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Title page

The blockade of adenosine deaminase ameliorates chronic experimental colitis through the recruitment of adenosine A_{2A} and A_3 receptors

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Running title page

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Abbreviations: APP, 4-amino-2-(2-hydroxy-1-decyl)pyrazole[3,4-d]pyrimidine; CSC, 8-(3-chlorostyryl) caffeine; DNBS, 2,4-dinitrobenzenesulfonic acid; dNTP, deoxynucleoside-5'-triphosphate mixture; EDTA, ethylenediaminetetraacetic acid; EHNA, erythro-9-(2-hydroxy-3-nonyl)adenine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; IBDs, inflammatory bowel diseases; IL-6, interleukin-6; MDA, malondialdehyde; MMLV, moloney murine leukaemia virus; MPO, myeloperoxidase; RIPA, radioimmunoprecipitation assay buffer; SDS-PAGE, sodium dodecyl sulphate- polyacrylamide gel electrophoresis; TNF-α, tumor necrosis factor-α

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Abstract

Adenosine modulates immune/inflammatory reactions. This study investigates the expression of adenosine deaminase in the inflamed colon, the effects of adenosine deaminase inhibitors on established colitis and the recruitment of adenosine receptors by endogenous adenosine following adenosine deaminase blockade. Adenosine deaminase expression was determined by Western blot. The effects of 4-amine-2-(2-hydroxy-1-decyl)pyrazole[3,4-*d*]pyrimidine (APP, novel adenosine deaminase inhibitor), *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA, reference adenosine deaminase inhibitor), dexamethasone and selective adenosine receptor antagonists were tested in rats with 2,4-dinitrobenzenesulfonic acid-induced colitis. Systemic (food intake, body and spleen weight) and colonic (macroscopic/microscopic damage, TNF-α, IL-6 and malondialdehyde) inflammatory parameters were assessed. Test drugs were administered i.p. for 6 days, starting at day 5 from colitis induction. Adenosine deaminase was detected in normal colon, and its expression was increased in inflamed tissues. Colitis was associated with decreased food intake and body weight, augmented spleen weight, and increased levels of colonic TNF-α, IL-6 and malondialdehyde. APP or EHNA, but not dexamethasone, improved food intake and body weight. APP, EHNA and dexamethasone counteracted the increments of spleen weight, ameliorated macroscopic and microscopic indexes of inflammation, and reduced TNF-α, IL-6 levels and malondialdehyde. The beneficial effects of APP and EHNA on inflammatory parameters were prevented by the pharmacological blockade of A_{2A} or A_3 receptors, but not A_1 or A_{2B}. The present results show that: a) bowel inflammation is associated with an enhanced adenosine deaminase expression; b) the anti-inflammatory actions of adenosine deaminase inhibitors against chronic established colitis depend on sparing of endogenous adenosine leading to enhanced A_{2A} and A_3 receptor activation.

Introduction

Inflammatory bowel diseases (IBDs) are chronic, idiopathic inflammations of the enteric wall associated with severe alterations of the enteric tissue architecture and functions (Neuman, 2007). These disorders are characterized by varying periods of active disease or flare-ups and remission, with great impact on well being and patient's quality of life (Neuman, 2007). Although the precise etiological factors leading to the onset of these chronic inflammatory conditions remain unclear, increasing evidence supports the concept that IBDs arise from altered and complex interactions of environmental, genetic and immunological factors (Torres and Rios, 2008). In the presence of inflammatory bowel disorders, the physiological balance of enteric immune system appears completely unsettled (Baumgart and Carding, 2007). In particular, it has been observed a marked polarization of the enteric immune response toward an uncontrolled increase in the production of pro-inflammatory mediators, which overrides the endogenous anti-inflammatory and/or healing pathways (Torres and Rios, 2008). Such mediators can trigger the biosynthesis of further downstream factors, deeply involved in the propagation and maintenance of the inflammatory process, resulting in an excessive degree of collateral injuries to the intestinal mucosa (Camuesco *et al.*, 2004).

As a consequence of massive bowel inflammation, the host attempts to counteract the uncontrolled immune reactions through the activation of various mechanisms. Several lines of evidence indicate that adenosine can take a significant part in the mitigation of abnormal inflammatory responses (Antonioli *et al.*, 2008a). Indeed, under inflammatory conditions adenosine, through dynamic changes in the expression and/or function of its ectoenzymes, participates actively to a fine tuning of immune cellular activities, thus playing an important role in limiting the inflammatory insults to host tissues (Desrosiers *et al.*, 2007; Antonioli *et*

al., 2008b). On the other hand, a close correlation has been found between the severity of inflammation and a local increase in both expression and activity of adenosine deaminase, the enzyme responsible for conversion of endogenous adenosine into inosine, which leads to a decreased availability of biologically active adenosine (Conlon and Law, 2004;; Desrosiers *et al.*, 2007). Based on this knowledge, the pharmacological inhibition of adenosine deaminase is being regarded as a novel therapeutic approach to counteract inflammation in several pathological conditions (Adanin *et al.*, 2002; Law *et al.*, 2003; Kayhan *et al.*, 2008). In line with this concept, we previously provided evidence on the protective effects exerted by drugs acting as adenosine deaminase inhibitors against the onset of experimental colitis. In particular, we observed that, when started prior the induction of colitis, the blockade of adenosine deaminase resulted in a significant amelioration of several inflammatory parameters (Antonioli *et al.*, 2007).

Our previous experience has risen a number of issues which deserve further investigation. In particular, it remains to be assessed: *a)* the extent of adenosine deaminase expression in inflamed colonic tissue; *b)* the anti-inflammatory efficacy of adenosine deaminase inhibitors in the setting of established colitis; *c)* the receptor pathways mediating the anti-inflammatory effects resulting from adenosine deaminase blockade. Accordingly, the present study has been designed to test the effects of adenosine deaminase inhibitors in a model of established chronic colitis as well as to clarify the mechanisms and adenosine receptor pathways involved in such anti-inflammatory activity.

Methods

Animals

Albino male Sprague-Dawley rats, 250-300 g body weight, were employed throughout the study. The animals were fed standard laboratory chow and tap water *ad libitum* and were not subjected to experimental procedures for at least one week after their delivery to the laboratory. Their care and handling were in accordance with the provisions of the European Union Council Directive 86-609, recognized and adopted by the Italian Government.

Induction of colitis, drug treatments and experimental design

Colitis was induced in accordance with the method previously described by Antonioli *et al.* (2007). Briefly, during a short anaesthesia with isoflurane (Abbott, Rome, Italy), 15 mg of 2,4-dinitrobenzenesulfonic acid (DNBS) in 0.25 ml of 50% ethanol were administered intrarectally via a polyethylene PE-60 catheter inserted 8 cm proximal to the anus. Control rats received 0.25 ml of 50% ethanol. Five and eleven days after DNBS or vehicle administration, rats were subjected to the evaluation of systemic and tissue inflammatory parameters as well as the expression of adenosine deaminase, in order to assess the inflammatory status and the expression pattern of the molecular target of adenosine deaminase inhibitors at the onset of drug treatments. Test drugs were administered intraperitoneally for 6 consecutive days, starting 5 days after the induction of colitis.

To select appropriate doses of test drugs, the potencies of *erythro*-9-(2-hydroxy-3-nonyl)adenine (EHNA) and 4-amine-2-(2-hydroxy-1-decyl)pyrazole[3,4-*d*]pyrimidine (APP) in blocking adenosine deaminase activity were assessed *in vitro* by an enzyme inhibition assay, performed as reported by Antonioli et al. (2007). The dose of dexamethasone was

selected on the basis of a previous study performed on a rat model of colitis (Nakase *et al.*, 2001).

