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REVIEW ARTICLE

Human Opiorphin an Endogenous Inhibitor of Enkephalin-Inactivating Ectopeptidases that Displays Antinociception: A Review

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Abstract: ***Background:*** The most important investigations since the first publication on opiorphin, in 2006, are summarized in this review. Opiorphin is a human endogenous pentapeptide (Gln-Arg-Phe-Ser-Arg) which inhibits both the Neutral EndoPeptidase (NEP) and aminopeptidase N (AP-N), protecting enkephalins from degradation by these ectopeptidases, thus activating endogenous enkephalin-related opioid pathways.

Objective: In this review we highlight the *in vitro* and *in vivo* effects of opiorphin.

Results: We examine in detail the structure-activity relationship studies addressed in order to identify the amino acids of opiorphin sequence required to functional interactions with its targets and to design its metabolically stable analogs. The modulation of emotional-related behaviors and effects on smooth muscle motility including their resulting therapeutic implication are also analysed. In addition, the *in vitro* potency of opiorphin and its metabolically stable analogs on opioid receptor binding by enkephalin-related peptides are also summarized. Finally, quantitative or semi-quantitative analytical methods of opiorphin on various biological samples are cited.

Conclusion: The advantages and difficulties of therapeutic applications of opiorphin peptide and its synthetic derivatives are discussed.

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1. INTRODUCTION

To our knowledge, since the first publication on the discovery of opiorphin by Wisner *et al.*, in 2006 [1], more than 30 papers have been published within 10 years. This wealth of scientific data justifies, to summarize, discuss and evaluate, the results obtained so far. To appreciate the importance of opiorphin discovery as a potent antinociceptive peptide we must remind of the most therapeutically applied analgesic drug, the morphine, which has a lot of side effects, among them, nausea, dyspnoe, constipation, tolerance and dependence [2].

The opiorphin era began with the above-cited paper of Rougeot and coworkers [1], where they announced, for the first time, that human opiorphin is "a natural antinociceptive modulator of opioid-dependent pathways". Opiorphin is a peptide of five amino acids present under 2 forms of sequence Gln-Arg-Phe-Ser-Arg and pGlu-Arg-Phe-Ser-Arg (Fig. **1**). It was first extracted, purified and characterized by a functional biochemical approach from human saliva. The gene, currently named *PROL1* gene, coding the PRL1 precursor protein of opiorphin mature peptide product, was identified by Dickinson *et al*. in 1996; authors also reported that the gene is expressed in human lachrymal and salivary glands [3]. Almost 20 years later, opiorphin peptide was found and quantified in addition to saliva and human lacrimal tears, in seminal fluid, milk, blood and urines of young adult volunteers, showing that it is synthetized, secreted and distributed as a paracrine/autocrine and/or

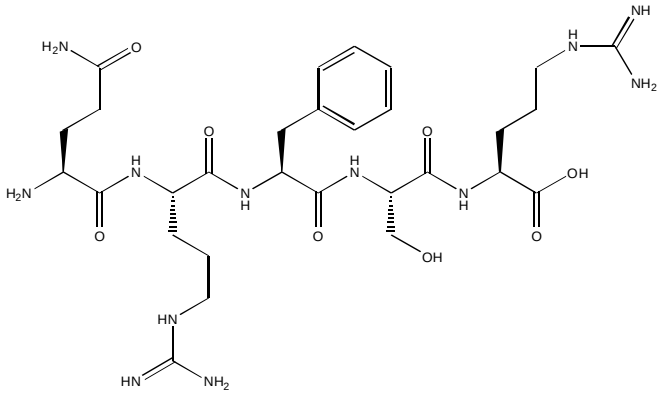

Fig. (1). Chemical structure of opiorphin.

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exocrine peptide messenger [4]. It is also interesting to note that database searches of the human transcriptome reveal that the *PROL1* gene is expressed in the male and female reproductive systems, as well as in human brain as a neuroendocrine messenger.

Previously to the characterization of human opiorphin, two similarly acting natural inhibitors of the rat membrane-bound neutral endopeptidase with analgesic activity were also detected: spinorphin, a heptapeptide extracted from bovine spinal cord [5] and the sialorphin QHNPR-peptide, the functional homolog of human opiorphin in rat [6].

