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Discovery of Novel Multifunctional Ligands with μ/δ Opioid Agonist/Neurokinin-1 (NK1) Antagonist Activities for the Treatment of Pain

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Abstract

Multifunctional ligands with agonist bioactivities at μ/δ opioid receptors (MOR/DOR) and antagonist bioactivity at the neurokinin-1 receptor (NK1R) have been designed and synthesized. These peptide-based ligands are anticipated to produce better biological profiles (e.g., higher analgesic effect with significantly less adverse side effects) compared to those of existing drugs and to deliver better synergistic effects than coadministration of a mixture of multiple drugs. A systematic structure–activity relationship (SAR) study has been conducted to find multifunctional ligands with desired activities at three receptors. It has been found that introduction of Dmt (2,6-dimethyl-tyrosine) at the first position and *N*MePhe at the fourth position (ligand **3**: H-Dmt-D-Ala-Gly-*N*MePhe-Pro-Leu-Trp-NH-Bn(3',5'-(CF₃)₂)) displays binding as well as functional selectivity for MOR over DOR while maintaining efficacy, potency, and antagonist activity at the NK1R. Dmt at the first position with Phe(4-*F*) at the fourth position (ligand **5**: H-Dmt-D-Ala-Gly-Phe(4-*F*)-Pro-Leu-Trp-NH-Bn(3',5'-(CF₃)₂)) exhibits balanced binding affinities at MOR and DOR though it has higher agonist activity at DOR over MOR. This study has led to the discovery of several novel ligands including **3** and **5** with excellent in vitro biological activity profiles. Metabolic stability studies in rat plasma with ligands **3**, **5**, and **7** (H-Tyr-D-Ala-Gly-Phe(4-*F*)-Pro-

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Author Contributions

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Notes

An international patent application has been filed with this discovery. A part of this work has been presented as a poster during the American Peptide Symposium (APS)-2015 and is going to be published in the 24th American Peptide Symposium Proceedings as a short communication.

The authors declare no competing financial interest.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmed-chem.5b01170.

HPLC conditions, chromatogram, general experimental description for ICR/MS analysis, and HRMS sheets (PDF)

Molecular formula strings, ALOGPs, and biochemical/biological data (XLS)

Leu-Trp-NH-Bn(3',5'-(CF₃)₂)) showed that their stability depends on modifications at the first and fourth positions (**3**: $T_{1/2} > 24$ h; **5**: $T_{1/2} \approx 6$ h; **7**: $T_{1/2} > 2$ h). Preliminary in vivo studies with these two ligands have shown promising antinociceptive activity.

Graphical Abstract

INTRODUCTION

The Institutes of Medicine (IOM) and the American Pain Society estimate that pain affects >100 million American adults and costs \$100 billion or more each year in medical treatment and lost productivity. Current novel analgesics are limited in their clinical utility as a consequence of significant adverse effects, preventing “dosing to effect” to achieve adequate management of chronic pain. Still today, the primary drugs of choice for both acute and chronic pain are opioids, in particular μ opioid analgesics such as morphine. Opioids which are well accepted for their clinical analgesic efficacy often result in escalating doses to achieve a similar analgesic effect for chronic pain.¹ Opioids are limited by the development of adverse events (e.g., nausea, constipation, and dependence) with decreased efficacy at tolerable doses over time.² Recently, the risks associated with opioid addiction have enhanced concerns in both patients (36%) and physicians (68%).³ In the US, prescribed opioids have become some of the most highly abused drugs as measured by treatment center admission/cause of overdose with a NIDA Abuse survey reporting a 13% increase in prescription drug abuse in 2009.⁴ While many studies in the last 15 years concerning opioid abuse examined the idea that both injury and sustained MOR therapy can induce neuroplastic adaptations, no drug-development strategies have targeted such changes. Adaptations that may contribute to the development of opioidergic adverse events include altered opioid receptor regulation, trafficking, activation, signaling, and interactions with nonopioid receptors.⁵ Adaptations in pronociceptive systems are linked to “anti-opioid”

effects including expression and function of substance P (SP) and its primary receptor, the neurokinin-1 receptor (NK1).⁶ This system is altered following injury and after sustained exposure to opioids including changes in SP content and release with enhanced activity at their respective receptors suggesting antiopioid activity.⁷ In addition to such systems acting as endogenous antiopioids, SP-NK1 is implicated in the mechanisms underlying opioid antinociceptive tolerance, withdrawal, and reward.⁸ Chemical ablation of NK1 expressing neurons in rodents attenuated hyperalgesia, reward/anxiety, and reduced symptoms of physical withdrawal.⁹ No existing drug counteracts these induced neuroadaptations. Thus, there is a lingering, unmet need for novel medications which are effective for these pathological conditions that do not result in the numerous unwanted side effects.

Of interest to our group are interactions between opioid and neurokinin signaling.¹⁰ Co-administrations of a μ/δ opioid agonist and a neurokinin-1 (NK1) antagonist increases the antinociceptive effect such as enhanced potency in acute pain models¹¹ while inhibiting opioid-induced tolerance in chronic tests using rats.¹² A study also revealed that mice lacking a NK1R did not show the rewarding properties of morphine.^{8a} These observations directed us to anticipate that a ligand which was an agonist at opioid receptors (δ/μ) and an antagonist at an NK1 receptor might have synergistic effects in the management of prolonged pain states that involve higher substance P activity. The use of drug cocktails as therapeutics is restricted by their reduced patient compliance, poor ADME properties, and possible drug–drug interactions. A novel approach has been taken to combine these activities in one ligand which should have better ADME properties. The ligand would have potent analgesic affects in both acute pain and in neuropathic pain states without the development of unwanted side effects.¹³ The design of our drugs of interest is based on the adjacent and overlapping pharmacophores, in which the opioid agonist pharmacophore is placed at the *N*-terminus, and the NK1 antagonist pharmacophore sits at the *C*-terminus of a single peptide derived ligand. The designed multifunctional ligands are expected to have additional rewards over a cocktail of individual drugs for easy administration, simple ADME properties, and no drug–drug interactions. Concentration at the biological target is also expected to be higher than that in the coadministration of drug cocktails. As the expression of the NK1 and opioid receptors as well as the neurotransmitters show a significant degree of overlap in the central nervous system, it is predictable that these ligands would show better potency and efficacy.¹⁴ Previous studies from our group showed that the lead ligands **1a** (Figure 1)¹⁵ could reverse neuropathic pain in a rodent model with blood–brain barrier (BBB) permeability, no development of opioid-induced tolerance, and no development of reward liability. A recent study in our laboratories with **1b** (Figure 1) has shown that this multifunctional ligand is highly efficacious in rodent models of acute and neuropathic pain following multiple routes of administration while reducing unwanted side effects associated with opioid therapy, including antinociceptive tolerance, reward liability, gastrointestinal impairment, and physical dependence over a long duration.¹⁶ In vivo study with another ligand **1c** (Figure 1), which has shown improved bioactivities and half-life (>6 h) in rat plasma compared to those of **1a** and **1c**, is now in progress in our laboratories.¹⁷ These results support our hypothesis that a single ligand containing opioid agonist/NK1 antagonist activities is effective against acute and neuropathic pain and that structural modifications can enhance stability and efficacy.

