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Engineering endomorphin drugs: state of the art

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Abstract

Importance of the field—Although EM-1 (H-Tyr-Pro-Phe-Trp-NH_2) and EM-2 (H-Tyr-Pro-Phe-Phe-NH_2) are primarily considered agonists for the μ-opioid receptor (MOR), systematic alterations to specific residues provided antagonists and ligands with mixed μ/δ-opioid properties suitable for application to health related topics.

Areas covered in this review—This review attempts to succinctly provide insight on the development and bioactivity of endomorphin analogues during the past decade. Rational design approaches will focus on the engineering of endomorphin agonists, antagonists and mixed ligands for their application as a multi-target ligand.

What the reader will gain—While the application of endomorphins as antinociceptive agents and numerous biological endpoints were experimental delineated in laboratory animals and in vitro, clinical use is currently absent. However, structural alterations provide enhanced stability, formation of MOR antagonists or mixed and dual μ/δ-acting ligands could find considerable therapeutic potential.

Take home message—Aside from alleviating pain, EM analogues open new horizons in the treatment of medical syndromes involving neural reward mechanisms and extraneural regulation effects on homeostasis. Highly selective MOR antagonists may be promising to reduce inflammation, attenuate addiction to drugs and excess consumption of high caloric food, ameliorate alcoholism, affect the immune system and combat opioid bowel dysfunction.

Keywords

endomorphins; opioid receptors; rational drug design; agonists; antagonists; peptide synthesis

1. Introduction and background

Endomorphins-1 and -2 (EM-1 and EM-2), potent MOR agonists, came into existence through the diligent investigative and rational design efforts with Tyr-W-MIF-1 peptides by Zadina et al. and their isolation from bovine [1,2] and human brain tissue [3]. Like all opioid ligands characterized by a N-terminal Tyr, except nociceptin/orphanin-FQ with a Phe group [4], the EM tetrapeptide backbone has the potential to be chemically mutated into ligands exhibiting enhanced specificity for MOR and selectivity towards δ-opioid receptors (DOR). Furthermore, since these two receptors consist of a closely related structural class of G protein coupled receptors [5,6] formulations of new opioid analogues developed as specific

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agonists, antagonists, or as mixed or chimeric ligands to both receptors simultaneously to elicit different biological endpoints.

Opioid peptides and alkaloid opiates [7] specifically interact with MOR to produce drug-induced tolerance and physical dependency with continued use. Since the existence of natural occurring opioid antagonists are unknown, the initial development of antagonists were formulated on the structural nucleus of morphine, which yielded naloxone [8] and naltrexone [9], among a large variety of compounds [10]; however, these morphinan-derived antagonists have numerous and often adverse side effects, such as withdrawal symptoms in the treatment of drug addiction and alcoholism due to suppression of the basal activity of opioid receptors [11], termed inverse agonism [12]. The unique sequence of the endomorphins, on the other hand, permitted judicious substitutions that provided not only more potent agonists, but also and importantly in our discussion on the attenuation of non-pain medical syndromes, the appearance of selective or mixed antagonists. Our summary focuses on the chemistry and biology of EM ligands that were patented and supported, for the most part, on published articles in the literature. For a comprehensive and recent review of EM chemistry, the reader is directed to the article by Keresztes et al. [13].

1.1 Endomorphin properties

Endomorphins (EM-1 and EM-2) are, beyond question, the most selective peptidic compounds interacting with MOR [1]. While their level changes during neuropathic pain models [22], they are detected in tissues throughout the mammalian body [23–25] and affect divergent physiological parameters: e.g., asthma [26] and antitussive effects [27], since endomorphins are found in nerves in esophageal smooth and striated muscle [28] to modulate cholinergic neurotransmission [29]; reduce inflammation [30–33]; decrease cardiac output [34] with vasodepressor activity on systemic arterial pressure [35,36]. Considering their selectivity for MOR, they must play a central role in the activity of the neural reward mechanism. As endogenous compounds with the innate ability to alter the activity of MOR with high specificity becomes a challenge for medicinal chemists to design more potent analgesic drugs that readily cross membrane barriers, exhibit greater half-lives, and limit or modify the deleterious problems associated with opiates. The patents discussed in this review have attempted to broach this objective with varying degrees of success.

1.2 Distribution of opioid receptors

Briefly, opioid receptors represent an evolutionarily conserved family of membrane receptors (known as μ, δ, κ, and nociceptin/orphanin-FQ) and exhibit sequence similarity and a common tertiary structure—the basic seven-transmembrane topography of G protein-coupled receptors. They exist in virtually every tissue and organ system throughout the body, but mainly associated with neural tissue to regulate physiological functions [14–19]. Their integration in a neuroregulatory system exceeds a perceived association for analgesia towards pain [20] or the induction of euphoria among opiate addicts. In fact, even placebo effects appear to be mediated by MOR [21]. In other words, MOR is important and its activity can be modulated not only by using addictive narcotic drugs, such as morphine and derivatives, but also by the consumption of high caloric foods and inflammation, which mediate homeostatic mechanisms.

