ELSEVIER

Life Sciences, Vol. 60, No. 15, pp. 1263-1269, 1997

0024-3205/97 $17.00 + .00

PII S0024-3205(97)00069-6

STRUCTURE-ACTIVITY RELATIONSHIP OF BIPHALIN. THE SYNTHESIS AND BIOLOGICAL ACTIVITIES OF NEW ANALOGUES WITH MODIFICATIONS IN POSITIONS 3 AND 4.

Aleksandra Misicka[1,3], Andrzej W. Lipkowski[1,4], Robert Horvath[2], Peg Davis[2], Frank Porreca[2], Henry I. Yamamura[2], and Victor J. Hruby[1*]

Departments of [1]Chemistry and [2]Pharmacology, University of Arizona, Tucson AZ 85721, [3]Department of Chemistry, Warsaw University, 02-093 Warsaw, Poland, and [4]Medical Research Centre, Polish Academy of Sciences, 00-784 Warsaw, Poland

(Received in final form January 13, 1997)

Summary

New analogues of biphalin [(Tyr-D-Ala-Gly-Phe-NH-)$_2$] with modifications of amino acid residues in positions 3,3' and 4,4' have been synthesized. The potency and selectivity of these analogues were evaluated by competitive radioreceptor binding assay in the rat brain using [^{3}H]CTOP (mu ligand) and [^{3}H][p-Cl-Phe^4]DPDPE (delta ligand) as ligands, and by bioassay in the mouse vas deferens (MVD, delta receptor assay) and guinea pig ileum (GPI, mu receptor assay). The symmetrical substitution of phenylalanine in positions 4 and 4' with p-fluorophenylalanine or p-nitrophenylalanine resulted in an enhancement of the affinity at both delta and mu receptors, with some increase of the selectivity for delta opioid receptors. The analogue containing p-chlorophenylalanine in positions 4 and 4' is the most selective to the delta receptors in this series, with a selectivity ratio about 5. The symmetrical substitution of the glycine-3 residue with phenylalanine resulted in a decrease of binding affinities and biological potencies at both μ & δ receptors.

Key Words: biphalin, opioid peptides

Biphalin is a dimeric enkephalin analogue in which the C-terminal methionine or leucine residues are replaced by a second active fragment of the enkephalin analogue, and the two fragments are connected by a hydrazide bridge (Figure 1)(1).

Tyr-Gly-Gly-Phe-Met (or -Leu)

(Tyr-D-Ala-Gly-Phe-NH-)$_2$

Figure 1. A comparison of enkephalins and biphalin sequences

*Corresponding Author: Dr. Victor J. Hruby, Department of Chemistry, University of Arizona, Tucson, AZ 85721 USA

The higher probability of successful interaction of the molecule with two active sites on the receptor, and of possible unique interactions with proteolytic systems, were the original rationales for synthesis of biphalin. The complementary idea of synthesizing enkephalin analogues that are highly active because of their ability to bind with more than one receptor per molecule, was originally proposed by Coy et al. (2) who synthesized N-α, N-ϵ-bis[D-Ala^2-Met-enkephalin]-Lys-amide, and was explored later by Shimohigashi et al. (3) who synthesized a number of analogues with diamine bridged enkephalin dimers.

Of all the synthesized dimeric enkephalin analogues, biphalin expresses uniquely high potencies in both *in vitro* and *in vivo* tests, which qualify this compound as a potential new analgesic drug (4). Biphalin expresses high, nearly equal affinity to both mu and delta receptors (5). This compound is much more potent than morphine after i.c.v. (6) and i.t. (7) administration, equally active after i.v. administration (6), but practically inactive after s.c. administration (8). These unique properties of biphalin are probably partially the result of the rigidity of the hydrazide bond which conformationally constrains the two hydrophobic active peptide chains. In the cases of more flexible methylene bridges, self-aggregation of the two peptide chains can take place and this leads to reduced interaction with target receptors (9). To prevent such self-aggregation, Stepinski et al. (10) used hydrophilic hydroxymethylene bridges which, with addition of water molecules, probably formed a hydrophilic sphere isolating the two hydrophobic peptide chains.

To investigate the influence of amino acid residues in positions 3 and 4 of biphalin on potency and selectivity of the parent biphalin, several 3 and 4 position substituted analogues have been synthesized and studied for their binding and biological activity profiles.

