New peptide inhibitor of dipeptidyl peptidase IV, EMDB-1 extends the half-life of GLP-2 and attenuates colitis in mice after topical administration

M Salaga, A Mokrowiecka, M Zielinska, E Malecka-Panas, R Kordek, E Kamysz, J Fichna*

Department of Biochemistry, Department of Digestive Tract Diseases, Department of Pathology, Faculty of Medicine, Medical University of Lodz, Lodz, Poland: MS, AM, MZ, EM, RK, JF

Department of Molecular Biotechnology, Faculty of Chemistry, University of Gdansk, Gdansk, Poland: EK
Running title: EMDB-1 in colitis

* To whom correspondence should be addressed:

Prof. Jakub Fichna, PhD, DSc, Department Biochemistry, Faculty of Medicine, Medical University of Lodz, Mazowiecka 6/8, 92-215 Lodz, Poland

Phone: ++48 42 272 57 07, Fax: ++48 42 272 56 94, Email: jakub.fichna@umed.lodz.pl

Number of pages: 34

Number of tables: 0

Number of figures: 8

Number of references: 34

Number of words in the Abstract: 250

Number of words in the Introduction: 841

Number of words in the Discussion: 1286

Recommended section: Gastrointestinal, Hepatic, Pulmonary and Renal

List of non-standard abbreviations:

AMC - aminomethylcoumarin
CD - Crohn’s disease
CNS - central nervous system
DMSO - dimethyl sulfoxide
DPP IV – dipeptidyl peptidase IV
DSS - dextran sulfate sodium
EM-2 - endomorphin-2
GAPDH - glyceraldehyde-3-phosphate dehydrogenase
GI - gastrointestinal
GLP-1 – glucagon-like peptide 1
GLP-2 – glucagon-like peptide 2
GLP2R – glucagon-like peptide 2 receptor
JPET #242586

GPCR - G-protein coupled receptor
HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC - high-performance liquid chromatography
HEX - hexamethonium
HPRT - hypoxanthine-guanine phosphoribosyltransferase
HRP - horseradish peroxidase
HTAB - hexadecyltrimethylammonium bromide
IBD – inflammatory bowel diseases
i.c. - intracolonic
IHC - immunohistochemistry
IL – interleukin
MOR - µ-opioid receptor
MPO – myeloperoxidase
NLX - naloxone
NSAID - non-steroidal anti-inflammatory drug
NF-κB - nuclear factor κB
PBS - phosphate buffered saline
PETIR - PEptidase-Targeted ImunoRegulation
TFA - trifluoroacetic acid
TNBS - 2,4,6-trinitrobenzene sulfonic acid
TNFα - tumor necrosis factor α
UC – ulcerative colitis
Abstract

Protease inhibition has become a new possible approach in the inflammatory bowel disease (IBD) therapy. A serine exopeptidase, dipeptidyl peptidase IV (DPP IV) is responsible for inactivation of incretin hormone, glucagon-like peptide 2 (GLP-2), a potent stimulator of intestinal epithelium regeneration and growth. Recently we showed that the novel peptide analog of endomorphin-2, EMDB-1 is a potent blocker of DPP IV and has an inhibitory effect on gastrointestinal (GI) smooth muscle contractility. The aim of this study was to characterize the anti-inflammatory effect and mechanism of action of EMDB-1 in the mouse GI tract. We used two models of experimental colitis (induced by TNBS and DSS). The anti-inflammatory effect of EMDB-1 was assessed by determination of macroscopic score, ulcer score, colonic wall thickness as well as myeloperoxidase activity. Additionally, we measured the expression of GLP-2, GLP-2 receptor and DPP IV in the colon of control and colitic animals treated with the test compound. Expression of GLP-2 and GLP2R in the serum and colon of IBD patients and healthy controls has been assessed. We showed that EMDB-1 elevates the half-life of GLP-2 in vitro and attenuates acute, semichronic and relapsing TNBS-as well as DSS-induced colitis in mice after topical administration. Its anti-inflammatory action is associated with changes in the level of colonic GLP-2 but not DPP IV expression. Our results validate DPP IV as a pharmacological target for the anti-IBD drugs and its inhibitors based on natural substrates, such as EMDB-1, have the potential to become valuable anti-inflammatory therapeutics.
Introduction

Inflammatory bowel disease (IBD) is a group of chronic inflammatory bowel disorders mainly represented by Crohn's disease (CD) and ulcerative colitis (UC). The main symptoms of IBD include abdominal pain and other clinical symptoms, such as diarrhea and rectal bleeding. Attenuation of these ailments can be achieved through the use of non-steroid anti-inflammatory drugs, corticosteroids or biologics, such as anti-tumor necrosis factor α (TNFα) or anti-α4β7 integrin antibodies depending on the stage and the severity of the disease (Raine, 2014; Rogler, 2015). However, all available treatment options have debatable efficiency (significant fraction of non-responders), while their use, especially in a prolonged administration, may bring major side effects.

