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Development and *in Vitro* Characterization of a Novel Bifunctional μ-Agonist/δ-Antagonist Opioid Tetrapeptide

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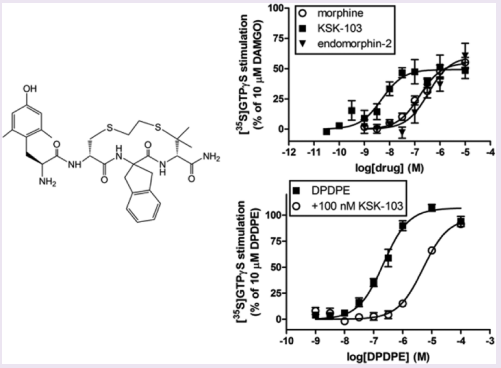
ABSTRACT: The development of tolerance to and dependence on opioid analgesics greatly reduces their long-term usefulness. Previous studies have demonstrated that co-administration of a μ-opioid receptor (MOR) agonist and δ-opioid receptor (DOR) antagonist can decrease MOR agonist-induced tolerance and dependence development after chronic exposure. Clinically, a single ligand displaying multiple efficacies (*e.g.*, MOR agonism concurrently with DOR antagonism) would be of increased value over two drugs administered simultaneously. Guided by modeling of receptor−ligand complexes we have developed a series of potent non-selective opioid tetrapeptides that have differing efficacy at MOR and DOR. In particular, our lead peptide (KSK-103) binds with equal affinity to MOR and DOR but acts as a MOR agonist with similar efficacy but greater potency than morphine and a DOR antagonist in cellular assays measuring both G protein stimulation and adenylyl cyclase inhibition.



Opioid drugs such as morphine are the primary treatment for postoperative and chronic pain conditions through their actions at the μ-opioid receptor (MOR). However, development of tolerance to and dependence on these drugs limits their usefulness. Thus, a novel ligand with analgesic properties but lacking tolerance and dependence liability would be of great value in clinical settings. Several published reports have documented that blockade of the δ-opioid receptor (DOR), through either antagonism or knockdown of the receptor, leads to decreased development of tolerance or dependence in rodents chronically treated with the MOR agonist morphine.[1−4] Evidence for interactions between MOR and DOR leading to altered signaling profiles has also been discussed in reference to locomotor sensitization,[5,6] while other research has shown MOR and DOR cell-surface receptor expression levels to be linked.[7,8] The studies described above have highlighted roles for both MOR and DOR in the development of morphine-provoked analgesic tolerance and/or dependence.

For pain relief, an optimal therapeutic would be a single drug containing two opioid receptor actions: MOR agonism to promote analgesia along with DOR antagonism to prevent MOR tolerance and dependence development during chronic administration. Co-administration of two drugs acting separately at each individual receptor could be hampered by increased "off-target" effects, differences in pharmacokinetic profiles, and user compliance.[9,10] We have therefore focused on the development and characterization of peptide ligands that simultaneously display MOR agonism and DOR antagonism while binding with equivalent affinity to each receptor.

The development of such bifunctional or mixed-efficacy ligands has become a topic of increasing interest in several therapeutic areas.[10,11] For example, bifunctional ligands have been proposed with DOR-KOR efficacy[12] or MOR-cholecystokinin receptor activities,[13] targeting tolerance liability,[14] or toward novel ligands for treatment of cocaine abuse.[15−17] Similarly, early studies indicating that blockade of DOR reduces the development of tolerance to MOR agonists have stimulated several investigations into mixed MOR agonist/DOR antagonist compounds.[18−20]

Small peptides and in particular receptor-specific ligands provide a means to determine structural or conformational requirements of binding to a particular membrane-bound receptor. We have previously used molecular modeling and conformationally restricted cyclic peptide ligands as tools to analyze determinants of ligand binding to MOR, DOR, and κ-opioid receptors (KOR). Through this methodology, we developed peptides that bind selectively to different opioid receptors[21−23] and have expanded these concepts to develop peptides that bind non-selectively but with differing efficacy profiles. Recent work[19] described the development of a non-selective opioid cyclic pentapeptide that displayed MOR agonism, DOR partial agonism, and KOR agonism. The pentapeptides characterized in that study were designed to have decreased DOR efficacy compared with the parent ligand due to steric interactions inferred from ligand docking to a model of the active state of DOR. Indeed, replacement of Phe residues in position 3 or 4 of the pentapeptide with bulkier 1-naphthylalanine (1-Nal) or 2-naphthylalanine

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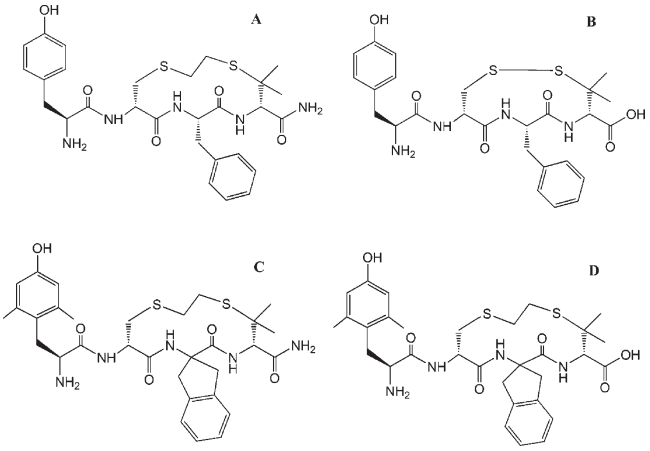

Figure 1. Structures of parent peptides (A) JOM-6 and (B) JOM-13 and new analogues (C) KSK-102 and (D) KSK-103.

