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Mini-Reviews in Medicinal Chemistry, 2013, *13*, 11-33

Structure-Activity Relationships of Biphalin Analogs and their Biological Evaluation on Opioid Receptors

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Abstract: Biphalin (Tyr-D-Ala-Gly-Phe-NH-NH<-Phe<-Gly<-D-Ala<-Tyr) is an opioid octapeptide with a dimeric structure based on two identical pharmacophore portions, derived from enkephalins, joined "tail to tail" by a hydrazide bridge. This particular structure enhances the antinociceptive activity of the native enkephalins with an unknown mechanism, probably based on a cooperative binding and improved enzymatic stability. Biphalin has excellent binding affinity for μ and δ receptors and it is a highly potent analgesic, as potent as or more than ethorphine. A definitive explanation of the extraordinary *in vivo* potency shown by this compound, which has pronounced efficacy in pain modulation, is still not available; it has been suggested, however, that the high agonist activity may be related to its binding mode at both μ and δ opioid receptors. Biphalin has significantly higher potency than other analgesics with novel biological profiles; in particular, most recent data show that biphalin is unlikely to produce dependency in chronic use. In the past 20 years, there have been many attempts to modify its structure to obtain products unaffected by the action of enkephalinases, to enhance its antinociceptive activity and to modify the BBB penetration. In addition, structure-activity relationship studies (SAR) were performed in order to understand the elements responsible for biphalin's high activity. The aim of the studies reported in this review was to clarify: i) the role of the hydrazide bridge, ii) the role of residues in position 4, 4' and 3, 3', iii) the consequences of molecular simplifications (truncation, delection), iv) the consequences of cyclization through a disulfide bridge, v) conjugation with PEG and fluorescet residues, and vi) radiolabeling on Tyr^1.

Keywords: Analgesic activity, Biphalin, Dimeric ligands, Enkephalins, μ/δ Mixed opioid agonist, Opioid receptors, peptidomimetics.

1. INTRODUCTION

The discovery, in 1975, of the endogenous opioid peptides, enkephalins (Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu) [1] opened up a whole new area of research on opioid ligands [2]. Enkephalins, originally isolated from pig and cow brain [1] are endogenous δ ligands of the opioid multiple receptor family located in neuronal cell membranes which express the extensively studied μ-, δ- and κ-opioid receptors. Enkephalins are highly sensitive to enzymatic degradation and exhibit scarce binding selectivity and minimal capacity to cross the phospholipid bilayer [3]. In order to obtain compounds with higher selectivity, potency, enzymatic resistance and improved biological profile, the enkephalins have been the most extensively modified among the natural opioid peptides [2]. These studies showed that: i) the amino-terminal tyrosine residue is essential for the compound's activity [4]; this gives a reason for assuming an interaction between the peptide N-terminus and its counterpart in the receptor site [5]; ii) the replacement of the glycine residue in position 2 by D-alanine enhances the activity of enkephalin analogs, the enhanced activity was not observed for other D-amino acid residues [6,7]; iii) C-terminal methionine or leucine residues can be replaced by a large number of substituents, not only by L- or D- amino acids, without decreasing the compound's activity [8,4].

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2. BIPHALIN: GENERAL INFORMATIONS

A type of modification leading to very potent analogs of opioid peptides is the "bivalent" ligand approach [9]. Bivalent ligands often contain two pharmacophores linked by a spacer, whose constitution plays an important role in modulating selectivity and potency. Basis of this model is the consideration that the opioid receptor is located on a dimeric or oligomeric subunit, whose supramolecular organization contains a unique array of recognition sites. Among the bivalent ligands, biphalin, first synthesized by Lipkowski *et al.* [10], was found to be a very effective compound, with strong analgesic activity. Biphalin is a dimeric enkephalin analogue with a palindromic structure, in which the C-terminal amino acid is replaced by a second tetrapeptide active fragment of an enkephalin analogue and the two fragments are connected "tail to tail" by a hydrazine bridge [11] (Fig. **1**). Hydrazine protects the C-terminus of biphalin from enzymatic hydrolysis [10]. However, in the event of enzymatic degradation it is possible to have one remaining enkephalin fragment that may still bind to the

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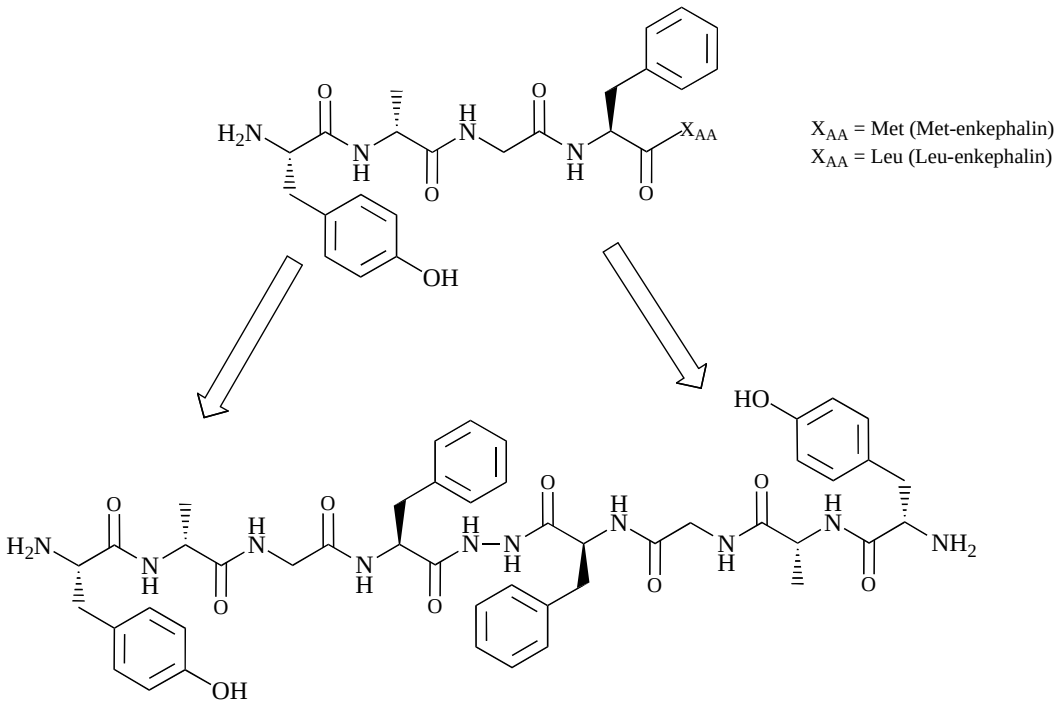


Fig. (1). Biphalin.

opioid receptor [12]. Biphalin displays a high affinity for μ and δ opioid receptor types and a lower affinity for κ opioid receptor [10,13]. Several studies indicate the existence of physical and functional interactions between the opioid receptors, particularly between the μ and δ receptors [14]. These receptors exist on overlapping populations of neurons in pain-modulating regions of the central nervous system (CNS), and the presence of both μ and δ receptors, within the same neuron, has been demonstrated [15] and it was found that μ and δ receptors form functionally distinct hetero-oligomeric complexes [16-19]. Several early studies using co-administration of μ and δ agonist ligands demonstrated that both the potency and efficacy of μ agonists can be increased by δ agonists. The activation of δ opioid receptors has been reported to have synergistic effect on μ opioid functional activities in cells transfected with μ and δ receptors [15, 16, 20]. Treatment of rats or mice for several days with μ agonists leads to the translocation of δ opioid receptors to neuronal plasma membranes and enhances δ-receptor-mediated nociception [21]. These observations imply that addition of a δ agonist may allow for the treatment of pain with lower doses of μ agonists, and ligands possessing dual agonist activities at the δ and μ receptors may allow for the effective treatment of pain with lessened μ-receptor-mediated side effects [22, 23].

3. PHARMACOLOGICAL PROPERTIES

Biphalin has been shown to be one of the most potent opioids ever synthesized in eliciting antinociception after central administration in the mouse and its potency in the tail-flick test was almost seven times greater than that of i.c.v. (intracerebroventricular administration) etorphine and three orders of magnitude greater than the antinociceptive potency of i.c.v. morphine [24]. It has been demonstrated that this compound crosses the blood brain barrier BBB through diffusional and carrier-mediated mechanism and also blood-cerebrospinal fluid barriers, [12, 25] and was found to be remarkably active in eliciting antinociception after peripheral administration, with an i.p. (intraperitoneal administration) antinociceptive potency similar to that of morphine. Direct measurement of the penetration of [^{125}I] biphalin into the brain reveals that only a small fraction of this compound penetrates after i.p. administration, apparently the antinociceptive efficacy of i.p. biphalin may be the result of the remarkable antinociceptive potency of this compound in the brain. When administered i.t. (intrathecal administration) biphalin produced only 60% of the maximal antinociceptive effect in the tail-flick test even when given at doses up to 3 orders of magnitude higher than those effective i.c.v. [24]. When given s.c. (subcutaneous administration) biphalin displayed limited analgesic activity in comparison to morphine and i.v. (intravenous administration) produced significant analgesia, although less potent than morphine *via* this route [26]. Biphalin induces less physical dependence with respect to morphine, Yamazaki *et al.* [27] compared the tendency of biphalin to exert physical dependence with that of morphine in equipotent intravenous doses. They found that the group of rats treated with morphine for five days showed classical withdrawal signs after naloxone injection and infusion of an equipotent antinociceptive dose of biphalin did not produce significant withdrawal signs. Such differences between biphalin and morphine may result from their receptor selectivity of each ligands and interactions among the types of opioid receptors. Co-administration of SPA (undecapeptide substance P antagonist) at the dose of 0.25

μg [28] and ketamine [29] significantly enhanced and prolonged the antinociceptive effect of i.t. biphalin. Analgesic profile of i.v. biphalin was also enhanced by co-administration of 0.01% Pluronic P85 block copolymer [30].

3.1. Non-Antinociceptive-Related Activity of Biphalin

Biphalin has also been shown to act as immunomodulator. It was found in fact that this bivalent ligand stimulated human T cell proliferation, natural killer (NK) cell cytotoxicity *in vitro* and interleukin-2 (IL-2) production. It also releases chemokine like factor in the culture supernatant that was responsible for increased chemotaxis of monocytes; furthermore, biphalin inhibited tumor necrosis factor (TNF-α) production in lipopolysaccharide (LPS) stimulated peripheral blood mononuclear cells (PBMC) and nitric oxide (NO) production in mouse macrophage cells [31]. Biphalin also exhibited an inhibitory effect on human T98G glioma cell proliferation, the findings are encouraging and indicate that biphalin application in chronic pain treatment for cancer might produce in addition the beneficial effect of limiting tumor progression [32]. Maksymowicz *et al.* found that i.v. administration of biphalin increases lymphocyte extravasation, but decreases lymphocyte homing to lymph nodes and their release to the lymph; i.t. administration has a similar effect on lymphocyte migration and distribution [33]. Biphalin also exhibits anti-retroviral activity. In fact, when given in non-cytotoxic concentrations, suppressed in a dose-dependent fashion the replication of Friend Leukemia Virus (FLV) and when combined with 3'-azido-3'-deoxythymidine (AZT), splenocytes or cytokine acted synergistically in inhibiting FLV replication compared to either used alone. Using a reverse transcriptase (RT) assay, FLV RT levels were also noted to be reduced in the presence of biphalin. Antiviral property exhibited by biphalin suggests that this peptide may be a candidate for the combined therapy with AZT and possibly other drugs for the retroviral infections, including the human immunodeficiency virus [34,35]. In addition to these properties biphalin showed: i) a remarkable antitussive effect with lower respiratory-depressant effect than morphine after systemic administration [36] and ii) neuroprotective effects: reduction in edema and infarction ratios and improved neurologic score when administered i.p. one hour post ischemia [37] and a strongest neuroprotective effect than morphine in the organotypic hippocampal cultures challenged with NMDA [38].

4. CONFORMATION OF BIPHALIN SOLID STATE

Crystal structure of biphalin sulphate and an analysis of it with respect to interaction with various biological targets have been reported [39]. The solid-state conformation of biphalin was determined by the X-ray diffraction (Fig. **2**) and showed that both halves of the molecule have a folded backbone conformation. The backbone fold is more open for residues 1-4 than for residues 5-8. The side-chains of both tyrosine residues are *trans* to the backbone and both phenylalanine side chains are *gauche* in a g(-)orientation.

