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Strategies to Improve Bioavailability and *In Vivo* Efficacy of the Endogenous Opioid Peptides Endomorphin-1 and Endomorphin-2

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Abstract: Morphine and the other alkaloids found in the opium poppy plant still represent the preferred therapeutic tools to treat severe pain in first aid protocols, as well as chronic pain. The use of the opiate alkaloids is accompanied by several unwanted side effects; additionally, some forms of pain are resistant to standard treatments (*e.g.* neuropathic pain from cancer). For these reasons, there is currently renewed interest in the design and assay of modified versions of the potent endogenous opioid peptides endomorphin-1 and endomorphin-2. This review presents a selection of the strategies directed at preparing highly stable peptidomimetics of the endomorphins, and of the strategies aimed at improving central nervous system bioavailability, for which increased *in vivo* antinociceptive efficacy was clearly demonstrated.

Keywords: Blood brain barrier, endomorphins, enzymatic stability, lipophilicity, opioid, peptidomimetic.

1. INTRODUCTION

The opiates, *i.e.* the alkaloids such as morphine (**1**) and codeine (**2**) (Fig. **1**) extracted from the opium poppy plant, are widely utilized alone or in combination with adjuvants [1], as analgesics for sedating acute pain [2] or for prolonged treatment of chronic pain caused by cancer [3], nerve damage, arthritis, and other diseases [4-6].

Despite of the availability of these potent painkillers, some forms of pain remain undertreated [7]. Chronic pain may become resistant to single-agent therapy over time, necessitating a multimodal approach to therapy. The degree to which these therapies are successful varies from patient to patient, and several factors have been identified or hypothesized to be the cause for this large variability. For instance, receptor mutations cause inter-individual variability of the clinical effects of opioids. With the recent advances in genetic research, inherited causes of the variability of opioid therapy can be investigated [8].

Besides, prolonged administration of potent opiates produces several undesired side-effects, in particular tolerance and dependence. Tolerance is the decrease of drug efficacy with repeated administration, causing patients to require escalating doses of opioids to maintain the same level of analgesia; dependence is a physical, behavioural, and cognitive syndrome in which the craving of the drug takes on a high priority; other drawbacks are constipation, dry mouth, edema, headache, insomnia, itching and skin rash, nausea and vomiting, urinary retention, weight gain, *etc.*

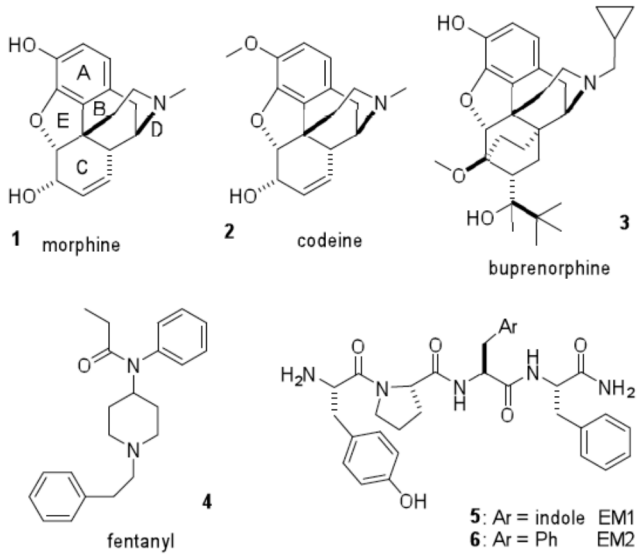

Fig. (1). Structures of morphine (**1**) and codeine (**2**), buprenorphine (**3**), and fentanyl (**4**); structures of the endogenous MOP receptor ligands EM1 (**5**) and EM2 (**6**).

As a consequence, there is presently an urgent need for novel therapeutic protocols, as well as for innovative compounds that can inhibit the transmission of pain signals in the central nervous system (CNS) based on alternative action mechanisms, not involved in the arising of harmful side-effects.

Opiates elicit their powerful effects by activating the opioid receptors (ORs), widely distributed throughout the brain and peripheral tissues, in particular the gut. These receptors are members of the seven transmembrane G-protein coupled receptor (GPCR) family. Three different types of

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Ors were cloned in the early 1990s, beginning with the mouse δ-opioid peptide (DOP) and followed by μ-opioid peptide (MOP) and κ-opioid peptide (KOP) receptors. All these receptors share extensive structural homologies [9]. The orphan opioid-like receptor (ORL1) shares around 60% of the sequence homology with ORs, but it produces different, and even opposite, pharmacological effects [10].

In a similar way as for many other members of the GPCR family [11], ORs exist in homo-oligomeric or hetero-oligomeric complexes [12], so the pharmacological responses of the receptors are cross-modulated and cross-regulated. ORs are quite promiscuous and can form heterodimers also with nonopioid receptors, for example, MOP with α^2a-adrenoceptors [13]. In addition, opioid receptors also interact with a variety of other proteins, which alter ligand binding or functional responses, as well as receptor localization and processing [14].

Pure MOP agonists, such as morphine, exert the strongest analgesia but produce a number of serious side effects. Therefore, their use is usually limited to short-term "rescue" analgesia, though chronic pain, especially in case of cancer, may require their continuous use in the later stages of the disease. Mixed agonist/antagonists, such as buprenorphine (**3**) (Fig. **1**), are not considered useful in the management of chronic pain, but rather to treat opioid addiction.

Apart from morphine (**1**), the majority of opiates currently used in hospital practice are MOP agonists derived from it. The progressive simplification of morphine structure led to morphinans, such as butorphanol, benzomorphans, (*e.g.* pentazocine, ketocycloazocine, bremazocine, and piperidines), *e.g.* fentanyl (**4**) (Fig. **1**) [15].

DOP agonists possess a lower antinociceptive efficacy, but they might have a reduced addictive potential. A few DOP-selective analgesics have been identified through the modification of morphine, including TAN-67 and SB 213698 or the piperazine derivatives, BW 373U86 and SNC 80 [16].

Finally, KOP agonists trigger a lesser analgesic response and may cause miosis, sedation, and dysphoria. Among the KOP-selective analgesics, the arylacetamide derivative U-50,488 and its derivatives spiradoline (U-62,066) enadoline (CI-977), asimadoline, EMD-61753 are worth mentioning [17].

When the appropriate agonist binds to the receptor at the extracellular side of the membrane, a conformational switch is transmitted to the cytoplasmic G-protein, which consists of the three subunits α, β, and γ. GDP dissociates from the α subunit of the G-protein; GTP then binds to the empty guanine nucleotide-binding pocket of the G-protein, causing the release of the α subunit. The GTP-bound α- and βγ-subunits activate a number of signalling pathways: activation of an inwardly rectifying K^+-channels, inhibition of voltage operated K^+ channels, and inhibition of adenylyl cyclase. Other responses of an unknown mechanism include activation of PLA, PLC, MAPK, and some Ca^{2+}-channels.

Until 2012, the precise 3D structures of ORs were unknown. The first crystal structure of a GPCR appeared in the literature was that of *Bos taurus* Rhodopsin (Bt_Rho) [18], while the first crystal structures of a non-rhodopsin GPCR, the *Homo Sapiens* β^2-adrenergic receptor (Hs_Adrb2) bound to partial inverse agonist carazolol, were obtained at the end of 2007. Homology modelling based on the rhodopsin crystal structure gave the opportunity to investigate the structural features of ORs, both in the active and inactive states [19-21].

In 2012 Kobilka *et al.* disclosed the crystallographic structures of murine MOP and DOP receptors in complex with their antagonists, using the fusion protein T4 lysozyme (T4L) to replace the floppy helix III [15, 16]. This strategy was used by Stevens *et al.* in the same year, for the crystal structure of KOP [17]. Naltrindole was used as an antagonist of DOP receptor, and JDTic was used for co-crystallization with KOP.

As for the MOP receptor, the structure of the mutated murine receptor covalently bound to a morphinan antagonist, β-fulnatrexamine (β-FNA), is sketched in Fig. (**2**). The position of β-FNA inside the binding pocket of the receptor is shifted in comparison to the position of naltrindole within DOP receptor. β-FNA makes contacts with TM3, TM5, TM6 and TM7, and the electron density observed in the structure confirms previous data identifying the $K^{5.39}$ side chain as the site of covalent attachment. There are 14 residues within 4Å of β-FNA. $D^{3.32}$ engages in a charge-charge interaction with the amine moiety of the ligand and hydrogen bonds with $Y^{7.43}$. $H^{6.52}$ interacts with the phenol of the morphinan group, but it is not directly hydrogen bonded to β-FNA. However, the electron density suggests the presence of two water molecules that form a hydrogen-bonding network between $H^{6.52}$ and the phenolic hydroxyl of the morphinan group (Fig. **2**) **[**15].

