

Anti-Inflammatory Effects of Novel P2X4 Receptor Antagonists, NC-2600 and NP-1815-PX, in a Murine Model of Colitis

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Abstract

The pharmacological blockade of P2X4 receptors has shown potential benefits in the management of several immune/inflammatory diseases. However, data regarding the involvement of P2X4 receptors in the pathophysiological mechanisms of action in intestinal inflammation are not well defined. We aimed to evaluate the anti-inflammatory effects of two novel and selective P2X4 receptor antagonists, NC-2600 and NP-1815-PX, and characterize the molecular mechanisms of their action in a murine model of 2,4-dinitrobenzene sulfonic acid (DNBS)-induced colitis. These two drugs and dexamethasone (DEX) were administered orally for 6 days, immediately after the manifestation of DNBS. The body weight decrease, resulting from colitis, was attenuated by NC-2600 and NP-1815-PX, but not DEX. However, all three drugs attenuated the increase in spleen weight and ameliorated macroscopic and microscopic colonic tissue damage. Furthermore, all three compounds decreased tissue IL-1 β levels and caspase-1 expression and activity. Colonic tissue increase of tumor necrosis factor was downregulated by DEX, while both NC-2600 and NP-1815-PX were ineffective. The reduction of occludin associated with colitis was ameliorated by NC-2600 and NP-1815-PX, but not DEX. In THP-1 cells, lipopolysaccharide and ATP upregulated IL-1 β release and NLRP3, caspase-1, caspase-5 and caspase-8 activity, but not of caspase-4. These changes were prevented by NC-2600 and NP-1815-PX treatment.

For the first time, the above findings show that the selective inhibition of P2X4 receptors represents a viable approach to manage bowel inflammation *via* the inhibition of NLRP3 inflammasome signaling pathways.

Introduction

Inflammatory bowel diseases (IBDs), comprising ulcerative colitis (UC) and Crohn's disease (CD), are chronic intestinal inflammatory disorders, characterized by massive inflammation of various parts of the digestive tract [1]. In UC the inflammatory process are limited to the intestinal mucosa and submucosa in a continuous pattern, while in CD the tissue inflammation phlogistic process is generally transmural and often discontinuous [1]. Clinically, UC and CD share similar symptoms, including diarrhea, weight loss, rectal bleeding and abdominal pain, which may be debilitating and lead to life-threatening complications [1].

It is widely recognized that in genetically susceptible hosts, pathologic immune responses with an imbalanced interactions with luminal microbes, compromise the intestinal epithelial barrier, triggering the development of chronic intestinal inflammation [2].

With the ever increasing incidence of IBD, a large body of evidence has highlighted the often insufficient efficacy and/or safety of traditional drugs (including corticosteroids, immunosuppressants and salicylates [2–4]. Likewise, more recent biotechnological drugs including anti-TNF alpha and other biologic treatments can prove ineffective or over the years of administration often loose effectiveness in many IBD patients. [2–4]. It has become clear that the currently available treatment options for IBD are

far from being truly satisfactory, spurring the interest of several pharmaceutical companies to find novel molecular targets for clinical treatment options with even better benefit/risk profiles [5, 6].

A number of evidences revealed a the critical involvement of extracellular adenosine triphosphate (ATP) in modulating immune responses [7]. It has been widely demonstrated that during inflammation, ATP is released massively from immune and parenchymal cells, acting as a “danger” and a “find-me” signal aimed to fine-tune phagocyte activation and migration via autocrine or paracrine pathways [8]. Over the years, several purinergic receptors have been identified as critical players in orchestrating the pro-inflammatory activity of ATP [9].

Recently, increasing attention has been focused on the P2X4 receptor, a trimeric ATP-gated cation channel, that is widely expressed in central and peripheral neurons, microglia, immune cells, endothelial cells and even in glandular tissues [9]. This purinergic receptor, once activated by ATP in nanomolar concentrations, elicits a pro-inflammatory response with the release of pro-inflammatory cytokines, such as IL-1 β and IL-18, with consequent exacerbation of the inflammatory response [9, 10].

Several evidences show the involvement of P2X4 receptors in many disorders characterized by immune dysfunctions, including post-ischemic inflammation, rheumatoid arthritis, asthma, neurodegenerative and cardiovascular diseases as well as metabolic syndrome [11, 12]. At present, the involvement of P2X4 receptors in the pathophysiological mechanisms of intestinal inflammation is not well characterized. However, the pivotal role of this receptor subtype in driving IL-1 β and IL-18 release, and other pro-inflammatory cytokines in gut tissue of IBD patients [13, 14], lead us to hypothesize a significant role of P2X4 in the pathophysiology of IBD.

Therefore, the present study was designed to investigate the role of P2X4 receptors in a murine model of 2,4-dinitrobenzene sulfonic acid (DNBS)-induced colitis, and to evaluate the potentially beneficial therapeutic effect of two novel P2X4 receptor antagonists, NC-2600 and NP-1815-PX.

Materials And Methods

Experiments on animals

Animals

Albino male Sprague–Dawley rats, 200–225 g in body weight, were employed throughout the study. The animals were fed with standard laboratory chow and tap water *ad libitum* and were given at least one week to acclimatize after their delivery to the animal facility. They have been housed, three in a cage, in temperature-controlled rooms at 22–24°C and 50–60% humidity with a 12-h light cycle. Animal care and handling were in accordance with the provisions of the European Community Council Directive 210/63/UE, recognized and adopted by the Italian Government. The experiments have been approved by the Ethical Committee for Animal Experimentation of the University of Pisa and by the Italian Ministry of Health (authorization n 674/2016-PR).

Induction of Colitis and Drug Treatments

Rats were weighed and randomly distributed into separate experimental groups of six rats in each. Colitis was induced in accordance with the method described previously by Pellegrini et al., in 2018 [15]. Briefly, during a short anesthesia with isoflurane (Abbott Labs, Pomezia, Italy), 15 mg of 2,4-dinitrobenzenesulfonic acid (DNBS) in 0.25 ml of 50% ethanol has been administered intrarectally via a polyethylene PE-60 catheter inserted 8 cm proximal to the anus. Control rats received 0.25 ml of 50% ethanol. Following the DNBS administration, rats developed full scale colonic inflammation, that became evident 6 days later.

Immediately after the DNBS treatment, test drugs (NC-2600 3, 10, 30 mg/kg; NP-1815-PX 3, 10, 30 mg/kg and Dexamethasone (DEX) 1 mg/kg) were orally administered to the animals for 6 days. DNBS-untreated animals (control group) and DNBS-treated rats (DNBS group) received only methocel (drug vehicle). When these treatments were initiated, at the same time we started the daily monitoring of body weight of the rats.

The doses of NC-2600 (3, 10, 30 mg/kg) and NP-1815-PX (3, 10, 30 mg/kg) were selected based on our preliminary experiments designed to measure the effects of increasing doses of these compounds on food intake, body weight, spleen weight and macroscopic score in the model of DNBS-induced colitis. Effective dosing of NC-2600 (10 mg/kg) and NP-1815-PX (10 mg/kg) was then chosen to further study the effects of these compounds on inflammasome pathway and tight junction expression. The dose of DEX was selected according to a previous study using a rat model of colitis [16]. Macroscopic and histological scores were assessed on the entire length of colon, while biochemical assays were performed on colonic segments collected from inflamed regions adjacent and distal to grossly necrotic tissue areas.