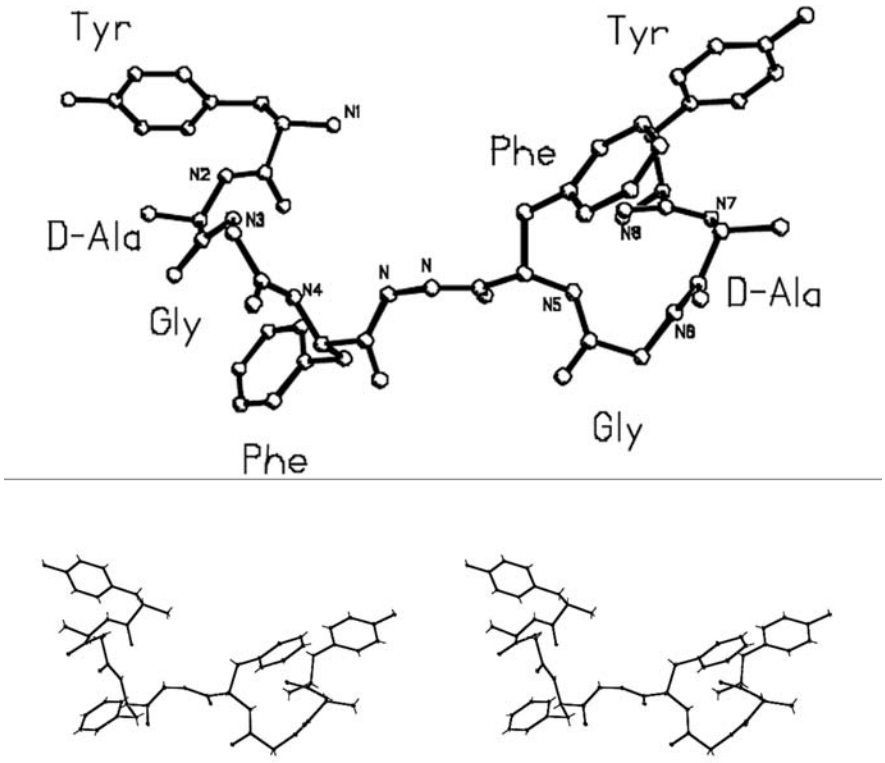


Fig. (2). Upper part: results of the X-ray study on biphalin drawn using the experimentally determined coordinates with arbitrary thermal parameters. Hydrogen atoms have been omitted for clarity (from ref. [39]). Lower part: stereoview of a single molecule of biphalin extrapolated from the original X-ray data of ref. [39] deposited at the Cambridge Crystallographic Data Center by using Vesta 3.0 Software. Stereoview was originated by using Maestro 9.2v Software.

The distances between the centroids of the aromatic rings are quite different, 8.6 Å for Tyr[1] and Phe[4], and 6.0 Å for Phe[5] and Tyr[8]. The tyrosine N to phenylalanine distances, however, are similar at 7.2 Å for residues 1-4 and 6.7 Å for residues 5-8. The two phenylalanine residues are disposed on opposite sides of the N-N bridge. The two halves of the molecule fold differently off the N-N bridge with residue 1-4, making a *trans* turn. The CO-NH groups at the bridge are planar and perpendicular to one another. Biphalin conformation was compared with that found for the solid state conformation of ligands showing strong preference for a single receptor site, DADLE ([D-Ala[2],D-Leu[5]]Enkephalin) [40,41] was chosen as the δ selective model and D-TIPP-NH_2 (Tyr-D-Tic-Phe-Phe-NH_2) [42,43] as the μ selective model. There is a good correlation between the backbone torsions for residues 1-4 of biphalin and those of the somewhat δ selective ligand DADLE. There is a good overall fit for residues 1-3 and good alignment of the Phe[4] aromatic ring in biphalin with Phe[4] ring in DADLE. The peptide backbone in biphalin is more open than that found in DADLE, the more open conformation is most likely due to interactions with the sulphate anion. The second aromatic ring in biphalin does not fit well with any of the three remaining rings in D-TIPP-NH_2. This is not unexpected given the large differences in backbone torsion angles for the two molecules. Despite these differences, the pharmacophoric distances showed good agreement between biphalin and D-TIPP-NH_2, and a better fit for the two molecules can be obtained by matching the Tyr residue in biphalin to the D-Tic residue in D-TIPP-NH_2. Biphalin as well as the other opioid peptide analogs whose X-ray structures were used for comparison are very flexible molecules. Their final conformation in the solid state is strongly dependent on co-crystallizing elements and packing energy [44]. Biphalin conformation also showed structural similarities with two naltrexone analogs that are specific to μ and κ receptor sites: naltrexonazine [45] and norbinaltorphamine [46], respectively.

5. SAR-STUDIES ON BIPHALIN

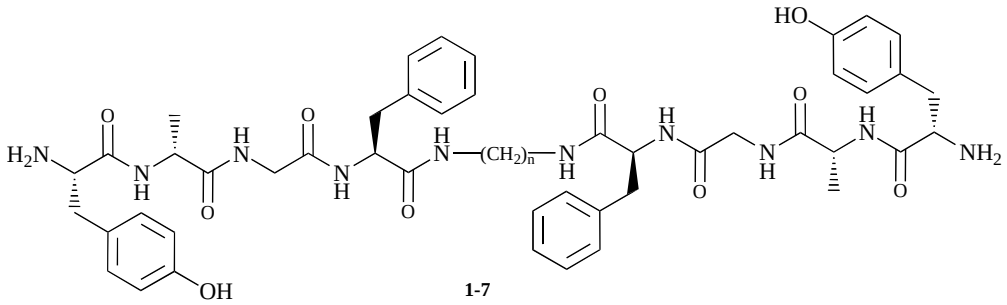
The aim of the studies reported in this review was to elucidate: i) the role of the hydrazide bridge; ii) the role of residues in position 4,4' and 3,3'; iii) the consequences of molecular semplifications (truncation, delection); iv) the consequences of cyclization through a disulfide bridge; v) conjugation with PEG and fluorescent residues and vi) radiolabeling on Tyr[1]. It's worth noting here that a comprehensive discussion of all the binding and functional activity data of biphalin and its derivatives is not always straightforward, especially regarding the early works, when the biological data were made comparing the biphalin activity to linear compounds such as DADLE, DALEA (Tyr-D-Ala Gly-Phe-Leu-NH_2), DAPEA (Tyr-D-Ala-Gly-Phe-NH_2), and not to DPDPE (*c*[D-Pen[2],D-Pen[5]]Enkephalin) like all the new studies. For these reasons, the SAR analysis will be circumscribed to a specific series of modifications. Moreover, in the early studies biphalin has still not been recognized as a standard product, so sometimes it was not tested together with the other derivatives.

5.1. Modifications of Hydrazide Bridge

5.1.1. Substitution of Hydrazide Linker by Diamine Bridge of Variable Length

Biphalin analogs with increased distances between the two pharmacophores were synthesized by Lipkowski and Shimohigashi *et al.* [10,47,48] (Fig. **3**). In these compounds, the hydrazide linker was replaced by a diamine bridge of variable length ($(CH_2)_n$ were n = 2, 3, 4, 6, 8, 10 12, compounds **1**-**7**).

The binding data reported in Table **1** were evaluated by radiolabeled displacement of [^{3}H]DADLE and [^{3}H]naloxone for δ- and μ- opioid receptors, respectively. These data have shown that dimeric tetrapeptide enkephalins were very active for the δ receptors with IC_{50} values of 1-4 nM, compounds **6** and **7** (with cross-linking methylene chains of n = 10 and 12, respectively) were 30-fold more potent than the monomer DAPEA and were as active as DADLE and DALEA. In the μ receptor assay dimers with n = 2, 3, 4, 6, 8 (**1**-**5**) showed higher potencies than the monomer DAPEA, Surprisingly, the dimer of tetrapeptide enkephalins with n = 12 (**7**) showed an extremely low affinity for μ receptors: approximately 28-, 12- and 80-fold lower affinity than DAPEA, DADLE and DALEA, respectively. Compounds **1** and **3** are non-selective (selectivity ratio SR~1), while **4** and **5** slightly favour the μ and δ receptor sites respectively; the dimer **6** favorsδ receptors (SR = 4.7). The dimeric tetrapeptide enkephalin **7** shows an extraordinary selectivity for δ receptors (SR = 91). This peptide shows a 30-fold greater affinity for the δ receptor and a 30-fold weaker affinity for the μ receptor, relative to the monomer DAPEA; it is thus a δ-selective ligand [47].



1-7

Compound	n
1	2
2	3
3	4
4	6
5	8
6	10
7	12

Fig. (3). Biphalin analogs with diamines of variable length (*n = 2, 3, 4, 6, 8, 10 12*) as spacers.

In the MVD (mouse vas deferens) assays dimeric tetrapeptides **1**, **3**, **4**, **5**, **6**, **7** are consistently more potent than the monomer. The most active compound is **1** (relative activity = 10), while **3**, **4**, **5**, **6** and **7** have shown only a 3- or 4- fold increase in potency compared to the monomer. In the GPI (guinea pig ileum), compounds with spacers of 2, 4, 6, 8 and even 10 methylene units are nearly equipotent with the monomer: the most potent appears to be **3** (1.6-fold more potent than monomer) and the least potent is **6** (50% of monomer activity). A spacer of 12 carbons produces a sharp fall in GPI activity, in fact **7** retains only 8% of monomer activity. This is in marked contrast to the retention of potency by **7** in the MVD and δ-binding assays. MVD activity and μ-binding were coincident with only a minor discrepancy for **1.** In contrast, activities in δ-binding are consistently higher than in the MVD and the discrepancy appears to be increasing with the spacer length: while **1** has a 14-fold increase in binding and a 10-fold increase in bioassay relative potency, **7** is 15 times as potent as monomer for δ-receptor binding but only 3 times in the MVD assay. According to Table **1**, the discrepancy between the two data sets doesn't concern only the dimer **1** but also, and in a more relevant measure, the dimer **7**. The increases in activity and affinity are limited to the δ-type of opiate receptor, and μ-activity and affinity were either little changed or significantly decreased, depending on the spacer length. There was a close correspondence between potencies in GPI and the ^{3}H-naloxone (μ) binding assay, this was in contrast with the discrepancies between potencies in MVD and δ-binding assays. The discrepancy is also related to the spacer chain length: the divergence in the two assays appears to be a function of the spacer length, being smaller for the shorter and larger for the longer spacers, bioassay seems to be much more sensitive to changes in hydrophobicity than the binding assay. Compound **7** as compared to monomer showed a 13 fold increase in binding activity but only a threefold increase in bioactivity. This compound was also tested *in vivo* but it was found that it produces no effect after i.c.v. administration [49].

Dimerization *per se* (moving from DAPEA to **7**) results in about a ten-fold increase in δ-selectivity, while μ activity remains about the same. Increasing chain length from 2 to 8 methylene groups has relatively little effect. For **6** and **7,** there is a marked increase in δ selectivity, by virtue of a loss of μ activity. Compound **7** shows the highest affinity and δ selectivity of the series. Affinity and selectivity vary systematically with chain length (n); **7** shows an IC_{50} of 1 to 2 orders of higher magnitude, despite its high affinity for the δ receptors. This peptide also showed a weak activity in the MVD assay, but was more potent than the corresponding monomer (DAPEA) [50]. Compound **7** was the most potent of the series [47]. Compounds **1** and **7** were also examined to determine their antinociceptive potency using the cutaneous-thermal (tail flick (TF) and hot plate (HP)) and visceral-chemical (writhing) response tests [51, 52]. Compounds **1** and **7** produced a dose-dependent suppression of cutaneous-thermal responses, but they failed to significantly alter the visceral-chemical evoked responses. These agents showed a significant effect on the HP and TF which lasted longer than the interval during which the visceral-chemical evoked response were examined [53].

Table 1. Binding Affinities and Bioassays of DAPEA, DALEA, DADLE and Compounds 1-7

Compound	Binding IC_{50} (nM)		Bioassay EC_{50} (nM)	
	δ	μ	MVD	GPI
DAPEA	33.2[a]	3.48[a]	126[b]	89[b]
DALEA	1.24[a]	1.20[a]	8.8[b]	16.2[b]
DADLE	1.15[a]	8.22[a]	0.52[b]	28.6[b]
1	1.68[a]	1.81[a]	12[b]	130[b]
2[c]	--	--	--	46.8 ± 9.1
3	2.87[a]	2.79[a]	39[b]	56[b]
4	3.67[a]	2.28[a]	43[b]	74[b]
5	1.46[a]	2.66[a]	31[b]	138[b]
6	1.04[a]	4.84[a]	37[b]	180[b]
7	1.06[a]	96.3[a]	38[b]	1033[b]

[a] Data from ref. [47].

[b] Data from ref. [49].

[c] Data from ref. [10].

Lipkowski *et al.* [10] found that compound **2** (with cross-linking methylene chains of n = 3) was 3.7 times more potent than Met-enkephalin but twenty-four times less potent than biphalin on GPI, a weaker biological activity of **2** with respect to biphalin may be related to a greater size of the diamine bride in its molecule. Introduction of alkyl diamines could lead to a reduced activity, probably because of the higher degree of freedom around the diamide bridge [10,54].

5.1.2. Substitution of Hydrazide Linker by Hydrophilic Diamines

In early studies of the synthesized bivalent opioid peptides, linear diaminoalkyl chains were used as spacers [10,47,48]. In an aqueous medium, such flexible and liphopilic chains probably have a tendency to form folded conformers in which two vicinal, also lipophilic, opioid pharmacophores form an aggregate [55]. Only for the shorter distance between pharmacophores is it possible to use a rigid spacer which may prevent folding. Stepinski *et al.* used multihydroxyalkyl-spacers instead of polymethylene spacers for connecting pharmacophores with larger distances between them [56]. Interaction of water with a hydrophilic spacer should effectively isolate vicinal pharmacophores. During interaction with opioid receptors, such spacers may interact, not with lipophilic lipid membranes, but with hydrophilic sites of receptor glycoproteins, if such sites exist. As spacers for bridging two opioid pharmacophores, 1,4-diamino-(2*S*,3*S*)-butanediol (1,4-diamino-1,4-dideoxy-L-threitol) (**8**) and 1,6-diamino-1,6-dideoxy-galactitol (**9**) have been used (Fig. **4**).

