**Final Report**

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# Imidazoline 1 receptor - sphingosine 1-phosphate interaction in the gut : novel targets for the therapy of colonic inflammation?

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## 1. Introduction

Our knowledge of inflammatory bowel diseases (IBDs) has greatly expanded over the last years, and it has become evident that they result from a combination of different environmental, microbial, immunological and genetic factors (1,2). Unfortunately, due to the complex and still insufficiently understood pathogenesis a causal therapy for IBDs is lacking and a lot of effort is currently put into finding new therapeutic approaches.

The main aim of this study was to find novel targets to treat colonic inflammation. Based on our preliminary findings (clonidine aggravates dextran sulfate sodium (DSS)-induced colitis in mice) and on literature data (imidazoline receptors and ligands are abundant in the gastrointestinal tract, imidazoline drugs influence gastric mucosal integrity, imidazoline receptors may be linked to the sphingosine 1-phosphate (S1P) receptors, which have a pivotal role in immune cell trafficking) (3-8) we hypothesized that imidazoline I1 receptors (I1-IRs) might be involved in the pathomechanism of intestinal inflammation and might represent promising targets to treat IBDs.

Thus, our original aim was to clarify whether synthetic or endogenous I1-IR ligands are able to modulate the development and/or restoration of murine colonic inflammation, and in case of positive findings to analyse whether the effects of imidazoline ligands are - at least partly - mediated by S1P receptors.

## 2. Results

### 2.1. Analysing the role of I1-IRs in the pathomechanism of DSS-induced colitis

Our first goal was to establish the appropriate acute DSS protocol, because according to our experience and to literature data the DSS-induced inflammatory reaction strongly depends on multiple factors, including the concentration of DSS, the duration of DSS exposure or the genetic background of animals (9). After testing various protocols we have decided to use C57BL/6 mice and to treat them with 2.5 % DSS for 7 days, which was followed by 2 days

recovery. With this protocol DSS induced diarrhea, rectal bleeding and weight loss, which were accompanied by typical histopathological signs of inflammation and tissue damage, significant reduction of colon length and elevation of myeloperoxidase (MPO) and interleukin-6 (IL-6) levels measured in the colon wall and serum, respectively.

After choosing the appropriate DSS protocol a wide range of I1-IR ligands were tested, including moxonidine and rilmenidine (synthetic I1-IR agonists), AGN 192403 and efaroxan (synthetic I1-IR partial agonist and antagonist, respectively) as well as agmatine and harmane (endogenous I1-IR agonists) (10). All applied dose ranges were chosen based on our previous studies and on literature data. In general, doses of test compounds were in the range of 0.01 - 10 mg/kg, but agmatine was tested up to a dose of 100 mg/kg, which is the most commonly reported effective systemic dose of this compound (11). Although our first results suggested that some of these drugs may have some influence on the development of colonic inflammation, the observed effects were weak, not dose-dependent and/or not reproducible. Similar negative results were obtained after changing the route of administration (intraperitoneal, intragastric) or the treatment regimen (once or twice daily injection). Thus, our comprehensive analysis revealed that - in spite of the premises - I1-IRs are not involved in the pathomechanism of DSS-induced intestinal inflammation, and also supported some previous studies suggesting that these receptors may have limited (patho)physiological importance in the digestive tract.

On the other hand, from a clinical aspect, these results also suggested that imidazoline drugs probably do not have any harmful effect on gut inflammation. This is important and clinically relevant, because I1-IR agonists moxonidine and rilmenidine are widely used as antihypertensive drugs (12, 13), and agmatine is becoming increasingly popular as a bodybuilding supplement (14), but to our best knowledge there is no preclinical or clinical evidence that they have any beneficial or detrimental effect on patients with IBDs.

*Publication*

*Fehér Á., Tóth V.E., Al-Khrasani M., Balogh M., Lázár B., Helyes Z., Gyires K., Zádori Z.S. Analysing the effect of I1 imidazoline receptor ligands on DSS-induced acute colitis in mice. Inflammopharmacology, under minor revision (IF: 2.304)*

**2.2. Analysing the effect of clonidine on DSS-induced colitis**

The hypothesis that I1-IRs might be involved in the pathomechanism of colitis was based partly on our preliminary findings that clonidine, an I1-IR agonist aggravated DSS-induced intestinal

inflammation. After excluding the role of I1-IRs in the pathomechanism of DSS-colitis, in the second half of the project we aimed to identify the exact receptor mediating the damaging effect of clonidine. Because this compound has comparable affinity for I1-IRs and alpha$_2$-adrenoceptors (15), we focused on the latter ones and by using genetically engineered animals (alpha$_{2A}$-, alpha$_{2B}$- and alpha$_{2C}$-adrenoceptor subtype knockout mice) and alpha$_2$-adrenoceptor subtype selective antagonists we were able to identify the alpha$_{2A}$ subtype, which mediates the observed proinflammatory action. We also demonstrated that pharmacological blockade of this receptor significantly delays the development of colitis, reduces the colonic levels of MPO and chemokine (C-C motif) ligand 3, chemokine (C-X-C motif) ligand 2 (CXCL2), CXCL13, and granulocyte colony stimulating factor, and elevates that of tissue inhibitor of metalloproteinases-1 (TIMP-1).

