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Endomorphin-1 analogs with enhanced metabolic stability and systemic analgesic activity: Design, synthesis, and pharmacological characterization

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Abstract—We synthesized four new analogs of endomorphin-1 by systematic chemical modifications. To identify the best possible drug candidates for clinical pain management and to investigate the potential contribution of these alterations to the biological activity, their pharmacological properties were determined. All of the analogs showed significantly enhanced metabolic stability. The fact that centrally mediated analgesia following peripheral administration was observed with one of the analogs suggested the approach design undertaken here had validity in the development of endomorphin-1 as a successful opioid drug for the clinic.

1. Introduction

Many peptides are potential neuropharmaceuticals, and the study of naturally occurring peptides provides a rational and potentially powerful approach in the design of peptide therapeutics. In 1997, two potent endogenous opioid peptides, endomorphin-1 (Tyr-Pro-Trp-Phe-NH_2) and endomorphin-2 (Tyr-Pro-Phe-Phe-NH_2), which have high affinity and selectivity for the μ-opioid receptor, were isolated from bovine.[1] Since their first description, these two neuropeptides have been extensively characterized pharmacologically. They are found to elicit equipotent analgesia to morphine but without some of its undesirable side effects. Furthermore, there are evidences that endomorphin-1 has a more favorable therapeutic profile than endomorphin-2 and other μ-opioids,[2–4] thus providing a new structural motif for more effective analgesics with increased therapeutic potential.

It is widely accepted that the mediation of opioid analgesic effects occurs exclusively within the central nervous system (CNS). Unfortunately, exogenous application of native opioid peptides in general has a limited in vivo efficacy, owing to their poor metabolic stability and limited delivery to the CNS. Therefore, in spite of the strong analgesic activities of endomorphins, some of their unfavorable pharmacological properties have hindered their development for clinical use. So far, although a large number of structural analogs of endomorphins have been synthesized,[5–8] only a very few are reported to be able to gain access to the CNS and produce analgesia after peripheral administration.[9–11] Very recently, potent and long-lasting analgesic effects were observed with five endomorphin-1 analogs developed by our research group when given subcutaneously.[12]

Previous studies have shown that it is the presence of an active saturable brain-to-blood efflux system at the blood–brain barrier (BBB) that limits the entry of endomorphin-1 into the brain.[13,14] Although specific transport mechanisms are perhaps the best targets to enhance their bioavailability to the brain, appropriate physicochemical modification to endomorphins remains a simple and viable method that may ultimately provide a vital increment in their CNS permeability, thus

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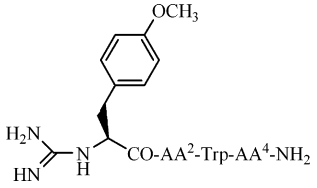
modifying their pharmacological properties following peripheral administration. Presently, two analogs of endomorphin-1 with modifications at both the amino- and the carboxyl-terminus have been synthesized and extensively investigated in attempts to develop novel peptide analgesics as well as to determine their pharmacological properties. Their corresponding analogs without C-terminal modification together with endomorphin-1 were also synthesized for comparison purpose. The modifications introduced into the molecular structure included N^{α}-guanidino-addition and *O*-methylation on Tyr[1], D-Pro-Gly substitution in position 2, and C-terminal chloro-halogenation. Several of their pharmacological properties based on the design rationale were determined and compared.

2. Results and discussion

2.1. Peptide synthesis

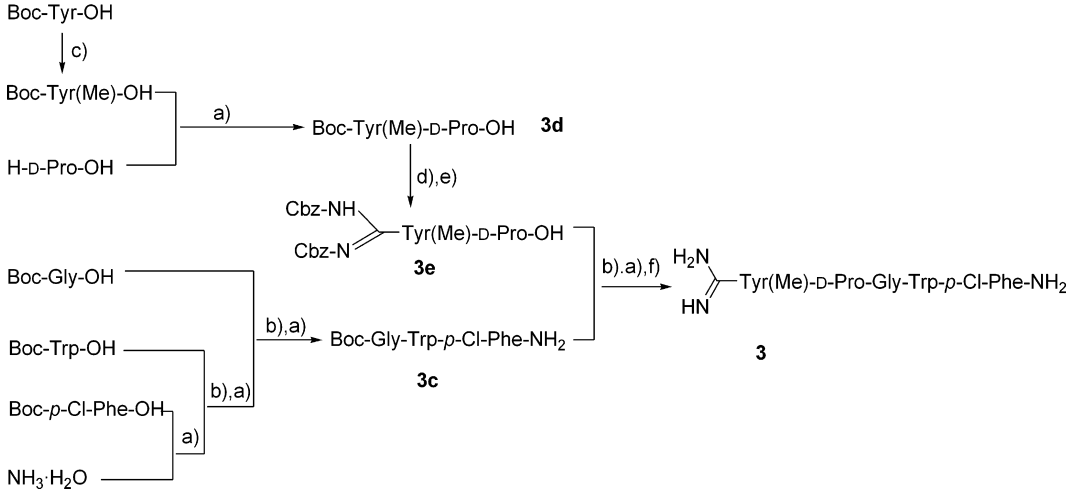
Endomorphin-1 and its four analogs, listed in Table 1, were synthesized by solution-phase method (Scheme 1). Initially, starting with H-Pro-OH/H-D-Pro-OH or Boc-Phe-NH_2/Boc-*p*-Cl-Phe-NH_2, dipeptide/tripeptide segments at the N-/C-terminus were prepared by using *N*,*N*′-dicyclohexylcarbodiimide (DCC)/*N*-hydroxysuccinimide (HOSu) coupling via a stepwise elongation of the peptide chain in the C to N direction. Most previous guanylation of peptides was carried out on the entire peptide. Herein, we had improved this procedure by combining the step-by-step elongation of the peptide chain with segment-coupling strategy for a fast and straightforward synthesis of four guanylated peptides. This strategy not only resulted in excellent yield but also reduced the risk of wasting materials. The guanylation was performed on the N-terminal dipeptide segment by treatment with a mixture of 1-(bis-benzyloxycarbonylguanyl) pyrazole and diisopropylethylamine (DIEA) in DMF. Then, the guanylated dipeptides and the C-terminal dipeptides/tripeptides were assembled in THF with excellent yield. The protecting groups were removed by catalytic hydrogenation to give the desired product. All of the purified peptides were characterized by electrospray ionization (ESI)-MS, RP-HPLC, and TLC.

