



## Opioid activity of C8813, a novel and potent opioid analgesic

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### Abstract

Compound *trans*-4-(*p*-bromophenyl)-4-(dimethylamino)-1-(2-thiophen-2-yl-ethyl)-cyclohexanol (C8813), structurally unrelated to morphine, is a novel analgesic. The present study examined the antinociception, opioid receptor selectivity and *in vitro* activity of C8813. The antinociceptive activity was evaluated using mouse hot plate and acetic acid writhing tests. In mouse hot plate test, the antinociceptive ED<sub>50</sub> of C8813 was 11.5 µg/kg, being 591 times and 3.4 times more potent than morphine and fentanyl respectively. In mouse writhing test, the antinociceptive ED<sub>50</sub> of C8813 was 16.9 µg/kg, being 55 times and 2.3 times more active than morphine and fentanyl respectively. In the opioid receptor binding assay, C8813 showed high affinity for µ-opioid receptor (K<sub>i</sub> = 1.37 nM) and δ-opioid receptor (K<sub>i</sub> = 3.24 nM) but almost no affinity for κ-opioid receptor (at 1 µM). In the bioassay, the inhibitory effect of C8813 in the guinea-pig ileum (GPI) was 16.5 times more potent than in the mouse *vas deferens* (MVD). The inhibitory effects of C8813 in the GPI and MVD could be antagonized by µ-opioid receptor antagonist naloxone and δ-opioid receptor antagonist ICI174,864 respectively. However, the inhibitory effect of C8813 in the rabbit *vas deferens* was very weak. These results indicated that C8813 was a potent analgesic and a high affinity agonist for the µ- and δ-opioid receptors.

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**Keywords:** C8813; Antinociception; Opioid receptor; Binding assay; Bioassay

### Introduction

Opium-derived alkaloids, in particular morphine, are the standard strong analgesics for the treatment of acute and chronic severe pain. But the adverse reactions limit their prolonged use.

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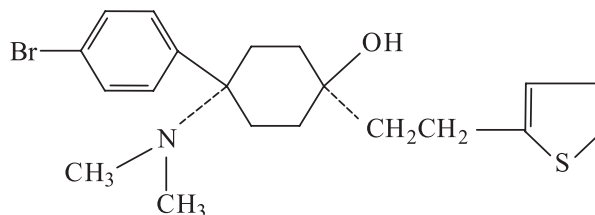


Fig. 1. Chemical structure of compound C8813.

The development of new analgesics with high potency but reduced side effects is still one of challenges of opioid research. To search more effective analgesic agents, our department had synthesized a series of novel 3-methylfentanyl derivatives and found that ohmefentanyl was an extremely potent representative in 3-methylfentanyl derivatives with lower dependence liability (Jin et al., 1981, 1996; Huang et al., 1984; Zhou et al., 1986; Zhao et al., 1991). Subsequently, we synthesized 4-arylcylohexanol derivatives and found that *trans*-4-(*p*-bromophenyl)-4-(dimethylamino)-1-(2-thiophen-2-yl-ethyl)-cyclohexanol (C8813) (Fig. 1) showed potent antinociceptive activity. In the present study, the antinociception, opioid receptor selectivity and in vitro activity of compound C8813 were evaluated in detail.

## Materials and methods

### Animals

Male or female Kunming strain mice (18–22 g) and guinea pigs (300–350 g) were supplied by the Shanghai Experimental Animal Center, Chinese Academy of Sciences. All animals were kept on a 12/12 h light-dark cycle in temperature and humidity controlled rooms. The animals were fed with standard laboratory food and water ad libitum. All experiments were in accordance with the recommendation in the Guide for the Care and Use of Laboratory Animals published by the National Institute of Health, USA.

