Thienorphine: Receptor Binding and Behavioral Effects in Rhesus Monkeys

Jun-Xu Li, Ginger L. Becker, John R. Traynor, Ze-Hui Gong, and Charles P. France

Departments of Pharmacology (J.-X.L., G.L.B., C.P.F.) and Psychiatry (C.P.F.), The University of Texas Health Science Center at San Antonio, San Antonio, Texas; Department of Pharmacology, University of Michigan Medical School, Ann Arbor, Michigan (J.R.T.); and Department of Pharmacology, Beijing Institute of Pharmacology and Toxicology, Beijing, People’s Republic of China (Z.-H.G.)

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ABSTRACT

Thienorphine is an oripavine with long-lasting antinociceptive effects in mice that are thought to be mediated by μ-opioid receptors. This study examined the receptor binding of thienorphine in cell membrane homogenates and its behavioral effects in rhesus monkeys (Macaca mulatta). Affinity and potency for these receptors, both in vitro and in vivo.

Thienorphine-induced antinociception. Thienorphine enhanced the antinociceptive effects of morphine and U50,488 [trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide] with 50°C water; with 55°C water, thienorphine enhanced the effects of morphine and attenuated the effects of U50,488. In other monkeys, thienorphine decreased responding in both components of a multiple schedule of food presentation and stimulus shock termination for up to 8 days; naltrexone and nor-BNI partially prevented these rate-decreasing effects. In morphine-treated monkeys discriminating naltrexone and nor-BNI partially prevented rate-decreasing effects. In morphine-treated monkeys discriminating naltrexone, thienorphine, and U50,488 neither substituted for nor modified the naltrexone discriminative stimulus. Thienorphine and U50,488 produced the same directly observable signs. These results show that thienorphine has long-lasting effects that seem to be mediated by low-efficacy agonism at μ-opioid receptors, both in vitro and in vivo.

Opioids, specifically μ opioids, continue to be the drugs of choice in the treatment of moderate-to-severe pain, despite the well established abuse liability of most compounds in this pharmacological class. Notwithstanding the need for strong analgesics that have reduced abuse liability, there has been relatively little success in the effort to develop alternative pharmacological approaches for treating moderate-to-severe pain.

In the search for alternative analgesics to morphine, research interests have focused on modifications of well known alkaloids, including the oripavines. One example is bu-buprenorphine (Temgesic) (Heel et al., 1979), which has potential antinociceptive effects in various species (Cowan et al., 1977; Heel et al., 1979). Buprenorphine is used as an alternative to methadone for replacement therapy and detoxification in heroin abusers (Bickel et al., 1988; Kosten and Kleber, 1988); however, it reportedly is abused (San et al., 1993). Whereas buprenorphine is a low-efficacy agonist at μ-opioid receptors and an antagonist at δ-opioid receptors, its effects at κ-opioid receptors remain less clear (Pick et al., 1997). Buprenorphine acts as an antagonist at κ-opioid receptors under some (Leander, 1987) but not all (Pick et al., 1997) conditions. Furthermore, buprenorphine has poor oral bioavailability (Heel et al., 1979; Chawarski et al., 2005), which might contribute to low compliance. Thus, compounds that are similar to buprenorphine, but with better oral bioavailability, could be useful for treating pain and opioid abuse.

N-Cyclopropylmethyl-7α-[1-(R)-1-hydroxy-1-methyl-3-(thien-2-yl) propyl]-6, 14-endooethanotetrahydro-orpavine

ABBREVIATIONS: CHO, Chinese hamster ovary; GTPγS, guanosine 5′-O-[3-32P]triphosphate; U69,593, (5α,7α,8β) (+)-N-methyl-N-[7-[1-pyrrolidinyl]-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide; DAMGO, [D-Ala², N-Me-Phe⁴,Gly⁵-ol]-enkephalin; SNCG80, [(-)-4-[αR]-α-[2S,5R]-2,5-dimethyl-4-(2-propenyl)-1-piperazinyl]-[3-methoxyphenyl][methyl]-N,N-diethylbenzamide; nor-BNI, nor-binaltorphimine; FR, fixed ratio; SST, stimulus-shock termination; MPE, maximal possible effect; U50,488, trans-3,4-dichloro-N-methyl-N-[2-(1-pyrrolidinyl)-cyclohexyl]-benzeneacetamide.
Dulbecco’s modified Eagle’s medium (C6 cells) or Dulbecco’s modified Eagle’s medium/nutrient mix F-12 (CHO cells) supplemented with 10% fetal calf serum. For subculture, one flask from each passage was grown in the presence of 1 mg/ml Geneticin (G-418; Invitrogen, Carlsbad, CA). Once cells had reached confluence, they were harvested in 20 mM HEPES-buffered saline, pH 7.4, containing 1 mM EDTA, dispersed by agitation, and collected by centrifugation at 1600 rpm. The cell pellet was suspended in 50 mM Tris-HCl buffer, pH 7.4, and homogenized with a tissue tearor (Biospec Products, Bartlesville, OK). The resultant homogenate was centrifuged for 20 min at 15,000 rpm at 4°C, and the pellet was collected, resuspended, and recentrifuged. The final pellet was resuspended in 50 mM Tris-HCl buffer, pH 7.4, separated into 0.5-ml aliquots (0.75–1.0 mg of protein), and frozen at −80°C. Protein concentration was determined by the method of Bradford (1976), using a bovine serum albumin standard.

