The standardized herbal combination BNO 2103 contained in Canephron N alleviates inflammatory pain in experimental cystitis and prostatitis

Bernhard Nausch[a,*], Simona Pace[b], Helmut Pein[b], Andreas Koeberle[b], Antonietta Rossi[c], Gerald Künstle[a], Oliver Werz[b,*]

[a] Bionorica SE, Kerschensteinerstrasse 11-15, 92318 Neumarkt, Germany

[b] Department of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University Jena, Philosophenweg 14, 07743 Jena, Germany

[c] Department of Pharmacy, School of Medicine, University of Naples Federico II, Via D. Montesano 49, I-80131 Naples, Italy

[*] Corresponding authors: Dr. Bernhard Nausch, Bionorica SE, Kerschensteinerstrasse 11-15, 92318 Neumarkt, Germany. Tel.: +49 (0) 9181 231-210; Fax: +49 (0) 9181 231-6210. E-mail address: Bernhard.Nausch@bionorica.de

Prof. Oliver Werz, Department of Pharmaceutical/Medicinal Chemistry, Institute of Pharmacy, Friedrich-Schiller-University Jena, Philosophenweg 14, 07743 Jena, Germany. Tel.: +49 (0) 3641-949801; Fax.: +49 (0) 3641-949802. E-mail address: Oliver.werz@uni-jena.de.

Abstract

Background: Urinary tract infections are among the most common types of infections and give rise to inflammation with pain as one of the main symptoms. The herbal medicinal product Canephron® N contains BNO 2103, a defined mixture of pulverized rosemary leaves, centaury herb, and lovage root, and has been used in the treatment of urinary tract infections for more than 25 years.

Purpose: To test the hypothesis that BNO 2103 reduces pain in cystitis and prostatitis by virtue of anti-inflammatory properties, and to reveal potential mechanisms underlying the anti-inflammatory features.

Study Design: BNO 2103 was studied for anti-inflammatory and analgesic properties in three animal models *in vivo*, and the mode of action underlying the anti-inflammatory features was investigated in human leukocytes and cell-free assays *in vitro*.

Methods: To assess the anti-inflammatory and analgesic efficacy of BNO 2103 we employed cyclophosphamide-induced cystitis and carrageenan-induced prostatitis in rats, and zymosan-induced peritonitis in mice. Human neutrophils and monocytes as well as isolated human 5-lipoxygenase and microsomal prostaglandin E_2 synthase-1-containing microsomes were utilized to assess inhibition of leukotriene and/or prostaglandin E_2 production by HPLC and/or ELISA.

Results: When given orally, BNO 2103 reduced inflammation and hyperalgesia in experimental cystitis in rats, while individual components of BNO 2103 also reduced hyperalgesia. Furthermore, BNO 2103 reduced hyperalgesia in rats with carrageenan-induced prostatitis. Cell-based and cell-free studies implicate inhibition of prostaglandin E_2 and leukotriene B_4 biosynthesis as potential mechanisms underlying the analgesic and anti-inflammatory effects.

Conclusion: Our data support the hypothesis that BNO 2103 reduces pain by virtue of its anti-inflammatory properties, possibly related to suppression of prostaglandin E_2 and leukotriene B_4 formation, and suggest that this combination has the potential to treat clinical symptoms such as inflammatory pain. Thus BNO 2103 may represent an alternative to reduce the use of antibiotics in urinary tract infections.

Keywords

Uncomplicated urinary tract infection; inflammation; pain; prostaglandin E_2; leukotriene; antibiotic resistance

Abbreviations

5-H(P)ETE, 5-hydro(pero)xyeicosatetraenoic acid; 5-LO, 5-lipoxygenase; ANOVA, analysis of variance; AUC, area under the curve; COX, cyclooxygenase; cycP, cyclophosphamide; H&E, hematoxylin & eosin; (RP-) HPLC, (reversed phase) high performance liquid chromatography; IL, interleukin; LPS, lipopolysaccharide; LT, leukotriene; mPGES-1, microsomal prostaglandin E_2 synthase-1; PBS, phosphate-buffered saline; PBMC, peripheral blood mononuclear cells; PG, prostaglandin; UTI, urinary tract infection.

Introduction

Urinary tract infections (UTIs) are among the most common bacterial infections, affecting 150 million people each year worldwide (Flores-Mireles et al., 2015; Geerlings, 2016). While women are preferentially at risk for UTI, with 50-60% of women experiencing at least one UTI in their lifetime, UTIs can affect men and women at all ages causing significant morbidity in infants, older men, and females at all ages (Flores-Mireles et al., 2015). Clinically, UTIs are considered uncomplicated in otherwise healthy individuals with no structural or neurological urinary tract abnormalities. While a variety of microorganisms can cause UTIs, infections with *E. coli* are most common (Asadi Karam et al., 2019).

The most bothersome symptom of acute uncomplicated UTIs is pain, which is part of inflammation associated with the immune response (Kidd and Urban, 2001). Depending on the site, these can be urethritis, cystitis, pyelonephritis, or prostatitis in males. For uncomplicated UTIs, treatment with antibiotics is recommended (Grabe et al., 2015). However, recent guidelines indicate non-antibiotic treatment (e.g. non-steroidal anti-inflammatory drugs, NSAIDs) as option for patients with mild to moderate symptoms in order to avoid fostering antibiotic resistance development (AWMF, 2017).

Canephron® N has a long-standing use for the treatment and prophylaxis of uncomplicated UTIs and of urinary stones (Naber, 2013). It is an herbal medicine containing BNO 2103, a standardized mixture of pulverized rosemary leaves, centaury herb, and lovage root. BNO 2103 is proposed as an attractive alternative to antibiotics due to its efficacy in the

treatment of pain and inflammation associated with UTIs, its good safety and tolerability (Naber, 2013), and its intrinsic ability to avoid problems associated with bacterial antibiotic resistance.

To test the hypothesis that BNO 2103 reduces pain by suppressing inflammation, we employed cyclophosphamide (cycP)-induced cystitis in female rats and carrageenan-induced prostatitis in male rats. In addition, we investigated biosynthesis of prostaglandin E_2 (PGE_2) and leukotrienes (LTs), which promote inflammation and inflammatory pain, as possible points of attack.

Materials and methods

Test item

Canephron® N contains BNO 2103, a 1:1:1 (w:w:w) mixture of pulverized rosemary leaves (*Rosmarinus officinalis* Linné), centaury herb (*Centaurium erythraea* Rafn), and lovage root (*Levisticum officinale* Koch) as active pharmaceutical ingredient. BNO 2103 was produced, controlled for quality, and provided by Bionorica SE (Neumarkt, Germany). An UPLC-based fingerprint (UV detection) at a wavelength of 205 nm of the aqueous ethanolic extract of BNO 2103 can be found as Supplemental material.