1.3 Mechanisms of endomorphin action

Recent and extensive investigations in opioid chemistry resides on several important issues: (i) development of potent opioids for analgesic remediation of pain specific either for central or peripheral mode of action, the latter in particular to circumvent the limitations by the acquisition of tolerance, dependence and potential addiction associated with central-acting morphinans [37]; (ii) provide molecules with high biological efficacy and/or hydrophobicity

through SAR analyses in order to effectively pass through membrane barriers with reasonable proteolytic resistance [38,39]; proteolysis leads to unknown metabolites that might have secondary consequences or toxicological properties; and (iii) conformational analysis of the ligands and subsequent docking in MOR which provides evidence on the topography of the ligand-bind site [13]. Opioids face considerable biological hurdles in the body in order to act as therapeutic agents [40]: membrane barriers prevent passage of charged molecules in the molecular weight range > 500 Daltons and exclusion by membrane-bound molecular pumps, such as multidrug resistance P-glycoprotein-1. Owing to the highly selective interaction of EM-1 and -2 to MOR, the majority of the published data ([13] and references therein) and many of the patents discussed herein underscore the analgesic aspects in the spectrum of their bioactivity.

To overcome these limitations, judicious alterations in the sequence of EM-2 modified their properties and enhanced biostability [41,42], increased hydrophobicity and reduced overall charge with minimal alteration of its bioactivity. Structural modification included peptide cyclization [2,43,44], residue substitution by an unnatural amino acid within its sequence and/or at the N- or C-termini [41,42,45–53]; e.g., heteroaromatic compounds [54,55], formation of 1,5-enediol derivatives [56–58], a reduced bond [59], or modification with lipoidyl or glycosyl groups [60,61]. These analogues also revealed an inherent ability to alter the chemistry and side chains of the EM backbone while retaining activity and the promiscuity of MOR permitting binding of unique EM analogues to elicit a biological response.

Synthesis of EM analogues with mixed receptor properties, namely μ agonism/δ antagonism, as well as an unusual δ antagonist (*infra vide*) and formation of potent MOR antagonists by the *N*-allylation of [Dmt^1]EM-1 and -2 [48,62,63] provides myriad opioids for potential therapeutic and clinical applications. In particular, the μ antagonists exhibited neutral antagonism (absence of inverse agonist properties) that eliminated or greatly reduced morphine-induced withdrawal symptoms in mice [11] and substantially attenuated the enhancement of GABAergic neurotransmission by alcohol [62,63].

Studies elucidated the conformation of EM-1 and EM-2 [64], and NMR and CD analyses in solution provided an assessment of the contribution of *cis/trans* rotamers at the Dmt-Pro amide or Tyr-Pro amide bond. [46,64]. Data revealed a marked preference for the *cis* conformer that might contribute to the elevated activity of Dmt-containing analogues [46]. The *cis* conformer provides greater flexibility in [Dmt^1]EM-2, permitting the peptide to attain lower energy conformers [64] that might auger for enhanced interaction within the ligand-binding domain of the MOR.

2. Rational design of endomorphin analogues

2.1 General procedures for synthesis of endomorphin-2 analogues

Essentially two basic methods are used in the synthesis of opioids; namely, solution and solid phase methods. To describe each of the minor variations in each method would be outside the scope of this review; therefore, we present only two specific examples: one for the solution synthesis of [Dmt^1]EM-2 and a general outline for solid phase synthesis using as an example the formation of the unusual EM-2 analogues containing a 1,5-enediol in lieu of the Pro residue [56,57,58].

2.1.1 Solution synthesis—Syntheses of EM-2 analogues were performed by standard solution peptide synthesis methods in various patents and are principally the same for all EM analogues [2,42,48,50–53]. Although the patent of Carr et al. [65] does not stipulate the method of EM synthesis, their publication describes a solution procedure [66].

In an overview of the solution synthesis of EM-2 analogues [48], we use as an example the procedures developed for Dmt derivatives. Dmt was the residue to choice due to the enhanced MOR affinity and functional pharmacological activity of endomorphins [46,54]. This decision was based on the efficacy of Dmt on the induction of extraordinary DOR selectivity and unique pharmacological properties of the Dmt-Tic pharmacophoric opioids [67–71] and described in another series of EM-2 stereoselective 1,5-enediol analogues [57]. Dmt can be either prepared according to the method of Dygos et al. [72] and its chirality assessed by HPLC, or purchased commercially (RSP Amino Acids LLC, Shirley, MA USA).

As outlined in Figure 1, (a) a mixed anhydride method used IBCF, NMM, or (c) PyBop was employed as a coupling reagent. The Boc group protected the *N*-terminal amine function. Deprotection (b) was performed in 6 N HCl/dioxane or TFA. During the course of synthesis, Boc protected peptide intermediates were identified by NMR and elemental analysis. The final product was purified by semi-preparative reversed phase-HPLC. Purified opioids exhibited > 98% purity by analytical HPLC (absorbance at 220 nm), and characterized by NMR, MALDI-TOF mass spectrometry and elemental analysis.

Unlike EM-2, EM-1 contains tryptophan (H-Tyr-Pro-Trp-Phe-NH_2) which contains a 3-alkylindole side chain that is easily attacked by an electrophile under acidic conditions, especially at the 2-position, and is susceptible to oxidative degradation [73,74]. In the presence of strong bases the indole system reacts as an anion, mainly at the indole nitrogen, necessitating its protection. Considering these circumstances, it is easy to quite understand that the synthesis of EM-2 analogues in large quantity and diversity is preferable to those of EM-1 [13,75,76]; nonetheless, biologically active EM-1 analogues were patented [48,50–53].

