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Novel peptide inhibitor of dipeptidyl peptidase IV (Tyr-Pro-D-Ala-NH_2) with anti-inflammatory activity in the mouse models of colitis

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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 (Tyr-Pro-D-ClPhe-Phe-NH_2) is a potent blocker of DPP IV and exhibits an anti-inflammatory activity in vivo. The aim of this study was to design, synthesize and characterize the therapeutic activity and mechanism of action of a series of novel EMDB-1 analogs. The inhibitory potential of all peptides was evaluated using the fluorometric screening assay employing Gly-Pro-Aminomethylcoumarin (AMC) to measure DPP IV activity. Consequently, one compound, namely DI-1 was selected and its therapeutic activity evaluated using mouse models of experimental colitis (induced by TNBS and DSS). Macro- and microscopic score, ulcer score, colonic wall thickness as well as myeloperoxidase activity were measured. We showed that DI-1 blocks DPP IV in vitro (IC_{50} = 0.76 ± 0.04 nM) and attenuates acute, semi-chronic and relapsing TNBS- as well as DSS-induced colitis in mice after topical administration. Its anti-inflammatory action is associated with the increase of colonic GLP-2 but not GLP2 receptor or DPP IV expression. Our results validate DPP IV as a pharmacological target for the anti-IBD drugs and its inhibitors, such as DI-1, have the potential to become valuable anti-inflammatory therapeutics.

1. Introduction

Inflammation is a complex biological response to pathogens and other irritant stimuli that engages primarily the immune system, but also involves the activity of the nervous system and hormonal, paracrine and autocrine signaling. One of the examples of chronic inflammation are inflammatory bowel diseases (IBD), a group of chronic bowel disorders represented by Crohn's disease (CD) and ulcerative colitis (UC). The pathogenesis of IBD is still not fully understood and it is associated with generalized immune imbalance characterized by extensive infiltration of macrophages, neutrophils and T cells into the intestinal epithelium and overproduction of proinflammatory cytokines (e.g. tumor necrosis factor α; TNFα, interleukin 1β; IL-1β) [1]. In IBD, a prolonged cytokine imbalance leads to a chronic intestinal inflammation, which results in clinical manifestations and inflammatory lesions in the intestinal wall. Both, pharmacological treatment (non-steroid anti-inflammatory drugs (NSAID), corticosteroids or anti-TNFα and anti-$\alpha_4\beta_7$ integrin antibodies) as well as surgical procedures may be applied in IBD patients [1,2]. However, all available treatment options, especially in a prolonged administration, may bring major side effects.

Recent studies suggest that the etiology of IBD also involves disruption of the intestinal epithelial barrier. Intestinal epithelial cells constitute a specific form of a physical, chemical, and immune barrier between the external and internal environment. In healthy individuals, the mucus layer protects the epithelium and the layers below from luminal bacteria [3]. Any damage to this layer may lead to an increased inflammatory process. Disturbance in the epithelial barrier seems to be the

Abbreviations: AMC, aminomethylcoumarin; CD, Crohn's disease; DIP, diprotin; DMSO, dimethyl sulfoxide; DPP, IV dipeptidyl peptidase IV; DSS, dextran sulfate sodium; EM-2, endomorphin-2; GAPDH, glycerylaldehyde-3-phosphate dehydrogenase; GI, gastrointestinal; GLP-1, glucagon-like peptide 1; GLP-2, glucagon-like peptide 2; GLP2R, glucagon-like peptide 2 receptor; HRP, horseradish peroxidase; HTAB, hexadecyltrimethylammonium bromide; IBD, inflammatory bowel diseases; i.c., intracolonic; IL, interleukin; MPO, myeloperoxidase; NSAID, non-steroidal anti-inflammatory drug; PBS, phosphate buffered saline; PETIR, PEptidase-Targeted ImunoRegulation; TNBS, trinitrobenzene sulfonic acid; TNFα, tumor necrosis factor α; UC, ulcerative colitis

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key element in the onset of IBD and, subsequently, in the frequent relapses. One of the factors responsible for the maintenance of epithelial barrier integrity are incretin hormones, including glucagon-like peptide 1 (GLP-1) and 2 (GLP-2) produced by the enteroendocrine L-cells of the small intestine and the colon [4]. GLP-2, a 33-amino acid peptide released by the neuroendocrine convertase 1 from proglucagon is one of the modulators of the gut function which primarily affects the intestinal weight gain, mucosal development, and intestinal integrity [4]. It is a potent intestinotrophic growth factor, which stimulates crypt cell proliferation and inhibits crypt cell apoptosis [5]. GLP-2 has been also shown to attenuate small intestinal and colonic injury in various animal models of inflammation, such as trinitrobenzene sulfonic acid (TNBS)-induced ileitis, NSAID-induced enteritis, necrotic colitis, postoperative ileus and dextran sulfate sodium (DSS)-induced colitis [6–10].

In both rodents and humans GLP-2 is rapidly degraded by specific protease which renders its half-life after administration relatively short ($t_{1/2}$ = 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) [11]. DPP IV is ubiquitously expressed on the surface of epithelial cells including human intestine, bone marrow and kidney. Soluble DPP IV has also been detected in human plasma and other body fluids [11,12].

Here, we have undertaken to design and synthesize a series of novel peptide inhibitors of DPP IV that target this enzyme in the colon and lead to the increase of GLP-2 level thus restoring the immunological balance and alleviating macro- and microscopic damage of the large intestine.

