ORIGINAL ARTICLE

Multiple Mechanisms Underlying the Long Duration of Action of Thienorphine, A Novel Partial Opioid Agonist for the Treatment of Addiction

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SUMMARY

Aims: It is considered that a long-acting therapy would be advantageous in the treatment of addiction. In a search for novel buprenorphine analogues, thienorphine was demonstrated to be an extremely long-acting orally active partial opioid agonist. This study explored the mechanisms underlying the long-lasting effects of thienorphine. **Methods:** The binding kinetics of [³H]thienorphine were measured in membrane preparations expressing cloned rat opioid receptors. Flow cytometric analysis was used to determine the effect of thienorphine on the surface opioid receptor number. The long-lasting effects of thienorphine were also confirmed at the tissue level and *in vivo*. **Results:** At 37°C, [³H]thienorphine showed rapid association with μ - and κ -opioid receptors, while its dissociation was sluggish and biphasic ($K_{-1} = 0.21 \text{ min}^{-1}$, $K_{-2} = 0.0078 \text{ min}^{-1}$ for the μ -receptor; $K_{-1} = 0.17 \text{ min}^{-1}$, $K_{-2} = 0.0042 \text{ min}^{-1}$ for the κ -receptor). Treatment with thienorphine for 24, 48, and 72 h downregulated surface μ -receptor in a dose- and time-dependent manner. The inhibitory effect of thienorphine on guinea pig ileum persisted for more than 120 min after prolonged washing. *In vivo*, thienorphine exhibited significant antagonism of morphine-induced antinociception for more than 7 days. **Conclusions:** These results indicate that multiple factors, including persistent receptor occupation and enhanced receptor downregulation, may contribute to the long-lasting effects of thienorphine that would be beneficial for its application in addiction treatment.

Introduction

It has been proposed that a long-acting therapy would be desirable in some clinical situations, as it allows good acceptance by patients, stable control of disease, and prevention of relapse. It has also been considered that a long duration of action offers the advantage of a reduction in medical burden and may assist patients to get back to a normal life. This is especially the case for the treatment of addiction. Addiction is a chronic brain disease characterized by a compulsion to seek and take a drug, and by a loss of control over drug consumption. Because of the life-long duration of the drug craving, addicts cannot maintain abstinence from drugs of abuse and are very likely to relapse in spite of their best efforts and intentions [1]. More importantly, stress, negative mood states, and drug-related environmental stimuli prompt relapse [2].

Buprenorphine was approved by the United States Food and Drug Administration for the detoxification and maintenance treatment of opioid addiction. Compared with full agonists such as

methadone, buprenorphine carries less risk of overdose because of its partial agonist nature, and it is the first medication for the addiction treatment that is authorized for prescription by certified physicians. In addition, because it is a partial agonist, buprenorphine can attenuate the subjective effects of illicit opioids while having a low potential for abuse [3,4]. However, although it has a longer duration of action than many other opioids, buprenorphine in its typical sublingual formulation still requires daily or alternate-day dosing [5,6], which increases patient inconvenience and overall medical costs.

Thienorphine is a novel buprenorphine analogue under development as a treatment for opioid dependence [7]. Competitive receptor binding and [³⁵S]GTP γ S binding assays showed that thienorphine was a potent partial agonist at μ - and κ -opioid receptors [8,9]. In rodent models, thienorphine showed long-lasting agonist and antagonist effects. The antinociceptive effect of thienorphine lasted for more than 8 h after subcutaneous administration. In addition, the antagonist effect of thienorphine on morphine-induced lethality remained significant 15 days after a single

intragastric administration, whereas the effect of buprenorphine lasted for less than 2 days [8]. In an attempt to better understand the unique pharmacological profile of thienorphine, [^3H]thienorphine was synthesized, and its association and dissociation kinetics were determined in membrane preparations expressing cloned rat μ - and κ -opioid receptors. We hypothesized that slowed receptor kinetics may contribute to the enhanced duration of action of thienorphine. In addition, we measured the ability of thienorphine to modulate the number of membrane μ -receptors after prolonged exposure. Finally, tissue bioassay and *in vivo* experiments were performed to confirm the long duration of thienorphine action.