### Drugs

Compound C8813 and fentanyl were synthesized in the Chemical Group, 2nd Department of Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Morphine hydrochloride was purchased from Qinghai Pharmaceutical Factory, China. Naloxone hydrochloride was obtained from Medical Center of Fudan University, Shanghai, China. [*D*-Ala<sup>2</sup>, *D*-Leu<sup>5</sup>]enkephalin (DADLE) was provided by Peninsula Laboratories (USA). *Trans*-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (U50488H) and normorphine (Upjohn Co.) were devoted by Prof. Kosterlitz (University of Aberdeen, UK). ICI174,864 was purchased from RBI, USA. [<sup>3</sup>H]ohmefentanyl (50 Ci/mmol) was labeled by Shanghai Institute of Nuclear Research, Chinese Academy of Sciences. [<sup>3</sup>H][*D*-Pen<sup>2</sup>, *D*-Pen<sup>5</sup>]enkephalin ([<sup>3</sup>H]DPDPE, 36 Ci/mmol) and [<sup>3</sup>H][5 $\alpha$ , 7 $\alpha$ , 8 $\beta$ ]-(+)-*N*-methyl-*N*-(7-[1-pyrrolidinyl]-1-oxaspiro[4,5]dec-8-yl)-benzeneacetamide ([<sup>3</sup>H]U69593, 39.7 Ci/mmol) were purchased from Dupont and NEN (New England Nuclear Co.), respectively.

### *Antinociceptive assay*

Antinociceptive activity was examined using the mouse hot plate assay (Chao and Tsoh, 1956) and the mouse writhing assay (Koster et al., 1959). In the mouse hot plate assay, female mice were placed on a zinc plate heated to  $55 \pm 1$  °C. The latency time of licking hind-paw was recorded as nociceptive response. Each mouse was tested twice before drug administration and the latencies were averaged to obtain the baseline latency. Only mice with baseline latencies between 10–20 s were chosen for further studies. The efficiency of antinociceptive activity was defined as doubling of the baseline latency (Huang et al., 1984). The antinociceptive activity of drugs was measured 5 min after intraperitoneally injection. In the writhing assay, the male mice were received 0.2 ml of 1% acetic acid intraperitoneally and they were observed for 15 min for writhing. Different doses of C8813, morphine or fentanyl were subcutaneously administered before acetic acid administration. The absence of writhing response was scored as antinociception. Ten animals were used at each dose group.

### *Membrane preparation*

Homogenate of male mouse brain was prepared as previously described (Jin et al., 1996). The mice were decapitated and the brains were rapidly removed. Brains without cerebellum were homogenized in 0.32 M ice-cold sucrose. The homogenates were centrifuged at  $1000 \times g$  for 10 min. The supernatants were centrifuged at  $40,000 \times g$  for 30min. The pellets were suspended in ice-cold 50 mM Tris-HCl buffer (0 °C, pH 7.4) and centrifuged at  $40,000 \times g$  for 30 min. The pellets were resuspended in ice-cold Tris-HCl buffer and recentrifuged at  $40,000 \times g$  for 30 min. The pellets were homogenized in ice-cold Tris-HCl buffer. The protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

### *Receptor binding assay*

The binding for  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors was determined with the highly selective  $\mu$ -opioid receptor ligand [ $^3\text{H}$ ]ohmefentanyl (Jin et al., 1981; Xu et al., 1985), the highly selective  $\delta$ -opioid receptor ligand [ $^3\text{H}$ ]DPDPE and  $\kappa$ -opioid receptor ligand [ $^3\text{H}$ ]U69593, respectively. Nonspecific binding was measured by incubation in the presence of 10  $\mu\text{M}$  naloxone. The binding assays were conducted at 30 °C in 50 mM Tris-HCl buffer (pH 7.4). Aliquots (containing about 2 mg protein in the final volume of 1 ml) of crude membrane preparation were incubated with tested drugs and tritiated ligand at 30 °C for 40 min. After the incubation, the mixture was immediately cooled in an ice bath and then filtered rapidly through Whatman GF/B glass fiber filter in Millipore harvester. The filters were washed 3 times with 4 ml aliquots of ice-cold 50 mM Tris-HCl buffer, dried and transferred to counting vials. Four ml hydrophilic scintillation cocktail was added. The radioactivity was counted with Beckman LS6500 liquid scintillation analyzer. The  $\text{IC}_{50}$  value of tested drugs, defined as the concentration produced a 50% inhibition of the specific binding of radioligand, was estimated by linear regression from concentration-probit semi-logarithmic plot. The  $K_i$  (the equilibrium dissociation constant of inhibitor) values were calculated from the formula:  $K_i = \text{IC}_{50}/[1 + (\text{L})/K_d]$ , where L is the concentration of labeled ligand and  $K_d$  is the dissociation constant of labeled ligand (Cheng and Prusoff, 1973).