The etiology of IBD is not fully understood, there is substantial evidence that immunological, genetic and environmental factors are the main contributors in its pathogenesis. Both disorders are characterized by an excessive activation of common inflammatory pathways, what results in an enhanced secretion of pro-inflammatory cytokines, such as interleukins (IL-1β, -2, -6, -8, -12, and -17), TNFα, and an imbalance in the levels of pro- and anti-inflammatory factors in the tissue (Jump and Levine, 2004). Recent studies suggest that the etiology of IBD also involves factors (both genetic and environmental) that cause dysfunction of the epithelial barrier with consequent aberrations in the mucosal responses to gut microbiota (Baumgart and Carding, 2007; Geuking, et al., 2014). Intestinal epithelial cells constitute a specific form of a physical, chemical, and immune barrier between the external and internal environment. Any damage to these cells may lead to an increased inflammatory process. Disturbance in the epithelial barrier seems to be the key element in the onset of IBD and, subsequently, in the frequent relapses. One of the factors responsible for the regeneration and growth of the epithelium are incretin hormones, such as glucagon-like peptide 2 (GLP-2) produced by the enteroendocrine L-cells of the small intestine and the colon. GLP-2, a 33-
amino acid peptide released by the neuroendocrine convertase 1 from the proglucagon is one of the most potent modulators of the intestinal function. GLP-2 signals through a G protein-coupled receptor (GLP2R), expressed predominantly in the small intestine and colon (Yazbeck, et al., 2008). It is a potent intestinotrophic growth factor, which stimulates crypt cell proliferation and inhibits crypt cell apoptosis (Hartmann, et al., 2000). GLP-2 has also been shown to improve epithelial barrier function and to increase mucosal hexose and glucose transport. It is also known to alleviate the symptoms in animal models of small intestinal and colonic injury, such as a non-steroidal anti-inflammatory drug (NSAID)-induced enteritis, necrotic colitis, postoperative ileus and dextran sulfate sodium (DSS)-induced colitis (Bank, et al., 2006; Hartmann, et al., 2000; Mimura, et al., 2013; Moore, et al., 2010; Nakame, et al., 2016; Salaga, et al., 2013; Yazbeck, et al., 2008).

GLP-2 undergoes swift enzymatic degradation and thus its potential therapeutic action rapidly ceases after administration ($t_{1/2}=5$-$7$ min in humans). The enzyme mainly responsible for decomposition of GLP-2 is dipeptidyl peptidase IV (DPP IV), which cleaves the protein from its bioactive form, GLP-2(1-33), to inactive GLP-2(3-33) (Salaga, et al., 2013). DPP IV is ubiquitously expressed on the surface of epithelial cells and the highest levels in humans have been reported to occur in the intestine, bone marrow and kidney, although there are also soluble DPP IV forms in plasma and other body fluids (Salaga, et al., 2013).

There have been some attempts to target proteases, including DPP IV, by synthetic inhibitors as a novel form of treatment of gut inflammation. One of the strategies commonly used to design a novel enzyme inhibitor is to modify the structure of its natural substrate to obtain a compound that actively binds to the enzyme active site and blocks its catalytic activity. Using this approach our group designed and synthesized a series of peptide DPP IV inhibitors based on the structure of endomorphin-2 (EM-2), which is a potent endogenous µ-opioid receptor (MOR) agonist and DPP IV substrate. We found that one of the compounds (Tyr-Pro-D-
ClPhe-Phe-NH$_2$; EMDB-1) significantly extends the half-life of EM-2 and does not exhibit affinity towards MOR in vitro (Fichna, et al., 2006b). The results of the experiments with isolated proteolytic enzymes suggest that EMDB-1 is a competitive and selective inhibitor of DPP IV (Fichna, et al., 2010). Cravezic et al. (2011) demonstrated that EMDB-1 significantly prolonged the analgesic and antidepressant-like effects, induced by exogenous EMs, by blocking EM degrading enzymes. Fichna et al. (2010) reported that EMDB-1 has a significant influence on the intestinal tissue showing inhibitory effects on the smooth muscle contractility of the rat ileum. Encouraged by the previously reported results, here we aimed at testing the hypothesis that EMDB-1 attenuates experimental colitis in mice by elevation of the intestinal GLP-2. Non-selective opioid antagonist naloxone (NLX) was employed to exclude the role of opioid receptors in the action of EMDB-1. Moreover, we characterized the expression of GLP-2, GLP2R and DPP IV in the control and colitic EMDB-1-treated animals. To translate our results to clinical conditions, we measured the expression of GLP-2 and GLP2R in the serum and colon of IBD patients and healthy controls.
Materials and methods

Determination of EMDB-1 inhibitory activity in vitro

Potency of EMDB-1 was determined using the DPP IV Inhibitor Screening Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), which utilizes the fluorogenic substrate, Gly-Pro-Aminomethylcoumarin (AMC) to measure DPP IV activity. Cleavage of the peptide bond by the enzyme releases free AMC group whose fluorescence can be measured using excitation wavelength of 350-360 nm and emission wavelength of 450-465 nm (Victor 3 Multilabel Microplate Reader, Perkin Elmer, Waltham, MS, USA). Multiple concentrations of EMDB-1 ranging from $10^{-10}$ to $10^{-4}$ M were tested to obtain a concentration-response curve. Curve fitting analysis has been performed to obtain IC$_{50}$ value. A well-known DPP IV inhibitor sitagliptin was used as a positive control for the assay.

GLP-2 degradation studies and HPLC analysis

The degradation studies were performed using purified DPP IV isolated from porcine kidney, according to the modified method described elsewhere (Fichna, et al., 2006b). Briefly, the lyophilized enzyme was reconstituted in 50 mM Tris–HCl (pH 7.4). The aliquots (100 µL, 0.2 mg protein/mL) were incubated with 50 µL of either GLP-2 (0.042 mM) and 50 µL of EMDB-1 (0.21 mM) or Tris, over 0, 7.5, 15, 22.5 and 30 min at 37 °C in a final volume of 200 µL. The reaction was stopped at the required time by placing the tube on ice and acidifying with 20 µL of 1 M aqueous HCl solution. The aliquots were centrifuged at 20 000 g for 10 min at 4 °C. The obtained supernatants were filtered through Millex-GV syringe filters (Millipore) and analyzed by high-performance liquid chromatography (HPLC) on a Gemini-NX C18, 4.6 x 150 mm, particle size 5µm, pore size 110 Å, Phenomenex, CA, USA), using the solvent system of 0.1% trifluoroacetic acid (TFA) in water (A) and 0.1% TFA in acetonitrile (B) and a linear gradient of 3–80% B over 20 min with a flow rate 2.5
ml/minute. Three independent experiments for each assay were carried out in duplicate. The rate constants of degradation (k) were obtained by a least-square linear regression analysis of logarithmic endomorphin peak areas \([\ln(A/A_0)]\), where \(A\) – amount of peptide remaining, \(A_0\) – initial amount of peptide, versus time. Degradation half-lives \((t_{0.5})\) were calculated from the rate constants as \(\ln2/k\).

