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Adenosine Receptors: New Targets to Protect Against Tissue Damage in Inflammatory Bowel Symptoms

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1. Introduction

Irritable bowel syndrome (IBS) is a disease in which, typically, alterations in intestinal motility and visceral hypersensitivity appear to exist, apparently without any organic alteration (Thompson, 1991). Several pathogenic factors responsible for IBS have been suggested. It seems that there are cell factors, which give reason to believe that there is a low-grade intestinal inflammation in this pathology (Ortiz-Lucas et al., 2010). Several cytokines, such as tumour necrosis factor α (TNFα), interleukin 1, and interleukin 6, contribute to the pathogenesis (Ardizzone and Bianchi Porro, 2005; Pizarro et al., 2006). Macrophages are the major producers of TNFα, and, interestingly, they are also highly responsive to TNFα. TNFα has been shown to play a pivotal role in activating the cytokine cascade in many inflammatory diseases and it has been proposed as a therapeutic target for a number of diseases. Consequently, recent strategies for the treatment of intestinal inflammation have primarily targeted the immunopathogenic processes that mediate intestinal inflammation at the cytokine level (Bamias et al., 2003; Sandborm and Targan, 2002). 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 adenosine regulates immunity and inflammation (Amann and Peskar, 2002; Montesinos et al., 2007). The wide distribution of adenosine receptors (AR) as well as enzymes for purine metabolism in different gut regions suggests a complex role for this mediator in the regulation of gastrointestinal functions (Antonioli et al., 2008). 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 having a unique pharmacological profile, tissue distribution and signalling pathway (Jacobson and Gao, 2006). All known ARs

contribute to the modulation of inflammation, as demonstrated by many *in vitro* and *in vivo* pharmacological studies (Hasko and Cronstein, 2004; Montesinos and Constein, 2001). The involvement of adenosine pathways in the anti-inflammatory and immunomodulating effects becomes evident. These observations have stimulated the research of novel drugs suitable for treatment of intestinal inflammatory disorders through the pharmacological modulation of adenosine pathways (Cavalcante et al., 2006; Guzman et al., 2006; Odashima et al., 2005).

One medication which is successfully used in functional dyspepsia and IBS is the fixed herbal combination product STW 5 (Iberogast®; Madisch et al., 2004; Perez and Youssef, 2007; Raedsch et al., 2007; Schmulson, 2008). There is growing evidence that STW 5 besides being effective in functional dyspepsia, also improves IBS symptoms (Madisch et al., 2004; Krueger et al., 2009). STW 5 and its fresh plant component *Iberis amara* (STW 6) show a powerful reduction of morphological and contractile damages observed after experimental inflammation within the small intestine, and may thus have a promising therapeutic value as anti-inflammatory drug (Michael et al., 2009).

We therefore investigated the effect of STW 5 and its main component STW 6 on experimentally induced inflammation in rat ileum/jejunum preparations and the mechanisms of action. Using RT-PCR the expression of the ARs mRNA was determined and an interaction between the receptors and STW 5 as well as STW 6 was characterized. The results were confirmed by receptor binding experiments and pharmacological use of selective receptor antagonists.

2. Materials and methods

2.1 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 (12 h light/12 h dark) controlled environment with food and water ad libitum. The rats were anaesthetized with CO_2 and killed by decapitation. The abdomen was immediately opened; intestinal segments (ileum and distal part of the jejunum) of about 15 cm were rapidly removed and placed in a dish containing aerated modified Krebs solution at 37 °C.

2.2 Materials

STW 5 contains *Iberis amara* totalis (STW 6) fresh plant extract and eight dried plants as drug extracts (Table 1).

STW 5 and STW 6 were kindly provided by Steigerwald Arzneimittelwerk GmbH, Darmstadt, Germany, in 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 as described before (Hohenester et al., 2004). STW 6 was used in equivalent concentrations to its proportion in STW 5.

ACh (1 M) was prepared as fresh 1:10 dilution 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*-sulfophenylxanthine) was synthesized at the PharmaCenter Bonn,

Department of Pharmaceutical Chemistry I, University of 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 %.

Plant extract	Drug-extract ratio	ml/100ml
Iberis amara totalis (STW 6)	1:1.5-2.5	15
Menthae piperitae folium	1:2.5-3.5	5
Matricariae flos	1:2.0-4.0	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

Table 1. Constituents of STW 5

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. The reverse transcription (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 (PBS) contained (mM): NaCl (15.0), NaH_2PO_4 (4.0), 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. Primers were from Invitrogen. Enzymes used for reverse transcription were from Fermentas GmbH. The PCR reaction kit was from Bio-Rad Laboratories GmbH, Munich, Germany. All other substances were purchased from Sigma-Aldrich Chemie GmbH, Steinheim, Germany.

2.3 Radioligand binding assays

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

A_1R was taken from rat cortical tissue homogenates. Boards with 48 or 96 cavities were used. Each cavity carried 30 µg proteins in 200 µl final volume. 2-Chloro-N^6-[^{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 adenosine deaminase resistant adenosine analogue 2-chloroadenosine (CADO 10 µM). The extracts were dissolved in water. Incubation of boards took place at room temperature for 1.5 h. After that all samples were filtered on a cell harvester (Brandel) with ice-cold tris-HCl and filled into scintillation tubes. After 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).

$A_{2A}R$ was taken from rat brain striatal tissue homogenates. Boards with 48 or 96 cavities were used. Each cavity carried 50 µg protein 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 was used as medium. Unspecific binding was determined with complete displacement by broad spectrum AR agonist 5'-N-ethylcarboxamidoadenosine (NECA 50 µM).

The extracts were dissolved in water. Incubation of boards took place at room temperature for 1.5 h. The final procedure was the same as for A_1R preparations.

The results were analyzed and displayed with GraphPad PRISM®.

2.4 Induction of inflammation and drug application

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

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

2.5 Recording of mechanical activity

The preparations were suspended in 20 ml organ baths containing oxygenated (95 % O_2, 5 % CO_2) modified Krebs solution maintained at 37 ºC. Then they 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 difference between the basal tone and the first maximum contraction after drug application. For each experiment an untreated preparations (control 100%) and TNBS-treated preparation were used from the same animal. The TNBS-induced alterations of ACh-induced contractions differed strongly between several experimental series. To evaluate the experimental series the damage was calculated as an internal standardisation of the TNBS-induced alterations of the ACh-contractions. 100% damage represents the effect of TNBS on ACh-contractions under control conditions.

