PHARMACOLOGICAL ACTIVITIES OF SOME SYNTHETIC PEPTIDES RELATED TO DERMORPHIN

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SUMMARY

Objectives: To investigate the relationship between the structure of dermorphins (DM) and their pharmacological properties, six analogues of [Hyp']DM and [Pro']DM were synthesised and their biological activities were studied.

Methods: The peptides were synthesised by the solid phase method using 9-fluorenylmethoxycarbonyl amino acid trichlorophenyl esters as coupling agents and Merrifield resin as solid support. The opioid agonist activity was studied using co-axially, electrically stimulated contraction of isolated guinea pig ileum (GPI, in vitro). Their analgesic activity was assessed in mice using Eddy’s hot plate method and tail-flick method. The antidiarrhoeal activity was determined by the charcoal meal test in mice.

Results: In the GPI assay, the synthetic analogues possess agonistic activity that are less pronounced than morphine. Peptides I and II (substitution of ser at position 7 and Gly at position 4 in [Hyp']DM series respectively) possess considerable analgesic activity but are almost inactive in the GPI assay. Peptide III ([Pro', Sar]DM) possess only analgesic activity. In GPI assay, peptide IV was inactive. Peptide V and VI had equipotent analgesic and antidiarrhoeal activity.

Conclusion: Peptides with various structures can possess specificities that may prove useful in biological applications. Among them [Sar4, Pro6, Tyr7]DM, [Hyp5, Pro6, Sar]DM and [Phg3, Pro4]DM exhibited a high degree of selectivity in their activities.

KEY WORDS Dermorphin solid phase peptide synthesis biological activity selectivity

INTRODUCTION

[Pro6]dermorphin (DM), a heptapeptide (Tyr-D-Ala-Phe-Gly-Tyr-Pro-Ser-NH2) isolated from the methanol extracts of the skin of the South American frog of Phyllomedusa species'2, possesses exceptionally intense and long lasting peripheral, endocrine and central opioid activities both in vitro and in vivo2. Together with this peptide, another heptapeptide, [Hyp5]DM, which differs from dermorphin only by the presence of hydroxyproline (Hyp) residue instead of proline at position 6 was also isolated2,4. To investigate the relationship between the structure of dermorphins and their pharmacological properties, several analogues have been synthesized in several laboratories5-10. In a series of analogues synthesized by us, D-Ala at position 2 in [Pro6]DM was replaced by other aromatic and aliphatic D-amino acids like D-phenylalanine, D-p-hydroxyphenylglycine (D-Hpg), D-serine, D-ethionine, D-leucine, D-norleucine and D-norvaline11. In another series, Ser at position 7 was replaced by either Ala, Tyr or Thr12. Among them [Pro6, Tyr7]DM was found to have the most pronounced opiate agonistic activity in the guinea pig ileum (GPI) assay being nearly two times as potent as [Pro6]DM. Another analogue, [Pro6, Thr7]DM is almost as potent as [Pro6]DM.

In continuation with these studies, six more analogues of [Hyp5]DM and [Pro5]DM have now been synthesized in which Phe at position 3 was replaced by phenylglycine (Phg), D-Phg or N-methylphenylalanine (MePhe), and Gly at position 4 was replaced by sarcosine (Sar). Further, Pro at position 6 was replaced by Hyp, and Ser at position 7 was replaced by Pro, Sar or Tyr and the biological activities of the resulting analogues have been studied.

MATERIALS AND METHODS

All amino acids used, except Gly and Sar unless otherwise specified are of L-configuration. Melting points were determined using Leitz-Wetzlar melting point apparatus and are uncorrected. Optical rotations were recorded using automatic digital AA-10 polarimeter (Optical Activity, U.K.). Thin layer
Table 1. Physical characteristics of the synthetic peptides related to dermorphin°