2. OPIORPHIN, AN ENHANCER OF ENKEPHALIN-MEDIATED CONTROL OF PAIN PERCEPTION

The inhibitory effect of opiorphin on both enkephalin-catabolizing Zn-ectopeptidases, the human neutral endopeptidase (hNEP; EC 3.4.24.11) and human aminopeptidase (hAP-N; EC 3.4.11.2) was first reported [1]. The overall *in vitro* data shows that the inhibitory potency of opiorphin for hNEP and hAP-N was half-maximal at doses ranging from 10 to 50 µM depending on enzyme conformation (native membrane-anchored or recombinant soluble enzymes), on substrates (physiological or fluorogenic synthetic substrates) and detection methods (FRET peptide-based or radiometric-based assays) [1, 7]. This inhibitory action of opiorphin made it possible to protect Met-enkephalin from degradation by these membrane-anchored enzymes [1] and thus to prolong the short lasting activation of enkephalin-like peptides on the opioid receptors. As a result, *in vitro* opiorphin significantly improves the specific binding and affinity of enkephalin-related peptides to rat brain membrane opioid receptors without directly interacting with opioid receptors [8, 9] and *in vivo*, the opiorphin exerts an antinociceptive effect as efficiently as morphine. Indeed, on homologous binding experiments it has been demonstrated that opiorphin increases by 40-60% the maximal specific binding and affinity of opioid labeled peptides [H^3]MERF (Tyr-Gly-Gly-Phe-Met-Arg-Phe) and [H^3]MEGY (Met-enkephalin-Gly^6-Tyr^7) to rat brain opioid receptors compared to the classic inhibitor cocktail containing bestatin, captopril, thiorphan and bacitracin [8]. And, *in vivo* using various standard rodent models of pain the analgesic action of 1mg/kg *i.v.* or 5 µg/kg *i.c.v.* opiorphin is equivalent to 2-3 mg/kg *i.v.* or 10 µg/kg *i.c.v.* morphine [1, 10, 11]. These statements were completed by *in vivo* experiments showing that antinociceptive effects of opiorphin require activation of endogenous enkephalin-related MOR (mu-opioid receptor) and DOR (delta-opioid receptor) pathways and that opiorphin is non-addictive, neither produces tolerance or antiperistalsis effects after subchronic administration [10-12].

3. STRUCTURE ACTIVITY RELATIONSHIP (SAR) INVESTIGATIONS AND FUNCTIONAL MIMETIC DESIGN OF OPIORPHIN

The major limitation on the *in vivo* use of peptide drugs is their rapid degradation by circulating peptidases (metabolic half-life of opiorphin evaluated at 5 min, [7]). The following step was, therefore, to search for functional derivatives of opiorphin with improved metabolic stability. And for design them it was necessary to first determine which residues of the opiorphin molecule are required for its ecto-enkephalinase inhibitory potency. Overall SAR data demonstrated that any change in the intra-peptide sequence inhibits or even abolishes at least one of the two inhibitory activities [7, 13]. These findings highlight the existence of a tight structural selectivity in the functional interaction of opiorphin with both human NEP and AP-N targets. One exception concerned the hydroxyl group of the Ser^4 side chain that does not seem to play a critical role for interaction with targets [7].

Among the investigated set of analog peptides the [Cys^0]-opiorphin derivative was more effective on both hAPN and hNEP, than the original peptide, i.e., opiorphin IC_{50}: 8 µM and 30 µM, [Cys^0]-opiorphin IC_{50}: 0.8 µM and 7 µM for hAPN and hNEP, respectively [7]. The Ser^4-O-$[CH_2]_8$ hydrophobic residue substitution makes also the opiorphin molecule 10 more potent against hNEP (IC_{50}: 2 µM) without affecting its inhibitory potency for hAP-N [7]. Otherwise, the biochemical functional screening conjugated to the *in silico* comparative conformational analysis and NMR studies provided a structural explanation for the dual hAP-N and hNEP inhibitory activity of opiorphin and showing that an inborn flexibility of the molecule is required to its activity [13].

Structure-activity relationship (SAR) studies also revealed the key role played by the aromatic side chain of Phe^3 residue of opiorphin for its inhibitory potency toward both hNEP and hAP-N. Indeed, its substitution by an Ala^3 residue led to a compound with completely diminished inhibitory potency toward both hNEP and hAP-N. In addition, replacing L-Phe^3 for D-Phe^3 increased the hAPN inhibition potency by one order of magnitude, however, the inhibition of hNEP was abolished [7, 14].

