The buprenorphine analogue BU10119 attenuates drug-primed and stress-induced cocaine reinstatement in mice.

Todd M. Hillhouse$^{1,2*}$, Keith Olson$^2*$, James E. Hallahan$^2$, Lauren G. Rysztak$^2$, Bryan F Sears$^2$, Claire Meurice$^2$, Mehrnoosh Ostovar$^3$, Peyton O. Koppenhaver$^1$, Joshua West$^2$, Emily M Jutkiewicz$^2$, Stephen M Husbands$^3$, and John R Traynor$^{2,4}$.

$^1$Department of Psychology, University of Wisconsin Green Bay, Green Bay, Wisconsin, USA
$^2$Department of Pharmacology and Edward F Domino Research Center, University of Michigan Medical School, Ann Arbor, Michigan, USA
$^3$Department of Pharmacy and Pharmacology, and Center for Therapeutic Innovation, University of Bath, Bath, UK
$^4$Department of Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, Michigan, USA

*These authors contributed equally
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Corresponding Author:

John R Traynor, PhD
jtraynor@umich.edu
Department of Pharmacology
University of Michigan
1150 West Medical Center Drive
1301 MSRB III
Ann Arbor, MI, 48109
Phone: +1 734 764 6186
Fax: +1 734 763 4450

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Abstract

There are no Food and Drug Administration-approved medications for cocaine use disorder, including relapse. The mu-opioid receptor (MOPr) partial agonist buprenorphine, alone or in combination with naltrexone, has been shown to reduce cocaine positive urine tests and cocaine seeking in rodents. However, there are concerns over the abuse liability of buprenorphine. Buprenorphine’s partial agonist and antagonist activity at the nociception-receptor (NOPr) and kappa-opioid receptor (KOPr), respectively, may contribute to its ability to inhibit cocaine seeking. Thus, we hypothesized that a buprenorphine derivative that exhibits antagonist activity at MOPr and KOPr with enhanced agonist activity at the NOPr could provide a more effective treatment. Here we compare the pharmacology of buprenorphine, and two analogues BU10119, and BU12004, in assays for antinociception, and for cocaine- and stress-primed reinstatement in the conditioned place preference paradigm. In vitro and in vivo assays showed that BU10119 acts as an antagonist at MOPr, KOPr, and delta-opioid receptor (DOPr) and a partial agonist at NOPr, whereas BU12004 showed MOPr partial agonist activity, and DOPr, KOPr and NOPr antagonism. BU10119 and buprenorphine, but not BU12004 lessened cocaine-primed reinstatement. In contrast, BU10119, BU12004, and buprenorphine blocked stress-primed reinstatement. The selective NOPr agonist SCH221510, but not naloxone, decreased cocaine-primed reinstatement. Together, these findings are consistent with the concept that NOPr agonism contributes to the ability of BU10119 and buprenorphine to attenuate reinstatement of cocaine conditioned place preference in mice. The findings support the development of buprenorphine analogues lacking MOPr agonism with increased NOPr agonism for relapse prevention to cocaine addiction.
**Significance Statement:** There are no FDA-approved medications for cocaine use disorder. Buprenorphine has shown promise as a treatment for cocaine relapse prevention; however, there are concerns over the abuse liability of buprenorphine. Here we show a buprenorphine analogue BU10119, which lacks mu-opioid receptor agonism, inhibits cocaine-primed and stress-primed reinstatement in a conditioned place-preference paradigm. The results suggest the development of BU10119 for the management of relapse to cocaine-seeking.

**Keywords:** buprenorphine, BU10119, cocaine, reinstatement, nociception receptor, mu-opioid receptor, conditioned place preference
Introduction

Cocaine use increased among adults (age 18-24) between 2014-2018 and contributed to approximately 24,000 psychostimulant-induced deaths per year by 2018 (Hedegaard H 2018). Despite this increasing use and death toll, there are no Food and Drug Administration (FDA) approved drugs to manage cocaine addiction and reduce relapse. Buprenorphine is a crucial tool for the clinical management of opiate addiction, and preclinical and human studies suggest it may also help reduce relapse to cocaine use (Gerra, Fantoma et al. 2006, McCann 2008, Wee, Vendruscolo et al. 2012, Cordery, Taverner et al. 2014). Buprenorphine is used as an agonist-based therapy for individuals suffering from opioid use disorder, and its clinical effects can be ascribed to partial mu-opioid receptor (MOPr) agonist activity as well as its long duration of action (Sorge, Rajabi et al. 2005, Gerra, Fantoma et al. 2006, McCann 2008, Ling, Hillhouse et al. 2016, Zoorob, Kowalchuk et al. 2018). However, the implementation of buprenorphine in the clinical population for the management of cocaine use disorder has been limited due to abuse liability associated with MOPr agonists (Gerra et al., 2006; Montoya et al., 2004). The abuse liability of buprenorphine can be minimized through co-treatment with a non-selective opioid receptor antagonist, such as naltrexone or naloxone (e.g., as in Suboxone) (Gerra, Fantoma et al. 2006, McCann 2008). Unfortunately, current combinations retain some degree of MOPr agonism and associated abuse liability, thereby limiting their clinical utility (Cordery, Taverner et al. 2014); (Johanson, Arfken et al. 2012).

