Translation of Structure-Activity Relationships from Cyclic Mixed Efficacy Opioid Peptides to Linear Analogues

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ABSTRACT:

*Most opioid analgesics used in the treatment of pain are mu opioid receptor (MOR) agonists. While effective, there are significant drawbacks to opioid use, including the development of tolerance and dependence. However, the coadministration of a MOR agonist with a delta opioid receptor (DOR) antagonist slows the development of MOR-related side effects, while maintaining analgesia. We have previously reported a series of cyclic mixed efficacy MOR agonist/DOR antagonist ligands. Here we describe the transfer of key features from these cyclic analogs to linear sequences. Using the linear MOR/DOR agonist, Tyr-DThr-Gly-Phe-Leu-Ser-NH_2 (**DTLES**), as a lead scaffold, we replaced Phe^4 with bulkier and/or constrained aromatic residues shown to confer DOR antagonism in our cyclic ligands. These replacements failed to confer DOR antagonism in the **DTLES** analogs, presumably because the more flexible linear ligands can adopt binding poses that will fit in the narrow binding pocket of the active conformations of both MOR and DOR. Nonetheless, the pharmacological profile observed in this series, high affinity and efficacy for MOR and DOR with selectivity relative to KOR, has also been shown to reduce the development of unwanted side effects. We further modified our lead MOR/DOR agonist with a C-terminal glucoserine to improve bioavailability. The resulting ligand displayed high efficacy and potency at both MOR and DOR and no efficacy at KOR. © 2013 Wiley Periodicals, Inc. Biopolymers (Pept Sci) 102: 107–114, 2014.*

Keywords: *mixed efficacy; mu opioid receptor; delta opioid receptor; homology modeling*

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INTRODUCTION

It has long been assumed that the more specific a ligand is for its therapeutic target, the fewer negative side effects it will elicit. This seems intuitive, as there will be fewer off-target interactions and, theoretically, fewer unintended effects. However, when the development of negative side effects is mediated through the same receptor as the desired effects, as in the case of opioid analgesics, the simultaneous modulation of multiple targets often generates a more desirable drug profile.[1–3] The coadministration of a mu opioid receptor (MOR) agonist with a delta opioid receptor (DOR) agonist[4–7] or antagonist[8–12] retains MOR mediated analgesia and, interestingly, displays reduced tolerance and dependence liabilities, features that limit the clinical use of MOR agonist opioid analgesics.[13]

For pharmacokinetic simplicity, it is preferable to incorporate the desired MOR and DOR functionalities into a single compound.[11–24] Consequently, opioid ligands that interact simultaneously with both receptors have been widely pursued and many peptide,[11,13,22–24] peptide-like,[16,17,20,25] and nonpeptide[14,15,26] ligands have been described. Using homology

models that we have developed for the active and inactive conformations of MOR, DOR, and the kappa opioid receptor (KOR),[11,13,27–31] we have successfully designed cyclic mixed efficacy MOR agonist/DOR antagonist ligands[11,24] based on our previously described cyclic, tetra- and pentapeptide agonists. In our cyclic pentapeptide series we used the nonselective opioid agonist Tyr-c(S-S)[DCys-Phe-Phe-Cys]NH_2 (compound **1** in Refs. 11 and 24) as a starting point, where c(S-S) denotes cyclization through a disulfide linkage via the thiol containing cysteine side chains. Our homology models suggest that the binding pocket in the active state conformations of MOR and DOR differ in size and shape in the area accommodating Phe^3 of "Tyr^1-X-Phe^3" cyclic tetrapeptides or Phe^3 and Phe^4 of "Tyr^1-X-Phe^3-Phe^4" cyclic pentapeptides: the MOR active state binding site is slightly larger than that in the DOR active state binding site, which has bulkier residues occluding the ligand binding pocket (Lys^{108} and $Met^{199}$of DOR in place of Asn^{127} and Thr^{218} of MOR).[24,27–31] We exploited these differences to selectively modulate efficacy by incorporating bulky aromatic replacements for the Phe^3or Phe^4 residues of the ligand which can be accommodated in the MOR active state binding site, but which produce a steric clash in the comparatively narrower DOR active state binding site. The ligand binding site of the DOR inactive conformation, however, is larger than that of the DOR active conformation and can tolerate these bulkier Phe replacements. Successful design of ligands that favor interactions with the MOR active and DOR inactive conformation would be expected to display the desired MOR agonist/DOR antagonist profile.

