Pergamon

Bioorganic & Medicinal Chemistry Letters 9 (1999) 2763–2766

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

BIOLOGICAL ACTIVITY OF FRAGMENTS AND ANALOGUES OF THE POTENT DIMERIC OPIOID PEPTIDE, BIPHALIN

Andrzej W. Lipkowski,[a,c] Aleksandra Misicka,[a,d] Peg Davis,[b] Dagmar Stropova,[b] Jacqueline Janders,[b] Magdalena Lachwa,[c] Frank Porreca,[b] Henry I. Yamamura,[b] and Victor J. Hruby[a*]

Departments of [a]Chemistry and [b]Pharmacology, University of Arizona, Tucson, AZ 85721, U.S.A., [c]Medical Research Centre, Polish Academy of Sciences, Warsaw, PL02106, and [d]Department of Chemistry, Warsaw University, Warsaw, PL02093

Received 14 June 1999; accepted 18 August 1999

Abstract: The synthesis and biological activity of two fragments of the very potent opioid peptide biphalin, showed that Tyr-D-Ala-Gly-Phe-NH-NH<-Phe 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 analogues.

One of the major goals of modern medicinal chemistry of peptides is to develop analogues with high potency, stability, and selectivity for a particular biological function. The most general approach is to develop compounds with high selectivity for a particular type of biological receptor. The other, less explored approach, is to develop compounds that will interact with a broad spectrum of the receptors which are engaged in a particular biological effect[1] (balanced agonists). In the second approach, the final biological effect will be not only a sum of the separate interactions with particular receptors, but also effects of synergistic cross interactions between them. Searching for new opioid analgesics is a good illustration of both approaches. It was postulated over twenty years ago that endogenous opioids and opiate drugs block pain signals through interactions with specific multiple receptors located in neuronal cell membranes, of which the μ, δ, and κ receptors are the most commonly accepted. The endogenous enkephalin opioid peptides, which are natural ligands to these receptors, are not very selective for any of the opioid receptors.[2] The very low half-life of endogenous peptides in biological systems disqualifies them as potential drugs. The synthetic opioid peptide biphalin[3] is a highly potent enkephalin analogue with a biological profile similar to endogenous enkephalins, but with a 1000-fold plus increased potency and a pharmacological profile that makes it a good candidate for further development as an analgesic drug.[1,4-6]

Biphalin is a dimeric enkephalin analogue in which the C-terminal amino acid is replaced by a second tetrapeptide active fragment of the enkephalin analogue, and the two fragments are connected "tail-to-tail" by a hydrazide bridge (Figure 1).

0960-894X/99/$ - see front matter
PII: S0960-894X(99)00464-3

Tyr^1-D-Ala^2-Gly^3-Phe^4-NH-NH<-**Phe$^{4'}$<-Gly$^{3'}$<-D-Ala$^{2'}$<-Tyr$^{1'}$**

Figure 1. Amino acid sequence of biphalin

A study of the metabolism of biphalin indicates that des (Tyr-D-Ala-Gly)biphalin, **3**, may be one of the major metabolites of biphalin. Therefore, we have synthesized *de novo* this peptide and its analogues for evaluation of their biological activities and for structural studies.

Materials and Methods

The syntheses were performed in solution with the "Boc-strategy" by coupling of Boc-Tyr-D-Ala-Gly [3] to Phe-OMe. Then, the Boc-tetrapeptide methyl ester was dissolved in *iso*-propanol and a 4 molar excess of $H_2NNH_2.H_2O$ was added. In two days Boc-Tyr-D-Ala-Gly-PheNH-NH_2 precipitated from the reaction mixture in 98% yield. The crude hydrazide was filtered off and washed with citric acid (2 times) and water. After drying over P_2O_5 the compound was used for further reactions. In the case of peptides **3 – 8** (Table 1), the respective Boc-amino acids was coupled by the method previously described, using HBTU.[7] The Boc-protection was removed from the respective peptide using 2 N HCl in acetic acid. The crude products were purified by RP-HPLC and characterized by FABMS and amino acid analysis. The purity of the products was assessed by HPLC (one single peak, UV detection at 225, 254, and 280 nm, using two different linear gradients).

Receptor binding and biological *in vitro* activities were performed by methods previously described.[8] Opioid receptor binding affinities were determined using [^{3}H] DAMGO or [^{3}H] CTOP, and [^{3}H][*p*-ClPhe^4] DPDPE or [^{3}H] Deltorphin II for μ and δ receptors, respectively. The receptor binding and *in vitro* biological values (Table 1) presented are the mean of triplicate experiments; the standard errors were less than 20% of the presented values.