As reported in Table **2**, the length of hydrophilic spacer did not improve the affinity for δ receptors. It is possible that the distance was too short to reach two δ receptor sites. Both analogs containing hydrophilic spacers express 4-5 times

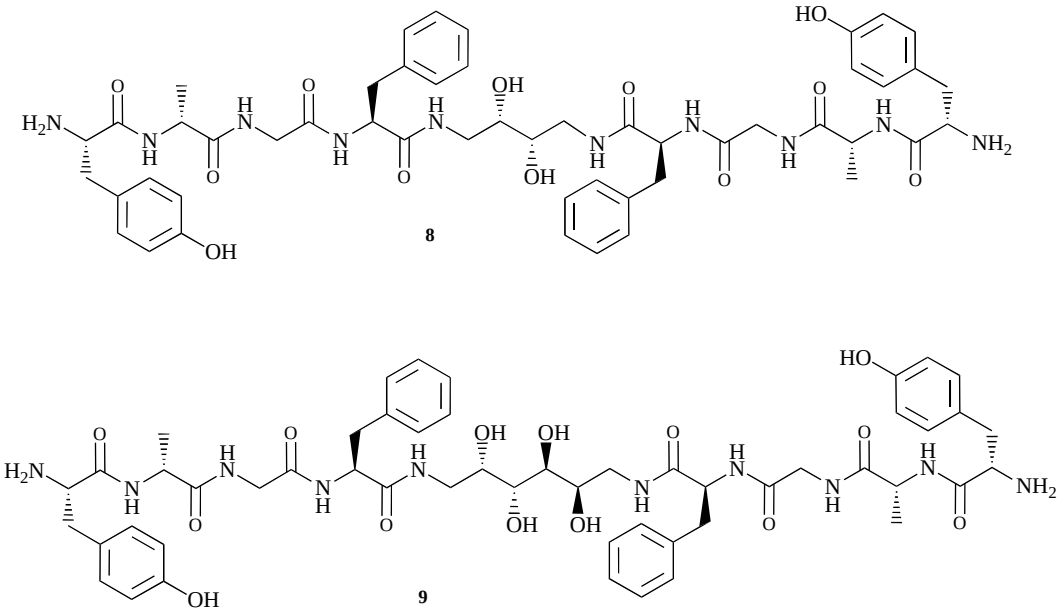


Fig. (4). Biphalin analogs containing hydrophilic diamines as linkers.

lower affinity for δ receptors compared to biphalin. This may be explained by a possible hydrophilic interaction at the receptor or cell surface. Extension of the spacer length between the pharmacophores of biphalin by four carbons containing two hydroxyl groups (**8**) increased the affinities for κ receptors by 150 times and μ receptors by 4 times. In contrast, the affinity for δ receptors was decreased by 4 times. Further lengthening of the hydrophilic spacer by the addition of two hydroxymethyl groups (**9**) resulted in a 10 times decrease in the affinity for μ receptors and a dramatic decrease of 250 times for κ receptors. The affinity for δreceptor was not affected by this increase in hydrophilic spacer length. The results of this study demonstrate that the use of hydrophilic spacers creates new possibilities in the modulation of activity and selectivity of opioid peptide bivalent ligands [56].

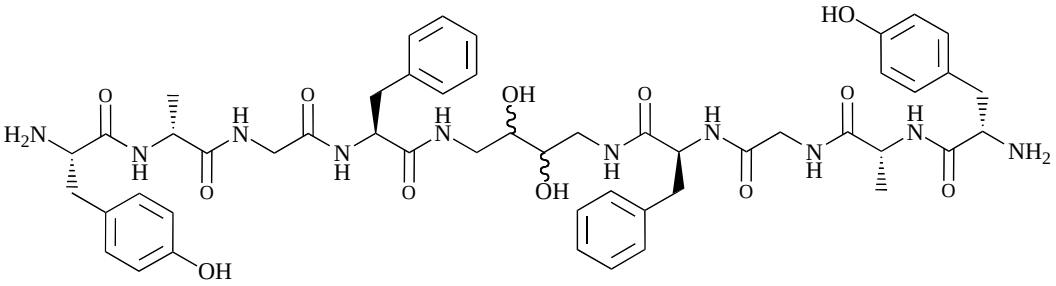
Table 2. Affinities (K_i (nM)) of Opioid Analogues for μ, δ, and κ Binding Sites in Guinea Pig Brain Membrane Preparation

Compound	μ	δ	κ
8	3.2 ± 1.2	18 ± 5	1.79 ± 1.0
9	35 ± 5	24 ± 9	460 ± 100
Biphalin	12 ± 2	4.6 ± 0.2	270 ± 15
Morphine	38 ± 4	510 ± 55	1900 ± 93
Dynorphin (1-13)	31 ± 9	12 ± 0.9	0.98 ± 0.12
DADLE	150 ± 21	1.8 ± 0.4	10000

Subsequently, Stepinski *et al.* synthesized three new analogs within this series. The short spacers having one or two hydroxyl groups of various configuration (1,4-diamino-(2*R*,3*R*)-butanediol (**10**), 1,4-diamino-(2*R*,3*S*)-butanediol (**11**) and 1,3-diamino-propan-2-ol (**12**) have been used for bridging two peptide pharmacophores [57] (Fig. **5**). The effect of length as well as configuration of a spacer on selectivity of a bivalent opioid ligand was of interest in this study.

Data reported in Table **3** showed that all three analogs exhibit different affinity profiles toward opioid receptors. Extension of the spacer length between the tetrapeptide pharmacophores of biphalin by three or four carbons containing one and two hydroxyl groups, respectively (compounds **10**, **11** and **12**) in general increased κ receptor affinity and decreased δ and μ receptor affinity. The *R*,*S* spacer configuration produced a non-selective compound (**11**) with moderate affinity for all 3 opioid receptor types. The *R*,*R* spacer configuration produced a compound (**10**) with high affinity and relative δ selectivity. The change of spacer configuration *R*,*R* (**10**) to *S*,*S* (**8**) (or D to L, compounds **10** and **8,** respectively) resulted in an about 20 times increase in the affinity for μ receptors and an even more spectacular increase of 40 times for κ receptors, while the affinity for δ receptors was not significantly affected. Thus, both the length and configuration of the spacer are important factors in determining receptor potency and selectivity within this series [57].

Zajaczowski *et al.* [58] also synthesized new analogs within this series, the five carbons spacers or bridges bearing three hydroxyl groups (n = 3) of different configuration have been employed for linking two tetrapeptide fragments: 1,5-diamino-1,5-dideoxyribitol (1,5-diamino 2*S*,3*s*,4*R* pentanetriol) (**13**), 1,5-diamino-1,5-dideoxy-xylitol (1,5-diamino-2*S*,3*r*,4*R*-pentanetriol) (**14**), 1,5-diamino-1,5-dideoxy-D-arabinitol (1,5-diamino-2*R*,3,4*R*-pentanetriol) (**15**) and 1,5-diamino-1,5-dideoxy-L-arabinitol (1,5-diamino-



Linker configuration: (2*R*,3*R*) **10**
(2*R*,3*S*) **11**

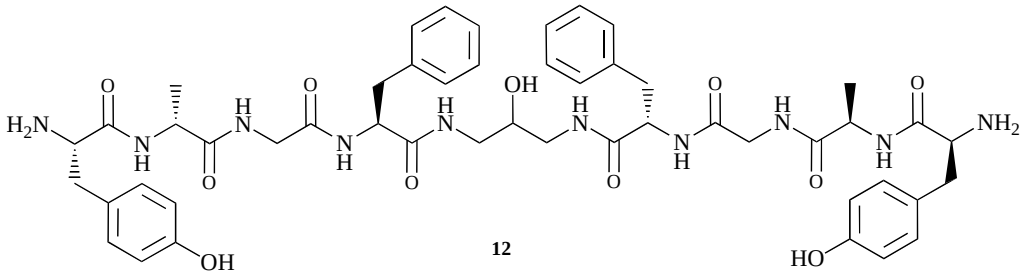


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Fig. (5). Biphalin analogs containing hydrophilic diamines as linkers.

2*S*,3,4*S*-pentanetriol) (**16**). In addition, one analogue with longer spacer, 1,6-diamino-1,6-dideoxy-D-mannitol (1,6-diamino-2*R*,3*R*,4*R*,5*R*-hexanetetraol) (**17**) with D-manno configuration and one reference compound of monomeric nature having tetrapeptide terminated with ethanolamide (**18**) have been synthesized [58] (Fig. **6**).

Table 3. Affinities (Ki (nM)[a]) of Biphalin and Compounds 8, 10-12 for μ, δ and κ Opioid Receptors.

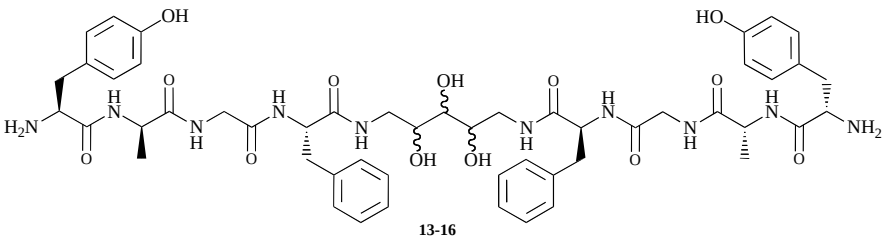
Compound	Bridge Configuration	μ	δ	κ
Biphalin[b]		12 ± 2	4.6 ± 0.2	270 ± 15
12		62 ± 11	82 ± 7	90 ± 7
11	erythro (*R*,*S*)	69 ± 17	44 ± 6	137 ± 23
10	D-threo (*R*,*R*)	71 ± 23	10 ± 2	74 ± 8
8[c]	L-threo (*S*,*S*)	3.2 ± 1.2	18 ± 5	1.8 ± 1

[a] Data represent mean ± S.E.M. of 3-4 experiments in duplicate.

[b] Data from ref. [13].

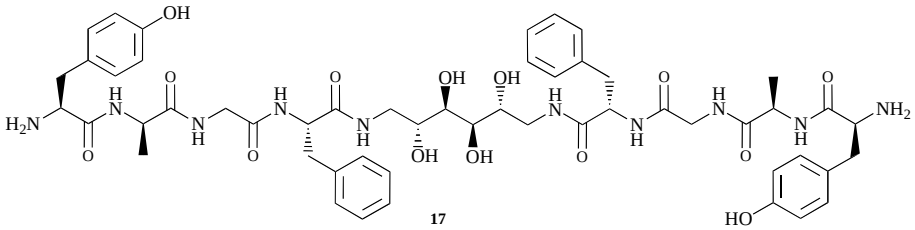
[c] Data from ref [56].

Biological results (Table **4**) were compared to those obtained for the previous series [56,57] (Table **2** and **3**), and it was found that analogs with multihydroxyl spacers (except of only compound **12**) possessed similar δ affinity comparable to that for dynorphin (1-13) but lower than that for DADLE and biphalin. No marked dependence of the affinity on the length of the hydrophilic linker and only minor effects of the various configurations of the spacers were observed. It is possible that the distance between two peptide ligands in these analogs is too short to bridge two δ receptor sites. It is in accord with observations of Shimohigashi *et al.* [47] that the optimum distance for a spacer chain was 10-12 carbons. Nevertheless, three of four newly synthesized analogs with bridges bearing three hydroxyl groups, namely compounds **13**, **15** and **16** exhibited certain δ selectivity. Affinities toward μ receptor sites of the analogs exhibited grater diversity. Almost all compounds expressed higher activity than DADLE or reference monomeric peptide **18** but lower with respect to biphalin (except compound **8**). The most active analogue (**8**) possessed four-carbon spacer with two vicinal hydroxyl groups of *L-threo* configuration. It is possible to assume that the configuration of this compound meets in the best manner for the stereochemical requirements of μ binding site. Two analogs of the five membered bridge (compound **13** and **14**) and both of the six membered bridge (compound **17** and **9**) also showed high μ affinities compared to that of dynorphin (1-13) and morphine but always lower with respect to biphalin. Probably these compounds (**13**, **14**, **17** and **9**) may act as bivalent opioids, by binding not very strongly but simultaneously to two closely situated μ receptor sites. An alternative explanation is that after the binding of one pharmacophore to the receptor pocket, the other pharmacophore can interact with a certain domain of the receptor, thus enhancing affinity. In either cases the optimum distance for a spacer would be 5-6 carbons atoms. The most impressive and consistent results were obtained for affinity toward κ receptor binding sites: all analogs (except compounds **9** and **18**) exhibited higher affinity than biphalin. Compound **8** appeared to have high affinity comparable to dynorphin (1-13). This analogue represents the first opioid peptide derived from enkephalins showing a preference for κ receptors. The other dimeric analogs possessed about two order lower affinity but displayed that an interesting dependence of activity occurred for four carbon linkers and depended on the bridge configuration. Probably biphalin and



Compound	Linker configuration
13	(2*S*,3*s*,4*R*)
14	(2*S*,3*r*,4*R*)
15	(2*R*,3,4*R*)
16	(2*S*,3,4*S*)

13-16



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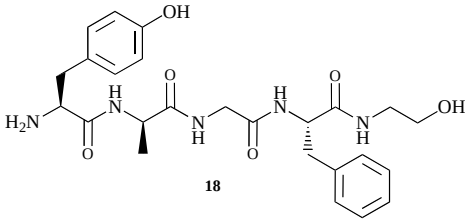

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Fig. (6). Biphalin analogs containing hydrophilic diamines as linkers.