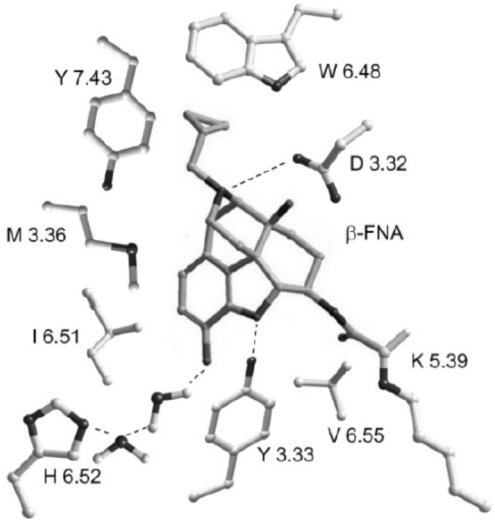

Fig. (2). Sketch of the β-FNA-MOP receptor complex as determined by Kobilka [15] (ligand-covalently bound to the receptor).

The endogenous ligands of opioid receptors in mammals are opioid peptides. Most of these peptides were isolated in the 1970-ies, including enkephalins (YGGFM, YGGFL), dynorphins (YGGFLRRIRPKLKWDNQ, YGGFLRRQFKVVT), and β-endorphin (YGGFMTSEKSQTPLVTLFKNAIIKNAYKKGE), derived from the propeptides proenkephalin, prodynorphin, and proopiomelanocortin, respectively [4,22]. The length of these endogenous opioid

peptides vary from few to more than 30 amino acids, nevertheless their structures are strongly correlated. They all show a common fragment at the *N*-terminus, the "message", while the variable *C*-terminus is known as the "address", and is responsible for receptor selectivity. Indeed, enkephalins show a certain selectivity for DOP over MOP, β-endorphin binds equally to both, DOP and MOP, while dynorphin A and its truncated fragments show a preference for KOP.

More recently, a couple of novel peptides having an unusual and short sequence have been discovered in the brain and named endomorphin-1 (EM1, YPWF-NH_2) and endomorphin-2 (EM2, YPFF-NH_2) [23]. EMs show a noteworthy potency and selectivity for MOP, and are currently regarded as the endogenous MOP agonists in the mammals, with a strong antinociceptive effect against neuropathic pain and acute pain (Fig. **1**). The neuroanatomical distribution of EMs and the MOP receptors in the CNS reflects their potential role in many major biological processes. These include perception of pain, responses related to stress, and complex functions such as reward, arousal, and vigilance, as well as autonomic, cognitive, neuroendocrine, and limbic homeostasis [24].

Since their discovery, EMs have been studied extensively aiming at developing effective drugs for the treatment of pain in humans [4,25]. The increasing interest spurred the development of modern green chemistry protocols for the production of large quantities of EMs [26-28]. Unfortunately, their clinical applications as painkillers remain unrealistic due to their poor metabolic stability, inability to cross the blood-brain barrier (BBB), and efficient efflux [29,30]. In this review we focus on the recent strategies to improve EM bioavailability, stability, and *in vivo* efficacy.

2. ENZYMATIC INACTIVATION OF OPIOID PEPTIDES

An important limitation of native peptides as drug is their scarce enzymatic stability. Native peptides are rapidly degraded *in vivo* by peptidase such as dipeptidyl peptidase III and IV (DPP III, DPP IV), [4]. The DPPs are glycoproteins present in the kidney, liver, placenta. Good substrates for these enzymes are enkephalins, dynorphins, β-endorphins, substance P, EMs, β-casomorphins, NPY, PYY, bradykinin, and others. DPPs remove dipeptides from the amino terminus of sequences containing proline as the penultimate amino acid [31].

EMs are relatively more stable than other opioid peptides due to the presence of the proline residue at the position 2, which not only influences the conformation of the peptide chain, but also confers stability against some proteases [32]. To be degraded, a peptide bond must be in the *trans* configuration. Proline is responsible for the *cis-trans* isomerisation, and changes the direction of a peptide chain, resulting in a protection from peptidase. However, nature has developed a group of enzymes that can specifically hydrolyze peptide bonds involving proline residue [32].

Studies performed on EM1 by molecular modeling and two-dimensional NMR in different environments, indicated that proline is the key residue that confers the proper spatial orientation for the best ligand-receptor interaction. Moreover, the introduction of modified proline could play a special role in the peptide stability *in vivo*, since the activity of DPP IV is particularly directed towards the degradation of proline-containing peptides.

EMs are subject to DPP IV that cleaves the *N*-terminal Tyr-Pro. EMs are degraded also by aminopeptidase P and aminopeptidase M (APP and APM, respectively) which hydrolyze the Tyr-Pro peptide bond, releasing Tyr and the tripeptides [31].

Other enzymes targeting EMs are carboxypeptidase Y and proteinase A. The amidated *C*-terminus of EMs increases stability against carboxypeptidases. However, the carboxypeptidase Y is an exception, since it exhibits preference for peptides containing hydrophobic *C*-terminal amino acid residues (apart from Pro) and hydrolyzes peptide amides releasing ammonia. Proteinase A is a non-pepsin-type endopeptidase. It cleaves small peptide acids or amides at only one site and the rate of hydrolysis differs markedly, depending on the peptide [32].

3. EM ANALOGUES WITH MEASURABLE ENZYMATIC STABILITY

In the last years, many structural modifications have been introduced into EMs to improve their pharmacological properties, including incorporation of unnatural amino acids, introduction of structural constraints, basic nitrogen quaternization, alteration of lipophilicity, replacement of the natural L-amino acids by their D-enantiomers, *N*-alkylation, introduction of α-substituted α-amino acids, β-substituted α-amino acids, proline analogues, γ- and β-amino acids, and substituted α- or β-amino acids [33-35]. These issues have been extensively reviewed [4, 24, 33-37]. The following sections focus on the recent publications reporting modifications aimed at overcoming the problems of enzymatic degradation and inability to cross the BBB, directly correlated to a well-documented, measurable *in vivo* activity.

3.1. Analogues Containing Unusual or Substituted Amino Acids

Six analogues of tyrosine, containing alkyl groups, single or multiple, at positions 2', 3', and 6' of the aryl ring, were incorporated at the *N*-terminus of EM2. Analogues containing 2′,6′-diethyltyrosine (Det), 2′-ethyl-6′-methyltyrosine (Emt), and 2′,3′,6′-trimethyltyrosine (Tmt) were the most potent in the binding assays. These modifications produced analogues with high MOP receptor affinity and a potent functional agonism, as determined by the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays. However, bulkier moieties, such as the isopropyl groups of Dit, substantially interfered with ligand-receptor interactions [38].

The analgesic effects of [Dmt^1]EM2 and [Det^1]EM2 were determined by the tail-flick test (spinally mediated mechanism) and the hot-plate test (supraspinal effects) in comparison to the effect produced by EM2. Intracerebroventricular (i.c.v.) administration of the compounds produced a dose-dependent antinociception response in mice. [Dmt^1]EM2 was the most potent analogue in terms of the duration of the analgesic response (60 min compared with 20 min for EM2).

[Dmt[1]]EM1 obtained by Jinsmaa *et al.* had a weak selectivity for MOP over DOP receptors. It exhibited MOP agonism, and, unlike [Dmt[1]]EM2, turned out to be a potent DOP antagonist. Therefore, Dmt can not only affect a ligand interaction with a specific receptor but also change its agonist/antagonist properties. *In vivo*, [Dmt[1]]EM1 was 1.6-2.3 and 1.5-1.6 times more potent than [Dmt[1]]EM2 in tail-flick and hot-plate tests, respectively [39].

The cyclic asparagine derivative, 6-oxo-2-phenyl hexahydropyrimidine-4-carboxylic acid (cycloAsn), was introduced as a six-membered proline mimic into EM2 structure [40]. Peripheral administration of [cycloAsn][2]EM2 (**7**) (Fig. **3**) was more effective than EM2 in blocking abdominal constrictions elicited by AcOH (77% versus 62% at the same dose, respectively) [40]. The analgesic effect of **7** occurred later than that of aspirin in AcOH-induced mice writhing test after i.p. injection. [CycloAsn][2]EM2 showed the highest inhibition (97%) 30-35 min. after i.p. injection of AcOH.

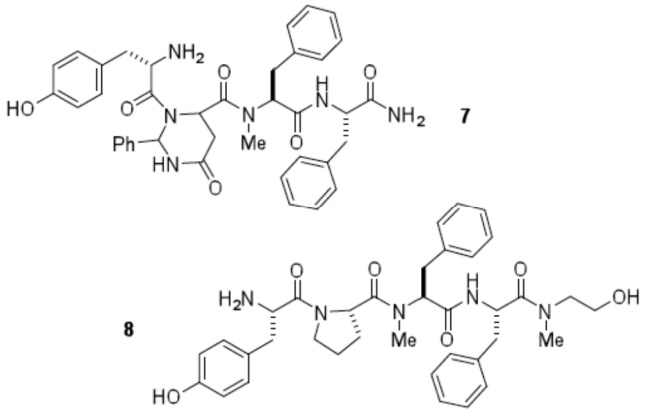

Fig. (3). Structures of the EM2 analogues **7** and **8**.

