Adenosine A_{2A} agonist and A_{2B} antagonist mediate an inhibition of inflammation-induced contractile disturbance of a rat gastrointestinal preparation

Sebastian Michael · Claudia Warstat · Fabien Michel ·
Luo Yan · Christa E. Müller · Karen Nieber

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Abstract Adenosine can show anti-inflammatory as well as pro-inflammatory activities. The contribution of the specific adenosine receptor subtypes in various cells, tissues and organs is complex. In this study, we examined the effect of the adenosine A_{2A} receptor agonist CGS 21680 and the A_{2B}R antagonist PSB-1115 on acute inflammation induced experimentally by 2,4,6-trinitrobenzenesulfonic acid (TNBS) on rat ileum/jejunum preparations. Pre-incubation of the ileum/jejunum segments with TNBS for 30 min resulted in a concentration-dependent inhibition of acetylcholine (ACh)-induced contractions. Pharmacological activation of the A_{2A}R with CGS 21680 (0.1–10 μ M) pre-incubated simultaneously with TNBS (10 mM) prevented concentration-dependently the TNBS-induced inhibition of the ACh contractions. Stimulation of A_{2B}R with the selective agonist BAY 60-6583 (10 μ M) did neither result in an increase nor in a further decrease of ACh-induced contractions compared to the TNBS-induced inhibition. The simultaneous pre-incubation of the ileum/jejunum segments with TNBS (10 mM) and the selective A_{2B}R antagonist PSB-1115 (100 μ M) inhibited the contraction-

decreasing effect of TNBS. The effects of the A_{2A}R agonist and the A_{2B}R antagonist were in the same range as the effect induced by 1 μ M methotrexate. The combination of the A_{2A}R agonist CGS 21680 and the A_{2B}R antagonist PSB-1115 at subthreshold concentrations of both agents found a significant amelioration of the TNBS-diminished contractility. Our results demonstrate that the activation of A_{2A} receptors or the blockade of the A_{2B} receptors can prevent the inflammation-induced disturbance of the ACh-induced contraction in TNBS pre-treated small intestinal preparations. The combination of both may be useful for the treatment of inflammatory bowel diseases.

Keywords Adenosine receptors · A_{2A}R agonist · A_{2B}R antagonist · Inflammation · TNBS · Small intestine · Rat

Introduction

The purine nucleoside adenosine, which is involved in a variety of physiological functions, regulates a wide variety of immune and inflammatory responses and acts also as a modulator of gut function. Although it is present at low concentrations in the extracellular space, stressful conditions, such as inflammation and hypoxia, can markedly increase its extracellular level up to the micromolar range [13, 24, 27, 28, 44, 48]. Adenosine binds to four different types of G protein-coupled cell surface receptors referred to as A₁R, A_{2A}R, A_{2B}R and A₃R, each of which has a unique pharmacological profile, tissue distribution and signalling pathway [18]. There is growing evidence that adenosine plays an important role in the regulation of inflammation [6, 39]. All known adenosine receptors contribute to the modulation of inflammation, as demonstrated by many in vitro and in vivo pharmacologic studies [14, 33]. Depending on the tissue or cell type,

S. Michael · C. Warstat · F. Michel · K. Nieber
Institute of Pharmacy, Pharmacology for Natural Sciences,
University of Leipzig,
Leipzig, Germany

L. Yan · C. E. Müller
PharmaCenter Bonn, Pharmaceutical Institute,
Pharmaceutical Chemistry I, University of Bonn,
Bonn, Germany

K. Nieber (✉)
Institut für Pharmazie, Pharmakologie für Naturwissenschaftler,
Talstrasse 33,
04103 Leipzig, Germany
e-mail: nieber@uni-leipzig.de

adenosine may produce either pro-inflammatory or anti-inflammatory effects [21]. Recent evidence suggests a prominent role of $A_{2A}R$ and $A_{2B}R$ in the pathophysiology of inflammation [39, 43].