Table 4. Affinities (Ki (nM)[a]) of Opioid Analogues for μ, δ, and κ Binding Sites in Guinea Pig Brain Membrane Preparation

Compound	Bridge Configuration	μ	δ	κ
13	ribo	37.6 ± 15	15.4 ± 2.2	195 ± 49
14	D-arabino	28.3 ± 1.6	46.3 ± 23	126 ± 12
15	L-arabino	96.5 ± 57	26.3 ± 3.9	232 ± 58
16	xylo	168 ± 60	22 ± 7	168 ± 23
17	D-manno	26.6 ± 5.7	30.1 ± 3.1	246 ± 27
18		216 ± 57	163 ± 41	> 10000
Biphalin[b]		12 ± 2	4.6 ± 0.2	270 ± 15
Morphine[b]		38 ± 4	510 ± 55	1900 ±93
Dynorphin[c]		31 ± 9	12 ± 0.9	0.98 ± 0.1
DADLE[c]		150 ± 21	1.8 ± 0.4	> 10000

[a] Data represent mean ± S.E.M. of 3-4 experiments in duplicate or triplicate.

[b] Data from ref. [13].

[c] Data from ref. [56].

analogue **12** are "too short" to display a high affinity, whereas the five- and six-membered linkers (compounds **13**-**17** and **9**) are "too long". In the optimum activity point of four-carbon linkers, the dependence on the configuration of the spacer becomes important. Again, the most active and k selective compound (**8**) have *L-threo* configuration [58].

5.1.3. Introduction of Different Diamines Containing an Aromatic or an Aliphatic Cyclic Structure as Linkers

Further modifications on hydrazide linker of biphalin were performed recently by Mollica *et al.* by designing three biphalin analogs in which the hydrazide bridge was replaced with three different diamines containing an aromatic or an

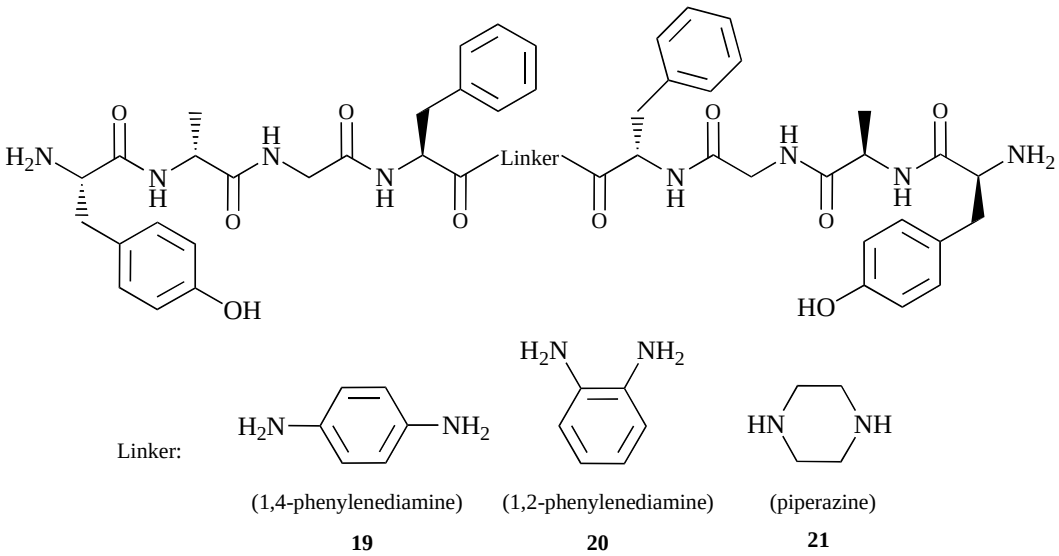

Fig. (7). Biphalin analogs with different diamines containing an aromatic or an aliphatic cyclic structure as linkers.

aliphatic cyclic structure: 1,4-phenilenediamine (**19**), 1,2-phenylenediamine (**20**) and piperazine (**21**) [59] (Fig. **7**).

As reported in Table **5**, compounds **19-21** showed exceptionally good binding affinity and bioactivity (Table **5**). Analogue **19** was comparable with biphalin and compound **21** binds to the receptors with three to five times higher affinity than biphalin, with the *in vitro* bioassay potency reflecting the same pattern. Analogue **20** shows a 1:10 selectivity for the δ versus μ opioid receptors binding. This preference is more evident in the bioassays where the bioactivity for the δreceptors is 50 times higher than at the μ receptor. As compared with the activity of biphalin, all the above reported results show how a reduced degree of freedom between the two pharmacophore moieties and their consequent relative position can influence the binding affinity and selectivity toward different receptors. Whereas the CO-NH of the linker fragment in compounds **19** and **20** should adopt the usually more favorable *trans* conformation, this limitation is not present in compound **21** which contains tertiary amide bonds at the two piperazine nitrogen atoms and thus are free to choose between equivalent conformers. The remarkable activity of compound **21** leads to the hypothesis that the NH moiety of hydrazine in biphalin is not related to the binding at the opioid receptors. We can conclude that the hydrazine linker is not fundamental for activity or binding, and it can be conveniently substituted by different conformationally constrained cycloaliphatic diamine linkers [59].

5.2. Modifications in Positions 3,3'

Position 3 of enkephalins is very intolerant to substitution, therefore enkepalin derivatives generally retain Gly at this position [2]. To investigate the influence of amino acid residues in position 3 of biphalin on potency and selectivity of the parent biphalin position 3 substituted analogs have been synthesized and studied for their binding and biological activity profiles. Misicka *et al*. synthesized a biphalin analogue (**22**) in which Gly residues in position 3,3' were replaced by Phe residues [60] (Fig. **8**).

Binding assays (Table **6**) have shown that the symmetrical substitution of the Gly^3 residue with phenylalanine resulted in a decrease of binding affinities at both μ and δ receptors and biological potency at δ receptor and in a weak improvement in GPI value [60].

Table 5. Binding Affinities and Bioassays of Biphalin and Compounds 19-21

Compound	Binding IC_{50}[a] (nM)		Bioassay IC_{50}[a] (nM)	
	δ	μ	MVD	GPI
Biphalin[b]	2.6 ± 0.4	1.4 ± 0.2	27 ± 1.5	8.8 ± 0.3
19	3.17 ± 0.6	1.27 ± 0.08	35.6 ± 6.4	40 ± 16.0
20	0.19 ± 0.04	1.93 ± 0.20	0.72 ± 0.20	40 ± 13.0
21	0.65 ± 0.30	0.48 ± 0.06	9.3 ± 0.30	2.5 ± 0.6

[a] ± S.E.M.

[b] Data from ref. [13].

Table 6. Binding Affinities and Bioassays of Biphalin and Compound 22

Compound	Binding IC_{50} ± S.E.M. (nM)		Bioassays IC_{50} (nM)	
	δ[a]	μ[b]	MVD	GPI
Biphalin	2.6 ± 0.3	1.4 ± 0.4	27 ± 1.5	8.8 ± 0.3
22	65 ± 31	6.5 ± 2.7	32 ± 9.5	3.14 ± 0.37

[a] Vs [^{3}H][*p*-Cl-Phe^4]DPDPE.

[b] Vs [^{3}H]CTOP (D-Phe-Cys-Tyr-D-Trp-Orn-Thr-Pen-Thr-NH_2).

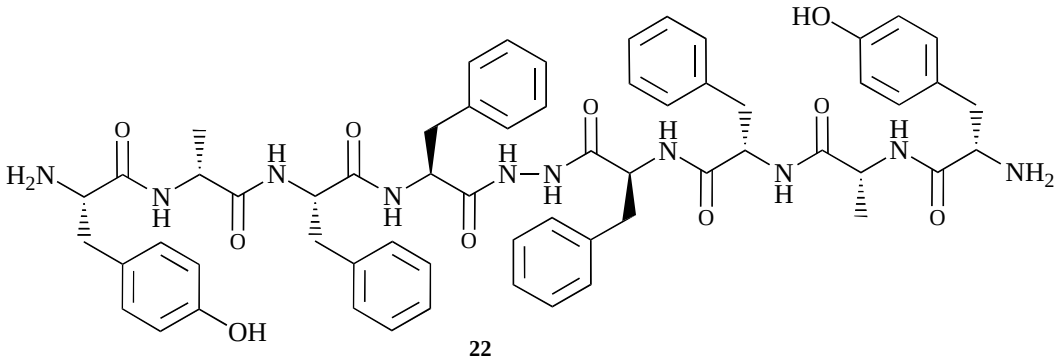

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Fig. (8). Biphalin analogue with Phe residues in potitions 3,3'.

5.3 Modifications in Positions 4,4'

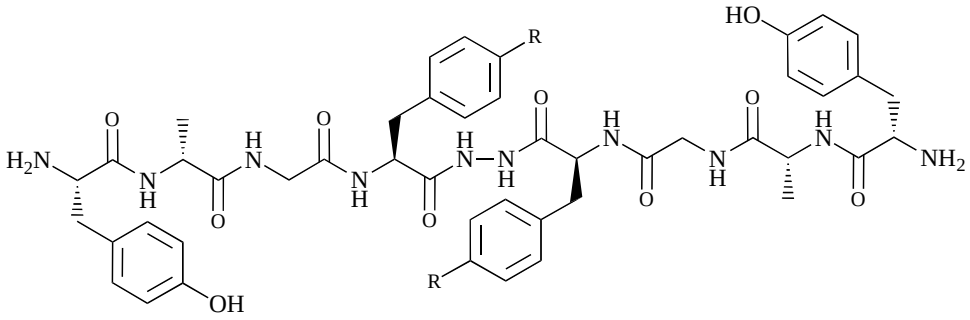
It has been shown that the phenylalanine residue in position 4 of enkephalins can significantly modulate binding to the μ- and δ- opioid receptors [61-63]. To investigate the influence of amino acids residues in positions 4,4' on potency and selectivity of biphalin, several 4,4' positions substituted analogs have been synthesized and studied for their binding and biological activity profiles. Misicka *et al.* synthesized biphalin analogs in which various substituents were introduced in *para* position of the aromatic ring of Phe residues (-NO_2 for compound **23**, -Cl for compound **24**, -F for compound **25**, -I for compound **26**, -NH_2 for compound **27**) [60] (Fig. **9**).

Data reported in Table **7** show that the introduction of a *p*-fluoro-phenylalanine residue in position 4 in both peptide chains (**25**) increases affinity for δ receptors 8 fold, with a much lower (2 fold) significant increase in the affinity to the μ receptor, compared to the parent compound. The large increase in affinity on introducing fluorine in the *para* position of phenylalanines in positions 4 and 4', is progressively lost on increasing of the size of the halogen atom. Replacing fluorine by a chlorine or an iodine atom resulted in a reduced affinity for δ receptors respectively 1.7 and 17 fold, and to μ receptor respectively 3.8 and 38 fold. Substitution in the same position by a nitro-group, resulted in affinity values between analogs with *p*-fluoro- and *p*-chlorophenylalanine containing residues. Interestingly, the di(*p*-nitrophenylalanine) biphalin (**23**) is the most δ selective biphalin analogue in bioassays. Introducing a basic amino group in the *para* position of phenylalanine greatly reduced affinity to δ receptors (46 fold compared to biphalin), and practically eliminated affinity for the μ receptors, though interestingly **27** still retained modest activity on the GPI assay. Probably biphalin forms an active complex with only one receptor molecule. It has been postulated [10] that the second "peptide arm" can play a role in the processes of receptor recognition and also may provide some protection from enzymatic degradation. The binding affinities obtained at the CNS receptors (rat brain homogenates), correlate well with the bioassay results done on respective smooth muscle (MVD and GPI). These data suggest that biphalin itself and its analogs do not discriminate between central and peripheral opioid receptor types [59].

To aid in understanding the mechanism of the transmembrane movement, the permeability and partition coefficients of biphalin and the series of analogs where -F, -Cl, -I, -NO_2 or -NH_2 were placed in the *para* position of the aromatic rings of Phe^4 and $Phe^{4'}$ were determined and analyzed [64]. The observed changes in cross-membrane permeation and water-membrane partition of biphalins generally correlate with the electron affinities of the groups substituted at the *para* position of the benzene ring of Phe^4 and $Phe^{4'}$. The permeability coefficient increases in the following order of substituents: *p*-NO_2 < *p*-Cl, lowest for the electron withdrawing substitution and largest for π-electron donating substitution [64]. Chlorohalogenation of biphalin has been shown to improve CNS entry, most likely through an enhancement in lipophilicity, and increase in biological stability [12].

