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## Binding affinity to and dependence on some opioids in Sf9 insect cells expressing human $\mu$ -opioid receptor<sup>1</sup>

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**KEY WORDS** narcotics; mu opioid receptor; competitive binding; cyclic AMP; analgesia; opioid-related disorders

### ABSTRACT

**AIM:** To investigate the receptor binding affinity and naloxone-precipitated cAMP overshoot of dihydroetorphine, fentanyl, heroin, and pethidine in Sf9 insect cells expressing human  $\mu$ -opioid receptor (Sf9- $\mu$  cells). **METHODS:** Competitive binding assay of [<sup>3</sup>H]ohmefentanyl was used to reveal the affinity for  $\mu$ -opioid receptor in Sf9- $\mu$  cells. [<sup>3</sup>H]cAMP RIA was used to determine cAMP level. Antinociceptive activity was evaluated using 55 °C mouse hot plate test. Naloxone-precipitated withdrawal jumping was used to reflect physical dependence in mice. **RESULTS:** All drugs displayed antinociceptive activity and produced physical dependence in mice. The  $K_i$  values of dihydroetorphine, fentanyl, heroin, and pethidine in competitive binding assay were (0.85±0.20) nmol, (59.1±11.7) nmol, (0.36±0.13)  $\mu$ mol, and (12.2±3.8)  $\mu$ mol respectively. The binding affinities of these drugs for  $\mu$ -opioid receptor in Sf9- $\mu$  cells were paralleled to their antinociceptive activities in mice. After chronic pretreatment with these drugs, naloxone induced cAMP withdrawal overshoot in Sf9- $\mu$  cells. The dependence index in Sf9- $\mu$  cells was calculated as  $K_i$  value in competitive binding assay over  $EC_{50}$  value in naloxone-precipitated cAMP assay. The physical dependence index in mice was calculated as antinociceptive  $ED_{50}$ /withdrawal jumping cumulative  $ED_{50}$ . There was a good linear correlation between dependence index in Sf9- $\mu$  cells and physical dependence index in mice. **CONCLUSION:** The Sf9- $\mu$  cells could be used as a cell model to evaluate the receptor binding affinity and physical dependent liability of analgesic agents.

### INTRODUCTION

The cyclic AMP (cAMP) second messenger system played a key role in opioid dependence<sup>[1]</sup>. Acute administration of opiates inhibited cAMP system. Chronic treatment with opiates led to up-regulation of

cAMP system and addition of opioid antagonist caused cAMP overshoot in the locus coeruleus<sup>[2]</sup> and several cell lines such as neuroblastoma×glioma NG108-15 cells<sup>[3]</sup>, human neuroblastoma SH-SY5Y cells<sup>[4]</sup>, Chinese hamster ovary (CHO) cells and pituitary GH<sub>3</sub> cells expressing opioid receptors<sup>[5,6]</sup>. So up-regulation of cAMP system has been supposed at least partly responsible for the cellular and molecular mechanisms of opioid dependence<sup>[7]</sup>.

The baculovirus/insect cell system is a useful tool for the overexpression of recombinant proteins<sup>[8,9]</sup>. We had successfully expressed human  $\mu$ -opioid receptor in

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baculovirus-infected *Spodoptera frugiperda* (Sf9) cells and demonstrated that the  $\mu$ -opioid receptor expressed in Sf9 cells was functionally coupled to endogenous  $G_{i/o}$  proteins<sup>[10,11]</sup>. It provided a useful tool for studying pharmacological properties and mechanism of opioid dependence.

In the present study, we investigated the receptor binding affinity and naloxone-precipitated cAMP overshoot of dihydroetorphine, fentanyl, heroin, and pethidine in Sf9 cells expressing human  $\mu$ -opioid receptor (Sf9- $\mu$  cells). Furthermore, we investigated the antinociception and physical dependence of these drugs in mice and analyzed the correlation between dependence in Sf9- $\mu$  cells and physical dependence in mice.

