中国药理学报 *Acta Pharmacologica Sinica* 1989 May; **10** (3) : 205-210

# P-7521 —— a new irreversible opioid ligand

JIN Wen-Qiao, FAN Li-Qun, CHEN Xin-Jian, CHI Zhi-Qiang

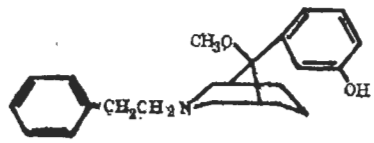
*(Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 200031, China)*

**Abstract** In the receptor binding assay, P-7521 was a potent opioid ligand which acted mainly on μ receptor. The relative affinity ratio at μ, δ and κ sites was 66 : 8: 1. The inhibitory effects of P-7521 were 1868 and 6060 times more potent than morphine on the electrically evoked contractions in guinea pig ileum and mouse vas deferens, respectively and were readily antagonized by naloxone and Mr 2266. These results indicate that P-7521 acted on μ receptor in guinea pig ileum and mouse vas deferens. In rabbit vas deferens, the compound had no agonist activity, but could antagonize the inhibitory effect of U-50488 H, a κ agonist, showing the antagonistic characterization was on κ receptor. The dissociation of P-7521 binding to opioid receptor were very difficult in μ binding assay and bioassays.

**Key words** azabicyclononane; analgesics; endorphin receptors; radioligand assay; ileum; vas deferens; P-7521

P-7521 [3-(β-phenylethyl)-9 β-methoxy-9 α-(*m*-hydroxyphenyl)-3-azabicyclo (3, 3, 1)-nonane] is a novel and potent analgesic[1]. In previous work[2], we have reported that this compound has higher binding affinity and lower GTP + NaCl response ratio in binding assay, and long analgesic duration and weak antagonist activity *in vivo*. To delineate more clearly the characterization we study further the binding and agonist or antagonist properties of this compound to μ, δ and κ opioid receptor subtype *in vitro*, and examine binding tightness of the compound to opioid receptor.

Received 1988 Jul 1 Accepted 1988 Dec 1




P-7521

## Materials and methods

**Chemicals** Ohmefentanyl and P-7521 were synthesized in our institute. U-50488 H {trans-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide (Upjohn Co) and Mr 2266 [(-)2-(3-furylmethyl)-5, 9-diethyl-2′-hydroxy-6,7-benzomorphan] were devoted by Prof Kosterlitz (University of Aberdeen, UK). Naloxone, normorphine and [$^3$H] etorphine (1.70 TBq/mmol) were obtained from Shanghai Medical University. (D-Ala$^2$, D-Leu$^5$) enkephalin (DADLE) and dynorphin A (1-13) were provided from Peninsula Laboratories (USA), and (D-Ser$^2$, Thr$^6$)enkephalin from Prof Roques (INSERM, France). [$^3$H]DPDPE[(D-Pen$^2$, D-Pen$^5$) enkephalin, 1.04 TBq/mmol] was purchased from Amersham. [$^3$H]ohmefentanyl (1.49 TBq/mmol) was labelled by Shanghai Institute of Atomic Nucleus, Chinese Academy of Sciences.

**Preparation of brain homogenate** Male mice (20.8 ± SD 2.5 g) were decapitated and the brains were rapidly removed. Brains without cerebellum were homogenized in Tris-HCl buffer 50 mmol/L (0°C, pH 7.4) and centrifuged at 49 000 × *g* for 30 min. The pellets were suspended in ice-cold Tris-HCl buffer (0°C, pH 7.4), and then

recentrifuged at 49 000 × g for 10 min. The pellets were resuspended in ice-cold Tris-HCl buffer 50 mmol/L (30℃, pH 7.4) and homogenized. Protein was determined by the method of biuret reaction using bovine-serum albumin as a standard. Concentration of protein was diluted to about 10 mg/ml and stored at −40℃.