To date, our lead ligands are selective for delta opioid receptor (DOR) over mu opioid receptor (MOR) though some ligands with μ -selectivity have been discussed and presented in some conferences from our group.¹⁸ From our previous studies, it is now clear that these three activities are important to achieve our desired biological activity profile. However, it is not clear what ratio of activities at these opioid receptors is optimal for potent analgesia without toxic side effects. We assume that it might be patient dependent. We thus need to develop ligands with different ratios of these activities. Herein, we report our study in discovering novel multifunctional ligands with binding and functional selectivity for MOR over DOR and NK1 antagonist activity.

The design of structures of the present ligands is based on our previously studied molecules. A close look at the structures of two lead compounds **1a** and **1b** shows that there is a functional group difference in their *C*-terminus (Figure 1). Compound **1a** having the ester at the *C*-terminus has a short half-life in rat plasma (about 1 min), while compound **1b** having a *C*-terminal amide had a half-life of about 4.8 h. Thus, we have chosen to continue with a *C*-terminus with amide modification.^{17,19} Since we observed high binding affinity and antagonist activity at NK1R by both **1b** and **1c**, the corresponding NK1 pharmacophore (i.e., Pro-Leu-Trp-NH-Bn(3',5'-(CF₃)₂)) was adopted for our ligands.^{17,19} Removal of Met from the fifth position of **1b** resulted in **1d** (Figure 1), and it showed good binding selectivity for MOR over DOR though functional assays displayed higher agonist activity at DOR compared to that at MOR.²⁰

N-Methylated α -amino acids are common in nature, as part of larger peptidic natural products. They also have broad application in designing biologically active substances in medicinal chemistry. Further, N-methylation of amino acids is identified to increase BBB permeability, proteolytic stability, and conformational firmness.²¹ Dmt (i.e., 2,6-dimethyl tyrosine) is known for its contribution in increasing the potency and stability of opioid drug candidates.¹⁷ The presence of halogens is common in natural products.²² In general, they play similar kinds of roles as played by methyl group. Among all of the halogens, fluorine is less common in nature, but it has been found to be very useful in drug discovery.²³ In search of further improvement in biological activities, metabolic stability, and BBB permeability, we studied the effects of introducing Dmt, N-methylated, and halogenated amino acid residues in the opioid pharmacophore moiety in the biological profiles of our ligands (Figure 2).

In spite of being highly potent, many ligands fail to show their expected antinociceptive activity in animal models because of their poor bioavailability. These drugs can act most effectively if they interact with corresponding receptors in the central nervous system, which is possible only when they cross the BBB. As higher lipophilicity enhances a molecule's BBB permeability, the above modifications could increase bioavailability leading to effective analgesics. Table 1 shows the ALOGPs (calculated with the help of <http://www.vcclab.org/lab/alogps/start.html>)²⁴ and RP-HPLC retention times of our new designed ligands. The higher the ALOGPs or HPLC retention time, the higher is the lipophilicity. Some of the literature reports might suggest that these ligands cannot cross the BBB because of higher ALOGPs values. However, our previously published ligand **1b** having an ALOGP value of 5.45 does cross the BBB.^{16,25} In vivo studies with another lead ligand **1c** (Figure

1)¹⁷ having an ALOGP value of 5.77 are in progress in our laboratories, and they are showing excellent results.

The multifunctional ligands **2–10** were synthesized using our previously described methods with some modifications (Scheme 1). N-Methylation on solid phase was conducted following the literature procedure (Scheme 2).²⁶ Binding affinities of these ligands were measured on radioligand binding assays.^{15a} Our well-established methods with isolated tissue-based functional assays using guinea pig ileum (GPI) and mouse isolated vas deferens (MVD) were employed for evaluating functional activities of the ligands **2–10**.^{19,20} Metabolic stability of selected ligands was examined by incubating the ligands in rat plasma at 37 °C.²⁷ Please see the Experimental Section for the details of all of these methods.

RESULTS AND DISCUSSION

Biological Activity

In our present investigation, we designed multifunctional ligands which would show higher binding affinity and agonist activity at the μ opioid receptor compared to those at the δ opioid receptor while maintaining their affinity and antagonist activity at the NK1 receptor. To achieve our goal, we introduced unnatural amino acids like Dmt (2,6-dimethyl tyrosine), D-alanine, and N-methylated and halogenated amino acids in the opioid pharmacophore part. Our previous research showed that if the linker (i.e., Met) between the opioid and NK1 pharmacophores is removed from the ligand **1b** (H-Tyr-D-Ala-Gly-Phe-Met-Pro-Leu-Trp-NH-Bn(3',5'-(CF₃)₂)), then the resulting ligand **1d** (H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-Bn(3',5'-(CF₃)₂)) became μ -selective in binding assays. However, functional assays with this ligand showed higher agonist activity at DOR compared to that at MOR (Table 3). To achieve our goal, we initiated our investigation with ligand **2** (**AKG117**), which is a ligand produced by the replacement of Phe at the fourth position of ligand **1d** (**TY012**) by Δ MePhe. It showed 10-fold binding selectivity for MOR over DOR receptors ($K_i^\mu = 27$ nM; $K_i^\delta = 260$ nM; Table 2) while showing good binding affinity at NK1 receptors ($K_i^{hNK1} = 3.4$ nM; $K_i^{tNK1} = 61$ nM; Table 2), meaning that no appreciable change in binding affinities were present compared to that in **1d** ($K_i^\mu = 9.5$ nM, $K_i^\delta = 72$ nM, $K_i^{hNK1} = 0.6$ nM, and $K_i^{tNK1} = 33$ nM; Table 2). Functional assays with ligand **2** also showed no major change in agonist activities at opioid receptors and antagonist activity at NK1R ($IC_{50}^\mu = 230$ nM, $IC_{50}^\delta = 102$ nM, and $K_e^{NK1} = 21$ nM; Table 3) compared to those for **1d** ($IC_{50}^\mu = 350$ nM, $IC_{50}^\delta = 45$ nM, and $K_e^{NK1} = 8.5$ nM; Table 3). So, introduction of Δ MePhe alone at the fourth position has minimum impact in altering the in vitro biological profiles. Dmt is well-known to increase the binding affinities at opioid receptors. Ligand **3** (**AKG115**), where Tyr at the first position of ligand **2** was replaced by Dmt, showed 5 times the binding selectivity for MOR ($K_i^\mu = 1$ nM; $K_i^\delta = 5$ nM; Table 2) and slightly more agonist activity at MOR over DOR ($IC_{50}^\mu = 21$ nM; $IC_{50}^\delta = 31$ nM; Table 3), while showing high binding affinity and antagonist activity at the NK1 receptor ($K_i^{hNK1} = 2.2$ nM; $K_i^{tNK1} = 48$ nM; Table 2; $K_e^{NK1} = 9.7$ nM; Table 3). This indicates that the presence of Dmt at the first position played a role in increasing binding affinities and agonist activities at μ/δ opioid receptors and antagonist activity at NK1R as well. To cross-check whether N-methylated Phe at the fourth position in **3** (**AKG115**) had any impact in binding affinities and functional activities, ligand **4**