<table>
<thead>
<tr>
<th>No.</th>
<th>Peptide sequence**</th>
<th>M.P. (°C)</th>
<th>$\left[\alpha\right]_D^{25}$ (C1, methanol)</th>
<th>$R_f$ values</th>
<th>Molecular formula</th>
<th>Elemental analysis Calc. (Found) %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>$R_f$ A</td>
<td>$R_f$ B</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>Tyr-D-Ala-Phe-Sar-Tyr-Hyp-Tyr-NH,</td>
<td>213-215</td>
<td>+8.0°</td>
<td>0.65</td>
<td>0.58</td>
<td>$C_{46}H_{56}O_{11}N_{8}$</td>
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<td></td>
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<tr>
<td>II</td>
<td>Tyr-D-Ala-Phe-Gly-Tyr-Hyp-Pro-NH,</td>
<td>159-161</td>
<td>-20.0°</td>
<td>0.51</td>
<td>0.40</td>
<td>$C_{43}H_{52}O_{10}N_{8}$</td>
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<td></td>
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<tr>
<td>III</td>
<td>Tyr-D-Ala-Phe-Gly-Tyr-Pro-Sar-NH,</td>
<td>186-l 89</td>
<td>-20.0°</td>
<td>0.46</td>
<td>0.51</td>
<td>$C_{40}H_{50}O_{9}N_{8}$</td>
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<td></td>
</tr>
<tr>
<td>IV</td>
<td>Tyr-D-Ala-D-Phe-Gly-Tyr-Pro-Ser-NH,</td>
<td>165-l 67</td>
<td>-10.0°</td>
<td>0.68</td>
<td>0.39</td>
<td>$C_{39}H_{48}O_{10}N_{8}$</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>V</td>
<td>Tyr-D-Ala-D-Phe-Gly-Tyr-Pro-Ser-NH,</td>
<td>166-l 69</td>
<td>-10.0°</td>
<td>0.63</td>
<td>0.48</td>
<td>$C_{39}H_{48}O_{10}N_{8}$</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VI</td>
<td>Tyr-D-Ala-MePhe-Sar-Tyr-Pro-Ser-NH,</td>
<td>189-191</td>
<td>-8.0°</td>
<td>0.58</td>
<td>0.61</td>
<td>$C_{42}H_{51}O_{11}N_{8}$</td>
</tr>
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</table>

°Amino acid analyses were carried out using Waters Pica-Tag amino acid analysis system after hydrolysing the peptides at 110° with 6N HCl for 24 h under nitrogen atmosphere and were found to be satisfactory.

°Abbreviations used are in accordance with the recommendations of the IUPAC-IUB Commission for amino acids and peptides (J Biol Chem 1972;257:977-983).
Figure 1. Schematic representation of the solid phase synthesis of [D-Phga] dermorphin.

\[ \text{Boc-Ser} + \text{Cl-CH}_2-\text{C}_6\text{H}_4-\text{Resin} \]

\[ \downarrow \text{TEA in EtOH, 90° for 24h} \]

\[ \text{Boc-Ser-CH}_2-\text{C}_6\text{H}_4-\text{Resin} \]

\[ (i) \text{1N HCl-AcOH} \]

\[ (ii) \text{TEA in DMF} \]

\[ (iii) \text{Fmoc-Pro-OTcp (3 equiv.) / HOBt (1 equiv.)} \]

\[ \text{Fmoc-Pro-Ser-CH}_2-\text{C}_6\text{H}_4-\text{Resin} \]

\[ (i) \text{60% DEA in DMF} \]

\[ (ii) \text{Appropriate Fmoc-amino acid active ester (3 equiv.)/HOBt (1 equiv.)} \]

\[ \text{Fmoc-D-Ala-D-Phg-Gly-Tyr-PwSer-CH}_2-\text{C}_6\text{H}_4-\text{Resin} \]

\[ \text{Ammonolysis (NH}_3\text{-MeOH)} \]

\[ \text{Boc-Tyr-D-Ala-D-Phg-Gly-Tyr-Pro-Ser-CH}_2-\text{C}_6\text{H}_4-\text{Resin} \]

\[ (i) \text{98-100% formic acid/anisole} \]

\[ (ii) \text{Gel filtration (Sephadex G-15)} \]

\[ \text{Tyr-D-Ala-D-Phg-Gly-Tyr-ProckSer-NH}, \]

by the solid phase method using the conventional Merrifield resin (chloromethylated polystyrene - 1% divinylbenzene, Cl content 40-50 mg/g resin) as solid support and employing mainly 9-fluorenylmethoxycarbonyl (Fmoc) amino acid 2,4,5-trichlorophenyl esters (OTcp) in the presence of 1-hydroxynbenzotriazole (HOBt) as outlined in Figure 1. Cleavage of Fmoc group after each step was carried out by 60% diethylamine (DEA) in dimethylformamide (DMF). However, MePhe (at position 3 of peptide IV) was introduced using its t-butoxycarbonyl derivative (Boc-MePhe)\textsuperscript{13} in the presence of dicyclohexyl-carbodiimide/HOBt procedure since its active ester could not be obtained in a pure crystalline form. The acidolytic cleavage of Boc group was effected using 50% trifluoroacetic acid in dichloromethane and the resulting trifluoroacetate salt was neutralized using 10% triethylamine (TEA) in DMF. The N-terminal amino acid, Tyr (in all the analogues) was protected by Boc group instead of Fmoc group to avoid partial cleavage during the subsequent ammonolysis step. Each of the protected peptide resins were subjected to ammonolysis separately and the resulting protected peptide amide was treated with 98-100% formic acid to get the free peptide amide which was then purified by gel filtration on Sephadex G-15. The purity of each peptide was checked by TLC, HPLC, amino acid analysis and elemental analysis.

