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ORIGINAL ARTICLE

Adenosine A_{2A} receptor contributes to the anti-inflammatory effect of the fixed herbal combination STW 5 (Iberogast®) in rat small intestinal preparations

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Abstract STW 5 (Iberogast®), an established herbal combination, was effective in randomized, double blind clinical studies in functional dyspepsia and irritable bowel syndrome. Since STW 5 was found to influence intestinal motility and has anti-inflammatory properties, this study investigated the expression of adenosine receptors and characterized their role in the control of the anti-inflammatory action of STW 5 and its fresh plant component STW 6 in inflammation-disturbed rat small intestinal preparations. The inflammation was induced by intraluminal instillation of 2,4,6-trinitrobenzene sulfonic acid (TNBS, 0.01 M). The effects of coincubation with selective receptor agonists and antagonists, STW 5, STW 6, or combinations of these compounds on acetylcholine (ACh)-evoked contraction of ileum/jejunum preparations were tested. Adenosine receptor mRNA expression was examined by reverse transcription-polymerase chain reaction (RT-PCR). In untreated preparations, RT-PCR revealed the presence of all adenosine receptor subtypes. Suppressed expression was detected for all subtypes in inflamed tissues, except for A_{2B}R mRNA, which was unaffected. STW 5 reversed these effects and enhanced A_{2A}R expression above control levels. Radioligand binding assays confirm the affinity of STW 5 to the A_{2A}R, and the A_{2A}R antagonist was able to prevent the effect of STW 5 on TNBS-induced attenuation of the ACh contraction. Our findings provide evidence that STW 5, but not STW 6 interacts with A_{2A}R, which is involved in the anti-inflammatory action of STW 5. STW 6 did not contribute to adenosine A_{2A}R-mediated anti-inflammatory effect of STW 5. Other signaling pathways could be involved in the mechanism of action of STW 6.

Keywords Iberogast · Inflammation · Adenosine receptors · TNFα · Isometric contraction · TNBS

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Introduction

Inflammatory bowel diseases are characterized by recurrent and serious inflammation of the enteric mucosa, with significant alterations of gastrointestinal function, mainly as a consequence of marked changes in the enteric nervous system (Abraham and Cho 2009; Lomax et al. 2005). Several cytokines, such as tumor necrosis factor α (TNFα), interleukin 1, and interleukin 6, contribute to their pathogenesis (Ardizzone and Bianchi-Porro 2005; Pizarro et al. 2006), and the resulting inflammatory responses are mediated predominantly by activated neutrophils and macrophages. Consequently, recent strategies for the treatment of intestinal inflammation have primarily targeted the immunopathogenic processes that mediate chronic intestinal inflammation at the cytokine level (Sandborn and Targan 2002; Bamias et al. 2003). At present, pharmacotherapy represents the mainstay of inflammatory bowel disease management (Stein and Hanauer 1999).

Some anti-inflammatory or immuno-modulating drugs, including salicylates and methotrexate, are able to decrease intracellular adenosine 5′-triphosphate concentrations and raise extracellular adenosine levels. It has been proposed that such properties can significantly contribute to the drugs' pharmacological actions in inflammatory diseases (Cronstein et al. 1999).

Several lines of evidence suggest that the purine nucleoside adenosine regulates immunity and inflammation 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 to micromolar range (Guieu et al. 1998; Sullivan 2003; Wood 2004; Kuno et al. 2006; Kong et al. 2006). Adenosine binds to four different types of G-protein-coupled cell surface receptors referred to as A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R, each of which has a unique pharmacological profile, tissue distribution, and signaling pathway (Jacobson and Gao 2006). All known adenosine receptors contribute to the modulation of inflammation, as demonstrated by many in vitro and in vivo pharmacological studies (Hasko and Cronstein 2004). There is growing evidence that adenosine plays an important role in the regulation of inflammation (Antonioli et al. 2008; Bilkei-Gorzo et al. 2008; Palmer and Trevethick 2008), but supporting data are often conflicting. The involvement may produce either pro-inflammatory or anti-inflammatory effects, depending on the types of receptors stimulated (Akkari et al. 2006).

Intestinal inflammation can be induced in vivo by administration of an enema containing contact sensitizing allergens, 2,4,6-trinitrobenzenesulfonic acid (TNBS) or 2,4-dinitrobenzenesulfonic acid. These compounds have been used in rats, rabbits, and mice (Antonioli et al. 2011a; Elson et al. 1995). Acute inflammation can also be generated by intraluminal instillation of the acid into preparations of the rat small intestine in vitro (Michael et al. 2009; 2010). As previously observed in this model, the fixed herbal combination product STW 5 (Iberogast®) and its fresh plant component *Iberis amara* (STW 6) show a powerful reduction of morphological and contractile abnormalities observed after TNBS treatment and may thus have a promising therapeutic value as anti-inflammatory drugs. Moreover, the in vivo and in vitro findings are of clinical relevance. Multi-center, randomized, double-blind, placebo-controlled studies showed that STW 5 is effective in patients with irritable bowel syndrome (Madisch et al. 2004a, b) and functional dyspepsia (von Arnim et al. 2007). The results of Kelber et al. (2006) allowed concluding that STW 5 possesses a good bioavailability, which is in accordance with the rapid onset of its therapeutic effect and explains its known pharmacological effects and clinical efficacy in terms of multiple drug action.

The present study was designed to examine the expression of adenosine receptor mRNA in rat ileum/jejunum preparations using reverse transcription-polymerase chain reaction (RT-PCR) and to characterize the interaction between STW 5, as well as between STW 6 and adenosine receptors in untreated and TNBS-inflamed ileum/jejunum preparations. The involvement of adenosine receptors in the anti-inflammatory action of STW 5 pharmacologically was confirmed by receptor binding experiments and by using selective receptor antagonists to block the effect of STW 5 on acetylcholine (ACh)-induced isometric contractions.