The aim of SAR investigations was also to improve the metabolic stability of opiorphin against serum peptidases without loss or better with increasing its inhibitory potency against both the hAPN and hNEP. This was achieved in three steps: first by the addition of a Cys-thiol group, then by replacement of the first labile peptide bond by a $[CH_2]_6$ linker, third by substitution of the Ser^4 by Ser-O-$[CH_2]_8$ hydrophobic residues. As a result, the C-[$(CH_2)_6$]-QRF[S-O-$CH_2]_8$]-R peptidomimetic compound displays increased metabolic stability in human plasma. Furthermore, this designed opiorphin analog shows reinforced inhibitory potency toward human AP-N (more than 10-fold increase) and NEP (more than 40-fold increase) activities relative to the QRFSR native peptide. It also retains full analgesic activity in the behavioral formalin-induced rat pain model as it inhibits dose-dependently inflamed paw licking and flinches and body tremors, which were recorded over a 60 min period [7].

Another study reported earlier also showed that changes in the structural conformation of N- and C- terminal amino acids by substitution with non-natural ß-amino acids (ß2hGln-Arg-Phe-Ser-ß3hArg), increase by about 7-fold the metabolic half-life of the modified opiorphin in human plasma while unfortunately reducing by up to 10-fold its inhibitory potency toward both targets [15].

The most recent SAR experiments on opiorphin involved modifications on the polar side chains of Arg^2 and Arg^5 residues and glycosylation at Ser^4 position. None of such modified compounds displayed inhibitory activity for hNEP and hAPN superior to the native opiorphin. Thus, confirming previous results [7] lateral chains of $Arg^{2,}$ Arg^5 and Ser^4 residues appear to be important for inhibition of both hNEP and hAPN. The authors also made NMR conformational studies and presented a computational model structure of a glycoopiorphin [16].

A structural and thermochemical characterization of the conformational change of opiorphin and its derivatives (qRFSR, QrFSR, QRfSR, QRFsR, QRFSr, where the lower case amino acid code indicates its carbon α atom is in D configuration) was explored using a theoretical study of molecular mechanic methods [17]. Among these conformers only the QrFSR epimer (r) had a ß-turn formed between glutamine (Q^1) and serine (S^4) residues. The most energetically common intra-molecular H-bonds of opiorphin occur for the donor-acceptor pair: arginine (R^2) and glutamine (Q^1) [17]

Among the synthetic analogs of human opiorphin previously designed [7] in order to extend the metabolic stability of opiorphin in human plasma, the Cys-[$(CH_2)_6$]-QRF-[Ser-O-octanoyl]-R peptide (monomeric CC_6-opiorphin) and its cystine-dipeptide (dimeric CC_6-opiorphin) derivatives were further investigated in homologous competition experiments [9]. The affinity of [H^3]MERF was significantly increased with both compounds compared to that measured with the classic inhibitor cocktail. In addition, ten times less concentrations of monomeric and dimeric CC6-opiorphin derivatives were sufficient to produce the same effect than native opiorphin in binding experiments (5 µM instead of 50 µM for opiorphin). Similarly, in heterologous displacement experiments with unlabeled dynorphin$_{(1\text{-}10)}$ the affinity and binding increased with both compounds [9].

4. OPIORPHIN, A MODULATOR OF ENKEPHALIN-MEDIATED EMOTION AND SMOOTH MUSCLE CONTRACTION

Most of the opiorphin investigations were based on its anti-nociceptive action via activation of mu-opioid and/or delta-opioid pathways. These data are summarized in the first part of the present review. Since enkephalin-dependent opioid pathways are also implicated in the modulation of emotion-related behaviors (such as depression, panic) and contraction of smooth muscles, soon after the discovery of its analgesic property, a potential action of opiorphin in these behaviors was therefore explored.

The first papers described the antidepressant-like effect of human opiorphin in the standard rat model of forced swim test [18]. The antidepressant-like effect was elicited with 1-2 mg/kg human opiorphin *i.v.* doses and was reversible by the selective delta-opioid receptor antagonist, naltrindole (10 mg/kg *i.p.*). Some other behavioral tests were also performed demonstrating that by modulating the concentrations of endogenous enkephalin released in response to psychological stimuli, opiorphin can improve the certain form of mood disorders, particularly depression without inducing hypo- or hyper-active, anxiogenic- nor anxiolytic-like and amnesic behavioral responses [18].

