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Design of high affinity cyclic pentapeptide ligands for κ-opioid receptors

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Abstract: Using results from our previously reported cyclic opioid peptide series and reliable models for μ-, δ-, and κ-opioid receptors (MOR, DOR, and KOR, respectively) and their complexes with peptide ligands, we have designed and synthesized a series of cyclic pentapeptides of structure Tyr-c[D-Cys-Phe-Phe-*X*]-NH_2, cyclized via disulfide, methylene, or ethylene dithioethers, and where X = D- or L-Cys; or D- or L-penicillamine (Pen; β,β-dimethylcysteine). Determination of binding affinities to MOR, DOR, and KOR revealed that members of this series with X = D- or L-Cys display KOR affinities in the low nanomolar range, demonstrating that a 'DPDPE-like' tetrapeptide scaffold is suitable not only for DOR and MOR ligands, but also for KOR ligands. The cyclic pentapeptides reported here are not, however, selective for KOR, rather they display significant selectivity and high affinity for MOR. Indeed, peptide **8**, Tyr-c[D-Cys-Phe-Phe-Cys]-NH_2-cyclized via a methylene dithioether, shows picomolar binding affinity for MOR (K_i^{μ} = 16 pM) with more than 100-fold selectivity for MOR vs. DOR or KOR, and may be of interest as a high affinity, high selectivity MOR ligand. Nonetheless, the high affinity KOR peptides in this series represent excellent leads for the development of structurally related, selective KOR ligands designed to exploit structurally specific features of KOR, MOR, and DOR.

Abbreviations: CHO, Chinese hamster ovary; C6, rat C6 glioma; EL, extracellular loop; Pen, penicillamine; RP-HPLC, reverse phase high-performance liquid chromatography; TM, transmembrane α-helix.

Introduction

Understanding the differences in structure and function of opioid receptors and of the modes of their interactions with ligands is fundamental for rational design of safer analge-

sics. Small peptides and truncated endogenous ligands can serve as tools for this task. The cloning of the human opioid receptor types: δ (DOR) (1,2), μ (MOR) (3–5), and κ (KOR) (3,6–8) in the early 1990s has enabled a more thorough study of the ligand-binding site and development of receptor selective ligand pharmacophores. Sequence variability of homologous MOR, DOR, and KOR in the region of ligand binding predisposes the opioid receptor types for structurally dissimilar ligands.

Because of an inherently restricted number of conformations, short cyclized peptides have been extensively used as probes of receptor-binding sites, which has allowed more detailed exploration of spatial requirements for ligand binding at opioid receptors. Previously, we have successfully merged two approaches to examine receptor–peptide interactions: design of small cyclic peptides and modeling of ligand–receptor complexes. This methodology has resulted in the development of ligand–receptor interaction models for two closely related peptides that are selective for DOR and MOR, JOM-13 [Tyr-c(SS)[D-Cys-Phe-D-Pen]-OH, cyclized through the residues 2 and 4 side chain thiols to a disulfide] and JOM-6 [Tyr-c(SEtS)[D-Cys-Phe-D-Pen]-NH_2, cyclized through the side chain thiols to an ethylene dithioether], respectively (9,10; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=pubmed&dopt=Abstract&list_uids=12464111).

The challenge remains, however, to decipher the specific pharmacophore of KOR. It is known that endogenous Leu-enkephalin (YGGFL) binds and activates only MOR and DOR, but not KOR, while the larger endogenous peptide, dynorphin A (YGGFLRRIRPKLKWDNQ), is a potent KOR agonist (11). Truncation and substitution studies on dynorphin A demonstrated that Arg^7, Lys^{11}, and Lys^{13} are essential for high affinity and potency of dynorphin A to KOR (12), while the basic character of Arg^6 is important for KOR-selectivity (13). Therefore, it was assumed that the extension of the 'address' moiety enriched by positively charged residues is required for KOR-activity of enkephalin-like peptides. Consequently several dynorphin-related undecapeptides either linear or cyclized by lactam bridge between residues 2 and 5 were designed, which demonstrated high κ-binding affinity and moderate selectivity (14).