Materials and methods

Materials

STW 5 contains *I. amara* totalis (STW 6) fresh plant extract and eight drug extracts from dried plants (Table 1). STW 5 and STW 6 were kindly provided by Steigerwald Arzneimittelwerk GmbH, Darmstadt, Germany in the form of ethanol-free lyophilisates (58.0 mg resp. 18.2 mg corresponding to 1 ml of the fluid extract). STW 5 and STW 6 were dissolved in water. The concentrations of STW 5 were used according to Hohenester et al. (2004). STW 6 was used in concentrations equivalent to its proportion in STW 5.

ACh (1 M) was prepared as fresh 1:10 dilutions from a 10-M stock solution. The final concentration in the organ baths was 1 mM. ACh (1 mM) was used as positive control. PSB-1115 (1-propyl-8-p-sulfophenulxanthine) was synthesized at the PharmaCenter Bonn, Department of Pharmaceutical Chemistry I, University Bonn, Germany, according to previously described procedures (Kirfel et al. 1997; Müller et al. 1993; Yan and Müller 2004) and purified by preparative HPLC to obtain a purity of >98%. The non-purinergic A_{2B} 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. [^{3}H]CCPA and [^{3}H]CGS21680 were purchased from Perkin

Table 1 The herbal components of STW 5 and its composition

Plant extract	Drug–extract ratio	ml/100 ml
Iberis amara totalis (STW 6)	1:1.5–2.5	15
Menthae piperitae folium	1:2.5–3.5	5
Matricariae flos	1:2–4	20
Liquiritiae radix	1:2.5–3.5	10
Angelicae radix	1:2.5–3.5	10
Carvi fructus	1:2.5–3.5	10
Silybi mariani fructus	1:2.5–3.5	10
Melissae folium	1:2.5–3.5	10
Chelidonii herba	1:2.5–3.5	10

Elmer, Waltham, MA, USA and [^{3}H]PSB11 from Amersham, Munich, Germany.

The modified Krebs solution contained (in 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. The RT buffer contained 250 mM Tris–HCl (pH 8.3 at 25°C), 250 mM KCl, 20 mM $MgCl_2$, and 50 mM DTT. Phosphate buffered saline contained (in mM): NaCl (15.0), NaH_2PO_4 (4.0), and Na_2HPO_4 (1.0) adjusted to a pH of 7.4. Tris–HCl was obtained from Carl Roth GmbH & Co KG, Karlsruhe, Germany.

The RNA preparation kit was from Qiagen GmbH, Germany. Primers were from Invitrogen, Hilden, Germany. Enzymes used for reverse transcription were from Fermentas GmbH, Germany. The PCR reaction kit was from Bio-Rad Laboratories GmbH, Munich, Germany. All other substances were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

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 of Leipzig and were maintained at room temperature in a light-controlled (12-h light/12-h dark) environment with free access to food and water ad libitum. The rats were anesthetized with CO_2 and killed by decapitation. The abdomen was immediately opened, and intestinal segments (ileum and distal part of the jejunum) of about 10–15 cm were rapidly removed and placed in a dish containing aerated modified Krebs solution at 37°C. The segment of each animal was dissected in four preparations. Each experiment was repeated using preparations of at least four animals.

Induction of inflammation and drug application

Inflammation was induced according to Michael et al. (2009). In brief, an ileum/jejunum segment approximately 10–15 cm long was prepared as described above, cleaned, and divided into four 1.5 cm long preparations. One end of each preparation was tied up with a thread and in the other end a cannula was inserted through which TNBS (0.01 M) or test substances were instilled. Thereafter, the cannula was removed, and the end was closed with a thread. The preparations were suspended for 30 min in a 10 ml incubation chamber containing aerated modified Krebs solution. After preincubation, the threads were removed and the preparations were rinsed with modified Krebs solution.

Four preparations per animal were used to test the effects of STW 5 and STW 6 in the same experiment. Modified Krebs solution (control), TNBS (0.01 M) alone, TNBS together with STW 5 (512 μg/ml) or STW 6 (24.1 μg/ml), respectively, were instilled and incubated for 30 min.

In a second set of experiments, the involvement of A_1R and $A_{2A}R$ in TNBS-induced inflammation was investigated, by preincubation of the ileum/jejunum preparations with TNBS together with different specific AR agonists and antagonists for 30 min, followed by mechanical recording of ACh-induced contractions.

Recording of mechanical activity

The preincubated ileum/jejunum were suspended in 20-ml organ baths containing oxygenated (95% O_2, 5% CO_2) modified Krebs solution maintained at 37°C. The preparations were attached to fixed pins in the bath and to isometric transducers (TSE Systems, Bad Homburg) using polyester threads. 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 preparations. Thereafter, ACh was applied into the organ bath every 20 min. A washout and equilibration period followed after registration of the maximum contraction.

The ACh-evoked contraction was defined as the difference between the basal tone and the first maximum of contraction after drug application.

RNA isolation and reverse transcription

Total RNA from ileum/jejunum segments was extracted after preincubation for 3 h using the RNeasy Mini kit® (Qiagen) according to the protocol of the manufacturer. Ten microliters of the RNA eluates were activated with 1 μl of oligo-dT(20) 500 μg primers in a 5-min incubation step at 70°C in the Crocodile III cycler. Reverse transcription was performed with 200 U of RevertAID (Fermentas) and dNTP (1 mM) in the Crocodile III cycler in RT buffer. The final volume was 20 μl. The reaction was stopped by heating at 70°C for 10 min.