For *in vivo* experiments, BNO 2103 or individual herbal components were suspended in water for injection, ultrasonicated and administered orally. For *in vitro* experiments, BNO 2103 or individual herbal components were extracted with 50% ethanol (40 mg/ml) in an ultrasonic bath, and insoluble material was removed by centrifugation.

Comparison of animal and human dosages of BNO 2103 is based on body surface: 33 mg/kg for rats and 66 mg/kg for mice are equivalent to the recommended human daily dose of BNO 2103.

Animals

All procedures complied with applicable rules and provisions for ethical use of animals in research. Female (200-250 g) and male (300-325 g) Sprague-Dawley rats (Janvier Labs,

Saint-Berthevin, France) were housed in groups of 2 to 4 animals (12 hour dark and light cycle, 22°C, food and water *ad libitum*) for at least 3 days prior to experiments for acclimatization. All experimental procedures were in accordance with the European Community Council Directive 2010/63/UE or 86/609/EEC and the French Ministry for Agriculture, Agrifood and Forestry (Decree 2013-118), procedures were reviewed by CEEA-122 Ethical Committee for Protection of Animals used for Scientific Purposes and approved by the French Ministry for National Education, Higher Education and Research.

Male CD-1 mice (33–39 g, 8-9 weeks, Charles River Laboratories, Calco, Italy) were housed in a controlled environment (21±2°C) and provided with standard rodent chow and water *ad libitum*. Prior to experiments, mice were allowed to acclimate for 5 days and were kept at 12 h light–dark schedule; experiments were performed during the light phase. Animal care was in compliance with Italian regulations on protection of animals used for experimental and other scientific purpose (Ministerial Decree 116/92) and with the European Economic Community regulations (Official Journal of E.C. L 358/1 12/18/1986). Animal studies were approved by the ethical committee of the University of Naples Federico II (approval number 2014/18760).

At the end of experiments animals were sacrificed by pentobarbital overdose (experimental prostatitis), cervical dislocation following pentobarbital (experimental cystitis) or in a saturated CO_2 atmosphere (experimental peritonitis).

Dosing for in vivo experiments

The application route for *in vivo* experiments was *per os (p.o.)*. The range of doses applied corresponds to approximately 0.4 to 40 times the recommended human daily oral dose, given as mg drug/kg body weight (mg/kg).

Experimental cystitis

The model of experimental cystitis is described in detail by Augé et al. (Auge et al., 2013). BNO 2103 was given *p.o.* twice a day for three days before and once on the day of induction of cystitis (6.6, 66, or 666 mg/kg, equivalent to 0.4, 4, or 40 times the recommended

human daily dose). Animals in the vehicle group received saline. For experiments with individual herbal components, treatments (including BNO 2103) were given once prior to induction of cystitis at a dose of 66 mg/kg. Experimental cystitis was induced in female Sprague-Dawley rats using a single intraperitoneal injection of cycP (150 mg/kg). Sham rats received saline instead of cycP.

Nociceptive parameters were determined as described below using the response to von Frey filaments of increasing strength (1, 2, 4, 6, 8, 10, 15 and 26 g), applied to the lower abdomen 2 h after induction of cystitis. Ibuprofen (100 mg/kg, *p.o.*) was used as positive control.

Experimental prostatitis

BNO 2103 was given *p.o.* twice per day for two days before, and once at the day of induction of prostatitis (666 mg/kg, equivalent to 40 times the recommended human daily dose). Animals of the vehicle group received saline. Following pretreatment, adult male Sprague-Dawley rats were anesthetized with isoflurane and the ventral lobes of the prostate were exposed surgically. Then, carrageenan (30 mg/ml) was injected into the prostate; sham rats received physiological saline. Subsequently, the muscle and skin layers were closed and nociceptive parameters were determined 24 hours after induction of prostatitis using von Frey filaments of increasing strength (0.16, 0.4, 0.6, 1, 1.4 and 2 g) applied in the scrotal area. Ibuprofen (100 mg/kg, *p.o.*) was used as positive control.

Nociceptive parameters

The response to a given von Frey filament strength was scored on a scale from 0 to 3 (no reaction: 0, reaction of the animal (*e.g.* retraction of the abdomen/testes): 1, reaction of the animal and change of position/jump: 2, reaction of the animal, change of position/jump and licking of the stimulated site and/or vocalization: 3).

The overall nociceptive score is given as sum of 3 applications of the same strength filament; area under the curve (AUC) was calculated to allow for comparison of different treatments.

Histology

Following cystitis experiments, rats were euthanized and bladders were inflated with 4% formalin solution *in situ*, excised, placed in 4% formaldehyde, and stored in 70% ethanol at 4°C. Slices of approximately 4 µm thickness were cut after embedding in paraffin and the following parameters were scored in hematoxylin & eosin (H&E)-stained slices: urothelial hyperplasia, urothelial erosion, hemorrhage, inflammatory infiltrate (poly- and mononuclear cells), congestion (excess of blood in vessels), and edema. Each parameter was scored between 0 and 4 and the scores of the six individual parameters were added to yield an overall inflammation score.

Prostaglandin E_2 and LTB_4 release in vivo

PGE_2 and LTB_4 release was measured in experimental peritonitis, a well-established general model of inflammation. Peritonitis was induced in adult male CD-1 mice by intraperitoneal injection of zymosan (0.5 ml/mouse, 2 mg/ml in saline, boiled and washed; Sigma, Milan, Italy). Vehicle (water for injection), BNO 2103 (13 mg/kg, 133 mg/kg, 1333 mg/kg) and the positive control indomethacin (10 mg/kg) were administered orally 1 hour before peritonitis induction. Animals were sacrificed 4 hours after zymosan injection, peritoneal exudates were collected using 2 ml phosphate-buffered saline (PBS) and centrifuged (4° C, 20,000×g, 20 min). PGE_2 and LTB_4 were measured in supernatants using enzyme immunoassay (Cayman Chemical and Biotrend).

Preparation of human monocytes

Human monocytes were isolated from freshly withdrawn peripheral blood of healthy adult donors with written informed consent who had not taken anti-inflammatory drugs for the last 10 days (University Hospital Jena, Germany) as described (Schaible et al., 2013). For analysis of PGE_2 biosynthesis, peripheral blood mononuclear cells (PBMC) were isolated using Accuspin® tubes (Sigma) following the manufacturer's instructions.

