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The biological activity and metabolic stability of peptidic bifunctional compounds that are opioid receptor agonists and neurokinin-1 receptor antagonists with a cystine moiety ☆

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ABSTRACT

In order to improve metabolic stability, a ring structure with a cystine moiety was introduced into TY027 (Tyr-D-Ala-Gly-Phe-Met-Pro-Leu-Trp-NH-[3′,5′-$(CF_3)_2$Bzl]), which is a lead compound of our developing bifunctional peptide possessing opioid agonist and NK1 antagonist activities. TY038 (Tyr-*cyclo*[D-Cys-Gly-Phe-Met-Pro-D-Cys]-Trp-NH-[3′,5′-$(CF_3)_2$Bzl]) was found as a highly selective δ opioid agonist over μ receptor in conventional tissue-based assays, together with an effective NK1 antagonist activity and good metabolic stability with more than 24 h half life in rat plasma.

1. Introduction

The opioid drugs are strong analgesics widely used following major surgery and controlling the pain of terminal diseases such as cancer, but their use is limited by several undesired side effects, including the development of tolerance, sedation, euphoria, constipation, respiratory depression and physical dependence.[1–3] It has been reported that prolonged pain as well as sustained opioid administration develops neuroplastic changes in the central nerve system (CNS) in which pain-enhancing neurotransmitters, such as substance P, and their corresponding receptors are up-regulated to lead to more pain and tolerance.[4–6] Therefore, it is not surprising that current medications, which can modulate only at single receptor, cannot control severe and sustained neuropathic pain states.

To effectively address this problem we have proposed that treatment of these pain states will require the design of new kinds of multiple ligands.[7–12] One of the approaches we have taken involves the design and synthesis of novel bifunctional peptide ligands that can act as agonists at the δ/μ opioid receptors and that can also act as antagonists for the neurokinin-1 (NK1) receptor. The important advantage of these bifunctional ligands is that they could counteract against the increased expression of substance P and NK1 receptors in CNS, this generally has not been considered in current drug design. In fact, several pharmacological studies have suggested an effective role for substance P antagonists to enhance analgesic potency of opiates as well as to eliminate the development of opioid-induced tolerance, and that the combination of these two bioactivities could be expected to show an enhanced analgesic effect without showing undesirable side

Abbreviations: AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; BuOH, butanol; BSA, bovine serum albumin; Cl-HOBt, 1-hydroxy-6-chlorobenzotriazole; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; DCM, dichloromethane; DIEA, diisopropylethylamine; DMF, *N,N*-dimethylformamide; DMSO, dimethylsulfoxide; DPC, dodecylphosphocholine; DPDPE, c[D-Pen^2, D-Pen^5]-enkephalin; DQF-COSY, double quantum filtered correlation spectroscopy; DAMGO, [D-Ala^2, NMePhe^4, Gly^5-ol]-enkephalin; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; FAB, fast-atom bombardment; Fmoc, fluorenylmethoxycarbonyl; GPI, guinea pig isolated ileum; HCTU, 1H-benzotriazolium-1-[bis(dimethylamino)methylene]-5-chloro-hexafluorophosphate-(1-),3-oxide; HRMS, high-resolution mass spectroscopy; LMMP, longitudinal muscle with myenteric plexus; MVD, mouse vas deferens; NK1, Neurokinin-1; RP-HPLC, reverse phase-high performance liquid chromatography; SPPS, solid-phase peptide synthesis; TFA, trifluoroacetic acid; TLC, thin-layer chromatography, TOCSY, total correlation spectroscopy; TOCSY, total correlation spectroscopy; Trp-NH-[3′,5′-$(CF_3)_2$Bzl], 3′,5′-(bistrifluoromethyl)-benzyl amide of tryptophan.

☆ Abbreviations used for amino acids and designation of peptides follow the rules of the IUPAC-IUB Commission of Biochemical Nomenclature in *J. Biol. Chem.* **1972**, *247*, 977.

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effects.[9,10,12–18] Therefore, the developing bifunctional compounds could be a useful research tool, and are expected to act as potent drug candidates for severe pain control. In fact, our lead bifunctional compounds, TY005 (Tyr^1-D-Ala^2-Gly^3-Phe^4-Met^5-Pro^6-Leu^7-Trp^8-O-[3′,5′-$(CF_3)_2$Bzl]) and TY027 (**1**: Tyr^1-D-Ala^2-Gly^3-Phe^4-Met^5-Pro^6-Leu^7-Trp^8-NH-[3′,5′-$(CF_3)_2$Bzl]), have been shown to reverse neuropathic pain without producing the opioid-induce tolerance, to prove our opioid agonist/NK1 antagonist bifunctional concept in the animal models of neuropathic pain.[19,20]

In this article, we report on a new class of novel bifunctional compounds possessing good resistance against metabolic degradation to improve their therapeutic potential as analgesics. A common and effective drug-design strategies to improve metabolic stability is the introduction of a disulfide ring. Through the limitation of the conformational flexibility with stabilization in terms of secondary structures and orientations of important amino-acid residues, this approach could reduce chances to be recognized by the undesirable receptors and proteins, including peptide degrading enzymes.

Another focus of this report is the δ/μ opioid selectivity of the bifunctional ligands. It is well known that the strong pain-controlling effect of morphine mainly comes through μ opioid receptor stimulation,[1–3] but the activation of the μ opioid receptor is also associated with several undesired side effects. While, the δ opioid receptor-selective agonists may possess potential clinical merits compared to the μ selective agonists: these are greater relief of neuropathic pain,[21] reduced respiratory depression,[22] and no constipation,[23] as well as a reduced potential for the development of physical dependence.[24,25] Therefore, a bifunctional compound with selective agonist activity at the δ opioid receptor is an interesting approach in which we could find novel analgesics possessing strong pain-controlling effects without development of tolerance, physical dependence and other toxicities. It should be noted that the cyclic constraint could decrease the unfavorable entropy loss on binding at the targeting receptor, and thus often results in the improved selectivity and a change in its mode of action for a targeted receptor. In fact, it was reported that cyclic structure in the derivatives of enkephalin leads to structured conformation for the backbone atoms as well as for the important pharmacophores in binding at the opioid receptors, including the two aromatic amino-acid residues Tyr^1 and Phe^4, and the protonated N-terminal nitrogen.[26]