The first part of the study was aimed at evaluating the efficacy of adenosine deaminase inhibitors are effective against established colitis as well as the involvement of adenosine receptor subtypes in mediating the anti-inflammatory effects resulting from adenosine deaminase blockade. For this purpose, the effects of APP were tested, either in the absence or in the presence of selective A_1, A_{2A}, A_{2B} and A_3 adenosine receptor antagonists, which were administered by intraperitoneal route concomitantly with APP, EHNA or dexamethasone, on systemic inflammatory parameters (food intake, body weight, spleen weight) and macroscopic score. Effective and selective doses of the receptor antagonists were chosen on the basis of previous reports (Liem *et al.*, 2001; McLaughlin *et al.*, 2006; Wakeno *et al.*, 2006; Ilie *et al.*, 2009), and they were then validated by means of preliminary experiments in the model of DNBS-induced colitis. In this setting, increasing doses of adenosine receptor antagonists were tested against effective doses of adenosine receptor agonists on colonic myeloperoxidase (MPO) levels, a reliable index of tissue inflammatory response, thus allowing the selection of effective and selective doses of antagonists to be employed in subsequent experiments on adenosine deaminase inhibition. The mean findings on the effects of adenosine receptor antagonists versus the respective agonists are summarized in Table 1.

The second part of the study was designed in order to gain more detailed information on the role played by adenosine receptor subtypes involved significantly in the anti-inflammatory effects exerted by the adenosine deaminase inhibitors. For this purpose, the effects of test drugs were evaluated on microscopic colonic damage as well as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and malondialdehyde (MDA) tissue levels.

The macroscopic score was evaluated on the whole colon, whereas microscopic and biochemical analyses were performed on tissue specimens taken from a region of inflamed colon immediately adjacent and distal to the gross necrotic damage. Therefore, at the time of sacrifice, portions of colonic tissues were immediately snap frozen in liquid nitrogen and stored at -80°C for subsequent western blot analysis, MPO, cytokine and MDA assays, or fixed in cold 4% paraformaldehyde for the assessment of microscopic damage score.

Assessment of colitis

At the end of treatments, colonic tissues were excised, rinsed with saline, and scored for macroscopic and histological damage, in accordance with the criteria previously reported by Antonioli *et al.* (2007). The criteria for macroscopic scoring of colonic damage were as follows: i) presence of adhesions between colonic tissue and other organs (0 none, 1 minor, 2 major adhesions); ii) consistency of colonic faecal material (0 formed, 1 loose, 2 liquid stools); iii) presence of ulceration (0 none, 1 hyperaemia, 2 ulceration without hyperaemia, 3 ulceration with inflammation at 1 side; 4 ≥ 2 sites of ulceration and inflammation; 5 major sites of damage; 6 major sites of damage extending >2 cm). The score was then increased of 1 unit for each mm of colonic wall thickness. Microscopic damage and inflammation were assessed by light microscopy on haematoxylin/eosin-stained histological sections obtained from whole gut specimens. The histological criteria included: mucosal architecture loss (0-3); cellular infiltrate (0-3); muscle thickening (0-3); crypt abscess (0 absent, 1 present); goblet cell depletion (0 absent, 1 present). All parameters of macroscopic and histological damages were recorded and scored for each rat by two observers blinded to the treatment. At the time of experiment, the weight of spleen was also measured.

Western blot analysis

The colonic specimens were weighed and homogenized in radioimmunoprecipitation assay buffer (RIPA) lysis buffer containing: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 10 mmol/L, NaCl 30 mmol/L, ethylenediaminetetraacetic acid (EDTA) 0.2 mmol/L, phenylmethylsulfonyl fluoride 2 mmol/L, leupeptin 10 µg/ml, aprotinin 10 µg/ml, sodium fluoride 1 mmol/L, sodium orthovanadate 1 mmol/L, glycerol 2%, $MgCl_2$ 0.3 mmol/L and Triton-X 100 1%, using a polytron homogenizer (Cole Palmer homogenizer). The homogenates were spun by centrifugation at 20000 r/min for 15 min at 4°C, and the resulting supernatants were then separated from pellets and stored at -20°C. Protein concentration was determined in each sample by the Bradford method (Protein Assay Kit, Bio-Rad, Hercules, CA, USA). To perform western blot analysis of adenosine deaminase, aliquots of 30 µg of protein were separated by electrophoresis on 8% SDS-PAGE and transferred onto a poly(vinylidene difluoride) membrane. The blots were then blocked for 2 hours with 1% bovine serum albumin in Tween-20 phosphate-buffered saline (PBS-T), and incubated overnight at room temperature with a rabbit polyclonal primary antibody raised against adenosine deaminase (anti-ADA, Santa Cruz Biotechnology, CA, U.S.A., 1:1000). After repeated washings with 0.1% PBS-T, a peroxidase-conjugated donkey anti-rabbit secondary antibody (Santa Cruz Biotechnology, CA, U.S.A, dilution 1:10000) was added for 1 hour at room temperature. After repeated washings with PBS-T, immunoreactive bands were visualized by incubation with chemiluminescent reagents (Immobilon reagent, Millipore USA) and exposed to Kodak Image Station 440 for signal and densitometric image analysis. To ensure the equal loading and accuracy of changes in protein abundance, the protein levels were normalized to β-actin.

Determination of tissue myeloperoxidase

In order to validate the doses of adenosine receptor antagonists to be employed against the adenosine deaminase inhibitor under study, preliminary experiments were performed to determine colonic MPO levels, as previously reported by Antonioli et al. (2007). Briefly, colonic samples (300 mg) were homogenized three times (30 s each) at 4°C with a polytron homogenizer (Qiagen, Milan, Italy) in 1 ml of ice-cold 50 mM phosphate buffer, pH 6.0, containing 0.5% of hexadecyltrimethylammonium bromide to prevent the pseudoperoxidase activity of hemoglobin as well as to solubilize membrane-bound MPO. The homogenate was sonicated for 10 s, frozen-thawed three times, and spun by centrifugation for 20 min at 18,000*g*. The supernatant was then recovered and used for determination of MPO by means of a kit for enzyme-linked immunosorbent assay (Bioxytech; Oxis International Inc., Portland, OR). All samples were assayed within 2 days from collection. The results were expressed as nanograms of MPO per 100 mg of tissue.

Evaluation of tissue cytokine levels

Tissue TNF-α and IL-6 levels were measured by means of kits for enzyme-linked immunosorbent assay (Biosource International, Camarillo, CA, USA). For this purpose, tissue samples, previously stored at -80°C, were weighed, thawed and homogenized in 0.3 ml of phosphate buffered saline (PBS, pH 7.2)/100 mg of tissue at 4°C, and centrifuged at 13,400 *g* for 20 min. One hundred μl aliquots of the supernatants were then used for assay. Tissue TNF-α and IL-6 levels were expressed as pg per mg of tissue.

Evaluation of tissue malondialdehyde levels

Malondialdehyde (MDA) concentration in colonic specimens was evaluated to obtain quantitative estimation of membrane lipid peroxidation. Colonic tissues were weighed, minced by forceps, homogenized in 2 ml of cold buffer (Tris-HCl 20 mmol/L, pH 7.4) by a polytron homogenizer (Qiagen, Milan, Italy), and spun by centrifugation at 1,500 *g* for 10 min at 4°C. Colonic MDA concentrations were determined by means of a kit for colorimetric assay (Calbiochem-Novabiochem Corporation, San Diego, CA, USA), and the results were expressed as µmol of MDA per mg of colonic tissue.

Drugs and reagents

2,4-dinitrobenzenesulfonic acid (DNBS), dexamethasone, 3-ethyl-5-benzyl-2-methyl-4-phenylethynyl-6-phenyl-1,4-(±)-dihydropyridine-3,5-dicarboxylate (MRS 1191), chloroform and TRIzol® were purchased from Sigma Aldrich (St Louis, Mo, USA). *Erythro*-9-(2-hydroxy-3-nonyl)adenine hydrochloride (EHNA), 8-cyclopentyl-1,3-dipropylxanthine (DPCPX), N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl) phenoxy]-acetamide (MRS 1754) and 8-(3-chlorostyryl) caffeine (CSC) were purchased from Tocris (Bristol, UK). The synthesis of APP was performed as previously reported (Da Settimo *et al.*, 2005). APP, EHNA, dexamethasone, DPCPX, CSC, MRS 1754 and MRS 1191 were dissolved in sterile dimethylsulfoxide (DMSO) and further dilutions were made with sterile saline. The solutions were frozen into aliquots of 2 ml and stored at -80°C until use.

Statistical analysis

The results are given as mean ± standard error of the mean (S.E.M). The statistical significance of data was evaluated by one way analysis of variance (ANOVA) followed by *post hoc* analysis by Student-Newman-Keuls test, and *P* values lower than 0.05 were

considered significant. All statistical procedures were performed using GraphPad Prism 3.0 software (GraphPad, San Diego, CA, USA).