Real-time fluorescence PCR of adenosine receptor mRNA

Adenosine receptor mRNA expression was measured quantitatively by a ready-to-use real-time fluorescence PCR assay. SYBR Green® Mix reaction (BioRad) was used in a MyIQ® cycler (BioRad) according to the manufacturer's protocol. β-Actin was used as a housekeeping gene. The primers for β-actin and the adenosine A_1R were self-designed, whereas the primers for the adenosine $A_{2A}R$, $A_{2B}R$, and A_3R were found in literature (Chen et al. 2004). The specific primers are shown in Table 2.

Table 2 Primers for RT-PCR

Primer	Sequence	Origin
β-Actin sense	5′-TGTCACCAACTGGGACGATA-3′	Designed by the authors
β-Actin antisense	5′-GGGGTGTTGAAGGTCTCAAA-3′	
A_1 sense	5′-CTGCTCCTCATGGTCCTCAT-3′	Designed by the authors
A_1 antisense	5′-GGGCAGAAGAGGGTGATACA-3′	
A_{2A} sense	5′-CTCACGCAGAGTTCCATCTT-3′	Chen et al. (2004)
A_{2A} antisense	5′-TCCATCTGCTTCAGCTGTCT-3′	
A_{2B} sense	5′-CTTCTGCACGGACTTTCACA-3′	Chen et al. (2004)
A_{2B} antisense	5′-GGTGGCACGGTCTTTACTGT-3′	
A_3 sense	5′-ATATGGCTATTCCTGGGCCT-3′	Chen et al. (2004)
A_3 antisense	5′-ACCAGAAACAGGGACTTAGC-3′	

Each sample contained 10 µl of the SYBR Green® SuperMix, 1 µl sense primer, 1 µl antisense primer, 1 µl complementary DNA, and 7 µl sterile water in a volume of 20 µl. The results were analyzed using the $\Delta\Delta c_t$-method (Pfaffl et al. 2002) and expressed as relative gene expression.

Radioligand binding assays

The radioligand binding assays were performed according to methods established by Klotz et al. (1989), and Müller (2000), and Müller et al. (2002). All studies were carried out as competition assays.

For determination of A_1R binding, rat cortical tissue homogenates were utilized. Each well of the 48- or 96-well plates used carried 30 µg proteins in 200 µl final volume. 2-Chloro-N6-[^{3}H]cyclopentyladenosine ([^{3}H]CCPA, specific activity 42.6 Ci/mmol, K_D 0.2 nM) was used as standard A_1R agonist in a final concentration of 1 nM. Tris–HCl was used as medium. Unspecific binding was determined with complete displacement by the adenosine deaminase-resistant adenosine analogue 2-chloroadenosine (10 µM). The extracts were dissolved in water. Incubation of all plates took place at room temperature for 1.5 h. Samples were filtered on a cell harvester (Brandel) with ice-cold Tris–HCl and filled in scintillation tubes. After the addition of 40 µl ultima gold cocktail (Perkin Elmer) for the amplification of ^{3}[H] signal, the radiation intensity was measured in a LS counter (Packard).

Rat striatal tissue homogenates were used to determine A_{2A}R binding; 48- or 96-well plates were used. Each well carried 50 µg proteins in 200 µl final volume. [^{3}H]CGS-21680 (specific activity 41 Ci/mmol, K_D 15.5 nM) was used as standard A_{2A}R agonist in a final concentration of 5 nM. Tris–HCl buffer was used as medium. Unspecific binding was determined with complete displacement by the broad spectrum agonist 5′-*N*-ethylcarboxamidoadenosine (50 µM). The extracts were solved in water. Incubation of plates took place at room temperature for 1.5 h. The final procedure was the same as for A_1R preparations.

To determine A_{2B}R binding, cell membranes of stable transfected CHO cells were taken. Each well carried 30 µg proteins in 200 µl final volume. [^{3}H]PSB 603 (specific activity 73 Ci/mmol, K_D 0.41 nM) was used as standard A_{2B} agonist in a final concentration of 0.3 nM. Tris–HCl was used as medium. Unspecific binding was determined with complete displacement by 8-cyclopentyl-1,3-dipropylxanthine (DPCPX 10 µM). The extracts were dissolved in water. Incubation of plates took place at room temperature for 75 min. The final procedure was the same as for A_1R preparations.

Binding to A_3R was determined using cell membranes of stable transfected CHO cells; 48- or 96-well plates were used. Each well carried 75 µg proteins in 200 µl final volume. [^{3}H]PSB 11 (specific activity 53 Ci/mmol, K_D 4.9 nM) was used as standard A_3R agonist in a final concentration of 1 nM. Tris–HCl was used as medium. Unspecific binding was determined with complete displacement by (*R*(−)N6-(2-phenylisopropyl) adenosine (100 µM). The extracts were dissolved in water. Incubation of plates took place at room temperature for 1 h. The final procedure was the same as for A_1R preparations. The results were analyzed and displayed with GraphPad PRISM®.

Statistics

Experimental data are presented as the means ± SEM of the number (*n*) of experiments. Multiple comparisons with a control value were performed by one-way analysis of variance followed by Student's *t* test. A probability level of 0.05 or less was considered statistically significant. K_i values were calculated by nonlinear correlation.

Results

Gene expression

Reverse transcription of total RNA followed by RT-PCR was used to study the expression of receptor mRNA for ARs in intact and TNBS-treated ileum/jejunum preparations.

A_1R, $A_{2A}R$, $A_{2B}R$, and A_3R mRNA were identified in intact preparations (Fig. 1a–d, control). Incubation of the preparations with TNBS (10 mM, 30 min) resulted in a significant suppression of A_1R, $A_{2A}R$, and A_3R mRNA (Fig. 2a, b, d), whereas $A_{2B}R$ mRNA was unaffected (Fig. 1c). STW 5 (512 µg/ml) coincubated with TNBS (10 mM, 30 min) protected from TNBS-induced suppression of the A_1 and A_3R mRNA. The gene expression remained at control levels (Fig. 1a, d). For $A_{2A}R$ mRNA, even a significant induction by the factor 6.9±0.5 was detectable (Fig. 1b). STW 5 (512 mg/ml) did not influence the gene expression of the $A_{2B}R$ (Fig. 1c).

