New Dimeric Tetrapeptide Enkephalin Analogues with Five- or Six-Carbon Hydrophilic Spacers

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Bivalent Opioids, Enkephalin Analogues, Opioid Receptor Selectivity

To investigate the role of distance between two opioid peptide pharmacophores on binding activities to delta, mu and kappa receptor in vitro, a number of new dimeric enkephalin analogues were synthesized in which two identical tetrapeptides: Tyr-D-Ala-Gly-Phe were connected together at their C-termini by $\alpha,\omega$-diamino-$\alpha,\omega$-dideoxyalditols. The length of the hydrophilic spacer (3–6 carbons) did not affect the affinities for delta receptors, whereas the affinities for mu and kappa receptors were dependent on the length as well as the configuration of the spacer. The analogues had high affinities, and some of them possessed moderate delta selectivity.

Introduction

In the development of chemistry for enkephalin analogues, it was found [1–3] that some glycosylated opioid peptides showed higher biological activity than the parent peptide compound itself. It can be assumed that the incorporation of sugar moieties in the enkephalin sequence could alter conformational properties, enzymatic stability and the affinity of the molecule toward opioid receptors.

On the other hand, a number of laboratories [4–7] introduced a new class of opioid analogues which contained two peptide pharmacophores in one molecule. In most of the synthesized dimeric enkephalin analogues, diaminoalkyl chains were used as spacers connecting two peptide fragments at the C-termini.

In light of the above, we have proposed [8,9] the use of hydrophilic sugar derived multihydroxyl spacers for bridging two tetrapeptides. The resulted dimeric analogues possessed the following structures:

$$\text{Tyr-D-Ala-Gly-Phe-NH-CH}_2$$

$$\mid$$

$$(\text{CHOH})_n$$

$$\mid$$

$$\text{Tyr-D-Ala-Gly-Phe-NH-CH}_2$$

where $n = 1, 2$ or $4$. The tetrapeptide Tyr-D-Ala-Gly-Phe, the minimum enkephalin-related sequence necessary for high affinity for opioid receptors, was used as an opioid pharmacophore. Various configurations of the multihydroxyl linkers were used, and our previous studies [8, 9] demonstrated that both the length and the configuration of the bridge were important factors in receptor potency and selectivity.

In the present study we report the syntheses of new analogues within this series. The five-carbon spacers or bridges bearing three hydroxyl groups ($n = 3$) of different configuration have been employed for linking two tetrapeptide fragments. In addition we present one analogue with longer spacer ($n = 4$) with D-manno configuration and one reference compound of "monomeric" nature having tetrapeptide terminated with ethanolamide. The effect of length as well as configuration of a spacer on selectivity of a bivalent opioid ligand was of interest in this study.

Experimental

The $^{13}$C NMR spectra were obtained with a Jeol FX90Q spectrometer. IR spectra were recorded...
with a UR-20 Zeiss spectrophotometer. Optical rotations were measured with a Perkin-Elmer 241 polarimeter. The liquid matrix secondary ions mass spectrometry (LSIMS) was performed on an AMD-604 Intectra GmbH spectrometer. Analytical HPLC analyses were performed on a Spectra-Physics SP 8800 liquid chromatograph, utilizing an analytical Supelco LC-18-T (25 cm with a 2 cm guard) reversed phase column. The solvent system was buffer A (0.01 M KH₂PO₄, pH 3.0) and buffer B (50% buffer A / 50% acetonitrile, v/v). The linear gradient was 40–100% B in 20 min, the flow rate 2.0 ml/min, and monitoring was performed at 210 nm.

Chemical Synthesis

1. 1,5-Diamino-1,5-dideoxyribitol (1,5-diamino-2S,3S,4R-pentanetriol, 1), 1,5-diamino-1,5-dideoxyxylitol (1,5-diamino-2S,3R,4R-pentanetriol, 2), and 1,6-diamino-1,6-dideoxy-D-mannitol (1,6-diamino-2R,3R,4R,5R-hexanetetraol, 5) were obtained as their dihydrochlorides according to Kiely et al. [10] starting from the appropriate aldoses: D-ribose, D-xylose and D-mannose, respectively. Analytical data, which were in good accord with the reported ones [10], and in addition IR and ¹³C NMR spectra confirmed the proposed structures.

