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Letters

Covalently Induced Activation of the δ Opioid Receptor by a Fluorogenic Affinity Label, 7′-(Phthalaldehydecarboxamido)naltrindole (PNTI)

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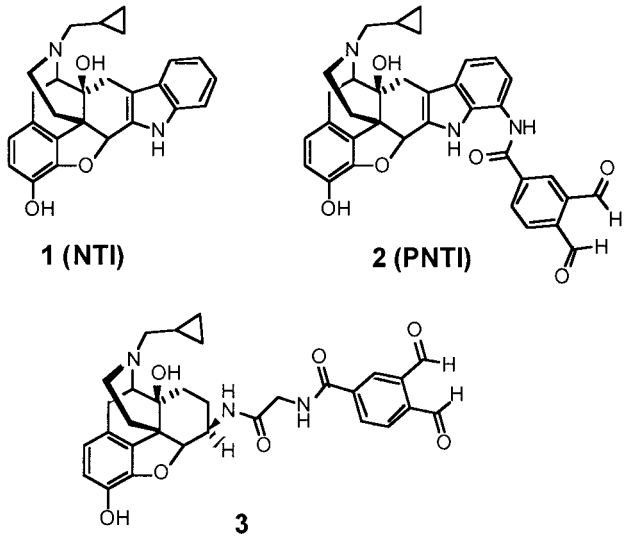
The prototypical nonpeptide δ opioid receptor antagonist, naltrindole[1] (**1**, NTI), is widely employed as a pharmacologic tool in opioid research. It is one of the most potent δ antagonists, with potency in the subnanomolar range.[2] Here we report that modification of NTI (**1**) with an electrophilic *o*-phthalaldehyde (OPA) moiety to afford **2** (PNTI) results in an unprecedented change in activity from antagonist to a highly potent agonist upon covalent binding to the δ opioid receptor.

We have previously reported on a new approach to the design of an affinity label **3** whose OPA moiety binds covalently to opioid receptors.[3] The covalent binding involves the generation of an isoindole fluorophore,[4] presumably as a consequence of cross-linking neighboring lysine and cysteine residues by the OPA moiety. This class of fluorogenic ligands has been termed "reporter affinity labels" because the generation of fluorescence implicates covalent cross-linking to specific amino acid residues.[3]

The rationale for the design of PNTI (**2**) was based on prior structure−activity relationship (SAR) studies which revealed that 7′-substitution on the indole ring of NTI is well-tolerated.[2,5,6] Also, computer-aided docking of PNTI onto the δ opioid receptor revealed that an OPA moiety attached to NTI through a 7′-carboxamide



group may be in the vicinity of Lys214 and Cys216 residues located on TM 5 close to the extracellular surface of the δ opioid receptor (Figure 1A).

PNTI (**2**) and its nonelectrophilic analogue **4** were synthesized from 7′-aminonaltrindole[7] (**5**) as illustrated in Scheme 1. Coupling of **5** with benzoic acid or 3,4-bis-[2-(1,3-dioxolanyl)]benzoic acid[3] in the presence of 1-hydroxybenzotriazole and dicyclohexylcarbodiimide gave the corresponding amido esters **6** or **7** which were selectively hydrolyzed with potassium carbonate to afford the amides **4** or **8**. Target compound **2** was then obtained by hydrolysis of **8** under acidic conditions.

Receptor binding of **2** to membranes from CHO cells that possessed stably expressed μ (83 fmol/mg protein), δ (51 fmol/mg protein), and κ (36 fmol/mg protein) opioid receptors revealed that it was δ-selective, with apparent K_i[8] values of 14.9 ± 0.8, 1.9 ± 0.1, and 20.1 ± 0.8 nM for μ, δ, and κ receptors, respectively. Pretreatment of δ receptors with low (20 nM) or high (1 μM) concentrations of **2** (37°C, 15 min incubation in HEPES buffer, pH = 7.5) followed by extensive washing reduced the binding of [^{3}H]diprenorphine in a concentration-dependent fashion (Figure 2). In contrast, pretreatment of δ