2.1.2 Solid phase synthesis of endomorphin-2 analogues—The majority of EM and patented analogues thereof were synthesized using solid-phase methodology [2,49,58,60,78] or undefined “by chemical or enzymatic synthesis” [79]. Among some EM patents, the method of peptide synthesis was absent due to a focus on their application as prophylaxis compounds [80], anti-inflammation agents [31], cosmetic formulations [81], or merely referenced [82].

This procedure is principally similar to that for solution phase methodology except for the protection of the C-terminus by a solid support. There are two main strategies in solid phase methods: either Boc or Fmoc chemistry [83]. The synthesis of EM analogues can produce a multiplicity of diastereoisomers of EM-2 using a Fmoc amide resin as follows: the solid support, Fmoc-D- or L-Tyr(Bu^t)-OH, Fmoc-D- or L-Pro-OH and Fmoc-D- or L-Phe-OH as the protected amino acids, and HBTU/HOBT/DMF, DIEA/NMP for each reaction in a peptide synthesizer [84]. After each coupling reaction, the Fmoc group was removed with piperidine/NMP. In the final deblocking step, the dried protected peptide resin was suspended in TFA/H_2O and the reaction mixture stirred at room temperature. The material was filtered, ether added to the filtrate, and the precipitate collected by filtration and lyophilized from HCl.

The procedure for the synthesis of stereoisomers of Tyr^1- or [Dmt^1]EM-2 analogues containing a 1,5-enediol moiety [56,57] used Boc-Tyr(Bu^t)-OMe or Boc-Dmt(Bu^t)-OMe as the starting material and only one stereoisomer (**1**) (Figure 2) is described briefly as a specific example. As shown in Figure 2, Boc-Dmt(Bu^t)-OMe (**2**) [(*S*)-**2**] was reduced to the aldehyde with DIBAL-H and allylated with allylmagnesiumbromide to give (*S*,*S*)-**3** and (*S*,*R*)-**3** and separated by flash chromatography. (*S*,*S*)-**3** was coupled with excess (*S*,*R*)-**4** using the olefin cross-metathesis approach [85] with a $Cl_2(PCy_3)(IMesH_2)$RuCHPh catalyst

[86] by refluxing in CH_2Cl_2, followed by hydrolysis with LiOH and H_2O_2 to give (*S*,*S*,*S*,*R*)-**5**. Compound (**1**) was prepared by coupling (*S*,*S*,*S*,*R*)-**5** and solid supported Phe by HBTU/HOBT, followed by TFA deprotection and HPLC purification with high purity

2.2 Agonists and agonism

The bulk of the patents issued are pertinent to EM-2 analogues as analgesic agents in the management of pain through MOR [48]. To re-emphasize, EM-1 and EM-2 are the most highly selective natural occurring opioids with remarkable specificity for only MOR [1,2]. One interesting patent seeking to overcome the limitations of externally supplied opioids for pain remediation constructed a library of DNA sequences encoding precursors of C-terminal amidated peptides, such as EM-2; however, data on its application were unavailable [87]. Endomorphins were suggested as potential compounds for neuropsychiatric applications, but the patent lacked experimental or patient data on EM-2 in combating these symptoms despite the inclusion of its binding affinity to rat spinal cord membrane ($K_i\mu$ = 8.7 nM) [78].

The patent by Persons *et al*. [49] focused on the modification of the N-terminal Tyr and/or Pro^2 and the C-terminal residue Phe^4 in EM-2 with substitution of peptide bonds. However, MOR affinity (IC_{50} values) or functional pharmacological activity (guinea-pig ileum bioassay) cannot be fully ascertained since the data were only presented as < 1 μM, < 10 μM or < 30 μM relative to DAMGO; it terms of MOR agonism, the value for DMAGO was given as 0.03 μM. The amended claims section mentioned acceptable receptor affinities having an IC_{50} < 100 nM, however, without supplying details on specific EM analogues [49].

Similarly, extensive work by Wang et al. [50,51,52,53] explored modification of EM-1 and EM-2 in Chinese language patents, the data of which was detailed in numerous published articles and briefly discussed herein relative to EM bioactivity: (i) Phenylalanine mimetics at positions Phe^3 and Phe^4 in EM-2 [88,89] weakly interacted with MOR despite a gain in enzymic stability [88]. Structural analysis by NMR and molecular modeling paradigms were published in two virtually identical papers [90,91]. C-Terminal analogues containing oligoarginine derivatives yielded essentially inactive peptides [92]. Those data support earlier interpretations that C-terminal modifications are detrimental for optimum activity; however, retention of bioactivity and/or change in receptor selectivity was clearly identified in EM analogues in which various aromatic, heteroaromatic or aliphatic groups replaced the Phe^4-NH_2 group [54] due to, perhaps, a more effective stacking with a hydrophobic side chain in the receptor, such as Trp, Tyr or Phe, through van de Waals forces, π−π bonds, cation-π interactions, atomic volumes or spatial dimensionality. And that interaction produces an alteration in the dynamically fluid 3-dimensional topography of the receptor leading to shifts in bioactivity from agonism to antagonism, from μ to δ selectivity.