**Animals**

Experimentally naive male C57B1/6 mice were obtained from the vivarium at the University of Lodz, Poland. All animals used in the experiments weighed 22–26 g (6-8 weeks of age). Mice were housed at a constant temperature (22°C) and maintained under a 12-hour light/dark cycle (lights on at 6.00 am) in sawdust-lined plastic cages. Chow pellets (Agropol S.J., Motycz, Poland) and tap water were available *ad libitum*. All animal protocols were approved by the Medical University of Lodz Animal Care Committee (Protocol 36/LB670/2015) and complied with the European Communities Council Directive of 22 September 2010 the EU (2010/63/EU). All efforts were made to minimize animal suffering and to reduce the number of animals used. Groups of 6-10 animals were used in all in vivo experiments.

**Induction of colitis**

**TNBS model**

Colitis was induced by intracolonic (i.c.) administration of 2,4,6-trinitrobenzene sulfonic acid (TNBS), as described before (Salaga, et al., 2014b). Briefly, mice were weighed and lightly anesthetized with 1% isoflurane (Baxter Healthcare Corp., IL, USA) and TNBS (0.1 mL of 30% ethanol in saline; acute and semichronic models: 4 mg\(_{TNBS}\)/animal; chronic relapsing model: first dose – 150 mg\(_{TNBS}\)/kg, second dose – 75 mg\(_{TNBS}\)/kg) was injected into the colon through a catheter inserted 3 cm proximally from the anus. Mice were then maintained in an
inclined position for 1 min to ensure the proper distribution of the inductor in the colon. Next, recovery was allowed with food and water supplied. Control animals received vehicle alone (30% ethanol in saline; TNBS replaced with equivolume water). Preliminary experiments demonstrated that the dose of TNBS used in this study resulted in reproducible colitis manifested by body weight decrease as well as macroscopic damage and biochemical alterations characteristic for the disease.

DSS model

Colitis was induced by the addition of DSS to drinking water starting from day 0 to day 5 (3% wt/vol; molecular weight 40,000; MP Biomedicals, Aurora, OH, Lot No. 5237K). On days 6 and 7 the animals received water without DSS. Control animals received tap water. Animal body weight as well as general health and disease symptoms were monitored daily.

Pharmacological treatments

In this study we used three different therapeutic regimens in the TNBS model. In the acute paradigm the effect of EMDB-1 was evaluated as follows: colitis was induced on day 0 and EMDB-1 was administered twice daily from day 0 to day 2 at the doses ranging from 0.1 to 3 mg/kg intracolonic (i.c.) with the first treatment 30 minutes before the TNBS instillation. Animals were euthanized on day 3 by rapid cervical dislocation and the evaluation of disease parameters was performed (Fig. 2A). Non-selective opioid receptor antagonist NLX was administered i.p. 30 minutes before EMDB-1 (3 mg/kg) at the dose of 1 mg/kg, which has been selected based on the preliminary studies and previously published data (Salaga, et al., 2015).

In the semichronic TNBS model, a curative treatment mode was tested. Inflammation was induced on day 0 and animals received EMDB-1 (1 mg/kg, i.c., twice daily) starting from day
3 to day 6 (Fig. 3A). On day 7 mice were euthanized by rapid cervical dislocation and the evaluation of colonic damage was performed.

The therapeutic activity of EMDB-1 in the chronic, relapsing colitis was evaluated in the model described earlier by Martin et al. (Martin, et al., 2014) with minor modifications.

Briefly, colitis was induced on day 0 by i.c. administration of TNBS at the dose of 150 mg/kg. Between days 7 and 13 mice were injected with EMDB-1 at the dose of 1 mg/kg twice daily (i.c.). The relapse of colitis was induced on day 11 by the second administration of TNBS solution at the dose of 75 mg/kg. Three days later mice were sacrificed (D14, endpoint) and the evaluation of colonic damage was performed (Fig. 4A). Doses of TNBS used in this experiment were selected based on the preliminary experiments involving titration and evaluation of disease parameters as well as mice survival rate. In all experiments control animals received vehicle (100 µl i.p.) alone.

In the DSS-model animals were treated with EMDB-1 (1 mg/kg, i.c. twice daily) from day 3 to day 6 (Fig. 6A). On day 7 mice were sacrificed and the evaluation of colonic damage was performed. In all experiments control animals received vehicle (100 µl i.c.) alone.

**Evaluation of colonic damage**

**TNBS-induced colitis**

After euthanasia the colon was rapidly removed, opened longitudinally, rinsed with phosphate buffered saline (PBS), and immediately examined. Macroscopic colonic damage was determined by an established semiquantitative scoring system by adding individual scores for ulcer, colonic shortening, wall thickness, and presence of hemorrhage, fecal blood, and diarrhea, as described before (Salaga, et al., 2014a). For scoring ulcer and colonic shortening the following scale was used: ulcer: 0.5 points for each 0.5 cm; shortening of the colon: 1 point for >15%, 2 points for >25% (based on a mean length of the colon in untreated mice of
7.87 ± 0.12 cm; n = 8). The wall thickness was measured in millimeters, a thickness of n mm corresponded to n scoring points. The presence of hemorrhage, fecal blood, or diarrhea increased the score by 1 point for each additional feature.