2.6 Van Gieson staining and morphometric analysis

The middle part (0.5 cm) of each preparation was fixed in phosphate buffered paraformaldehyde (4 %), washed with PBS, dehydrated in sequential ethanol baths with increasing concentrations of ethanol (50 % to 99.8 %) and embedded in paraffin wax. Slices of 7 µm were cut with a Jung Biocut microtome (Leica). The slices were dewaxed with xylene and rehydrated in three sequential ethanol baths with decreasing concentrations that ranged from 99.8 % to 70 % ethanol. For histological studies, the sections were stained with haemalaun solution (Mayer, cell nuclei) and van Gieson solution (picric acid with sour fuchsine) according to the method of Romeis (Mikroskopische Technik, 1989). The slides were examined qualitatively under a light microscope at 20x magnification. For analysing of

the histological photographs the method of calibrated ocular micrometer gauge was used. Largest and smallest diameters of the tissue layers were determined to calculate the area of mucosa as well as muscularis (longitudinal and circular muscle layers). Three positions were implemented in the calculation. Ten objects from at least three animals were used for statistics.

2.7 RNA isolation and reverse transcription

Following the manufacturers protocol total RNA from ileum/jejunum segments was extracted after preincubation for 3 h using the RNeasy Mini kit® (Qiagen). 10 µl 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 units of RevertAID (Fermentas) and dNTP (1 mM) in the Crocodile III cycler in the RT-buffer. The final volume was 20 µl. The reaction was stopped by heating at 70 °C for 10 min.

2.8 Real-time fluorescence PCR

AR mRNA expression as well as the expression of TNFα and IL-10 mRNA were measured quantitatively by a ready-to-use real-time fluorescence polymerase chain reaction (PCR) assay. SYBR Green® Mix reaction (BioRad) was used in a MyIQ® cycler (BioRad) according to manufacturers protocol. β-actine was used as a housekeeping gene. The primers for β-actin and the adenosine A$_1$R were self-designed, whereas the primers for the A$_{2A}$R, TNFα and IL-10 were found in literature (Chen et al. 2004). Table 2 summarizes the sequences of the primers.

primer	sequence	origin
ß-Actin sense	5'-TGTCACCAACTGGGACGATA-3'	designed by the authors
ß-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, 2004
A$_{2A}$ antisense	5'- TCCATCTGCTTCAGCTGTCT-3'	
TNFα sense	5'-TCAGCCTCTTCTCATTCCTG-3'	designed by the authors
TNFα antisense	5'-GGCTACGGGCTTGTCACTCG-3'	
IL-10 sense	5'- TTTAAGGGTTACTTGGGTTGC-3'	Klingenberg, 2006
IL-10 antisense	5'- GCTCCACTGCCTTGCTTTTA-3'	

Table 2. Primers for RT-PCR.

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.

2.9 Maceration

STW 6 (24.2 µg/ml) was extracted three times each with hexane, chloroform and ethyl acetate (water saturated). Emulsions were centrifuged. The solvent phase was siphoned off. The solvent phases of the three extractions of each solvent were combined and vacuum evaporated. The fractions as well as the remaining aqueous fraction were lyophilised. The

lyophylisates were analysed qualitatively by thin layer chromatography using standard protocols (Wagner, 1996) and quantitatively by high pressure liquid chromatography (HPLC, Kroll, 2006). The results of HPLC are shown in table 3.

fraction	Cucurbitacin E	Cucurbitacin I	mass percentage
STW 6	44.4 µg/ml	31.6 µg/ml	100 %
STW 6-CHCl$_3$	31.4 µg/ml	14.9 µg/ml	3.2 %
STW 6-EA	1.2 µg/ml	0.65 µg/ml	2.1 %
STW 6-H$_2$O	n.d.	n.d.	93.5 %

Table 3. Quantitative results of the fraction chromatography (n.d. not detected).

2.10 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.

3. Results

3.1 Effects of STW 5 and STW 6 on ACh-contractions in inflamed preparations

The ileum/jejunum preparations were pre-incubated with TNBS for 30 min. During this time a marked inflammation developed, manifested by a constant inhibition of the ACh-induced contractions. As shown previously TNBS (1 mM - 1 M) resulted in a concentration-dependent inhibition of ACh-induced contractions (damage) with an IC_{50} value of 63 mM. 100 mM TNBS reduced the ACh-induced contraction to approximately 35 % in comparison to intact preparation of the same animal (Michael et al., 2010). The combined preincubation of the ileum/jejunum preparation with TNBS (0.01 M, 30 min) and STW 5 (64-512 µg/ml) or STW 6 in equivalent concentrations (3-24.1 µg/ml) diminished concentration-dependently the TNBS-induced damage of the ACh-contraction. STW 5 (128-512 µg/ml) prevented up to 30 % of the TNBS-induced damage, whereas STW 6 at the highest concentration of 24.1 µg/ml had even a stronger effect of 56.8±6.6 % (Fig. 1A).

To study whether the TNBS-induced damage is linked with morphological alterations van Gieson staining was done followed by detailed morphometric analysis. Histological assessment revealed tissue damages in preparations pre-incubated with TNBS. As seen in Fig. 1 B, the mucosal area of control preparations was 22,150±100 µm². It was reduced to 16,080±810 µm² by TNBS (0.1 M). The combined pre-incubation with TNBS and STW 5 (29,980±2,150 µm²) or STW 6 (20,690±1,010 µm²) preserved the mucosa. The gradual reduction of the muscularis area (longitudinal and circular muscle layers) was even greater after TNBS incubation. The decrease was from 5,770±300 µm² to 2,750±140 µm². STW 5 and STW 6 also prevented this TNBS-induced morphological disturbance (5,530±450 µm² and 5590±360 µm², Fig. 1C).

