of the perfusate samples were assayed, and a 100-µl aliquot was assayed for tissue radioactivity. Radioactivity was determined in a liquid scintillation counter (Packard 1900; Packard, Meriden, CT). The outflow of tritium was expressed as fractional release (FR), i.e., as the percentage of the amount of radioactivity in the tissue at the time of the release. To calculate electrical field stimulation-induced overflow, the mean of the basal release determined before and after the stimulation was subtracted from the total efflux of radioactivity from the tissue in response to electrical stimulation.

Statistical analysis

Results are presented as mean ±SD; statistical analysis was performed using one-way ANOVA followed by unequal N HSD post hoc analysis, or Fisher's exact test as appropriate, with a *P* value of <0.05 considered significant.

Results

Effect of CGS 21680 on the clinical signs of colitis

Treatment of NMRI mice with 5 % DSS in their drinking water for 10 days resulted in many clinical signs of colitis compared with mice receiving regular drinking water. At earlier stages, all mice, independently from the presence or absence of DSS in their drinking water, gained weight. After *day 8*, DSS-treated animals exhibited progressive weight loss (Fig. 1). Treatment with CGS 21680 starting on *day 1* could not reverse this weight loss; moreover, it slightly increased it (Fig. 1).

We next recorded the effect of CGS 21680 on colon length. DSS-treated mice had significantly shortened colons than those of control counterparts (3.62 ± 0.51 cm in DSS-treated mice vs. 5.96 ± 0.56 cm in control group, *P* < 0.001). CGS 21680 did not reverse this shortening effect of DSS on the colon length (3.62 ± 0.51 cm without CGS 21680 vs. 3.69 ± 0.47 cm with CGS 21680, *P* > 0.05; Fig. 2).

Rectal bleeding first appeared on *day 4* or *5*, and its incidence progressively increased during DSS-treatment. Although this increase in the incidence of rectal bleeding was moderated by CGS 21680 at earlier points ($P = 0.0642$ on *day 6*, $P = 0.0516$ on *day 7*, $P = 0.049$ on *day 8*), there was no difference in the incidence of rectal bleeding between CGS 21680-treated and untreated groups at the end of the experiment ($P > 0.1$ on *day 9 and 10*, Fig. 3).

Effect of CGS 21680 on chemokine and cytokine production in colon during DSS-induced colitis

Since the underlying mechanism by which colitis is induced involves increased production of chemokines and proinflammatory cytokines, we determined levels of the chemokines macrophage inflammatory protein (MIP)-1 α and MIP-2, as well as four proinflammatory cytokines, which included interferon gamma (IFN γ), interleukin (IL)-1 β , IL-12 and tumour necrosis factor-alpha (TNF- α). DSS-treated mice showed elevated levels of MIP-1 α , MIP-2 and IL-1 β (Fig. 4), while the concentration of IFN γ , IL-12 and TNF- α did not change in these animals compared to control ones (Table 1). CGS 21680 failed to decrease the DSS-induced elevations of MIP-1 α , MIP-2 and IL-1 β levels (Fig. 4). In addition, CGS 21680 did not have any effect on the levels of IFN γ , IL-12 and TNF- α (Table 1).

[³H]ACh release from longitudinal muscle strip preparation of mice with colitis

As cholinergic mechanisms play an important role in the regulation of gastrointestinal motility, we studied the release of ACh, the major neurotransmitter of the enteric nervous system, from longitudinal muscle strip preparation of mice with colitis. No change could be observed in release of ACh in DSS-induced colitis both in the presence and in the absence of CGS 21680 (Table 2).

Discussion

In this work, we investigated the effect of activation of adenosine A_{2A} receptors by the selective agonist CGS 21680 in DSS-induced colitis in mice. CGS 21680 failed to affect DSS-induced changes in bodyweight and colon length, incidence of rectal bleeding as well as the excessive production of MIP-1 α , MIP-2, IL-1 β , and the release of [³H]ACh. Thus, despite promising results with A_{2A} agonists in other IBD models [38], A_{2A} receptor stimulation is not protective in the DSS model of colitis in mice.