Activation of $A_{2A}R$ produced various responses that can be characterised as anti-inflammatory effects [3]. A key molecular mechanism is a suppression of the nuclear factor- κB pathway activated by cytokines such as tumour necrosis factor (TNF)- α , interleukin (IL)-1 β as well as pathogen-derived Toll-like receptor agonists such as lipopolysaccharide [15, 29, 38, 40]. For example, the $A_{2A}R$ agonist ATL-146e has been demonstrated to suppress the inflammation in the intestinal mucosa. The leucocytes' infiltration and the levels of the pro-inflammatory cytokines TNF- α , interferon- γ and IL-4 were reduced [37]. Moreover, activation of $A_{2A}R$ on human monocytes and mice macrophages inhibits the secretion of the pro-inflammatory cytokines IL-12 and TNF- α [15, 29].

$A_{2B}R$ is located on immune cells, and it is the predominate adenosine receptor expressed in the colonic epithelia cells [21]. $A_{2B}R$ is up-regulated in models of intestinal inflammatory diseases. The role of this receptor subtype has been largely unexplored because of the lack of specific $A_{2B}R$ agonists. As demonstrated indirectly by antagonists, the $A_{2B}R$ might be an important target to modulate inflammatory responses in the colon [20]. Furthermore, $A_{2B}R$ antagonists were potent in reducing inflammatory pain and inflammatory hyperalgesia [6] as well as in attenuating pulmonary inflammation [36, 45]. Contrary to this line of evidence, a recent report pointed out that $A_{2B}R$ knockout mice showed a phenotype with increased inflammatory responses [50]. BAY 60-6583 was the first specific non-adenosine-like $A_{2B}R$ agonist. The EC_{50} values of BAY 60-6583 are 3–10 nM for human $A_{2B}R$ and >10 μM for A_1 and A_{2A} receptors [9] and had no agonistic effect in the adenosine A_3 -G α 16 assay up to a concentration of 10 μM [26].

Two different agents were normally used to induce inflammatory processes in the small or large intestine, dextran sodium sulphate (DSS) and 2,4,6-trinitrobenzenesulfonic acid (TNBS). Disturbed intestinal activity induced in vitro by TNBS is characterised by an unspecific damage and the presence of inflammation, which is manifested by crypt and muscle destruction, mucosal damage and infiltration of inflammatory cells into the mucosal tissue [31, 47]. Additionally, TNBS increased the gene expression of the pro-inflammatory cytokine TNF- α but not of the anti-inflammatory cytokine IL-10. These effects are accompanied by lowering the phasic and tonic activity and suppression of acetylcholine (ACh)-induced contraction [32]. Contrary to TNBS, the DSS-induced colitis is independent of lymphocytes [8].

Therefore, in the current study, we investigate for the first time whether the combination of the selective A_{2A}

agonist CGS 21680 together with the selective A_{2B} antagonist PSB-1115 can affect inflammation-induced disturbed intestinal contractile response in the TNBS in vitro model. To pursue this goal, we first assayed the effects of the selective ligands CGS 21680, BAY 60-6583, and PSB-1115 alone on the ACh-induced contractions in TNBS-damaged rat ileum/jejunum preparations and compared the effects with methotrexate, a standard medication of inflammatory bowel diseases. Additionally, we evaluated for the first time the combination of CGS 21680 and PSB-1115 both in ineffective concentrations.

Materials and methods

Materials

PSB-1115 (1-propyl-8-p-sulfophenulxanthine) was synthesised at the PharmaCenter Bonn, Department of Pharmaceutical Chemistry I, University Bonn, Germany, according to previously described procedures [19, 35, 49] and purified by preparative high-performance liquid chromatography to obtain a purity of >98%. The non-purinergic $A_{2B}R$ agonist BAY 60-6583 2-[6-amino-3,5-dicyano-4-[4-(cyclopropylmethoxy)phenyl]pyridin-2-ylsulfanyl]acetamide was a gift from Bayer HealthCare AG, Wuppertal, Germany. All other substances were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

ACh (1 M) was prepared as fresh 1:10 dilution in water from a 10 M aqueous stock solution. ACh was applied directly into the organ bath. Therefore, the final concentration was 1 mM. The adenosine receptor ligands were made up in stock solutions in dimethylsulfoxide, and methotrexate was dissolved in water. The final dimethyl sulfoxide concentration was 0.1%, which was without effect in any experiments. All stock solutions were stored as frozen aliquots at $-20^{\circ}C$. Dilutions of these stock solutions and appropriate solvent controls were made. TNBS was purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany, in a 1 M stock solution in water, and stored at $-20^{\circ}C$ after aliquotation. Dilutions were freshly prepared before experiments.