Results

Adenosine deaminase expression

Western-blot analysis showed a detectable expression of adenosine deaminase in colonic tissues from vehicle-treated rats (Fig. 1). In tissues from animals with colitis, the expression of adenosine deaminase was increased significantly at day 11 after the administration of DNBS (Fig. 1).

Evaluation of colonic inflammation under control conditions

Five days after intracolonic DNBS administration, there was a significant decrease in food intake and body weight, with concomitant increments of spleen weight and bowel inflammatory parameters (macro- and microscopic damage, TNF-α, IL-6 and MDA), as compared to vehicle-treated animals (Table 2). Likewise, at day 11 after the induction of colitis, food intake and body weight gain were lower, while other systemic and colonic indexes of inflammation were significantly increased in comparison with vehicle-treated animals, and these parameters were similar to those recorded in inflamed animals at day 5 (Table 2).

Effects of test drugs on colonic inflammation

Food intake, body weight and spleen weight

Following a course of six-day administration to animals with established colitis, APP (15 μmol/kg/day) or EHNA (90 μmol/kg/day) ameliorated food consumption, while no significant effects on food intake were observed in rats receiving dexamethasone (0.25 μmol/kg/day) (Table 3). Under the same conditions, the delay in body weight gain associated with colitis was counteracted after treatment with APP or EHNA (Table 3), while dexamethasone was

without effect on this parameter (Table 3). Spleen weight was significantly increased in DNBS-treated rats, and such an increment was reduced by administration of APP, EHNA or dexamethasone (Table 3).

The beneficial effects exerted by APP on food intake and body weight in rats with established colitis were counteracted by administration of CSC (6 μmol/kg/day) and, to a lower extent, by MRS 1191 (7 μmol/kg/day), while no significant effects were obtained with the A_1 or A_{2B} receptors antagonists DPCPX (4 μmol/kg/day) and MRS 1754 (2 μmol/kg/day), respectively (Fig. 2). In addition, the effects of APP administration on spleen weight were blunted by the A_{2A} receptor antagonist, while no significant effects were observed in the presence of the other receptor antagonists (Fig.2). Moreover, the adenosine receptor antagonists did not exert any significant influence on food intake, body weight or spleen weight when tested alone (Fig. 2).

Macroscopic damage

Animals with colitis treated with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day) for six days displayed a significant reduction in the macroscopic damage score (Fig. 3A). The adenosine receptor antagonists did not modify the macroscopic damage associated with chronic colitis when tested alone (Fig 3B). However, CSC (6 μmol/kg/day) prevented the ameliorative effects exerted by APP, while no appreciable effects were observed with the administration of DPCPX (4 μmol/kg/day), MRS 1754 (2 μmol/kg/day) or MRS 1191 (7 μmol/kg/day) (Fig. 3B).

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Microscopic damage

Colonic specimens obtained from inflamed rats treated with APP (15 μmol/kg/day) showed a significant improvement in microscopic scores (Fig. 4A). Similarly, EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day) induced a significant amelioration of histological damage (Fig. 4A). The A_{2A} or A_3 receptor antagonist did not affect the microscopic damage of inflamed colon when tested alone (Fig 4B). The beneficial effects exerted by APP or EHNA on the microscopic patterns of colonic inflammation were markedly counteracted by the concomitant administration of CSC and, to a lower extent, by MRS 1191 (Fig. 4B). The A_{2A} or A_3 receptor antagonist did not show significant effects in dexamethasone-treated rats (Fig. 4B).

TNF-α and IL-6 levels in colonic tissues

Treatments with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day) significantly decreased the concentrations of TNF-α and IL-6 in the inflamed colonic tissues (Fig. 5A and 6A). CSC or MRS 1191 were without effects on TNF-α and IL-6 when administered alone to inflamed rats. However, the blockade of A_{2A} or A_3 receptors counteracted the decrease in TNF-α and IL-6 tissue levels induced by APP or EHNA in animals with colitis (Fig. 5B and 6B). The ameliorative effects of dexamethasone were unaffected by treatment with CSC or MRS 1191 (Fig. 5B and 6B).

MDA levels in colonic tissues

Treatments with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day) significantly attenuated the increase in colonic MDA levels associated with established colitis (Fig. 7A). The blockade of A_{2A} receptors with CSC or treatment with the

A_3 receptor antagonist MRS 1191 did not influence the oxidative injury of inflamed colon when tested alone, but CSC significantly reversed the anti-oxidant actions promoted by both adenosine deaminase inhibitors (Fig. 7B). CSC or MRS 1191 did not exert significant effects in rats treated with dexamethasone (Fig. 7B).

Discussion

The present study was performed to investigate the efficacy of adenosine deaminase blockade against established colitis, as well as to characterize the adenosine receptors which are recruited by endogenous adenosine following its preservation from catabolic inactivation. In order to pursue these aims, the effects of the novel adenosine deaminase inhibitor APP and its standard comparator EHNA were assayed during the chronic phase of colitis elicited by DNBS, and dexamethasone was used as a comparator with known anti-inflammatory activity to assess the relative anti-inflammatory potency of both adenosine deaminase inhibitors. In this curative dosing protocol, the administration of test drugs was started five days after DNBS administration, when chronic colonic inflammation was fully established (Tran *et al.*, 1999). At this stage, and up to day 11, the inflammation was associated with reduced weight gain, diarrhea, mucosal ulceration and bowel wall thickening. Significant increments of inflammatory cytokines (TNF-α, IL-6) and oxidation products (MDA) were also recorded in the inflamed colon. All these observations support the evidence that from day 5 to 11 after DNBS administration there was a steadily active inflammatory response of colon resulting from an abnormal activity of inflammatory cells infiltrating the intestinal tissue.

Our molecular analysis provided the first demonstration that a significant increase in adenosine deaminase expression occurs at level of the inflamed colonic tissue. In keeping with our observation, previous studies have reported increments of adenosine deaminase expression and activity in different models of inflammation, including sepsis, small bowel enteritis and hypoxia (Cohen *et al*., 2002; Eltzschig *et al*., 2006), as well as in the serum of patients with rheumatoid arthritis (Sari *et al.*, 2003) or pancreatitis (Ibis *et al.*, 2007). However, the cellular localization of adenosine deaminase in normal or inflamed colonic tissues was not investigated in the present study, and it remains to be established whether the

induction of adenosine deaminase in the setting of colitis occurs at intra- or extracellular sites, as well as whether such an increment depends on its increased expression in resident cells and/or accumulating immune cells. Based on previous study, both these circumstances appear to be conceivable, as it has been shown that adenosine deaminase was expressed in mucosal cells of normal colon (Dinjens et al., 1989), and the expression of this enzyme was found to be increased in different populations of immune cells, including macrophages and lymphocyte T, during inflammatory responses (Martín *et al*., 1995; Conlon and Law, 2004). Of note, the increase in adenosine deaminase expression, occurring in concomitance with the inflammatory bowel reaction, might enhance the catabolic degradation of adenosine, thus resulting in an impairment of immune modulation by endogenous adenosine pathways and a consequent worsening of inflammation and tissue injury.

Previous reports suggest that adenosine deaminase may act as a critical regulatory factor, endowed with the ability of limiting bioactive adenosine concentrations and recruitment of downstream receptors in the microenvironment of inflamed tissues (Hashikawa *et al*., 2006). For example, adenosine deaminase has been linked to the hyperreactivity of some immune cells (dendritic and T cells) during inflammatory reactions (Dong *et al*., 1997; Desrosiers *et al*., 2007). When considering bowel inflammation, our present experiments demonstrated that the pharmacological blockade of adenosine deaminase promotes a significant attenuation of colonic injury in a model of established colitis. The ameliorative effects resulting from the six-day treatment with APP or EHNA were testified by improvements of all inflammatory parameters, including systemic indexes (food intake, body weight, spleen weight), macroscopic and histological scores, tissue cytokines (TNF-α and IL-6) and tissue oxidation (MDA). In this study, another relevant finding was that the improvement of colitis and related inflammatory indexes, associated with adenosine deaminase inhibition, were prevented by the

blockade of A_{2A} and to minor extent by A_3 receptors, while A_1 and A_{2B} receptors did not appear to be significantly involved. Taken together these data support the concept that: a) inhibitors of adenosine deaminase are effective not only in protecting bowel tissues from the onset of inflammation, but also in blunting the inflammatory response in the setting of established colitis; b) the anti-inflammatory actions resulting from inhibition of the catabolic breakdown of endogenous adenosine are driven by recruitment of A_{2A} and A_3 receptors.