**DSS-induced colitis**

After euthanasia on day 7 following addition of DSS to the drinking water the entire colon was isolated and weighed with fecal content. Then, colon was opened along the mesenteric border and cleaned from the fecal material. A total macroscopic damage score was calculated for each animal based on the (i) stool consistency (where 0 means normal well-shaped fecal pellets and 3 means diarrhea), (ii) colon epithelial damage considered as a number of ulcers (0 - 3), (iii) colon length and weight scores considered as a percentage loss of each parameter in relation to the control group (0 points, ≤5% weight/length loss; 1 point, 5–14% weight/length loss; 2 points, 15–24% weight/length loss; 3 points, 25–35% weight/length loss; and 4 points, ≥35% weight/length loss). Total score = 0 means no inflammation (Salaga, et al., 2014a). The presence (score = 1) or absence (score = 0) of fecal blood was also recorded.

**Determination of tissue myeloperoxidase activity**

The method described by Salaga and collaborators (Salaga, et al., 2014a) was used to quantify the myeloperoxidase (MPO) activity. Briefly, 0.5 cm segments of colon were weighed and homogenized in hexadecyltrimethylammonium bromide (HTAB) buffer (0.5% HTAB in 50 mM potassium phosphate buffer, pH 6.0; 50 mg of tissue/mL) immediately after isolation. Next, homogenate was centrifuged for 15 min (13,200 g, 4°C) and supernatant was used in the subsequent steps. On a 96-well plate, 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% hydrogen peroxide was added to 7 µl of the supernatant. Absorbance was measured at 450 nm (iMARK
Microplate Reader, Biorad, United Kingdom) at 0, 30 and 60 seconds after initiation of reaction. All measurements were performed in triplicate. MPO was expressed in milliunits per gram of wet tissue, 1 unit being the quantity of enzyme able to convert 1 µmol of hydrogen peroxide to water in 1 min at room temperature. Units of MPO activity per 1 min were calculated from a standard curve using purified peroxidase enzyme.

Histology

After the macroscopic scoring, segments of the distal colon were stapled flat, mucosal side up, onto cardboard and fixed in 10% neutral-buffered formalin for 24 h at 4 °C. After subsequent dehydration samples were embedded in paraffin, sectioned at 5 µm and mounted onto slides. Next sections were stained with hematoxylin and eosin 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). A microscopic total damage score was determined in a blinded fashion based on the presence (score = 1) or absence (score = 0) of goblet cell depletion, the presence (score = 1) or absence (score = 0) of crypt abscesses, the destruction of mucosal architecture (normal = 1, moderate = 2, extensive = 3), the extent of muscle thickening (normal = 1, moderate = 2, extensive = 3), and the presence and degree of cellular infiltration (normal = 1, moderate = 2, transmural = 3).

Study population for the evaluation of human GLP-2 and GLP2R expression

To quantify the relative expression of human GLP-2 and GLP2R, forceps biopsy samples and serum were analyzed. In total 13 specimens prepared from human colon biopsies and 29 serum samples were used for the study. The study population comprised 9 patients with Crohn’s disease (CD, age 21-53), 12 patients with ulcerative colitis (UC, age 25-73) and 8
healthy, unrelated controls (age 25-65) recruited from January 2014 to January 2015 (for more detailed information see supplementary Table 1). The specimens were frozen shortly after isolation and kept at -80°C until further analysis. The diagnosis of CD and UC in patients was assessed accordingly to established clinical criteria using endoscopic, radiologic, and histopathologic criteria. This human part of the study was approved by the Ethics Committee of the Medical University of Lodz. All patients gave written, informed consent prior to the analysis.

**Determination of GLP-2 protein level by ELISA**

For determination of GLP-2 in the mouse colon and human serum the competitive inhibition enzyme immunoassay kit was used (Mybiosource, San Diego, CA, USA). Briefly, mouse tissue segments were rinsed in ice-cold PBS to remove excess blood and weighed before the homogenization. Next, tissues were minced using motor cordless tissue grinder (Fisher scientific, Goteborg, Sweden) in the 20 volumes of mammalian cell lysis buffer (50 mM Tris-HCl, pH 7.5; 1 mM EDTA, 150 mM NaCl; 0.1% SDS; 0.5% deoxycholic acid; 1% Igepal CA-630; Sigma-Aldrich, Poznan, Poland) containing protease inhibitor cocktail (AEBSF, pepstatin A, bestatin, leupeptin, aprotinin). Then, the homogenates were centrifuged for 12 minutes at 10,000 g, 4°C. The supernatant was used for the procedure following manufacturers instructions. Human serum has been diluted 5 times with PBS before assaying. The amount of GLP-2 in the colonic samples was calculated from the standard curve prepared with the purified GLP-2 standard provided in the kit.

