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Communications to the Editor

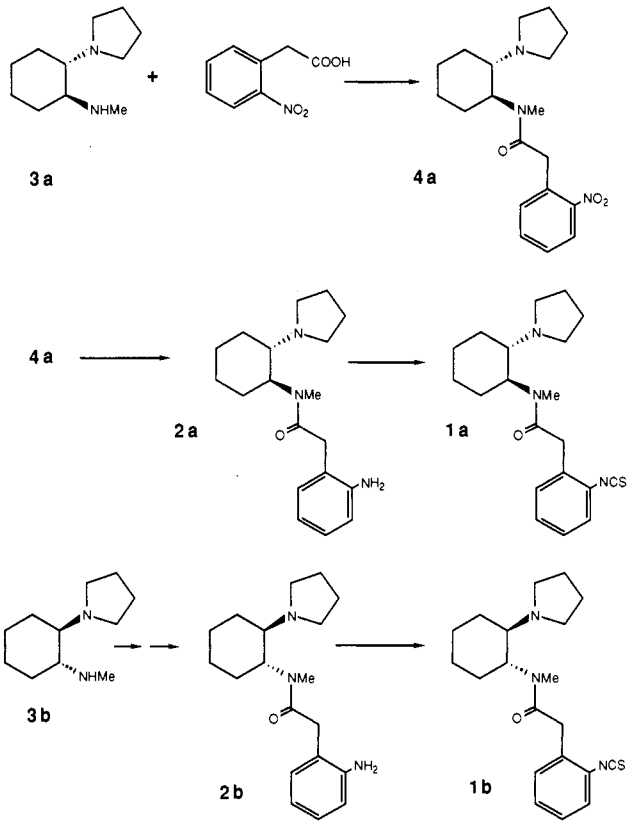
Selective and Enantiospecific Acylation of κ Opioid Receptors by (1*S*,2*S*)-*trans*-2-Isothiocyanato-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide. Demonstration of κ Receptor Heterogeneity

Sir:

Progress in many areas of medicinal chemistry and pharmacology depends on the availability of highly selective molecular probes. With ever increasing evidence that the actions of opioids are mediated through at least three major opioid receptor types, namely μ, δ, and κ,[1-3] it has become apparent that selective ligands are required to study these receptor types. Because irreversible ligands can bind covalently to a particular receptor type, these drugs make it possible to study the characteristics of a given receptor type either by selective depletion of the remaining types or direct action on one receptor type.[3-5] The highly selective μ receptor irreversible ligands BIT[6] and β-FNA[3,7] and δ receptor selective FIT[6] and SUPERFIT[8] have been described. Such ligands have made possible substantial advances in elucidation of the structure and function of individual receptors,[9,10] as in the case of [^{3}H]SUPERFIT, which we employed for purification to homogeneity of the δ opioid receptor from NG108-15 neuroblastoma × glioma hybrid cells.[11] Enantioselectivity of irreversible action is confirmatory evidence of receptor labeling as opposed to interaction with a nonspecific binding site.[12] Irreversible agents are also finding utility in mapping receptor distribution in living brain.[4]

To our knowledge, there are no published reports of an irreversible specific agent for the κ receptor, although there are reports of irreversible agents that will nonspecifically

Scheme I



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inhibit κ receptors.[3,13] To gain further insight into the biological role of κ receptors, we directed our attention toward developing a κ selective irreversible ligand. Our irreversible ligand is based on U50,488,[14] which is a member of a family of highly selective κ agonists that also includes U69,593[15] and PD117302.[16,17] We have recently shown, by single-crystal X-ray analysis, that the enantiomer of U50,488 that is most active at κ receptors has the 1*S*,2*S* absolute configuration.[18] This has subsequently been confirmed in an independent study.[17] On the basis of this observation, we synthesized (1*S*,2*S*)-*trans*-2-isothiocyanato-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]-benzeneacetamide (**1a**) (Scheme I). Enantiomerically pure **1a** was synthesized in order to reduce any nonspecific acylation that might be caused by the less potent enantiomer (**1b**).[12]

The ability of **1a** and **1b** to function as irreversible inhibitors of opioid binding sites was tested in vitro by incubating brain membranes with 1 μM of each compound for 60 min at 25 °C, followed by a washing procedure sufficient to remove unreacted, reversibly bound drug. As reported in Table I, incubation of guinea pig brain membranes with **1a** reduced the binding of the selective κ agonist [^{3}H]-U69,593 to 11.2% of control, while incubation with its enantiomer **1b** had no effect. Guinea pig brains were used since κ binding in the rat is too low too be useful.

