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Anti-inflammatory effect of dual nociceptin and opioid receptor agonist, BU08070, in experimental colitis in mice

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Abstract

Endogenous opioid and nociceptin systems are widely distributed in the gastrointestinal tract where they seem to play a crucial role in maintaining homeostasis. The aim of our study was to assess whether activation of nociceptin (NOP) and μ -opioid (MOP) receptors by a mixed NOP/MOP receptor agonist, BU08070, induces anti-inflammatory response in experimental colitis.

The anti-inflammatory effect of BU08070 (1 mg/kg i.p.) was characterized in the mouse model of 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis, based on the assessment of the macroscopic and microscopic total damage scores and determination of MPO activity and TNF- α level in the colon. The effect of BU08070 on cell viability and NF- κ B was characterized in THP-1 Blue cell line. The antinociceptive activity of BU08070 was examined in mustard oil-induced mouse model of abdominal pain.

A potent anti-inflammatory effect of BU08070 (1 mg/kg i.p.) was observed as indicated by decrease in macroscopic damage score (1.88 ± 0.39 vs. 5.19 ± 0.43 units in TNBS alone treated mice), MPO activity (2.29 ± 0.37 vs. 9.64 ± 2.55 units) and TNF- α level in the colon (35.85 ± 2.45 vs. 49.79 ± 3.81 pg/ml). The anti-inflammatory effect of BU08070 was reversed by selective NOP and

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Disclosures

SH, JF designed the research study
MZ, TBH, GCK, MSa, AJ, JF, MP performed the research
MZ, TBH, GCK, MSa, AJ, RK, MSp, SH, JF analyzed the data
MZ, JF wrote the paper
SH, MSp critical revised the paper for important intellectual content

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MOP receptor antagonists. BU08070 produced concentration-dependent inhibition of TNF- α and LPS-induced NF- κ B activation. BU08070 exerted antinociceptive action in mice with experimental colitis.

In conclusion, BU08070 significantly reduced the severity of colitis in TNBS-treated mice compared with controls. These results suggest that BU08070 is a potential therapeutic agent for IBD therapy.

Keywords

Crohn's disease; inflammatory bowel diseases; mixed opioid agonists; visceral pain

1. Introduction

The endogenous nociceptin system is composed of nociceptin receptors (NOP) and nociceptin, and the endogenous opioid system consists of three types of receptors: μ (MOP), κ (KOP) and δ (DOP) and their endogenous ligands (Meunier et al., 1995; Reinscheid et al., 1995; Snyder et al., 1974). While both systems share structural and functional homology, the effects induced by nociceptin are not mediated by naloxone-sensitive pathways (Cremins et al., 2012). Nociceptin and opioid receptors are widely distributed in the central and peripheral nervous system, but also in peripheral tissues, among others in the gastrointestinal (GI) tract, on muscle, nerve and immune cells (Mani and Moore, 2009; Osinski et al., 1999). In the GI tract, NOP receptors participate in the maintenance of homeostasis by affecting secretion and motility (Sobczak et al., 2013). Opioid receptors, besides a well-established role in pain modulation are also known to be involved in the modulation of GI functions by impairing peristalsis, decreasing epithelial secretion, and promoting fluid and electrolyte absorption (Lang et al., 1996; Malarchik et al., 1988). Of all three opioid receptor types, MOP receptors seem to play the most important role in the GI tract and constitute the major target for immune modulation (Holzer, 2009; Sharp, 2006). Chronic administration of opioids triggers several adverse effects, including physical dependence, tolerance development, respiratory depression, euphoria, nausea and constipation (Martin and Forrester, 2013; Porreca and Ossipov, 2009; Wiffen et al., 2014). In contrast, NOP receptor activation attenuates MOP receptor-mediated reward and tolerance development (Khroyan et al., 2007). Taken together, mixed NOP/MOP receptor ligands may induce MOP receptor-mediated antinociceptive action with reduced rewarding properties. The mechanisms underlying the cross-talk between the opioid and nociceptin systems are still poorly understood and currently the design of successful candidates based on this concept is critically hampered.