**Receptor binding assays** Binding experiments were performed as previously described[3]. The μ and δ binding sites were determined with the highly selective μ ligand, [$^3$H]ohmefentanyl, and the highly selective δ ligand, [$^3$H]DPDPE, respectively. For the estimation of κ binding, the [$^3$H]etorphine was used in the presence of unlabelled ohmefentanyl 30 nmol/L and DADLE 100 nmol/L to suppress bindings to the μ and δ sites. Specific binding was calculated from the difference of the counts in the absence and presence of unlabelled etorphine (1 μmol/L) or levorphanol (10 μmol/L). In competition experiments, the concentrations of [$^3$H]ohmefentanyl, [$^3$H]DPDPE and [$^3$H]etorphine were 0.5, 3.4 and 0.5 nmol/L, respectively. The $IC_{50}$ values were estimated by linear regression from probit-log plots with 5-7 concentrations of unlabelled ligand. The corresponding $K_I$ values were calculated according to the formula $K_I = IC_{50}/(1 + L/K_d)$ in which L is the concentration and $K_d$ the dissociation constant of labelled ligand. Relative affinity is calculated by ($K_I^{-1}$ for μ, δ or κ)/($K_I^{-1}$ for μ + $K_I^{-1}$ for δ + $K_I^{-1}$ for κ). In binding tightness experiments, the incubation mixture contained 200 μl of mouse brain membrane fraction (about 2 mg protein), 150 μl of the ligand at the stated concentration, in Tris-HCl buffer 50 mmol/L (30℃, pH 7.4) in a final volume of 3 ml. After 45 min incubation at 30℃, the samples were diluted with 37 ml ice-cold buffer and centrifuged at 40 000 × g for 10 min. The pellets were suspended in 40 ml buffer, incubated for 15 min at 20℃ and centrifuged again. This washing step was repeated 4 times. The final pellets were homogenized in 3 ml of the Tris-HCl buffer 50 mmol/L (25℃, pH 7.4) and used immediately for binding assays.

All experiments were performed at least three times with trilicate determinations at each point.

**Bioassays** The myenteric plexus-longitudinal muscle from guinea pig ileum was prepared as reference (4). The vas deferens from mice or rabbits were prepared as reference (5) and (6), respectively. The preparations were suspended in the organ bath containing 6 ml Krebs solution. The resting tension was maintained at 250 mg for the mouse vas deferens and at 500 mg for the guinea pig ileum and the rabbit vas deferens. After equilibration for 30 min, longitudinal contractions were evoked by field stimulation through Pt-electrodes at the upper and lower ends of the bath. For the guinea pig ileum and the rabbit vas deferens, single pulses were used (50 V, 1.0 ms duration, 15 s interval). For the mouse vas deferens the trains consisted of 3 pulses at intervals of 200 ms (40 V, 1.0 ms duration, 15 s interval). The contractions were recorded by means of a force displacement transducer and auto-balancing recorder.

The agonist potencies of the compounds were obtained from dose-response curves by calculating the concentration of the compound that reduced the height of the contractions by 50% ($IC_{50}$). The antagonist equilibrium constant, $K_e$ values were determined by the single dose method[7].

## Results

**Binding characteristics of [$^3$H]DPDPE in homogenates of mouse brain** [$^3$H]DPDPE binding to mouse brain homogenates was found to be concentration-dependent and saturable. In the Scatchard plot

shown in Fig 1, the affinity was $4.4 \pm 0.5$ nmol/L($K_d$) and the maximum binding capacity was $4.2 \pm 0.4$ pmol/g protein ($B_{max}$). [$^3$H]DPDPE 3.4 nmol/L near the equilibrium dissociation constant was used in competitive inhibition study.

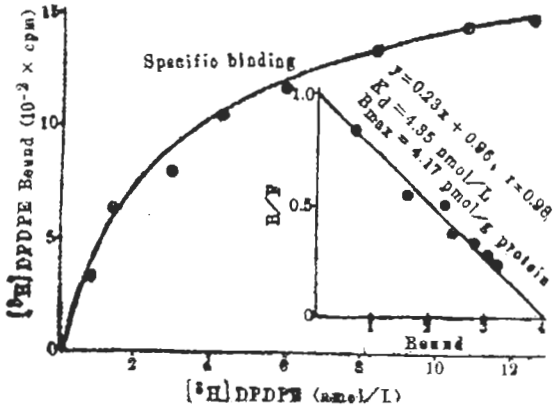



Fig 1. Specific binding of [$^3$H]DPDPE to the homogenates of the mouse brain and Scatchard analysis.