Biological activity

The opioid agonistic activity of the synthetic peptides was studied using co-axially, electrically stimulated contractions of the isolated GPI longitudinal muscle-myenteric plexus (in vitro)\textsuperscript{14}. Their central opioid activity (in vivo) was assessed by analgesic test using Eddy's hot plate method\textsuperscript{15} and also by tail-flick method\textsuperscript{16}. The antidiarrhoeal activity (in vivo) was determined by the charcoal meal test\textsuperscript{17}. All the experiments were repeated at least three times.

GPI assay

Guinea pigs of either sex weighing 250-300 g were fasted for 24 h and water given ad libitum. The ileum was removed and about 2-3 cm of the ileum was mounted in an aerated organ bath maintained at 37 ± 1°C and equilibrated for 30 min in modified Kreb’s physiological solution, containing (µmoles per litre) NaCl 118, NaHCO\textsubscript{3} 25, KCl 4.75, KH\textsubscript{2}PO\textsubscript{4} 1.19, CaCl\textsubscript{2} 2.25, dextrose 11, MgSO\textsubscript{4} 0.12. The bath volume (40 mL) was kept constant throughout. The ileum was stimulated co-axially by two platinum electrodes at 0.1 Hz, 1 µsec duration, 1 µsec delay.
Table 2. Biological activity of synthetic dermorphin analogues

<table>
<thead>
<tr>
<th>Peptide</th>
<th>GPI</th>
<th>Analgesic</th>
<th>Antidiarrhoal</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean IC₅₀M</td>
<td>Relative potency</td>
<td>Hot plate (ip)</td>
</tr>
<tr>
<td>Morphine sulphate</td>
<td>—</td>
<td>1.0000</td>
<td>1.0000</td>
</tr>
<tr>
<td>[Pro⁶]DM₁¹,²¹</td>
<td>1.567 x 10⁻¹¹</td>
<td>42.57</td>
<td>10.15</td>
</tr>
<tr>
<td>[Hyp⁵]DM</td>
<td>—</td>
<td>87.00**</td>
<td>100.00**</td>
</tr>
<tr>
<td>[Pro⁶,Tyr⁷]DM¹¹</td>
<td>0.083 x 10⁻⁹</td>
<td>78.71</td>
<td>14.38</td>
</tr>
<tr>
<td>[Pro⁶, Thr⁷]DM¹¹</td>
<td>0.1533 x 10⁻¹¹</td>
<td>44.5</td>
<td>8.85</td>
</tr>
<tr>
<td>[Pro⁶, Gly⁷]DM¹⁸</td>
<td>—</td>
<td>75.00**</td>
<td>20.00**</td>
</tr>
<tr>
<td>[Sar⁴, Hyp⁶, Tyr⁷]DM</td>
<td>3.2 x 10⁻⁴ (±0.208)</td>
<td>0.0544</td>
<td>0.4261</td>
</tr>
<tr>
<td>[Hyp⁵, Pro⁷]DM</td>
<td>18.26 x 10⁻⁵ (±0.08)</td>
<td>0.0962</td>
<td>0.4883</td>
</tr>
<tr>
<td>[Pro⁶, Sar⁷]DM</td>
<td>7.7 x 10⁻⁴ (±0.1732)</td>
<td>0.0242</td>
<td>0.5426</td>
</tr>
<tr>
<td>[Phg³, Pro⁶]DM</td>
<td>7.9 x 10⁻³ (±0.0577)</td>
<td>0.0022</td>
<td>0.3909</td>
</tr>
<tr>
<td>[D-Phg³, Pro⁶]DM</td>
<td>42.26 x 10⁻⁹</td>
<td>0.5260</td>
<td>0.5802</td>
</tr>
<tr>
<td>[MePhe³, Sar⁴, Pro⁵]DM</td>
<td>32.91 x 10⁻⁹</td>
<td>0.7664</td>
<td>0.5896</td>
</tr>
<tr>
<td>[Sar⁴, Pro⁶]DM⁷</td>
<td>20.6</td>
<td>28.00**</td>
<td>—</td>
</tr>
<tr>
<td>[Pro⁶, Ser(Bzl)]²⁷]DM¹⁸</td>
<td>—</td>
<td>80.00**</td>
<td>20.00**</td>
</tr>
<tr>
<td>[Pro⁶, Abu⁷]DM¹⁸</td>
<td>—</td>
<td>75.00**</td>
<td>—</td>
</tr>
<tr>
<td>[Pro's, D-Ser⁷]DM¹⁸</td>
<td>12.11</td>
<td>47.00**</td>
<td>—</td>
</tr>
</tbody>
</table>