In peptides from "Tyr^1-X-Phe^3-Phe^4" cyclic series, replacement of Phe^3 with a bulkier 1- or 2-napthylalanine (1- and 2-Nal, respectively) successfully reduced the agonist character of the resulting ligands at DOR without drastically reducing MOR agonist activity.[11,24] In a related series of "Tyr^1-X-Phe^3" tetrapeptides[13] we found that a ligand, **KSK-103** (Dmt-c(S-Et-S)[DCys-Aic-DPen]OH, where c(S-Et-S) denotes cyclization through an ethylene dithioether linkage via the side chains thiols), containing a constrained 2-aminoindan-2-carboxylic acid (Aic) residue within the cycle, adopted a conformation that would fit easily in the more open MOR active state binding site, but formed a steric clash in the DOR active state binding site. In short, we found that through careful modulation of the constraint and steric bulk of these cyclic peptides we were able to selectively reduce DOR efficacy to yield a MOR agonist/DOR antagonist ligand.[11,13,24]

While the cyclic ligands described above display the desired binding and efficacy profiles, their syntheses are low yielding and often require complicated, inefficient purification procedures. Hence, we sought to examine whether we could translate the structure-activity relationships found in our cyclic ligands to linear compounds. As noted above, a key feature for conferring DOR antagonism in our cyclic peptide series is replacement of a Phe residue in the third or fourth position of the lead, agonist peptide with a bulkier and/or more constrained aromatic residue.[11,13,24] In this report we examined whether DOR antagonism can be conferred upon linear opioid peptides by similar replacements (Figure 1). As a starting point for these substitutions, we chose a linear opioid hexapeptide developed by Roques with a "Tyr^1-X-Gly-Phe^4" motif, Tyr-DThr-Gly-Phe-Leu-$SerNH_2$ (**DTLES**).[32] **DTLES** has been shown to have high affinity for both MOR and DOR and display agonist character at both receptors in the guinea pig ileum (GPI) and mouse vas deferens (MVD) assays, respectively.[33] A further advantage of **DTLES** is that the C-terminal serine residue can be glycosylated to improve bioavailability, as demonstrated by Polt and co-workers.[33]

RESULTS AND DISCUSSION

The *in vitro* binding and efficacy data for our set of linear analogues using the **DTLES** scaffold with constrained and/or bulky substitutions for Phe^4 are presented in Table I. In an attempt to translate the structure activity relationships from our cyclic peptides to linear ligands, we initially installed an Aic in place of Phe^4 in the **DTLES** scaffold to mimic **KSK-103**. As previously reported, **KSK-103** displays low nanomolar binding for MOR and DOR, with moderate agonist activity at MOR and no stimulation of DOR.[13] Unfortunately, the resulting compound, **1**, displayed micromolar binding to all three opioid receptors and was not pursued further. In contrast to **KSK-103**, peptide **1** with Aic at the fourth position cannot be accommodated in the binding pocket of any opioid receptor. Reasoning that the quaternary α-carbon of Aic might adversely affect the backbone conformation resulting in the observed low affinity, we next replaced Phe^4 with an indanylglycine (Idg) in which the side chain is one carbon further removed from the peptide backbone. This ligand, **2**, exhibited relatively weak binding to MOR (200 ± 20 n*M*) and DOR (300 ± 55 n*M*) and acts as a low potency agonist at MOR (maximal stimulation 70 ± 9% of standard) with no agonist activity at DOR. In an effort to increase the affinity and potency of **2** we next replaced the Tyr^1 with a 2',6' dimethyltyrosine (Dmt) as this replacement often confers improved binding affinity at opioid receptors.[34] The resulting compound, **3**, displayed the expected increase in affinity (MOR 3.4 ± 0.7 n*M*; DOR 2.1 ± 0.3 n*M*) and retained selectivity relative to KOR (270 ± 12 n*M*), however, the substitution of a Dmt^1 for a Tyr^1 also completely abolished MOR efficacy (Table I).

