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Novel delta opioid receptor agonists exhibit differential stimulation of signaling pathways

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ABSTRACT

A novel family of 1,3,5-trisubstituted 1,2,4-triazoles was discovered as potent and selective ligands for the δ opioid receptor by rational design. Compound **5b** exhibited low-nanomolar in vitro binding affinity (IC_{50} = 5.8 nM), excellent selectivity for the δ opioid receptor over the alternative μ and κ opioid receptors, full agonist efficacy in receptor down-regulation and MAP kinase activation assays, and low-efficacy partial agonist activity in stimulation of GTPγS binding. The apparent discrepancy observed in these functional assays may stem from different signaling pathways involved in each case, as found previously for other G-protein coupled receptors. More biological studies are underway to better understand the differential stimulation of signaling pathways by these novel compounds.

1. Introduction

Opioid receptors belong to class A within the superfamily of G-protein coupled receptors (GPCRs). It has been well documented that at least three distinct opioid receptors (δ, μ, and κ) exist in the central nervous system and periphery.[1–4] Opioid agonists are very effective analgesics for acute and postoperative pain through activation of the μ opioid receptor. Many serious side effects have been reported with opioid analgesics, including respiratory depression, physical dependence, constipation, nausea, and vomiting.[5] A significant body of evidence has suggested that δ selective opioid receptor agonists and antagonists offer great potential as therapeutic agents devoid of the adverse side effects associated with μ agonists (e.g., morphine).[6–9] δ selective agonists have been reported to produce many pharmacological effects including analgesia,[9] cardioprotection,[10] neuroprotection,[11,12] antidepression,[13,14] and anti-Parkinson activities.[15] On the other hand, δ selective antagonists have been shown to modulate the development of tolerance[16,17] and dependence on μ agonists such as morphine,[18] to act as antitussive agents[19] and to elicit anti-alcoholism[20] and anti-obesity[20] effects. The δ selective opioid ligands thus represent attractive candidates for a broad range of pharmaceutical applications.

The interactions between opioid receptors and their ligands have been described in terms of the 'message–address' concept,[21,22] illustrated here using the δ opioid receptor selective antagonist naltrindole,[23] agonists SNC80,[24] TAN67,[25] and SB219825[26] (Fig. 1). The 'message' (red in Fig. 1) represents structural features involved in binding to all opioid receptors (δ, μ, and κ), while the 'address' (blue in Fig. 1) refers to specific structural features that confer high selectivity for a particular opioid receptor subtype (e.g., δ).[23,26] Lipophilic, sterically bulky δ 'address' groups, such as diethylcarbamoyl in SNC80 and SB219825 and phenyl in naltrindole and TAN67, have been reported to confer δ receptor selectivity (Fig. 1). More recently, Bernard et al.[27] developed quantitative pharmacophore models for δ opioid ligands that reinforce the importance of the hydrophobic 'address' moiety in conferring selective δ receptor activity.

In an earlier study,[28] we reported a novel family of 3,4,5-trisubstituted 1,2,4-triazoles as δ selective opioid receptor antagonists that were discovered by ligand-based drug design approaches in view of the 'message–address' concept (Fig. 1).[28–30] In this series, the 4-*tert*-butyl was chosen as the hydrophobic 'address' to confer δ selectivity. When published, these compounds represented the first nonpeptidic δ receptor selective ligands lacking a highly basic

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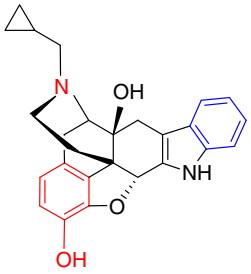
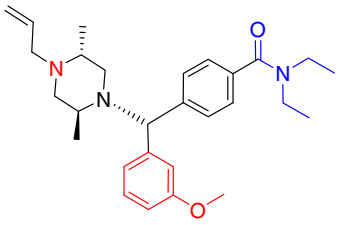
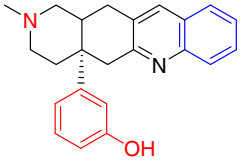


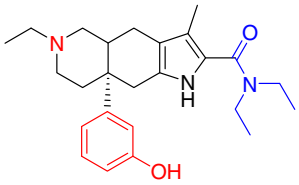
Naltrindole



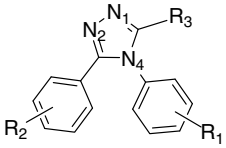
SNC80



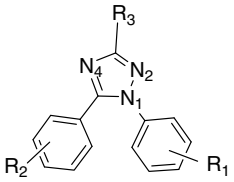
TAN-67



SB219825



3,4,5-trisubstituted 1,2,4-triazole



1,3,5-trisubstituted 1,2,4-triazole

8 R_1 = 3-OH, R_2 = 4-*tert*-butyl, R_3 = $N(Me)_2$
16 R_1 = 3-OH, R_2 = 3-*tert*-butyl, R_3 = $CH_2N(Me)_2$
17 R_1 = 3-OH, R_2 = 4-*tert*-butyl, R_3 = $CH_2N(Me)_2$

5a R_1 = 3-OH, R_2 = 3-*tert*-butyl, R_3 = $CH_2N(Me)_2$
5b R_1 = 3-OH, R_2 = 4-*tert*-butyl, R_3 = $CH_2N(Me)_2$
5c R_1 = 3-OH, R_2 = 2,4-$(Me)_2$, R_3 = $CH_2N(Me)_2$
5d R_1 = 3-*tert*-butyl, R_2 = 3-OH, R_3 = $CH_2N(Me)_2$
5e R_1 = 4-*tert*-butyl, R_2 = 3-OH, R_3 = $CH_2N(Me)_2$

Figure 1. Structures of δ selective opioid receptor ligands. Structures of our previous 3,4,5-trisubstituted triazoles are exemplified by compounds (**8**, **16**, **17**) mentioned in this article.

N atom. The most potent compound (**8** in Fig. 1) described in this previous study[28] exhibited δ binding affinity of 50 nM and moderate selectivity for δ over the μ and κ opioid receptors (selectivity ratio: $\delta/\mu = 80$; $\delta/\kappa > 200$).