Other analogs of biphalin modified at 4,4' position were synthesized by Li *et al.* [65]. These compounds contain (2*S*,3*R*)-β-methylphenylalanine (**28**), (2*S*,3*S*)-β-methylphenylalanine (**29**), 1-naphthylalanine (1-Nal) (**30**), 2-naphthylalanine (2-Nal) (**31**) and pentafluoro-L-phenylalanine (**32**) in position 4,4' (Fig. **10**).

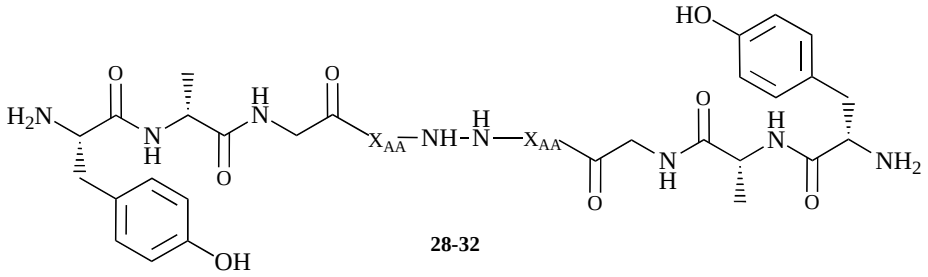
As shown in Table **8**, all of these topographical modifications of Phe^4 and $Phe^{4'}$ residues resulted in higher selectivity for the μ opioid receptor and, in addition, the binding affinity also has been improved (or remained unchanged). The (2*S*,3*R*)-β-methylphenylalanine analogue **28** was 45 times more selective than native biphalin, and is among the most μ-receptor selective biphalin derivatives examined thus far. (2*S*,3*S*)-β-Methylphenylalanine modification **29**, resulted in only a two-fold enhanced selectivity relative to biphalin analogue, while the binding affinity to the μ opioid receptor remained unchanged. These results suggest that biphalin selectivity could be enhanced by topographical constraints in the side-chain moieties, and that the (2*S*,3*R*) *threo*-L stereochemistry in the 4 and 4' phenylalanine positions is favorable for specific stereochemical interactions with the active sites on μ-opioid receptor. 1-Nal and 2-Nal substituted analogs, and the analogue with an aromatic moiety with low electron density (the $F_5Phe^{4,4'}$-containing analogue **32**) can result in greater binding selectivities and increased binding affinities for μ opioid receptors relative to biphalin. Interestingly, biphalin analogs which prefer the δ receptors were obtained when



Compound	R
23	$-NO_2$
24	-Cl
25	-F
26	-I
27	$-NH_2$

23-27

Fig. (9). Biphalin analogs with various substituents in *para* position of Phe[4,4'].



Compound	X_{AA}
28	(2*S*,3*R*)-b-methylphenylalanine
29	(2*S*,3*S*)-b-methylphenylalanine
30	1-naphthylalanine
31	2-naphthylalanine
32	pentafluoro-L-phenylalanine

28-32

Fig. (10). Biphalin analogs with modified residues in positions 4,4'.

Table 7. Binding Affinities and Bioassays of Biphalin and Compounds 23-27

Compound	Binding IC_{50} ± S.E.M. (nM)		Bioassay IC_{50} (nM)	
	δ[a]	μ[b]	MVD	GPI
Biphalin	2.6 ± 0.3	1.4 ± 0.4	27 ± 1.5	8.8 ± 0.3
23	0.63 ± 0.10	0.94 ± 0.12	0.54 ± 0.13	2.84 ± 0.27
24	0.54 ± 0.05	2.44 ± 1.8	2.80 ± 0.93	2.56 ± 0.43
25	0.31 ± 0.12	0.64 ± 0.38	1.30 ± 0.11	2.14 ± 0.66
26	5.20 ± 0.30	24.5 ± 5.3	11 ± 0.31	13 ± 3.0
27	120 ± 34	10 μM (0%)	200 ± 29	4200 ± 680

[a] Versus [^{3}H][*p*-Cl-Phe^4]DPDPE.

[b] Versus [^{3}H]CTOP.

Table 8. Binding Affinities and Bioassays of Biphalin and Compounds 28-32

Compound	Binding Affinity IC_{50}		μ/δ	Bioassay IC_{50} (nM) ± S.E.M.		GPI/MVD
	δ[a]	μ[b]		GPI	MVD	
Biphalin	5.2 ± 0.3[c]	2.8 ± 0.4[c]	0.54	8.8 ± 0.3	27 ± 1.5	0.33
28	110 ± 13	1.3 ± 0.19	0.012	21 ± 7.7	180 ± 78	0.12
29	11 ± 1.7	3.0 ± 1.0	0.27	41 ± 17	120 ± 73	0.34
30	6.4 ± 2.6	0.79 ± 0.16	0.12	1.7 ± 0.33	17 ± 3.5	0.10
31	7.4 ± 1.9	1.7 ± 0.52	0.23	2.2 ± 0.56	9.3 ± 2.4	0.24
32	7.8 ± 2.5	0.91 ± 0.21	0.12	8.9 ± 2.0	25 ± 4.1	0.36

[a] Versus [^{3}H][*p*-Cl-Phe^4]DPDPE.

[b] Versus [^{3}H]CTOP.

[c] Estimated from K_i.

phenylalanine-4 *para*-substituted analogs with electron-withdrawing groups (*para* NO_2 and F) were used for 4,4'-modifications [60]. It seems that both *para* electron-withdrawing and *para* electron-donating groups are not desirable for the design of μ receptor selective biphalin [60]. The *in vitro* biological activities from the guinea pig ileum and mouse vas deferens showed that both the (2*S*,3*R*)- and (2*S*,3*S*)-β-methylphenylalanine-4 substituted analogs **28** and **29**, though they bind as well or better than biphalin, had lower potency at the μ receptor than biphalin, and a much lower potency in the δ assay as compared to native biphalin. The two other extended aromatic modifications in position 4 (**30** and **31**) resulted in higher potencies in both the μ and δ assay system, with 5 times greater potency in the GPI assay, and 1.6 times greater potency in the MVD for the 1-naphthylalanine modification. The biological activities at both μ and δ assays were similar to the native biphalin when the pentafluorophenylalanine was examined. The 4,4'-replacement with (2*S*,3*R*)-β-methylphenylalanine resulted in a 2.4 times higher partition coefficient than biphalin itself. It was suggested that the interaction of biphalin with the bilayer membranes involved a conformational change that allowed the formation of intramolecular hydrogen bonds and aromatic ring pair interaction [66]. (2*S*,3*R*)-β-methylphenylalanine substitution might provide favorable conformational constraints for diffusion through membranes. The increased hydrophobicity from introducing an extra methyl group into the β-position of phenylalanine side-chain may also be an important factor which is responsible for the enhanced partition coefficient across phospholipid bilayers. Membrane permeability studies currently are in progress with the other four unusual amino acid modified biphalin analogs. In summary, the 4,4'-positions have been confirmed as important for biphalin molecular design. The asymmetric (2*S*,3*R*)-β-methylphenylalanine modification in the 4,4' positions provided the highest μ opioid binding selectivity, and it is among the most selective biphalin analogs designed so far. This modification also has resulted in greater ability to cross phospholipid bilayer membranes. In addition, the 1-naphthylalanine modification resulted in both greater binding selectivity and improved potency for the μ-opioid receptor [65].

5.4. Truncations

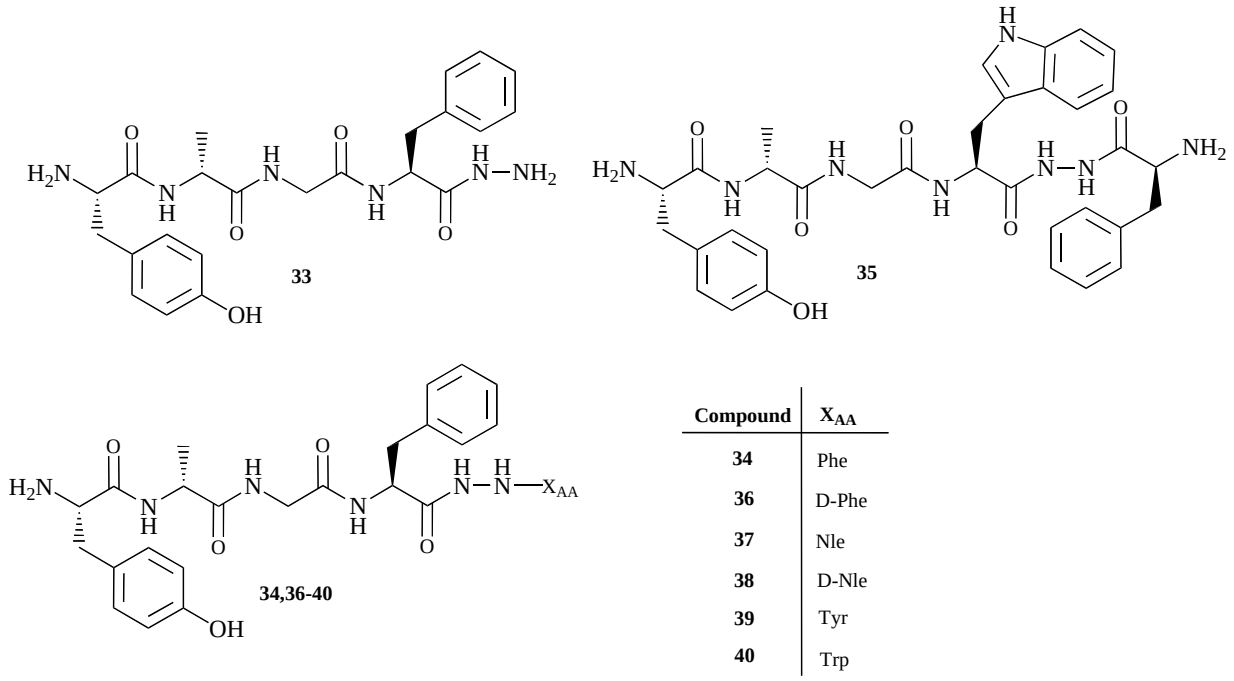
Fragments of biphalin and their analogs were synthesized for evaluation of their biological activities and for structural studies and also to evaluate the possible importance of biphalin metabolities, which may contain one pharmacophore [67]. Lipkowski *et al.* [67] synthesized biphalin fragments in which the second arm of the biphalin was completely deleted (**33**) or replaced by a Phe (**34**) residue and also analogs of these fragment in which Phe^4 residue was replaced by Trp (**35**) and Phe^5 by: D-Phe (**36**), Nle (**37**), D-Nle (**38**), Tyr (**39**), Trp (**40**) (Fig. **11**).

The biological activities of biphalin fragments (Table **9**) indicate that at least for μ receptor binding, the presence of two pharmacophores is not necessary. Even the hydrazide tetrapeptide **33** shows good affinity for the μ receptor similar to the affinity of biphalin. However, this peptide had 100-times lower affinity for δ receptors. The affinity for δ receptors can be significantly restored by acylation of the hydrazide with phenylalanine so as to become more balanced agonists. Surprisingly, replacing the aromatic phenylalanine with non-aromatic, but lipophilic, amino acids did not greatly change the binding properties of these analogs. Also, changing the chirality of the amino acid in this position (**34** vs **36**; **37** vs **38**) led to only modest reduced affinity by a factor of four or less. Interestingly, the binding properties of the analogs did not fully correlate with *in vitro* biological properties. The binding data of the ligand measures the binding interaction *per se*, but does not distinguish between binding to the receptor in its active or in its inactive form. The measured biological activity is a result of binding to respective receptors followed by the activation of transduction mechanism. The ratio of the *in vitro* data and the receptor binding data, though in different systems, may provide some measure of the efficacy of the compounds. In the case of biphalin, the K_i/IC_{50} are 1:10 and 1:4, for δ/MVD and μ/GPI, respectively. For the compound **34**, the respective values are 1:2 and 1:3, which may suggest that compound **34**, interacting with δ receptors, is more efficacious than biphalin. Data obtained showed that even at the same level of binding, compounds synthesized may express significant differences in efficacy. Compound **34** is the minimal fragment necessary to express equal affinities and the same biological activity profile as the parent biphalin; the replacement of N'-Phe with other L- or D-lipophilic amino acids showed the possibility of modification of receptor efficacy of the analogs [67].

5.5. Reduction of Distances between Aromatic Key Amino Acids

To investigate the role of distance between two aromatic moieties, bivalent opioid analogs have been synthesized in which the dipeptide Tyr-D-Phe was connected by diamine moieties. Lipkowski *et al.* synthesized [13] a compound in which two Tyr-D-Phe moieties are connected by a diaminomethane moiety (**41**), it is a symmetrical molecule that preserves the main elements responsible for high affinities and biological activities: Tyr as N-terminal amino acid residue, a D-amino acid residue in position 2, and the aromatic (D-Phe) ring in position corresponding to $L\text{-}Phe^4$ in opioid peptides. Two other analogs were also synthesized in which diaminomethane was replaced by a shorter bridge, hydrazine (**42**) and a longer bridge, diaminoethane (**43**) [13] (Fig. **12**).

