Pergamon

Bioorganic & Medicinal Chemistry, Vol. 5, No. 9, pp 1867–1871, 1997

PII: S0968-0896(97)00117-X
0968-0896/97 $17.00 + 0.00

Libraries of Opiate and Anti-opiate Peptidomimetics Containing 2,3-Methanoleucine

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Abstract—A library of 96 peptides/peptidomimetics was prepared, in which half was based on the YGGFL-NH_2 sequence, while the remainder were derivatives of a presumed anti-opiate peptide, YGGFLRF-NH_2. Of the 48 compounds in each half of the library, 32 contained a stereoisomer of 2,3-methanoleucine substituted for Leu^5. Binding of the YGGFL-NH_2 derivatives to the μ- and δ-opioid receptors, and to the anti-β-endorphin monoclonal antibody (clone 3E7), indicated any change at the Leu^5 had little effect on the binding when compared with modifications to the YGGF-sequence. Conversely, cyclo-Leu residues did alter the binding of YGGFLRF-NH_2 derivatives when substituted for Leu^5. Of these 32 peptidomimetics, three derivatives of *2S,3S*-cyclo-Leu had relatively low K_i values for binding to an NPFF receptor. Differences between the outcome of the screens were interpreted in terms of the position of the cyclo-Leu residue in the two sequences. © 1997 Elsevier Science Ltd.

Introduction

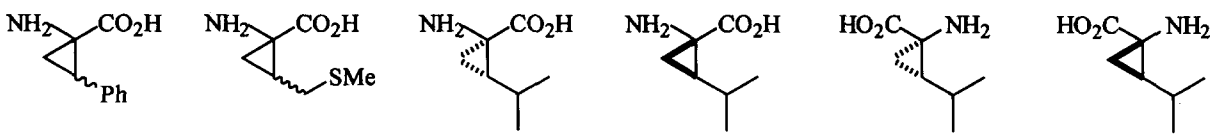
Peptidomimetics incorporating 2,3-methanoamino acids[1,2] can have conformational biases[3] and proteolytic stabilities[4–7] that are not characteristic of the parent peptide sequence from which they are derived. For these reasons, 2,3-methanoamino acids can be used to enforce orientations that approximate to the bioactive conformation, and to enhance bioavailability.

2,3-Methanoamino acids are not easy to prepare in optically pure form, so previous investigations into the biological activities of peptidomimetics containing these amino acids have been limited by the amount of material available. Consequently, small numbers of compounds have been screened (typically less than five) in the few studies reported so far. For instance, four enkephalin analogues wherein Phe^4 is replaced by 2,3-methanophenylalanine have been prepared and tested as opioids,[4,8,9] and four diastereoisomers of F{cyclo-M}RF-NH_2 (where cyclo-M represents the four 2,3-methanomethionine stereoisomers) have been synthesized and screened for their anti-opiate properties.[10–12] It would be desirable to test larger groups of compounds, but syntheses of even small libraries tend to require gram amounts of starting materials. Studies of libraries containing 2,3-methanoamino acids are therefore limited by the availability of the optically pure starting materials. Recently, however, two of us devised a gram scale asymmetric synthesis of all four cyclo-Leu stereoisomers (shown below);[13] that synthetic work provides an opportunity for more extensive testing.

In the research described in this paper, we sought to prepare a small library of peptidomimetics containing 2,3-methanoamino acids, and test for opiate and anti-opiate properties. It was anticipated that consideration of a larger number of compounds than in previous studies would facilitate rapid evaluation of the effects of the constrained amino acids. Patterns in the biological data might then be correlated with the 2,3-methanoamino acid stereochemistries. Simultaneously, changes in the peripheral amino acid sequence could be made and compared with a series of control peptides from natural amino acids. Consequently, a small library format should reveal the importance of the 2,3-methanoamino acid stereochemistry relative to modifications elsewhere in the sequence. Our objectives were to prepare and test a small library of this kind and to obtain accurate biological data for the peptidomimetics that appeared to be most active in the high-throughput screen.