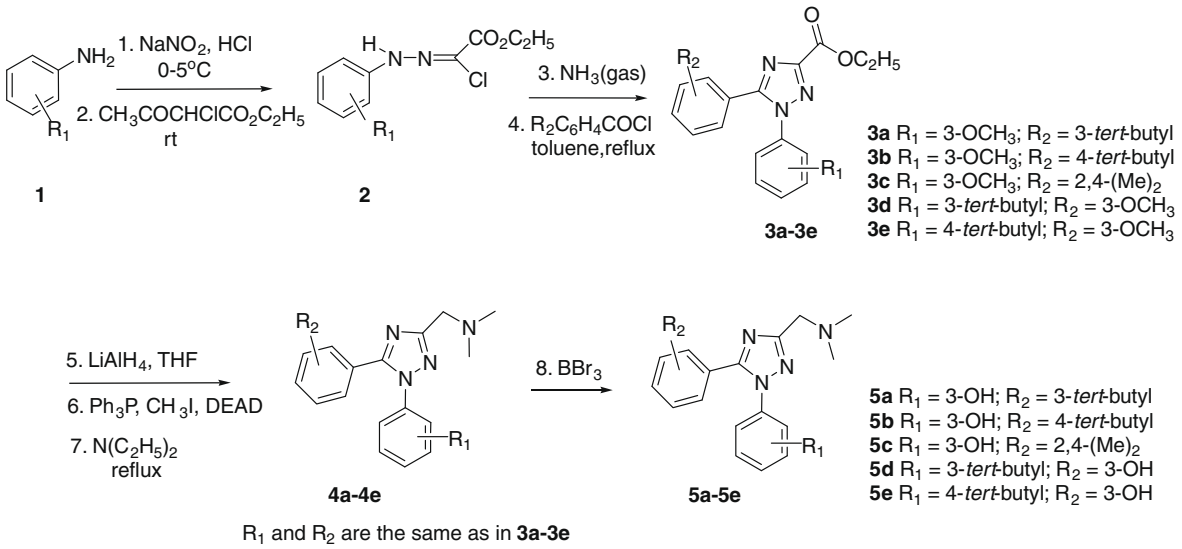
Our next aim was to improve the binding affinity and selectivity for the δ opioid receptor. Guided by computational molecular modeling, we reconfigured the substitution pattern around the triazole ring to yield the 1,3,5-trisubstituted 1,2,4-triazole series (Fig. 1). The majority of known potent opioids contain a basic N atom which would be expected to exist predominantly in its protonated form at physiological conditions (pH 7.4); this protonated N atom has been suggested to form a salt bridge with a highly conserved Asp residue in TM3 of opioid receptors.[31,32] We incorporated this feature by judiciously adding a basic amino group ($-CH_2N(Me)_2$) on the core triazole ring of the 1,2,4-triazole compounds. A limited series of 10 compounds (**4a**–**4e** and **5a**–**5e** in Scheme 1) was then synthesized to establish proof of concept. Here we report the chemical synthesis and initial biological testing of this series of 1,3,5-substituted 1,2,4-triazoles as δ selective opioid receptor ligands.

2. Chemistry

The synthesis of 1,3,5-substituted triazoles **4a**–**4e** and **5a**–**5e** (Table 1) is depicted in Scheme 1. Reaction of amines **1** with $NaNO_2$ in 2.0 M HCl at 5 °C gave diazonium salts, which were reacted with ethyl-2-chloro-3-oxobutanoate to yield intermediates **2**. Amination of **2** with ammonia produced amidrazones in 100% yield typically within 2 h. The 1,2,4-triazole core ring was then closed by refluxing of amidrazones with acyl chlorides in toluene to give the key intermediates **3**.[33] The ethyl esters in **3** were converted to alcohols using $LiAlH_4$ as reducing reagent, which were further converted to iodide by Mitsonobu reaction.[34] The iodides, without isolation, were substituted by *N*,*N*-dimethylamine in refluxing chloroform to produce **4**. Yields of **4** from **3** were excellent (80–90%). Compounds **4a**–**4e** were then converted to phenols **5a**–**5e** by BBr_3 with yields greater than 95%.

3. Results and discussion

Structural alignment between our triazole-based compounds with known δ opioid ligands SNC80 and naltrindole was performed to explore their degree of overlap in three-dimensional space (Fig. 2). For clear comparison, compounds 1,3,5-substituted **5b** and 3,4,5-substituted **17**, which have identical substitutions at R_1, R_2, and R_3, are both shown in Fig. 2. In general, compound **5b** aligns very well with both SNC80 and naltrindole in terms of the 'message' (the basic N atom and the phenolic moiety) and the 'address' (respectively, *tert*-butyl vs diethylcarbamoyl; and *tert*-butyl vs phenyl). In contrast, the 'address' group (*tert*-butyl) of



Scheme 1.

Table 1
Binding affinity of the subject compounds to the opioid receptors

Compd	IC_{50} (nM) ± SEM			Selectivity	
	δ[a]	μ[b]	κ[c]	μ/δ	κ/δ
4a	2195 ± 354	>10,000	>10,000	>4.6	>4.6
5a	48.9 ± 8.3	2447 ± 661	341 ± 20	50	7.0
4b	2307 ± 393	>10,000	>10,000	>4.3	>4.3
5b	5.8 ± 1.2	>10,000	3851 ± 311	>1724	664
4c	>10,000	>10,000	>10,000	N/A	N/A
5c	273 ± 82	2435 ± 372	351 ± 95	8.9	1.3
4d	1139 ± 311	>10,000	>10,000	>8.8	>8.8
5d	28.8 ± 3.3	245 ± 20	46.3 ± 8.2	8.5	1.6
4e	>10,000	>10,000	>10,000	N/A	N/A
5e	121 ± 6.1	>10,000	760 ± 127	>80	6.1
16[d]	2600	>10,000	>10,000	>3.9	>3.9
17[d]	460	>10,000	>10,000	>21.6	>21.6
SNC80	0.8 ± 0.1	6066 ± 478	3601 ± 523	7583	4501

[a] IC_{50} for inhibition of [^{3}H]DPDPE binding to HEK 293 cells expressing the murine δ receptor.
[b] IC_{50} for inhibition of [^{3}H]DAMGO binding to HEK 293 cells expressing the murine μ receptor.
[c] IC_{50} for inhibition of [^{3}H]U69593 binding to HEK 293 cells expressing the rat κ receptor.
[d] K_i values using [^{3}H]bremazocine as the radiolabeled ligand, Ref. 28.

compound **17** deviates somewhat from the orientation of the diethylcarbamoyl in SNC80 and the phenyl in naltrindole. Clearly, reconfiguring the substitution pattern on the core triazole ring improves the pharmacophore alignment with known opioids of the 1,3,5-substituted compounds compared with the 3,4,5-substituted compounds. In addition, one of the triazole N atoms (N4 in Fig. 2) appears to correspond to the C-14 hydroxyl of naltrindole, which has been proposed to hydrogen bond with the side chain of Tyr129.[35] This high degree of structural alignment of the subject compounds with naltrindole and SNC80 strengthened our confidence in their likelihood as δ opioid receptor ligands.

Docking of **5b** to a homology model of the δ opioid receptor based on the X-ray crystal structure of bovine rhodopsin[36] revealed that the basic N atom is well positioned to form a salt bridge with the side-chain carboxylate group of Asp128 in TM3 (Fig. 3). Likewise, the 3-hydroxyl at R_1 of **5b** is situated within hydrogen bonding distance (d_{O-O} = 3.31 Å) of the backbone carbonyl O atom of Ala221 in TM5. The 4-*tert*-butyl phenyl moiety, that is, the delta 'address' in this series, is appropriately buried in a hydrophobic envelop lined by the side-chains of residues of Phe202 (EL2); Val217 (TM5); and Phe218 (TM5); Val281 (TM6), Trp284 (TM6) and Thr285 (TM6); and Leu300 (TM7). The N4 atom (Fig. 3) in the triazole ring is located near the side-chain hydroxyl group of Tyr129 ($d_{N\cdots O}$ = 4.52 Å) in TM3, suggesting a potential hydrogen bond or an electrostatic interaction.