Based on the above discussion, the disulfide ring was introduced into the previously reported bifunctional compound **1**, which had moderately δ-selective affinities in radioligand binding assay as well as in classical isolated tissue-based assays (Table 1).[10] For the design of novel cyclic peptide derivatives, D-Cys was introduced into the second position of **1**, since it is well known that the introduction of several types of D-amino acids in the second position of enkephalin analogues is well accepted.[2,27–29] Residues 5 and 7 were selected as the other sites to be cyclized, since Tyr^1, Gly^3, Phe^4 and Trp^8 were considered as the major 'message' residues of each pharmacophore,[10] and Pro^6 had a positive effect on affinity at the NK1 receptor (Fig. 1).[30] Since two β-turn structural elements in **1** was observed at the D-Ala^2-Met^5 and Pro^6-*C*-terminal benzyl moiety,[10] cyclization between residues 2 and 5 ([D-Cys^2, Cys^5]TY027, **2** and [D-Cys^2, D-Cys^5]TY027, **3**) was expected to stabilize the corresponding β-turn of **1**, while the introduction of a disulfide bond for residues 2 and 7 ([D-Cys^2, D-Cys^7]TY027, **4** and [D-Cys^2, Cys^7]TY027, **5**) might eliminate the turn structure in the Pro^6-*C*-terminal benzyl moiety.

2. Chemistry

The peptide derivatives **2**–**5** were synthesized using previously published methods (the detailed experimental condition is avail-

Table 1
Binding affinity, GTP binding assay and E_{max} % for bifunctional peptide derivative ligands at opioid and neurokinin receptors

No.	Radioligand binding[a,b]								[^{35}S]GTPγS binding[a]					
	δ[c,d]		μ[c,e]		hNK1[f,g]		rNK1[f,h]		δ[c]			μ[c]		
	Log IC_{50}	K_i (nM)	Log IC_{50}	K_i (nM)	Log IC_{50}	K_i (nM)	Log IC_{50}	K_i (nM)	Log EC_{50}[i]	EC_{50} (nM)[j]	E_{max}[k] (%)	Log EC_{50}[i]	EC_{50}[j] (nM)	E_{max}[k] (%)
1[l]	−8.84 ± 0.07	0.66	−7.44 ± 0.05	16	−10.9 ± 0.10	0.007	−7.61 ± 0.03	7.3	−8.07 ± 0.11	8.6	60 ± 2	−8.16 ± 0.17	7.0	51 ± 3
2	−7.65 ± 0.16	5.0	−6.37 ± 0.10	200	−8.14 ± 0.04	3.7	−5.75 ± 0.09	560	−7.29 ± 0.12	51	36 ± 2	−6.93 ± 0.51	120	16 ± 3
3	−7.81 ± 0.11	7.8	−6.94 ± 0.03	52	−9.00 ± 0.02	0.52	−6.86 ± 0.08	45	−7.01 ± 0.13	98	22 ± 1	−6.77 ± 0.47	170	7 ± 1
4	−8.57 ± 0.15	1.3	−6.78 ± 0.09	79	−9.73 ± 0.03	0.10	−7.02 ± 0.09	30	−10.76 ± 0.51	0.02	11 ± 1	−7.44 ± 0.25	36	36 ± 3
5	−6.93 ± 0.14	54	−6.50 ± 0.09	160	−9.31 ± 0.02	0.25	−7.65 ± 0.09	7.1	−7.88 ± 0.31	13	110 ± 9	−7.66 ± 0.33	22	9 ± 1
Tyr-DAla-Gly-Phe-Met-NH_2	−8.86 ± 0.05	0.66	−8.90 ± 0.16	0.50										
DCLCE-NH_2[m]		1.6		1.7										
DCDCE-NH_2[m]		0.82		0.55										
Biphalin		2.6[n]		1.4[n]					−8.95 ± 0.17	1.1	83 ± 4			
DAMGO	−8.84 ± 0.07	0.66	−7.44 ± 0.05	16								−7.44 ± 0.19	37.0	150
L-732,138					−8.83 ± 0.02	0.73	−6.40 ± 0.03	134						

[a] The experiments were run in at least two independent experiments performed in duplicate.
[b] The log IC_{50} ± standard errors were determined from nonlinear least squares regression analysis. The K_i values are calculated using the Cheng and Prusoff equation to correct for the concentration of the radioligand used in the assay.
[c] The analyses were carried out using membrane preparations from transfected cells that constitutively expressed human δ opioid receptor or rat μ opioid receptors.
[d] K_d = 0.45 ± 0.1 nM.
[e] K_d = 0.50 ± 0.1 nM.
[f] Competition analyses were carried out using membrane preparations from transfected cells that constitutively expressed human or rat NK1 receptors.
[g] K_d = 0.16 ± 0.03 nM.
[h] K_d = 0.40 ± 0.17 nM.
[i] Log EC_{50} ± standard errors were determined from nonlinear regression analysis.
[j] Anti-logarithmic value of the respective log EC_{50}.
[k] The E_{max} value is the saturable, maximum level of [^{35}S]GTPγS binding in the cell membranes upon incubation with the peptide, expressed as [Net [^{35}S]GTPγS bound/basal [^{35}S]GTPγS bound] × 100 ± standard error.
[l] Ref. 10.
[m] Ref. 35.
[n] Ref. 43.