(**AKG116**) having Phe in place of *N*MePhe was designed and synthesized keeping Dmt at the first position. This ligand showed strong binding affinities for both μ and δ opioid receptors ($K_i^\mu = 3$ nM; $K_i^\delta = 1$ nM; Table 2). Its functional assays showed 26-fold less agonist activity at MOR compared to that at DOR ($IC_{50}^\mu = 81$ nM; $IC_{50}^\delta = 3.1$ nM; Table 3). It produced slightly increased binding affinity but a small decrease in antagonist activity at the NK1R ($K_i^{hNK1} = 1.4$ nM; $K_i^{rNK1} = 27$ nM; Table 2; $K_e^{NK1} = 25$ nM; Table 3). From the results observed for ligands **2**, **3**, and **4**, it is evident that the presence of Dmt at the first position and *N*-methylated Phe at the fourth position is required for higher agonist activity at MOR than that at DOR. These results also are consistent with our previous observations that structural change at opioid pharmacophores can have an impact on the biological profiles at NK1 receptors. The presence of halogens in drug candidates is known to play influential roles in their affinity and activities at biological targets. In ligands **5** (**AKG127**), **6** (**AKG128**), **7** (**AKG190**), **8** (**AKG191**), **9** (**AKG192**), and **10** (**AKG193**), we examined the effects of the presence of halogens. Though among halogen containing natural products, the presence of fluorine is less common, it has been found that the presence of single or multiple fluorine atoms in synthetic drug candidates can have profound effects on their biological profiles. In ligands **5**, **6**, and **7**, we studied the effect of Phe(4-F) at the fourth position. When we replaced the Phe from ligand **4** by 4-fluorophenylalanine, i.e., Phe(4-F), to produce ligand **5** (**AKG127**), it showed balanced binding affinities at MOR over DOR ($K_i^\mu = 1$ nM; $K_i^\delta = 1$ nM, Table 2) while showing high affinity for NK1 receptors ($K_i^{hNK1} = 1$ nM; $K_i^{rNK1} = 29$ nM; Table 2). However, the functional assay results displayed 21 times higher agonist activity at DOR compared to that at MOR while exerting high antagonist activity at the NK1 receptor ($IC_{50}^\mu = 42$ nM, $IC_{50}^\delta = 2$ nM, and $K_e^{NK1} = 5.3$ nM; Table 3). This kind of selectivity might be due to the fact that all bonded ligands to MOR are not involved in its activation. To check the effect of combination of *N*-methylation and the presence of fluorine, we synthesized ligand **6** (**AKG128**), which contains *N*-methylated 4-fluorophenylalanine (*N*Me-Phe(4-F)) as its fourth residue. It showed good binding affinity at all three receptors with 10-fold selectivity for MOR over DOR ($K_i^\mu = 0.4$ nM, $K_i^\delta = 4$ nM, $K_i^{hNK1} = 2.6$ nM, and $K_i^{rNK1} = 34$ nM; Table 2). However, functional assays showed nearly 7 times lower agonist activity at MOR than that at DOR while maintaining antagonist activity at the NK1R ($IC_{50}^\mu = 77$ nM, $IC_{50}^\delta = 11$ nM, and $K_e^{NK1} = 11$ nM; Table 3). To examine whether the combination of Dmt at the first position and Phe(4-F) at the fourth position had an impact on the in vitro biological profile of **5** (**AKG127**), we substituted Dmt at the first position by Tyr, which produced ligand **7** (**AKG190**). This modification did not lead to an improvement in the biological profile ($K_i^\mu = 4$ nM, $K_i^\delta = 7$ nM, $K_i^{hNK1} = 5.6$ nM, and $K_i^{rNK1} = 34$ nM; Table 2) as well as in functional assays ($IC_{50}^\mu = 65$ nM, $IC_{50}^\delta = 12$ nM, and $K_e^{NK1} = 5.8$ nM; Table 3). Then, to investigate the effect of other halogens we synthesized ligands **8** containing Phe(4-Cl), **9** containing Phe(4-Br), and **10** containing Phe(4-I) as the fourth residue. All of them showed reduced binding affinities (Table 2) as well as functional activities (Table 3) at opioid receptors compared to those of the parent ligand **5** (**AKG127**), but they displayed comparable binding affinities (Table 2) as well as functional activities (Table 3) at NK1 receptors. Iodine containing ligand **10** (**AKG193**) was much less active at the MOR though it showed good affinity for the same receptor. This again indicates that the binding of a ligand to a receptor is not necessarily correlated with its functional activities.

In Vitro cAMP Functional Assays

Three of our ligands, i.e., **3** (**AKG115**), **4** (**AKG116**), and **5** (**AKG127**) were taken and further examined for their agonist activities at MOR/DOR and antagonist activity at NK1R using cAMP functional assays. The in vitro opioid agonist activity was assessed by measuring cAMP inhibition in MOR-HEK293 and DOR-CHO cells treated with 10 mM forskolin and various ligand concentrations. Compound **3** (**AKG115**) displayed high and almost equipotency at both of these receptors ($IC_{50}^{\mu} = 39$ nM; $IC_{50}^{\delta} = 30$ nM; Table 4). Compound **4** (**AKG116**) showed high potency at both MOR and DOR with IC_{50} values of 46 nM and 7.2 nM, respectively (Table 4), meaning that it was ~6 times more active at DOR. **AKG127** showed high potency at opioid receptors with 3 times more activity at DOR compared to that at MOR (Table 4). These results are consistent with those observed during the smooth muscle stimulation assay (Table 3). Small differences are likely attributable to differences in cell type such as the differential expression of effector proteins or other receptors; or the moderate SEM calculation in the camp assays. Nonetheless, taken together the potency differences are minimal when considering cell type, assay differences, and standard deviations of each experiment. Therefore, these ligands display more balanced MOR and DOR agonist activities compared to those of previous ligands in the series.

NK1R antagonist activity was assessed by treating NK1-CHO cells with varying concentrations of the ligands to inhibit substance P (SP) dose–response curves. These yielded K_e values indicative of the amount of ligand required to double the EC_{50} value of SP. Compounds **3** (**AKG115**) and **4** (**AKG116**) have shown very similar antagonist activity at NK1R (K_e values of 13 and 11 nM, respectively), while compound **5** (**AKG127**) appears nearly 10 times less potent with a K_e of 125 nM (Table 4).

