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Brief Article

Development of Potent μ and δ Opioid Agonists with High Lipophilicity

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An SAR study on the Dmt-substituted enkephalin-like tetrapeptide with a *N*-phenyl-*N*-piperidin-4-ylpropionamide moiety at the C-terminal was performed and has resulted in highly potent ligands at μ and δ opioid receptors. In general, ligands with the substitution of D-Nle[2] and halogenation of the aromatic ring of Phe[4] showed highly increased opioid activities. Ligand **6** with good biological activities in vitro demonstrated potent in vivo antihyperalgesic and antiallodynic effects in the tail-flick assay.

Introduction

Many studies have shown that coadministration of a small amount of a δ opioid agonist can reduce serious side effects of morphine, a μ agonist, such as tolerance, while it increases the potency and efficacy.[1−3] Furthermore several studies have observed synergistic antinociceptive effects between μ agonists and δ agonists.[4] These observations imply that the modulation of μ and δ receptors can allow treatment of pain with lower doses of μ agonists, therefore lessening μ receptor-mediated side effects. Our group previously sought to design nonselective bifunctional ligands possessing μ and δ agonist activities and as a result developed the very potent μ and δ opioid ligand **12** (K_i of 0.36 and 0.38 nM at hDOR[a] and rMOR, respectively; IC_{50} of 1.8 and 8.5 nM in MVD and GPI assays, respectively) with the C-terminus of an enkephalin-like tetrapeptide (H-Dmt-D-Ala-Gly-Phe-OH) linked to a *N*-phenyl-*N*-piperidin-4-ylpropionamide moiety, which is a part of the fentanyl structure.[5] We found that the propionamide moiety in the ligand resulted in a great increase in lipophilicity (aLogP = 2.96) which can improve the bioavailability of the ligand.

Despite their high potency and important role in combating pain, natural opioid peptides are limited as clinically viable drugs because of poor bioavailability, mainly due to their inability to penetrate the blood−brain barrier (BBB) and rapid degradation in vivo by peptidases. Increasing the lipophilicity can improve analgesia with a concomitant enhancement of bioavailability, since the lipophilicity is a key factor in determining the rate at which a drug crosses the BBB. Methylation and halogenation enhance the lipophilicity and cell permeability by reducing overall hydrogen bonding. For example, addition of a chlorine on the Phe[4] residue of [D-Pen[2],D-Pen[5]]enkephalin (DPDPE) led to a significant increase in cell permeability in both in vivo and in vitro studies.[6,7]

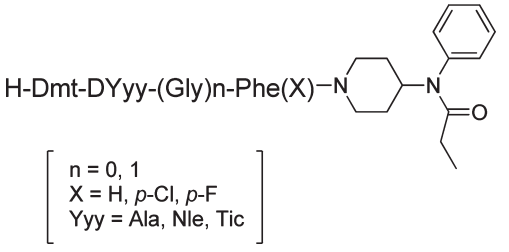

Figure 1. Design of opioid ligands.

In the pursuit of enhanced bioavailability at the μ and δ receptors by increasing lipophilicity, a systematic structure−activity relationship (SAR) study on ligand **12** was performed (Figure 1). The propionamide moiety and the Dmt[1] residue were conserved to increase cell permeability and potency, respectively. As a result, a series of highly lipophilic (aLogP of 3.01−4.74 in Table 1) nonselective μ and δ opioid agonists were obtained.

Results and Discussion

As published before, the ligands designed were prepared by stepwise solution-phase peptide syntheses using N^{α}-Boc-chemistry and the crude products were isolated by preparative RP-HPLC to afford >98% pure compounds in 40−50% overall yields.[5] During the chain elongations, pure peptide intermediates were obtained by simple precipitation from appropriate organic solvents, usually diethyl ether. Their in vitro bioassays were carried out as previously described.[8]

Opioid binding affinities at the human δ opioid receptor (hDOR) and the rat μ opioid receptor (rMOR) were determined by competition analyses against [^{3}H]DPDPE (δ) and [^{3}H]DAMGO (μ) using membrane preparations from transfected HN9.10 cells that constitutively express the respective receptors. Opioid agonist efficacy was examined by monitoring