Table 1. Opioid receptor binding affinities and functional bioactivities of endomorphin-1 and its analogs



Peptide no.	AA^2	AA^4	$K_i(\mu)$ (nM)	$K_i(\delta)$ (nM)	$K_i(\delta)/K_i(\mu)$	IC_{50} (nM)	
						GPI	MVD
1	L-Pro	*p*-ClPhe	538 ± 177	1368 ± 104	2.5	845 ± 173	1288 ± 268
2	L-Pro	Phe	792 ± 165	4789 ± 640	6.0	988 ± 259	1343 ± 345
3	D-Pro-Gly	*p*-ClPhe	919 ± 108	1478 ± 316	1.6	>1000	>10,000
4	D-Pro-Gly	Phe	1062 ± 121	>10,000	>9.4	>1000	>10,000
Endomorphin-1	Tyr-Pro-Trp-Phe-NH_2		4.55 ± 0.16[a]	5093 ± 660[a]	1121[a]	11.4 ± 1.13[a]	25.6 ± 3.47[a]

[a] Values are cited from our previous report.[12]



Scheme 1. Synthesis of analog **3**. Reagents: (a) DCC-HOSu; (b) 2.5 N HCl in EtOAc; (c) NaH, CH_3I in THF; (d) TFA/CH_2Cl_2 (v/v = 1:1); (e) 1-(Bis-benzyloxycarbonylguanyl)pyrazole, DIEA in DMF; (f) H_2/Pd–C in MeOH.

We have also investigated another approach for the preparation of guanylated dipeptide, in which the direct guanylation on the H-Tyr(Me)-OH was studied. However, the guanylated-Tyr(Me)-OH was proved to be unable to couple with H-Pro-OH/H-D-Pro-OH. Therefore, we did not consider this route for obtaining guanylated dipeptide.

2.2. Radioligand binding and in vitro bioactivity assays

The affinity and selectivity of endomorphin-1 and its four analogs were evaluated by radioligand binding assay using rat brain membranes and by bioassays using guinea pig ileum (GPI) and mouse vas deferens (MVD) preparations. These results are summarized in Table 1. In the radioligand binding assay, all of the four analogs showed low affinities to μ-opioid receptor, and showed no substantial δ-opioid receptor binding activity, with $K_i(\delta)$ higher than 1000 nM. In agreement with predictions from the binding data, these analogs showed low GPI and MVD potencies.

It is believed that the activity of opioid peptides requires an amino-terminal tyrosine residue that contains a free hydroxyl group.[15] However, recent studies suggest that the para hydroxyl group on the aromatic nucleus of Tyr^1 is not an absolute requirement for the receptor interaction.[16–20] In our previous report, we described the synthesis of a variety of analogs of endomorphin-1 and their pharmacological properties.[12] In the present study, the four analogs of endomorphin-1 in which the alterations in the molecular structure were essentially similar to four of the compounds disclosed previously except for the *p*-methoxyphenylalanine moiety at the cationized N-terminal. By comparing the four pairs of compounds, our data indicated that *O*-methylation decreased both the μ-opioid receptor affinity and functional bioactivity but with variable effects on δ-opioid receptor affinity; namely, for the two pairs of analogs with halogenation modification at the C-terminus, *O*-methylation increased the δ-opioid receptor affinity by approximately 2-fold, whereas for the other two pairs of corresponding non-halogenated forms, it resulted in a decrease in δ-opioid receptor affinity. These data suggested that this additional group may prevent or hinder the binding of peptide into its receptor site, thus being unfavorable for ligand-receptor interaction at the μ-opioid receptor. On the other hand, although only four analogs were analyzed here, it appeared that with the same modified Tyr in position 1, the C-terminal chloro-halogenation slightly increased the μ-affinity in reference to the corresponding non-halogenated analogs, which was distinct from the results observed previously.[12] Overall, these data led to the hypothesis that besides the undesired effect of cationization on the receptor binding ability of peptides, the impact of *O*-methylation at the N-terminus on μ-affinity was more significant than that of the C-terminal modification.