**Displacement of [$^3$H]ohmefentanyl binding by unlabelled ohmefentanyl and [$^3$H]DPDPE binding by uniabelled DADLE** Ohmefentanyl has previously been shown to bind selectively to μ opioid receptor[8,9]. While DADLE shows some selectivity for δ opioid receptor[10]. The displacement curves of [$^3$H]ohmefentanyl by ohmefentanyl and [$^3$H]DPDPE by DADLE were shown in Fig 2 A, B, respectively. Hill analysis of both displacement curves indicated that ohmefentanyl had Hill coefficient of 0.94 whereas DADLE had Hill coefficient of 0.75. Unlabelled ohmefentanyl in 30 nmol/L concentration inhibited 95% of the binding of [$^3$H]ohmefentanyl (μ binding site), unlabelled DADLE in 100 nmol/L concentration inhibited 90% of the binding of [$^3$H]DPDPE (δ binding site) (Fig 2). Therefore, in the determination of κ binding, the cross-reactivity of [$^3$H]etorphine to both the μ and δ binding sites would be reduced by addition of unlabelled ohmefentanyl 30 nmol/L and unlabelled DADLE 100 nmol/L.

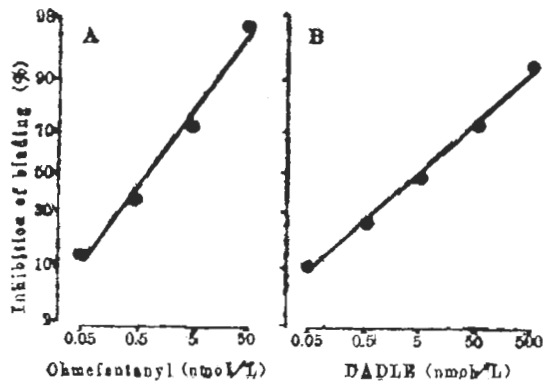



Fig 2. Inhibition of specific binding of [$^3$H] ligand by unlabelled ligand. A) ohmefentanyl displaced specific binding of [$^3$H]ohmefentanyl. B) DADLE displaced specific binding of [$^3$H] DPDPE.

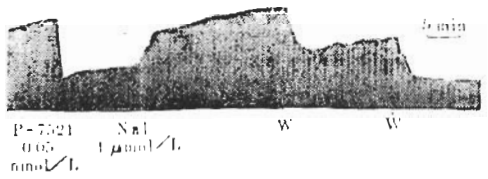



Fig 3. Inhibitory action of compound P-7521 and antagonism by naloxone on guinea pig ileum (NaI-naloxone, W-washing)

**Binding affinity of P-7521 for opioid receptor subtype** The ability of P-7521 to compete with the binding of [$^3$H] ohmefentanyl, [$^3$H]DPDPE or [$^3$H]etorphine was examined over a wide concentration range (Tab 1). The results were expressed as $IC_{50}$ and $K_I$ values. As can be seen in Tab 1, P-7521 had the highest affinity for μ receptor ($K_I$ only 0.025 nmol/L), intermediate affinity for δ receptor($K_I$ 0.20 nmol/L) and low affinity for κ receptor ($K_I$ 1.64 nmol/L). The relative affinity ratio for μ, δ and κ was 66:8:1. These data indicated that P-7521, which readily displaced μ binding, exhibited a 12% cross-reactivity with the δ receptor and to a lesser extent with the κ receptor (1.5%).