There is an increasing evidence in literature indicating a marked involvement of A_{2A} and A_3 receptors in the modulation of immune cell activity and inflammation (Antonioli *et al*., 2008a; Haskò *et al*., 2008). In particular, previous works have shown that A_{2A} is the primary receptor responsible for the inhibitory effect of adenosine on the production of pro-inflammatory cytokines and reactive oxygen species at sites of inflammation in different experimental models, including asthma, chronic obstructive pulmonary disease and arthritis (Haskò et al., 2000; Haskò and Cronstein, 2004; Haskò *et al*., 2008). Furthermore, studies from other groups have shown that the direct stimulation of A_{2A} receptors, via selective agonists, produced beneficial effects in different models of bowel inflammation (Odashima *et al*., 2005). In addition, a study by Kreckler *et al.* (2006) showed that cytokine release from murine peritoneal macrophages was predominantly suppressed via A_{2A} receptor activation.

In recent years, special attention has been paid to the role played by A_3 receptors in the regulation of inflammatory responses. Pharmacological agents targeting this receptor pathway are being tested in preclinical models of inflammation or hypoxic damage with encouraging results (Haskò et al., 1998; Szabò et al., 1998; Van der Hoeven *et al.*, 2008), and some A_3 receptor agonists have already entered the phase of clinical development for treatment of rheumatoid arthritis (Silverman *et al.*, 2008). Several lines of evidence suggest that the activation of A_3 receptors can favourably affect the outcome of intestinal inflammation, thus

representing a promising target for the development of a novel therapeutical strategy against IBDs (Mabley *et al.*, 2003). However, in the present study the relevance of A_3 receptors in mediating the beneficial effects resulting from adenosine deaminase inhibition appear to be less consistent in comparison with A_{2A} receptors. In particular, the involvement of A_3 receptors in mediating the anti-inflammatory effects resulting from adenosine deaminase blockade appear to be appreciable for few phlogistic parameters. One explanation for this differential recruitment of receptor subtypes could be ascribable to an increase in the activity of nucleoside transporters arising from the activation of A_{2A} receptors (Pinto-Duarte *et al.*, 2005; Ye and Rajendran, 2009). In this regard, the greater stimulation of A_{2A} receptors by the pharmacological blockade of adenosine deaminase could be related to an increased activity of nucleoside transporters to avoid a spread of extracellular adenosine, sufficient to activate A_3 receptors, as well as the low affinity A_{2B} receptor.

In the present study the A_{2A} or A_3 receptor antagonists did not exacerbate the severity of bowel inflammation when tested alone. This finding might be explained by the reduced bioavailability of endogenous adenosine resulting from the enhanced catabolic activity of adenosine deaminase at level of the inflamed colon. Consistently with this view, Lee *et al.* (2006) showed that in murine microglial cells stimulated by exposure to lipopolysaccaride, A_{2A} or A_3 receptor antagonists did not worsen inflammatory response in comparison with vehicle. Moreover, Van der Hoeven *et al*. (2008) demonstrated that two pro-inflammatory actions of murine neutrophils (i.e. superoxide production and chemotaxis) did not differ when comparing *wild type* with A_{2A} or A_3 knockout animals. However, McColl *et al.* (2006) observed that, in leukocytes migrated into air pouches of mice lacking A_{2A} receptors, the expression of TNF-α was significantly higher in comparison with *wild type* animals, thus suggesting that different models of inflammation can be associated with different degrees of

adenosine-dependent anti-inflammatory activity mediated by tonic A_{2A} or A_3 receptors stimulation. As far as A_{2B} receptors are concerned, previous reports showed conflicting evidence about their anti/pro-inflammatory role during intestinal inflammation (Kolachala *et al.*, 2008; Frick *et al.*, 2009). In our study, A_{2B} receptors did not appear to be significantly involved in the modulation of colonic inflammatory response. This discrepancy could depend on a number of differences between the present work and those mentioned above. In particular, there are differences in species, experimental model of bowel inflammation, and drug administration protocol. Moreover, in our preliminary experiments the administration of NECA was associated with an increase in colonic myeloperoxidase levels, thus suggesting that the exogenously induced activation of A_{2B} receptors is likely to promote pro-inflammatory effects (Antonioli *et al.*, unpublished data). For these reasons, it is conceivable that in the setting of the present study, A_{2B} receptors were not able to take a significant part to the anti-inflammatory response resulting adenosine deaminase blockade.

Overall, the present results suggest that the pharmacological modulation of adenosine deaminase represents a novel and promising therapeutic strategy for the management of bowel inflammation. Indeed, the specific and selective inhibition of this enzyme, markedly expressed in inflamed bowel tissues, produced ameliorative effects against an established inflammatory environment, acting on several aspects of the inflammatory reaction. Most importantly, the enhanced levels of endogenous adenosine, resulting from adenosine deaminase inhibition, down-regulated the production of key pro-inflammatory cytokines, counteracted tissue oxidative injury, and attenuated the severity of colonic injury via recruitment of the A_{2A}- and A_3-receptor pathways.

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Footnotes

The results of this work have been presented as oral communication at the Digestive Disease Week 2009 in Chicago (IL, U.S.A.).

Legends for figures

Figure 1. Western blot analysis of adenosine deaminase (ADA) in colonic tissues obtained from vehicle-treated animals (V) or from rats with DNBS-induced colitis at day 5 and day 11. Each column represents the value±SEM obtained from 6 animals. **P*<0.05, significant difference versus vehicle.

Figure 2. Systemic inflammatory parameters estimated in rats with colitis treated with APP alone or upon combined administration with DPCPX (4 μmol/kg/day, i.p.), CSC (6 μmol/kg/day, i.p.), MRS 1754 (2 μmol/kg/day, i.p.) or MRS 1191 (7 μmol/kg/day, i.p.). Treatments with test drugs were started at day 5 from the induction of colitis with DNBS. Systemic inflammatory parameters were estimated at day 11. Each column represents the mean value±SEM obtained from 8 experiments. $^{*}P$<0.05, significant difference vs control; ^{a}P<0.05, significant difference vs DNBS alone; $^{§}P$<0.05, significant difference vs DNBS plus APP.

Figure 3. (A) Macroscopic damage score estimated in rats with DNBS-induced colitis, either in the absence or in the presence of treatment with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day, i.p.). **(B)** Effects of DPCPX (4 μmol/kg/day, i.p.), CSC (6 μmol/kg/day, i.p.), MRS 1754 (2 μmol/kg/day, i.p.) or MRS 1191 (7 μmol/kg/day, i.p.) on macroscopic damage either in the absence or in the presence of concomitant APP-administration. Treatments with test drugs were started at day 5 from the induction of colitis with DNBS. Macroscopic and microscopic scores were estimated at day 11. Each column represents the mean value±SEM obtained from 8 experiments. $^{*}P$<0.05,

significant difference vs control; [a]*P*<0.05, significant difference vs DNBS alone; [§]*P*<0.05, significant difference vs DNBS plus APP.

Figure 4. (A) Microscopic damage scores estimated in rats with DNBS-induced colitis, either in the absence or in the presence of treatment with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day). **(B)** Effects of DPCPX (4 μmol/kg/day, i.p.), CSC (6 μmol/kg/day, i.p.), MRS 1754 (2 μmol/kg/day, i.p.) or MRS 1191 (7 μmol/kg/day, i.p.) on microscopic damage, either alone or in combination with APP, EHNA or dexamethasone. Treatments with test drugs were started at day 5 from the induction of colitis with DNBS. Macroscopic and microscopic scores were estimated at day 11. Each column represents the mean value±SEM obtained from 8 experiments. [*]*P*<0.05, significant difference vs control; [a]*P*<0.05, significant difference vs DNBS alone; [§]*P*<0.05, significant difference vs DNBS plus APP; [#]*P*<0.05, significant difference vs DNBS plus EHNA.