Materials and methods

Animals

Male and female guinea pigs weighing 250–300 g and female Kunming mice weighing 18–22 g were supplied by Beijing Animal Center (Beijing, China) and maintained on a 12-h light/dark cycle. Animals had free access to food and water. All experimental procedures were conducted in accordance with the guidelines for the use of experimental animals approved by the local ethical committee and the Institutional Review Committee on Animal Care and Use.

Drugs and Materials

Thienorphine, buprenorphine, and naltrexone were synthesized in our institute. [^3H]thienorphine (52.0 Ci/mmol, 2.0 mCi/mL) was synthesized by the China Institute of Atomic Energy (Beijing, China). The purity of [^3H]thienorphine, as determined by HPLC, was >95%. Rabbit anti- μ -receptor antibody was developed in our institute. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was purchased from Zhongshan Goldenbridge Biotechnology Co., Ltd. (Beijing, China). The following materials were obtained as indicated: Dulbecco's modified Eagle's medium and geneticin (Gibco, Grand Island, NY, USA); fetal bovine serum (HyClone, South Logan, UT, USA); morphine (Qinghai Pharmaceutical Factory, Xining, China); and Tris (Sigma, St. Louis, MO, USA).

Cell Culture and Membrane Preparation

Chinese hamster ovary (CHO) cells stably expressing the rat μ - and κ -opioid receptors were cultured in Dulbecco's modified Eagle's medium supplemented with 100 U/mL penicillin, 100 U/mL streptomycin, 200 $\mu\text{g}/\text{mL}$ geneticin, and 10% fetal bovine serum at 37°C with humidified atmosphere consisting of 95% air and 5% CO_2 .

Cell membranes were prepared as previously [10], with minor modifications. In brief, cells were harvested in Versene solution (EDTA 0.54 mM, NaCl 140 mM, KCl 2.7 mM, Na_2HPO_4 8.1 mM, KH_2PO_4 1.46 mM, and glucose 1 mM) and collected by centrifugation (500 *g*, 5 min). The cell pellets were suspended in lysis buffer (5 mM Tris-HCl, 5 mM EDTA, 5 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4), passed through a 26G3/8-syringe needle five times, and then centrifuged at 24,000 *g* for 25 min. The pellets were resuspended in lysis buffer and then passed through the syringe needle and centrifuged again as described above. The final pellets were resuspended in a 50 mM Tris-HCl buffer (pH

7.4), and protein concentration was determined by Bradford method. All procedures were performed at 4°C.

Saturation Binding Assays

Membrane protein prepared from CHO- μ or CHO- κ cells was incubated in 50 mM Tris-HCl buffer (pH 7.4) containing a series of concentrations of [^3H]thienorphine in a total volume of 0.5 mL for 30 min at 37°C. Nonspecific binding was determined with 10 μM unlabeled thienorphine. The binding was terminated by inserting the assay tubes into ice-cold water, and the free and membrane-bound radioligands were separated by rapid filtration through GF/C filters. The filters were washed with 4 mL of ice-cold Tris-HCl buffer three times, and filter-bound radioactivity was counted by a liquid scintillation counter.

Binding Kinetics Assays

In association experiments, membrane protein was incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 1.0 nM [^3H]thienorphine in a total volume of 0.5 mL at 37°C for various times up to 60 min. The incubations were terminated by rapid filtration through GF/C filters. In dissociation experiments, membrane protein was incubated in 50 mM Tris-HCl buffer (pH 7.4) containing 1.0 nM [^3H]thienorphine in a total volume of 0.5 mL at 37°C for 30 min to reach equilibrium binding. Dissociation was initiated by addition of 10 μM unlabeled thienorphine. The membrane-bound [^3H]thienorphine was determined at different times by rapid filtration.

