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PSB 603 – a known selective adenosine A2B receptor antagonist – has anti-inflammatory activity in mice

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ABSTRACT

A_{2B} adenosine receptors are present in a wide spectrum of tissues, especially on cells of the immune system. Since these particular receptors have the lowest, of all adenosine receptor subtypes, affinity for adenosine they are believed to play a special role in immunological processes associated with elevated adenosine levels such as inflammation.

The aim of this preliminary study was to determine the potential anti-inflammatory properties of compound PSB-603, a potent and selective adenosine A_{2B} receptor antagonist, in two different experimental models of local and systemic inflammation.

In a model of inflammation induced by local carrageenan administration paw edema was measured using a pletysmometer. Additionally, levels of C-reactive protein (CRP), interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α) and reactive oxygen species (ROS) were determined in the inflamed paw. Using the mouse model of peripheral inflammation induced by intraperitoneal (*ip*) administration of zymosan A, the influence of the A_{2B} antagonist on the infiltration of neutrophils into the peritoneum and its effect on the plasma levels of CRP, TNF-α, and IL-6 were investigated.

The results showed that PSB-603 administered at a dose of 5 mg/kg b.w. *ip* significantly reduced inflammation in both tested models. Particularly, it significantly decreased levels of the inflammatory cytokines IL-6, TNF-α and of ROS in the inflamed paw and reduced inflammation of the peritoneum by significantly decreasing the infiltration of leukocytes. Additionally, in the latter model, no statistically significant difference was observed in the CRP level between the control group without inflammation and the group which has been treated with the PSB-603 compound. Thus, the results may indicate the anti-inflammatory activity of adenosine A_{2B} receptor antagonists in two different models of inflammation.

1. Introduction

Inflammation is defined as one of the defence mechanisms against external factors interfering with the body's natural homeostasis. It is associated with unpleasant feelings, often causes suffering, and it might lead to the state of severe, life-threatening disorders [1]. Inflammation is observed in the course of various pathological conditions e.g. infection, trauma, acute ischemia, burns, poisoning or obesity and it is considered to be a hallmark of many important diseases such as cardiovascular, metabolic, intestinal and pulmonary disorders [2–6]. Regardless of the cause, the same mediators are involved in all inflammatory responses, and their systemic effects lead to similar disorders. The search for new anti-inflammatory drugs is therefore extremely important considering the wide group of patients struggling with inflammation and its consequences.

In recent years adenosine has been considered to be one of the most important mediators and modulators of the immune system response. There are four known subtypes of adenosine receptors namely A_1, A_{2A},

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A_{2B} and A_3 [6]. All of them belong to the group of cell surface receptors called GPCRs (G-protein-coupled receptors). These receptors, activated by extracellular adenosine, are present on almost all immune cells and via its action adenosine may modulate various aspects of both immune and inflammatory responses. As to the regulation of immune activity adenosine effects are mostly anti-inflammatory and are mainly concentrated on the tissue protection [7].

Adenosine receptors have different affinity to its ligand and thus they are activated at the different adenosine concentrations. Receptor A_{2B} is the one with the lowest affinity to adenosine (μM concentrations) therefore it mostly remains silent, since under resting conditions adenosine levels are only between ten and few hundred nM [8]. The affinities of other adenosine receptor subtypes are much higher, which makes them active at significantly lower adenosine concentrations. However, in pathophysiological conditions when adenosine concentrations are the highest, the role of A_{2B} receptor becomes more significant [9]. In such states actions mediated by A_{2B} receptor signalling include, among others, reduction of the acute inflammation, increase of the ability of tissues to adapt to hypoxia, as well as increase of tolerance to ischemia under conditions of an acute insult. Long-term elevated adenosine levels and prolonged A_{2B} receptor signalling are characteristic of a number of chronic diseases, ex.: inflammatory and fibrotic lung diseases (idiopathic pulmonary fibrosis, chronic obstructive pulmonary disease), asthma, bronchiolitis obliterans, pulmonary hypertension, sickle cell disease, cardiac remodelling after myocardial infarction, cancer (metastasis), chronic kidney disease and diabetes [6,10,11].