The modified Krebs solution contained (mM): NaCl (130.5), KCl (4.86), $MgCl_2$ (1.2), NaH_2PO_4 (1.97), Na_2HPO_4 (4.63), $CaCl_2$ (2.4) and glucose (11.4). The pH value was adjusted to 7.3.

Animals

All procedures used throughout this study were conducted according to the German Guidelines for animal Care and approved by the Institutional Review Board of Animal Care Committee.

Adult male Wistar-rats (8–10 weeks old, 150–220 g body weight) were obtained from the Biomedical Centre, Medical Faculty University Leipzig, and were maintained at room temperature in a light (12 h light/12 h dark)-controlled environment with free access to food and water *ad libitum*. The rats were anaesthetized with ether and killed by decapitation. The abdomen was immediately opened, a small intestinal segment (ileum and distal part of the jejunum) of about 15 cm was rapidly removed and placed in a dish containing aerated modified Krebs solution at 37°C.

Induction of inflammation

Inflammation was induced as described previously [31]. In brief, an ileum/jejunum segment approximately 2.5 cm long was prepared and cleaned. A thread closed one end, and in the other end, a canula was installed through which TNBS (10 mM) or the control solution was applied. After application, the canula was removed, and the segment was closed by a thread. The preparation was suspended for 30 min in a 10 ml incubation chamber containing aerated modified Krebs solution. After incubation, the threads were removed, and the preparation was rinsed with modified Krebs solution. Sections of 1.5 cm in length were prepared for the experiments.

Drug application

ACh was applied directly into the organ bath. The A_{2A}R and A_{2B}R ligands, methotrexate or the control were instilled intraluminal 3 min before instillation of TNBS and were then incubated together with TNBS for 30 min. In some preliminary experiment, the A_{2A}R agonist or the A_{2B}R antagonist was applied directly into the organ bath 3 min before ACh application to test the direct effect or the ACh contraction.

Isometric tension recording

A thread was attached to each end of a segment, and then it was suspended in 20 ml organ bath containing oxygenated (95% O₂ and 5% CO₂) modified Krebs solution maintained at 37°C. One end of the preparation was anchored to a stationary hook on the bottom of the organ bath. The other end was connected to an isometric transducer (TSE Systems, Bad Homburg) for continuous recording of the isometric tension. The preparations were allowed to equilibrate for 40 min under a tension of 10 mN interrupted by a wash out before starting the experiment. ACh (1 mM) was applied at the beginning of each experiment to test the sensitivity of the preparation used. The concentration of 1 mM was used according to concentration-response curves

recorded on intact ileo-jejunal segments in the range from 10 μM to 2.5 mM with an ED₅₀ value of 750 μM [46]. The concentration of 1 mM induced reproducible contractions with amplitudes suitable to examine inhibitory effects of TNBS.

Statistics

A relative comparison of the ACh contraction between preparations instilled with solvent (control=100%) and with TNBS or TNBS in combination with the receptor ligands was used to determine functional differences. Data are expressed as the means ± SEM of *n* experiments. Multiple comparisons with a control value were performed by one-way analysis of variance followed by unpaired Student's *t* test. A probability level of 0.05 or less was considered to represent a statistically significant difference. The concentration-response curve was constructed using nonlinear regression. The IC₅₀ was calculated with SigmaPlot.

Results

Effects of the A_{2A}R agonist and the A_{2A}R antagonist on the TNBS-induced inhibition of the ACh contraction

The ileo-jejunal segments were pre-incubated with TNBS for 30 min. During this time, an equilibrium inhibition of the ACh-induced contractions developed. TNBS (1 mM–1 M) resulted in a concentration-dependent inhibition of ACh-induced contractions (Fig. 1a) with an IC₅₀ value of 63 mM calculated from the concentration-response curve (Fig. 1b). TNBS in a concentration of 100 mM reduced the ACh contraction to 35.2±2.9% (*n*=9). The moderate disturbance induced by 10 mM was used for further experiments.