Flow Cytometric Analysis

Flow cytometric analysis was used to determine the effects of compounds on the surface μ -opioid receptor number. The CHO- μ cells were treated with Dulbecco's modified Eagle's medium alone or with various opioid compounds for 24, 48, and 72 h at 37°C. The cells were then chilled on ice and harvested in 1 mM EDTA/PBS. Cells were then stained with rabbit anti- μ -receptor antibody (1:100 dilution) in 3% BSA/PBS for 2 h at 4°C and then with FITC-conjugated goat anti-rabbit IgG (1:50 dilution) for 1 h at 4°C. Finally, cells were washed twice with PBS and fixed with 4% paraformaldehyde, and receptor immunofluorescence was analyzed by FACScan (BD Biosciences, Palo Alto, CA, USA). The mean fluorescence intensity of 10,000 cells is used for comparison of different samples.

Isolated Tissue Bioassay

Ileal segments were prepared from drug-naïve guinea pigs as described previously [11]. The preparations were suspended under 1 g tension in a 12-mL organ bath containing Krebs' solution at 37°C and continuously bubbled with 95% O_2 and 5% CO_2 . Tension changes were recorded using an isometric transducer coupled to a multichannel polygraph. A basal response to acetylcholine (1.0 μM) was recorded at the start of each experiment. Following 5-min contact of preparations with opioid compounds, their responses to acetylcholine were measured at different times after prolonged washing, and the time course curves were generated.

Antinociceptive Test

The prolonged antagonist effects of thienorphine were assessed in mice using hot plate test as described previously [8]. In brief, female mice were individually placed on the surface of a hot plate (HUGO SACHS Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) maintained at $55 \pm 0.1^\circ\text{C}$, and the latency was recorded as the duration from starting to the endpoint of jumping, licking, or shaking the hind paws. A cutoff time of 60 s was imposed to prevent the possibility of tissue damage. Mice were injected subcutaneously with thienorphine 1.8 mg/kg or morphine 6.6 mg/kg, which produced equivalent antinociception in a preliminary study, and then the antinociceptive effects of morphine 20 mg/kg s.c. were measured at different times thereafter (1, 3, 7, and 10 days).

Data Analysis

All binding data were analyzed using nonlinear regression analysis with Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). For the saturation binding assay, the K_d and $B_{\max}$ values were determined using a one-site binding equation. For the binding kinetics assay, the observed association rate constant (K_{ob}) was determined by fitting the specific binding data to the monoexponential equation $Y = Y_{\max} (1 - \exp(-K_{\text{ob}} t))$, where t is the time from the onset of association and $Y_{\max}$ is the maximum specific binding at this concentration. The fast dissociation rate constants (K_{-1}) and slow dissociation rate constants (K_{-2}) were calculated from two-phase exponential decay analysis: $Y = \text{Span1} \times \exp(-K_{-1} t) + \text{Span2} \times \exp(-K_{-2} t) + \text{Plateau}$, where t is the time from the onset of dissociation. Antinociceptive data obtained in the hot plate test are presented as a percentage of the maximum possible effect (% MPE) following the equation of % MPE = (postdrug latency – predrug latency)/(cutoff time – predrug latency) $\times 100\%$. Multiple comparisons were performed by one- or two-way ANOVA followed by Bonferroni post tests. The level of statistical significance was defined as $P < 0.05$.

Results

Saturation Binding of [^3H]thienorphine to μ - and κ -Opioid Receptors

The specific binding of [^3H]thienorphine to μ - and κ -opioid receptors was saturable. A one-site binding model provided a good fit for both binding curves (Figure 1A,B). [^3H]thienorphine exhibited high affinities for μ - and κ -opioid receptors with K_d values of 0.52 and 0.48 nM, respectively (Table 1). These results are consistent with the reported K_i values of thienorphine as determined by competitive binding assays [8]. The $B_{\max}$ values of [^3H]thienorphine binding to μ - and κ -opioid receptors were 5.89 and 6.87 pmol/mg protein, respectively (Table 1).