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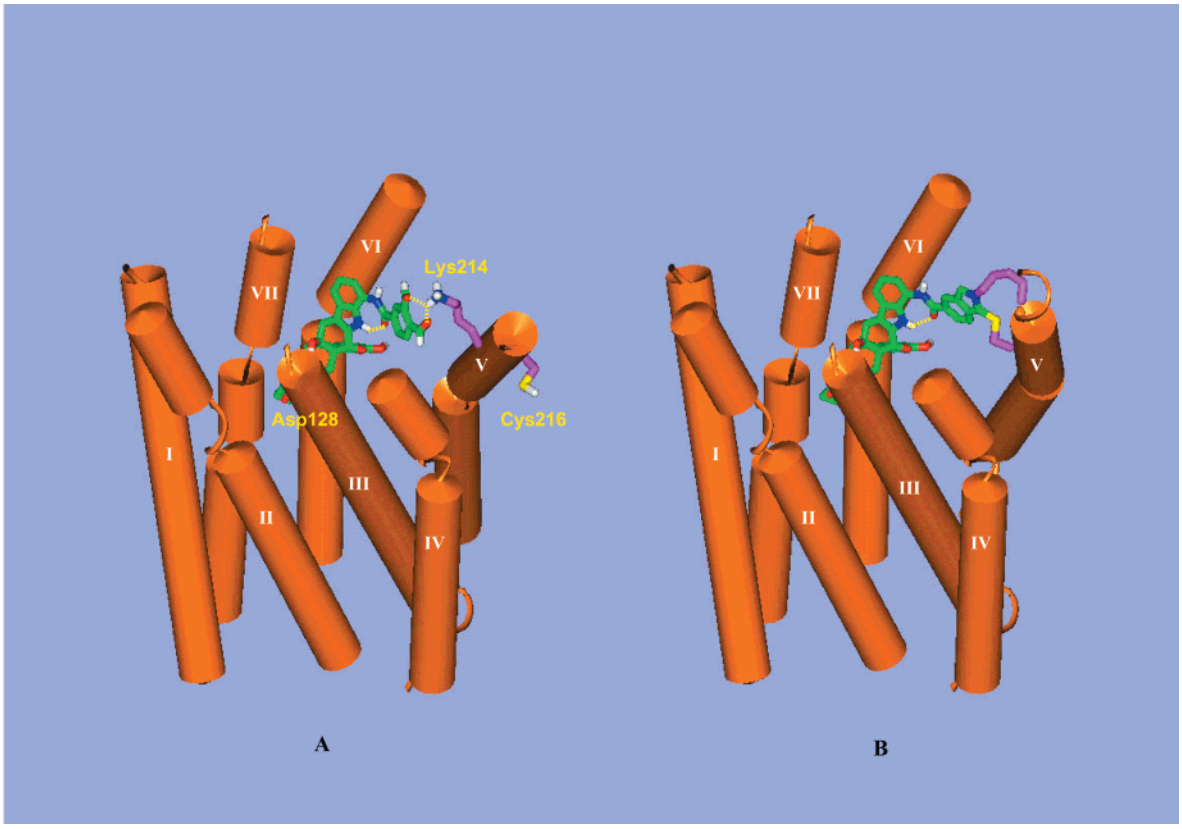
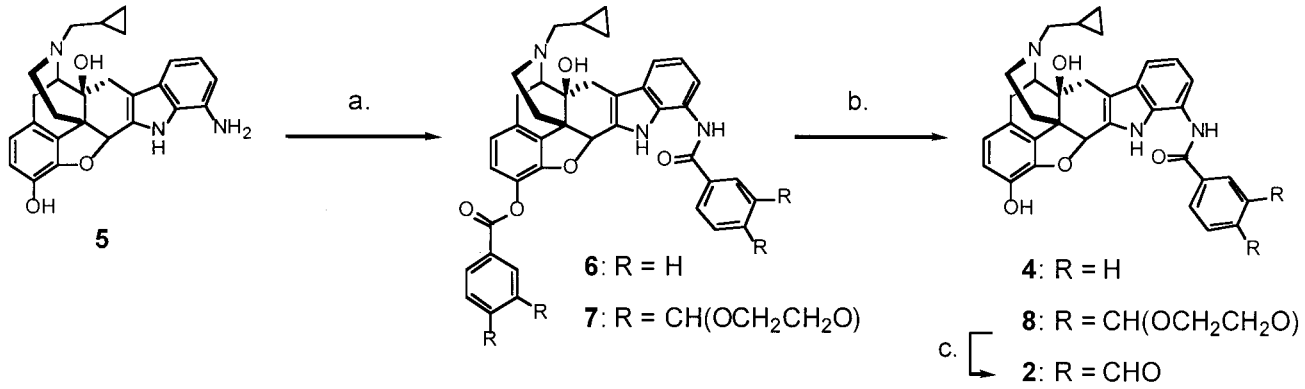


Figure 1. Modeled interaction between PNTI (**2**) (green) and the δ opioid receptor (orange) before (A) and after (B) covalent binding. (A) The OPA moiety of **2** is hydrogen-bonded to the ϵ-$NH_3^{(+)}$ group of Lys214 as a reversibly bound complex. (B) Formation of the fluorescent isoindole after covalent binding of the OPA moiety to the side chains of Lys214 and Cys216 (magenta) in TM 5 leads to receptor activation triggered by clockwise rotation (when viewed from the extracellular side) of TM 5.