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[a] Abbreviations: BBB, blood−brain barrier; Boc, *tert*-butyloxycarbonyl; BOP, (benzotriazole-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate; CHO, Chinese hamster ovary; DMF, *N*,*N*-dimethylformamide; hDOR, human δ opioid receptor; DPDPE, c[D-Pen[2], D-Pen[5]]enkephalin; DAMGO, [D-Ala[2],NMePhe[4],Gly[5]-ol]enkephalin; Dmt, 2,6-dimethyltyrosine; GPI, guinea pig ileum; HOBt, 1-hydroxybenzotriazole; IACUC, Institutional Animal Care and Use Committee; IASP, International Association for the Study of Pain; ith, intrathecal; rMOR, rat μ opioid receptor; MVD, mouse vas deferens; NMM, *N*-methylmorpholine; RP-HPLC, reverse phase high performance liquid chromatography; SAR, structure−activity relationship; SNL, spinal nerve ligation; TFA, trifluoroacetic acid; Tic, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid.

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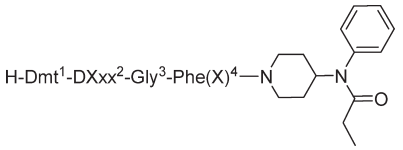
Table 1. Structure and Analytical Data of Opioid Ligands



							HRMS[a] (M − TFA + H)$^+$		HPLC,[b] t_R (min)	
compd	1	2	3	4	aLogP[11]	molecular formula	obsd	calcd	A	B
1	Dmt	D-Nle	Gly	Phe	3.66	$C_{42}H_{56}N_6O_6$	741.4325	741.4340	19.3	10.8
2	Dmt	D-Nle		Phe	4.20	$C_{40}H_{53}N_5O_5$	684.4109	684.4125	19.8	11.9
3	Dmt	D-Ala		Phe	3.52	$C_{37}H_{47}N_5O_5$	642.3665	642.3655	18.3	8.9
4	Dmt	D-Nle	Gly	Phe(Cl)	4.18	$C_{42}H_{55}ClN_6O_6$	775.3995	775.3950	20.7	12.5
5	Dmt	D-Nle	Gly	Phe(F)	3.74	$C_{42}H_{55}FN_6O_6$	759.4247	759.4245	20.0	12.0
6	Dmt	D-Ala	Gly	Phe(Cl)	3.47	$C_{39}H_{49}ClN_6O_6$	733.3494	733.3480	19.2	10.3
7	Dmt	D-Nle		Phe(Cl)	4.74	$C_{40}H_{52}ClN_5O_5$	718.3713	718.3735	22.2	14.0
8	Dmt	D-Ala		Phe(Cl)	3.97	$C_{37}H_{46}ClN_5O_5$	676.3272	676.3266	19.8	11.0
9	Dmt	D-Ala	Gly	Phe(F)	3.01	$C_{39}H_{49}FN_6O_6$	717.3797	717.3776	17.7	9.5
10	Dmt	D-Tic	Gly	Phe(F)	4.02	$C_{46}H_{53}FN_6O_6$	805.4055	805.4089	20.3	12.0
11	Dmt	D-Tic	Gly	Phe(Cl)	4.46	$C_{46}H_{53}ClN_6O_6$	821.3799	821.3793	21.2	13.1
12	Dmt	D-Ala	Gly	Phe	2.96	$C_{39}H_{50}N_6O_6$	699.3852	699.3870	20.1	12.7

[a] FAB-MS (JEOL HX110 sector instrument) or MALDI-TOF. [b] Performed on a Hewlett-Packard 1100 [C-18, Microsorb-MV, 4.6 mm × 250 mm, 5 μm]. (A) 10−90% of acetonitrile containing 0.1% TFA within 40 min and up to 100% within an additional 5 min, 1 mL/min. (B) 25−65% of acetonitrile containing 0.1% TFA within 20 min and up to 100% within an additional 5 min, 1 mL/min.