These same ligand–receptor interactions of **5b**, viz., the salt bridge, the hydrogen bonds and the hydrophobic interaction, are well conserved in the binding of known δ receptor selective opioid agonists. Ala221 but not His278 has been reported to form a hydrogen bond with the tyramine hydroxyl of nonpeptidic opioid agonists,[35] which is the case for our compounds. Some of the residues (Tyr129, Phe218, Val281, Trp284, and Leu300) in contact with **5b** have been reported using mutagenesis studies to play important roles in the binding of δ selective opioid agonists (BW373U86, DPDPE, SNC80, and TAN67).[37–39] The similarity in ligand–receptor interactions between the subject compounds and known δ opioids offers compelling structural evidence of their potential binding affinity to the δ opioid receptor.

Competitive binding assays were conducted against the three opioid receptors δ, μ, and κ (Table 1). The results revealed that structural modification from 3,4,5-substituted 1,2,4-triazoles to 1,3,5-substituted 1,2,4-triazoles significantly improves the binding affinity and selectivity for the δ opioid receptor, which supports the molecular modeling studies and our intuitive deductions. For example, **5a** and **5b** in the present 1,3,5-substituted series demonstrated binding affinities of 48.9 and 5.8 nM for the δ receptor. In comparison, their corresponding compounds (**16** and **17** in Fig. 1) from our previously reported 3,4,5-substituted series exhibited weak δ binding affinity (K_i = 2600 and 460 nM, respectively, Table 1).[28] For all compounds in this series, the presence of a hydroxyl (e.g., **5a**–**5c**) rather than a methoxyl (e.g., **4a**–**4c)** at R_1 dramatically enhanced the binding affinity for the δ opioid receptor, which is consistent with the structure–activity relationship (SAR) of morphine-like opioids[40–43] and SNC80 derivatives.[44] Likewise, the presence of the sterically bulky *tert*-butyl group (**5a** and **5b**) rather than methyl (**5c**) at R_2 greatly improved the binding affinity and selectivity for the δ opioid receptor, in agreement with the 'address' requirement for lipophilic, sterically bulky groups at the position. Shifting the *tert*-butyl group at R_2 from the *meta* position to *para* position on the phenyl ring improved both the binding affinity and selectivity for the δ opioid receptor. Interchanging the points of attachment of the phenolic and 4-*tert*-butylphenyl

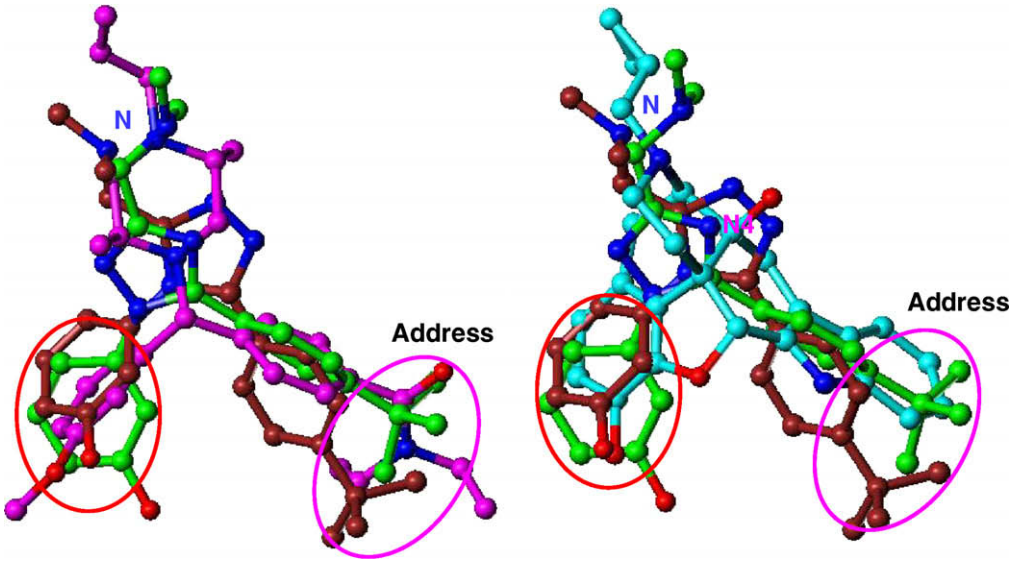


Figure 2. Structural alignment of **5b** (green) and **17** (brown) with SNC80 (purple) and naltrindole (cyan). The compounds are rendered in ball-and-stick mode. Nitrogen: blue; oxygen: red; carbon: green (**5b**), purple (SNC80), cyan (naltrindole) and brown (**17**). The N4 atom of **5b** is labeled in purple text. The phenolic moiety and 'address' are indicated with red and purple ovals, respectively.

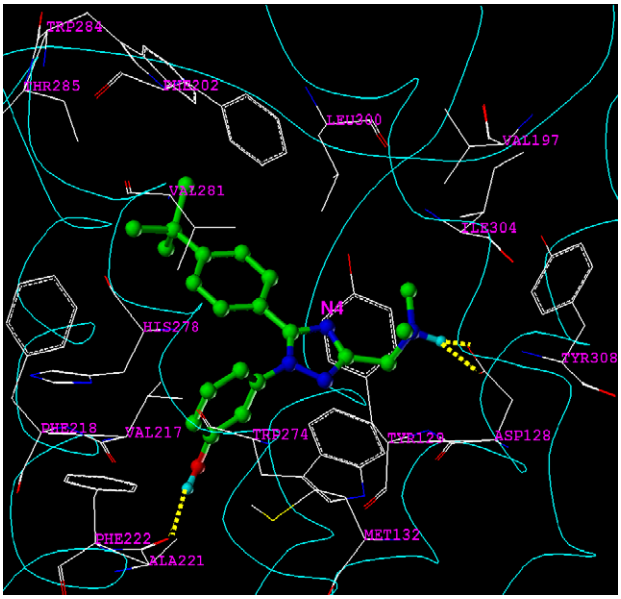


Figure 3. Docked conformation of compound **5b** in the active site of the δ opioid receptor structural model. Compound **5b** is rendered in ball-and-stick mode and colored by atom-type (C: green; N: blue; O: red; H: pale blue). The opioid receptor is rendered as a line ribbon and colored cyan. Residues in contact with **5b** are rendered in stick mode and colored by Sybyl v7.2 atom-type. Hydrogen bonds are depicted as yellow dotted lines. The N4 atom of **5b** is labeled in purple text. To obtain further experimental confirmation of the structures of these ligands, X-ray crystallographic analysis was conducted on compounds **5b** and **5e** in this series (Fig. 4). (CCDC 728550 (**5b**) and CCDC 728551 (**5e**) contain the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif.)

groups on the triazole ring (**5a** vs **5d**; **5b** vs **5e**) altered both the binding affinity and selectivity for the δ receptor. Although the small number of examples in this study precludes us from discerning a clear SAR, these encouraging results have motivated the synthesis and biological evaluation of an expanded series of analogs for further structural optimization of these triazole-based δ selective opioids.