Crohn's disease belongs to a group of inflammatory bowel diseases (IBD) (Actis et al., 2014). IBD are chronic relapsing ailments of the GI tract with numerous extraintestinal manifestations, affecting more than 1 million people in the United States alone and several million worldwide (Kaistha and Levine, 2014). The etiology of IBD is multifactorial, what makes it relatively difficult to develop efficient therapies. Throughout the years, many drugs have been approved by FDA and EMA for the management and treatment of IBD, including corticosteroids, antibiotics and biological therapy (for review see: (Sands et al., 2015 and

Amiot and Peyrin-Biroulet, 2015). However, developing novel efficient therapeutics, which would induce and maintain clinical remission for long term, is still a common interest for both, pharmacologists and patients.

The interactions between nociceptin and opioid receptors indicate that molecules with mixed nociceptin and opioid activity could be more effective as drugs compared with “classical” ligands acting at a single target (Khroyan et al., 2011). In our previous studies we showed that an analogue of buprenorphine, BU08070, displayed higher binding affinity to MOP and NOP receptors in comparison with parent compound. BU08070 was active in the GI tract and exerted antinociceptive effect in the mouse models of visceral pain (Cami-Kobeci et al., 2011; Sobczak et al., 2014). The aim of our study was to assess the effect of BU08070, a buprenorphine analogue with dual activity at NOP and MOP receptors, on the severity of intestinal inflammation in the mouse model of TNBS-induced colitis and to determine its mechanism of action.

2. Material and methods

2.1. Animals

Male Balb/C mice (Animal Facility of Nofer Institute of Occupational Medicine, Lodz, Poland), weighing 22–24 g, were used for the study. The animals were housed in a room controlled for temperature (22 ± 1 °C) and humidity ($70\pm5\%$) and light/dark cycles conditions (12/12 h). Animals were group-housed in sawdust coated transparent cages and had a free access to chow and tap water. All procedures were approved by the Local Ethical Committee for Animal Research with the following number: #589/2013.

2.2. Drugs and pharmacological treatment

BU08070 was synthesized at the Department of Pharmacy and Pharmacology, University of Bath, Bath, United Kingdom as described previously (Cami-Kobeci et al., 2011). J-113397 and β -funaltrexamine (β -FNA) were purchased from Tocris Bioscience (Ellisville, MO, USA). Tumour necrosis factor- α (TNF- α) and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Zeocin was obtained from Invivogen (San Diego, CA, USA). Cell culture media and supplements were from Sigma-Aldrich Chemicals (St. Louis, MO, USA) or Invitrogen (Paisley, UK). All other reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Poznań, Poland).

For animal studies, BU08070 was dissolved in dimethyl sulfoxide (DMSO), further diluted with 0.9% NaCl solution to the final concentration of 5% DMSO. Animals without treatment received vehicle alone. BU08070 was injected intraperitoneally (i.p.) at the dose of 1 mg/kg in the final volume 100 μ l. The dose of BU08070 used in all experiments was selected based on preliminary studies. β -FNA and J-113397 were injected i.p. (at the dose of 1 mg/kg and 12 mg/kg, respectively) 15 min before BU08070.

2.3. Induction of colitis and assessment of colonic damage

In the mouse model of Crohn's disease, colitis was induced by intracolonic (i.c.) instillation of trinitrobenzenesulfonic acid (TNBS), as described previously (Salaga et al., 2014). TNBS

causes ulcerations, marked thickening of the intestinal wall and mucosal and submucosal infiltration by immune cells, including polymorphonuclear leukocytes, macrophages and lymphocytes (Ballinger, 2002).

Briefly, mice (n=6–8 per group) were anesthetized with 1% isoflurane (Aerrane, Baxter, Deerfield, USA). TNBS at the dose of 4 mg in 30% ethanol in 0.9% NaCl (in the final volume of 100 µl) was slowly administered into the distal colon using a catheter inserted 2.0 cm proximally to the anus. Mice were weighted daily and observed for clinical symptoms of colonic inflammation, including diarrhea and bloody stools.