Figure 5. (A) TNF-α levels in colonic tissues obtained from rats with DNBS-induced colitis, either in the absence or in the presence of treatment with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day). **(B)** Effects of DPCPX (4 μmol/kg/day, i.p.), CSC (6 μmol/kg/day, i.p.), MRS 1754 (2 μmol/kg/day, i.p.) or MRS 1191 (7 μmol/kg/day, i.p.) on TNF-α levels either alone or in combination with APP, EHNA or dexamethasone. Treatments with test drugs were started at day 5 from the induction of colitis with DNBS. TNF-α levels were assayed at day 11. Each column represents the mean value±SEM obtained from 8 experiments. [*]*P*<0.05, significant difference vs control; [a]*P*<0.05, significant difference vs DNBS alone; [§]*P*<0.05, significant difference vs DNBS plus APP; [#]*P*<0.05, significant difference vs DNBS plus EHNA.

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Figure 6. (A) IL-6 levels in colonic tissues obtained from rats with DNBS-induced colitis, either in the absence or in the presence of treatment with APP (15 μmol/kg/day), EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day). **(B)** APP, EHNA or dexamethasone were tested alone or upon combined administration with DPCPX (4 μmol/kg/day, i.p.), CSC (6 μmol/kg/day, i.p.), MRS 1754 (2 μmol/kg/day, i.p.) or MRS 1191 (7 μmol/kg/day, i.p.). Treatments with test drugs were started at day 5 from the induction of colitis with DNBS. IL-6 levels were assayed at day 11. Each column represents the mean value±SEM obtained from 8 experiments. $^{*}P<0.05$, significant difference vs control; $^{a}P<0.05$, significant difference vs DNBS alone; $^{\S}P<0.05$, significant difference vs DNBS plus APP; $^{\#}P<0.05$, significant difference vs DNBS plus EHNA.

Figure 7. (A) MDA levels in colonic tissues obtained from rats with DNBS-induced colitis, either in the absence or in the presence of treatment with APP (15 μmol/kg/day) EHNA (90 μmol/kg/day) or dexamethasone (0.25 μmol/kg/day). **(B)** Effects of APP, EHNA or dexamethasone, tested alone or upon combined administration with DPCPX (4 μmol/kg/day, i.p.), CSC (6 μmol/kg/day, i.p.), MRS 1754 (2 μmol/kg/day, i.p.) or MRS 1191 (7 μmol/kg/day, i.p.). Treatments with test drugs were started at day 5 from the induction of colitis with DNBS. MDA levels were assayed at day 11. Each column represents the mean value±SEM obtained from 8 experiments. $^{*}P<0.05$, significant difference vs control; $^{a}P<0.05$, significant difference vs DNBS alone; $^{\S}P<0.05$, significant difference vs DNBS plus APP; $^{\#}P<0.05$, significant difference vs DNBS plus EHNA.

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Table 1. Effects of increasing doses of adenosine receptor antagonists on colonic MPO levels (ng/100 mg tissue) in rats treated with adenosine receptor agonists

	Veh	**CCPA (0.4)**	**CCPA (0.4)+ DPCPX (2)**	**CCPA (0.4)+ DPCPX (4)**	**CCPA (0.4)+ DPCPX (8)**
CON	4.8 2.6	4.2 3.8	5.4 2.4	3.1 3.2	6.7 4.3
DNBS	23.6 4.5*	15.7 2.9[a]	16.4 3.5[a]	21.6 3.1[$]	22.8 2.8[$]
	Veh	**CGS21680 (4)**	**CGS 21680 (4)+ CSC (3)**	**CGS 21680 (4)+ CSC (6)**	**CGS 21680 (4)+ CSC (12)**
CON	3.5 1.9	5.1 2.8	6.1 3.1	4.3 3.6	6.4 4.5
DNBS	25.8 4.6*	8.8 2.6[a]	9.3 2.5[a]	21.7 5.1[$]	22.8 3.3[$]
	Veh	**NECA (2)**	**NECA (2)+ MRS 1754 (1)**	**NECA (2)+ MRS 1754 (2)**	**NECA (2)+ MRS 1754 (4)**
CON	5.4 2	6.5 2.8	4.1 3.6	6.2 4.4	6.5 2.7
DNBS	21.9 4.4*	30.4 2.8[a]	31.5 4.3[a]	17.3 3.5[$]	16.2 4.6[$]
	Veh	**IB-MECA (1)**	**IB-MECA (1)+ MRS 1191 (3.5)**	**IB-MECA (1)+ MRS 1191 (7)**	**IB-MECA (1)+ MRS 1191 (14)**
CON	5.1 3.4	4.8 3	5.7 2	6.4 3.5	3.8 2.4
DNBS	24.9 4.7*	9.7 2.9[a]	11.5 2.5[a]	21.4 3[$]	22 4.1[$]

CON: control; Veh: vehicle.
The doses (in brackets) of adenosine receptor ligands are expressed in µmol/kg/day.
[*]P<0.05 significant difference vs control
[a] P<0.05 significant difference vs DNBS alone
[$] P<0.05 significant difference vs the respective agonist alone
n=4 for each drug and dose level of antagonist

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Table 2. Systemic and tissue inflammatory parameters in animals treated with DNBS or vehicle at day 5 or 11

	Food intake (g)	*Body weight variation (%)*	*Spleen weight (%)*	*Macroscopic damage*	*Microscopic damage*	*TNF-α (pg/mg)*	*IL-6 (pg/mg)*	*MDA (μmol/mg)*
Vehicle (day 5)	34.4 3.7	+15.2 1.9	100 5	1.4 0.4	1.5 0.3	4.1 2.7	31.4 3.9	170 22.4
DNBS (day 5)	16.8 4.1*	-5.2 1.8*	130 3.5*	8.1 1.4*	4.5 0.9*	15.7 4.2*	125 6.8*	580.4 29.6*
Vehicle (day 11)	38 2.9	+22 3.5	100 6.5	1.2 0.5	1.3 0.5	5.5 3	27 4.1	172.5 37
DNBS (day 11)	22 3.5*	+6.5 2.9*	141.7 5.8*	6.9 1.2*	3.8 0.7*	24 4.8*	136 8.3*	530.6 30.8*

*P<0.05 significant difference vs the respective control value.

TNF- α: tumor necrosis factor-α ; IL-6: interleukin-6; MDA: malondialdehyde

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Table 3. Effects of APP (15 μmol/kg/day), EHNA (90 μmol/kg/day) or dexamethasone (DEX, 0.25 μmol/kg/day) on food intake, body weight and spleen weight.

Inflammatory parameters	***DNBS (day 11)***	***+APP***	***+EHNA***	***+DEX***
Food intake (g)	20 4	33.5 4.5*	28 3.5*	20.5 5
Body weight variation (%)	+4.9 2.5	+18 3*	+16.8 3.2*	+1.2 1.7
Spleen weight (%)	137.4 6.2	108.7 3.5*	115 4.8*	105.4 3.7*

Treatments with test drugs were started at day 5 from the induction of colitis with DNBS; systemic parameters of inflammation were assessed on day 11.

*P<0.05 significant difference vs DNBS.