## MATERIALS AND METHODS

**Animals** Kunming mice (Grade II, Certificate: SCXK No 2002-002, 18-22 g,  $\hat{\alpha}$  for physical dependence test,  $\hat{\alpha}$  for mouse hot plate test) were supplied by Medical Center of Fudan University, Shanghai, China. All animals were kept on a 12/12-h light-dark cycle in temperature and humidity controlled room. The animals were fed with standard laboratory food and water *ad libitum*.

**Drugs and reagents** Ohmefentanyl, heroin, fentanyl, and naloxone hydrochloride were synthesized by the Chemical Group, 2nd Department of Pharmacology, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, China. Morphine hydrochloride was purchased from Qinghai Pharmaceutical Factory, China. Pethidine hydrochloride was purchased from Shenyang First Pharmaceutical Factory, China. Dihydroetorphine hydrochloride was obtained from Beijing Sihuan Pharmaceutical Factory, China. [<sup>3</sup>H]ohmefentanyl (1.85 TBq/mmol) was labeled by Shanghai Institute of Nuclear Research, Chinese Academy of Sciences, China. Forskolin and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma, USA. The [<sup>3</sup>H]cAMP assay kit was purchased from Shanghai Second Medical University, China.

**Cell culture** The Sf9 cells were grown in monolayer culture in TNM-FH medium (Sigma, USA) supplemented with 10 % heat-inactivated (56 °C, 30 min) fetal calf serum, benzylpenicillin (100 kU/L) and streptomycin (100 mg/L) at 27 °C. Infection was performed in monolayer at a cell density of  $2 \times 10^6$  by recombinant baculoviruses containing human  $\mu$ -opioid receptor cDNA with a tag of six consecutive histidines at its carboxyl

terminus. Cells were harvested at the peak time of production (60-64 h after infection)<sup>[10,11]</sup>.

**Competition binding assay** Sf9- $\mu$  cells were harvested, washed and resuspended in ice-cold Tris-HCl buffer (50 mmol/L, pH 7.4). About  $5 \times 10^5$  cells were incubated in Tris-HCl buffer with [<sup>3</sup>H]ohmefentanyl 0.8 nmol/L in the presence of increasing concentrations of drugs. Nonspecific binding was determined in the presence of 10  $\mu$ mol/L unlabeled ohmefentanyl. Binding reactions were conducted in triplicate at 37 °C for 30 min, in a volume of 0.2 mL. The reaction was terminated by adding 3 mL ice-cold Tris-HCl buffer, followed by rapid filtration through Whatman GF/B glass fiber filters using a Millipore harvester. Filters were washed three times with 3 mL ice-cold Tris-HCl buffer, dried, and transferred to counting vials. Scintillation cocktail (0.5 mL) was added and the radioactivity was counted with Beckman LS6500 liquid scintillation analyzer. The IC<sub>50</sub> value, defined as the concentration of tested drugs that produced 50 % inhibition of the [<sup>3</sup>H]ohmefentanyl binding, was determined.

**cAMP assay** Sf9- $\mu$  cells were pretreated with increasing concentrations of drugs for 6 h. Before the end of pretreatment, naloxone 1  $\mu$ mol/L was added and incubated for 10 min. Then, cells were harvested, washed, and incubated in PBS containing IBMX 0.5 mmol/L, forskolin 10  $\mu$ mol/L, and naloxone 1  $\mu$ mol/L at 37 °C for 5 min. The reactions were terminated with perchloric acid 1 mol/L and neutralized with KOH 2 mol/L. The intracellular cAMP concentration was measured using [<sup>3</sup>H]cAMP RIA kit as described in protocol. The EC<sub>50</sub> value, defined as the concentration of tested drugs that produced 50 % increase of naloxone-precipitated cAMP overshoot, was determined.

**Antinociceptive assay** The hot-plate test was used for assessing antinociceptive response. The plate temperature was kept at (55 $\pm$ 1) °C. Female mice were used. The latency time of licking hind-paw or jumping off the plate was recorded as nociceptive response. Each mouse was tested twice before drug administration and the latencies were averaged to obtain the baseline latency. The antinociceptive ED<sub>50</sub> values were calculated by antinociceptive efficiency at 15 min after drug sc injection. A cut-off time of 60 s was employed as the antinociceptive efficiency.

