Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 1847–1850
Bioorganic & Medicinal Chemistry Letters

Structure–activity relationship of the novel bivalent and C-terminal modified analogues of endomorphin-2

Yanfeng Gao,[a] Xin Liu,[a] Jie Wei,[a] Beibei Zhu,[a] Qiang Chen[a] and Rui Wang[a,b,*]

[a]*Department of Biochemistry and Molecular Biology, School of Life Sciences, Lanzhou University, Lanzhou 730000, China*
[b]*State Key Laboratory for Oxo Synthesis and Selective Oxidation, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou 730000, China*

Received 16 January 2005; revised 5 February 2005; accepted 7 February 2005

Abstract—Endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2) is a putative endogenous μ-opioid receptor ligand. To develop potent analgesics with less side effects related to it, we used the methods of dimerization and C-terminal modification. Through dimerization we got the 'balanced agonists' with potent analgesic activity and we have developed the structure–activity relationship between the selectivity and the distance of the two tyrosine pharmacophores. Modification at the C-terminal increased the selectivity of endomorphin-2 to μ-opioid receptor with binding affinity conserved.

It is widely accepted that there are at least three opioid receptor types, μ, δ, and κ. These receptors and their endogenous ligands, the enkephalins (δ), dynorphins (κ) and endomorphins (μ) appear to be involved in the modulation and perception of pain.[1,2] To study the structure–activity relationships against their receptors and develop potent analgesics without the well-known side effects, such as tolerance, physical dependence, respiratory depression, and adverse gastrointestinal effects, hundreds of analogues of opioid peptides were synthesized and evaluated.[3]

To develop opioid analgesics without side effects, the most general approach is to develop compounds with high selectivity for a particular type of the opioid receptors. The other, less explored approach, is to develop 'balanced agonists' (which will interact with a broad spectrum of the opioid receptors).[4–6] In the second approach, developing bivalent ligands is a useful tool. Compounds that contain two pharmacophores linked by a connecting spacer have been termed 'bivalent ligands'. The most successful example of bivalent opioid ligands is biphalin, it is a dimeric opioid peptide composed of two tetrapeptides connected 'tail to tail' by a hydrazide bridge. It has a moderate affinity for both μ- and δ-opioid receptors. When administered intracerebroventricularly, it has been shown to be more potent than morphine and etorphine at eliciting antinociception. Biphalin has also been shown to cross both the blood–brain and blood–cerebrospinal barriers, and is currently undergoing intensive preclinical studies.[7] In this paper, we adopted the methods of dimerization and C-terminal modification to modulate the selectivity of endomorphin-2 and to study the structure–activity relationship.

In 1997, Zadina and his colleagues isolated Endomorphin-1 (EM-1: Tyr-Pro-Trp-Phe-NH_2) and Endomorphin-2 (EM-2: Tyr-Pro-Phe-Phe-NH_2) from bovine brain and human brain. EM-1 and EM-2 have shown high selectivity towards μ-opioid receptor with potent analgesic activity,[8–10] EM-2 is more selective for μ-opioid receptor than EM-1. Unfortunately, the exogenous application of these opioid peptides generally met with failure, owing to their biological instability and inability to be transmitted through the blood–brain barrier (BBB).[3] In order to avoid these disadvantages and increase their affinity and selectivity, a number of their analogues were synthesized and evaluated. They were mainly focused on modification at the C-terminal or N-terminal and using conformational restrained or flexible unnatural amino acids to substitute or scan the four residues,[11–20] but no one studied the bivalent analogues of endomorphins. And there was less knowledge for us

Keywords: Endomorphin-2; Dimerization; Modification; Structure–activity relationship; Opioid peptide.
* Corresponding author. Tel.: +86 931 8912567; fax: +86 931 8912561; e-mail: wangrui@lzu.edu.cn

0960-894X/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2005.02.021

to modulate the selectivity of endomorphin-2. One purpose of this paper was to study the structure–activity relationship of the bivalent and C-terminal modified analogues of endomorphin-2.