In a first series of experiments, the standard A_{2A}R agonist CGS 21680 applied directly into the organ bath was tested on the ACh-induced contraction. CGS 21680 (10 μM) increased the ACh-induced contractions by 20±7% compared to the control (*P*<0.05, *n*=5). The increase was prevented by the A_{2A}R antagonist 1,3,7-trimethyl-8[3-chlorostyryl]xanthine (CSC, 0.2 μM; data not shown). Thereafter, CGS 21680 (0.1–10 μM) was pre-incubated simultaneously with TNBS (10 mM). It restored concentration-dependently the TNBS-induced inhibition of the ACh contractions. Whereas CGS 21680 in a concentration of 0.1 μM was without significant effect compared to the TNBS-control, concentrations of 1 and 10 μM significantly affected the ACh-induced contraction respectively by 50.5±3.14% and 79.3±2.6% (*n*=9, Fig. 2a). The A_{2A}R antagonist CSC (0.2 μM) was without effect on the TNBS-reduced contractions (52.3±2.6% vs. 46.3±6.8%),

Fig. 1 Effects of 2,4,6-trinitro benzenesulfonic acid (TNBS; 1–100 mM) on the ACh-induced contraction in rat ileum/jejunum segments. **a** Representative original registrations. ACh was used in a concentration of 1 mM. **b** Concentration-response curve of the TNBS-induced inhibition of the ACh-induced contraction (1 mM). Each point represents $n=21$ experiments

but it antagonised the protective effect of CGS 21680 (10 μ M; $79.3 \pm 2.6\%$ vs. $52.3 \pm 3.7\%$; Fig. 2b).

Effects of the A_{2B}R agonist and A_{2B}R antagonist on the TNBS-induced inhibition of the ACh contraction

BAY 60-6583 (10 μ M) applied directly into the organ bath reduced the ACh-induced contractions by $22.7 \pm 6.4\%$ $n=11$,

data not shown). In the same concentration (10 μ M), BAY 60-6583 did not affect the TNBS-induced inhibition of ACh-induced contraction ($54.6 \pm 6.1\%$ vs. $64.5 \pm 3.8\%$, $n=12$; Fig. 3). The simultaneous pre-incubation of the ileum/jejunum segments with TNBS (10 mM) and the selective A_{2B}R antagonist PSB-1115 (100 μ M), which was without effect on intact preparation, inhibited the contraction-decreasing effect of TNBS ($80.6 \pm 5.0\%$, $n=12$). The combined pre-

Fig. 2 Effects of the A_{2A}R ligands on the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced decrease of the ACh (1 mM) contractions. **a** Concentration-dependent effect of the selective A_{2A}R agonist CGS 21680 on the TNBS-induced attenuation of the ACh (1 mM)-induced contractions. **b** Effects of TNBS, the A_{2A}R agonist CGS 21680

(10 μ M) and the A_{2A}R antagonist CSC (0.2 μ M) as well as the simultaneous pre-incubation on the ACh-induced contraction (1 mM) in rat ileum/jejunum segments. Means $\pm$ SEM of $n=9$ experiments. Asterisk: $p<0.05$ vs. control (open column); Number sign: $p<0.05$ vs. previous column; Plus sign: $p<0.05$ vs. CGS 21680

incubation of both the agonist BAY 60-6583 (10 μ M) and the antagonist PSB-1115 (100 μ M) together with TNBS (10 mM) did not significantly affect the TNBS-dependent inhibition of ACh-induced contraction compared with TNBS alone ($64.5 \pm 3.8\%$ vs. $62.5 \pm 7.3\%$; $n=12$; Fig. 3).

The effect of PSB-1115 was concentration-dependent in the range of 1 to 100 μ M. The inhibition of the ACh-induced contraction by TNBS (10 mM) in the presence of 1 μ M PSB-1115 was not significantly changed ($98.0 \pm 6.6\%$), whereas the simultaneous pre-incubation of the ileum/jejunum segments with TNBS (10 mM) and PSB-1115 (10 and 100 μ M) reduced the TNBS-induced decrease of the ACh-induced contractions significantly by $77.0 \pm 10.7\%$ and $29.5 \pm 6.7\%$, respectively.

