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SUMMARY OF DOCTORAL DISSERTATION

"Synthesis and biological studies of endogenous enkephalinase inhibitors and their analogues as potential therapeutic substances in therapy of inflammatory bowel disease and visceral pain"

Pharmacological treatment and/or maintenance of remission in inflammatory bowel disease (IBD) which includes Crohn's disease (CD) and ulcerative colitis (UC) is currently one of the biggest challenges in the field of gastroenterology. The main goal in anti-IBD therapy includes management of inflammation and alleviation of other clinical symptoms like intestinal motility disorder or visceral pain. One of the recent trends in the research of new forms of IBD therapy focuses on the endogenous opioid system. Endogenous opioid peptides such as enkephalins (Met-enkephalin and Leu-enkephalin) participate in the antinociception [1], regulation of gastrointestinal motility [2], regulation of the immune system [3, 4], cardiovascular system [5, 6] anti-inflammatory, hormonal and behavioural responses [7,8]. The action of endogenous opioids in the living organism is strongly regulated by their metabolism and the half-life of enkephalins in the human plasma can be measured within minutes [9], which significantly hampers their pharmacological application. Aminopeptidase N (APN), neutral endopeptidase (NEP), dipeptidyl peptidase III (DPP III) and angiotensin-converting enzyme (ACE) are the major enkephalin-degrading enzymes. These proteases are widely distributed in the human body and are significantly involved in physiological modulation and pathophysiological processes in the gastrointestinal tract [10]. Rat sialorphin (Gln-His-Asn-Pro-Arg), human opiorphin (Gln-Arg-Phe-Ser-Arg) and bovine spinorphine (Leu-Val-Val-Tyr-Pro-Trp-Thr) are endogenous inhibitors of enkephalin-degrading enzymes. Studies have confirmed the efficacy of sialorphin, opiorphin and spinorphine in blocking the activity of enkephalinase both *in vitro* and *in vivo*. It has been demonstrated that these

inhibitors have a strong analgesic, anti-inflammatory, immunological and metabolic effect either directly or indirectly by affecting the level of enkephalins [11 – 14]. Indirect activation of opioid receptors by sialorphin, opiorphin and spinorphine has two major advantages: enkephalinase inhibition does not produce any undesired effects accompaniying systemic application of opioids and restritcs opioid activity in time and space enabling a better control of pharmacological activity. Following the literature reports related to the topic of sialorphin, opiorphin and spinorphin, it can be noticed that the discovery and understanding of endogenous enkephalinase inhibitors have been carried out to design strong and stable APN and NEP inhibitors.

As part of the PhD thesis I synthesised Leu/Met-enkephalin, endogenous enkephalinase inhibitors and their analogues. I conducted a study on the influence of sialorphin, opiorphin, spinorphin as well as their analogues on the degradation of enkephalins, evaluation of susceptibility of peptide inhibitors of enkephalinase to metabolic degradation in human plasma and enzymatic stability relative to NEP. A study on molecular modelling *in silico* interactions of endogenous inhibitors of enkephalinase and their analogues with APN and NEP was then carried out as well as an anti-inflammatory characteristic of the endogenous enkephalinase inhibitors and their analogues. Furthermore, it involved a research on antinociceptive activity of enkephalins and enkephalinase inhibitors in a mouse model of visceral pain.

In the first stage of my dissertation, I synthesised Leu-/Met-enkephalin, peptides inhibitors of enkephalinase: sialoriphin, opiorphin, and spinorphin and I designed and synthesised as many as 74 analogues of enkephalinase inhibitors. The peptides were synthesised on the 2-chlorotrityl chloride resin and amide resin by standard solid phase synthesis (SPPS, Fmoc/tBu strategy). The products were cleaved from the resin in strong acidic conditions, purified with RP-HPLC and identified using MALDI-TOF MS and RP-HPLC. I tested the collected peptides (purity above 95% by RP-HPLC) for their effect on the degradation of enkephalins by NEP and APN using RP-HPLC *in vitro*. In the next step, I determined a metabolic stability characteristic in the human plasma against sialorphin, opiorphin and spinorphin and their five analogues. Moreover, I established an *in vitro* characteristic of the enzymatic stability relative to NEP for sialorphin and its synthetic analogue (Palm-Lys-Lys-Gln-His-Asn-Pro-Arg) which displayed a higher inhibitory potency against NEP than the parent compound and resulted in a stronger anti-inflammatory effect in a CD mouse model (acute model).

In the next phase of the research, the molecular modelling of *in silico* interactions of endogenous inhibitors of enkephalinase and their analogues with APN and NEP was developed under cooperation with Dr. Giełdoń from the University of Gdansk. The computer simulations allowed to explain the reason behind the growth and/or depressed activity of the peptides in relation to APN and NEP.

The research was conducted using different animal models of IBD for tsialorphin, opiorphin, spinorphin and their four analogues of the best pharmacological profile among the compounds. It was conducted in cooperation with Prof. Dr. hab. Jakub Fichna and Dr. Maciej Sałaga from the Medical University of Lodz.

Sialorphin in the mouse model of CD (acute, semichronic and relapsing models) revealed the anti-inflammatory effects. The activity of the sialorphin in the acute model was stronger than the classic one used in the IBD (5-ASA) and was blocked by MOR and KOR receptor antagonists. Sialorphin has no effect on the anti-inflammatory activity in the mouse model of UC. Among the *in vivo* analogues of the endogenous enkephalinase inhibitors in the mouse; acute model of CD, a strong anti-inflammatory activity was exhibited by the Palm-Lys-Gln-His-Asn-Pro-Arg peptide. Injection of the Palm-Lys-Lys-Arg-Phe-Ser-Arg opiorphin analogue did not affect the inflammatory state.

In addition, to emphasize the importance of research and translate observations from animals to human conditions, the levels of APN and NEP expression were assessed in patients with IBD. It has been demonstrated that the relative expression of APN mRNA in the colonic tissue increases for patients with CD and UC. On the other hand, the expression of NEP mRNA in the colonic tissue does not change in CD and tends to decrease for UC patients. Expression in the colon did not show any significant variations in the level of APN. In contrast, the expression of NEP protein was elevated in CD patients. Determination of the APN and NEP levels in the serum did not show any differences.

In the studies of the animal model of visceral pain, only enkephalins and sialorphin demonstrated tanalgesic effects.

The results of this research show that the modulation of enkephalin levels by inhibiting enzymes involved in their degradation may be an attractive alternative to currently used anti-inflammatory drugs. Sialorphin and its analogue, Palm-Lys-Lys-Gln-His-Asn-Pro-Arg, alleviate symptoms of functional and inflammatory diseases of the gastrointestinal tract and

provide a solid foundations for further research on enkephalinase as a potential therapeutic target in IBD therapy.

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