Kinetics of [^3H]thienorphine Binding to μ - and κ -Opioid Receptors

A 1.0 nM concentration of [^3H]thienorphine was used in the association and dissociation kinetics studies. Saturation binding studies showed that this concentration of [^3H]thienorphine resulted in

60–70% occupancy of both receptors. The kinetic studies revealed rapid association of [^3H]thienorphine when incubated with CHO- μ and CHO- κ cell membrane proteins at 37°C . The specific binding of [^3H]thienorphine reached equilibrium after a 10-min incubation, and the data could be fitted by a monoexponential function. The K_{ob} values for μ - and κ -opioid receptors were determined to be 0.34 and 0.57 min^{-1} , respectively (Figure 1C,E; Table 2).

Dissociation of specifically bound [^3H]thienorphine was initiated by the addition of 10 μM unlabeled thienorphine. Analysis of the dissociation kinetics revealed a biphasic behavior. The dissociation rate constants for the fast phase and the slower phase, respectively, were 0.21 ($t_{1/2} = 3.3 \text{ min}$) and 0.0078 ($t_{1/2} = 89.4 \text{ min}$) for the μ -receptor and 0.17 ($t_{1/2} = 4.1 \text{ min}$) and 0.0042 ($t_{1/2} = 166.6 \text{ min}$) for the κ -receptor. The slow dissociation of [^3H]thienorphine from both receptors accounted for ~60% of the total binding (Figure 1D,F; Table 2). These results indicated that the dissociation of [^3H]thienorphine was sluggish, which to some extent explains the long-lasting effects of thienorphine in *in vivo* rodent experiments.

Regulation by Thienorphine of Surface μ -Opioid Receptor Number

To exclude the competitive occupation of receptor binding sites by residual opioid compounds, which may occur when determining receptor number by radioligand binding assays, the effects of prolonged exposure to compounds on the number of cell surface μ -receptor were studied by fluorescence flow cytometry. In CHO- μ cells, 24 h treatment with morphine induced a 37% decrease in surface receptor number, whereas buprenorphine or naltrexone significantly increased receptor number (Figure 2A). These results are in accordance with a previous study that reported that 18-h treatment of cells with buprenorphine or naloxone produced a significant increase in surface receptor number [12]. Despite its similar activity at the μ -receptor, the effect of 24 h thienorphine treatment on receptor regulation differed from that of buprenorphine. At concentrations of 0.1–10 μM , thienorphine dose-dependently decreased the surface receptor number, with a maximum decrease of 34%. In cells treated with various opioid compounds for 48 h, the fluorescence intensity was similar to that after 24 h treatment, with the exception that there was a significant decrease in fluorescence intensity in buprenorphine-treated cells (Figure 2B). After 72 h treatment, buprenorphine-treated cells showed a significant decrease in surface receptor number, whereas the effects of morphine and naltrexone were stable. Longer treatment with thienorphine resulted in a greater decrease in surface receptor number. The maximum effect of thienorphine (43% decrease in receptor number) was reached in cells treated for 72 h with a concentration of 10.0 μM , and treatment with 0.1 μM thienorphine also induced a significant decrease after 72-h incubation (Figure 2C).