However, we have recently shown that KOR-active peptides can be developed using a relatively rigid cyclic tetrapeptide scaffold similar to that of JOM-13 (15). A cyclic tetrapeptide, Tyr-c[D-Cys-Phe-D-Cys]-NH_2 (MP-133)-cyclized via a disulfide, exhibited high binding affinity toward all opioid receptors, including KOR ($K_i^\kappa = 38.7$ nM). We observed that D-Cys, rather than D-Pen, in the fourth position was essential for high KOR-binding affinity. Indeed, the substitution of D-Cys to a larger β-gem-dimethylcysteine (D-Pen) precluded binding to KOR, but did not affect binding to MOR and DOR. Moreover, the presence of small aromatic side chain in the third position (Phe^3) of the cyclic tetrapeptides was also required for KOR activity, while larger aromatic and aliphatic side chains were well tolerated only by MOR and DOR. Comparison of MOR, DOR, and KOR homology models allowed the suggestion that the high bias of KOR toward Phe^3 and D-Cys^4 is related to the smaller size of the KOR-binding pocket in the region that accommodates the third and fourth peptide residues. Modeling studies demonstrated that the binding pocket for the cyclic opioid peptides is located between transmembrane (TM) helices 3–7, and is partially filled by extracellular loop 2 (EL-2), which in KOR is three-residues longer than in DOR or MOR, resulting in less space available for ligand. The requirement for an amidated vs. anionic C-terminus in these tetrapeptide ligands is a consequence of proximal acidic residues from EL-2 and EL-3 of KOR.

Although Tyr-c[D-Cys-Phe-D-Cys]-NH_2(SS) binds relatively well to KOR, its MOR affinity is approximately 30-fold higher, a consequence of the constrained KOR-binding pocket which requires a binding conformation of the peptide tripeptide cycle that is approximately 2 kcal/mole higher than its lowest energy state. Here, we describe alternate scaffolds designed to accommodate the requirements of the KOR-binding site with a reduced energetic penalty. In particular we describe a series of pentapeptides of the form: Tyr-c[D-Cys-Phe-Phe-X]-NH_2-cyclized through S-$(CH_2)_n$-S (n = 0, 1, 2), where X represents D- or L-Cys or D- or L-Pen, which display significantly improved KOR affinity.

Experimental Procedures

Materials

All Fmoc-protected amino acid were obtained from Advanced ChemTech (Louisville, KY, USA) or Chem-Impex International (Wood Dale, IL, USA). All other reagents were from Sigma-Aldrich (Milwaukee, WI, USA) unless otherwise indicated.

Solid phase peptide synthesis

All peptides were synthesized by solid phase methods on an ABI Model 431A solid phase peptide synthesizer (Applied

Biosystems, Foster City, CA, USA). Rink resin (Advanced ChemTech) was used as the solid support for C-terminal carboxamide peptides. Peptide elongation on the peptide-resin involved treating resin with piperidine (Aldrich) to cleave the Fmoc-protecting group, followed by coupling of the next amino acid with *o*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyl uronium hexafluorophosphate (HBTU) and 1-hydroxybenzotriazole (HOBt; Applied Biosystems). A solution of trifluoroacetic acid/H_2O/thioanisole/ethylene-dithiol (9 : 0.5 : 0.25 : 0.25, v/v/v/v) was used to cleave the linear peptide from the resin and simultaneously remove the side chain-protecting groups. The peptide solution was filtered from the resin and then subjected to preparative reverse phase high-performance liquid chromatography (RP-HPLC) to afford the linear disulfhydryl-containing peptide. Final product confirmation was obtained by ESI-LC-MS (ThermoFinnigan, San Jose, CA, USA).

General method for disulfide cyclization of peptides

To obtain disulfide-cyclized peptide, linear disulfhydryl-containing peptide was dissolved in a 1% (v/v) acetic acid (HOAc) in H_2O solution (saturated with N_2) at 5 °C (1 mg linear peptide/mL of aqueous HOAc solution). The pH of the peptide solution was raised to 8.5 using NH_4OH, followed by the addition of 4 mEq of $K_3Fe(CN)_6$. The reaction mixture was stirred for 1 min then quenched by adjusting the pH to 3.5 with HOAc. The mixture was then subjected to preparative RP-HPLC to afford the disulfide-cyclized peptide.

General method for dithioether cyclization of peptides

To form dithioether-containing cyclic peptides, linear disulfhydryl peptide was added to dimethylformamide and maintained at 5 °C under a N_2 atmosphere (0.1 mg linear peptide/mL dimethylformamide). About 10 mEq of potassium *t*-butoxide were added to the peptide solution, followed by the addition of 10 mEq of Br-$(CH_2)_n$-Br ($n = 1$ or 2). The reaction was quenched with 5 mL HOAc after 2 h and the solvent was removed in vacuo. The residue was dissolved in water, filtered, and then subjected to preparative RP-HPLC to afford the alkyl dithioether-cyclized peptide.