Compound **5b** was submitted to the NIMH Psychoactive Drug Screening Program (PDSP)[45] to screen against a panel of G-protein coupled receptors and transporters. The screening results (Supplementary data, Table 1) revealed that compound **5b** is highly selective for the δ opioid receptor over 5HT receptors, adrenergic receptors, dopamine receptors, histamine receptors, muscarinic subtype receptors, and transporters of DAT, NET and SERT.

The functional activities of these novel compounds at the opioid receptors were determined by ligand-induced receptor down-regulation (Table 2), stimulation of GTPγS binding (Table 2), and by phospho-MAP kinase assays (for SNC80 and **5b**, Fig. 5). In the receptor down-regulation assays, incubation of HEK293 cells expressing the δ opioid receptor with **5a**–**5e** produced a decrease in receptor binding activity, due to proteolysis of the receptor.[46] The level of down-regulation for **5b**, **5d**, and **5e** was nearly equal to SNC80, suggesting that the subject compounds at 10 μM act as full δ agonists in this assay. In the GTPγS binding assay, however, the level of stimulation was much less for **5b**, **5d**, and **5e** than for SNC80. Even **5b**, the most potent compound in this series, was at least threefold less stimulatory than SNC80 when both were assayed at 10 μM. Consequently, results from the GTPγS binding assay indicate that the subject compounds act as low-efficacy partial opioid agonists at this concentration. To further explore the functional efficacy of **5b**, its activity was compared with SNC80 for agonist-induced phosphorylation and activation of MAP kinase.[47] In this assay, like the agonist-induced down-regulation assay, **5b** was equally efficacious as SNC80 for activation of MAP kinase (Fig. 5).

The apparent discrepancy between the receptor down-regulation, GTPγS binding, and phospho-MAP kinase assays for **5b** may stem from different signaling pathways involved in each case. This phenomenon has also been observed for other GPCRs, for example, serotonin 5HT receptors,[48] adrenergic receptors,[49] and angiotensin receptors.[50] It has been reported that activation of GPCRs can induce differential cellular signaling patterns by both G-protein dependent and G-protein independent mechanisms, such as through arrestin.[49,51–54] Thus, a molecule classified as an agonist based on one functional assay may behave differently in another assay. For example, a well-established β2 adrenergic receptor inverse agonist (ICI118551) for adenylyl cyclase stimulated the activation of ERK that depends on β-arrestin-2 expression.[49] Similarly,

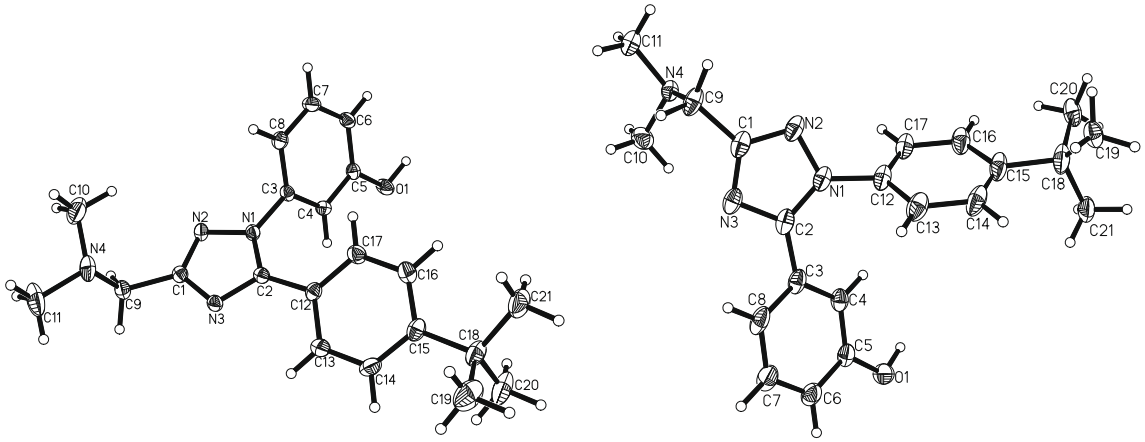


Figure 4. X-ray crystal structures of compounds **5b** and **5e**. Atoms are depicted as 50% thermal probability ellipsoids.

Table 2
Functional activity (±SEM) of the subject compounds at 10 μM for the δ opioid receptor

Compd	Down-regulation control%	GTPγS control%
Control	100.0 ± 2.9	101.7 ± 2.2
SNC80	16.0 ± 2.3	824.3 ± 31.3
Naloxone	103.3 ± 1.8	101.0 ± 2.6
5a	70.0 ± 5.8	120.0 ± 7.5
5b	15.0 ± 2.9	251.0 ± 27.7
5c	82.0 ± 5.8	125.7 ± 8.8
5d	20.0 ± 2.9	210.7 ± 24.3
5e	19.2 ± 2.6	183.7 ± 10.9

Wei et al.[50] have shown that a modified angiotensin peptide could stimulate arrestin-dependent activation of ERK1/2 but not activation of G-protein. More recently, it was reported that the δ receptor agonists DPDPE and TIPP inhibited adenylyl cyclase activity and activated MAP kinase; however, only DPDPE, but not TIPP or morphine, induced desensitization and internalization of the δ receptor.[55] These preliminary results for our novel compounds provide another example that agonist efficacy is dependent on the cellular signaling pathway. However, the underlying mechanism for the differential stimulation remains to be determined although this phenomenon has been observed for a variety of GPCRs. Currently, more biological studies are underway in our laboratory to better understand signaling mechanisms of the subject compounds.