**Assessment of physical dependence** Male mice were induced dependence on tested drugs by sc injections of progressively increasing doses twice a day for 7 d according to previously described method<sup>[12]</sup>. With-

drawal was precipitated by injecting naloxone (2 mg/kg, ip) 2 h after the last administration of tested drugs. Mice were placed individually into round glass boxes with a white floor immediately after naloxone injection. The number of jumps was counted for 30 min. The positive jumping response was defined as jumping more than 4 times within 30 min. The degree of physical dependence was evaluated by the cumulative dose of tested drugs that induced 50 % positive jumping response ( $ED_{50}$ ).

**Data analysis** All data of competitive binding assay and measurements of intracellular cAMP levels were expressed as % of control values.  $IC_{50}$  and  $EC_{50}$  values were determined by nonlinear regression analysis with GraphPad Prism 3.0 (GraphPad software, USA). The  $K_i$  value was calculated from the formula:  $K_i = IC_{50} / (1 + [L] / K_d)$ , where  $L$  is the concentration of [ $^3H$ ]ohmefentanyl and  $K_d$  is the dissociation constant of [ $^3H$ ]ohmefentanyl<sup>[13]</sup>. Data were expressed as mean  $\pm$  SD. The  $ED_{50}$  and 95 % confidence intervals were calculated by probit analysis. The correlation coefficient was analyzed by Pearson's two-tailed test.

## RESULTS

**Binding affinity for  $\mu$ -opioid receptor in Sf9- $\mu$  cells** Sf9 cells, which do not possess constitutive  $\mu$ -opioid receptor, were transfected with recombinant baculoviruses containing human  $\mu$ -opioid receptor<sup>[10]</sup>. Competition of [ $^3H$ ]ohmefentanyl binding with increasing concentrations of tested drugs was carried out in these Sf9- $\mu$  cells. The potency order of binding affinity for  $\mu$ -opioid receptor of these drugs was dihydroetorphine > fentanyl > heroin > pethidine as determined by the  $K_i$  value (Tab 1).

**Naloxone-precipitated cAMP overshoot in Sf9- $\mu$  cells** After pretreating Sf9- $\mu$  cells with increasing concentrations of tested drugs for 6 h, addition of 1  $\mu$ mol/L naloxone caused cAMP withdrawal overshoot, indicating that Sf9- $\mu$  cells produced dependence on these drugs. The  $EC_{50}$  values of these drugs-induced naloxone-precipitated cAMP overshoot were calculated. To evaluate the relative dependence potency of these drugs, dependence index was introduced as described previously with minor modification<sup>[12,14]</sup>. The dependence index in Sf9- $\mu$  cells was calculated as  $K_i$  value in competitive binding assay over  $EC_{50}$  value in naloxone-precipitated cAMP assay (Tab 1).

**Antinociceptive activity in mice** All tested drugs showed an antinociceptive activity. The order of antinociceptive potency was dihydroetorphine > fentanyl > heroin > pethidine (Tab 1).

**Physical dependence in mice** All tested drugs produced physical dependence characterized by naloxone-precipitated withdrawal jumping in mice. The  $ED_{50}$  values of cumulative dose of tested drugs in naloxone-precipitated jumping were determined. To evaluate the relative dependent liability, physical dependence index was calculated as the antinociceptive  $ED_{50}$ /withdrawal jumping cumulative  $ED_{50}$ . Higher index indicated relatively higher physical dependent potency. Fentanyl showed the highest and pethidine showed the weakest physical dependent liability among these drugs (Tab 1).

The binding affinity of dihydroetorphine, fentanyl, heroin, and pethidine for  $\mu$ -opioid receptor in Sf9- $\mu$  cells was paralleled to the antinociceptive activity ( $r=0.99$ ,  $P<0.01$ , Fig 1). A good linear relationship was also obtained between dependence index ( $K_i$  in binding assay/ $EC_{50}$  in cAMP assay) in Sf9- $\mu$  cells and physical

**Tab 1. The physical dependence index in mice and dependence index in Sf9- $\mu$  cells for dihydroetorphine, fentanyl, heroin, and pethidine. A and B: 95 % confidence intervals were given in parentheses.  $n=10$  mice each group. C and D: mean  $\pm$  SD.  $n=3-5$  experiments in triplicate.**