Therefore, we have decided to investigate the anti-inflammatory effect of compound PSB-603 – a known selective adenosine A_{2B} receptor antagonist, in two mice models of inflammation (local and systemic) and determine its antioxidant activity as well as effect on the levels of selected inflammatory cytokines. The specific models were selected based on our previous experience and after thorough study of the available literature on the subject [12–15]. The adenosine A_{2B}receptor antagonist chosen for this study displays greater than 17,000-fold selectivity over other adenosine receptor subtypes in humans ($K_i$values are 0.553, >10,000, >10,000 and >10,000 nM for adenosine A_{2B}, A_1, $A_{2A}$and A_3receptors, respectively) [16] and is similarly potent and selective in rats and mice [17]. PSB-603 has been used in various studies as a standard antagonist for adenosine A_{2B} receptors [18–21], but so far there are no reports of its activity in models of inflammation.

2. Materials and methods

2.1. Animals

Adult male Albino Swiss mice, CD-1, weighing 25–30 g were used in this study. Animals were kept in environmentally controlled rooms, in standard cages lit by an artificial light for 12 h each day. Animals had free access to food and water, except for the time of the acute experiment. The randomly established experimental groups consisted of 8 mice. All animal care and experimental procedures were carried out in accordance with European Union and Polish legislation acts concerning animal experimentation, and were approved by the Local Ethics Committee at the Jagiellonian University in Cracow, Poland (Permissions No: 256/2015, 55/2017).

2.2. Drugs, chemical reagents and other materials

Ketoprofen was used as a standard anti-inflammatory compound and was purchased from Sigma-Aldrich (Poland). Compound PSB-603 was synthesized at the PharmaCenter Bonn, Pharmaceutical Institute, Bonn, Germany as previously described [16] (Borrmann et al., 2009). Identity and purity of the final product was assessed by NMR and LC–MS techniques.

2,2-Diphenyl-1-picrylhydrazyl and 2,4,6-tripyridyl-*s*-triazine were purchased from Sigma-Aldrich (Poland) and $FeCl_3{\cdot}6H_2O$, ethanol, $C_2H_3NaO_2{\cdot}3H_2O$, $C_2H_4O_2$, HCl from POCH S.A. Gliwice (Poland). Carrageen was purchased from FCM Corporation (USA) and zymosan A from Sigma-Aldrich (Poland).

2.3. In vitro methods

2.3.1. In vitro antioxidant activity

The antioxidant properties of PSB-603 compound were tested *in vitro* in two different assays: the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and in the $FeCl_3$ reduction activity assay (FRAP – ferric reducing antioxidant power).

2.3.1.1. DPPH assay. DPPH is a molecule containing a stable free radical. The reduction of DPPH in ethanol solution takes place in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form of DPPH-H. This transformation results in colour change from purple to yellow, which can be measured spectrophotometrically. Discoloration of DPPH indicates the scavenging potential of a tested antioxidant. The investigated compound was tested at different concentrations ranging from 0.01 mM to 1 mM. To determine antioxidant capacity, 5 μl of the PSB-603 solution (dissolved in 96 % ethanol) was mixed with 95 μl of 0.3 mM ethanolic DPPH solution. The change in the absorbance was detected at 517 nm after 30 min of incubation. Results were expressed as percent decrease in absorbance of the tested sample compared to the sample containing the solvent. L-Ascorbic acid was used as a reference compound.

2.3.1.2. FRAP assay. A modified method of Benzie and Strain [22] was adopted for the FRAP assay [17]. The investigated compound was tested at different concentrations ranging from 0.01 mM to 1 mM. Readings of the coloured product (ferrous tripyridyltriazine complex) were taken at 593 nm against ethanol. Results for the tested compound were expressed as an increase in absorbance of the tested sample compared to a sample containing the solvent. L-Ascorbic acid was used as a reference.

