New Trends in the Development of Opioid Peptide Analogues as Advanced Remedies for Pain Relief

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Abstract: The search for new peptides to be used as analgesics in place of morphine has been mainly directed to develop peptide analogues or peptidomimetics having higher biological stability and receptor selectivity. Indeed, most of the alkaloid opioid counterindications are due to the scarce stability and the contemporary activation of different receptor types. However, the development of several extremely stable and selective peptide ligands for the different opioid receptors, and the recent discovery of the μ-receptor selective endomorphins, rendered this search less fundamental. In recent years, other opioid peptide properties have been investigated in the search for new pharmacological tools. The utility of a drug depends on its ability to reach appropriate receptors at the target tissue and to remain metabolically stable in order to produce the desired effect. This review deals with the recent investigations on peptide bioavailability, in particular barrier penetration and resistance against enzymatic degradation; with the development of peptides having activity at different receptors; with chimeric peptides, with propeptides, and with non-conventional peptides, lacking basic pharmacophoric features.

Key Words. Opioid peptide analogues; opioid receptors; pain; antinociception; peptide stability; bioavailability.

INTRODUCTION. OPIOID PEPTIDES, RECEPTORS, AND PAIN

The endogenous opioid peptides have been studied extensively since their discovery aiming to develop effective drugs for the treatment of pain in humans [1-4]. Pain is modulated by a circuit that includes amygdala, PAG, DLPT, and RVM in the brainstem. This circuit controls spinal and trigeminal dorsal horn pain transmission neurons and mediates endogenous analgesics. Pain can be defined mild, moderate, and severe, on the basis of its severity. In terms of temporality, it can be acute or chronic. Pain can be also defined in terms of its etiology. Mechanistically, it can be considerd spontaneous, or hyperalgesia, an exaggerated response to normally mild painful stimulus, or allodynia, which occurs in response to normally non-noxious stimulus.

Substance P is a tachykinin neurotransmitter and neuromodulator acting as a prototypic spinal excitatory peptide. The interaction of endogenous peptides with substance P determines the regulation of analgesic responses to nociceptive stimuli. Therefore, opioid peptides are significantly implicated in antinociceptive processes. Their action is modulated by opioid receptors, widely distributed in the CNS [5,6]. They belong to the class of G-protein coupled receptors, and can be divided into three types, μ, δ, and κ; each type can be further divided into several subtypes. They have been recently cloned, allowing the studies of ligand-receptor interaction by structure-function studies of recombinant receptors and chimera receptors [7,8]. Experiments performed on mutant mice gave new information about the mode of action of opioids, receptor heterogeneity and interactions [9].

The cloning of each of the opiate receptors allowed the comparison of the amino acid sequences. The different receptors share a large number of amino acid sequences, and therefore show a large degree of homology. Among the other members of the G-protein coupled receptor superfamily, opioid receptors share the highest homology with the somatostatin and angiotensin receptors.

The role of opioid peptides as endogenous analgesics suggests a possible use as pharmacological tools for pain relief, devoid of undesired secondary effects [10,11]. The prolonged use of morphine or other alkaloid analgesics induces tolerance and dependence. Patients must receive escalating doses of opioid to maintain the same level of analgesia. The decrease of drug efficacy with repeated administration is defined tolerance. Actually, during the course of drug administration, the dose can increase up to 1000-fold. It can be explained by loss of surface receptors and signaling desensitization, or receptor internalization [12]. Dependence is defined in terms of the ability of a drug to produce a physical or psychological withdrawal syndrom upon its removal.

It is generally accepted that the μ-receptor agonists possess the most potent antinociceptive activity, accompanied by the highest abuse liability. On the other hand, δ-receptor agonists possess a lower antinociceptive efficacy, but might have a reduced addictive potential. κ-Receptor agonists are believed to be potential analgesics for

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peripheric use only, since they cause psychotomimetic and strong dysphoric effects.

β-Endorphin and other peptides act as strong peripheral analgesic in the hyperalgesia caused by inflammation [1]. Enkephalins are easily degraded by proteolytic enzymes, therefore their action is short-lasting, unless enzyme inhibitors are used in concomitance. Several enkephalin analogues show more resistance to hydrolysis (e.g. DPDPE), but their poor blood-brain barrier penetration renders their peripheral administration unpracticable. Dynorphins posses slight analgesic properties, in particular associated to neuropathic pain, accompanied by neurotoxic effects. Besides their nutritive role, a number of peptides derived from milk act at the opioid receptors. Casoxins and lactoferroxins have antagonistic properties, while certain casomorphins are agonists [13]. In particular, β-casomorphin -7 and β-casomorphin-5, which are released by enzymatic hydrolysis of β-casein in the gut of adults and newborns, can act as hormone-like substances. Endomorphin-1 and endomorphin-2 [14,15] display a strong antinociceptive effect in acute pain similar to that of morphine. They are also more effective than the majority of the opioid peptides against neuropathic pain even at low doses, opening the possibility of using the two peptides as drugs [16]. On the contrary, they seem less potent and shorter acting than morphine in inflammatory pain. Endomorphins are considered to be the real endogenous analgesics in mammalians, being released in response to pain stimuli. The two peptides play different roles. Indeed, endomorphin-1 is widely distributed throughout the brain, while endomorphin-2 is mostly present in the terminal regions of primary afferent neurons in the dorsal horn of the spinal cord and in the medulla. Therefore, endomorphin-2 modulates pain at an earliest stage of perception than endomorphin-1 [17].

Several papers have recently appeared in the literature supporting the concept that the immunomodulatory, [18] cardiovascular, respiratory, and analgesic effects of endomorphin-1 agonists can be dissociated, [19,20] due to the involvement of different μ-opioid receptor subtypes [1].

In addition to their analgesic effects, endogenous peptides are involved in the regulation of stress response [21], of several behavioral [22] and emotional effects, such as dependence, learning, memory, reward, eating and drinking, alcohol and other drugs consumption, mental illness and mood, seizures. They are also effective in a large number of physiological functions: locomotion, gastro-intestinal, renal, and hepatic function, cardiovascular responses, respiration and thermoregulation, immunological responses, sexual activity, pregnancy, etc [23].

SELECTIVITY OF OPIOID PEPTIDES TOWARDS THE OPIOID RECEPTORS

One of the major drawbacks of using natural opioid peptides as analgesics is their poor receptor specificity. Receptor selectivity has been attributed to a number of factors, primarily to the chemical structure and conformation [24]. The relatively high conformational freedom of opioid peptides is responsible for their low selectivity towards the different receptor types and subtypes, which share extensive structural homologies, although they are differently distributed in the neuronal nociceptive system.

From the structural point of view, naturally occurring opioid peptides consist of two parts, a biologically important N-terminal tri or tetrapeptide fragment, the message sequence, and the remaining C-terminal fragment, the address sequence. The comparison of several endogenous and synthetic opioid ligands showed that the message sequence needed strict requirements for a good interaction with opioid receptors. In particular, the presence of amino and phenolic groups of Tyr in position 1, an appropriate spacer such as Pro or D-Ala in position 2, lipophilic and aromatic residue in positions 3 and 4, and amidation at the C-terminus, seem to be very important features [25].

[Met]-enkephalin, H-Tyr-Gly-Gly-Phe-Met-OH, and [Leu]-enkephalin, H-Tyr-Gly-Gly-Phe-Leu-OH possess a slight preference for δ-receptors over μ-receptors. The family of deltorphins, isolated from frog skin, is based on the sequence of the parent peptide deltorphin, H-Tyr-D-Met-Phe-His-Leu-Met-Asp-NH_2, and display high selectivity for δ-receptors. β-Endorphin, a 31 amino acid peptide, binds both to δ- and μ-opioid receptors. β-Casomorphins such as β-casomorphin-7, H-Tyr-Pro-Phe-Pro-Gly-Pro-Ile-OH, and β-casomorphin-5, H-Tyr-Pro-Phe-Pro-Gly-OH, and dermorphin, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2, show only a modest μ-selectivity. Dynorphin A (DynA), H-Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Trp-Asp-Asn-Gln-OH, and its truncated fragments, e.g. DynA(1-11) and DynA(1-13), show a certain preference for κ-opioid receptors. Nociceptin/orphanin, is considered the endogenous ligand of a orphan opioid-receptor-like (ORL1) receptor [26]. The newly discovered endomorphin-1, H-Tyr-Pro-Trp-Phe-NH_2, and endomorphin-2, H-Tyr-Pro-Phe-Phe-NH_2, [14] are considered the endognous ligands for μ-opioid receptors. They are unique in comparison with other opioid peptides by atypical structure and high selectivity, in contrast with the other endogenous peptides, which display only moderate preferences for some receptor types over the others [27].

Many studies attempted to determine the bioactive conformation of natural opioid peptides in different solvents that mimic in part the different environments in which the peptides exert their action. None of the structural investigations provide a convincing model, but the global conformation of longer peptides in biomimetic environments can shed light on the interaction with receptors [28]. Useful information can be also deduced from the solid state structure determined by X-ray diffraction [29]. The studies of the structure-activity relationships of receptor-selective peptides which have been systematically modified are of particular interest for the development of therapeutic agents. In certain cases the comprehension of the ligand-receptor interaction has been obtained by means of constrained ligands.

INACTIVATION OF OPIOID PEPTIDES BY PEPTIDASES

Beside a partial receptor selectivity, a second important limitation to the use of native peptides as pharmacological tools is their rapid enzymatic degradation. Proteolytic

cleavage of a biologically active peptide generally results in a modification of its biological activity, or inactivation. In certain cases, it results in a bioavailability alteration by modification of the binding properties to matrix or other proteins.

Natural opioid peptides are rapidly degraded *in vivo* by several peptidases, such as aminopeptidases, dipeptidyl peptidase III and IV (DPP III, DPP IV), etc. DPP IV is a non-classical serine protease which appears to be a major physiological regulator [30] for some neuropeptides, regulatory peptides, circulating hormones and chemokines [31]. This glycoprotein is widely present in the kidney, the liver, the placenta. In the CNS it is found mostly in the circumventricular organs and on leptomeningeal cells. It is active towards peptides with N-penultimate proline or alanine up to 80 residues in length. Good substrates for DPP IV are enkephalins, dynorphins, β-endorphins, substance P, endomorphins, β-casomorphins, NPY, PYY, and bradykinin. Another important enzyme involved in peptide metabolism is the angiotensin-converting enzyme [32]. Peptidases are present in the serum, on capillary endothelial cells, and on the different organs. The presence of several peptidases in the blood-brain barrier can also constitute a metabolic barrier for opioid peptides [32].