3.2 Effects of STW 5 and STW 6 on the gene expression of TNFα and IL-10

Human monocyte is an established model to study the inflammatory or anti-inflammatory drugs (Linden, 2006; Sitkovsky et al., 2004). In previous studies we have shown that treatment of monocytes with STW 5 (128 µg/ml) or STW 6 (6.0 µg/ml) had no effect on the

TNFα release whereas LPS, a well described inflammatory mediator, stimulated the release of TNFα from 0.007±0.007 ng/ml to 1.4±0.1 ng/ml. STW 5 prevented the LPS-induced TNFα release by depressing the release to 0.4±0.2 ng/ml. No inhibitory effect was found with STW 6 (Michael et al., 2009).
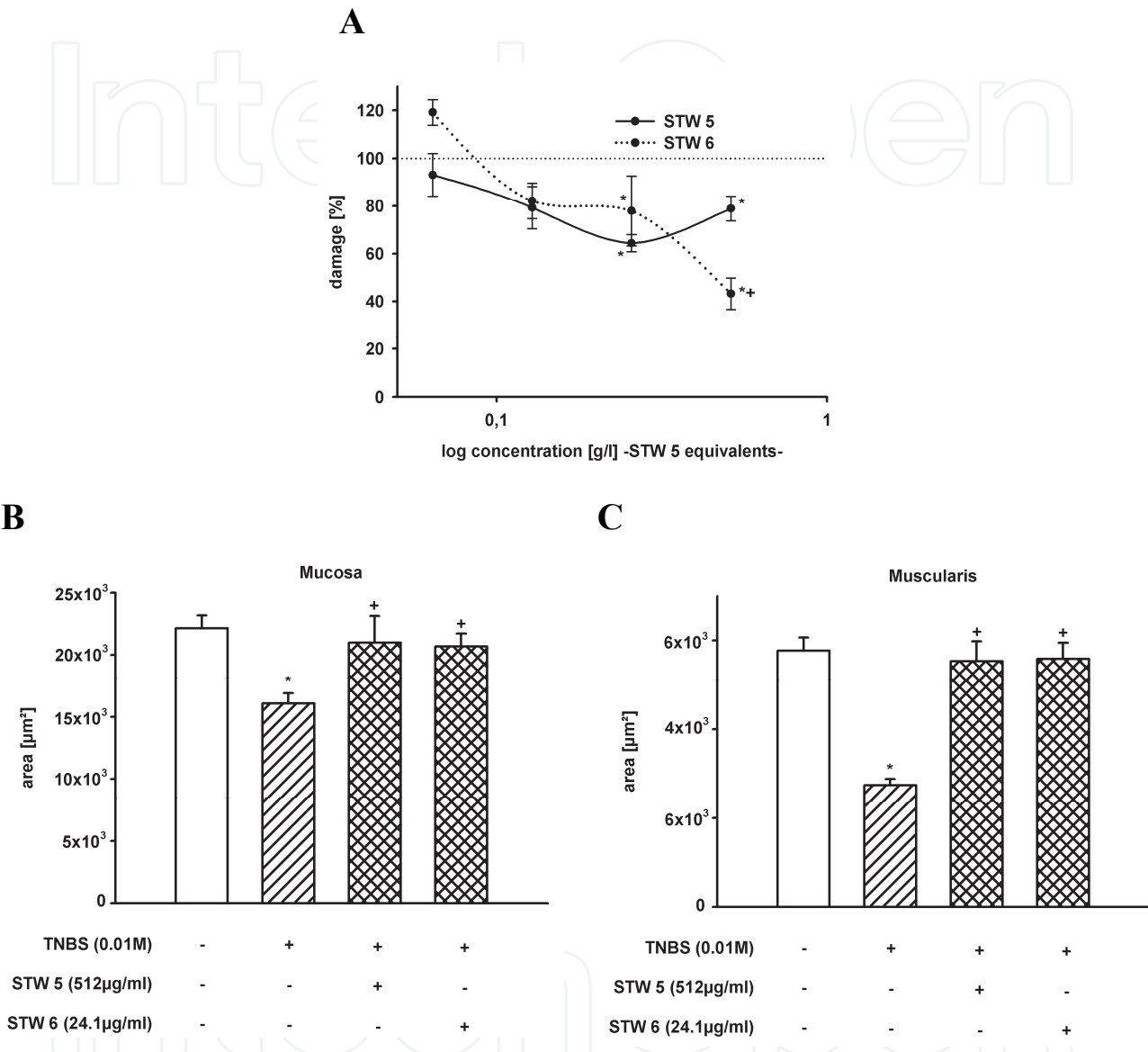