Changing the amide ($CONH_2$) group at the C-terminal of EM-2 into carboxylic acid (COOH) made more than 500-folds decrease in the binding affinity of EM-2 towards μ-opioid receptor, but alcoholic terminal (CH_2OH) increased the binding affinity. The results indicated that a neutral but not acidic group is needed at the C-terminal of EM-2 for its binding to μ-opioid receptor.[11] Changing the carboxylic acid group at the C-terminal of deltorphin A (1–4)-OH into $CONH_2$, COOMe and $CONHNH_2$ increased its affinity towards μ-opioid receptor for 6.6-, 67- and 149-folds, respectively.[21] Therefore, we changed the C-terminal amide group of EM-2 into COOMe and $CONHNH_2$ to see if they could increase the selectivity of it.

Many studies have shown that N-terminal sequence of the opioid peptides especially the Tyr^1 residue is critical for their binding to the receptors,[22] so we designed the bivalent ligands 'tail to tail' and linked the two pharmacophores of endomorphin-2 by various lengths of diamines as the spacers. In order to study the role of Phe^3 ($Phe^{3'}$) and Phe^4 ($Phe^{4'}$) in their binding activities, we synthesized bivalent ligands with deletion of Phe^4 ($Phe^{4'}$) or both of Phe^3 ($Phe^{3'}$) and Phe^4 ($Phe^{4'}$) (Fig. 1).

All the compounds in this study were synthesized by solution methodology for peptide synthesis using Boc-amino protection groups and using *N*-methyl morpholine (NMM) and isobutyl chloroformate (IBCF) as coupling reagents. TFA and anisole (v/v = 9:1) were used as deprotection reagents. All the analogues were synthesized by the modified methods reported previously.[23,24] After deprotection, the compounds were precipitated with ether, filtered, the crude peptides were obtained as TFA salts and then purified using RP-HPLC, purity of all peptides was greater than 95%. The analytical data were listed in Table 1.

Tyr^1-Pro^2-Phe^3-Phe^4-NH-CH_2-CH_2-NH<-$Phe^{4'}$<-$Phe^{3'}$<-$Pro^{2'}$<-$Tyr^{1'}$

Figure 1. Amino acid sequence of bivalent ligand **8**.

The opioid receptor binding assays were performed in 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 0.5 mL containing 250–400 μg of protein (Synaptosomal brain membrane P2 was prepared from Wistar rats). In competition experiments, the following conditions were used for incubations: [^{3}H]DAMGO (0.5 nM, 25 °C, 1 h), [^{3}H]DPDPE (1 nM, 25 °C, 3 h). Nonspecific binding was determined in the presence of 10 μM naxolone. K_d values of [^{3}H]DAMGO and [^{3}H]DPDPE were 0.533 and 2.75 nM, respectively. K_i values were calculated according to the equation of Cheng and Prusoff.[25] The K_i values of EM-2 in [^{3}H]DAMGO and [^{3}H]DPDPE assays agreed with Toth and co-workers[16] and Okada et al.[14], respectively. The data were listed in Table 2. Biological activities of some analogues were also evaluated in guinea pig ileum (GPI) and mouse vas deferens (MVD) assays. Stand compounds (endomorphin-2 for the GPI and deltorphin I for the MVD) were assayed in each preparation to permit estimation of relative potencies. The data were listed in Table 3. The data shown in Tables 2 and 3 were the means of at least three experiments.

From the opioid receptor binding data shown in Table 2, we can see that modified the C-terminal amide group of endomorphin-2 into COOMe (**1**) and $CONHNH_2$ (**2**) both increased the selectivity of endomorphin-2, and conserved its binding affinity to μ-opioid receptor. It was indicated that neutral or weak basic group at the C-terminal of endomorphin-2 might increase its binding selectivity.

The dimeric analogues **4** and **5** increased the binding affinity of its monomer **3** for 4.6- and 5.4-folds to μ-opioid receptor, respectively, to δ-opioid receptor, increased more than 5.3- and 10.8-folds, respectively. Deletion of Phe^3 ($Phe^{3'}$) of **5** produced compound **6**, it decreased the affinity of **5** both to μ- and δ-opioid receptors for 113- and more than 108-folds, respectively. But **6** increased the μ affinity of its monomer, Tyr-Pro-NH_2[14]