Prolonged Inhibition by Thienorphine of Acetylcholine-Induced Contraction in Guinea Pig Ileum

The duration of action of thienorphine was compared with that of buprenorphine and morphine in isolated preparations of guinea

Figure 1 Radioligand binding analysis of $[^3\text{H}]$ thienorphine with membranes prepared from CHO cells stably expressing rat μ - or κ -opioid receptors. Saturation binding to μ - (A) or κ -opioid (B) receptors was performed with increasing $[^3\text{H}]$ thienorphine concentrations (0.1–3.0 nM). Nonspecific binding was determined with 10 μM unlabeled thienorphine. (C) Association of $[^3\text{H}]$ thienorphine with μ -opioid receptors was determined by incubating membrane protein with 1.0 nM $[^3\text{H}]$ thienorphine for various times up to 60 min. (D) Dissociation of $[^3\text{H}]$ thienorphine from μ -opioid receptors was initiated by the addition of 10 μM unlabeled thienorphine after 30 min incubation. (E) Association of $[^3\text{H}]$ thienorphine with κ -opioid receptors was determined by incubating membrane protein with 1.0 nM $[^3\text{H}]$ thienorphine for various times up to 60 min. (F) Dissociation of $[^3\text{H}]$ thienorphine from κ -opioid receptors was initiated by the addition of 10 μM unlabeled thienorphine after 30 min incubation. Data are expressed as the mean $\pm$ SEM of three separate experiments. The K_d and B_{max} are summarized in Table 1. The observed association rate constant (K_{ob}) and dissociation rate constants K_{-1} and K_{-2} are summarized in Table 2.

Table 1 Saturation binding of [3 H]thienorphine to membranes prepared from CHO- μ and CHO- κ cells. Membranes were incubated with [3 H]thienorphine (0.10–3.000 nM) for 30 min at 37°C. Nonspecific binding was determined by including 10 μ M unlabeled thienorphine. K_d and B_{max} were calculated by fitting the data to a one-site binding model using nonlinear regression

	K_d (nM)	B_{max} (pmol/mg protein)
CHO- μ	0.52 ± 0.06	5.89 ± 0.24
CHO- κ	0.48 ± 0.09	6.87 ± 0.41

Data are expressed as the mean $\pm$ SEM of three separate experiments.

Table 2 Association and dissociation of specific [3 H]thienorphine binding in membranes prepared from CHO- μ and CHO- κ cells. The association of [3 H]thienorphine was determined by incubating [3 H]thienorphine with cell membranes at 37°C for various times up to 60 min. The observed association rate constant (K_{ob}) was calculated by fitting the data to the monoexponential equation using nonlinear regression. The dissociation of the binding of [3 H]thienorphine was initiated by the addition of 10 μ M unlabeled thienorphine after 30 min incubation. The fast dissociation rate constant (K_{-1}) and the slow dissociation rate constant (K_{-2}) were calculated from two-phase exponential decay analysis using nonlinear regression. P_{-1} and P_{-2} represent the percentage of specific binding that dissociates with K_{-1} and K_{-2} , respectively

	K_{ob} (min^{-1})	K_{-1} (min^{-1})	P_{-1} (%)	K_{-2} (min^{-1})	P_{-2} (%)
CHO- μ	0.34 ± 0.03	0.21 ± 0.08	41 ± 7	0.0078 ± 0.0022	59 ± 6
CHO- κ	0.57 ± 0.08	0.17 ± 0.04	41 ± 5	0.0042 ± 0.0016	59 ± 5

Data are expressed as the mean $\pm$ SEM of three separate experiments.

pig ileum. As shown in Figure 3, 4.0 mM morphine, 0.16 mM buprenorphine, or 0.1 mM thienorphine produced comparable inhibition of acetylcholine-induced contraction at the start of washout. After prolonged washing, the effects of morphine diminished rather rapidly, with no detectable effect remaining at 10 min. The duration of action of buprenorphine was longer than that of morphine, but its effect also diminished in less than 40 min. Compared with morphine and buprenorphine, the effect of thienorphine persisted much longer, with acetylcholine-induced contraction returning quite slowly toward the pretreatment level, and more than 30% of the effect remaining after 120 min washing. Therefore, in this model, the duration of action of thienorphine is more than three times longer than that of buprenorphine.