Fig. 1. Effects of STW 5 and STW 6 on TNBS-induced damage of ACh-induced contractions in ileum/jejunum preparations. (A) Concentration response curves of the effect of STW 5 (64-512 μg/ml) and STW 6 in equivalent concentrations (3-24 μg/ml) after coincubation with TNBS (0.01 M, 30 min). STW 5 and STW 6 prevented concentration dependently the TNBS-induced damage. STW 6 at maximum concentration mediated a <20 % stronger effect than STW 5. Mean±SEM of nine experiments, *p<0.05, significant vs. control; +p<0.05 significant vs. previous value. Morphometric analysis of the effects of STW 5 and STW 6 on mucosa (B) and muscularis (C). STW 5 (512 μg/ml) and STW 6 (24.1 μg/ml) prevented the TNBS-induced effects. Mean±SEM of 30 measurements (B and C), *p<0.05, significant vs. control; +p<0.05 significant vs. TNBS.
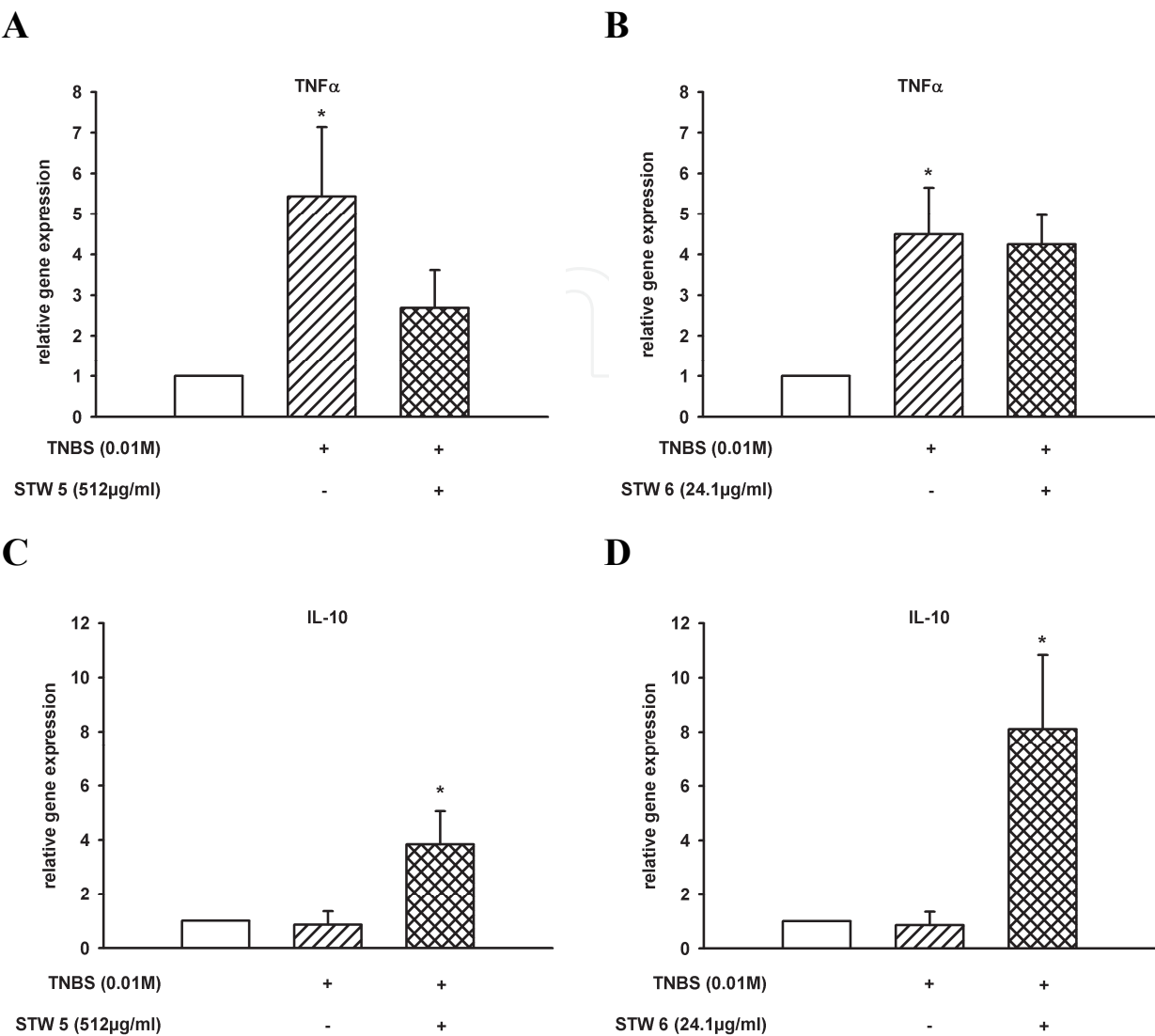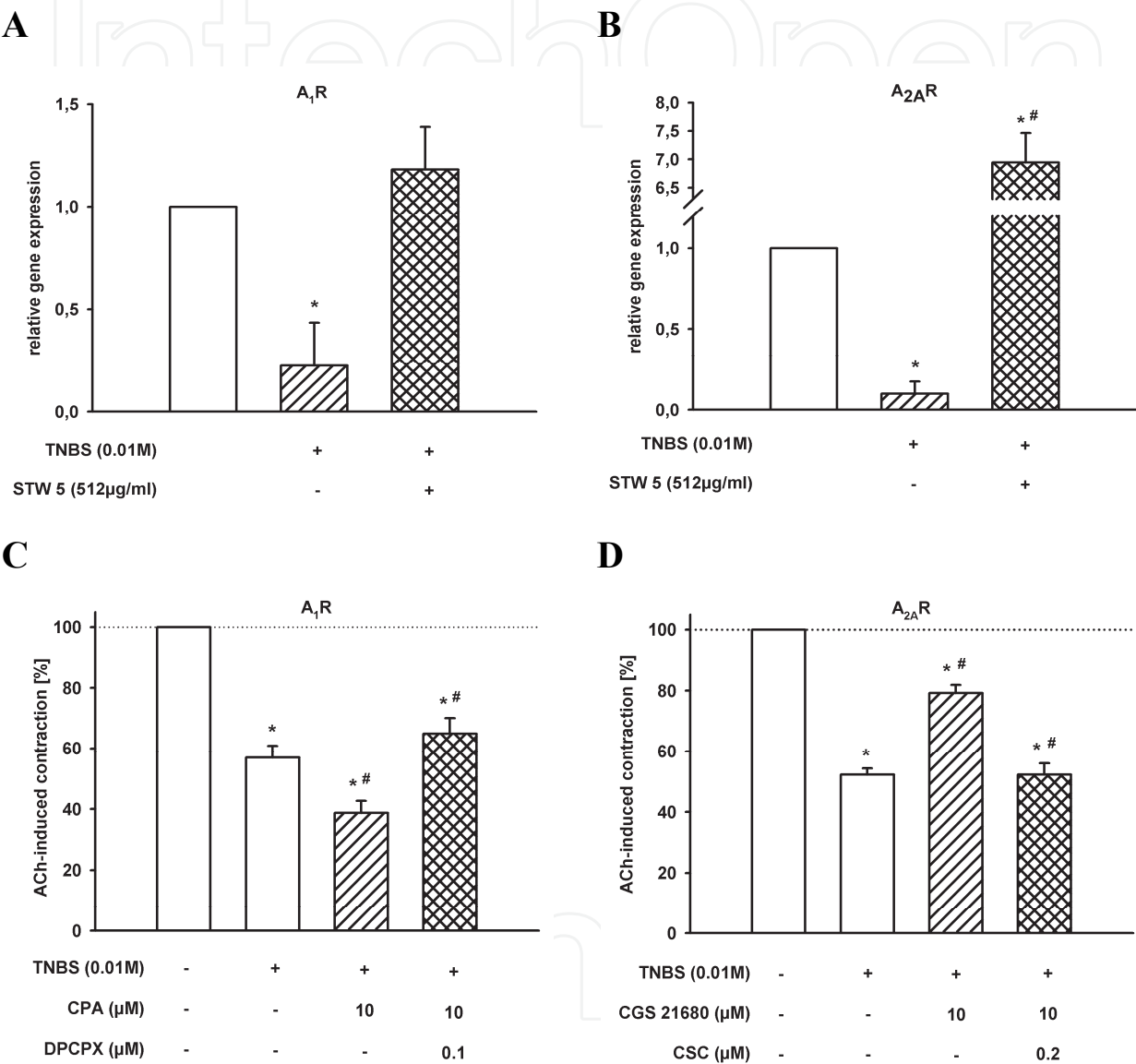
Fig. 2. Effects of TNBS alone and after coincubation of TNBS with STW 5 or STW 6 on TNFα and IL-10 gene expression in ileum/jejunum preparations. TNBS (0.01 M) increased significantly the TNFα gene expression. STW 5 (512 µg/ml, A) but not STW 6 (24.1 µg/ml, B) prevented the gene activation. The IL-10 gene expression was not affected significantly by TNBS (0.01 M). It was increased by coincubation of TNBS and STW 5 (512 µg/ml, C) and this effect was more pronounced with STW 6 (24.1 µg/ml, D). Mean±SEM of five experiments, * p<0.05 significant vs. control (blank column).

