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Synthesis of an affinity ligand ('UPHIT') for in vivo acylation of the κ-opioid receptor

Brian R. de Costa, Linda Band°, Richard B. Rothman°, Arthur E. Jacobson, Victor Bykov°, Agu Pert+ and Kenner C. Rice*

Laboratory of Medicinal Chemistry, National Institute of Digestive, Diabetes, and Kidney Diseases, National Institutes of Health, °Laboratory of Clinical Science and *+ Biological Psychiatry Branch, National Institute of Mental Health, Bethesda, MD 20892, USA*

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The isothiocyanate analog (1*S*,2*S*-*trans*-2-isothiocyanato-4,5-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide, **3a**) of the highly selective κ-opioid receptor agonist, U50,488, was prepared as a potential site-directed affinity ligand for acylation of κ-opioid receptors in vivo. The isothiocyanate (**3a**) which we have designated UPHIT and its enantiomer (**3b**) were synthesized in 3 steps starting from optically pure (1*S*,2*S*)-(+)-*trans*-2-pyrrolidinyl-*N*-methylcyclohexylamine (**4a**) and its enantiomer (**4b**), respectively, thus defining their absolute stereochemistry. Binding in vitro of the 1*S*,2*S* enantiomer **3a** to κ receptors labelled by [^{3}H]U69,593 was shown to occur with an IC_{50} value of 25.92 ± 0.36 nM, whereas 827.42 ± 5.88 and 115.10 ± 1.23 nM were obtained for the IC_{50} value of the 1*R*,2*R* enantiomer (**3b**) and (±)-**3** respectively. Intracerebroventricular (ICV) injection of 100 μg of (±)-**3** into guinea-pig brain followed by analysis of remaining κ-binding sites 24 h later revealed that (±)-**3** depleted 98% of the κ receptors that bind [^{3}H]U69,593 and 40% of those that bind [^{3}H]bremazocine. These preliminary data suggest exciting uses for these compounds in furthering our knowledge of the κ-opioid receptor.

Opioid agonist, κ-; Opioid receptor agonist, U50,488; Site-directed affinity ligand; Receptor, κ-; Intracerebroventricular administration; Isothiocyanate analog

1. INTRODUCTION

Site-directed electrophilic affinity ligands have been utilized with much success as tools for characterization of opioid receptors [1]. Efforts in our laboratory led to the synthesis and use of the isothiocyanate-derived irreversible ligands BIT [2], FIT [2] and SUPERFIT [3] for the characterization of μ- and δ-opioid receptors. Electrophilic affinity ligands such as chlornaltrexamine (CNA, a nitrogen mustard analog of naltrexone) have been used as irreversible ligands in vivo [4]. In that study [4], intracerebroventricular (ICV) injection of CNA into mice resulted in long-lasting narcotic antagonist effects as a result of non-selective depletion of opioid receptors. In contrast, ICV injection of the less reactive β-funaltrexamine (β-FNA) [5] into rats resulted in a selective wash-resistant inhibition of μ-receptor binding [6]. Compounds which selectively deplete opioid receptor subtypes in vivo can allow one to perform direct observation of the physiological effects associated with these receptor types [1].

We recently reported the synthesis of 1*S*,2*S*-*trans*-2-isothiocyanato-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (**1**, fig.1) [7], an electrophilic ligand for the κ-opioid receptor in vitro. Although this compound could irreversibly inhibit κ receptors with an IC_{50} value of 100 nM, it failed to function as an irreversible inhibitor of κ-opioid receptors after intracerebroventricular

Correspondence address: K.C. Rice, Building 8, Room B1-23, Laboratory of Medicinal Chemistry, NIDDK, NIH, Bethesda, MD 20892, USA

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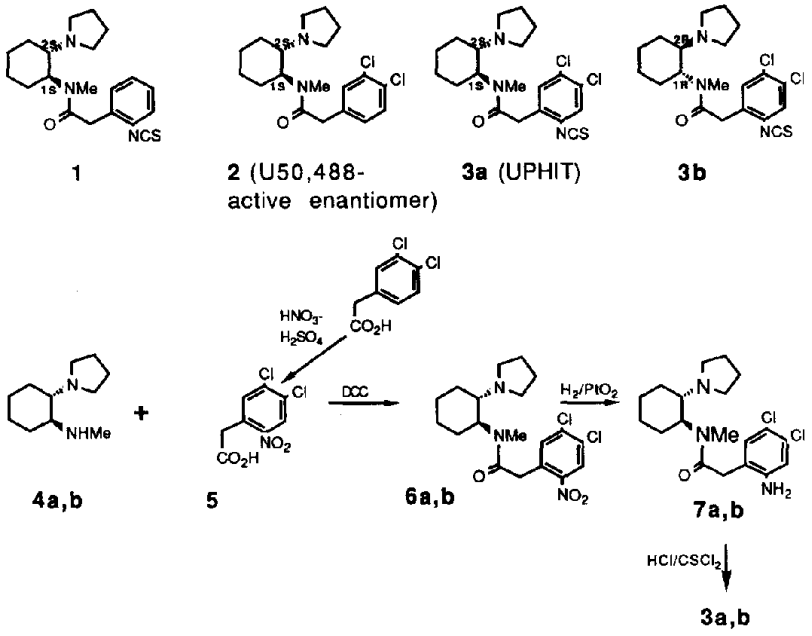