4. Conclusion

We report here a novel family of 1,3,5-trisubstituted 1,2,4-triazoles as potent and selective δ opioid receptor ligands discovered by rational design. Among the compounds tested thus far, **5b** exhibited potent binding affinity of 5.8 nM and excellent selectivity for the δ opioid receptor (selectivity ratio: δ/μ >1712; δ/κ = 664). Screening assays performed by PDSP reveal that **5b** exhibits high selectivity for the δ opioid receptor over a panel of GPCRs and transporters. The subject compounds exhibited differential agonistic efficacy in different functional assays: receptor down-regulation, stimulation of GTPγS binding, and MAP kinase activation. This discrepancy between different functional assays has been observed for the δ receptor and other GPCRs like serotonin, angiotensin, and adrenergic receptors. It has been suggested that different signaling pathways are involved in each functional assay. Currently, more biological studies are underway to better understand the mechanism of differential stimulation by these novel compounds. A broader range of analogs will be synthesized to more fully explore the structure–activity relationships of this series of compounds. This task will be facilitated by an integrated approach that encompasses both ligand-based and structure-based drug design methods. Structural models for the δ opioid receptor will be constructed using homology modeling approaches based on the recently published X-ray crystal structures of several GPCRs.[49,56–60]

5. Experimental

5.1. General information

^{1}H NMR spectra were recorded at 400 MHz on Varian Gemini-400 spectrometer, in deuterated chloroform ($CDCl_3$) or DMSO (DMSO-d_6) solution at room temperature, using TMS (0.00 ppm)

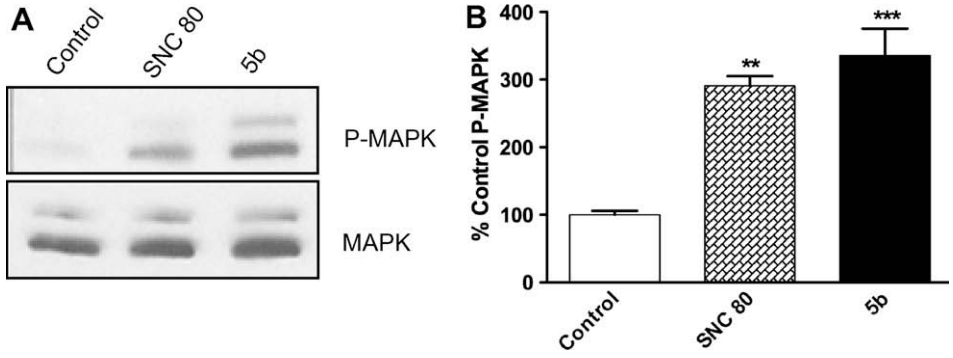


Figure 5. HEK293 cells expressing the δ receptor were treated with saline (control), SNC80 (10 μM)) or **5b** (10 μM) for 10 min. Whole cell lysates were prepared and analyzed by western blot analysis using antibodies selective for phosphorylated MAP kinase or total (phosphorylated and nonphosphorylated) MAP kinase. Statistical analysis was performed using one-way ANOVA (**, p<0.01; ***, p<0.001) Newman–Keuls multiple comparison test (n = 5).

as internal standards and were reported in parts per million (ppm). ^{13}C NMR spectra were recorded on a Varian Gemini 100 MHz NMR spectrometer at room temperature in $CDCl_3$ and were internally referenced to $CDCl_3$ (77.23 ppm). Abbreviations for signal coupling are as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; w, wide. Coupling constants, *J*, are reported (Hz). Analytical thin layer chromatography (TLC) was performed on pre-coated plastic backed plates purchased from Aldrich (Silica Gel 60 F_{254}; 0.25 mm thickness). Flash column chromatography was conducted with Silica Gel 60 (230–400 mesh) from Natland Co. HPLC was performed using the Waters ACQUITY™ HPLC system, which was equipped with a Synergi 4u Fusion-RP80A column (2.0 mm × 50 mm) and a PDA detector. All samples were run under gradient conditions with CH_3CN/H_2O modified with 0.1% formic acid. All samples had purity >95% under 254 nm. MS and MS–MS were conducted on a Finnigan LCQ DUO Mass Spectrum (Thermo Quest Co.) Gas chromatographic analyses were performed on a Hewlett–Packard 6890 GC–MS instrument with a FID detector using 25 m × 0.20 mm capillary column with cross-linked methylsiloxane as a stationary phase. Melting points were taken on a Meltemp melting point apparatus in open capillary tubes without calibration.

All reactions were carried out with anhydrous solvents in oven-dried and argon-charged glassware. All anhydrous solvents except as mentioned were freshly distilled and stored in 4 Å molecular sieves. All solvents used in workup, extraction procedures, re-crystallization process, and chromatography were used as received from commercial supplier without further purification. All reagents were purchased from Aldrich Chemical Company.

5.2. Ethyl 2-chloro-2-(2-(3-methoxyphenyl)hydrazono)acetate (2a)

The 3-methoxylaniline 2.46 g (20.0 mmol) was dissolved in 50 mL 2.0 N hydrochloride to give a clear solution. The solution was cooled to 5 °C and $NaNO_2$ 1.38 g (20.0 mmol) which was dissolved in 5 mL water was added dropwise. The temperature was kept <5 °C. Thirty minutes later, the solution was cooled to <1 °C and ethyl 2-chloro-3-oxobutanoate 3.28 g (20.0 mmol) was added dropwise multiple portions. The solution was stirred overnight at room temperature. Chloroform 30 mL was added and the organic layer was dried over $MgSO_4$. After concentrating, the product was purified by chromatography using EtOAc and hexane as eluent to obtain 3.53 g (69%) **2a** as colorless liquid which became a solid while standing. ^1H NMR (400 MHz, $CDCl_3$): δ 1.39 (t, *J* = 5.5 Hz, 3H), 3.82 (s, 3H), 4.37 (dd, *J* = 5.5 Hz, 2H), 6.59 (d, *J* = 5.7 Hz, 1H), 6.76 (d, *J* = 6.3 Hz, 1H), 6.83 (s, 1H), 7.19–7.25 (m, 1H), 8.32 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 14.2, 55.3, 62.8, 100.4, 107.0, 108.7, 116.1, 130.3, 142.8, 160.8, 197.9.

5.3. Ethyl 2-(2-(3-*tert*-butylphenyl)hydrazono)-2-chloroacetate (2b)

Prepared as **2a** to get 3.61 g (72%) as yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ 1.31 (s, 9H), 1.41(t, *J* = 4.9 Hz, 3H), 4.39 (dd, *J* = 7.2 Hz, 2H), 7.15 (d, *J* = 5.9 Hz, 2H), 7.20 (s, 1H), 7.24 (t, *J* = 6.1 Hz, 1H), 8.35 (s, 1H).

5.4. Ethyl 2-(2-(4-*tert*-butylphenyl)hydrazono)-2-chloroacetate (2c)

Prepared as **2a** to get 3.97 g (76%) as yellow solid. ^1H NMR (400 MHz, $CDCl_3$): δ 1.29 (s, 9H), 1.41(t, *J* = 7.2 Hz, 3H), 4.38 (dd, *J* = 7.2 Hz, 2H), 7.16 (d, *J* = 5.9 Hz, 2H), 7.36 (d, *J* = 4.5 Hz, 2H), 8.31 (s, 1H).