2.3. Metabolic stability

Using in vitro metabolic stability studies, we have assessed the impact of these particular chemical modifications on the stability of peptides. Table 2 summarizes the half-lives determined for all of the test peptides in twice washed 15% mouse brain membrane homogenate and 100% mouse serum. All of the analogs showed a significant increase in brain and serum stability over the parent, with the half-lives in brain exceeding 3 h. As expected, the introduction of D-Pro significantly increased the half-lives of peptides in both biological media, with effects being more significant in the serum. This finding paralleled other reports indicating that the introduction of D-amino acid in general increased the stability of peptides. An additional observation of note was that the half-lives of those halogenated analogs were shorter than those of the non-halogenated forms in the brain homogenates, whereas in the serum, the former were found to be more stable than the latter.

In the face of enhancing the metabolic stability of endomorphin-1, the unique approach undertaken here relied upon utilizing N-terminal cationization by guanidino-addition and D-Pro substitution in position 2. Previous studies have shown that cationization can improve the CNS entry of many peptides which is related to the presence of anionic sites on the endothelial cell membranes of the BBB and the ability of cationized peptides to use absorptive-mediated endocytosis (AME) to cross the BBB.[21] The highly basic nature of the guanidino function may be an important factor that controls the translocation of peptides. Besides, guanidino-addition to Tyr^1 may result in an enhanced resistance against enzymatic degradation, as seen previously.[9,12] In 2002, Paterlini et al. showed in a study that drastic loss of biological activity was observed by replacement of L-Pro with D-Pro in endomorphin-1 which may be due to a resulted change in peptide conformation.[22] Similarly, Okada et al. found that the inversion of chirality at Pro^2 in endomorphin-2 also significantly attenuated the μ affinity,[23] but this modification resulted in an

Table 2. Stability half-lives in brain and serum, octanol/buffer coefficients and percentage of protein binding in Ringer's solution for endomorphin-1 and its analogs

Peptide no.	AA^2	AA^4	Stability (half-lives, min)		Octanol/buffer coefficient (*D*)	Protein binding (%)
			Brain	Serum		
1	L-Pro	*p*-ClPhe	190 ± 3.5	48.2 ± 4.3	21.2 ± 4.8	65.57 ± 9.59
2	L-Pro	Phe	258 ± 27	31.3 ± 1.3	1.63 ± 0.21	40.58 ± 3.49
3	D-Pro-Gly	*p*-ClPhe	409 ± 34	216 ± 26.2	43.3 ± 3.56	62.52 ± 3.47
4	D-Pro-Gly	Phe	447 ± 27	165 ± 21.3	6.04 ± 0.25	45.15 ± 2.94
Endomorphin-1	Tyr-Pro-Trp-Phe-NH_2		21.2 ± 1.8[a]	9.4 ± 1.3[a]	12.5 ± 0.32[a]	32.9 ± 4.46[a]

[a] Values are cited from our previous report.[12]

analog that produced more potent and longer-lasting antinociception after ventricular administration.[24] Presently, the introduction of D-Pro in position 2 was mainly for stability enhancement purpose. However, it must be considered that the potential biologically active structure of endomorphins was suggested to be a β-turn conformation.[25] Since Gly essentially lacks a side chain and is believed to have a strong turn-forming propensity due to its flexibility and its small size, herein, we introduced Gly as a spacer residue in position 3 with the hope of ensuring a β-turn conformation of the peptide.

As for the influence of *O*-methylation on the stability, by comparison with our previous results, it was found that this structural modification resulted in a slight increase of the serum stability but had little effect on the brain stability.

2.4. Octanol/buffer distribution

The relative lipophilicity of peptides as determined by octanol/buffer distribution is shown in Table 2. Analog **3** exhibited the highest lipophilicity. Analogs **1** and **3** were found to be more lipophilic than their corresponding non-halogenated forms (analogs **2** and **4**), with *D* values being increased by 13- and 7.2-fold, respectively, indicating that halogenation of a peptide indeed increase its overall lipophilicity.[26–29] Additionally, comparisons of *D* values between analogs **1** and **3** as well as between analogs **2** and **4** revealed that the substitution of L-Pro in position 2 by D-Pro-Gly also resulted in a slight increase of lipophilicity, which was in agreement with our previous observations.[12]

The introduction of hydroxymethyl on Tyr was mainly for hydrogen bonding reduction and lipophilicity improvement purpose, which was modeled after the effect methylation has on morphine (i.e., codeine).[30] The effect of *O*-methylation on the lipophilicity can be assessed by comparing the four pairs of compounds in each of which one member differs from the other only in the N-terminal Tyr^1 of the phenolic hydroxyl by methylation. Our results indicated that this single modification increased the overall lipophilicity by approximately 2- to 5-fold. Herein, both analog **2** and analog **4** showed significantly decreased lipophilicity compared with endomorphin-1, indicating that the N-terminal cationization drastically decreased the lipophilicity despite the expected positive effect of *O*-methylation, an observation previously reported for similar analogs.[9,12] Taken together, we concluded that C-terminal chloro-halogenation contributed a greater part than *O*-methylation to the overall lipophilicity enhancement, and these two modifications in combination effectively overcame the undesired impact of N-terminal cationization on lipophilicity.