Fig.1. Structures of compounds and reaction sequence: (a series) 1*S*,2*S*; (b series) 1*R*,2*R*.

(ICV) injection [8]. A number of factors, including the lower affinity of **1** for the κ-opioid receptor compared with the parent molecule (U50,488) (**2**) (fig.1), could account for the inactivity of this compound in vivo. A good site-directed affinity ligand should exhibit high affinity, specificity and reactivity towards the receptor with minimal reactivity towards water [1]. In our hands, the isothiocyanate function has served this purpose well in a number of studies [2,3,7,9].

Since the aromatic chlorine atoms on **2** have been identified as being important for high affinity and selectivity at the κ-opioid receptor [10,11], we felt that **3a** (an analog of **1** with the aromatic chlorine substituted as in **2**) would have improved specificity for the κ-opioid receptor compared with the dechloro compound, **1**, and thus have a better chance of functioning in vivo as a κ receptor acylator. Since it has been well established that drug enantiomers can exert different, and in some cases opposite effects [12], it was imperative to synthesize both enantiomers of (±)-**3**. We report here the synthesis and characterization of an electrophilic ligand, 1*S*,2*S*-*trans*-2-isothiocyanato-4,5-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (**3a**) as well as its enantiomer **3b** and racemate (±)-**3**. The 1*S*,2*S* enantiomer **3a** bears the same stereochemical relation to the active enantiomer of U50,488 (**2**). The optical purity of **3a** and **3b** follows from the previously demonstrated [13] optical purity of the precursors **4a** and **4b**.

2. MATERIALS AND METHODS

2.1. *Chemical synthesis*

The synthesis of (±)-**3**, **3a** and **3b** is shown in fig.1. (1*S*,2*S*)-(+)-*trans*-2-Pyrrolidinyl-*N*-methylcyclohexylamine (**4a**) and its enantiomer (**4b**) (fig.1) were synthesized as described [13]. Spectra from NMR (Varian XL300 spectrometer), infrared (Beckmann 3001 instrument), and chemical ionization (CI) mass spectroscopy (Finnegan Mat-311 spectrometer) were in accord with the assigned structures. Gas chromatographic analysis (GC) was performed on a Hewlett-Packard 5880A instrument with a carbowax capillary column and a flame ionization detector. Melting points were obtained using a Thomas-Hoover Unimelt apparatus, and are uncorrected. Specific rotation values were recorded at the sodium-D line using a Perkin-Elmer 241 MC polarimeter. Thin-layer chromatographic analysis (TLC) was performed on Analtech silica gel (GHF) plates, elution being carried out with a solvent system consisting of chloroform/methanol/conc. ammonium hydroxide (90:9:1). All new compounds gave combustion analyses for carbon, hydrogen and nitrogen within ± 0.4% of

the calculated value and were performed at Atlanta Microlabs (Atlanta, GA).

2.2. *2-Nitro-4,5-dichlorophenylacetic acid (5)*

To a stirred solution (at 0°C) of 3,4-dichlorophenylacetic acid (100 g, 0.488 mol) in a mixture of 200 ml fuming sulfuric acid (11% SO_3) and 300 ml acetic acid was added dropwise during a 10 min period, nitric acid [(90%; 1.5 equiv.) caution!], and the reaction mixture stirred at room temperature for a further 30 min at 20°C. The mixture was poured into ice-water (2000 ml), and the crystalline precipitate filtered and washed with 1000 ml distilled water. Recrystallization from 300 ml aqueous ethanol (1:1) afforded 66.1 g (54%) of **5** (m.p. 130–132°C).