All experiments were performed three times, each in triplicate, and the values represented the means of three determinations. The error of the triplicate determinations was less than 10%.

### *In vitro bioassays*

The myenteric plexus-longitudinal muscle from the guinea pig ileum (GPI) (Kosterlitz and Waterfield, 1975), mouse vas deferens (MVD) (Hughes et al., 1975) and rabbit vas deferens (LVD) (Oka et al., 1981) were prepared as described. The preparations were suspended in an organ bath containing 4 ml Krebs solution. The bath fluid was kept at  $37 \pm 1$  °C and gassed continuously with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. The resting tension was maintained at 250 mg for the MVD and 500 mg for the GPI and LVD. The composition of Krebs solution was (in mM): NaCl 119, KCl 4.7, CaCl<sub>2</sub> 2.55, KH<sub>2</sub>PO<sub>4</sub> 1.6, MgSO<sub>4</sub> 1.18, NaHCO<sub>3</sub> 25, glucose 11 and mepyamine maleate 0.00013. Hexamethonium bromide 70 µM and choline chloride 20 µM were added to the Krebs solution for the GPI. MgSO<sub>4</sub> was not included in the Krebs solution for the MVD. After equilibration for 45 min, longitudinal contractions were evoked by field stimulation through Pt-electrodes at the upper and lower ends of the bath. The parameters of stimulation were as follows. For the GPI and LVD, single pulses of 1 ms were used (50 V, 1.0 ms duration, 15 s interval). For the MVD, the trains were consisted of 4 pulses at intervals of 200 ms (40 V, 1.0 ms duration, 15 s interval). The contractions were recorded with a force displacement transducer and an autoequilibrium recorder. The agonist potencies of the compounds were obtained from dose-response curves by calculating the concentration of the compounds that reduced the height of the contractions by 50% (IC<sub>50</sub>).

The antagonist equilibrium constant, Ke values were determined by the single dose method of Kosterlitz and Watt (1968).  $Ke = a/(DR - 1)$ , where a is the molar concentration of antagonist and DR is the dose-ratio.  $DR = M_3/(M_2 - M_1)$ , where M<sub>1</sub> is the concentration of test compound which would be expected to have the same depressant effect produced by antagonist on the twitch in the absence of the antagonist. M<sub>2</sub> is the concentration of test compound which would have a depressant effect equal to the combined actions of the antagonist and the test compound (in higher concentration, M<sub>3</sub>) in the absence of the antagonist.

### *Data analysis*

The antinociceptive ED<sub>50</sub> values and 95% confidence limits were calculated by the method of Bliss (1938). The receptor binding data were analyzed by the EBDA/LIGAND compute program. Data were expressed as mean  $\pm$  S.E.M.

## **Results**

### *Antinociceptive activity*

Compound C8813 showed potent antinociceptive activity (Table 1). The antinociceptive ED<sub>50</sub> of C8813 in the mouse hot plate was 11.5 µg/kg (i.p.), being 591 times and 3.4 times more active than morphine and fentanyl respectively. In the mouse writhing test, the antinociceptive ED<sub>50</sub> of C8813 was 16.9 µg/kg (s.c.), being 55 times and 2.3 times more active than morphine and fentanyl respectively.