(2-Nal) residues produced analogues with decreased DOR efficacy, in agreement with our modeling studies.[19]

We have extended this approach by re-examining previously synthesized, non-selective opioid peptides. As our earlier studies were aimed at development of selective opioid ligands for MOR, DOR, or KOR, resulting non-selective cyclic peptides were not evaluated beyond binding affinity. Re-evaluation of these previously synthesized ligands has led to the identification of potential leads with MOR agonist/DOR antagonist properties. Among these ligands were several analogues of the MOR-selective tetrapeptide JOM-6 (Tyr-c(SCH_2CH_2S)[DCys-Phe-DPen]NH_2)[24] and the DOR-selective tetrapeptide JOM-13 (Tyr-c(SS)[DCys-Phe-DPen]OH[25] (where DPen is D-penicillamine (β,β-dimethyl-D-cysteine) and c(SCH_2CH_2S) and c(SS) denote cyclization through the side-chain sulfurs of DCys and DPen *via* an ethylene dithioether or a disulfide, respectively) in which Phe^3 was replaced with a bulkier or more constrained aromatic residue that might be expected to bind differently to the active and inactive states of opioid receptors. Evaluation and further modification of the most promising candidates led to the two new analogues reported here, KSK-102 (Dmt-c(SCH_2CH_2S)-[DCys-Aci-DPen]NH_2) and KSK-103 (Dmt-c(SCH_2CH_2S)-[DCys-Aci-DPen]OH), where Dmt is 2′,6′-dimethyltyrosine and Aci is 2-aminoindane-2-carboxylic acid (Figure 1). Of these peptides, KSK-103 displayed the desired bifunctional profile and behaved *in vitro* as a MOR agonist with greater potency than the clinical standard morphine. KSK-103 was also found to be a DOR antagonist at the level of receptor-G protein stimulation and at inhibition of the downstream effector enzyme adenylyl cyclase. By comparison, DIPP(Ψ)NH_2 (Dmt- Tic(Ψ)[CH_2NH_2]Phe-$PheNH_2$ (where Tic is tetrahydroisoquinoline-3-carboxylic acid))[20] and UFP-505 (Dmt-Tic-GlyNH-benzyl),[18,26] two previously described MOR agonist/DOR antagonist bifunctional peptides with reported decreased propensity to produce tolerance relative to morphine, displayed partial DOR agonism in the adenylyl cyclase assay and had less desirable receptor binding properties.

RESULTS AND DISCUSSION

For development of the bifunctional peptides described here, we examined alterations to the tetrapeptide JOM-6 scaffold[22,23] that included replacement of Tyr^1 with 2′,6′-dimethyltyrosine (Dmt) and Phe^3 with the conformationally constrained

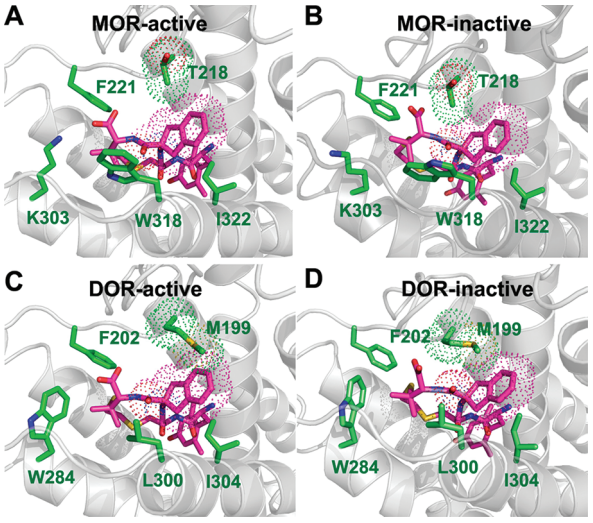

Figure 2. Computational modeling of KSK-103 in MOR and DOR ligand binding pockets reveals structural determinants of ligand efficacy. KSK-103 can be docked without steric hindrances into the ligand binding pocket of the MOR models in the active (A) and inactive (B) conformations but displays significant overlap between Aci^3 of the ligand and Met^{199} of the receptor in the DOR active conformation (C). This overlap is removed in the DOR inactive conformation, where Met^{199} is shifted away from the ligand binding pocket (D).