All final product peptides were >95% pure as assessed by RP-HPLC on a Vydac 218TP C-18 column (The Nest Group, Southboro, MA, USA) using the solvent system 0.1% trifluoroacetic acid (TFA) in water/0.1% TFA in acetonitrile by a gradient of 0–70% organic component in 70 min, monitored at 230 nm, and all peptides displayed the appropriate molecular weights as determined by mass spectrometry (Table 1).

Table 1. **Analytical data for peptides 1–12**

Analog	Molecular weight, theoretical (MW)	Molecular weight, [MW + H^+][a]	HPLC (min; R_t)[b]
1	706.3	707.2	30.1
2	720.3	721.1	35.0
3	734.3	735.2	30.2
4	706.3	707.2	30.4
5	720.3	721.2	32.2
6	734.3	735.3	32.6
7	678.2	679.1	30.2
8	692.3	693.1	30.6
9	706.3	707.1	31.1
10	678.2	679.1	28.9
11	692.3	693.2	31.4
12	706.3	707.1	32.2

a. Molecular weight was determined by ESI-LCMS, positive mode.
b. Retention time assessed by analytical RP-HPLC: 0–70% ACN w/0.1% TFA in 70 min, 230 nm, samples in H_2O w/0.1% TFA (elution column heated at 35 °C).
RP-HPLC, reverse phase high-performance liquid chromatography; TFA, trifluoroacetic acid.

Radioligand-binding assays

Opioid ligand-binding assays were based on the displacement by the test compounds of radiolabeled ^{3}H-diprenorphine from membrane preparations containing opioid receptors [human KOR stably expressed in Chinese hamster ovary (CHO) cells (16) or rat MOR and DOR receptors stably expressed in C6 cells (17)]. The assay mixture, containing membrane suspension in 50 mM Tris buffer (pH 7.4), radiolabeled ligand (0.2 nM), and test compound, was incubated at 25 °C for 1 h to allow binding to reach equilibrium. Subsequently, the samples were filtered rapidly, and the radioactivity retained was determined by liquid scintillation counting. Inhibition of radiolabeled ligand binding by the test compounds was determined from maximal specific binding, measured with an appropriate excess of unlabeled naloxone (10 μM). IC_{50}-values were determined by nonlinear regression analysis to fit a logistic equation to the competition data using GRAPHPAD PRISM Software. The results presented are the mean ± SEM from at least three separate assays, each performed in duplicate.

Modeling of complex of KOR with cyclic pentapeptide

The comparative modeling of the active conformation of human KOR (residues 55–348, accession code P41145) in complex with cyclic pentapeptide **10**, Tyr-c[D-Cys-Phe-Phe-D-Cys]-NH_2(SS), was done using the distance geometry program DIANA (18) and the structural template of the active conformation of MOR, as described previously (15). The active conformation of MOR was calculated from the rhodopsin crystal structure (1gzm) (19) and structural constraints corresponding to the active states of different G protein-coupled receptors (GPCRs) (10). The position of the pentapeptide ligand inside KOR was restricted by H-bond distance constraints (O…N and O…O distances of 3.5 Å) between Tyr^1 (N^+) of the peptide and Asp^{138} (COO-) and Glu^{209} (COO-) of KOR, and between Tyr^1 Oη of peptide and Ala^{234} (C=O) and His^{291} (Nε2) of KOR. Moreover, the arrangement of the pentapeptide depends on the orientations of several residues in the binding pocket of KOR, such as Phe^{212}, Lys^{227}, Glu^{297}, and Tyr^{312}. The rotamers of these side chains that provided favorable interactions with ligand, devoid of hindrances, were defined during calculations.