Mollica *et al.* [41] used the combination of the structures of EM2 and DAMGO, [Tyr-D-Ala-Gly-(*N*-Me)Phe-Gly-ol], both highly MOP selective. DAMGO was more than 100-fold potent than morphine, in mouse writhing nociception test. In addition, DAMGO displayed an increased enzymatic stability compared to other opioid peptides. Further, the hybrids were *N*-methylated. The position to be *N*-methylated for peptide give protection from proteolysis. The *N*-alkyl group eliminates the predominance of *trans vs cis* peptide bond configuration. Besides, the substitution of NH by *N*-alkyl groups eliminates some inter- and intramolecular hydrogen bonds. Finally, the adjacent carbonyl group increases basicity and decreases polarity [35].

The hybrid compound **8** (Fig. **3**) showed *in vivo* a AD_{50} 3.77 and 2.65 nmol in the hot plate test and tail flick assay respectively, compared to EM2 (AD_{50} 2.29 and 1.49 nmol). This data indicate that, the hybrid showed a better plasma stability ($t_{1/2}$ is about 40 min.) than parent peptide [41]. Metabolic stability studies, carried out on product **8**, confirmed that multi-*N*-methylation and *C*-terminal modification with ethanolamide moiety, gave for compound **8** a better plasma stability than parent peptide, with $t_{1/2}$ around 40 min., and could be a useful tool to develop stable candidates as opioid drugs [41].

3.2. Analogues Containing β-Amino Acids

β-Peptides composed of β-amino acids have been studied for years and shown to posses stable well-ordered secondary structures [42]. There are different kinds of β-amino acids, the β^2- or β^3-versions, which can be further distinguished in homologated β-amino acids possessing an extra C atom, or isomeric β-amino acids, which maintain the same molecular weight of the corresponding α-amino acids. The β^3-amino acids are much more utilized than the β^2 ones [43]. Almost all appropriately protected β^3-residues with proteinogenic side chains are commercially available. The enzymatic resolution of racemates with immobilized enzymes or with cell cultures, constitutes a cheap and easy method to obtain optically active β-amino acids [35].

In this context, Gentilucci *et al.* introduced β-amino acids in the sequence of EM1 [44]. In particular, the substitution of Pro with (*R*)-pyrrolidine-3-carboxilic acid, (*R*)-β^2-Pro, gave the tetrapeptide **9** (Figure 4) with the appropriate conformation to bind to MOP receptors. This modification resulted in an affinity for MOP receptor (*K*i 0.33 nM, IC_{50} 1.80 nM) comparable to that of the parent peptide EM1 (Ki 0.16 nM, IC_{50} 0.5 nM). Subsequently, the same authors prepared the stereoisomers **10**, and **11**, having affinity in the nanomolar range (**10**: *K*i 3.8 nM, IC_{50} 180 nM; **11**: *K*i 10.4 nM, IC_{50} 72.0 nM) [45].

The β^2-Pro analogues of EM1 showed significantly higher enzymatic stability compared to the native EM1. Indeed, the digestion of EM1 with α-chymotrypsin proceeded very rapidly, and gave a mixture of the tripeptide Tyr-Pro-Trp and Phe-NH_2 after 3 h. The digestion of EM1 (**5**) with aminopeptidase-M gave a mixture of Tyr-Pro and Trp-Phe-NH_2; after 3 h, further hydrolysis of the latter dipeptide to Trp and Phe-NH_2 was evident. The enzymatic digestion of EM1 by carboxypeptidase-Y began with hydrolysis of the *C*-terminal amide Phe-NH_2, to give the tetrapeptide Tyr-Pro-Trp-Phe, which was in turn hydrolyzed to the tripeptide Tyr-Pro-Trp upon cleavage of the Trp-Phe bond. After 24 h only 23% of the initial amount of native **5** was detected.

On the contrary, under the same conditions the β^2-Pro analogue **10** remained almost completely intact (>90%), while **11** and **9** remained intact in about 80 and 20%, respectively. The degradation of the peptides with carboxypeptidase-Y was slower; peptides **9** and **11** were strongly degraded (18-25% of the initial amount), while **10** was still present in about 90%. Finally, the peptides **9-11** were scarcely degraded by aminopeptidase-M, being still present in 85-90%.

The stabilities displayed by **9-11** were of interest, since the presence of just a (*S*)- or a (*R*)-β^2-proline in a sequence of natural residues was sufficient to ensure a good stability to the entire structure [45]. The comparison of the affinities displayed by the different peptides for MOP receptors, and the comparison of the degradation rates in the presence of proteolyic enzymes, confirmed proline at the position 2 of the EM sequence as the key residue both for biological activity and enzymatic stability [34, 45].

In yet another series of analogues, Gentilucci *et al.* consecutively substituted all residues in the EM1 sequence with the corresponding β^3-homologues. In a competitive binding assay, compound **12** with L-β-homo-proline displayed a low nanomolar affinity, with Ki 2.1 nM and IC_{50} 4.0 nM. This analogue was less potent compared to the reference parent peptide [46]. The introduction of D-β-homo-Pro gave peptide **13** (Fig. **4**) which had somewhat lower MOP receptor affinity (Ki 67 nM, IC_{50} 79 nM). The substitution of Phe, Trp, and Tyr with homo-Phe, homo-Trp, and homo-Tyr, respectively, caused a significant loss of affinity. These results were explained by an unfavorable change in their spatial disposition that was detrimental for a proper ligand-receptor fit.

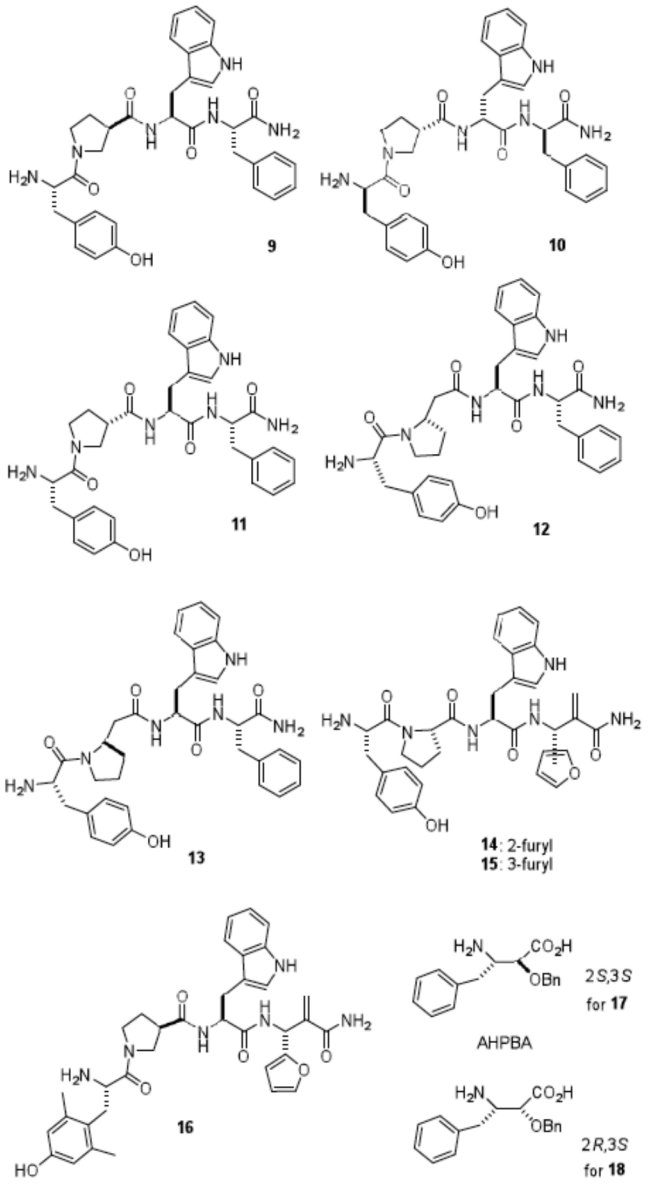

Fig. (4). Structures of EM analogues containing β^2-Pro (**9**-**11**) or β^3-homo-Pro (**12**, **13**), Map β-amino acids (**14**-**16**), and the β-amino acid AHPBA (**17**, **18**).

EM1-[β-Pro]^2 derivatives **12** and **13**, as well as EM1, were shown to act predominantly as MOP agonists, and do not interact significantly with DOP and KOP. Indeed, they inhibited the accumulation of cAMP induced by forkoline in the cAMP test, with IC_{50} values of 6.5 and 45.0 nM, respectively (for DAMGO 1.1 nM).

The stability of the compound **12** compared to EM1 was estimated by enzymatic assays. As commented above, EM1 was almost completely degraded in a few hours after digestions with either α-chymotrypsin, carboxypeptidase-Y, or aminopeptidase-M. On the other hand, **10** was much more resistant under the same conditions, being 93% intact after 3 h in the presence of α-chymotrypsin. After 24 h, 29% of the **12** was found in carboxypeptidase-Y. These results indicate that the presence of homo-Pro conferred the peptides a good resistance [46].