**Western blot**

Fragments of tissues were mixed with the mammalian cell lysis buffer (50 mM Tris-HCl, pH 7.5; 1 mM EDTA, 150 mM NaCl; 0.1% SDS; 0.5% deoxycholic acid; 1% Igepal CA-630;
Sigma-Aldrich, Poznan, Poland) containing protease inhibitor cocktail (4-benzenesulfonyl fluoride, pepstatin A, bestatin, leupeptin, aprotinin). Then, samples were homogenized using motor cordless tissue grinder (Fisher scientific, Goteborg, Sweden). The homogenate was cleared by centrifugation at 10,000 g for 12 min. Concentration of total protein pool was evaluated in each sample (triplicate) using the Pierce 660 nm protein assay (Thermo Scientific, Rockford, IL, USA). Electrophoresis of the samples (15 μg protein/well) was performed on precast 4-20% SDS-PAGE gel (Bio-Rad, Warsaw, Poland) in electrophoretic buffer (0.1% SDS, 192 mM glycine, 25 mM Tris, pH 8.3). Separated proteins were transferred using a semi-dry system onto PVDF membranes (pore size, 0.45 μm; Life Technologies, Carlsbad, CA, USA) in transfer buffer containing 15% (v/v) methanol, 192 mM glycine, 25 mM Tris, pH 8.3. The PVDF membranes were incubated at room temperature for 1 h in 5% non-fat dry milk in PBS with Tween 20 (PBST; PBS, 0.1% Tween 20) to saturate non-specific protein binding sites. Subsequently, the membranes were incubated with specific primary antibodies diluted in 1% non-fat dry milk in PBST for 80 minutes at room temperature for immunodetection of the studied proteins. The primary rabbit anti-mouse GLP2R polyclonal antibody (H-57; sc-99092; dil. 1:1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), rat anti-mouse DPP IV monoclonal antibody (MBS690035; dil. 1:1000 Mybiosource, San Diego, CA, USA) and mouse anti-glycerylaldehyde-3-phosphate dehydrogenase (GAPDH; MAB374; dil. 1:15 000; Merck, Warsaw, Poland) were used. After wash steps (6 times, 2 minutes) using PBST, membranes were incubated with appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature and then the bands were visualized using Super Signal west pico western blotting substrate (Thermo Scientific, Rockford, IL, USA) as a substrate for the localization of HRP activity. Qualitative and quantitative analysis was performed by measuring integrated optical density (IOD) by ImageLab v.5.2.1 for WindowsTM program (Bio-Rad SA, Warsaw, Poland). For
determination of protein weight, we have used 5 µl/lane of Precision Plus Protein Standards (Bio-Rad SA, Warsaw, Poland).

**Statistics**

Statistical analysis was performed using Prism 5.0 (GraphPad Software Inc., La Jolla, CA, USA). The data are expressed as means ± SEM. Student t-test or one-way ANOVA followed by Newman-Keuls post-hoc test were used for analysis. P values < 0.05 were considered statistically significant.

**Drugs**

All drugs and reagents, unless otherwise stated, were purchased from Sigma-Aldrich (Poznan, Poland). DSS (MW 40,000) was purchased from MP Biomedicals (Solon, OH, USA). NLX was purchased from Tocris Bioscience (Bristol, UK). EMDB-1 was synthesized by solid-phase method using Fmoc chemistry. Particulars concerning the synthesis, purification and physicochemical characteristic of this peptide are as described previously (Fichna, et al., 2006a). All drugs were dissolved in 5% dimethyl sulfoxide (DMSO) in saline, which was used as vehicle. The vehicles in the used concentrations had no effects on the observed parameters.
Results

EMDB-1 is a potent inhibitor of DPP IV and protects GLP-2 against enzymatic degradation in vitro

In vitro experiments employing the Gly-Pro-AMC demonstrated that, in line with previous report (Fichna, et al., 2006b), EMDB-1 exhibits a potent inhibitory activity towards DPP IV (IC$_{50}$=89.7 ± 3 nM; Fig. 1A). Furthermore, the degradation half-life of GLP-2 incubated with EMDB-1 was increased approx. 4-fold vs. GLP-2 incubated alone (Fig. 1B). However, the difference between the half-lives did not reach statistical significance (p=0.11).

EMDB-1 exhibits anti-inflammatory effect in TNBS- and DSS-induced colitis

To evaluate the anti-inflammatory activity of EMDB-1 in the mouse GI tract, we used a well-established mouse model of acute colitis induced by TNBS. The i.c. administration of TNBS resulted in a reproducible colitis in mice, as indicated by elevated macroscopic colon damage scores and MPO activity. EMDB-1 administered twice daily (i.c.) at the doses of 0.1, 1 and 3 mg/kg significantly improved colitis in a dose-dependent manner, as shown by decreased macroscopic and ulcer scores, colonic wall thickness and MPO activity (Fig. 2B-E).

To examine the possible involvement of opioid receptors in the effect of EMDB-1 we used a non-selective opioid antagonist NLX (1 mg/kg, i.p.). As shown in Fig. 2B-E, NLX did not block the anti-inflammatory effect of EMDB-1. NLX administered alone did not affect the disease parameters, besides the ulcer score, which was significantly reduced in this experimental group. EMDB-1 at the dose of 1 mg/kg i.c. (twice daily) was used in all subsequent experiments.

Our previous observations as well as literature data show that the severity of colitis in mice manifested by its clinical symptoms, such as body weight loss, reaches its maximum at day 3 after TNBS injury (Monteleone, et al., 2012; Sans, et al., 2001). Hence, to investigate the healing effect of EMDB-1 on established colitis, we administered the compound twice daily
from day 3 post-TNBS. Treatment with EMDB-1 resulted in the significant improvement of colitis as indicated by reduced macroscopic score, ulcer score, colonic wall thickness and MPO activity (Fig. 3B-E). To mimic the pattern of symptoms that occur in humans (including the acute phase of colitis, the recovery and relapse periods), we evaluated the effect of EMDB-1 in the chronic, relapsing colitis paradigm. EMDB-1 (1 mg/kg, i.c.) administered twice daily from day 7 to day 13 significantly attenuated the clinical and molecular parameters of the disease as shown by reduced macroscopic and ulcer scores, colonic wall thickness, MPO activity as well as decreased body weight loss and expression of TNFα and IL-1β (Fig. 4B-G). Histological evaluation of the mouse colon specimens supported the macroscopic observations. Pathological changes induced by TNBS were reversed after treatment with EMDB-1 (Fig. 5). Analysis of sections of distal colon from untreated animals showed intact epithelium, absence of edema, and normal muscle architecture (Fig. 5A). Severe microscopic damage, characterized by loss of mucosal architecture, thickening of smooth muscle, presence of crypt abscesses, and extensive cellular infiltration was observed in TNBS-treated colon specimens (Fig. 5B). The histological damage was reduced after i.c. EMDB-1 (1 mg/kg twice daily from day 7 to day 13; Fig 5C).