In addition to its partial MOPr agonist activity, buprenorphine is a nociceptin receptor (NOPr) partial agonist, and an antagonist at delta-opioid (DOPr) and kappa-opioid receptors (KOPr) (Lufty and Cowan, 2004; Olson, Duron et al. 2019; also see Tables 1 and 2), resulting in a complex pharmacology. Hence, opioid receptors other than MOPr may play an important role
in the potential of buprenorphine to inhibit cocaine self-administration (Gerra, Fantoma et al. 2006, McCann 2008, Wee, Vendruscolo et al. 2012, Cordery, Taverner et al. 2014). In particular, rodent models have demonstrated that NOPr and KOPr ligands can modify the rewarding properties of cocaine. For example, the endogenous NOPr agonist nociceptin (also known as orphanin or OFQ) attenuates the acquisition and expression of conditioned place preference (CPP) to cocaine (Kotlinska et al., 2002; Sakoori and Murphy 2004), while KOPr antagonists reduce stress-primed reinstatement, but not drug-primed reinstatement, following the extinction of cocaine CPP (Marton-Popovici et al. 2003, Carey, Borozny et al. 2007, Carlezon, Beguin et al. 2009). These findings suggest that the MOPr agonist activity of buprenorphine, while important for managing opioid abuse, may not be necessary for inhibiting cocaine use.

Therefore, we hypothesized that a “mixed efficacy opioid” that has antagonist activity at MOPr and KOPr, but partial agonist activity at NOPr could provide for a safer and more effective treatment for the prevention of relapse to cocaine use disorder. To test this hypothesis, we evaluated the effects of two buprenorphine analogues BU10119 (Fig. 1; also known as OREX-1019) and BU12004 with varying levels of NOPr agonism (Table 1) on cocaine-primed and stress-primed reinstatement following extinction of cocaine CPP. BU10119 and BU12004 are members of a series of C7b-methyl orvinols reported previously as compounds 15a and 15b, respectively in Cueva et al.(2015). BU10119 decreases opioid intake in a self-administration model in rhesus monkeys and prevents drug-plus-cue-induced remifentanil and heroin seeking (Maguire et al., 2020). Buprenorphine and BU12004 (Fig. 1), were compared to BU10119 to probe the possible mechanism(s) responsible for the in vivo effects of BU10119.
Material and Methods

In Vitro Assays

Cell Culture. Chinese hamster ovary (CHO) cells expressing human (h) MOPr, DOPr, KOPr, or NOPr were grown in 50:50 DMEM/F12 media with 10% heat-inactivated FBS and 1X penicillin/streptomycin and 500 μg/mL G418 (all Gibco) in a 37°C humidified incubator with 5% CO2 atmosphere. Cells were harvested with 5 mM EDTA in 50 mM Tris HCl pH 7.4, re-suspended in 50 mM Tris HCl pH 7.4, and homogenized with a tissue grinder for 15-30 seconds on ice. Homogenates were centrifuged at 15,000 g for 30 min, washed, and re-centrifuged. Final homogenates were stored at -80°C.

Radioligand Binding. Homogenates of CHO cells (5-15 μg/mL protein) expressing hMOPr, hDOPr, hKOPr, or NOPr were incubated with 0.2-0.5 nM 3H-diprenorphine (or 3H-nociceptin for CHO-hNOPr) and varying concentrations of a competitor ligand at room temperature for 1-3 h to reach equilibrium. Reactions were terminated by rapid filtration through GF/B filter mats (PerkinElmer) and washed with cold 25 mM Trish HCl pH 7.4 buffer, dried, and ECOLUMNETM scintillation cocktail (MP Biomedicals, Santa Cruz, CA, USA) added. Bound radioactivity was measured using a MicroBeta2 scintillation counter (PerkinElmer). Assays were performed on at least three separate occasions in duplicate.