Data reported in Table **10** showed that the two bivalent analogs **41** and **43**, in which two dipeptide elements (Tyr-D-Phe) were connected with diamine bridges, both show lack of affinity for κ receptors, and similar but moderate affinities to μ and δreceptors. On the contrary, the analogue **42** in which two dipeptides are connected with hydrazide bond, was much more active with respect to the other two analogs, but less active with respect to biphalin. This analogue shows significant affinity to all three opioid receptor types, but has preference for μ receptors. Its affinity to μ receptors is similar to that of the morphine. Nevertheless, this analogue is less μ selective than morphine. Since this is a small peptide, it is not clear whether its selectivity and activity is a result of binding to one receptor or the bridging of vicinal



Compound	X_{AA}
34	Phe
36	D-Phe
37	Nle
38	D-Nle
39	Tyr
40	Trp

Fig. (11). Fragments of biphalin and their analogs.

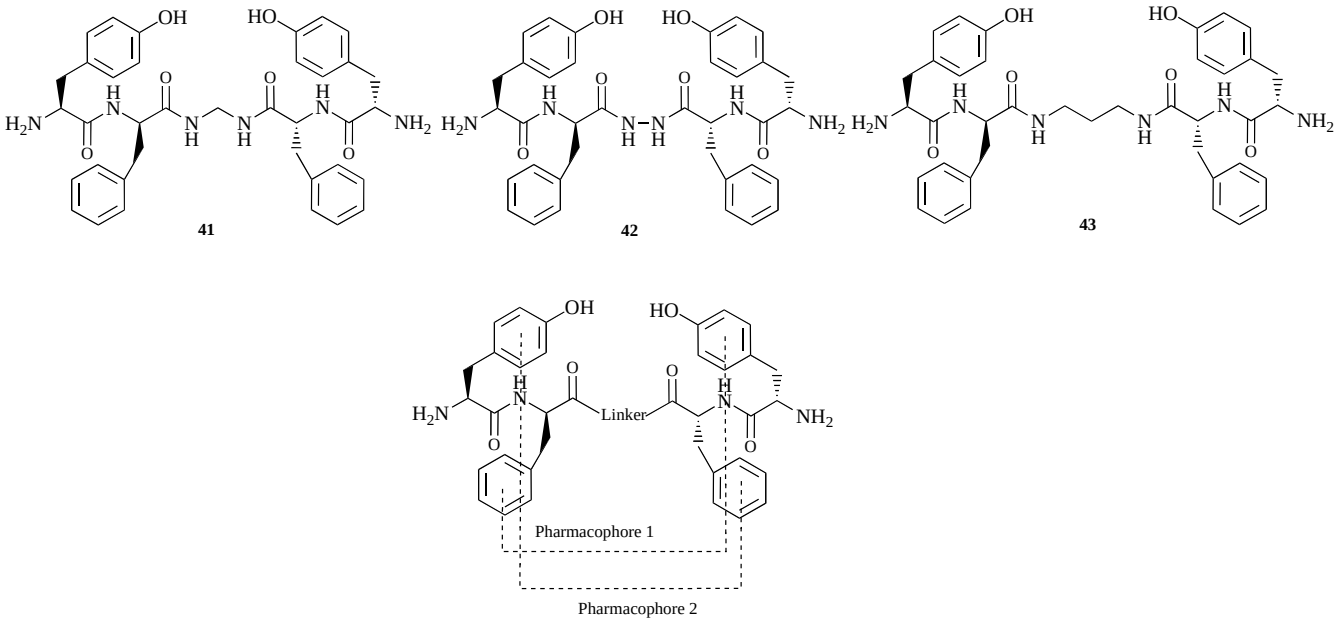


Fig. (12). Bivalent opioid peptide analogs with reduced distances between aromatic key amino acids.

receptors. If the bridging of vicinal receptors took place, the location of opioid receptor pockets in the complex of two macromolecular receptor subunits should be close to the junction between subunits. It is possible that the much higher activity of the bivalent peptide with a hydrazine bridge (**42**) over **41** and **43** is a result of the rigidity of the dihydrazide bond and the presence of two overlapped pharmacophore moieties close to that present in biphalin [68] which reduces the number of possible conformers of analogue. The analogue **42**, which possesses the highest receptor affinities, was tested for its antinociceptive effects after i.p. and i.t. administration. Intraperitoneal administration resulted in a very high antinociceptive activity in both visceral and thermal, nociceptive tests similar to biphalin. Compound **42** and biphalin have similar affinities to μ and κ receptor types, but biphalin has 40 times higher affinity over compound **42** for δ receptor types [13].

Mollica *et al.* [59] have synthesized three analogs of compound **42** in which the hydrazide bridge has been

Table 9. Binding Affinities and Bioassays of Biphlin, and Compounds 33-40

Compound	Binding IC_{50} (nM)*		Bioassay EC_{50} (nM)*	
	δ	μ	MVD	GPI
Biphalin	2.6	1.4	27	8.8
33	230	4.7	290	90
34	15	0.74	27	2.4
35	46	8.3	130	26.0
36	30	0.88	32	9.8
37	71	5.9	95	5.2
38	21	1.3	20	24
39	16	1.6	45	15
40	29	2.0	15	7.1

* The standard errors were less than 20% of the presented values.

Table 10. Relative Affinities (Ki (nM)) of Biphalin, Morphine and Compounds 41-43 for μ, δ and κ Opioid Receptors

Compound	μ	δ	κ
Biphalin	12 ± 2	4.6 ± 0.2	270 ± 15
Morphine	38 ± 4	510 ± 55	1900 ± 93
41	690 ± 48	1480 ± 49	14000 ± 390
42	31 ± 2	187 ± 15	360 ± 34
43	720 ± 170	820 ± 97	14000 ± 1200

replaced with three different diamines containing an aromatic or an aliphatic cyclic structure: 1,4-phenylenediamine (**44**), 1,2-phenylenediamine (**45**) and piperazine (**46**) [58] (Fig. **13**).

As shown in Table **11**, compounds **44-46** showed weak binding affinity and *in vitro* activity at the opioid receptors, probably because the Tyr and Phe moieties are not in a favorable position to accomplish the overlapping of the pharmacophore [59].

5.6. Non-Hydrazine Linker Combined with Modification in 4,4' Positions

Modification of 4,4' residues of biphalin, by symmetrical incorporation of *para*-fluoro or *para*-nitro phenylalanine residues, leads to analogs which show enhancement of affinity towards δ- and μ-opioid receptors accompanied by an increase of δ/μ selectivity [60,65]. Replacement of hydrazide bridge by 1,4-phenylenediamine, 1,2-phenylenediamine and piperazine led to analogs with better affinity and *in vitro* bioactivity than biphalin itself [59]. With the aim of investigate the effects of the para substitution at the Phe^4 aromatic ring combined with hydrazide bridge replacements, new fluorinated biphalin analogs containing 1,2-phenylenediamine (**47**) and piperazine (**48**) as linkers were synthesized [69] (Fig. **14**).

Data reported in Table **12** showed that, when compared to biphalin, the fluorinated piperazinic derivative **48** shows improved binding values for both μ- and δ-receptors. Furthermore, the GTP binding value reveals an extremely high capacity to trigger the transduction mechanism with an efficacy three to six times higher than biphalin for both receptors. In particular, the E_{max} exhibited by **48** is, to the best of our knowledge, the highest value so far reported for a non-cyclic biphalin analogue. Compounds **47** and **48** are both more active than biphalin: the IC_{50} of **48** is in fact 1:10 for μ/GPI receptors and ca. 1:20 for δ/MVD. The corresponding values of **47** are ca. 1:30 for μ/GPI and ca. 1:2 for δ/MVD. Moreover, *in vivo* antinociceptive efficacy of **47** was evaluated in male rats using the hot-plate test by Leone and coworkers [70]. I.c.v. (1 nmol/kg) and i.v. (1200 nmol/kg) administrations in rats clearly showed that **47** has a greater potency and a more extended antinociceptive effect respect to biphalin, with an analgesic response still upper than 50% of maximum 90 min after i.c.v injection and 180 min after i.v. injection [70]. These data are in according with binding and functional assays results of **47**, previously reported by Mollica *et al*. [69]. Results obtained indicate that the improvement of the activity due to the replacement of the native hydrazine linker and those derived from the *p*F substitution on Phe aromatic ring at positions 4,4' are in part

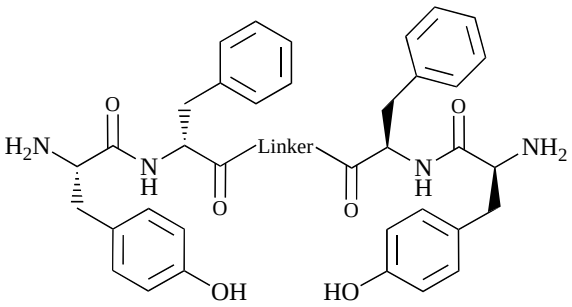


Fig. (13). Bivalent opioid peptide analogs with reduced distances between aromatic key amino acids containing different diamines with an aromatic or an aliphatic cyclic structure as linker.

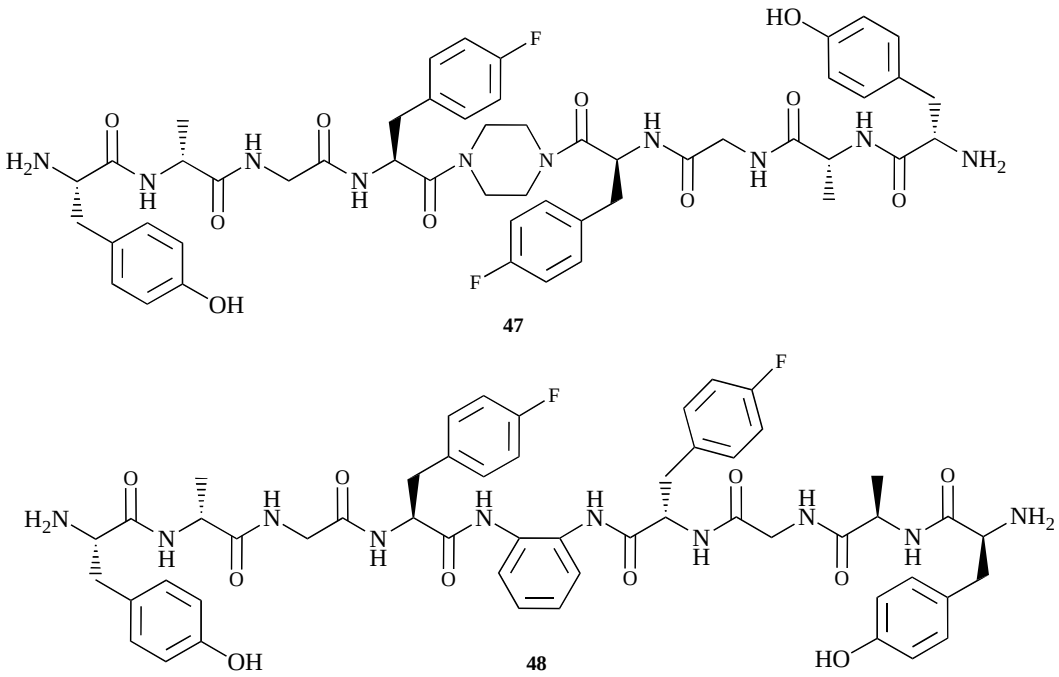


Fig. (14). Fluorinated biphalin analogs containing 1,2-phenylenediamine and piperazine as linkers.

Table 11. Binding Affinities and Bioassays of Biphalin and Compounds 44-46.

Compound	Binding IC_{50}[a] (nM)		Bioassay IC_{50}[a] (nM)	
	δ	μ	MVD	GPI
Biphalin[b]	2.6 ± 0.4	1.4 ± 0.2	27 ± 1.5	8.8 ± 0.3
44	2400 ± 1000	8200 ± 1800	17% at 1 μM	43% at 20 μM
45	640 ± 44	3010 ± 1300	47% at 10 μM	61% at 20 μM
46	400 ± 98	2700 ± 370	8.1% at 1 μM	25.5% at 20 μM

[a] ± S.E.M.

[b] Data from ref. [13].

Table 12. Binding Affinity, GTP Binding Assay, E_{max} (%) (Net Total bound/Basal Binding x 100) and *in vitro* activity of Biphalin and Compounds 47-48

Comp.	Binding K_i[a,b] (nM)		GTP Binding[b,c] (nM)				Bioassay[b] (nM)	
	δ	μ	EC_{50} (nM), δ	E_{max} (%)[d]	EC_{50} (nM), μ	E_{max} (%)[d]	MVD	GPI
Bph[e]	2.6 ± 0.4	1.4 ± 0.2	2.5 ± 0.5	27 ± 3	6.0 ± 0.2	25 ± 4	27 ± 1.5	8.8 ± 0.3
47	0.09 ± 0.01	0.11 ± 0.01	0.80 ± 0.05	94 ± 8	1.0 ± 0.02	77 ± 8	2.7 ± 1.7	0.48 ± 0.07
48	13 ± 1	0.51 ± 0.07	0.56 ± 0.04	72 ± 6	2.9 ± 0.05	44 ± 3	0.66 ± 0.17	3.7 ± 1.0

[a] Displacement of [^{3}H]DAMGO (μ-selective) and [^{3}H]DPDPE (δ-selective) using membrane preparations from transfected cells expressing rat μ-opioid receptor and human δ-opioid receptor, respectively.