Interestingly, compound **13** was found more stable with respect the stereoisomer **12** with carboxypeptidase-Y, (with 50% of the starting intact after 24 h), but less stable in α-chymotrypsin, (only 23% intact after 3 h incubation). As for aminopeptidase-M, **12** and **13** both showed excellent resistance, 89% and 96% after 3 h.

The analgesic efficacy of **12** was assayed *in vivo* by the tail flick and visceral pain tests. After s.c. injection in the mouse, **12** showed antinociceptive effect in the tail flick (ED_{50} 9.2 mg/kg) and acetic acid-induced abdominal constriction (ED_{50} 1.2 mg/kg) tests. The peripheral administration of MOP receptor agonists in the mouse, is more effective in visceral models of pain while the tail-flick test is less sensitive.

Moreover, s.c. **12** significantly decreases, in the mouse, the gastrointestinal propulsion (ED_{50} 10.0 mg/kg) [47]. Subcutaneous (s.c.) β-FNA (or a high dose of the MOP receptor-selective antagonist naloxonazine) prevents the antinociceptive and antitransit action. The antinociceptive activity of systemically administered **12** was partially blocked by both intracerebroventricular (i.c.v.) naloxone and i.p. naloxone methiodide; this latter compound does not readily cross the blood-brain barrier, thus implicating both central and peripheral mechanisms of action. On the contrary, the KOP receptor antagonist nor-binaltorphimine or the DOP receptor antagonist naltrindole are ineffective. Apparently, **12** acts preferentially through central and peripheral MOP receptors to produce antinociception and to inhibit gastrointestinal transit.

[β-Pro]EM1 was among the first EM1 analogues showing antinociceptive activity after systemic administration. This compound can be extremely useful for exploring the pharmacological profile of EMs *in vivo* and confirms the potential therapeutic interest of EM derivatives as analgesic agents [47].

A new class of EM1 analogues was synthesized by Wang and *co*-workers. They introduced α-methylene-β-amino acids (Map) at several positions [48]. Compared to classic β-amino acid with flexible backbone, these novel unnatural amino acids were highly constrained due to the double bond at the Cα. The most active and selective compounds **14** and **15** incorporating a furyl ring at position 4 showed EC_{50} of 0.0334 and 0.0342 nM, respectively (Fig. **4**). Antinociceptive potencies were studied in the mouse tail-flick test. The analogues displayed a higher analgesic effect compared EM1. In

particular compound **14** and **15** were 10.9- and 9.8-fold more potent than the parent peptide EM1.

The metabolic stability of these analogues was assessed in the mouse brain homogenate, showing that EM1 disappeared rapidly with a half life ($t_{1/2}$) of 16.9 min, while this value for [Map^4]EM1 was ranging from 62 to 90 min.

Subsequently, the same authors combined Dmt^1, (*R*)-β-Pro^2, and (Ph)-Map/(2-furyl)Map^4 modifications introducing them into the sequence of EM1. The resulting analogue Dmt-(R)-β-Pro-Trp-(2-furyl)Map-NH_2 **16** showed a picomolar affinity and subpicomolar potency for MOP (EC_{50} 0.0420 pM, Ki 3.72 pM) [49]. The forskolin-induced cAMP accumulation test indicated that this analogue displayed an extremely high agonistic potency, in the subpicomolar range (EC_{50} 0.0421 pM, Emax 99.5%).

The resistance of analogue **16** to enzymatic hydrolysis was examined in mouse brain membrane homogenate. The compound displayed high metabolic stability ($t_{1/2}$>600 min.). As a consequence, **16** induced a strong and consistent analgesic effect in the tail-flick assay, with an ED_{50} value of 0.532 nmol/kg. The metabolic stability of the analogue appeared to be an important factor in the high *in vivo* analgesic potency [49].

The introduction of α-hydroxy β-amino acids at position 3 or 4 of EMs was proposed by Yu *et al.* [50]. The synthesized analogues, Dmt-Tic-Trp-(2S,3S)AHPBA-NH_2 (**17**) and Dmt-Tic-(2R,3S)AHPBA-Phe-NH_2 (**18**) (where Tic is 1,2,3,4-tetrahydroisoquinoline carboxylic acid), (Fig. **4** showed a great increase in enzymatic stability ($t_{1/2}$>2 h).

The antinociceptive activity of **18** determined in the the mouse warm-water (55 °C) tail-withdrawal test was equel to that of morphine. It can be concluded that the replacement of Tyr^1-Pro^2 by Dmt^1-Tic^2 and introduction of α-amino acid provide a dramatic increase in enzymatic stability of EM analogues [50].

Tóth *et al.* studied another series of EM analogues, incorporating 2-amino cyclohexanecarboxylic acid (Achc) in combination with Dmt^1, pF-Phe^4, or βMe-Phe^4. Consistent with the earlier results, it was found that the analogues carrying Dmt^1 and Achc^2 residues displayed the highest MOP receptor affinities, which however depended on the configuration of Achc^2. Combined application of Dmt^1, *cis*-(1S,2R)Achc^2, and pF-Phe^4 resulted in the most potent analogue [51]. Ligand stimulated [^{35}S]GTPγS binding assays indicated that, the analogues retained their agonist activities and opioid receptor specificities [51]. EM analogues containing pF-Phe^4 retained the activity of the parent compound in receptor binding assays (confirming previous observations for derivatives incorporating para-halogenated amino acids). Introduction of (2S,3S)βMe-Phe^4 resulted in an increased affinity and potency as compared with the parent ligands, while (2R,3R)βMe-Phe^4 substitution decreased the affinity and selectivity toward the MOP receptor. These confirm that the modulation of side chain rotations is a very important feature determining the biological activity of a peptide [51].

These analogues demonstrated prolonged half-lives (>20 h) compared to the EMs ($t_{1/2}$ = 5-7 min), proving the enzymatic resistance of the new analogues.

3.3. End-Group Modifications

The end-group modifications of *N*- or *C*-terminus affording different amides, esters, hydrazides, etc., were also shown to play an important role in the regulation of opioid activities enzymatic stability.

Fujita *et al.* prepared analogues of EM2 by introduction of aromatic amines at the *C*-terminal amide. Only compounds with 1-naphthyl, 5-quinolyl, cyclohexyl, and 2-adamantyl exhibited MOP receptor affinity in the nanomolar range (*K*i 2.41-6.59 nM). Replacement of Tyr^1 by Dmt exerted profound effects. Antinociceptive effect of Dmt-Pro-Phe-NH-5-Isq (5-isoquinolyl) after intracisternal (i.c.) administration in the tail pressure test in mice, produced a dose-dependent antinociceptive effect and was antagonized completely by naltrexone. The maximal effect of the analogue (30 μg/mouse) was about one tenth of that obtained for morphine [52].

Enzymatic stability of the peptides containing Dmt at position 1 was examined by use of aminopeptidase and rat brain homogenates, which revealed that they were more stable than the original peptides. Therefore, Dmt-Pro-Phe-NH-5-Isq might be similarly resistant to enzymatic degradation by aminopeptidase or carboxypeptidase, although the Pro^2-Phe^3 bond is metabolized.

The modification of the last amino acid in EMs was studied by Wang *et al.* [53]. The analogues of EMs, in which Phe^4 was replaced by D-Ala and ended with -benzyl, exhibited 22- and 14-fold higher MOP affinity (Ki 4.56 and 8.67 nM, respectively), the replacement with D-Val in EM1, gave a compound which exhibited a MOP affinity about 2-fold (Ki 2.32 nM) higher, and a DOP affinity 1.6-fold higher, compared to parent peptide. Although both MOP and DOP affinity of the most potent EM2 analog increased in binding assays, instead the selectivity decreased about 3-fold compared to EM2.

The antinociceptive response was expressed as a percentage of maximal possible effect (% MPE). All the drugs produced a dose-related antinociceptive effect after i.c.v. administration, and the inhibition effect reached its peak 5 min after injection. The EM1 analog, with D-Val still displayed about 50% MPE (60 min after i.c.v. administration at the dose of 6.7 nmol/kg). Furthermore, the duration of hot plate response inhibition, induced by all the parents and analogs, appeared to be shorter than the corresponding duration in the case of morphine.

Introduction of methyl, ethyl, and *tert*-butyl ester at the *C*-terminus of EM2 gave compounds which were tested *in vivo* for contractions of the longitudinal muscle of distal colon. The ED_{50} values induced by analogs were about 1.5-fold higher, 2- and 8-fold lower than EM2, respectively [54]. This approach is interesting to reduce undesirable side effects. Antinociception was assessed using the 50°C warm water tail flick test after i.c.v. injection (0.67-20 nmol/kg). All analogs induced a dose-related antinociceptive effect, and the inhibition of the tail flick response reached its peak 5 min after injection. At the dose of 20 nmol/kg, the maximal %MPE values of analogs were 61, 73 and 61, respectively, similar to EM2 (58).