Materials and Methods

Peptide Synthesis. General Procedure. The syntheses of biphalin and its analogues were performed by classic solution phase synthesis using methods similar to those previously reported (1) which involves a (2x3) + 2 strategy. N$^\alpha$-t-butyloxycarbonyl (Boc) protected amino acids (Bachem) were used throughout the synthesis. The N-terminal tripeptides N$^\alpha$-Boc-Tyr-D-Ala-Gly or N$^\alpha$-Boc-Tyr-D-Ala-Phe were synthesized by stepwise elongation starting from glycine ethyl ester or phenylalanine ethyl ester, respectively. The N$^\alpha$-protecting groups were deblocked by HCl in ethyl acetate and the DCC/HOBt method was used throughout to give N$^\alpha$-Boc-Tyr-D-Ala-Gly-OEt (or N$^\alpha$-Boc-Tyr-D-Ala-Phe-OEt). The ethyl esters of the respective tripeptides were hydrolyzed by 4N aqueous KOH. The synthesis of the symmetric dihydrazide of N$^\alpha$-Boc-phenylalanine was accomplished in a one step synthesis using 2 equivalents of N$^\alpha$-Boc-Phe with 1 equiv. of hydrazine hydrate, and BOP as the coupling reagent (90% yield). The unsymmetrical dihydrazides were synthesized under the same conditions using one equiv. of N$^\alpha$-Boc-amino acid and hydrazine to give the monohydrazide, which was later acylated by the other amino acid to provide the unsymmetrical dihydrazide. After deprotection of N$^\alpha$-Boc-protecting group from the hydrazides using HCl in acetic acid, the final coupling to the tripeptide was performed by the BOP method. Then 1 equiv. of the deblocked symmetrical or unsymmetrical dihydrazides were coupled with 2 equivs. of the synthesized tripeptides. The octapeptides were deblocked by HCl in acetic acid to give the final coumpounds. Purification of the peptides was accomplished by preparative reverse phase high pressure liquid chromatography (HPLC, Rainin). The purity of the final products was assessed by analytical reverse phase liquid chromatography (RP-HPLC), thin layer chromatography (TLC), amino acid analysis and fast atom bombardment mass spectrometry (FAB-MS) (Table I). The $[M+1]^+$ molecular ions were obtained by FAB-MS and were in agreement with the calculated molecular weights for each peptide. RP-HPLC (Table I) was performed on a Hewlett Packard 1090 Liquid chromatograph and monitored at 230, 254, and 280 nm. TLC was performed on glass-back silica gel plates (DC-Fertigplatten Kieselgel 60, Merck); ninhydrin spray and iodine were used for detection. Amino acid analyses were

performed on a Beckman 7300 Amino Acid Analyzer after acid hydrolysis with 4M methanesulfonic acid for 24 hrs at 110°C. Analytical properties of the new peptides are presented in Table I.

As an example, the synthesis of (HCl.Tyr-D-Ala-Gly-Phe(p-F)-NH-)$_2$ will be described.

(Boc-Phe(p-F)-NH-)$_2$. To a stirred solution of 1.13 g (40mM) of Boc-Phe(p-F) in 30 mL of DMF 1.7 g (40mM) of BOP, 0.54 g (40mmol) HOBt and 1.9 mL (11mM) of DIPEA were added. Then 0.1 mL (20mM) of $NH_2NH_2.H_2O$ were added. In 30 min product started to precipitate from the solution. After 2 hrs, the product was precipitated out by addition of ethyl acetate, filtered, washed with ethyl acetate, and recrystallized from acetic acid. Yield 1 g (90%), m.p.184-186°C, R_f=0.94 (chloroform:methanol, 9:1), R_f=0.95 (I).

(HCl.Phe(p-F)-NH-)$_2$. (Boc-Phe(p-F)-NH-)$_2$, 2 g, was dissolved in 5 mL of acetic acid, then 5 mL of 2 N HCl in acetic acid were added and the solution was stirred 25 min at room temperature. To the reaction mixture was added 100 mL of ethyl ether. Precipitated crystals were filtered off and washed with ethyl ether. Yield 1.52 g (95%), m.p. 255-258 °C, R_f=0.62 (I), R_f=0.26(IV).

(Boc-Tyr-D-Ala-Gly-Phe(p-F)-NH-)$_2$. 0.44 g (1 mmol) of (HCl.Phe(p-F)-NH-)$_2$ were dissolved in 20 mL of DMF, and 0.82 g (2mM) of Boc-Tyr-D-Ala-Gly, 0.88 g (2mmol) of BOP, 0.27 g (2mM) of HOBt and 1.0 mL (mM) of DIPEA were added. The reaction mixture was stirred for 4 hrs at room temperature. Product was precipitated by aqueous 1N $NaHCO_3$. The precipitated crystals were washed by 1N citric acid and water, and dried. Yield 0.87 g (76%), m.p. 152-154 °C, R_f=0.63 (chloroform:methanol, 4:1), R_f=0.95 (I).