2-aminoindane-2-carboxylic acid (Aci). Additionally, C-terminal carboxamide (KSK-102) and carboxylic acid (KSK-103) containing analogues were compared. The computational docking of these peptides to the ligand binding pockets of models of active and inactive states of MOR and DOR, illustrated for KSK-103 in Figure 2, reveals a favorable interaction of Aci^3-containing peptides with the active and inactive states of MOR (Figure 2A and B) and the inactive state of DOR (Figure 2D), but a less favorable interaction with DOR in the active state (Figure 2C). In particular, the conformationally constrained Aci^3 displays steric overlap with the bulky side chain of Met^{199} from extracellular loop 2 (EL2) of the active state DOR model. The corresponding residue in MOR (Thr^{218}) has a smaller side chain, allowing favorable docking of Aci to the active MOR state. The different interactions of KSK-103 with distinct functional states of MOR and DOR predict different efficacy of the ligand at both receptors: agonist action at MOR and antagonist action at DOR. These predictions were tested in *in vitro* assays evaluating receptor binding, G protein activation, and inhibition of cAMP production by forskolin-stimulated adenylyl cyclase.

Opioid Receptor Binding. The binding affinity of each peptide was determined at MOR, DOR, and KOR from membrane preparations of C_6 rat glioma cells (MOR or DOR) or CHO cells (KOR) (Table 1). As reported previously, JOM-6 displays 100-fold MOR selectivity in binding to opioid receptors (K_i = 0.29 ± 0.04 nM affinity at MOR and 25 ± 1.5 nM at DOR, Table 1). Replacement of Tyr^1 with Dmt often results in decreased selectivity of the ligand by increasing the affinity at the less favored receptor.[27,28] Replacing the Tyr^1 residue with Dmt^1 and Phe^3 with Aci^3 while maintaining the same ring size with ethylene dithioether cyclization produced KSK-102. These alterations did not change the binding affinity at MOR (0.6 ± 0.1 nM) but significantly increased affinity at DOR (0.9 ± 0.2 nM) and at KOR (9.8 ± 3.6 nM). Incorporation of a C-terminal carboxylic acid in KSK-103 in place of the carboxamide group of KSK-102

Table 1. Opioid Receptor Binding Affinity of Peptides at MOR, DOR, and KOR [a]

peptide	sequence	K_i, nM MOR	DOR	KOR
JOM-6	Tyr-c(SCH_2CH_2S)[DCys-Phe-DPen]NH_2	0.3 ± 0.04[b]	25 ± 1.5[b]	9537 ± 622
KSK-102	Dmt[c]-c(SCH_2CH_2S)[DCys-Aci[c]-DPen]NH_2	0.6 ± 0.1	0.9 ± 0.2	9.8 ± 3.6
KSK-103	Dmt-c(SCH_2CH_2S)[DCys-Aci-DPen]OH	2.4 ± 0.7	2.3 ± 0.5	776 ± 149
DIPP(Ψ)NH_2	Dmt-Tic[c](Ψ)[CH_2NH_2] Phe-PheNH_2	0.4 ± 0.1	0.4 ± 0.04	3.9 ± 0.2
UFP-505	Dmt-Tic-GlyNH-Bzl	26 ± 8	0.2 ± 0.06	128 ± 42

[a] Opioid receptor binding studies in cell membrane preparations. Experiments were performed as described in Methods, and affinity was determined by nonlinear regression following displacement of 0.2 nM [^{3}H]diprenorphine from membrane preparations of opioid receptors individually expressed in C_6 rat glioma (MOR and DOR) or Chinese hamster ovary cells (KOR). Results reported as mean K_i ± standard error from at least three experiments performed in duplicate. [b] JOM-6 affinity at MOR and DOR taken from McFadyen *et al.*[24] [c] Abbreviations include Dmt for 2′,6′-dimethyltyrosine, Aci for 2-aminoindane-2-carboxylic acid, and Tic for 1,2,3,4-tetrahydroisoquinoline, 3-carboxylic acid. Cyclization of peptides reported as (SCH_2CH_2S) for ethylene dithioether linkage.

Table 2. Efficacy and Potency of Cyclized Peptides for Stimulation of [^{35}S]GTPγS Binding at Opioid Receptors

peptide	sequence	MOR % max[a]	MOR EC_{50}, nM[b]	DOR % max	DOR EC_{50}	KOR % max	KOR EC_{50}
KSK-102	Dmt-c(SCH_2CH_2S)[DCys-Aci-DPen]NH_2	58 ± 8	0.4 ± 0.02	37 ± 4	1.4 ± 0.4	n.s.[c]	
KSK-103	Dmt-c(SCH_2CH_2S)[DCys-Aci-DPen]OH	59 ± 11	4.7 ± 0.7	n.s.		n.s.	
DIPP(Ψ)NH_2	Dmt-Tic(Ψ)[CH_2NH_2] Phe-PheNH_2	18 ± 1	5.7 ± 3.3	n.s.		n.s.	
UFP-505	Dmt-Tic-GlyNH-Bzl	n.s.		n.s.		n.s.	
morphine		57 ± 5	194 ± 21	n.t.[d]	n.t.	n.t.	n.t.
endomorphin-2		49 ± 7	125 ± 31	n.t.	n.t.	n.t.	n.t.