**Comparison of binding tightness of P-7521 to μ, δ and κ receptors** The binding tightness of P-7521 with the membrane-bound opioid receptor subtypes

**Tab 1. Binding affinity of compound P-7521 for opioid receptor subtype in homogenate of mouse brain (nmol/L). $\bar{x}\pm SD$**

| | $IC_{50}$ | $K_I$ |
|---|---|---|
| [$^3$H]ohmefentanyl (μ) | 0.064±0.013 | 0.025±0.005 |
| [$^3$H]DPDPE (δ) | 0.38±0.06 | 0.20±0.03 |
| [$^3$H]etorphine*(κ) | 3.7±0.5 | 1.64±0.22 |
| Relative affinity | μ : δ : κ | 66:8:1 |

* In the presence of unlabelled ohmefentanyl 30 nmol/L and DADLE 100 nmol/L to suppress bindings to the μ and δ sites. The values were the averages of 3 experiments.
Relative affinity was calculated by ($K_I^{-1}$ for μ, δ or κ)/($K_I^{-1}$ for μ + $K_I^{-1}$ for δ + $K_I^{-1}$ for κ).

was determined. This reaction affinity was conducted in Tris-HCl buffer at pH 7.4 and at 0.1 μmol/L P-7521 concentration. After 4 washes of the membranes in the buffer, subtype-selective specific bindings were measured both in P-7521-treated and in control samples. Paralled determinations were also made for comparison with morphine and normorphine. Tab 2 showed the results of binding tightness of P-7521, morphine and normorphine. The dissociation of binding of P-7521 to μ receptor was extremely slow. After 4 washes P-7521 binding to the membranes still decreased the binding of [$^3$H]ohmefentanyl about 87%. The blockade of μ specific binding was significantly greater than that of δ binding or κ binding, indicating a persistent interaction between P-7521 and the μ receptor subtype. There was almost no inhibition of the 3 opioid receptor bindings using morphine or normorphine instead of P-7521 in the preincubation. It showed that the washes given can remove sufficiently reversibly bound opioid ligands.

**Effect of P-7521 on isolated organs** The results of P-7521 in the guinea pig ileum, the mouse and rabbit vas deferens were shown in Tab 3. The agonist potency in the guinea pig ileum and the

**Tab 2. Binding tightness of compound P-7521, morphine and normorphine to opioid receptor subtype. After 4 washes the remained binding sites were measured with [$^3$H]opioid as described in the methods. Data (% of control values) represent the means of triplicate samples in a single experiment. The assay was repeated twice.**

| Compound | [$^3$H]ohmefentanyl 0.5 nmol/L (μ site) | [$^3$H]DPDPE 4.0 nmol/L (δ site) | [$^3$H]etorphine* 0.5 nmol/L (κ site) |
|---|---|---|---|
| Control | 100% | 100% | 100% |
| P-7521 (0.5 μmol/L) | 13.7% | 47.7% | 88.8% |
| Morphine (5 μmol/L) | 92.1% | 93.3% | 103% |
| Normorphine (5 μmol/L) | 98.7% | 86.8% | 104% |

*Add unlabelled ohmefentanyl 30 and 100 DADLE nmol/L

mouse vas deferens was expressed by relative potency (normorphine = 1). Because the offset of action of P-7521 was very slow, so that assessement of its $IC_{50}$ value was difficult. P-7521 had very potent agonist activity, being 1868 and 6060 times more potent than normorphine in the guinea pig ileum and the mouse vas deferens, respectively. The concentration of the compound that reduced the height of the contractions by 50% was about 0.05-0.10 nmol/L in both preparations. The inhibitory effects of P-7521 were readily antagonized by μ antagonist naloxone and (μ + κ) antagonist Mr 2266. Their $K_e$ values were 1.3 and 1.5 nmol/L in the guinea pig ileum, 1.7 and 2.9 nmol/L in the mouse vas deferens, respectively. There was no significant difference statistically between the $K_e$ values of naloxone and Mr 2266, indicating that P-7521 mainly acted on μ receptor in the guinea pig ileum and the mouse vas deferens. P-7521 had no antagonistic effect on the action of normorphine or DADLE in the guinea pig ileum and the demouse vas deferens. In the rabbit vas deferens which contains only κ receptor,