Table 1. Analytical data of EM-2 analogues

Compound		MS [M + H]$^+$		TLC[c] (R_f)	$[\alpha]_D$[d] (°)
		Calculated	Found		
EM-2	Tyr-Pro-Phe-Phe-NH_2	572.3	572.3[a]		
1	Tyr-Pro-Phe-Phe-OMe	587.3	587.5[a]	0.78	
2	Tyr-Pro-Phe-Phe-$NHNH_2$	587.3	587.2[a]	0.39	−19
3	Tyr-Pro-Phe-NH_2	425.2	425.4[a]	0.65	
4	[Tyr-Pro-Phe-NH-]$_2$	847.4	847.5[b]	0.55	−25
5	[Tyr-Pro-Phe-NH-CH_2-]$_2$	875.5	875.8[b]	0.52	−29
6	[Tyr-Pro-NH-CH_2-]$_2$	581.3	581.5[b]	0.32	
7	[Tyr-Pro-Phe-Phe-NH-]$_2$	1141.6	1141.4[b]	0.48	−30
8	[Tyr-Pro-Phe-Phe-NH-CH_2-]$_2$	1169.6	1169.6[b]	0.54	−56
9	[Tyr-Pro-Phe-Phe-NH-CH_2-CH_2-]$_2$	1197.6	1197.6[b]	0.55	−57

[a] FAB-MS.
[b] ESI-MS.
[c] Solvent system: ethyl acetate/MeOH/ammonia (8:1:1).
[d] MeOH (20 °C), c = 0.3.

Table 2. Opioid receptors binding assay of EM-2 analogues

Compound	[^{3}H]DAMGO (μ)		[^{3}H]DPDPE (δ)		Selectivity
	K_i ± SE (nM)	Relative potency	K_i ± SE (nM)	Relative potency	(δ/μ)
EM-2	8.23 ± 0.48	1	8360 ± 1314	1	1016
1	9.07 ± 0.77	0.91	>10,000		>1109
2	9.35 ± 2.43	0.88	>10,000		>1070
3	140 ± 24.0	0.059	>10,000		>71.4
4	30.7 ± 7.07	0.27	1896 ± 325	4.41	61.8
5	26.1 ± 8.63	0.32	923 ± 95.5	9.06	35.4
6	2956 ± 467	0.0028	>10,000		>3.38
7	96.4 ± 8.77	0.085	539 ± 192	15.5	5.59
8	86.3 ± 10.6	0.095	613 ± 50.8	13.6	7.10
9	168 ± 43.3	0.049	7025 ± 1693	1.19	41.8

Table 3. In vitro biological assay of EM-2 analogues

Compound	GPI (μ) IC_{50} ± SE (nM)		MVD (δ) IC_{50} ± SE (nM)		Ratio
	IC_{50}	Relative potency	IC_{50}	Relative potency	(MVD/GPI)
EM-2	14.7 ± 0.72	1	48.2 ± 24.4	1	3.28
1	42.1 ± 16.1	0.35	37.2 ± 15.0	1.30	0.88
2	132 ± 42.4	0.11	185 ± 79.7	0.26	1.40
4	1135 ± 41.1	0.013	3203 ± 347	0.015	2.82
5	218 ± 62.0	0.067	1012 ± 160	0.048	4.64
7	518 ± 232	0.028	721 ± 184	0.067	1.39
8	3544 ± 640	0.0041	29.0 ± 14.6	1.66	0.0082
9	Not determined		>10,000		

(K_i = 26,107 ± 1339 nM). Dimeric compounds **7**, **8**, and **9** all decreased the μ affinity of their monomer EM-2 for about 10–20-folds, but they all increased the δ affinity of EM-2. So we got the desired 'balanced agonists'. The relationship between the selectivity and the distance of the two Tyr pharmacophores are shown in Figure 2. The results demonstrated that we can use dimerization approach to increase the binding affinity of the fragments of endomorphin-2 and modulate the selectivity of the bivalent analogues by controlling the distance between the two Tyr pharmacophores. Phe^4 and Phe$^{4'}$ are important for us to get the 'balanced agonists'. This was backed by the bioassay data shown in Table 3.