The parent peptides chosen for this study were YGGFL-NH_2 and YGGFLRF-NH_2. Of these, the first is the Leu-enkephalin sequence but with a carboxamide *C*-terminus. Selection of YGGFLRF-NH_2 provides a derivative of Leu-enkephalin in which the –RF-NH_2 *C*-terminus was anticipated to impart anti-opiate properties.[14] This heptapeptide has hardly been studied, for any purpose,[15–17] but it is related to Leu-enkephalin

Abbreviations: BOP, benzotriazol-1-yloxytris(dimethylamino)-phosphonium hexafluorophosphate; DMF, dimethylformamide; Fmoc, 9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; HPLC, high-performance liquid chromatography; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; MOPS, 4-morpholinepropanesulfonic acid; NMM, *N*-methylmorpholine (4-methylmorpholine); TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.



cyclo-Phe **cyclo-Met** ***2R,3S*-cyclo-Leu** ***2S,3R*-cyclo-Leu** ***2S,3S*-cyclo-Leu** ***2R,3R*-cyclo-Leu**

in that same way that the anti-opiate peptide YGGFMRF-NH_2[18] is related to Met-enkephalin. Substitution of cyclo-Leu stereoisomers for the Leu residue in YGGFL-NH_2 and in YGGFLRF-NH_2 facilitates evaluation of the effect of 2,3-methanoleucine in *C*-terminal and near-central positions. In both cases, the cyclopropane amino acid would be proximal to, but not part of, the active sequence. In the pentamers the cyclo-Leu would be appended to the core opiate sequence YGGF–, and in the heptamers it would precede –RF-NH_2, the putative anti-opiate pharmacophore.[14]

Results

A library of 96 compounds was made on Geysen's pins apparatus (Multipin™ method),[19] then cleaved into solution, as described in the experimental section. Couplings on the pin apparatus are from the *C*- to the *N*-terminus (as is standard for solid phase peptide synthesis);[20] consequently, half the pins were coupled with Phe then Arg to produce the anti-opiate mimics, then the rest of the synthesis was performed using all the pins in parallel couplings. The purities of the peptides in the library were checked by HPLC and MALDI–MS. They were deliberately not separated from residues derived from the cleavage cocktail to avoid any variable depletion of the peptide in each well. Peptides synthesized with this method are proved to be suitable for biological studies.[21]

Tables 1–4 give results from the biological assays on the YGGFL-NH_2 (Tables 1–3) and YGGFLRF-NH_2 (Table 4) mimics, presented according to the following format. Data for the parent sequence are presented in the top left of each table. Rows in the tables represents one particular amino acid at residue-5 (Leu or cyclo-Leu stereoisomers). Columns in the tables depict any other changes that were made in the rest of the sequence (none in the left-most column, D-Ala for Gly at residue 2 in the second, etc). Lower-case letters represent D-amino acids.

The YGGFL-NH_2 mimics were screened in three assays for their opiate activities: binding to the μ-opioid receptor (Table 1), to the δ-opioid receptor (Table 2), and in an assay using the monoclonal E7-antibody (mAb) which is selective for the sequence YGGF. Tables 1 and 2, describing the receptor binding assays, give percent binding inhibition for the most active compounds at 100 nM concentration; activities of the other materials are quoted in terms of percent binding inhibition at 10 μM concentration. Table 3 gives binding inhibition data from the radioimmunoassay.