Table 1

Antinociceptive ED<sub>50</sub> (μg/kg) of compound C8813, morphine and fentanyl in mouse hot plate and acetic acid writhing tests

Drug	Antinociceptive ED <sub>50</sub> (95% CL, μg/kg) <sup>a</sup>	
	Hot plate (i.p.)	Writhing (s.c.)
C8813	11.5 (8.2–16.1)	16.9 (13.8–20.8)
Morphine	6800 (5520–8360)	930 (490–1760)
Fentanyl	39 (27–55)	39 (31–49)

<sup>a</sup> 95% CL = 95% confidence limits.*Affinity for μ-, δ- and κ-opioid receptors*

The inhibition of specific [<sup>3</sup>H]ohmefentanyl (μ), [<sup>3</sup>H]DPDPE (δ) and [<sup>3</sup>H]U69593 (κ) bindings by C8813 was examined in membrane preparation from mouse brain. The ability of C8813 to compete with the bindings of [<sup>3</sup>H]ohmefentanyl, [<sup>3</sup>H]DPDPE and [<sup>3</sup>H]U69593 was examined over a wide concentration range. All competitive binding curves were best fitted using a one-site model. The hill coefficients of C8813 in the competitive [<sup>3</sup>H]ohmefentanyl binding and [<sup>3</sup>H]DPDPE binding were 1.07 and 0.98 respectively. As shown in Table 2, C8813 had high affinity for μ-opioid receptor (K<sub>i</sub> = 1.37 nM) and for δ-opioid receptor (K<sub>i</sub> = 3.24 nM) but almost no affinity for κ-opioid receptor at concentration of 1 μM. The affinity of C8813 for μ-opioid receptor was somewhat stronger, while the affinity for δ-opioid receptor was much stronger than that of morphine and fentanyl.

*Effects on isolated organs*

The properties of C8813 were investigated using three in vitro bioassays (Table 3). In the GPI, compound C8813 exhibited strongly inhibitory action on the electrically evoked contractions. The IC<sub>50</sub> value of C8813 was much less than that of morphine and fentanyl. The inhibitory effect of C8813, morphine and fentanyl was readily reversed by μ-opioid receptor antagonist naloxone. In the MVD, the IC<sub>50</sub> value of C8813 was 0.38 ± 0.06 nM, which was 16.5 times higher than that in the GPI. The antagonism of the inhibitory effect of C8813 by δ-opioid receptor antagonist ICI174,864

Table 2

Binding affinity of compound C8813, morphine and fentanyl for μ-, δ- and κ-opioid receptors in homogenate of mouse brain

Drug	K <sub>i</sub> (nM)		
	μ	δ	κ
C8813	1.37 ± 0.46	3.24 ± 0.76	>1000
Morphine <sup>a</sup>	1.8 ± 0.26	90 ± 16	317 ± 68
Fentanyl <sup>a</sup>	7.0 ± 0.83	151 ± 21	470 ± 68

K<sub>i</sub> represented the dissociation equilibrium constant of inhibitor. Values were expressed as the mean ± S.E.M. of 3 experiments performed in triplicate. Labeled ligands were [<sup>3</sup>H]ohmefentanyl (μ), [<sup>3</sup>H]DPDPE (δ) and [<sup>3</sup>H]U69593 (κ) in C8813 assay, and [<sup>3</sup>H][D-Ala<sup>2</sup>, MePhe<sup>4</sup>, Gly-ol<sup>5</sup>]enkephalin (μ), [<sup>3</sup>H][D-Ala<sup>2</sup>, D-Leu<sup>5</sup>]enkephalin (δ) and [<sup>3</sup>H](–)ethylketazocine (κ) in morphine and fentanyl assays.