Here, we investigated the gene expression of TNFα and additionally the gene expression of the anti-inflammatory cytokine IL-10. Preincubation of ileum/jejunum preparations with TNBS (0.01 M) resulted in a significant increase of the relative gene expression of TNFα by factor 5.4±1.7 (Fig. 2A) and 4.5±1.1 (Fig. 2B) compared to the gene expression in control preparations. The combined preincubation with TNBS and STW 5 (512 µg/ml) reduced this factor to 2.7±0.9 (Fig. 2A), whereas STW 6 had no significant effect (4.2±0.7) on the TNBS-increased gene expression (Fig. 2B). In contrast, TNBS did not influence the relative gene expression of IL-10 (factor 0.86±0.50). After combined preincubation with STW 5 (512 µg/ml), the relative gene expression was significantly increased by factor 3.85±1.21 compared to the gene expression in

control preparations (Fig. 2C). The increase was even more pronounced after combined preincubation with STW 6 (24.1 µg/ml, factor 8.10±2.73, Fig. 2D).

3.3 Gene expression and functionality of adenosine receptors

RT-PCR was used to study the expression of receptor mRNA for A_1R and $A_{2A}R$ in intact and TNBS-treated ileum/jejunum preparations.

Fig. 3. Gene expression of A_1R and $A_{2A}R$ as well as their alteration after incubation with TNBS and STW 5 (A and B). AR-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. Effect of the A_1R agonist CPA (10 µM) and the A_1R antagonist DPCPX (0.1 µM) on the TNBS-induced decrease of the ACh (1 mM) -induced contractions (C). Effect of the $A_{2A}R$ agonist CGS 21680 (10 µM) and the $A_{2A}R$ antagonist CSC (0.2 µM) on TNBS-induced decrease of the ACh (1 mM) contractions (D). Mean±SEM of 12 experiments, *p<0,05 vs. control (open column), #p<0.05 vs. previous column.

A_1R and $A_{2A}R$ mRNA were identified in intact preparations. Incubation of the preparations with TNBS (10 mM,) resulted in a significant suppression of the mRNA expression. STW 5 (512 µg/ml) coincubated with TNBS (10 mM) protected from TNBS-induced suppression of the A_1R (Fig. 3A). The gene expression remained at control levels. For $A_{2A}R$ mRNA even a significant induction by factor $6.9±0.5$ was detectable (Fig. 3B).

The functionality of the adenosine A_1R and $A_{2A}R$ was tested pharmacologically. Ileum/jejunum preparations were incubated with TNBS together with specific receptor agonists and antagonists for 30 min. After that ACh (1 mM)-induced contractions were recorded. TNBS (10 mM) decreased the ACh-induced contraction to $57.9±3.9$ % ($p<0.05$ vs. control, n=12). After preincubation together with the A_1R agonist CPA (10 µM), the decrease to $38.8±3.26$ % (n=12, $p<0.05$ vs. control). DPCPX (0.1 µM), described as inverse agonist on A_1R, enhanced slightly the TNBS-reduced contractions but prevented the CPA-induced inhibition ($65.0±5.1$ %, $p<0.05$ vs. CPA, n=12, Fig. 3C). 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 $A_{2A}R$ antagonist CSC (0.2 µM) had negligible 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 enhanced contraction ($52.3±3.7$ %, $p<0.05$ vs. CGS 21680, n=9, Fig. 3D).
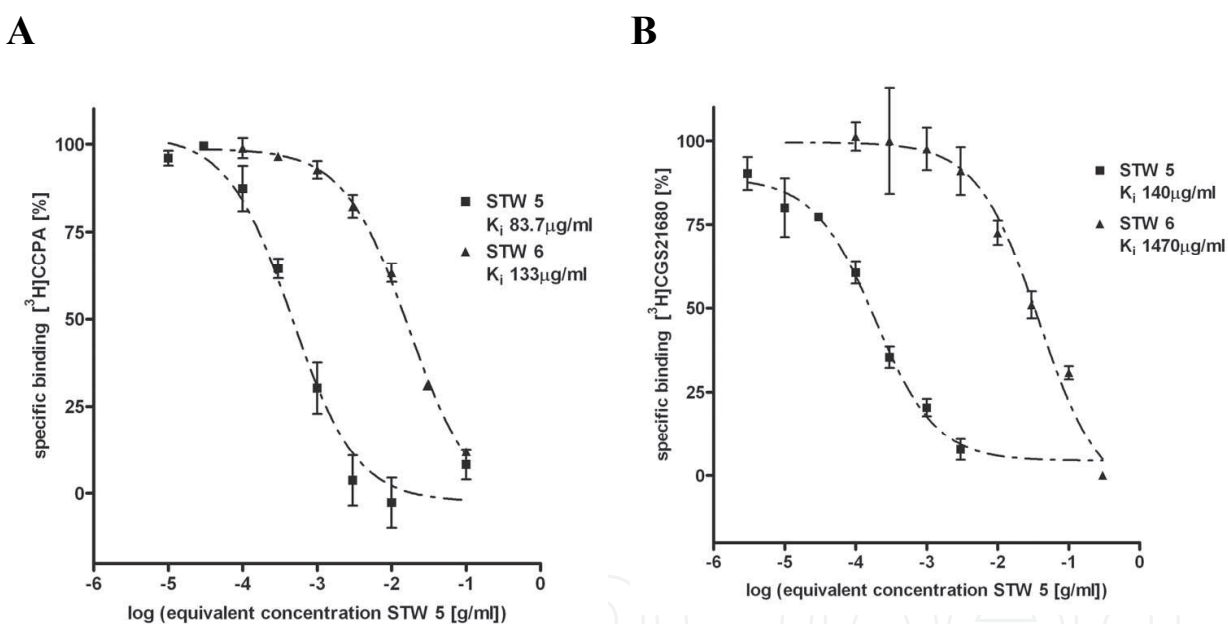
A   **B**

Fig. 4. Competition curves of STW 5 and STW 6 at the A_1R and the $A_{2A}R$. Displacement of (A) the radioactive labelled A_1R agonist [3H]CCPA and (B) the radioactive labelled $A_{2A}R$ agonist [3H]CGS 21680. Half logarithmic line graphs of mean ± SEM of specific binding of [3H]CCPA and [3H]CGS 21680 from 3 independent experiments, respectively. A decreased binding of the radioactive labelled agonist represents competitive displacement by STW 5 or STW 6. The concentrations of STW 6 were calculated as equivalent to STW 5. Mean±SEM of three independent experiments.

