Pharmacological mechanisms underlying the antinociceptive and tolerance effects of the 6,14-bridged oripavine compound 030418

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Aim: To investigate possible pharmacological mechanisms underlying the antinociceptive effect of and tolerance to N-methyl-7α-[((R)-1-hydroxy-1-methyl-3-(thien-3-yl)-propyl]-6,14-endo-ethanotetrahydronororipavine (030418), a derivative of thienorphine.

Methods: The binding affinity and efficacy of 030418 were determined using receptor binding and guanosine 5′-O-[3-[35S]thio]triphosphate ([35S]GTPγS) assays in CHO-μ, CHO-κ, CHO-δ, and CHO-ORL1 cell membranes. The analgesic activity of and tolerance to 030418 were evaluated in thermal nociceptive tests in mice. The effects of 030418 on opioid receptors were further investigated using in vivo pharmacological antagonist blockade and in vitro tissue preparations.

Results: The compound 030418 displayed high binding affinity to all subtypes of opioid receptors with \( K_i \) values in the nanomolar range. In \([35S]GTP\gamma\S\) binding assay, the maximal stimulation of 030418 to μ-, κ-, δ-receptors and the ORL1 receptor was 89%, 86%, 67% and 91%, respectively. In hot-plate test, the antinociceptive effect of 030418 was more potent and longer than morphine. The nonselective opioid receptor antagonist naloxone could completely block 030418-induced antinociception, while both the μ-opioid receptor antagonist β-FNA and the κ-opioid receptor antagonist nor-BNI attenuated 030418-induced antinociception. In contrast, the ORL1 receptor antagonist J-113397 enhanced the antinociceptive effect of 030418. Additionally, chronic treatment with 030418 resulted in a dramatic development of tolerance that could not be effectively prevented by J-113397. In guinea pig ileum preparation, the existing action of 030418 could be removed with difficulty after prolonged washing.

Conclusion: The compound 030418 is a novel agonist of opioid receptors with high efficiency, long-lasting effect and liability to tolerance, which may be closely correlated with the methyl group at the N17 position and the high hydrophobicity of the C7-thiophene group in its chemical structure.

Keywords: 030418; opioid receptor; oripavine; antinociception; drug tolerance

Introduction

Although opioid-derived alkaloids, particularly morphine, are the standard analgesic drugs of choice in the treatment of moderate to severe pain, these compounds cause various adverse reactions\(^5,\,2\). This observation implies that developing other analgesics that exhibit high potency and fewer side effects as alternatives to morphine is still a challenge that remains for pharmacologists and medicinal chemists\(^6\). The 6,14-bridged oripavines, which were originally derived from the Diels-Alder adducts of thebaine in the 1960s by Bentley and Hardy\(^4,\,5\), have been studied extensively to yield a rich source of resultant high-affinity opioid ligands\(^6\). Among these ligands, a number of important opioid drugs have emerged, such as etorphine/dihydroetorphine, diprenorphine, and particularly buprenorphine. In fact, buprenorphine is still used as an analgesic and is an effective alternative to methadone in the treatment of opiate dependence\(^7-10\).

Many compounds obtained from the structural modification of buprenorphine have also been extensively developed\(^6,\,11,\,12\). Inspired by these reports, N-cyclopropylmethyl-7α-[(R)-1-hydroxy-1-methyl-3-(thien-3-yl)-propyl]-6,14-endo-ethanotetrahydronororipavine (thienorphine, Figure 1) was synthesized in our institute\(^13\). Previous studies have found that thienorphine has a higher potency, longer duration of action, and better oral bioavailability than buprenorphine\(^14,\,15\). Therefore, thienorphine has a pharmacological profile that indicates its use as a potential treatment for opiate abuse. However, thienorphine has been demonstrated to be a partial κ-opioid receptor agonist with low efficacy at the μ-opioid receptor\(^16\),
which might cause poor compliance in addicts.

To obtain more highly efficacious compounds, a series of thienophene derivatives that possess a thiophene group at the C7 position have been further synthesized and assessed. In primary animal experiments, one of these derivatives, N-methyl-7α-[(R)-1-hydroxy-1-methyl-3-(thien-3-yl)-propyl]-6,14-endo-ethanotetrahydronororipavine (030418, Figure 1), seemed to show powerful antinociceptive effects. In addition, 030418 exhibited a very low dependence liability in naloxone precipitated withdrawal, conditioned place preference in mice and a self-administration model in rats (unpublished data).