The data in Table 1 and 2 indicate very similar trends: highest binding inhibition for the compounds with D-Ala at position 2, while modifications at the Leu[5] residue have relatively small effects except that the 2*R*,3*R*-cyclo-Leu isomer is detrimental to binding in both μ- and δ-opioid receptor assays. Weaker binding inhibitions were observed for the compounds with Gly[2]

Table 1. Percentage binding inhibition (at the dose in parentheses) for YGGFl-NH_2 peptidomimetics relative to ^{3}H-DAGO at the μ-opioid receptor from rat membrane[a]

Deviation from sequence YGGFX-NH_2; where X is b	YGGFX-NH_2	YzGFX-NH_2	YGaFX-NH_2	YaaFX-NH_2	YGGfX-NH_2	YaGfX-NH_2	YGafX-NH_2	YaafX-NH_2
L-Leu	91 (100 nM)	94 (100 nM)	50 (100 nM)	56 (100 nM)	4 (100 nM)	0 (100 nM)	55 (10 μM)	63 (10 μM)
D-Leu	69 (100 nM)	89 (100 nM)	0 (100 nM)	7 (100 nM)	76 (10 μM)	4 (100 nM)	13 (10 μM)	4 (10 μM)
2*R*,3*S*-cyclo-Leu	65 (100 nM)	88 (100 nM)	1 (100 nM)	8 (100 nM)	2 (100 nM)	6 (100 nM)	66 (10 μM)	66 (10 μM)
2*S*,3*R*-cyclo-Leu	75 (100 nM)	90 (100 nM)	0 (100 nM)	53 (100 nM)	70 (10 μM)	0 (100 nM)	6 (10 μM)	0 (10 μM)
2*S*,3*S*-cyclo-Leu	76 (100 nM)	85 (100 nM)	5 (100 nM)	2 (100 nM)	81 (10 μM)	9 (100 nM)	3 (10 μM)	0 (10 μM)
2*R*,3*R*-cylco-Leu	50 (100 nM)	10 (100 nM)	55 (10 μM)	5 (100 nM)	59 (10 μM)	5 (100 nM)	0 (10 μM)	2 (10 μM)

[a]Assays were carried out in 50 mM Tris–HCl, pH 7.4, at 25 °C for 1 h. 2.0 nM ^{3}H-DAGO was incubated with frozen rat membrane in the presence of 100 nM or 10 μM of the test compound (as indicated).
[b]The difference between various rows represents changes from the Leu[5], and the variation between columns is indicative of other changes.

Table 2. Percentage binding inhibition (at the dose in parentheses) for YGGFL-NH_2 peptidomimetics relative to ^{3}H-DADL at the μ-opioid receptor from rat membrane[a]

Deviation from sequence YGGFX-NH_2; where X is b	YGGFX-NH_2	YzGFX-NH_2	YGaFX-NH_2	YaaFX-NH_2	YGGfX-NH_2	YaGfX-NH_2	YGafX-NH_2	YaafX-NH_2
L-Leu	78 (100 nM)	88 (100 nM)	15 (100 nM)	21 (100 nM)	10 (100 nM)	11 (100 nM)	0 (10 μM)	5 (10 μM)
D-Leu	32 (100 nM)	74 (100 nM)	7 (10 μM)	66 (10 μM)	56 (10 μM)	14 (100 nM)	0 (10 μM)	11 (10 μM)
2*R*,3*S*-cyclo-Leu	31 (100 nM)	76 (100 nM)	4 (10 μM)	3 (100 nM)	3 (100 nM)	6 (100 nM)	0 (10 μM)	73 (10 μM)
2*S*,3*R*-cyclo-Leu	77 (100 nM)	86 (100 nM)	2 (100 nM)	2 (100 nM)	63 (10 μM)	13 (100 nM)	0 (10 μM)	1 (10 μM)
2*S*,3*S*-cyclo-Leu	40 (100 nM)	68 (100 nM)	50 (10 μM)	2 (100 nM)	50 (10 μM)	4 (100 nM)	0 (10 μM)	0 (10 μM)
2*R*,3*R*-cylco-Leu	16 (100 nM)	23 (100 nM)	50 (10 μM)	0 (100 nM)	1 (10 μM)	4 (100 nM)	0 (10 μM)	0 (10 μM)

[a]Assays were carried out in 50 mM Tris–HCl, pH 7.4, at 25 °C for 1 h. 2.0 nM ^{3}H-DADL was incubated with frozen rat membrane in the presence of 100 nM or 10 μM of the test compound (as indicated). 100 nM DAGO was used for the blockage of ^{3}H-DADL binding to the μ-receptor.
[b]The difference between various rows represents changes from the Leu^5, and the variation between columns is indicative of other changes.