NK1 pharmacophore

1 (TY027): H-Tyr-*D*-Ala-Gly-Phe-Met-Pro-Leu-Trp-NH-[3',5'-$(CF_3)_2$Bzl]

opioid pharmacophore

2 (TY039): H-Tyr-c[*D*-Cys-Gly-Phe-Cys]-Pro-Leu-Trp-NH-[3',5'-$(CF_3)_2$Bzl]

3 (TY037): H-Tyr-c[*D*-Cys-Gly-Phe-*D*-Cys]-Pro-Leu-Trp-NH-[3',5'-$(CF_3)_2$Bzl]

4 (TY035): H-Tyr-c[*D*-Cys-Gly-Phe-Nle-Pro-Cys]-Trp-NH-[3',5'-$(CF_3)_2$Bzl]

5 (TY038): H-Tyr-c[*D*-Cys-Gly-Phe-Nle-Pro-*D*-Cys]-Trp-NH-[3',5'-$(CF_3)_2$Bzl]

DPDPE: H-Tyr-c[*D*-Pen-Gly-Phe-D-Pen]

DCLCE-NH_2: H-Tyr-c[*D*-Cys-Gly-Phe-Cys]-NH_2

DCDCE-NH_2: H-Tyr-c[*D*-Cys-Gly-Phe-*D*-Cys]-NH_2

L-732,128: Ac-Trp-O-[3',5'-$(CF_3)_2$Bzl]

Figure 1. Sequences of opioid and NK1 receptor peptides.

able in the Section 5.2).[10,12,31] Briefly, the linear peptides, Boc-Tyr(tBu)-D-Cys(Trt)-Gly-Phe-Cys(Trt)-Pro-Leu-Trp(Boc)-OH, Boc-Tyr(tBu)-D-Cys(Trt)-Gly-Phe-D-Cys(Trt)-Pro-Leu-Trp(Boc)-OH, Boc-Tyr(tBu)-D-Cys(Trt)-Gly-Phe-Nle-Pro-Cys(Trt)-Trp(Boc)-OH, and Boc-Tyr(tBu)-D-Cys(Trt)-Gly-Phe-Nle-Pro-D-Cys(Trt)-Trp(Boc)-OH were synthesized using N^{α}-Fmoc chemistry with HCTU as a coupling reagent on a 2-chlorotrityl resin, followed by cleavage using 1% TFA in DCM. The C-terminal amide bond formation was performed using standard EDC/Cl-HOBt coupling chemistry with 2 equiv of reactant amine with the protected C-terminal-free peptide. All the acid-labile protecting groups were removed with the cleavage cocktail treatment (82.5% v/v TFA, 5% water, 5% thioanisole, 2.5% 1,2-ethanedithiol, and 5% phenol) to quench the highly stabilized carbocations released from permanent protecting groups, to give the linear crude peptides which were directly cyclized using the classical oxidizing agent $K_3Fe(CN)_6$. Oxidation was performed by slowly adding a solution of the linear peptide into a reaction vessel containing an excess of the oxidizing agent in aqueous solution. The concentration of the reduced peptide was regulated by controlling the speed of addition of the peptide with the help of an automated syringe pump. The obtained crude oxidized peptides were concentrated using the solid-phase extraction technique with C-18 reversed-phase silica gel column, and then purified with RP-HPLC followed by lyophilizion (>99%). The final purified peptides were characterized by analytical HPLC, ^{1}H NMR, HRMS, and TLC. ^{1}H NMR studies showed *cis/trans* isomerization at the Pro^6 residue. These analytical data as well as the ratios of two amide rotamers are available in the Supplementary data.

3. Pharmacological studies

3.1. Biological activities

The biological evaluation of synthesized cyclic peptide derivatives were performed as previously described.[9,10,12] The binding affinities of the synthesized bifunctional peptide derivatives for the human δ-opioid receptors and rat μ-opioid receptors were determined by competitive binding against [^{3}H]-c[D-Pen^2, D-Pen^5]-enkephalin ([^{3}H]DPDPE) and [^{3}H]-[D-Ala^2, NMePhe^4, Gly^5-ol]-enkephalin ([^{3}H]DAMGO) in cells that stably express these receptors. For functional characterization of the peptide derivatives at the opioid receptors, [^{35}S]GTPγS binding assays in transfected cells and dose effects of these compounds in inhibiting smooth muscle contraction using the guinea pig ileum (GPI assay for μ receptors) and mouse vas deferens (MVD assays for δ receptors) were used. The binding affinities at the human NK1 receptor (hNK1) were also evaluated. Because species difference between rat and human NK1 receptors has been described for many small molecule NK1 antagonists,[32,33] binding assays at the rat NK1 (rNK1) receptor were conducted as well. The antagonist action of the peptide derivatives was determined for tissue bioassays using GPI in the presence of naloxone to block all the opioid-mediated activities.

As seen in Table 1, compound **2**, which has a disulfide bond between D-Cys^2 and Cys^5 (Fig. 1), retained good affinity at the δ receptor but weaker affinity at the μ receptor (K_i = 5.0 and 200 nM, respectively), resulting in enhanced selectivity for the δ receptor. The cyclization in **2** also reduced agonist efficacies at both receptor types when compared with that of **1**, reflected by both functional assays in cells (Table 1) and smooth muscle preparations (Table 2). The other cyclic derivative of **1** at the second and fifth residues, *c*[D-Cys^2, D-Cys^5]TY027 (**3**) showed a similar right shift in binding affinities as seen for **2**. While the functional activity of **3** was similar to that of **2** in the GTPγS binding assay, its agonist activity in the tissue assays was found to be comparable to that of **1**. Thus, cyclization with a disulfide bond between the second and fifth residues of **1** resulted in a similar right shift in the affinity of the peptide at both δ and μ receptors by about one order of magnitude. It is interesting that while both **2** and **3** exhibited similar lower potencies in the cell assays as compared with that of **1**, only derivative **2** had reduced efficacy in the tissue preparation. The differential effect of D-Cys^5 may reflect receptor conformational differences either in different environments (tissue vs cells) or in different species. Interestingly, **2** had characteristic physicochemical properties: low solubility in organic solvents and in aqueous media even with a 40-fold excess of lipid-like surfactant dodecylphosphocholine. In the tissue-based assay, only **2** showed the unusual slow washout from tissues. Since the rest of the cyclic derivatives did not show such physicochemical and biological properties, perhaps the ring size and configuration of **2** was responsible for the observed solubility and tissue-specific interactions, both of which might also influence its bioactivity.