2.4. In vivo methods

2.4.1. Carrageenan-induced edema model

To induce local inflammation, 0.1 mL of 1 % carrageenan solution in water was injected into the hind paw subplantar tissue of mice, according to the modified method of C. A. Winter and P. Lence [23,24], as described previously [25]. The development of paw edema was measured with a plethysmometr (Plethysmometr 7140, Ugo Basile). Prior to the administration of the tested substances (PSB-603 or ketoprofen used as a reference standard), paw diameters were measured and recorded. The PSB-603 compound was administered at doses of 1, 5 or 10 mg/kg b.w., intraperitoneally (*ip*), prior to carrageenan injection, similarly ketoprofen was administered at the dose of 5 mg/kg b.w. [26]. Both compounds were administered as suspensions in 1 % Tween 80, therefore 1 % Tween 80 (vehicle) was injected *ip* to the control group (it had no effect on edema, data not shown). Results were presented as changes in the hind paw volume registered 3 h after carrageenan administration. Immediately after paw volume measurement, mice were injected *ip* with heparin (2500 units/mice) and 20 min later the blood was collected from animals sacrificed by decapitation, and centrifuged at 600 × *g* (15 min, 4 °C) in order to obtain plasma for the quantification of C-reactive protein (CRP) levels. Then, the paws were isolated, placed in liquid nitrogen and stored at − 80 °C until biochemical tests were performed i.e. quantification of tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6) and reactive oxygen species (ROS). Samples were prepared by homogenization of 1 g of the tissue in 4 mL of 0.1 M phosphate buffer (pH 7.4) using an IKA-ULTRA-TURRAX T8 homogenizer.

2.4.2. Zymosan A-induced peritoneal inflammation

Peritoneal inflammation was induced as described previously [27]. Zymosan A solution was freshly prepared (2 mg/mL) in sterile 0.9 % NaCl and 30 min after *ip* injection of the investigated compounds (PSB-603 or ketoprofen, both at the dose of 5 mg/kg), zymosan A was injected via the same route. Both compounds were administered as suspensions in 1 % Tween 80, therefore 1 % Tween 80 (vehicle) was injected *ip* to the control group. Four hours later the animals were killed by decapitation. Mice plasma was collected in a heparin-containing tubes to determine the CRP, TNF-α and IL-6 levels. The peritoneal cavity was lavaged with 1.5 mL of PBS and after 30 s of gentle manual massaging the exudates were retrieved. Cells were counted using an optical microscope (DM1000, Leica) and Bürker hemocytometer following staining with Turk's solution.

2.5. Biochemical analysis

To determine IL-6 and TNF-α levels LANCE® Ultra Detection Kits (PerkinElmer, Inc, USA, catalogue numbers: TRF1505, TRF1504C/TRF1504 M) were used. Standard enzymatic spectrophotometric tests (Shanghai Sunred Biological Technology Co., Ltd, China, catalogue number: 201-02-0219) were used for determination of CRP levels. Reactive oxygen species (ROS) were assayed using 2′,7′-dichlorofluorescein diacetate [28]. Calibration curve was prepared by dilution of 10 μM ethanolic 2′7′-dichlorofluorescein stock solution. The highest calibration point equaled to 1 nM (0.4012 ng/mL). The subsequent 10 calibration points were prepared by serial dilution of the highest concentration. Thus, the final calibration curve range was from 0 (pure solvent) to 0.4012 ng/mL. Fluorescence was monitored on a Spectrofluorimeter (Multiskan GO, Thermo Scientific), with excitation wavelength at 488 nm and emission wavelength at 525 nm.

2.6. Statistical analysis

The results were analysed using a one-way variance analysis (ANOVA), followed by a Dunnett post-hoc test or by a Tukey post-hoc test, with the significance level set at 0.05. They were expressed as the means ± standard error of the mean (SEM), n = 8. Graph Pad Prism 6.0 was used for data analysis.

3. Result

3.1. In vitro antioxidant activity

Even at the highest concentration tested, in both *in vitro* assays, the investigated compound PSB-603 did not display any antioxidant activity. The results are presented in Fig. 1.

