Eur. J. Med. Chem. (1990) 25, 29–33

Original paper

Bivalent opioid peptides synthesized from μ selective monomers display preferential selectivity for δ receptors

Giuseppe RONSISVALLE[1]*, Maria S. PAPPALARDO[2], Lorella PASQUINUCCI[2], Franco VITTORIO[2], Severo SALVADORI[3], Santi SPAMPINATO[4], Emanuela CAVICCHINI[4] and Sergio FERRI[4]

[1]*Istituto di Chimica Farmaceutica e Tossicologica, Università di Trieste, P. le Europa, 1, 34127 Trieste ;*
[2]*Istituto di Chimica Farmaceutica e Tossicologica, Università di Catania, V. le A. Doria, 6, 95125 Catania ;*
[3]*Dipartimento di Scienze Farmaceutiche, Università di Ferrara, Via Scandiana, 21, 44100 Ferrara;*
[4]*Istituto di Farmacologia, Università di Bologna, Via Irnerio, 48, 40126 Bologna, Italy*

(Received February 6, 1989; accepted June 6, 1989)

Summary – A series of dimeric opioid peptides derived from μ selective compounds was synthesized to investigate whether μ and δ receptors coexist as distinct recognition sites on the same receptor complex. Some compounds were several times more potent than the corresponding monomers in the MVD (mouse vas deferens) smooth muscle preparation, which is rich in δ receptors, but yet retaining substantial activity in the GPI (guinea pig ileum). It is suggested that δ receptors might differ from μ receptors in that they possess an additional accessory recognition site, to which could bind a particular amino acid residue present in the second halves of these bivalent ligands. This residue could play the role of "address" at δ opioid receptors.

Résumé – **Les peptides opioïdes bivalents synthétisés à partir de monomères à activité μ sélective présentent une sélectivité préférentielle vis-à-vis des récepteurs δ.** *Une série de peptides opioïdes dimères dérivés de composés μ sélectifs ont été synthétisés dans le but d'évaluer si les récepteurs μ et δ coexistent comme sites distincts de reconnaissance sur le même complexe récepteur. Certains composés s'avèrent plusieurs fois plus puissants que les monomères correspondants, vis-à-vis de la préparation de muscle lisse du vas deferens de souris qui est très riche en récepteurs δ, mais conservaient une activité notable sur l'iléon de cobaye. Les récepteurs δ pourraient différer des récepteurs μ par la possession d'un site accessoire de reconnaissance, auquel pourrait se lier un reste aminoacide présent dans la seconde moitié de ces ligands bivalents. Ces restes pourraient jouer un rôle directionnel vis-à-vis des récepteurs δ.*

opioid receptors / opioid peptides / bivalent opioid ligands / guinea pig ileum / mouse vas deferens / μ-receptors / δ-receptors

Introduction

The synthesis of compounds with high selectivity for one of the three subpopulations of opioid receptors (μ, δ and κ) has represented an invaluable tool to further characterize the pharmacological activity of opiates and to better understand possible physiological roles of endogenous opioids [1, 2]. In recent years, new insights have been obtained synthesizing opioid ligands with two pharmacophores connected by a spacer chain ("bivalent ligands" [3]) to investigate possible interactions between the opioid receptor to which the relative pharmacophore preferentially binds and vicinal opioid and/or non-opioid recognition sites [4–6]. In particular, studies on hybrid bivalent ligands containing two pharmacophores preferentially binding one (morphine-like) to the μ and the other (enkephalin-like) to the δ site have further strengthened the hypothesis that μ and δ receptors may be functionally coupled [7]. However, it is still questioned if μ and δ receptors coexist as distinct recognition sites on the same receptor complex [7–10] and, although sharing a similar high affinity binding site, they differ in the accessory site ("message-address" concept [11, 12]). Thus, to better clarify the above questions, we decided to prepare bivalent peptidergic ligands from μ selective monomers and evaluate the μ/δ selectivity ratio of the corresponding dimers.

To this purpose, we selected as pharmacophores some analogues of dermorphin, a μ selective peptide [13], and synthesized a series of bivalent ligands. The activities of the compounds for opioid receptors were determined by

**Author to whom correspondence should be addressed.*
Abbreviations: **TFA:** trifluoroacetic acid; **AcOH:** acetic acid; **Boc:** tert-butyloxycarbonyl; **[Leu]Enk:** Tyr-Gly-Gly-Phe-Leu; **Phg:** phenylglycine; **HPLC:** high performance liquid chromatography; **TLC:** thin layer chromatography; other abbreviations are in accordance with recommendations of the IUPAC-IUB Joint Commission of Biochemical Nomenclature: *Biochem. J.* (1984) 219, 345.