CGS 21680 did not exacerbate weight loss in our DSS-treated mice. Chronic administration of CGS 21680 does not appear to have other toxic effects. For example, administering CGS 21680 to transgenic mice suffering from Huntington's disease did not have any adverse effects and attenuated symptoms of the disease [39]. Also, repeated administration of CGS 21680 for 3 weeks was without toxic side effects in spontaneously hypertensive rats, and it normalized the blood pressure of the animals [40]. Finally, our results indicate that injection of CGS 21680 for 3 weeks to mice with streptozotocin-induced diabetes has no systemic toxic effects (data not shown).

Our finding of a lack of efficacy of A_{2A} receptor stimulation in DSS-induced colitis is somewhat unexpected, because adenosine A_{2A} receptors are generally considered to have a non-redundant role in the attenuation of inflammation and tissue damage [41]. Previous studies, including ours [42] and a study by Gomez and Sitkovsky [43] have shown that 0.5 mg/kg CGS 21680 effectively reduces inflammatory cytokine (TNF) production. The selectivity of the effect of CGS 21680 in decreasing TNF production was confirmed by using A_{2A} knockout animals, because CGS 21680 was no longer able to inhibit TNF production in these animals, whereas CGS 21680 decreased TNF production in wild-type mice [43]. Furthermore, Odashima et al. have recently demonstrated that the activation of A_{2A} adenosine receptors with

ATL-146e, a novel selective A_{2A} receptor agonist [44], significantly reduces inflammation in the intestinal mucosa of SAMP/YitFc mice, a strain that develops disease spontaneously [38]. One obvious explanation of the differential effect of A_{2A} receptor stimulation in DSS-induced mice and SAMP/YitFc mice is the difference in the pathophysiology of the two models. While colitis in SAMP/YitFc mice appears to be dependent on T lymphocytes and the anti-inflammatory action of A_{2A} is targeted to this cell compartment [38], DSS-induced colitis is independent of lymphocytes, because DSS-induced colitis occurs in severe combined immunodeficient mice [45].

On the other hand, evidence exists that A_{2A} receptor stimulation with CGS 21680 does not moderate all aspects of inflammation, and its effect may depend on the tissue environment or the function studied. Support for this notion comes from a recent study in which we have demonstrated that CGS 21680 ameliorates shock-induced lung injury but fails to prevent gut injury [46]. In addition there is evidence that while A_{2A} receptor stimulation with CGS 21680 had no effect on the bronchoalveolar lavage fluid inflammatory cell influx or TNF- α , KC and MIP-2 levels, CGS 21680 potentially inhibited neutrophil activation, as measured by bronchoalveolar lavage fluid elastase levels in a lipopolysaccharide-driven model of lung inflammation [47]. This observation indicates that CGS 21680 does not inactivate macrophage inflammatory processes in every model *in vivo*. Since macrophages appear to be central to the pathogenesis of DSS-induced colitis [7,48], the inefficacy of CGS 21680 to suppress macrophage activation *in vivo* may provide an explanation for its lack of protective effect in DSS-induced mice.

While A_{2A} receptor stimulation does not prevent colitis in DSS-induced mice, the A₃ receptor agonist IB-MECA (*N*⁶-(3-iodo-benzyl)adenosine-5'-N-methyl-uronamide) is protective in this model [49]. In addition, we have shown previously that inosine, a naturally occurring anti-inflammatory purine [50–55] that can bind A₃ receptors [56], effectively suppressed the development of DSS-induced colitis in mice [57]. Inosine attenuated disease parameters, increased survival, and it reduced colon levels of chemokines (MIP-1 α and MIP-2) and Th1 cytokines (IL-1, IL-6, IL-12, and TNF- α). These observations underscore the proposition that different adenosine receptors might be responsible for alleviating inflammation in different experimental models [16].