In order to test the anti-inflammatory activity of EMDB-1 in the mouse model mimicking UC, a DSS-induced colitis was used. Animals treated with 3% DSS in drinking water developed severe colonic injury (Fig. 6). The i.c. administration of EMDB-1 (1 mg/kg, twice daily) between day 3 and 6 alleviated the disease as demonstrated by macroscopic colon damage score (Fig. 6B), colon weight (Fig. 6C), colon length (Fig. 6D), MPO activity (Fig. 6E) and body weight loss (Fig. 6F).

**EMDB-1 alters the expression of GLP-2, GLP2R but not DPP IV in control and TNBS-treated mice**
For further characterization of possible mechanisms of the anti-inflammatory action of EMDB-1, changes in the colonic levels of GLP-2, GLP2R and DPP IV were characterized in naive (“healthy”) and TNBS-treated animals. In naive animals, the level of GLP-2 was significantly elevated (approx. 9-fold increase) after treatment with EMDB-1 (1 mg/kg, i.c.). However, in the TNBS-treated mice this effect was substantially stronger (approx. 19-fold increase; Fig. 7A).

Expression of GLP2R in the naive animals treated with EMDB-1 has significantly decreased compared to vehicle treated mice. Similar pattern was observed in the TNBS-treated animals, however the reduction in expression was weaker and did not reach statistical significance (Fig. 7B).

Treatment with EMDB-1 did not alter the expression of DPP IV in both control and TNBS-treated mice (Fig. 7C).

Expression of GLP-2 and GLP2R is altered during CD and UC

To translate our observations from animals to human conditions, we examined GLP-2 and GLP2R protein expression levels in serum and colon specimens from patients with CD, UC and healthy controls. We found that the expression of GLP-2 in the human serum is significantly decreased in patients with CD (Fig. 8A). Western blot analysis of GLP2R protein expression in the colon showed significant decrease in the expression of this receptor in UC patients (Fig. 7C).
Discussion

IBD is a chronic, relapsing inflammatory disorder of the GI tract affecting several millions of patients worldwide. High incidence of IBD was reported in developed countries, however the number of cases in the regions formerly absent from IBD incidence maps is rapidly increasing (Molodecky, et al., 2012). The lack of fully effective and safe treatment strategy against IBD forces the pursuit of novel compounds targeting proteins that modulate function of the gut in pathophysiological conditions. In this study, we aimed at testing the concept that the inhibition of DPP IV attenuates colonic inflammation via increased GLP-2.

We showed that new, highly potent, peptide inhibitor of DPP IV, EMDB-1 displays anti-inflammatory activity in TNBS- and DSS-induced mouse models of experimental colitis. Moreover, we demonstrated that this effect is associated with the striking elevation of the colonic GLP-2. We also showed that EMDB-1 is effective in the model of chronic, relapsing colitis that recapitulates the course of IBD in humans, a finding of high clinical relevance. Finally, we quantified the expression of GLP-2 as well as GLP2R in human serum and colonic specimens to evaluate the potential involvement of the incretin signalling in the pathophysiology of IBD.

The significant anti-inflammatory action of EMDB-1 validates therapeutic use of DPP IV inhibitors in the treatment of IBD and corroborates with the concept of protease inhibition as a way to combat diseases characterized by immunological imbalance. Proteases are widely distributed in the human body (to date 500-600 different proteases have been identified), including the GI tract where they serve not only for the digestion purposes but also maintenance of gut homeostasis (e.g. immune cells activation and interaction with gut microbiota) (Vergnolle, 2016). In pathophysiological conditions, such as inflammation, the content of proteases in the gut increases due to the infiltration of immune cells (e.g. neutrophils release significant amounts of elastase and proteinase-3) which use these enzymes
to degrade tissues and absorbed biological molecules, thereby increasing phagocytic properties of these cells (Vergnolle, 2016). In line, the expression of a very large number of proteases is increased in IBD. Enzymatic activity of these upregulated proteases is not balanced by endogenous inhibitors since their expression often remains unchanged. Thus, re-equilibration of the protease-antiprotease balance with exogenous inhibitors may be a useful therapeutic strategy (Vergnolle, 2016).

Recently, a novel therapeutic strategy, referred to as PEptidase-Targeted ImunoRegulation (PETIR™) has been proposed, which takes for the purpose restoration of immune balance by limiting the activation of immune cells and induction of endogenous immunosuppressive mechanisms, such as transforming growth factor β and regulatory T cells through inhibition of peptidase-dependent pathways (Bank, et al., 2006; Salaga, et al., 2013). Experimental data indicate that PETIR results in the suppression of cell proliferation and reduced synthesis of pro-inflammatory cytokines without affecting cellular vitality (Bank, et al., 2006). Moreover, protease inhibitors seem to be superior to exogenous receptor ligands since they allow for finer tuning of the signaling pathways which are transduced through the elevation of endogenous mediators. Furthermore, their action may be limited in time and space ensuring greater pharmacological control. Here we show that targeting DPP IV attenuates experimental colitis after topical administration.