C-terminal amide to hydrazide conversion of EMs did not markedly change their MOP binding affinities. Nevertheless, EM2-$NHNH_2$ showed decreased GPI and MVD potencies (10- and 5-fold compared to the parent compound, respectively) [55]. It is noteworthy that EM1-$NHNH_2$ exhibited the highest antinociception effect after i.c.v. injection, about 1.5-fold more potent than EM1, but with moderate colonic contractile and expulsive effects, comparable with EM1 [55]. Additionally, EM2-$NHNH_2$ showed a slightly lower antinociceptive effect than EM2, at higher doses (i.c.v., 1.5 and 5 nmol/mouse). The inhibitory effects of colonic propulsion were significantly attenuated, which would be helpful in the development of suitable MOP receptor therapeutics, but without some undesirable side effects.

Varamini *et al.* modified the *N*-terminus of EM1 by acylation of the Tyr amino group with succinamic acid, esterified in turn on the *C*-terminus with lactose. This modification led to a drop in receptor binding affinity and agonist activity at the MOP receptor, but resulted in increased stability in human plasma and significant pain relieving effect in rats, after both i.v. and oral administration [56]. Indeed, the glycopeptide produced a dose-dependent antinociceptive activity, following intravenous administration in a chronic constriction injury (CCI) rat model of neuropathic pain with an ED_{50} of 8.3 μmol/kg (for morphine, 2.6 μmol/kg), and produced dose-dependent pain relief after oral administration in CCI rats, ED_{50} 19.6 μmol/kg, which was comparable with that of morphine (20.7 μmol/kg). Interestingly, antinociception was not accompanied by constipation, a major side effect of existing opioid analgesics.

The combined insertion of guanidinium on Tyr^1, halogenation, and reversal of stereochemistry at some residues, was utilized to increase the metabolic stability of opioid peptides. The first attempt of cationization in opioid peptides was reported by Hau *et al.* [57] who introduced a guanidine group to the structure of EM2. This modification decreased the MOP receptor affinity but slightly increased metabolic stability, BBB permeability (see next paragraph) and analgesic profile after i.c.v. and i.v. administration in the tail-flick test.

The analog N^{ω}-amidino-Tyr(Me)-D-Pro-Gly-Trp-*p*-Cl-Phe-NH_2 was found by Wang *et al.* to display a potent and prolonged antinociceptive activity upon s.c. administration through a central mechanism. The analogs showed a significant increase in brain and serum stability over the parent, with the half-lives in brain exceeding 3 h. The half-lives of those halogenated analogs were shorter than those of the non-halogenated forms in the brain homogenates, whereas in the serum, the former were found to be more stable than the latter [58]. Apparently, cationization of EM1 by guanidino-addition on Tyr^1 led to a significant increase in metabolic stability in both brain and serum.

To increase the lipophilicity and stability of EMs, Koda *et al.* [59] modified both ends of the sequences by introduction of lipoamino acids (Laas). The diasteriomers of Laa-conjugated peptides can behave differently *in vivo*, particularly in terms of their stability to enzymatic degradation and bioavailability (see next paragraph). Indeed, the introduction of short-chain lipo-amino acids at the *N*-terminus of the peptide provided significant protection to metabolic enzymes, while having minimal effect on receptor affinity or agonist activity.

Introduction of lipoamino acids and the replacement of Tyr^1 by Dmt, gave the pentapeptide EM1 analogue [C8Laa-DMT^1]EM1, that exhibited the $t_{1/2}$ of 43.5 min in a solution of digestive enzymes, which was >8 times that of the parent peptide. Remarkably, this stable, permeable peptide exhibited potent *in vitro* MOP receptor agonist affinity with a Ki of 0.08 nM, which is 140-fold greater than that of the parent peptide, and a subnanomolar inhibition of cAMP production [59].

3.4. Cyclization

Cyclic peptides are widespread throughout nature [60]. One may ask what the advantages of the circular form are. Actually, there are several. Cyclization removes a major degradation pathway, as free ends are targeted by exopeptidases. Cyclic peptides can be resistant even to endopeptidases, as their rigid structures are less susceptible to all hydrolases. Elimination of charged termini increases lipophilic character and therefore enhances membrane permeability, so some cyclic peptides can cross the biological barriers much better than their linear counterparts (see next sections).

Cyclization also minimizes conformational entropy losses upon binding to target receptors. Linear peptides are flexible and need to adopt appropriate conformations upon binding and that requires energy. On the contrary, cyclic peptides may already have the conformation necessary for binding to a specific receptor [61].

Finally, constrained peptides are useful in identifying possible bioactive conformations, and in developing pharmacophoric models for receptor-ligand interactions. Therefore, cyclization can be considered an important step forward in the design of non-peptide mimetics which can be viewed as a new generation of peptide-based drugs.

Peptides can be cyclized through a variety of strategies and at various positions [62]. In opioid peptides, the presence of the pharmacophoric *N*-terminal Tyr residue is considered an absolute requirement for their pharmacological activity (albeit a few exceptions have been documented [63]). Therefore, the most often used strategies involve side-chain to *C*-terminus or side-chain to side-chain cyclization.

The cyclization of EM2 has been amply studied by Janecka and *co*-workers. In 2005 Janecka's group tested cyclic analogues of morphiceptin (Tyr-Pro-Phe-Pro-NH_2) and EM2. The resulting compounds Tyr-c[Asp-Phe-Phe-Lys-NH_2 and Tyr-c[Asp-Phe-D-Pro-Lys)-NH_2 produced, after i.c.v. administration, a stronger and longer lasting analgesic effect in the hot-plate test than EM2 [64]. This effect was dose-dependent and was reversed by naloxone, which indicated that it was mediated by the opioid receptors [64].

Subsequently, Janecka *et al.* introduced D-amino acids in position 2. The incentive to introduce a D-amino acid was to increase metabolic stability of the analogues [65]. The receptor binding results showed that the cyclic analogs Tyr-c(D-Lys-Phe-Phe-Asp)-NH_2 and Tyr-c(D-Asp-Phe-Phe-Lys)-NH_2, containing an unmodified Tyr^1 residue, retained very high affinity for MOP receptor (IC_{50} 0.56 and 1.14 nM, respectively), compared to the parent peptide (IC_{50} 0.79 nM).

Enzymolysis of EM2 was rapid, with about 16% of the peptide left after 30 min. After 30 min of incubation, the two analogs Tyr-c(D-Lys-Phe-Phe-Asp)-NH_2 and Tyr-c(D-Asp-Phe-Phe-Lys)-NH_2 remained intact in 94 and 87%, respectively. After peripheral administration, the most potent cyclic analog Tyr-c(D-Lys-Phe-Phe-Asp)-NH_2 produced a significant analgesic effect, which was reversed by the i.c.v. injection of β-FNA, indicating that it was mediated by the MOP receptors in the brain. The cyclic peptide was resistant to degradation and had a highly lipophilic character as reflected by the value of log *P* 2.13.

Introduction of Dmt instead of Tyr in the cyclic analogues of EM-2 increased, as expected, MOP affinities [66]. The methyl groups on the aromatic ring of Dmt undoubtedly play a dominant role in the interaction within the opioid binding domain by either direct interaction with hydrophobic side-chains of receptor residues in order to align the critical OH group, or by stabilization of a favored *cis*-conformer prior to and during binding. Tetrapeptide analogs of dermorphin/deltorphin cyclized by the ureido bridge between two basic amino acids in positions 2 and 4, synthesized by Izdebski and *co*-workers induced, when given i.c.v., an antinociceptive effect by activation of the MOP and DOP receptors [67]. The extension of the amidated *C*-terminus produced the analog **19** (Fig. **5**) which showed stronger than morphine antinociceptive effect after systemic administration [68].

Biphalin, (Tyr-D-Ala-Gly-Phe-NH-NH<-Phe<-Gly<-D-Ala<-Tyr), an opioid octapeptide with a dimeric structure based on two identical pharmacophore portions, derived from enkephalins, joined "tail to tail" by a hydrazide bridge was recently cyclized by Mollica *et al.* [69]. The cyclization was performed through a disulfide bond between two D-penicillamide residues introduced instead of D-Ala in positions 2', 2' of the biphalin sequence (Fig. **5**). In the *in vivo* tests, the cyclic analog **20** showed several times higher antinociceptive activity than morphine after i.c.v. administration. Following i.v. injection it displayed a greater and longer lasting activity than biphalin, though lower than morphine, probably due to a reduced BBB penetration.

Interesting cyclic analogs based on the structure of enkephalin, containing thiourea (**21**) or *N*-methyl-guanidine (**22**) bridges were recently reported by Verbeken *et al.* (Fig. **5**) [70]. These peptides displayed high metabolic stability in mouse serum and brain homogenate and affinities for the MOP receptor in the nanomolar range. In the tail immersion test in mice, both analogs showed higher antinociceptive activity relative to morphine, but the peptide with a positively charged *N*-methylguanidine bridge was more potent than the one with a neutral thiourea group.