Prostaglandin E_2 biosynthesis in vitro

Freshly isolated human PBMCs were incubated with test items for 15 minutes and then stimulated with lipopolysaccharide (LPS, 1 µg/ml, 18 h, 37°C, 5% CO_2). After centrifugation, PGE_2 was analyzed in the supernatant by enzyme immunoassay (Enzo Life Sciences GmbH, Lörrach, Germany) according to manufacturer's instructions.

The activities of cyclooxygenase (COX)-2 and microsomal prostaglandin E_2 synthase (mPGES)-1 in cell-free assays were investigated using recombinant human COX-2 and microsomal fractions of human A549 cells containing mPGES-1 as described (Koeberle et al., 2008).

5-Lipoxygenase (5-LO) product formation in vitro

Human monocytes (2×10^6 cells/ml) were resuspended in PBS (pH 7.4, 1 mg/ml glucose, 1 mM $CaCl_2$) and incubated for 15 minutes at 37°C with test items, and then stimulated with Ca^{2+}-ionophore A23187 (5 µM) at 37°C. After 10 minutes the reaction was stopped on ice and samples were centrifuged (500×g, 10 minutes, 4°C). Then, 750 µL supernatant were mixed with 750 µl methanol, and 22.5 µl of 1 N HCl, 150 ng PGB_1, and 375 µL of PBS were added. 5-LO products (LTB_4, *trans* isomers of LTB_4, 5-hydro(pero)xyeicosatetraenoic acid (5-H(p)ETE)) were extracted and analyzed by HPLC as previously described (Schaible et al., 2013). Analysis of the test compounds to inhibit the activity of human recombinant 5-LO was performed as described before (Schaible et al., 2013).

Data and statistics

Data are presented as mean ± standard error of the mean (SEM). Graphpad Prism 7 (Graphpad Inc., La Jolla, USA) was used for statistical analysis: nonlinear fit (log(inhibitor) *vs.* normalized response) to determine absolute IC_{50} values, One-way ANOVA (followed by Dunnett's multiple comparisons test) to compare three or more groups, or Kruskal-Wallis test (followed by Dunn's multiple comparisons test) to compare scoring data (inflammation *in vivo*). Statistical significance was considered at $p<0.05$.

Results

BNO 2103 reduces nociception in experimental cystitis

Since pain is a prominent symptom of cystitis, we studied whether BNO 2103 could reduce pain in cycP-induced cystitis in female rats. CycP-treated animals displayed pronounced hyperalgesia as evidenced by reduced nociceptive threshold (4.1 ± 0.60 g) compared to sham animals (16.4 ± 2.6 g). Animals pretreated with BNO 2103 exhibited a dose-dependent reduction in nociceptive responses (Fig. 1A,B). For comparison, the area under the curve (AUC) was calculated for each treatment (Fig. 1B): 79.5 ± 6.04 (vehicle), 50.0 ± 3.92 (6.6 mg/kg), 43.2 ± 2.39 (66 mg/kg), 37.7 ± 5.16 (666 mg/kg), and 28.7 ± 2.81 g × score (ibuprofen, positive control). When compared to the vehicle group, BNO 2103- and ibuprofen-treated groups showed significantly reduced AUC.

Since BNO 2103 contains rosemary leaves, lovage root, and centaury herb, we tested these individual components for their contribution to the observed anti-nociceptive effects. Animals treated with the single herbal components of BNO 2103 (66 mg/kg each) displayed lower nociceptive scores than the vehicle control group over the 1-26 g stimulus range (Fig. 1C,D). Compared to vehicle, BNO 2103, rosemary, and lovage groups significantly reduced AUC values, while for the centaury group the AUC was numerically lower than for the vehicle group, although without statistical significance. Among the three herbal components, lovage caused the most pronounced decrease in AUC, yet did not fully reach the low AUC value obtained with BNO 2103.

BNO 2103 reduces nociception in experimental prostatitis

24 hours after induction of prostatitis, carrageenan-injected animals exhibited significant hyperalgesia, i.e. lower nociceptive threshold than sham animals (0.54 ± 0.18 g and 1.18 ± 0.17 g, respectively). Treatment with 666 mg/kg BNO 2103 reduced nociceptive scores compared to the vehicle group (Fig. 1E). BNO 2103 and ibuprofen (reference drug) significantly reduced AUC values compared to vehicle-treated animals (Fig. 1F).

BNO 2103 suppresses inflammation in experimental cystitis

While cycP-induced cystitis may differ from bacterial UTI, both share a number of inflammatory signs, including edema, hyperemia, hemorrhage, and ulceration (Szigeti and Wheeler, 2014). Histological markers typical for cycP-induced cystitis are inflammatory infiltrate, edema, urothelial hyperplasia and erosion, hemorrhage, and congestion; these are absent in normal rat bladder (Fig. 2A,B). An inflammation score comprising these markers was determined in order to assess the severity of inflammation. BNO 2103 (66 mg/kg) significantly reduced inflammatory infiltrate (Fig. 2D), edema (Fig. 2E), and congestion (Fig. 2F) compared to vehicle-treated controls. The highest dose tested, *i.e.* 666 mg/kg, caused significant effects only on congestion (Fig. 2C). At 6.6 and 66 mg/kg, BNO 2103 significantly reduced the inflammation score (Fig. 2C). No statistically significant effects were observed on hemorrhage, urothelial erosion and hyperplasia (Fig. 2G-I). Interestingly, 100 mg/kg ibuprofen had no or only weak effects on inflammation parameters, except urothelial hyperplasia, and on the inflammation score, which was not significantly different from vehicle control.

BNO 2103 interferes with PGE_2 and LT biosynthesis

PGE_2, produced by the COX/PGES pathway, and LTs, produced by the 5-LO pathway, are well-known lipid mediators related to inflammation and pain (Funk, 2001). Therefore, we analyzed PGE_2 and LTB_4 biosynthesis as possible targets of BNO 2103 in zymosan-induced peritonitis. Oral treatment of mice with BNO 2103 decreased PGE_2 levels in peritoneal exudates (Fig. 3A). Indomethacin (10 mg/kg, positive control) caused a significant reduction of PGE_2 levels as expected.