In Vitro Binding Assay. Ligand binding assay. Cell membranes (20 μg of protein) were incubated at 25°C in 50 mM Tris-HCl buffer, pH 7.4, for 1 h with 0.2 nM [3H]diprenorphine in the presence or absence of eight concentrations of competing ligand ranging from 10^{-12} to 10^{-10} M to give a final volume of 250 μl. Nonspecific binding was defined with 10 μM naltrexone. Reactions were terminated by filtration through a glass-fiber filtermat mounted in a cell harvester (Brandel Inc., Gaithersburg, MD) and rinsed three times with ice-cold 50 mM Tris-HCl. Filtermats were dried, and 0.1 ml of EcoLume scintillation cocktail was added to each sample area. Each filtermat was placed in a polyethylene bag, and the bag was heat-sealed. Radioactivity retained in each sample area was counted in a Wallac 1420 MicroBeta liquid scintillation and luminescence counter (PerkinElmer Wallac, Gaithersburg, MD). 

Materials and Methods

Subjects

Three adult rhesus monkeys (Macaca mulatta) (one male, two females) were used for studies of thermal nociception, three adult rhesus monkeys (two males, one female) were used for studies of schedule-controlled responding, and five adult rhesus monkeys (one male, four females) were used for drug discrimination and observation procedures. All monkeys had received drugs (primarily benzodiazepines and opioids) in previous studies. Monkeys weighed between 5 and 8 kg, and they were maintained at 95% of their free-feeding weight with monkey chow (Harlan Teklad High Protein Monkey Diet; Harlan Teklad, Madison, WI), fresh fruit, and peanuts provided after daily sessions. All monkeys were individually housed on a 14:10-h light/dark cycle with unlimited access to water. Monkeys in the schedule-controlled responding procedure also received food pellets during experimental sessions. The monkeys used in these studies were maintained in accordance with the Institutional Animal Care and Use Committee, The University of Texas Health Science Center at San Antonio, and with the 1996 Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animals Resources on Life Sciences, National Research Council, National Academy of Sciences).

Procedures

Cell Culture and Membrane Preparation. C6 glioma cells stably transfected with μ- or δ-opioid receptors (C6μ, C6δ) (Lee et al., 1999) and CHO cells expressing human κ-opioid receptors (CHOκ) (Zhu et al., 1997) were cultured under a 5% CO₂ atmosphere in Dulbecco’s modified Eagle’s medium (C6 cells) or Dulbecco’s modified Eagle’s medium/nutrient mix F-12 (CHO cells) supplemented with 10% fetal calf serum. For subculture, one flask from each passage was described in detail previously (Lelas et al., 1999). In brief, monkeys were seated in chairs, and the lower part of their shaved tail (approximately 15 cm) was immersed in a thermal flask containing water maintained at 40, 50, or 55°C. Temperatures were presented in a nonsystematic order among monkeys and across cycles. When a subject failed to remove its tail within 20 s, the experimenter removed the tail from the water, and a latency of 20 s was recorded.

Thermal Nociception. A warm water, tail-withdrawal procedure was described in detail previously (Lelas et al., 1999). In brief, monkeys were seated in chairs, and the lower part of their shaved tail (approximately 15 cm) was immersed in a thermal flask containing water maintained at 40, 50, or 55°C. Temperatures were presented in a nonsystematic order among monkeys and across cycles. When a subject failed to remove its tail within 20 s, the experimenter removed the tail from the water, and a latency of 20 s was recorded.
Test sessions began with control determinations at each temperature. Tail withdrawal latencies were measured at each of the three temperatures with approximately 1 min between tests; consecutive drug injections were separated by 15-min periods during which tail withdrawal latencies were determined, beginning 10 min after drug administration. An acute injection of thienorphine (0.032–0.32 mg/kg) was administered after the control determination and then tested for 10 cycles (150 min). Thereafter, tail withdrawal latency was retested in two cycles daily or every 3rd day until the latency was not different from control (no drug) values. The ability of naltrexone or nor-BNI to antagonize the antinociceptive effects of thienorphine was evaluated by administering a dose of 1.0 mg/kg naltrexone 30 min or 3.2 mg/kg nor-BNI 24 h before the injection of 0.032 mg/kg thienorphine on the first of 10 cycles. Because nor-BNI antagonized the effects of thienorphine under these conditions, antagonism at κ-opioid receptors was further tested by obtaining a dose-effect curve for antinociceptive effects of the prototypic κ-opioid receptor agonist U50,488 3 days after the administration of nor-BNI.

No antinociception was apparent after the administration of 0.0032 mg/kg thienorphine; to test whether this ineffective dose of thienorphine modified the antinociceptive effects of morphine (enhance or diminish), a morphine dose–effect curve was determined 24 h after administration of this dose of thienorphine, using a multiple-dosing procedure whereby the cumulative dose of morphine increased 0.25 log units per cycle (injection). Because this dose of thienorphine enhanced the effects of morphine, a morphine dose–effect curve was also determined 4 and 6 days later (i.e., 5 and 7 days after the administration of 0.0032 mg/kg thienorphine).