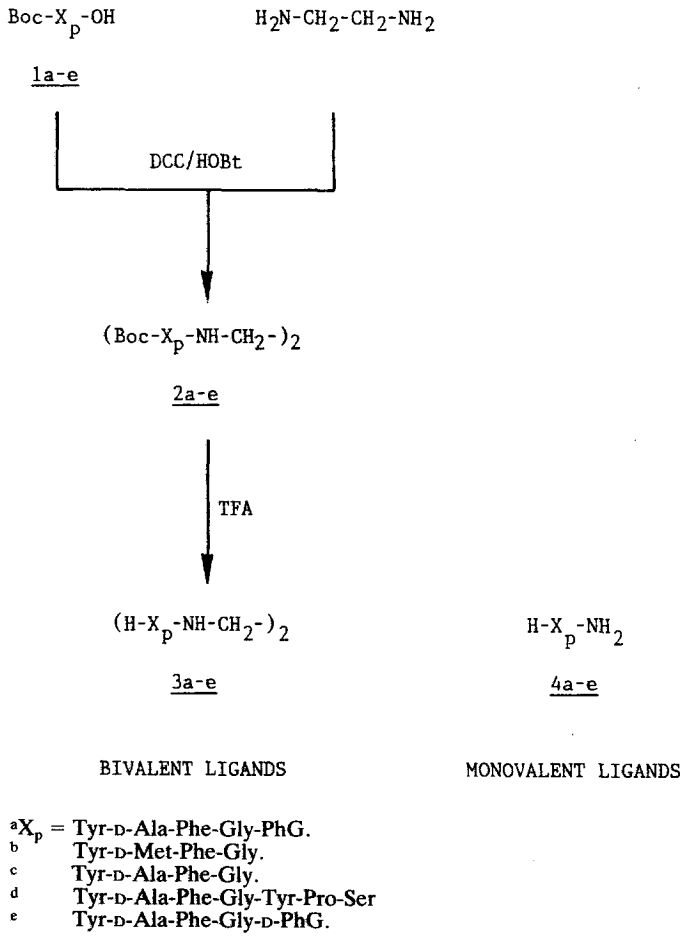
two different *in vitro* bioassays, the guinea pig ileum (GPI) [14] and the mouse vas deferens (MVD) [15].

Chemistry

A consideration in the selection of the μ peptides (Scheme 1) was the various μ/δ selectivity ratios they possess. In view of the well established fact that the connection of δ selective peptidic pharmacophores through the terminal carboxyl group conferred a greater degree of δ relative to μ receptor activity when very short spanner chains were used [16], only an ethylendiaminic spanner chain was employed in this study. Thus, in order to determine whether or not bivalency of μ selective peptides could actually afford compounds with enhanced δ opioid activity, the selectivity ratios of the resulting ligands, **3a–e,** were compared with the corresponding monovalent opioids **4a–e** [17–23].

All the bivalent ligands, **3a–e,** were synthesized using a standard peptide coupling procedure (Scheme 1), employing dicyclohexylcarbodiimide (DCC) and 1-hydroxybenzotriazole (HOBt) to form intermediate active esters of the boc-protected starting peptides **1a–e,** which upon reaction with one equivalent of 1,2-ethylendiamine afforded the corresponding bivalent ligands **2a–e.** Dichloromethane was employed satisfactorily as the reaction medium. The crude products obtained after the solvent was evaporated *in vacuo* were washed successively with 4% sodium bicarbonate, 10% citric acid and water. Deprotection was then afforded by treating compounds **2a–e** with 1.5 eq of TFA at 0°C for 30 min. Final purification was accomplished by partition chromatography on Sephadex G-25 or when necessary by HPLC. The homogeneity of intermediate peptides and target compounds was checked by TLC, HPLC and elemental analyses. All the derivatives **3a–e** were proven to be dimers on the basis of the molecular weights detected by FAB mass spectrometry (Tables I and II).

Scheme 1. Synthesis of bivalent ligands and structures of the corresponding monovalent analogues [18–22].



[a] X_p = Tyr-D-Ala-Phe-Gly-PhG.
[b] Tyr-D-Met-Phe-Gly.
[c] Tyr-D-Ala-Phe-Gly.
[d] Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser
[e] Tyr-D-Ala-Phe-Gly-D-PhG.