3.2. Carrageenan-induced edema model

Activity of the tested compound in the paw edema model was examined at three doses (1, 5 and 10 mg/kg b.w.). Ketoprofen at a dose of 5 mg/kg b.w. was used as a reference compound. In the control group (receiving 1 % Tween only) the mouse paw edema reached its peak at 3 h after the carrageenan injection (increase by 97.7 % of the initial volume). As seen in Fig. 2A the increase in paw oedema was significantly inhibited in all groups receiving PSB-603. The dose of 5 mg/kg b.w turned out to be the most active and was chosen for further studies. In the plasma of mice treated with PSB-603 the decrease in CRP level however visible did not reach statistical significance (Fig. 2B).

In the paw of mice treated with PSB-603, the TNF-α level was only slightly lower than in the control group with inflammation (Fig. 3A). However, the IL-6 level declined significantly and was comparable to the one observed in the ketoprofen treated group (Fig. 3B). Similarly, the level of ROS in the mice paw was significantly lower in the group treated with PSB-603 as compared to the control group (Fig. 3C).

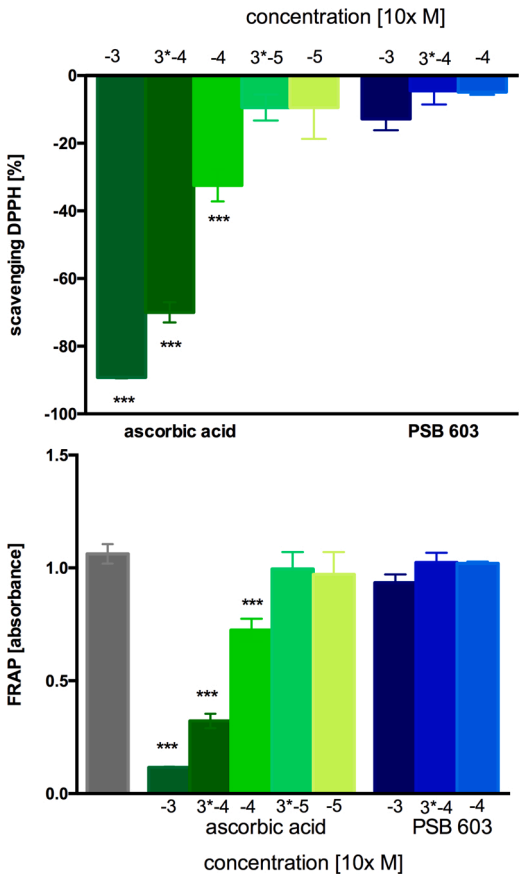

Fig. 1. Anti-oxidant in vitro test.
Results are mean ± SEM, n = 6. Comparisons were performed by one-way ANOVA Dunnet's post hoc. * - Significant against control. ***p < 0.001.

3.3. Zymosan A-induced peritoneal inflammation

In the model of systemic inflammation, the early infiltration of neutrophils measured 4 h after zymosan-induced peritonitis was significantly inhibited in the group receiving PSB-603 vs untreated control group with inflammation, and results were comparable to the ones observed in the group receiving ketoprofen (Fig. 4A). CRP concentration in mice plasma was also slightly decreased in the group receiving PSB-603, however changes did not reach statistical significance (Fig. 4B). TNF-α or IL-6 levels in plasma of mice treated with zymosan A and PSB-603 did not differ significantly from the levels in both control groups – with or without induced inflammation (Fig. 4C and D). In the group receiving ketoprofen, similarly to the results after PSB-603 administration, levels of CRP were virtually unchanged compared to the ones measured in the control group which was given zymosan A alone, however levels of IL-6 and TNF-α were significantly increased.

4. Discussion

The present study aimed to investigate whether a known, selective adenosine A_{2B} adenosine receptor antagonist can exert positive, significant influence on inflammatory processes. In the literature, there is some data available on the benefits of using adenosine A_{2B} receptor antagonists in inflammatory processes and their anti-inflammatory activity [11,29–31], in addition to analgesic effects [32]. However, there is no information regarding this particular ligand [33,34], which is known to be a highly potent and selective adenosine A_{2B} receptor antagonist in different species including humans and rodents commercially available, and used in various studies as a reference standard [17,20,21].