[^{35}S]GTP-γ-S binding (Table 2). Functional assays to evaluate their opioid agonist activities were performed using the stimulated isolated mouse vas deferens (MVD, δ) and guinea pig ileum (GPI, μ) bioassays. In general, the functional assay results correlated with those from the [^{35}S]GTP-γ-S binding assay.

Our SAR study showed that replacement of D-Ala^2 with D-Nle^2 and halogenation at the 4 position of the aromatic ring of Phe^4 increased opioid activitiy at both receptors in vitro, which was in agreement with the other observations,[9,10] along with increased lipophilicity (aLogP increase: 0.7 for D-Nle, 0.5 for Cl).[11] First, replacement of D-Ala in **12** with D-Nle led to ligand **1**, which showed increased opioid agonist activities [IC_{50} = 0.69 nM (3-fold increase) in the MVD assay; IC_{50} = 1.6 nM (5-fold increase) in the GPI assay] and efficacies [EC_{50} = 0.10 nM (8-fold increase) with E_{max} = 47% at hDOR; EC_{50} = 0.11 nM (8-fold increase) with E_{max} = 77% at rMOR in the [^{32}S]GTP-γ-S assay], whereas its binding affinities at both receptors were not affected distinctly. The same trend was observed in the other ligands in which the D-Ala residue was replaced by D-Nle. The D-Nle substitution caused greater effects on the functional activities and efficacies than on the binding affinities, suggesting that the change of functional efficiency was due to increased lipophilicity.

To reduce pharmacophore size but increase lipophilicity, the Gly^3 residue in ligands **1**, **4**, **6**, and **12** was truncated, yielding ligands **2**, **7**, **8**, and **3**, respectively. The resulting ligands had decreased bioactivities at both receptors but raised μ selectivity because δ receptor activity was further reduced (Table 2). Even though **3** had increased binding affinity (K_i = 0.15 nM) and agonist activity (IC_{50} = 3.6 nM) at the μ opioid receptor of 2-fold by the truncation, in general, the truncation of Gly was not tolerated for both opioid receptors. It has been known that the relative proximity of the Phe^4 aromatic ring and Tyr^1 aromatic ring is critical for δ versus μ receptor selectivity.[11] In our case, shortening their proximity by the truncation of Gly^3 turned out to reduce δ receptor activity much more than μ receptor activity and subsequently to increase μ selectivity. All the Gly truncated ligands showed μ opioid receptor selectivity in the binding and functional assays.

All of the ligands with Cl (or F) at the 4′ position of the aromatic ring of Phe^4 showed highly increased opioid activity in binding and functional assays. Ligand **5**, in which D-Nle^2 and Phe(F)^4 were substituted, showed the most potent subnanomolar opioid agonist activity (IC_{50} of 0.37 and 0.26 nM in the MVD and GPI assays, respectively) with excellent efficacy (EC_{50} = 0.07 nM, E_{max} = 48% at hDOR; EC_{50} = 0.29 nM, E_{max} = 98% at rMOR) at both receptors. The binding affinity at the μ receptor was subnanomolar (K_i = 0.02 nM), and the E_{max} at rMOR in the [^{35}S]GTP-γ-S binding assay was 98% which represents high efficacy at the receptor. These results suggest that Cl (or F) in the opioid ligands could increase cell permeability and enhance potency, which is more pronounced for the μ receptor, thus producing very well balanced ($IC_{50}^{\delta}/IC_{50}^{\mu}$ < 2 in ligands **4**, **5**, and **9**) mixed agonist activities for both receptors, respectively. On the contrary, the *p*-Cl substitution in the Gly truncated ligands **7** and **8** decreased opioid activity in all of the assays, especially the functional assays, up to 47-fold (IC_{50} of 250 and 220 nM for **7**, IC_{50} of 180 and 93 nM for **8** at MVD and GPI, respectively) but retained μ opioid receptor selectivity in ligands **2** and **3**.