As opposed to all mentioned above cyclic analogs, in which a positively charged amino group of Tyr in position 1 was maintained as a fundamental requirement for opioid receptor binding, several analogs with *N*- to *C*-terminus cyclization scheme were also reported [71]. The best analog c[Tyr-D-Pro-D-Trp-Phe-Gly] (**23**) (Fig. **5**), whose affinity was in the nanomolar range, was more lipophilic and resistant to enzymatic degradation than EM1, and produced preemptive antinociception in a mouse visceral pain model when given i.p. or s.c. [72]. Docking simulations to analyze the molecular determinants of the interaction of **23** with the MOP receptor were performed and allowed for the optimization of its structure, giving the analogue c[Tyr-Gly-D-Trp-Phe-Gly] (**24**), which dispayed a 10-fold improved MOP receptor affinity [73]. It was shown that these cyclic analogs, which lack the *N*-terminal amino group, can still interact [74] and activate the opioid receptor as agonists, and trigger the antinociceptive pathways by alternative mechanisms, producing a considerable part of antinociception *via* the peripheral opioid receptors [72]. Such peripheral antinociception could be produced by inhibition of the excitability of sensory nerves and/or the release of proinflammatory neuropeptides.

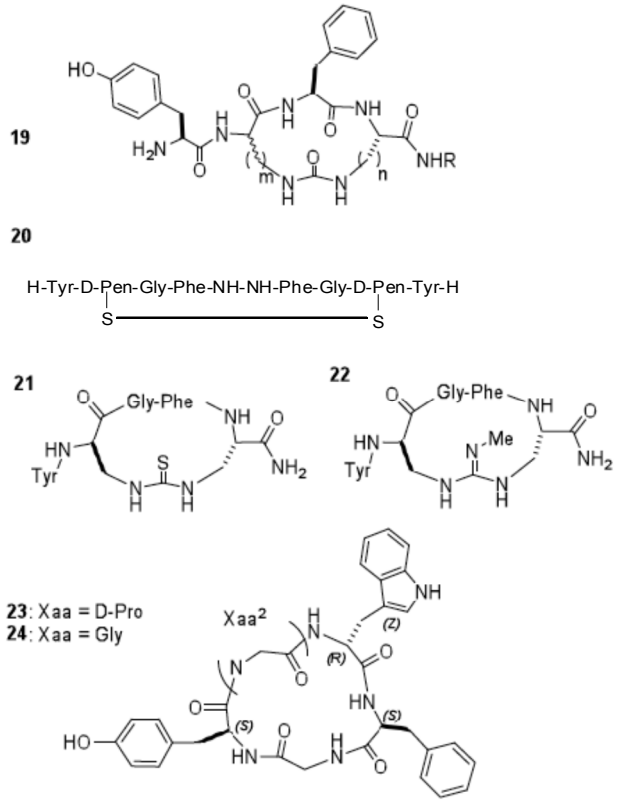

Fig. (5). Selected examples of cyclic EM-like opioid peptides.

4. CHEMICAL MODIFICATIONS DESIGNED TO INCREASE BLOOD-BRAIN-BARRIER PERMEABILITY

The BBB, situated at the level of the endothelial cells of the brain microvascular capillaries, is characterized by tight junctions and thus by a reduced vesicular transport, high electrical resistance, proteolytic activity, and low paracellular diffusion, excluding most of the molecules from reaching the brain. The surface area of the human BBB is almost five thousand times larger than that of the blood-cerebrospinal fluid barrier (BCSFB), and thence the BBB plays a unique role managing the export of materials from the CNS (efflux transport) and the import of materials from the blood (influx transport).

However, the BBB is not an absolute physical barrier but rather a regulatory tool that controls delivery of compounds to the CNS. Specific transporters are present that permit nutrients to enter the brain and toxicants/waste products to exit. These transporters are potential routes for mimetic designed drugs [75].

Also peptides can cross this barrier either by passive diffusion or saturable active or facilitated transport systems [76,77]. In the last decade numerous structural modifications have been developed for enhancing delivery of opioid peptides to the CNS [78,79].

The BBB is not only a physical but also a metabolic barrier. Indeed, many peptidases can be found in the brain microcapillaries of the BBB, such as: gamma-glutamyl transpeptidase alkaline phosphatase, monoamine oxidase catechol-*O*-methyl transferase, butyrylcholinesterase and aromatic-L-amino-acid carboxylase (or Dopa-decarboxylase or aromatic-L-amino-acid decarboxylase), epoxidehydrolase (or epoxide hydrolase), UDP-glucuronosyl-transferase, benzyloxyresorufin-*O*-deethylase (cytochrome P-450 CYP2B1), NADPH cytochrome P-450 reductase and glutathione-Stranferase. The protein-disulfide reductase, is also present in the brain and can alter peptide structures stable in plasma [35].

The ability of a drug to cross the BBB and enter the brain relies on specific compositional elements, involving molecular weight, hydrogen bonding potential, lipophilicity, flexibility, enzymatic stability, biochemical properties of amino acids, and amino acid arrangement. To improve the CNS penetration of EMs and subsequently achieve greater analgesic activity, the most generally used strategy was introduction of chemical modifications of the parent peptide structures (*e.g.* lipidization, cationization of the *N*-terminal end, cyclization, glycosylation), and the use of hybrid peptides in which EM sequence is chemically linked to a transportable lipids. It is important to note that structural modifications designed to reduce enzymatic degradation, are also an effective approach widely used to enhance peptide transportation to the CNS [33].

4.1. Lipidization

Peptides characterized by low molecular weight and high lipid solubility can cross the BBB *via* the passive diffusion [80,81]. Lipid solubility depends on the lipophilicity of a compound which is one of the crucial parameters used in drug discovery [82]. Lipophilicity not only determines the permeability of peptides through biological membranes but it also affects solubility and may contribute to potency and selectivity.

The simplest method to increase lipophilicity of peptides is by addition of lipophilic groups, for example methyl groups. Methylation of amide bonds is an important modification that can regulate biological functions. This small structural change reduces conformational flexibility which is usually an undesired property of a ligand and inhibits the action of proteolytic enzymes. *N*-Methylation of amino acids reduces hydrogen bonding potential thus increasing the BBB transport of small peptides [75].

Systematic investigation of EM2 analogs containing *N*-methylated amino acid residues consecutively in each position, was described by Kruszynski *et al.* [83]. [Sar^2]EM2 (Sar = *N*-methylglycine) was equipotent with the parent peptide in triggering antinociception in mice after central administration but showed also a significant pain-relieving effect when given peripherally.

Methylation of aromatic rings in peptides with Tyr or Phe residues in their structure can also increase lipophilicity [38]. In opioid peptides, the replacement of the Tyr residue by 2',6'-dimethyltyrosine (Dmt) resulted in achieving greatly increased bioactivities [84], however the transit of such modified analogs into the CNS after peripheral administration was still not always sufficient.

To increase the lipophilicity of EMs, Koda *et al.* [59] introduced lipoamino acids at both ends of EMs. Such amino acid derivatives combine properties of amino acids (contain amino and carboxyl groups) and lipids (long, unbranched hydrocarbon chains). The introduction of the short-chain lipo-amino acids at the *N*-terminus of the peptide increased the passive diffusion of the peptide across Caco-2 cell which was of 30 times that of EM1.

Applying the same approach, Varamini *et al.* [85] synthesized EM1 and [Dmt^1]EM1 modified by introduction of 2-aminodecanoic acid residue at the *N*-terminus. Both compounds showed promising potential for the treatment of neuropathic pain, exhibiting strong analgesic activity after systemic administration, without producing constipation, a major side-effect of morphine.

Opioid agonists such as morphine and DAMGO, significantly influence the respiratory system and generally produce respiratory depression. Varamini *et al.* [86] observed that the hypercapnic-stimulated respiratory ventilation was not affected significantly by lipid-conjugated opioid peptides.

Increased lipophilicity was also achieved when Pro^2 residue in the sequence of EM2 was replaced by 2-aminocyclopentane- or cyclohexanecarboxylic acid residues (Acpc and Achc, respectively, see also above) [87]. The new analogs, [(1*S*,2*R*)Acpc^2]EM2 and [(1*S*,2*R*)Achc^2]EM2 penetrated across the rat brain cells (co-culture of primary cerebral ECs and glial cells) *via* passive diffusion and the permeability coefficient of the analogs was significantly higher than that of EM2, suggesting increased BBB permeation properties.