(ii) Analogues modified at positions 2 and/or 4 with D-Pro-Gly or *p*-ClPhe, respectively, exhibited enhanced stability in brain homogenates and serum, but only weak receptor affinities or in vitro bioactivity data were published; the antinociception of the most active analogue [$N^{\acute{\alpha}}$-guanidino-$Tyr(Me)^1$,D-$Pro\text{-}Gly^2$,*p*-$ClPhe^4$]EM-1 was only partially (50%) suppressed by naloxone [89], indicative of other receptor-ligand attributes. However, several N-terminal guanidylated-modified EM-1 in combination with D-Ala^2, Sar^2 and/or D-Ala^2,*p*-$ClPhe^4$ revealed either enhanced MOR affinity or a reduction by about two-thirds, while antinociception was similar to EM-1 following intracerebroventricular injection in mice due perhaps to marked an increase in stability [93]. (iii) Bivalent and C-terminal esterified analogues in which -OMe and -$NHNH_2$ groups revealed $K_i\mu$ values comparable to EM-2 but exhibited weak bioactivity [94,95]. (iv) Incorporation of a reduced amide bond ψ (CH_2NH) between Tyr^1 and Pro^2 (EM-1[ψ] and EM-2[ψ]) were devoid of MOR bioactivity but exhibited a modest DOR activity that was substantially less than the parent peptides [59]. (v)

Substitutions at positions 2 and 4 of [N^{α}amidino-Tyr^{1}]EM-1 were investigated for their cardiovascular effects using rats in comparison to naloxone, which reversed the recorded changes initiated by the peptide analogues [96]. Complete protection of the N-terminal amine obliterates biological activity, such as for example *N*,*N*-disubstituted allyl groups [62] among other N-terminal modifications [60], while a *N*-monosubstituted allyl group is readily accommodated in MOR ligands [62].

Other publications that may be irrelevant to these patents but are mentioned only for the sake of thoroughness, namely the efficacy of EM-1 and/or EM-2 on physiological parameters [29,97–99], their release from nanoparticles [100], or dimeric analogues of EM-2 with or without alkane spacers, which were basically devoid of receptor affinities or functional bioactivities [94].

2.2.1 Cyclic endomorphin analogues—In a series of patented linear and cyclic analogues by Zadina et al. were based on their precursor model peptide for MOR, Tyr-W-MIF-1 [2]; additional cyclic EM-2 analogues were published as well [44,101]. Cyclization utilized the DPPA method [77]. The patent, however, only described the pharmacological properties of one of the 14 cyclic analogues, namely H-Tyr-c-[D-Lys-Trp-Phe] and studied in comparison to EM-1. It exhibited ca. 15-fold higher MOR affinity, 4-fold greater efficacy on GPI (Table 1) and a 10-fold increased antinociception (IC_{50} = 300 ng) in vivo, which had ca. twice the potency of morphine following intravenous injection.

The patent issued to Maione [82] describes the application of the same cyclic derivative of EM-1 as Zadina et al. [2]. This patent centered on producing a pharmacological effective peptide as an organic or inorganic salt, safe for human consumption, presumably without negative toxicological side effects [82]. In essence, the author states that the peptide salt, suitable for “external, enteral or parenteral applications” would produce analgesia and relief from gastrointestinal distress from diarrhea resulting from disease or drugs implemented in cancer therapy, act as an anti-inflammatory substance (see [31]) and combat “drug dependence in patients” [82]. Nonetheless, considering the potent MOR agonism of this compound [2], it is difficult to visualize any role in alleviating drug addiction! (Even methadone used to treat drug dependency is itself addictive.) Aside from the detailed physicochemical characterization o f various peptide salt complexes, the patent lacked biological and/or pharmacological/physiological data to support the claims [82].

2.2.2 Lipid and glycosylated modified EM-2 analogues—Modification of the backbone of EM-1 and EM-2 with one or more lipid or saccharide moieties arose from the observation that these derivatives improved cell permeability and peptide stability through enhanced hydrophobic properties without abolishing, for the most part, MOR binding properties [60]. Moreover, as shown elsewhere [46], replacement of Tyr by Dmt in EM-2 in these analogues enhanced μ-receptor affinity (Table 1) but had no significant effect on stability in plasma; however, other N-terminal [Dmt^{1}]EM-1 lipid-modified analogues, containing 2-aminododecanoyl and 2-aminodecanoyl enhanced stability ca. 5- and 10-fold, respectively, while maintaining < 1 nM μ-receptor affinity (Table 1) [60]. In general, the *N*-substituted lipid derivatives exhibited greater biological activity than the corresponding glycosylated compounds [60]. A series of EM-1 analogues containing lipo-, glyco- and liposaccharide moieties internally or at the N and/or C termini generally exhibited weak MOR affinity based on whole SH-SY5Y cells or rat brain synaptomes, yielding comparable values. A high MOR affinity was recorded with the [*N*-2-aminodecanoyl-Dmt^{1}]EM-1 analogue [61], once again providing substantial evidence for the efficacy of Dmt replacement of Tyr to improve an opioid bioactivity profile and containing only a single substituent on the N-terminal amine [62].

2.3 Antagonists and antagonism

The transformation of the μ agonists EM-1 and EM-2 into high affinity, potent MOR antagonists was described [48] and its pharmacological and physiological actions delineated in considerable detail elsewhere [11,62,63]. These substances, [*N*-allyl-Dmt^1]EM-1 and [*N*-allyl-Dmt^1]EM-2 (Table 2), are defined as neutral μ antagonists due to their lack of inverse agonist properties by functional guanosine 5'-*O*-(3-[^{35}S]thiotriphosphate) assays in vitro from membranes of cells grown in the presence of morphine or alcohol [11]. More importantly, they completely inhibited naloxone- and naltrexone-evoked withdrawal symptoms following acute morphine dependency in mice [11]. Similarly, [*N*-allyl-Dmt^1]EM-2 effectively reversed the ethanol-induced enhancement of GABAergic neurotransmission in CA1 pyramidal cells of rat hippocampus through inhibition of evoked or spontaneous inhibitory postsynaptic currents (IPSC) [63]. Interestingly, while naltrexone is considered the Food and Drug Administration approved drug of choice in combating alcohol [102,103] and drug addiction (along with naloxone) [104], [*N*-allyl-Dmt^1]EM-2 is > 100-fold more potent in modulating the effects of alcohol [63]. This portends a potential therapeutic role in combating addiction from both alcohol and the morphinans.