Dmt-Tic is a highly potent selective δ opioid antagonist, and this modification has been recognized as leading to significant alterations in opioid activities and selectivities.[12] In utilizing this potent pharmacophore to alter agonist functions at both opioid receptors, the Tic residue was modified to D-Tic in ligands **10** and **11**. These ligands showed biological activities analogous to those of the other ligands, which confirmed that the constrained and very hydrophobic D-Tic residue could be replaced for the flexible D-Nle and D-Ala residues in the enkephalin structure. Both ligands showed high δ opioid receptor selectivities (δ/μ = 0.03 for **9**; δ/μ = 0.04 for **10**) in the functional assays, while ligand **10** gave the most potent agonist activity at the δ opioid receptor (IC_{50} = 0.029 nM in the MVD). No δ opioid antagonist activity was observed for either of the D-Tic-containing ligands.

On the basis of the in vitro bioassay results showing balanced high μ and δ opioid activities (K_i = 0.14 nM, EC_{50} = 0.16 nM at hDOR; IC_{50} = 0.70 nM in MVD, K_i = 0.14 nM,

Table 2. Bioactivities of the Opioid Ligands

					[^{35}S]GTP-γ-S binding							
	hDOR[a] [^{3}H]DPDPE[b]		rMOR[a] [^{3}H]DAMGO[c]		hDOR[d]			rMOR[d]			IC$_{50}$ (nM)[e]	
compd	log IC$_{50}$[f,g]	K_i, nM[h]	log IC$_{50}$[f,g]	K_i, nM[h]	log EC$_{50}$[f]	EC$_{50}$, nM[i]	E_{max}, %[j]	log EC$_{50}$[f]	EC$_{50}$, nM[i]	E_{max}, %[j]	MVD(δ)	GPI(μ)
1	−9.42 ± 0.08	0.18	−9.06 ± 0.06	0.39	−10.0 ± 0.21	0.10	47	−9.95 ± 0.20	0.11	77	0.69 ± 0.09	1.6 ± 0.1
2	−8.62 ± 0.08	1.1	−9.16 ± 0.10	0.36	−8.55 ± 0.07	2.8	61	−8.73 ± 0.16	1.9	29	8.5 ± 1.7	4.7 ± 1.2
3	−8.44 ± 0.10	1.7	−9.47 ± 0.20	0.15		nr[k]		−9.03 ± 0.19	0.94	33	15 ± 1	3.6 ± 0.8
4	−9.73 ± 0.04	0.08	−9.71 ± 0.03	0.10	−10.19 ± 0.20	0.07	37	−9.85 ± 0.43	0.14	58	1.9 ± 0.5	1.3 ± 0.3
5	−9.07 ± 0.05	0.40	−10.50 ± 0.10	0.02	−10.15 ± 0.16	0.07	48	−9.53 ± 0.33	0.29	98	0.37 ± 0.12	0.26 ± 0.14
6	−9.52 ± 0.08	0.14	−9.41 ± 0.06	0.14	−9.79 ± 0.25	0.16	58	−9.46 ± 0.13	0.31	55	0.70 ± 0.38	2.6 ± 0.8
7	−7.40 ± 0.09	19	−7.97 ± 0.11	5.0	−7.52 ± 0.12	30	36	−7.72 ± 0.25	19	15	250 ± 90	220 ± 40
8	−8.49 ± 0.11	1.6	−9.13 ± 0.04	0.33	−7.59 ± 0.46	26	27	−8.33 ± 0.33	4.7	29	180 ± 50	93 ± 30
9	−10.19 ± 0.10	0.03	−10.81 ± 0.07	0.01	−9.92 ± 0.25	0.12	23	−9.16 ± 0.21	0.69	50	1.8 ± 0.8	1.0 ± 0.1
10	−8.97 ± 0.14	0.48	−9.15 ± 0.13	0.35	−10.07 ± 0.37	0.09	24	−9.14 ± 0.23	0.72	51	0.029 ± 0.007	0.96 ± 0.13
11	−9.59 ± 0.06	0.11	−9.55 ± 0.04	0.15	−9.69 ± 0.17	0.20	31	−10.23 ± 0.27	0.06	37	0.21 ± 0.06	4.8 ± 0.7
12	−9.10 ± 0.10	0.36	−9.08 ± 0.22	0.38	−9.12 ± 0.17	0.77	24	−9.05 ± 0.18	0.88	50	1.8 ± 0.2	8.5 ± 3.3
YDAGF-NH$_2$	−6.14 ± 0.16	300	−8.24 ± 0.13	2.8	−6.72 ± 0.17	190	44	−7.98 ± 0.22	13	99	120 ± 10	47 ± 9
DAMGO								−7.44 ± 0.19	37	150		
DPDPE					8.80 ± 0.25	1.6	69					