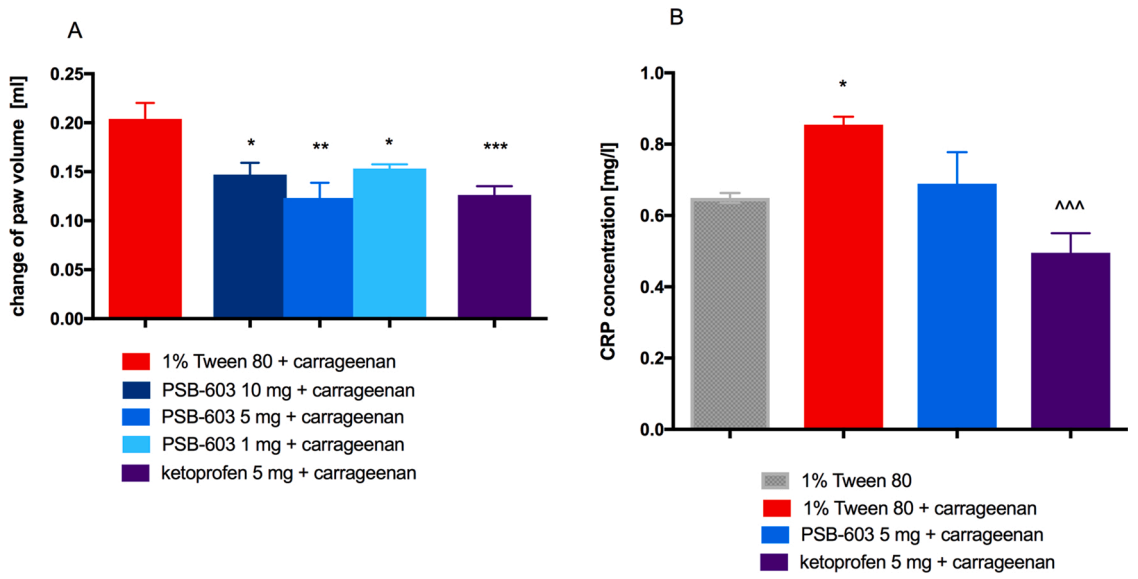

Fig. 2. Anti-inflammatory effects of the compounds in the carrageenan-induced paw edema test.
(**A**) Changes in paw volume in relations to the initial volume during 3 h (before carrageenan injection), Results are mean ± SEM, n = 8. Comparisons were performed by one-way ANOVA Dunnet's post hoc. * - Significant against control mice administered with carrageenen; *p < 0.05, **p < 0.01, ***p < 0,001. (**B**) Concentration of C-reactive protein in plasma. Results are mean ± SEM, n = 8. Comparisons were performed by one-way ANOVA Tukey's post hoc. * – Significant against control mice, ^ – Significant against control mice administared with carrageenen; *p < 0.05, ^^^p < 0.001.