The backbone dihedral angles for residues 2–5 of pentapeptide **10** were restricted to the L-, R-, R-, and P-domains of the Ramachandran plot, respectively, consistent with the crystal structure of tetrapeptide cycle of the DPDPE analog, Tyr-c[D-Pen-Ala-Phe-D-Pen]OH(SS) (20), because we assumed that these analogous pentapeptides, which possess high binding affinity to DOR, are likely to adopt similar conformations in the DOR-binding pocket. The pentapeptide **10**, which binds with high affinity to all opioid receptors (Table 2), presumably adopts comparable conformations (at least for the 14-membered ring) in complexes with all opioid receptors. Indeed, the comparison of complexes of opioid receptors with selective cyclic tetrapeptides demonstrated good superposition of their cycles and of their major exocyclic pharmacophore elements, the Tyr^1 amine and side chain (9,15,21), as well as the Phe^3 side chain in MOR and KOR tetrapeptide ligands (15). Moreover, we concluded that aromatic side chains of Tyr^1 and Phe^3 in **10** would occupy *trans*-rotamers, similar to the orientation of Tyr^1 and Phe^3 in the tetrapeptide, Tyr-c[D-Cys-Phe-D-Cys]-NH_2(SS) in complex with KOR (15), while the Phe^4 side chain would favor the *gauche*-rotamer, to provide favorable interactions with Phe^3 side chain of the pentapeptide. These assumptions were supported by subsequent distance geometry calculations, which resulted in well-defined structures (r.m.s.d. between all Cα-atoms of 10 best calculated models of ligand–receptor complexes was <0.7 Å) that satisfied the spatial constraints (target function <30), imposed on the active conformations of KOR and on the receptor-bound conformation of pentapeptide **10**.

Table 2. **Opioid receptor-binding affinity of cyclic pentapeptide analogs**

			K_i (nM; ±SEM)		
Peptide sequence	Bridge[a]	Analog	μ	δ	κ
[*N*-Met-Tyr^1, *N*-Met-Arg^7-D-Leu^8]-DynA(1–8)-EtNH_2	–	E2078	0.1 ± 0.01	1.5 ± 0.06	0.67 ± 0.08
Tyr-c[D-Cys-Phe-D-Cys]-NH_2	SS	MP-133	1.26 ± 0.25	16.1 ± 3.8	38.7 ± 1.84
Tyr-c[D-Cys-Phe-Phe-D-Pen]-NH_2	SS	**1**	0.11 ± 0.02	2.0 ± 1.0	151 ± 54.4
Tyr-c[D-Cys-Phe-Phe-D-Pen]-NH_2	SMeS	**2**	0.4 ± 0.07	26 ± 7	31 ± 9
Tyr-c[D-Cys-Phe-Phe-D-Pen]-NH_2	SEtS	**3**	0.14 ± 0.06	40 ± 6.0	42.0 ± 18.0
Tyr-c[D-Cys-Phe-Phe-L-Pen]-NH_2	SS	**4**	0.40 ± 0.22	2.6 ± 1.0	66.3 ± 18.1
Tyr-c[D-Cys-Phe-Phe-L-Pen]-NH_2	SMeS	**5**	0.11 ± 0.02	3.5 ± 1.0	21.7 ± 9.39
Tyr-c[D-Cys-Phe-Phe-L-Pen]-NH_2	SEtS	**6**	0.41 ± 0.02	34 ± 6.7	42.0 ± 20.0
Tyr-c[D-Cys-Phe-Phe-L-Cys]-NH_2	SS	**7**	0.27 ± 0.20	0.8 ± 0.3	8.63 ± 5.0
Tyr-c[D-Cys-Phe-Phe-L-Cys]-NH_2	SMeS	**8**	0.016 ± 0.01	1.8 ± 0.8	2.5 ± 1.5
Tyr-c[D-Cys-Phe-Phe-L-Cys]-NH_2	SEtS	**9**	0.19 ± 0.01	5.4 ± 0.7	4.59 ± 1.8
Tyr-c[D-Cys-Phe-Phe-D-Cys]-NH_2	SS	**10**	0.05 ± 0.01	0.4 ± 0.09	1.6 ± 0.5
Tyr-c[D-Cys-Phe-Phe-D-Cys]-NH_2	SMeS	**11**	0.03 ± 0.01	2.5 ± 0.7	2.7 ± 0.7
Tyr-c[D-Cys-Phe-Phe-D-Cys]-NH_2	SEtS	**12**	0.12 ± 0.06	5.9 ± 2	14 ± 3

a. Bridge between the second and the last amino acids: SS, disulfide bridge; SMeS, S-CH_2-S; SEtS, S-CH_2-CH_2-S.