[a] Competition analyses were carried out using membrane preparations from transfected HN9.10 cells that constitutively expressed the respective receptor types. [b] K_d = 0.50 ± 0.1 nM. [c] K_d = 0.85 ± 0.2 nM. [d] Expressed in CHO cells. [e] Concentration at 50% inhibition of muscle contraction in electrically stimulated isolated tissues. [f] Logarithmic values determined from the nonlinear regression analysis of data collected from at least two independent experiments. [g] Competition against radiolabeled ligand. [h] Antilogarithmic value of the respective IC$_{50}$. [i] Antilogarithmic value of the respective EC$_{50}$. [j] (Net total bound/basal binding ×100) ± SEM. [k] nr: no response.

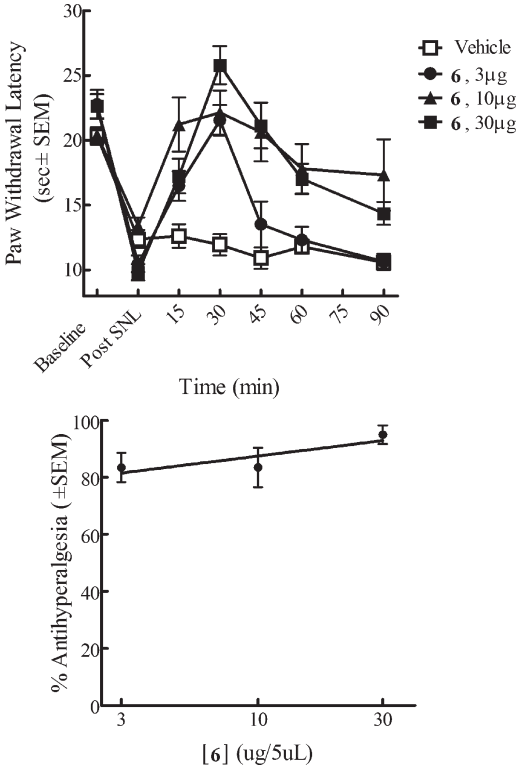


Figure 2. Antihyperalgesic effects of **6** (ith) using radiant heat in L$_5$/L$_6$ SNL-operated male SD rats: (top) thermal hypersensitivity by paw withdrawal; (bottom) antinociceptive dose-response curve at 30 min).

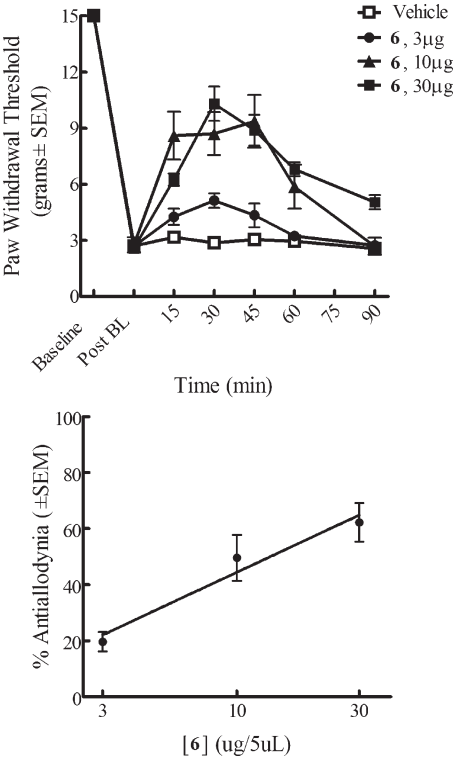


Figure 3. Antiallodynic effects of **6** (ith) using von Frey filaments in L$_5$/L$_6$ SNL-operated male SD rats: (top) tactile allodynia by paw withdrawal thresholds; (bottom) antinociceptive dose-response curve at 30 min.