Similarly, it has been demonstrated that after central administration of 1-6 µg *i.c.v.*/mouse, opiorphin produced an antidepressant-like effect by activating both endogenous MOR and DOR (mu and delta-opioid receptor, respectively) in the forced swim test in mice [19]. Indeed, the opiorphin antidepressant effect is abolished by co-administering the selective delta-opioid receptor antagonist, naltrindole, or the selective mu-opioid antagonist, ß-funaltrexamine. In addition, mice treated with opiophin do not display any convulsive behavior [19].

More recently a panicolytic-like effect was also attributed to opiorphin when given centrally in the dorsal periaqueductal gray (5 nmol, intra-dPAG) or systemically (2 mg/kg *i.v.*) [20]. The behavioral escape responses were investigated using the elevated T-maze and dPAG electrical stimulation tests in rats. The antipanic-like effect of oporphin is mediated by MOR as it is antagonized after local pretreatment with the selective MOR antagonist, CTOP (1 nmol) [20].

Opiorphin effects on motility of smooth muscles were demonstrated in the gastrointestinal and urogenital tracts. Thus, using *in vitro* bioassay, it has been reported that opiorphin causes colonic contraction in a concentration dependent manner (from 10^{-6} to 10^{-4} M concentrations). This opiorphin effect is blocked by naloxone and partially inhibited by ß-funaltrexamine and naltrindole, the MOR and DOR antagonists, respectively. In addition, opiorphin significantly enhanced the Met-enkephalin-induced contractile response, demonstrating the involvement of enkephalin-dependent opioid pathways in the opiorphin-evoked colonic motility [11].

Three years later another team investigated the comparative effect of human opiorphin, and rat sialorphin each of them under their respective 2 natural forms: glutamine 1 (Gln1-Arg-Phe-Ser-Arg and Gln1-His-Asn-Pro-Arg) and pyroglutamate 1 (pGlu1-Arg-Phe-Ser-Arg and pGlu1-His-Asn-Pro-Arg), on mouse ileum motility [21]. According to the *in vitro* assays, only the glutamine1 forms of opiorphin and sialorphin significantly increased electrical field-stimulated ileum contractions in a DOR-dependent manner. This demonstrates that glutamine in position 1 is crucial for their pharmacological action on gastro-intestinal motility [21]. Met-enkephalin and rat sialorphin co-administered intravenously (1mg/kg) significantly inhibited the upper gastrointestinal transit of mouse, but not the same dose of human opiorphin [21].

Effects of human opiorphin and rat sialorphin were also reported on male genital tissues, in particular corporal smooth muscles [22, 23]. In addition, the level of expression of the *PROL1* gene coding opiorphin precursor in corpora cavernosa tissue samples of men with erectile dysfunction is severely down-regulated as compared to normal volunteers [24, 25]. Thus, gene encoding opiorphin precursor could be applied as biomarker of erectile dysfunction. Intracorporal injection of plasmids expressing sialorphin precursor in aging rats or diabetic rats or injection of sialorphin peptide improves relaxation of corporal smooth muscle and thus

erectile function [25, 26]. Applied to preclinical and clinical studies on pre-priapic sickle cell disease that is recognized as a disease of hypoxia, opiorphin-related peptides seem to be regulators of the hypoxic response in corporal smooth muscle cells [23, 27].

Finally, owning to the physiological role of NEP and/or AP-N in turning off hormonal peptides involved in the regulation of cardiovascular system, a recent study was performed to investigate the potential peripheral cardiovascular effects of opiorphin in anesthetized rats. Data showed that opiorphin (300 nmol/200 µg/kg *i.v.*) induces transient blood pressor and tachycardic responses in anesthetized rats (about 40 mmHg and beats/min, respectively). These effects are mediated through the renin-angiotensin system, the sympathetic ganglia and adrenal medulla, but not through the opioid system [28].

5. OPIORPHIN, AN HUMAN ENDOGENOUS MEDIATOR CIRCULATING IN VARIOUS BIOLOGICAL FLUIDS

The first attempt to quantify opiorphin in human saliva by a liquid chromatography-tandem mass spectrometry method (LC-MS) was published in 2011 [29]. Applying this method in human saliva from 14 young healthy subjects (8 females and 6 males) the opiorphin saliva levels ranged from 2.8 to 25.9 ng/ml.