Results and Discussion

In order to improve the KOR affinity displayed by the cyclic tetrapeptide Tyr-c[D-Cys-Phe-D-Cys]-NH_2(SS) (MP-133), at first we chose to examine cyclic pentapeptides patterned on a DPDPE-like scaffold. Lead compound **1**, Tyr-c[D-Cys-Phe-Phe-D-Pen]-NH_2(SS), can be viewed as a DPDPE analog with expected low-energy conformations of its tetrapeptide cycle that are similar to [Ala^3]DPDPE (see Experimental Procedures). Such cycle conformations allow approximate fit to the binding pockets of DOR, MOR, and KOR and thus could avoid the conformational energy penalty imposed on MP-133. Moreover, **1** can be viewed as analogous to the MP-133 (and JOM-13, JOM-6) tetrapeptide series with an extra Phe residue inserted as residue 4. Indeed, previous studies have shown that cyclic pentapeptides of structure Tyr-c[D-Cys-Phe-X-D-Pen]-NH_2(SS) display high opioid receptor affinity and behave as though JOM-13/JOM-6 analogs, i.e. their critical pharmacophore elements are the Tyr^1 and Phe^3 residues (22). Of the pentapeptides in this series, analogs with X = Phe displayed the most promising KOR affinity (unpublished observations) and hence served as the starting point for the present study. Pentapeptides-like **1** can be viewed as retaining the essential features that allow good KOR affinity of MP-133, while 'relaxing' the ring conformational constraints to allow binding of a low-energy peptide conformer. In order to further probe the optimal features for KOR binding in this series, we examined a series of cyclic pentapeptides of structure Tyr-c[D-Cys-Phe-Phe-X]-NH_2, where X = D- or L-Pen, or D- or L-Cys, and where cyclization is effected via disulfide, methylene dithioether, or ethylene dithioether. Results are summarized in Table 2 which also contains reference data for MP-133 and the dynorphin analog, E2078, a KOR reference ligand.

Several straightforward conclusions regarding the KOR affinity of the cyclic pentapeptides are evident from Table 2. First and most significantly, the results indicate that the goal of enhancing KOR affinity by enlarging the ring cycle has been realized; analogs **8**–**11** all display KOR affinities <5 nM. As in the tetrapeptide series, a C-terminal Cys residue provides considerable improvement in KOR affinity relative to a C-terminal Pen residue. This improvement is especially profound in the case of analogs **1** vs. **10**, where replacement of D-Pen by D-Cys results in approximately 100 improvement in KOR affinity. By contrast, stereochemistry of the C-terminal residue has only a minor effect: KOR affinities are fairly similar for the corresponding diastereomers **1** and **4**; **2** and **5**; **3** and **6**, etc. Likewise, mode of cyclization has a relatively minor effect, with a slight preference for the smaller disulfide or methylene dithioether analogs.

In order to understand the mode of interaction of the cyclic pentapeptides with the KOR-binding site, the complex of the active conformation of KOR with pentapeptide **10** was calculated using distance geometry, as described in Experimental Procedures. The calculated model of KOR is close to the crystal structure of rhodopsin (19) with r.m.s.d. of 2.2 Å for 212 common Cα-atoms in the seven TM bundle. The difference is mostly attributed to the large movement of TM6, shift of EL-2 and smaller adjustments in the positions

Table 3. **Torsion angles (°) for the calculated receptor-bound conformation of pentapeptide 10, Tyr-c[D-Cys-Phe-Phe-D-Cys]-NH_2(SS) in comparison with X-ray crystal structure of [L-Ala^3]DPDPE (20)**

	Residue	
Torsion angle	Pentapeptide 10	[L-Ala^3]DPDPE
	Tyr^1	Tyr^1
ψ	111	131
ω	175	−175
χ^1	−177	−179
χ^2	−99	−101
	D-Cys^2	D-Pen^2
φ	65	73
ψ	35	18
ω	−180	−178
χ^1	−58	−60
χ^2	169	−174
SS bond	84	115
	Phe^3	Ala^3
φ	−85	−89
ψ	−47	−42
ω	−177	−168
χ^1	−171	
χ^2	90	
	Phe^4	Phe^4
φ	−94	−121
ψ	−109	−31
ω	−180	179
χ^1	−71	−46
χ^2	−74	−59
	D-Cys^5	D-Pen^5
φ	165	122
χ^1	−64	−86
χ^2	87	66