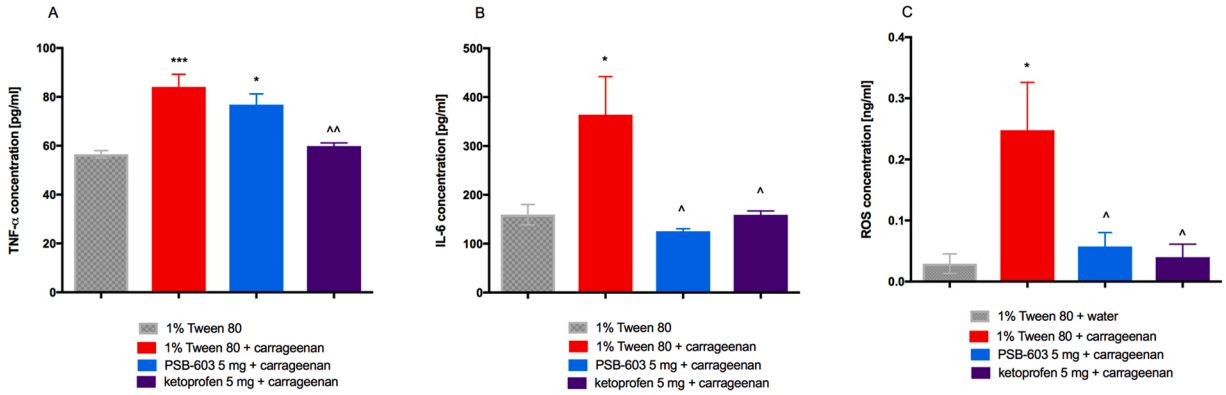

Fig. 3. Effect of administration of PSB-603 or ketoprofen on TNF-α (A), IL-6 (B) and ROS (C) levels in paws in the carrageenan-induced paw edema test.
Results are expressed as means ± SEM, n = 8. Comparisons were performed by one-way ANOVA Tukey's post hoc. * – Significant against control mice; ^ – significant against control mice administared with carrageenen; *,^ p < 0.05, ^^p < 0.01, ***p < 0.001.

The first in vivo experiment performed was the carrageenan inflammation model. We have started administration of the tested compound PSB-603 at a dose of 10 mg/kg b.w., and because it was effective, subsequently lowered the dose first 2 times (5 mg/kg) and then 10 times (1 mg/kg), expecting to obtain logical loss of the effect. However, surprisingly the effect was stronger at the dose of 5 mg/kg b. w. than at 10 mg/kg b.w., and it decreased at 1 mg/kg. Therefore it was suspected that at the higher dose (10 mg/kg b.w.) PSB-603 might exert some pro-inflammatory activity. It is known that sometimes compound might act in a "U" dependence, and at both high and low doses, the expected effect disappears [35]. Moreover, in the spontaneous activity test the dose of 10 mg/kg had undesirable sedative effect (data not included). Thus a dose of 5 mg/kg b.w. was chosen for the further research and in the second model – the inflammation induced by the administration of Zymosan A only that dose was tested.

The conducted tests clearly show that PSB-603 administered at a dose of 5 mg/kg b.w. *ip* significantly reduces inflammation in both the local and systemic inflammation model. This is an important finding considering the use of PSB-603, for several years now, as a selective adenosine A_{2B} receptor antagonist reference compound.

The inflammatory reaction occurs in two phases. Initially, TNF-α is released, which, along with cytokines such as IL-1β, IL-2, IL-6, IL-8, IL-15, and IFN-γ, belongs to the pro-inflammatory mediators. Immune system cells also produce free oxygen radicals and proteases and secrete lipid mediators (prostaglandins, leukotrienes). Subsequently, the increased activity of pro-inflammatory cytokines stimulates the synthesis of anti-inflammatory cytokines such as soluble TNF receptors, IL-1 and IL-4 antagonists, IL-10, IL-13, TNF-β. The next step is the post-inflammatory response [1]. There is a known correlation between CRP and inflammation, and therefore this protein has attracted wide attention as a non-specific marker used to evaluate and monitor the development of infection and inflammation [36]. Thus, compounds with anti-inflammatory activity also lower plasma CRP levels [37–39].

Consequently, in our studies, the levels of CRP were quantified in mice plasma as well as the levels of TNF-α and IL-6 in both plasma and paws with induced inflammation. Moreover, the ROS levels were also assayed in the paws of mice with induced inflammation.