Effects of methotrexate on the TNBS-induced inhibition of the ACh contraction

Methotrexate pre-incubated simultaneously with TNBS significantly counteracted the TNBS-induced inhibition of the ACh contractions (Fig. 4a). The decrease of the ACh-induced contraction by TNBS (10 mM) was $52.4 \pm 7.2\%$; after pre-incubation together with 1 μ M methotrexate, the decrease was reduced to $25.5 \pm 3.9\%$ ($n=12$). As can be seen from Fig. 4, the TNBS-preventing effect of methotrexate is comparable to the effect of the $A_{2A}R$ agonist CGS 21680 (10 μ M, Fig. 2) or $A_{2B}R$ antagonist PSB-1115 (100 μ M, Fig. 3).

Fig. 3 Effects of the $A_{2B}R$ agonist BAY 60-6583 and the $A_{2B}R$ antagonist PSB-1115 as well as the simultaneous pre-incubation with TNBS on the ACh (1 mM)-induced contractions. Means $\pm$ SEM of $n=12$ experiments. Asterisk: $p<0.05$ vs. control (open column); Number sign: $p<0.05$ vs. previous column

Effect of the combination of CGS 21680 and PSB-1115 on the TNBS-induced inhibition of the ACh contraction

Based on the findings that the $A_{2A}R$ agonist and the $A_{2B}R$ antagonist counteracted the TNBS-induced disturbance of contractile responses, the effect of the combination of CGS 21680 and PSB-1115, both in a concentration without significant effect in the experiments before, was tested. Thus, the ileum/jejunum segments were pre-incubated with the combination of 0.1 μ M CGS 21680 and 1 μ M PSB-1115 together with TNBS (10 mM). Under this condition, the pre-incubation resulted in considerable inhibition of the TNBS-induced decrease of the ACh-induced contractions ($66.6 \pm 6.6\%$ vs. $43.6 \pm 3.8\%$; $n=11$; Fig. 4b). The effect of the combination was in the same range as the action of each ligand applied separately in a high concentration or as induced by methotrexate.

Discussion

In this study, using an in vitro TNBS model, we demonstrate that the $A_{2A}R$ agonist and the $A_{2B}R$ antagonist or the combination of both effectively counteracted the development of TNBS-induced disturbance of the ACh contraction. The ligands exerted protective effects when treatment began simultaneously with the application of TNBS. Additionally, we have shown for the first time that a significant amelioration of the contractility disturbance by the combination of the $A_{2A}R$ agonist CGS 21680 and the $A_{2B}R$ antagonist PSB-1115 was found at subthreshold concentrations of both agents. The protective effect was in range as the effect induced by 1 μ M methotrexate, a concentration normally used in in vitro experiments [11].

These observations provide support to the view that activation of $A_{2A}R$ and the blockade of the $A_{2B}R$ could attenuate the inflammatory response and argue against an anti-inflammatory effect resulting from activation of $A_{2B}R$ as discussed by Yang et al. [50]. Therefore, our results confirm on ileo-jejunal segments results of in vivo experiments in which the blockade of adenosine A_{2B} receptors ameliorates DSS-induced disturbance in mice colon [22]. We could exclude an involvement of A_3 receptors. Meanwhile the receptor is expressed; neither the A_3R agonist IB-MECA (10 μ M) nor the A_3R antagonist MRS 1220 (0.1 mM) showed an influence on the TNBS-induced damage in additional experiments.

Recent evidence indicates that adenosine can have a beneficial role as immunomodulator in tissues subjected to injurious stimuli, including inflammation mainly by activation of $A_{2A}R$ [14].

Several lines of evidence from experimental data, published recently, confirm this assumption for gastrointes-

Fig. 4 Effect of the $A_{2A}R$ agonist and the $A_{2B}R$ antagonist as well as methotrexate on the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced decrease of the ACh (1 mM) contractions. **a** Effect of TNBS (10 mM) and the combined pre-incubation of TNBS and methotrexate (10 μM) on the ACh (1 mM) contraction. Means $\pm$ SEM of $n=12$

experiments. Asterisk: $p<0.05$ vs. control (open column); Number sign: $p<0.05$ vs. previous column. **b** Effect of the combined pre-incubation of the $A_{2A}R$ agonist CGS 21680 and the $A_{2B}R$ antagonist PSB-1115 both in a concentration which was without effect alone and simultaneously with TNBS (10 mM) on the ACh (1 mM) contraction