Prolonged Antagonism by Thienorphine of Morphine-Induced Antinociception

Morphine-pretreated mice, reinjected with 20 mg/kg morphine 1, 3, 7, and 10 days later, showed a consistent sharp increase in pain

Figure 2 Effects of prolonged treatment with thienorphine on surface μ -opioid receptor number. CHO- μ cells were pretreated with various concentrations of drugs (Thie: thienorphine, Bup: buprenorphine, NTX: naltrexone, Mor: morphine) for 24 h (A), 48 h (B), and 72 h (C) at 37°C. Surface receptor number was determined by FACSscan analyses as described in Materials and Methods. Data are expressed as the mean $\pm$ SEM of three separate experiments. * $P < 0.05$, compared with control cells. # $P < 0.05$, compared with the cells treated with the respective compound for 24 h.

thresholds, indicating that no tolerance to morphine developed under this experimental design. However, morphine-induced antinociception was attenuated in thienorphine-pretreated mice across the observation period. Significant decreases were evident 1, 3, and 7 days after thienorphine treatment (Figure 4).

Discussion

The extremely long duration of action of thienorphine is unique among opioid compounds. It was demonstrated previously that the agonist effect of thienorphine, which is reflected in its antinociceptive effect, lasted for more than 8 h in mice using hot plate test, and its antagonist effect remained significant 15 days after a single oral administration [8]. The long-lasting *in vivo* effect of thienorphine was confirmed in this study by the demonstration that thienorphine pretreatment antagonized morphine-induced antinociception for more than 7 days. Although thienorphine and its metabolites were demonstrated to have a relatively long residence time, pharmacokinetic factors are not sufficient to explain the

Figure 3 Time course of the inhibition by thienorphine of acetylcholine-induced contraction in guinea pig ileum during prolonged washing. Ileum contractions stimulated by acetylcholine ($1.0 \mu\text{M}$) were recorded as the basal responses. Following 5-min contact of preparations with 4.0 mM morphine (Mor), 0.16 mM buprenorphine (Bup), or 0.1 mM thienorphine (Thie), their responses to acetylcholine were measured at different times after prolonged washing. Data are expressed as the mean $\pm$ SEM of percent change in response, $n = 5$. * $P < 0.05$, compared with basal responses to acetylcholine (repeated one-way ANOVA followed by Bonferroni posttests).

long duration of action [13]. The present study explored the pharmacodynamic mechanisms, showing that thienorphine was reluctant to dissociate from opioid receptors and that prolonged treatment with thienorphine downregulated the number of μ -opioid receptors on the cell surface. These results are in good agreement with those obtained from isolated tissue and *in vivo* experiments. Therefore, the persistent occupation of opioid receptors and regulation of the μ -receptor number after prolonged exposure may account in part for the long-lasting effects of thienorphine.

Our analysis of the dissociation of [^3H]thienorphine from opioid receptors revealed that the curves fit better with biphasic kinetics rather than single-phase kinetics. In principle, biphasic kinetics could be due to either the existence of different receptor–ligand complexes or multiple binding sites on one receptor. However,

Figure 4 Time course of the antimorphine effects of thienorphine. Mice were pretreated with 1.8 mg/kg thienorphine or 6.6 mg/kg morphine subcutaneously, and the antinociception induced by 20 mg/kg s.c. morphine was tested at different times thereafter. Data are expressed as the mean $\pm$ SEM, $n = 10$. * $P < 0.05$, compared with morphine-pretreated mice (repeated two-way ANOVA followed by Bonferroni posttests).

the single-phase association of [^3H]thienorphine with μ - and κ -opioid receptors suggested that these opioid receptors are uniform and that there is only one binding site in each experimental system. Previous competitive binding results also lend support to this assumption, with only one K_i value being determined for each opioid receptor [8]. One possible explanation for our results is that part of the initially formed receptor–thienorphine complex could be converted to another form displaying a much slower dissociation rate. Thus, thienorphine association measurements primarily reflect the formation of the initial complex, whereas dissociation measurements reflect the contributions of two distinct receptor–ligand complexes.