(HCl.Tyr-D-Ala-Gly-Phe(p-F)-NH-)$_2$. (Boc-Tyr-D-Ala-Gly-Phe(p-F)-NH-)$_2$, 2 g, was dissolved in 5 mL of acetic acid, then 5 mL of 2N HCl in acetic acid were added and the solution was stirred 25 min at room temperature. To the reaction mixture was added 100 mL of ethyl ether. Precipitated crystals were filtered off and washed with ethyl ether. Purification of the final peptide was accomplished by preparative RP-HPLC. The analytical data are presented in Table I.

Radioreceptor Binding Assay. Adult male Sprague-Dawley rats (200-300 g) were sacrificed and their brains immediately removed and placed on ice. Whole brains were homogenized in 20 volumes of 50 mM Tris-HCl stock buffer (pH = 7.4) with a glass-teflon homogenizer. The homogenate was centrifuged (48,000 x g for 15 min), resuspended and preincubated (25°C for 30 min) to remove endogenous opioids. The homogenate was centrifuged and resuspended again (0.5% final conc.).

Binding affinities of the compounds at six to seven different concentrations were measured against [^{3}H][p-ClPhe^4]DPDPE (42.7 Ci/mmol) (11) and [^{3}H]CTOP (64.1 Ci/mmol) (12) (New England Nuclear, Boston, MA) by a rapid filtration technique. A 100 µL aliquot of the rat brain homogenate was incubated at 25°C for 180 min with either 0.75 nM [^{3}H] [p-ClPhe^4]DPDPE or 0.5 nM [^{3}H]CTOP in a total volume of 1 mL of 50 mM Tris-HCl pH (pH = 7.4) containing bovine serum albumin (1 mg/mL), bacitracin (50 µg/mL), bestatin (30 µM), and captopril (10 µM), and were done in duplicate. Naltrexone hydrochloride (10 µM) was used to define non-specific binding to tissue. The binding reaction was terminated by rapid filtration through presoaked (0.5% polyethylenimine solution) GF/B Whatman glass fiber strips with a Brandel Cell Harvester followed immediately by three rapid washes with 4 mL aliquots of ice-cold saline solution. The filters were removed and soaked in 10 mL scintillation fluid at 4°C for at least 6 hr before bound radioactivity was measured. At least two assays run in duplicate were performed. The data was analyzed by a non-linear least square regression analysis computer program. The receptor binding data are presented in Table II.

GPI and MVD *in vitro* Bioassays. The bioassays of all of the analogues were based on inhibition

TABLE I
Analytical Characterization of Biphalin and Analogues Synthesized in This Work

Peptide	TLC R_f Values[a]				RP HPLC	FAB-MS	
	I	II	III	IV	k'[b]	$[M+H]_{calc}$	$[M+H]_{obs}$
1	0.58	0.88	0.82	0.40	8.14	910	910
2	0.57	0.93	0.81	0.82	12.04	1089	1089
3	0.52	0.81	0.81	0.31	8.90	1000	1000
4	0.56	0.81	0.81	0.33	10.01	979	979
5	0.56	0.81	0.81	0.29	9.00	946	946
6	0.60	0.74	0.84	0.28	10.90	1162	1162
7	0.14	0.88	0.66	0.06	4.67	940	940

[a]Solvent systems as follows: **I**, n-butanol/acetic acid/water (4:1:1); **II**, n-butanol/acetic acid/pyridine/water (13:3:12:10); **III**, 2-propanol/ammonia/water (4:1:1); **IV**, n-butanol/acetic acid/ethyl acetate/water (5:1:3:1).
[b]Capacity factor (k') for the following systems: Vydac 218TP104C18 reverse phase (RP) column (25x0.46cm) with 0.1% trifluoroacetic acid/acetonitrile gradient (from 100/0 v/v to 60/40 v/v in 40 min) at a flow rate of 1.0 ml/min. All peptides were monitored at λ=230, 254, and 280nm).