In both models of inflammation, despite the fact that the differences

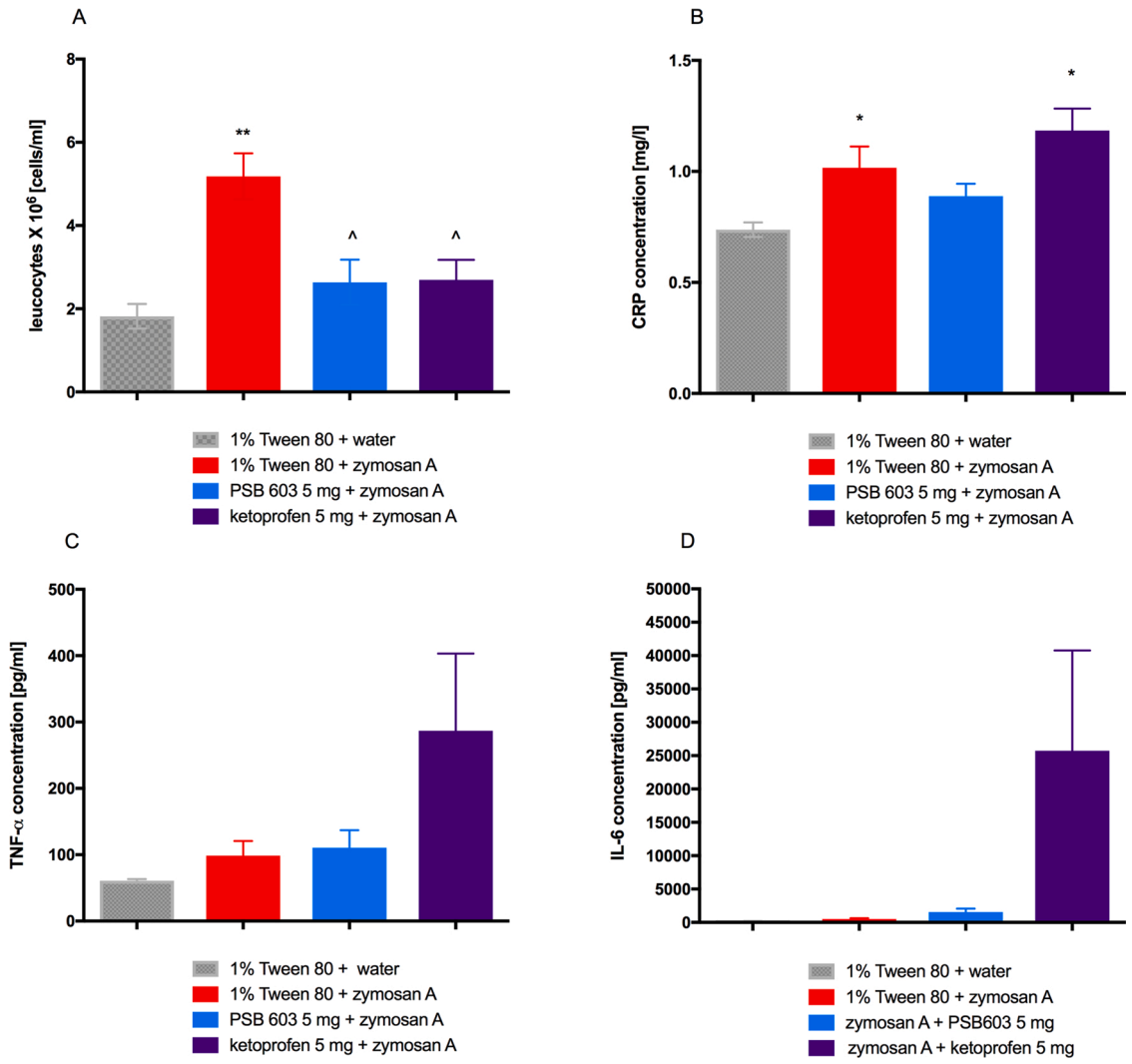

Fig. 4. Anti-inflammatory effect of PSB-603 or ketoprofen in model of zymosan-induced peritonitis in mice.
(**A**) Neutrophil infiltration during zymosan-induced peritonitis in mice, (**B**) Concentration of C-reactive protein in plasma, (**C**) Concentration of TNF-α in plasma, (**D**) Concentration of IL-6 in plasma. Results are mean ± SEM, n = 8. Comparisons were performed by one-way ANOVA Tukey's post hoc. * – Significant against control mice, ^ – Significant against control mice administered with zymosan. *,^p < 0.05, **,^^p < 0.01, ***p < 0.001.