In Vitro Metabolic Stability

To check the stability of our lead ligands, we conducted metabolic stability studies by incubating the ligands in rat plasma at 37 °C.^{27,28} Ligand **3** (**AKG115**) having Dmt at the first position and NMePhe at the fourth position showed high stability ($T_{1/2} > 24$ h, Table 5 and Chart 1). Ligand **5** (**AKG127**) containing Dmt and Phe(4-F) at first and fourth positions, respectively, showed higher stability ($T_{1/2} > 12$ h; Table 5 and Chart 1) compared to that with **TY027** ($T_{1/2}$: 4.8 h).¹⁷ Compound **7** (**AKG190**), which has Tyr and Phe(4-F) at first and fourth positions, respectively, was also tested for its metabolic stability to know the effect of Dmt in **5**. It showed a lower half-life ($T_{1/2} > 2$ h; Table 5 and Chart 1) compared to that for **3** and **5**. These results suggest that the presence of Dmt at the first position is playing a major role in enhancing metabolic stability. The presence of Dmt and NMePhe at first and fourth positions, respectively, might be responsible for the high stability of ligand **3** in rat plasma.

When all of the in vitro results are taken into account, ligands **3** (1.5 times higher agonist activity at MOR, Table 3) and **5** (21 times higher agonist activity at DOR, Table 3) were selected for in vivo study to compare their potential as analgesics. As a proof of concept, some preliminary in vivo studies were conducted with these two ligands as described in the following section.

In Vivo Study

Comparison of our in vitro results suggested that two compounds **3** (AKG115) and **5** (AKG127) may have good antinociceptive activity in vivo. Since one of them are μ -selective (ligand **3**) while the other one is δ -selective (ligand **5**), we anticipated that these two ligands for in vivo studies may have different in vivo antinociceptive activities. To assess antinociception, we used a radiant heat assay to elicit a paw withdrawal reflex.²⁹ The analgesic efficacy of spinal **3** or vehicle were evaluated in rats. Paw withdrawal latencies (PWLs) of rats after spinal administration of **3** (0.1 μ g in 5 μ L, i.t.) were not significantly higher than those of vehicle-treated rats and baseline values 60 min after the injection (Figure 3). The dose was increased to 10 μ g in 5 μ L; however, motor skills using the rotarod were impaired rendering analysis of PWLs inconclusive (data not shown). The structural modification made to compound **3** to create compound **5** indicated that in vivo activity may be more pronounced in the latter. Preliminary studies in a mouse model of acute thermal pain showed that tail flick latencies (TFLs) of mice administered with **5** (0.1 μ g in 5 μ L, i.t.) were significantly higher than those of vehicle-treated mice and baseline values 60 min after injection ($p = 0.04$ compared to that of the vehicle treatment group, $p = 0.02$ compared to the baseline value; Figure 4); follow up studies will determine if compound **5** retains activity in rats.

For both studies, maximal percent efficacy was calculated and expressed as

$$\% \text{ antinociception} = 100 \times (\text{test latency after drug treatment} - \text{baseline latency}) / (33 - \text{baseline latency})$$

(1)

Here, we showed limited in vivo activity of ligands **3** and **5** for acute thermal pain in two species. Despite having high binding affinity and in vitro functional activity at mouse and rat receptors, the maximal level of antinociception observed after the administration of **3** was minimal ($24.0 \pm 15.8\%$, $p = 0.99$). In a murine model of acute pain (i.e., tail flick), a single spinal injection of **5** was nearly 70% effective ($68.4 \pm 14.0\%$) and was statistically significant over that of the vehicle ($p = 0.02$).

CONCLUSIONS

In our ongoing effort to discover better analgesics for the treatment of prolonged and neuropathic pain, we concentrated on the opioid pharmacophore in part to achieve our desired biological profiles at μ/δ opioid receptors while maintaining potent antagonist activity at NK1 receptors. This study has led to the discovery of lead ligand **3** having Dmt at the first position and NMePhe at the fourth position. The presence of these two residues at the mentioned locations has a synergistic effect in functional selectivity at opioid receptors. As expected, structural changes in the opioid pharmacophore have changed the agonist activities at μ/δ opioid receptors. In addition, these modifications alter the antagonist activity at NK1R to some extent meaning opioid and NK1 pharmacophores are not completely

independent of each other in these ligands. Among all of the halogenated Phe used, the maximum increase in potency of ligands was found when fluorine containing Phe (i.e., Phe(4-F)) was incorporated as the fourth residue. Ligand **3** having Dmt and *N*MePhe at the first and fourth positions, respectively, showed the highest metabolic stability ($T_{1/2} > 24$ h) in rat plasma among the three ligands studied. Ligand **3** also showed higher agonist activity at MOR compared to that at DOR. Ligand **5** containing Dmt and Phe(4-F) at the first and fourth positions, respectively, and with appreciable metabolic stability ($T_{1/2} > 6$ h) showed higher agonist activity at DOR compared to that at MOR. Limited in vivo studies with these two multifunctional ligands showed that structural modifications at the first and fourth positions could lead to better analgesics. Future studies will investigate in vivo activity in models of acute and chronic pain.

EXPERIMENTAL SECTION

Materials

The amino acids, coupling reagents, and resins used for this study were purchased from AAPPTEC (USA), and Chem-Impex International (USA). ACS grade organic solvents were purchased from VWR and were used without further purification. HPLC grade acetonitrile was also purchased from VWR.

Synthesis of Ligands

Linear peptides were synthesized on solid phase using 2-chlorotrityl chloride resin (loading: 1.02 mmol/g) via Fmoc/^tBu approach. All steps during solid phase synthesis were performed in fritted syringes. N-Methylation on desired amino acids was performed on the solid phase. C-Terminal amidation was conducted in solution.

Loading of the First Amino Acid on the Resin

2-Chlorotrityl chloride resin (0.102 mmol) was swollen in dry dichloromethane (DCM) for 1 h at room temperature. After swelling, dry DCM was expelled from the syringe, and the resin was washed with DCM (1 mL, 3 × 1 min). It was then ready for the first amino acid coupling. The pregenerated (by treating with 5.0 equiv of DIPEA) carboxylate of Fmoc-Trp(Boc)-OH (1.2 equiv) in dry DCM (1.0 mL) was loaded onto the resin by displacing chloride from the resin. After the coupling of the first amino acid, methanol (0.1 mL) was added to the mixture and was shaken for 15 min in order to cap any unreacted chloride present in the resin. It was then washed with DCM (1 mL, 5 × 1 min) and DMF (1 mL, 4 × 1 min).

Deprotection

Following the washes, deprotection of the Fmoc group was performed. This was done by stirring the resin with 20% piperidine in DMF two times: first for 8 min, followed by a second treatment for 12 min. A DMF wash (1 mL, 1 min) was performed in between the two deprotection steps to remove side products. After the second time piperidine treatment, resin washes were performed with DMF (1 mL, 3 × 1 min), DCM (1 mL, 3 × 1 min), and DMF (1 mL, 3 × 1 min) before the next coupling. These steps were repeated after coupling of each Fmoc protected amino acid in the peptide sequence.