Varamini *et al.* [88] modified their earlier EM1 analog containing a C10-Laa at the *N*-terminus by attaching various substance P (SP) fragments at the *C*-terminus. The undecapeptide SP is a neurotransmiter associated with mediating the hyperalgesic response. However, low doses of SP were shown to produce a modest pain-releaving effect [89] and were capable of intensifying opioid-mediated analgesia [90]. It was demonstrated that different *C*-terminal SP fragments conjugated with opioid alkaloids or opioid peptides, produced strong antinociceptive response with little or no development of opioid tolerance or dependence in rats [91]. The EM1/SP hybrid peptides showed a significant improvement in permeability across Caco-2 monolayers, stability in cell homogenates, relatively high MOP receptor affinity, and agonist activity with nanomolar inhibition of forskolin-stimulated cAMP production.

Reduction of the number of peptide bonds in the opioid peptidomimetics while retaining the chemical characteristics and spatial orientation of the pharmacophores is yet another strategy employed to improve the bioavailability. The rationale for the synthesis of such peptidomimetics is to increase

the lipophilic character of the compounds while maintaining the aqueous solubility. The obtained structures contained an unbranched alkyl chain containing 2 to 8 methylene groups, with Dmt on both ends [37,92,93]. The best analog, 1,4-bis(Dmt-NH)butane, had high MOP receptor affinity and functional bioactivity, with *in vivo* central and systemic antinociceptive activity in mice. Further improvement was achieved by coupling two Dmt residues to a pyrazinone ring platform by means of alkyl chains to yield the class of 3,6-bis[Dmt-NH-$(CH_2)_n$]-2(1*H*)-pyrazinones. These new analogs displayed high MOP receptor affinity, selectivity and functional agonism. Moreover, 3,6-*bis*[Dmt-NH$(CH_2)_3$]-5-methyl-2(*1H*)-pyrazinone produced analgesia in mice in a naloxone reversible manner after central (i.c.v.) and also peripheral (s.b. and oral) administration.

These findings indicate that opioid mimetics may be useful candidates in the search for novel painkillers that pass through the epithelium of the gastrointestinal tract and the BBB to target brain receptors.

4.1.1. Lipophlicity Efficiency

Important biopharmaceutical properties that facilitate a drug's entry into the body and across various cellular barriers include its solubility, stability, permeability, and first pass effect, as well as pharmacokinetic properties (clearance rate, biological half-life, and volume of distribution among others) [94].

Lipophilicity is a fundamental chemical-physical parameter correlated to penetration across biological barriers, including gut and BBB [95]. The partition coefficient (log*P*) is a value that characterizes, at least to some extent, the lipophilicity of a compound and its ability to cross the BBB. Very often, the calculated log P (clog*P*) is utilized instead of the experimenal log*P* [96].

It is generally accepted that peptides can reach the brain if their log*P* > 2, but compounds with a log*P* > 3.5 usually have poor water solubility which reduces their bioavailability [97,98]. Indeed, an important limitation to lipophilicity enhancement as a strategy to improve bioavailability, is the solubility of analogs in aqueous media and their partition into the brain's interstitial fluid to exert an effect. Substances that are too lipid soluble cannot traverse the BBB because they are effectively trapped in the membrane [99,100]. In general, a good BBB permeability does not necessarily correspond to high *in vivo* efficacy. High BBB penetration allows rapid distribution between plasma and CNS compartments, but the unbound drug concentration in the CNS might be lowered by non-productive nonspecific binding to lipids and proteins, rapid metabolism, intrinsic clearance, efflux transport, and subsequent low unbound drug concentration [101,102].

Another widely used parameter which has been shown to correlate with drug transport properties is the topological polar surface area (tPSA). tPSA represents the sum of surface contributions of polar atoms in a molecule [103].

Starting from these considerations, this paragraph focuses on the concept of lipophilicity efficiency. Lipophilicity efficiency indexes such as LLE and LELP, can help the future development of opioid peptides with *in vivo* activity [104,105]. LLE can be used to identify low potency target compounds that are small in size and have low lipophilicity that would otherwise be overlooked. The ligand efficiency-dependent lipophilicity index (LELP) has recently been proposed to combine lipophilicity, molecular size and potency into one composite descriptor, thus overcoming the size limitation of LLE. Large LLE and low LELP values have been shown to correlate well with *in vivo* efficacy. The optimal LLE scores range from ~5-7 or greater based on an average oral drug clogP ~2.5 and potency in the range of ~1-10 nM. The lower limit of LLE is 0.3 and the lipophilicity range is -3< log P <3 which defines a range of optimal LELP scores between -10 and 10 [94].

In 2014 De Marco *et al.*, investigated for the first time the correlation between *in vivo* efficacy and lipophlicity efficiency physicochemical indicators in the field of opioid compounds, using short peptidic sequences derived from EM1. Initially, c[Tyr-D-Pro-D-Trp-Phe-Gly] (**23**) [71] and c[Tyr-Gly-D-Trp-Phe-Gly] (**24**) [73], were designed as cyclic analogues of EM1. These compounds showed weaker receptor affinity than EM1, correlated to the lack of the amino group at Tyr^1, which plays a relevant role in receptor interaction and signal transduction. Nevertheless, the cyclopentapeptides were still selective partial agonist of MOR. Starting from these cyclopeptides, the tripeptide Ac-D-Trp-Phe-GlyNH_2 (**25**) (Fig. **6**) was recognized as a minimal MOR active structure [106].

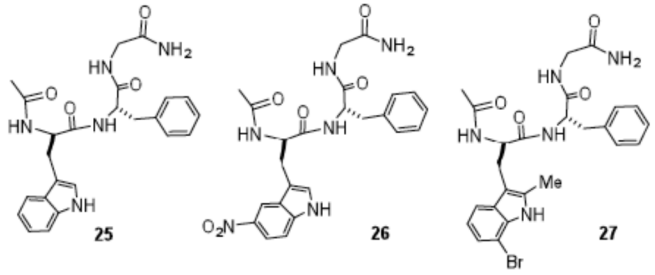

Fig. (6). Minimalist peptide sequence representative of the aromatic portion of EM1, utilized for the analysis of lipophilicity efficiency.

Subsequently, the introduction of nitro groups, halogens, alkyl groups, and combinations of different groups on the indole ring of the tripeptide sequence, gave two compounds with interesting behavior *in vitro* and *in vivo*. *In vitro*, the 5-nitro-indole tripeptide **26** showed a significant MOP affinity (Ki 51.9 nM), and the tripeptide **27** with two substituents each at the indole, 7-Br and 2-Me, was an excellent MOP ligand (Ki 4.03 nM), despite of the presence of the two sterically demanding groups and the absence of any cationic group to bind the receptor by strong ionic interaction [107].

Central analgesia *in vivo* was determined by the tail flick test. The parent cyclopeptide c[Tyr-Gly-D-Trp-Phe-Gly] (**24**) determined a mild analgesic effect, which peaked at 30 min of exposure with 32% MPE. The tripeptide Ac-D-Trp-Phe-GlyNH_2 (**25**) showed a similar analgesic profile, MPE 37% at 30 min. The tripeptide **26** with a nitro-group at the position five of indole ring displayed the highest analgesic effect *in vivo*, at tail flick test, 46% MPE at 30 min, while the 7-Br-2-Me-tripeptide **27**, under the same conditions, displayed 32% MPE at 15 min. Also the enzymatic stability

was increased, in particular in mouse serum after 3 h, the nitro-**26** showed a degradation <10%, and the 7-Br-2-Me-**27** ~20% [107].

Apparently, compounds with different structures showed similar onset of analgesic effects in the tail flick test in mice. To correlate the *in vivo* efficacy to molecular properties, we calculated and analyzed relevant physicochemical indicators of compounds **24** to **27**. The ranking of clog*P* was **27** >> **24** > **25** > **26**, and the calculated tPSA were **27** ~ **25** < **24** < **26**. These calculated values accounted very poorly for the experimental efficacy in the mouse tail flick assay. It is generally accepted that low clog*P* and/or higher tPSA correspond to poor BBB permeation. In particular, 5-nitro-**26** showed significantly higher antinociception compared to the other compounds despite its clearly less favorable clog*P* (too low) and tPSA (too large), and even despite of the reduced MOP affinity (10^{-8} M) compared to **27** (low nanomolar).

On the other hand, the lipophilic efficiency indices LLE and LELP nicely correlated to compounds efficacy in the mouse tail flick assay. The calculated LLE values were: **25** ~ **26** > **24** > **27**. For LELP: **26** << **25** < **24** < **27**. Clearly, the large LLE (>7) and the very low LELP calculated for **26** perfectly agree with the comparatively higher efficacy in the mouse tail flick assay. As for **27**, the halogen is the major contributor to the lipophilicity indices. Possibly, the favorable clog*P* and tPSA support that **27** is likely to cross the BBB very rapidly. Indeed, **27** peaked already at 15 min (33% MPE), while the other tested compounds showed their maximal effect at 30 min. However, the *in vivo* efficacy of **27** declined more rapidly (MPE at 30 min 32% *vs* 46% for **26**); this and the low MPE % could be correlated to the modest lipophilicity efficiency indices.