Our results are in good accordance with previous findings (16) and suggest that alpha$_2$-adrenoceptors are overactive in the acute phase of colitis and contribute to a proinflammatory mileu. We demonstrated for the first time that among the three alpha$_2$-adrenoceptor subtypes the alpha$_{2A}$ one is primarily involved in the modulation of inflammation and these findings suggest that selective pharmacological blockade of this subtype may serve as a novel therapeutic option to treat colitis.

*Publication*

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**2.3. Analysing the role of I1-IRs in the pathomechanism of TNBS-induced colitis**

In order to completely rule out the role of I1-IRs in the pathomechanism of IBDs, besides using the innate immunity-driven DSS-colitis model we also analysed the effect of imidazoline ligands on 2,4,6-trinitrobenzene sulphonic acid (TNBS)-induced inflammation, which mainly depends on the adaptive immune functions. These two models differ in several aspects, such as pathomechanism, clinical/histological picture and cytokine profile (17, 18), and it is possible that a drug is able to influence the development of inflammation in one but not in another model (19).

As in case of DSS-colitis, we first aimed to establish the appropriate TNBS protocol, because the inflammatory response strongly depends on the amount of applied TNBS, on the concentration of ethanol used as a vehicle, or on the genetic background of animals (20). After testing various protocols and monitoring the different signs of disease, including colitis-induced weight loss and increased mortality, we chose the highly susceptible BALB/c strain and treated them with 3 mg of TNBS dissolved in 40% ethanol after 24 h fasting. 72 h after the intracolonic injection of TNBS mice were sacrificed, the macroscopic signs of colonic and systemic inflammation were assessed and serum samples and full-thickness colon specimens were collected for further cytokine analysis.

After choosing this protocol we analysed the effect of different doses of moxonidine, rilmenidine, AGN 192403 and agmatine. Although animals treated with these drugs showed some tendency towards an aggravated inflammatory response (e.g. more pronounced weight loss), the observed changes did not reach statistical significance.

Because TNBS-induced acute inflammation resolved within 72 h in several animals who survived the first day after the exposure, we also analysed the effect of imidazoline compounds one day earlier, i.e. 48 h after the administration of TNBS, at the peak of the inflammatory reaction. However, similar results were found as with the other protocol, namely, none of the tested I1-IR ligands had any significant impact on the parameters of TNBS-evoked inflammatory disease.

Thus, these results provided further evidence against the role of I1-IRs in the pathomechanism of colitis and also suggested that pharmacological modulation of these receptors (e.g. with IR agonist antihypertensive agents) do not have any influence on the severity of gut inflammation in patients with IBDs.

Although earlier radioligand binding studies provided evidence for the presence of non-adrenergic imidazoline binding sites throughout the gastrointestinal tract (3, 4, 21) including the colon (22), the inability of I1-IR ligands to affect DSS- and TNBS-colitis in spite of their gastroprotective (8) and anti-inflammatory action (23) prompted us to determine the expression of I1-IRs in the large intestine. This task, however, was not easily achieved because despite of intensive research the molecular identity of IRs still remains to be identified. To date the best candidate protein for I1-IR is nischarin, a novel soluble intracellular protein that is capable of interacting with the cytoplasmic domain of the integrin α5 subunit of the fibronectin receptor (24). It was shown that nischarin is a functional I1-IR or is at least associated with its signalling

pathway (25, 26). Thus, we aimed to determine whether nischarin protein is expressed in the colon, which has never been addressed before. By using Western blot technique we identified nischarin in the brain (this was used as a positive control) and stomach of NMRI and C57BL/6 mice, but not in the colon. This result was somewhat surprising, because both nischarin mRNA and protein showed an ubiquitous expression pattern in the rat (27). The absence of nischarin in the mouse colon may provide a molecular explanation for the inability of I1-IR ligands to influence colitis. However, it should be borne in mind that due to uncertainty regarding the exact relationship between nischarin and I1-IR (28), the lack of nischarin signal in the colon only strongly suggests, but does not necessarily mean the absence of I1-IRs.

*Publication (under preparation) - Zádori Z.S. et al. Evidence against the role of I1 imidazoline receptor/nischarin in the pathomechanism of TNBS colitis*

**2.4. Analysing the effect of I1-IR ligands on intestinal peristalsis and contractility**

It is well-established that chronic inflammation of the gut causes wide-ranging clinical symptoms including nausea, anemia, diarrhea and abdominal pain (1). Although patients with IBDs are mainly treated with anti-inflammatory agents, alleviation of these accompanying symptoms may also be important in the therapy. There is some evidence that IRs might be involved in the regulation of intestinal motility, but the results are conflicting (29 - 32). Hence, in the second half of the project we also aimed to analyse whether I1-IR ligands can inhibit gut peristalsis, which could be exploited in the therapy of patients suffering from inflammation-associated hypermotility and diarrhea.