To study modulation of PGE_2 biosynthesis *in vitro*, freshly isolated human PBMCs were stimulated with LPS to induce PGE_2 formation. BNO 2103, lovage and rosemary inhibited PGE_2 release: the effectiveness of lovage (IC_{50} = 100 µg/ml) was comparable to BNO 2103 (IC_{50} = 110 µg/ml), rosemary displayed higher potency (IC_{50} = 8.5 µg/ml) than lovage or BNO 2103, and centaury had no consistent inhibitory effect (Fig. 3B). Because COX-2 and mPGES-1 are the major enzymes contributing to inducible PGE_2, we analyzed whether

BNO 2103 inhibits human COX-2 and mPGES-1. In fact, experiments with human recombinant COX-2 failed to demonstrate inhibitory effects of BNO 2103 up to 300 µg/ml (data not shown). However, using microsomal preparations as source for mPGES-1 and 20 µM of PGH_2 as substrate, BNO 2103 and rosemary potently inhibited mPGES-1 activity (IC_{50} = 50 and 87 µg/ml), while inhibition by lovage or centaury was less pronounced (IC_{50} = 260 and 486 µg/ml) (Fig. 3C). The reference drug indomethacin (0.3 µM) reduced PGE_2 production in PBMC by approx. 90% and MK886 (10 µM) inhibited mPGES-1 activity by approx. 67%.

In zymosan-induced peritonitis in mice BNO 2103 did not reduce LTB_4 (Fig. 3D), but BNO 2103 and rosemary potently reduced the biosynthesis of 5-LO products in ionophore-stimulated monocytes (IC_{50} = 12 and 6 µg/ml, respectively), higher IC_{50} were observed for lovage (68 µg/ml) and centaury (168 µg/ml) (Fig. 3E). BNO 2103 and rosemary potently inhibited isolated human recombinant 5-LO (IC_{50}: 7 and 2 µg/ml), again higher IC_{50} were obtained for lovage or centaury (25 and 33 µg/ml) (Fig. 3F). The reference drug zileuton inhibited 5-LO product formation in monocytes by approx. 20% (at 2 µM) and isolated 5-LO by approx. 61% (at 0.56 µM).

Discussion

UTIs are among the most common types of infection (Geerlings, 2016) and the body responds with pronounced inflammation (Wu et al., 2017). Acute inflammation is initiated by the release of various mediators, *e.g.* PGs, LTs, and cytokines (Funk, 2001; Newton and Dixit, 2012). The cardinal signs of inflammation are redness, heat, and swelling, as well as pain and reduced function, and PGs and LTs markedly contribute to the development of these signs (Funk, 2001). For patients with lower UTIs, the most noticeable and bothersome symptoms are pain, dysuria, and reduced function, *i.e.* impaired urine storage and thus frequent urination (Geerlings, 2016; Pietrucha-Dilanchian and Hooton, 2016). Based on the hypothesis that BNO 2103 reduces pain by virtue of anti-inflammatory properties we confirmed the analgesic and anti-inflammatory potential of BNO 2103 in

experimental cystitis and prostatitis. Moreover, our data suggest that suppression of PGE_2 and LT biosynthesis may be potential underlying mechanisms of these anti-inflammatory properties, since BNO 2103 inhibited PGE_2 and LT formation *in vitro*, and reduced PGE_2 levels *in vivo.*

We show that BNO 2103 reduced pain in experimental cystitis, attributed mainly to lovage root and rosemary leaves. While centaury reduced AUC numerically, this reduction did not achieve statistical significance. In the context of infection, pain is a consequence of the inflammatory response, mediated by sensitization of nociceptors (Cook et al., 2018). Besides anti-nociceptive properties, we show reduction of inflammation by BNO 2103 in cycP-induced cystitis. The dose of 66 mg/kg in rats corresponds to 2-times the recommended human daily dose of BNO 2103, and this dose had a clear and significant inhibitory effect on inflammation, while a dose that corresponds to 20-times the human dose, i.e. 666 mg/kg, had only little or no effect. Such bell-shaped dose-response curves are not unusual for herbal products, because these contain many compounds with various and sometimes opposing effects with varying efficacies, potencies and dynamics. Surprisingly, ibuprofen (100 mg/kg) had no significant effect on the overall inflammation score and parameters such as inflammatory infiltrate and edema, suggesting that BNO 2103 acts via other pathways besides COX-1/2, the classical targets of ibuprofen. Because the same dose of ibuprofen effectively reduced hyperalgesia in the same animals, a lack of effect due to insufficient dosage seems unlikely.

PGE_2 is a well-known mediator of inflammation and pain (Koeberle and Werz, 2009; Schaible et al., 2011). It is massively produced by the concerted action of COX-2 and mPGES-1 at sites of inflammation (Koeberle and Werz, 2009) and contributes to pain by sensitizing nociceptors (Schaible et al., 2011). The classic NSAIDs like ibuprofen reduce pain by inhibiting COX-1/2-mediated PGE_2 production.

While BNO 2103 had no effect on COX-2, our data implicate mPGES-1 as possible target and its inhibition as mechanism underlying the impaired PGE_2 production in LPS-stimulated PBMCs. Interestingly, inhibition of PGE_2 formation by lovage and rosemary appears to be more potent in cell-based versus cell-free assay, indicating that besides mPGES-1

additional targets of these agents are conceivable. Elucidating such additional targets warrants further investigations. Irrespective of the exact mechanism, BNO 2103 reduced PGE_2 levels in zymosan-induced peritonitis, an experimental *in vivo* model of inflammation. Since PGE_2 has pro-inflammatory activity and induces hyperalgesia (Koeberle and Werz, 2009; Schaible et al., 2011), reduction of PGE_2 by BNO 2103 presents a potential mechanism underlying the analgesic and anti-inflammatory activity.

The discrepancies between the effects of BNO 2103 and ibuprofen on pain and inflammation in experimental cystitis indicates that BNO 2103 targets additional mechanisms than PGE_2 production. LTs play well-known and prominent roles in initiation and maintenance of inflammation by increasing vascular permeability and recruiting pro-inflammatory immune cells, particularly neutrophils (Lammermann et al., 2013; Martel-Pelletier et al., 2003), which contribute to tissue damage by generating oxidative stress (Wu et al., 2017). Our data reveal potent inhibition of 5-LO, the key enzyme in LT formation, and marked reduction of LTB_4 release from stimulated human monocytes by BNO 2103 as well as by the single constituents rosemary, lovage, and centaury.

Inhibition of 5-LO by BNO 2103 is more potent than inhibition of mPGES-1 (IC_{50} = 7 µg/ml compared to 87 µg/ml). Suppression of the chemotactic LTB_4 may coincide with impaired recruitment of immune cells (Lammermann et al., 2013) which was indeed observed in the *in vivo* histology analysis. This in turn could explain the occurrence of fewer PGE_2-producing immune cells at the site of inflammation and thus lower PGE_2 levels. However, since LTs were not reduced by BNO 2103 under the conditions of our experimental peritonitis model, inhibition of LT production *in vivo* by BNO 2103 remains to be shown. Insufficient oral bioavailability of constituents of BNO 2103 that target 5-LO *in vivo* might be an explanation. Alternatively, increased availability of arachidonic acid for 5-LO, due to inhibition of mPGES-1 and consequent substrate shunting, might counteract inhibitory effects of BNO 2103 on LT production *in vivo*.