Drug	Mice			Sf9- $\mu$ cells		
	Antinociceptive $ED_{50}/mg \cdot kg^{-1}$ (A)	Withdrawal jumping cumulative $ED_{50}/mg \cdot kg^{-1}$ (B)	Physical dependence index (A/B)	Binding $K_i/\mu mol$ (C)	cAMP overshoot $EC_{50}/\mu mol$ (D)	Dependence index (C/D)
Dihydroetorphine	0.0005 (0.00036-0.00069)	0.0036 (0.0013-0.010)	0.14	0.00085 $\pm$ 0.00020	0.008 $\pm$ 0.004	0.10
Fentanyl	0.039 (0.028-0.055)	0.12 (0.071- 0.20)	0.33	0.059 $\pm$ 0.012	0.33 $\pm$ 0.16	0.18
Heroin	0.6 (0.5-0.7)	1.9 (1.2-3.2)	0.32	0.36 $\pm$ 0.13	2.5 $\pm$ 1.2	0.14
Pethidine	10.5 (9.0-12.2)	193 (163-229)	0.054	12 $\pm$ 4	224 $\pm$ 146	0.055

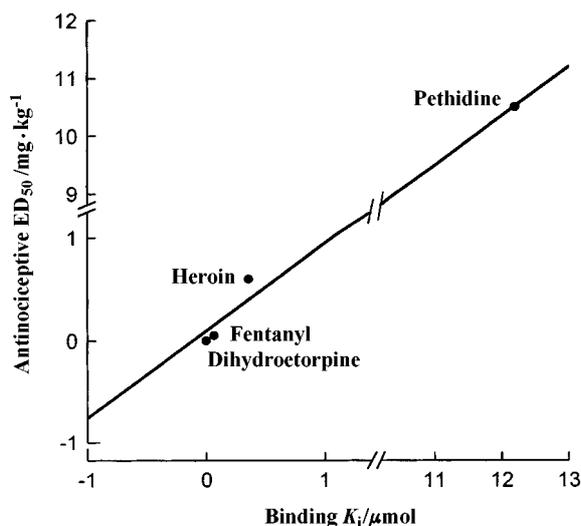


Fig 1. Correlation between binding  $K_i$  value in Sf9- $\mu$  cells and antinociceptive  $ED_{50}$  value in mice for dihydroetorphine, fentanyl, heroin, and pethidine.  $r = 0.99$  ( $P < 0.01$  by Pearson's two-tailed test).

dependence index (antinociceptive  $ED_{50}$ /withdrawal jumping cumulative  $ED_{50}$ ) in mice ( $r = 0.95$ ,  $P < 0.05$ , Fig 2).

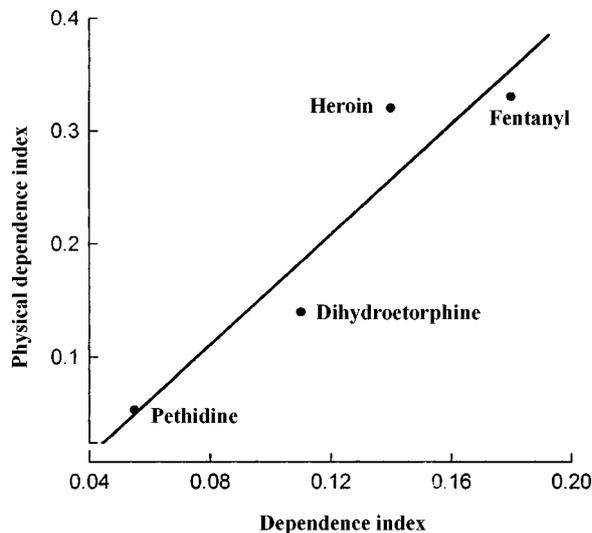


Fig 2. Correlation between dependence index in Sf9- $\mu$  cells and physical dependence index in mice for dihydroetorphine, fentanyl, heroin, and pethidine.  $r = 0.95$  ( $P < 0.05$  by Pearson's two-tailed test).