Assessment of Colitis

Colonic tissue samples were graded for macroscopic damage, as reported previously [15]. The criteria for macroscopic grading of colonic damage were as follows: (1) consistency of colonic fecal material (0, formed; 1, loose; 2, liquid stools); (2) presence of abdominal adhesions between colonic tissue and other organs (0, none; 1, minor; 2, major adhesions); and (3) presence of mucosal ulceration (0, none; 1, hyperemia; 2, ulceration without hyperemia; 3, ulceration with inflammation at one side; 4, ≥ 2 sites of ulceration and inflammation; 5, major sites of damage; and 6, major sites of damage extending > 2 cm). The score was then increased by one unit for each millimeter of colonic wall thickness. All parameters of macroscopic damage were recorded and scored for each rat by two observers blinded to the treatment. At the time of experiment, the weight of each spleen was also measured.

Microscopic tissue injury scores

Microscopic tissue injury and inflammation were assessed by light microscopy on hematoxylin/eosin-stained histological sections obtained from whole gut specimens. The histological criteria included mucosal architecture loss (0 - 3), cellular infiltration (0 - 3), muscle layer thickening (0 -3), crypt abscess

(0, absent; 1, present), and goblet cell depletion (0, absent; 1, present). All parameters of histological damages were recorded and scored for each rat by two observers blinded to the treatment.

Cytokine assays

Tumor necrosis factor (TNF) and interleukin (IL)-1 β were measured with enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, Camarillo, CA), as described previously [17, 18]. For this purpose, colonic tissue samples (40 mg), stored previously at -80°C , were weighed, thawed, and homogenized in 0.4 ml of PBS, pH 7.2 and centrifuged at 13,400*g* for 20 min. Aliquots (100 μL) of the supernatants were then used for assay. The concentrations of TNF- α and IL-1 β were expressed as picograms per milligram of tissue protein.

Protein extraction and Western blot analysis

The colon was collected from rats and flushed of fecal content with ice-cold phosphate-buffered saline (PBS), as described previously [19, 20]. Tissues were minced and homogenized using a Potter-Elvehjem Grinder homogenizer on ice in 20% (w/v) TNE lysis buffer (50mM Tris-HCl pH 7.4, 100mM NaCl, 0.1mM EDTA, 1% NP-40, 1% SDS, 0.1% DOC) with various protease s and phosphatase s inhibitors. Samples were then sonicated and boiled for 5 min at 95°C . Protein concentrations were measured with the Bradford assay. Total lysates were run on 4–20% Criterion™ TGX™ Precast Midi Protein Gels (Bio-Rad, Hercules, CA, USA) and then transferred to PVDF membranes (Trans-Blot Turbo™ PVDF Transfer packs, Biorad). Then, membranes were blocked with 3% bovine serum albumin (BSA) diluted in Tris-buffered saline (TBS, 20 mM Tris-HCl, PH 7.5, 150 mM NaCl) with 0.1% Tween 20. Primary antibodies against caspase-1 (ab1872, Abcam) and occludin (ab167161, Abcam) were used. Secondary antibodies were obtained from Abcam (anti-mouse ab97040 and anti-rabbit ab6721). Protein bands were detected with ECL reagents (Clarity Western ECL Blotting Substrate, Biorad). Densitometry was performed by IBright Analysis software.

Caspase-1 activity assay

Caspase-1 activity in colonic tissues was measured by using the colorimetric protease assay kit (BioVision Research Products, Mountain View, CA), following the protocols provided by the manufacturer. For this purpose, colonic tissue samples (40 mg), stored previously at -80°C , were weighed, thawed, and homogenized in 0.4 ml of PBS, pH 7.2 and centrifuged at 13,400 *g* for 20 min. Aliquots (100 μL) of these supernatants were then used for the assay.

Experiments on cell cultures

Cell culture

Human monocytic THP-1 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum (FBS) and 100 unit/ml penicillin-streptomycin at 37°C in a humidified atmosphere of 5% CO_2 .

Cell treatments

THP-1 cells were seeded at density of 5×10^5 cells/well in 24-well plates and treated with phorbol 12-myristate 13-acetate (PMA, 0.5 μ M). After 3 h, the medium was removed, fresh media was added, and cells were incubated overnight (37°C, 5% CO₂).

In the first series of experiments, lipopolysaccharide (LPS)-primed (1 μ g/mL) 3 h) cells were treated for 1h with vehicle (0.03% dimethyl sulfoxide, DMSO) before the addition of ATP (5 mM, 1h).

In the second series of experiments, LPS-primed (1 μ g/ml, 3h) cells were treated for 1h with NC-2600 (0.03 - 0.3 - 3 μ M, 1h) before the addition of ATP (5 mM, 1h).

In the third series of experiments, LPS-primed (1 μ g/ml, 3h) cells were treated for 1h with NP-1815-PX (0.1 - 1 - 10 μ M, 1h) before the addition of ATP (5 mM, 1h). Controls were run in parallel.

After LPS stimulation, THP-1 cells were lysed, and the medium were collected and centrifuged for 5 minutes at 800 rpm to obtain cell-free supernatants. Cell supernatants were used to evaluate IL-1 β release, while cells were lysed for analysis of inflammasome subunits and caspase-1/-4/-5 and -8 expression.

Western Blot

Cells were lysed as previously described [21, 22]. Proteins were quantified with the Bradford assay. Proteins were separated onto a pre-cast 4-20% polyacrylamide gel (Mini-PROTEAN® TGX gel, Biorad) and transferred to PVDF membranes (Trans-Blot® Turbo™ PVDF Transfer packs, Biorad). Membranes were blocked with 3% BSA diluted in Tris-buffered saline (TBS, 20 mM Tris-HCl, PH 7.5, 150 mM NaCl) with 0.1% Tween 20. Primary antibodies against β -actin (ab8227, Abcam), nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain containing protein 3 (NLRP3) (ab214185, Abcam), apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) (D2W8U, Cell Signaling), caspase-1 (ab1872, Abcam), caspase-4 (#4450, Cell Signaling), caspase-5 (#46680, Cell Signaling) and caspase-8 (ab227430, Abcam) were used. Secondary antibodies were obtained from Abcam (anti-mouse ab97040 and anti-rabbit ab6721). Protein bands were detected with ECL reagents (Clarity Western ECL Blotting Substrate, Biorad). Densitometry was performed by IBright Analysis software.

Assessment of IL-1 β release in THP-1 cells

The release of IL-1 β was measured by ELISA kit (R&D system), as previously described [23]. After cell stimulation, the medium was collected and centrifuged for 5 min at 800 rpm to obtain cell-free supernatants. Aliquots of 100 μ L were used for the test. IL-1 β concentration was expressed as percentage *versus* THP-1 control cells (Ctrl).

Drugs and reagents

ATP, DEX, DMSO, DNBS, FBS, LPS, PBS and PMA were purchased from Sigma Aldrich (St. Louis, MO, USA). The synthesis of NC-2600 and NP-1815-PX were performed by Nippon Chemiphar Co., Ltd. For cell

culture experiments, we dissolved the compound NC-2600 in sterile DMSO and subsequently diluted it in culture medium. For the experiments on animals, solid dispersion form of NC-2600 was used, as NC-2600 is hardly dissolved.