2. 1,5-Diamino-1,5-dideoxy-D-arabinobitol (1,5-diamino-2R,3R,4R-pentanetriol, 3), 2,3,4-Tri-O-acetyl-1,5-dichloro-1,5-dideoxy-D-arabinobitol [11] (315 mg, 1 mmol), sodium azide (1.3 g, 20 mmol) and DMF (8 ml) were heated to 90–100°C for 20 h with magnetic stirring. The mixture was filtered and, the filtrate was concentrated under diminished pressure (~1 mm Hg, oil pump). The residue was dissolved in ethyl acetate (30 ml), washed twice with water and dried over Na₂SO₄. The ethyl acetate solution was concentrated and the residue was dissolved in ethanol (20 ml). Palladium on charcoal (100 mg) was added to the solution, and hydrogen was passed through the mixture for at least 8 h. The catalyst was filtered off and washed twice with a small amount of water. The filtrate together with the washing water were concentrated to a small volume and treated with 40% HBr (1 ml). The mixture was heated gently to the boiling point and left overnight at room temperature. The solution was diluted with ethanol and concentrated on a rotatory evaporator. The procedure of dilution with ethanol and concentration was repeated a few more times to remove an excess of hydrobromic acid. The resulted precipitation of 3 dihydrobromide was filtered and crystalized from ethanol and recrystallized from isopropanol. Yield: 178 mg (57%); m.p. (3 dihydrobromide) 189–191°C; [α]D (3 ditosylate) + 6.8° (c 0.57, water).

Analysis for C₁₇H₂₀N₈O₁₇·H₂O (3 dipicrate hydrate)

Calcd C 32.60 H 3.54 N 17.89%;
Found C 32.63 H 3.46 N 17.86%;

3. 1,5-Diamino-1,5-dideoxy-L-arabinobitol (1,5-diamino-2S,3S,4R-pentanetriol, 4) was prepared by the same synthetic route as described above but starting from L-arabinobitol. Overall yield: 25.3%; m.p. (4 dihydrobromide) 197°C; [α]D (4 ditosylate) – 7° (c 0.5, water).

Analysis for C₁₇H₂₀N₈O₁₇·H₂O (4 dipicrate hydrate)

Calcd C 32.60 H 3.54 N 17.89%;
Found C 32.63 H 3.46 N 17.86%;

4. The tripeptide Boc-Tyr-D-Ala-Gly was prepared according to Lipkowski et al. [7].

5. 1,5-Dideoxy-1,5-di-(tyrosyl-D-alanyl-glycyl-phenylalanylamido)-ribitol (6), 1,5-Dideoxy-1,5-di-(tyrosyl-D-alanyl-glycyl-phenylalanylamido)-xylitol (7), 1,5-Dideoxy-1,5-di-(tyrosyl-D-alanyl-glycyl-phenylalanylamido)-D-arabinobitol (8), 1,5-Dideoxy-1,5-di-(tyrosyl-D-alanyl-glycyl-phenylalanylamido)-L-arabinobitol (9) and 1,6-Dideoxy-1,6-di-(tyrosyl-D-alanyl-glycyl-phenylalanylamido)-D-mannitol (10) were prepared according to the scheme depicted in Fig. 1. Experimental details for the syntheses were similarly described elsewhere [9]. The final crude products were purified on a Sephadex G 25 column using n-butanol – acetic acid – water (4:1:5, upper phase) as solvent. Appropriate fractions were pooled and lyophilized. Portions of each compound (a few mg) were repurified by semipreparative HPLC using a Supelco LC-18-DB reverse phase column with linear gradients of buffer A (0.03 M ammonium acetate, pH 5.0) and buffer B (33% buffer A / 67% acetonitrile, v/v) and monitoring at 230 or 260 nm. Collected fractions were lyophilized twice to obtain fine white powdery final products. The homogeneity and identity of the compounds were checked by analytical HPLC, LSIMS and TLC. Thin layer chromatography (TLC) was performed on silica gel (Kieselgel G, Merck) using the following solvent systems (v/v): (A) isopropanol – triethylamine – water = 7:1:2, (B) n-butanol – acetic acid – water = 4:1:2, (C) n-butanol – pyridine – acetic acid – water = 4:1:1:1. The prepared analogues gave correct amino acid analyses. Analytical data for compounds 6–10 are shown in Table I.
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Fig. 1. Synthesis of dimeric enkephalin analogues with hydrophilic spacers.

6. 2-(Tyrosyl-D-alanyl-glycyl-phenylalanylamido)-ethanol (11) was obtained utilizing ethanolamine as a starting material and using the same strategy as shown in Fig. 1. Purification procedures were the same as described above. Analytical data for the compound are shown also in Table I.