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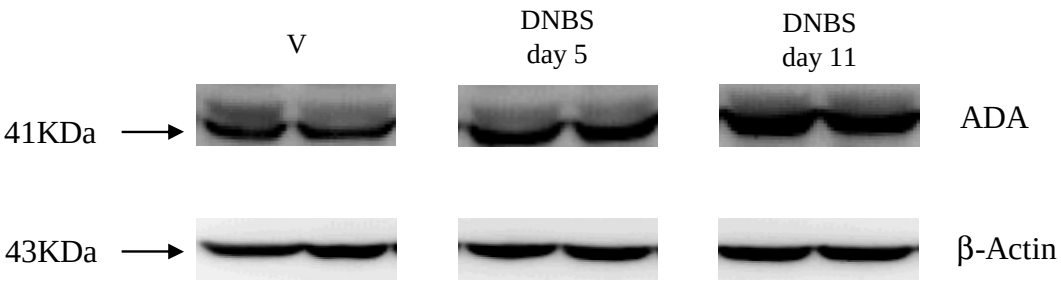

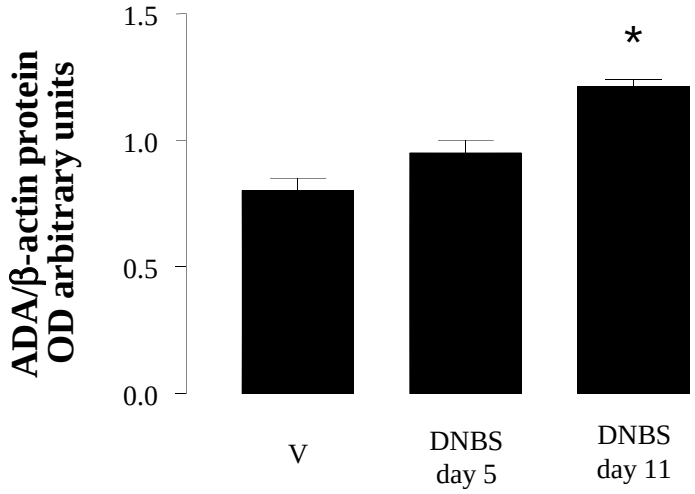

Figure 1

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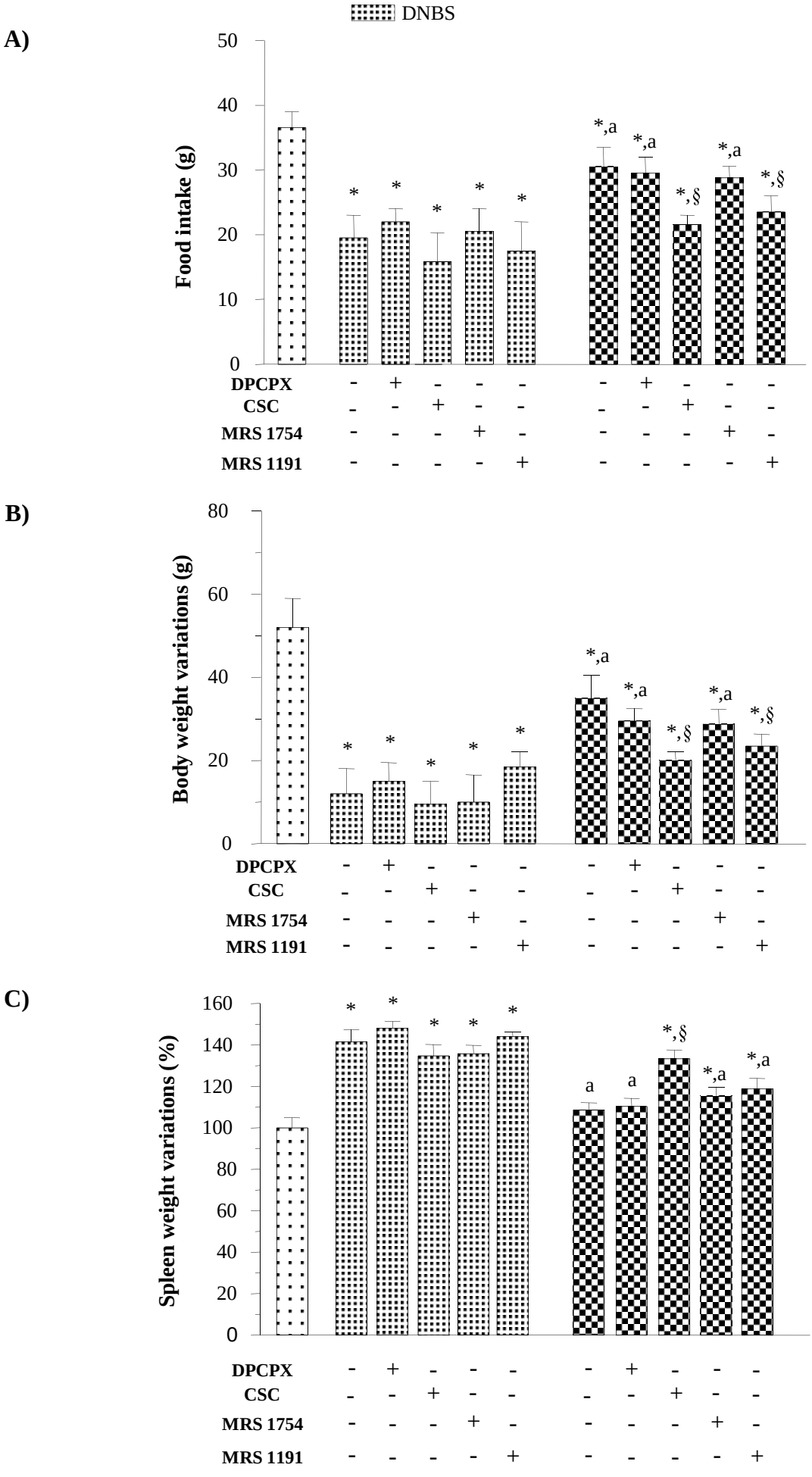

Figure 2

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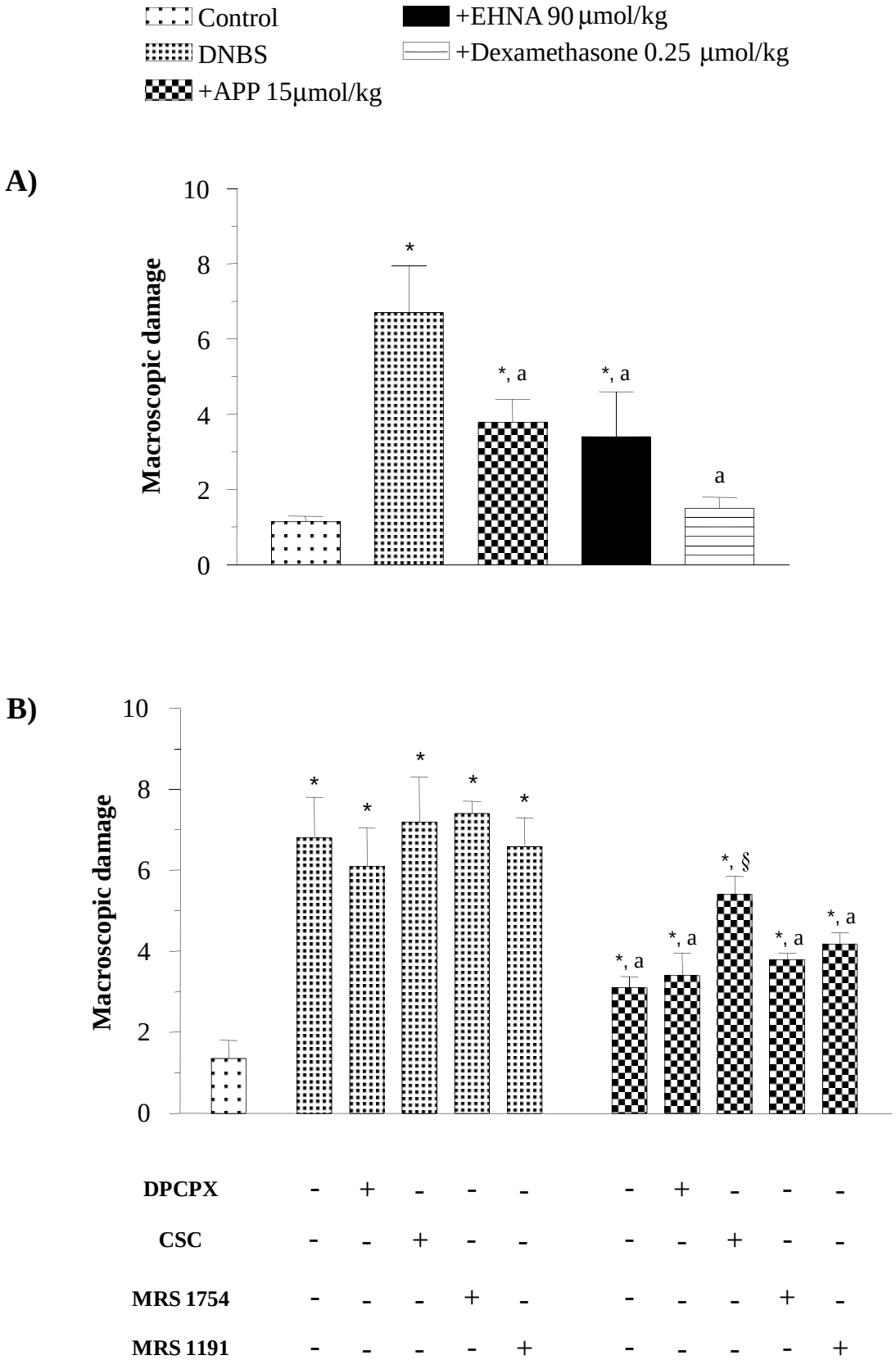