2.5. Protein binding

Protein binding also plays a major role in determining CNS uptake. We assessed the ability of peptides to bind to BSA in the mammalian Ringer's solution. As shown in Table 2, all of the analogs, with the exception of analog **2**, were found to bind to the BSA to a greater degree than endomorphin-1. It is likely that there will be less free fraction of analogs available in the circulation to gain access to the CNS and produce analgesia. However, although it is often assumed that plasma protein-bound drug is not available for transport through the BBB, studies in the past have shown that many plasma protein-bound drugs are available for transport through the brain capillary endothelial wall.[31] Therefore, extensive binding of peptides to serum protein might actually be protecting peptides from enzymatic degradation by systemic peptidases.

2.6. Assessment of antinociception

Antinociceptive activities of endomorphin-1 and its analogs were studied in the tail-flick test in mice after intracerebroventricular (icv) and subcutaneous (sc) administration. Figure 1 shows the time course of the antinociceptive activity of peptides. With icv administration, although the four analogs produced a less potent antinociceptive activity compared with the parent, with an analgesic potency being approximately 50% of that of endomorphin-1 at the same

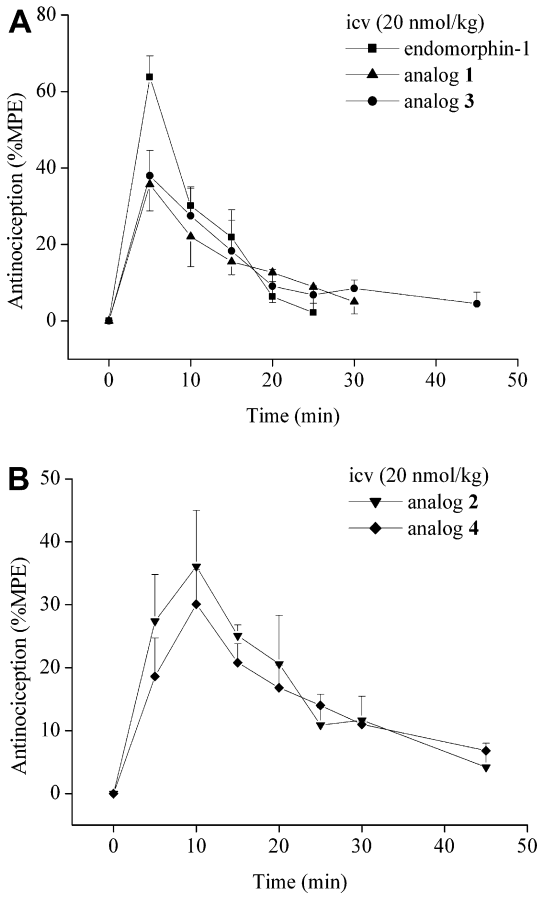


Figure 1. Time course of the antinociceptive effect of icv endomorphin-1, analogs **1** and **3** (A), and analogs **2** and **4** (B) in the mouse tail-flick test. Groups of mice were administered an injection of 20 nmol/kg of endomorphin-1 or its analogs. The tail-flick responses were measured at 5, 10, 15, 20, 25, 30, and 45 min after the injection. Each value represents the mean with SEM for 10 mice. Control mice treated with saline only did not show any significant change of nociceptive threshold, and these data are not shown.

dose, they showed longer durations of action. The peak analgesic effect of endomorphin-1 occurred at 5 min after injection, rapidly declined and returned to the pre-injection level 20 min after injection, whereas the antinociceptive effects of the analogs reached their peaks 5–10 min after injection, slowly declined, and returned to the pre-injection level 30 min after injection of analog **1** and approximately 45 min after injection of analog **2**, **3**, and **4**. We inferred that this may be related to decreased susceptibility to enzymatic degradation.

Although analog **3** had low binding to the μ- or δ-opioid receptor in rat brain membrane and was not active in the GPI and MVD assays, based on its highest lipophilicity and high stability in brain and serum among all of the peptides tested, analog **3** was selected for determination of antinociception after sc injection. Endomorphin-1 was injected as a positive control. Our results revealed that endomorphin-1 failed to induce any significant antinociception at a dose of 30 mg/kg, whereas, when equimolar dose of analog **3** was injected, low but significant antinociception was observed (Fig. 2). Significant antinociception could be detected for approximately 1 h. It is not known at present what mechanism is responsible for the improved analgesia, but we reasoned that possible explanations for this phenomenon were that the significantly enhanced serum and brain stability together with the increased lipophilicity might increase passive diffusion of peptide into the CNS, thus increasing the bioavailability of the peptide to the brain. Furthermore, it was surprising to find that with similar in vitro stability and an approximately 4-fold higher lipophilicity, analog **3** exhibited a less potent and shorter duration of action in the antinociception test after sc administration when compared to its non-methylated correlate.[12] We assumed that the lower potency was partly due to its decreased μ-opioid receptor affinity and functional bioactivity based on the current biological data tested. Besides, we found that despite its higher lipophilicity, analog **3** had a lower protein binding ability to BSA in the mammalian Ringer's solution than its non-methylated correlate (unpublished data). As illustrated in Figure 3, we have also shown that the antinociceptive effect of analog **3** after sc injection can significantly be antagonized by naloxone, but not by naloxone methiodide, an opioid antagonist that does not readily cross the BBB, indicating that the analgesia is mediated by μ-opioid receptor largely through a central mechanism.