Table I. Physical properties of protected bivalent ligands **2a–e.**

Compound No.	mp (°C)	TLC R_f	Yield (%)	Formula	Anal.
2a	206	0.78[a]	46	$C_{74}H_{90}N_{12}O_{16}$	C, H, N
2b	210	0.75[b]	70	$C_{62}H_{84}N_{10}O_{14}S_2$	C, H, N, S
2c[c]	215	0.70[b]	58	$C_{64}H_{92}N_{10}O_{14}$	C, H, N
2d[c]	195	0.11[b] 0.71[a]	15	$C_{100}H_{134}N_{16}O_{24}$	C, H,N
2e	217	0.75[b]	65	$C_{74}H_{90}N_{12}O_{16}$	C, H, N

[a] $MeOH/CHCl_3/AcOH$ (1:4:1).
[b] $CHCl_3/MeOH$ (4:1).
[c] The phenolic OH on the Tyr[1] residue is *t*-butylated.

Table II. Physical properties of bivalent ligands **3a–e.**

Compound No.	mp (°C)	TLC R_f[a]	Yield (%)	Formula	Anal.	Fab-ms MH^+
3a	147	0.80	78	$C_{64}H_{74}N_{12}O_{12} \cdot 2C_2HO_2F_3$	C, H, N	1203
3b	190	0.72	72	$C_{52}H_{68}N_{10}O_{10}S_2 \cdot 2C_2HO_2F_3$	C, H, N, S	1057
3c	198	0.68	75	$C_{48}H_{60}N_{10}O_{10} \cdot 2C_2HO_2F_3$	C, H, N	937
3d	145	0.73	62	$C_{82}H_{102}N_{16}O_{20} \cdot 2C_2HO_2F_3$	C, H, N	1631
3e	212	0.35	68	$C_{64}H_{74}N_{12}O_{12} \cdot 2C_2HO_2F_3$	C, H, N	1203

[a] *n*-BuOH / AcOH / H_2O (4:1:2).

Pharmacological results

The bivalent ligands **3a–e** and the corresponding monovalent ligands **4a–e** were tested as agonists on the guinea pig ileal longitudinal muscle (GPI) and mouse vas deferens (MVD) preparations. Results for the monovalent ligands were in agreement with those reported previously [17–23]. All the data are reported in Table III. In Table IV

Table III. Opioid agonist potencies of bivalent ligands, **3a–e**, and monovalent ligands, **4a–e**, on the guinea pig ileum (GPI) and mouse vas deferens (MVD) preparations[a].

Compound No.	Dimer			Compound No.	Monomer[b]	
	GPI IC_{50} (nM)	K_d	MVD IC_{50} (nM)	K_d		GPI IC_{50} (nM)	MVD IC_{50} (nM)
3a	21.0 ± 1.42	5.0	2.42 ± 0.62	16.4	**4a**	1.02 ± 0.1	16.0 ± 0.5
3b	91.2 ± 2.24	3.8	11.4 ± 0.92	14.2	**4b**	97.6 ± 7.1	501 ± 53
3c	92.0 ± 8.70	ND[c]	190 ± 10.5	ND[c]	**4c**	45.2 ± 3.71	510 ± 49
3d	331 ± 17.1	5.15	58.9 ± 6.30	13.4	**4d**	3.30 ± 0.22	29.0 ± 3.35
3e	19.9 ± 1.90	6.1	9.5 ± 0.74	16.9	**4e**	3.77 ± 0.3	1.36 ± 0.1
Morphine	78 ± 3.28	1.4	460 ± 66.6	2.6			
[Leu]Enk	280 ± 12.72	3.5	5.0 ± 0.32	12.5			

[a]Value represent the mean ±SEM of 3 determinations.
[b]See references [18–22].
[c]Not detected.

are reported the relative potencies of compounds **3a–e**, in comparison with those of the corresponding monovalent ligands set equal to unity, together with the selectivity ratios (μ / δ) calculated as IC_{50} in the MVD divided by IC_{50} in the GPI.

All bivalent ligands were more potent than the corresponding monovalent ligands in the MVD, except **3d** and **3e**. The greatest potency in this assay was associated with bivalent ligands **3a** and **3b**, which were 6.61- and 44-fold more potent than their monovalent analogues. Compound **3a** was twice as potent as [Leu]Enk.

In the GPI preparation, all the bivalent ligands were less potent than the corresponding monomers, except **3b**, which showed a comparable activity. Compounds **3a** and **3e** were approximately 4 times more potent than morphine and compounds **3b** and **3c** displayed comparable activity.