[a] Stimulation of [^{35}S]GTPγS binding in membrane preparations from cells stably expressing MOR, DOR, or KOR. % maximum (% max.) values represent percent of maximal [^{35}S]GTPγS binding obtained with 10 μM peptide compared to a 10 μM concentration of standard agonists DAMGO (MOR), DPDPE (DOR), and U69,593 (KOR). [b] EC_{50} values determined from nonlinear regression analysis of [^{35}S]GTPγS incorporation as described in Methods. Experiments were performed in duplicate at least three times and data reported is the mean ± standard error. [c] n.s. = no significant stimulation (less than 10% of standard compound), [d] n.t. = not tested. Abbreviations as in Table 1.

was designed to reduce KOR affinity, as a negative charge in this part of the ligand causes adverse electrostatic interactions at KOR,[29] and previous studies have shown a carboxamide to be beneficial in producing KOR affinity.[30] In agreement, a C-terminal carboxylic acid motif produced a 100-fold decrease in KOR affinity compared with KSK-102. Replacement of the carboxamide by the carboxylic acid in KSK-103 also resulted in a slight decrease in binding affinity to both MOR and DOR (2.4 ± 0.7 nM at MOR and 2.3 ± 0.5 nM at DOR) when compared to KSK-102.

DIPP(Ψ)NH_2 and UFP-505 were also analyzed for opioid receptor binding (Table 1). Our assays were performed using conditions slightly different from those under which these peptides were first reported.[18,20] DIPP(Ψ)NH_2 binds equally well at MOR and DOR with affinity values of 0.4 ± 0.1 nM at MOR and 0.4 ± 0.04 nM at DOR and has 10-fold selectivity for these receptors over KOR (3.9 ± 0.2 nM). UFP-505, on the other hand, is DOR-selective, with a binding affinity of 0.2 ± 0.06 nM. UFP-505 binding affinity at MOR is approximately 100-fold lower (26 ± 8 nM) and KOR affinity is reduced still further (128 ± 42 nM).

Stimulation of G Protein. The ability of the peptides to activate G protein at each receptor was assessed using the [^{35}S]-GTPγS binding stimulation assay (Table 2). The [^{35}S]GTPγS binding results are reported as both maximal stimulation, employed as a read-out of efficacy as a percentage of the [^{35}S]-GTPγS incorporation afforded by known opioid receptor agonist ([D-Ala^2-NMe-Phe^4-Gly^5-ol]-enkephalin (DAMGO) at MOR, D-Pen^{2,5}-enkephalin (DPDPE) at DOR, and U69,593 at KOR) and potency as EC_{50} values.

KSK-102 and KSK-103 behaved as partial agonists at MOR, giving maximal stimulation of 58 ± 8% and 59 ± 11%, respectively, compared to 10 μM DAMGO (Table 2, Figure 3A). By comparison, the clinically used analgesic morphine produced 57 ± 5% of DAMGO-induced stimulation, and the endogenous MOR-selective peptide endomorphin-2 gave 49 ± 7% stimulation. Both KSK-102 and KSK-103 were far more potent at G protein stimulation than morphine (480- and 47-fold, respectively) or endomorphin-2 (310- and 31-fold, respectively). DIPP(Ψ)NH_2 displayed very low efficacy partial agonism at MOR in this assay, giving a relative stimulation of 18 ± 1% compared with DAMGO (Table 2). UFP-505 displayed no significant efficacy at MOR (maximal stimulation <10%).

At DOR, KSK-102 had the highest efficacy of all ligands tested, yielding 37 ± 4% stimulation compared to the DOR peptide agonist DPDPE,[21] with an EC_{50} value of 1.4 ± 0.4 nM. In contrast, KSK-103 produced no significant stimulation of [^{35}S]GTPγS binding at DOR (Table 2). In confirmation of this result, KSK-103 acted as an antagonist in this assay and produced a 26-fold rightward shift in the concentration−response curve for DPDPE (Figure 3B). The EC_{50} for DPDPE was shifted from 246 ± 45 to 6300 ± 1000 nM upon the addition of 100 nM KSK-103. The antagonist affinity constant (K_e)[31] for KSK-103 calculated from this shift was 4.4 ± 1.4 nM.