Taken together, our results indicate that BNO 2103 has the potential to reduce inflammation and inflammatory pain in animal models of experimental cystitis and prostatitis. This anti-inflammatory effect could be mediated, at least in part, by reduction

of LTB_4 and PGE_2 as well as by impaired immune cell recruitment. Although pharmacological intervention with inflammation, which is part of the normal immune response to tissue damage or microbial infection, is a double-edged sword, the use of NSAIDs (*e.g.* ibuprofen) has been evaluated in recent clinical trials (Bleidorn et al., 2010; Bleidorn et al., 2016; Gagyor et al., 2015). Of interest, results of these trials suggest that symptomatic, anti-inflammatory treatment is sufficient for therapy of uncomplicated UTIs. Recently, Canephron® N, which contains BNO 2103, has been compared to the standard antibiotic treatment fosfomycin regarding the need for additional antibiotic treatment, and non-inferiority of Canephron® N has been shown (Wagenlehner et al., 2018). Notably, the most recent guidelines on UTIs include the option of symptomatic treatment as alternative to antibiotic therapy (AWMF, 2017). In practice, however, antibiotics are still the mainstay of therapy (Grabe et al., 2015). By affecting also the microflora, antibiotics have a high potential to cause adverse effects, such as GI disturbances (Ianiro et al., 2016), and excessive use of antibiotics contributes to the development of resistant bacteria. To prevent or at least retard future development of bacterial resistances, antibiotic stewardship is a bare necessity and symptomatic treatment option, such as NSAIDs or BNO 2103 offer valuable alternatives to the still overused antibiotics.

Conclusion

Our data highlight the efficacy of BNO 2103 in reducing hyperalgesia and inflammation in experimental inflammation models *in vivo* and *in vitro*. These results suggest that Canephron® N, which contains BNO 2103, has the potential to alleviate one of the most bothersome symptoms of UTIs, *i.e.* inflammatory pain. Thus, it presents a valuable alternative treatment option to antibiotics in uncomplicated cases of UTI and thereby facilitates antibiotic stewardship.

Acknowledgements

We thank Philippe Lluel and Endre Mikus for their experimental expertise; and Marietta Kaszkin-Bettag and Sean O'Shea for help with editing.

Funding

This work was supported by Bionorica SE.

Conflict of interest

BN and GK are employees of Bionorica SE; other authors declare no conflicts of interest.

Contributions of authors

BN, GK, OW planned and designed experiments; AK, SP, HP, AR conducted experiments, collected and analyzed data; BN, GK prepared figures; BN, GK, OW drafted and all authors reviewed, revised and approved the manuscript.

References

Asadi Karam, M.R., Habibi, M., Bouzari, S., 2019. Urinary tract infection: Pathogenicity, antibiotic resistance and development of effective vaccines against Uropathogenic Escherichia coli. Mol Immunol 108, 56-67.

Auge, C., Chene, G., Dubourdeau, M., Desoubzdanne, D., Corman, B., Palea, S., Lluel, P., Vergnolle, N., Coelho, A.M., 2013. Relevance of the cyclophosphamide-induced cystitis model for pharmacological studies targeting inflammation and pain of the bladder. Eur J Pharmacol 707, 32-40.

AWMF, 2017. Interdisziplinäre Leitlinie der Qualität S3 zur Epidemiologie, Diagnostik, Therapie, Prävention und zum Management unkomplizierter, bakterieller, ambulant erworbener Harnwegsinfektionen bei erwachsenen Patienten.

Bleidorn, J., Gagyor, I., Kochen, M.M., Wegscheider, K., Hummers-Pradier, E., 2010. Symptomatic treatment (ibuprofen) or antibiotics (ciprofloxacin) for uncomplicated urinary tract infection?--results of a randomized controlled pilot trial. BMC Med 8, 30.

Bleidorn, J., Hummers-Pradier, E., Schmiemann, G., Wiese, B., Gagyor, I., 2016. Recurrent urinary tract infections and complications after symptomatic versus

antibiotic treatment: follow-up of a randomised controlled trial. Ger Med Sci 14, Doc01.

Cook, A.D., Christensen, A.D., Tewari, D., McMahon, S.B., Hamilton, J.A., 2018. Immune Cytokines and Their Receptors in Inflammatory Pain. Trends Immunol 39, 240-255.

Flores-Mireles, A.L., Walker, J.N., Caparon, M., Hultgren, S.J., 2015. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. Nat Rev Microbiol 13, 269-284.

Funk, C.D., 2001. Prostaglandins and leukotrienes: advances in eicosanoid biology. Science 294, 1871-1875.

Gagyor, I., Bleidorn, J., Kochen, M.M., Schmiemann, G., Wegscheider, K., Hummers-Pradier, E., 2015. Ibuprofen versus fosfomycin for uncomplicated urinary tract infection in women: randomised controlled trial. BMJ 351, h6544.

Geerlings, S.E., 2016. Clinical Presentations and Epidemiology of Urinary Tract Infections. Microbiol Spectr 4.

Grabe, M., Bartoletti, R., Bjerklund Johansen, T.E., Cai, T., Çek, M., Köves, B., Naber, K.G., Pickard, R.S., Tenke, P., Wagenlehner, F., Wullt, B., 2015. Guidelines on Urological Infections. EAU Guideline.

Ianiro, G., Tilg, H., Gasbarrini, A., 2016. Antibiotics as deep modulators of gut microbiota: between good and evil. Gut 65, 1906-1915.

Kidd, B.L., Urban, L.A., 2001. Mechanisms of inflammatory pain. Br J Anaesth 87, 3-11.

Koeberle, A., Siemoneit, U., Buhring, U., Northoff, H., Laufer, S., Albrecht, W., Werz, O., 2008. Licofelone suppresses prostaglandin E2 formation by interference with the inducible microsomal prostaglandin E2 synthase-1. J Pharmacol Exp Ther 326, 975-982.

Koeberle, A., Werz, O., 2009. Inhibitors of the microsomal prostaglandin E(2) synthase-1 as alternative to non steroidal anti-inflammatory drugs (NSAIDs)--a critical review. Curr Med Chem 16, 4274-4296.