More recently, using a specific ELISA-based method in tandem with RP-HPLC chromatography, opiorphin levels in blood, urine, semen and milk of healthy young adults were precisely determined [4]. In human blood the opiorphin physiological concentrations range from 0.3 to 1.1 ng/ml, depending on gender (higher in males than in females) and on physiological hormonal status (lower in six month pregnant women than non-pregnant volunteers). Opiorphin also circulates in the reproductive system, notably in the semen of normozoospermic donors and in the milk of lactating women [4]. Opiorphin is also secreted in tears and saliva at the highest physiological levels.

The LC-MS analysis has been also used to resolve an important question: could the peripheral circulating opiorphin reach and act on the central nervous system to modulate pain transmission? Thus, the flux of opiorphin was analyzed by LC-MS on the blood-brain barrier (BBB) culture model consisting of rat brain endothelial, glial and pericyte cells [30]. The transfer of opiorphin through the blood-brain barrier was estimated at 3%, which is quite a high amount compared to that measured for other peptides by using a variety of methods [31]. Interestingly, a rapid cyclization of the N-terminal glutamine into pyroglutamate residue during the transfer of opiorphin was also observed [30].

Otherwise, very recently, a sensitive, reliable and improved extraction method for opiorphin detection by MS/MS was applied for quantitative measurement of opiorphin added to equine plasma and urines. The reason of the new determination of opiorphin levels in equine was to help prevent its potential illegal use in the horse racing industry [32].

DISCUSSION

Considering the paper cited in the introduction entitled "Pain and poppies: the good, the bad and the ugly of opioid analgesia" [2], when compared the side effects of morphine to those of opiorphin, the advantages of the latter are striking. Nevertheless, the effect of opiorphin is shorter owing to its rapid destruction by circulating peptidases [10]. However, compared to the other opioid peptides [31] the transfer of opiorphin through the BBB is relatively high [30].

The effort of most of the SAR publications was to find more stable functional mimetics than the native opiorphin peptide that could prolong the favorable effects of opiorphin [7, 13-16]. Few of them fulfilled all criteria. The most promising of them were the Cys-derivatives [7, 13]. Among them, the Cys-[$(CH_2)_6$]-QRF-[Ser-O-octanoyl]-R opiorphin peptidomimetic shows an increase in stability, increased affinity for human NEP and AP-N ectoenkephalinase [7] and also an increase of the maximal binding and affinity of enkephalin-related peptides to the opioid receptors [9], while retaining antinociceptive properties at similar doses than opiorphin native peptide (1-2 mg/kg *i.v.*) [7]. Although metabolically more resistant and ten more potent in its ability to inhibit enkephalin-degrading ectopeptidases, the opiorphin analog-induced pain reduction is similar to the opiorphin natural peptide, in terms of dose effect, delay and duration of action. Moreover, the cystine-dimeric form of this Cys-derivative failed to significantly inhibit pain behavioral responses at these doses [7]. In view of these results the explanation could be to the loss of a significant proportion of the active Cys-monomeric form by dimerization and/or by a rapid hepatic metabolism *in vivo* in rats. On the other hand, the *in vivo* acute toxicity of the Cys-monomeric derivative was also increased. Indeed, if we consider that the maximum effective analgesic dose for the two compounds (native and analog) is 1 mg/kg *i.v.*, the safety-effectiveness ratio is estimated at 30 for the designed analog while at 100 for the native peptide [7].

Otherwise, numerous chemical families of Dual ENKephalinase Inhibitors (DENKIs) have been designed by B. Roques and collaborators. Their recent publication [33] report the design and synthesis of new pro-drugs, derived from co-drugs combining an NEP and an APN inhibitors through a disulfide bond, with improving oral bioavailability. One of these new DENKIs, 19-IIIa, demonstrated efficacy in various animal models of acute and neuropathic pain and was therefore selected for clinical development [33].

CONCLUSION

Keeping in view the severe side effects of morphine therapeutic application and its use as narcotic drug we can find many famous poets and authors, who praised its euphoric effects. Nevertheless, one of them Charles Baudelaire indicated also its dark sides in his poem: Le Poison (Poison translated by Lewis Piaget Shanks in Flowers of Evil NY 1931): "opium widens all that has no bound in its unbounded sea; moments grow hours, pleasures cease to be in souls that over-worn, drown in its black abyss of lethargy".

In sharp contrast, human opiorphin endogenous peptide does not draw our soul in lethargy, instead of it ceases our pain and produces well-being. Unfortunately, it is not so easy to create an effective molecule on only several years what evolution produced during several millions of years.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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