At 1 μM the amine precursors of **1a** and **1b**, compounds **2a** and **2b**, inhibited [^{3}H]-U69,593 binding by 94% and 20%, respectively, in a reversible binding assay. However, the inhibition of [^{3}H]-U69,593 binding by **2a** and **2b** was not wash resistant. Thus, the protocol was sufficient to remove reversibly bound drugs (data not shown). As reported in Table I, **1a** had no effect on the binding of [^{3}H]DADLE (higher affinity (δ) and lower affinity (μ) binding sites)[19] or [^{3}H]FOXY (μ binding sites),[20] providing evidence for selective acylation of κ binding sites in vitro. Compound **1b**, which had no effect on [^{3}H]-U69,593 binding, inhibited [^{3}H]FOXY binding by 31%, illustrating the necessity for using enantiomerically pure acylating agents.

κ receptors were also assayed with [^{3}H]bremazocine in guinea pig membranes devoid of functional μ and δ receptors.[4] Neither **1a** nor **1b** irreversibly inhibited [^{3}H]-bremazocine binding, providing evidence that the κ binding sites labeled by [^{3}H]-U69,593 and [^{3}H]bremazocine are distinct.

Preincubation of membranes with varying concentrations of **1a** produced a dose-dependent decrease in the binding of a single concentration (2.4 nM) of [^{3}H]-U69,593 in a "reversible" binding assay, giving an apparent IC50 of 52 ± 7.5 nM (mean ± SEM, n = 3). Previous studies have shown that, using guinea pig brain membranes, [^{3}H]-U69,593 labels an apparent single class of binding sites.[15,21] Theoretically, inhibition of binding is attributable either to a reduction in the absolute number (B_{max}) of available binding sites or to decrease in affinity (increase in K_d) of receptors for their reversible ligands. To determine if **1a** altered the K_d or B_{max}, guinea pig brain membranes were pretreated with 100 nM of **1a** and binding surfaces generated and analyzed as described elsewhere.[4] The resulting data (Figure 1) were simultaneously fit to a one-site binding model for the best fit parameter estimates. The results indicated that treatment

Table I. Effects of Acylators 1a and 1b on κ, δ, and μ Binding[a]

[^{3}H]ligand	compd	specific binding, fmol/mg of protein	% of control
[^{3}H]-U69,593	control	15.1 ± 1.4	100.0
	1a	1.70 ± 0.38	11.2*
	1b	14.5 ± 0.98	95.9
[^{3}H]DADLE	control	56.2 ± 4.9	100.0
	1a	56.4 ± 1.6	100.0
	1b	49.9 ± 1.7	89.0*
[^{3}H]FOXY	control	35.2 ± 5.6	100.0
	1a	41.49 ± 0.89	119.0
	1b	24.4 ± 6.0	69.0**
[^{3}H]bremazocine	control	88.0 ± 3.6	100.0
	1a	86.0 ± 4.9	97.6
	1b	96.8 ± 5.0	110.0