The hydrolytic efficacy of peptidases can be antagonized by specific inhibitors. For example, DPP IV is inhibited by di-isopropyl fluorophosphate, and by metal ions such as Pb^{++}, Hg^{++}, Zn^{++}, while DPP III is inhibited by spinorphin, H-Leu-Val-Val-Tyr-Pro-Trp-Thr-OH, an endogenous factor derived from bovine spinal cord, and more efficiently by its truncated fragment tynorphin, H-Val-Val-Tyr-Pro-Trp-OH [33]. Cysteine enzymes are inhibited by leupeptin. Metalloproteases are often blocked by EDTA and phenanthroline [31]. Other recent and selected examples concerning the analgesic efficacy of enzymatic inhibitors are described here. Bestatin, an antibiotic of microbial origin, is a well studied potent inhibitor of some, but not all, aminopeptidases, and it is also ineffective against DPP IV. Bestatin-sensitive enzymes play an important role in the digestion and absorption of opioid peptides in the brush border of the intestine, in the kidney, and in the reproductive system [34]. The administration of p-hydroxymercuri-benzoate, or phosphoramidon in the presence of bestatin, gave rise to antinociceptive effects; the efficacy of p-hydroxymercuribenzoate may be due to the inhibition of a cysteine protease degrading endogenous dynorphins, whereas the combination phosphoramidon/bestatin probably blocks the degradation of enkephalins [35]. The antinociceptive effects of intracerebroventricularly administered DynA were examined in the presence of p-hydroxymercuribenzoate or phosphoramidon/bestatin. Cysteine proteases as well as endopeptidase 24.11 seem to be involved in a two-step degradation of DynA in the mouse brain, and phosphoramidon seems to inhibit the degradation of intermediary δ-opioid receptor active fragments which are formed from DynA [36]. The degradation of DynA(1-8) in rat cerebral membrane preparations is almost completely prevented by a mixture of three peptidase inhibitors, amastatin, captopril and phosphoramidon. The antinociceptive effect of DynA(1-8) was increased more than 100-fold by the pretreatment of rats with the three inhibitors. The efficacy of DynA(1-8)in rats pretreated with any combination of two inhibitors was significantly smaller than that in rats pretreated with three inhibitors, indicating that any residual single peptidase could inactivate significant amounts of DynA(1-8) [37].

OTHER ANTINOCICEPTIVE PEPTIDES

It might be worthwhile to mention briefly that also some non-opioid or anti-opioid peptides have been recently investigated as potential targets for analgesia. Nociceptin or orphanin FQ (noc/oFQ) is a recently discovered heptadeca-peptide, H-Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln-OH, that activates an opioid-like G protein-coupled receptor ORL1. These receptors share around 60% of the sequence homology with the other opioid receptors. The marked structural analogy between the ORL1 and opioid receptors, especially the κ-opioid receptor, and between the noc/oFQ and opioid peptides, particularly DynA, is not reflected anatomically since noc/oFQ and opioid peptides appear to be located in separated neuronal circuits. Noc/oFQ triggers the same G protein-mediated signaling pathways as do opioids, but it produces pharmacological effects that sometimes differ from, and even oppose, those of opioids [26]. Recent data indicate that the peptide can act as a spinal analgesic, supporting a hypothesis that considers the ORL-1 as a potential target for new analgesics [38]. Noc/oFQ has not yet been found to precipitate withdrawal in morphine-tolerant rats. Nor does it elicit motivational effects, suggesting it lacks abuse liability. Modified nociceptin analogues have been synthesized to investigate the interaction modes with the ORL1, in search for a better comprehension of its hyperalgesic vs. analgesic activity [39-45].

Reports on the antinociceptive properties of other opioid peptides, such as histogranin and related peptides, [46] neuropeptide FF, [47,48] cholecystokinin, [49] interleukin-2, [50] spinorphin, [51,52] rubiscolins, bioactive peptides derived from plant Rubisco, [50] etc. have been published.

MODIFIED OPIOID PEPTIDES AS POTENTIAL THERAPEUTIC AGENTS

To bypass the problems arising from the scarce selectivity and the scarce *in vivo* stability of native opioid peptides, an enormous number of modified peptides or peptidomimetics have been designed so far. The main synthetic strategies were classified by Peter W. Schiller, [54] and are: the development of analogues by way of insertion of unnatural amino acids in the natural sequence; the design of bivalent peptide ligands to interact with two receptors at the same time; the introduction of conformational constraints; cyclization; the modification of peptide bonds [55]. More recently, an increasing attention has been paid to the development of peptides having new features, such as peptides with contemporary activity at different receptor types, or peptides showing higher penetration of biological barriers, propeptides, non-conventional opioid peptides, etc.

Combinatorial chemistry has allowed the rapid synthesis and the biological investigation of a very large number of new opioid peptides. The obtained analogues are composed of L-amino acids, D-amino acids, or L-, D-, and unnatural

amino acids, and generally range from tetrapeptides to decapeptides. New compounds have been identified from peptidomimetic libraries, such as peptoids and alkylated dipeptides, or from acyclic libraries, e.g., polyamine, urea, etc. and heterocyclic libraries, e.g., bicyclic guanidine, etc [56].

This review mainly deals with opioid peptide analogues, and will skip the discussion of peptidomimetics in which the peptide character is not preponderant. Non-peptide ligands as opioid peptidomimetics can be designed in order to obtain high affinity and selectivity towards receptors and new features concerning stability to biodegradation and good bioavailability, including the ability to cross membrane barriers [57-59]. Starting from a complete NMR and computational analysis of a large number of correlated compounds, it is possible to propose nonpeptide scaffolds carrying the pharmacophoric groups in the proper 3D arrangement. Several studies are in progress to develop different aspects of conformational design that permit assembling of all components necessary for molecular recognition and transduction [60].

The complete description of the hundreds of examples which appeared in literature so far concerning the development of highly selective peptide analogues seems unpractical; several reviews by P. W. Schiller, V. J. Hruby, and others (see throughout) have been dedicated to this topic.

OPIOID PEPTIDE ANALOGUES

The most wanted achievements for modified opioid peptides and peptidomimetics are good receptor selectivity and stability. In several cases, however, the novel stable and receptor-selective peptidomimetics display long term toxicity or difficulties to penetrate the blood-brain barrier [61,62]. For this reason, the search for new peptide ligands with optimal properties is still in progress. The immense number of biologically active molecules derived from opioid peptides can be rationalized in terms of the different elements introduced in the peptide sequence which can be considered responsible of the new properties.

INTRODUCTION OF UNNATURAL AMINO ACIDS E.G. D-AMINO ACIDS, N-ALKYL AMINO ACIDS, ETC.

The introduction of D-amino acids in a sequence can give the opioid peptide an increased stability, since only a few enzymes that effectively hydrolyse peptide bonds involving D-amino acids have been discovered and characterized in multicellular organisms [63]. Moreover, D-residues often enforce a different conformation of the peptide, and strongly influence receptor affinity and selectivity.

Some of the first successes of this approach [55] have been the δ-receptor selective enkephalin analogues DADLE, H-Tyr-D-Ala-Gly-Phe-D-Leu-OH, and BUBU, H-Tyr-D-Ser(O*t*Bu)-Gly-Phe-Leu-Thr(O*t*Bu)-OH. The search for ligands composed of all D-amino acids having high selectivity for κ-opioid receptors has been performed using a combinatorial approach. A huge number of tetrapeptides was examined and the most potent and selective ligand with agonist properties was D-Phe-D-Phe-D-Nle-D-Arg-NH_2 (FE 200041) [64].

A series of diastereoisomeric endomorphin-1 [65] and endomorphin-2 [66] analogues containing a D-amino acid have been synthesized and their potency measured, but in general these peptides exhibited poor affinities towards μ-receptors, in particular when D-proline was introduced. In addition, the conformational analysis and the systematic study of the structure-affinity relationship gave only elusive indications about the bioactive conformation.

Alternatively or in addition to the substitution with D-amino acids, the introduction of unnatural residues proved to be an efficient method to obtain effective analogues. DAMGO, H-Tyr-D-Ala-Gly-MePhe-Glyol, including a N-methyl phenylalanine, shows a notable μ-receptor selectivity, and for this reason in the [^{3}H]-form it is widely used as a radioligand for binding experiments.

In a similar way, the presence of N-methylamino acid residues and D-amino acids rendered the DynA(1-8) analog [N-Me-Tyr^1,N-Me-Arg^7,D-Leu^8]DynA(1-8) ethylamide, E-2078, less prone to be biotransformed, when compared to the unmodified opioid peptide. E-2078 has been found to produce κ-opioid agonist effects [67]. DynA(1-11) and DynA(1-13) have been utilized as simplified DynA models since they maintain almost the same level of activity as the parent peptide. Several analogues carrying modifications at Tyr^1, at Gly^2, at Gly^3, as well as at positions 6-11 and 13, have been studied to determine the role of each residue in the determination of receptor affinity [68,69].

N,N-Dialkylated and N-monoalkylated tyrosine derivatives of [D-Pro^{10}]DynA(1-11), with alkyl: allyl, benzyl, and cyclopropylmethyl (Cpm), were synthesized to explore the structure-activity relationships for antagonist vs. agonist activity at κ-opioid receptors. In general, the N-monoalkylated derivatives exhibited much higher affinity and greatly enhanced selectivity compared to the N,N-dialkyl analogs. In particular, the N-Cpm analog exhibited potent *in vivo* antinociceptive activity [70].

Modified deltorphin I analogs were prepared by introduction of D- or L-N-methylalanine (MeAla), D- or L-proline, α-aminoisobutyric acid (Aib), sarcosine or D-tert-leucine (2-amino-3,3-dimethyl butyric acid) in place of D-Ala^2, or phenylalanine in place of Tyr^1. The D-MeAla^2-analogue was a slightly more potent δ-agonist and showed two-fold higher antinociceptive potency in the analgesic test in comparison with the parent peptide. Substitution of Aib in the 2-position led to a compound, H-Tyr-Aib-Phe-Asp-Val-Val-Gly-NH_2, which displayed lower δ-receptor affinity than deltorphin-I, but higher selectivity and, surprisingly, three times higher antinociceptive potency [71].