residues (i.e. no changes other than those at Leu^5, first column) than D-Ala^2 (second column). In the radioimmunoassays with the anti-β-endorphin mAb (Table 3) the percentage binding inhibition diminishes with every modification of the YGGF sequence; the most potent compounds are found in the first column, wherein no changes to the YGGF sequence were made, and the least potent compounds have two deviations from the YGGF sequence. These trends override any influence of changes at the Leu^5 residue, except that peptides containing L-Leu at this position are the most active in each column. In summary, *binding inhibition in each of the opiate activity assays is governed by the amino acids peripheral to the Leu^5 residue.*

Data in Table 4 indicate that the influence of modifications in the YGGF molecular segment has relatively small effects on the binding inhibitions recorded for the YGGFLRF-NH_2 peptidomimetics at an NPFF receptor (for anti-opiate properties). With regard to changes at the Leu^5 residue, L-Leu gives the most potent compounds. Most interestingly, however, several derivatives of 2*S*,3*S*-cyclo-Leu gave relatively high binding inhibitions within the series of 32 peptidomimetics incorporating cyclo-Leu residues. In fact, the data shown in Table 4 indicate that the three most active of the cyclo-Leu peptidomimetics were 2*S*,3*S*-cyclo-Leu derivatives. Thus, *the stereochemistry of the cyclo-Leu residue does have a significant effect on the binding inhibition in this series*. The peripheral sequence is also important; a single substitution, D-Phe for L-Phe at residue 4, emerges as favorable to binding.

The three cyclo-Leu-containing peptidomimetics with the highest binding inhibitions were prepared on a larger scale and retested to obtain the following K_i values (nM) using data from three experiments,[22] errors quoted represent standard error of the mean, SEM (values in parentheses are Hill coefficients, $n_H \pm$SEM): YGGF(2*S*,3*S*-cyclo-L)RF-NH_2, 6.28±0.11 (0.99±0.11); YGGf(2*S*,3*S*-cyclo-L)RF-NH_2, 16.21±0.15 (1.21 ±0.15); YGaf(2*S*,3*S*-cyclo-L)RF-NH_2, 47.18±0.05 (1.03±0.05). NPFF has a K_i of 0.44±0.06 nM under similar conditions (n_H = 1.04±0.06). Consequently, all three peptidomimetics show reasonably strong affinities to the NPFF receptor.

Discussion

The results presented in Tables 1–3 reveal that binding to the μ- and δ-opioid receptors and to the anti-β-endorphin mAb is relatively insensitive to the Leu^5

Table 3. Percentage binding inhibition for YGGFL-NH_2 peptidomimetics in the radioimmunoassay using anti-β-endorphin mAb[a]

Deviation from sequence YGGFX-NH_2; where X is b	YGGFX-NH_2	YzGFX-NH_2	YGaFX-NH_2	YaaFX-NH_2	YGGfX-NH_2	YaGfX-NH_2	YGafX-NH_2	YaafX-NH_2
L-Leu	95	80	85	18	51	19	9	0
D-Leu	89	79	57	0	5	0	0	0
2*R*,3*S*-cyclo-Leu	84	47	72	1	54	0	0	0
2*S*,3*R*-cyclo-Leu	76	41	16	0	13	0	0	0
2*S*,3*S*-cyclo-Leu	90	55	48	0	71	6	10	0
2*R*,3*R*-cylco-Leu	83	23	16	0	0	0	0	0

[a]Competition experiment with 42 μM of peptidomimetic in competition with 0.5 nM of ^{3}H-Leu^5-enkaphalin.
[b]The difference between various rows represents changes from the Leu^5, and the variation between columns is indicative of other changes.