[b] ± S.E.M.

[c] Reference compound: [^{35}S]GTP-γ-S.

[d] Net total bound/basal binding x 100 ± S.E.M.

[e] Data from ref. [67].

additive and synergistic, in particular for the stimulation of the second messenger system. The piperazine linker, as compared to the 1,2-phenylenediamine linker, confirms more favourable properties in bridging the two palindromic arms of biphalin. The absence in the piperazine moiety of the two CO-NH groups, available for H-bond donor interactions as well as its more flexible and less planar structure, is probably at the basis of the different behaviors observed. The influence on the activity of the analogue **48** appears remarkable as the introduction of the piperazine link and the *p*F substitution leads to the most potent non-cyclic biphalin analog so far described [69].

5.7. Cyclization

5.7.1. Disulfide Bridge Containing Analogs

Cyclization of peptides is a useful approach to develop diagnostic and therapeutic peptidic and peptidomimetic agents. Mollica *et al.* [71] synthesized the first cyclic biphalin analogs, obtained by closing a disulfide bridge between two cysteine residues located in position 2 and 2' of the backbone of biphalin. Two different structures were obtained by replacing the D-Ala residues of biphalin with two D-Cys or two L-Cys residues. The D-Cys containing cyclic model **50** maintain the original biphalin heterochiral structure (mixed L and D amino acids), whereas the Cys containing isomer **49** presents the unusual homochiral L-sequence. However, these new models, at variance with standard cyclopeptides, present an inversion of the direction of the amide bonds caused by the hydrazine bridge joining the two Phe residues which behave as *gem*-diamines in the retro-inverso peptide isomers. As a consequence of this structural feature, the two flanking backbone fragments, departing from NH-NH moiety of hydrazine, present the same direction of the peptide bonds (Fig. **15**).

The new cyclic ligands showed high *in vitro* bioactivity (Table **13**), compound **50**, containing D-Cys at the positions 2 and 2' in place of D-Ala residues of biphalin, revealed binding affinity and bioactivity higher than those of the product **49** built with Cys. Although the binding value of isomer **50** was close to that of biphalin, the GTP binding and E_{max} were surprisingly high. Compound **50** showed a capacity to activate the transduction to the δ receptor (E_{max} = 100%) higher than that to the μ receptor (E_{max} = 47%). This was in contrast with its binding values which were very similar for both the receptors. Biphalin and compound **49**

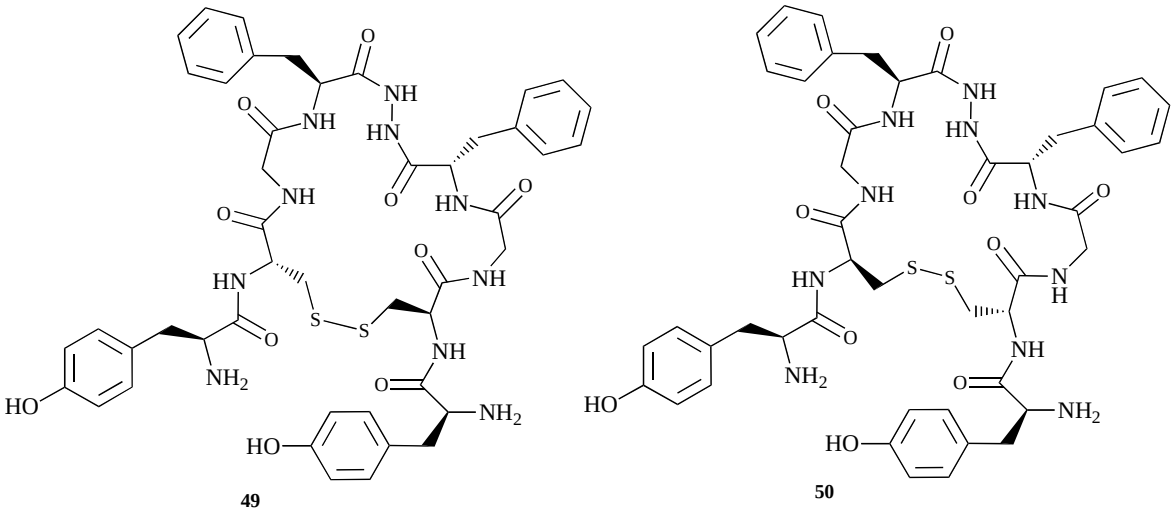


Fig. (15). Cyclic biphalin analogs.

Table 13. Binding Affinity, GTP Binding Assay, E_{max} (%) (Net Total Bound/Basal Binding x 100) and *in vitro* activity of Biphalin and Compounds 49-50

Comp.	Binding K_i[a] (nM)		GTP Binding[a,b] (nM)				Bioassay[a] (nM)	
	δ	μ	EC_{50} (nM), δ	E_{max} (%)[d]	EC_{50} (nM), μ	E_{max} (%)[d]	MVD	GPI
Bph	2.6 ± 0.4[c]	1.4 ± 0.2[c]	2.5 ± 0.5	27 ± 3.5	6.0 ± 0.2	25 ± 4	27 ± 1.5[c]	8.8 ± 0.3[c]
49	53 ± 10.5	130 ± 230	260 ± 100	58 ± 18	120 ± 34	57 ± 2.2	570 ± 130	420 ± 48
50	0.87 ± 0.1	0.60 ± 0.2	0.87 ± 0.3	100 ± 2.3	0.2 ± 0.1	47 ± 5.7	9.9 ± 1.3	25 ± 7.9

[a] ± S.E.M.

[b] Reference compound: cold [^{35}S]GTP-γ-S.

[c] Data from ref. [67].

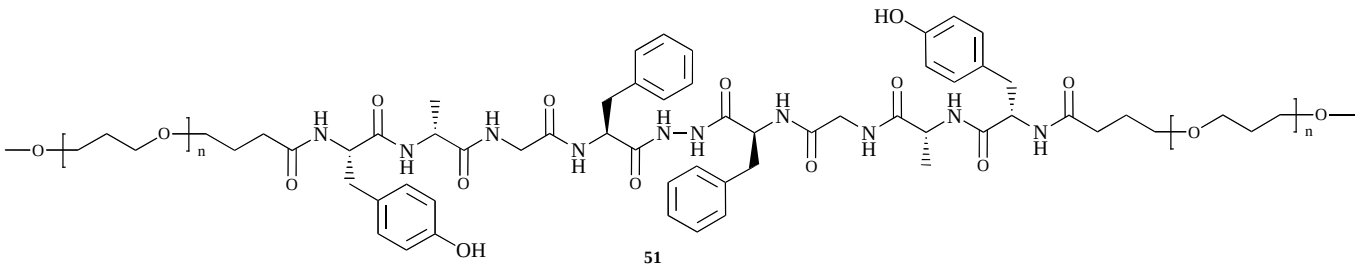
were partial agonists at both μ and δ receptors, compound **50** was a full agonist at the δ receptor and a partial agonist at the μ receptor. Its ability to partially stimulate the μ-opioid receptor was also appreciable by taking into account the fact that the high biphalin analgesic activity seems to be related to its ability to bind both δ- and μ-opioid receptors [24]. Furthermore, although a certain degree of selectivity was observed for both the isomers **49** and **50**, this is certainly low as already found in the case of biphalin [71].

5.8. Conjugated Derivatives

5.8.1. Conjugation with PEG

Poly(ethylene glycol) (PEG) conjugation to therapeutic proteins has been shown to be an effective tool for enhancing systemic drug delivery, as it improves circulating half-life and reduces proteolysis, clearance, and immunogenicity [72]. When PEG is properly conjugated to a protein or peptide, it alters the physicochemical properties while retaining biological activity. To obtain maximal therapeutic benefit, the optimal PEG size must be determined. A PEG chain that is too short offers little advantage over the parent compound; whereas, the use of a PEG conjugate too large may result in decreased biological effect. Biphalin was conjugated with linear PEG (**51**) (*X* kDa; *X* = 1, 2, 5, 12, or 20) on the terminal tyrosines [73] (Fig. **16**).

The effect of various PEG sizes, ranging from $(2\ kDA)_2$ to $(20\ kDa)_2$ on antinociceptive activity was investigated (Table **14**). Optimization of the PEG size attached to biphalin was determined using the antinociceptive profile produced compared to biphalin following i.v. injection. Results show that PEG-conjugated biphalin analogs appear to retain biological activity with $(2\ kDa)_2$, $(5\ kDa)_2$, and $(20\ kDa)_2$ analogs showing significantly enhanced antinociception compared to biphalin. $(2\ kDa)_2$ PEG-biphalin exhibited the most efficacious antinociceptive profile with analgesia extending out over 300 min post-injection. $(2\ kDa)_2$ PEG-biphalin was determined to be optimal sized PEG for biphalin attachment. The analgesic profiles of biphalin and $(2\ kDa)_2$ PEG-biphalin was examined *via* three parenteral routes of administration: i.v., intramuscular (i.m.) and subcutaneous. Results indicate that $(2\ kDa)_2$ PEG-biphalin enhanced the antinociceptive profile by all routes of administration in a dose-dependent manner. Nociceptive sensitivity was determined by converting the recorded analgesic tail-flick times to a percent maximal possible effect (% M.P.E.) and area under the curve (AUC) was calculated from the % M.P.E. plot and used to determine the time-response profile. The increased AUC following $(2\ kDa)_2$ PEG-biphalin administration demonstrates both an increased analgesic effect and enhanced time-response profile. Therapeutic potency of $(2\ kDa)_2$ PEG-biphalin was increased ~two fold following i.v. and s.c. administration compared to biphalin. A possible explanation for the antinociceptive activity seen with $(2\ kDa)_2$ PEG-biphalin is that it acts as a prodrug. With increased circulating free fraction, it is possible that over time the PEG moieties on biphalin are cleaved and biphalin or biphalin metabolites are transported across the blood-brain barrier into the brain. The results shown following i.c.v. administration demonstrate a delayed response of the $(2\ kDa)_2$ PEG-biphalin effect compared to biphalin, which seems to indicate a metabolic stage before full antinociceptive effects are possible. Another possible mechanism involves enzymatic degradation of biphalin, which is supported by a previous study containing three biphalin metabolites having equal or greater potency at the μ-opioid receptor than biphalin [67].



51

Fig. (16). Biphalin conjugated with PEG.

Table 14. Comparison of Antinociceptive Effect of Biphalin and PEG-Conjugated Biphalin with Varying Sized PEG Chains Following i.v. Administration (685 nmol Kg^{-1})

Compound	Antinociceptive Effect (AUC* ± S.E.M.)
Biphalin	7849 ± 467
$(1\ kDa)_2$ Biphalin	6330 ± 966
$(2\ kDa)_2$ Biphalin	18525 ± 726[b]
$(5\ kDa)_2$ Biphalin	12164 ± 636[b]
$(12\ kDa)_2$ Biphalin	10008 ± 794
$(20\ kDa)_2$ Biphalin	9741 ± 368[a]

Values are mean ± S.E.M. (n = 6 mice/drug).

[a] $p < 0.05$; [b] $p < 0.01$, indicate significantly different from biphalin using one-way analysis of variance.

* Area under the curve.

5.8.2. Conjugation of Biphalin Fragment with Fluorescent Residues

Structure-activity study of biphalin showed that the full dimeric sequence is not required for high biological potency, indeed elimination of the tripeptide from one arm of biphalin does not reduce the biological potency significantly [67]. Lipophilic amino acids [67] or other lipophilic elements [74] could replace the residue phenylalanine residue. Lipkowski *et al.* synthesized a biphalin fragment analogue in which the phenylalanine residue has been replaced with a dansyl (DNS) moiety (compound **52**) [75] (Fig. **17**). Fluorescent moiety can allow extensive studies of *in vivo* metabolism, permeability distribution and other studies. The presence of a fluorescent group makes compounds potentially very useful tools for pharmacokinetic and pharmacodynamic studies *in vivo*, as well as for detailed macromolecular studies of their interactions with opioid receptors [75].

As shown in Table **15**, compound **52** expressed high receptor binding affinity for the δ and μ receptor types. Its affinity profile more closely corresponds to biphalin than the original fragment. The high affinity to opioid receptors fully correlated with its antinociceptive activity. Even at dose of 0.2 nmol, the analogue produces strong (MPE > 50%) nociception similar to that reported for biphalin [12]. The antinociceptive effect is dose dependent. Increasing the dose to 0.5 nmol over increases both the level and duration of the observed antinocicption. An overdose of the compound, up to 1.0 nmol, produces reversible, long lasting antinociception, without any visible signs of side effects, such as rigidity or respiratory depression [75].