N-methylation and other modifications have been attempted also on endomorphin-2. Analogues with N-Me Phe^3 instead of Phe^3 or C-terminal modifications, showed decreased binding affinity and activity. The only modification tolerated at the C-terminus was the substitution of Phe^4-NH_2 with Phe^4-ol, which gave increased affinity [72].

H-Tyr-Tic-Phe-Phe-OH (TIPP) and H-Tyr-Tic-Phe-OH (TIP), which contain 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (Tic), have been the subject of intense structure-activity relationship studies, leading to the development of analogues that are of interest as potential therapeutic agents [73]. The conformational analysis and the

pharmacological investigation of the agonist or antagonist properties of constrained analogues of TIPP allowed the determination of the bioactive conformation at the binding site of δ-receptors. The results of the performed structure-activity studies revealed that the δ antagonist versus δ agonist behavior of this class of compounds depended on very subtle structural differences in diverse locations of the molecule [74].

Peptide analogues carrying positive net charges could display a good selectivity for μ-receptor over δ-receptors, since the latter are supposed to be present in a positive, and so repulsive, cationic membrane environment. The tetrapeptide H-Tyr-D-Arg-Phe-Lys-NH_2 (DALDA), was determined to be one of the most selective ligands towards μ-receptors [75]. The high affinity for μ-opioid receptors and potency of the tetrapeptide Tyr-D-Arg-Phe-Sar, Sar: N-methylglycine (TAPS), [76] may be explained in terms of a combination of two factors: a slow degradation or inactivation by proteases, owing to the presence of a D-residue and a methylglycine, and the presence of a positively charged D-Arg in position 2, which should permit the accumulation of the peptide in the vicinity of the receptors, as proposed for DALDA.

INCORPORATION OF MODIFIED PEPTIDE BONDS

The incorporation of reduced peptide bonds renders the native sequences of opioid peptides highly resistant towards enzymatic hydrolysis in the modified positions. Some DynA(1-11)-NH_2 analogues containing [CH_2-NH] bonds have been tested for their *in vitro* enzymatic stability in mouse brain homogenates. Among them, the analogue (Leu^{5}Ψ[CH_2-NH]Arg^6)DynA(1-11)-NH_2 exhibited an almost complete resistance, indicating that the Leu^5-Arg^6 and, to a lesser extent, the Arg^7-Ile^8 and Ile^8-Arg^9 peptide bonds are the more susceptible to enzymatic cleavage in the native peptide [77]. Reduced peptide bonds have been introduced also in the sequence of [D-Leu^8]Dyn(1-8)-NH_2. Each peptide bond was systematically replaced with a Ψ(CH_2NH) peptide bond [78].

Stable TIPP-derived δ-opioid antagonists with subnanomolar δ-receptor binding affinity and extraordinary δ receptor selectivity have been obtained by introduction of a reduced peptide bond between Tic^2 and Phe^3 residues, giving H-Tyr-TicΨ[CH_2NH]Phe-Phe-OH (TIPP[Ψ]) and H-Tyr-TicΨ[CH_2NH]Cha-Phe-OH, Cha: cyclohexylalanine (TICP [Ψ]). The modification confer the molecules a high stability against chemical and enzymatic degradation [73].

Another important and widely applied bond modification for peptides or proteins is the incorporation of false peptide bonds [79]. Peptide-bond reversal represents an important structural alteration for opioid peptides, and proved to be useful to reduce the degradation rate of the peptides by peptidases. Retro-inversion both reverses the primary sequence and replaces L-amino acids with D-amino acids. The synthesis of partially modified analogues can be accomplished by introduction of bilateral residues. For instance, the introduction of substituted malonates allowed the linear analogue to be synthesized by coupling of the two opposite strands at the reversed bond. The return of the normal direction of the amino acid sequence can be obtained by introduction of geminal diamino residues. Several examples have been reported in literature: partially modified enkephalins, dermorphin, deltorphins, morphiceptins, cyclic dermorphin, etc [80]. Many of these analogues exhibit high potency, selectivity, and metabolic stability.

INTRODUCTION OF AMINO ACIDS CARRYING SUBSTITUTED AROMATIC RINGS

The introduction of substituents in the aromatic ring of Tyr^1, Phe^3, or Phe^4, etc. in several cases produces a modulation of the opioid receptor affinity and selectivity. In general, this kind of modification influences the molecular conformation and lipophylicity, because of the increased bulkiness of the side chains. A large number of TIPP derivatives containing 2',6'-dimethyltyrosine (Dmt), tri-methyltyrosine (Tmt), or 3'-halo-tyrosine in the position 1 were investigate to define the δ agonists or δ antagonists properties [73]. Tyrosine and phenylalanine have been methylated also in β-position of DPDPE analogues [81]. Other examples have been recently reported, indicating that this kind of modification is currently under investigation. Methylated tyrosine is present in the series of β-methyl-2', 6'-dimethyltyrosine^1-substituted [D-Ala ^{2}, Asp ^{4}]deltorphin (DELT I) analogues, and allowed to determine that topographical modifications of the side chains strongly affect antinociceptive potency and opioid receptor selectivity [82]. Dmt is also present in the dermorphin-derived peptide H-Dmt-D-Arg-Phe-Lys-NH_2 ([Dmt^1]DALDA), an interesting and extraordinarily potent, systemically active peptide analgesic, which labels μ-opioid receptors with high affinity and selectivity [83]. [Dmt^1]DALDA appears to penetrate the blood-brain barrier better than the highly hydrophobic DALDA; in addition, it exhibits low cross-tolerance to morphine [84]. Several other DALDA analogues containing N,2',6'-trimethyltyrosine (Tmt), 2'-methyltyrosine (Mmt) or 2'-hydroxy 6'-methyltyrosine (Hmt) in place of Tyr^1, or Orn, or α,γ-diaminobutyric acid (A_2bu) in place of Lys^4, have been synthesized and tested [85].

Increased μ-opioid receptor affinity and selectivity has been observed in a Leu-enkephalin analogue upon introduction of Dmt and 2,6-dimethylphenylalanine (Dmp) in place of Tyr^1 and Phe^4 respectively, and in a dermorphin analogue by substitution of Phe^3 with Dmp, while the substitution of Phe^3 with Dmp in deltorphin-II gave increased δ-opioid receptor selectivity [86,87].

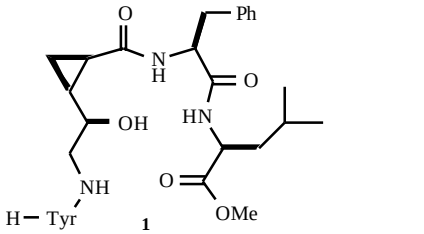
INTRODUCTION OF CONFORMATIONAL CONS-TRAINTS

The introduction of conformationally constrained surrogates in a peptide sequence is a typical strategy to gain molecular rigidity and to increase metabolic stability. Moreover, the topographical modifications of the side-chain conformation of critical structural moieties in a peptide can significantly modulate both the potency and receptor selectivity for ligands that have multiple sites of biological activity [60,73,81,82,88]. Many of the modified peptides mentioned in the previous sections actually contain conformational constraints. A large number of constrained enkephalin derivatives have been synthesized during the last years that are potent and selective, and the analysis suggests that a β-turn involving residues 5 and 2 might be an important conformational motif in the biologically active conformation. Recent examples of constrained Leu-enkephalin mimetics focused attention in particular to the Gly^2-Gly^3 peptide bond.

Enkephalin analogues with a branched peptide chain in position 2 were prepared by replacement of Gly^2 with D-ornitine and prosecution of the peptide chain by attachment of residues to the δ-amino group. They showed higher analgesic potency and longer duration of action as compared to linear and cyclic pentapeptides having the same amino acid sequence [89,90]. The constrained Gly^2-Gly^3 dipeptide surrogate (2S,6R,8S)-9-oxo-8-N-(Boc) amino-1-azabicyclo [4.3.0] nonane-2-carboxylic acid has been very recently introduced in the Leu-enkephalin sequence. The indolizidinone analog exhibited affinities for the μ- and δ-opioid receptors that were three orders of magnitude lower than that of Leu-enkephalin, as well as partial agonist character for both receptors. However, in *in vivo* assays the analogue showed significantly enhanced duration of action, indicating an increased metabolic stability [91]. Cis cyclopropanes have been incorporated as replace-ments of Gly^2-Gly^3 and/or Phe^4-Leu^5 subunits in enkephalin analogues, but only the first one **1**, Fig. (**1**), maintained a modest affinity [92].

Constrained deltorphin-I and -II analogues were synthesized by substitution of Ile instead of Val at positions 5 and 6 in the address domain, and 2-aminotetralin-2-carboxylic acid instead of Phe in the message domain [93].

Very recently, pseudoproline-containing analogues of morphiceptin, H-Tyr-Pro-Phe-Pro-NH_2, and endomorphin-2, H-Tyr-Pro-Phe-Phe-NH_2 **2**, Fig. (**1**), having an essentially rigid cis Tyr-Pro conformation resulted μ-opioid receptor agonists, even though with lower efficacy in respect to native compounds, suggesting an important clue in the investigation of the bioactive conformation of μ-receptor agonists [94].



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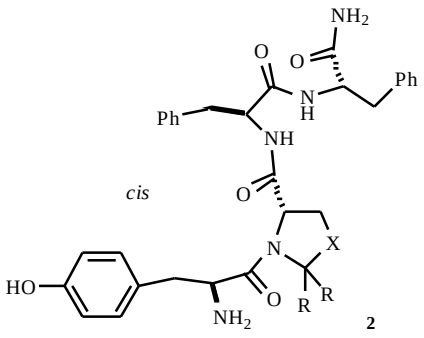

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X = O, S; R = H, Me

Fig. (1). Examples of modified peptides containing conformational constraints.