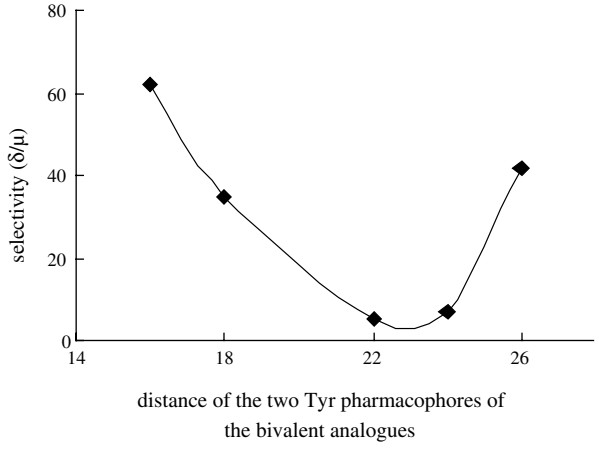

Figure 2. The relationship between the selectivity and the distance of the two Tyr pharmacophores of the bivalent analogues (The distance was shown by the number of the atoms in the peptide backbone between the Cα of the two Tyr. Compounds **4**, **5**, **7**, **8**, and **9** were chosen to be studied).

From the data shown in Table 3, it is interesting to note that compound **8** showed very low MVD/GPI selective ratio. Its selective ratio (0.0082) is lower than that of the classical δ selective compounds [D-Ala^2, D-Leu^5] enkephalin (DADLE)[26] and about 0.25-folds as that of DPE_2[27] (the MVD/GPI selective ratio are 0.011 and 0.033, respectively), which suggested its specific pharmacological properties. So we evaluated its analgesic activity administered intracerebroventricularly in mice by hot-plate test (data are not shown here) in two doses (20 and 41.6 nmol/kg), it was more potent than that of morphine.

As a conclusion, we have synthesized novel bivalent and C-terminal modified analogues of endomorphin-2. Dimerization increased the affinity of N-terminal fragments of endomorphin-2 both to μ- and δ-opioid receptors. Through this method, we got the 'balanced agonists' with moderate affinity both to μ- and δ-opioid receptors and with potent analgesic activity. And we have developed the structure–activity relationship between the selectivity and the distance of the two tyrosine pharmacophores. Modifying the classical C-terminal amide group into COOMe and $CONHNH_2$ increased the selectivity and conserved the affinity of endomorphin-2 towards μ-opioid receptor. Our results suggest that we can use these two methods to develop more potent analgesics with less side effects related to endomorphin-2. Further studies about these novel ligands will be reported subsequently by our laboratory.

Acknowledgements

We thank the National Natural Science Foundation of China (Nos. 20372028, 20472026) and the Ministry of Education of China for financial support. We are grateful to Dr. Dingjian Yang and Dr. Yuanming Qi for technical assistance.