Pharmacological studies of adenosine receptors

In the following settings, the functionality of the adenosine A_1R and $A_{2A}R$ was tested pharmacologically. Ileum/jejunum preparations were incubated with TNBS together with specific AR agonists and antagonist for 30 min. Then, isometric ACh (1 mM)-induced contraction was measured (Fig. 2). Preincubation with TNBS (10 mM) reduced the ACh contraction to 57.9±3.9% ($p<0.05$ vs. control, $n=12$). The A_1R agonist CPA (10 µM) inhibited the ACh-induced contraction further to 38.8±3.26% ($n=12$, $p<0.05$ vs. control). DPCPX (0.1 µM), described as inverse agonist on A_1R, slightly normalized the TNBS-reduced contractions from 43.5±5.7% to 66.6±6.9% ($p<0.05$, $n=12$). DPCPX in the same concentration prevented the CPA-induced inhibition (65.0±5.1%, $p<0.05$ vs. CPA, $n=12$, Fig. 2a). The activation of $A_{2A}R$ by CGS 21680 (10 µM) enhanced the TNBS-reduced contraction by 27% (79.3±2.6 vs. 52.3±2.1, $p<0.05$, $n=9$). The A_{2A} antagonist CSC (0.2 µM) was without effect on the TNBS-reduced contraction (52.3±2.6% vs. 46.4±6.6%, $p>0.05$, $n=9$), but it abolished completely the agonist-induced enhancement of contraction (52.3±3.7, $p<0.05$ vs. CGS 21680, $n=9$, Fig. 2b).

The $A_{2B}R$ agonist BAY 60–6583 did not affect the TNBS-induced inhibition of ACh-induced contraction (54.6±6.1% vs. 64.5±3.8%, $p>0.05$, $n=12$). The simultaneous preincubation of the ileum/jejunum segments with TNBS (10 mM) and the selective $A_{2B}R$ antagonist PSB-1115 (100 µM) inhibited the contraction-decreasing effect of TNBS (80.6±5.0%, $n=12$). The combined preincubation of both the agonist BAY 60–6583 (10 µM) and the antagonist PSB-1115 (100 µM) together with TNBS (10 mM) did not significantly affect the TNBS-dependent inhibition of ACh-induced contraction compared with TNBS alone (64.5±3.8% vs. 62.5±7.3%; $n=12$; Fig. 2c).

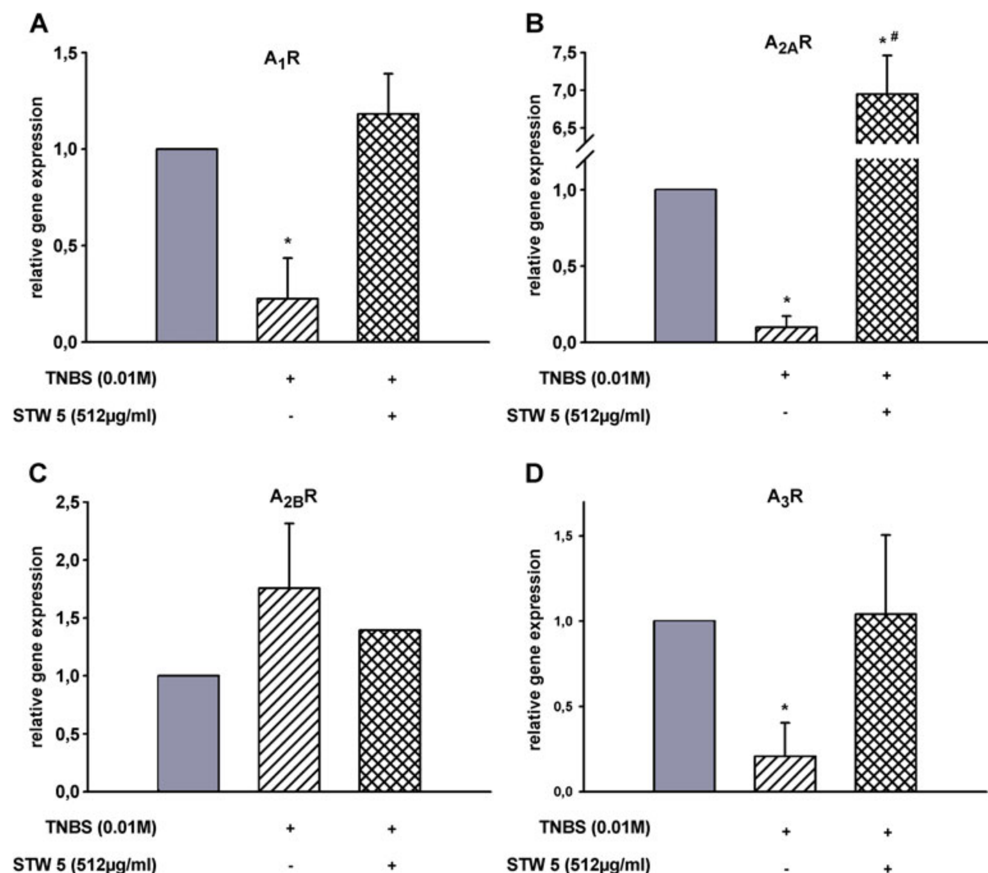

Fig. 1 Gene expression of adenosine receptor subtypes and their alteration after incubation with TNBS and STW 5. Adenosine receptor mRNA expression was measured quantitatively by a ready-to-use real-time fluorescence polymerase chain reaction (PCR) assay and expressed as relative gene expression using the $\Delta\Delta c_t$-method. **A** Gene expression of adenosine A_1 receptor (A_1R). **B** Gene expression of adenosine A_{2A} receptor ($A_{2A}R$). **C** Gene expression of adenosine A_{2B} receptor (A_{2B}). **D** Gene expression of adenosine A_3 receptor (A_3). Means ± SEM of relative gene expression from three independent experiments. *$p<0.05$ vs. control (*gray-colored column*), $^{\#}p<0.05$ vs. TNBS preincubation