Recently we have reported a novel peptide inhibitor of DPP IV (EMDB-1) based on the structure of a natural substrate of this enzyme, endomorphin-2 (EM-2) [12,13]. It effectively blocked the activity of DPP IV in vitro, significantly improved colitis in mice and caused a substantial increase in the GLP-2 level in the colon tissue. Encouraged by these results we used EMDB-1 as a scaffold for design and synthesis of novel peptides with similar activity (Table 1). We found that one of the compounds (Tyr-Pro-D-Ala-NH_2; DPP IV inhibitor 1; DI-1) significantly inhibited DPP IV in vitro and did not exhibit affinity towards μ- and δ-opioid receptors. Consequently, we performed in silico docking simulations to determine which amino acid residues in the molecule of DPP IV contribute to the binding to DI-1. To determine the therapeutic activity of DI-1 we used mouse models of colitis induced by TNBS and DSS. Moreover, we characterized the expression of GLP-2, GLP2 receptor (GLP2R) and DPP IV in the control, colitic and DI-1-treated animals.

2. Materials and methods

2.1. Radioligand binding studies

Receptor binding assay was performed as described previously [14] with some modifications. Crude membrane preparations, isolated from Wistar rat brains, were incubated at 25 °C for 60 min with 0.5 nM [^{3}H] DAMGO to μ-opioid receptor and [Ile^{5,6}]deltorphin-2 to δ-opioid receptors in a total volume of 1 ml of 50 mM Tris − HCl (pH 7.4) containing bovine serum albumin (BSA) (1 mg/ml), bacitracin (50 mg/ml), bestatin (30 mM) and captopril (10 mM). All reactions were carried out in duplicate, at 10 μM peptide concentration. Incubations were terminated by rapid filtration through GF/B Whatman glass fiber strips, using Brandel 24 Sample Semi-Auto Harvester. The filters were washed with 2 ml of ice-cold saline solution and the bound radioactivity was measured in the liquid scintillation counter MicroBeta LS, TriLux (ParkinElmer). Nonspecific binding was determined in the presence of naltrexone hydrochloride (10 mM). The data were analyzed by a nonlinear least square regression analysis computer program Prism Graph Pad.

2.2. Determination of the inhibitory activity in vitro

2.2.1. Isothermal calorimetry

Measurements were performed using Nano ITC Isothermal Titration Calorimeter (TA Instruments, Lindon, UT, USA) characterized by 950 μL of sample cell volume with a 250 μL dosing syringe. During all experiments, the reference cell was filled with degassed water. Before starting the measurement, the system was equilibrated at the desired temperature. The stirring rate provided by the injector paddle rotation was set to 250 rpm and it was kept constant. All experiments were carried at 37 °C using 50 mM TRIS − HCl pH 8.0, 5% DMSO. The measurements were performed according to the method described by Freire et al. [15]. In brief, the method links the instrument registered heat flow with the rate of enzymatic reaction taking place in the sample cell, according to the equation:

$$\frac{dQ}{dt} = \frac{d[P]}{dt} \times V_0 \times \Delta H$$

where dQ/dt stands for heat (Q) generated during time t which in practice is measured as a level of heat flow signal registered after the substrate injection, d[P]/dt represents the change in product concentration during time t, V_0 defines reaction volume which was equal to calorimeter measuring vessel volume and ΔH is the molar enthalpy of the particular reaction at used conditions. The technique was proven to be fast, sensitive, accurate, and straightforward method for determination of enzyme inhibitors potency [16,17]. The apparent molar enthalpy (ΔH) of the DPP IV (isolated from porcine kidney, Sigma) catalyzed cleavage of EM-2 as a substrate was determined by measuring heat generated after a single injection of 10 u L 2 mM EM-2 into measuring vessel filled with a solution containing 0.1 U of the enzyme. Enzyme saturation curve was constructed by measuring and averaging the heat flow (dQ/dt) signal registered at a series of different substrate concentrations. A range of substrate concentration (20–225 μM) was achieved during a single experiment by a series of cumulative, 10 u L injections of 2 mM EM2 into a solution containing 0.04 U of DPP IV. For inhibition analysis, both solutions were supplemented with 500 μM of tested inhibitors or 50 μM diprotin A (DIP) as a reference. The resulting

Table 1
Chemical data, inhibition of DPPIV in vitro as well as μ- and δ-opioid receptor binding affinities of EMDB-1 and its novel analogs.

Peptide number	Sequence	MW	μ-opioid receptor IC_{50} ± SEM (nM)[b]	δ-opioid receptor IC_{50} ± SEM (nM)[b]	DPP IV % Inhibition ± SEM[a]
	Tyr-Pro-D-Phe(4-Cl)-Phe-NH_2	605	> 1000	No data	63.37 ± 7.75 [12,13]
1	Tyr-Pro-D-Ala-NH_2 (DI-1)	348	> 2000	> 3000	60.36 ± 4.75
2	Tyr-NMeAla-D-Phe(4-Cl)-Phe-NH_2	594	175.30 ± 7.52	> 3000	60.76 ± 9.42
3	Tyr-Pro-D-Phe(4-Cl)-NMePhe-NH_2	620	420.90 ± 15.24	832.50 ± 74.26	3.14 ± 27.56
4	Tyr-NMeAla-Ala-NH_2	336	> 1000	> 5000	15.72 ± 22.51
5	Phe-Pro-D-Phe(4-Cl)-Phe-NH_2	590	No data	No data	< 1
6	Phe-Pro-Ala-NH_2	332	5.32 ± 0.19	> 2000	< 1

[a] Mean ± SEM of five independent reactions performed in triplicate at the concentration of 26 μM.
[b] Displacement of [^{3}H]DAMGO or [Ile^{5,6}]deltorphin-2 used as MOR and DOR-selective radioligands, respectively. Mean ± SEM of three independent experiments performed in duplicate.