Although 6,14-bridged oripavines that possess the complex substituents at the C7 position have been previously reported to have high binding affinities for opioid receptors, the pharmacological mechanisms of 030418, which also belongs to the 6,14-bridged oripavine compounds, have not been studied. The present study systematically examined the binding affinity, stimulating potency, and efficacy of 030418 to the µ-, κ-, δ-opioid receptors and the opioid receptor-like 1 receptor (ORL1 receptor) using receptor binding and a [35S]GTPγS assay in Chinese hamster ovary (CHO) cell membrane homogenates. In addition, the antinociceptive effects of and tolerance to 030418 and the gold standard morphine were comparatively evaluated in thermal stimuli-induced nociceptive tests in mice. The pharmacological properties of 030418 on opioid receptors were further investigated using in vivo pharmacological blockade of opioid receptor antagonists and in vitro guinea pig ileum preparations. These investigations illustrate the pharmacological properties and mechanisms of the antinociceptive effect of and tolerance to 030418.

Materials and methods

Materials

Compounds including 030418 hydrochloride (purity ≥99%) and thienophene hydrochloride were synthesized at our institute. Morphine hydrochloride, dihydroetorphine hydrochloride, and buprenorphine hydrochloride were purchased from Qinghai Pharmaceutical Factory (Xining, China). DAMGO, (±)U50488, SNC80, N/OFQ, naloxone, nor-BNI, and GDP were purchased from Sigma Chemical Co (St Louis, MO, USA), while J-113397 and β-FNA were purchased from Tocris Bioscience (Bristol, UK). [3H]diprenorphine (1.85 TBq/mmol) and [35S]GTPγS (46.25 TBq/mmol) were obtained from PerkinElmer Inc (Boston, MA, USA), and [3H]nociceptin/OFQ (5.70 TBq/mmol) was obtained from Amersham Corp (Buckinghamshire, UK). GTPγS was purchased from Biolog Life Science Institute (Bremen, Germany). F12 medium, fetal bovine serum, and G418 were obtained from Gibco (Grand Island, NY, USA) and GF/C filters from Whatman (Maidstone, UK).

Animals

Male and female Kunming mice (18–22 g, 7 weeks of age) and male Hartley guinea pigs (350–400 g, 3 months of age) were supplied by the Beijing Animal Center and maintained on a 12-h light/dark cycle (lights on between 7:00 AM and 7:00 PM). Animals were allowed access to food and water ad libitum. All experimental procedures were conducted in accordance with the Guidelines for the Use of Experimental Animals and were approved by the local ethical committee and the Institutional Review Committee on Animal Care and Use.

CHO cell line and membrane preparation

CHO cells stably expressing the rat µ-, human κ-, and human δ-opioid receptors and the human ORL1 receptor have previously been established in our laboratory. These cells were cultured in F12 medium supplemented with 100 units/mL penicillin, 100 µg/mL streptomycin, 200 µg/mL Geneticin (G418), and 10% fetal bovine serum at 37°C in a humidified atmosphere consisting of 95% air and 5% CO2. Cell membranes were prepared using a modified procedure of Zhu et al [20]. Briefly, the cells were harvested and centrifuged at 500×g for 5 min and the cell pellet was then suspended in Lysis buffer (5 mmol/L Tris, 5 mmol/L EDTA, 5 mmol/L EGTA, and 0.1 mmol/L phenylmethylsulfonyl fluoride, pH 7.4). The cells were passed through a 29-gauge 3/8 syringe needle five times and then centrifuged. The pellet was resuspended in Tris-HCl buffer (50 mmol/L Tris, pH 7.4) and centrifuged again, whereupon this process was repeated. Subsequently, the membrane pellet was resuspended in Tris-HCl buffer (50 mmol/L Tris, pH 7.4). The protein concentration was determined using the Bradford method [21] with bovine serum albumin as the standard. All the above procedures were performed at 4°C.