Since the constrained nature of Aic (cyclized through the backbone α-carbon) and Idg (β-branched) could be the cause

Table I In Vitro Binding and Efficacy Data for Linear Mixed Efficacy Peptides

	Sequence	Binding (n*M*)			Efficacy					
					MOR		DOR		KOR	
		MOR	DOR	KOR	%	EC50 (n*M*)	%	EC50 (n*M*)	%	EC50 (n*M*)
	DTLES	1.4 ± 0.08[a]	4.1 ± 0.41[a]	34 ± 2.2[a]	[b]		**			
1	Tyr-DThr-Gly-Aic-Leu-SerNH$_2$	>1000	>5000	>10,000	nd	nd	nd	nd	nd	nd
2	Tyr-DThr-Gly-Idg-Leu-SerNH$_2$	200 ± 20	300 ± 55	>10,000	70 ± 9	>2000	dns	nd	nd	nd
3	Dmt-DThr-Gly-Idg-Leu-SerNH$_2$	3.4 ± 0.7	2.1 ± 0.3	270 ± 12	dns	nd	dns	nd	nd	nd
4	Tyr-DThr-Gly-1Nal-Leu-SerNH$_2$	0.8 ± 0.2	0.9 ± 0.1	25 ± 3	93.0 ± 0.6	31 ± 7	144 ± 7	35 ± 6	75 ± 2	>1000
5	Tyr-DThr-Gly-2Nal-Leu-SerNH$_2$	2.7 ± 0.08	0.47 ± 0.07	125 ± 22	101 ± 3	13 ± 2	196 ± 2	11.3 ± 0.8	25 ± 4	>1000
6	Dmt-DThr-Gly-2Nal-Leu-SerNH$_2$	0.5 ± 0.1	0.7 ± 0.1	2.3 ± 0.5	81 ± 5	1.5 ± 0.6	164 ± 7	0.3 ± 0.05	dns	nd
7	Tyr-DThr-Gly-2Nal-Leu-Ser(Glc)NH$_2$	4.9 ± 0.3	0.87 ± 0.03	130 ± 10	93 ± 3	110 ± 11	144 ± 15	26 ± 11	<10	nd

Binding affinities (K_i) were obtained by competitive displacement of radiolabeled [^{3}H] diprenorphine. Efficacy data were obtained using [^{35}S] GTPγS binding assay. Efficacy is represented as percent maximal stimulation relative to standard agonists DAMGO (MOR), DPDPE (DOR), or U69,593 (KOR) at 10 μ*M* concentrations. All values are expressed as mean ± SEM of three separate assays performed in duplicate. nd, not determined; dns, does not stimulate.

[a] Binding data from Ref. 33.

[b] Acts as agonist in guinea pig ileum and mouse vas deferens bioassays.[33]

FIGURE 1 Structures of unnatural amino acids.

of the observed poor affinities of **1** and **2**, we next examined analogues with less constrained, but still bulky hydrophobic Phe[4] replacements. These ligands contained either a 1-Nal or 2-Nal in the fourth position (compounds **4** and **5**, respectively) to mimic the results observed in our previously reported cyclic pentapeptides.[24] Interestingly, both Nal-containing analogues displayed tight binding to MOR and DOR, with full agonist activity at both receptors (Table I). This is similar to the profile exhibited by **DTLES** (Table I) which binds with high affinity to MOR and DOR and is reported to be a potent agonist in the MVD and GPI assays.[33] Compound **4** displays significant agonist activity at KOR (75 ± 2% of standard), albeit with low potency, and low selectivity for MOR and DOR over KOR. Compound **5**, however, displays improved MOR agonist/DOR agonist potencies and a significant decrease in KOR binding and efficacy as compared to peptide **4**. This is consistent with our opioid receptor models; compound 5 fits neatly into the binding pocket of the active states of both MOR (Figure 2A) and DOR (Figure 2B), explaining the full agonist activity at both receptors. However, there is a significant steric clash between DThr[2] of **5** and Ile[294] of the KOR active state (Figure 2C) and inactive state (not shown) models which accounts for the decrease in efficacy and binding affinity. This steric hindrance is absent in the MOR and DOR active state binding site models which have a Val in the corresponding position.