tinal inflammation. (1) Reverse transcription-polymerase chain reaction showed an increase in $A_{2A}R$ expression in gastric tissues isolated from TNBS-inflamed animals [2]. (2) Inhibition of adenosine deaminase attenuated inflammation in rat experimental colitis [3]. (3) Inosine, a naturally occurring purine formed by the enzymatic breakdown of adenosine, has been shown to exert powerful anti-inflammatory effects both in vivo and in vitro, reduces inflammation and improved survival in a murine model of colitis [30]. (4) Drugs used in the management of inflammatory gastrointestinal diseases, such as methotrexate, sulfasalazine and related compounds, exert their anti-inflammatory actions through a variety of mechanisms, which include their ability to promote the accumulation of adenosine in the inflamed tissue [7, 34].

In our study, CGS 21680 was used as selective $A_{2A}R$ agonist. This compound effectively reduces inflammatory $TNF-\alpha$ production in vivo [12, 16], whereas in $A_{2A}R$ knockout animals, CGS 21680 was no longer able to inhibit cytokine production [12]. Contrary to the TNBS model, CGS 21680 failed to ameliorate the DSS-induced acute colitis in mice [42]. One explanation of the differential effect of CGS 21680 could be the difference in the pathophysiology of the two models. The DSS-induced colitis is independent of lymphocytes [8], and the anti-inflammatory action of $A_{2A}R$ is targeted to these cell components [37].

Recently, it has been shown indirectly by blockade of $A_{2B}R$ that this receptor subtype plays a pro-inflammatory

role in DSS-colitis [23]. Using the selective $A_{2B}R$ agonist BAY 60-6583, our results confirm this assumption. BAY 60-6583 was characterised in CHO cells expressing human adenosine receptors. The EC_{50} values were 3 nM for $A_{2B}R$ and $>10,000$ nM for A_1R and $A_{2A}R$ [25]. Soluble 5'-nucleotidase or $A_{2B}R$ agonist treatment mimicked cardio-protection by ischemic preconditioning because it was associated with significant attenuation of myocardial infarct sizes after ischaemia [9].

Mice fed the highly specific inhibitor of the $A_{2B}R$ 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) along with DSS showed a significantly lower extent and severity of colitis than mice treated with DSS alone [22]. Likewise ATL-801, in our model PSB-1115 attenuates TNBS-induced changes in ileo-jejunal segments. PSB-1115 is a peripherally acting $A_{2B}R$ antagonist, which probably cannot penetrate the blood-brain barrier due to its polar sulfonate group [5]. Sulfonates such as PSB-1115 will also not penetrate cell membranes and, therefore, the observed effects have to be due to an extracellular mechanism and are believed to be mediated by a blockade of $A_{2B}R$. The selectivity of PSB-1115 for the $A_{2B}R$ is confirmed by the determined K_i value of 53 nM for the $A_{2B}R$ in comparison to 24,000 nM for the $A_{2A}R$ [17] and by binding studies showing only low to negligible binding to 30 different receptors [1]. The anti-inflammatory effect obtained with a selective $A_{2B}R$ antagonist is in agreement with results

from previous in vitro studies, which indicate a pro-inflammatory function of A_{2B}R and anti-inflammatory actions of A_{2B}R antagonists [4, 6].

It should be noted that the subthreshold combination of the A_{2A}R agonist and the A_{2B}R antagonist showed a comparable effect with methotrexate. This combination investigated for the first time may be important for the development of novel therapeutic strategies. The anti-inflammatory and tissue-protective properties of A_{2A}R agonists have not yet been adequately tested in clinical trials because the A_{2A}R has a widespread tissue and cell-type distribution, and therefore, activating this receptor might lead to unwanted side effects. The A_{2A}R activation mediates inhibition of platelet aggregation, hypotension and a variety of effects within the central nervous system [10, 39, 41]. In this respect, considering the low concentration of the A_{2A}R agonist and the inability of the A_{2B}R antagonist to penetrate the cell membrane as well as the blood–brain barrier, the concept of the agonist/antagonist combination could overcome these therapy limiting side effects.

In conclusion, our results point out the concept that the selective activation of A_{2A}R and the blockade of the A_{2B}R would be potentially beneficial in the treatment of acute inflammatory processes in the gut.

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Conflict of interest The authors state no conflict of interest.

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