Statistical analysis

Data are presented as mean \pm SEM and analyzed by GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical significances were determined by one-way ANOVA followed by Tukey's post hoc test. A *P* value < 0.05 was considered statistically significant.

Results

Body Weight and Spleen Weight

Six days after DNBS administration, rats displayed a significant decrease in their body weight in comparison with control animals ($-36.67 \pm 4.41\%$ and $+ 26.62 \pm 3.22\%$, respectively) (Figure 1A). The body weight loss was significantly attenuated in the rats treated with NC-2600 (3, 10, 30 mg/kg) or NP-1815-PX (3, 10, 30 mg/kg), but not in animals with DEX treatment. (1 mg/kg) (Figure 1A).

The induction of colitis with DNBS resulted in a significant increase of spleen weight ($+ 44.33\%$ on average) (Figure 1B), that was counteracted by all tested drugs, such as NC-2600 (3, 10, 30 mg/kg), NP-1815-PX (3, 10, 30 mg/kg) and DEX (1 mg/kg) (Figure 1B).

Furthermore, the administration of the above compounds did not elicit any significant effects in DNBS-vehicle treated animals (Control group; data not shown).

Macroscopic Damage

The administration of DNBS was associated with colonic thickening and ulcerations, with marked areas of transmural inflammation. Adhesions were often present, and the bowel was occasionally dilated, with a macroscopic tissue injury grade of 7.83 ± 0.61 (Figure 1C). In this setting, the macroscopic colonic injury was reduced significantly by treatment with the indicated concentrations of NC-2600 (3, 10, 30 mg/kg), NP-1815-PX (3, 10, 30 mg/kg) and DEX (Figure 1C).

Microscopic Damage

Histological examination of colonic specimens from DNBS-treated rats showed that inflammation was evident and associated with large areas of mucosal necrosis, where the glandular architecture of tissue was completely destroyed, and only amorphous material could be observed. Submucosa was thickened due to the presence of oedema and marked infiltration with inflammatory cells associated with vasodilation. These processes occasionally involved the muscular layer which appeared to be thickened as well. The mucosa and submucosa surrounding the necrotic area exhibited inflammation marked with cellular infiltration, compared with tissues obtained from control animals (Figure 2). Colonic sections from rats with colitis displayed a severe tissue damage 6 days after DNBS treatment (Figure 2). NC-2600,

but not NP-1815-PX, dose-dependently reduced the histological damage in colonic tissues obtained from inflamed rats (Figure 2). DEX (1 mg/kg) determines a significant improvement in microscopic scores (Figure 2).

TNF and IL-1 β Levels in Colonic Tissues

Rats with colitis displayed a significant increase in colonic TNF levels (12.12 ± 1.78 pg/mg tissue), in comparison with control animals (5.39 ± 0.60 pg/mg tissue) (Figure 3A). Administration of NC-2600 or NP-1815-PX did not affect TNF concentration in colonic tissues from DNBS-treated rats (Figure 3A). By contrast, DEX significantly counteracted the increase in colonic TNF levels associated with experimental colitis (Figure 3A).

DNBS-induced colitis was associated with a significant increase in tissue IL-1 β levels (32.73 ± 1.88 pg/mg tissue), in comparison with control rats (9.53 ± 5.43 pg/mg tissue) (Figure 3B). This response was significantly downregulated by NC-2600 or DEX (Figure 3B). By contrast, NP-1815-PX failed to have any significant effects on IL-1 β levels (Figure 3B).

Pro-Caspase-1 and Cleaved Caspase-1 in Colonic Tissues

Expression of pro-caspase-1 in colonic tissues did not differ among the experimental groups (Figure 4A). In parallel, colonic tissues obtained from rats with colitis showed a significant increase in cleaved caspase-1 expression (p20, an auto-processed fragment of caspase-1) when compared with control animals (Figure 4B). Treatment with NC-2600 (10 mg/kg) or NP-1815-PX (10 mg/kg) did not exert significant effects on cleaved caspase-1 (Figure 4B). By contrast, DEX significantly counteracted the increase in cleaved caspase-1 expression associated with experimental colitis (Figure 4B).

Caspase-1 Activity in Colonic Tissues

Rats with DNBS-induced colitis displayed a significant increase in caspase-1 activity, in comparison with control animals (Figure 4D). Treatment with NC-2600 (10 mg/kg), NP-1815-PX (10 mg/kg) or DEX counteracted significantly the increment of colonic caspase-1 activity associated with DNBS administration (Figure 4D).

Occludin Expression in Colonic Tissues

Western blot analysis showed that the expression of occludin (integral plasma-membrane protein located at the tight junctions) was significantly reduced in colonic mucosa from DNBS-treated rats in comparison with control animals (Figure 4E). Rats with colitis administered with NC-2600 (10 mg/kg) or NP-1815-PX (10 mg/kg) displayed a significant increase in occludin expression, whereas those subjected to DEX administration did not experience this phenomenon (Figure 4E).

In vitro **assays on NLRP3 inflammasome: canonical and non-canonical pathways**

A set of *in vitro* experiments were performed to characterize the molecular mechanisms underlying the anti-inflammatory effects of P2X4 receptor antagonists, NC-2600 and NP-1815-PX. In particular, we focused our attention on the effects of P2X4 antagonists on the expression of inflammasome components, including NLRP3, ASC, caspase-1, caspase-4, caspase-5 and caspase-8, as well as IL-1 β release in THP-1, a human monocytic cell line.

Expression of NLRP3 and ASC

LPS-primed THP-1 cells incubated with ATP were characterized by a significant increase of expression of NLRP3 (114 KDa) whereas ASC (25 KDa) was not affected (Figure 5A and 5B). Treatment with NC-2600 or NP-1815-PX counteracted the increase in the expression of both NLRP3 and ASC in a dose-dependent fashion (Figure 5A and 5B).

Expression of pro-caspase-1 and cleaved caspase-1 (canonical NLRP3 pathway).

NC-2600 and NP-1815-PX did not affect the expression of pro-caspase-1 (45 KDa) in LPS-primed THP-1 cells incubated with ATP (Figure 5C and 5E). By contrast, both P2X4 antagonists significantly counteracted the increase in the expression of caspase-1 in LPS-primed THP-1 cells incubated with ATP (Figure 5D and 5E).

Expression of caspase-4, caspase-5 and caspase-8 (non-canonical NLRP3 pathway)

LPS-primed THP-1 cells incubated with ATP showed a significant increase of caspase-5 and caspase-8 expression, whereas no significant variations were observed for caspase-4, as compared with control cells (Ctrl) (Figure 6A, 6B and 6C). Treatment with NC-2600 and NP-1815-PX significantly counteracted the increase in the expression of caspase-5 and caspase-8 (Figure 6A, 6B and 6C).

IL-1 β processing and release

Incubation of LPS-primed THP-1 cells with ATP significantly increased the release of IL-1 β , as compared with control cells (Ctrl) (Figure 6D). Such an increase was dose-dependently reduced by treatment with NC-2600 (0.03-3 μ M) (Figure 6D). Of note, despite NP-1815-PX (0.1 and 1 μ M) did not alter IL-1 β processing and release, incubation with 10 μ M NP-1815-PX reduced significantly the IL-1 β production induced by the co-treatment with LPS and ATP (Figure 6D).