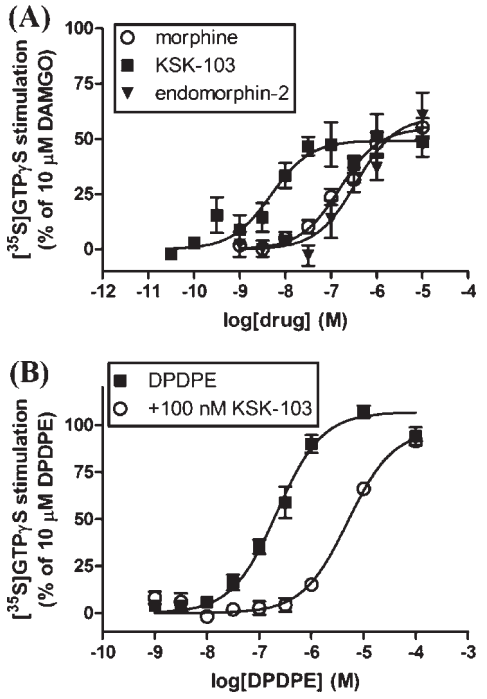

Figure 3. KSK-103 behaves as a MOR agonist and DOR antagonist in the [^{35}S]GTPγS stimulation assay. Incorporation of [^{35}S]GTPγS as a measure of G protein stimulation was analyzed in cell membrane preparations from C_6-rat glioma cells stably expressing either MOR or DOR. (A) At MOR, KSK-103 behaved as a partial agonist, producing 59 ± 11% stimulation of G protein compared to MOR agonist DAMGO (■). Morphine (○) and endomorphin-2 (▼) produced a similar percent stimulation; however, they were less potent than KSK-103. (B) Addition of 100 nM KSK-103 (○) produced a 26-fold rightward shift in the concentration−response curve for DPDPE at DOR, affording a K_e value for the antagonist of 4.4 ± 1.4 nM.

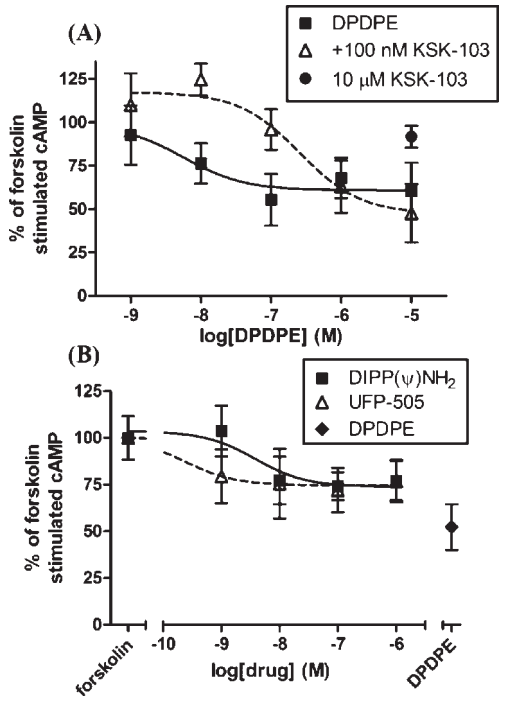

Figure 4. KSK-103 is a DOR antagonist in adenylyl cyclase inhibition, while reference peptides DIPP(Ψ)NH$_2$ and UFP-505 display partial agonist efficacies. Inhibition of forskolin-stimulated cAMP production was measured utilizing a whole cell assay in C_6-rat glioma cells stably expressing DOR. (A) In this assay, 10 μM KSK-103 (●) was unable to significantly inhibit forskolin-stimulated cAMP levels (91 ± 6%) while addition of 100 nM KSK-103 (△) produced a 9.5-fold rightward shift in the concentration−response curve for DPDPE. This afforded a K_e value for antagonism of 12 ± 3.3 nM. (B) Peptides DIPP(Ψ)NH$_2$ (■) and UFP-505 (△) behaved as partial agonists at DOR in this assay, able to reduce forskolin-stimulated cyclase to 65 ± 10% and 72 ± 2%, of controls, respectively. One μM DOR peptide agonist DPDPE (◆) inhibited forskolin stimulation to 52 ± 12%.

The DOR agonist activity of KSK-102, which like KSK-103 has the Aci[3] residue and might be expected to bind poorly to the DOR active state, is likely attributed to its slightly deeper positioning in the binding pocket due to the ability of the KSK-102 C-terminal amide to form an H-bond with the backbone carbonyl of Leu[200] (not shown). This slight readjustment of KSK-102 in the DOR binding pocket results in a small shift of Aci[3] that relieves the steric interaction with the Met[199] side chain in the active conformation of DOR.

Neither UFP-505 nor DIPP(Ψ)NH$_2$ caused detectable stimulation of [^{35}S]GTPγS binding at DOR. At KOR, none of the peptides produced significant stimulation of [^{35}S]GTPγS binding up to a concentration of 10 μM (Table 2).