Table 4. Percentage inhibition of radiolabeled NPFF binding to NPFF receptors in rat spinal cord[a]

Deviation from sequence YGGFXRF-NH_2; where X is b	YGGFXRF-NH_2	YzGFXRF-NH_2	YGaFXRF-NH_2	YaaFXRF-NH_2	YGGfXRF-NH_2	YaGfXRF-NH_2	YGafXRF-NH_2	YaafXRF-NH_2
L-Leu	84	76	75	65	98	60	81	58
D-Leu	31	35	28	63	48	40	48	69
2*R*,3*S*-cyclo-Leu	21	−7	22	35	30	20	34	32
2*S*,3*R*-cyclo-Leu	10	9	13	15	19	19	17	30
2*S*,3*S*-cyclo-Leu	**57**	14	27	27	**55**	37	**43**	32
2*R*,3*R*-cylco-Leu	35	22	29	13	42	38	37	34

[a]Average of two experiments using 90 nM of peptidomimetic in competition with 0.28 nM of radiolabeled NPFF (which has a K_i of 0.44±0.06 nM in this assay).
[b]The difference between various rows represents changes from the Leu[5], and the variation between columns is indicative of other changes.

modifications. This observation is unsurprising for the radioimmunoassay because anti-β-endorphin mAb was evolved to be specific for YGGF. However, the receptor binding data warrant further consideration. A cyclo-Leu residue at the *C*-terminus of a peptidomimetic restricts ϕ and χ rotations at that position. 2,3-Methanoamino acids placed within a peptide sequence influence conformations by constraining ϕ, χ, and ψ rotations, but the latter is not applicable to a 2,3-methanoamino acid at the *C*-terminus. Consequently, a 2,3-methanoamino acid is less likely to cause the peptide backbone to fold into any particular conformation when incorporated at either of the peptidomimetic termini. The terminal cyclo-Leu in the opiate peptidomimetics apparently has minimal direct influence in binding, and is unable to influence binding by enforcing any particular secondary structure. Consequently, *a C-terminal cyclo-Leu residue has less effect on the activities of these compounds than changes in the YGGF segment.*

Cyclo-Leu residues were placed at a position removed from the termini in the anti-opiate peptidomimetics. 2,3-Methanoamino acids in such positions can influence the conformational bias of peptidomimetics via a combination of ϕ, χ, and ψ constraints. Moreover, a cyclo-Leu at residue 5 in derivatives of the sequence YGGFLRF-NH_2 is proximal to the critical RF-NH_2 region. The binding data presented in this paper indicate that substitution of leucine by 2,3-methanoleucine stereoisomers generally reduces binding. Nevertheless, the stereochemistry of the cyclo-Leu residue is important indicating that this amino acid has significant effect on the local structures of the peptidomimetics which impacts their biological activities.

Experimental

Synthesis of the peptidomimetic library, and the peptidomimetics

The peptidomimetics were prepared via stepwise couplings of Fmoc-amino acid derivatives using BOP/HOBt/NMM[22] on Geysen pins (Chiron Mimotopes) functionalized with the Rink amide[23] handle. Deprotection of the Fmoc groups was performed using 20% piperidine in DMF. The 4-methoxy-2,3,6-trimethylbenzene sulfonyl (Mtr) group was used for side chain protection of the Arg residues, and the Tyr residues were side-chain protected as *tert*-butyl derivatives. Peptide synthesis was carried out at 25 °C in a plate of 96 wells without agitation. The coupling time for 2,3-methanoleucine derivatives was 48 h while it was 3 h for all the other amino acids. A DMF washing cycle (2 min) followed by methanol washing cycles (3 × 2 min) were used after each coupling and deprotection. The peptidomimetics were cleaved from the pins using TFA:1,2-ethanedithiol:anisole (38:1:1) for 24 h. Each peptide solution was concentrated using a gentle stream of dry nitrogen, then a vacuum centrifuge was employed to reduce the volumes to about 10% of their original values. A solvent mixture containing ether/petroleum ether/mercaptoethanol (approximately 1:1:0.01) was added to each solution, the solutions were centrifuged, and the solvent was decanted away. This procedure was repeated twice more. The resulting peptidomimetics were placed under vacuum for 24 h. MALDI-MS and HPLC traces were recorded for all the products. The corresponding molecular ion was observed for each peptidomimetic, and the HPLC traces indicated one major product in each case.