5.5. Ethyl 5-(3-*tert*-butylphenyl)-1-(3-methoxyphenyl)-1H-1,2,4-trazole-3-carboxylate (3a)

The crystal of **2a** 668 mg (2.6 mmol) was dissolved in 10 mL THF, and ammonia gas was bubbled through the solution at room temperature. Two hours later, TLC indicated all **2a** had been converted to amidrazone. The solvent and excess ammonia were evaporated, the residue was dissolved in 15 mL chloroform, and the clear solution was cooled to 0–5 °C. 3-*tert*-Butylbenzoyl chloride 510 mg (2.6 mmol) was dissolved in 5 mL chloroform and added dropwise carefully into the amidrazone solution. The mixture was refluxed overnight. After cooling to room temperature, 5 mL triethylamine was added and the solution was extracted by water (3 × 10 mL). The organic layer was dried over $MgSO_4$, and the product was purified by chromatography using EtOAc and hexane as the eluent to give 886 mg (90%) of **3a** as colorless liquid. ^1H NMR (400 MHz, $CDCl_3$): δ 1.16 (s, 9H), 1.44(t, *J* = 1.9 Hz, 3H), 3.78 (s, 3H), 4.52 (q, *J* = 2.9 Hz, 2H), 6.91 (d, *J* = 6.9 Hz, 2H), 6.96 (d, *J* = 7.3 Hz, 1H),7.24 (s, 1H), 7.27 (d, *J* = 7.2 Hz, 2H), 7.40–7.44 (m, 2H).

5.6. Ethyl 5-(4-*tert*-butylphenyl)-1-(3-methoxyphenyl)-1H-1,2,4-trazole-3-carboxylate (3b)

Prepared as **3a**, yield was 93%. ^1H NMR (400 MHz, $CDCl_3$): δ 1.17 (s, 9H), 1.43(t, *J* = 2.3 Hz, 3H), 3.74 (s, 3H), 4.48 (q, J_1 = 2.7 Hz, J_2 = 2.0 Hz, 2H), 6.86 (m, 3H), 7.22 (s, 1H), 7.32 (d, *J* = 6.9 Hz, 2H), 7.96 (d, *J* = 7.6 Hz, 2H).

5.7. Ethyl 5-(2,4-dimethylphenyl)-1-(3-methoxyphenyl)-1H-1,2,4-trazole-3-carboxylate (3c)

Prepared as **3a**, yield was 87%. ^1H NMR (400 MHz, $CDCl_3$): δ 1.22 (s, 9H), 1.56 (t, *J* = 6.4 Hz, 3H), 2.01 (s, 3H), 2.32 (s, 3H), 3.67 (s, 3H), 4.48 (q, J_1 = 5.9 Hz, 2H), 6.82 (d, *J* = 5.9 Hz, 2H), 7.01 (s, 1H), 7.16–7.20 (m, 2H), 7.51 (d, *J* = 5.1 Hz, 1H), 7.68 (d, *J* = 3.7 Hz, 1H).

5.8. Ethyl 1-(3-*tert*-butylphenyl)-5-(3-methoxyphenyl)-1H-1,2,4-trazole-3-carboxylate (3d)

Prepared as **3a**, yield was 89%. ^1H NMR (400 MHz, $CDCl_3$): δ 1.32 (s, 9H), 1.46 (t, *J* = 7.2 Hz, 3H), 3.67 (s, 3H), 4.53 (q, J_1 = 7.1 Hz, 2H), 6.92 (d, *J* = 4.7 Hz, 1H), 6.99–7.04 (m, 3H), 7.20–7.22 (m, 2H), 7.32 (s, 1H), 7.45 (d, *J* = 4.1 Hz, 1H).

5.9. Ethyl 1-(4-*tert*-butylphenyl)-5-(3-methoxyphenyl)-1H-1,2,4-trazole-3-carboxylate (3e)

Prepared as **3a**, yield was 92%. ^1H NMR (400 MHz, $CDCl_3$): δ 1.31 (s, 9H), 1.44 (t, *J* = 6.9 Hz, 3H), 3.71 (s, 3H), 4.49 (q, J_1 = 6.9 Hz, 2H), 6.92 (d, *J* = 4.7 Hz, 1H), 7.10 (d, *J* = 7.8 Hz, 2H),7.22 (d, *J* = 7.4 Hz, 1H), 7.32 (d, *J* = 4.6 Hz, 2H),7.45 (d, *J* = 4.6 Hz, 2H).

5.10. (5-(3-*tert*-Butylphenyl)-1-(3-methoxyphenyl)-1H-1,2,4-triazol-3-yl)-*N*,*N*-dimethylmethanamine (4a)

The ethyl ester **3a** 177 mg (0.46 mmol) was dissolved in 4 mL anhydrous ether and $LiAlH_4$ 41 mg (1.05 mmol) was added in small portions. The mixture was stirred for 1 h and ethyl acetate was added dropwise to quench reaction. Two milliliters of water and 10 mL chloroform were added and organic layer was dried and concentrated to yield the alcohol with 100% yield. The alcohol 146 mg (0.43 mmol) was dissolved in 4 mL THF and 143 mg CH_3I (1.0 mmol), and 174 mg PPh_3 (0.64 mmol) were added in sequence. The solution was then cooled to 0 °C. Diethyl diazocarboxylate (DEAD) 113 mg (0.64 mmol) was slowly added dropwise into the solution above, which was then stirred at room temperature. TLC

indicated that the reaction finished within 4 h. Diethylamine (2.0 M in THF) 6.0 mL was introduced to above solution, and the solution was heated to reflux overnight. Solvent and excess diethylamine were removed and residual was purified by chromatography using chloroform and methanol as eluent to give 167 mg **4a** as white solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.12 (s, 9H), 2.43 (s, 6H), 3.57 (s, 2H), 3.72 (s, 3H), 6.85–7.18 (m, 3H), 7.21 (d, J = 5.1 Hz, 1H), 7.26 (m, 2H), 7.35 (d, J = 4.9 Hz, 1H), 7.49 (m, 1H). MS (ESI): 365.5 (M^++1).

5.11. (5-(4-*tert*-Butylphenyl)-1-(3-methoxyphenyl)-1*H*-1,2,4-triazol-3-yl)-*N*,*N*-dimethylmethanamine (4b)

Prepared as **4a**, white solid, yield was 87% from 3b. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.28 (s, 9H), 2.41 (s, 6H), 3.67 (s, 2H), 3.74 (s, 3H), 6.92 (d, J = 5.9, 1H), 6.95 (s, 1H), 7.25–7.30 (m, 2H), 7.31 (d, J = 8.9 Hz, 2H), 7.45 (d, J = 7.8 Hz, 2H). MS (ESI): 365.5 (M^++1).

5.12. (5-(2,4-Dimethylphenyl)-1-(3-methoxyphenyl)-1H-1,2,4-triazol-3-yl)-*N*,*N*-dimethylmethanamine (4c)

Prepared as **4a**, white solid, yield was 89% from **3c**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 2.01 (s, 3H), 2.31 (s, 3H), 2.42 (s, 6H), 3.64 (s, 3H), 3.72 (s, 2H), 6.77–6.82 (m, 2H), 6.98 (d, J = 5.6 Hz, 1H), 7.12–7.17 (m, 3H). MS (ESI): 337.3 (M^++1).