Likewise, to test whether thienorphine modified the antinociceptive effects of a prototypic κ-opioid receptor agonist, a dose-effect curve was determined for U50,488 beginning 30 min after the administration of a behaviorally active dose (0.032 mg/kg) of thienorphine. This test allowed for examination of any interactions between thienorphine and U50,488 under a condition where these two drugs shared effects (50°C) and under a condition where they did not share effects (U50,488 and not thienorphine was effective with 55°C water). For comparison, control dose-effect curves were determined for morphine and for U50,488 on different days and in the absence of thienorphine. For antinociception studies, dosing terminated for an individual subject when a near maximum effect (20 s) was obtained in the first minute of the 10-min time-out period (e.g., first minute or four electric stimulus presentations, whichever occurred first.

Schedule-Controlled Responding. Monkeys responded under a multiple (FR10/FR10) schedule of food presentation and stimulus-shock termination (SST). For training conditions, two to eight cycle sessions were conducted daily with each cycle making up a 10-min pretreatment, during which the chamber was dark and presses on either lever had no programmed consequence. This was followed by a 5-min response session. The beginning of a 2-min food component was signaled by illumination of a green light located above each lever, and subjects could respond 10 times on the active lever (FR10) to receive a 300-mg banana-flavored pellet (Bio-Serv, Frenchtown, NJ). The green light was extinguished after 2 min or the delivery of 10 food pellets; and for the latter case, the remainder of the 2-min food component was a time-out period. The food component was followed by a 1-min time-out after which illumination of red stimulus lights above each lever signaled the beginning of a 2-min SST component. The subjects could respond 10 times (FR10) within 15 s (limited hold) on the same activated lever to postpone scheduled presentation of an electric stimulus. A cycle ended after 5 min (including a 2-min food component, a 1-min time-out, and a 2-min SST component) or after four electric stimulus presentations, whichever occurred first.

For training sessions, saline or “sham” injections were given during the first minute of the 10-min time-out period (e.g., first minute of the cycle) with the number of cycles varying nonsystematically across days. Test sessions did not commence until stable rates of responding were established for both components during training sessions, defined as 10 consecutive days with rates of responding for both components within ±20% of the mean rate for those days. According to the results obtained from antinociception studies, 0.032 mg/kg thienorphine was used in this study, because this dose produced intermediate antinociception at 50°C that lasted for several days. A larger dose of thienorphine (0.32 mg/kg) produced marked behavioral effects that persisted for at least 1 week. For antagonism studies, 3.2 mg/kg nor-BNI or 1.0 mg/kg naltrexone was administered 24 h or 30 min before an acute injection of 0.032 mg/kg thienorphine, respectively. The duration of antagonism was evaluated over eight subsequent cycles (2 h) on the day that thienorphine was administered and again in two cycle sessions (30 min) on subsequent days. Tests were conducted no more than once per week.

Drug Discrimination. Monkeys had been treated for at least 2 years twice daily with morphine (5.6 mg/kg at 6:30 AM and 6:30 PM) with sessions beginning 3 h after the morning injection. All monkeys were trained to press a lever under an FR schedule of SST while discriminating 0.0178 mg/kg naltrexone from saline (France and Woods, 1989). For training conditions, two to six cycle sessions were conducted 6 days a week with each cycle made up of a 10-min pretreatment, during which the chamber was dark and presses on either lever had no programmed consequence. This was followed by a 5-min response session, during which red stimulus lights located above each lever were illuminated, and subjects could respond within 15 s (limited hold), five consecutive times on the correct lever (FR5), to postpone scheduled presentation of an electric stimulus. The correct lever was determined by an injection of either saline or 0.0178 mg/kg s.c. naltrexone administered immediately before the start of the session. The left lever was designated as correct after saline, and the right lever was correct after naltrexone for three of the monkeys with the lever assignments reversed for the remaining two monkeys. Completion of an FR on the correct lever produced a 30-s time-out. Responses on the incorrect lever reset the response requirement on the correct lever. The response period ended after 5 min or four electric stimulus presentations, whichever occurred first, during which the lights were extinguished. For training days, monkeys received an injection of naltrexone followed by one sham (non-injection) cycle or an injection of saline followed by one to five sham cycles.

Monkeys had previously satisfied the following criteria for five consecutive or six of seven sessions: at least 80% of the total responses on the correct lever; and fewer than five responses on the incorrect lever before completion of the first FR on the correct lever. Monkeys were tested provided that these criteria were satisfied during at least two intervening training sessions. During test sessions, saline was administered in the first cycle followed by increasing doses of a test compound in subsequent cycles up to doses that occasioned greater than 80% naltrexone lever responding and resulted in delivery of an electric stimulus, or up to the largest doses that could be safely studied. Doses of test compounds studied using cumulative dosing procedures were as follows: naltrexone (0.001–0.032 mg/kg) and U50,488 (0.032–1.0 mg/kg). Thienorphine (0.0032–0.1 mg/kg) was not administered under cumulative dosing conditions due to its long duration of action; acute injections of thienorphine were administered on different days before 2-h sessions. To determine whether thienorphine or U50,488 would modify a naltrexone discriminative stimulus, behaviorally active doses (e.g., from the antinociception and observational studies) of 0.1 mg/kg thienorphine and 0.32 mg/kg U50,488 were administered before redetermining a naltrexone dose-effect curve (0.001–0.032 mg/kg). Thienorphine was tested not more than once weekly.