The pentapeptide enkephalin analogues incorporated with two cysteine residues Tyr-*c*[DCys-Gly-Phe-DCys]-NH_2 (DCDCE-NH_2) and Tyr-*c*[DCys-Gly-Phe-Cys]-NH_2 (DCLCE-NH_2) (Fig. 1) are important

Table 2
Functional assay result for bifunctional peptide derivative ligands at opioid and substance P receptors

No.	Opioid agonist		Substance P antagonist
	MVD (δ) IC_{50}[a] (nM)	GPI (μ) IC_{50}[a] (nM)	GPI Ke[b] (nM)
1[c]	15 ± 2	490 ± 29	10 ± 2
2[e]	73 ± 4	10% inh. at 1 μM[d]	430 ± 160
3	8.3 ± 1.8	280 ± 39	4.7 ± 0.6
4	85 ± 18	1000 ± 200	2.2 ± 0.6
5	19 ± 3	3% inh. at 1 μM[d]	12 ± 1
Tyr-DAla-Gly-Phe-Met-NH_2	36 ± 7	45 ± 8	
DCLCE-NH_2[f]	0.60	1.65	
DCDCE-NH_2[f]	0.56	1.30	
Biphalin	2.7 ± 1.5	8.8 ± 0.3	
L-732,138			250 ± 87

[a] Concentration at 50% inhibition of muscle contraction at electrically stimulated isolated tissues. Calculated from four isolated tissues (n = 4). All the tested compounds except for compound **2** and compound **5** at the μ opioid receptor were full agonists compared to the standards DPDPE and PL-017 for MVD and GPI assays, respectively.

[b] Inhibitory activity against substance P induced muscle contraction in the presence of 1 μM naloxone; Ke: concentration of antagonist needed to inhibit substance P to half its activity. Calculated from four isolated tissues (n = 4).

[c] Ref. 10.

[d] No antagonist activity was observed at the tested concentration.

[e] Unusually slow washout from the tissues was observed.

[f] Ref. 35.

cyclic opioid peptides, since they showed good activities on the opioid receptors through the imposition of global structural constraint by the cyclization at the 2- and 5-residues. However, they did not have δ/μ selectivity (Table 1).[34,35] The introduction of the NK1 antagonist pharmacophore (Pro-Leu-Trp-NH-[3′,5′-$(CF_3)_2$Bzl]) in **2** and **3** in comparison with their corresponding truncated N-terminal sequences, DCDCE-NH_2 and DCLCE-NH_2, suggests that the NK1 antagonist pharmacophore modifies significantly and to a much greater extent with the binding of the N-terminal sequence to the μ opioid receptor than to the δ receptor, thus conferring selectivity of **2** and **3** for the δ receptor (Table 1). A similar trend can be seen for **1**. The lower affinity of **1**–**3** at the μ receptor is reflected by the poor efficacy of these derivatives in the GPI assays. On the other hand, the differential agonist activity of these derivatives at the δ and μ receptors in the cell assays, while trending towards lower efficacy at the μ receptor, was not as pronounced when compared to that seen in tissue preparations (Tables 1 and 2). These results suggest that the C-terminal moiety of **2** and **3** likely alters the three dimensional conformation of the opioid pharmacophore or interferes with the docking of the N-terminal moiety at opioid receptors especially at μ receptors.

The cyclized derivative of **1** at the second and seventh positions *c*[D-Cys^2, Cys^7]TY027 (**4**) (Fig. 1) did not significantly alter the binding affinity at δ opioid receptors and shifted the affinity at μ receptors moderately by about fourfold (Table 1). However, this modification reduces the peptide's efficacies at both opioid receptor types, as seen in both cell and tissue functional assays (Tables 1 and 2). Thus, even though **4** retains binding selectivity for the δ receptor, the modification is likely to have introduced a constraint that diminishes its efficacy at the receptor. On the other hand, the derivative *c*[D-Cys^2, D-Cys^7]TY027 (**5**) (Fig. 1) exhibits lower affinity for both δ and μ opioid receptors, but this right shift in the binding affinity at the δ receptor did not significantly diminish its agonist activity at this receptor, as reflected by both functional assays. The effect of this derivative is similar in both binding affinity and functional activity at the μ receptor as compound **2**. Since the sequential difference between **4** and **5** was found only at position 7 which was located within the NK1 antagonist pharmacophore, it is not surprising that their affinities and activities at the NK1 receptors were different. However, these findings clearly suggest that the cysteine residue at position 7 critically influences the agonist conformation of the cyclic peptide at the δ receptor, where the binding of **4** may favor an inactive conformation for the δ receptor and that of **5** favors an active conformation for the δ receptor. On the other hand, both Cys^7 and D-Cys^7 reduces the affinity and activity of the peptide at the μ receptor.

For the NK1 antagonist activity, the peptide derivatives **1**–**3** had the common sequence in the NK1 antagonist pharmacophore (Pro-Leu-Trp-NH-[3′,5′-$(CF_3)_2$Bzl]) with different opioid agonist pharmacophore in their N-terminal halves. Thus, the difference in their activities may provide an insight on the biological influence of the N-terminal structure on the NK1 antagonist activity due to the disulfide-ring constraints between the second and fifth residues. *c*[D-Cys^2, Cys^5]TY027 (**2**) showed the largest decrease in its binding affinities at both human and rat NK1 receptors (Table 1). *c*[D-Cys^2, D-Cys^5]TY027 (**3**) also showed a right shift in the affinity at both hNK1 and rNK1 receptors compared to those of **1**. These results suggest that the disulfide ring of **2** and **3** act as the 'message structure' for the opioid agonist activities as well as the 'address structure' for the NK1 antagonist activities through the special and conformational interaction with the combining C-terminal sequence. Consistent with the decrease in the NK1 binding affinities, **2** showed weak antagonist activity against substance P stimulation on the GPI tissue, but **3** had improved Ke value compared to **1** in the GPI assay (Table 2). In fact, the binding affinities for **1**–**3** do not correlate with their respective Ke values, which could be due to species differences in the NK1 receptors used in the binding and functional assays.[32] Alternatively, **2** has similar affinity for the NK1 receptor as **3** but its binding site is distinct from that of **3**. Other factors, such as differences in pharmacokinetics and accessibility to the membrane of each ligand in each assay systems could also play a part in this inconsistency. The observed matching between the binding affinity at the NK1 receptors and the activity in the isolated tissue assay might be due to the total effect of these factors.