* Percentage inhibition of charcoal meal transit relative to morphine (= 35±3).
** Potency relative to dermorphin (=100) and the analgesic test was performed in rats

and 32V. The twitch-like contractions were recorded with the help of force transducer. The stimulation was 60 sec prior and 90 sec following the addition of the drug. A dose interval of 15 min was maintained between every stimulation. Each drug was added in a graded dosage. A log-dose response curve was plotted to determine the IC₅₀ of the drugs and compared with morphine sulphate as standard during the course of the bioassays. IC₅₀ of morphine sulphate was taken as 1 to express the relative potencies of the analogues.

**Analgesic activity**

The mice of either sex weighing between 18 and 30 g which showed reaction of 3-5 sec were selected for the experiment and the analgesic activity was
studied by using Eddy’s hot plate method (following i.v. administration) and by tail-flick method (following i.p. administration). Mice were divided into groups of six each and fasted for 24 h. Control group was given 0.2 mL saline, second group morphine sulphate (15 mg/kg) and the other groups were given DM analogues (15 mg/kg). The reaction time was recorded at 0, 15, 30, 45, 60, 90, 180 and 210 min after the drug administration.

**Eddy’s hot plate method**

The animal was placed on a hot plate which was maintained at 55 ±5°C. The time required by the animal to lick its hind paw was recorded. The recording was stopped when the reaction time reached the control value. The potencies of the analogues were calculated by dose-response curves. The maximal reaction time obtained for morphine sulphate was taken as 1 to express the relative potencies of the analogues.

**Tail-flick method**

It was carried out by placing the tip (last 1-2 cm) of the tail on the radiant heat source by using analgesiometer (Techno). The tail withdrawal from the heat (flicking response) was taken as the end point. The potencies of the analogues were calculated by dose-response curves. The maximal reaction time obtained for morphine sulphate was taken as 1 to express the relative potencies of the analogues.

**Antidiarrhoeal activity**

The charcoal meal was prepared as tragacanth (0.5%) and was fed orally with the help of catheter. Mice (18 to 30 g) of either sex were divided into groups of three each. The animals were fasted for 24 h. Control group was given 0.3 mL charcoal meal followed by the administration of 0.2 mL distilled water. Second group was treated with morphine sulphate (30 mg/kg) immediately by intraperitoneal (i.p.) route after the administration of charcoal meal. The other groups were injected with DM analogues by i.p. route immediately after the administration of charcoal meal. The animals were then maintained on a standard laboratory diet and water was given ad libitum for 30 min after which the peritoneal cavity was cut open. The distance travelled by the charcoal and the total length of the alimentary tract was measured and was expressed as percentage of total intestinal length. Mean percentage inhibition of morphine sulphate was taken as 1 to express the relative potencies of the analogues.

**RESULTS**

The relative potencies of the synthetic peptides in the GPI, analgesic and antidiarrhoeal assays with respect to morphine as standard are given in Table 2. In the GPI assay, the synthetic analogues possess agonistic activities that are less pronounced than morphine. Amongst them, analogue [MePhe³, Sar⁴, Pro⁶]DM (peptide VI) exhibits the highest potency of 75% of that of morphine. [D-Phe³, Pro⁶]DM (peptide V) has 50% activity while its L-isomer, [Phe³, Pro⁶]DM (peptide IV) is almost inactive. In the in vivo assays, the two peptides V and VI are equipotent (both analgesic and antidiarrhoeal). Substitution of Ser at position 7, and Gly at position 4 in the [Hyp⁵]DM series (peptides I and II) considerably reduces the activity in all the three assays. Note-worthy among these synthetic analogues are [Hype, Pro⁷]DM (peptide II) and [Pro⁶, Sar⁷]DM (peptide III) which are unique in possessing only analgesic activity to the virtual exclusion of the other two activities. Peptides I and II possess considerable analgesic activity but are almost inactive in the GPI assay, thus exhibiting a significant degree of selectivity. Since the analgesic activity of all the peptides tested by tail-flick method were almost similar to the results obtained by the hot-plate method, only one of the data is discussed.