<sup>a</sup> Data were from Magnan et al. (1982).

Table 3

Agonist potencies and the equilibrium constants of C8813, morphine, fentanyl and DPDPE in the guinea pig ileum (GPI), mouse vas deferens (MVD) and rabbit vas deferens (LVD)

Drug	GPI		MVD		LVD
	IC <sub>50</sub> (nM)	Ke (nM, Naloxone)	IC <sub>50</sub> (nM)	Ke (nM, ICI174,864)	IC <sub>50</sub> (nM)
C8813	0.023 ± 0.002	2.1 ± 0.46	0.38 ± 0.06	25.3 ± 4.8	500 (58%)
Morphine	312 ± 23	1.8 ± 0.3	1087 ± 590	61.1 ± 19.2	ND
Fentanyl	1.88 ± 0.03	1.33 ± 0.06	8.4 ± 0.1	60.4 ± 8.9	ND
DPDPE <sup>a,b</sup>	3000 ± 520	ND	3.4 ± 0.6	17.0 ± 12.8	>10000

IC<sub>50</sub> represented the concentration of agonists to inhibit 50% of contractions. Ke represented the antagonist equilibrium constant of antagonist. Values were expressed as the mean ± S.E.M. of 3 experiments. ND, not determined.

<sup>a</sup> Data of DPDPE in the GPI and LVD were from Corbett et al. (1984).

<sup>b</sup> Data of DPDPE in the MVD were from Berzetei et al. (1987).

was slightly weaker than that of  $\delta$ -opioid receptor agonist DPDPE, but stronger than that of morphine and fentanyl. In the LVD, the inhibitory effect of the compound was very weak. The concentration of the compound that reduced the height of the contractions in the LVD by 58% was about 500 nM. Compound C8813 had no antagonism against the inhibition of normorphine in the GPI, DADLE in the MVD and U50488H in the LVD.

## Discussion

The present study showed that compound C8813, one of 4-arylcylohexanol derivatives, was a potent opioid analgesic. In the hot plate test where nociception was induced by thermal stimuli, C8813 showed strong antinociceptive activity. The antinociception of C8813 was also obtained in the acetic acid writhing test where nociception was induced by chemical stimuli. The hot plate assay reflects acute nociception while acetic acid writhing assay reflects prolonged nociception (Abdel-Fattah et al., 2000). The difference of ED<sub>50</sub> values between C8813 and morphine in hot plate test was greater than that in writhing test. This might reflect that the more potent analgesic had better efficacy on acute nociception. Similar result was observed with fentanyl, since fentanyl was 174 times more active than morphine in hot plate test while 23 times in writhing test (Table 1). Another example is ohmefentanyl. Previous study suggested that ohmefentanyl was 7880 times more active than morphine in hot plate test while only 1788 times in writhing test (Huang et al., 1984). Taken together, the present study demonstrated that C8813 was a more potent analgesic than morphine and fentanyl.

Lednicer et al. (1981) had reported the antinociceptive effect of some 4-arylcylohexanone derivatives. Compound *trans*-4-(*p*-bromophenyl)-4-(dimethylamino)-1-phenylethyl-cyclohexanol was the most potent compound of this series. We had compared the antinociceptive effect and the binding affinity for  $\mu$ -opioid receptor between C8813 and *trans*-4-(*p*-bromophenyl)-4-(dimethylamino)-1-phenylethyl-cyclohexanol. The antinociceptive ED<sub>50</sub> in mouse hot plate of the two compounds were 11.5  $\mu$ g/kg and 13.4  $\mu$ g/kg respectively. The binding K<sub>i</sub> values for  $\mu$ -opioid receptor of the two compounds were 1.37 nM and 1.49 nM respectively. Therefore, C8813 was similar to *trans*-4-(*p*-bromophenyl)-4-(dimethylamino)-1-phenylethyl-cyclohexanol not only in chem-

ical structure but also in antinociceptive efficacy and affinity for  $\mu$ -opioid receptor. Compound C8813 was another new and potent analgesic synthesized by our laboratory.