Receptor binding assay

Competitive inhibition of [3H]diprenorphine (1 nmol/L) binding to the µ-, κ-, and δ-opioid receptors or [3H]nociceptin/OFQ (0.5 nmol/L) binding to the ORL1 receptor was performed in the absence or presence of various concentrations of 030418 or thienorphine. Binding was carried out in 50 mmol/L Tris-HCl buffer (pH 7.4) at 37°C for 30 min in duplicate in a final volume of 0.5 mL with 20–40 µg of membrane protein.

Figure 1. Chemical structures of the 6,14-bridged oripavine compounds 030418, thienorphine, and dihydroetorphine.
Naloxone (5 µmol/L) and N/OFQ (5 µmol/L) were used to define nonspecific binding. Subsequently, bound and free \[^{[3]}H\]diprenorphine or \[^{[3]}H\]nociceptin/OFQ were separated by filtration under reduced pressure with GF/C filters presoaked with 0.2% polyethyleneimine. The radioactivity on the filters was determined by liquid scintillation counting (LS6500, Beckman Inc, USA). Each experiment was performed in duplicate and repeated at least three times. The competitive binding data were fitted to sigmoidal curves for the determination of IC\(_{50}\) values. The K\(_{d}\) values of each drug were derived from the IC\(_{50}\) values using the following equation: K\(_{d}\)=IC\(_{50}\)/[1+\[^{[3]}H\] ligand/Km]. Based on our previous experiments, the K\(_{d}\) values of \[^{[3]}H\]diprenorphine to the \(\mu\)-, \(\kappa\)-, and \(\delta\)-opioid receptors and \[^{[3]}H\]nociceptin/OFQ to the ORL1 receptor were 0.56 nmol/L, 0.55 nmol/L, 0.46 nmol/L, and 0.29 nmol/L, respectively.

\[^{[25]}S\]GTP\(_{S}\) binding assay

Membrane protein (15–50 µg) was incubated with different concentrations of 030418, thienorphine, or dihydroetorphine (10\(^{-13}\) to 10\(^{-8}\) mol/L) in buffer A (100 mmol/L Tris, 100 mmol/L NaCl, and 5 mmol/L MgCl\(_2\) pH 7.4) containing \[^{[25]}S\]GTP\(_{S}\) (200 pmol/L) and GDP (20–40 µmol/L) in a total volume of 0.5 mL at 30 °C for 60 min. Nonspecific binding was defined by incubation in the presence of 20 µmol/L GTP\(_{S}\). Bound and free \[^{[25]}S\]GTP\(_{S}\) was separated by filtration with GF/C filters under reduced pressure and rinsed three times with ice-cold buffer B (50 mmol/L Tris-HCl, 50 mmol/L NaCl, and 5 mmol/L MgCl\(_2\) pH 7.4). The radioactivity on the filters was determined by liquid scintillation counting. The maximal stimulation of each of the selective agonists DAMGO (\(\mu\)), (\(\pm\))U50488 (\(\kappa\)), SNC80 (\(\delta\)), and N/OFQ (ORL1) was defined as the 100% effect in this experiment. Each experiment was performed in duplicate and repeated at least three times. The concentration-response data were fitted to sigmoidal curves for the determination of EC\(_{50}\) values and the maximal effects (E\(_{max}\)).

Tail-flick test

The mouse tail-flick test was adapted for measuring the latency of the tail-flick response\(^{[22]}\). Mice were gently held by hand with their tail positioned in an apparatus (Tail-flick Analgesia Meter, Columbia instruments, OH, USA) for radiant heat stimulus on the surface of the tail. The intensity of the heat stimulus was adjusted so that the animal flicked its tail after 3 to 5 s for a baseline reading. The inhibition of the tail-flick response was expressed as a percentage of maximal possible effect (%MPE), which was calculated as %MPE=[(T\(_{1}\)–T\(_{0}\))/(T\(_{2}\)–T\(_{0}\))]×100%, where T\(_{0}\) and T\(_{1}\) were the tail-flick latencies before and after the treatments, respectively, and T\(_{2}\) was the cut-off time, which was set at 10 s to avoid injury of the tail.