5.13. (1-(3-*tert*-Butylphenyl)-5-(3-methoxyphenyl)-1*H*-1,2,4-triazol-3-yl)-*N*,*N*-dimethylmethanamine (4d)

Prepared as **4a**, white solid, yield was 81% from **3d**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.22 (s, 9H), 2.43 (s, 6H), 3.66 (s, 3H), 3.70 (s, 2H), 6.90 (d, J = 6.5 Hz, 1H), 7.02 (s, 1H), 7.16–7.22 (m, 2H), 7.32 (s, 1H), 7.32–7.42 (m, 3H). MS (ESI): 365.3 (M^++1).

5.14. (1-(4-*tert*-Butylphenyl)-5-(3-methoxyphenyl)-1*H*-1,2,4-triazol-3-yl)-*N*,*N*-dimethylmethanamine (4e)

Prepared as **4a**, white solid, yield was 81% from **3e**. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.32 (s, 9H), 2.43 (s, 6H), 3.66 (s, 3H), 3.69 (s, 2H), 6.91 (d, J = 6.5 Hz, 1H), 7.04–7.09 (m, 1H), 7.09 (s, 1H), 7.19 (d, J = 8.9 Hz, 1H), 7.27 (d, J = 10.2 Hz, 2H), 7.41 (d, J = 9.8 Hz, 2H). MS (ESI): 365.3 (M^++1).

5.15. 3-(5-(3-*tert*-Butylphenyl)-3-((dimethylamino)methyl)-1H-1,2,4-triazol-1-yl)phenol (5a)

36.5 mg **4a** (0.1 mmol) was dissolved in 5 mL dichloromethane and 5.0 equiv of BBr_3 was added dropwise at −78 °C. The solution was stirred at room temperature; TLC indicated the reaction was finished within 5 h. Ten milliliters of chloroform and 10 mL water were added in sequence and mixture was extracted by water (3 × 5 mL). The organic layer was dried over $MgSO_4$ and concentrated. The final product **5a** was purified by chromatography using chloroform and methanol as the eluent to obtain 130 mg **5a** (78%) as white solid. Mp: 192–194 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.11 (s, 9H), 2.13 (s, 6H), 3.57 (s, 2H), 6.16 (s, 1H), 6.85 (d, J = 6.9 Hz, 1H), 6.92 (d, J = 7.1 Hz, 1H), 7.14(d, J = 5.6 Hz, 1H), 7.16 (d, J = 4.9 Hz, 1H), 7.25–7.30 (m, 4H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 30.9, 34.5, 44.7, 55.5, 112.6, 116.0, 116.8, 125.9, 126.0, 126.8, 127.1, 128.2, 130.3, 138.5, 151.5, 154.8, 158.0, 159.2. MS (ESI): 351.3 (M^++1).

5.16. 3-(5-(4-*tert*-butylphenyl)-3-((dimethylamino)methyl)-1H-1,2,4-triazol-1-yl)phenol (5b)

Prepared as **5a**, white solid, yield was 82% from **4b**. Mp: 191–193 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.25 (s, 9H), 2.23 (s, 6H), 3.60 (s, 2H), 6.27 (s, 1H), 6.82 (d, J = 5.9 Hz, 1H), 6.87 (d, J = 5.0 Hz, 1H), 7.21–7.25 (m, 6H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 31.1, 34.8, 44.9, 55.6, 112.8, 116.0, 116.9, 124.4, 125.5, 128.5, 130.3, 138.5, 153.3, 154.4, 158.0, 159.4. MS (ESI): 351.3 (M^++1).

5.17. 3-(3-((Dimethylamino)methyl)-5-(2,4-dimethylphenyl)-1H-1,2,4-triazol-1-yl)phenol (5c)

Prepared as **5a**, white solid, yield was 71% from **4c**. Mp: 167–169 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.96 (s, 3H), 2.28 (s, 3H), 2.40 (s, 6H), 3.72 (s, 2H), 6.54 (s, 1H), 6.63 (d, J = 7.9 Hz, 1H), 6.72 (d, J = 6.7 Hz, 1H), 7.01 (t, J = 7.1 Hz, 2H) 7.03–7.24 (m, 3H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 19.6, 21.3, 44.6, 55.2, 111.1, 114.8, 115.9, 125.2, 126.7, 129.9, 130.0, 131.4, 137.0, 138.3, 140.2, 157.3158.5, 161.9. MS (ESI): 323.4 (M^++1).

5.18. 3-(2-(3-*tert*-Butylphenyl)-5-((dimethylamino)methyl)-2H-1,2,4-triazol-3-yl)phenol (5d)

Prepared as **5d**, white solid, yield was 78% from **4d**. Mp: 177–178 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.19 (s, 9H), 2.45 (s, 6H), 3.79 (s, 2H), 6.82 (s, 1H), 6.85 (d, J = 6.0 Hz, 2H), 7.10–1.15 (m, 2H), 7.20 (s, 1H), 7.30 (t, J = 7.9 Hz, 1H), 7.37 (d, J = 6.7 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 31.0, 34.7, 44.8, 55.3, 95.4, 116.0, 117.9, 120.3, 121.9, 122.3, 125.6, 128.5, 128.9, 129.7, 137.4, 152.7, 153.5, 156.9. MS (ESI): 351.3 (M^++1).

5.19. 3-(2-(4-*tert*-Butylphenyl)-5-((dimethylamino)methyl)-2H-1,2,4-triazol-3-yl)phenol (5e)

Prepared as **5a**, white solid, yield was 72% from **4e**. Mp: 153–155 °C. ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.29 (s, 9H), 2.37 (s, 6H), 3.70 (s, 2H), 6.72 (s, 1H), 6.84 (d, J = 4.7 Hz, 1H), 6.90 (d, J = 5.1 Hz, 1H), 7.09–7.16 (m, 3H).7.34 (d, J = 4.5 Hz, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 31.2, 34.7, 44.9, 55.4, 115.9, 118.1, 124.5, 126.2, 128.3, 129.8, 135.2, 152.0, 154.4, 157.1, 159.3. MS (ESI): 351.3 (M^++1).

5.20. Receptor binding assay

Cell membrane preparations were obtained from HEK293-stable transfectants expressing either the murine δ receptor,[47] μ receptor,[46] or the rat κ receptor[61] by ultracentrifugation. Opioid receptor binding assays were conducted in duplicate at least three times on membrane preparations that had been re-suspended in 50 mM Tris–HCl, pH 7.5, utilizing [^{3}H]DPDPE, [^{3}H]DAMGO and [^{3}H]U69593 as radioligand for δ, μ, and κ opioid receptors, respectively. Cyclazocine (10 μM) was used to define nonspecific binding. Following 1-h incubation on ice, binding assays were terminated by filtration through Whatman GF/B filters. Filters were soaked in Ecoscint liquid scintillation solution (National Diagnostics, Manville, NJ) and filter-bound radioactivity was measured using a Packard Tri-Carb 2100 TR liquid scintillation analyzer. Receptor binding data were analyzed by nonlinear regression of saturation and competition curves using Prism 3.0 software (GraphPad Software, San Diego, CA). Protein concentrations were determined with the Bio-Rad Bradford protein assay using bovine serum albumin as the standard.