Receptor Binding Assays

Brains were dissected from decapitated male Hartley guinea pigs. Brain membranes preparation and receptor binding assays were performed as described previously [12,13]. The final concentration of labelled ligands used were: 0.5 nM [3H]naloxone (μ binding); 1 nM [3H]DADLE in the presence of 4 nM sufentanil (δ binding); and 1 nM (-)[3H]-ethylketocyclazocine (EKC) in the presence of 500 nM DADLE and 20 nM sufentanil (κ binding). Under these conditions, the apparent K_s for [3H]naloxone, [3H]DADLE, and (-)[3H]EKC were 0.98, 0.64 and 0.62, respectively. Binding was performed with 100 mM NaCl and bacitracin (50 mg/ml). IC_{50}s were calculated from log-logit plots. Apparent K_s were calculated from the equation, K_s = IC_{50}[1+(L/K_d)], where L is the concentration of the radioligand and K_d is its dissociation constant. The results are shown in Table II.
Table I. Physical data on new analogues.

<table>
<thead>
<tr>
<th>Comp.</th>
<th>HPLC retention times (min)*</th>
<th>LSIMS*</th>
<th>TLC data*</th>
<th>Amino acid Analyses data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>RfA</td>
</tr>
<tr>
<td>6</td>
<td>10.8</td>
<td>1027</td>
<td>0.58</td>
<td>0.49</td>
</tr>
<tr>
<td>7</td>
<td>10.7</td>
<td>1027</td>
<td>0.60</td>
<td>0.50</td>
</tr>
<tr>
<td>8</td>
<td>11.1</td>
<td>1027</td>
<td>0.58</td>
<td>0.48</td>
</tr>
<tr>
<td>9</td>
<td>10.7</td>
<td>1027</td>
<td>0.58</td>
<td>0.48</td>
</tr>
<tr>
<td>10</td>
<td>9.5</td>
<td>1057</td>
<td>0.57</td>
<td>0.48</td>
</tr>
<tr>
<td>11</td>
<td>4.3</td>
<td>500</td>
<td>0.71</td>
<td>0.57</td>
</tr>
</tbody>
</table>

* For details see Experimental; * (M+Na)+ and (M+K)+ ions were also observed.

Table II. Affinities of opioid analogues for mu, delta and kappa binding sites in guinea pig brain membrane preparation.

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Analogue Bridge</th>
<th>Ki(nM)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>structure configur.</td>
<td>δ</td>
</tr>
<tr>
<td>6</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH-CHOH} )b \text{ribo}</td>
<td>15.4 ± 2.2</td>
</tr>
<tr>
<td>7</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH-CHOH} ) \text{D-arabino}</td>
<td>46.3 ± 23</td>
</tr>
<tr>
<td>8</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH-CHOH} ) \text{L-arabino}</td>
<td>26.3 ± 3.9</td>
</tr>
<tr>
<td>9</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH-CHOH} ) \text{xylo}</td>
<td>22 ± 7</td>
</tr>
<tr>
<td>10</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH-CHOH} ) \text{D-manno}</td>
<td>30.1 ± 3.1</td>
</tr>
<tr>
<td>11</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CH}_2\text{-CHOH} ) \text{L-threo}</td>
<td>163 ± 41</td>
</tr>
</tbody>
</table>

Previously characterized:

<table>
<thead>
<tr>
<th>Comp.</th>
<th>Analogue Bridge</th>
<th>Ki(nM)a</th>
</tr>
</thead>
<tbody>
<tr>
<td>A\text{c}</td>
<td>(\text{Y-DA-G-F-NH-1} )</td>
<td>–</td>
</tr>
<tr>
<td>B\text{d}</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH} )</td>
<td>–</td>
</tr>
<tr>
<td>C\text{d}</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH} )</td>
<td>–</td>
</tr>
<tr>
<td>D\text{d}</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH} )</td>
<td>–</td>
</tr>
<tr>
<td>E\text{e}</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH} )</td>
<td>–</td>
</tr>
<tr>
<td>F\text{e}</td>
<td>(\text{Y-DA-G-F-NH-CH}_2\text{-CHOH} )</td>
<td>–</td>
</tr>
<tr>
<td>Morphine\text{e}</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Dynorphin(1-13e)</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>DADLE\text{e}</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

\text{a} – data represent mean ± S.E.M. of 3–4 experiments in duplicate or triplicate; \text{b} – \(\text{Y-DA-G-F-NH} = \text{Yr-Tyr-D-Ala-Gly-Phe} \); \text{c} – reference [14]; \text{d} – reference [9]; \text{e} – reference [8].