Discussion

For several years, pharmacological management of IBD was limited to aminosalicylates, thiopurines, and steroids, which, despite providing symptomatic relief, affected scarcely the disease course [2].

Subsequently, advances in understanding the molecular mechanisms underlying the pathogenesis of IBD allowed us to identify novel therapies based on biological entities. These therapies aimed at curbing the activity of pro-inflammatory cytokines pivotally involved in the onset and progression of IBDs [3]. Even though biological drugs undoubtedly represent an evolution in the therapeutic approach to IBD, these are

often characterized by a high degree of therapeutic failure and in many cases by heavy adverse effects [3]. For these reasons, the identification of new therapeutic targets with immunomodulating/anti-inflammatory properties represents a field of extreme interest for the scientific community.

In this regard, increasing attention has been paid to the design and synthesis of novel pharmacological entities acting on the purinergic system as viable way to regulate the development and course of several immuno-mediated inflammatory diseases (IMIDs) [8]. In particular, the active role of P2X4 receptor subtype has been well defined in driving the immunostimulating effects of extracellular ATP, eliciting pro-inflammatory responses in both myeloid or lymphoid cells [11]. Therefore, this receptor subtype represents an ideal target for pharmacological management several immune/inflammatory diseases [8, 9, 11, 12].

The aims of our study were: *a)* to evaluate the putative anti-inflammatory effects of NC-2600 and NP-1815-PX, two novel and selective P2X4 receptor antagonists, and *b)* to characterize the molecular mechanisms underlying their immunomodulatory effect in a murine model of DNBS-induced colitis. DEX was employed as a comparative agent with known anti-inflammatory activity to evaluate the relative anti-inflammatory effects of both P2X4 ligands.

The DNBS-induced colitis closely mimics the pathology of Crohn's disease as it is characterized by body weight loss, diarrhea, colon ulceration and bleeding, the depletion of goblet cells, and the formation of granulomas within the gut wall [24, 25]. This represents a predictive model to assess the efficacy of novel anti-inflammatory agents. In this regard, the suitability of this model was corroborated by the efficacy of DEX to ameliorate the macroscopic and histological features of colitis as well as to reduce tissue TNF and IL-1 β levels, in line with previous reports showing similar beneficial effects of this glucocorticoid drugs [16, 26].

In the first set of experiments, we observed that the pharmacological blockade of P2X4 receptor *via* NC-2600 and NP-1815-PX elicited a significant improvement in several inflammatory parameters, including body weight, spleen weight, macroscopic and microscopic tissue injury score, as well as down-regulated IL-1 β levels in DNBS-rats, thus providing evidence that the pharmacological modulation of this receptor is a viable option to manage enteric inflammatory processes. This is in line with a number of recent studies describing the critical role of P2X4 receptor in protecting against infections, inflammation, and organ injury [11]. Indeed, this receptor has been shown to regulate a variety of pathophysiological processes characterized by an excessive immune cell activity such as autoimmune and neuroinflammatory diseases [9]. In particular, P2X4, widely expressed on cells of both the innate and adaptive immune cells, is emerging as a pivotal pathway in mediating the immunostimulating/pro-inflammatory effect of ATP [11], thus representing an attractive therapeutic target in multiple diseases, especially in chronic inflammation and neuropathic pain [27–29].

Interestingly, in our experimental model, both P2X4 antagonists failed to reduce tissue TNF levels. By contrast, NC-2600 and NP-1815-PX were able to counteract the increase of IL-1 β concentration in colonic tissues from rats with DNBS-induced colitis. IL-1 β is a potent inflammatory cytokine centrally involved in

the development of colitis and T_H17-associated immune responses in the gut [30]. On the same line, patients with Crohn's disease showed a positive correlation between the severity of mucosal inflammation and the levels of IL-1 β , thus corroborating a relevant role for this cytokine in the pathophysiology of IBDs [31].

Despite the mechanism of IL-1 β release has proven to be complex a set of molecular and biochemical investigations allowed to demonstrate that the bioactive form of this cytokine is produced by the inflammasome NLRP3, a caspase-1 activating molecular platform, in response to selected danger-associated molecular patterns and pathogen-associated molecular patterns [32]. In particular, the NLRP3 inflammasome complex, including NLRP3, ASC and pro-caspase-1, through the processing and release of IL-1 β and IL-18, acts as a key player both in shaping the central and/or peripheral immune/inflammatory responses in several inflammatory diseases, including IBDs [33]. Such pathway is currently designated as "canonical NLRP3 inflammasome activation." In addition, a "non-canonical NLRP3 inflammasome activation," which depends on caspase-11 in mice (caspase-4 and caspase-5 in human) and caspase-8, has been reported to play a pivotal role in the pathophysiological events underlying bowel inflammation [34].

Based on these findings we designed several experiments to evaluate the putative effect of NC-2600 and NP-1815-PX in counteracting inflammasome NLRP3 activation, especially focusing on the effect of tested drugs on the expression and activity of caspase-1, the canonical activation pathway of NLRP3 complex. Our findings showed that treatment with NC-2600 and NP-1815-PX, despite not reducing the caspase-1 expression, was able to decrease its activity in colonic tissues from rats with colitis. This evidenced the anti-inflammatory activity of this novel P2X4 antagonists by inhibiting the canonical caspase-1-dependent NLRP3 inflammasome signaling pathway. This is in line with previous studies, performed in murine models of diabetic nephropathy, bladder inflammation and acute kidney injury, showing that the pharmacological blockade of P2X4 receptors blunted the IL-1 β release through the inhibition of NLRP3 inflammasome pathway [29, 35, 36].

In order to corroborate the results obtained from *in vivo* experiments, we designed a set of *in vitro* experiments to evaluate the effect of the P2X4 antagonists on modulating the canonical and non-canonical pathways involved in NLRP3 activation. Therefore, we performed experiments in LPS-primed and PMA-differentiated THP-1 cells treated with ATP, an established model to investigate the functions of purinergic receptors in monocyte/macrophage cells [37–39]. THP-1 cells were incubated with LPS, a well-recognized activator of the first step of NLRP3 signaling as well as a hallmark of altered intestinal permeability, a condition typically observed in the murine model of colitis and IBD patients [40, 41]. We observed that co-treatment THP-1 cell line with LPS and ATP determined an increase in the expression of inflammasome components, including NLRP3, ASC and caspase-1, followed by marked IL-1 β release. Under this condition, the incubation with NC-2600 and NP-1815-PX decreased all of the above mentioned parameters, indicating an inhibitory effect of test drugs on the canonical caspase-1-dependent NLRP3 inflammasome pathway. This result is in line with a previous report displaying an inhibitory effect on

canonical NLRP3 activation following the pharmacological blockade of P2X4 receptors in rat urothelial cells, neuronal dorsal horn cells and renal tubule epithelial cells incubated with ATP [29, 35, 36, 42].