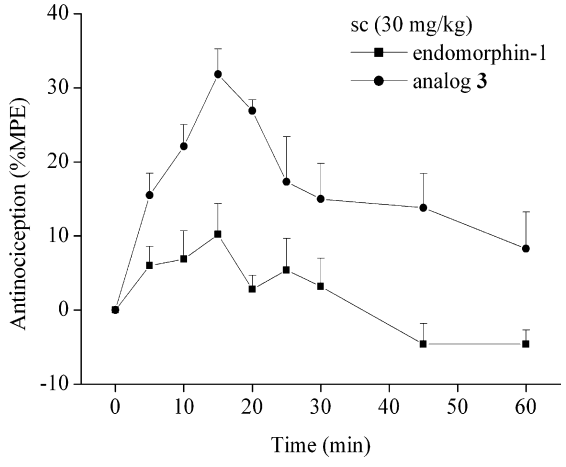


Figure 2. Time course of the antinociceptive effect of sc endomorphin-1 and analog **3** at a dose of 30 mg/kg in the mouse tail-flick test. The tail-flick responses were measured at 5, 10, 15, 20, 25, 30, 45, and 60 min after the injection. Each value represents the mean with SEM for six to eight mice. Control mice treated with saline only did not show any significant change of nociceptive threshold, and these data are not shown.

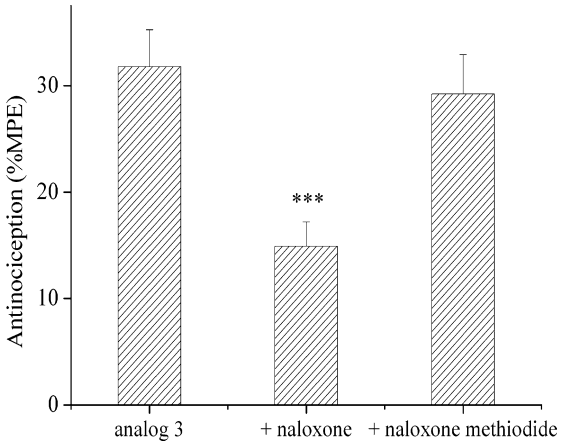


Figure 3. Antagonism of antinociception elicited by sc 30 mg/kg of analog **3** in the tail-flick assay by naloxone (10 nmol/mouse icv, administered 5 min before drug) and naloxone methiodide (10 mg/kg sc, administered 10 min before drug). Each value represents the mean with SEM for five to seven mice. The vertical line indicates the SEM of the mean. $^{***}p < 0.001$, compared with the drug-injected control.

3. Conclusion

The scope of the present study was to investigate the pharmacological characteristics of endomorphin-1 and a series of its analogs designed by systematic structural modification, and to identify the best possible drug candidates for clinical nociceptive pain management as well as to determine the potential contribution of these alterations to the biological activity, thus furthering our knowledge of the structural requirements necessary for pharmacological effects. All of the analogs had low binding to the μ- or δ-opioid receptor in rat brain membrane and showed low GPI and MVD potencies. Cationization of endomorphin-1 by guanidino-addition on Tyr^1 led to a significant increase in metabolic stability in both brain and serum. The introduction of D-Pro-Gly in place of L-Pro not only drastically enhanced their stability but also resulted in a slight increase in lipophilicity. C-terminal chloro-halogenation produced analogs that all showed an increase in lipophilicity and in protein binding affinity over their corresponding non-halogenated forms. By comparing the four pairs of analogs of cationized endomorphin-1 in each of which one member differed from the other only in the N-terminal Tyr^1 of the phenolic hydroxyl by methylation that were previously reported, we concluded that *O*-methylation increased the overall lipophilicity and serum stability with no significant influence on brain stability, while at

the same time decreasing μ-opioid receptor affinity and functional bioactivity but with variable effects on δ-receptor affinity. Antinociception studies showed that icv administration of the four analogs elicited less potent but longer-lasting analgesia. Inconsistent with its binding property, the selected analog **3**, N^{α}-amidino-Tyr(Me)-D-Pro-Gly-Trp-*p*-Cl-Phe-NH_2, was found to display potent and prolonged antinociceptive activity upon sc administration through a central mechanism. Overall, the results indicated that these combined chemical modifications in the molecular structure of endomorphin-1 resulted in alterations in receptor binding, lipophilicity, stability, protein binding affinity, and possibly some other physicochemical properties, which in combination contributed to the altered analgesic effects. Finally, due to its enzyme resistance and antinociceptive activity after peripheral administration, analog **3** can be regarded as a promising modified endomorphin model and may validate further study of developing a peptide analgesic based on the endomorphin sequence.

4. Experimental section

4.1. Chemicals

Radioligands [^{3}H]*H*-Tyr-D-Ala-Gly-MePhe-Gly-ol and [^{3}H]H-Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH were purchased from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Commercial N-Boc/Cbz-protected amino acids were obtained from GL Biochem (Shanghai) Ltd, China. 1H-Pyrazole-1-carboxamidine hydrochloride was purchased from Aldrich Chemical Co. (Milwaukee, WI). Naloxone hydrochloride and naloxone methiodide were purchased from Sigma–Aldrich (St. Louis, MO).