Hot-plate test

The mouse hot-plate test, as previously reported\(^{[23]}\), was used to assess the antinociceptive effects of the compounds. Female mice were individually placed on the surface of a hot plate (Hugo Sachs Elektronik-Harvard Apparatus GmbH, March-Hugstetten, Germany) maintained at 55±0.5 °C. The latency time for hind-paw licking was recorded as the nociceptive response. Mice with a baseline latency shorter than 5 s or longer than 30 s were eliminated. The antinociceptive data are also presented as %MPE calculated using the equation %MPE=[(T\(_{1}\)–T\(_{0}\))/(T\(_{2}\)–T\(_{0}\))]×100%, where T\(_{0}\) and T\(_{1}\) were the latency times of hind-paw licking before and after treatment, respectively, and T\(_{2}\) was the cutoff time, which was set at 60 s to avoid injury of the animal’s paw.

Dosage regimen and injection procedure in thermal nociception

To establish the dose-response curves in acute thermal nociception tests, mice (n=8–10 per group) received sc injections of 030418 alone (1.6–4.0 µg/kg for the tail-flick and 1.4–5.7 µg/kg for the hot-plate test). To compare the effects of 030418 with those of other opioid drugs, morphine (0.7–9.9 mg/kg/sc for tail-flick and 1.3–10.0 mg/kg/sc for hot-plate) and dihydroetorphine (0.25–0.9 µg/kg/sc for tail-flick and 1.3–2.9 µg/kg/sc for hot-plate) dose-response curves for thermal antinociception were determined using parameters similar to those described above. The animals were tested for response latency 30 min after administration of 030418, morphine, or dihydroetorphine.

To determine the duration of antinociceptive action during the hot-plate test, mice were administered 030418 (6.0 µg/kg, sc), dihydroetorphine (3.0 µg/kg, sc), or morphine (10 mg/kg, sc) and tested at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 10, and 12 h after injection. The doses of 030418, dihydroetorphine, and morphine were chosen because they produced the greatest level of antinociception of the doses tested.

To determine the effects of different opioid receptor antagonists on the analgesic effects produced by 030418 during the hot-plate test, the animals were pretreated with the nonselective opioid antagonist naloxone (1 mg/kg, sc), the \(\mu\)-opioid receptor antagonist β-FNA (10 µg/mouse, icv), and the \(\kappa\)-opioid receptor antagonist nor-BNI (3.7 µg/mouse, icv) before sc injection of 030418 (4.0 µg/kg, approximate ED\(_{50}\)). Animals were also pretreated with the ORL1 receptor antagonist J-113397 (4.0 µg/mouse, icv) before sc injection of 030418 (2.0 µg/kg, approximate ED\(_{50}\)). In the experiments, the opioid receptor antagonists naloxone, β-FNA, nor-BNI, and J-113397 were respectively administered 15 min, 24 h, 24 h, and 15 min prior to the agonists. The dosage and time intervals of these antagonists were based on previous experiments and reports\(^{[24–26]}\). The doses of the other opioid agonists were administered as follows: morphine (6.0 mg/kg, sc), DAMGO (50 ng/mouse, icv), (\(\pm\))U50488 (50 µg/mouse, icv), and buprenorphine (1.7 mg/kg, sc). Animals were tested for response latency 15 min after icv injection of DAMGO and (\(\pm\))U50488 or 30 min after sc injection of 030418, morphine, and buprenorphine. The control groups were given a corresponding volume of vehicle injection.

To establish a chronic analgesic tolerance model during the hot-plate test, animals received sc injections of high doses of 030418 (18.0 µg/kg, once daily), morphine (30.0 mg/kg, thrice daily), or vehicle for 7 consecutive days. The dosage regimen
was for the induction of drug tolerance, as previously reported by our laboratory \[^{27}\]. The antinociceptive effects were measured 30 min after administration of the opioid drugs every day to monitor the development of tolerance. In this chronic tolerance model, mice were pretreated with the ORL1 receptor antagonist J-113397 (4.0 µg/mouse, icv) or vehicle 15 min before the last injection of morphine or 030418 on d 7, and the animals were tested at different times after the administration of morphine (30.0 mg/kg, sc) or 030418 (18.0 µg/kg, sc) on d 1 and d 7, respectively.