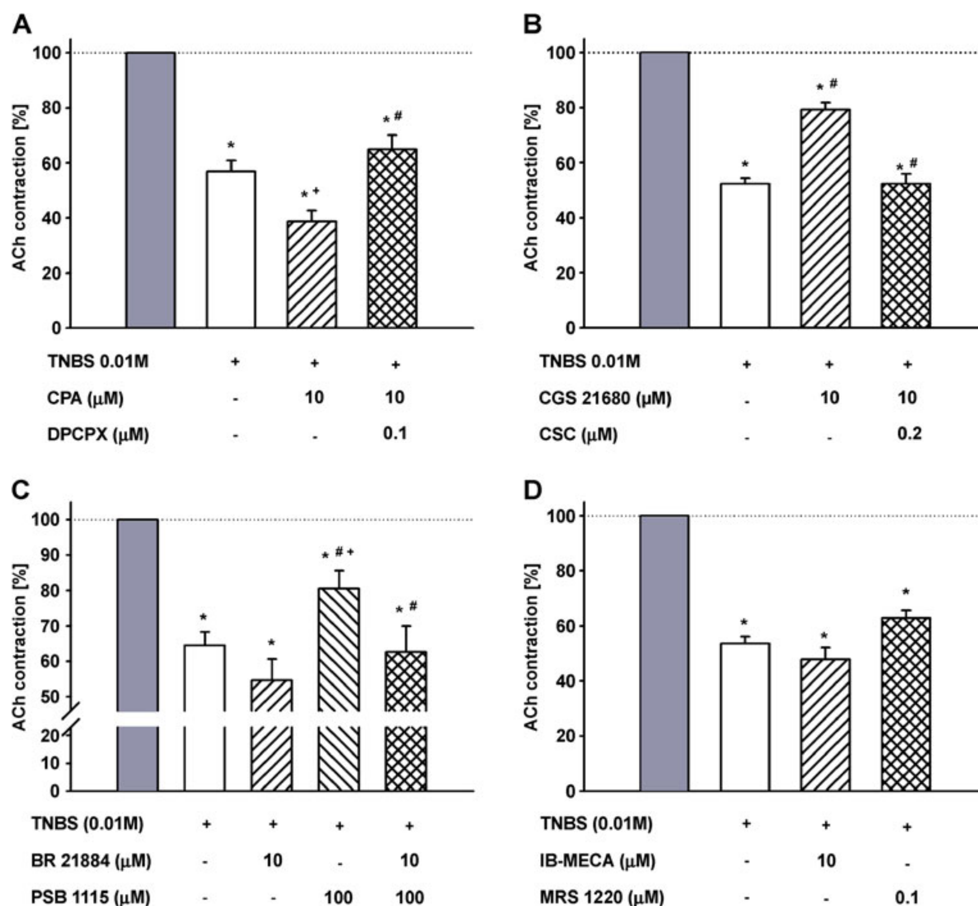

Fig. 2 Effect of adenosine receptor ligands as well as simultaneous preincubation on TNBS-induced attenuation of the ACh (1 mM) contractions in rat ileum/jejunum preparations. **A** A_1R agonist CPA (10 μM) and the A_1R antagonist DPCPX (0.1 μM). **B** $A_{2A}R$ agonist CGS 21680 (10 μM) and the $A_{2A}R$ antagonist CSC (0.2 μM). **C** $A_{2B}R$ agonist BR 21884 (10 μM) and the $A_{2B}R$ antagonist PSB 1115 (100 μM). **D** A_3R IB-MECA (10 μM) and the A_3R antagonist MRS 1220 (0.1 μM). $^*p<0.05$ vs. control (*gray-colored column*), $^{\#}p<0.05$ vs. vs. previous column; $^{+}p<0.05$ vs. TNBS

A_3R was activated by the selective agonist N^6-(3-iodobenzyl)adenosine-5′-*N*-methylcarboxamide (IB-MECA). IB-MECA (10 μM) as well as the A_3R antagonist MRS 1220 did not influence significantly ACh-induced contractions (Fig. 2d).

Receptor binding assays

STW 5 and STW 6 were able to displace the radioactive-labeled A_1 agonist [^{3}H]CCPA (Fig. 3a) as well as radioactive-labeled A_{2A} agonist [^{3}H]CGS-21680 (Fig. 3b) from their respective receptors in a concentration-dependent manner. The shift of the displacement curve of STW 6 to the right indicates a nonspecific binding of this extract. STW 6 inhibited the [^{3}H]CCPA binding by 37±3% and the [^{3}H]CGS-21680 binding by 27±3% in an equivalent concentration of 10 mg/ml STW 5. The results are summarized in Table 3. The data for STW 5 but not for STW 6 permit the interpretation as specific binding.

Displacement experiments with the radiolabeled specific $A_{2B}R$ agonist [^{3}H]PSB 603 indicated no affinity of STW 5 and STW 6 to the $A_{2B}R$. STW 5 but not STW 6 showed a binding to the A_3R when [^{3}H]PSB 11 was used as a specific labeled agonist. The K_i value was 43 μg/ml (Table 3).