4.2. General methods

Synthesis of all of the peptides was conducted by conventional solution methods with N-Boc and N-Cbz protection and DCC/HOSu coupling. *N*-α-*t*-Boc-*O*-methyl-tyrosine was prepared by simple methylation of Boc-Tyr-OH with sodium hydride (NaH) and iodomethane (CH_3I) in THF and used without further purification. 1-(bis-benzyloxycarbonylguanyl) pyrazole was prepared according to the reported method with Cbz-Cl, Cbz-OSu, and 1H-pyrazole-1-carboxamidine.[32] The first step in the synthesis of the two of chloro-halogenated analogs involved the preparation of the amide of Boc-*p*-Cl-Phe-OH that was easily achieved by DCC/HOSu coupling with ammonia. TLC was performed on silica gel 60 F_{254}-precoated glass plates with the following solvent systems: (I) ethyl acetate/petroleum ether (2:2), (II) ethyl acetate/petroleum ether (2:1), (III) ethyl acetate/petroleum ether (3:1), (IV) ethyl acetate/methanol (5:0.3), and (V) ethyl acetate/methanol/ammonia (6:2:1). UV light, I_2 vapor, and water were applied to visualize the TLC spots. Flush column chromatography for the intermediates and final products was performed using silica gel 60. Analytical HPLC was performed on a reverse phase Water Delta Pak C_{18} column (3.9 × 150 mm) with the absorbance monitored at 280 nm. An eluting system of a linear gradient of 20–80% acetonitrile in 0.1% aqueous trifluoroacetic acid (TFA) over 15 min was used. The flow rate was 0.6 mL/min.

4.3. Synthesis of Boc-*p*-Cl-Phe-NH_2 (3a)

To a stirred suspension of Boc-*p*-Cl-Phe-OH (900 mg, 3 mmol) and HOSu (380 mg, 3.3 mmol) in anhydrous THF (15 mL), a solution of DCC (742 mg, 3.6 mmol) in anhydrous THF (5 mL) was added at 0 °C. The reaction mixture was stirred for 30 min at the same temperature and 6 h at room temperature. After *N*,*N*′-dicyclohexylthiourea (DCU) was discarded by filtration, to the remained solution was added ammonia (2 mL) at 0 °C. The reaction mixture was stirred for 30 min at the same temperature and overnight at room temperature. Solvents were then evaporated in vacuo and the residue was solidified from ethanol and H_2O to give white powder (882 mg, 98%). ESI-MS: calculated for $C_{14}H_{19}ClN_2O_3$ $[M+H]^+$, 300.11; found, 300.29; TLC R_f (I) 0.35.

4.4. Synthesis of Boc-Trp-*p*-Cl-Phe-NH_2 (3b)

Boc-*p*-Cl-Phe-NH_2 (1.05 g, 3.5 mmol) was dissolved into 2.5 N HCl/EtOAc (20 mL) and stirred for 2 h at room temperature. The pH of the solution was adjusted to 9 with 2 N NaOH. To a stirred suspension of Boc-Trp-OH (1.12 g, 3.67 mmol) and HOSu (464 mg, 4.0 mmol) in anhydrous THF (20 mL), DCC (907 mg, 4.4 mmol) in THF (5 mL) was added to the solution at 0 °C. The reaction mixture was stirred for 30 min at the same temperature and 6 h at room temperature. DCU was discarded by filtration. The remained solution was added to the amine component at 0 °C. This mixture was stirred for 30 min at the same temperature and overnight at room temperature. Solvents were then evaporated in vacuo and the residue was diluted with EtOAc (200 mL) and washed with 5% citric acid, saturated $NaHCO_3$, and brine. The organic phase was dried over Na_2SO_4, filtered, and evaporated in vacuo. The residue was crystallized from EtOAc to give **3b** as white powder (1.58 g, 93%). ESI-MS: calculated for $C_{25}H_{29}ClN_4O_4$ $[M+H]^+$, 486.19; found, 486.18; TLC R_f (III) 0.32.

4.5. Synthesis of Boc-Gly-Trp-*p*-Cl-Phe-NH_2 (3c)

The same peptide coupling procedure as for compound **3b** for the synthesis of Boc-Gly-Trp-*p*-Cl-Phe-NH_2 was employed. The Boc-protected tripeptide was solidified from EtOAc to give **3c** as white powder with a yield of 95%. ESI-MS: calculated for $C_{27}H_{32}ClN_5O_5$ $[M+H]^+$, 543.21; found, 543.57; TLC R_f (IV) 0.57.

4.6. Synthesis of Boc-Tyr(Me)-D-Pro-OH (3d)

The same peptide coupling procedure as for compound **3b** for the synthesis of Boc-Tyr(Me)-D-Pro-OH was employed, but the saturated $NaHCO_3$ washing in the workup process was skipped. The residue was purified by flush chromatography eluted with petroleum ether/EtOAc/acetic acid (50:80:1) to give **3d** with a yield of 78%. ESI-MS: calculated for $C_{20}H_{28}N_2O_6$ $[M+H]^+$, 393.20; found, 393.27; TLC R_f (III) 0.42.