**Isolated tissue bioassay**

The longitudinal muscle of guinea pigs was gently separated from the underlying circular muscle of the ileum using the method described by Paton and Vizi \[^{28}\] and placed under 1 g tension in a 5 mL organ bath containing Krebs-Henseleit solution (118 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl\(_2\), 1.2 mmol/L MgSO\(_4\), 1.2 mmol/L K\(_2\)HPO\(_4\), 25 mmol/L NaHCO\(_3\), and 10 mmol/L glucose). The bath was maintained at 37 °C and continuously bubbled with a mixture of 95% O\(_2\) and 5% CO\(_2\). Muscle contractions were recorded using an isometric transducer coupled to a multichannel polygraph. The data acquired from the samples stimulated by acetylcholine chloride (1.0 µmol/L) was recorded as the ileum muscle contraction response. A single 030418 (0.1 mmol/L), morphine (5 mmol/L), dihydroetorphine (0.2 mmol/L), or vehicle was added to the organ bath containing the tissue preparations (n=3–4 samples per group). The response of ileum muscle contractions to opioid drugs was expressed as %inhibition, which was calculated as %inhibition=[1−(muscle contractions after treatment)/(muscle contractions before treatment)]×100%. The tissue preparations were measured at different times during prolonged washing, and the time-course curves were generated.

**Statistical analysis**

All the data in this study are expressed as mean±SEM. The ED\(_{50}\) values with 95% confidence limits in the antinociceptive assays were calculated using the method described by Bliss \[^{29}\]. The competition binding data and concentration-response data in \[^{35}\]S\]GTP\(_{\gamma}\)S assays were fitted by nonlinear regression analysis using Origin 6.0 program (Northampton, MA, USA). The effects of the opioid receptor agonists and antagonists in the hot-plate tests were analyzed using one-way ANOVA followed by the SNK test. The time-course of the drug effects was analyzed using two-way ANOVA for repeated measures followed by Bonferroni-corrected Student’s \(t\) test. All statistical analyses were performed using SPSS 13.0 (SPSS Inc, Chicago, IL, USA), and \(P<0.05\) was the level of statistical significance.

**Results**

**Binding affinity of 030418 to opioid receptors and the ORL1 receptor**

As shown in Table 1, 030418 showed high binding affinities to all four subtypes of opioid receptors with \(K_i\) values for inhibit-

| Compound | \(\mu\) | \(\kappa\) | \(\delta\) | ORL1 |
|----------|-------|------|------|-----|
| 030418   | 0.91±0.05 | 0.60±0.28 | 0.58±0.21 | 1.6±0.4 |
| Thienorphine | 1.4±0.1 | 0.34±0.15 | 0.63±0.48 | 36.5±3.0 |

**Potency and efficacy of 030418 in the \[^{35}\]S\]GTP\(_{\gamma}\)S binding assay**

As controls for each receptor, the subtype selective, highly efficacious agonists DAMGO (\(\mu\)), (±)U50488 (\(\kappa\)), SNC80 (\(\delta\)), and N/OFQ (ORL1) increased the binding of \[^{35}\]S\]GTP\(_{\gamma}\)S in a concentration-dependent manner and produced maximal stimulations of 174%, 204%, 162%, and 287% over the basal level, respectively. The EC\(_{50}\) values and maximal effects (\(E_{\text{max}}\)) are shown in Figure 2 and Table 2. The compound 030418 produced a maximal stimulation of 89% on the \(\mu\)-opioid receptor, 86% on the \(\kappa\)-opioid receptor, 67% on the \(\delta\)-opioid receptor, and 91% on the ORL1 receptor. The EC\(_{50}\) values of 030418 for the \(\mu\), \(\kappa\), and \(\delta\)-opioid receptors and the ORL1 receptor were 0.10, 0.15, 0.38, and 15 nmol/L, respectively. Comparatively, thienorphine only partially activated the \(\mu\)- and \(\kappa\)- receptors and showed a maximal response of 28% and 65%, respectively. The EC\(_{50}\) values for thienorphine were in the nanomolar range, but this compound was inactive at the \(\delta\)-opioid receptor and the ORL1 receptor. In addition, dihydroetorphine exhibited full agonism at the \(\mu\)-, \(\kappa\)-, and \(\delta\)-opioid receptors and moderate, low-potency agonism at the ORL1 receptor.