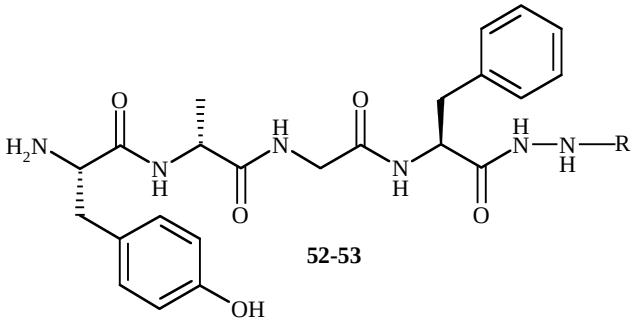
Table 15. Affinities (Ki (nM)) of Biphalin, Morphine and Compounds 34 and 52-53 for μ and δ Opioid Receptors

Compound	μ	δ
Biphalin[a]	1.4 ± 0.2	2.6 ± 0.4
Morphine	9.8 ± 3.5	112.2 ± 6
34	0.74[b]	15[b]
52	1.1[b]	2.0[b]
53	5.1 ± 3.6	389 ± 6

[a] Data from ref. [67].

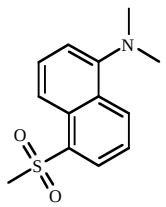
[b] The standard errors were less than 20 % of the presented values.

Lukowiak *et al.* synthesized a biphalin fragment analogue in which the phenylalanine residue has been replaced with fluorescent 7-succinylamido-4-methyl-coumarin (**53**) [76]

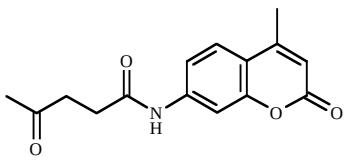


52-53

Compound	R
52	Dansyl
53	7-Succinylamido-4-methyl-coumarin



Dansyl



7-Succinylamido-4-methyl-coumarin

Fig. (17). Biphalin fragment conjugated with fluorescent residues.

(Fig. **17**). This compound displayed a μ receptor binding affinity in the range of biphalin and morphine, however it is much more μ receptor-selective, because its affinity to the δ receptor was over a hundred times lower than biphalin and ten times lower than morphine. Its antinociceptive activity was on the nanomolar level and in the same range as that of the morphine [77].

The compounds **52** and **53** are μ selective fluorescent analogs of biphalin with broad affinities for opiod receptors. They create a complementary pair of opioid ligands with promising applications in pharmacological and biochemical studies of opioid peptides [76].

5.9. Radiolabeled Derivatives

5.9.1. Iodination of Tyr[1]

As above mentioned, structure-activity relationship studies indicate that one fragment containing one tetrapeptide connected *via* a hydrazide bridge with an additional phenylalanine is a minimal necessary structural element responsible for biphalin's high biological activities. A Tyrosine residue could be used for radioactive iodination to obtain useful ligands for further pharmacological study. Mono-iodinated analogs of biphalin, nonradioactive [I-Tyr[1]]biphalin (**54**) and [^{125}I-Tyr[1]]biphalin (**55**) were synthesized [78] (Fig. **18**).

Radioligand binding profile of these compounds for two types of tissues (rat brain membranes and NG108-15 cell membranes) was described (Table **17**). Rat brain membrane preparations contain all three opioid receptor types, whereas NG108-15 cell membranes are known to express only the δ opioid receptor [79]. It was found that the iodination of one tyrosine residue of biphalin did not change its receptor affinity profile compared to parent biphalin. This is additional evidence for the hypothesis that only one tyrosine is necessary for the high binding affinity and bioactivity of biphalin. Although both biphalin and [I-Tyr[1]]biphalin have been shown to have a good binding affinity for both μ and δ receptors in rat brain membranes of the same order of magnitude (Table **16**), the iodinated radioligand, [^{125}I-Tyr[1]]biphalin, appears to be binding predominantly to the μ opioid receptor (Table **17**). However, [^{125}I-Tyr[1]]biphalin does bind well to δ receptors as shown in NG108-15 cell membranes. Thus, in rat brain membranes [^{125}I-Tyr[1]]biphalin binds predominantly to μ opioid receptors or at least was much more readily detected than [^{125}I-Tyr[1]]biphalin binding to δ opioid receptors in this preparation. The results clearly show that the binding of both biphalin and [I-Tyr[1]]biphalin to the two kinds of opioid receptors cannot be completely independent. Nevertheless, the binding data clarify the high potency of biphalin in the mouse and rat tail-flick tests. [^{125}I-Tyr[1]]biphalin appears to interact with both μ and δ opioid receptors, even at very low concentration. Thus biphalin could be expected to act at both opioid receptor types after i.c.v. or i.t. administration, in keeping with previous observations of its potency [78].

CONCLUSIONS

This review reports the structure-activity relationship studies made in order to understand the elements responsible for biphalin's high activity. These can be summarized as follows:

a) Modifications of hydrazide bridge: i) introduction of alkyl diamines could lead to reduced activity probably because of the higher degree of freedom around the diamide bridge which can lead to an incorrect

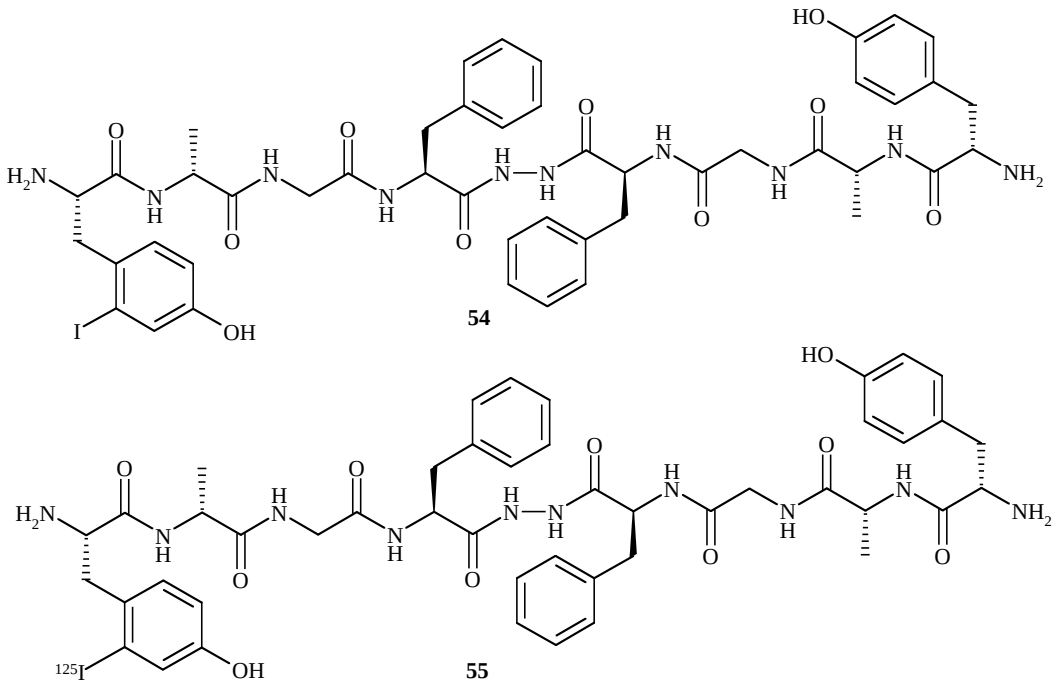


Fig. (18). Iodinated analogs of biphalin.

Table 16. Inhibition of Opioid Receptor Selective Ligand Binding to Rat Brain Membtranes

Compound	IC_{50} ± S.E.M. (nM)		
	[^{3}H]CTOP	[^{3}H][4'-Cl-Phe^4]DPDPE	[^{3}H]U-69,593[a]
Biphalin	0.74 ± 0.26	2.96 ± 0.22	35.1 ± 2.0
[I-Tyr^1]Biphalin	0.97 ± 0.48	3.39 ± 1.71	31 ± 11

[a] (5a, 7a, 8b)-(+)-N-methyl-N-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)-benzeneacetamide.

Table 17. Inhibition of [^{125}I-Tyr^1]Biphalin (10 pM) Binding to Rat Brain and NG 108-15 Cell Membranes

Inhibitor	Rat Brain	NG 108-15
	IC_{50} (nM) ± S.E.M.	IC_{50} (nM) ± S.E.M.
Biphalin	0.50 ± 0.16	0.71 ± 0.26
[I-Tyr^1]Biphalin	0.26 ± 0.07	1.26 ± 0.37
Morphine	1.20 ± 0.27	250 ± 94
Naltrindole	26 ± 11	--
Deltorphin II	1500 ± 580	1.5 ± 0.6
[4'-Cl-Phe^4]DPDPE	150 ± 48	0.71 ± 0.18

positioning of the two pharmacophores in the receptor pockets. However, the compound with cross-linking methylene chains with n = 12 (**7**) showed the highest activity and δ selectivity inside that series. For compounds with cross-linking methylene chains of n = 10 (**6**) and 12 (**7**), there was a marked increase in δ selectivity caused by a loss of μ activity. These findings are consistent with the hypothesis that the dimers of the tetrapeptide can serve as bivalent ligands, binding simultaneously to two distinct but closely clustered δ receptors, but failing to bridge two μ receptors [47]; ii) the use of hydrophilic spacers creates new possibilities in the modulation of activity and selectivity of opioid peptide bivalent ligands and both the length and configuration of these spacers are important factors in determining receptor potency and selectivity, but still accompanied by a loss of activity; iii) hydrazine linker is not fundamental for activity or the binding, and can be substituted by different conformationally constrained cycloaliphatic and cycloaromatic diamine linkers.

b) The symmetrical substitution of the Gly^3 residue with phenylalanine resulted in a reduction of binding affinity and biological potencies at both μ and δ receptors.

c) Modifications in position 4,4': i) the di(*p*-chlorophenylalanine) biphalin (**24**) is the most δ selective biphalin analogue evaluated and the di(*p*-nitrophenylalanine) biphalin (**23**) is the most δ selective biphalin analogue in bioassays. Introducing a basic amino group (-NH_2) in the *para* position of phenylalanine greatly reduced affinity to δ receptors (46 fold compared to biphalin), and practically eliminated affinity for the μ receptors, chlorohalogenation of biphalin has also been shown to improve CNS entry, most likely through an enhancement in lipophilicity, and increase in biological stability; ii) topographical modifications of Phe^4 and Phe$^{4'}$ residues resulted in higher selectivity for the μ opioid receptor and the binding affinity has been improved (or remained unchanged). (2*S*,3*R*)-β-Methylphenylalanine biphalin analogue (**28**) is among the most μ-receptor selective derivatives examined. This modification also resulted in greater ability to cross phospholipid bilayer membranes. In addition, the 1-naphthylalanine modification (**30**) resulted in both greater binding selectivity and improved potency for the μ-opioid receptor. These results suggest that biphalin selectivity could be modulated by topographical constraints [80] in the amino acid side-chain moieties.

d) The synthesis of fragments of biphalin and their analogs showed that Tyr-D-Ala-Gly-Phe-NH-NH<-Phe (**34**) is the minimal fragment necessary to express equal affinities and the same biological activity profile as the parent biphalin.

e) Compound in which two Tyr-D-Phe moieties are connected by a hydrazide bridge (**42**) showed significant affinity to all three receptor types and an affinity to receptors similar to that of morphine. After i.p. administration it showed antinociceptive activity similar to that of biphalin.

f) The replacement of the native hydrazine linker and the *p*F substitution on Phe aromatic ring at positions 4,4' are in part additive and synergistic in determining improvement of the activity. These modifications led in fact to the most potent non-cyclic biphalin analogue so far described.

g) Cyclization through a disulfide bridge between two D-Cys residues located in position 2 and 2' led to a very high capacity to activate the transduction to the δ receptor (E_{max} = 100%, the most potent biphalin analogue ever synthesized).

h) Conjugation with $(2\ kDa)_2$ led to an enhancement of the antinociceptive profile by intravenous, intramuscular and subcutaneous administration in a dose-dependent manner. Further investigation of $(2\ kDa)_2$ PEG-biphalin needs to focus on establishing pharmacodynamic and pharmacokinetic profiles and to elucidate the mechanism by which these effects occur. Conjugation of biphalin fragment with fluorescent residues led to μ selective fluorescent biphalin analogs with promising applications in pharmacological and biochemical studies of opioid peptides.

i) Iodination of one tyrosine residue of biphalin did not change its receptor affinity profile compared to parent biphalin. This is an additional evidence for the hypothesis that only one tyrosine is necessary for the high binding affinity and bioactivity of biphalin.

At present, the pharmacological management of severe chronic pain remains a difficult achievement with currently available analgesic drugs and is still a large unmet therapeutic need. Since it has been shown that the development of potent analgesics must be associated with reduced tolerance [81], dependence [82], respiratory depression [36] and other unwanted side effects [27,62], the development of new biphalin analogs could be of great value in the clinical treatment of chronic pain.

CONFLICT OF INTEREST

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

ACKNOWLEDGEMENTS

Declared none.

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Received: September 18, 2011 Revised: February 03, 2012 Accepted: February 08, 2012