2.4 Endomorphin analogues with multiple receptor properties

2.4.1 Bifunctional analogues—This category embraces EM-2 analogues described as having mixed μ agonism/δ antagonism or δ antagonism [54] and/or dual μ/δ agonist properties [76] as an outgrowth of a patent [48]. Although not patented, these analogues are discussed in light of the biological importance of heterodimeric μ- and δ-opioid receptor complexes, which enhance or attenuate receptor function [105–108] through allosteric changes to their three-dimensional topology. Heterodimeric receptors regulate receptor turnover, translocation and endocytotic mechanisms [108]. In addition, heterodimers complex with non-opioid receptors altering the innate function of each component [109–111] that has a direct bearing on opioid dependency, tolerance [112–114] and withdrawal symptoms [115–119].

The receptor-mediated properties of representative analogues of EM-2 are listed in Tables 1–3. Interestingly, while the replacement of the C-terminal Phe amide in [Dmt^1]EM-2 with various hydrophobic groups had minimal disruptive effect on MOR affinities [54], the spatial orientation of the quinolyl and naphthyl groups determined by molecular modeling paradigms indicated that the positioning of the heteroaromatic ring was responsible for the highly effective MOR agonism (5- > 6- > 8-quinoline) and the acquisition of DOR agonism; i.e., 5-quinolyl and 1-naphthyl had potent DOR bioactivity [54]. These data further support the concept that increased hydrophobicity at the C-terminus of EM in lieu of Phe^4-NH_2 in [Dmt]EM-2 exerts substantial effect on the determination of biological activity. In addition, increasing the hydrophobic nature of Phe^3, such as replacement by Tmp or even Dmp, produced potent opioid ligands with mixed μ agonist/δ antagonist properties [76] (Table 3).

Recently, Zhang et al. [120] expanding upon earlier published studies [54,75,76] on hydrophobic residues substituted in the sequence of [Dmt^1]EM-2. They reported acquisition of a high dual μ/δ affinity and non-receptor selectivity in one compound, H-Dmt-Pro-Tmp-Tmp-NH_2 (Table 1), that has yet to be biologically assessed but obviously needs to be addressed. These data compliment a large body of evidence that verifies that enhancing the hydrophobicity of residues 3 and/or 4 affects both MOR and DOR affinities in EM.

2.4.2 Hybrid analogues—Hybrid formulation covalently coupled EM-2 and one of two SP analogues [ESP7 ($SP_{7–11}$) and ESP6 ([Pro^9]$SP_{7–11}$)] to the C-terminal Phe-amide of EM-2 [65]. The basis of this patent arose from studies on the potentiation of antinociception of morphine by SP in the spinal cord [121–123] and published in considerable detail [66].

An earlier patent on a morphine-SP hybrid discussed the potential clinical value to induce analgesia and reduce tolerance using a hybrid composed of morphine and SP separated by a flexible organic acid spacer; however, no data were presented on the claims for analgesic potentiation [122]. In terms of the EM-SP hybrid molecules, only ESP7 was thoroughly assessed in the patent [65]. The hybrid, however, displayed considerably reduced receptor affinity relative to DAMGO and SP, exhibited ca. 100-fold decrease in binding to MOR and ca. 3,000-fold loss towards the NK_1 receptor, respectively. On the other hand, the inhibition of forskolin-stimulated cAMP activity, ESP7 had ca. 10% the potency of either DAMGO or EM-2 [66]. In addition, the intracerebroventricular injected ESP7 produced moderate analgesia; however, control data against an equivalent molar concentration of morphine was absent, nonetheless naloxone completely inhibited analgesia induced by a low dose of ESP7 indicative of an opioid-mediated effect [65].

A hybrid molecule formed by coupling the C-termini of EM-1 and Dmt-Tic-NH-$(CH_2)_2$-NH_2 (a moderate DOR antagonist) enhanced MOR affinity by 50% while elevating DOR affinity > 6000-fold to yield a formidable DOR antagonist (pA_2 = 8.9), while retaining about half the MOR agonist activity [124] (Table 3). Like genetic hybrids, this F1 offspring, a mixed μ agonist/δ antagonist, had new biological properties considerably different than either parent opioid molecule. The question of efficacy for this bivalent molecule is unknown, but the potential application in pain remediation needs to be addressed.