Contrary to the other examined compounds, bivalency provoked a relevant loss of potency for the ligand derived from dermorphin, **3d**. In fact, it displayed only one-hundredth and one half of the potency of the monomer in the GPI (IC_{50} = 331 ± 17.1 nM *vs* 3.30 ± 0.22 nM) and in the MVD (IC_{50} = 58.9 ± 6.30 nM *vs* 29.0 ± 3.35 nM), respectively.

Estimates of the apparent dissociation constant for naloxone in antagonizing the effects elicited by bivalent ligands in the GPI and MVD are also given in Table III. While naloxone was effective in antagonizing morphine in the GPI (K_d = 1.4) and the MVD (K_d = 2.6), it was less potent as an antagonist of bivalent compounds effects in the GPI (K_d in the range 3.8–6.1) and in the MVD (K_d in the range 13.4–16.9).

Discussion

In this study, we have employed the GPI and MVD assays to evaluate the potency and receptor selectivity of this series of bivalent opioid peptides derived from dermorphin analogues. Contrary to what was observed in previous studies [17–23] on the monomers, **4a–e**, which were extremely potent in the GPI, a tissue where μ receptors are predominantly present [24], the corresponding dimers, **3a–e**, displayed a relative higher opioid agonist potency in the MVD, a preparation which is preferentially sensitive to δ agonists [25]. Moreover, the highest apparent equilibrium constant for naloxone (as a preferential antagonist for the μ receptor [26]) showed by the bivalent ligands is comparable to that of [Leu]Enk and notably higher than that of morphine and further supports the hypothesis that these peptides bind preferentially to δ sites.

In comparing the selectivity ratios (μ / δ) of the bivalent opioid peptides (Tables IV), it can be noted that they are all comparable, ranging between 0.11–0.48, with exception of **3c**, which displays a slight preference for μ receptors. This suggests that bivalency increases significantly increases δ selectivity. Moreover, the data reveal that the most significant increases of δ selectivity are observed as

Table IV. Selectivity and potency ratios of bivalent ligands at μ and δ receptors on the GPI and MVD preparation.

Compound No.	Selectivity ratio (μ / δ)[a]		Potency ratio[b]	
	Dimer	Monomer	GPI	MVD
3a	0.11	15.7	0.05	6.61
3b	0.12	5.1	1.07	44
3c	2.06	11.3	0.49	2.68
3d	0.18	8.8	0.01	0.49
3e	0.48	0.36	0.19	0.14
Morphine		5.9		
[Leu]Enk		0.02		

[a]IC_{50} in the MVD divided by IC_{50} in the GPI.
[b]IC_{50} of the dimer divided by IC_{50} of the correspondent monomer in the same tissue preparation.

a consequence of dimerization of the most μ selective peptides. In particular, compound **3b** was 44 times more potent in the MVD than the corresponding monomer.

It is also interesting that the bivalent ligand **3e,** which differs from **3a** only in the chirality of the PhG residue, did not exhibit increase in δ selectivity. On the contrary, it displayed a moderate loss of potency in both the GPI and the MVD in comparison with the corresponding monovalent ligand **4e.**

It is noteworthy that the increase of δ selectivity is also associated with a significant retention of μ activity in the GPI, at least for compounds **3a** and **3b,** thus supporting the possibility that such bivalent ligands still bind to the μ receptor subpopulation.

These results seems to support the hypothesis that δ receptors might differ from μ receptors in that they possess an additional accessory recognition site (II_δ) in the same receptor complex, but, perhaps on a different receptor protein (G'), which could be present only in certain tissue preparations (Fig. 1). This site could bind a particular amino acid residue, which is present in the second halves of these bivalent ligands, and which plays the role of "address" for δ receptors, in accordance with the "message–address" concept proposed by Schwyzer [11]. The "message" segment, which confers μ activity, is to be found in the first segment corresponding to the first pharmacophore [27].

Of course, conformational restrictions of the "message" segment could also play an important role in the "inversion" of selectivity showed by these molecules.

