Communications to the Editor

Reporter Affinity Labels: An *o*-Phthalaldehyde Derivative of *β*-Naltrexamine as a Fluorogenic Ligand for Opioid Receptors

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Affinity labels derived from opioid ligands have provided a useful approach to aid in the pharmacologic and structural characterization of opioid receptors.[1] Electrophilic affinity labels have been particularly useful in this regard. Because the location of covalently bound residues can be determined through site-directed mutagenesis and peptide mapping,[2,3] the binding locus of the ligand can be identified, as exemplified by *β*-funaltrexamine[4] (*β*-FNA, **1**) which has been reported to bind covalently to Lys233 of the *μ* receptor.[5]

Here we describe a unique type of opioid receptor affinity label **2** that offers an advantage over conventional affinity labels, in that it cross-links neighboring lysine and cysteine residues through a highly selective mechanism[6] (Figure 1). We have named such ligands "reporter affinity labels", because cross-linking leads to a fluorescent, covalently bound product whose fluorescence suggests the involvment of the *ε*-amino group of lysine and the sulfhydryl group of cysteine in the formation of an isoindole moiety. Another feature of this type of reporter affinity label is that cross-linking neighboring residues should restrict the translational mobility of the tethered ligand to a greater degree than an affinity label that is bound through only one residue.
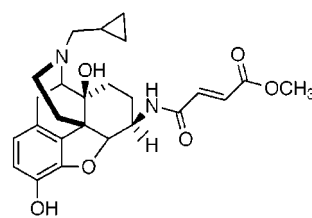
1 (β-FNA)
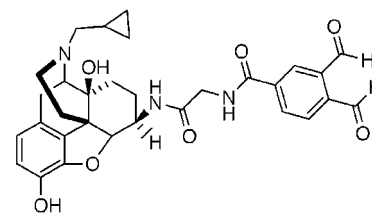
2

Consequently, modeling of the pharmacophore recognition locus of a specifically cross-linked ligand should, in principle, require less effort.

The chemical basis for the design of **2** is derived from the known reactivity of *o*-phthalaldehyde (OPTA) with primary amines and thiols to form fluorescent isoindoles.[6–8] In fact, the facility and selectivity of this reaction has found utility in the analysis of amino acids using OPTA as a fluorogenic reagent in the presence of a thiol.[9] Also, OPTA has proven to be useful in probing the catalytic site of different enzymes.[10] Given that opioid receptors contain multiple lysine and cysteine residues in the upper region of the transmembrane domain, it is possible a similar reaction can occur if such receptor-based residues are in proper juxtaposition to the OPTA moiety of **2** in the reversibly bound state.

Compound **2** (in its hydrated dihydroxyphthalan form) has been synthesized in seven steps from 3,4-dimethylbenzoic acid and *β*-naltrexamine (**3**).[11] Synthon **8**, which was required for coupling to *β*-naltrexamine (**3**), was prepared as follows (Scheme 1): 3,4-Dimethylbenzoic acid was converted to its tetrabromo derivative **4** in the presence of *N*-bromosuccinimide and benzoyl peroxide.[12] Treatment of **4** with a hot aqueous solution

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Scheme 1[a]
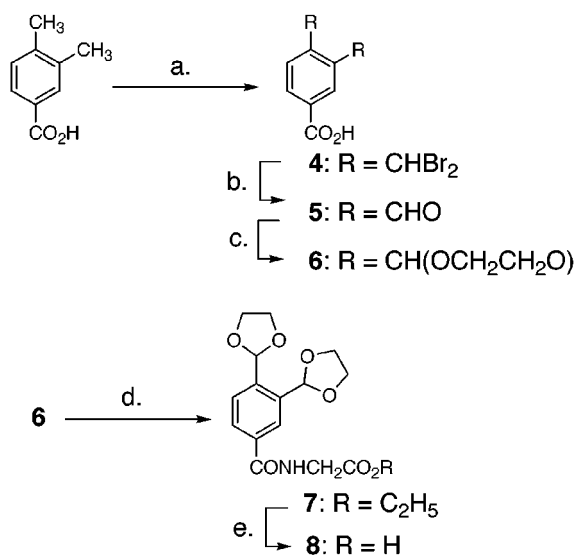
[a] Reagents and conditions: (a) *N*-bromosuccinimide, benzoyl peroxide, CCl$_4$, reflux, 10 h, 48%; (b) Na$_2$CO$_3$, H$_2$O, 60 °C, 4 h, 80%; (c) HO(CH$_2$)$_2$OH, *p*-TsOH, benzene, Dean–Stark, 15 h, 67%; (d) HCl·H$_2$NCH$_2$CO$_2$C$_2$H$_5$, N(C$_2$H$_5$)$_3$, DCC, HOBt, THF, rt, 10 h, 90%; (e) (1) NaOH, H$_2$O, rt, 5 h, (2) HCl (1 N), 75%.