EC$_{50}$ = 0.31 nM at rDOR; IC$_{50}$ = 2.6 nM in GPI), ligand **6** was chosen for in vivo assays. Neuropathic pain was induced by L$_5$/L$_6$ spinal nerve ligation (SNL) in male Sprague−Dawley rats.[13] Thermal hypersensitivity and tactile allodynia were determined by paw withdrawal latencies to infrared radiant heat and paw withdrawal thresholds from von Frey filaments, respectively, which were applied to the plantar aspect of the hind paw. The time course of paw withdrawal latency or threshold after intrathecal microinjections of **6** at 3 μg/5 μL, 10 μg/5 μL, and 30 μg/5 μL were examined (Figures 2 and 3). A dose-response curve was generated at the time of peak effect (30 min). The results showed that **6** attenuates tactile allodynia and thermal hyperalgesia when administered via ith injection. The L$_5$/L$_6$ SNL model was used to evaluate efficacy against tactile allodynia or thermal hyperalgesia compared to baseline. Significant efficacy for **6** was observed at doses of 3, 10,

and 30 μg. A dose-response curve was generated at peak effect which occurred 30 min after ith administration. The A_{50} values at this time point were 11.0 (6.5−21.1 μg; 95% CI for allodynia) and 1.04 (1.0−1.2 μg/5 μL; 95% CI for hyperalgesia). No effect was seen in vehicle treated animals.

Conclusions

In the present study, a systematic SAR study on the enkephalin analogue **12** with conservation of the propionamide moiety and Dmt[1] residue resulted in the development of nonselective potent opioid agonists for μ and δ receptors with high lipophilicity. These analogues can produce the desired physiological effects without many of the undesirable side effects of selective μ opioid agonist, for example, **5** (K_i of 0.40 and 0.02 nM at hDOR and rMOR; IC_{50} of 0.37 and 0.26 nM in MVD and GPI, respectively). In summary, the substitution of D-Nle[2] and the halogenation of the aromatic ring of Phe[4] increased opioid activities at both receptors along with the lipophilicity. Ligand **6** which possessed highly potent opioid activities at both receptors (K_i = 0.14 nM at hDOR and rMOR; IC_{50} of 0.70 and 2.6 nM in MVD and GPI assays, respectively) showed potent antihyperalgesic and antiallodynic effects in the in vivo assays, demonstrating that increase of lipophilicity can be a good approach to develop a potent ligand with good bioavailability.

Experimental Section

All amino acid derivatives were purchased from Novabiochem, ChemImpex International, and RSP. *N*-Phenyl-*N*-piperidin-4-ylpropionamide was prepared as previously described.[14] Coupling reactions were monitored by TLC using the following solvent systems: (1) $CHCl_3$/MeOH/AcOH = 90:10:3 with ninhydrin spray used for detection. Analytical HPLC was performed on a Hewlett-Packard 1090 [C-18, Vydac, 4.6 mm × 250 mm, 5 μm] and preparative RP-HPLC on Hewlett-Packard 1100 [C-18, Microsorb-MV, 10 mm × 250 mm, 10 μm]. ^{1}H NMR spectra were recorded on a Bruker Advance-300 spectrometer. Mass spectra were taken in the positive ion mode under ESI methods.