461 Lammermann, T., Afonso, P.V., Angermann, B.R., Wang, J.M., Kastenmuller, W., Parent,
462 C.A., Germain, R.N., 2013. Neutrophil swarms require LTB4 and integrins at sites of
463 cell death in vivo. Nature 498, 371-375.
464 Martel-Pelletier, J., Lajeunesse, D., Reboul, P., Pelletier, J.P., 2003. Therapeutic role of
465 dual inhibitors of 5-LOX and COX, selective and non-selective non-steroidal anti-
466 inflammatory drugs. Ann Rheum Dis 62, 501-509.
467 Naber, K.G., 2013. Efficacy and safety of the phytotherapeutic drug Canephron(R) N in
468 prevention and treatment of urogenital and gestational disease: review of clinical
469 experience in Eastern Europe and Central Asia. Res Rep Urol 5, 39-46.
470 Newton, K., Dixit, V.M., 2012. Signaling in innate immunity and inflammation. Cold
471 Spring Harb Perspect Biol 4.
472 Pietrucha-Dilanchian, P., Hooton, T.M., 2016. Diagnosis, Treatment, and Prevention of
473 Urinary Tract Infection. Microbiol Spectr 4.
474 Schaible, A.M., Traber, H., Temml, V., Noha, S.M., Filosa, R., Peduto, A., Weinigel, C.,
475 Barz, D., Schuster, D., Werz, O., 2013. Potent inhibition of human 5-lipoxygenase
476 and microsomal prostaglandin E(2) synthase-1 by the anti-carcinogenic and anti-
477 inflammatory agent embelin. Biochem Pharmacol 86, 476-486.
478 Schaible, H.G., Ebersberger, A., Natura, G., 2011. Update on peripheral mechanisms of
479 pain: beyond prostaglandins and cytokines. Arthritis Res Ther 13, 210.
480 Szigeti, R., Wheeler, T., 2014. Pathology of cystitis: overview, etiology, gross findings,
481 in: Cheng, L. (Ed.).
482 Wagenlehner, F.M., Abramov-Sommariva, D., Holler, M., Steindl, H., Naber, K.G., 2018.
483 Non-Antibiotic Herbal Therapy (BNO 1045) versus Antibiotic Therapy (Fosfomycin
484 Trometamol) for the Treatment of Acute Lower Uncomplicated Urinary Tract
485 Infections in Women: A Double-Blind, Parallel-Group, Randomized, Multicentre, Non-
486 Inferiority Phase III Trial. Urol Int, 1-10.
487 Wu, J., Miao, Y., Abraham, S.N., 2017. The multiple antibacterial activities of the bladder
488 epithelium. Ann Transl Med 5, 35.
489

Figure Legends

Figure 1: Effects of BNO 2103 on pain.

BNO 2103 reduced the nociceptive score in cycP-induced cystitis (A, B, n = 10 – 12 rats). The components of BNO 2103, rosemary, centaury, and lovage, reduced the nociceptive score at 66 mg/kg (C, n = 6 rats). For comparison, AUC was calculated and reveals significant reduction for BNO 2103, rosemary and lovage (D, n = 6 rats). BNO 2103 reduced the nociceptive score in carrageenan-induced prostatitis (E, n = 10 – 12 rats) and AUC reveals a significant reduction in AUC for BNO 2103 (F, n = 10 - 12). * $p<0.05$, ** $p< 0.01$, *** $p<0.001$ *vs.* vehicle, one-way ANOVA followed by Dunnett's multiple comparisons test, Ibu: Ibuprofen (100 mg/kg).

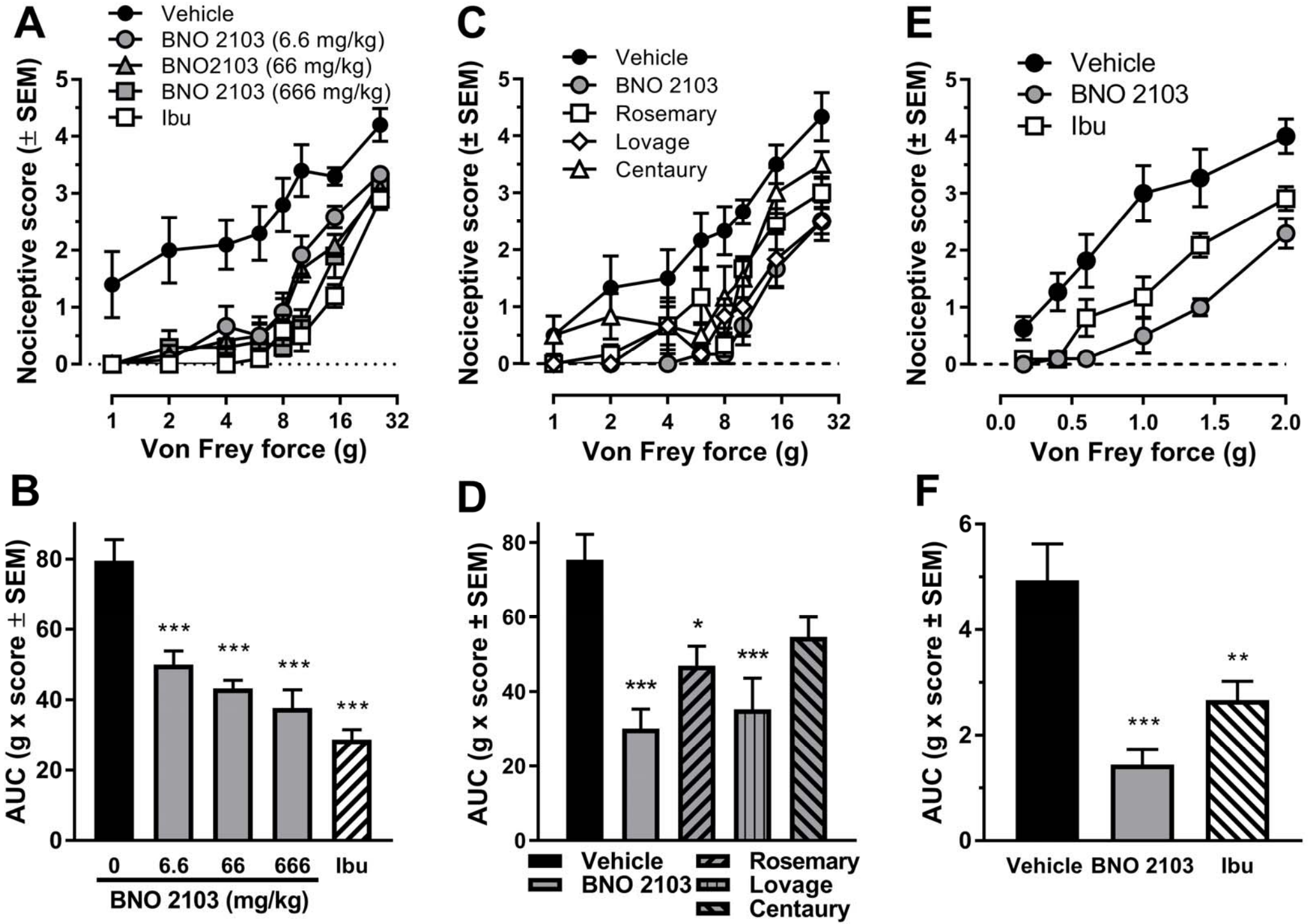
Figure 2: Effects of BNO 2103 on inflammation in cycP-induced cystitis.