## DISCUSSION

We have previously reported a method of quantitative comparison of opioid dependence using dependence index<sup>[10]</sup>. In the present study, physical dependence index in mice was calculated as the  $ED_{50}$  value of

antinociception over the  $ED_{50}$  value of cumulative dose of tested drugs in withdrawal jumping. Higher index indicated relatively higher physical dependent potency. According to the index, fentanyl and heroin were more liable to develop physical dependence than pethidine. By using the  $ED_{50}$  value in withdrawal jumping alone, it would be drawn that dihydroetorphine was the most liable to develop physical dependence. In fact, studies had suggested that dihydroetorphine had more potent antinociception and lower physical dependent liability than fentanyl and heroin<sup>[15,16]</sup>. Therefore, the physical dependence index that considered both antinociceptive  $ED_{50}$  and withdrawal jumping cumulative  $ED_{50}$  had advantage in quantitative comparison of opioid dependence.

Investigations for the cellular and molecular mechanisms underlying drug dependence *in vitro* have widely based on the use of cell lines. Many studies used the neuronal cell lines. But these cell lines had some disadvantages because of existence of multiple constitutive opioid receptors<sup>[3,4]</sup>. The insect cells, which devoid of constitutive opioid receptors, are one of the candidates to express cloned opioid receptors. We and other laboratories had successfully expressed human  $\mu$ -opioid receptor in Sf9 cells and demonstrated that the  $\mu$ -opioid receptor expressed in Sf9 cells was functionally coupled to endogenous  $G_{i/o}$  proteins<sup>[10,11,17]</sup>. The cloned opioid receptors transfected in insect cells were very useful for the study of mechanisms of opioid tolerance and dependence.

In the present study, the competitive binding assay in Sf9- $\mu$  cells revealed that dihydroetorphine, fentanyl, heroin, and pethidine had different affinity for  $\mu$ -opioid receptor. Combined with the antinociceptive  $ED_{50}$  of these drugs, the binding affinities of these drugs for  $\mu$ -opioid receptor in Sf9- $\mu$  cells were paralleled to their antinociceptive activities in mice. Since  $\mu$ -opioid receptor played a major role in opioid analgesic effect<sup>[18]</sup>, the different binding affinity for  $\mu$ -opioid receptor of these drugs might be responsible for their different analgesic activity.

After pretreatment of Sf9- $\mu$  cells with tested drugs for 6 h, addition of opioid antagonist naloxone resulted in an increase of cAMP levels. Similar increase of cAMP levels was also reported in SH-SY5Y and NG108-15 cells by either rapid removal of morphine, or addition of naloxone to cells pretreated with morphine<sup>[3,4]</sup>. This rebound in intracellular cAMP levels was thought to be a compensatory process for the decrease of cAMP lev-

els caused by chronic opioid exposure, and was thought to reflect the dependence phenomena in cells. These results further proved the important role of cAMP system in opioid dependence<sup>[1]</sup>. Other studies are needed to elucidate the mechanism underlying the cellular adaptation in Sf9- $\mu$  cells after chronic treatment with opioids.

Similar to physical dependence index in mice, we introduced the dependence index to quantitatively analyze the dependent potency in the Sf9- $\mu$  cell model. The dependence index in Sf9- $\mu$  cells was calculated as  $K_i$  value in competitive binding assay over  $EC_{50}$  value in naloxone-precipitated cAMP assay. Higher dependence index infers relatively higher dependent potency. Fentanyl had the highest and pethidine had the lowest dependence index, indicating that fentanyl had the highest and pethidine had the lowest dependent potency. A good linear correlation was obtained between dependence index in Sf9- $\mu$  cells and physical dependence index in mice. It suggested that the dependent potency of opioids could be analyzed using the dependence index in Sf9- $\mu$  cells. However, further demonstrations are needed to verify this dependence index using more opioid analgesics.

In conclusion, after pretreating Sf9- $\mu$  cells with dihydroetorphine, fentanyl, heroin, and pethidine, naloxone led to cAMP withdrawal overshoot. Quantitative analysis of opioid dependence could be done using dependence index, ie,  $K_i$  value in competitive binding assay over  $EC_{50}$  value in naloxone-precipitated cAMP assay. The Sf9- $\mu$  cells might be served as a cell model to study the receptor binding affinity and dependence of opioids.

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