Results and Discussions

Several explanations have been proposed for biphalin's high potency. Most of interpretations focused on the role of two pharmacophores in one molecule (e.g. simultaneous binding to two receptor sites) or a higher probability of interaction with the receptor of a molecule containing two pharmacophores.[3] The other possibility discussed previously takes into account the broad spectrum of receptor affinities of biphalin, and the possible synergistic interactions between these receptors and/or interactions with the proposed μ/δ complex.[5] In addition to this, the possible importance of biphalin metabolites, which may contain one pharmacophore, also has been suggested.[3]

The biological activities of fragments of biphalin (Table 1) indicate that at least for μ receptor binding, the presence of two pharmacophores is not necessary. Even the hydrazide tetrapeptide **2** 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

Table 1. Binding Affinities and Bioassays of Biphalin Fragments and Analogues

	Compound	Binding IC_{50} (nM)		Bioassay EC_{50} (nM)	
		δ	μ	MVD	GPI
1	(Tyr-D-Ala-Gly-Phe-NH-)$_2$ Biphalin	2.6	1.4	27	8.8
2	Tyr-D-Ala-Gly-Phe-NH-NH_2	230	4.7	290	90
3	Tyr-D-Ala-Gly-Phe-NH-NH<-Phe	15	0.74	27.	2.4
4	Tyr-D-Ala-Gly-Phe-NH-NH<-D-Phe	30	0.88	32.	9.8
5	Tyr-D-Ala-Gly-Phe-NH-NH<-Nle	71	5.9	95	5.2
6	Tyr-D-Ala-Gly-Phe-NH-NH<-D-Nle	21	1.3	20	24
7	Tyr-D-Ala-Gly-Phe-NH-NH<-Tyr	16	1.6	45.	15
8	Tyr-D-Ala-Gly-Phe-NH-NH<-Trp	29	2.0	15	7.1
9	Tyr-D-Ala-Gly-Trp-NH-NH<-Phe	46	8.3	130	26.0

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 lipophylic, amino acids did not greatly change the binding properties of these analogues (Table 1). Also changing the chirality of the amino acid in this position (**3** vs **4**; **5** vs **6**) led to only modest reduced affinity by a factor of 4 or less.

Interestingly, the binding properties of the analogues did not fully correlate with *in vitro* biological properties (Table 1). 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. On the other hand, the measured biological activity is a result of binding to respective receptors followed by activation of transduction mechanisms. 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 compound. For example, in the case of biphalin **1**, the K_i/IC_{50} are 1:10 and 1:4, for δ/MVD and μ/GPI, respectively. For the compound **3**, the respective values are 1:2 and 1:3, which may suggest that compound **3**, interacting with δ receptors, is more efficacious than biphalin. Thus as discussed in the Introduction, balanced agonists may be an important approach to take. Examination of all the data in Table 1 suggests that even at the same level of binding, the obtained compounds may express significant differences in efficacy. Further studies of efficacy by quantitative measurement of the ability of the analogues to stimulate second messenger systems[9] are in progress.

Acknowledgement: We thank The National Institute of Drug Abuse, U.S. Public Health Service Grant DA 06284 and the Medical Research Centre of Polish Academy of Sciences for supporting this work. The views expressed here are those of the authors and do not necessarily reflect those of the USPHS.

References

1. Lipkowski, A. W.; Misicka, A.; Hruby, V. J.; Carr, D. B. *Polish J. Chem.* **1994**, *68*, 907.
2. For a review, see: Hruby, V. J.; Gehrig, C. A. *Med. Res. Rev.* **1989**, *9*, 343.
3. Lipkowski, A. W.; Konecka, A. M.; Sroczynska I. *Peptides* **1982**, *3*, 697.
4. Silbert, B. S.; Lipkowski, A. W.; Cepeda, M. S.; Szyfelbein, S. K.; Osgood, P. F.; Carr, D. B. *Agents Actions* **1991**, *33*, 382.
5. Horan, P. J.; Mattie, A.; Bilsky, E. J.; Weber, S. J.; Davis, T. P.; Yamamura, H. I.; Malatynska, E.; Appleyard, S. M.; Slaninova, J.; Misicka, A.; Lipkowski, A. W.; Hruby, V. J.; Porreca, F. *J. Pharmacol. Exp. Ther.* **1993**, *265*, 1446.
6. Tang, J.-L.; Lipkowski, A. W.; Specter, S. *Int. J. Immunopharmacol.* **1998**, *20*, 457.
7. Li, G.; Haq, W.; Xiang, L.; Lou, B-S.; Hughes, R.; De Leon, I. A.; Davis, P.; Gillespie, T. J.; Romanowski, M.; Zhu, X.; 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.
8. Misicka, A.; Lipkowski, A. W.; Horvath, R.; Davis, P.; Kramer, T. M.; Yamamura, H. I.; Hruby, V. J. *Life Sci.* **1992**, *51*, 1025.
9. Burkey, T. H.; Ehlert, F. J.; Hosohata, Y.; Quock, R. M.; Cowell, S.; Hosohata, K.; Varga, E.; Stropova, D.; Li, X.; Slate, C.; Nagase, H.; Porreca, F.; Hruby, V. J.; Roeske, W. R.; Yamamura, H. I. *Life Sci.* **1998**, *62*, 1531.