4.7. Synthesis of N^{α}-(*N*, *N*′-Bis-benzyloxycarbonyl) amidino-Tyr(Me)-D-Pro-OH (3e)

Dipeptide Boc-Tyr(Me)-D-Pro-OH (250 mg, 0.64 mmol) was dissolved in TFA/CH_2Cl_2 (v/v = 1:1, 2 mL) at 0 °C and stirred for 1 h. Solvents were then evaporated by EtOAc in vacuo to give Boc-removed dipeptide as colorless oil (178 mg, 0.61 mmol). Under argon, this amine component and (benzyloxycarbonylimino-pyrazol-1-yl-methyl) carbamic acid benzyl ester (193 mg, 0.51 mmol) were dissolved in DMF (1.5 mL). The pH of the solution was adjusted to 8 with DIEA at 0 °C. The mixture was stirred for 72 h at room temperature, and DIEA was added in the middle of the reaction to keep the pH 8. The reaction mixture was poured into 5% citric acid, and the precipitated oil was extracted with EtOAc (150 mL). The EtOAc solution was washed with brine, dried over Na_2SO_4, filtered, and evaporated in vacuo. The residual oil was purified by flush chromatography eluted with petroleum ether/ethyl acetate/acetic acid (10:17:1) to give colorless oil (235 mg, 77%). ESI-MS: calculated for $C_{32}H_{34}N_4O_8$ $[M+H]^+$, 603.24; found, 603.11; TLC R_f (II) 0.43.

4.8. Synthesis of N^{α}-(*N*, *N*′-Bis-benzyloxycarbonyl) amidino-Tyr(Me)-D-Pro-Gly-Trp-*p*-Cl-Phe-NH_2 (3f)

The same peptide coupling procedure as for compound **3b** for the synthesis of N^{α}-(*N*, *N*′-bis-benzyloxycarbonyl) amidino-Tyr(Me)-D-Pro-Gly-Trp-*p*-Cl-Phe-NH_2 was employed. The residual oil was purified by flush chromatography eluted with petroleum ether/ethyl acetate/methanol/acetic acid (20:80:4:1) to give colorless oil with a yield of 68%. ESI-MS: calculated for $C_{54}H_{56}ClN_9O_{10}$ $[M+Na]^+$, 1049.53; found, 1049.70; TLC R_f (IV) 0.32.

4.9. Synthesis of N^{α}-amidino-Tyr(Me)-D-Pro-Gly-Trp-*p*-Cl-Phe-NH_2 (3)

To the solution of protected pentapeptide in methanol [6:1, w/v (mg/mL)] was added 20% Pd–C. This solution was vigorously stirred under H_2 atmosphere for 2 h at room temperature. The catalyst was removed by filtration, and the filtrate was concentrated in vacuo. The residue was purified by flush chromatography eluted with ethyl acetate/methanol/ammonia (45:20:8) to give a white powder with a yield of 51%. ESI-MS: calculated for $C_{38}H_{44}ClN_9O_6$ $[M+H]^+$, 758.31; found, 758.21; TLC R_f (V) 0.35. RP-HPLC t_R = 10.37 min.

4.10. N^{α}-amidino-Tyr(Me)-Pro-Trp-*p*-Cl-Phe-NH_2 (1)

ESI-MS: calculated for $C_{36}H_{41}ClN_8O_5$ $[M+H]^+$, 701.29; found, 701.25; TLC R_f (V) 0.34; RP-HPLC t_R = 10.25 min.

4.11. N^{α}-amidino-Tyr(Me)-Pro-Trp-Phe-NH_2 (2)

ESI-MS: calculated for $C_{36}H_{42}N_8O_5$ $[M+H]^+$, 667.33; found, 667.24; TLC R_f (V) 0.31; RP-HPLC t_R = 9.74 min.

4.12. N^{α}-amidino-Tyr(Me)-D-Pro-Gly-Trp-Phe-NH_2 (4)

ESI-MS: calculated for $C_{38}H_{45}N_9O_6$ $[M+H]^+$, 724.35; found, 724.28; TLC R_f (V) 0.32; RP-HPLC t_R = 9.86 min.

4.13. Tyr-Pro-Trp-Phe-NH_2 (endomorphin-1)

ESI-MS: calculated for $C_{34}H_{38}N_6O_5$ $[M+H]^+$, 611.29; found, 611.30; TLC R_f (V) 0.63; RP-HPLC t_R = 9.19 min.

4.14. Radioligand binding and in vitro bioactivity assays

The affinity and selectivity of peptides had been investigated by radioligand binding and functional in vitro bioassay. Competition binding studies of endomorphin-1 and its analogs for the μ-opioid receptor and δ-opioid receptor were performed on synaptosomal brain membranes according to the literature method.[33] All binding experiments were performed in 50 mM Tris–HCl buffer, pH 7.4, at a final volume of 0.5 mL containing 300–500 μg/mL protein. The following conditions were used for incubations: [^{3}H]Tyr-D-Ala-Gly-MePhe-Gly-ol, 0.5 nM, 1 h; [^{3}H]Tyr-c[D-Pen-Gly-Phe-D-Pen]-OH, 1 nM, 3 h. The extent of non-specific binding was determined in the presence of 10 μM naloxone. All experiments were carried out in duplicate assays and repeated at least three times. K_i values were calculated according to the equation of Cheng and Prusoff.[34]

In vitro opioid activities of peptides were tested in the guinea pig ileum (GPI) and mouse vas deferens (MVD) bioassays as reported previously.[12] Briefly, the GPI tissue and MVD tissue were mounted in a 10-mL bath that contained aerated (95% O_2, 5% CO_2) Krebs-Henseleit solution at 37 and 36 °C, respectively. Twitch contractions were evoked by rectangular pulses with the following parameters: 0.1-Hz, 50-V, 0.5-ms pulse width for GPI assay; pairs (100-ms pulse distance) of rectangular impulses (1-ms pulse width, 9 V/cm, i.e., supramaximal intensity) were repeated by 10 s for MVD assays. Isometric responses were recorded using a train gauge transducer linked to a recorder system (model BL-420E$^+$, Taimeng technology Corporation of Chengdu, China). Dose–response curves were constructed, and IC_{50} values were calculated graphically. The values were arithmetic means of five to eight measurements.