Mice were killed by cervical dislocation on day 4. The colon with caecum was immediately removed, opened longitudinally, washed with phosphate buffered saline (PBS) and examined. The total macroscopic colonic damage was assessed using established scoring system (Fichna, Diczaj et al., 2012). The total macroscopic score was defined as a sum of the following parameters: ulcer area (for ulcer score 0.5 points for each 0.5 cm), colonic shortening (0–2), the adhesion (0–2), the wall thickness measured in mm, the presence of diarrhea, hemorrhage and fecal blood (increased the score by 1 point for each additional feature).

2.4. Histology

The sections of the distal colon were stapled flat, mucosal side-up, onto cardboard strips and fixed in Zamboni fixative for at least 24 h at 4°C. Sections were dehydrated, embedded in paraffin, sectioned at 5 µm and mounted onto slides and stained with hematoxylin and eosin. The slides were blinded and examined using Motic AE31 microscope (Ted Pella, Sweden). Photographs were taken using a digital imaging system consisting of a digital camera (Moticam 2300, Ted Pella, Sweden) and image analysis software (Motic Images Plus 2.0, Germany).

The microscopic total damage score was determined using established scoring system (Fichna et al., 2012) and following parameters were assessed: the extent of muscle thickening and the presence and degree of cellular infiltration (normal=1, moderate=2, transmural=3) and the destruction of mucosal architecture (normal =1, moderate=2, extensive= 3), the goblet cell depletion and crypt abscesses (presence=1, absence=0).

2.5. Determination of tissue myeloperoxidase (MPO) activity

The colon sections (20–30 mg) were isolated from mice and immediately washed with PBS and homogenized in hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM potassium phosphate buffer, pH 6.0; 50 mg of tissue/ml) using Ika Ultra Turrax Disperser T25 Digital 2 (Sigma Aldrich, Poznań, Poland). Then, homogenates were centrifuged (15 min, 13–200g, 4°C) and the supernatants were transferred to test tubes. Seven µl of supernatant was added on a 96-well plate, followed by 200 µl of 50 mM potassium phosphate buffer (pH 6.0), containing 0.167 mg/ml of O-dianisidine hydrochloride and 0.05 µl of 1% H₂O₂. The absorbance was measured at 450 nm after 30 and 60 sec (iMARK Microplate Reader, Biorad, United Kingdom). All measurements were performed in triplicate.

MPO activity was expressed in milliunits per gram of wet tissue, 1 unit being the quantity of enzyme, which was able to convert 1 μmol hydrogen peroxide to water in 1 min at room temperature. MPO activity units were calculated from a standard curve using purified peroxidase enzyme.

2.6. Determination of TNF- α level in the colon

The sections of the colon (15–20 mg) were isolated, washed with PBS and kept at -80°C until further analysis. The tissue homogenates were prepared using Mammalian Cell Lysis Kit – MCL1 according to manufacturer's protocol (MCL1-1KT, Sigma Aldrich, Poznań, Poland). The levels of TNF- α in the colon were measured with a commercially available enzyme-linked immunosorbent assay (ELISA) kit that specifically detects TNF- α in mouse material (Quantikine TNF-alpha, R&D Systems, USA) according to the manufacturer's instructions. The absorbance was measured at 450 nm. The level of TNF- α was calculated from a standard curve and values were expressed as pg/ml.

2.7. Cell culture

The human myelomonocytic THP1-Blue cell line (Invivogen, San Diego, CA, USA) was kindly provided by Dr. F. Überall (Biocenter, Innsbruck Medical University, Austria).

THP1-Blue cells are stably transfected THP-1 cells containing a nuclear factor- κB (NF- κB)/activator protein 1 (AP-1) inducible secreted embryonic alkaline phosphatase (SEAP) reporter construct (Invivogen, San Diego, CA, USA). THP1-Blue cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 200 $\mu\text{g/ml}$ zeocin. Cell cultures were maintained at 37°C in 5% CO_2 humidified air.