Elongation of Peptide via Coupling Reactions

For the coupling of the remaining amino acids, Fmoc-AA-OH (3.0 equiv, AA = amino acid of interest), HCTU (3.0 equiv and in the case of primary amine), or HATU/HOAt (3.0 equiv of each, in the case of secondary amine) was used as coupling reagents and DIPEA (6.0 equiv) as base. All couplings involving primary amines were carried out in DMF (1 mL/0.3 mmol of amino acid) while the coupling of secondary amine was performed in NMP (1 mL/0.1 mmol of amino acid). Between each coupling, resin washes were performed with DMF (1 mL, 3 × 1 min), DCM (1 mL, 3 × 1 min), and DMF (1 mL, 3 × 1 min).

After each coupling or deprotection, a Kaiser or chloranil test was performed to determine whether or not amino acid coupling or Fmoc deprotection was successful. Kaiser tests were run for primary amino acids and chloranil tests for secondary amino acids (e.g., proline and methylated amino acids). A negative test after each coupling suggests that the reaction was complete. After deprotection, the same test would be positive.

N-Methylation of Amino Acid Derivatives

After Fmoc deprotection of the desired amino acid that will be N-methylated, *o*-NBS protection, *N*-methylation, and then *o*-NBS deprotection were performed. *o*-NBS protection: After Fmoc deprotection, the resin was washed with DMF, DCM, then NMP (3 × 1 min each). NMP was drained out from the syringe. NMP (1 mL) was added to the resin followed by the addition of *o*-NBS-Cl (4 equiv) and sym-collidine (10.0 equiv). It was stirred for 15 min. The same step was repeated one more time after filtering and washing the resin with NMP (1 mL, 1 × 1 min) in between. It was then washed with NMP (1 mL, 5 × 1 min) and then used for N-methylation. N-Methylation (DBU mediated method): DBU (1,8-diazabicyclo(5,4,0)undec-7-ene) (3.0 equiv) in NMP (1 mL) was treated with the resin for 3 min. Afterward and without filtering, DMS (10.0 equiv) was added directly to the syringe containing resin and DBU solution and stirred for another 3 min. The resin was then filtered and washed with NMP (1 × 1 min). This step was repeated once followed by filtration and washing with NMP (5 × 1 min). The resultant resin bound peptide with N-methylation on amino acid was used for *o*-NBS deprotection. *o*-NBS deprotection: NMP (1 mL), 2-mercaptoethanol (10.0 equiv), and DBU (5.0 equiv) were added to the syringe, and the resin was treated for 5 min. The resin was filtered and washed with NMP (1 mL, 1 × 1 min). The procedure was repeated one more time, and then the resin was filtered and washed with NMP (5 × 1 min).

Cleaving Peptide from the Resin

DIPEA (0.200 mL) was added to a centrifuge tube to trap excess TFA while collecting the peptide. The resin was stirred on a shaker with 1% TFA (2 mL/0.102 mmol of starting resin) in DCM (3 × 5 min) on the shaker. The resin was rinsed in between cleavage with small amounts of DCM. The peptide containing solution was collected in the centrifuge tube. The resin became darker with each TFA treatment. Volatiles were evaporated from the centrifuge tube by flushing the resulting solution with argon.

Amidation

The crude peptide was dissolved in dry DMF (1 mL) followed by the addition of HATU (1.0 equiv), HOAt (1.0 equiv), DIPEA (4.0 equiv), and 3,5-bis(trifluoromethyl)benzylamine (1.1 equiv), and the mixture was stirred overnight. Workup: KHSO₄ (0.5 M in H₂O, 5 mL) was added to reaction mixture followed by extraction with DCM (3 × 15 mL). The combined organic extract was taken into a separatory funnel and was washed with brine (1 × 15 mL). The organic part was washed with NaHCO₃ (1 × 15 mL) followed by another brine wash. The final organic solution was dried over anhydrous sodium sulfate, gravity filtrated, and then evaporated under pressure to remove DCM in a round-bottomed flask (RBF).

Removal of Boc/^tBu Protecting Groups

The crude peptide was treated for 1 h with a cleavage cocktail containing 82.5% TFA, 5% H₂O, thioanisole, 5% phenol, and 2.5% 1,2-ethanedithiol to remove Boc/^tBu protecting groups. After 1 h, the solution was flushed with argon to evaporate volatiles.

Precipitation

Hexane wash (3 × 15 mL) was performed to remove low polar materials by vortexing the mixture with hexanes followed by centrifugation at 3.3 rpm (3 × 5 min), each time replacing the hexane layer. Washes with hexanes and diethyl ether mixture (30:70, 3 × 15 mL) gave a white precipitate in 90–100% as a crude yield.

Characterization and Purification

Synthesis of all peptides was confirmed by mass-spectrometry (ESI-MS) data. HRMS data were taken only for the final purified products. Purification of crude products using semipreparative RP-HPLC (Vydac 218TP1010 C18 column) furnished the pure ligands in 20–40% overall yield. Analytical purity of the final ligands was checked by RP-HPLC (Vydac 218TP C18 10 μ column, length 250 mm, ID 4.6 mm). Run time was 40 min using a linear gradient (solvent A, 0.1% aq TFA; solvent B, acetonitrile; 10–90% of solvent B in 40 min). All ligands were >95% pure when submitted for biological study.

General Experimental Description for ICR/MS Analysis

The samples were dissolved in 0.1% formic acid in water/acetonitrile (50/ 50) and then infused into a Bruker Apex 9.4T Fourier transform/ion cyclotron resonance (FT/ICR) mass spectrometer using electrospray ionization (ESI) equipped with a dual ion funnel interface. The ESI needle was maintained at 3.5 kV, and nitrogen at 1.4 L/min was employed to produce a spray which was further desolvated by an opposing stream of nitrogen at 2.5 L/min heated to 200 °C. Positive ions were analyzed. The FT/ICR was scanned from m/z 200 to 2000 in 1 s, and 20 scans were accumulated for each data file. The FT/ICR was calibrated immediately prior to sample analysis by infusion of an Agilent Technologies Tuning Mix (P/N G2421A) under the same conditions and then comparison to the reference masses for the calibration mix. The data files were processed with Bruker Data-Analysis software v. 1.4.