Inhibition of Forskolin-Stimulated Adenylyl Cyclase. The [^{35}S]GTPγS binding assay requires a compound to activate G protein under stringent conditions. As such, it is possible to misclassify a compound with downstream partial agonist activity as a pure antagonist in this assay.[19] Therefore, we examined the inhibition of adenylyl cyclase (measured as a decrease in forskolin-stimulated cAMP levels) by DPDPE in the presence or absence of KSK-103. KSK-103 maintained the same DOR antagonist profile at the downstream cellular effector adenylyl cyclase (Figure 4A). Thus KSK-103 was unable to significantly reduce forskolin-stimulated cAMP accumulation even at a 10 μM concentration (91 ± 6% forskolin stimulation, or 9% inhibition) and at 100 nM KSK-103 produced a 9.5-fold rightward shift in the DPDPE concentration−response curve (EC_{50} DPDPE alone: 29 ± 7 nM; EC_{50} DPDPE + 100 nM KSK-103: 276 ± 69 nM) giving a calculated K_e value in the nanomolar range (12 ± 3.3 nM), similar to results in the [^{35}S]GTPγS assay.

DIPP(Ψ)NH$_2$ and UFP-505 were also characterized further in the adenylyl cyclase inhibition assay. Both pseudopeptides were observed to have significant partial agonism at DOR. DIPP(Ψ)NH$_2$ decreased forskolin stimulation of cAMP levels by 30 ± 4% with a potency (IC_{50}) of 2.2 ± 0.9 nM, while UFP-505 decreased cAMP levels by 28 ± 2% with an IC_{50} value of 0.7 ± 0.2 nM. (Figure 4B).

Since both DIPP(Ψ)NH$_2$ and UFP-505 displayed very low MOR partial agonist activity in the GTPγS binding assay, they were further examined for the ability to inhibit adenylyl cyclase through activation of MOR. Both DIPP(Ψ)NH$_2$ (64 ± 10% maximal inhibition with an IC_{50} value of 5.1 ± 3.8 nM) and UFP-505 (30 ± 10% maximal inhibition with an IC_{50} value of 45 ± 13 nM) showed significant MOR partial agonism in this assay (data not shown), underscoring again the need to evaluate downstream signaling when stimulation of GTPγS binding yields equivocal results. As expected from the [^{35}S]GTPγS data, KSK-102 and KSK-103 were more efficacious in this assay, able to inhibit the forskolin-stimulated cAMP production by 76 ± 0.9% and 77 ± 2.4%, respectively. For reference, the full MOR

agonist DAMGO produced 86 ± 4.3% maximal inhibition of cAMP production.

Conclusions. The desired profile for a MOR/DOR bifunctional ligand would combine high affinity binding to MOR and DOR with much lower affinity for KOR together with agonism at MOR but antagonism at DOR. In this study we have synthesized and characterized a new cyclic tetrapeptide, KSK-103, with these properties. Utilization of a conformationally constrained Aci[3], along with replacement of Tyr[1] by Dmt[1], and a C-terminal carboxylic acid produced, in KSK-103, a ligand displaying the desired characteristics. This is in agreement with predictions from computational modeling of receptor−ligand complexes, providing further evidence for the value of receptor models for structure-based drug design.

In contrast to KSK-103, neither DIPP(Ψ)NH_2 nor UFP-505 display the desired profile. While DIPP(Ψ)NH_2 had equivalent binding affinities to MOR and DOR, it was only 10-fold selective for these receptors over KOR. UFP-505, on the other hand, was determined to be DOR-selective, having a 130- or 640-fold lower affinity at MOR and KOR, respectively. Moreover, both DIPP(Ψ)NH_2 and UFP-505 display partial agonism at DOR, as indicated by their ability to inhibit adenylyl cyclase, rather than the desired DOR antagonism. Thus, KSK-103 represents a step forward in the development of novel ligands potentially lacking tolerance and dependence liability. Future studies will investigate the *in vivo* actions of KSK-103 after both acute and chronic administration. These studies will determine tolerance liability of KSK-103 versus standard opioid ligands including DAMGO, morphine, and endomorphin-2. The propensity of KSK-103 to produce dependence using *in vitro* and *in vivo* models will also be used to further investigate hypothesized interactions between MOR and DOR that result in adaptive side effects.

METHODS

Materials. Reagents for peptide synthesis and characterization were from Sigma-Aldrich unless otherwise indicated. Fetal bovine serum, cell culture media, and additives were purchased from Gibco Life Sciences. [D-Ala[2], NMePhe[4], Gly[5]-ol]-enkephalin (DAMGO) and other biochemicals were obtained from Sigma-Aldrich. [^{35}S]-Guanosine-5′-*O*-(3-thio)triphosphate ([^{35}S]GTPγS; 1250 Ci (46.2TBq)/mmol) and [^{3}H]-diprenorphine were purchased from Perkin-Elmer. DIPP(Ψ)NH_2 was a gift from the National Institute on Drug Abuse (NIDA) Drug Supply Program.