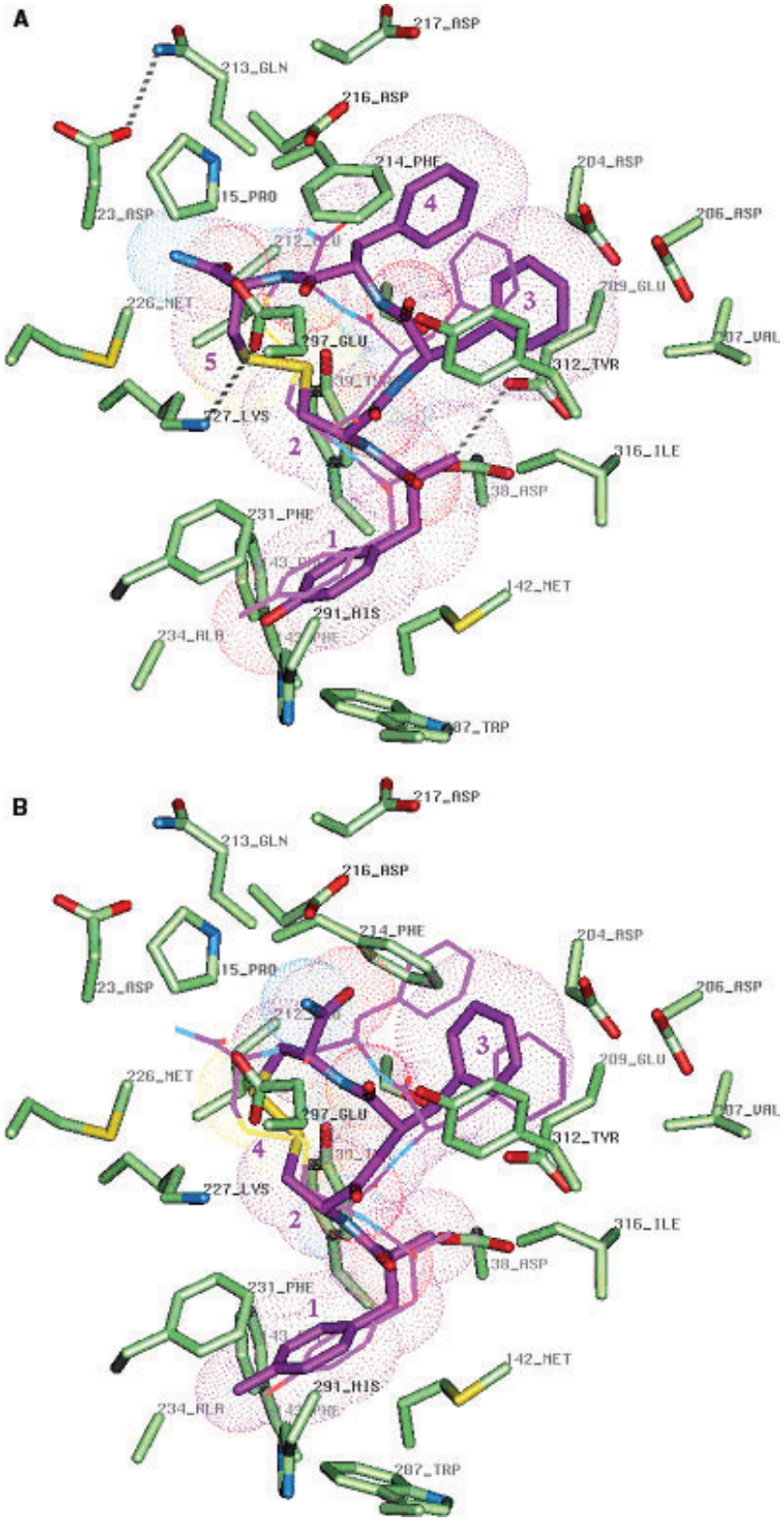

Figure 1. The superposition of pentapeptide **10**, Tyr-c[D-Cys-Phe-Phe-D-Cys]-NH_2 (SS bridge), and tetrapeptide MP-133, Tyr-c[D-Cys-Phe-D-Cys]-NH_2 (SS bridge), in the binding pocket of human κ-opioid receptor (KOR). Pentapeptide **10** is shown by thick lines and MP-133 by thin lines in (A) and vice versa in (B). Ligand and receptor residues are colored by atom type: oxygen is denoted by red, nitrogen by blue, sulfur by yellow, carbon by purple (ligand) or green (receptor residues). Both pentapeptide and tetrapeptide are positioned in the KOR-binding pocket similarly, except for the orientation of the C-terminal amide and the presence of Phe^4 in the pentapeptide, which requires the rotation of Phe^{214} from extracellular loop (EL)-2 of KOR.

of other TM helices, which are the characteristic features of the activated MOR template (10).