Behavioral Observations. Monkeys were observed in their home cages 30 min after receiving doses of naltrexone or U50,488. It was determined in the schedule-controlled responding experiments that thienorphine had a long duration of action; therefore, monkeys were observed 2 and 3 h after receiving doses of thienorphine (data presented are for the 3-h time-point only). Drug (or vehicle) was tested once daily in a Latin-square design. The frequency with which
signs occurred was recorded by a nonblinded, experienced observer who was familiar with the behavior of individual subjects. A list of 21 withdrawal signs incorporated those previously reported to occur as a result of μ-opioid withdrawal (e.g., vocalizations, grimace, saliva-
tion, tremor, and wet-dog shakes) (Katz, 1986; Sell et al., 2005) or the administration of a κ-opioid receptor agonist in nonhuman primates (e.g., eye closing, muscle relaxation, and stupor) (Dykstra et al., 1987, Butelman et al., 2001), including signs that were observed in preliminary studies with the same group of monkeys used for these studies (e.g., lip protrusion and unusual tongue movements). Mon-
keys were observed for a 2-min period, and the occurrence of a sign was recorded as present or absent every 30 s to give a maximum frequency score of 4 for each sign (i.e., the sign occurred in all the four 30-s observation periods). Stupor was scored when monkeys did not respond to loud claps or tactile stimulation; muscle relaxation was scored when monkeys leaned against the cage wall or if they seemed to lose their balance while sitting upright; and a grimace was scored when monkeys opened their mouth, withdrawing their lips wide to reveal the sides of their teeth.

Drugs

[3H]Diprenorphine (53 Ci/mmol) and [35S]GTPyS (1250 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Bos-
ton, MA). All tissue culture materials were from Invitrogen. DAMGO and all other analytical grade biochemicals were purchased from Sigma-Aldrich (St. Louis, MO). The compounds used included thieno-
phrine hydrochloride (synthesized at The Beijing Institute of Phar-
macology and Toxicology, Beijing, China), morphine sulfate, naltrexone hydrochloride, SNC80, U69,593, U50,488, and nor-binal
torphine dihydrochloride (The Research Technology Branch, Na
tional Institute on Drug Abuse, Bethesda, MD). In behavioral assays, all drugs were administered s.c. in a volume of 0.1 to 1.0 ml, and doses were expressed as the forms indicated above. All drugs were dissolved in either saline or sterile water except thienophrine, which was dissolved in 20% dimethyl sulfoxide in water.

Data Analyses

Competition binding data and concentration-response data in the [35S]GTPyS assay were fitted to sigmoidal curves using GraphPad Prism (GraphPad Software Inc., San Diego, CA) for determination of IC50 values and EC50 values, respectively. Data from at least three separate experiments, each carried out in duplicate, are presented as the mean ± S.E.M. Ki values at the three receptors were derived from IC50 values for the displacement of [3H]diprenorphine using the Cheng and Prusoff equation Ki = IC50/[1 + ([3H]diprenorphine/Ki)] (Cheng and Prusoff, 1973) using K50 values for [3H](diprenorphine of 0.25 nM (κ), 0.15 nM (μ), and 0.45 nM (δ) (Traynor and Woods, 2005).

In the thermal nociception studies, latency was expressed as the percentage of maximal possible effect (PME) using the following formula: %PME = ([test latency – control latency]/20 s – control latency]) × 100, where the control latency was defined as the average latency of tail withdrawal determined in the absence of drug. The percent maximal possible effect was calculated for each individual and then averaged to obtain a group mean. In the schedule-con
trolled responding studies, the rates of responding of individual monkeys for each session were calculated separately for each com-
ponent of the multiple schedule by averaging rates of responding for all cycles. Control response rate was the mean rate of 10 training sessions. Rates of responding during tests were expressed as a per-
centage of the control response rate. The response rates during tests were considered to be significantly different from control when the mean values for rate did not overlap the 95% confidence limit of control rates. Drug discrimination data are expressed as the percent-
age of total responses occurring on the naltrexone lever averaged across monkeys (±S.E.M.). For the behavioral observations, the fre-
quency with which a sign was observed was averaged among mon-
keys (±S.E.M.) and plotted as a function of dose. For potency com-
parisons among drugs in the observational study, ED50 values were estimated using linear regression.

Results

In Vitro Binding Assay. Thienophine had affinity for all three opioid receptors, with Ki (nanomolar) values being 0.14 ± 0.06 (κ), 0.22 ± 0.07(μ), and 0.69 ± 0.03 (δ), respectively (Table 1). Agonist-induced stimulation of G proteins was measured in homogenates of cell membranes expressing κ-, μ-, and δ-opioid receptors (Table 1; Fig. 2). All subtype-selective high-efficacy agonists of κ- (U69,593), μ- (DAMGO), and δ (SNC80)-opioid receptors increased the binding of [35S]GTPyS over basal level in a concentration-dependent manner and produced maximal effects at a concentration of 10 μM (data not shown). Thienophine produced a maximal stimulation of 75.40 ± 4.90% on κ-opioid receptors (in comparison with U69,593), 19.30 ± 3.60% on μ-opioid receptors (in comparison with DAMGO), and little to no stimulation on δ-opioid receptors (in comparison with SNC80; Table 1; Fig. 2). The EC50 values of thienophine on κ- and μ-opioid recep-
tor stimulation were 0.34 ± 0.23 and 1.90 ± 0.40 nM, respec-
tively.