Solid-Phase Peptide Synthesis and Cyclization of Peptides. KSK-102 and KSK-103 were synthesized in a sequential fashion by solid-phase methods. Synthesis of the carboxylic acid terminal KSK-103 utilized chloromethylated polystyrene (Merrifield) resin cross-linked with 1% divinylbenzene (AdvancedChemTech), while synthesis of the carboxamide terminal analogue, KSK-102, employed *p*-methylbenzhydrylamine resin (AdvancedChemTech). *tert*-Butyloxycarbonyl (Boc) protection of the α-amino function was used throughout. The labile sulfhydryl groups of the D-Cys and D-Pen were protected with the *p*-methylbenzyl function. Both peptides were cleaved from the resin and deprotected by treating with 10 mL of anhydrous HF in the presence of 0.5 g of thiocresol and 0.5 g of cresol. After 1 h of stirring at 0 °C, the solvent was removed under vacuum, and the resin was washed several times with dry diethyl ether. The peptide was extracted from the resin with 5 mL washes of dimethylformamide (DMF)/80% acetic acid (9:1, v/v), diluted 20-fold with aqueous HPLC component, filtered, and subjected directly to semipreparative HPLC (Waters Corporation). Purification of the resulting free sulfhydryl-containing linear peptides was effected by semipreparative HPLC (Waters Corporation) on a Vydac Protein & Peptide C-18 column (2.2 cm × 25 cm) with the solvent system 0.1% trifluoroacetic acid (TFA) in H_2O/0.1% TFA in CH_3CN, using a 0−50% gradient of organic component. The identity and purity of the linear peptides were determined by electrospray ionization mass spectrometry (ES-MS; Agilent Technology, 6130 Quadrupole LC-MS) in positive mode. Dmt-Tic-GlyNH-Bzl (UFP-505) was synthesized according to the reported protocol.[18]

Table 3. Analytical Data for KSK-102 and KSK-103

	molecular weight[a]		
peptide	calcd (MW)	obsd (MW + 1)	HPLC[b] (min; t_R)
KSK-102	627.1	628.1	28.8
KSK-103	628.1	629.1	30.3

[a] Observed molecular weights determined by electrospray ionization mass spectrometry (ES-MS). [b] Retention times assessed by analytical high-performance liquid chromatography (HPLC) using the solvent system 0.1% trifluoroacetic acid (TFA) in water (w/v)/0.1% TFA in CH_3CN with a gradient of 0−50% organic component in 50 min, monitored at 230 nm with samples dissolved in a mixture of aqueous and organic HPLC components. The column was maintained at 35 °C. Both peptides were found to be 98−99% pure.

Dithioether Cyclization of Peptides. A DMF solution of the linear peptide (15 mg/40 mL) containing 10 molar equiv of 1,2-dibromoethane was added dropwise to a cooled round-bottom flask containing 10 molar equiv of potassium *tert*-butoxide in 100 mL of anhydrous DMF. The stirring was continued for 2 h, and the reaction was quenched with 5 mL of acetic acid. Solvents were removed *in vacuo*, and the residue was purified using preparative RP-HPLC to afford the alkyl dithioether-cyclized peptide.

The purity of final peptides was determined using a Waters Alliance 2690 Analytical HPLC and molecular weight confirmed using ES-MS (KSK-102 [M + 1] = 628.1; KSK-103 [M + 1] = 629.1; Table 3).

Cell Lines and Membrane Preparations. C_6-rat glioma cells stably transfected with a rat μ (C_6-MOR) or rat δ (C_6-DOR) opioid receptor[32] and Chinese hamster ovary (CHO) cells stably expressing a human κ (CHO-KOR) opioid receptor[33] were used for all *in vitro* assays. Cells were grown to confluence at 37 °C in 5% CO_2 in either Dulbecco's Modified Eagle's Medium (DMEM; C_6 cells) or DMEM-F12 Medium (CHO cells) containing 10% fetal bovine serum and 5% penicillin-streptomycin. Membranes were prepared by washing confluent cells three times with phosphate-buffered saline (0.9% NaCl, 0.61 mM Na_2HPO_4, 0.38 mM KH_2PO_4, pH 7.4). Cells were detached from the plates by incubation in harvesting buffer (20 mM HEPES, 150 mM NaCl, 0.68 mM EDTA, pH 7.4) and pelleted by centrifugation at 200*g* for 3 min. The cell pellet was suspended in ice-cold 50 mM Tris-HCl buffer, pH 7.4 and homogenized with a Tissue Tearor (Biospec Products, Inc.) for 20 s at setting 4. The homogenate was centrifuged at 20,000*g* for 20 min at 4 °C, and the pellet was rehomogenized in 50 mM Tris-HCl with a Tissue Tearor for 10 s at setting 2, followed by recentrifugation. The final pellet was resuspended in 50 mM Tris-HCl to 0.5−1.0 mg mL^{-1} protein and frozen in aliquots at −80 °C.[34] Protein concentration was determined using the BCA protein assay (Thermo Fisher Scientific) using bovine serum albumin as the standard.

Radioligand Binding Assays. Opioid ligand-binding assays[30] were performed using competitive displacement of 0.2 nM [^{3}H]diprenorphine by the test compound from membrane preparations containing opioid receptors. The assay mixture, containing membrane suspension (20−40 μg protein/tube) in 50 mM Tris-HCl buffer (pH 7.4), [^{3}H]diprenorphine, and various concentrations of peptide, was incubated at 25 °C for 1 h to allow binding to reach equilibrium. The samples were rapidly filtered through GF/C filters (Whatman) using a Brandel harvester and washed three times with 50 mM Tris-HCl buffer.