P-7521 had marked antagonism against the inhibition of κ ligand, U-50488 H, the $K_e$ value being 8.6 nmol/L, but with no agonist action (Fig 3). Moreover, although effect of P-7521 can be antagonized by naloxone in the guinea pig ileum and the mouse vas deferens, but the inhibitory effect reappeared after washing off naloxone (Fig 4). The recovery of the depressant action of P-7521 in the guinea pig ileum was only 20% after repeated washes for 3 h at interval of 15 min. Therefore the effect of P-7521 is extra-long lasting.

**Tab 3. The agonist potency of compound P-7521 and the equilibrium constant of the antagonism ($K_e$) in the guinea pig ileum, mouse and rabbit vas deferens ($\bar{x}\pm SD$).**

| | Guinea pig ileum | Mouse vas deferens | Rabbit vas deferens |
|---|---|---|---|
| Agonist potency (normorphine = 1) | 1900±300 | 6100±800 | |
| Naloxone $K_e$ (nmol/L) | 1.30±0.20 | 1.70±0.10 | |
| Mr 2266 $K_e$ (nmol/L) | 1.5±0.6 | 2.9±1.0 | |
| $K_e$* | | | 8.6±1.5 |

*Antagonism of P-7521 against U-50488 H, a κ agonist. The values were averages of 3 experiments.

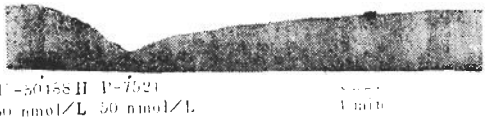


**Fig 4. Antagonistic action of compound P-7521 against U-50488 H on rabbit vas deferens.**

## Discussion

The present study demonstrated that an azabicyclononane derivative, P-7521 is a new and very potent mixed agonist-antagonist with μ agonist and κ antagonist activities. P-7521 inhibits all of the [$^3$H]opioid ligand bindings in a concentration dependent manner. Since this blockade particularly in μ receptor binding still exists after extensive washes of the membrane fractions, it appears irreversible. The irreversibility was exhibited more markedly in the bioassays. The inhibition on the contraction in the guinea pig ileum and the mouse vas deferens by P-7521 can be reversed by naloxone. However, after washing the inhibition of the twitch returned to nearly the same level before naloxone was added. From the phenomenon that naloxone can antagonize the effect of P-7521, but not replace the binding of P-7521, we postulated that opioid receptor is a two-site model that contains an agonist site and a neighboring regulatory recognition site for the antagonist. Binding of the antagonist to this neighboring site allosterically inhibits agonist-receptor interaction, thereby antagonizing agonist effect. After washing, the antagonist is selectively removed, thereby restoring the agonism of the bound ligand. The persistent binding of P-7521 to receptor could result from a greater lipophilicity of P-7521 relative to that of naloxone. P-7521 could be proposed as a powerful probe in the study of opioid ligand-receptor interactions and receptor purification.

We have investigated the pharmacological actions of P-7521 in rabbit[11]. P-7521 in analgesic dose displays slight respiratory depressant effect. Even when doses as large as $1200\times ED_{50}$ were given, the respiration was never completely depressed and the animal did not die. It was different from those affected by fentanyl derivatives. We considered it is possible that, at high dose, P-7521 exhibits κ antagonist activity, preventing respiratory further depression. This antagonistic property provides perhaps an explaination of why P-7521 can slightly antagonize the analgesic action of morphine (unpublished results).

Our experiments show that [$^3$H]

ohmefentanyl synthesized by our laboratory is a good probe for the μ binding site. The ligand is much more stable than DAGO which is the best μ selective ligand at present. The determination of κ receptor affinity is hindered by the lack of labelled ligand of high selectivity. In this paper we used [$^3$H]etorphine in the presence of unlabelled ohmefentanyl 30 nmol/L and DADLE 100 nmol/L to suppress bindings to the μ and δ sites as a selective κ ligand. The results indicated that this estimation of the κ receptor affinity is available. The P-7521 has high affinity for the κ receptor in the binding assay, displays also potent κ antagonist activity in the rabbit vas deferens which contain only κ receptor.