Thermal Nociception. Thienophine dose-dependently increased the latency for monkeys to remove their tails from 50°C water (Fig. 3): 0.0032 mg/kg thienophine had no clear effects. Concentrations of 0.0138 and 0.0414 mg/kg decreased latency 
over control by 100%, where the control latency was defined as the average latency of tail withdrawal determined in the absence of drug. The percent maximal possible effect was calculated for each individual and then averaged to obtain a group mean. In the schedule-con
trolled responding studies, the rates of responding of individual monkeys for each session were calculated separately for each com-
ponent of the multiple schedule by averaging rates of responding for all cycles. Control response rate was the mean rate of 10 training sessions. Rates of responding during tests were expressed as a per-
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keys (±S.E.M.) and plotted as a function of dose. For potency com-
parisons among drugs in the observational study, ED50 values were estimated using linear regression.
effect; 0.032 mg/kg produced a maximal effect of 68% and continued to produce a measurable effect for 3 days; and 0.32 mg/kg produced the maximal possible effect (i.e., 20 s) that persisted for 7 days, gradually dissipating thereafter. Thirteen days after 0.32 mg/kg thienorphine, tail withdrawal latency returned to control (predrug) values. Over the dose range tested, thienorphine did not affect tail withdrawal latency from 55°C water (data not shown).

Acute administration of 3.2 mg/kg nor-BNI produced weak (maximum of 41% MPE) antinociceptive effects with 50°C water, which was evident throughout the 2-h session, and no effect with 55°C water (data not shown). Twenty-four hours after administration, no antinociceptive effects were observed with nor-BNI; however, this dose of nor-BNI prevented the antinociceptive effects of 0.032 mg/kg thienorphine for at least 4 days (Fig. 4, triangles). Furthermore, the dose-effect curve for U50,488-induced antinociception with 50°C water was shifted 0.5 log unit to the right 3 days after administration of nor-BNI (data not shown). A dose of 1.0 mg/kg naltrexone administered 30 min before thienorphine also prevented the antinociceptive effects of 0.032 mg/kg thienorphine (Fig. 4, squares). In contrast to the lingering antagonism of thienorphine obtained with nor-BNI, 24 h after the administration of naltrexone and thienorphine the antinociceptive effects of thienorphine reemerged and persisted for 4 days (Fig. 4, squares, right).

Morphine alone increased the latency for monkeys to remove their tails from 50 or 55°C water (Fig. 5, circles, top and bottom, respectively). The antinociceptive effects of morphine were enhanced by thienorphine (0.0032 mg/kg s.c.) (Fig. 5). One and 5 days after the administration of thienorphine, the morphine dose-effect curve was shifted 0.25 to 0.5 log unit to the left for 50 and 55°C water. A morphine dose-effect curve determined 7 days after the administration of thienorphine was not different from a control morphine dose-effect curve.

U50,488 alone increased the latency for monkeys to remove their tails from 50 or 55°C water with a dose of 3.2 mg/kg producing 100% and 72% MPE for 50 and 55°C water (Fig. 6, filled circles, top and bottom, respectively). A dose of 0.032 mg/kg thienorphine had moderate antinociceptive effects (51% MPE) with 50°C water and no effect with 55°C water (Fig. 6, points above T). This dose of thienorphine enhanced the antinociceptive effects of U50,488 with 50°C water (Fig. 6, top), producing a greater than 0.5 log unit shift to the left. In the U50,488 dose-effect curve, and it attenuated the effects of U50,488 with 55°C water (Fig. 6, bottom). Under control conditions, a dose of 3.2 mg/kg U50,488 produced 71% of the MPE, whereas in the presence of 0.032 mg/kg thienorphine this dose of U50,488 produced only 30% of the MPE.

Schedule-Controlled Responding. Thienorphine (0.032 mg/kg) had marked and long-lasting rate-decreasing effects in both the food presentation and the SST components of the multiple schedule. In the food presentation component, thienorphine decreased response rate in a time-dependent manner. One hour after drug administration, response rate decreased to near zero, and monkeys did not obtain any food pellets. Marked rate-decreasing effects persisted for 5 days, gradually recovering thereafter (Fig. 7). In the SST component, thienorphine decreased response rate in a time-dependent manner, and 2 h after drug administration response rate had decreased to an average of 34% of control. Response rate recovered partially (63% of control) 24 h later and fully 5 days later (Fig. 7). The nor-BNI (3.2 mg/kg) alone was without effect on rate of responding (data not shown), and,

![Fig. 3. Time course of the antinociceptive effects of three doses of thienorphine in monkeys (n = 3). Symbols represent the mean ± S.E.M.; if not shown, S.E.M. values are contained by the symbol. The 55°C data are not shown because thienorphine did not show antinociceptive effects in 55°C water. All data were converted to %MPE as indicated under Materials and Methods. Ordinate, %MPE; abscissa, time.](https://example.com/fig3.png)