3.4 Receptor binding assays

To evaluate an interaction between STW 5 and STW 6 and A_1R and $A_{2A}R$, receptor binding studies were designed. STW 5 and STW 6 were able to displace the radioactive labelled A_1R

agonist [3H]CCPA (Fig. 4A) as well as radioactive labelled $A_{2A}R$ agonist [3H]CGS 21680 (Fig. 4B) from these receptors in a concentration dependent manner. The shift of the displacement curve of STW 6 to the right indicates a very weak binding of this extract, which may be due to nonspecific interactions.

3.5 Interaction of STW 5 and $A_{2A}R$ in inflamed preparations

According to data from gene expression in further experiments the role of A_1R and $A_{2A}R$ in STW 5 responses is indicative for an anti-inflammatory action and was therefore investigated in TNBS-treated ileum/jejunum preparations. STW 5 (512 µg/ml) was able to prevent the TNBS-induced damage of the ACh-contraction by 19.0 % ($p<0.05$ vs. control, n=18) in ileum/jejunum preparations. The $A_{2A}R$ antagonist CSC (0.2 µM) was significantly effective in blocking the effect of STW 5 ($p<0.05$, n=18, Fig. 5). The experiments were repeated in the presence of A_1R antagonist DPCPX. The additional preincubation with DPCPX (0.1 µM) enhanced the damage of the ACh-induced contractions but did not affect the effective blockade by CSC (Fig. 5).The experiments indicate that the protective effect of STW 5 in TNBS-inflamed preparations is mediated primarily by activation of $A_{2A}R$.
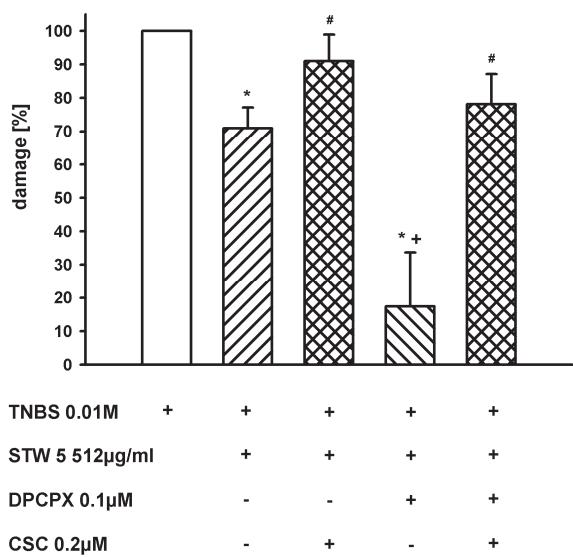

Fig. 5. Impact of $A_{2A}R$ antagonist CSC and A_1R antagonist DPCPX on the STW 5 mediated effect on the TNBS-induced damage of the ACh contraction. STW 5 (512 µg/ml) was incubated together with TNBS (0.01 M), CSC (0.2 µM) and DPCPX (0.1 µM,) or the combination of the antagonists. Means±SEM from 12 to 18 experiments. *$p<0.05$ vs. TNBS (open column), # $p<0.05$ vs. previous column, + $p<0.05$ vs. STW 5.

3.6 Mechanism of action of STW 6 in inflamed preparations

The experiments described above clearly indicate that the protective effect of STW 6 was not mediated by activation of $A_{2A}R$ or interaction with TNFα pathway rather than by activation of IL-10 pathway. Therefore, the next set of experiments was designed to study

the mechanisms involved in the action of STW 6. Bio-guided isolation of active ingredients within STW 6 was performed using solvents of different polarities. The hexane fraction had only a marginal mass portion (1.3 %) and was, therefore, excluded from further experiments. The chlorophorm, ethyl acetate and water fractions were concentrated to the equivalent concentration of STW 6 (24.1 µg/ml), respectively, and used for simultaneous incubation of ileum/jejunum preparations with TNBS. The chloroform (37.9±12.8%, p<0.05, n=9) and ethyl acetate fraction (29.7±9.9%, p<0.05, n=9), which contained the cucurtacines E and I (Fig. 6A), prevented the TNBS inhibition of the ACh-induced contractionsrespectively. The water fraction, devoid of cucurbitacins, was without effect. This observation was further supported by the fact that purified cucurbitacin E and I could antagonize the TNBS-induced damage of ACh- stimulated contraction with EC_{50} of 0.12 µM and 0.04 µM, respectively (Fig. 6B). Additionally, cucurbitacins were able to stimulate the IL-10 gene expression. TNBS was without effect on the relative IL-10 gene expression. Cucurbitacin E (10 mM) coincubated with TNBS (10 mM, 30 min) significantly induced IL-10 mRNA by the factor 7.0±2.8 (p<0.05 vs. control, n=3, Fig. 6C).

4. Discussion

Gastrointestinal inflammation is accompanied by structural and functional changes of the gut, leading to gastrointestinal motility disturbances during both acute and chronic inflammation (Collins, 1996). Motility disturbance may persist in the period following an episode of gastrointestinal inflammation, resulting in the development of IBS or functional dyspepsia, which are suggested to be part of a single syndrome (Holtmann et al., 1997). Both IBS and functional dyspepsia are associated with disturbed gastric motor function and decreased gastric emptying (Caballero-Plasencia et al., 1999). It is well established that inflammatory bowel diseases are chronic immune-mediated intestinal disorders. Both subtypes, Crohn's disease (CD) and ulcerative colitis (UC), are considered to arise as a consequence of an aberrant intestinal immune response in genetically predisposed individuals (Xavier and Podolsky, 2007). In inflamed segments of the bowel, persistent overproduction of pro-inflammatory cytokines, such as TNFα and IL-6 and impaired production of anti-inflammatory cytokines are characteristic features (Strober and Fuss, 2006). Furthermore, gut inflammation is associated with extensive structural and functional alterations of the enteric nervous system and dysregulation in neuroimmune interactions (Bischoff and Gebhardt, 2006). Different experimental animal models are used to address these clinical findings.