2.8. Measurement of NF- κB activity and cells viability in THP1-Blue Cells

THP1-Blue cells were seeded in 96-well plates at a density of 100 000 cells per well, and were incubated with BU08070 (5–25 μM) or vehicle for 30 min at 37°C and 5% CO_2 , before the addition of TNF- α (10 ng/ml) or LPS (1 $\mu\text{g/ml}$). Vehicle treated TNF- α or LPS stimulated cells and non-stimulated cells were used as controls. After 24 h incubation, 10% (v/v) of cell supernatant was mixed with 90% (v/v) of Quanti-Blue reagent, and absorbance was measured at 620 nm using a spectrophotometric plate reader (Thermo Electron Corporation, Multiskan EX., USA) (Schroecksnadel et al., 2010).

The effect of BU08070 (5–25 μM) on THP-1 Blue cells viability was assessed using the Cell-Titer BlueR (CTB) assay (Promega, Vienna, Austria), performed according to the manufacturer's instructions. The CTB viability assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin). THP-1 Blue cells were seeded in 96-well plates at a density of 100 000 cells per well, and were treated with BU08070 (5–25 μM) or vehicle for 24 h at 37°C and 5% CO_2 . CTB reagent was added to each well, plates were incubated for 3 h at 37°C and 5% CO_2 and the number of viable cells was quantified by measuring fluorescence using a fluorometer (Fluoroskan Ascent, Labsystems Inc., USA). All experiments were performed in triplicates and repeated at least three times.

2.9. Mustard-oil induced pain responses

To assess the antinociceptive effect of BU08070 in mice with acute colitis, intestinal inflammation was induced by TNBS instillation four days prior to proper experiment (Sobczak et al., 2014). On day 4, animals were divided into two groups: BU08070-treated and vehicle-treated. BU08070 or vehicle were injected i.p. and 15 min later behavioral response to pain, induced by the i.c. instillation of 1% mustard oil (MO, allyl isothiocyanate) in 70% EtOH in 0.9% NaCl, was measured. Briefly, mice were separated into clear plastic boxes (20x20x15 cm) and allowed a 5-minute recovery after MO administration. Spontaneous behaviors were then observed and counted for 20 min. The following behavioral pain responses were counted as 1: licking of the abdomen, stretching the abdomen, squashing of lower abdomen against the floor, and abdominal retraction.

2.10. Statistical analysis

The results are expressed as mean $\pm$ standard error of the mean (S.E.M.). Statistical analysis was performed using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). ANOVA followed by Newman-Keuls post-hoc testing was used for multiple comparisons. P values < 0.05 were considered statistically significant.

3. Results

3.1. BU08070 displays anti-inflammatory effect in experimental colitis

BU08070 injected i.p. at the dose of 1 mg/kg twice daily significantly reduced the severity of the intestinal inflammation, as indicated by macroscopic total damage score (1.88 ± 0.39 vs. 5.19 ± 0.43 units for TNBS-treated mice), Fig. 1A. The major indicator of neutrophil infiltration, the MPO activity level was decreased after BU08070 treatment (2.29 ± 0.37 vs. 9.64 ± 1.55 units for TNBS-treated mice), Fig. 1B. The ulceration was also attenuated in BU08070-treated group in comparison with TNBS-treated mice (1.00 ± 0.00 vs. 1.92 ± 0.08 units for TNBS-treated mice), Fig. 1C. The shortening of the colon occurs normally in TNBS-treated animals; BU08070 administration improved also this parameter (8.30 ± 0.61 vs. 7.33 ± 0.45 cm for TNBS-treated mice), Fig. 1D. Of note, mice treated with BU08070 regained their body weight faster than the TNBS-treated group, Fig. 1E.

3.2. Anti-inflammatory action of BU08070 is mediated by NOP and MOP receptors

In vitro BU08070 binds with high affinity to NOP and MOP receptors (Cami-Kobeci, Polgar, Khroyan, Toll, and Husbands, 2011). To assess whether BU08070 exerts anti-inflammatory effect through NOP and MOP receptors *in vivo*, we used respective selective antagonists: J-113397 (NOP receptor antagonist) and β -FNA (MOP receptor antagonist). Both antagonists given alone did not influence the observed parameters (data not shown).