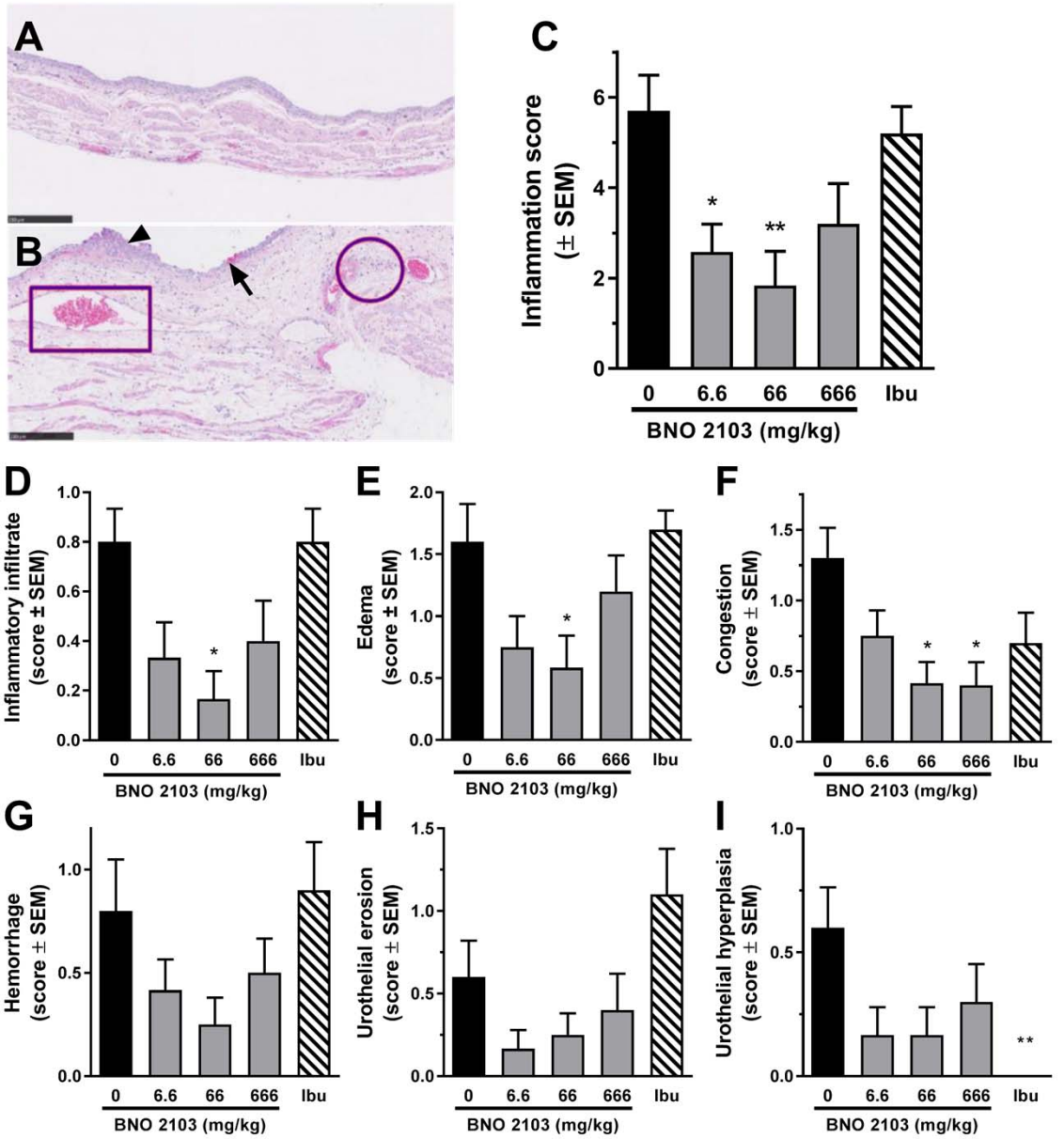
Bladder of BNO 2103-treated rat that was classified as normal (A). Bladder of vehicle-treated rat showing characteristics of cycP-induced inflammation: inflammatory infiltrate (circle), edema, urothelial hyperplasia (arrow head), hemorrhage (arrow), congestions (rectangle) (B). BNO 2103 reduced the overall inflammation score at 6.6 and 66 mg/kg (C). BNO 2103 reduced inflammatory infiltrate (D), edema (E), and congestion (F). No significant effects were observed for hemorrhage (G), urothelial erosion (H) and hyperplasia (I); significant effects of ibuprofen were observed only for urothelial hyperplasia (I). (n = 10 - 12). * $p<0.05$, ** $p< 0.01$ *vs.* vehicle, Kruskal-Wallis test followed by Dunn's multiple comparisons test, Ibu: Ibuprofen (100 mg/kg)