of sodium carbonate followed by acidic hydrolysis gave 3,4-diformylbenzoic acid (**5**).[12] Bis-acetalization of **5** with ethylene glycol under Dean–Stark conditions afforded

the carboxylic acid **6**. Coupling of **6** with glycine ethyl ester in the presence of 1-hydroxybenzotriazole and dicyclohexylcarbodiimide gave the amido ester **7** which was saponified to afford the key intermediate **8**. Coupling of β-naltrexamine (**3**) with **8** afforded the corresponding amide **9** (Scheme 2). Hydrolysis of **9** under acidic conditions gave the hydrochloric salt of **2** as the hydrated dihydroxyphthalan.[13]

Receptor binding of **2** was investigated in membranes from CHO cells stably expressing μ (83 fmol/mg protein), δ (51 fmol/mg protein), and κ (36 fmol/mg protein) opioid receptors. Compound **2** inhibited [³H]diprenorphine binding to μ, δ, and κ receptors with apparent K_i [14] values of 0.45 ± 0.12, 2.57 ± 0.46, and 0.70 ± 0.11 nM, respectively. Pretreatment of μ, δ, and κ receptors with low (1 nM) or high (1 μM) concentrations of **2** (25 °C, 1 h incubation in HEPES buffer, pH = 7.5) followed by extensive washing reduced the binding of [³H]diprenorphine in a concentration-dependent fashion (Figure 2). In contrast, pretreatment of opioid receptors with naloxone (1 μM) followed by washing did not reduce radioligand binding. These results demonstrated that **2** binds irreversibly to μ, δ, and κ opioid receptors. The in vitro pharmacological profile of **2** was investigated on the electrically stimulated guinea-pig ileal longitudinal muscle[15] (GPI) preparation. Incubation of the GPI with **2** (20 nM) for 20 min followed by thorough washing led to a parallel shift of the morphine dose–response curve
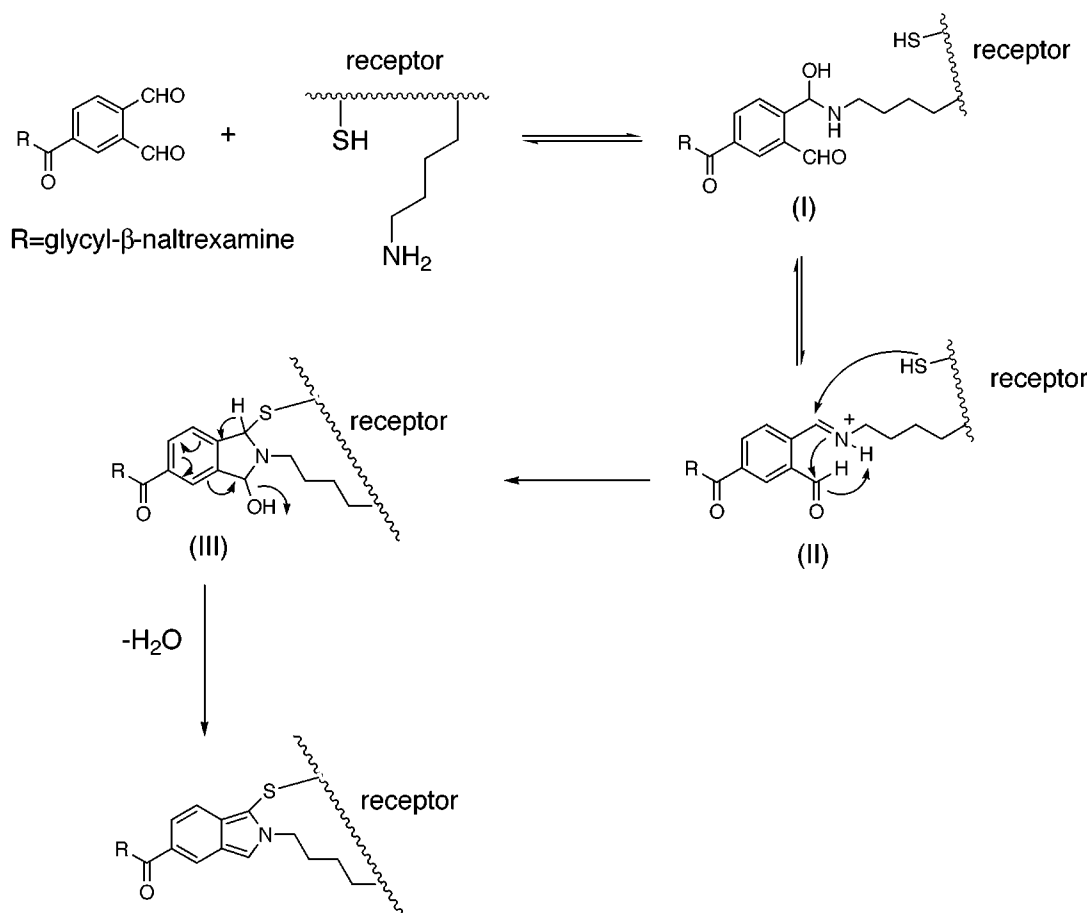