The anti-inflammatory effect of BU08070 was reversed principally by the MOP receptor antagonist and – to a lesser degree – by the NOP receptor antagonist, as indicated by macroscopic total damage score: 2.58 ± 0.23 for BU08070-treated group vs. 6.37 ± 0.96 and 4.95 ± 0.90 for β -FNA- and J-113397-treated group, respectively, Fig. 2A. The MPO level was decreased after BU08070 treatment in comparison with TNBS-treated group (7.78 ± 2.61 vs. 13.85 ± 3.40); the co-administration of BU08070 with the MOP receptor antagonist

increased neutrophil infiltration (19.32 ± 9.32), while the NOP receptor antagonist had no effect (6.90 ± 2.86), Fig. 2B. The ulceration was improved after BU08070 administration and worsened in both antagonist-treated groups (1.08 ± 0.15 for BU08070 alone vs. 2.08 ± 0.15 and 2.25 ± 0.14 for J-113397 and β -FNA-treated group, respectively), Fig. 2C. The colon length was improved after BU08070 treatment in comparison with TNBS-treated group (9.25 ± 0.15 vs. 8.18 ± 0.38 , respectively). Both antagonists blocked the effect of BU08070 on the colon length (8.12 ± 0.15 and 7.35 ± 0.30 for J-113397 and β -FNA-treated group, respectively) Fig. 2D. Finally, J-113397 and β -FNA-treated group did not regain their body weight, Fig. 2E.

Microscopically, the induction of inflammation by TNBS was associated with a destruction of the intestinal wall (including architecture of mucosa and the presence of goblet cells and crypts), infiltration of immune cells and thickening of the muscle layer, as compared with naive animals (9.25 ± 0.31 vs. 3.50 ± 0.19). The administration of BU08070 at the dose of 1 mg/kg significantly improved the microscopic inflammation score (5.67 ± 0.25). The co-administration of BU08070 with NOP and MOP receptor antagonists aggravated microscopic score (9.00 ± 0.55 and 7.70 ± 0.66 for J-113397 and β -FNA-treated group, respectively), Fig. 3.

3.3. TNF- α level is decreased after BU08070 administration

TNF- α is one of the major pro-inflammatory cytokines released during colitis and its level is increased in the colon in TNBS-treated mice in comparison with naïve mice (49.67 ± 3.92 vs. 28.73 ± 3.54 pg/ml, respectively). The i.p. injection of BU08070 at the dose of 1 mg/kg twice daily over three days during TNBS-colitis significantly decreased the level of TNF- α in the colon (35.83 ± 2.45 pg/ml).

3.4. BU08070 inhibits NF- κ B in vitro

To investigate the possible mechanism involved in the anti-inflammatory action of BU08070 in experimental colitis, the effect of BU08070 on NF- κ B activity was assessed in vitro using THP1-Blue cells transfected with a NF- κ B/AP-1-inducible reporter system. Using TNF- α and LPS, inducers of NF- κ B, we found a concentration-dependent inhibition of NF- κ B activation by BU08070, Fig. 5A,B. In TNF- α -stimulated cells, a significant effect was noticed after exposure of cells to BU08070 at the concentration of 10 and 25 μ M; in LPS-stimulated cells, concentrations of 5, 10 and 25 μ M produced a considerable decrease in NF- κ B activity. BU08070 (5–25 μ M) did not affect NF- κ B activity in the absence of TNF- α or LPS stimulation, Fig. 5C, or THP1-Blue cell viability (data not shown).

3.5. Antinociceptive effect of BU08070 during acute colitis

Abdominal pain is one of the major symptoms occurring in IBD. Here, the anti-nociceptive action of BU08070 was assessed in the behavioral model of visceral pain evoked by i.c. instillation of mustard oil and performed in mice with TNBS-induced colitis. BU08070 at the dose of 1 mg/kg injected i.p. significantly decreased the number of pain-induced behaviors as compared to mice treated with TNBS alone (2 ± 1 vs. 65 ± 7 , respectively), Fig. 6.

4. Discussion

In this study, we observed that simultaneous activation of the opioid and nociceptin systems with a mixed NOP/MOP receptor agonist BU08070 produced anti-inflammatory response in mice with TNBS-induced colitis. This potent anti-inflammatory action of BU08070 was reversed by selective NOP and MOP receptor antagonists, confirming dual activity of the ligand *in vivo*.