![Fig. 4. Effects of 3.2 mg/kg nor-BNI and 1.0 mg/kg naltrexone on 0.032 mg/kg thienorphine-induced antinociceptive effects. Data for 0.032 mg/kg thienorphine-induced antinociceptive effects are replotted from Fig. 2. Naltrexone and nor-BNI were administered 30 min and 24 h, respectively, before thienorphine. See Fig. 3 for other details.](https://example.com/fig4.png)
when administered 24 h before thienorphine, it failed to alter the rate-decreasing effects of thienorphine in either component of the multiple schedule (Fig. 7). In the food presentation component, naltrexone attenuated the rate-decreasing effects of thienorphine during the first hour of the 2-h session; however, response rate gradually decreased to 22% of the control response rate by the end of the 2-h session. In the SST component, 1.0 mg/kg naltrexone partially antagonized the rate-decreasing effects of thienorphine during the first 2 h of the test. Rate-decreasing effects were evident for several days after administration of 0.032 mg/kg thienorphine in combination with nor-BNI or with naltrexone.

**Drug Discrimination.** Administration of vehicle or the smallest dose of naltrexone (0.001 mg/kg) produced little to no responding on the naltrexone-associated lever; however, larger doses of naltrexone (0.0032–0.032 mg/kg) occasioned responding predominantly on the naltrexone-associated lever (Table 2). Thienorphine (0.0032–0.032 mg/kg) did not occasion naltrexone lever responding in any monkey up to doses (0.032 mg/kg) that decreased responding and produced marked stupor (Table 2). Likewise, U50,488 (0.032–1.0 mg/kg) did not occasion naltrexone lever responding up to doses (1.0 mg/kg) that markedly decreased responding (Table 2). Doses of thienorphine (0.1 mg/kg) and U50,488 (0.32 mg/kg) that markedly decreased rates of responding did not modify the naltrexone discriminative stimulus when administered before redetermination of a naltrexone dose-effect curve (Fig. 8). When administered alone over a five-cycle test session, 0.32 mg/kg U50,488 decreased response rates to 30 to 50% of
TABLE 2
Percentage of responses on the naltrexone lever (%DR) and rate of responding expressed as a percentage of the control rate (vehicle training days) in monkeys treated with morphine (5.6 mg/kg/12 h) and discriminating naltrexone (0.0178 mg/kg; n = 5)

<table>
<thead>
<tr>
<th>Dose</th>
<th>Thienorphine</th>
<th>Naltrexone</th>
<th>U50,488</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%DR ± S.E.M.</td>
<td>Rate ± S.E.M.</td>
<td>%DR ± S.E.M.</td>
</tr>
<tr>
<td>Vehicle</td>
<td>0.4 ± 0.4</td>
<td>103.3 ± 0.4</td>
<td>0 ± 0</td>
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<td>0.001 mg/kg</td>
<td>2.2 ± 1.7</td>
<td>96.5 ± 1.6</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.0032 mg/kg</td>
<td>25.3 ± 13.4</td>
<td>88.2 ± 7.1</td>
<td>0 ± 0</td>
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<tr>
<td>0.01 mg/kg</td>
<td>81.3 ± 7.9</td>
<td>73.7 ± 12.9</td>
<td>0 ± 0</td>
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<td>0.032 mg/kg</td>
<td>97.1 ± 1.4</td>
<td>71.6 ± 11.9</td>
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<tr>
<td>0.1 mg/kg</td>
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<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>0.32 mg/kg</td>
<td>N.D.</td>
<td>N.D.</td>
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<tr>
<td>1.0 mg/kg</td>
<td>N.D.</td>
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N.D., not determined.

![Fig. 8. Effects of thienorphine and U50,488 on the discriminative stimulus effects of naltrexone in morphine-treated monkeys discriminating 0.0178 mg/kg naltrexone. A cumulative dosing procedure was used to determine the dose-effect curve for naltrexone in the presence and absence of thienorphine and U50,488. Response rate is presented as the mean ± S.E.M. response rate expressed as a percentage of control (vehicle training days) rates. Ordinate, top, percentage of responding on the naltrexone lever; bottom, percentage of control of response rate; abscissa, drug dose (milligrams per kilogram).](image)

![Fig. 9. Effects of thienorphine and U50,488 on the discriminative stimulus effects of naltrexone in morphine-treated monkeys discriminating 0.0178 mg/kg naltrexone. A cumulative dosing procedure was used to determine the dose-effect curve for naltrexone in the presence and absence of thienorphine and U50,488. Response rate is presented as the mean ± S.E.M. response rate expressed as a percentage of control (vehicle training days) rates. Ordinate, top, percentage of responding on the naltrexone lever; bottom, percentage of control of response rate; abscissa, drug dose (milligrams per kilogram).](image)

Behavioral Observation. Thienorphine had directly observable effects in monkeys receiving morphine daily. When vehicle was administered, these behavioral signs were rarely observed (Fig. 9, points above V). Naltrexone dose-dependently increased salivation, vocalization, unusual tongue movement, and, to a lesser extent, grimacing; however, naltrexone did not produce stupor, muscle relaxation, or eye closing at any dose (0.0032–0.032 mg/kg). In contrast, thienorphine produced a near maximum frequency score for stupor, muscle relaxation, salivation, and, to a lesser extent, eye closing. Thienorphine did not produce grimacing, vocalization, unusual tongue movement or tongue protrusion. Similar to thienorphine, U50,488 (0.32–1.0 mg/kg) produced muscle relaxation, salivation, eye closing, and stupor; U50,488 was 130-fold less potent than thienorphine (e.g., ED50 for thienorphine- and U50,488-induced stupor was 0.004 and 0.537 mg/kg, respectively). Like thienorphine, U50,488 did not produce unusual tongue movement, tongue protrusion, grimace, or vocalization (Fig. 9).