2.3. *(±)-trans-2-Nitro-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide [(±)-6]*

To a stirred solution of (±)-**4** (10.00 g, 54.8 mmol), **5** (14.89 g, 82.3 mmol) and dry pyridine (2.17 g, 27.4 mmol) in 200 ml anhydrous methylene dichloride was added (in one portion), *N,N*-dicyclohexylcarbodiimide (22.63 g, 109.6 mmol) and the solution stirred for 10 min at 20°C or until TLC indicated that the reaction was complete. The precipitated *N,N*-dicyclohexylurea was filtered and the solvent evaporated from the filtrate. The residue was partitioned between 500 ml of 10% aqueous citric acid and ether (500 ml). The ether layer was discarded and the aqueous layer was washed with a further 2 × 500 ml of ether, being then made alkaline by addition of excess aqueous concentrated ammonia solution. The alkaline solution was extracted with methylene dichloride (3 × 200 ml) and the organic extract dried by filtering through Na_2SO_4, the solvent being evaporated in vacuo. Treatment of crude **6** with a solution of HCl gas in 2-propanol and crystallization of the salt from 2-propanol (200 ml) afforded (±)-**6**·HCl [23.2 g, 94%; m.p. 259–260°C (dec.)].

2.4. *1S,2S-trans-2-Nitro-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (6a)*

10.00 g (54.8 mmol) of **4a** [13] was coupled with **5** as described above and the HCl salt crystallized from 500 ml EtOAc to give **6a**·HCl [(23.8 g, 96%), m.p. 239–240°C (dec.), $[\alpha]_D$ − 14° (*c* 0.74, MeOH)].

2.5. *1R,2R-trans-2-Nitro-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (6b)*

10.00 g (54.8 mmol) of **4b** [13] was coupled with **5** as described above and the HCl salt recrystallized from 500 ml EtOAc to give **6b**·HCl [(22.8 g, 92%), m.p. 239–240°C (dec.), $[\alpha]_D$ 12.7° (*c* 0.52, MeOH)].

2.6. *(±)-trans-2-Amino-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide [(±)-7]*

A solution of (±)-**6**·HCl (16.00 g, 35.5 mmol) in a mixture of 95% ethanol (340 ml) and distilled water (110 ml) containing 1.6 g PtO_2 was stirred under an atmosphere of hydrogen for 35 min at room temperature and atmospheric pressure. The catalyst was filtered through a pad of celite, and after evaporation of the solvent in vacuo, the product was crystallized from 500 ml of hot 2-propanol to afford (±)-**7**·HCl [(9.60 g, 64%), m.p. 251–252°C (dec.)].

2.7. *1S,2S-trans-2-Amino-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (7a)*

A solution of **6a**·HCl (15.0 g, 33.3 mmol) in methanol (100 ml) containing PtO_2 (1.5 g) was stirred under an atmosphere of hydrogen for 60 min at room temperature and atmospheric pressure. The catalyst was filtered through a pad of celite and evaporation of the solvent left the product which was crystallized from 300 ml ethyl acetate to yield **7a**·HCl [(10.8 g, 77%), m.p. 185–188°C, $[\alpha]_D$ −25.6° (*c* 0.40, MeOH)].

2.8. *1R,2R-trans-2-Amino-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (7b)*

6b·HCl (20.0 g, 44.4 mmol) was hydrogenated as described for **7a** (section 2.7) and recrystallized from ethyl acetate to afford **7b**·HCl [(12.33 g, 66%), m.p. 185–189°C, $[\alpha]_D$ 23.1° (*c* 0.55, MeOH)].

2.9. *(±)-trans-2-Isothiocyanato-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide [(±)-3]*

(±)-**7**·HCl (0.40 g, 0.95 mmol) was suspended in 100 ml dry chloroform and the solution acidified by addition of an excess of HCl gas dissolved in ethyl acetate (to diprotonate (±)-**7**). The solution was brought to reflux and a solution of 145 μl (1.90 mmol) of freshly redistilled thiophosgene ($CSCl_2$) in 5 ml dry chloroform was added in one portion. Refluxing was continued for a further 20 min when TLC indicated that all of the starting material had reacted. The solvent and excess $CSCl_2$ were evaporated in vacuo to give a white crystalline residue (0.38 g, 86%). Crystallization of the HCl salt from 10 ml 2-propanol afforded (±)-**3**·HCl [m.p. 230–231°C (dec.)].

2.10. *1S,2S-trans-2-Isothiocyanato-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (3a)*

7a·HCl (0.40 g, 0.95 mmol) was reacted with $CSCl_2$ as described in section 2.9 for (±)-**7**·HCl to afford **3a**·HCl. This crystallized from 20 ml 2-propanol/ether, yielding 0.31 g (70%) of **3a**·HCl [(m.p. 198–200°C (dec.), $[\alpha]_D$ −48.3° (*c* 0.31, MeOH)].

2.11. *1R,2R-trans-2-Isothiocyanato-4,5-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (3b)*

7b·HCl (0.40 g, 0.95 mmol) was reacted with $CSCl_2$ as described above and crystallized from 20 ml 2-propanol/ether to afford 0.27 g (61%) of **3b**·HCl [m.p. 196–200°C (dec.), $[\alpha]_D$ 50.2° (*c* 0.41, MeOH)].