It has been evidenced that the endogenous nociceptin system is implicated in the colitis development; however, because of high plasticity of this system currently available data are often conflicting. Kato et al. (Kato et al., 2005) showed that NOP receptor-deficient mice were more resistant to DSS-induced colitis than wild type controls, suggesting that the endogenous nociceptin system may play a derogatory role in inflammation. In line, Alt et al. (Alt et al., 2012) demonstrated that NOP receptor inhibition with a selective NOP receptor antagonist, SB612111, injected i.p. at the dose of 30 mg/kg caused anti-inflammatory effect in DSS-induced colitis in mice. The attenuation of disease progression was correlated with decreased production of pro-inflammatory cytokines in the colon, including IL-1 β , IL-6, and TNF- α . In contrast, in our earlier study we showed that SCH 221510, a selective NOP receptor agonist, induced anti-inflammatory response in TNBS-induced colitis in mice in a NOP receptor-reversible manner (Sobczak et al., 2014). We also observed a decrease in the relative NOP receptor mRNA expression in biopsies collected from IBD patients in comparison with healthy subjects. Accordingly, as shown by Petrella et al. (Petrella et al., 2013), repeated peripheral injection of nociceptin (at low doses) in rats significantly attenuated TNBS-induced colitis as indicated by decreased MPO activity and the improvement of microscopic inflammatory damage in the colon. Moreover, administration of UFP-101, the peptide antagonist selective against NOP receptor reversed the protective effect of nociceptin on intestinal inflammation, suggesting that the activation of peripheral NOP receptors participates in the anti-inflammatory action during development of TNBS-induced colitis.

The anti-inflammatory action of opioid ligands is more apparent and extensively reported. For example, DALDA and DAMGO, selective MOP receptor agonists, were shown to significantly attenuate experimental colitis and MOP receptor-deficient mice were more susceptible to TNBS-induced inflammation than wild type animals (Philippe, Dubuquoy et al., 2003). Furthermore, it was found that mRNA expression of MOP receptor was increased in biopsies collected from IBD patients (in inflamed areas) as compared to non-inflamed tissues in the same patients (Philippe et al., 2006). Finally, Saccini et al. (Saccini et al., 2012) showed that the activation of opioid receptors attenuated intestinal inflammation induced by mesenteric ischemia/reperfusion in mice. Namely, DAMGO decreased microscopic damage score in comparison with vehicle-treated group, reduced TNF- α expression at mRNA level in the colon, and decreased the MPO activity.

In our study we showed that BU08070 exerted anti-inflammatory activity, as it improved clinical symptoms, including loss of body weight, rectal bleeding and diarrhea. Similar anti-inflammatory effect was found for macroscopic and histological scores. Moreover, we observed a decrease in MPO activity after BU08070 treatment and this effect was mediated

principally by MOP receptors, but not NOP receptors. These findings elegantly support the concept that the activation of opioid and NOP receptors induces anti-inflammatory response during intestinal inflammation and are in line with our earlier observations for classical and non-classical NOP and MOP receptor agonists (Sobczak et al., 2014).

Finally, we observed that after BU08070 treatment TNF- α level was decreased in the colon. TNF- α is a pro-inflammatory and immunoregulatory molecule, which enhances leukocyte migration, promotes transcription of numerous inflammatory genes and induces apoptosis of intestinal epithelial cells (Nielsen et al, 2000). Anti-TNF- α antibodies, which neutralize TNF- α (both transmembrane and soluble form) constitute a large group of therapeutics. For example infliximab, a monoclonal antibody binds to TNF- α and prevents its action through a specific receptor and induction of pro-inflammatory molecular pathways. The development of anti-TNF- α antibodies was a significant progress in control of IBD; however, there is a large group of anti-TNF- α non-responders. Namely, up to 30% of IBD patients do not respond to anti-TNF- α antibodies (primary non-responders) and many patients lose the response over time (Allez et al., 2010; Ben-Horin et al., 2011). Furthermore, treatment with anti-TNF- α is often combined with occurrence of extraintestinal manifestations, abscess and fistula formation and other serious side effects (McLean et al., 2014). The design of a new class of anti-TNF- α agents, with higher efficiency and fewer side effects could be a breakthrough in IBD treatment. Whether BU08070 or derivatives become such an alternative, requires further studies on these compounds.