Inflamed rat gastrointestinal preparation

According to the data obtained from gene expression and binding studies and the results of the pharmacological studies, further experiments were performed to investigate the role of A_1R and A_2R in STW 5 responses as indicative of anti-inflammatory action in TNBS-treated ileum/jejunum preparations. Previous studies with pharmacological and morphological methods revealed that preincubation of tissue preparations with TNBS (10 mM, 30 min) induced a damaging effect accompanied by lowering of the phasic and tonic activity and suppression of ACh-evoked contractions (Michael et al. 2010). The combined preincubation of the tissue preparations with TNBS and STW 5 (64–512 mg/ml) or STW 6 (3–24.1 μg/ml) reduced concentration-dependently the TNBS-induced attenuation of the ACh-induced contractions (Michael et al. 2009). Following the described protocol, ileum/jejunum preparations were preincubated with TNBS for 30 min. During this time, a marked inflammation developed, manifested by an equilibrium inhibition of the ACh-induced contraction. In the following experiment, STW 5 and STW 6 were used in a concentration which was previously effective in motility modulation experiments (Ammon et al. 2006; Michael et al. 2010). Figure 4a demonstrates that STW 5 (512 μg/ml)

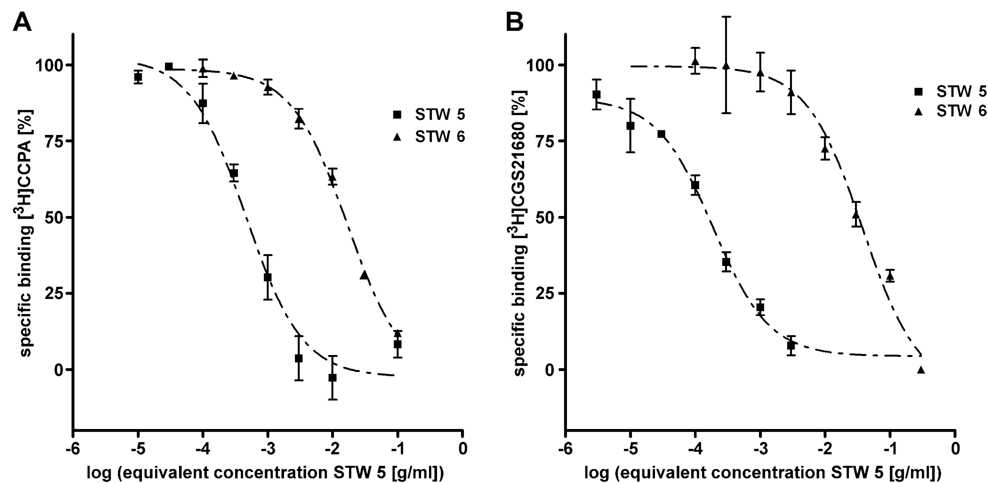

Fig. 3 Competition curves of STW 5 and STW 6 in the adenosine A_1 receptor and the adenosine A_{2A} receptor. Displacement of **A** the radioactive-labeled A_1R agonist [^{3}H]CCPA and **B** the radioactive-labeled $A_{2A}R$ agonist. *Half logarithmic line graphs* of means ± SEM of specific binding of [^{3}H]CCPA and [^{3}H]CGS 21680 from three independent experiments, respectively. A decreased binding of the radioactive-labeled agonist represents competitive displacement by STW 5 or STW 6. The concentrations of STW 6 were calculated as equivalent to STW 5

was able to prevent the TNBS-induced attenuation of the ACh-induced contraction by 19.0% ($p<0.05$ vs. control, $n=18$). The A_{2A} antagonist CSC (0.2 µM) was significantly effective in blocking the STW 5-induced enhancement of ACh-induced contraction (49.1±4.8% vs. 41.0±7.0%; $p<0.05$, $n=18$, Fig. 4a). The experiments were repeated in the presence of the A_1R antagonist DPCPX. The additional preincubation with DPCPX (0.1 µM) did not influence the effect of STW 5. Under these conditions, the enhancement of the ACh-induced contraction by STW 5 (512 µg/ml) amounted to 29.1% ($n=12$) and was completely blocked by 0.2 µM CSC (97.3±6.1% vs. 67.0±4.05%, $p<0.05$, $n=12$, Fig. 4b). The experiments indicated that the protective effect of STW 5 in TNBS-inflamed preparations is mediated primarily by the activation of $A_{2A}R$.

Discussion

The herbal extract STW 5 (Iberogast®) has been shown in clinical trials with irritable bowel syndrome (IBS) patients to be significantly superior to placebo using both an abdominal pain scale and an IBS symptom score after 4 or 8 weeks of treatment (Gundermann et al. 2003; Madisch et al. 2004a, b; Rosch et al. 2002, 2006). Although STW 5 is successfully used in the treatment of IBS, studies to determine the mechanisms underlying an anti-inflammatory effect are lacking. Some facts focused our interest to investigate an interaction of STW 5 and adenosine receptors:

- The mechanisms behind the pathogenesis of IBS are not clear, but important roles played by inflammation and immunological alterations in the development of symptoms compatible with IBS have become evident. An increased innate immune activity in the intestinal mucosa and in blood is found in subpopulations of patients with IBS. Mast cells and monocytes seem to be particularly important (Ohman and Simren 2010).
- Previously, we found that STW 5 concentration-dependently reduced the tone and decreased the ACh-induced contractions in healthy rat intestinal preparations. However, STW 6 in equivalent concentrations neither

Table 3 Mean inhibition of radioligand binding through the herbal extracts STW 5 and STW 6 at adenosine A_1 receptor (A_1R) and A_{2A} receptor ($A_{2A}R$)

Extract	A_1R		$A_{2A}R$		$A_{2B}R$		A_3R	
	Inhibition (%)	K_i (µg/ml)	Inhibition (%)	K_i (µg/ml)	Inhibition (%)	K_i (µg/ml)	Inhibition (%)	K_i (µg/ml)
STW 5	103±7	83.7±11.1	92±3	140±15	n.d.	n.d.	82±1	43±15
STW 6	37±3	133±15	27±3	1,470±255	n.d.	n.d.	n.d.	n.d.