The presence of amino acid residues carrying two substituents in α-position has been largely utilized to gain molecular rigidity. Endomorphin-2 analogs containing α,α-disubstituted glycines, [Aib_2]-endomorphin-2 and [Ac5c2]-endomorphin-2, exhibited considerably high affinity for the μ-opioid receptor, and were deaminated by carboxy-peptidase-Y less rapidly than endomorphin-2 [95]. Other endomorphin-2 analogues included unusual amphiphilic amino acids, (R)- and (S)-α-hydroxymethyltyrosine in position 1 and (R)- and (S)-α-hydroxymethylphenylalanine in the positions 3 and 4 [96]. The DALDA analogue containing alpha-hydroxymethylamino acids, [(R)-$HmPhe^3$)] DALDA, displayed full agonistic activity and turned out to be a δ-receptor-selective opioid agonist [97]. Optically pure aminotetralin-2-carboxylic acid has been introduced as a constrained mimic of Phe in the position 4 of the opioid peptide [D-Ala^8]DynA(1-11)NH_2 [98].

The introduction of alkyl groups in the β-position of aromatic amino acid residues gave constrained residues; some recent examples are described here. The substitution of a β-MePhe for Phe^3 in deltorphin I and dermenkephalin has a large and variable effect on the bioactivities of the synthesized analogues. The stereochemistry of β-MePhe plays a main role in the determination of potency and selectivity [99]. The introduction of an isopropyl group in Phe gave the highly hydrophobic novel amino acid β-isopropylphenylalanine (β-iPrPhe). Replacement of Phe^3 in deltorphin I with the four stereoisomers of β-iPrPhe produced four pharmacologically different deltorphin-I peptidomimetics. The presence of the (2S,3R)-$iPrPhe^3$ residue provided the highest affinity, the highest potency and an exceptional selectivity for the δ-opioid receptor over the μ-opioid receptor [100].

CONIUGATION OF TWO PEPTIDE SEQUENCES

A special kind of modification planned to develop highly selective non natural opioid peptides is constituted by the coupling of two identical peptide sequences to form dimers [55]. Generally, the two opioid peptides are identical and are connected by a short spacer through their C-terminus. The main idea is based on the possibility that a contemporary interaction of the dimeric ligand with two receptors should increase both selectivity, and affinity. This possibility has been formerly verified for (H-Tyr-D-Ala-Gly-NH-CH_2-)$_2$, $(DTRE)_2$, whose affinity and selectivity at μ-opioid receptors was much higher than that of the monomeric peptide. More recently, biphalin arose a great interest, for the newly discovered properties. In biphalin, (H-Tyr-D-Ala-Gly-Phe-NH-)$_2$, the two pharmacophores are linked by a hydrazine bridge. When administered intracerebroventricularly it is more potent than morphine and etorphine at eliciting antinociception. In a cross-dependence study, biphalin induced less physical dependence than morphine [101]. Biphalin has also been shown to cross both the blood-brain and blood-cerebrospinal fluid barriers; this good bioavailability in comparison to similar linear sequences is correlated to a lipop-hilicity enhancement due to the presence of intramolecular hydrogen bonds. Tyr-D-Ala-Gly-Phe-NH-NH-Phe is the minimal fragment necessary to express the same biological activity profile as the full dimer. The replacement of N'-Phe with other L- or D-lipophilic amino acids showed the possibility of modification of the receptor efficacy [102]. Halogenated biphalin analogues are described in a following section.

INTRODUCTION OF β-AMINO ACIDS

Peptides formed by homologated β-amino acids have been studied for years to discover stable secondary structures [103-107]. In several cases, the substitution of α-amino acids by their β-isomers in biologically active peptides resulted in increased activity and enzymatic stability [108,109]. The introduction of β-amino acids in native opioid peptide sequences has been not investigated till now as widely as it could have been. Nevertheless, the few examples reported show that this introduction allows an improvement of peptide stability and, in certain cases, also of affinity.

The simple introduction of homo-Phe in place of Phe^3 in β-casomorphin-5 and β-casomorphin-7 gave two new peptides with a 5-fold and 2-fold enhanced affinity towards μ-opioid receptors, respectively, compared to the natural peptides. Although the two peptides are less potent than other cyclic β-casomorphin derivatives, the presence of a homo-amino acid remains of interest for the possibility of enhanced stability against enzymatic hydrolysis [110]. The peptide H-Tyr-D-Arg-Phe-β-Ala-OH (TAPA), an analogue of dermorphin N-terminal peptide which contains β-alanine, was designed to examine the interactions with μ-opioid receptor subtypes, and produced potent antinociception [111]. Constrained analogs of dermorphin were synthesized by replacing $D\text{-}Ala^2$ with stereoisomers of β-amino-cycloalkane or cycloalkene carboxylic acids. All of the new derivatives displayed highly attenuated binding to both μ- and δ-receptors, [112] albeit the decrease in their potency seemed to be less in the case of δ-binding. Trans position of the β-amino groups resulted in higher binding affinities than that of the corresponding cis isomers, the latter being more flexible than the former [112].

Very recently, a series of endomorphin-1 analogues containing β-amino acids have been synthesized and their receptor affinities have been measured. Endomorphin-1 and endomorphin-2 can be regarded as the most promising native opioid peptides to be tested for the development of peptide analgesics, for the high μ-receptor affinity, the high selectivity, the outstanding antinociceptive action, and the low incidence of undesired side-effects [15]. The proline at the position 2 has a large influence on the conformation of the whole molecule, and determines the correct position of the pharmacophoric aromatic groups in the bioactive conformation. It also restricts the risk of attack by many proteases, even those with broad specificity. Peptides having proline in penultimate position cannot be degraded by non-specialized exopeptidases. Nevertheless, endomorphins are easily degraded, although slowly compared with $[Leu^5]$ enkephalin, [113] by dipeptidyl peptidase IV, and by some X-Pro aminopeptidases, prolyl aminopeptidases, and carboxypeptidases which specifically or selectively attack proline bonds [31]. Several of endomorphin-1 degradation products have been isolated from the central nervous system. None of the detected degradation products had an effect on GTP binding, nor was able to produce analgesia, suggesting that degradation suppresses the biological activity [114]. Some modified endomorphins have been synthesized and tested, and have been briefly discussed, [72,95,96,115] including the already mentioned diastereoisomers containing a D-amino acid [65,66].

The introduction of β-isomeric amino acids in the native sequence of endomorphin-1 gave analogues whose affinity for the opioid receptors largely varied depending on the β-amino acid present [116]. In particular, the affinity of the modified enomorphins H-Tyr-β-(*R*)-Pro-Trp-$PheNH_2$ **3**, Fig. (**2**), and H-Tyr-β-(*S*)-Pro-Trp-$PheNH_2$ were in the nanomolar range. Further, each amino acid was replaced in turn by its homologue, and the best affinity was displayed by the peptide containing homo-proline H-Tyr-(*S*)-homo-Pro-Trp-$PheNH_2$ **4**, Fig. (**2**) [117]. This homo-tetrapeptide possess agonist properties, even though slightly weaker than the native endomorphin-1. It has been also determined that the modifications introduced allowed an enzymatic stability enhancement with respect to endomorphin-1, by comparing the degradation rates upon incubation with commercially available proteolytic enzymes, α-chimotrypsin, carboxy-peptidase-Y, aminopeptidase-M. Interestingly, the modified peptides showed a certain resistance enhancement, and in particular aminopeptidase-M was ineffective in the degradation of **4**, Fig. (**3**), while endomorphin-1 was degraded to a mixture of the two dipeptides H-Tyr-Pro-OH and H-Trp-$PheNH_2$ in a few hours under the same experimental conditions. The introduction of modified proline could be of particular interest in the peptide stability *in vivo*, since aminopeptidases [118] and DPP IV are strongly active in the degradation of proline-containing peptides.

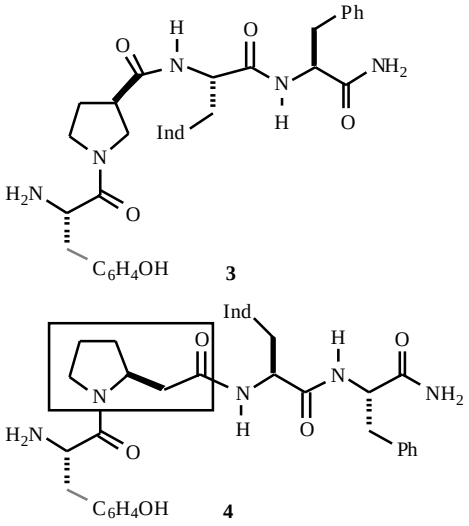

Fig. (2). Endomorphin-1 analogues containing isomeric (**3**) or homo (**4**) β-proline.

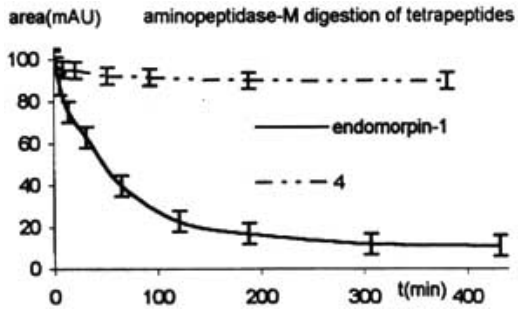

Fig. (3). Comparison of the stability of enomorphin-1 and **4** upon incubation with aminopeptidase-M.

CYCLIC OPIOID PEPTIDES

The cyclization of linear peptides represents a widely used strategy utilized to provide a remedy for the major obstacles to the use of opioid peptides as pharmacological tools: poor receptor selectivity, rapid enzymatic degradation, and scarce penetration of biological barriers [55,60]. Indeed, cyclization reduces the molecular conformational freedom, which is responsible of the contemporary activation of different receptors, increases the metabolic stability, and generally increases the molecular lipophilicty. The first cyclic enkephalin analogue, Tyr-c[-D-A_2bu-Gly-Phe-Leu-] **5**, Fig. (**4**), has been reported by P. W. Schiller, [55] and displayed a slight μ-receptor preference. Cyclization was obtained via a lactam involving the D-diaminobutyric residue and Leu. After this early success, a number of different enkephalin analogues have been designed, using different cyclization approaches [81]. The introduction of natural or unnatural amino acids carrying an amino function in the side chain proved to be an efficient procedure to obtain cyclic analogues containing lactam bridges. In several cases, the ring size was a determinant for the biologically activity of the cyclic peptide. Here will be mentioned some selected recent results.