While, **4** and **5** had the half cystine residue at the seventh position in the middle of their NK1 pharmacophores. Since **1** had the β-turn structure between Pro^6 and C-terminal benzyl moiety in the presence of membrane-mimicking DPC micelles,[10] the ring formation at residues 2–7 might eliminate the corresponding β-turn structural element in **1**. Thus, the importance of this turn structural element on the bioactivities could be examined through the evaluation of **4** and **5**, both of which appears to have significantly reduced binding affinities at hNK1 receptor as that exhibited by **1** (Table 1). However, at the rNK1 receptor, the reduction in the binding affinity was only 4.1-fold for **4** and **5** had the comparable affinity to **1**. In the isolated tissue assays for the estimation of substance P antagonist activity, **5** also had the comparable activity and the activity of **4** was 4.5-fold effective than that of **1**. These results suggest that the β-turn structural element in the C-terminus of **1** might be essential only for the hNK1 binding. It should be noted that although the binding affinities of **3**–**5** for hNK1 receptor were significantly lower than that of **1**, they are still comparable or better than that of the standard NK1 antagonist L-732,138[33,36,37] (Table 1). These compounds are also more effective in inhibiting muscle contraction induced by substance P than L-732,138.

Collectively, the ring structure in **2**–**5** affects both binding affinities and functional activity not only at the opioid receptors but also at the NK1 receptors. Among the tested cyclic peptide derivatives, **5** has the most interesting biological activities as an efficacious δ opioid agonist with good antagonist activity at NK1 receptors in conventional tissue-based assays.

3.2. Metabolic stability in rat plasma

The difference in the biological stability due to the introduction of disulfide ring was evaluated for **2**–**5** in comparison with the linear peptide derivative **1** by incubating them in rat plasma at 37 °C.[11] Aliquots were withdrawn at various time points and analyzed by HPLC to determine the concentration of remaining peptide. As seen in Figure 2, the linear peptide **1** had good stability with a half life ($T_{1/2}$) of 4.8 h. DCDCE-like cyclic peptide [D-Cys^2, D-Cys^5]TY027 (**3**), which is the only derivative possessing D-amino acid residue at the fifth position among the cyclic peptides **2**–**5**, showed almost equivalent stability, though it has a the disulfide ring in the structure ($T_{1/2}$ = 4.9 h). The stability of **2**, **4** and **5** were found to be improved from **1**, and their half lives were more than 6 h. Among them, **5** with the best bifunctional activities was tested for 24 h incubation, and 70% was found intact. Thus, the introduction of disulfide bond was an effective strategy to prevent metabolic degradation in the bifunctional octapeptide derivatives, but not for all cases. The chirality of the fifth residue in the peptidic sequence plays a critical role to be recognized by splitting enzymes.

4. Conclusion

As a result of the cyclization with D-Cys^2 and D-Cys^7 in **1**, **5** was found to have significantly improved stability, and its half live was more than 24 h, suggesting that introduction of this disulfide-ring structure is effective against metabolic degradation. **5** also was an effective activator at the δ opioid receptor, leading to highly selec-

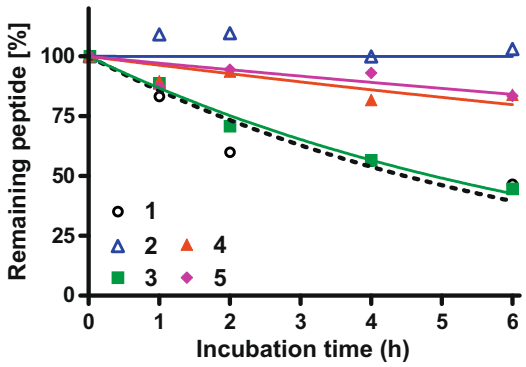

Figure 2. Comparison of the in vitro metabolic stability for **1** (black), **2** (blue), **3** (green), **4** (red) and **5** (purple) incubated in rat plasma at 37 °C. Calculated half lives of peptide derivatives ($T_{1/2}$) were 4.8 h for **1**, 4.9 h for **3** and >6 h for **2**, **4** and **5**. 70 ± 1% of **5** was found intact after 24 h incubation. The samples were tested in at least two independent experiments and the mean values were displayed.

tive δ opioid agonists over μ receptor in the conventional MVD and GPI assays, although its δ opioid selectivity in terms of the binding affinity was relatively lower. Together with the potent NK1 antagonist activity, **5** can be considered as an interesting research tool to seek next generation analgesics which can control severe to moderate pain states.

5. Experimental section

5.1. Materials

All amino acid derivatives and coupling reagents were purchased from EMD Biosciences (Madison, WI), Bachem (Torrance, CA), SynPep (Dublin, CA) and Chem Impex International (Wood Dale, IL). 2-Chlorotrityl resin was acquired from Iris Biotech GmbH (Marktredwitz, Germany). ACS grade organic solvents were purchased from VWR Scientific (West Chester, PA), and other reagents were obtained from Sigma–Aldrich (St. Louis, MO) and used as obtained. The polypropylene reaction vessels (syringes with frits) were purchased from Torviq (Niles, MI). Myo-[2-^{3}H(N)]-inositol; [tyrosyl-3,5-^{3}H(N)] D-Ala^{2}-MePhe^{4}-Glyol^{5}-enkephalin (DAMGO); [tyrosyl-2,6-^{3}H(N)]-c(2-D-penicillamine, 5-D-penicillamine)enkephalin (DPDPE); [^{3}H]-substance P; and [^{35}S]-guanosine 5′-(γ-thio)triphosphate were purchased from Perkin–Elmer (Wellesley, MA). Bovine serum albumin (BSA), protease inhibitors, Tris and other buffer reagents were obtained from Sigma (St. Louis, MO). Culture medium (MEM, DMEM, and IMDM), penicillin/streptomycin and fetal calf serum (FCS) were purchased from Invitrogen (Carlsbad, CA).