**Antinociceptive effect of 030418**

According to the data shown in Figure 3, 030418 presented full opioid agonist characteristics with typical sigmoid dose-response curves in thermal stimuli-induced nociceptive models. In the mouse tail-flick test and hot-plate test, the antinociceptive ED\(_{50}\) (95% confidence limits) values of 030418 were calculated to be 2.39 µg/kg sc (2.26–2.52) and 2.90 µg/kg sc (2.42–3.37), respectively. In comparison, the ED\(_{50}\) (95% confidence limits) values of the gold standard morphine were 3.74 mg/kg sc (2.42–5.05) and 3.20 mg/kg sc (3.02–3.39), respectively. The ED\(_{50}\) (95% confidence limits) values of another pure full agonist, dihydroetorphine, were 0.66 µg/kg sc (0.61–0.72).
and 1.71 µg/kg sc (1.62–1.80), respectively. As shown in Figure 4, the maximally effective dose of 030418 (6.0 µg/kg, sc) had the longest duration of the antinociceptive effect in the mouse hot-plate test compared to that of dihydroetorphine (3.0 µg/kg, sc) and morphine (10.0 mg/kg, sc). This dose of 030418 maintained the maximal antinociceptive activity for at least 2 h after administration and was ineffective 10 h after administration.

### Effects of opioid receptor antagonists on antinociception of 030418

In the mouse hot-plate test, 030418 (4.0 µg/kg, sc), morphine...
(6.0 mg/kg, sc), DAMGO (50 ng/mouse, icv), and (±)U50488 (50 µg/mouse, icv) produced a %MPE of approximately 80%. The antinociceptive effects of 030418 and morphine could both be blocked by the nonselective opioid receptor antagonist naloxone (Figure 5A). The selective opioid antagonists β-FNA (10 µg/mouse, icv) and nor-BNI (3.7 µg/mouse, icv) could partially block the DAMGO- and (±)U50488-induced antinociception, respectively (Figure 5B and 5C). Pretreatment with the same doses of β-FNA and nor-BNI decreased the antinociceptive response to 030418 (4.0 µg/kg, sc) to 26% and 50%, respectively. As shown in Figure 5D, moderate doses of 030418 (2.0 µg/kg, sc) and buprenorphine (1.7 mg/kg, sc) respectively produced 64% and 33% of MPE in the hot-plate test. Co-administration of the ORL1 receptor antagonist J-113397 (4.0 µg/mouse, icv) enhanced the %MPE of 030418 to 90% and that of buprenorphine to 56%. In addition, these opioid antagonists administered alone did not alter the latency time in the hot-plate test because the mice that received these compounds exhibited similar levels of %MPE relative to vehicle control mice (data not shown).

Development of 030418 tolerance and the effect of pretreatment with J-113397
The development of tolerance to 030418 and morphine across 7 d in the mouse hot-plate test is shown in Figure 6A. Many previous studies have used high doses of opioid drugs to induce and study tolerance [27, 30, 31]. As expected, repeated treatment with morphine (30 mg/kg, sc, thrice daily) induced the rapid development of analgesic tolerance. The mice started to develop tolerance on d 4 of morphine injection and had only 45.7% %MPE on d 7. The 18 µg/kg dose of 030418 also produced a significant decrease in %MPE over time. Compared with d 1, 030418 produced a significant reduction in %MPE on d 4, 5, 6, and 7. Moreover, the decrease in antinociception observed after repeated administration of 030418 was more dramatic than that of morphine.

Subsequently, the effects of the ORL1 receptor antagonist J-113397 on analgesic tolerance to morphine and 030418 were further investigated. As mentioned above, repeated treatments with morphine led to tolerance on d 7, and a single pretreatment with J-113397 (4.0 µg/mouse, icv) before the injection of morphine could significantly attenuate the analgesic tolerance to morphine (Figure 6B). In contrast, 030418 tolerance on d 7 was not significantly affected by coadministration of J-113397 over the entire 8-h session (Figure 6C).