Discussion
This study shows thienorphine to be a long-acting, low efficacy κ-opioid receptor agonist. In vitro, thienorphine had affinity for κ-, μ-, and δ-opioid receptors, partially activating κ-opioid receptors, with less activity at μ-opioid receptors and little to no activity at δ-opioid receptors. Thienorphine had dose-dependent and long-lasting antinociceptive effects that were antagonized by the κ-opioid receptor antagonist nor-BNI. A large dose of the nonselective opioid antagonist naltrexone prevented but did not reverse (data not shown) thienorphine-induced antinociception. Under conditions where thienorphine was not effective (55°C), it enhanced the effects of morphine and attenuated the effects of U50,488, further indicating its limited efficacy at κ-opioid receptors. Rate-decreasing effects of thienorphine were only partially blocked by naltrexone and nor-BNI, suggesting that these effects are mediated by opioid and nonopioid receptors. Thienorphine produced directly observable signs that were similar to those produced by a prototypic κ-opioid receptor agonist.

In Vitro Binding of Thienorphine. Consistent with a previous report (Yu et al., 2006), thienorphine bound to κ-, μ-, and δ-opioid receptors. In the previous study, thienorphine produced 62% of maximal stimulation at μ-receptors (compared with morphine), functioning as a partial μ-receptor agonist. In contrast, thienorphine only weakly (19% compared with DAMGO) stimulated μ-receptors in the current study. Procedural details (e.g., different receptor expressing system or standard drugs) might account for this difference. For example, morphine is less effective than DAMGO in stimulating G proteins in C6 glioma cells expressing μ-opioid receptors (Traynor et al., 2002). In the absence of data with thienorphine and selective opioid receptor antagonists, it is not possible to confirm whether earlier reported effects (Yu et al., 2006) were due to actions at μ-opioid receptors. Thienorphine protected mice from lethal effects of morphine, as would be expected for a low-efficacy agonist in combination with a more efficacious agonist (morphine); however, possible κ-opioid receptor activity for thienorphine was not directly...
tested in the previous study. The current study suggests that in vitro thienorphine acts as a partial agonist (low efficacy) in stimulating κ-opioid receptors, to a lesser extent μ-opioid receptors, and not δ-opioid receptors, findings that are supported by behavioral studies discussed below.

**Effects of Thienorphine on Thermal Nociception.** Thienorphine had dose-dependent antinociceptive effects in 50°C water, producing the maximal possible effect at 0.32 mg/kg and being 30-fold more potent than morphine (Fig. 3) (Gerak et al., 2003). Thienorphine did not have antinocicep-
tive effects with a more intense stimulus (55°C); loss of antinociceptive effect as stimulus intensity increases supports the in vitro data that thienorphine has limited efficacy. To directly test this possibility, the high-efficacy κ-opioid receptor agonist U50,488 was studied with and without thienorphine. Under conditions where thienorphine and U50,488 shared effects (50°C), thienorphine enhanced the effects of U50,488, shifting the dose-effect curve leftward. However, under conditions where thienorphine and U50,488 did not share effects (55°C), thienorphine attenuated the effects of U50,488. This differential effect of thienorphine in the same study with two stimulus intensities provides direct evidence for thienorphine having low efficacy at κ-opioid receptors. In contrast to the differential effect of thienorphine in modifying the effects of U50,488, thienorphine enhanced the antinociceptive effects of the μ-opioid receptor agonist morphine at both stimulus intensities. These leftward shifts in the morphine dose-effect curve likely reflect the addition of μ (morphine) and κ (thienorphine) activity, because even larger doses of thienorphine were ineffective in a drug discrimination assay that is exceptionally sensitive to μ-receptor activity (see below). Given the adverse subjective effects generally reported with κ-opioid receptor agonists (Kumor et al., 1986), it seems unlikely that combined treatment with μ- and κ-opioid receptor agonists would prove useful in the clinic.

Yu et al. (2006) reported that 1.25 mg/kg thienorphine was maximally effective in a hot-plate assay in mice; however, these effects might reflect long-lasting behavioral suppression by thienorphine that occurs in this strain of mice under these conditions (Zhao et al., 2004). There are other examples where effects in the hot-plate assay were due to behavioral suppression and not to antinociception (Porsolt et al., 2002). In a warm water, tail withdrawal assay, latency generally is not affected by drugs without antinociceptive effects (e.g., pentobarbital), even at sedating doses (Dykstra and Woods, 1986).