2.12. *Binding assays and in vivo injection procedure*

In vitro κ receptor binding assays were performed as in [15]. In vivo κ receptor binding assays were carried out as follows: male Hartley guinea pigs weighing 300 g were anesthetized with halothane. Following placement in a stereotaxic apparatus, an incision was made through the neck muscles, exposing the occipital membrane. A 10 μl Hamilton syringe used to deliver agents was inserted through the membrane into the cisterna magnum and 100 μg of various drugs, each dissolved in 10 μl dimethyl sulfoxide (DMSO) were injected. 24 h after injection, animals were decapitated and brain tissue immediately frozen in isopentane at −80°C. The frozen tissue was later prepared for binding assay as described above [15]. All data were the results of duplicate experiments, each performed in triplicate.

3. RESULTS

Radioligand binding experiments with (±)-**3**, **3a** and **3b** indicate that they are potent displacers of [^{3}H]U69,593 and weak displacers of [^{3}H]bremazocine (table 1), thus indicating κ receptor subtype selectivity as has been observed previously for **1** [7]. Compound **3a** displaced [^{3}H]U69,593 with an IC_{50} value of 25.92 ± 0.36 nM, whereas **3b** exhibited an IC_{50} value of 827.42 ± 5.88 nM. These results are consistent with the enantioselectivity observed in vitro for the enantiomers of **2** [13]. In contrast to what was anticipated, the affinity of **3a** was only marginally improved as compared with **1** (table 1).

Examination of (±)-**3** in vivo (ICV) showed that it had the capacity to deplete completely κ-opioid receptors labelled by the κ-opioid receptor ligand [^{3}H]U69,593 [14] (table 2) while having no effect on μ and δ receptors [14]. Thus, ICV injection of 100 μg (±)-**3** into guinea pigs resulted in 98% depletion of κ receptors labelled by [^{3}H]U69,593, and 40% in the case of [^{3}H]bremazocine; injection of **2** under the same conditions failed to affect any of the above receptor systems (not shown). The results for enantiomers **3a** and **3b** are included in a more extensive examination of the in vivo acylation of the κ-opioid receptor by these and related compounds [14].

Table 1

Displacement of κ-opioid receptor radioligands by (±)-**3**, **3a** and **3b**

Compound	IC_{50} (nM)	
	[^{3}H]U69,593	[^{3}H]Bremazocine
1	33.70 ± 0.82	27000 ± 2601
(±)-**3**	115.10 ± 1.23	10338 ± 1159
3a	25.92 ± 0.36	5248 ± 398
3b	827.42 ± 5.88	82624 ± 4866

Table 2

Effect of 100 μg intracerebroventricular injection of (±)-**3** on [^{3}H]U69,593 and [^{3}H]bremazocine binding in guinea pig brain

Compound	Specific binding (fmol/mg protein)	% of control
Control	22.87 ± 1.23	100
[^{3}H]U69,593	0.32 ± 0.30	1.30 ± 1.30
Control	246.30 ± 27.10	100
[^{3}H]Bremazocine	148.10 ± 11.90	54.90 ± 4.43

4. DISCUSSION

Synthesis of **3a**, **3b** and (±)-**3** was successfully accomplished in three steps. It was hoped that **3a** and (±)-**3** would exhibit improved potency as acylators as compared to the previously reported 1*S*,2*S*-*trans*-2-isothiocyanato-*N*-methyl-*N*-[2-(1-pyrrolidinyl)cyclohexyl]benzeneacetamide (**1**, fig.1). The rationale for these compounds was a combination of the high-affinity κ selective agonist U50,488 (**2**) and the lower affinity in vitro κ receptor irreversible agent (**1**). In a previous study [15], **2** displaced [^{3}H]U69,593 with a K_i of 0.89 nM while its enantiomer exhibited a K_i of 299 nM. **3a** showed only slightly improved affinity for the κ-opioid receptor (IC_{50} = 25.92 ± 0.36 nM vs the value for **1** of 33.70 ± 0.82 nM [4]; table 1). In a previous study [8], evaluation of **1** in vivo as a κ selective irreversible agent indicated that it was inactive. However, evaluation of the isothiocyanate derivative (±)-**3** indicated that it was a potent and selective κ receptor acylator in vivo. Increased reactivity of the isothiocyanate function of (±)-**3** towards nucleophiles as compared with **1** could be one factor accounting for the difference between **1** and **2** in vivo. The conclusion drawn here is that the isothiocyanate derivative (±)-**3** is a valuable complement to **1** as a selective agent for in vivo inhibition of κ-opioid receptors.

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