[a] Frozen brain membranes were thawed, resuspended in 10 mM MOPS (3-morpholinopropanesulfonic acid) buffer, pH 7.4, containing 3 mM $MnCl_2$, and then incubated for 60 min at 25 °C in the presence or absence of 1 μM of various drugs. The incubation was terminated by centrifugation (11000*g* for 10 min), and membranes were washed three additional times by resuspension and centrifugation. The pellets were then resuspended in 50 mM Tris·HCl, pH 7.0, incubated for 60 min at 25 °C, and washed twice by centrifugation. The final pellets were resuspended in the appropriate buffer for assay. This washing protocol was sufficient to prevent any inhibition of [^{3}H]-U69,593 binding when the membranes were incubated with 1 μM of the amine precursors of **1a** and **1b**, **2a** and **2b**, respectively. (1) κ binding sites were measured with [^{3}H]-U69,593 (1.18 nM, sp act. = 40 C_i/mmol).[21] Incubations took place for 60 min at 37 °C (equilibrium) in 50 mM Tris-HCl, pH 7.4, containing 3 mM $MnCl_2$, 0.1 mg/mL bovine serum albumin, and several protease inhibitors: bacitracin (0.1 mg/mL), bestatin (0.01 mg/mL), leupeptin (0.004 mg/mL), chymostatin (0.002 mg/mL), and captopril (0.001 mg/mL). Incubations were terminated by rapid filtration over glass fiber filters presoaked in 1% polyethylenimine. The nonspecific binding was determined by incubations in the presence of 1 μM U69,593. (2) μ binding sites were measured with [^{3}H]FOXY (1.16 nM, sp act. = 53 C_i/mmol) as previously described.[20] (3) [^{3}H]DADLE (1.34 nM, sp act. = 46.9 Ci/mmol) binding sites (higher and lower affinity binding sites) were measured by using previously described methods.[19] With this ligand, 76% of the cpm were to the higher affinity (δ) site while 24% of the cpm were due to the μ binding site. (4) κ binding sites were also measured with [^{3}H]bremazocine (1.31 nM, sp act. = 21.3 Ci/mmol) with the following modifications of published procedures:[4] incubations were in the absence of NaCl, and the same protease inhibitors used in the [^{3}H]-U69,593 binding assay were included in the assay. Guinea pig membranes used for the [^{3}H]-U69,593 and [^{3}H]bremazocine binding assays were pretreated with the site-directed acylating agents 2-(*p*-ethoxybenzyl)-1-[(diethylamino)ethyl]-5-isothiocyanatobenzimidazole hydrochloride (BIT) and *N*-phenyl-*N*-[1-[2-(*p*-isothiocyanatophenyl)ethyl]-4-piperidinyl]propanamide hydrochloride (FIT), with minor modifications of published methods.[26] BIT and FIT completely eliminate μ and δ binding sites, respectively.[4] [^{3}H]DADLE and [^{3}H]-FOXY binding assays used frozen, lysed P2 membranes prepared from rat brain as described elsewhere.[21,26] Each value is the mean ± SD (fmol/mg of protein) of three independent experiments. (*) $p < 0.001$ when compared to control. (**) $p < 0.05$ when compared to control.

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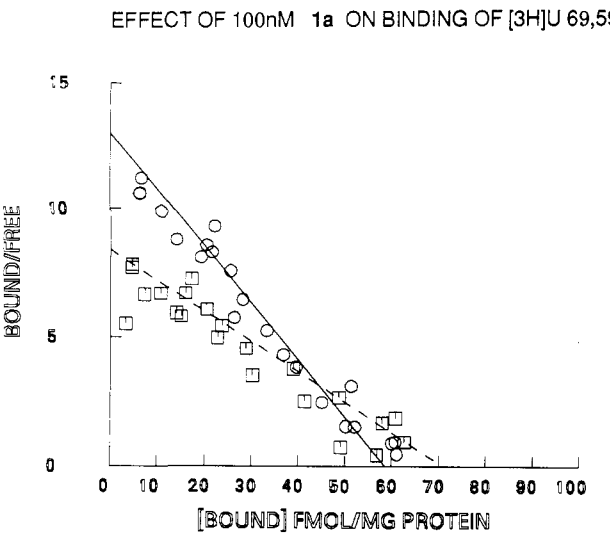