In parallel with the canonical NLRP3 inflammasome activation, a “non-canonical NLRP3 inflammasome activation,” depending on caspase-11, has been described to be pivotal in the maintenance of intestinal immune homeostasis [43, 44]. Recent studies have also reported the involvement of caspase-8 in regulating the expression and release of IL-1 β by NLRP3 inflammasome activation, highlighting a novel non-apoptotic role of caspase-8 in the context of inflammation [45, 46]. In the present study, sets of experiments were devoted to evaluate the modulatory effect of NC-2600 and NP-1815-PX also on the non-canonical NLRP3 inflammasome pathway. The pharmacological blockade of P2X4 receptors affects also the non-canonical NLRP3 inflammasome pathways, reducing the caspase-5 and caspase-8 expression in THP-1 cells treated with LPS and ATP. Of note, no effect for both tested drugs was observed on caspase-4 expression. However, since uncleaved caspase-4 can promote the release of IL-1 β through activation of the canonical NLRP3-ASC-caspase-1 signaling [47, 48], we could rule out that the inhibition of IL-1 β release resulting from the blockade of P2X4 receptors would depend, at least in part, in an uncleaved caspase-4 manner.

Taken together, these results provide convincing evidence that the blockade of P2X4 receptors exerts an inhibitory effect on both the canonical and non-canonical pathways involved in NLRP3 activation. This represents the novelty of the present study, and further studies aimed at better characterizing the molecular mechanisms underlying the P2X4 receptors/NLRP3 inflammasome interplay represent the logical continuation of this research topic.

IL-1 β , beyond being involved in the initiation and amplification of inflammatory responses, plays a critical role in the apoptosis of epithelial cells causing tissue damage and barrier dysfunction. This, in turn, leads to increased intestinal permeability typically associated with IBDs [49]. Consistently with this evidence, our experimental model showed a decreased expression levels of colonic tight junctions. Interestingly, treatment with NC-2600 and NP-1815-PX counteracted the reduction of tight junction expression associated with experimental colitis, suggesting that the pharmacological blockade of P2X4 receptors can exert beneficial effect in the maintenance of intestinal epithelial barrier integrity. Despite the fact that no data are available about the molecular mechanisms underlying the beneficial effects of this novel P2X4 antagonists in the maintenance of intestinal epithelial barrier, it is conceivable that these likely depend, at least in part, on the action of these drugs on the NLRP3/caspase-1/IL-1 β axis.

In conclusion, the present study expands current knowledge about the beneficial effects of the P2X4 receptor blockade in modulating immune/inflammatory responses. Our results demonstrated here for the first time that the direct and selective inhibition of P2X4 receptors represent a viable approach for the management of bowel inflammation *via* the inhibition of the canonical and non-canonical NLRP3 inflammasome signaling pathways. According to the present results, the blockade of P2X4 receptors allows an effective control of experimental intestinal inflammation, and novel and selective P2X4

antagonists can provide a basis for development of anti-inflammatory drugs suitable for treatment of IBD.

Declarations

Ethics approval and consent to participate (Human Ethics, Animal Ethics or Plant Ethics): The experiments have been approved by the Ethical Committee for Animal Experimentation of the University of Pisa and by the Italian Ministry of Health (authorization n 674/2016-PR)

Consent for publication: All the authors have read the manuscript and agreed to submit the paper to the journal.

Availability of data and materials: All data generated or analyzed during the current study are included in this article

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Author contributions: VD, CP and LA write original draft preparation; VD, CP, LB, CDS, GS performed the research; LB, MF, CDS, GS collected and analyzed the data. VD, CP, MF, ZHN interpreted the data; GH, CB, LA coauthored the writing of the manuscript and edited the manuscript. All authors read and approved the final manuscript.

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Author's information: the use of P2X4 antagonists as a medication for preventing or treating irritable bowel syndrome or inflammatory bowel diseases has been patented (PCT/IB2020/053313)

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Figures

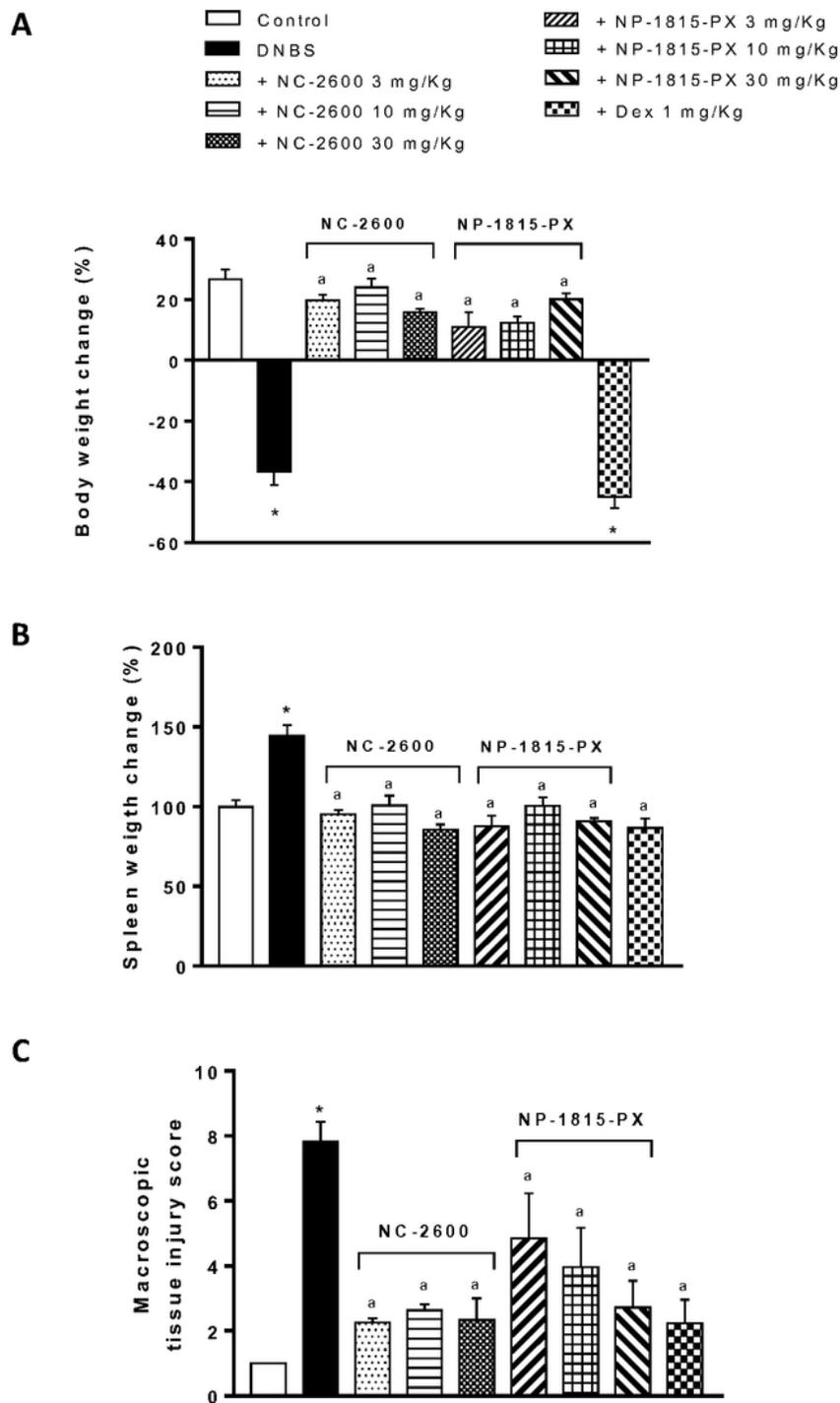


Figure 1

Figure 1

Effect of NC-2600 (3, 10, 30 mg/kg), NP-1815-PX (3, 10, 30 mg/kg) and DEX (1 mg/kg) on body weight (A), spleen weight (B) and the macroscopic tissue injury score (C) at day 6 after the induction of colitis with DNBS. Each column represents the mean \pm SEM (n = 6). One-way ANOVA followed by Tukey post hoc test results: * $p < 0.05$, significant difference vs. the control group; a $p < 0.05$, significant difference vs. the DNBS group.