In turn, in a more recent study, A_{2A} receptors were shown to mediate inhibitory actions of endogenous adenosine on colonic motility in a rat model of experimental colitis induced by DNBS (2,4-dinitrobenzenesulfonic acid). In this model, CGS 21680 exerted inhibitory effects on electrical cholinergic contractions, acting with greater efficacy on colonic preparations from inflamed rats [58]. As intestinal motility is impaired in IBD patients, and intestinal dysmotility may result in abnormal growth of intestinal flora [59], which plays a role in the pathogenesis of IBDs [60], the inhibitory effect of CGS 21680 on intestinal motility may interfere with its anti-inflammatory effect. However, in our model, the release of acetylcholine was not affected by DSS-induced colitis either in the presence or in the absence of CGS 21680.

Our further interesting observation is that CGS 21680 can decrease the incidence of rectal bleeding at the early but not late stages. It is possible that the early inflammatory response is decreased by CGS 21680 thereby decreasing the incidence of rectal bleeding. It is not clear why CGS 21680 loses its efficacy later during the inflammatory process. One possibility is that A_{2A} receptor expression is down-regulated on inflammatory cells, as has been shown in both monocytes [36] and endothelial cells [61] that have been exposed to an inflammatory environment.

In conclusion, our results demonstrate that the adenosine A_{2A} receptor selective agonist CGS 21680 fails to ameliorate symptoms of DSS-induced colitis in NMRI mice. These observations

indicate the necessity of further investigations to clarify the exact role of adenosine receptors in the regulation of the pathogenesis of IBDs.

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Fig. 1.

DSS induces a progressive decrease in bodyweight both in the presence and in the absence of CGS 21680. Mice were exposed to DSS *ad libitum* for 10 days, treatment with CGS 21680 (0.5 mg/kg/day) started on *day 1*. Results are expressed as means $\pm$ SD from 8–14 mice. * $P < 0.05$, *** $P < 0.001$ when compared DSS-treated animals vs. control animals; # $P < 0.05$, ### $P < 0.001$ when compared CGS+DSS-treated animals vs. control animals.

Fig. 2.

CGS 21680 does not prevent shortening of colon length during DSS-induced colitis. Mice were exposed to DSS *ad libitum* for 10 days, treatment with CGS 21680 (0.5 mg/kg/day) started on *day 1*. Results are expressed as means $\pm$ SD from 9–12 mice. *** $P < 0.001$ vs. control.

Fig. 3.

The increase of the incidence of rectal bleeding is more moderate at early but not late stages of colitis in CGS 21680-treated animals. Mice were exposed to DSS *ad libitum* for 10 days, treatment with CGS 21680 (0.5 mg/kg/day) started on *day 1*. * $P < 0.05$.

Fig. 4. Effect of CGS 21680 on colon MIP-1 α (A), MIP-2 (B) and IL-1 β (C) levels in colitis. Cytokine levels were determined in colon biopsies following treatment for 10 days with DSS + CGS 21680 (0.5 mg/kg/day) or DSS + vehicle. Results are expressed as means $\pm$ SD from 9–12 mice. * $P < 0.05$ vs. control.

Table 1Unchanged colonic production of IFN γ , IL-12p70, and TNF- α in DSS-induced colitis.

	Cytokines (pg/mg protein)		
	IFN γ	IL-12p70	TNF- α
control	1.749 $\pm$ 0.693	2.655 $\pm$ 1.243	0.947 $\pm$ 0.423
DSS *	1.492 $\pm$ 0.538	2.388 $\pm$ 0.501	1.291 $\pm$ 0.734
CGS+DSS **	2.123 $\pm$ 1.454	2.908 $\pm$ 0.893	1.375 $\pm$ 0.455

*
5 % (w/v)**
0.5 mg/kg/dayResults are expressed as means $\pm$ SD from 9–12 mice.

Table 2

[³H]ACh release from longitudinal muscle strip preparation of mice with colitis.

	FRS2/FRS1 ratio
control	0.94 ± 0.18
DSS [*]	0.95 ± 0.24
CGS+DSS ^{**}	0.98 ± 0.20

^{*}
5 % (w/v)

^{**}
0.5 mg/kg/day

Results are expressed as means ±SD from 3–4 samples.