**References**


1 Zhou DH, Fan SN, Ge BL, *et al.* Synthesis and analgesic activity of derivatives of azabicycloalkanes. *Acta Pharm Sin* 1982; 17 : 503

2 Wang DY, Chi ZQ. Relationship between analgesic activity and opiate receptor binding affinity for 5 derivatives of 3-azabicyclo [3,3,1] nonanes. *Acta Pharmcol Sin* 1984; 5 : 158

3 Wu SW, Jin WQ, Chi ZQ. Characterization of opioid receptors in golden hamster brain. *Ibid* 1986; 7 : 495

4 Kosterlitz HW, Lydon RJ, Watt AJ. The effects of adrenaline, noradrenaline and isoprenaline on inhibitory α-and β-adrenoceptors in the longitudinal muscle of the guinea-pig ileum. *Br J Pharmacol* 1970; 39 : 398

5 Hughes J, Kosterlitz HW, Leslie FM. Effect of morphine on adrenergic transmission in the mouse vas deferens : Assessment of agonist and antagonist potencies of narcotic analgesics. *Ibid* 1975; 53 : 371

6 Oka T, Negishi K, Suda M, Matsumiya T, Inazu T, Ueki M. Rabbit vas deferens: A agocific bioassay for opioid kappa-receptor spenists. *Eur J Pharmcol* 1981; 73 : 235

7 Kosterlitz HW, Watt AJ. Kinetic parameters of narcotic agonists and antagonists, with particular reference to *N*-allylnoroxymorphone (naloxone). *Br J Pharmacol* 1968; 33 : 266

8 Xu H, Yao YH, Zhu YC, Chen J, Chi ZQ. Potent 3-methylfentanyl analogs: morphine-like catalepsy and receptor binding characteristics. *Acta Pharmacol Sin* 1987; 8 : 289

9 Jin WQ, Chen XJ, Chi ZQ. The choice of opioid receptor subtype in isolated preparations by ohmefentanyl. *Sci Sin* [B] 1987; 30 : 176

10 Chang KJ, Cooper BR, Hazum E, Cuatrecasas P. Multiple opiate receptors: different regional distribution in the brain and differential binding of opiates and opioid peptide. *Mol Pharmacol* 1979; 16 : 91

11 Zhou J, Zheng WJ, Chi ZQ. Analgesic activity and respiratory inhibition of 3-(β-phenylethyl)-9 β-methoxy-9 α- (*m*-hydroxyphenyl)-3-azabicyclo [3, 3, 1]-nonane (P-7521) in rabbits. *Acta Pharmacol Sin* 1987; 8 : 10


# P-7521 — 一个新的不可逆阿片配体

金文桥、范力群、陈新建、池志强 （中国科学院上海药物研究所，上海 200031，中国）

**提要** P-7521 系氮杂二环壬烷类化合物。受体结合试验中，P-7521 和脑内 μ、δ、κ 受体的相对亲和力为 66 : 8 : 1。该化合物作用于豚鼠回肠和小鼠输精管 μ 受体，其作用分别比去甲吗啡强 1868 和 6060 倍。在含单一 κ 受体的兔输精管中，P-7521 无激动活性，但能拮抗 κ 激动剂 U-50488 H 的抑制作用，呈现 κ 受体拮抗性质。P-7521 和脑内阿片受体的结合很难解离，经 4 次洗涤后仍能抑制 87%的[$^3$H]羟甲芬太尼专一性结合。在豚鼠回肠和小鼠输精管中，反复洗涤 3 h 后，该化合物的抑制作用仅恢复 20%。结果表明，P-7521 是一个主要作用于 μ 受体的强力、超长效阿片配体，对 μ 受体激动，对 κ 受体拮抗。

**关键词** 氮杂二环壬烷；镇痛药；内啡肽受体；放射配位体测定；回肠；输精管；P-7521