We next substituted the Tyr[1] of **5** with a Dmt[1] in an attempt to further improve affinity and potency at MOR and DOR, as more potent ligands require lower doses of drug to be effective, reducing the incidence of negative side effects.[35] The resulting ligand, **6**, displayed higher binding affinity and potency at both MOR and DOR, and, surprisingly, no efficacy at KOR. However, KOR binding affinity was also greatly improved, with the result that **6** shows similar high affinity for MOR, DOR, and KOR. The observed high KOR affinity but no efficacy suggests that **6** is a KOR antagonist. This was confirmed by the

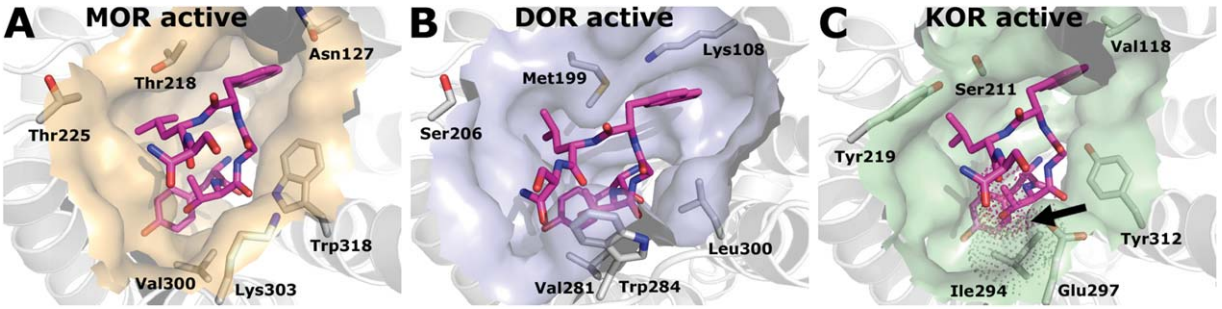

FIGURE 2 Comparison of 5 in the MOR, DOR, and KOR active state binding sites – 5 (Tyr-DThr-Gly-2Nal-Leu-SerNH$_2$) docked in the active state models of MOR (A), DOR (B), and KOR (C). 5 fits neatly into the active state binding pockets of MOR and DOR, allowing for full agonist character at both receptors. In the active state of KOR, however, DThr^2 of 5 forms a steric clash (highlighted by an arrow) with Ile^{294} of the receptor, reducing agonist activity at KOR.

observation that **6** causes a shift in the stimulation of [^{35}S]GTPγS binding evoked by the KOR agonist U69,593, with a K_e (antagonist equilibrium constant) of 63 ± 11 n*M*. The combination of high affinity but no efficacy at KOR, although not one of our design goals, may be advantageous as KOR antagonists have been shown to possess anti-depressant like effects and reduce stress induced drug reinstatement behavior, features that prevent relapse in addicted individuals.[36]

As noted above, Tyr^1-containing ligands with more constrained residues in the fourth position, **1** (Aic^4) and **2** (Idg^4), displayed significantly lower affinity for MOR and DOR than other Tyr^1 compounds in this series. Perhaps unsurprisingly, **2** is the only ligand in this series which displays a MOR agonist/DOR antagonist profile. This is likely due to the fact that some conformational restriction is necessary to selectively produce a steric clash in the DOR active state binding site to reduce DOR efficacy; more flexible residues are able to assume conformations that can be accommodated in the DOR active state binding site and display DOR agonism. When we replaced the Tyr^1 of **2** with a Dmt^1 (**3**), we greatly improved affinity at MOR and DOR, but lost all agonist activity. Our homology models suggest that the phenolic hydroxyl of the Tyr^1 is anchored between Tyr^{148} and Ile^{296} in the MOR binding site. However, the 2',6' methyl groups of the Dmt form a steric clash with these residues (Figure 3B). This steric clash is relieved in the more open MOR inactive site, allowing for tight binding, but no efficacy at MOR.