Prolonged washing of 030418 from the guinea pig ileum preparation
In the isolated guinea pig ileum preparation, stimulation induced by acetylcholine chloride (1.0 µmol/L) produces ileum muscle contraction, which can be inhibited by the action of an opioid drug added into the organ bath. The final concentrations of 0.1 mmol/L 030418, 5 mmol/L morphine, or 0.2 mmol/L dihydroetorphine were chosen in the experiments because they could produce an approximately 80% inhibition. As shown in Figure 7, 030418, morphine and dihydro-
etorphine all significantly inhibited ileum muscle contraction stimulated by acetylcholine chloride (1.0 µmol/L) at time zero. During prolonged washing, the action of morphine and dihydromorphine could be easily removed within 5 min, which was a relatively rapid process. In comparison, the course of prolonged washing of 030418 was much slower, and the inhibitory effect of 030418 remained at 24% until 45 min after the attempted washout. Moreover, the time course curve of 030418 was biphasic with an initial rapid phase followed by a slower phase.

**Discussion**

The 6,14-bridged oripavines, which possess a C₆-methoxy group part of the ring system and a long alkyl chain at the
npg

030418 was in the microgram range and was calculated as a full agonist dihydroetorphine. Moreover, the eD50 value of 030418 was similar to that of the typical receptor with a cyclopropylmethyl substituent, have been reported to have high affinity to opioid receptors[47]. As expected, the receptor binding assay revealed that 030418, a novel 6,14-bridged oripavine compound, displayed nonselective binding affinities to the μ-, κ-, and δ-opioid receptors and the ORL1 receptor with Ki values in the nanomolar range, as did thienorphine. Moreover, our observation that thienorphine highly bound to the μ-, κ-, and δ-opioid receptors is in accord with previous reports[15, 16].

Consistent with Li et al[14], thienorphine exhibited partial agonist activity at the κ-opioid receptor and at the μ-opioid receptor (to a lesser extent) but not at the δ-opioid receptor in vitro. In contrast, 030418, which has a methyl group at the Nγ substituent, was more efficacious in stimulating [35]S[GTPγS binding at the μ-, κ-, and δ-opioid receptors and the ORL1 receptor than its parent congener, thienorphine. This compound was also equally as efficacious as dihydroetorphine at all subtypes of the receptors except at the ORL1 receptor. Several structure-activity relationship (SAR) studies have shown that the 6,14-bridged oripavines with a cyclopropylmethyl group at Nγ, such as diprenorphine, buprenorphine, and nal-trexone, might possess some morphine-antagonist characteristics. Replacing this group with a methyl group has been found to greatly increase the efficacy of compounds, particularly at the μ- and κ-opioid receptors[11, 12]. We also considered that the mixed ORL1/μ-opioid receptor activity of 030418 may have an attractive profile for the treatment of pain and addiction, similar to SR16435 and buprenorphine[32, 33]. However, explaining the nonselective agonism of 030418 at opioid receptors will be difficult, and further studies are required to provide insights into the SAR of 030418 and the 6,14-bridged oripavine scaffold.

In the mouse tail-flick test and hot-plate test, the antinociceptive potency of 030418 was similar to that of the typical full agonist dihydroetorphine. Moreover, the ED50 value of 030418 was in the microgram range and was calculated approximately 1100–1500 times that of the standard morphine hydrochloride. Experimental data regarding the potency of dihydroetorphine and morphine were consistent with those of Aceto et al[34]. In addition, 030418 had more potent and powerful antinociceptive effects in vivo than its parent compound thienorphine, which has previously been demonstrated to be a partial opioid agonist, with an ED50 value in the milligram range in the mouse antinociceptive tests[15]. Compared with morphine, 030418 shows a rather long-lasting duration of the antinociceptive effect in the hot-plate test, which is similar to thienorphine. This similarity could occur because 030418 and thienorphine may have tight lipophilic interactions with the binding pocket of opioid receptors given the high liposolubility of the C7 substituent. This feature may lead to the persistent occupation of the opioid receptors by 030418. Several early reports have also demonstrated that 6,14-bridged oripavine compounds have very powerful binding interactions with opioid receptors and thus slow receptor kinetics, which causes a long-lasting effect[35–37].

Using pharmacological blockade in the hot-plate tests, the antinociception mediated by 030418 could be fully reversed by systemic administration of the classic opioid receptor antagonist naloxone, suggesting that the antinociceptive effect of 030418, similar to morphine, is likely related to the opioid receptor system. In addition, icv pretreatment with either β-FNA (μ-opioid receptor antagonist) or nor-BNI (κ-opioid receptor antagonist) was found to decrease 030418-induced antinociception. These results indicate that the activation of supraspinal μ- and κ-opioid receptors appears to be involved in the antinociceptive effect of 030418. The μ-opioid receptor is generally considered to represent the major molecular gate for opioid analgesia, and the activation of the κ-opioid receptor also evokes an analgesic action[39]. More interestingly, icv pretreatment with J-113397 (ORL1 receptor antagonist) could enhance antinociception induced by a moderate dose of 030418. In summary, the experimental results demonstrate that systemic administration of 030418 can simultaneously activate the μ- and κ-opioid receptor and the ORL-1 receptor in vivo.