Inhibition values are compared with the STW 5 equivalent concentration of 10 mg/ml. K_i values were calculated from the individual competition curves. Means ± SEM from three independent experiments

n.d. not determined

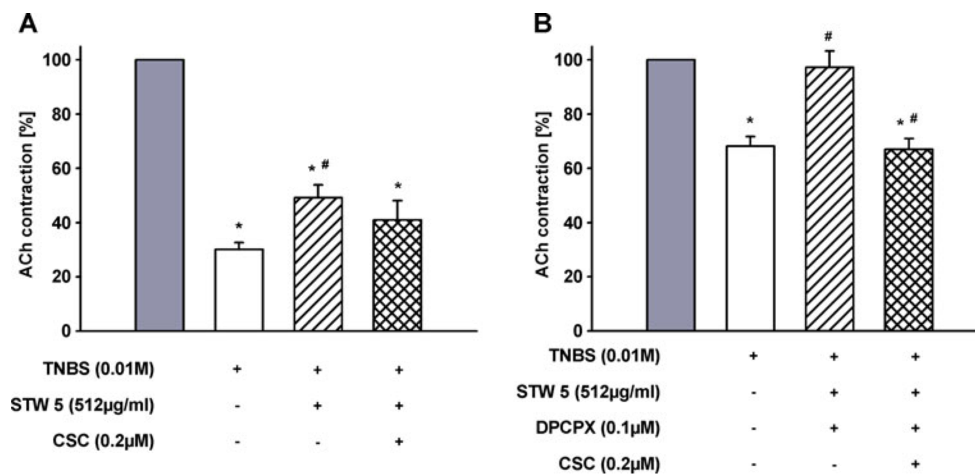

Fig. 4 Effect of A_{2A} receptor antagonists CSC and A_1 receptor antagonist DPCPX on the STW 5 enhancement of ACh contraction during incubation of ileum/jejunum preparations with TNBS. STW 5 (512 µg/ml) was incubated together with TNBS (0.01 M), CSC (0.2 µM), and DPCPX (0.1 µM). **A** Blockade of A_{2A}R with CSC. **B** Blockade of both A_1R with DPCPX and A_{2A}R with CSC. Means ± SEM from 18 (**A**) or 12 (**B**) experiments. *$p<0.05$ vs. control (*gray-colored* column), $^{\#}p<0.05$ vs. previous column

affected the tone nor the contractility of the preparations (Voß et al. 2011), and Heinle et al. (2006) published comparable results on histamine-induced contraction of guinea pig ileum preparations.

- Recent evidence suggests a role of adenosine in the pathophysiology of inflammation (Antonioli et al. 2011b; Michael et al. 2010; Palmer and Trevethick 2008; Sitkovsky and Lukashev 2005). A marked up-regulation has been observed in various models of inflammation, including colitis (Rogachev et al. 2006).
- The diverse distribution and expression of adenosine receptor genes and their translational products suggest a prominent and complex role for adenosine in the modulation of gastrointestinal functions (Christofi et al. 2001).

Therefore, the present experimental settings were designed to examine the expression of adenosine receptors as well as to characterize an interaction of the herbal medicine STW 5 and its component STW 6 with these receptor subtypes, both under healthy conditions and in the presence of inflammation.

For this purpose, our experiments were performed on rat ileum/jejunum preparations. TNBS-induced inflammation is characterized by irregular crypts and mucosal damage. These inflammatory alterations are associated with significant changes in motor function (Michael et al. 2009).

Two studies have examined the expression and localization of adenosine receptor subtypes in the human gastrointestinal tract (Puffinbarger et al. 1995; Christofi et al. 2001). The results using RT-PCR or immunohistochemical analysis have demonstrated a wide distribution of adenosine receptors in the neuromuscular compartment, mucosal and submucosal layer of the small intestine. Additionally, adenosine receptors are also located on epithelial cells and immune cells. Activation of these receptors in epithelial cells as well as in immune cells recruited to the inflamed intestinal mucosa determines the overall effect, ranging from a protective, anti-inflammatory modulation to a strong pro-inflammatory induction (Estrela and Abraham 2011). Changes in purinergic receptor expression appear to contribute to the modulation of intestinal inflammatory responses. This is supported by studies indicating that A_1R and A_{2A}R mediate the inhibitory effects of adenosine on the motor activity of human gastrointestinal preparations (Antonioli et al. 2008). Additionally, Sundaram et al. (2003) found an up-regulation of 1.34-fold for A_1R and 5.40-fold for A_3R in inflamed gut in a rabbit model of chronic ileitis, and Kolachala et al. (2008) demonstrated that the intestinal epithelial A_{2B}R is an important mediator of pro-inflammatory responses in dextran sodium sulfate-treated mice and piroxicam-treated IL-$10^{-/-}$ mice. mRNAs of all adenosine receptor subtypes were expressed in rat ileum/jejunum preparations, and the incubation with TNBS resulted in a significant suppression of A_1R, A_{2A}R, and A_3R mRNA. We focused on the A_1R and A_{2A}R because STW 5 did not bind to the A_{2B}R. Moreover, the activation of the A_{2B}R resulted in a further reduction of the ACh-induced contraction indicating that the attenuating effect of TNBS was pronounced. STW 5 was also shown to bind to A_3R; however, neither the selective agonist nor antagonist had any effect on ACh-induced contractions; therefore, functionality could be excluded. The protective effect of STW 5 against inflammation in the intestine is mainly mediated by activation of A_{2A}R. STW 5 normalized the TNBS-diminished A_{2A}R gene expression. Although the underlying mechanism may not be understood at this time, it might be possible that the occurrence of the up-regulatory mechanism phenomenon in our study may be due to DNA methylation,

since DNA methylation plays a role in $A_{2A}R$ gene transcription and, subsequently, in constitutive $A_{2A}R$ cell surface levels (Buira et al. 2010).