Methods for in Vitro Study

hNK1/CHO Cell Membrane Preparation and Radioligand Binding

Assay.^{15a,19,20}—Recombinant hNK1/CHO cells were grown to confluency in 37 °C, 95% air, and 5% CO₂, humidified atmosphere, in a Forma Scientific (Thermo Forma, OH) incubator in Ham's F12 medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 500 µg/mL Geneticin. The confluent cell monolayers were then washed with Ca²⁺, Mg²⁺-deficient phosphate-buffered saline (PD buffer) and harvested in the same buffer containing 0.02% EDTA. After centrifugation at 2700 rpm for 12 min, the cells were homogenized in ice-cold 10 mM Tris-HCl and 1 mM EDTA, pH 7.4, buffer. A crude membrane fraction was collected by centrifugation at 18000 rpm for 12 min at 4 °C, the pellet was suspended in 50 mM Tris-Mg buffer, and the protein concentration of the membrane preparation was determined by using the Bradford assay. The same protocol was followed for rNK1/CHO cell membrane preparation and the radioligand binding assay.

Bradford Assay—This assay was performed as per the manufacturer's instructions (Bio-Rad). Six different concentrations of the test compound were each incubated, in duplicate, with 20 µg of membrane homogenate, and 0.5 nM [³H] SP (135 Ci/mmol, PerkinElmer, United States) in a 1 mL final volume of assay buffer (50 mM Tris, pH 7.4, containing 5 mM MgCl₂, 50 µg/mL bacitracin, 30 µM bestatin, 10 µM captopril, and 100 µM phenylmethylsulfonylfluoride). SP at 10 µM was used to define the nonspecific binding. The samples were incubated in a shaking water bath at 25 °C for 20 min. The reaction was terminated by rapid filtration through Whatman grade GF/B filter paper (Gaithersburg, MD) presoaked in 1% polyethylenimine and washed four times each with 2 mL of cold saline, and the filter bound radioactivity was determined by liquid scintillation counting (Beckman LS5000 TD).

In Vitro cAMP Functional Assays at MOR and DOR and NK1R—All cAMP assays were performed with the GloMAX cAMP kit from Promega according to the manufacturer's instructions. In vitro opioid agonist activity was assessed by measuring cAMP inhibition in MOR-HEK293 and DOR-CHO cells after treatment with 10 mM forskolin for 15 min and then between 0.1 nM and 1 µM ligand. NK1R antagonist activity was assessed by treating NK1-CHO cells with varying concentrations of the ligands to inhibit substance P (SP) dose-response curves. These yielded *K_e* values indicative of the amount of ligand required to double the EC₅₀ value of SP.

Data Analysis—Analysis of data collected from three independent experiments performed in duplicate is done using GraphPad Prism 4 software (GraphPad, San Diego, CA). Log IC₅₀ values for each test compound were determined from nonlinear regression. The inhibition constant (*K_i*) was calculated from the antilogarithmic IC₅₀ value by the Cheng and Prusoff equation.

Guinea Pig Isolated Ileum/Longitudinal Muscle with Myenteric Plexus (GPI/LMMP).^{15a,19,20}—Male Hartley guinea pigs under CO₂ anesthesia were sacrificed by decapitation and a nonterminal portion of the ileum removed. The longitudinal muscle with

myenteric plexus (LMMP) was carefully separated from the circular muscle and cut into strips as described previously (Porreca and Burks).³⁰ These tissues were tied to gold chains with suture silk and mounted between platinum wire electrodes in 20 mL organ baths at a tension of 1 g and bathed in oxygenated (95:5 O₂/CO₂) Kreb's bicarbonate buffer at 37 °C. They were stimulated electrically (0.1 Hz, 0.4 ms duration) at supramaximal voltage. Following an equilibration period, compounds were added cumulatively to the bath in volumes of 14–60/L until maximum inhibition was reached. A dose–response curve of PL-017 was constructed to determine tissue integrity before analog testing.

Mouse Isolated Vas Deferens Preparation.^{15a,19,20}—Male ICR mice under CO₂ anesthesia were sacrificed by cervical dislocation and the vasa differentia removed. Tissues were tied to gold chains with suture silk and mounted between platinum wire electrodes in 20 mL organ baths at a tension of 0.5 g and bathed in oxygenated (O₂/CO₂ = 95:5) magnesium free Kreb's buffer at 37 °C. They were stimulated electrically (0.1 Hz, single pulses, 2.0 ms duration) at supramaximal voltage as previously described.³⁰ Following an equilibration period, compounds were added to the bath cumulatively in volumes of 14–60/L until maximum inhibition was reached. A dose–response curve of DPDPE was constructed to determine tissue integrity before analogue testing.

Agonist and Antagonist Testing—Compounds were tested as agonists by adding cumulatively to the bath until a full dose–response curve was constructed or to a concentration of 1 M. Compounds were tested as antagonists by adding to the bath 2 min before beginning the cumulative agonist dose–response curves of the δ (DPDPE) or μ (PL-017) opioid agonists.

Analysis—Percentage inhibition was calculated using the average tissue contraction height for 1 min preceding the addition of the agonist divided by the contraction height 3 min after exposure to the dose of agonist. IC₅₀ values represent the mean of not less than 4 tissues. IC₅₀ and $E_{\max}$ estimates were determined by computerized nonlinear least-squares analysis (FlashCalc).

In Vitro Metabolic Stability.^{27,28}—A stock solution (50 mg/mL in DMSO) of each compound in the study was made. It was diluted 1000-fold into rat plasma (Lot 32432, Pel-Freez Biologicals, Rogers, AK) resulting in an incubation concentration of 50 μ g/mL. Incubation temperature was 37 °C. Two hundred microliters of aliquots were pipetted out at different time points (i.e., 0.5 h, 1 h, 2 h, 4h, 6 h, and 8 h). Then, 300 μ L of acetonitrile was added to it and vortexed followed by centrifugation at 15000 rpm for 15 min. The supernatant was taken and analyzed for the remaining amount of parent compound using RP-HPLC (Vydac 218TP C18 10 μ m; length, 250 mm; ID, 4.6 mm). Each sample was run twice and each time in duplicate.

Methods for in Vivo Study

Animals.¹⁷—Adult male Sprague–Dawley rats (225–300 g; Harlan, Indianapolis, IN) and ICR mice (15–20 g; Harlan, Indianapolis, IN) were kept in a temperature-controlled environment with lights on 07:00–19:00 with food and water available ad libitum. All

animal procedures were performed in accordance with the policies and recommendations of the International Association for the Study of Pain, the National Institutes of Health, and with approval from the Animal Care and Use Committee of the University of Arizona for the handling and use of laboratory animals.

Surgical Methods—Rats were anesthetized (ketamine/xylazine anesthesia, 80/12 mg/kg i.p.; Sigma-Aldrich) and placed in a stereotaxic head holder. The cisterna magna was exposed and incised, and an 8 cm catheter (PE-10; Stoelting) was implanted as previously reported, terminating in the lumbar region of the spinal cord.³¹ Catheters were sutured (3–0 silk suture) into the deep muscle and externalized at the back of the neck. After a recovery period (7 days) after implantation of the indwelling cannula, the vehicle (10% DMSO/ 90% MPH₂O), or AKG115 (0.1 μ g; n = 6/treatment) was injected in a 5 μ L volume followed by a 9 μ L saline flush. Catheter placement was verified at the completion of experiments.