Abbreviations: DEX, Dexamethasone; DNBS, 2,4-dinitrobenzene sulfonic acid.

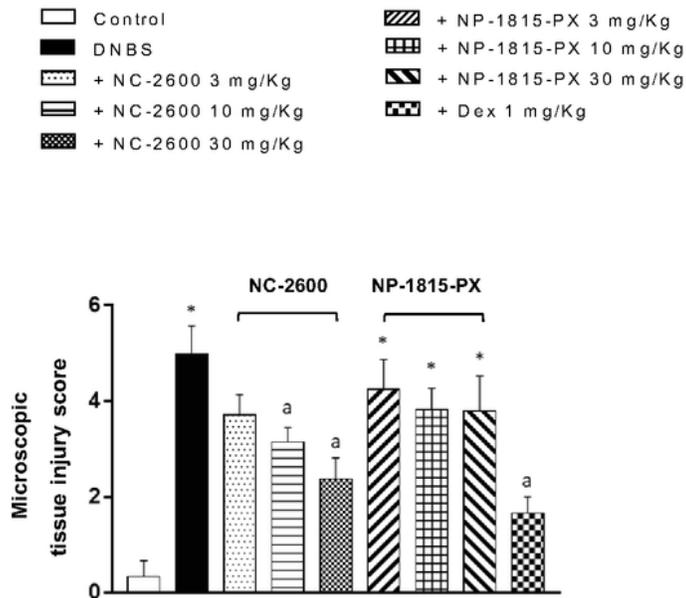


Figure 2

Figure 2

Microscopic tissue injury score estimated for colon in rats under normal conditions or with DNBS-induced colitis, either alone or after treatment with NC-2600 (3, 10, 30 mg/kg), NP-1815-PX (3, 10, 30 mg/kg) and DEX (1 mg/kg). Each column represents the mean \pm S.E.M (n = 6). One-way ANOVA followed by Tukey

post hoc test results: * $p < 0.05$, significant difference vs. the control group; a $p < 0.05$, significant difference vs. the DNBS group.

Abbreviations: DEX, Dexamethasone; DNBS, 2,4-dinitrobenzene sulfonic acid.

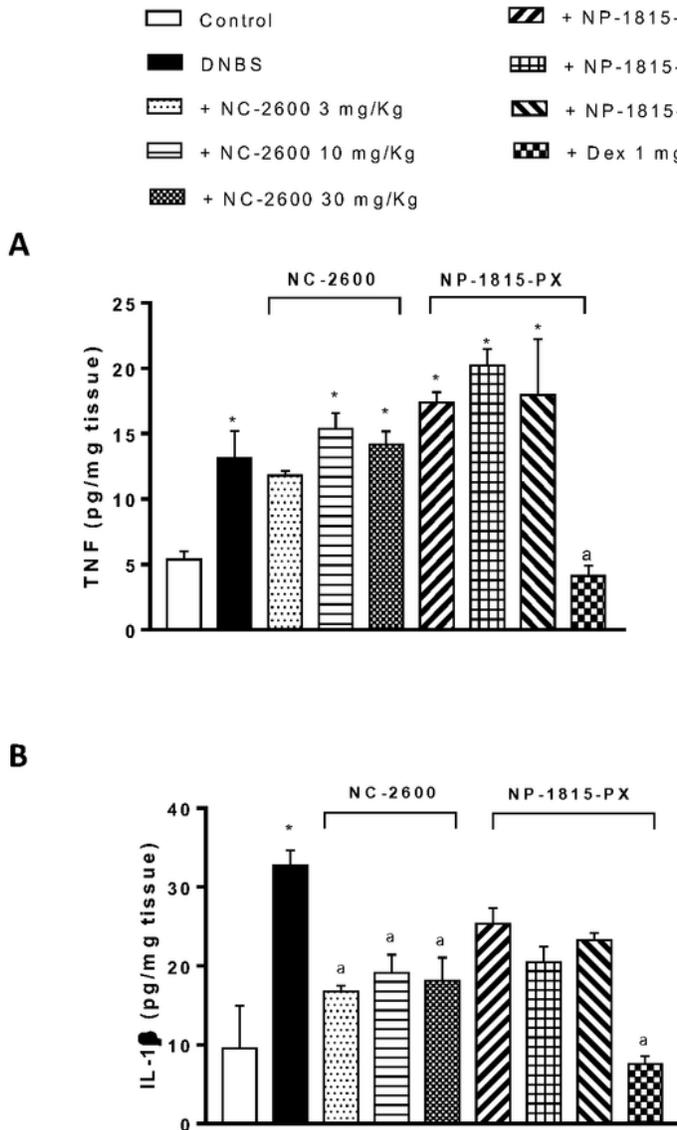


Figure 3

Figure 3

TNF (A) and IL-1 β levels (B) in colonic tissue from control rats and animals treated with DNBS alone or in combination with NC-2600 (3, 10, 30 mg/kg), NP-1815-PX (3, 10, 30 mg/kg) and DEX (1 mg/kg). Each column represents the mean \pm S.E.M. (n = 6). One-way ANOVA followed by Tukey post hoc test results: * p < 0.05, significant difference vs. the control group; a p < 0.05, significant difference vs. the DNBS group.

Abbreviations: DEX, Dexamethasone; DNBS, 2,4-dinitrobenzene sulfonic acid; IL-1 β , interleukin 1 β ; TNF, tumor necrosis factor.