Although 030418 showed remarkable antinociceptive effects, high doses of this compound unfortunately produced significant and rapid development of analgesic tolerance. In chronically administered animal models, 030418 produced a 78% decrease in %MPE on d 7 relative to d 1; in comparison, morphine produced a 54% decrease in %MPE. Although repeated administration of opioid agonists unavoidably produces analgesic tolerance associated with adaptive changes on opioid receptors and complex molecular events in cellular signaling pathways[39], our observation of the dramatic development of tolerance to 030418, which activates both the ORL1 and the μ-opioid receptors, seems contradictory to earlier reports. These studies have hypothesized that a compound that contains both μ-opioid receptor and ORL1 receptor agonist activities has reduced tolerance development[32, 33]. Thus, we next investigated the effect of pretreatment with the selective ORL1 antagonist J-113397 on 030418 tolerance. The experimental
results suggested that pharmacological blockade with J-113397 significantly prevented analgesic tolerance to morphine on d 7, which confirms earlier findings[30, 31]. In contrast, pretreatment with J-113397 could not effectively alter the development of tolerance to 030418. These findings highlight the complicated relationship between ORL1 receptor activation and μ-opioid receptor mediated tolerance. Therefore, additional studies are necessary to explain the issue.

Previous [32]GTPγS assays have revealed the high efficacy of 030418 at μ- and κ-opioid receptors; therefore, the inhibition by 030418 on muscle contraction in the guinea pig ileum (a tissue with enriched populations of functional μ- and κ-opioid receptors) seen in this study may have been primarily mediated through opioid receptor activity. Notably, the action of 030418 could be removed (with difficulty, compared to morphine and dihydroetorphine) by repeated, prolonged washing. This finding, taken together with the observation that 030418 had high binding affinity to opioid receptors, clearly indicates that 030418 has slow receptor dissociation kinetics. Considering that the only difference between 030418 and dihydroetorphine exists in the C7 substituent structure (Figure 1), the persistent binding affinity of 030418 to the opioid receptors may result from the high hydrophobicity caused by the structure of the C7-thiophene group. Similar examples of slow receptor dissociations of buprenorphine and BU74 have been observed and have been explained by powerful lipophilic binding interactions with the opioid receptors[37, 40]. The pharmacological property of slow receptor dissociation kinetics may account for the long duration of the agonist effect of 030418 in vivo. Additionally, the persistent occupation of the opioid receptors, particularly the μ-opioid receptors, can easily lead to the development of tolerance to 030418 by either receptor phosphorylation or a combination of receptor phosphorylation and desensitization. Woolf and Linderman[41] have believed that the ligand receptor dissociation rate constant and the conformational selectivity factor, which roughly corresponds to a drug’s efficacy, positively conspire to regulate receptor phosphorylation.

In conclusion, the present study demonstrates that 030418, a 6,14-bridged oripavaine compound, is a nonselective, high-affinity, and full opioid receptor agonist in vitro. This compound has highly potent and long-lasting antinociceptive effects, and tolerance to 030418 rapidly develops in vivo. The pharmacological properties of 030418 closely correlate with a high activity of the methyl group at the N1 position and high hydrophobicity of the C7-thiophene group in the 030418 chemical structure. The results of this study indicate that 030418 may be a candidate for development in the management of acute pain or as a ‘universal’ opioid ligand to be used as a pharmacological tool. Although more efforts are needed to discover the possible mechanisms of the unique effects of 030418, the current findings help to further understand the SAR of 6,14-bridged oripavines and provide insights into the design of ideal opioid drugs.

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Author contribution
Ze-hui GONG and Ling-di YAN designed the research; Quan WEN and Yu-lei LI performed the research; Quan WEN and Gang YU analyzed the data; and Quan WEN wrote the paper.

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