**DISCUSSION**

Replacement of the C-terminal amino acid residue (Ser⁷) in DM by other amino acids (aromatic and non-aromatic) greatly alters the opioid activity⁷,⁸,¹². Thus, [Pro⁶, Tyr⁷]DM has been reported to have the most pronounced opiate agonistic activity being two times as potent as [Pro⁶]DM¹². Further, substitution of Gly at position 4 in DM by the N-methylamino acid, Sar increases the potency considerably due to enhanced stability against peptidases¹⁰. In view of these observations, Gly at position 4 in [Hyp⁵, Tyr⁷]DM was replaced by Sar and the resulting peptide, [Sar⁴, Hyp⁵, Tyr⁷]DM (peptide I), exhibited decreased analgesic and antidiarrhoeal activity; in the GPI assay it has insignificant activity.

The introduction of N-alkylamino acids in the case of another opioid pentapeptide, enkephalin (Tyr-Gly-Gly-Phe-Leu/Met) results in overall increase of the lipophilic character of the molecule leading to increase in their analgesic activities. This may mainly be due to resistance to attack by carboxypeptidases¹⁹. Further, in the dermorphin series, replacement of Gly at position 4 by Sar, and Ser at position
7 by MeSer results in retention of their opiate activity. Consequently, we decided to introduce Sar in place of Ser at position 7 in one peptide (peptide III) and this virtually has no opiate or antidiarrhoeal activity but retains 50% of the in vivo activity (analgesic). In another peptide, MePhe and Sar were incorporated in place of Phe and Gly respectively leading to [MePhe, Sar, Pro]DM (peptide VI) which was found to have only about 70-80% activity in the GPI assay and about 60-70% in the analgesic and antidiarrhoeal assays. As reported earlier by others (18), either the protection of -OH group in Ser by benzyl group or the introduction of α-aminobutyric acid in place of Ser at position 7, in general, results in the retention of activity in the resulting peptides. However, in the present series, the replacement of Ser by an amino acid Pro results in [Hyp, Pro]DM (peptide II) which is almost inactive in GPI and antidiarrhoeal assays but retains nearly 50% of analgesic activity. It indicates that the nature of the C-terminal amino acid has an important bearing on the biological activity of the molecule.

In peptide IV, Phe is substituted by its lower homologue, phenylglycine (Phg) and it has very little GPI activity but has about 40% analgesic and 70% antidiarrhoeal potency of morphine whereas its D-isomer, [D-Phg, Pro]DM (peptide V) possesses nearly 50% of the GPI activity and about 55-70% potency in the analgesic and antidiarrhoeal assays. This confirms the fact that the replacement of Phe by other aromatic amino acids results in reduced activity indicating that the presence of this amino acid is of crucial importance for biological activity. In short, the structural modifications incorporated into [Pro, Tyr]DM and [Hyp, Pro]DM at positions 3, 4, 6 and 7 largely diminish their activity. Therefore, the amino acids in these positions in the original peptides play a vital role and are essential structural elements for their activities.

From the biological activities listed in Table 2 it is clear that there is a certain degree of selectivity in their activities. Noteworthy among them are [Hyp, Pro]DM (peptide II) and [Pro, Sar]DM (peptide III) which are inactive in both GPI and antidiarrhoeal assays but have considerable analgesic potency. It has thus been possible to retain one type of activity while suppressing the others. Further, [Sar, Hyp, Tyr]DM (peptide I) and [Phg, Pro]DM (peptide IV) have considerable antidiarrhoeal and analgesic but very little GPI activity. This demonstrates that peptides with various structures can possess specificities that may prove useful in biological applications.

ACKNOWLEDGEMENTS

The authors are thankful to the Council of Scientific and Industrial Research, Govt. of India for financial support.

REFERENCES


BLOOD DONATION REDUCES THE RISK OF HEART ATTACKS

A cohort study involving 2682 middle aged men in Eastern Finland found that "the blood donors" risk of acute myocardial infarction was 86% less than that of the "non-donors". Probably, the risk of heart attack is reduced due to depletion of body iron stores (mild iron deficiency).

Source: BMJ, 15 March, 1997, p.793