In this study, using an *in vitro* inflammation model (Michael et al., 2009), we demonstrated that the action of STW 5 resulted in a reduction of TNBS-induced disturbance of contractions possibly by attenuation of the TNBS-induced morphological damages. The mechanism of action of STW 5 is based at least on two distinctive mechanisms. STW 5 inhibited the pro-inflammatory TNFα pathway by activation of $A_{2A}R$ and STW 6, the fresh plant component of STW 5, stimulated the anti-inflammatory IL-10 pathway. It seems that cucurbitacins are involved in influencing this pathway. Both pathways effectively contribute to the overall effect of STW 5. It is known from numerous studies carried out with plant extracts that the whole extract has in most cases a better efficacy than

a single substance isolated from the extract (Wegener and Wagner, 2006). Our findings are in accordance with other pharmacological studies showing different effects of a single
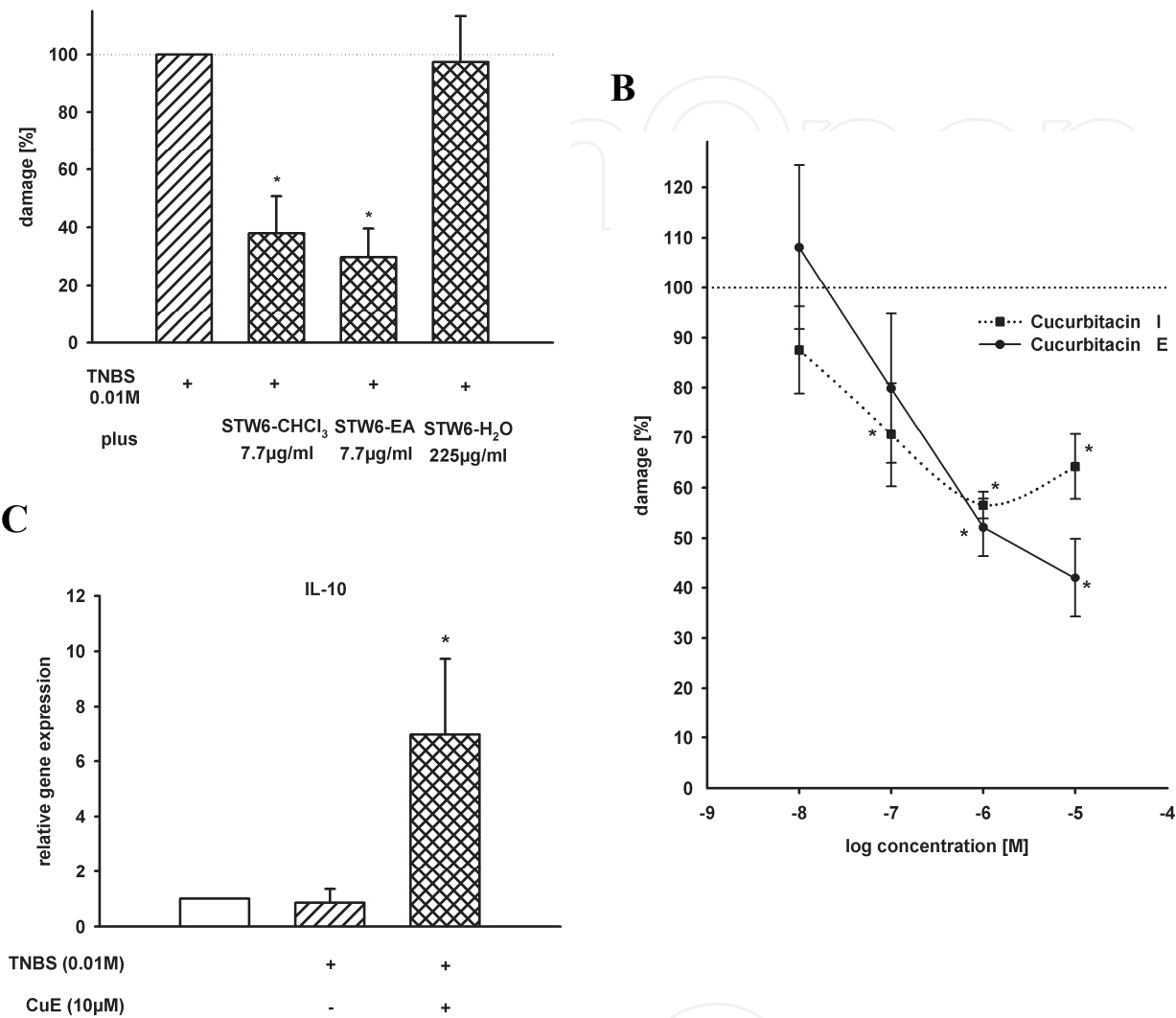
A

B

C

Fig. 6. Effects of the fractions of STW 6 and the cucurbitacins E and I on the TNBS-induced damage and the influence of cucurbitacin E on IL-10 gene expression. (A) The chloroform and ethyl acetate fractions containing the cucurbitacins E and I but not the water faction without cucurbitacins antagonized the damage of the ACh-contraction. (B) cucurbitacin E and I coincubated wit TNBS reduced concentration dependently the TNBS-induced damage of the ACh contraction. (C) Cucurbitacine E coincubated with TNBS enhanced the IL-10 gene expression. Means±SEM from 9 (A), 9 (B) and 3 (C) experiments. *p<0.05 significant vs. control.

constituent of STW 5 on mechanisms which are discussed as underlying the manifestation of IBS (Heinle et al., 2006, Ammon et al., 2006). At present it is not understood in detail how this multi-target principle of STW 5 arises (Wagner, 2006; Wagner and Ulrich-Merzenich, 2009). TNFα is a pro-apoptotic cytokine, which is produced by a wide variety of cell types in response to various inflammatory stimuli. It promotes the pathogenesis of several health

disorders, in particular those related to ulcerative colitis and Crohn's disease. In both diseases there is an increased synthesis of pro-inflammatory cytokines, including TNFα and an influx of nonspecific inflammatory cells into the mucosa. The cytokines contribute to tissue damage either directly or indirectly (Heuschkel, 2000; Pallone and Monteleone, 2001). The main sources of TNFα *in vivo* are stimulated monocytes, fibroblasts, and endothelial cells. The signals triggering the TNFα secretion are not completely understood. LPS, the endotoxin of Gram-negative bacteria, stimulates *in vitro* these cells including cells of the monocytic lineage (Rietschel et al., 1996). Human monocytes are characterised by their high level production of TNFα and by their propensity to preferentially develop into potent dendritic cells. Therefore, the effect of STW 5 was not only determined on the gene expression in TNBS-pretreated tissue but also on the TNFα release from untreated and LPS-stimulated human monocytes. Our data clearly show that TNBS increased the TNFα gene expression in rat intestinal tissue, which was reduced by STW 5. Additionally, STW 5 inhibited the increased TNFα release in LPS-stimulated monocytes (Michael et al., 2009). These results point out that those inflammatory processes contribute to the damaged morphology and to the reduced contractility and provide evidence that the protective effect of STW 5 in TNBS-pretreated preparations is largely related to inflammatory processes. Interestingly, STW 6, the fresh plant component of STW 5, neither stimulated the gene expression nor the release of TNFα (Michael et al., 2011). On the other hand, STW 6 enhanced drastically the gene expression of the anti-inflammatory cytokine IL-10 whereas STW 5 did it moderately. These results suggest that at least two different processes contribute to the protective effects of STW 5 in the gastrointestinal inflammation, and therefore, we focused to the mechanisms underlying the regulation of TNFα and IL-10.