While we were not able to selectively reduce DOR efficacy while retaining tight binding to MOR and DOR, compound **5** remains promising as a MOR agonist/DOR agonist profile is

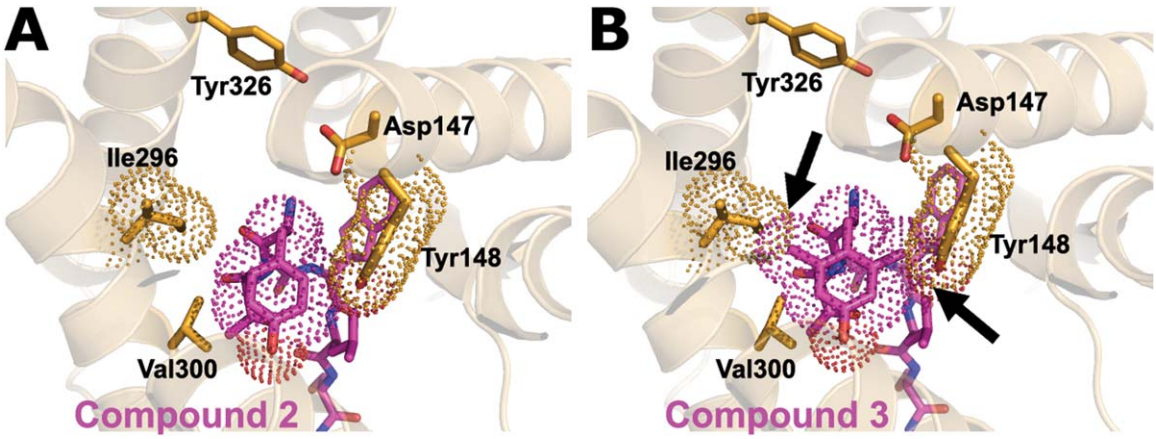

FIGURE 3 Comparison of 2 and 3 in the MOR active state binding site – 2 (Tyr-DThr-Gly-Idg-Leu-SerNH$_2$) (A) and 3 (Dmt-DThr-Gly-Idg-Leu-SerNH$_2$) (B) docked in the active state model of MOR. The 2, 6 methyl groups of Dmt^1 in 3 form a steric clash in the MOR active state with Tyr^{148} and Ile^{296} (highlighted by arrows). This clash prevents 3 from binding to the MOR active site, causing it to be an antagonist.

also desirable. It has been demonstrated that the co-administration of a MOR agonist with a DOR agonist potentiates the binding and antinociceptive potency of MOR agonists.[4–7] The synergistic effect between MOR and DOR agonists has been shown to lessen the development of tolerance and dependence, as well as other unwanted side effects.[7,33,37] Unfortunately, **5**, like most unmodified peptides, is expected to have low membrane permeability and therefore low bioavailability.[33,38,39] In fact, blood/brain barrier (BBB) penetration is considered one of the biggest hurdles to viable peptide therapeutics.[40,41] This is particularly problematic for opioid peptides as they must cross membranes in the digestive tract to be orally active, and the BBB to reach the central nervous system (CNS) where opioids exert their major impact on the management of moderate to severe pain.[42–44] To improve the "druggability" of peptide ligands, several approaches have been developed to increase stability and peptide penetration of biological membranes.[33,38,39] Polt and co-workers have demonstrated that the glycosylation of opioid peptides renders them enzymatically resistant and CNS active after peripheral administration.[7,33,45–48] Among these are peptides based upon **DTLES**, in which the C-terminal Ser side chain is the site of glycosylation. We therefore glycosylated the C-terminal serine of **5** with an O-linked β-D-glucose to determine if this modification maintains this analog's in vitro profile. The resulting ligand, **7**, does indeed display similar binding and efficacy as its parent compound, **5**, with the exception of somewhat reduced MOR potency.