5.2. Peptide synthesis

5.2.1. Linear peptide synthesis

The peptides were synthesized manually by the N^{α}-Fmoc solid-phase methodology using HCTU as the coupling reagents as previously reported.[10,12] 2-Chlorotrityl resin (2.0 g, 1.56 mmol/g) was placed into a 50 mL polypropylene syringe with the frit on the bottom and swollen in DMF (20 mL) for 1 h. The resin was washed with DMF (3 × 15 mL) and then with DCM (3 × 15 mL). Fmoc-Trp(Boc)-OH (1.2 equiv) was dissolved in 30 mL of DCM, and then DIEA (5 equiv) was added. The reaction mixture was transferred into the syringe with the resin then shaken for 2 h. The resin was washed three times with DMF (15 mL) and three times with DCM (15 mL), and then with DMF (3 × 15 mL). The N^{α}-Fmoc protecting group was removed by 20% piperidine in DMF (20 mL, 1 × 2 min and 1 × 20 min). The deprotected resin was washed with DMF (3 × 15 mL), DCM (3 × 15 mL) and then with DMF (3 × 15 mL). The protected amino acid (3 equiv) and HCTU (2.9 equiv) were dissolved in 30 mL of DMF, then DIEA (6 equiv) was added. Fmoc-D-Cys(Trt)-OH, Fmoc-Cys(Trt)-OH, Fmoc-Leu-OH, Fmoc-Pro-OH, Fmoc-Nle-OH, Fmoc-Phe-OH, Fmoc-Gly-OH, and Boc-Tyr(tBu)-OH were used for respective coupling as protected amino acids. The coupling mixture was transferred into the syringe with the resin, and then shaken for 2 h. All the other amino acids were consecutively coupled using the procedure described above, using the TNBS test or chloranil test to check the extent of coupling. In case of a positive test result, the coupling was repeated until a negative test result was obtained. The resulting batch of the resin-bound protected peptide was carefully washed with DMF (3 × 15 mL), DCM (3 × 15 mL), DMF (3 × 15 mL), and DCM (3 × 15 mL), and dried under reduced pressure. The peptide was cleaved off the solid support with 1% v/v TFA in DCM (30 mL) for 30 min, and most of the organic solvent was removed under reduced pressure. The obtained protected peptide with free C-terminal was precipitated out by the addition of chilled petroleum ether (45 mL) to give a white precipitate. The suspensions were centrifuged for 20 min at 7000 rpm, and then the liquid was decanted off. The crude peptide was washed with petroleum ether (2 × 50 mL), and after another centrifugation, the peptides were dried under vacuum (2 h) to obtain the corresponding crude protected peptide. The purity of the protected peptide with free C-terminal (>90%) was checked by analytical RP-HPLC using a Hewlett Packard 1100 system (230 nm) on a reverse phase column (Waters NOVA-Pak C-18 column, 3.9 × 150 mm, 5 μm, 60 Å). The peptide was analyzed with a linear gradient of aqueous 0.1% TFA in H_2O/CH_3CN at a flow rate of 1.0 mL/min. The crude peptide was used for the subsequent reactions without further purification.

The protected peptide with free C-terminal (300 mg, 0.173 mmol) and Cl-HOBt (33.4 mg, 0.208 mmol) were dissolved in DMF (3 mL). 3,5-Bistrifluoromethylbenzyl amine (84.1 mg, 0.346 mmol) and EDC (39.7 mg, 0.208 mmol) were added to the solution at rt and stirred until the starting material was not detected by TLC; then saturated aqueous sodium bicarbonate (100 mL) was added. The reaction mixture was extracted with ethyl acetate (100 mL) three times. The combined organic phases were washed with 5% aqueous citrate and saturated aqueous sodium chloride (100 mL each), then dried over sodium sulfate. The solvent was evaporated off and the crude protected peptide was precipitated in cold petroleum ether (45 mL). The product was twice dispersed in cold petroleum ether, centrifuged and decanted, then dried under reduced pressure. The obtained protected peptide was treated with 82.5% v/v TFA, 5% water, 5% thioanisol, 2.5% 1,2-ethanedithiol, and 5% phenol (1.5 mL, 1 h). The crude peptide was precipitated out by the addition of chilled diethyl ether (45 mL) to give a white precipitate. The resulting peptide suspension was centrifuged for 20 min at 7000 rpm, and the liquid was decanted. The crude peptide was washed with diethyl ether (2 × 45 mL), and after a final centrifugation, the peptide was dried under vacuum (2 h). The resulting white residue (225 mg, quantitative) was directly used for cyclization.

5.2.2. Oxidative cyclization to disulfides[31,38]

A solution of $K_3Fe(CN)_6$ was prepared as follows: 1 mmol (330 mg) of $K_3Fe(CN)_6$ was dissolved in a mixture of water (100 mL) and CH_3CN (100 mL), a saturated solution of ammonium acetate (20 mL) was added to it, and the pH was adjusted to 6.0 with glacial acetic acid. A solution of the linear peptide (0.173 mmol) in the mixture of CH_3CN (40 mL), DMSO (5 mL), and H_2O (5 mL) was added to the above solution dropwise overnight with the help of a syringe pump.[38] After the overnight reaction, glacial acetic acid was added to the reaction mixture to obtain

pH 5.0, followed by 20 mL of Amberlite IRA-68 anion-exchange resin (pre-equilibrated with 1 M HCl and extensively washed with distilled water), and the suspension stirred for 30 min until the solution turned colorless and the resin turned yellow. The resin was suction-filtered and the filtrate rotoevaporated to remove most of the organic solvent. The remaining solution was concentrated on Sep-Pak C18 cartridge (10 g, Waters, Milford, MA), then eluted with CH_3CN. The obtained yellow solution was concentrated under reduced pressure for the final purification by preparative RP-HPLC, and then lyophilized. Preparative RP-HPLC was performed on Waters Delta Prep 4000 with Waters XTerra C-18 column (19 × 250 mm, 10 μm, a linear gradient of 33–53% or 40–60% acedtonitrile/0.1% TFA at a flow rate of 15.0 mL/min).