A limitation of this study is the lack of a direct comparison between [^3H]thienorphine and [^3H]buprenorphine in the receptor binding kinetics study. According to a previous report, the half-lives of the dissociation of [^3H]buprenorphine from a rat brain membrane preparation were 5.6 min for the fast phase and 166.4 min for the slow phase, and the author speculated that the sluggish dissociation of buprenorphine from the opioid receptors accounted for its long-acting effect [14]. However, it should be noted that these experiments were conducted at 25°C , and it has been shown that temperature affects receptor binding kinetics. For example, the half-lives of the fast and slow dissociation of [^3H]fentanyl were 0.4 min and 6.8 min , respectively, at 25°C [14], whereas at 37°C , the dissociation rates were markedly enhanced, with half-lives of $\sim 5 \text{ sec}$ for the fast phase and 1.15 min for the slow phase [15]. Taking this into account, the dissociation of thienorphine is quite likely to be more sluggish than that of buprenorphine, as was suggested by the present and previous *in vivo* observations [8].

According to drug receptor theory, changes in the receptor number on the cell surface directly impact the effect of a drug [16]. Therefore, downregulation of the number of μ -receptors may contribute to a decrease in the effect of agonists. It has been demonstrated that chronic treatment with opioids such as morphine and etorphine altered the μ -receptor number in cell lines and in *in vivo* models and that the reduction in μ -receptor number represented one important mechanism of opioid tolerance [17–21]. In this study, incubation with morphine for 24, 48, and 72 h downregulated the receptor number in CHO- μ cells, while naltrexone and buprenorphine upregulated the receptor number after 24 h incubation. These results are in agreement with previous reports [12,18]. We also found that buprenorphine could induce receptor downregulation when the incubation time was extended to 72 h. Despite their similarity in chemical structure and pharmacological activities, the effects of thienorphine and buprenorphine on surface μ -receptor number differed. Thienorphine induced dose-dependent downregulation of μ -receptor after 24-h treatment, and its effect increased with incubation time. The effect of thienorphine on surface receptor number was consistent with the rapid tolerance observed in the analgesic study [8]. Therefore, thienorphine tends to downregulate μ -receptor, which may play a role in the decrease in its agonist effects and the persistence of its antagonist effects.

In the treatment of addiction, a long duration of therapeutic action gives power, not only in terms of persistent protection of patients from illicit drugs and therefore reducing the incidence of related infection and crime, but also in terms of

induction of a normalization of body functions disrupted by addictive drugs [22]. Duration of action is even more important for an antagonist-based treatment such as naltrexone, because it greatly affects adherence to treatment. These considerations have led to the development of extended release formulations for addiction treatment. Implantable formulations of naltrexone or buprenorphine can provide 6-month sustained release of the drug, allowing patients to reduce the frequency of visits to the clinic or pharmacy [23–25]. However, these formulations have their limitations as medications for the treatment of addiction. Many patients experience local adverse effects, such as pain, infection, and allergic reactions, which can result in discontinuation of treatment [24,25]. More importantly, the implant can be felt, and the possibility of deliberate removal still exists. As an alternative strategy to lengthen the duration of action of a treatment, continuous efforts have been made to lengthen the intrinsic duration of action of opioid compounds by chemical modifications. Long-acting effects were observed for some structures based on buprenorphine [26–28], and it was speculated that a strengthened ligand–receptor interaction and hence slowed dissociation kinetics could be the possible mechanism. Thienorphine, created by a thiophene substitution, provides another example of the development of long-acting buprenorphine analogues for the treatment of addiction.

In conclusion, the present study showed that thienorphine exhibits a long duration of action and that multiple factors may contribute to this. In addition to the pharmacokinetic factors reported previously, persistent receptor occupation and downregulation of surface receptors presented two pharmacodynamic mechanisms at the receptor level. The extremely long-acting effect of thienorphine may be beneficial for its application in the treatment of addiction.

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Conflict of Interest

The authors declare no conflict of interests.

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