As noted above, the Polt group has successfully employed glycosylation of **DTLES** to generate bioavailable, mixed efficacy MOR agonist/DOR agonist peptide ligands, and have investigated the in vivo actions of their key ligand, **MMP-2200** (Tyr-DThr-Gly-Phe-Leu-Ser-(*O*-β-D-lactose)-NH_2).[7,33,46] **MMP-2200** displays reduced development of tolerance and dependence compared to morphine[7] and does not produce reinforcing effects,[49] suggesting that this mixed efficacy profile is an answer to the negative neurochemical adaptations and addiction liability problems associated with MOR agonist analgesics. However, the lack of self-administration may be influenced by the residual KOR activity that **MMP-2200** displays. **MMP-2200** was reported by Polt and co-workers to have high KOR affinity, but no KOR efficacy data were presented. In our hands, this compound displays full agonist behavior at KOR (82 $\pm$ 11% stimulation of KOR relative to U69,593 standard at 10 μM). The KOR agonist activity of the Polt ligands is a potentially significant drawback as KOR agonists have been shown to cause dysphoria[50] and act as psychotomimetics[51] and therefore display aversive properties.[52] These actions could conceivably contribute to the observed reduced self-administration[52] of **MMP-2200**, rather than this reflecting a MOR agonist/DOR antagonist profile.

Ligands described in this report provide a possible advantage, in that they display a preference for binding to MOR and DOR over KOR (**5**,**7**), or act as KOR antagonists (**6**). In particular, the glycosylated peptide, **7**, exhibits considerably lower KOR affinity compared to MOR or DOR, and very low KOR efficacy. This lack of KOR activity and the anticipated improved bioavailability may help to clarify the effects that DOR agonist activity has on the self-administration of mixed efficacy MOR/DOR ligands and provide a more acceptable drug profile for use in human subjects.

MATERIALS AND METHODS

Materials

All reagents and solvents were purchased from commercial sources and used without further purification. All chemicals and biochemicals were purchased from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Hudson, NH), unless otherwise noted. All tissue culture reagents were purchased from Gibco Life Sciences (Grand Island, NY). Radioactive compounds were purchased from Perkin-Elmer (Waltham, MA). Peptide synthesis reagents, amino acids, and Rink resin were purchased from Advanced Chem Tech (Louisville, KY), except for Fmoc-2-aminoindan-2carboxylic acid, which was purchased from Chem Impex (Wooddale, IL). Wang resins were purchased from Nova Biochem, EMD (Gibbstown, NJ). **MMP-2200** was a kind gift from Dr. Robin Polt. Fmoc-Ser (β-Glc$(Ac)_4$)-OH (the glycosylated serine building block) was synthesized accordingly to previously published protocols.[53]

Solid Phase Peptide Synthesis

Peptides were synthesized using solid phase Fmoc (fluorenylmethyloxycarbonyl) chemistry as described above on a Discover S-Class CEM microwave using Synergy software. Deprotection of the first Fmoc protecting group was performed using a 20% solution (*v*/*v*) of piperidine in *N*-methyl-2-pyrrolidone (NMP) with "Fmoc deprotection" program (power: 20 W, Time: 1:30 min, Temperature: 75°C, Δ Temperature: 0°C) on the microwave synthesizer followed by three washes of NMP. Double coupling was then preformed using a 4.0x equivalence of the amino acids, 0.4*M* *O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HATU) and 1-hydroxy-7-azabenzotriazole (HOAt) in dimethylformamide (DMF) (2.5 mL), 0.125*M* diisopropylethylamine (DIEA) in NMP (1 mL), NMP (2.5 mL), and the "Coupling" program (power: 20W, Time: 5:00 min, Temperature: 75°C, Δ Temperature: 5°C) on the microwave synthesizer. After each double coupling the resin was washed with three times with NMP. After the final "Fmoc deprotection" the resin was washed three times with NMP, then three times with methylene chloride (DCM) and dried under vacuum.