3. Potential therapeutic applications

One interesting application of EM-2 is its inclusion in cosmetics for people with sun sensitive skin and its accompanying manifestations [79,81]. The authors claim that the presence of EM-2 might attenuate the symptoms sufficiently to allow people with these sensitivities to use a host of topical cosmetic preparations. In particular, preparations containing the beneficial effects of α-hydroxyl acids and retinol (vitamin A), known to combat the photoaging process in skin, to smooth out wrinkles or acne scars through increased rate of epithelial cell turnover with a concomitant enhancement of collagen and elastin synthesis. The rationale for the use of EM in a topical ointment was previously substantiated by animal studies: (i) In the absence of MOR, mice undergo epidermal hypertrophy suggesting opioid involvement in skin homeostasis [125]; and (ii) the attenuation of pruritus by a dermatological cream containing 1% naltrexone [126], a non-selective opioid antagonist [127], indicated interaction with opioid receptors since skin also contains DOR and KOR [125,128,129], the former affecting differentiation and wound healing [129]. Thus, stimulation of MOR by EM might be theoretically applicable to alleviate skin sensitivities in cosmetic emulsions, assuming, of course, a charged, hydrophilic peptide, such as an EM, can sufficiently penetrate the epidermal barrier to interact with dermal MOR even with the assistance of emulsifiers, hydrotropic compounds or swelling agents [79].

In other applications with EM-2, such as a therapeutic agent for the “prophylaxis and/or treatment of cancers” and numerous diseases, were not substantiated by the data supplied in the patent [80]. The inventors provided a series of formulations, such as gel, lotion and mother's milk for infants to use with or in the delivery of EM-2. Nonetheless, EM-2 exhibited very minimal or no effects on cell viability or blood cell proliferation, prevention of apoptosis, marginal effects of cytokine profiling and ineffective on lipopolysaccharide induction of TNFα in spleen cells, and lacked inhibition of bacterial growth [80]. Only a modest 15.6% inhibition of hepatitis B virus replication in HEP-G2 cells was observed as well as a 9.5% reduction in human cytomegalovirus plaque formation relative to the positive controls of 92.0% and 100%, respectively [80].

Involvement of EM-1 and EM-2 in immune function was the basis of a patent for the treatment of existing inflammation or prophylaxis of inflammation [31]. The patent was based on their discovery and isolation of immunoreactive EM-1 and EM-2 from immune tissues (spleen, thymus) [30] and in the synovial tissue from rats with adjuvant-induced arthritis [32]; in normal human subjects, both opioids were detected in spleen [30,32]. Other supporting data revealed the presence of immunohistochemical staining of both EM-1 and EM-2 in distinct cells in rat spleen [25]. In the patent, the authors provided experimental evidence that both opioids are secreted upon stimulation with concanavalin A from cultured human lymphocytes (T- and B-cells, macrophages). While individual data points were statistically relevant (20% reduction of inflammation in a rat paw model with induced adjuvant arthritis following systemic injection of EM-1), the supporting data failed to demonstrate a linear dose response and lacked data on preclinical trials [31].

Considering that EM-1 and EM-2 act on cardiac output as vasodepressor agents by decreasing systemic arterial blood pressure in a naloxone-reversible manner in both rat [34,36,130] and rabbit [131], it is interesting that these opioids or analogues thereof were not investigated in human subjects exhibiting hypertension. This physiological assessment of the action of EM-2 fully supports the involvement of MOR agonists in homeostatic mechanisms [16,19].

4. Conclusions

Despite the enormity of the investigative studies undertaken with the endomorphins [13] and the potential to exacerbate various health syndromes, neither EM-2 nor any of the analogues patented or published in the research literature have apparently reached the level of a therapeutic drug. While the authors are unaware of clinical trials utilizing EM, trademarked names and proprietary formulations of encapsulated or time-released drugs and inclusion into cosmetic creams or ointments would obscure detection. On-line searching of patents further proved futile to uncover clinical applications.

As major pharmaceutical firms continue to retrench from actively developing new drugs in-house with the termination of patent protection, the lack of foresight to invest in medicinal chemistry will be to the detriment of society. In particular, the epidemic of narcotic addiction [132], which is wholly dependent on MOR [16], is facing a crisis in the absence of reliable antagonists with reduced or the absence of side effects in contrast to withdrawal symptoms often accompanying treatment by naltrexone or naloxone [37,133]. Furthermore, no publications reveal the application of EM-2 antagonists to address the obesity pandemic —which is considered another form of addiction of the neural reward system [134,135]—or towards ameliorating osteoporosis due to the presence of opioid receptors on osteoblast-like cells which are stimulated by MOR antagonists [136]. Similarly, non-addictive or dual functioning EM-2 agonists could be tested to mitigate the negative effects observed in geriatric patients under treatment for pain management with opiates among other drugs [137]. Moreover, multiple direct and indirect interactions occur between opioids and neuroregulatory peptides as well as their receptors forming heterodimeric complexes with opioid receptors to directly impinge on the opioid system to mediate change [138,139].

5. Expert opinion

Despite extensive laboratory studies and projected or potential preclinical applications for EM and analogues thereof [13], their application as therapeutic drugs to alleviate acute, chronic or neuropathic pain symptoms as pure μ agonists or mixed μ agonists/δ antagonists has yet to materialize. Equally, the shortcoming to ameliorate opiate addiction and obesity [135] with neutral or pure MOR antagonists [62,63] fails to ignite interest in either the

pharmaceutical industry or medical community. Their agendas seem to be at odds with providing creative solutions for long-term goals of developing an equitable society [140]. “Valuable breakthroughs…[are]…driven by the curiosity of individuals,” Ahmed H. Zewail emphasized, when “…dreamers must be willing, and allowed, to take risks.” The potential application of selective MOR antagonists to reduce the loss of bone mass density in obesity-associated osteoporosis [141,142] serves as one example to overcome the serious and deleterious side effects of bisphosphonate-based drugs [143–147], products heavily marketed by pharmaceutical firms.