The radioactivity retained on dried filters was determined by liquid scintillation counting after saturation with EcoLume liquid scintillation cocktail (MP Biomedicals) in a Wallac 1450 MicroBeta (Perkin-Elmer). Nonspecific binding was determined using 10 μM 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]GTPγS binding was measured as described previously.[35] Briefly, membranes (20−40 μg of protein/tube) were incubated 1 h at 25 °C in GTPγS buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$, pH 7.4) containing 0.1 nM [^{35}S]GTPγS, 100 μM GDP, and varying concentrations (0.001−10,000 nM) of peptides. Peptide stimulation of [^{35}S]GTPγS was compared with 10 μM standard compounds DAMGO, D-Pen^{2,5}-enkephalin (DPDPE), or U69,593. The reaction was terminated by rapidly filtering through GF/C filters and washing three times with GTPγS buffer, and retained radioactivity was measured as described above. Experiments were performed at least three times in duplicate, and EC_{50} values were determined using nonlinear regression analysis with GraphPad Prism. To determine antagonist properties of peptides at DOR, [^{35}S]GTPγS binding was determined for DPDPE in the presence or absence of a single concentration of peptide.[31] The EC_{50} value for DPDPE in the presence of peptide was divided by the EC_{50} value for DPDPE alone, and this ratio (DR) was employed to calculate the K_e value using the equation K_e = ([antagonist]/(DR − 1)).

Whole Cell Acute Inhibition of Adenylyl Cyclase. Inhibition of adenylyl cyclase by opioid standards or peptides was measured in C_6-DOR or -MOR cells grown to confluence in 96-well plates. Cells were washed in serum-free DMEM at least 30 min prior to the start of the assay and incubated with various concentrations (1−1000 nM) of DPDPE or peptide in serum-free media containing 5 μM forskolin (FSK) and 0.25 mM 3-isobutyl-1-methylxanthine (IBMX) for 30 min at 37 °C. For assays in C_6-MOR cells, the reaction was quenched with addition of 3% perchloric acid and neutralized with 2.5 M $KHCO_3$. Samples were diluted 1:5 in Elisa Immunoassay (EIA) buffer and analyzed for cAMP accumulation using a kit from Cayman Chemical according to manufacturer's instructions (Ann Arbor, MI) using a VersaMax ELISA microplate reader (Molecular Devices, Sunnyvale, CA). For adenylyl cyclase inhibition assays performed in C_6-DOR cells, the assay was quenched during a 30 min incubation at 4 °C by replacing media with 0.1 mL of lysis buffer (0.3% Tween-20, 5 μM HEPES in dH_2O, pH 7.4). Antagonism of DPDPE-mediated inhibition was measured by addition of a single concentration of KSK-103 in C_6-DOR cells, as described above. Cyclic AMP (cAMP) was measured from samples in a 384-well plate with a BioTek MultiMode Microplate Reader using the AlphaScreen cAMP detection kit from Perkin-Elmer according to manufacturer's instructions. Inhibition of cAMP accumulation was calculated as a percent of FSK-stimulated cAMP accumulation in vehicle-treated cells. EC_{50} values were calculated for each compound using GraphPad Prism. Experiments were performed in triplicate and repeated a minimum of three times.

Modeling. The homology models of human MOR (UniProt ID: P35372, residues 67−354), in the inactive and active states were developed using crystal structures of bovine rhodopsin in the inactive (PDB ID: 1U19) and photoactivated (PDB ID: 3DQB) conformations, respectively. The models were generated as previously described for gonadotropin-releasing hormone receptor modeling.[36] The receptor models were refined using distance geometry calculations with structural restraints that involved receptor-specific H-bonds, natural and engineered disulfide bonds, and metal-binding clusters described in our earlier publications,[37,38] as well as contacts between receptor residues and the native MOR agonist, endomorphin-1 (Tyr-Pro-Trp-PheNH2) or synthetic MOR-antagonist, antanal-2 (Dmt-Pro-Phe-2NalNH$_2$)[39] docked similarly to cyclic tetrapeptides.[29,30] The models of human DOR (UniProt ID: P41143, residues 48−336) and human KOR (UniProt ID: P41145, residues 58−348) were developed from MOR active and inactive state models by residue substitution followed by energy minimization with CHARMm potentials (QUANTA, Accelrys), dielectric constant, ε = 10, and the adopted basis Newton−Raphson minimization method (100 iterations). Coordinates of MOR (active and inactive states) and DOR (inactive state) models with KSK-103 can be downloaded from our Web site (http://mosberglab.phar.umich.edu/resources/).

Statistical Analysis. Data were analyzed using Student's two-tailed *t* test or analysis of variance followed by Bonferroni's posthoc test using GraphPad Prism where appropriate. A *p* value less than 0.05 was used to determine significance.

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