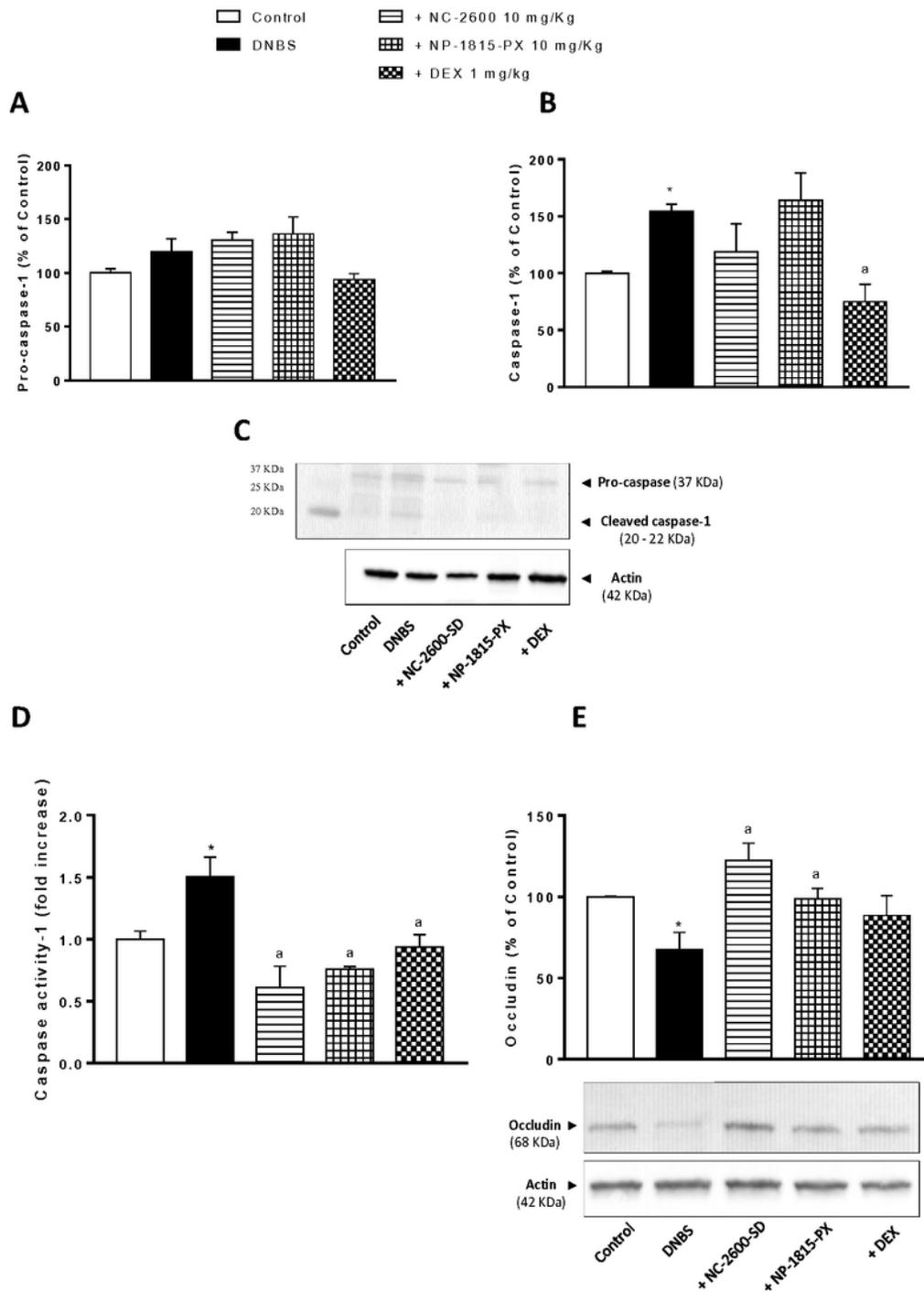


Figure 4

Figure 4

Representative blots and densitometric analysis of the expression of pro-caspase-1 (**A, C**) and cleaved caspase-1 (**B, C**) in colonic tissues from control rats and animals treated with DNBS alone or in combination with NC-2600 (10 mg/kg), NP-1815-PX (10 mg/kg) and DEX (1 mg/kg). (**D**) Caspase-1 activity in colonic tissue from control rats and animals treated with DNBS alone or in combination with NC-2600 (10 mg/kg), NP-1815-PX (10 mg/kg) and DEX (1 mg/kg). (**E**) Representative blots and densitometric analysis of the expression of occludin in colonic tissues from control rats and animals treated with DNBS alone or in combination with NC-2600 (10 mg/kg), NP-1815-PX (10 mg/kg) and DEX (1 mg/kg). Each column represents the mean \pm S.E.M. (n = 6). One-way ANOVA followed by Tukey post hoc test results: * p < 0.05, significant difference vs. the control group; a p < 0.05, significant difference vs. the DNBS group.

Abbreviations: DEX, Dexamethasone; DNBS, 2,4-dinitrobenzene sulfonic acid.

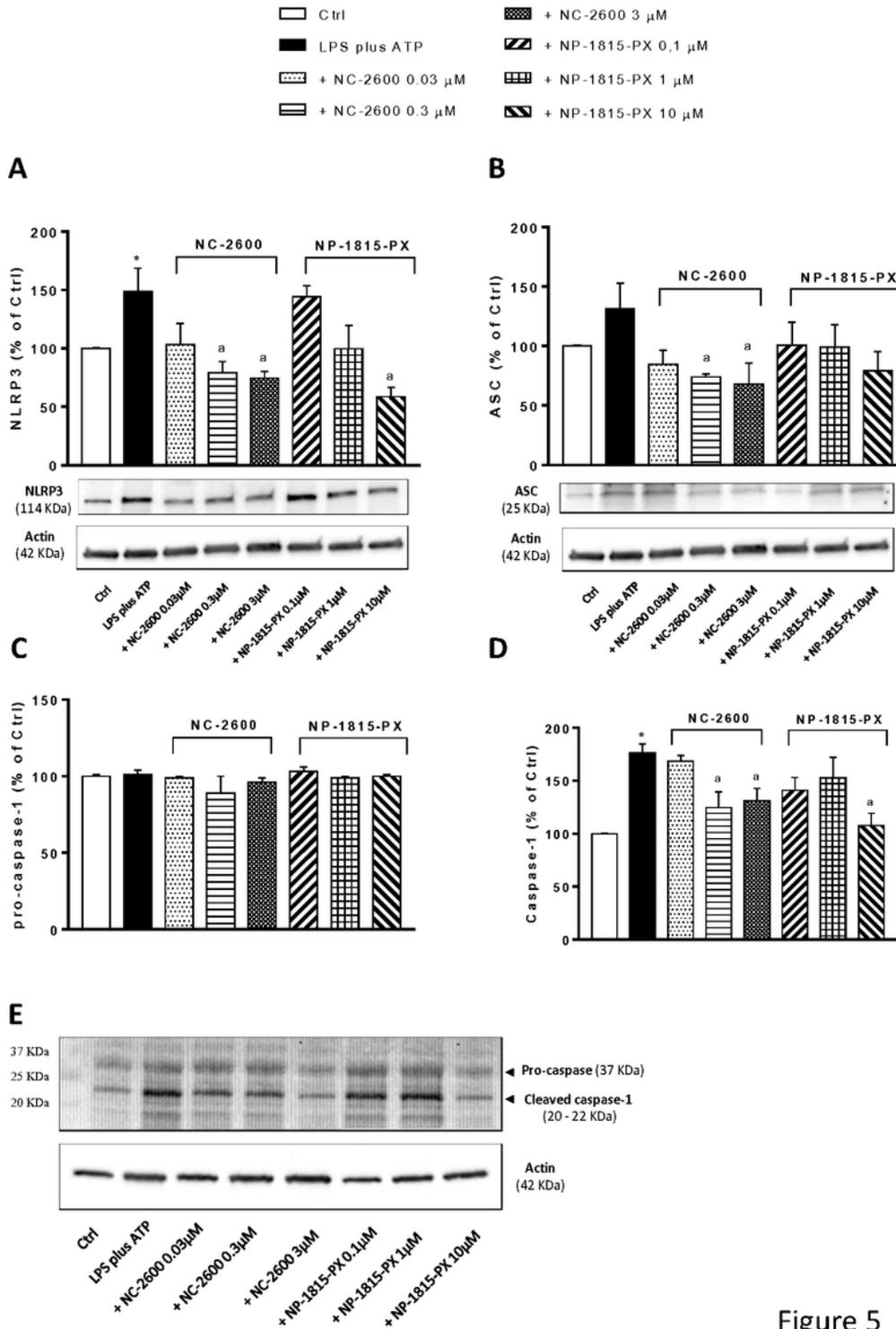


Figure 5

Figure 5

Representative blots and densitometric analysis of the expression of NLRP3 (A), ASC (B), pro-caspase-1 (C, E) and cleaved caspase-1 (D, E) assessed by Western blot assay in cultured THP-1 cells treated with LPS plus ATP, either alone or in combination with NC-2600 (0.03 - 0.3 - 3 μ M) and NP-1815-PX (0.1 - 1 - 10 μ M). Each column represents the mean \pm S.E.M. (n = 4). One-way ANOVA followed by Tukey post hoc test