Figure 1. Mechanism for isoindole formation. The reaction of the OPTA moiety with a primary amine (NH$_2$ group of lysine) affords a carbinolamine (I), which then dehydrates to give the highly reactive protonated imine (II). This, in turn, is rapidly attacked by a neighboring nucleophile (SH group of cysteine) to form an intermediate (III) which undergoes facile dehydration to yield the fluorescent isoindole.

Scheme 2[a]
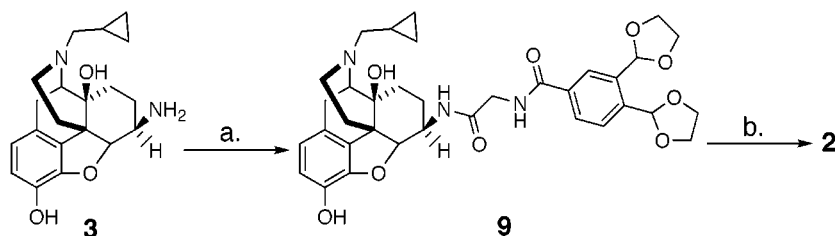
[a] Reagents and conditions: (a) **8**, DCC, HOBt, THF, rt, 12 h, 64%; (b) HCl (1 N), N_2, acetone, rt, 7 days, 38%.
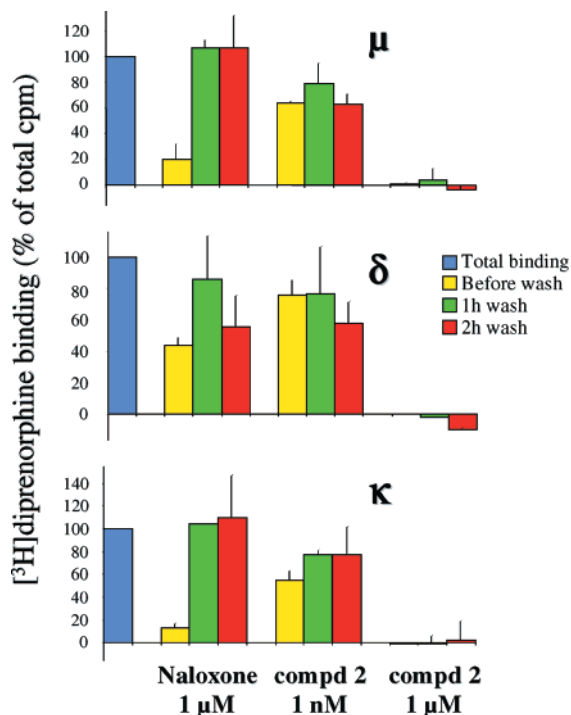

Figure 2. Irreversible binding of **2** to μ, δ, and κ opioid receptors. Membranes of CHO cells stably transfected with μ, δ, and κ opioid receptors were pretreated with or without **2** (1 nM or 1 μM) or with naloxone (1 μM, control) at 25 °C for 1 h. Free receptor sites were determined in the presence of [^{3}H]-diprenorphine (1 nM) before and after washing. The values represent means $\pm$ SE of three independent experiments performed in triplicate.
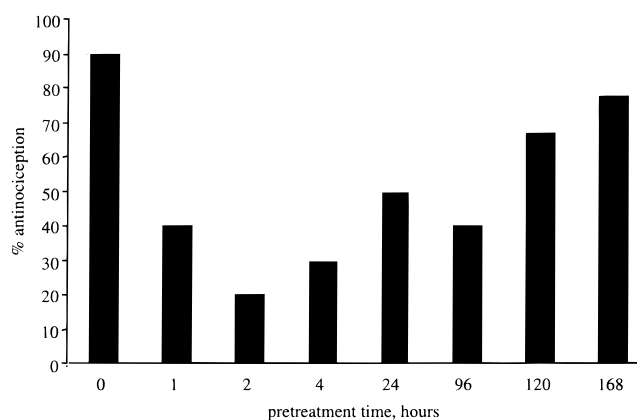

Figure 3. Antinociceptive effect of morphine (112 μmol/kg, sc) in mice pretreated with a single icv dose of **2** (1.2 nmol) at indicated time periods in the mouse tail-flick assay. At least 10 animals were used at each time period to determine the percentage of antinociception.

to the right (IC_{50} ratio = 191).[16] These data indicated that the observed antagonism by **2** was irreversible.