Opioid receptor binding assays

The membranes were prepared in the following way. Eighty frozen rat brains (including cerebellum) (Pel Freeze, Rogers, AZ) were homogenized (after defrosting at room temperature for 10 min), using a polytron in 10 mL/g wet weight of ice-cold 10 mM Tris–HCl, pH 7.4. Following centrifugation at 25,000*g* for 15 min (all centrifugations took place at 4 °C), the membrane pellets were resuspended in an equal volume of buffer and recentrifuged. The pellets were resuspended at 25 °C with 10 mM MOPS, pH 7.4, containing 3 mM $MnCl_2$, and brought to final volume of 880 mL. After incubation for 60 min at 25 °C, the homogenate was washed three times by centrifugation and resuspension in ice-cold 10 mM Tris–HCl, pH 7.4. The final pellets were stored at −70 °C.

The μ- and δ-opioid receptor binding assays were conducted with minor modifications of published methods.[24] Ligand binding assays were conducted at 25 °C for 1 h, in 50 mM Tris–HCl, pH 7.4. Membrane aliquots (0.75 mL) resuspended in 50 mM Tris–HCl (pH 7.4) were added to 12 × 75 mm polystyrene test tubes prefilled with 0.10 mL drug in 50 mM Tris–HCl, pH 7.4, 0.10 mL [^{3}H]-ligand in a protease inhibitor cocktail (bacitracin (100 μg/mL), bestatin (10 μg/mL) leupeptin (4 μg/mL), and chymostatin (2 μg/mL)), and 50 μL of 50 mM Tris–HCl, pH 7.4, yielding a final assay volume of 1 mL. μ-Opioid receptors were labeled with 2 nM [^{3}H]DAGO ([^{3}H][d-Ala^2, MePhe^4, Gly-ol^5]enkephalin) and δ-opioid receptors were labeled with 2 nM [^{3}H]DADL ([^{3}H][d-Ala^2, D-Leu^5]enkephalin) using 100 nM DAGO to block binding to μ-opioid receptors. Triplicate samples were filtered over Whatman GF/B glass-fiber filters presoaked in 2% PEI, using a Brandell cell harvester, and washed twice with 4 mL of ice-cold 10 mM Tris–HCl, pH 7.4. Nonspecific binding was defined using 20 μM levallorphan. The tritium retained on the filters were counted in 5 mL CytoScint (ICN) using a Taurus beta counter with 44% efficiency after an overnight extraction.

Competitive radioimmunoassay with monoclonal anti-β-endorphin antibody (mAb)

A liquid phase radioimmunoassay was used for the competitive ligand binding analysis of each peptidomimetic ligand; details of this assay have been described elsewhere.[25] Briefly, the assay employs 1 mL syringe barrel mini-columns of Sephadex G-25 that separates the mAb-bound ligand from the free-ligand containing supernatant. The mAb used in these assays was anti-β-endorphin (clone 3E7) which binds the *N*-terminal five residues of the peptide, which is equivalent to the enkephalin sequence.[26] In these competition assays we used tritiated Leu-enkephalin (^{3}H-Leu-Enk; specific activity 44.5 Ci/mmol; Bachem Bioscience, Co.) as the radiolabeled ligand. Statistical analyses and plotting of data were accomplished with the programs EBDA and LIGAND.[27]

Acknowledgements

We thank the The National Institutes of Health (GM 50772 and DA 06554 and the Robert A. Welch Foundation for direct support for this research, and K.B. thanks the NIH for a Research Career Development Award and the Alfred P. Sloan Foundation for a fellowship.

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(*Received in U.S.A.* 5 *December* 1996; *accepted* 13 *May* 1997)