The results clearly show an affinity of STW 5 to $A_{2A}R$. In accordance with these binding experiments, the $A_{2A}R$ antagonist CSC was effective in blocking the STW 5-induced enhanced ACh contraction in inflamed gastrointestinal rat preparations. Our results are in contrast to previous findings indicating a role in the inhibitory control of motor activity on colonic motility in a rat model of experimental colitis (Antonioli et al. 2006). $A_{2A}R$ modulate the activity of colonic excitatory cholinergic nerves via facilitatory control on inhibitory nitrergic pathways, and such a regulatory function is enhanced in the presence of bowel inflammation. Interestingly, in colonic preparations, neither $A_{2A}R$ agonist nor $A_{2A}R$ antagonist affected carbachol-induced contractions (Antonioli et al. 2006). It cannot be excluded that different $A_{2A}R$ subtypes are expressed in ileum and colon preparations as described for basal ganglia. These three different types of $A_{2A}Rs$ can be pharmacologically dissected by their ability to bind ligands with different affinities (Ferre et al. 2011).

A key molecular mechanism that has emerged as being critical for the inhibitory effects of the $A_{2A}R$ on inflammatory and immune responses is suppression of the nuclear factor-κB pathway, activated by cytokines such as TNFα and IL-1β as well as pathogen-derived Toll-like receptor agonists such as lipopolysaccharide (Hasko et al. 2000; Link et al. 2000; Odashima et al. 2006; Sands et al. 2004).

Activation of $A_{2A}R$ produced various responses that can be characterized as anti-inflammatory effects (Antonioli et al. 2007). For example, the stimulation with the $A_{2A}R$ agonist ATL-146e was associated with a reduction of the inflammation in the intestinal mucosa. The leucocytes' infiltration and the levels of the pro-inflammatory cytokines TNFα, IFN-γ, and IL-4 were reduced (Odashima et al. 2005). Moreover, activation of $A_{2A}R$ on human monocytes and mice macrophages inhibited the secretion of the pro-inflammatory cytokines IL-12 and TNFα (Hasko et al. 2000; Link et al. 2000).

Previously, we have demonstrated that the $A_{2A}R$ agonist and the $A_{2B}R$ antagonist or the combination of both effectively counteracted the development of TNBS-induced derangement of the ACh contraction. The ligands exerted protective effects when treatment began simultaneously with the application of TNBS. The effect was in the same range as the effect induced by 1 μM methotrexate (Michael et al. 2010), which is known to suppress inflammation by activation of $A_{2A}R$ (Montesinos et al. 2006). Here we showed that the gene expression of the $A_{2B}R$ was not affected by STW 5, and additionally, the activation of this receptor subtype did not enhance the TNBS-attenuated ACh contraction. The role of A_{2B} receptors has been largely unexplored because of the lack of specific $A_{2B}R$ agonists. $A_{2B}Rs$ are up-regulated in models of intestinal inflammatory diseases (Kolachala et al. 2005). Stimulation of $A_{2B}R$ in a human mast cell line increased production of pro-inflammatory cytokines like IL-6 (Ryzhov et al. 2004, 2008) and increased IL-6 release from airway smooth muscle cells and fibroblasts (Zhong et al. 2004, 2005). Contrary to this line of evidence, a recent report pointed out that $A_{2B}R$ knockout mice showed a phenotype with increased inflammatory responses (Yang et al. 2006). BAY 60–6583, the first $A_{2B}R$ agonist, was characterized in CHO cells expressing human $A_{2B}R$. The EC_{50} values were 23 nM for $A_{2B}R$ and >10,000 nM for A_1R and $A_{2A}R$. Soluble 5′-nucleotidase or $A_{2B}R$ agonist treatment mimicked cardioprotection by ischemic preconditioning because it was associated with significant attenuation of myocardial infarct size after ischemia (Eckle et al. 2007). Our present data provide no evidence for an affinity of STW 5 to $A_{2B}R$ in receptor binding studies. Therefore, it is suggested that the protective effect of STW 5 on TNBS-induced damage is mediated by activation of adenosine $A_{2A}R$. STW 5 interacted with A_1R as demonstrated in receptor binding studies. Nevertheless, the involvement of A_1R in the protective action of STW 5 on the TNBS-diminished ACh contraction could be excluded since DPCPX did not block the effect of STW 5 whereas CSC in the presence of DPCPX did.

A general question in herbal drug combinations is whether the action of a single main substance can explain the effects of the whole extract or the action results from a multi-extract effect. For STW 5, the multi-target concept was developed and comprises multiple effects on gastrointestinal motility, anti-inflammatory action, inhibitory effects on gastric acid production, and anti-oxidative and radical-inhibiting properties (Wegener and Wagner 2006). In order to study this aspect, STW 6, the fresh plant component in STW 5, was assayed and compared with STW 5. The binding studies confirmed that STW 6 did not bind to A_1R or $A_{2A}R$, and consequently, its anti-inflammatory action may be based on other mechanisms than those mediated by AR (Michael et al. 2009).

In conclusion, the present study described a new mechanism for understanding the contribution of AR to the anti-inflammatory action of STW 5. In particular, our findings provide evidence that STW 5 binds to $A_{2A}R$. This pathway may inhibit the pro-inflammatory cytokine TNFα resulting in a reduction of the inflammation-induced impairment of the contractility. STW 6, the fresh plant component of STW 5, did not contribute to the $A_{2A}R$-mediated anti-inflammatory effect. Other signaling pathways could be involved in the mechanism of action of STW 6.

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