5.2.3. Characterization of peptides

The purified peptides were characterized by HRMS, TLC, analytical HPLC and ^{1}H NMR. Sequential assignment of proton resonances was achieved by 2D-TOCSY NMR experiments.[39] High-resolution MS were taken in the positive ion mode using FAB methods at the University of Arizona Mass Spectrometry Facility. TLC was performed on aluminum sheets coated with a 0.2 mm layer of Silica Gel 60 F_{254} Merck using the following solvent systems: (1) $CHCl_3$/MeOH/AcOH = 90:10:3; (2) EtOAc/*n*-BuOH/water/AcOH = 5:3:1:1; and (3) *n*-BuOH/water/AcOH = 4:1:1. TLC chromatograms were visualized by UV light and by ninhydrin spray followed by heating (hot plate). Analytical HPLC was performed on a Hewlett Packard 1100 or Hewlett Packard 1090 m with Waters NOVA-Pak C-18 column (3.9 × 150 mm, 5 μm, 60 Å) or Vydac 218TP104 C-18 column (4.6 × 250 mm, 10 μm, 300 Å). ^{1}H-1D-NMR spectra were obtained on Bruker DRX-500 or DRX-600 spectrometer. 2D-TOCSY NMR spectra were performed on a Bruker DRX-600 spectrometer equipped with a 5 mm Nalorac triple-resonance single-axis gradient probe. The NMR experiments were conducted in DMSO-d_6 solution at 298 K. Spectra were referenced to residual solvent protons as 2.49 ppm. The processing of NMR data was performed with the XWINNMR software (Bruker BioSpin, Fremont, CA). In the TOCSY experiments, the TPPI mode[40] with MLEV-17 Mixing Sequence[41] were used with a mixing time of 62.2 ms, at a spin-lock field of 8.33 kHz. TOCSY spectra were acquired with 2k complex pairs in t_2 and 750 FIDs using a 90°-shifted sine-squared window function in both dimensions.

5.3. In vitro pharmacology

5.3.1. Radioligand labeled binding assay, [^{35}S]GTPγS binding assay, GPI and MVD in vitro bioassay

The methods were carried out according to that previously described.[9,10,12] Briefly, the evaluation of the binding affinities of the synthesized bifunctional peptide derivatives at the human δ-opioid receptors and rat μ-opioid receptors were performed on the cell (HN9.10) membranes that stably express these corresponding receptors using [^{3}H]-c[D-Pen^2, D-Pen^5]-enkephalin ([^{3}H]DPDPE) and [^{3}H]-[D-Ala^2, NMePhe^4, Gly^5-ol]-enkephalin ([^{3}H]DAMGO) as the radioligands, respectively. The [^{35}S]GTPγS binding assays were used to estimate the functional activities for δ and μ opioid agonist efficacies on the same cell membrane. The isolated tissue-based functional assays also were used to evaluate opioid agonist activities in the GPI (δ) and MVD (μ). For the affinity at the human NK1 (hNK1) receptors, binding assays utilized membranes from transfected CHO cells that stably express hNK1 receptors, using [^{3}H]-substance P as the standard radioligand. The binding assay at the rat NK1 (rNK1) receptors also were performed using transfected CHO cells that stably express rNK1 receptors. To evaluate antagonistic activities against substance P stimulation, isolated tissue bioassays using GPI were performed in the presence of naloxone to block any opioid activities.

5.3.2. In vitro stability of peptide derivatives in rat plasma[42]

Stock solution of compounds (50 mg/mL in DMSO) were diluted 1000-fold into rat plasma (Lot 24927, Pel-Freez Biologicals, Rogers, AK) to give an incubation concentration of 50 μg/mL. All samples were incubated at 37 °C and 200 μL of aliquots were withdrawn at 1, 2, 4, 6, and 24 h. Then 300 μL of acetonitrile was added and the proteins were removed by centrifugation. The supernatant was analyzed for the amount of remaining parent compound by HPLC (Hewlett Packard 1090 m with Vydac 218TP104 C-18 column; 4.6 × 250 mm, 10 μm, 300 Å). The samples were tested in three independent experiments (n = 3) for **1** and **5**, and in two independent experiments (n = 2) for **2**–**4**. The mean values were displayed. The statistical significances were evaluated for **1** and **5** with the Student t-test (displayed in the Graphical Abstract).

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Supplementary data

Supplementary data (^{1}H NMR, HPLC, and MS data of the cyclic peptide derivatives **2**–**5** and their intermediates) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.08.035.

References and notes

1. Ananthan, S.; Khare, N. K.; Saini, S. K.; Seitz, L. E.; Bartlett, J. L.; Davis, P.; Dersch, C. M.; Porreca, F.; Rothman, R. B.; Bilsky, E. J. *J. Med. Chem.* **2004**, *47*, 1400.
2. Yaksh, T. L. *Pain* **1981**, *11*, 293.
3. Ossipov, M. H.; Lai, J.; King, T.; Vanderah, T. W.; Porreca, F. *Biopolymers* **2005**, *80*, 319.
4. Mantyh, P. W.; Allen, C. J.; Ghilardi, J. R.; Rogers, S. D.; Mantyh, C. R.; Liu, H.; Basbaum, A. I.; Vigna, S. R.; Maggio, J. E. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 2622.
5. Kalso, E. *Eur. J. Pain* **2005**, *9*, 131.
6. King, T.; Ossipov, M. H.; Vanderah, T. W.; Porreca, F.; Lai, J. *Neurosignals* **2005**, *14*, 194.
7. Agnes, R. S.; Lee, Y. S.; Davis, P.; Ma, S. W.; Badghisi, H.; Porreca, F.; Lai, J.; Hruby, V. J. *J. Med. Chem.* **2006**, *49*, 2868.
8. Lee, Y. S.; Agnes, R. S.; Badghisi, H.; Davis, P.; Ma, S. W.; Lai, J.; Porreca, F.; Hruby, V. J. *J. Med. Chem.* **2006**, *49*, 1773.
9. Yamamoto, T.; Nair, P.; Davis, P.; Ma, S. W.; Navratilova, E.; Moye, M.; Tumati, S.; Vanderah, T. W.; Lai, J.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. *J. Med. Chem.* **2007**, *50*, 2779.
10. Yamamoto, T.; Nair, P.; Jacobsen, N. E.; Davis, P.; Ma, S. W.; Navratilova, E.; Lai, J.; Yamamura, H. I.; Vanderah, T. W.; Porreca, F.; Hruby, V. J. *J. Med. Chem.* **2008**, *51*, 6334.
11. Yamamoto, T.; Nair, P.; Jacobsen, N. E.; Vagner, J.; Kulkarni, V.; Davis, P.; Ma, S.-w.; Navratilova, E.; Yamamura, H. I.; Vanderah, T. W.; Porreca, F.; Lai, J.; Hruby, V. J. *J. Med. Chem.* **2009**, *52*, 5164.
12. Yamamoto, T.; Nair, P.; Vagner, J.; Davis, P.; Ma, S. W.; Navratilova, E.; Moye, M.; Tumati, S.; Vanderah, T. W.; Lai, J.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. *J. Med. Chem.* **2008**, *51*, 1369.
13. Ripley, T. L.; Gadd, C. A.; De Felipe, C.; Hunt, S. P.; Stephens, D. N. *Neuropharmacology* **2002**, *43*, 1258.
14. King, T.; Gardell, L. R.; Wang, R.; Vardanyan, A.; Ossipov, M. H.; Malan, T. P., Jr.; Vanderah, T. W.; Hunt, S. P.; Hruby, V. J.; Lai, J.; Porreca, F. *Pain* **2005**, *116*, 276.
15. Ma, W.; Zheng, W. H.; Kar, S.; Quirion, R. *Neuroscience* **2000**, *99*, 529.
16. Powell, K. J.; Quirion, R.; Jhamandas, K. *Eur. J. Neurosci.* **2003**, *18*, 1572.
17. Misterek, K.; Maszczynska, I.; Dorociak, A.; Gumulka, S. W.; Carr, D. B.; Szyfelbein, S. K.; Lipkowski, A. W. *Life Sci.* **1994**, *54*, 939.
18. Gu, G.; Kondo, I.; Hua, X. Y.; Yaksh, T. L. *J. Pharmacol. Exp. Ther.* **2005**, *314*, 1362.
19. Largent-Milnes, T. M.; Yamamoto, T.; Davis, P.; Ma, S. W.; Hruby, V. J.; Yamamura, H. I.; Lai, J.; Porreca, F.; Vanderah, T. W. In Neuroscience: San Diego, CA., 2007, Poster 725. Visceral Pain: Transmitters and Receptors.
20. Largent-Milnes, T. M.; Yamamoto, T.; Nair, P.; Navratrilova, E.; Davis, P.; Ma, S.-W.; Hruby, V. J.; Yamamura, H. I.; Lai, J.; Porreca, F.; Vanderah, T. W. In International Association for the Study of Pain/12th World Congress on Pain: Glasgow, Scotland, 2008.