In the radioligand binding assay, the  $K_i$  values of C8813 for  $\mu$ -,  $\delta$ - and  $\kappa$ -opioid receptors were 1.37 nM, 3.24 nM and more than 1000 nM respectively. These observations suggested that C8813 had high affinity for  $\mu$ - and  $\delta$ -opioid receptors and almost no affinity for  $\kappa$ -opioid receptor. The affinity for  $\mu$ -opioid receptor was the highest. It seemed to be that the potent antinociceptive effect of C8813 was related to its high affinity for  $\mu$ - and  $\delta$ -opioid receptors.

To functionally determine the property of the effect of C8813 for opioid receptors, the isolated tissues were used: the GPI mainly for  $\mu$ -opioid receptor (Kosterlitz and Waterfield, 1975), the MVD for  $\delta$ -opioid receptor (Hughes et al., 1975) and the LVD for  $\kappa$ -opioid receptor (Oka et al., 1981). The present study suggested that compound C8813 exhibited strongly inhibitory action on the electrically evoked contractions in the GPI and the effect was readily antagonized by  $\mu$ -opioid receptor antagonist naloxone. It indicated that C8813 acted on the  $\mu$ -opioid receptor in the GPI. To clarify the possibility of involvement of  $\delta$ -opioid receptor in the effect of C8813, the MVD that mainly contains  $\delta$ -opioid receptor was used. In the MVD, C8813 could inhibit the electrically induced contraction, but weaker than in the GPI. Moreover, this inhibitory effect could be antagonized by the  $\delta$ -opioid receptor antagonist ICI174864. These results indicated C8813 also acted on  $\delta$ -opioid receptor but less potent than on  $\mu$ -opioid receptor. In the LVD that contains only  $\kappa$ -opioid receptor, the inhibitory effect of C8813 was very weak. It discarded the involvement of  $\kappa$ -opioid receptor on the action of C8813. In addition, C8813 did not antagonize the inhibition of normorphine in the GPI, DADLE in the MVD and U50488H in the LVD. This observation indicated that C8813 did not exhibit antagonistic activity for opioid receptors. Taken together, the *in vitro* results demonstrated that C8813 was an agonist for  $\mu$ - and  $\delta$ -opioid receptors.

It is interesting to note that the potency of C8813 in the antinociceptive assays and *in vitro* bioassays was relatively higher than that would be expected on the basis of the binding assay. It suggested the complex mechanism of actions of this compound. The actions of C8813 might not be explained completely by independent action on either  $\mu$ - or  $\delta$ -opioid receptors alone. Previous studies had suggested the possibility of an interaction between  $\mu$ - and  $\delta$ -opioid receptors (Rothman and Westfall, 1982). Moreover, Vaught et al. (1982) had proposed a “self-potentiating” mechanism. As a  $\mu/\delta$  receptor agonist, its ability to bind to the  $\delta$ -opioid receptor will potentiate the antinociception resulting from its action on  $\mu$ -opioid receptor. This will result in an antinociceptive potency greater than that predicated by its affinity for the  $\mu$ -opioid receptor. A similar interpretation was considered for the actions of biphalin, a dimeric enkephalin analog with both  $\mu$ - and  $\delta$ -opioid receptors agonistic action (Horan et al., 1993). It was speculated that the effect of C8813 might be mediated by a hypothesized functional  $\mu/\delta$  opioid receptor complex, or by subtypes of these receptors. Further experiments are needed to clarify the mechanism of actions of C8813.

## Conclusion

The present study demonstrated that compound C8813 was a potent analgesic with high affinity for  $\mu$ - and  $\delta$ -opioid receptors, almost no affinity for  $\kappa$ -opioid receptor. Further pharmacological and neurobiology studies of compound C8813 will be made.

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