Analogues of morphiceptin have been obtained by cyclization of tetrapeptide sequences Boc-Tyr-Xaa-Phe-Pro-OH containing lysine, ornitine, or diaminobutyric acid in the Xaa position [119]. Modifications of the native sequence of β-casomorphins included cyclization and/or substitution of positions 1-3. For example several analogues of the β-casomorphin-5 cyclic derivative H-Tyr-c[D-Orn-2-Nal-D-Pro-Gly-] (2-Nal: 2-naphthylalanine) have been synthesized by coupling ornitine and glicine, to investigate the effects of different aromatic substituents in position 3 [120]. Lactam bridges have been used also for the cyclization of DynA analogues. The compounds have been studied to define the conformation of the message and address sequences [121].

The small cyclic peptide Tyr-c[D-Lys-Phe-Ala] (YkFA) **6**, Fig. (**4**), is a very potent opioid agonist at both μ- and δ-receptors. YkFA is cyclized through an amide bond between the side chain of D-Lys^2 and the carboxylate of Ala^4. The same authors that earlier proposed YkFA have recently made some modifications of the ring size and introduced new substituents in position 4 with aliphatic hydrophobic residues. Contraction of the ring from 13 to 11 atoms by incorporation of diaminobutyric acid in position 2 resulted in a compound with decreased potency relative to YkFA. However, a decrease in activity at the δ-receptor makes the new compound more than twice as selective as YkFA. Probably the larger ring system present in YkFA may allow the molecule to adopt conformations that more closely complement the topography of the receptor binding sites [122].

In many enkephalin derivatives, cyclization has been achieved by means of a disulfide bond. In the δ-receptor selective Tyr-c[-D-Pen-Gly-Phe-D-Pen-]-OH (DPDPE) **7**, Fig. (**4**), and Tyr-c[-D-Pen-Gly-Phe-L-Pen-]-OH (DPLPE), the two D-pennicillamine residues were connected by a

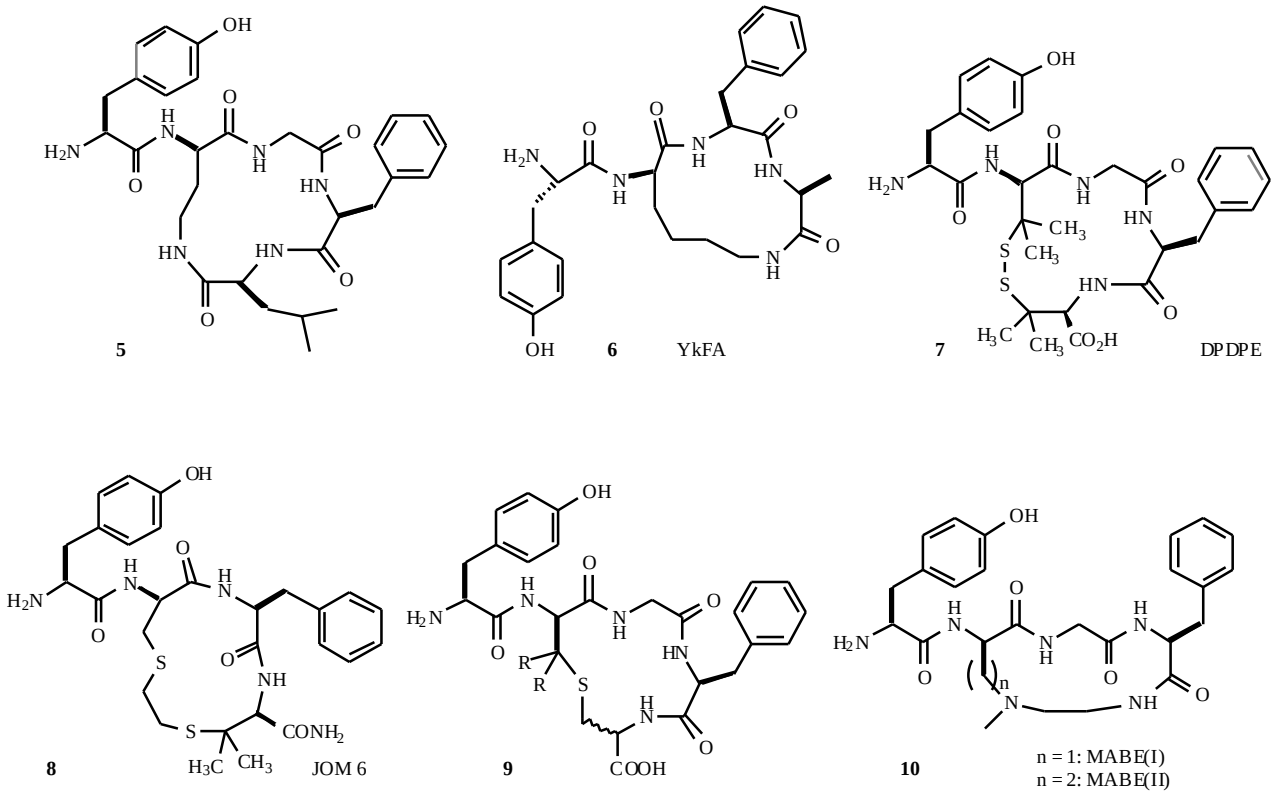

Fig. (4). Examples of cyclic opioid peptides.

disulfide bond. The presence of two constrained Pen residues in D-configuration in place of natural amino acids confer these compounds an excellent resistance against enzymatic degradation, still maintaining a low toxicity *in vivo*. DPDPE has been deeply investigated through NMR and molecular dynamics in order to gain conformational and topographical information for the determination of the bioactive conformation [81]. Despite of the cyclic structure and the presence of constrained moieties, the determination of the spatial disposition of the pharmacophores aromatic rings in the bioactive conformation remained troublesome. A series of β-methyl-2',6'-dimethyltyrosine (Tmt)-substituted cyclic [D-Pen^2,D-Pen^5] enkephalin, analogues of DPDPE, has been investigated to demonstrate the topographical dependence of antinociceptive activity and opioid receptor selectivity. In particular, the topology of Tmt group resulted to be crucial for the δ_1-opioid receptor recognition [82].

A disulfide bond has been used also for the cyclization of a series of cyclic somatostatin analogues. One of them, H-D-Phe-c[Cys-Tyr-D-Trp-Lys-Thr-Pen]Thr-NH_2 (CTP) had high potency and selectivity for the μ-opioid receptor. Two further modifications were obtained by introduction of Arg and Orn in place of Lys^5, giving CTAP and CTOP respectively. While they were μ-receptor antagonists, some CTOP and CTAP analogues were mixed δ-agonists and μ-antagonists, and in particular [D-Tca^1]CTAP, where D-Tca is a cyclic D-tryptophan analogue, showed unexpected analgesic activity [123].

Other sulfur-containing bridges have attracted the interest of the researchers. The cyclic μ-opioid receptor-selective tetrapeptide Tyr-c[D-Cys-Phe-D-Pen]NH_2 (Et) (JOM-6) **8**, Fig. (**4**), contains a -S-CH_2-CH_2-S- bridge. It has been recently modified at residues 1 and 3 by substitution with various natural and synthetic amino acids, and/or by alteration of the cyclic system [124]. The introduction of a lanthionine gave a novel, highly δ-receptor selective cyclic enkephalin analogue **9**, Fig. (**4**), containing a monosulfide bridge. **9** Was more stable than DPDPE towards enzymatic hydrolysis, and exhibited a good antinociceptive activity *in vivo* [125,126].

The use of diamines for the synthesis of cyclic peptides gave the possibility to introduce further functionalitizations at the protonable nitrogen. This possibility was explored for the synthesis of the potent methylamine-bridged enkephalins **10**, MABE(I) [127] and MABE(II), [125] Fig. (**4**), which displayed selectivity for μ and δ-receptors over κ-receptors.

Cyclization of linear enkephalin analogues containing basic amino acid residues in position 2 and 5 was achieved by means of a carbonyl bridge. Some of the compounds exhibit unusually high μ-opioid activity. The 18-membered analogue cyclo (N_ε, N_β-carbonyl-D-Lys^2, Dap^5)-enkephalinamide turned out to be one of the most potent μ-agonists reported so far [128].

PEPTIDES WITH μ-AGONIST AND δ-ANTAGONIST MIXED ACTIVITY

A recent review described the functional interaction among opioid receptor types that contribute to opioid dependence. In addition to respiratory depression, constipation, etc., μ-agonists are known to induce physical and psychological dependence and dependence on morphine. Several data suggest that also δ-opioid receptors are critically involved [129].

The opportunity do develop ligands capable to interact with more than one receptor at the same time opens the possibility that they could induce synergic or modulation effects. A special attention has been paid to the development of single compounds that are able to activate one type of receptor while blocking the activation of the others. The interaction of a μ-selective ligand with other receptor types could give additional antinociceptivity with reduced undesirable effects. For these reasons, several studies of ligands having a mixed activity both at μ- and δ-opioid receptors has been performed, in particular by P. W. Schiller and co-workers. Preliminary experiments showed that morphine development of tolerance and dependence in mice was reduced by the pretreatment with natrindole, a δ-opioid receptor antagonist [130]. The observation was confirmed using the δ-opioid receptor antagonist H-Tyr-TicΨ[CH_2NH]Phe-Phe-OH (TIPP[Ψ]) [131]. In addition, it was observed that morphine retained analgesic activity without producing tolerance upon a long period administration in δ-opioid receptor knockout mice [132].

Two cyclic β-casomorphin-5 analogues, namely H-Tyr-c[D-Orn-Phe-Pro-Gly-] and H-Tyr-c[D-Orn-Phe-D-Pro-Gly-], have been shown to possess high affinity towards both μ- and δ-opioid receptors and a potent antinociceptive activity. The substitution of Phe^3 in the latter compound with 2-naphthylalanine (2-Nal) gave H-Tyr-c[D-Orn-2-Nal-D-Pro-Gly-], which turned out to be a μ-agonist and a moderate δ-antagonist. A modulation or a reversal of the δ-agonist/antagonist properties was observed by substitution of 2-Nal with 1-Nal or by modification of the peptide ring size [133]. The 2-Nal^3 residue in this peptide was also replaced by residues carrying different aromatic rings. The δ-receptor tolerates bulky aromatic side chains in the 3-position, giving the peptides either δ agonist or δ antagonist properties. However, these compounds displayed drastically reduced μ-receptor affinity in nearly all cases [134].