Ligands 1−12. These ligands were prepared by stepwise synthesis using N^{α}-Boc chemistry starting from *N*-phenyl-*N*-piperidin-4-ylpropionamide. *N*-Phenyl-*N*-piperidin-4-ylpropionamide (1 equiv) and N^{α}-Boc-Phe-OH (1.1 equiv) were dissolved in DMF and cooled in an ice bath for 10 min. Bop (1.1 equiv), HOBt (1.1 equiv), and NMM (2 equiv) were added to the reaction mixture and stirred for 2 h at room temp. After a check for the disappearance of the starting amine by TLC, the reaction mixture was concentrated under reduced pressure. The concentrated mixture was diluted with EtOAC and washed with 5% $NaHCO_3$, 5% citric acid, brine, and water consecutively. The organic layer was dried under anhydrous Na_2SO_4 and filtered. The solution was concentrated under reduced pressure and triturated with diethyl ether to give a solid. The N^{α}-Boc group was deprotected by 100% TFA at 0 °C for 20 min. After completion of the chain elongation by subsequent coupling and deprotection, the mixtures were evaporated under reduced pressure. The residues were solidified by diethyl ether and purified by preparative RP-HPLC (20−50% acetonitrile within 20 min for **1**, **6**, and **7**, 20−60% acetonitrile within 15 min for **5** and **10**) to give pure ligands as white powders in overall yields of 20−42%. The purity of the ligands was determined as ≥95% by analytical HPLC (10−90% acetonitrile in 40 min, 25−65% acetonitrile in 20 min). For analytical data, see Supporting Information.

Radioligand Labeled Binding Assay, [^{35}S]GTP-γ-S Binding Assay, GPI and MVD in Vitro Bioassay. The methods were carried out as previously described.[8]

In Vivo Assay. Animals. The experiments contained herein were carried out using male Sprague−Dawley rats (250−350 g; Harlan; Indianapolis, IN). All animals were maintained on a 12/12 h light/dark cycle (lights on at 07:00 a.m.) and provided food and water ad libitum except as noted during the experimental procedures. All the experiments were performed under a protocol approved by Institutional Animal Care and Use Committee (IACUC) of the University of Arizona and in accordance with policies and guidelines for the care and use of laboratory animals as adopted by International Association for the Study of Pain (IASP) and the National Institutes of Health (NIH).

L_5/L_6 Spinal Nerve Ligation Surgery. SNL injury was induced as described by Chung and colleagues.[13]

Intrathecal Catheter Surgery. All rats were prepared for intrathecal drug administration by placing anesthetized (ketamine/xylazine 100 mg/kg, ip) animals in a stereotaxic head holder. The cisternum magnum was exposed, an incision was made, and animals were implanted with a catheter (PE: 10, 8 mm) that terminated in the lumbar region of the spinal cord. The animals were allowed to recover 5−7 days after surgery before any pharmacological manipulations were made.

Drug Administration. 6 was dissolved in 100% methanol. For ith drug administration, 5 μL of drug was injected followed by a 9 μL saline flush. Testing took place 15, 30, 45, 60, 75, and 90 min after drug injection, and dose-response curves were generated from data gathered at the time of peak effect.

Behavioral Assessment. Thermal Hypersensitivity. Thermal hypersensitivity was assessed using the rat plantar test (Ugo Basile, Italy) following a modified method of Hargreaves et al.[15] Paw withdrawal latencies were recorded in seconds. An automatic cut-off point of 33 s was set to prevent tissue damage. The apparatus was calibrated to give a paw withdrawal latency of approximately 20 s on the uninjured paw. The radiant heat source was activated with a timer and focused onto the plantar surface of the hindpaw. Paw-withdrawal latency was determined by a motion detector that halted heat source and timer when the paw was withdrawn.

Mechanical Hypersensitivity. The assessment of mechanical hypersensitivity consisted of measuring the withdrawal threshold of the paw ipsilateral to the site of nerve injury in response to probing with a series of calibrated von Frey filaments. Each filament was applied perpendicularly to the plantar surface of the ligated paw of rats kept in suspended wire-mesh cages. Measurements were taken before and after administration of drug or vehicle. The withdrawal threshold was determined by sequentially increasing and decreasing the stimulus strength ("up−down" method) analyzed using a Dixon nonparametric test[16] and expressed as the mean withdrawal threshold.

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Supporting Information Available: ^{1}H NMR data of the ligands **1−12**. This material is available free of charge via the Internet at http://pubs.acs.org.

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