Behavioral Assay—Paw-flick latency was collected as follows.²⁹ Rats were allowed to acclimate to the testing room for 30 min prior to testing. Basal paw withdrawal latencies (PWLs) to an infrared radiant heat source were measured (intensity = 40) and ranged between 16.0 and 20.0 s. A cutoff time of 33.0 s was used to prevent tissue damage. After a single, intrathecal injection (i.t.) of **3** (AKG115) or the vehicle, PWLs were reassessed up to 8 times postinjection.

In follow-up studies with **5** (AKG127), we chose a mouse model of acute thermal pain (tail flick latency-TFL) and administered our compound by lumbar puncture to eliminate the need for intrathecal catheters.³² Briefly, the latency to tail withdrawal (TFL) from a 52 °C water bath were measured before (baseline) intrathecal injection of **5** (0.1 μ g in 5 μ L volume, n = 6–8/treatment). Tail flick latencies were reassessed at up to 8 time points after administration. A cutoff latency of 10.0 s was implemented to prevent tissue damage to the distal third of the tail. Mice with baseline TFLs <3 s or >6 s were excluded from the study.

For both studies, maximal percent efficacy was calculated and expressed as

$$\% \text{ antinociception} = 100 \times (\text{test latency after drug treatment} - \text{baseline latency}) / (\text{cutoff} - \text{baseline latency})$$

Statistics

Between group data were analyzed by nonparametric two-way analysis of variance (ANOVA; post hoc, Neuman–Kuels) in FlashCalc (Dr. Michael H. Ossipov, University of Arizona, Tucson, AZ, USA). Within group data were analyzed by nonparametric one-way analysis of variance (ANOVA; post hoc, Bonferroni) in FlashCalc (Dr. Michael H. Ossipov, University of Arizona, Tucson, AZ, USA). Differences were considered to be significant if P 0.05. All data were plotted in GraphPad Prism 6.

Compounds **3** and **5** were prepared in 10% DMSO in 90% MPH₂O.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

An international patent application (PCT Int. Appl. (2015), WO 2015127451 A1 20150827) has been filed with this discovery. A part of this work has been presented as a poster during the American Peptide Symposium (APS)-2015 and is published in the American Peptide Symposium Proceedings as a short communication (<http://dx.doi.org/10.17952/24APS.2015.069>).³³ We thank US Public Health Service, National Institutes of Health (NIH) for supporting this project (Grant Nos.: 2P01 DA 006284 & R01 DA 013449). We thank the University of Arizona Mass Spectrometry Facility for the mass spectra measurements. We are grateful to Christine Hiner Kasten for her assistance with this manuscript.

ABBREVIATIONS USED

ADME	absorption, distribution, metabolism, and excretion
Bn	benzyl
DBU	1,8-diazabicyclo[5.4.0]undec-7-ene
DCM	dichloromethane
DIEA	<i>N,N</i> -diisopropylethylamine
DMF	<i>N,N</i> -dimethylformamide
DOR	delta opioid receptor
hNK1	human neurokinin-1
HATU	1-[bis(dimethylamino)-methylene]-1 <i>H</i> -1,2,3-triazolo[4,5- <i>b</i>]pyridinium 3-oxid hexafluorophosphate
HCTU	<i>O</i> -(6-chlorobenzotriazol-1-yl)- <i>N,N,N',N'</i> -tetramethyluronium hexafluorophosphate
HOAt	1-hydroxy-7-azabenzotriazole
HRMS	high resolution mass spectrometry
NMP	<i>N</i> -methyl-2-pyrrolidinone
MOR	mu opioid receptor
NK1R	neurokinin-1 receptor
<i>o</i>-NBS	2-nitrobenzenesulfonyl chloride
rNK1	rat neurokinin-1
TFA	trifluoroacetic acid

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- 1a (TY005):** H-Tyr-D-Ala-Gly-Phe-Met-Pro-Leu-Trp-O-Bn(3',5'-(CF₃)₂)
1b (TY027): H-Tyr-D-Ala-Gly-Phe-Met-Pro-Leu-Trp-NH-Bn(3',5'-(CF₃)₂)
1c (TY032): H-Dmt-D-Ala-Gly-Phe-Met-Pro-Leu-Trp-NH-Bn(3',5'-(CF₃)₂)
1d (TY012): H-Tyr-D-Ala-Gly-Phe-Pro-Leu-Trp-NH-Bn(3',5'-(CF₃)₂)

Figure 1.

Structures of some of our previously published multifunctional ligands.

Figure 2.
 Design of multifunctional ligands.

Figure 3.
Paw withdrawal latency after i.t. administration of ligand **3** (AKG115).

Figure 4.
Tail flick latency after i.t. administration of ligand **5** (AKG127).

N.B. When there was secondary amine to be coupled, 3.0 equivalent of each of HATU and HOAt were used instead of HCTU during solid phase synthesis.

Scheme 1.

General Path for the Synthesis of Multifunctional Ligands

Fmoc-deprotection followed by

o-NBS Protection (for 0.1 mmol resin bound primary amine)

1. **o-NBS-Cl** (4.0 equiv) in 1.0 mL NMP
2. **sym-Collidine** (10.0 equiv.)
3. Mix with resin and stir for 15 min,
4. Filter the resin and wash with NMP (1 × 1 min)
5. Repeat 1-2 and stir for 10 min,
6. Filter and wash the resin (5 × 1 min)

N-methylation

DBU-mediated method

1. **DBU** (3.0 equiv.) in 1.0 mL NMP
2. Treat the resin with DBU soln for 3 min
3. Add **DMS** (10.0 equiv.) in 0.5 mL NMP
4. Treat the resin with the DMS solution for 2 min,
5. Filter the resin and wash with NMP (1 × 1 min)
6. Repeat 1-4, 7. Filter and wash the resin with NMP (5 × 1 min)

o-NBS deprotection

1. **2-mercatoethanol** (10.0 equiv.) and **DBU** (5.0 equiv) in 1.0 mL of NMP
2. Treat the resin with the soln for 5 min,
3. Filter and wash the resin with NMP (1 × 1 min)
4. Repeat steps 1-2 and filter the resin,
5. Wash the resin with NMP (5 × 1 min)

Scheme 2.