Among the many components of the TIPP family, [73] the derivatives having a C-terminal carboxamide, H-Tyr-Tic-Phe-Phe-NH_2 (TIPP-NH_2), displayed a mixed μ-agonist/δ-antagonist profile, [135] and thus were expected to be analgesics with a low propensity to produce tolerance and physical dependence. Aiming to strengthen the μ-agonist component of TIPP-NH_2 without compromising the μ-agonist/δ-antagonist properties, modified tyrosines were inserted in place of Tyr^1. Interesting results were obtained with, 2',6'-dimethyltyrosine (Dmt) or N,2',6'-trimethyltyrosine (Tmt). The most effective compounds, H-Dmt-Tic-Phe-Phe-NH_2 (DIPP-NH_2), and H-Dmt-TicΨ[CH_2NH]Phe-Phe-NH_2 (DIPP-NH_2[ψ]),a pseudopeptide containing a reduced peptide bond between Tic^2 and Phe^3 residues, retained a mixed μ-agonist/δ-antagonist profile, whereas H-Tmt-Tic-Phe-Phe-NH_2 was a partial μ-agonist/δ-antagonist and H-Tmt-Ticψ[CH_2NH]Phe-Phe-NH_2 was a μ-antagonist/δ-antagonist. DIPP-NH_2 [ψ], showed binding affinities in the subnanomolar range for both μ-and δ-receptors, thus representing the first example of a balanced μ-agonist/δ-antagonist

with high potency, and produced a potent analgesic effect, being about 3 times more potent than morphine. It produced less acute tolerance than morphine but still a certain level of chronic tolerance. Unlike morphine, DIPP-NH_2[ψ] produced no physical dependence whatsoever upon chronic administration at high doses within a 1 week period [136].

A certain interest has been paid also to the investigation of analogs that yield analgesia via δ-agonist effects, lacking side effects associated with μ-activity. Some isomers of the somatostatin analog H-D-Phe-c[-Cys-Tyr-D-Trp-Arg-Thr-Pen-]-Thr-NH_2 (CTAP) were synthetized by introduction of β-MePhe in position 1. These analogs displayed varying potencies and biological activities including a simultaneous δ-receptor agonism/μ-receptor antagonism, or a μ-receptor antagonism or, finally, a δ-receptor agonism [138].

INCREASED BIOAVAILABILITY OF MODIFIED OPIOID PEPTIDES

Apparently, the search for opioid peptides to be used as pharmacological tools having higher performances in terms of analgesic potency and duration of the effects needs other investigations, by taking into account also different biochemical and physiological mechanisms in addition to receptor selectivity and stability. Important features to pursue are also an easy drug administration, which can be oral, intravenous, or intramuscular, a good penetration of gut-blood or blood-brain barriers, and possibly, the inactivity of metabolic products. In several cases, peptide analogues have been found to possess much higher biological activity than that expected on the simple basis of binding studies or tissue bio-essays. A complementary approach to the search for new drugs which obtained a growing interest in the last years concerns the synthesis of analogues having higher permeability of the biological barriers [61,62]. In contrast, it has been also proposed that the low permeability and the enzymatic instability of peptide drugs could be regarded as useful properties for the direct application in the spinal cord, for the reduced risk to produce side effects in other organs [138].

Many of the methods developed to enhance peptide entry into the central nervous system have been very recently and exhaustively reviewed by Davis *et al*. [139]. For this reason, this paragraph will mainly deal with modified opioid peptides in terms of antinociception enhancement.

Several strategies to manipulate peptide blood-brain barrier (BBB) transport processes have been developed including lipidization, chemical modifications of the N-terminal end, coupling of transport with post-BBB metabolism and formation of potent neuroactive peptides, up-regulation of putative peptide transporters, use of chimeric peptides in which a non-transportable peptide is chemically linked to a transportable peptide, use of monoclonal antibodies against peptide receptors, and binding of circulating peptides to apolipoproteins [140].

The BBB is located at the endothelial cells of the cerebrovascular capillary beds. The physiological function of the BBB is to maintain optimal conditions for neuronal function and prevent harmful substances from reaching the CNS. Unlike endothelial cells of the peripheral capillaries, BBB endothelial cells have cell/cell tight junctions, and an high electrical resistance. Such intercellular tight junctions prevent undesirable substances in blood from leaking into the brain tissue. Electrolytes and metabolic materials can still cross the barrier, thus it acts as a semi-permeable barrier that regulates substance exchange between the blood and the CNS in a bi-directional manner.

The design of a peptide modification which could ensure metabolic stability, reduced clearance, or a better transport through blood-brain barriers, could be a good method to gain improved opioid bioavailability, and therefore, a better analgesic activity. Many peptidomimetics are able to cross the BBB via transmembrane diffusion. Peptides cross the BBB and enter the brain by means of several transport mechanisms (i.e., receptor-mediated, absorptive-mediated, carrier-mediated and non-specific passive diffusion), as well as non-transport processes (i.e., endocytosis without transcytosis, absorption and metabolism) [140]. Biphalin, for instance, is thought to be transported by the large neutral amino acid carrier [141]. The [D-Pen^2,D-Pen^6]-enkephalin has been found to be probably transported by the organic anion-transporting peptide transporter [142]. Many peptides and large glycoproteins enter barriers through adsorptive endocytosis. Other peptides use a receptor-mediated endocytotic mechanism. In a few cases, transcytosis could be involved. The receptor for advanced glycation end products (RAGE), [143] is present in brain endothelium and transports glycosilated peptides and proteins, as well as β-amyloid.

The possible role of MDR1 P-glycoprotein as a transporter of opioid peptides has been investigated. MDR1 P-glycoprotein is known as an efflux pump for amphipatic toxic compounds. It could be involved also in the tissue distribution of endogenous opioid peptides such as adrenorphin, endomorphins, neurokinin, Substance P, etc., or in their elimination [144]. However, for endomorphin-1 and -2, a new efflux system across BBB which saturably transports the peptides from brain to blood, not requiring the P-glycoprotein unlike endorphin and morphine, [145] has been recently examined [146].

The comprehension of the interactions between the physiological barriers and opioid peptides is still incomplete, and several membrane models are presently under investigation to evaluate the interaction mechanisms in a simplified manner. Liposomes of a mixed composition consisting of zwitterionic lipids and cholesterol served as the model membranes for the study of the permeability coefficients of a series of analogues of c[D-Pen^2, D-Pen^5]enkephalin [147]. A cell line of human origin, the BeWo choriocarcinoma cell line, was used for the study of the transport and metabolism of opioid peptides across the *in vitro* model of the placental barrier [148]. A mixture of zwitterionic phospholipids with cholesterol was used as a model membrane to obtain information about the effect of membrane surface charge on the permeability and interaction of analgesic peptide ligands. These experiments suggest that the negative charge naturally present in cell membranes may hamper the membrane transport of some peptide drugs, especially cationic ones, unless there are cationic transporters present [149].

The BBB constitutes also a metabolic barrier, for the presence of several enzymes, such as aminopeptidase-A, aminopeptidase-M, angiotensin-converting enzyme, etc. Since the number of enzymes that degrade peptides is very large, the systematic inhibition as a general method to increase the penetration of peptides does not seem applicable. In certain cases, the coadministration of insulin allowed the enhancement of the transport of peripheral substances across BBB. Analyses of DPDPE transport revealed significant increases in DPDPE uptake across the BBB during coadministration of insulin. However, the analgesic efficacy of DPDPE was shown to decrease with increasing concentrations of insulin; such reduction probably occurs within the central nervous system rather than at the BBB [150].

PEPTIDE ANALOGUES WITH ENHANCED LIPOPHILICITY

In several cases, a good barrier permeability has been obtained by means of chemical modification. Lipophilicity is considered one of the most important properties connected to peptide penetration across membranes, giving the peptide the possibility to cross in a passive fashion. In a generalized rough correlation of the molecular weight with lipid solubility and permeability, it could be assumed that higher lipophilicity gives higher permeability below a molecular weight of around 400. Compounds with higher molecular weight are considered to have a restricted diffusion through BBB. Lipophilicity could be also predicted by evaluation of the molecular hydrogen bonding potential. A large number of hydrogen bonds will reduce the lipid solubility of the compound, and would be expected to a low BBB permeability. The presence of β-turns or cyclic structures often gives a good lipophilicity, since the number of hydrogen bonds directed to give interactions with the environment is reduced. Lipid solubility could also be estimated by way of partition coefficients in octanol/saline mixtures. Another important parameter is constituted by the charge distribution within the molecule. Zwitterionic peptides, for instance, should show a low membrane permeability. Cationic or policationic peptides, on the contrary, generally show a good membrane penetration via absorptive-mediated endocytosis, for the attraction exerted by the anionic sites present on BBB endothelium [151]. Many of the opioid peptides contain polar residues, and for this reason, several chemical modifications have been explored to increase the overall lipophilicity. For example, hydroxyl groups can be simply methylated, and terminal amino groups can be alkylated or acylated. A couple of N-acyl-DADLE derivatives have been reported to show good activity and improved permeability [152,153]. Leu-enkephalin analogues with different lipophilicities have been prepared by attachment of a sugar and a lipoamino acid. These modifications improved the apparent permeability across Caco-2 cell monolayers [154]. DPDPE, which has an excellent enzymatic stability, was modified by the trimethylation of the Phe^4 residue to give beta-methyl-2', 6'-dimethylphenylalanine analogue. Octanol/buffer distribution analysis showed enhanced lipophilicity of all four resulting conformations [155].

Topographical modification in a conformationally restricted peptide can significantly modulate not only receptor selectivity, binding capacity, and enzymatic stability, but also lipophilicity, P-glycoprotein affinity, and BBB permeability, resulting in a change of bioavailability.

Several studies demonstrated that halogenation significantly enhanced *in vitro* BBB permeability, providing evidence for improved delivery to the central nervous system. While DPDPE produced only a small analgesia upon subcutaneous administration, indicating a limited distribution to the brain, analogues containing halogens were able to enter the brain in a larger extent [156]. In a similar way, halogenated Met-enkephalin analogues were also investigated, and showed greater analgesic effect compared to DPDPE [157]. Other Met-enkephalin and DPDPE analogues containing the partially fluorinated amino acid 4,4-difluoro-2-aminobutyric acid in positions 2 or 3 of the peptide sequence were synthesized [158]. To improve central nervous system entry of the opioid analgesic [$D\text{-}Pen^2$, $L\text{-}Pen^5$, Phe^6]enkephalin (DPLPE-Phe), analogs with chloro, bromo, fluoro, and iodo halogens in the para positions of the phenylalanine-4 residue were synthesized. Halogenation affected stability, lipophilicity, and *in vitro* BBB permeability. Lipophilicity was greater for chloro derivatives, while *in vitro* permeability was greater for chloro and bromo derivatives [159].