The in vivo activity of **2** was evaluated using the mouse tail-flick antinociceptive assay.[17] The data show that pretreatment of mice with **2** (1.2 nmol, icv) produced a time-dependent antagonism of morphine-induced antinociception (Figure 3). This antagonism was observed at 1 h and lasted at least 5 days after administration of **2** with a maximal effect occurring at 2 h. This potent and long-lasting antagonism is similar to that observed for β-FNA.[17]

While the aforementioned data indicate irreversible binding of **2** to opioid receptors, it did not reveal whether the covalent association between **2** and opioid receptors was due to the formation of a fluorescent isoindole adduct. This was accomplished with a Becton Dickinson Facs Vantage equipped with a multiwavelength UV laser for excitation using a band-pass filter of 530 $\pm$ 30 nm.[18] When CHO cells with stably expressed μ opioid receptors were incubated with **2** in HEPES buffer (pH

= 7.5), the fluorescence intensity rapidly (30 s) increased relative to the autofluorescence of untreated cells. Nonspecific staining was evaluated by incubation of **2** (1 μM) with nontransfected CHO cells. In this case, the fluorescent distribution of total and nonspecific staining overlapped (data not shown). To reduce the nonspecific staining, the reaction of **2** with the cell surface was quenched after 1 min incubation by adding a mixture of lysine and mercaptoethanol at relatively high concentrations (10 μM), followed by several washings. This permitted us to visualize the specific fluorescent staining of μ opioid receptors (Figure 4).

In conclusion, the fluorogenic OPTA derivative of β-naltrexamine (**2**) binds irreversibly and with high affinity to the cloned μ, δ, and κ opioid receptors. The in vitro and in vivo pharmacological experiments have provided additional evidence of covalent association of **2** with μ opioid receptors. The fluorescence generation that accompanied binding suggests that **2** has reacted with neighboring lysine and cysteine residues of the μ opioid receptor thereby generating an isoindole fluorophore via the pathway outlined in Figure 1. Given that the specific generation of fluorescence reports the covalent association with neighboring lysine and cysteine residues, reporter affinity labels offer an advantage over conventional electrophilic affinity labels (e.g., Michael acceptors, isothiocyanates, nitrogen mustards) that are not as chemically selective and are nonfluorogenic. Furthermore, the participation of two neighboring residues (lysine and cysteine), in principle, affords the opportunity to more accurately localize the interaction of the pharmacophore of **2** with the receptor. Site-directed mutagenesis experiments are currently in
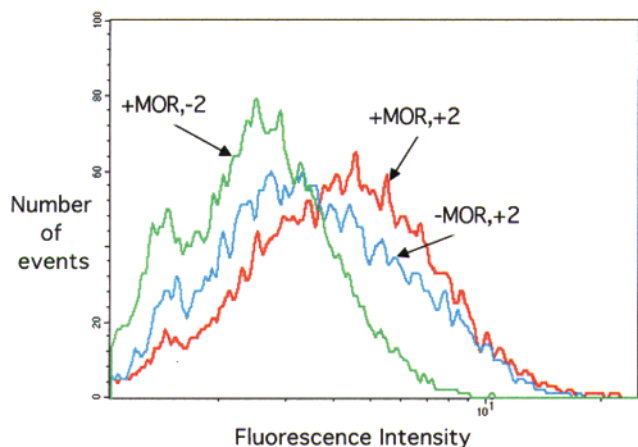
Figure 4. Representative flow cytometric analysis of fluorescent opioid labeling of CHO cells. Untransfected CHO cells (−MOR) or CHO cells transfected with μ opioid receptor (+MOR) were incubated with (+**2**) or without (−**2**) compound **2** (1 μM) at 25 °C for 1 min. The median fluorescence intensity values for each curve are as follows: green (2.44), blue (3.34), red (4.14).

progress to determine which neighboring amino acid residues (lysine and cysteine) are involved in the covalent association of **2** with opioid receptors. Such information should be useful in exploring the recognition of ligands by opioid receptors and will provide additional constraints for molecular modeling studies of opioid receptors.

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