Our results are in line with the report by Mimura et al. (2013) who showed that synthetic DPP IV inhibitor anagliptin improves body weight loss, disease activity index as well as histological score in the model of DSS-induced colitis. Moreover, in this study we show for the first time that a peptide compound targeting DPP IV could be potentially used as an anti-inflammatory agent in the GI tract. Of note, EMDB-1 was effective in two animal models that mimic both major types of IBD, namely CD recapitulated in TNBS- and UC recapitulated in the DSS-induced colitis.
In order to investigate the mechanism of action of EMDB-1 we characterized its effect on the level of GLP-2, GLP2R and DPP IV in control and TNBS-treated mice. These experiments undoubtfully demonstrated that increase in the colonic GLP-2 is associated with the anti-inflammatory effect of EMDB-1. Moreover, no change in the expression of DPP IV has been observed, hence the elevation of GLP-2 may be attributed solely to the inhibition of this protease. Particularly interesting is the fact that the protective effect of the DPP IV inhibitor on GLP-2 was substantially stronger in the TNBS-treated vs. control animals, leading to the approx. 19-fold increase of this incretin hormone in the colon. This phenomenon may be explained by the increase in the number of L cells that secrete GLP-2 in the colonic mucosa exposed to the TNBS as previously demonstrated by O’Hara et al. (2007). The presence of large amount of GLP-2 in the colon likely leads to the improvement of colitis by stimulation of cells that express GLP2R via various, previously described mechanisms, such as stimulation of subepithelial myofibroblasts to release growth factors and transforming growth factor β, inhibition of pro-inflammatory cytokines (e.g. TNFα, IL-1β) and enhancement of mesenteric blood flow (for comprehensive review please see: Hornby et al. (2011)).

Moreover, treatment with EMDB-1 significantly reduced the expression of GLP2R in control but not TNBS-treated mice. It is probably caused by a common adaptive mechanism regulating GPCR signaling based on the reduction of the number of receptors in response to the steep increase in the concentration of the ligand. Of note, this effect was weaker (insignificant) in the colitic animals suggesting that expression of GLP2R is maintained at the higher level by some additional mechanism in order to sustain the restorative GLP-2-mediated signaling. This phenomenon together with increased bioavailability and prolonged half-life of GLP-2 further support the concept of aiming at DPP IV in the IBD therapy.

Translational experiments on human tissues showed that GLP-2 is strongly reduced in the serum of CD but not UC patients. Recently, Sigalet et al. (2013), in a pilot study, showed that
postprandial GLP-2 is reduced in CD patients in the acute phase of the disease but not in the remission. This observation points to the conclusion that increase of GLP-2 level would restore the colonic damage in CD sufferers. Furthermore, our results suggest that GLP-2 signaling is also impaired in the UC, due to the low receptor expression, what further supports the concept of targeting incretin system in the anti-IBD therapy. Here we tested the i.c. administration of the EMDB-1 and obtained encouraging results in different models of colonic inflammation. Consequently, we envisage the use of enema consisting of DPP IV inhibitor(s) in CD and UC, especially since our test compound ameliorated both TNBS- and DSS-induced colitis. At this point topical administration of compounds modulating incretin signaling seems to be the most rational since the therapeutic potential of GLP-2 in GI diseases is based on the multiple indirect effects in the gut, where this compound is locally secreted. Of note, one of the natural substrates of DPP IV are endogenous opioid peptides, such as endomorphins that activate MOR-dependent pathways in the GI tract. Immune cells express opioid receptors and thus opioids may be involved in the regulation of inflammatory processes. It has been demonstrated that activation of MORs located on the peripheral immune and nerve cells exert anti-inflammatory effect (Borzsei, et al., 2008; Sobczak, et al., 2013; Sobczak, et al., 2014). Moreover, it was observed that MOR-/- mice are more susceptible to inflammation than their wild type litters (Philippe, et al., 2003). Here, to exclude the possible involvement of opioid peptides in the anti-inflammatory activity of EMDB-1 we used a non-selective opioid antagonist NLX which did not alter any of the measured parameters. Based on this observation, together with the fact that EMDB-1 does not exhibit affinity towards opioid receptors we conclude that MOR signaling cannot be taken into consideration regarding the EMDB-1 mechanism of action.
Conclusions and future perspectives

In the present study we showed that novel peptide inhibitor of DPP IV, EMDB-1 exerts potent anti-inflammatory effect in the mouse models of colitis, which is associated with the increase of the colonic GLP-2. These findings offer a new alternative in the pharmacological strategies of IBD treatment. EMDB-1 may now be used as a pattern for design and synthesis of peptides displaying not only low binding to opioid receptors and high inhibitory activity towards DPP IV but also resistance to degradation by proteases located in the upper GI tract. Such modification would allow the oral administration of the compound, which is preferable in the context of clinical application. Design and synthesis of peptide DPP IV inhibitors is thus a promising direction in the search for innovative anti-IBD drugs. Such therapy could be particularly appealing for the group of patients, in case of which other therapies did not provide sufficient relief.

Concerns about the side-effects of the GLP-2 that may hamper its therapeutic utility mainly relate to its potential carcinogenic effect. It has been raised that long-term stimulation of GLP2R may contribute to the increased proliferation and protection of the tumor cells. Indeed, it has been shown that exogenous GLP-2 elevates the number of aberrant crypt foci and leads to the formation of adenocarcinomas in the azoxymethane treated mice (Iakoubov, et al., 2009). On the other hand, it was reported that chronic GLP-2 treatment has no effect on the growth of human colon cancer cells in nude mice, neither on the number and size of polyps in Apc^{Min/+} mice (Koehler, et al., 2008). Given these contradictory data it is difficult to provide a clear-cut answer on the potential threat of GLP2R agonists. Hence nowadays, one of the greatest challenges of the field is to clearly estimate the risk of carcinogenesis after treatment with GLP-2.
Disclosures: The authors have nothing to disclose.