results: * $p < 0.05$, significant difference vs. THP-1 control cells (Ctrl); a $p < 0.05$, significant difference vs. LPS-primed THP-1 treated with ATP.

Abbreviations: ASC, adaptor protein apoptosis-associated speck-like protein containing caspase-recruitment domain; ATP, adenosine triphosphate; LPS, lipopolysaccharide; NLRP3, nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain containing protein 3.

Figure 6

Representative blots and densitometric analysis of the expression of caspase-4 (**A**), caspase-5 (**B**) and caspase-8 (**C**) assessed by Western blot assay in cultured THP-1 cells treated with LPS plus ATP, either alone or in combination with NC-2600 (0.03 - 0.3 - 3 μM) and NP-1815-PX (0.1 - 1 - 10 μM). Each column represents the mean \pm S.E.M. ($n = 4$). (**D**) IL-1 β levels in the supernatants of LPS-primed THP-1 cells treated with ATP in the presence or in the absence of NC-2600 (0.03 - 0.3 - 3 μM) or NP-1815-PX (0.1 - 1 - 10 μM). Each column represents the mean \pm S.E.M. ($n = 4$). One-way ANOVA followed by Tukey post hoc test results: * $p < 0.05$, significant difference vs. THP-1 control cells (Ctrl); a $p < 0.05$, significant difference vs. LPS-primed THP-1 treated with ATP.

Abbreviations: ATP, adenosine triphosphate; IL-1 β , interleukin-1 β ; LPS, lipopolysaccharide.

Intestinal inflammation

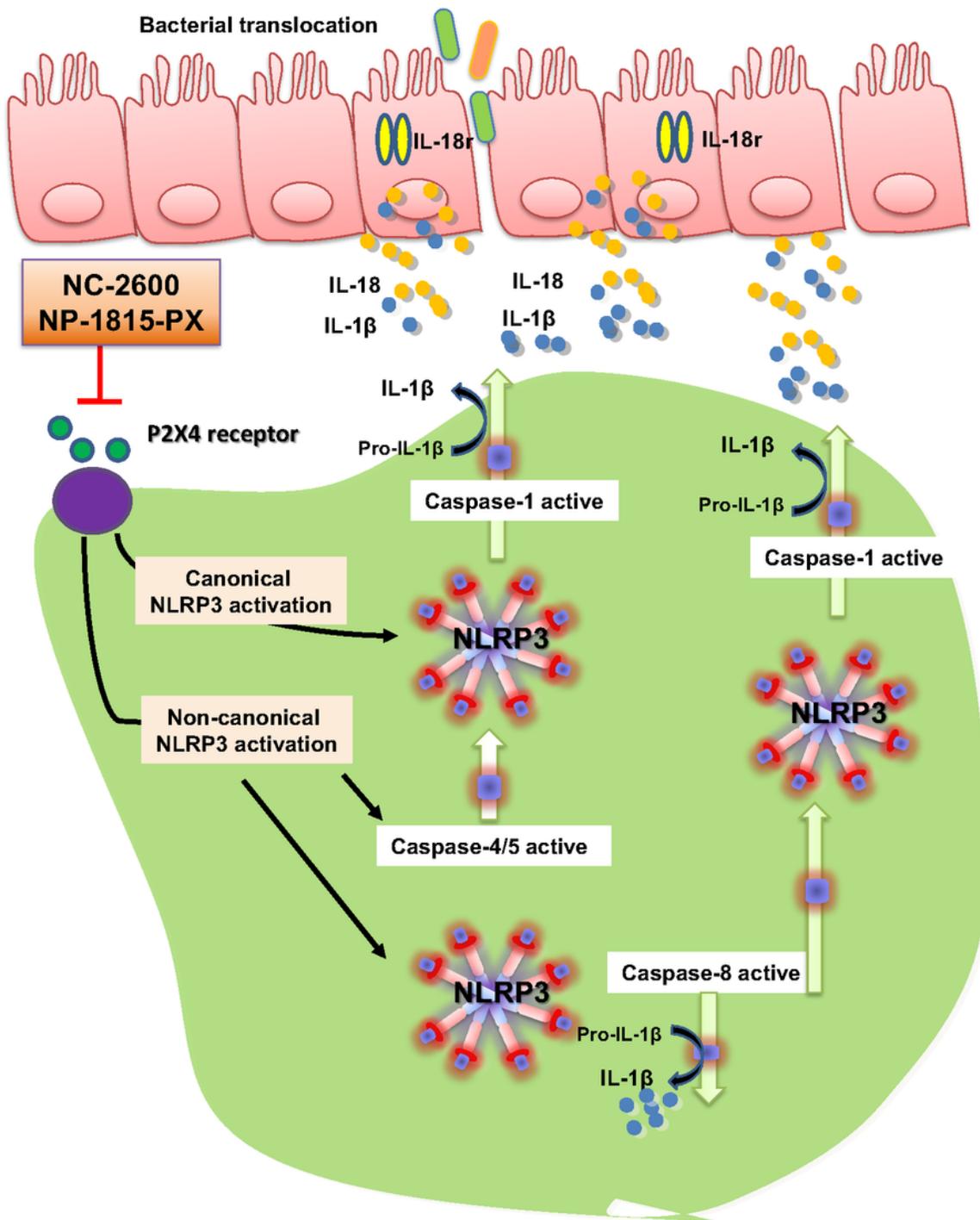


Figure 7

Diagram showing the molecular mechanisms through which NC-2600 and NP-1815-PX inhibit NLRP3 inflammasome signaling and counteract intestinal inflammation. In the chronic phase of inflammation, canonical and non-canonical pathways of NLRP3 inflammasome are associated with an increase in IL-1 β and IL-18 release that are harmful to the host. In this setting, NC-2600 and NP-1815-PX are able to

counteract the increase of IL-1 β release through the inhibition of canonical (NLRP3/Caspase-1/IL-1 β) and non-canonical (NLRP3/Caspase-4-5-8/IL-1 β) pathways of NLRP3 inflammasome.

Abbreviations: IL-1 β , interleukin-1 β ; IL-18, interleukin-18; IL-18r, interleukin-18 receptor; NLRP3, nucleotide-binding oligomerization domain leucine rich repeat and pyrin domain containing protein 3; pro-IL-1 β , pro-interleukin-1 β .