of electrically induced smooth muscle contractions of mouse vas deferens and strips of guinea pig ileum longitudinal muscle myenteric plexus (13). Tissues came from male ICR mice weighing 25-30 g and from male Hartley guinea pigs weighing 150-400 g. The tissues were first tied to gold chains with suture silk, suspended in 20 mL baths containing 37°C oxygenated (95% O_2, 5% CO_2) Krebs bicarbonate solution (magnesium-free for the MVD), and allowed to equilibrate for 15 min. The tissues were then stretched and allowed to equilibrate for 15 min. The tissues were stimulated transmurally between platinum plate electrodes at 0.1 Hz, 0.4 ms pulses (2.0 ms pulses for MVD) at supramaximal voltage. Drugs at five to seven different concentrations were added to the baths in 14-60 uL volumes to produce cumulative dose-response curves. The peptidase inhibitor cocktail contained 3.1 mg/mL bestatin, 5 mg/mL bacitracin and 2.2 mg/mL captopril. Twenty µL of each inhibitor was added to the bath three minutes prior to the initial aliquot of the test drug. Percent inhibition was calculated by using an average contraction height for 1 min preceding the addition of the peptide divided by contraction height 3 min after the exposure to the peptide. IC_{50} values are the mean of not less than four separate assays. IC_{50} estimates and their associated standard errors were determined by fitting the mean data to the Hill equation using a computerized least-squares method (14). The bioassay data are presented in Table III.

Result and Discussion

The general strategy for the synthesis of biphalin and its analogues was by fragment coupling (2x3) + 2, similar to the procedure originally described by Lipkowski et al. (1). However, since than much progress has been made in formation of peptide bonds and in protection of functional aminogroups. We have upgraded the original procedures by using BOP for peptide bond and hydrazide formations,

TABLE II
Binding Affinities of Biphalin and Its Analogues

Compound	IC_{50}±SEM (nM)	
	Delta*	mu**
1. [Tyr-D-Ala-Gly-Phe-NH-]$_2$ (biphalin)	2.6 ± 0.3	1.4 ± 0.4
2. [Tyr-D-Ala-Phe-Phe-NH-]$_2$	65 ± 31	6.5 ± 2.7
3. [Tyr-D-Ala-Gly-Phe(NO_2)-NH-]$_2$	0.63 ± 0.10	0.94 ± 0.12
4. [Tyr-D-Ala-Gly-Phe(pCl)-NH-]$_2$	0.54 ± 0.05	2.44 ± 1.8
5. [Tyr-D-Ala-Gly-Phe(pF)-NH-]$_2$	0.31 ± 0.12	0.64 ± 0.38
6. [Tyr-D-Ala-Gly-Phe(pI)-NH-]$_2$	5.20 ± 0.30	24.5 ± 5.3
7. [Tyr-D-Ala-Gly-Phe(pNH_2)-NH-]$_2$	120 ± 34	10 uM(0%)

*versus [^{3}H][p-Cl-Phe^4]DPDPE; **versus [^{3}H]CTOP

and Boc for protection of all a-amino groups. The high reactivity of BOP allowed us to obtain the diacylhydrazide in one step by coupling 2 equivalents of N^a-Boc-Phe with one equivalent of hydrazine with an isolated yield over 90%. Under the same conditions, one equivalent of N^a-Boc-amino acid and hydrazine resulted in a practically pure monohydrazide, which was later acylated by a second amino acid to give the unsymmetrical dihydrazides.

The introduction of a p-fluoro-phenylalanine residue in position 4 in both peptide chains (**5**, Table II) increases affinity for delta receptors 8 fold, with a much lower (2 fold) significant increase in the affinity to the mu receptor, compare to the parent compound. Such changes are similar to those observed for enkephalin analogues, such as DPDPE (15). 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 reduced affinity for delta receptors respectively 1.7 and 17 fold, and to mu receptor respectively 3.8 and 38 fold (Table II). Based on its binding affinity, the di(p-chlorophenylalanine) biphalin is the most delta selective biphalin analog evaluated thus far. Substitution in the same position by a nitro-group, resulted in affinity values between analogues with p-fluoro- and p-chlorophenylalanine containing residues (Table II). Interestingly, the di(p-nitrophenylalanine) biphalin (**3**) is the most delta selective biphalin analogue in bioassays (Table III). Previously it has been shown (16) in enkephalin and its analogues that p-nitro-substitution of Phe^4 produced significant potency increases at both the μ and δ receptor. Introducing a basic amino group in the para position of phenylalanine greatly reduced affinity to delta receptors (46 fold compared to biphalin), and practically eliminated affinity for the μ receptor (Table II), though interestingly **7** still retained modest activity on the GPI assay (Table III).