Several studies have been performed to increase biphalin permeability. After systemic administration of biphalin, only a moderate amount was detected in the brain, but analgesia was observed. Since halogenation of enkephalin analogs has been shown to increase the brain uptake after systemic administration, both p-[$Cl\text{-}Phe^{4,4'}$]biphalin and p-[$F\text{-}Phe^{4,4'}$]biphalin were synthesized. Chlorohalogenation of biphalin was shown to both improve CNS entry, most likely through an enhancement in lipophilicity, and increase biological stability [160].

A particular class of dipeptides H-Tyr-Tic-NH-X, X: arylalkyl, showed a lipophilic character albeit having a low molecular weight, and demonstrated to be potent and very selective δ-agonists [73].

OPIOD PEPTIDES INCORPORATING SUGAR MOIETIES

Many peptide analogues have been prepared by incorporation of carbohydrate moieties, and in several cases the analogues showed enhanced analgesic activity. Attachment of simple sugars to peptides generally confer the peptide a decreased lipophilicity, reducing the passive diffusion. However, this modification often increases their penetration of the BBB and allows the resulting glycopeptide to function effectively as drugs, [161] probably involving typical glucose carriers. For example, the cyclic glycopeptide H-Tyr-c[D-Cys-Gly-Phe-D-Cys]-Ser(β-Gluc)-$GlyNH_2$ showed modest μ- and δ-activity, but displayed high and prolonged analgesic efficacy after administration into brain [162]. O- and C-glycosylation of dermorphin halved the peptide affinity for brain μ-opioid receptors and the biological potency. Despite their lower affinity, the O-β-glucosylated analogs [Ser^7-O-β-Glc]dermorphin and [Ser^7-O-β-$Glc(Ac)_4$]-dermorphin, and the C-α-galactosylated analogs [Ala^7-C-α-

Gal]dermorphin and [Ala^7-C-α-Gal(Ac)$_4$]-dermorphin, were two times more potent analgesics than dermorphin. The permeability of the glycodermorphins was significantly higher than that of dermorphin, indicating a facilitated entry into the brain [163]. The higher analgesic potency displayed by dermorphin and deltorphin-I glucosylated and galactosylated at the seventh amino acid residue has been explained in terms of protection of the peptides towards enzymatic degradation and consequent increase in their blood and CNS concentration [164]. A series of lipo-, glyco- and glycolipo-conjugates of Leu-enkephalin have been also reported. Conjugation of a single lipoamino acid at the C-terminal of Leu-enkephalin retains biological activity. Conjugation of a glucuronic acid analog in an analogous position, however, increases activity 40-fold when compared to the native peptide and induces a high degree of δ-opioid receptor selectivity [165]. A D-glucopyranosyl moiety has been beta-O-glycosidically linked to a Thr^4 or Thr^7 side chain of deltorphin, H-Tyr-D-Ala-Phe-Asp-Val-Val-Gly-NH_2, and dermorphin, H-Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH_2. The Thr^7-glycosylated peptides retained high δ- or μ-selectivity and remarkable activity *in vivo*, and were more potent than the parent peptide, showing a high blood to brain rate of influx, which may be due to the glucose transporter GLUT-1 [166]. In other cases, short peptides glycosylated with p-(succinylamido)phenyl α- or β-D-glucopyranoside has been proposed to enter the biological barriers by means of the sodium ion-dependent transporter Glut-2 [167]. The δ-selective glycosylated Leu-enkephalin analogue H-Tyr-D-Thr-Gly-Phe-Leu-Ser(β-D-Glc)-$CONH_2$ (SAM 1095) **11**, Fig. (**5**), produces analgesic effects similar to morphine, even when administered peripherally, yet possesses reduced dependence liability. The bioavailability of SAM 1095 has been compared to that of the same nonglycosilated peptide (SAM 995) [168]. The glycosilated analogue was 10-times less lipophilic, and displayed a similar binding to serum proteins. Nevertheless, it demons-trated a higher barrier penetration, and higher metabolic resistance. These features could be responsible for the observed enhancement both in maximal analgesia and analgesia duration. The two peptides SAM 995 and 1095 do not share a common transport mechanism, and the glycosilated peptide is probably transported more efficiently [169]. Analogues of the cyclic [D-Cys^{2,5},Ser^6,Gly^7] enkephalin carrying a glucose or a xylose sugar moiety displayed enhanced analgesia associated with improved transport [170].

OPIOID PEPTIDE PRODRUGS

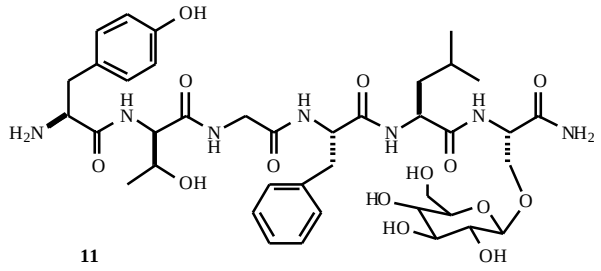

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Fig. (5). The glycosylated Leu-enkephalin analogue SAM 1095.

A peptide prodrug is constituted by the combination of a biologically active peptide and an additional moiety which gives the whole molecule a better BBB permeability and/or a better resistance against enzymatic hydrolysis. As above mentioned, several peptidases are present at the BBB. Proteolytic cleavage of the additional molecule, especially a short truncation at the N-terminus, directly at the BBB, or in a closer proximity of the site of action, releases the pharmacologically active drug. Prodrugs or prodrug-enzyme inhibitor combinations may optimize the delivery of peptide or protein drugs to the central nervous system [139,171].

Prodrug strategies applied to opioid peptides have tended to focus on modification of a single functional group. For example, the N-terminal 4-imidazolidinone prodrugs of Leu-enkephalin, being metabolically stable and bioreversible, has been proposed as a suitable prodrug candidates for delivery of Leu-enkephalin to the brain [172].

A different approach introduced the concept of making cyclic prodrugs of peptides as a way to modify their physicochemical properties sufficiently to allow them to permeate biological barriers (i.e., intestinal mucosa). Specifically, acyloxyalkoxy-, phenylpropionic acid- and coumarinic acid-based cyclic prodrugs of [Leu^5]-enkephalin and its metabolically stable analog DADLE have been prepared by means of chemical linkers. The cyclic prodrugs of these opioid peptides were shown to have favorable physicochemical properties, such as increased lipophilicity, for membrane permeation, unique solution structures (e.g., β-turns) that reduce their hydrogen bonding potential, and metabolic stability to exo- and endopeptidases [173]. Other cyclic prodrugs of [Leu^5]-enkephalin and DADLE have been prepared using a coumarinic acid or a phenylpropionic acid linker. These cyclic prodrugs exhibited excellent trans-cellular permeation characteristics across Caco-2 cell monolayers. The enhanced permeation is apparently due to the alteration of their lipophilicity and hydrogen bonding potential, but not their molecular sizes [174].

The cellular permeation characteristics and the chemical and enzymatic stability of the coumarinic acid-based cyclic prodrugs have been further investigated. In various biological media, the opioid peptides were released from the prodrugs by an esterase-catalyzed reaction [175].

In other cases, propeptides have been obtained by way of modification at the C-terminal end. Various prodrug forms of DPDPE and [D-Pen^2, L-Cys^5]enkephalin (DPLCE) were synthesized to increase lipophilicity and drug delivery to the brain. The prodrugs with carboxy-terminal phenylalanine residues (DPDPE-Phe and DPLCE-Phe) had significantly longer metabolic conversion times in both mouse serum and brain homogenates than did the prodrugs with amino-terminal phenylalanine residues [176]. Very recently, O-acetyl, O-propionyl and O-pivaloyl prodrugs of the Leu-enkephalin analogue Tyr-D-Ala-Gly-Phe-Leu-NH_2 were synthesized, and the chemical and enzymatic stability of the prodrugs has been investigated in detail. The prodrugs studied are quite stable chemically [177].

Phe(o)-analogues of a variety of opioid peptides expressed both receptor binding affinities and *in vitro* biological activities at least at the level of the primary opioid

peptides. Some of the analogues expressed even a slightly higher activity. Nevertheless, no significant shift in receptor selectivity was observed, which indicate that these analogues are propeptides [178].

A growing technology used to enhance efficacy of therapeutics is constituted by conjugation of proteins and peptides with polyethylene glycol (PEG) [139]. Polymer conjugation allows an increased peptide stability, and reduce elimination. PEG-conjugated DPDPE seems to act as a prodrug, enhancing peripheral pharmacokinetics, while undergoing hydrolysis in the brain and allowing nonconjugated DPDPE to act at the receptor [179].

Finally, following a retrometabolic drug design strategy, DADLE on its N-terminus has been connected via a spacer amino acid to a targetor, a 1,4-dihydrotrigonellyl group, and on its C-terminus to a bulky, lipophilic ester group . After its entry through the membrane by passive diffusion, the prodrug **12** has been locked *in vivo* by quaternarization, obtained in turn by oxidation, and finally DADLE has been delivered by means of specific enzymes, [180] as shown in Fig. (**6**).

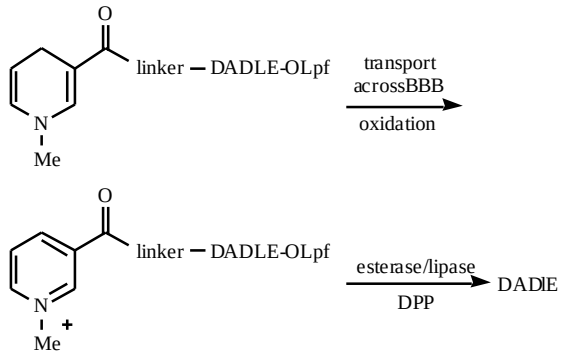

Fig. (6). Brain delivery of DADLE by way of prodrug **12**.

CHIMERIC OPIOID PEPTIDES

The combination of different peptide sequences or moieties belonging to diverse molecules is a widely applied method to develop new bioactive compounds. The newly generated compounds in some cases display new properties and enhanced performances in respect to the parent compounds. For instance, a series of hybrid ligands were synthetized linking various C-terminal fragments of DynA (1-8) to MPCB and its p-chloro-phenyl analogue CCB, two normetazocine derivatives that specifically binds κ-opioid receptors. In particular, MPCB-Arg-Arg-Ile-OMe **13**, Fig. (**7**), and MPCB-Gly-Arg-Arg-Ile-OMe showed an affinity higher than that of the original MPCB and CCB [181].