The purine nucleoside adenosine has been recognized for its regulatory functions in situations of cellular stress like ischemia, hypoxia and inflammation. The importance of agonists or antagonists of AR as modulators in the immune system is of great interest of the field of gastrointestinal inflammation (Estrela and Abraham, 2011). RT-PCR and immunohistochemical analysis demonstrated a wide distribution of AR in the neuromuscular compartment and mucosal/submucosal layers of both small and large intestine (Christofi et al., 2001; Puffinbarger et al., 1995). The protective effect of STW 5 against inflammation in ileum/jejunum preparations is mainly mediated by activation of $A_{2A}R$. Activation of $A_{2A}R$ by a selective agonist protects against TNBS-induced damage of the ACh-induced contractions and the results of binding studies clearly show an affinity of STW 5 to $A_{2A}R$. In accordance with the present experiments the $A_{2A}R$ antagonist CSC was shown recently to be effective in blocking the STW 5-induced enhanced ACh contraction in inflamed rat small intestinal preparation (Michael et al., 2011). Although, STW 5 enhanced the gene expression of by A_1R. The contribution of this receptor subtype could be excluded. STW 5 did not bind significantly to A_1R and the activation of A_1R by the selective agonist CPA enhanced the damage induced by TNBS.

We found that both STW 5 and STW 6 protected from TNBS-induced damage but STW 6 did not interact with $A_{2A}R$ and therefore it did not inhibit the TNFα pathway. Others than this pathway must be involved. Based on recently published results we focused on the anti-inflammatory cytokine IL-10. IL-10 was first identified as a cytokine, secreted by CD4^{+}Th2-cells, which inhibit cytokine production in antigen-presenting cells (Fiorentino et al., 1989). Its main function within the gastrointestinal tract is limitation and ultimately termination of immune responses. It acts as a key mediator for maintaining gut homeostasis (Paul et al., 2011). IL-10 has long been known for its substantial role in regulating gut immunity, but its

contribution to inflammatory gut diseases was somewhat elusive. A recent study identified mutations in either IL-10 receptor subunits that are associated with early-onset enterocolitis, a severe phenotype of IBD. Other than genetic variants of IL-10 receptors, IL-10 and STAT3 genes are also associated with IBD, emphasizing the involvement of the IL-10 signalling cascade in the pathogenesis of CD and UC. Interestingly, cucurbitacin E and I are inhibitors of STAT3. Cucurbitacin E inhibits tumor angiogenesis through VEGFR2-mediated Jak2-STAT3 signaling pathway (Dong et al., 2010). Cucurbitacin I potently induces apoptosis in leukemia cell lines and in primary chronic lymphocytic leukemia cells and was associated with a reduction in serine 727 phosphorylation of STAT3 (Ishdorj et al., 2010). In our experiment the fractions of STW 6 containing cucurbitacin E and I prevented significantly the TNBS-mediated disturbance of the ACh-induced contraction. The involvement of the cucurbitacins was confirmed by coincubation of cucurbitacins with TNBS. Under these conditions the cucurbitacins reduced the TNBS-induced disturbance of the ACh-induced contractions in a concentration-dependent manner. Additionally, they increased significantly IL-10 mRNA. Therefore, it appears, that the induction of the anti-inflammatory cytokine IL-10 by cucurbitacins is a second mechanism underlying the protective action of STW 5 in our *in vitro* inflammation model.

In conclusion, STW 5 exhibits significant anti-inflammatory properties which contribute to the reduction of TNBS-induced morphological and contractile changes. Its mode of action seems to be twofold: inhibition of the TNFα pathway by activation of $A_{2A}R$ and activation of the IL-10 pathway. These anti-inflammatory mechanisms appear to be involved in the multi-target action of STW 5 as a new therapeutic approach in IBS.

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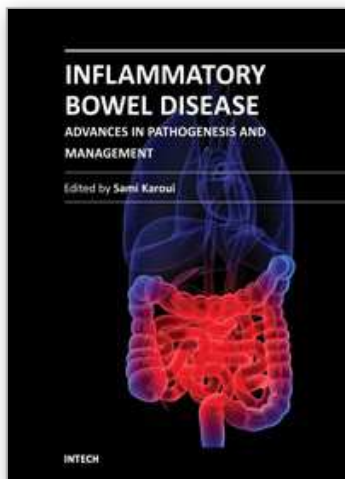
Inflammatory Bowel Disease - Advances in Pathogenesis and Management
Edited by Dr. Sami Karoui

ISBN 978-953-307-891-5
Hard cover, 332 pages
Publisher InTech
Published online 27, January, 2012
Published in print edition January, 2012

This book is dedicated to inflammatory bowel disease, and the authors discuss the advances in the pathogenesis of inflammatory bowel disease, as well as several new parameters involved in the etiopathogeny of Crohn's disease and ulcerative colitis, such as intestinal barrier dysfunction and the roles of TH 17 cells and IL 17 in the immune response in inflammatory bowel disease. The book also focuses on several relevant clinical points, such as pregnancy during inflammatory bowel disease and the health-related quality of life as an end point of the different treatments of the diseases. Finally, advances in management of patients with inflammatory bowel disease are discussed, especially in a complete review of the recent literature.

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In order to correctly reference this scholarly work, feel free to copy and paste the following:

Sebastian Michael, H.-W. Rauwald, Haba Abdel-Aziz, Dieter Weiser, Christa E. Müller, Olaf Kelber and Karen Nieber (2012). Adenosine Receptors: New Targets to Protect Against Tissue Damage in Inflammatory Bowel Symptoms, Inflammatory Bowel Disease - Advances in Pathogenesis and Management, Dr. Sami Karoui (Ed.), ISBN: 978-953-307-891-5, InTech, Available from: http://www.intechopen.com/books/inflammatory-bowel-disease-advances-in-pathogenesis-and-management/adenosine-receptors-new-targets-to-protect-against-tissue-damage-in-inflammatory-bowel-disease

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