Scheme 1[a]



[a] Reagents and conditions: (a) benzoic acid or 3,4-bis[2-(1,3-dioxolanyl)]benzoic acid, DCC, HOBt, DMF, rt, 5 days, 72−67%; (b) K_2CO_3, CH_3OH, rt, 1 h, 52−48%; (c) 1 N HCl, N_2, acetone, rt, 5 days, 67%.

opioid receptors with naloxone (1 μM) followed by washing did not reduce radioligand binding. The nonelectrophilic analogue **4** could be partially washed out from the membrane preparation.[9]

Covalent binding of **2** to δ opioid receptors via the formation of a fluorescent isoindole was established using flow cytometry. This was accomplished with a Becton Dickinson Facs Vantage equipped with a multiwavelength UV laser for excitation using a band-pass filter of 530 ± 30 nm as described previously.[3] When CHO cells with stably expressed δ opioid receptors were incubated with **2** (1 μM) in HEPES buffer (pH = 7.5), the fluorescence intensity rapidly (50 s) increased relative to the autofluorescence of untreated cells. Nonspecific fluorescence was evaluated by incubation of **2** (1 μM) with nontransfected CHO cells. This permitted us to visualize the specific fluorescent staining of δ opioid receptors (Figure 3).

The in vitro pharmacological profile of **2** was investigated on the electrically stimulated mouse vas deferens[10] (MVD) and the guinea-pig ileal longitudinal muscle[11](GPI) preparations as previously described.[12] Compound **2** behaved as an extremely potent full agonist in the MVD assay, with an IC_{50} of 0.12 ± 0.03 nM. This represented a 2-fold greater potency than the prototypical δ agonist [D-Ala^2,D-Leu^5]enkephalin[13] (DADLE) when tested in the same tissue.[14] This potent agonist effect of **2** could not be reversed by extensive washing. The finding that pretreatment with NTI (100 nM) in the MVD afforded a 160-fold shift of the

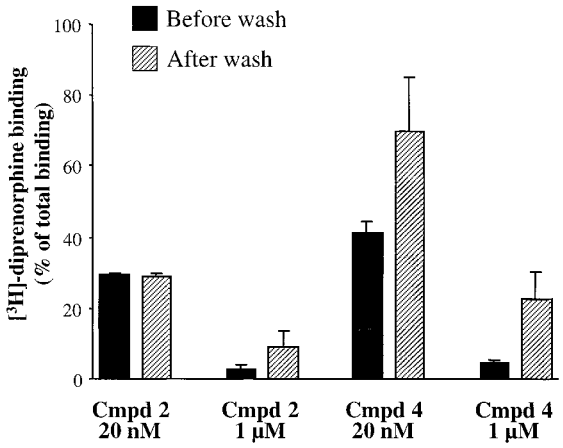


Figure 2. Irreversible binding of **2** to the δ opioid receptor. Membranes of CHO cells stably transfected with the δ opioid receptor were pretreated with **2** (20 nM or 1 μM) or **4** (20 nM or 1 μM) at 37 °C for 15 min. Free receptor sites were determined in the presence of [^{3}H]diprenorphine (1 nM) before and after wash. Data are reported as percent of the total [^{3}H]-diprenorphine binding of the treated sample. The values represent mean ± SE of three independent experiments performed in triplicate.

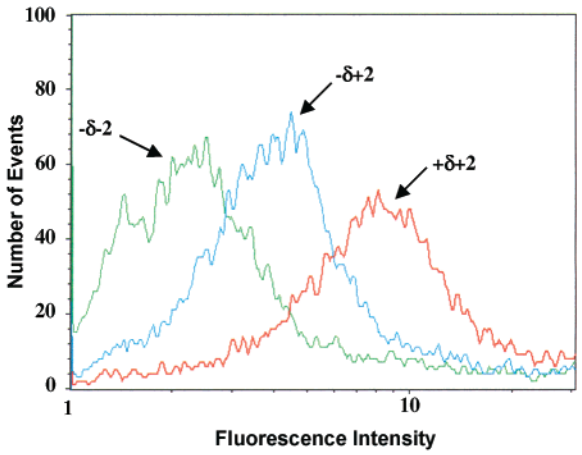


Figure 3. Representative flow cytometric analysis of fluorescent opioid labeling of CHO cells. Untransfected CHO cells (−DOR) or CHO cells transfected with the δ opioid receptor (+DOR) were incubated with (+**2**) or without (−**2**) compound **2** (1 μM) at 25 °C for 50 s. The median fluorescence intensity values for each curve are as follows: green (2.71), blue (4.26), red (7.84).