did not reach statistical significance, the level of CRP in plasma was visibly lowered by the administration of the tested adenosine A_{2B} receptor antagonist PSB-603, which indicates its anti-inflammatory effect. In the model of local inflammation PSB-603 administration significantly reduced (similarly to ketoprofen) levels of both TNF-α and IL-6 in the paws of mice with induced inflammation. In the model of systemic inflammation administration of PSB-603 did not influence the plasma levels of TNF-α and IL-6. Contrarily, in the case of ketoprofen administration, after zymosan A-induced peritonitis, plasma TNF-α and IL-6 levels significantly increased. However, this is in line with literature reports. Non-steroidal anti-inflammatory drugs (NSAIDs) acting by inhibiting cyclooxygenase have long been known to augment TNF-α production in various *in vitro* and *in vivo* models including human volunteers [40]. It has been shown that *S*-ketoprofen efficiently inhibited carrageenan-induced edema formation, but it could also amplify the LPS-induced production of inflammatory cytokines such as TNF-α and IL-1, in close correlation with its ability to inhibit prostaglandin synthesis [41]. Upregulation of TNF-α production by inhibiting the negative feedback mechanism represented by PGE_2 can increase the production of IL-6 [40] which in turn enhances the liver production of CRP [42]. In another study ketoprofen reduced inflammation by inhibiting cyclooxygenase, but again, the compound paradoxically increased TNF-α levels in mice with surgically-induced lymphedema [26]. TNF-α is also a potent inducer of IL-1 and IL-6, and the kinetics of this process suggest that just the increase in the TNF-α levels might be an early step in the cytokine cascade [43].

PSB-603 not only significantly reduced the levels of inflammatory interleukins in paws with induced local inflammation, but what is more, and this should be especially emphasized, it reduced also levels of ROS.

Oxidative stress is a hallmark of inflammation and is accompanied by a change in the cell redox balance with subsequent activation of redox-sensitive intracellular signalling pathways, which can be pro- or anti-inflammatory [50]. Adenosine inhibits the superoxide burst produced by the NADPH complex in response to activating agents including host- and bacterial-derived formylated peptides, a process that is essential for killing phagocytosed bacteria but that also promotes oxidant-induced tissue injury in inflammatory diseases such as asthma, arthritis, inflammatory bowel disease, and ischemia/reperfusion injury [44,45]. It is not known, however, whether this effect is mediated through the adenosine A_{2B} receptor. There is one report in the literature that PSB-603 being a selective adenosine A_{2B} receptor antagonist increased both mitochondrial oxygen consumption and intracellular ROS levels in *in vitro* tests [34]. However, *in vitro* tests performed in this study did not show a significant effect of the tested compound on oxidative processes.

The reduction of oxygen free radicals may depend on the dose/-concentration used [46–48] since there are many reports confirming that an antioxidant used at too high dose/concentration facilitates the induction of free oxygen radicals [49]. What is more important, our *ex vivo* studies showed that *ip* administration of PSB-603 reduced the amount of ROS in the paws into which the inflammatory factor was injected. This anti-inflammatory effect of the PSB-603 compound might be associated with the blockade of adenosine A_{2B} receptor, which inhibits the formation of ROS in the paws during the inflammatory reaction (the compound was administered immediately before the inflammatory factor). However, those results might also be associated with an additional mechanism of anti-inflammatory action of PSB-603 besides blocking the adenosine A_{2B} receptor, which requires further study.

Preliminary results described in this paper confirm the anti-inflammatory effect of the known highly potent and extraordinarily selective adenosine A_{2B} receptor antagonist in acute models of local and systemic inflammation. They are promising and encourage us to perform further, expanded research using this interesting adenosine A_{2B} receptor antagonist.

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Ethical approval

All animal care and experimental procedures were carried out in accordance with European Union and Polish legislation acts concerning animal experimentation, and were approved by the Local Ethics Committee at the Jagiellonian University in Cracow, Poland (Permissions No: 256/2015, 55/2017).

Author contribution statement

Magdalena Kotańska conceived and designed research. Magdalena Kotańska, Anna Dziubina, Marek Bednarski conducted experiments. Christa E. Müller, Katarzyna Kieć-Kononowicz contributed new reagents or analytical tools. Magdalena Kotańska, Małgorzata Szafarz, Kamil Mika, Jacek Sapa analysed data. Magdalena Kotańska, Małgorzata Szafarz, Kamil Mika, Anna Dziubina wrote the manuscript. All authors read and approved the manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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