The coupling of a peptide to a substance that is normally transported through the BBB via receptor mediated transcytosis or absorptive-mediated endocytosis is regarded as a promising strategy to enhance the peptide penetration [182]. These 'vectors', modified proteins or receptor-specific monoclonal antibodies, allow the shuttling of opioid peptides through the BBB *in vivo*. Once in the brain, these 'vector-mediated peptides' release the bioactive peptide after cleavage by enzymes such as disulfide reductases, which are abundant in brain. DALDA was coupled with avidin-biotin, and the resulting highly stable bio-DALDA showed an 18-fold increase in brain uptake compared to the peptide without vector [183]. Peptide vectors carrying cationic sites contribute to the penetration of the peptide into BBB by absorptive-mediated endocytosis. This effect have been explored for β-endorphin, connected with cationized albumin, or for morphiceptin and endomorphin-2, which have been derivatized at the N-terminus with guanidine, assuming a cationic state at physiologic pH [139].

A special kind of chimeric peptide is represented by molecules containing in their sequence assorted portions belonging to different biologically active peptides which could produce interesting effects due to the simultaneous activation of two distinct opposing spinal systems. As already mentioned in the introduction, the repeated administration over the course of few days of a wide variety of μ-opioids, including morphine and endomorphins, leads to a rapid development of tolerance. To examine the effects of simultaneous activation of two distinct opposing spinal systems on opioid tolerance, hybrid peptides were synthesized. The administration of a single chimeric compound instead of the simple administration of a specific ratio of the two peptide components offers several advantages. The combination of different moieties into a

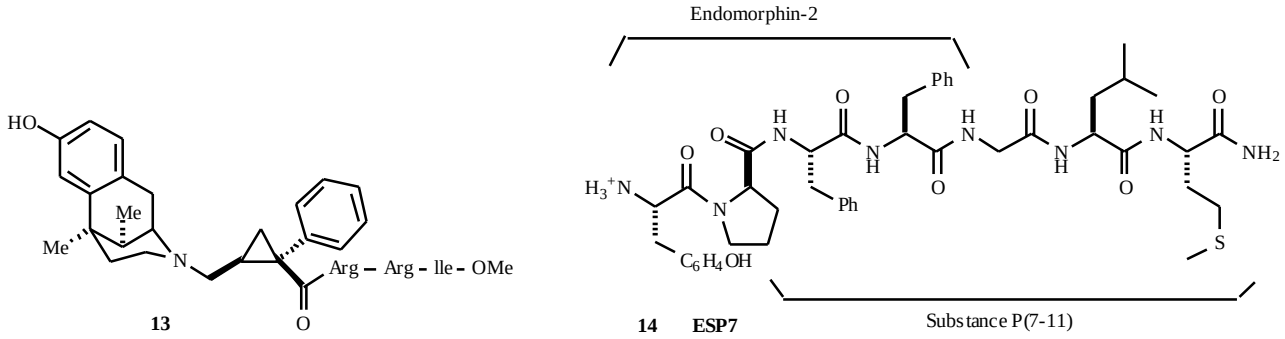

Fig. (7). Chimeric opioid peptides.

peptide gives a more kinetically favourable situation. Indeed, the same number of different receptors is targeted and activated, if the anatomical receptor distribution is similar. The combination could also avoid differences in drug distribution, time course of action, side effects, and metabolism of two different agents.

Recently, some results provided support for a role of amphiactive sequences in antinociception and its modulation. A synthetic chimeric peptide of Met-enkephalin and FMRFamide, Tyr-Gly-Gly-Phe-Met-$(Lys)_3$-Phe-Met-Arg-Phe-NH_2 (YFa), based on MERF was synthesized. The peripherally administered chimeric peptide can produce dose dependent, naloxone reversible, antinociception; potentiate morphine antinociception and attenuate morphine tolerance [184]. The enzymatically stable YFa analog [D-Ala^2]YFa produced modest to good antinociception [185].

The heptapeptide H-Tyr-Pro-Phe-Phe-Gly-Leu-Met-NH_2 **14**, Fig. (**7**), designated ESP7, contains overlapping domains of endomorphin-2 and substance P, respectively, and produces opioid-dependent analgesia without tolerance development. ESP7 can interact both with μ-opioid receptors and substance P receptors. The authors suggest that the simultaneous activation of the receptors mimics a state of reciprocal activation and inhibition in a similar way as happens during nociceptive processing [186].

A second substance P-opioid chimera, H-Tyr-Pro-Phe-Phe-Pro-Leu-Met-NH_2, designated ESP6, is distinguished from ESP7 by a glycine to proline substitution at position 5. Administration of morphine sulfate with ESP6 leads to a prolongation of MS analgesia over a 5-day period. The presence of a proline in ESP6 appears to reduce its conformational flexibility, limit its potency at the μ-opioid receptor, and hinder its analgesic effectiveness alone. However, ESP6 represents a novel adjuvant for the maintenance of opioid analgesia over time [187].

NON-CONVENTIONAL OPIOID PEPTIDES

The presence of a cationic amine, a phenol group, and an additional hydrophobic group, all having a proper spatial orientation, are considered necessary features to manifest biological activity through interaction with opioid receptors. However, some opioid analogues lacking a positive charge have been reported to show a good receptor affinity, with antagonist activity. To this class belong the cyclic peptide **15** containing all amide bonds, or the β-casomorphin derived peptide **16** in which the terminal amino group was eliminated or formylated, [188] the enkephalin analogue containing a modified tyrosine, the (2S)-2-methyl-3-(2',6'-dimethyl-4'-hydroxyphenyl)-propionic acid (Mdp), [189] and the DynA analogue after replacement of Tyr^1 with 3-(2,6-dimethyl-4-hydroxyphenyl)propanoic acid (Dhp) or Mdp [190]. The lack of a positively charged N-terminal amino group is considered primarily responsible for the antagonist behavior of these compounds, which is in agreement with the results of previous studies indicating that the N-terminal amino group of opioid peptide ligands plays a crucial role in signal transduction at μ-and δ-receptors.

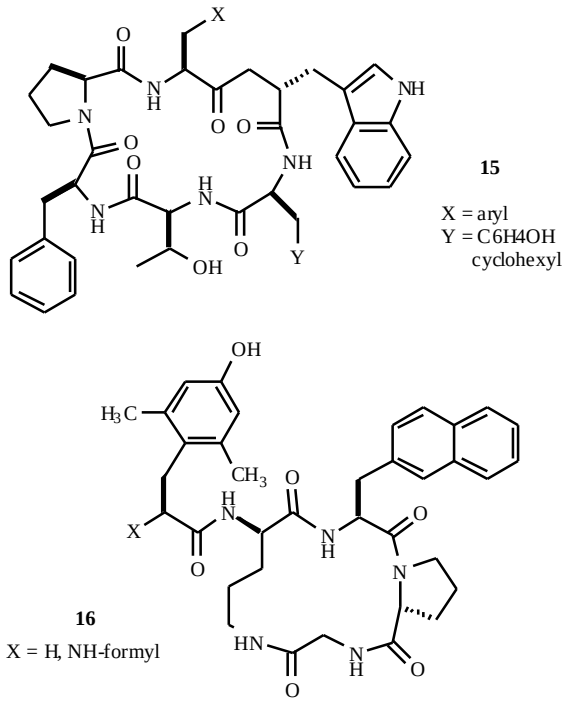

Fig. (8). Opioid peptides lacking a N-terminal amino group.

Interestingly, the analgesic efficacy of a peptidomimetic can be maintained even in the absence of N-terminal amino group. A series of highly constrained 6,6-bicyclic system analogues **17**, Fig. (**9**), incorporating functionality at the *i* to *i* + 3 positions was investigated to represent an enkephalin 5→2 β-turn model with one methylene length shorter at the *i* position and an endomorphin type III 4→1 β-turn model with one methylene length longer at the *i* position. The compounds are lipophilic and have no cationic amino group in the molecule. Among them a couple of compounds were found to be μ-opioid receptor agonists and displayed an initial level of analgesic activity of similar to that of morphine, although the *in vivo* half-life was shorter than that of morphine [191].

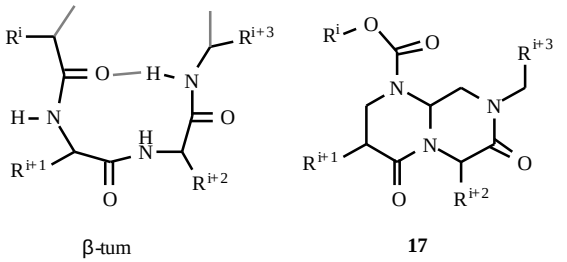

Fig. (9). Enkephalin and endomorphin analogues with non-peptidic beta-turn scaffold.

Also the traditional consideration of the *p*-hydroxy substituent of the N-terminal residue as a critical pharmacophoric element for opioid peptides has been recently re-discussed. The investigation of analogues of DynA carrying modifications at Tyr^1 indicated that the

phenolic group may not be as important for binding at the κ-opioid receptors as for binding to the other receptors [70].

A couple of Tyr-c[D-Lys-Phe-Ala] (YkFA) analogues were prepared by transposition of the Tyr^1-Phe^3 combination. The compounds were agonists both at μ- and δ-receptors, with lower affinity but with higher selectivity than YkFA [192]. Substitution of Tyr with Phe in the cyclic peptide Tyr-c[D-Cys-Phe-D-Pen]NH_2 (Et) (JOM-6) gave a μ-opioid receptor agonist (JH-54) with only 4-fold reduced affinity [193]. In a similar way, Tyr can be substituted in the DPDPE sequence with a variety of related aromatic residues lacking hydroxy group without drastic loss of potency and affinity [194,195]. Thus, although the tyrosyl hydroxyl group does play a role in the interaction of peptides with the μ-opioid receptor, this role is not critical. The maintaining of the high affinity of analogues can be due to a shift of the ligand's N-terminal residue within the μ-receptor binding pocket, which diminishes the importance of the usual hydrogen bond between the tyrosine phenolic moiety and the receptor.

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