References and notes

1. Przewlocki, R.; Przewlocka, B. *Eur. J. Pharmacol.* **2001**, *429*, 79–91.
2. Narita, M.; Funada, M.; Suzuki, T. *Pharmacol. Ther.* **2001**, *89*, 1–15.
3. Fichna, J.; Jean-Claude do-Rego; Costentin, J.; Chung, N. N.; Schiller, P. W.; Kosson, P.; Janecka, A. *Biochem. Biophys. Res. Commun.* **2004**, *320*, 531–536.
4. Lipkowski, A. W.; Misicka, A.; Davis, P.; Stropova, D.; Janders, J.; Lachwa, M.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2763–2766.
5. Lipkowski, A. W.; Konecka, A. M.; Sroczynska, I. *Peptides* **1982**, *3*, 697–700.
6. Yamazaki, M.; Suzuki, T.; Narita, M.; Lipkowski, A. W. *Life Sci.* **2001**, *69*, 1023–1028.
7. Egleton, R. D.; Abbruscato, T. J.; Thomas, S. A.; Davis, T. P. *J. Pharm. Sci.* **1998**, *87*, 1433–1439.
8. Zadina, J. E.; Hackler, L.; Ge, L. J.; Kastin, A. J. *Nature* **1997**, *386*, 499–502.
9. Hackler, L.; Zadina, J. E.; Ge, L. J.; Kastin, A. J. *Peptides* **1997**, *18*, 1635–1639.
10. Sakurada, S.; Zadina, J. E.; Kastin, A. J.; Katsuyama, S.; Fujimura, T.; Murayama, K.; Yuki, M.; Ueda, H.; Sakurada, T. *Eur. J. Pharmacol.* **1999**, *372*, 25–30.
11. Lengyel, I.; Orosz, G.; Biyashev, D.; Kocsis, L.; Al-Khrasani, M.; Ronai, A. Z.; Tomboly, C.; Furst, Z.; Toth, G.; Borsodi, A. *Biochem. Biophys. Res. Commun.* **2002**, *290*, 153–161.
12. Fujita, Y.; Tsuda, Y.; Li, T. Y.; Motoyama, T.; Takahashi, M.; Shimizu, Y.; Yokoi, T.; Sasaki, Y.; Ambo, A.; Kita, A.; Jinsmaa, Y.; Bryant, S. D.; Lazarus, L. H.; Okada, Y. *J. Med. Chem.* **2004**, *47*, 3591–3599.
13. Sasaki, Y.; Sasaki, A.; Niizuma, H.; Goto, H.; Ambo, A. *Bioorg. Med. Chem.* **2003**, *11*, 675–678.
14. Okada, Y.; Fujita, Y.; Motoyama, T.; Tsuda, Y.; Yokoi, T.; Li, T. Y.; Sasaki, Y.; Ambo, A.; Jinsmaa, Y.; Bryant, S. D.; Lazarus, L. H. *Bioorg. Med. Chem.* **2003**, *11*, 1983–1994.
15. Cardillo, G.; Gentilucci, L.; Tolomelli, A.; Spinosa, R.; Calienni, M.; Qasem, A. R.; Spampinato, S. *J. Med. Chem.* **2004**, *47*, 5198–6203.
16. Tomboly, C.; Kover, K. E.; Peter, A.; Tourwe, D.; Biyashev, D.; Benyhe, S.; Borsodi, A.; Al-Khrasani, M.; Ronai, A. Z.; Toth, G. *J. Med. Chem.* **2004**, *47*, 735–743.
17. Keller, M.; Boissard, C.; Patiny, L.; Chung, N. N.; Lemieux, C.; Mutter, M.; Schiller, P. W. *J. Med. Chem.* **2001**, *44*, 3896–3903.
18. Harrison, B. A.; Pasternak, G. W.; Verdine, G. L. *J. Med. Chem.* **2003**, *46*, 677–680.
19. Shi, Z. J.; Harrison, B. A.; Verdine, G. L. *Org. Lett.* **2003**, *5*, 633–636.
20. Harrison, B. A.; Gierasch, T. M.; Neilan, C.; Pasternak, G. W.; Verdine, G. L. *J. Am. Chem. Soc.* **2002**, *124*, 13352–13353.
21. Lazarus, L. H.; Salvadori, S.; Tomatis, R.; Wilson, W. E. *Biochem. Biophys. Res. Commun.* **1991**, *178*, 110–115.
22. Flippen-Anderson, J. L.; Deschamps, J. R.; George, C.; Hruby, V. J.; Misicka, A.; Lipkowski, A. W. *J. Pept. Res.* **2002**, *59*, 123–133.
23. Li, G. G.; Haq, W.; Xiang, L.; Lou, Bih-Show.; Hughes, R.; De Leon, I. A.; Davis, P.; Gillespie, T. J.; Romanowski, M.; Zhu, X. Y.; Misicka, A.; Lipkowski, A. W.; Porreca, F.; Davis, T. P.; Yamamura, H. I.; O'Brien, D. F.; Hruby, V. J. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 555–560.
24. Okada, Y.; Tsuda, Y.; Fujita, Y.; Yokoi, T.; Sasaki, Y.; Ambo, A.; Konishi, R.; Nagata, M.; Salvadori, S.; Jinsmaa, Y.; Bryant, S. D.; Lazarus, L. H. *J. Med. Chem.* **2003**, *46*, 3201–3209.
25. Cheng, Y. C.; Prusoff, W. H. *Biochem. Pharmacol.* **1973**, *22*, 3099–3108.
26. Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. *Proc. Natl. Acad. Sci. USA* **1983**, *80*, 5871–5874.
27. Costa, T.; Wuster, M.; Herz, A.; Shimohigashi, Y.; Chen, H. C.; Rodbard, D. *Biochem. Pharmacol.* **1985**, *34*, 25–30.