Authorship Contributions:

Participated in research design: Fichna, Salaga

Conducted experiments: Salaga, Zielinska, Kamysz

Contributed new reagents or analytic tools: Mokrowiecka, Malecka-Panas, Kordek

Performed data analysis: Salaga, Zielinska, Kamysz

Wrote or contributed to the writing of the manuscript: Salaga, Fichna, Kamysz
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Footnotes

This work was supported by the grants from the Medical University of Lodz [#502-03/1-156-04/502-14-298 to MSalaga and #503/1-156-04/503-11-001 to JF], National Science Centre [#UMO-2013/11/N/NZ7/02354 to MSalaga and #UMO-2013/11/B/NZ7/01301 to JF] and by the Iuventus Plus program of the Polish Ministry of Science and Higher Education (IP2015 068774 to MS).
Legend to figures:

**Figure 1.** EMDB-1 inhibited DPPIV activity in a dose-dependent manner and extended the half-life of GLP-2 in vitro. (A) Concentration-response curve showing the inhibitory effect of EMDB-1 on the DPPIV activity. (B) Effect of EMDB-1 on the half-life of GLP-2 in the presence of purified DPPIV. Data represent mean ± SEM of three independent experiments performed in triplicate.

**Figure 2.** EMDB-1 injected i.c., twice daily over 3 days at the doses ranging from 0.1 to 3 mg/kg attenuated TNBS-induced colitis in mice. The effect of EMDB-1 (3 mg/kg, i.c. was not blocked by non-selective opioid receptor antagonist NLX (1 mg/kg, i.p.). (A) Scheme illustrating the protocol and treatment regimen used in this experiment. Figure shows data for macroscopic score (B), ulcer score (C), colonic wall thickness (D) and MPO activity (E). &&P < 0.05, &&&P < 0.01, &&&&P < 0.001, as compared to control mice. *P < 0.05, **P < 0.01 ***P < 0.001, as compared to TNBS-treated mice. Data represent mean ± SEM of 6–10 mice per group.

**Figure 3.** The i.c. administration of EMDB-1 (1 mg/kg, twice daily) alleviates established TNBS-induced colitis in mice. (A) A schematic illustration of the protocol and treatment regimen used in this experiment. Figure shows data for microscopic score (B), ulcer score (C), colonic wall thickness (D) and MPO activity (E). &&&P < 0.001, as compared to control mice. **P < 0.01, ***P < 0.001, as compared to TNBS-treated mice. Data represent mean ± SEM of 6–10 mice per group.

**Figure 4.** The effect of EMDB-1 (1 mg/kg, i.c.) injected twice daily over 7 days on chronic, relapsing colitis in mice. (A) A scheme illustrating the experimental protocol and treatment
regimen used in this model. Figure shows data for macroscopic score (B), ulcer score (C), colonic wall thickness (D), MPO activity (E), body weight changes (F) as well as TNFα and IL-1β expression (G). &P < 0.05, &&P < 0.01, &&&P < 0.001, as compared to control mice. **P < 0.01, ***P < 0.001, as compared to TNBS-treated mice. Data represent mean ± SEM of 6–10 mice per group.

**Figure 5.** Microscopic total damage score and representative micrographs of hematoxylin and eosin-stained sections of distal colon obtained from the chronic-relapsing colitis model. Figure shows pictures of (A) control, (B) TNBS, (C) TNBS + EMDB-1 (1 mg/kg, i.c., twice daily from D7 to D13)-treated mice, Scale bar = 100 μm. &&&P < 0.001, as compared with control mice, ***P < 0.001, as compared to TNBS-treated mice. Data represent mean ± SEM of 6 mice per group.

**Figure 6.** EMDB-1 (1 mg/kg, i.c.) injected twice daily over 3 days (D3 to D6) exhibits healing effect on DSS-induced colitis in mice. (A) A scheme illustrating experimental protocol and treatment used in this model. Figure shows data for macroscopic score (B), colon weight (C), colon length (D), MPO activity (E) and body weight changes (F). &&&P < 0.01, &&&P < 0.001, as compared to control mice. *P < 0.05, **P < 0.01, ***P < 0.001, as compared to DSS-treated mice. Data represent mean ± SEM of 6–10 mice per group.

**Figure 7.** Effect of EMDB-1 (1 mg/kg i.p.) administered twice daily on the expression of GLP-2, GLP2R and DPPIV in the distal colon of control and TNBS-treated mice. Figure shows quantitative analysis by ELISA and Western blot of (A) GLP-2, (B) GLP2R and (C) DPPIV expression. Western blot data were normalized to GAPDH which was used as internal
control for protein loading. \&P < 0.05, as compared to control mice. \*\*\*P < 0.001, as compared to TNBS-treated mice. Data represent mean ± SEM of 6–7 mice per group.

**Figure 8.** Determination of GLP-2 expression in serum and colon specimens obtained from patients with diagnosed UC and CD vs. healthy controls. Figure shows data for the concentration of GLP-2 (A), determined by ELISA, in the serum and relative expression of GLP2R (B) measured by Western blot in the colonic specimens. \&P < 0.05, \&\&P < 0.01, as compared to the healthy control. HC – healthy control, CD – Crohn’s disease, UC – ulcerative colitis.
Figure 1
Figure 2
Figure 3
Figure 4

(A) Timeline of the experimental protocol

- **D0 – TNBS 150 mg kg\(^{-1}\)**
- **D11 – TNBS 75 mg kg\(^{-1}\)**
- **Severe period**
- **Recovery period**
- **Relapse**
- **Endpoint D14**

(B) Macroscopic score

- **Control**
- **TNBS**
- **TNBS + EMDB-1**

(C) Ulcer score

(D) Bowel thickness (mm)

(E) MPO activity [μU/g tissue]

(F) Body weight change [% of day 0]

(G) Relative OD

- **TNFα**
- **IL-1β**
Figure 5
Figure 7

A) GLP-2 [pg/mg tissue]

B) Relative expression of GLP2R

C) Relative expression of DPP IV
Figure 8