4.15. Metabolic stability

Enzymatic degradation studies of endomorphin-1 and its analogs were carried out using 15% mouse brain homogenate and 100% mouse serum that were prepared as described previously.[35] A final protein concentration of 2.3 mg/mL in 50 mM Tris buffer, pH 7.4, was used for all incubations. About 10 μL of a 10 mM peptide stock solution was added to 190 μL of the matrix. To resuspended 15% brain homogenate or serum were added peptides to a final concentration of 500 μM. Incubations were carried out at 37 °C for 0, 60, 120, 180, 240, and 300 min in triplicate. Aliquots of 20 μL were withdrawn from the incubation mixtures and

enzyme activity was terminated by precipitating proteins with 90 μL of glacial acetonitrile, vortexing, and maintaining the sample on ice for a couple of minutes. Samples were then diluted with 90 μL of 0.5% acetic acid to prevent further enzymatic breakdown and centrifuged at 13,000*g* for 15 min. The supernatants were collected for analysis by RP-HPLC as described below. The rate constants of degradation (k) were obtained by least square linear regression analysis of logarithmic peptides, peak areas [ln (A_t/A_0)] versus time courses, using a minimum of five points. Degradation half-lives ($t_{1/2}$) were calculated from the rate constants as $\ln 2/k$.

4.16. Octanol/buffer distribution

Partition coefficients for peptides were expressed as the ratio of peptide found in the octanol phase to that found in the aqueous phase. Equal volumes of octanol and 0.05 M Hepes buffer in 0.1 M NaCl, pH 7.4, were mixed and allowed to equilibrate for 12 h. The layers were then separated and stored at 4 °C. At testing, 50 μg of peptide was added to 500 μL of the Hepes buffer and mixed with 500 μL of octanol by vortexing for 2 min. The octanol/buffer solution was centrifuged in a Beckman microfuge (Beckman Coulter, Fullerton, CA) for 1 min at 4000 rpm. After separation into aqueous and octanol phases, peptide content of the aqueous phase was quantified by RP-HPLC. A portion of the octanol phase was lyophilized and reconstituted in methanol before RP-HPLC analysis. All octanol/buffer distribution studies were performed in triplicate. The octanol/buffer distribution coefficient (D) was calculated as the ratio of octanol layer to the buffer layer.

4.17. Protein binding

The binding affinity of the peptides to BSA in the mammalian Ringer's solution [117.0 mM NaCl, 4.7 mM KCl, 0.8 mM $MgSO_4$, 24.8 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 10 mM D-glucose, 3.9% dextran (mol. wt. 70,000), and BSA, 10 g L^{-1}, pH 7.4] was determined by ultrafiltration centrifugal dialysis. Peptides were added to 0.5 mL of Ringer's solution prewarmed to 37 °C and then ultrafiltered using a Centrifree™ micropartition device (cut-off mol. wt. = 30,000; Millipore, Americon) by centrifugation at 2000*g* for 10 min to separate the protein-bound drugs from free drugs. For control samples, drugs were pipetted into filtered blank Ringer's solution as 100% recovery standards. The total concentration (T) of peptides introduced into the system and the amount found in the ultrafiltrate (F) were determined by RP-HPLC analysis as described below. All protein binding studies were performed in triplicate. Percentage of peptide bound to BSA was calculated as: % bound = $(T - F)/T \times 100$.

4.18. RP-HPLC analysis

Samples from stability, octanol/buffer distribution, and protein binding studies were analyzed by RP-HPLC on a Water Delta Pak C_{18} column (3.9 × 150 mm; Milford, MA) with the absorbance monitored at 280 nm. The solvents for analytical HPLC were as follows: A, 0.1% trifluoroacetic acid in water; and B, 0.1% trifluoroacetic acid in acetonitrile. The column was eluted at a flow rate of 0.6 mL/min with a linear gradient of A:B = 80:20 to A:B = 20:80 for 12 min and A:B = 20:80 to A:B = 80:20 for 3 min.

4.19. Assessment of antinociception

Antinociceptive activities of endomorphin-1 and its analogs were assessed using the 50 °C warm water tail-flick test in mice after icv (4 μL) and sc (100 μL) administration. Nociception was evoked by immersing the mouse's tail in hot water (50 ± 0.2 °C) and measuring the latency to withdrawal. Before treatment, each mouse was tested, and the latency to tail-flick was recorded [control latency (CL)]. Mice not responding within 5 s were excluded from further testing; the tail-flick responses were measured at different times after icv or sc injection of drugs. The latency to tail-flick was defined as the test latency (TL); a cut-off of 10 s was adopted; 0.9% saline was used as control. The antinociceptive response was expressed as percentage of maximal possible effect (%MPE), calculated by the following equation: $\%MPE = 100 \times (TL - CL)/(10 - CL)$. For the study involving the opioid antagonists, animals were pretreated with naloxone (10 nmol/mouse icv) or naloxone methiodide (10 mg/kg sc) before sc challenge with peptide (30 mg/kg).

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