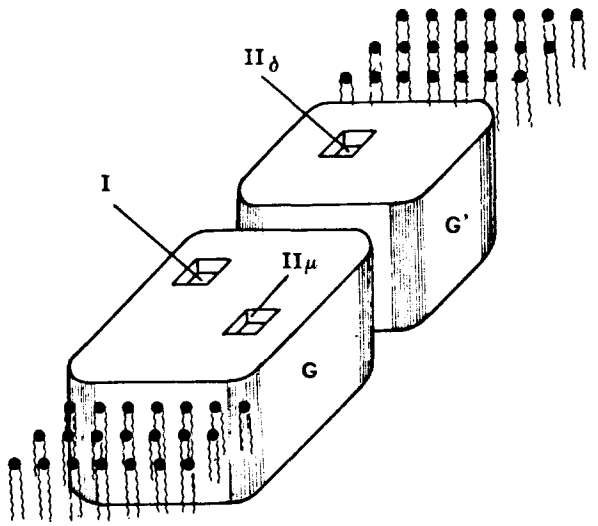


Fig. 1. A schematic illustration of the opioid receptor complex. A δ selective ligand could be capable of binding to both the accessory recognition sites II_μ and II_δ, while a μ ligand could interact only with the II_μ site. The primary recognition site (I) could be the same for both μ and δ ligands. G and G· indicate different receptor protein subunits.

The possibility of contemporary bridging of two distinct receptor sites by this series of bivalent ligands seems to be ruled out by the results of the present study, which indicate that all the tested compounds, although strictly structurally correlated, possess a similar profile of activity, but differ significantly in potency.

Experimental protocols

Chemistry

Melting points were determined in open capillary tubes with Büchi melting point apparatus and are uncorrected. Reversed phase HPLC was performed on a Varian 5020 liquid chromatograph equipped with a Varian UV-100 variable wavelength detector utilizing a LiChrosorb RP C-18 column (250 × 4.5 mm, 10-μm particle size). TLC was performed on precoated plates of silica gel F254 (from Merck) with the use of the following solvent systems: (i) *n*-BuOH / AcOH / H_2O (4:1:2); (ii) MeOH / $CHCl_3$ / AcOH (1:4:1); (iii) $CHCl_3$ / MeOH (4:1). Ninhydrin (1%) (Merck) and/or chlorine reagent were used as spray reagents. Samples were considered pure when they showed a single spot with more than one solvent system. Elemental analyses indicated by the symbols of the elements refer to data within ± 0.40% of the theoretical values. Analyses were carried out after the products were dried for 24 h at 50°C (0.2 Torr). Molecular weights of the dimeric compounds were determined by FAB-MS on a Kratos MS-50 mass spectrometer using a DS55 computer system.

Preparation of (Boc-X_p-NH-CH_2-)$_2$ **2a–e**
To a solution of Boc-protected peptides **1a–e** [18–22] (0.25 mmol) and 1,2-ethylendiamine (0.14 mmol) in CH_2Cl_2 (3 ml) were added HOBt (0.27 mmol) and DCC (0.27 mmol) at −10°C. The reaction mixture was stirred for 24–48 h at 0°C. The solvent was evaporated *in vacuo* and the residue which was collected washed 3 times with a 4% solution of $NaHCO_3$ and successively with a 10% solution of citric acid and water. Analytical data are reported in Table I.

Preparation of free bivalent ligands **3a–e**
The Boc-protected dimers **2a–e** were dissolved in 1.5 eq of TFA at −10°C. After 30 min a 0°C, the solution was evaporated *in vacuo* to leave an oil, which was solidified by the addition of anhydrous ethyl ether, yielding the TFA salts. Analytical data are reported in Table II.

Pharmacology

Myenteric-plexus preparations of guinea pig ileum were set up as described by Cox and Padhya [14] in a 5-ml bath. Tissues were stimulated electrically with 80 V, 0.25 ms shocks delivered at 0.1 Hz. Responses were recorded isometrically. Mouse vas deferens preparations were performed according to Hugues *et al.* [15]. Tissues were stimulated with 80 V, 1 ms shocks delivered at 0.1 Hz and the responses were registered isometrically. After a 1-h period of equilibration, a dose –response curve covering the range 15–75% of maximal effect was constructed and the relative IC_{50} was evaluated [14]. Each drug was left in contact with the tissue for 3 min before the solution of the bath was changed. The next dose was applied to the tissue 15 min after the second of two further washes given at 10-min intervals.

Specific effects of opioids were defined by the ability of naloxone to antagonize the opioid-effected inhibition of twitch tension. The apparent naloxone equilibrium constant (K_d) was calculated from the Schild equation, essentially as described by Kosterlitz and Watt [28], $K_d = a/(DR-1)$, where a = molar conc of naloxone hydrochloride (10 nM) and DR = the ratio of the IC_{50} for each compound evaluated in presence or in absence of the antagonist.

Acknowledgments

This research has been supported by the Italian CNR and in part by the Ministero della Pubblica Istruzione (Rome).

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