Figure 3

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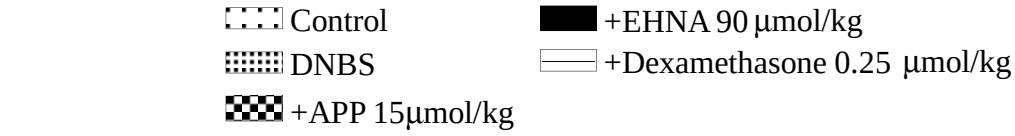

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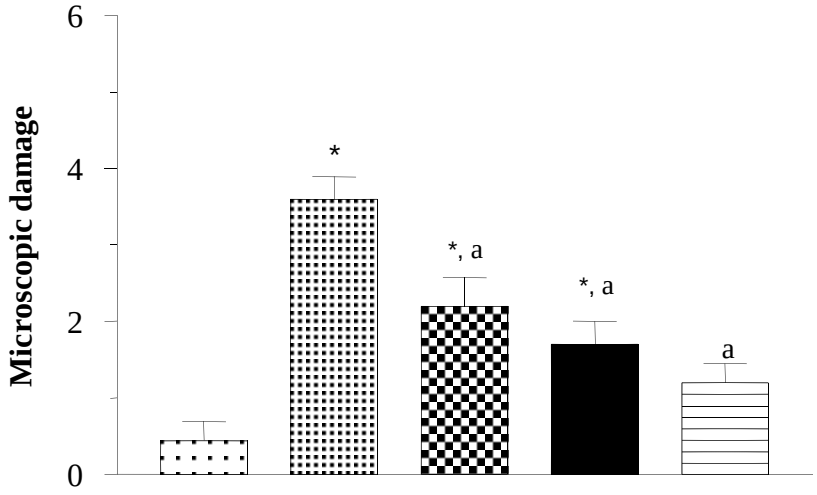

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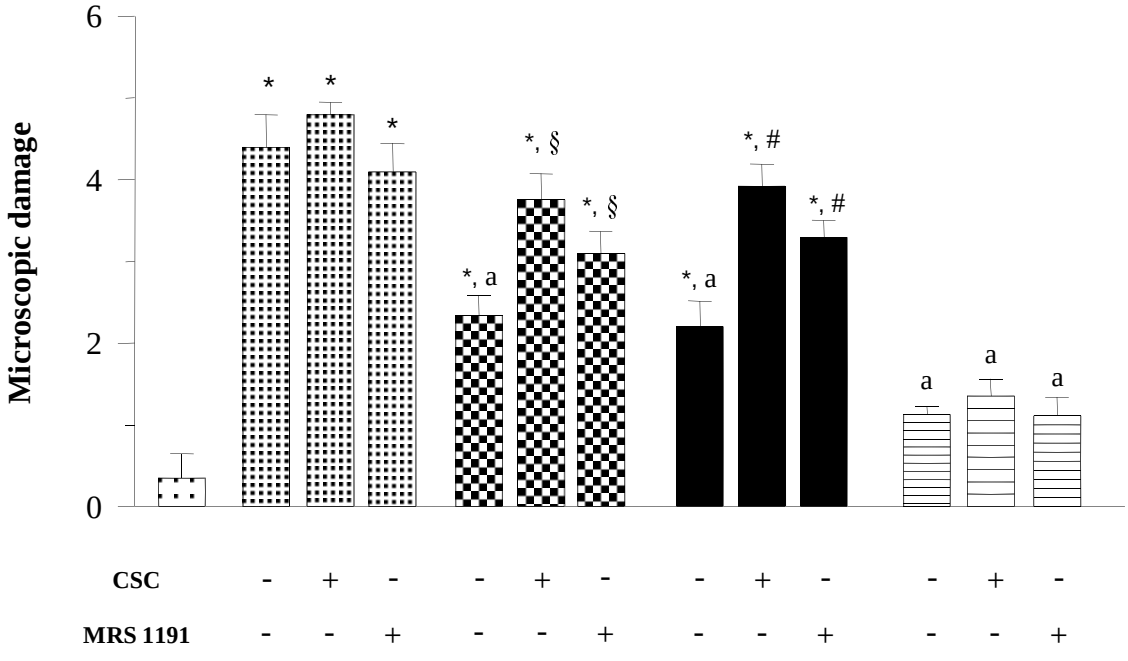

Figure 4

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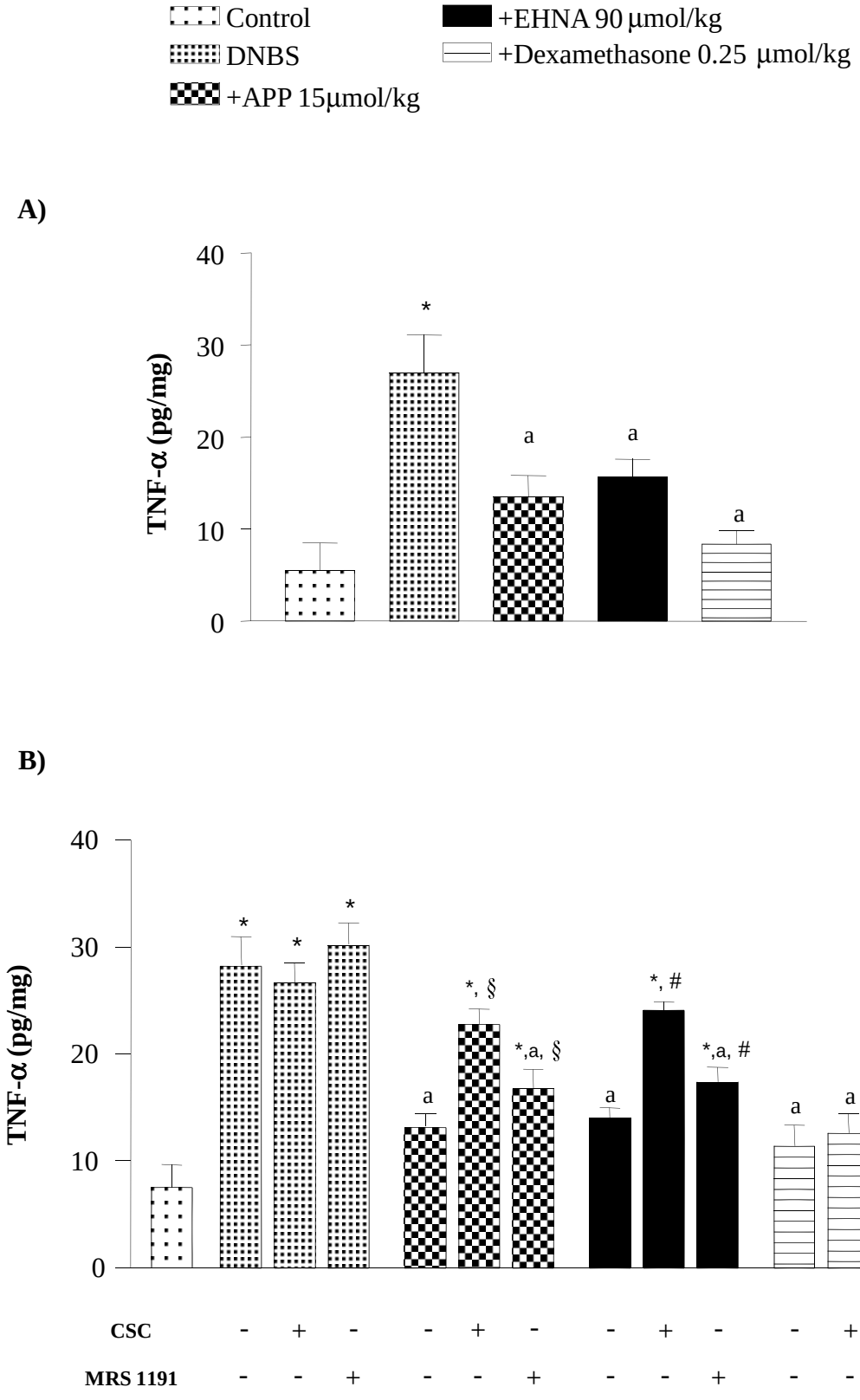