The inherent ease in the ability to alter the basic peptidic structure of EM, sometimes quite radical while maintaining receptor selectivity and enhancing receptor affinities, should provide a further impetus to seek and develop uniquely bioactive analogues, perhaps even specifically designed to differentiate between supraspinal and peripheral sites, or even relieve specific symptom modalities. Selective targeting symptoms could be accomplished with judicious studies on the efficacy of the types of EM analogues discussed in this review. Furthermore, it should be noted that in addition to clinical intervention, veterinary medicine could be an equal beneficiary in the application and development of new EM-based drugs. While the long road ahead remains challenging, fraught with the pitfalls of discarded failures, Goethe reaches out over the centuries to continually challenge and admonish society: “Nothing is more terrible than ignorance in action.”

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Abbreviations and definitions

Ac_3c	1-aminocyclopropanecarboxylic acid, an α,α-disubstituted glycine
Boc	*tert*-butyloxycarbonyl
Bzl	benzyl
cAMP	cyclic adenosinemonophosphate
$Cl_2(PCy_3)(IMesH_2)RuCHPh$	a ruthenium catalyst used in the olefin metathesis reaction in solid phase peptide synthesis [56,57]
CD	circular dichroism
Det	2',6'-diethyl-L-tyrosine
DAIBAL-H	diisobutylaluminium hydride
DAMGO	[D-Ala^2,*N*MePhe^4,Glyol^5]enkephalin, a MOR agonist
DIPEA	diisopropylethylamine
Dmp	2',6'-dimethyl-L-phenylalanine
Dmt	2',6'-dimethyl-L-tyrosine
DOR	δ-opioid receptor
DPPA	diphenylphosphoryl azide
EM	endomorphin

Emt	2'-ethyl-6'-methyl-L-tyrosine
Et_2O	diethyl ether
Fmoc	9-fluroenylmethyloxycarbonyl
Fmoc amide resin	4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamidoethyl resin
GPI	guinea pigileum to determine MOR pharmacological activity
GTP	guanosinetriphosphate
HBTU	*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium-hexafluorophosphate
HOBt	1-hydroxybenzotriazole
HPLC	high performance liquid chromatography
Hyp	hydroxyproline
IBCF	isobutyl chloroformate
IC_{50}	the molar concentration required for 50% inhibition of electrically induced contraction of GPI strips or whole MVD from a single animal
5-Isq	5-isoquinolyl
K_i	the binding constant of ligand to opioid receptors [148]
KOR	κ-opioid receptor
MALDI-TOF	matrix-assisted laser desorption ionization time-of-flight
MOR	μ-opioid receptor
NK_1	neurokinin 1 receptor
NMM	*N*-methylmorpholine
Mmp	2'-monomethyl-L-phenylalanine
Mmt	2'-monomethyl-L-tyrosine
MVD	mouse vas deferns used for assessment of DOR activity in vitro
NMP	*N*-methylpyrrolidone
NMR	nuclear magnetic resonance
1-Nph	1-naphthyl
pA_2	is the negative log of the molar concentration of the analogue required to double the EM-2 concentration to elicit the original response
opioid	a peptide compound capable of eliciting analgesia
opiate	a plant derived alkaloid belonging to the general class of morphinans, which exert biological addiction
Ph	phenyl
Phe-NH-Rink amide AM resin	the solid support used in EM synthesis

PyBop	benzotriazol-1-yloxytripyrrolidinophosphonium hexafluorophosphate
5-Qln	5-quinolyl
SAR	structural-activity relationship
SP	substance P
TFA	trifluoroacetic acid
Tmp	2',3',6'-trimethyl-L-phenylalanine
Tmt	2',3',6'-trimethyl-L-tyrosine
TNFα	tumor necrosis factor-alpha. Single letter designation for amino acids: F, phenylalanine
K	lysine
P	proline
W	tryptophan
Y	tyrosine

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Article highlights

- Discovery of EM-1 and -2 in 1997 provided new opioid ligands to combat various medical symptoms, most notably pain relief.
- To date, many patents were published from around the world and through the US Patent Office.
- The voluminous number of publications on the formation of EM analogues provides insights on their innate structure for the development of therapeutic drugs.
- Selective analogues of EM-2 enhanced its biological half-life and provided ligands with potential clinical application.
- While EM agonists are antinociceptive, antagonists are applicable to alleviating drug and alcohol addiction, and suppressing reward mechanisms.
- Experimental data point to potential applications to ameliorate numerous disease states, including but not limited to inflammation, alcoholism, opioid bowel dysfunction, osteoporosis and obesity, while enhancing the immune system.
- Formulation of mixed and dual μ/δ ligands acts as a multi-target therapeutic agent to reduce pain while alleviating possible detrimental side effects.

This box summarizes key points contained in the article.

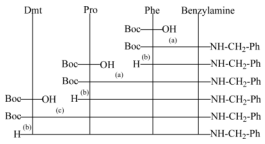

Figure 1. Synthetic scheme for the solution synthesis of [Dmt^{1}]endomorphin-2 analogues

(a) A mixed anhydride method using IBCF, NMM, or (c) PyBop with DIPEA were employed as coupling reagents. The Boc group was used for *N*-terminal protection. Deprotection (b) was performed in HCl in dioxane (6 N HCl/dioxane) or TFA.