The calculated model of KOR with pentapeptide **10** is nearly identical to the model of KOR with the tetrapeptide MP-133 (15), except for different orientations of several side chains in the binding pocket (Phe^{212}, Lys^{227}, and Glu^{297}). The dihedral angles of the calculated receptor-bound conformation of peptide **10** are close to those in the X-ray structure of [L-Ala^3]DPDPE (Table 3). Tyr^1 and Phe^3 of peptide **10** and MP-133 are similarly positioned in the binding pocket (Fig. 1). Specifically, the N-terminal nitrogen and the phenolic oxygen of Tyr^1 in both peptides form multiple H-bonds with Asp^{138} (COO-), Glu^{209} (COO-), His^{297} (Nε2), and Ala^{234} (backbone carbonyl) of KOR, and the Tyr^1 phenolic ring is surrounded by aromatic and sulfur-containing sides chains from TM3 (Tyr^{139}, Met^{142}, Phe^{143}), TM5 (Phe^{231}), and TM6 (Trp^{287}). Phe^3 of both peptides interacts with the aromatic side chain of Tyr^{312} and with Ile^{316} from TM7 and is enclosed by side chains of mainly acidic residues from EL-2 (Asp^{204}, Asp^{206}, Val^{207}, Glu^{209}). The peptide disulfide bridge is located in a tight pocket between EL-2 (Leu^{212}) and the extracellular ends of TM5 (Lys^{227}) and TM6 (Glu^{297}). The size of this area is much smaller in KOR than in MOR and DOR, due to the presence of an extra-residue in EL-2 near TM5. The small size of the binding pocket in this area cannot accommodate the bulky β,β-dimethyl groups of D-Pen^5 (or D-Pen^4)-containing enkephalin analogs without substantial hindrances with receptor residues, particularly with Pro^{215}, Lys^{227}, and Glu^{297}. This is consistent with the strong preference of C-terminal D-Cys over D-Pen for KOR recognition. Minor differences in the position of pentapeptides vs. tetrapeptides within the KOR-binding site results from the presence of Phe^4 in pentapeptide **10**, which interacts with backbone and side chains of EL-2 (Arg^{202}, Asp^{204}, Ser^{211}, and Phe^{214}), and from the relocation of the C-terminal amide group of the peptide ligand: -$CONH_2$ of MP-133 is located in the open space between EL-2 (Phe^{214}) and TM6 (Glu^{297}), while -$CONH_2$ of peptide **10** is more buried between EL-2 (Gln^{213}, Pro^{215}) and the end of TM5 (Asp^{223}, Met^{226}).

Our results demonstrate that very similar cyclic scaffolds can be utilized for high affinity peptide ligands for all three opioid receptors, a result that diverges from existing SAR that suggests only longer dynorphin-like peptides are suitable for KOR binding. In particular, analogs **8**–**11** exhibit low nanomolar KOR affinity, comparable with standard, dynorphin-based ligands. However, although high KOR affinity is exhibited within this series, none of the analogs is selective for KOR. Indeed, peptides **8**, **10**, and **11**, display extremely high MOR affinity ($K_i^{\mu} = 16$ pM, $K_i^{\mu} = 50$ pM, and $K_i^{\mu} = 30$ pM, respectively), higher even than Schiller and co-workers' [Dmt]DALDA [Ki ~ 150 pM (23)], and may be of future interest as MOR ligands. Further modifications of these pentapeptides are required to obtain KOR selectivity. Modeling of KOR in complex with the highest affinity KOR pentapeptide **10** suggests κ-specific interactions between receptor residues and pentapeptide that can be targeted to enhance selectivity. For example, Phe^3 of the peptide ligand is surrounded by several acidic residues from EL-2. Therefore, Phe^3 could be modified to a side chain with more basic properties to enhance KOR-specific ionic interactions with acidic residues from EL-2 (Asp^{204}, Asp^{206}). These and other modification aimed at improving KOR selectivity by increasing KOR affinity, while reducing MOR and DOR affinity are in progress.

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