Figure 5

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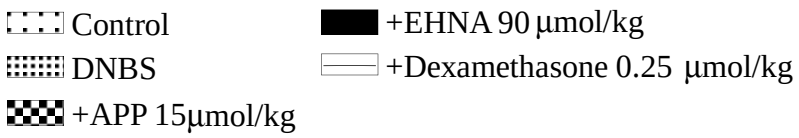

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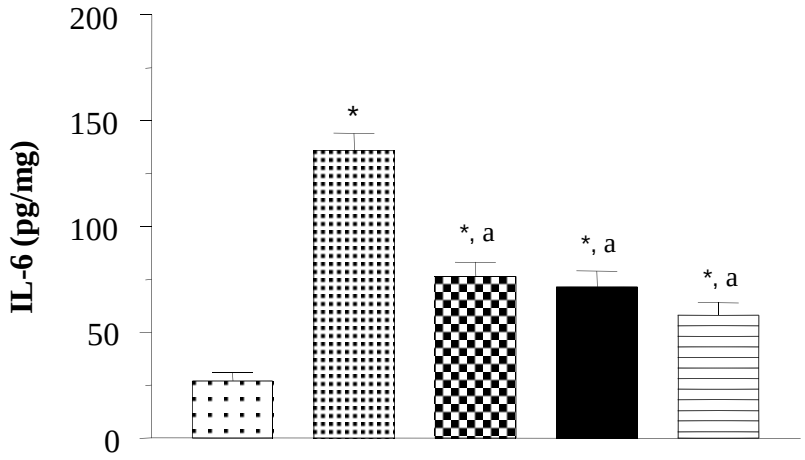

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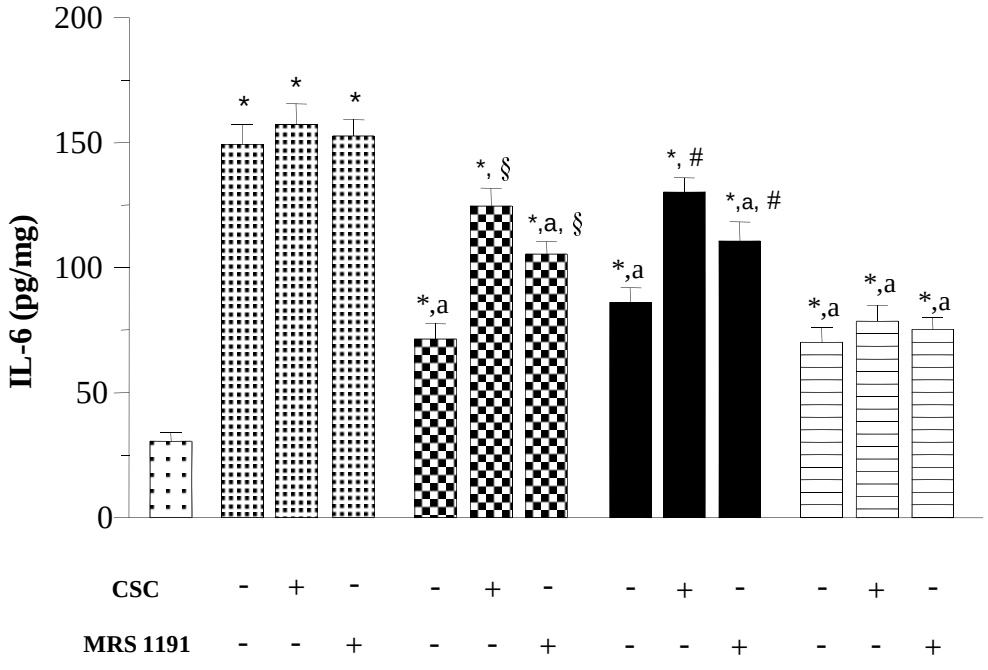

Figure 6

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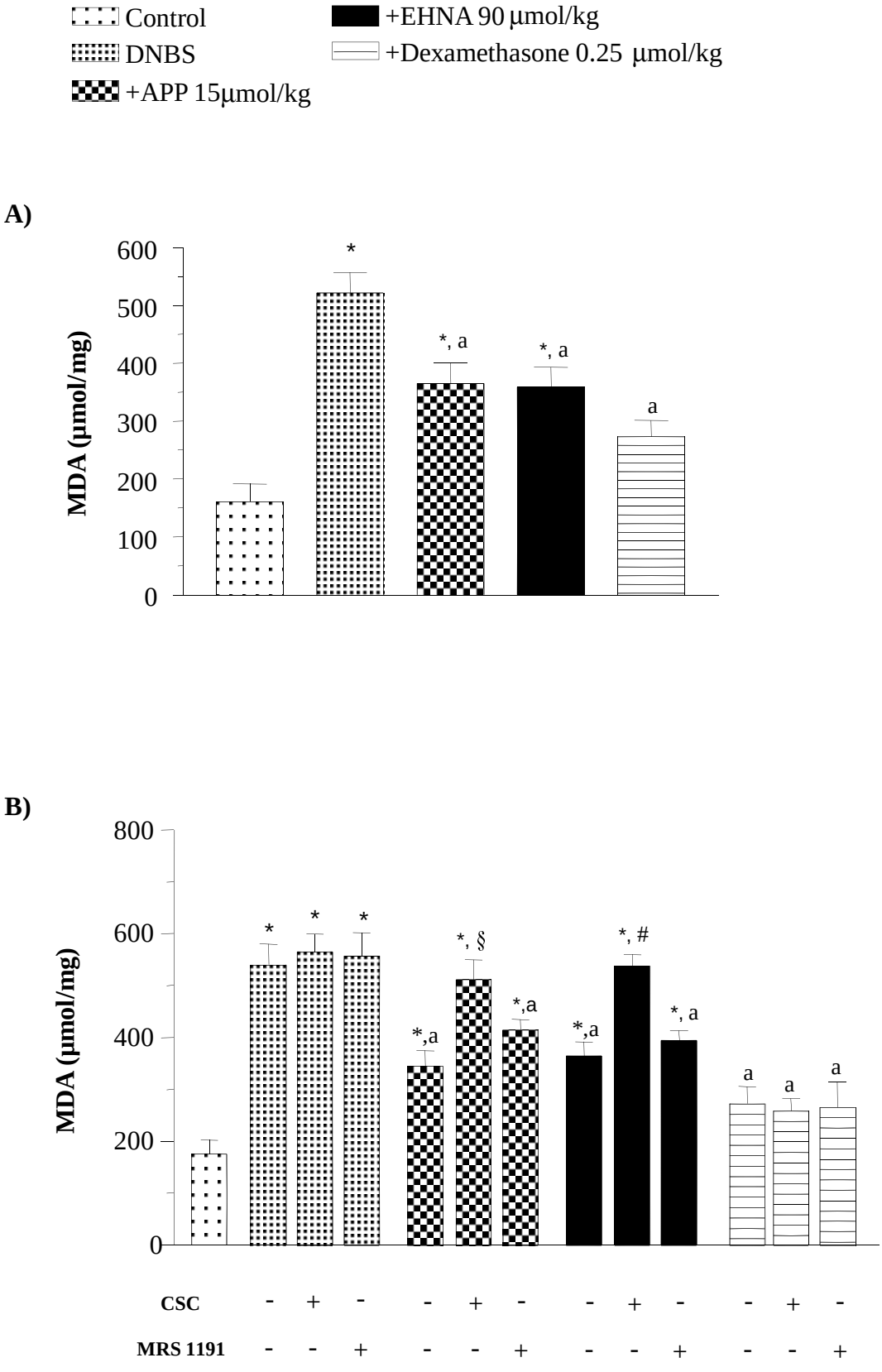

Figure 7