4.2. Cyclization

Examples of cyclic opioid peptides have been discussed in the previous sections concerning enzymatic stability. Elimination of charged termini also increases lipophilic character and therefore may enhance membrane permeability [108]. Some cyclic peptides (for example cyclosporine A), can cross the cell membrane much better than their linear counterparts. Such increased permeability might often be due to the intramolecular hydrogen bonds keeping hydrophilic groups from the surface of the molecule [109].

4.3. Cationization

Cationized peptides can bind to the anionic sites of the BBB endothelial membranes, and initiate absorptive-mediated endocytosis [110]. Very often, cationization is obtained by addition of a guanidine group. The highly basic nature of the guanidino function may be an important factor that controls the translocation of peptides. Besides, guanidilation to Tyr^1 may result in an enhanced resistance against enzymatic degradation [57, 58].

The *N*-terminal amidination of dermorphin tetrapeptides produced analogs with strong and long-lasting activity after oral administration [111], showing the usefulness of this method for the bioavailability enhancement. However, serious side-effects such as the induction of membranous nephropathy and increased cerebral and peripheral vascular permeability, discouraged further studies onthe cationized opioid peptides for pain control [33, 59].

Short oligoarginine peptides are a kind of cationic cell-penetrating peptides. The key structural feature of arginine implicated in the internalization is the guanidine moiety. The guanidine group is thought to form bidentate hydrogen bonds with the anionic groups on the surface of the cell, similar to the hydrogen bonds formed between arginine and the phosphate backbones of RNA. The cationic oligoarginine are able to strongly absorb to the cell surface though non-specific electrostatic interactions with the negative charges present.

Wang and *co*-workers synthesied a library of analogs of EM1 with *C*-terminus linked to oligoarginine in order to improve their antinociceptive effects. The authors observed that the increased number of arginine residues decreased MOP receptor affinity gradually, probably because of the steric hindrance at the *C*-termnus [112].

4.4. Glycosylation

A well-studied approach to obtain systemically active peptide analogs is conjugation with sugar moieties [113]. Glycosylation is responsible for changing interactions of a compound with biological membranes and has proven to be a useful method for enhancing BBB penetration, leading to increased biodistribution of opioid peptides to the brain and therefore improved analgesia after i.v. administration [114].

Initially, it was proposed that the bioavailability enhancement is due to the improved diffusion of peptides from peripheral sites into the bloodstream and their active transport *via* glucose transporter 1 (GLUT1) [115]. Currently, it is believed that the enhanced BBB transport of glycosylated peptides is rather due to adsorptive endocytosis [116].

Carbohydrate units not only assist peptides in their transport across membranes but also increase their water solubility. Glycopeptides showed up to a 3-fold increase in the rate of brain delivery when compared with the unglycosylated parent compounds. This improved potency is due to changes in the pharmacokinetic factors, rather than changes in opioid receptor binding affinity which is usually drastically reduced.

Incorporation of various sugar moieties such as glucose, galactose, lactose, and xylose on the Ser, Thr or Tyr residues *via* an *O*-linkage was successfully applied to obtain several analogs of opioid peptides [117]. The group of Blisky synthesized sugar analogs of enkephalin with a DOP/MOP affinity profile [114,118]. The best analog of the series, Tyr-D-Thr-Gly-Phe-Leu-Ser-(O-β-D-lactose)NH_2 (MMP-2200) produced dose-depended antinociception after various routes of administration [118].

Recently, some research has also been done to synthesize sugar derivatives of EMs. Glycosylation of EMs was first reported by Biondi *et al.* [119]. In order to carry out the glycosylation, Pro^2 in EM2 sequence was replaced by a hydroxyproline (Hyp) residue and then glucose or tetracetylated glucose was linked to the hydroxyl group of Hyp *via* a β-*O*-glycosidic bond. These modifications completely abolished biological activity of EM2

The attachment of a simple glucose unit to the *C*-terminus of EM1 or to the *N*-terminus *via* the succinamic

acid spacer, reported by Koda *et al.* [59] also did not provide significant improvement to epithelial permeability of this peptide, while the bulky sugar moiety decreased affinity of new analogs to the MOP receptor.

Varamini *et al.* [56] synthesizer new analog to EM1 (see previous paragraph) with *N*-terminus acylation on the Tyr^1 and esterification on acid terminal with sugar. The higher permeability of this analog through the BBB could have offset its lower binding affinity. Additionally, this sugar-modified EM1 derivative exhibited strong antineuropathic activity without producing constipation.

Most recently, EM2 sugar analog, Tyr-Pro-Tyr-(*O*-β-glucose)-PheNH_2, was obtained by glycosylation of the phenolic function of Tyr introduced into position 3 instead of Phe. The improved BBB permeability and enhanced central antinociception after i.v. administration was observed [120].

The described examples of the sugar-modified opioid peptides indicate that introduction of a carbohydrate moiety may or may not increase the BBB permeability. It seems that such factors as the type of sugar, the linkage used, the position of glycosylation and the use of an appropriate spacer that would enable the contact of the pharmacophoric groups with a receptor, keeping the bulky sugar moiety at some distance from these groups influence the activity of the glycoconjugates.

4.5. Halogenation

Incorporating of halogen moieties [121] can significantly modify serum and brain stability, and can exert different effects on protease breakdown. Unfortunately, very often this modification also leads to a certain decline of the opioid receptor binding [33,122]. Halogenation of amino acid side-chains can also enhance lipophilicity and the BBB permeability (See also paragraph *4.1.1.*). In an example discussed above, chlorination of EM1 at the *para*-position of Phe^4 gave rise to an analog with improved activity *in vivo* [58].

As part of continuing studies on the structure-activity relationships of EM2 analogs cyclized through the side-chains, Janecka and *co*-workers [123] reported the synthesis and biological activities of a series of cyclic analogs, incorporating fluorinated amino acids: 4-fluorophenylalanine (4-F-Phe), 2,4-difluoro phenylalanine (2,4-F-Phe) or 4-trifluoromethyl phenylalanine (4-CF_3-Phe) instead of the Phe residue in position 3 or 4.

Depending on the fluorinated amino acid residue and its position in the sequence, analogs were either mixed, high affinity MOP/KOP receptor agonists, MOP/DOP/KOP agonists or selective KOP agonists. The most potent analogs, Dmt-c[D-Lys-Phe-4-F-Phe-Asp]NH_2 and Dmt-c[D-Lys-Phe-2,4-F-Phe-Asp]NH_2 (**28**), tested in mouse hot-plate test, produced strong antinociceptive effect after i.c.v. but also after i.p. injection, indicating that they were able to cross the BBB.

4.6. Combined Modifications

Since several strategies have been developed for enhancing peptide delivery to the CNS, some researchers tried to combine them to achieve better results. For instance, the cyclopeptide **28** exploits cyclization and fluorination to achieve better stability and CNS biodistribution.

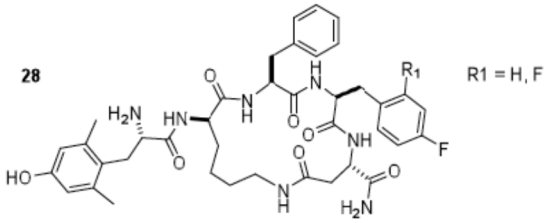

Fig. (7). Fluoro-substituted cyclic derivative of EM2.

To improve the physicochemical characteristics of EM1 Liu *et al.* [124] synthesized a series of analogs modified by *N*-terminal cationization achieved by guanidino-addition, *C*-terminal chloro-halogenation, and introduction of D-amino acids (D-Ala, Sar, D-Pro-Gly) in position 2. Guanidino-addition and chloro-halogenation attenuated MOP receptor affinity but increased stability. Chloro-halogenation compromised the decreased lipophilicity resulting from guanidine-addition. Analogs with $D\text{-}Ala^2$ or $D\text{-}Pro\text{-}Gly^2$ and *p*-$ClPhe^4$ showed strong antinociceptive activity after i.c.v. but also after s.c. administration in the tail-flick test in mice. Further modification of the most promising analog, GU-Tyr-D-Ala-Trp-*p*-ClPhe-NH_2, achieved by *O*-methylation of Tyr^1 to give GU-Tyr(*O*Me)-D-Pro-Gly-Trp-*p*-ClPhe-NH_2 resulted in a compound with significant and prolonged central-mediated analgesia upon s.c. administration [58].

CONCLUSION

This review discusses a variety of modifications introduced into the structure of EMs, which conferred analogues with increased stability and/or bioavailability, and thus a measurable *in vivo* analgesic activity. The survey of the literature and the correlated discussion highlighted the fact that several peptidomimetics with significant *in vivo* efficacy have been actually identified in the last years.

Nevertheless, it seems that renewed effort has to be dedicated to raise the interest of the pharmaceutical companies to develop and exploit these peptidomimetics as painkillers. Possibly, the acquisition of all possible information from the peptidomimetic field will open a new era of future development of innovative drugs.

CONFLICT OF INTEREST

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

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