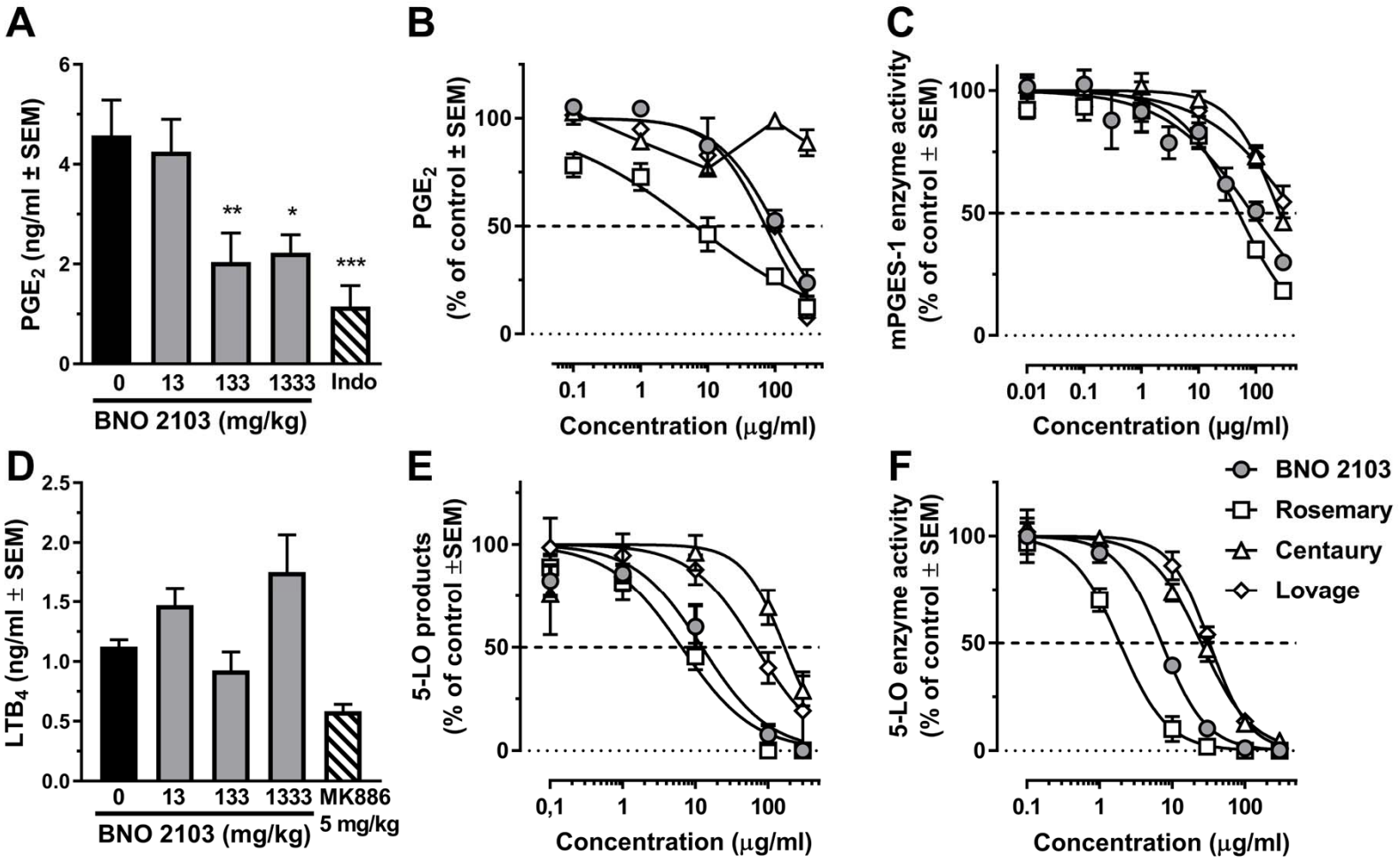
Figure 3: Effects of BNO 2103 on prostaglandin E_2 and leukotriene biosynthesis.

BNO 2103 lowered PGE_2 in the peritoneal exudate in zymosan-induced peritonitis in mice (A, n = 12, * $p<0.05$, ** $p< 0.01$, *** $p< 0.001$ *vs.* vehicle, one-way ANOVA followed by Dunnett‘s multiple comparisons test, indo: indomethacin 10 mg/kg) and inhibited PGE_2 production in LPS-stimulated human PBMCs with IC_{50} = 110 µg/ml (B, n = 3). PGE_2 production was also inhibited by rosemary and lovage with IC_{50} = 8.5 and 100 µg/ml (B, n = 3). BNO 2103, rosemary, centaury, and lovage inhibited mPGES-1 with IC_{50} = 87, 50,

260, and 486 (extrapolated) µg/ml (C, n = 3). While BNO 2103 did not reduce levels of LTB_4 in zymosan-induced peritonitis in mice (D), BNO 2103, rosemary, centaury and lovage reduced LTB_4 release from Ca^{2+}-ionophore-stimulated human monocytes with IC_{50} = 12, 6, 168, and 68 µg/ml (E, n = 3). Human isolated 5-LO was inhibited by BNO 2103 and by rosemary, centaury, and lovage with IC_{50} = 7, 2, 25, and 33 µg/ml (F, n = 3). Data are given as mean ± s.e.m.


A
Vehicle
BNO 2103 (6.6 mg/kg)
BNO2103 (66 mg/kg)
BNO 2103 (666 mg/kg)
Ibu
Nociceptive score (± SEM)
Von Frey force (g)
C
Vehicle
BNO 2103
Rosemary
Lovage
Centaury
Nociceptive score (± SEM)
Von Frey force (g)
E
Vehicle
BNO 2103
Ibu
Nociceptive score (± SEM)
Von Frey force (g)
B
AUC (g x score ± SEM)
0
6.6
66
666
Ibu
BNO 2103 (mg/kg)
D
AUC (g x score ± SEM)
Vehicle
BNO 2103
Rosemary
Lovage
Centaury
F
AUC (g x score ± SEM)
Vehicle
BNO 2103
Ibu


A
B
C
Inflammation score
(± SEM)
0
2
4
6
*
**
0
6.6
66
666
Ibu
BNO 2103 (mg/kg)
D
Inflammatory infiltrate
(score ± SEM)
0.0
0.2
0.4
0.6
0.8
1.0
*
0
6.6
66
666
Ibu
BNO 2103 (mg/kg)
E
Edema
(score ± SEM)
0.0
0.5
1.0
1.5
2.0
*
0
6.6
66
666
Ibu
BNO 2103 (mg/kg)
F
Congestion
(score ± SEM)
0.0
0.5
1.0
1.5
*
*
0
6.6
66
666
Ibu
BNO 2103 (mg/kg)
G
Hemorrhage
(score ± SEM)
0.0
0.5
1.0
0
6.6
66
666
Ibu
BNO 2103 (mg/kg)
H
Urothelial erosion
(score ± SEM)
0.0
0.5
1.0
1.5
0
6.6
66
666
Ibu
BNO 2103 (mg/kg)
I
Urothelial hyperplasia
(score ± SEM)
0.0
0.5
1.0
**
0
6.6
66
666
Ibu
BNO 2103 (mg/kg)


A
PGE2 (ng/ml ± SEM)
6
4
2
0
**
*
***
0
13
133
1333
Indo
BNO 2103 (mg/kg)
B
PGE2
(% of control ± SEM)
100
50
0
0.1
1
10
100
Concentration (µg/ml)
C
mPGES-1 enzyme activity
(% of control ± SEM)
100
50
0
0.01
0.1
1
10
100
Concentration (µg/ml)
D
LTB4 (ng/ml ± SEM)
2.5
2.0
1.5
1.0
0.5
0.0
0
13
133
1333
MK886
5 mg/kg
BNO 2103 (mg/kg)
E
5-LO products
(% of control ±SEM)
100
50
0
0,1
1
10
100
Concentration (µg/ml)
F
5-LO enzyme activity
(% of control ± SEM)
100
50
0
0.1
1
10
100
Concentration (µg/ml)
BNO 2103
Rosemary
Centaury
Lovage