5.21. Receptor down-regulation assays

HEK293 cells expressing the FLAG-tagged δ receptor were grown to near confluence in 100 mm dishes in DMEM media supplemented with 10% fetal calf serum and 0.25 mg/ml G418. Media was replaced with serum-free media prior to overnight incubation with 10 μM of **5a**–**5e** or control compounds. Media was aspirated

and cell washed twice with cold PBS. Cell homogenates were collected and further washed thrice with 50 mM Tris–HCl, pH 7.5. Protein concentration was measured using the Bio-Rad Bradford reagent, and an equal amount of protein (50 μg) was used for the binding assays. Binding assays were performed using [^{3}H]diprenorphine as the radioactive ligand. Binding assays were conducted as described above. The assays were run in duplicate at least three times.

5.22. GTPγS assays

Cell membranes were prepared from HEK293-stable transfectants expressing murine δ opioid receptors using ultracentrifugation. Protein concentration was measured using the Bio-Rad Bradford reagent. Ten micrograms of membrane protein expressing the delta opioid receptor was incubated at room temperature for 90 min with assay buffer containing [S-35]GTPγS and GDP in presence or absence of 10 μM of each compound except for compound **5c** which was used at 50 μM concentration. Following incubation, samples were filtered through Whatman GFB filters and washed three times with ice cold 50 mM Tris–HCl pH 7.4. Filters were soaked in Ecoscint liquid scintillation solution (National Diagnostics, Manville, NJ) and filter-bound radioactivity was measured using a Packard Tri-Carb 2100 TR liquid scintillation analyzer for S-35 isotope. The assays were performed in duplicate at least three times.

5.23. MAP kinase assays

HEK293 cells expressing the δ receptor were placed in serum-free media overnight to reduce basal MAP kinase signaling. The following day, the media was replaced with fresh serum-free media with or without SNC80 or **5b** at 10 μM and cells were incubated at 37 °C for 10 min. Reactions were terminated by aspiration of the media and solubilization of the cells on the dish with 1% dodecyl-β-D-maltoside, 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10% glycerol, protease inhibitor cocktail (Sigma, 1:100) and phosphatase inhibitor cocktail 1 and 2 (Sigma, each 1:100). The detergent lysate was centrifuged at 16,000*g* for 20 min, and the supernatant was recovered. The protein concentration in the supernatant was determined using the Dc protein assay (Bio-Rad). Protein (60 μg per lane) was resolved using 12% SDS/PAGE at 120 V for approximately 1.5 h and transferred to Immobilon P polyvinylidene difluoride membranes (Millipore, Bedford, MA) at 100 V for 1 h. Immunoblots were incubated in blocking buffer followed by overnight incubation with either mouse monoclonal anti-phospho-MAP kinase antibody (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-MAP kinase antibody that recognizes total (phosphorylated and nonphosphorylated) MAP kinase (1:3000, Upstate Biotechnology, Charlottesville, VA). Goat anti-mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology) or goat anti-rabbit IgG conjugated with horseradish peroxidase (Santa Cruz Biotechnology) were used as secondary antibodies. Membranes were developed using ECL Western blotting substrate (Pierce Biotechnology, Rockford, IL) and HyBlot CL autoradiography film (Denville Scientific, Metuchen, NJ). Blots were quantified using SynGene Software (Synoptics Ltd, Cambridge, England).

5.24. Molecular modeling

All molecular modeling operations were performed on Linux workstations and Solaris server. The crystal structures of (−)SNC80[62] and morphine[63] were used as the starting conformations to build the structures of (+)SNC80 and naltrindole using Sybyl v7.2.[64] Compounds **5b** and **17** were constructed with Sybyl7.2 as well. The MMFF94 force field and partial atomic charges were used to optimize the geometries of all compounds with protonated N atoms. Three atoms (basic nitrogen atom N, centroid of phenolic group, and centroid of pyrrole at naltrindole, or phenyl at SNC80, **5b** and **17**) were selected for the structural alignment using the Atom Fit scheme within Sybyl7.2.

The amino acid sequences of the human δ opioid receptor (hDOR accession number: P02699) and bovine rhodopsin were retrieved from the Swiss-Prot database.[65] The sequence alignment was performed by using ClustalW.[66] The default matrix BLOSUM and a gap open penalty 25 were chosen for the sequence alignment. The residues indicated in red in Fig. 1 (Supplementary data) include Asn67, Asp95, D/ERY motif in TM3, Trp173, Pro225, Pro276, NPXXY motif in TM7, Cys121 and Cys198. The conserved two Cys residues are involved in formation of the conserved disulfide bridge between TM3 and extracellular loop 2 (EL2). The same boundaries of the TM domains were applied to the opioid receptors as identified in the X-ray crystal structure for the corresponding sequence of bovine rhodopsin.

After the optimal alignment was achieved, the 3D structural models of the δ opioid receptor were constructed using the HOMOLOGY module in the Insight package[67] based on the X-ray crystal structure of bovine rhodopsin (pdb ID: 1F88) as the template.[36] The coordinates of TMs were copied from the corresponding TMs in the template structure. Loop searches in the PDB were performed on all extracellular and intracellular loops. The loop hits showing the highest sequence identity and lowest rms deviation were selected for the corresponding regions. The N and C termini, located, respectively, in the periplasm and cytosol, are distant from the ligand binding pocket and therefore were excluded in the final models. Energy minimization was performed with AMBER8.0 using AMBER99 force field on initial models for about 500 steps to relax close interactions.[68] The δ receptor selective agonist (+)SNC80 and antagonist naltrindole were manually docked to the putative active site based on the published mutation data and molecular modeling studies. The resulting ligand–receptor complexes were submitted to energy minimization and 1.5 ns molecular dynamics simulations at constant NTP (T = 25 °C; P = 1 atm) using AMBER8.0. The averaged structure was energy-minimized. Removing the ligands from the minimized complexes yielded one final set of coordinates for the agonist-bound and antagonist-bound δ opioid receptors, which were used for docking of compound **5b** with the GOLD docking program (version 3.1).[69] The number of poses for each compound was set to 100. After docking, all binding poses of each ligand were visually checked. Only the poses that exhibited a high GOLDscore and formed a salt bridge with Asp128 and hydrogen bond to TM5 or TM6 were selected as realistic docked conformations. The resulting receptor–ligand complexes were submitted to energy minimization with AMBER8.0. The binding interactions were analyzed with Sybyl v7.2. The binding pose of **5b** shown in Fig. 2 is from the agonist-bound structure of the δ opioid receptor. The key contact residues with **5b** are the same for agonist-bound and antagonist-bound structures.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2009.07.007.

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