To further understand the mechanism of action of BU08070, we characterized its effect *in vitro* in the THP-1 Blue cell line upon stimulation with two robust activators of NF- κ B, TNF- α and LPS. Our crucial observation is that BU08070 concentration-dependently inhibited TNF- α - and LPS-induced NF- κ B activation, which is a key transcription factor involved in promoting the production of pro-inflammatory molecules, downstream inducible pro-inflammatory enzymes, including iNOS and COX-2 and exacerbates the intestinal inflammation by affecting the integrity of the colon mucosa (Schwartz et al., 1999; Richmond et al., 2002). This finding elegantly explains the molecular basis for the anti-inflammatory effect of BU08070 and is in good agreement with earlier studies. Namely, it was shown that morphine affects production of pro-inflammatory cytokines by macrophages after LPS induction in a NF- κ B dependent manner (Martucci et al., 2007; Roy et al., 1998). Moreover, nociceptin inhibited LPS-induced release of pro-inflammatory molecules, including IL-1 β , TNF- α and IL-6 from cultured rat astrocytes (Fu et al., 2007), and modulates NF- κ B activity in human neuroblastoma cells (Donica et al., 2011).

Abdominal pain is one of the primary symptoms in 50–70% IBD patients. During inflammation, cytokines and chemokines, such as IL-1 β and TNF- α , are released and affect visceral afferent function. It has been clearly evidenced that MOP receptor agonists produce antinociceptive effect; however, the role of NOP receptor in visceral pain signaling remains ambiguous. For example, in a rat model of post-inflammatory colitis, activation of NOP receptor using nociceptin (2 nmol/kg) reduced TNBS-induced hypersensitivity in the periphery, but not in the central nervous system. Moreover, NOP receptor antagonist UFP-101, injected alone, exacerbated visceral hyperalgesia to colorectal distension compared with control group (Agostini et al., 2009). In our study we observed that

BU08070, injected peripherally (i.p.) at the dose of 1 mg/kg exerted an antinociceptive effect in the mouse model of visceral pain induced by mustard oil instillation. Importantly, we performed our assay in mice with acute TNBS-induced colitis, what shows potential applicability of BU08070 as antinociceptive agent also in pathophysiological conditions. In addition, in our previous study we observed that the antinociceptive effect induced by BU08070 in healthy mice was reversed by β -FNA, what indicated MOP receptor-dependent mechanism of antinociception (Sobczak et al., 2014). Therefore, a similar MOP receptor-dependent antinociceptive action may also be expected in inflamed animals.

The question that still needs to be answered is the molecular basis of the NOP-MOP receptor crosstalk in view of earlier reports and our current observations. Ko and Naughton showed that nociceptin injected i.t. in co-administration with morphine potentiated morphine-induced analgesia in monkeys, which may result from simultaneous activation of NOP and MOP receptors and a synergistic antinociceptive effect (Ko and Naughton, 2009). Accordingly, subcutaneous injection of SR14150, a partial NOP/MOP receptor agonist, but not SR16835, a full NOP receptor agonist in mice after spinal nerve ligation increased tail-flick latency (Khroyan et al., 2011). Consequently, the effect was inhibited by the classical opioid antagonist naloxone, but not the NOP receptor antagonist (Khroyan, et al., 2011). Recently, Linz et al. (Linz et al., 2014) characterized the effect of a mixed NOP/MOP receptor agonist, cebranopadol in several rat models of pain, including acute, inflammatory and bone cancer pain. Moreover, cebranopadol was more potent than morphine and its action was partially reversed by NOP and MOP receptor antagonists. However, none of these studies gave a deeper insight into the NOP-MOP receptor cross talk. We may hypothesize that the antinociceptive and anti-inflammatory effect of mixed NOP/MOP receptor ligands may be associated with the presence of NOP/MOP receptor heterodimers (Evans et al., 2010). These dimers have been demonstrated to attenuate MOP receptor-mediated activity in transfected HEK cells (Wang et al., 2005). NOP and MOP receptors may interact directly at the level of individual cells and also indirectly, at the neuronal circuitry level (Schroder et al., 2014).