Figure 1. Guinea pig membranes were incubated in the absence (three membrane preparations) and presence (three membrane preparations) of 100 nM **1a** as described in the footnote to Table I. [^{3}H]-U69,593 binding surfaces were generated by displacing two concentrations of [^{3}H]-U69,593 (0.6 and 2.4 nM) by eight concentrations each of U69,593 between 0.5 and 128 nM, yielding 18 data points/membrane preparation. The data of the control (54 data points) and treated (54 data points) were simultaneously fit to a one-site binding model for the best fit parameter estimates as described elsewhere.[4,27] The best fit parameter estimates for the control membrane preparations were K_d = 4.47 ± 0.20 nM and B_{max} = 58.5 ± 4.4 (mean ± SD, n = 3). The best fit parameter estimates for membranes treated with **1a** were K_d = 8.46 ± 0.41 and B_{max} = 71 ± 9.2 fmol/mg of protein. The B_{max} values were not significantly different (Student's t test). Each of these values is the mean of three determinations, which differed by less than 10% (open circles, control; open squares, treated with **1a**). The solid (control) and dashed (treated with **1a**) lines in the figure were generated by the best fit parameter estimates.

with 100 nM **1a** increased the K_d from 4.47 ± 0.21 nM to 8.45 ± 0.41 nM without a significant alteration in the B_{max}. The data (Figure 1) show that **1a** produces a graded increase in the K_d of [^{3}H]-U69,593 for the κ binding site. We have obtained similar results in vivo using the irreversible μ antagonist β-FNA: it increases the K_d of μ binding sites without altering the B_{max}.[22] Analogous results have been observed in vitro with DIGIT, an isothiocyanate derivative of 1,3-di-*o*-tolylguanidine (DTG). The use of DIGIT resulted in a reduction in affinity (K_d) of σ ligands for the σ site with no apparent change in the number of binding sites (B_{max}).[23] Determining the exact site acylated by **1a** must await purification and structural elucidation of the purified receptor. Additional experiments (data not shown) using 300 nM **1a** demonstrated larger graded increases in the K_d without significant change in the B_{max}, thus confirming the result observed at 100 nM.

In light of these results, we conclude that, at 1 μM, **1a** selectively and enantiospecifically acylates a population of κ binding sites labeled by [^{3}H]-U69,593 in vitro in guinea pig membranes (depleted of μ and δ receptors by pretreatment with BIT and FIT[4]). Furthermore, the selective action of **1a** on inhibiting binding of [^{3}H]-U69,593 compared with [^{3}H]bremazocine provides independent evidence for heterogeneity of κ binding sites.[24] We have also noted that U69,593 and U50,488 are weak displacers of [^{3}H]bremazocine (data not shown).

The starting material for synthesis of **1a** was enantiomerically pure (1*S*,2*S*)-(+)-*trans*-2-pyrrolidinyl-*N*-methylcyclohexylamine (**3a**) described previously (Scheme I).[18] DCC coupling of **3a** with 2-nitrophenylacetic acid afforded (1*S*,2*S*)-*trans*-2-nitro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide (**4a**), mp 236–237 °C, in 93% recrystallized (IPA/Et_2O) yield as its HBr salt. Catalytic hydrogenation of **4a**·HBr over 10% Pd/C at 50 psi gave after recrystallization (IPA) a 97% yield of **2a**·HBr as its 2-propanol solvate, mp 127–129 °C. Treatment of a solution of **2a**·HBr in a 1:1 mixture of saturated $NaHCO_3$/$CHCl_3$ with 1.1 equiv of freshly redistilled thiophosgene gave a 58% yield of **1a** isolated as its HCl salt, mp 190–192 °C.

The 1*R*,2*R* enantiomer **1b** was synthesized by using an identical procedure to that noted for **1a**, except that enantiomerically pure (1*R*,2*R*)-(−)-*trans*-2-pyrrolidinyl-*N*-methlycyclohexylamine (**3b**) was used as the starting material.[25]

The results of our work with **1a** indicate that it is the first site-directed acylating agent to specifically and irreversibly bind to the κ opioid receptor subtype in guinea pig membranes. Further studies with **1a** are in progress to investigate its irreversible acylation at the κ receptor.

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(25) All compounds in the 1*R*,2*R* series were identical by melting point, IR, ^{1}H NMR, mass spectrometry, and TLC to their enantiomers in the 1*S*,2*S* series. The only difference was in the sign of optical rotation. All compounds gave combustion analyses within ±0.4% of the calculated values, and the IR, ^{1}H NMR, and mass spectra were consistent with their assigned structure.

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