For the synthesis of **7**, the acetates on the glycosylated residue on the peptide were removed using 80% of hydrazine monohydrate in methanol before cleaving the peptide from the resin, following previously reported protocols.[54] All nonglycosylated peptides and acetate-deprotected glycosylated peptides were cleaved from the resin and side-chain-protecting groups removed by treatment at room

temperature for 2 h with a cleavage cocktail consisting of 9.5 mL trifluoroacetic (TFA) acid and 0.5 mL water. The solution was concentrated in vacuo, and peptides were precipitated using cold diethylether. The filtered crude material was then purified using a Waters semipreparative HPLC (Waters Corporation, Milford, MA) with a Vydac Protein and Peptide C18 column (10 micron particle size, 10 x 150 mm), using a linear gradient 10% Solvent B (0.1% TFA acid in acetonitrile) in Solvent A (0.1% TFA acid in water) to 60% Solvent B in Solvent A, at a rate of 1% per minute at 10 mL per minute. The identity of all peptides was determined using ESI-MS performed on an Agilent Technologies LC/MS system using a 1200 Series LC and 6130 Quadrupole LC/MS (Agilent Technologies, Santa Clara, CA) in positive mode with 50–100 μL injection volume and a linear gradient of 0% Solvent D (0.02% TFA and 0.1% acetic acid (AcOH) in acetonitrile) in Solvent C (0.02% TFA and 0.1% AcOH in water) to 60% Solvent D in Solvent C in 15 min. The purity of all peptides was determined using a Waters Alliance 2690 Analytical HPLC (Waters Corporation, Milford, MA) and Vydac Protein and Peptide C18 reverse phase column (5 micron particle size, 5 × 220 mm), using a linear gradient of 0–70% Solvent B in Solvent A at a rate of 1% per minute. Linear peptides were purified to ≥95% purity by UV absorbance at 230 nm.

Cell Lines and Membrane Preparations

C6-rat glioma cells stably transfected with a rat μ (C6-MOR) or rat δ (C6-DOR) opioid receptor[55] and Chinese hamster ovary (CHO) cells stably expressing a human κ (CHO-KOR) opioid receptor[56] were used for all in vitro assays. Cells were cultured and membranes prepared as previously described.[24]

Radioligand Binding Assays

Radioligand binding assays were performed as previously described.[24] In brief, assays were performed using competitive displacement of 0.2 n*M* [^{3}H]diprenorphine (250 μCi, 1.85TBq/mmol) by the test compound from membrane preparations containing opioid receptors. The assay mixture, containing membrane suspension (20 μg protein/tube) in 50 m*M* Tris-HCl buffer (pH 7.4), [^{3}H]diprenorphine, and various concentrations of test peptide, was incubated at room temperature for 1 h to allow binding to reach equilibrium. The samples were filtered through Whatman GF/C filters and washed three times with 50 m*M* Tris-HCl buffer (pH 7.4). The radioactivity retained on dried filters was determined by liquid scintillation counting after saturation with EcoLume liquid scintillation cocktail in a Wallac 1450 MicroBeta (Perkin-Elmer, Waltham MA). Nonspecific binding was determined using 10 μ*M* naloxone. K_i values were calculated using nonlinear regression analysis to fit a logistic equation to the competition data using GraphPad Prism version 5.01 for Windows. The results presented are the mean ± standard error from at least three separate assays performed in duplicate.