21. Dickenson, A. H. *Behav. Brain Sci.* **1997**, *20*, 392.
22. Chang, K. J.; Rigdon, G. C.; Howard, J. L.; McNutt, R. W. *J. Pharmacol. Exp. Ther.* **1993**, *267*, 852.
23. Sheldon, R. J.; Riviere, P. J.; Malarchik, M. E.; Mosberg, H. I.; Burks, T. F.; Porreca, F. *J. Pharmacol. Exp. Ther.* **1990**, *253*, 144.
24. Cowan, A.; Zhu, X. Z.; Mosberg, H. I.; Omnaas, J. R.; Porreca, F. *J. Pharmacol. Exp. Ther.* **1988**, *246*, 950.
25. Quock, R. M.; Burkey, T. H.; Varga, E.; Hosohata, Y.; Hosohata, K.; Cowell, S. M.; Slate, C. A.; Ehlert, F. J.; Roeske, W. R.; Yamamura, H. I. *Pharmacol. Rev.* **1999**, *51*, 503.
26. Hruby, V. J.; Gehrig, C. A. *Med. Res. Rev.* **1989**, *9*, 343.
27. Sewald, N.; Jakubke, H. D. In *Peptides—Chemistry and Biology*; Wiley-VCH: Weinheim, Germany, 2002, p 320.
28. Mosberg, H. I.; Hurst, R.; Hruby, V. J.; Gee, K.; Yamamura, H. I.; Galligan, J. J.; Burks, T. F. *Proc. Natl. Acad. Sci. U.S.A.* **1983**, *80*, 5871.
29. Shimohigashi, Y. *NIDA Res. Monogr.* **1986**, *69*, 65.
30. Millet, R.; Goossens, L.; Bertrand-Caumont, K.; Chavatte, P.; Houssin, R.; Henichart, J. P. *Lett. Pept. Sci.* **1999**, *6*, 255.
31. Balse-Srinivasan, P.; Grieco, P.; Cai, M.; Trivedi, D.; Hruby, V. J. *J. Med. Chem.* **2003**, *46*, 3728.
32. Datar, P.; Srivastava, S.; Coutinho, E.; Govil, G. *Curr. Top. Med. Chem.* **2004**, *4*, 75.
33. Cascieri, M. A.; Macleod, A. M.; Underwood, D.; Shiao, L. L.; Ber, E.; Sadowski, S.; Yu, H.; Merchant, K. J.; Swain, C. J.; Strader, C. D.; Fong, T. M. *J. Biol. Chem.* **1994**, *269*, 6587.
34. Schiller, P. W.; DiMaio, J.; Nguyen, T. M. In *Proc. 16th FEBS Meeting, Part B*, Utrecht, 1985; Ovchinnikov, Y.A., Ed.; VNU Science Press: Utrecht, 1985; pp 457–462.
35. Berezowska, I.; Chung, N. N.; Lemieux, C.; Wilkes, B. C.; Schiller, P. W. *J. Med. Chem.* **2007**, *50*, 1414.
36. Kuo, H. P.; Hwang, K. H.; Lin, H. C.; Wang, C. H.; Liu, C. Y.; Lu, L. C. *Br. J. Pharmacol.* **1998**, *125*, 711.
37. Cascieri, M. A.; Bull, H. G.; Mumford, R. A.; Patchett, A. A.; Thornberry, N. A.; Liang, T. *Mol. Pharmacol.* **1984**, *25*, 287.
38. Misicka, A.; Hruby, V. J. *Pol. J. Chem.* **1994**, *68*, 893.
39. Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 2820.
40. Marion, D. W. K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967.
41. Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521.
42. Hu, X.; Nguyen, K. T.; Jiang, V. C.; Lofland, D.; Moser, H. E.; Pei, D. *J. Med. Chem.* **2004**, *47*, 4941.
43. Lipkowski, A. W.; Misicka, A.; Davis, P.; Stropova, D.; Janders, J.; Lachwa, M.; Porreca, F.; Yamamura, H. I.; Hruby, V. J. *Bioorg. Med. Chem. Lett.* **1999**, *9*, 2763.