We evaluated the effect of clonidine, rilmenidine and AGN 192403 on small intestinal peristalsis *in vivo* in mice (by using the charcoal meal method) and *ex vivo* in guinea pigs, as well as on colonic contractility *ex vivo* in mice. We found, that clonidine and rilmenidine induced a dose-dependent inhibitory effect on intestinal peristalsis in both mice and guinea pigs, whereas AGN 192403 was ineffective and also failed to influence the effect of clonidine. On the other hand, pharmacological or genetic blockade of the alpha$_{2A}$-adrenoceptors inhibited completely the effect of both clonidine and rilmenidine. Thus, our results clearly demonstrated that I1-IRs are not involved in the regulation of peristalsis at the level of small intestines and the inhibitory effect of mixed I1-IR/alpha$_2$-adrenoceptor ligands is mediated entirely by alpha$_{2A}$-adrenoceptors.

Interestingly, at the level of colon we found that besides alpha$_{2A}$-adrenoceptors other receptors may also contribute to the inhibitory action of clonidine, because it was still able to significantly

reduce the amplitudes of electric field stimulation (EFS)-induced colonic contractions in alpha$_{2A}$-adrenoceptor knockout mice, or in the presence of different alpha$_2$/IR antagonists. Because there is a strong homology in the amino acid sequence between alpha$_2$-adrenoceptors and 5HT$_{1A}$ receptors (33) and several alpha$_2$-adrenoceptor ligands bind with a significant affinity to the latter receptors (34), we aimed to investigate whether 5HT$_{1A}$ receptors contribute to the inhibitory effect of alpha$_2$-adrenoceptor ligands on colonic contractility. This question has never been addressed before and the exact role of 5HT$_{1A}$ receptors in the control of colonic motility is still enigmatic (35). By analysing the effect of clonidine and a novel alpha$_2$-adrenoceptor ligand (allyphenyline) endowed with peculiar pharmacological profile and provided by Prof. Maria Pigini (Camerino, Italy) we demonstrated that activation of 5HT$_{1A}$ receptors induces potent gastroprotective action and inhibits gastric (fundic) contractions, but does not modulate cholinergic contractions in the murine colon. Hence, although the exact receptor mediating the inhibitory effect of clonidine and allyphenyline on colonic contractions still remains to be identified, we provided evidence for the important role of central and peripheral 5HT$_{1A}$ receptors in the regulation of different upper gastrointestinal functions.

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*Fehér Á., Holzer-Petsche U., Liebmann I., Holzer P., Gyires K., Zádori Z.S. Analysing the role of imidazoline receptors in the regulation of intestinal peristalsis. Central European Journal of Gastroenterology and Hepatology 2(Suppl.1):84, 2016 (abstract)*

*Zádori Z.S., Fehér Á., Tóth V.E., Al-Khrasani M., Köles L., Sipos S., Del Bello F., Pigini M., Gyires K. Dual Alpha2C/5HT1A Receptor Agonist Allyphenyline Induces Gastroprotection and Inhibits Fundic and Colonic Contractility. Dig Dis Sci 61:1512-1523, 2016 (IF: 2.516)*

**2.5. Review articles on the pathomechanism and novel therapeutic options of IBDs**

Our studies with different colitis models and the exponentially rising number of published papers in this field have prompted us to review and summarize the current state of knowledge on the pathomechanism of IBDs. We also aimed to discuss the newest and most promising therapeutic avenues for these chronic disorders, including various biologics (e.g. inhibitors of proinflammatory cytokines or different dowstream signalling pathways, growth factors, etc.), modulators of the endocannabinoid system or stem cells. The review on the potential role of stem cells in the therapy of various inflammatory diseases, including IBDs, contains our latest results showing that human dental pulp stem cells (hDPSCs, provided by Prof. Gábor Varga)

injected intravenously delay the development of DSS-induced colitis and may represent a promising approach to treat IBDs.

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*Gyires K., Tóth É.V., Zádori S.Z. Gut inflammation: current update on pathophysiology, molecular mechanism and pharmacological treatment modalities. Curr Pharm Des 20:1063-1081, 2014. (IF: 3.452)*

*Gyires K., Zádori Z.S. Role of cannabinoids in gastrointestinal mucosal defense and inflammation. Curr Neuropharmacol, in press (IF: 3.753)*

*Földes A., Kádár K., Kerémi B., Zsembery Á., Gyires K., Zádori Z.S., Varga G. Mesenchymal stem cells of dental origin - their potential for anti-inflammatory and regenerative actions in brain and gut damage. Curr Neuropharmacol, in press (IF: 3.753)*

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