Results and Discussion

The reaction pathways leading to the synthesis of compounds 6–10 are shown in Fig. 1. The presented route followed exactly our previously reported strategy [9]. The presence of free hydroxyl groups in multihydroxyl substrates (1–5) and consecutive intermediates, as well as the one on tyrosine moiety did not interfere during coupling reactions. Compound 11 was prepared in a similar manner starting from ethanolamine and equimolar peptide intermediates. The crude products (6–11) were purified by gel filtration over Sephadex G-25 and then, for analytical and receptor binding assay purposes, samples of each compound were obtained by semipreparative HPLC. Analytical data for the final enkephalin analogues are shown in Table I. The analytical HPLC was the ultimate criterion for purity of the compounds. Single peaks were detected for all 6–11 final products. Furthermore, the obtained mass spectra (LSIMS) gave proper molecular ions with predicted isotopic patterns. Thus, the dimeric structures of compounds 6–10 were confirmed.
The newly synthesized enkephalin analogues were tested for receptor binding to \( \delta \), \( \mu \) and \( \kappa \) receptors in guinea pig brain homogenates by the methodology reported elsewhere [14]. The bioassay conditions were the same as used previously for characterization of other dimeric analogues [8,9,14]. The obtained \( K_i \) values for dimeric compounds 6–10 are presented in Table II and compared with the results for the earlier prepared dimeric hydrophilic analogues (B–F), as well as for other referable compounds, including “monomeric” analogue 11.

**Delta affinity and selectivity.** As presented in Table II, analogues with multihydroxyl spacers (except of only compound B, which bore one hydroxyl group) possessed similar \( \delta \) affinity comparable to that for dynorphin(1–13) but lower than that for DADLE. We observed no marked dependence of the affinity on the length of the hydrophilic linker and only minor effects of the various configurations of the spacers. Thus, we conclude that none of the compounds acts as a real bivalent opioid, *i.e.* binding simultaneously to two distinct but closely clustered \( \delta \) receptors. It is possible that the distance between two peptide ligands in our analogues is too short to bridge two \( \delta \) receptor sites. It is in accord with earlier Shimohigashi *et al.* [4] observations that the optimum distance for a spacer chain was 10–12 carbons. Nevertheless, three of four our newly synthesized analogues with bridges bearing three hydroxyl groups, namely compounds 6, 8 and 9 exhibited relatively \( \delta \) selectivity.

**Mu affinity.** Affinities toward \( \mu \) receptor sites of the analogues exhibited bigger diversity. Almost all compounds expressed higher activity than DADLE or reference “monomeric” peptide 11. The most active analogue (E) possessed four-carbon spacer with two vicinal hydroxyl groups of \( L \)-threo configuration. It is possible to assume that the very configuration of this compound meets in the best manner for the stereochemical requirements of \( \mu \) binding site. It is interesting that two analogues of five-membered bridge (compounds 6 and 7) and both of the six-membered bridge (compounds 10 and F) showed also high \( \mu \) affinities comparable to that of dynorphin(1–13) and morphine. We tentatively postulate that these compounds (6, 7, 10 and F) can act as bivalent opioids, binding not very strongly but simultaneously to two closely situated \( \mu \) receptor sites. An alternative explanation is that after the binding of one pharmacophore to the receptor pocket, the other pharmacophore can interact with a certain domain of the receptor to enhance affinity. In either case the optimum distance for a spacer would be 5–6 carbons.

**Kappa affinity and selectivity.** The most impressive and consisted results were obtained for affinities toward \( \kappa \) receptor binding sites. All analogues exhibited a few orders higher affinity than DADLE or tetrapeptide opioids (for example the analogue 11). The compound E appeared to have high affinity comparable to dynorphin(1–13). This analogue represents the first opioid peptide derived from enkephalin showing a preference for \( \kappa \) receptors. The other dimeric analogues possessed about two order lower affinity but displayed an interesting dependence of activity on the length of the spacer. The optimum of activity occurred for four carbon linkers and depended on the bridge configuration. In contrast to conclusions about \( \mu \) affinity this dependence cannot be explained on the basis of bivalent ligands interactions. Instead, we assume that during binding to the \( \kappa \) receptor site one tetrapeptide ligand appear to be “message” [15, 16] and the rest of the molecule including the second tetrapeptide fragment and especially the hydrophilic spacer play the role of the “address”. In this context we conclude that the analogues A and B are “too short” to display a high affinity, whereas the five- and six-membered linkers (compounds 6–10 and F) are “too long”. In the optimum activity point of four-carbon linkers the dependence on the configuration of the spacer becomes important. Again, the most active and \( \kappa \) selective compound (E) have \( L \)-threo configuration.

The results of the study demonstrate that the use of hydrophilic spacers creates new possibilities in the modulation of activity and selectivity of opioid peptide bivalent ligands.

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