Thienorphine had a very long duration of action in monkeys, with 0.32 mg/kg producing near maximal antinociceptive effects (50°C) for 1 week. These long-lasting effects in monkeys are consistent with thienorphine protecting mice from morphine-induced lethality for as long as 15 days (Yu et al., 2006), and they are in contrast to the shorter duration of action of most κ-opioid receptor agonists (Mello and Negus, 2000). The possibility that thienorphine is redistributed to fatty tissue where it is slowly released due to its high lipophilicity is partially supported by data from this study. Although naltrexone (1.0 mg/kg) prevented thienorphine-induced antinociception, 24 h later the antinociceptive effects of thienorphine had emerged and persisted similarly to what was observed with thienorphine in the absence of naltrexone. Naltrexone is 2.5-fold selective for μ over κ-opioid receptors (Emmerson et al., 1994), and at larger doses, it blocks the effects of both μ- and κ-agonists. For example, 0.1 mg/kg naltrexone shifts a U50,488 dose-effect curve for antinociception 10-fold to the right (Ko et al., 1998). In the current study, a 10-fold larger dose (1.0 mg/kg) of naltrexone probably occupied κ-opioid receptors as well and antagonized thienorphine in a manner similar to what was observed with nor-BNI. These data cannot exclude a possible role for metabolites in the long-lasting behavioral effects of thienorphine.

Effects of Thienorphine in the Assay of Schedule-Controlled Responding. Thienorphine also had long-lasting rate-decreasing effects in both components of a multiple schedule, although it seems as though these effects are not mediated exclusively by κ-opioid receptors. For example, nor-BNI only partially antagonized the rate-decreasing effects of thienorphine, even at a dose that completely blocked the antinociceptive effects of thienorphine in this study and antinociceptive effects of U50,488 in another study (Butelman et al., 1993). The inability of nor-BNI to fully antagonize the rate-decreasing effects of thienorphine could be due to selective activation of the κ2-subtype of κ-opioid receptors, because κ2-receptors are reported to be resistant to nor-BNI antagonism (Butelman et al., 1993; Ko et al., 1998). However, naltrexone only partially antagonized the rate-decreasing effects of thienorphine, particularly in the SST component. Collectively, these results suggest that in addition to low efficacy at opioid receptors, thienorphine might also have nonopioid actions.

Effects of Thienorphine on Drug Discrimination. Drug discrimination procedures display significant pharmacological selectivity (Colpaert, 1999); in morphine-treated animals that discriminate an opioid agonist, only drugs with little or no efficacy at μ-opioid receptors occasion responding on the antagonist lever (Gellert and Holtzman, 1979; France and Woods, 1989). Likewise, the discriminative stimulus effects of μ-opioid receptor antagonists in the same animals are attenuated by an acute pretreatment with a μ-opioid agonist (Sell et al., 2003; McMahon et al., 2004). This pharmacological selectivity was used to examine whether thienorphine exerts μ-antagonist (i.e., substitute for naltrexone) or μ-agonist (i.e., attenuate naltrexone) actions in morphine-treated monkeys. That thienorphine failed to substitute for naltrexone and failed to attenuate naltrexone strongly indicates that at the doses studied, thienorphine has no activity at μ-opioid receptors; however, if larger doses could be studied, μ-receptor activity would be expected to emerge. Other κ-opioid agonists also fail to substitute for or attenuate the discriminative stimulus effects of naltrexone (i.e., U50,488 (this study) and others (France and Woods, 1989)). Although the relatively nonselective binding of thienorphine to opioid receptors might predict activity in this (μ-sensitive) discrimination assay, it is possible that the emergence of κ-agonist actions (e.g., rate suppression) precluded the expression of any behavioral effects of thienorphine that are mediated at μ-opioid receptors.

Effects of Thienorphine on Directly Observable Signs. Many drugs elicit unconditioned effects that are readily observed and easily quantified. For example, in subjects treated chronically with an agonist, either discontinuation of treatment or administration of an antagonist can elicit characteristic behavioral effects, which for μ-opioid receptor drugs can include unusual oral movements and vocalization (Brandt and France, 1998; Sell et al., 2005). Likewise, in normal subjects, agonists with actions at particular receptors can elicit characteristic behavioral effects. Acute administration of a μ-opioid receptor agonist produces behavioral effects that are, in most respects, qualitatively different from those observed after acute administration of a κ-opioid receptor agonist (Dykstra et al., 1987; Butelman et al., 2001). In monkeys thienorphine and U50,488, and not naltrexone, produced similar behavioral...
effects, including stupor, salivation, eye closing, and muscle relaxation. These data support the view that these behavioral effects of thienorphine are mediated by κ-opioid receptors.

In summary, using in vitro and behavioral methods, these studies showed that thienorphine is a long-acting, low-efficiency κ-opioid receptor agonist, which stands in contrast to previous reports that thienorphine is a partial µ-opioid receptor agonist. To the extent that thienorphine exerts other behavioral (e.g., subjective) effects over the same dose range that exerts antinociceptive effects, its clinical utility might be limited by induction of κ-opioid receptor-mediated dysphoria and presumably, poor compliance.

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References


Cheng Y-C and Prusoff WH (1973) Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50% inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22:3099–3108.


Address correspondence to: Dr. Charles P. France, Department of Pharmacology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Dr., Mail Code 7792, San Antonio, TX 78229-3000. E-mail: france@uthscsa.edu