concentration−effect curve of **2** suggested that it acted through δ receptors. Compound **4** did not display significant agonist activity (3.4% inhibition at 100 nM) and was a weak antagonist (DADLE IC_{50} ratio = 2.6) in the MVD.[9,15] In the GPI, PNTI (**2**) (100 nM) did not display any agonist activity and only weak antagonist activity at μ (morphine IC_{50} ratio = 2.5) and κ (ethylketazocine IC_{50} ratio = 2.1) receptors. Thus, the bioassay experiments have shown that **2** prefers δ receptors and possesses potent agonist potency in the MVD.

In the mouse tail-flick antinociceptive assay[16] PNTI (**2**) produced a dose- and time-dependent antinociception after icv administration. The antinociceptive peak effect was reached 20 min after icv administration, with an ED_{50} value of 2.06 (1.59−2.64) nmol/mouse. To determine the selectivity of **2** at peak activity (20 min), mice were pretreated sc with NTI[2] (4 μmol/kg), β-funaltrexamine[17] (β-FNA) (20 μmol/kg), and norbinaltorphimine[18] (nor-BNI) (12 μmol/kg), which are selective antagonists for δ, μ, and κ opioid receptors, respectively. The antinociception produced by **2** was antagonized by pretreatment with NTI [ED_{50} ratio = 13.99[19] (10.60−18.64)] but only marginally by β-FNA [ED_{50} ratio = 2.67 (1.65−4.09)] or nor-BNI [ED_{50} ratio = 2.14 (1.19−3.64)]. These data confirmed that the antinociceptive action of **2** was mediated primarily through δ opioid receptors.

Antinociceptive activity was undetectable after 90 min. Given that continued exposure of the δ opioid receptor[20] to agonist gives rise to rapid, reduced responsiveness as a consequence of desensitization and down-regulation, antinociceptive testing of the δ agonist, [D-Pen^2,D-Pen^5]enkephalin[21] (DPDPE), was conducted 2 h after administration of **2** (2 nmol icv), when the tail-flick latency time had returned to normal values. Under these conditions, the antinociceptive effect of DPDPE was diminished, as indicated by its ED_{50} ratio of 4.98 (3.99−6.30). We believe that this "antagonism" was a consequence of desensitization and down-regulation caused by the persistent agonism due to covalent binding of **2** to the δ opioid receptor recognition site.

In conclusion, PNTI (**2**) has been shown to bind covalently to cloned δ opioid receptors, as evidenced by the generation of fluorescence that is characteristic of isoindole cross-linked lysine and cysteine residues. That PNTI acts as a potent δ opioid receptor agonist is counterintuitive, given that affinity labels derived from potent antagonists generally exhibit irreversible antagonist activity.[1]

Since its nonelectrophilic analogue **4** did not display agonist activity in the MVD preparation, it appears likely that covalent binding to the δ receptor by PNTI (**2**) may be responsible for promoting a conformational change of the receptor from an inactive to an active state. A possible explanation for this unusual transformation is that the isoindole that is formed from covalent binding of PNTI to the neighboring Lys214 and Cys216 residues (Figure 1B) promotes a conformational change of TM 5. Recently, evidence for a rigid body motion of TM 6 has been observed in light-activated bacteriorhodopsin[22] and constitutively activated G protein-coupled receptors.[23] The involvement of TM 5 in the activation of the α_2-adrenergic receptor has been also reported.[24] If perturbation of TM 5 or TM 6 leads to a conformational change of intracellular loop 3, which is known to be involved in the activation of G proteins, it is possible that the δ agonism of **2** is initiated through a similar mechanism. Accordingly, cross-linking of the neighboring Lys214 and Cys216 residues may lead to axial rotation of TM 5 due to the torsion created by the formation of the isoindole fluorophore, as illustrated in Figure 1B. PNTI should be a useful tool to investigate the δ receptor recognition site and the conformational transitions that take place in receptor activation.

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(8) The apparent K_i reflects both the reversible and irreversible binding components.

(9) Compound **4** inhibited [^{3}H]diprenorphine binding to δ receptors with a K_i value of 1.7 ± 0.4 nM. Like many morphinans and NTI analogues in particular, compound **4** is a "sticky" ligand and could not be washed out completely from the membrane preparation.

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(14) DADLE: IC_{50} = 0.28 nM in the same tissue.

(15) The IC_{50} ratio represents the IC_{50} of DADLE in the presence of **4** (100 nM) divided by the control IC_{50}.

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