Figure 2. Scheme for the solid phase synthesis of stereoisomers of 1,5 enediol endomorphin-2 analogues

Reagents and conditions: (a) DAIBAL-H, toluene, at −78 °C. (b) allylMgBr, THF, Et_2O, at 0 °C. (c) $Cl_2(PCy_3)(IMesH_2)RuCHPh$, CH_2Cl_2, at 40 °C. (d) LiOH, H_2O_2, THF, H_2O. (e) HBTU, HOBt, DIPEA, NMP, Phe-NH-Rink amide AM resin, then 95% TFA.

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Table 1

Receptor affinity and bioactmty of cndormorphin-1 and -2 agonists.

	Affinity constant K_i (nM)			**Bioactivity, IC_{50} (nM)**		
Sequence	**K_i μ**	**K_i δ**	**δ/μ**	**GPI (μ)**	**MVD (δ)**	**Ref.**
YPWF-NH_2 (EM-1)	0.36	1506	4183	3.6	-	[1]
YPFF-NH_2 (EM-2)	0.69	9233	13381	4.0	-	[1]
Dmt-PFF-NH_2	0.15	28	188	0.07	1.87	[46]
Dmt-PF-NH-C_2H_4-Ph	0.51	18.0	35	5.03	>10000*	[54]
Dmt-PF-NH-Bzl	0.52	13.8	27	22.0	>10000*	[54]
Dmt-PF-NH-1-Nph	0.29	20	68	0.49	5.47	[54]
Dmt-PF-NH-5-Qln	0.11	30	288	0.26	0.62	[54]
Dmt-PF-NH-5-Isq	0.19	98	517	0.94	>10000*	[54]
YP-Dmp-F-NH_2	0.03	1063	34967	0.38	1.39	[47]
Mmt-PFF-NH_2	0.13	529	4005	0.92	28.7*	[75]
Emt-PFF-NH_2	0.06	56	884	0.62	1.08*	[75]
Det-PFF-NH_2	0.08	70	830	0.90	47.1*	[75]
Tmt-PFF- NH_2	0.11	594	5347	2.31	46.4*	[75]
YPF-D-F-NH_2	8.4	>10000	>1200	25	390	[150]
YPFF-OCH3	9.1	>10000	>11000	22.6†	-	[95]
Dmt-P-Tmp-Tmp-NH_2	0.47	1.63	3.5	-	-	[120]
Dmt-P-Tmp-F-NH_2	0.18	1.83	10	0.21	>10000*	[76]
Y-Hyp-FF-NH_2	16.1	-	10	2550	-	[43]
Dmt-1,5-enediol-F-CO-O-F-H (*SSSR*)	0.24	56	233	‡	-	[57]
Dmt-1,5-enediol-F-NH-$CONH_2$-F (*SSRR*)	0.42	36	86	‡	-	[57]
Y-c-[D-KWF]	~0.024	-	-	~0.9	-	[2]
Dmt-PWF-NH_2	0.054	5.60	104	0.27	>10000	[62]
N-2-aminooctanoyl-Dmt-PWF-NH_2	0.071	-	-	‡‡	-	[60]
N-2-aminodecanoyl-Dmt- PWF-NH_2	0.47	-	-	‡‡	-	[60]

	Affinity constant K_i (nM)			Bioactivity, IC_{50} (nM)		
Sequence	**K_i μ**	**K_i δ**	**δ/μ**	**GPI (μ)**	**MVD (δ)**	**Ref.**
N-2-aminododecanoyl-Dmt- PWF-NH_2	0.735	-	-	‡‡	-	[60]
Y-Ac_3c-FF-NH_2	0.53	364	687	-	-	[42]
Y-Ac_3c-Dmp-F-NH_2	2.58	3140	1220	-	-	[42]

Table 2

Endomorphin-1 and -2 analogues as μ antagonists

	Affinity constant K_i (nM)			**Bioactivity, IC_{50} (nM)**			
Sequence	**K_i μ**	**K_i δ**	**δ/μ**	**GPI (μ)**	**MVD (δ)**	**μ pA_2**	**Ref.**
[*N*-allyl-Dmt]-PWF-NH_2	**0.26**	**10.3**	40	>10000	>10000	8.18	[62]
[*N*-allyl-Dmt]-PFF-NH_2	0.45	560	1244	>10000	>10000	8.59	[62]

Table 3

The δ Antagonist properties of endomorphin-1 and -2 analogues

	Affinity constant K_i (nM)			**Bioactivity, IC_{50} (nM)**			
Sequence	**K_i μ**	**K_i δ**	**δ/μ**	**GPI (μ)**	**MVD (δ)**	**δ pA_2**	**Ref.**
Dmt-PF-NH-C_2H_4-Ph	0.51	18.0	35	5.03	>10000	7.05	[54]
Dmt-P-Mmp-F-NH_2	0.18	4.61	26	0.16	>10000	6.59	[76]
Dmt-P-Dmp-F-NH_2	0.07	2.27	33	0.12	>10000	8.15	[76]
Dmt-P-Dmt-F-NH_2	0.09	80.8	878	1.94	>10000	7.06	[76]
Dmt-P-Tmp-F-NH_2	0.18	1.83	10	0.21	>10000	9.05	[76]
[*N*-allyl-Dmt]-PWF-NH_2	0.26	10.3	40	>10000	>10000	7.32	[62]
[*N*-allyl-Dmt]-PFF-NH_2	0.45	560	1244	>10000	>10000	6.32	[62]
YPFF-NH-$(CH_2)_2$-NH-Tic-Dmt	1.03	1.45	1.41	25.1	-	8.9	[124]