Stimulation of [^{35}S]GTPγS Binding

Agonist stimulation of [^{35}S] guanosine 5'-O-[gamma-thio]triphosphate ([^{35}S]GTPγS, 1250 Ci, 46.2TBq/mmol) binding was measured as described previously.[24] Briefly, membranes (10–20 μg of protein/tube) were incubated 1 h at room temperature in GTPγS buffer (50 m*M* Tris-HCl, 100 m*M* NaCl, 5 m*M* $MgCl_2$, pH 7.4) containing 0.1 n*M* [^{35}S]GTPγS, 30 μ*M* guanosine diphosphate (GDP), and varying concentrations of test peptides. Peptide stimulation of [^{35}S]GTPγS was compared with 10 μ*M* standard compounds [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) at MOR, D-Pen^{2,5}- enkephalin (DPDPE) at DOR, or U69,593 at KOR. The reaction was terminated by rapidly filtering through GF/C filters and washing 10 times with GTPγS buffer, and retained radioactivity was measured as described above. The results presented are the mean ± standard error from at least three separate assays performed in duplicate; maximal stimulation was determined using nonlinear regression analysis with GraphPad Prism version 5.01 for Windows.

Determination of K_e for Compound 6

Agonist stimulation of [^{35}S]GTPγS binding by the known standard agonist U69,593 at KOR was measured as described in the previous section. This was then compared with [^{35}S]GTPγS binding stimulated by U69,593 in the presence of 500 n*M* compound **6**. Both conditions produced 100% stimulation relative to U69,593. The difference between the EC_{50} of U69,593 alone and in the presence of test antagonist (compound **6**) is the shift in dose reponse. The K_e for compound 6 was then calculated as K_e = (concentration of compound **6**)/ (Dose response shift – 1). The results presented are the mean ± standard error from at least three separate assays performed in duplicate; maximal stimulation was determined using nonlinear regression analysis with GraphPad Prism version 5.01 for Windows.

Receptor Modeling

Models for the opioid receptors were generated using the recently obtained X-ray structures for mouse MOR (PDB ID: 4dkl),[57] mouse DOR (PDB ID: 4ej4),[58] and human KOR (PDB ID: 4djh).[59] Modeling of active conformations of MOR, DOR, and KOR was performed as previously described[11,24] using crystal structures of KOR together with active conformations of bovine rhodopsin (PDB ID: 3dqb)[60] and bovine β_2-adrenergic receptor (PDB ID: 3sn6).[61] Coordinates of active conformations of opioid receptors with docked cyclic peptides can be downloaded from our website (http://mosberglab.phar.umich.edu/resources/).

X-ray structures of opioid receptors in inactive conformations and homology models of active receptor conformations were used for docking high affinity peptide antagonists and agonists, respectively. Conformations of linear peptides were generated to reproduce backbone conformations of previously reported cyclic peptides, as well as spatial positions of their pharmacophore elements, Tyr^1 and Phe^3 residues.[11,24,62] After minimization with CHARMm implemented in QUANTA (Accelrys, Inc), low-energy conformations of peptides were docked to the binding pockets of corresponding receptors in accordance with mutagenesis-derived peptide-protein interactions.[28] These interactions included hydrogen-bonding between peptide N^+ and Asp from transmembrane domain 3 (Asp^{128} in DOR, Asp^{147} in MOR, and Asp^{138} in KOR), water-mediated hydrogen-bonding between OH-group of peptide Tyr and His from transmembrane domain 6 (His^{278} in DOR, His^{297} in MOR and His^{291} in KOR), and interactions of the peptide X^3 aromatic sidechain with residues from extracellular loop 1, extracellular loop 2, and transmembrane domain 7, of receptors: Lys^{108}, Met^{199}, Leu^{300} of DOR, Asn^{127}, Thr^{218} and Trp^{318} of MOR, Val^{118}, Ser^{211}, and Tyr^{312} of KOR. The position of the aromatic ring of Tyr^1 of peptide antagonists generally followed the position of

aromatic rings of cocrystallized non-peptide antagonists, though positions of peptide N-termini were shifted by ~3 Å toward the extracellular surface. To minimize steric hindrances, manual docking of peptides in low-energy conformations was followed by the automated rigid docking implemented in QUANTA and subsequent minimization with CHARMm (Adopted-Basis Newton-Raphson method, 100 steps, $\varepsilon = 10$).

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