Based on our current knowledge regarding the molecular nature of opioid receptors, we can predict

TABLE III
Bioassays of Biphalin and Its Analogues

Compound	IC_{50} (nM)	
	MVD	GPI
1. [Tyr-D-Ala-Gly-Phe-NH-]$_2$ (biphalin)	27. ± 1.5	8.8 ± 0.3
2. [Tyr-D-Ala-Phe-Phe-NH-]$_2$	32. ± 9.5	3.14 ± 0.37
3. [Tyr-D-Ala-Gly-Phe(NO_2)-NH-]$_2$	0.54 ± 0.13	2.84 ± 0.27
4. [Tyr-D-Ala-Gly-Phe(pCl)-NH-]$_2$	2.80 ± 0.93	2.56 ± 0.43
5. [Tyr-D-Ala-Gly-Phe(pF)-NH-]$_2$	1.30 ± 0.11	2.14 ± 0.66
6. [Tyr-D-Ala-Gly-Phe(pI)-NH-]$_2$	11. ± 0.31	13. ± 3.0
7. [Tyr-D-Ala-Gly-Phe(pNH_2)-NH-]$_2$	200 ± 29	4,200 ± 680

with high probability that biphalin forms an active complex with only one receptor molecule. It has been postulated (1) that the second "peptide arm"can play a role in the processes of receptor recognition and also may provide some protection from enzymatic degradation. If this is the case, then interaction of the second arm with the receptor can be viewed as an extension of the peptide "address" chain. Therefore symmetrical substitution in two "arms", has to be taken into account as substitutions in two different positions in relation to each individual pharmacophore ("message").

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 analogues do not discriminate between central and peripheral opioid receptor types.

Acknowledgements

This work was supported by grants from the National Institute of Drug Abuse, DA 06284, and the Polish Scientific Committee, 663379203.

References

1. A.W.LIPKOWSKI, A.M. KONECKA, I. SROCZYNSKA, Peptides 3 697-700 (1982).
2. D.H. COY, A.J. KASTIN, M.J. WALKER, R.F. McGIVERN, C.A. SANDMAN, Biochem. Biophys. Res. Commun. 73 632-638 (1976).
3. Y. SHIMOHIGASHI, T. COSTA, H.C. CHEN and D. RODBARD, Nature 297 333-335 (1982).
4. A.W. LIPKOWSKI, A. MISICKA, V.J. HRUBY, D.B. CARR, Polish J. Chem. 68 907-912 (1994).
5 A.W. LIPKOWSKI, A.M. KONECKA, I. SROCZYNSKA, R. PRZEWLOCKI, L. STALA S.W. TAM, Life Sci. 40 2283-2288 (1987).
6. P.J. HORAN, A. MATTIA, E. BILSKY, S. WEBER, P. DAVIS, H.I. YAMAMURA,

E. MALATYNSKA, S.M. APPLEYARD, J. SLANINOVA, A. MISICKA, A.W. LIPKOWSKI, V.J. HRUBY, F. PORRECA, J. Pharmacol. Exp. Ther. 265 1446-1454 (1993).

7. K. MISTEREK, I. MASZCZYNSKA, A. DOROCIAK, S.W. GUMULKA, D.B. CARR, S.K. SZYFELBEIN, A.W. LIPKOWSKI, Life Sci. 54 939-944 (1994).
8. B.S. SILBERT, A.W. LIPKOWSKI, M.S. CEPEDA, S.K. SZYFELBEIN, P.F. OSGOOD, D.B. CARR, Agents Action 33 382-387 (1991).
9. A.W. LIPKOWSKI, Polish J. Pharmacol. Pharm. 39 153-164 (1987).
10. J. STEPINSKI, I. ZAJACZKOWSKI, D. KAZEM-BECK, A. TEMERIUSZ, A.W. LIPKOWSKI, S.W. TAM, Int. J. Pep. Prot. Res. 30 588-592 (1991).
11. L.K. VAUGHN, R.J. KNAPP, G. TOTH, J.-P. WAN, V.J. HRUBY, H.I. YAMAMURA, Life Sci. 45 1001-1008 (1989).
12. J.E. SHOOK, J.T. PELTON, W.S. WIRE, L.D. HIRNING, V.J. HRUBY, T.F. BURKS, J. Pharmacol. Exp. Ther. 240 772-777 (1987).
13. H.W. KOSTERLITZ, F.A.H. LORD, S.J. PATERSON, A.A. WATERFIELD, Br. J. Pharmacol. 68 333-342 (1980).
14. STATISTICAL CONSULTANTS INC., Am. Stat. 48 52 (1986).
15. G. TOTH, T.H.KRAMER, R. KNAPP, G. LUI, P. DAVIS, T.F.BURKS, H.I.YAMAMURA, V.J. HRUBY, J. Med. Chem. 33 249-253 (1990).
16. P.W. SCHILLER, T.M.-D. NGUYEN, J. DIMAIO, C. LEMIEUX, Life Sci. 33 319-322 (1983).