5. Conclusion

In summary, we evidenced that activation of opioid and nociceptin systems using BU08070 produced anti-inflammatory response in experimental colitis mouse model. Consequently, mixed NOP/MOP receptor agonists could now become the focus of future preclinical studies, as they may constitute a promising therapeutic strategy in the course of IBD.

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Fig. 1. BU08070 (1 mg/kg, i.p., twice daily) ameliorated acute intestinal inflammation in mice with TNBS-induced colitis, as indicated by: (A) macroscopic score, (B) MPO activity, (C) ulcer score, (D) colon length, (E) body weight. * $P < 0.05$, *** $P < 0.001$, as compared with control group. # $P < 0.05$, ### $P < 0.001$, vs. TNBS-treated animals. Data represent mean $\pm$ S.E.M. of 6–8 mice per group.

Fig. 2. The anti-inflammatory effect of BU08070 was mediated by NOP and MOP receptors BU08070 (1 mg/kg i.p., twice daily) improved TNBS-induced colitis in mice. The effect of BU08070 was reversed by the NOP receptor antagonist J-113397 (12 mg/kg, i.p.) and the MOP receptor antagonist, β-FNA (1 mg/kg, i.p.). Figure shows data for (A) macroscopic score, (B) MPO activity, (C) ulcer score, (D) colon length, (E) body weight. * $P < 0.05$, ** $P < 0.01$ *** $P < 0.001$, as compared with control, # $P < 0.05$, ### $P < 0.001$ vs. TNBS-treated mice, @ $P < 0.05$, @@ $P < 0.01$, @@@ $P < 0.001$, as compared with BU08070-treated animals. Data represent mean $\pm$ S.E.M of 6–8 mice per group.

Fig. 3. BU08070 significantly improved microscopic damage score in TNBS-induced colitis in mice

Representative micrographs of hematoxylin and eosin-stained sections of distal colon from naive (A) and TNBS-treated (B–E) and microscopic total damage score (F). A) control, B) TNBS alone, C) TNBS + BU08070 (1 mg/kg, i.p., twice daily), D) TNBS + BU08070 (1 mg/kg, i.p., twice daily) administered with J-113397 (12 mg/kg, i.p.). E) TNBS + BU08070 (1 mg/kg, i.p., twice daily) administered with β -FNA (1 mg/kg, i.p.). Scale bar = 100 μ m. *** P < 0.001, as compared with control, ### P < 0.001 vs. TNBS-treated mice, @@@ P < 0.001, as compared BU08070-treated animals. Data represent mean $\pm$ S.E.M. of 6–8 mice per group.

Fig. 4.
TNF- α level was decreased in the colon samples collected from TNBS-treated mice after administration of BU08070 (1 mg/kg, twice daily). ** $P < 0.01$, as compared with control, # $P < 0.05$ vs. TNBS-treated mice. Data represent mean $\pm$ S.E.M. of 6 mice per group.

Fig. 5. BU08070 produced concentration-dependent inhibition of TNF-α and LPS induced NF-kB activation in THP-1 cell line

A) B) BU08070 produced concentration-dependent inhibition of TNF-α-induced NF-kB activation, B) BU08070 produced concentration-dependent inhibition of LPS-induced NF-kB activation. C) BU08070 (5–25 μM) had no significant effect on basal NF-kB activity in the absence of TNF-α and LPS stimulation. Optical density absorbance units (OD) at 620 nm from three independent experiments performed in triplicates are shown as mean ± S.E.M., ***P < 0.001 vs. TNF-α or LPS only-stimulated cells; ###P < 0.001 vs. BU08070-treated cells.

Fig. 6. BU08070 exerted antinociceptive effect in TNBS-treated mice

The effect of BU08070 (1 mg/kg, i.p.) on mustard oil (MO)-induced pain-related behaviors in mice. *** $P < 0.001$, as compared with TNBS-treated mice. Data represent mean $\pm$ S.E.M. of 6 mice per group.