Steps for N-Methylation on Solid Phase Synthesis

Chart 1.
Comparison of Metabolic Stability of Ligands 3 (AKG115), 5 (AKG127), and 7 (AKG190) in Rat Plasma

Table 1

Physicochemical Properties of the Ligands

ligand no. (ID)	molecular formula	ALOGPs ^a	HPLC RT (min) ^b	ESI (M + H) ⁺	
				obsd	calcd
1 (TY012)	C ₅₄ H ₆₁ F ₆ N ₉ O ₈	5.32	26.1	see ref 18	1077.4547
2 (AKG117)	C ₅₅ H ₆₃ F ₆ N ₉ O ₈	5.56	26.0	1092.4782	1092.4782
3 (AKG115)	C ₅₇ H ₆₇ F ₆ N ₉ O ₈	5.80	26.6	1120.5091	1120.5095
4 (AKG116)	C ₆₆ H ₈₁ F ₆ N ₉ O ₁₂	5.60	26.7	1106.4937	1106.4939
5 (AKG127)	C ₅₆ H ₆₄ F ₇ N ₉ O ₈	4.42	26.8	1124.4844	1124.4844
6 (AKG128)	C ₅₇ H ₆₆ F ₇ N ₉ O ₈	5.82	26.6	1138.4995	1138.5001
7 (AKG190)	C ₅₄ H ₆₀ F ₇ N ₉ O ₈	5.24	26.1	1096.4530	1096.4531
8 (AKG191)	C ₅₆ H ₆₄ ClF ₆ N ₉ O ₈	5.75	28.2	1140.4543	1139.4471
9 (AKG192)	C ₅₆ H ₆₄ BrF ₆ N ₉ O ₈	5.52	28.4	1184.4040, 1186.4032	1184.4044, 1186.4023
10 (AKG193)	C ₅₆ H ₆₄ F ₆ IN ₉ O ₈	5.73	28.9	1232.3894	1232.3905

^a ALOGPs were calculated with the help of <http://www.vcclab.org/lab/alogs/start.html>.^b Retention times of the samples were recorded by running them through an analytical RP-HPLC (Vydac 218TP C18 10 μ column, length 250 mm, ID 4.6 mm) column. Run time was 40 min using a linear gradient (solvent A, 0.1% aq. TFA; solvent B, acetonitrile; 10–90% of solvent B in 40 min).

Table 2

Binding Affinities of the Multivalent Ligands at MOR, DOR, and NK1R ^a

Ligand no. (ID)	K _i ^M (nM)	log[IC ₅₀ ±]	K _i ^D (nM)	log[IC ₅₀ ±]	K _i ^{NK1} /K _i ^D	K _i ^{NK1} (nM)	K _i ^{NK1} (nM)	K _i ^{NK1} /K _i ^{NK1}
1d (TY012)	9.5	-7.7 ± 0.21	72	-6.8 ± 0.08	1/8	0.6	33	1/54
2 (AKG117)	27	-7.05 ± 0.04	260	-6.35 ± 0.13	1/10	3.4 ± 0.74	61 ± 2.0	1/18
3 (AKG115)	1	-8.78 ± 0.05	5	-7.92 ± 0.07	1/5	2.2 ± 0.07	48 ± 8.32	1/22
4 (AKG116)	3	-8.63 ± 0.04	1	-8.66 ± 0.03	3/1	1.4 ± 0.09	27 ± 1.98	1/19
5 (AKG127)	1	-8.72 ± 0.08	1	-7.18 ± 0.04	1/1	1 ± 0.07	29 ± 1.5	1/29
6 (AKG128)	0.4	-8.55 ± 0.18	4	-8.19 ± 0.08	1/10	2.6 ± 0.51	34 ± 6.2	1/13
7 (AKG190) ^b	4	-8.08 ± 0.10	7	7.81 ± 0.09	1/2	5.6 ± 0.65	34 ± 2.8	1/6
8 (AKG191)	4	-8.33 ± 0.09	5	-8.02 ± 0.03	1/1	2.9 ± 0.53	26 ± 5.3	1/9
9 (AKG192)	4	-7.96 ± 0.12	14	-7.71 ± 0.06	1/3	2.5 ± 0.21	47 ± 12.6	1/19
10 (AKG193)	6	-7.88 ± 0.07	13	-7.53 ± 0.08	1/2	3.3 ± 0.6	39 ± 3.6	1/12

^a All samples were run three times at each receptor and each time in duplicate unless otherwise mentioned.

^b The number of runs is two at MOR. Please see the Experimental Section for details.

Table 3Functional Activities of the Multivalent Ligands at MOR, DOR, and NK1R ^a

Ligand no. (ID)	GPI (MOR)		MVD (DOR)		GPI/MVD		GPI/MLMP (NK1R)	
	IC ₅₀ ^a (nM) (agonism)	IC ₅₀ ^a (nM) (agonism)	IC ₅₀ ^a (nM) (agonism)	IC ₅₀ ratio	agonism	K _e ^{NK1} (nM) ± (antagonism)		
1d (TY012)	350 ± 91	45 ± 6.3	8/1			8.5 ± 9.2		
2 (AKG117)	230 ± 53	102 ± 34	2/1		none at 100 nM	21 ± 9.2		
3 (AKG115)	21 ± 3.5	31 ± 7.5	1/1.5		none at 30 nM	9.7 ± 1.2		
4 (AKG116)	81 ± 18.1	3.1 ± 1.0	26/1		none at 100 nM	25 ± 3.6		
5 (AKG127)	42 ± 9.7	2 ± 0.680	21/1		none at 30 nM	5.3 ± 1.64		
6 (AKG128)	77 ± 15	11 ± 5.6	7/2		none at 30 nM	11 ± 2.7		
7 (AKG190)	65 ± 9.2	12 ± 4.0	5/1		none at 30 nM	5.8 ± 1.9		
8 (AKG191)	166 ± 72	25 ± 7.7	7/1		none at 300 nM	44 ± 7.7		
9 (AKG192)	460 ± 114	43.0 ± 12	11/1		none at 100 nM	23 ± 8.9		
10 (AKG193)	41% at 1 μM	97.2 ± 20.5			none at 300 nM	42 ± 5.9		

^aEach sample was run four times. Please see the Experimental Section for details.

Table 4Functional Activities (*c*AMP Assay) of the Multivalent Ligands at MOR, DOR, and NK1R

ligand no. (ID)	MOR-agonism	DOR-agonism	MOR/DOR	NK1R-antagonism
	IC ₅₀ (nM) ± SD	IC ₅₀ (nM) ± SD	IC ₅₀ ratio	K _e (nM) ± SD
Met-Enk	13 ± 10	6.4 ± 9.1	2/1	
3 (AKG115)	39 ± 13	30 ± 19	1.3/1	13 ± 17
4 (AKG116)	46 ± 9	7.2 ± 9.4	6/1	11 ± 10
5 (AKG127)	59 ± 23	21 ± 14	3/1	125 ± 31

Table 5Metabolic Stability of Ligands 3 (AKG115), 5 (AKG127), and 7 (AKG190) in Rat Plasma^a

incubation time (h)	amount of the remaining ligands (%)		
	3 (AKG115)	5 (AKG127)	7 (AKG190)
0	100	100	100
0.5	95	92	84
1	90	81	68
2	87	73	52
4	82	64	28
6	78	58	14
8	75	50	0.16

^a Each sample was run for two independent experiments ($n = 2$), and the average of the two was taken for the half-life calculation.