[35S]-GTPγS binding. MOPr-, DOPr-, KOPr- or NOPr-CHO cell homogenates (10-15mg/mL protein) were incubated with 50 pM [35S]-GTPγS (Perkin Elmer) in a buffer comprising 50 mM Tris HCl pH 7.4, 125 mM NaCl, 5 mM MgCl2, 1 mM EDTA and 30 μM GDP, for 50 min at 30°C. Reactions were terminated by vacuum filtration through GF/C filters using a Brandell Cell harvester and washed five times with ice-cold GTPγS buffer. Filters were dried and bound [35S]-
GTPγS was measured as above, under radioligand binding. Assays were performed on at least three separate occasions in duplicate.

**β-arrestin2 Recruitment.** The PathHunter® assay was used to measure β-arrestin2 recruitment to MOPr in CHO cells (DiscoveRx, Fremont, CA) according to the manufacturer’s protocol and read using a Synergy 2 plate reader (BioTek, Winooski, VT). Assays were performed on at least three separate occasions in triplicate.

**Forskolin-Stimulated Adenylyl Cyclase.** Forskolin (1μM)-stimulated cAMP inhibition was measured in CHO-MOPr cells using the AlphaScreen cAMP Detection Kit (PerkinElmer) in the presence of increasing ligand concentrations following the manufacturer’s instructions. cAMP levels were quantified on a Varioskan MultiMode Plate Reader (Thermofisher). Assays were performed on at least four separate occasions in triplicate.

**Docking Studies.** Performed using the Molecular Operating Environment (MOE) suite of programs (2019.01) and the NOPr crystal structure with the antagonist C-24 bound (PDB 4EA.3). MOE “Quick Prep” was used to determine the protonation state, calculate partial charges, and relax strain in atoms within 3 A of the orthosteric ligand. Ligands were prepared by calculating the dominant protonation species at pH 7, partial charges, and using the lowest energy conformation. The docking pocket was defined by residues within 4.5 A of C-24. Binding poses required a cation within 1.5 A of the tertiary N of C-24 that forms a salt bridge with Asp130. Ligand poses of the docked and co-crystallized C-24 were compared using DockRMSD (Zhang 2019).

**In Vivo Behavioral Assays**

**Subjects.** Adult C57BL/6 mice (Envigo Laboratories or bred in-house at University of Michigan) 8-16 weeks of age were used for all behavioral experiments. There were no
behavioral differences in mice purchased or bred at University of Michigan. For the antinociceptive assays, male and female C57BL/6 mice were used; our preliminary studies identified no differences between the sexes in baseline responses or in drug responses across assays. Baseline responses showed no differences between the sexes in the tail withdrawal assay \( t(18)=0.03, p=0.98 \), the acetic acid stretch assay \( t(12)=0.14, p=0.89 \), or the von Frey assay \( t(12)=0.14, p=0.89 \). The effects of morphine or EKC in the warm water tail withdrawal assay did not differ significantly between males and females (morphine, repeated measures two-way ANOVA interaction: \( F(2,14)=3.3, P=0.07 \), main effect of sex: \( F(1,7)=0.05, P=0.84 \); EKC, repeated measures two-way ANOVA interaction: \( F(2,14)=0.6, P=0.6 \), main effect of sex: \( F(1,7)=0.08, P=0.8 \)). The effects of SNC80 or vehicle did not differ between males and females in the acetic acid stretch assay (two-way ANOVA interaction: \( F(1,8)=0.9, P=0.4 \), main effect of sex: \( F(1,8)=2, P=0.2 \)). Finally, the effects of BU10119 on mechanical nociception did not differ between males and females (two-way ANOVA interaction: \( F(1,16)=3, P=0.1 \), main effect of sex: \( F(1,16)=0.2, P=0.6 \)). Since no sex differences were identified in these antinociception assays, treatment groups were comprised of 50% males/50% females or all males or all females, depending on the availability of animals in our colonies. However, male C57BL/6 mice were exclusively used for the reinstatement experiments to reduce variability, since there is a reported sex difference in cocaine conditioned place preference (Carroll et al., 2004; Feltenstein et al., 2011; Hilderbrand and Lasek, 2014).

All mice were group housed on a 12-h/12-h light/dark cycle (lights on 0600 h) at 20 ± 2°C with all training and test sessions conducted during the light portion of the cycle. Food and water were freely available in the home cage. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Michigan and the University
of Wisconsin Green Bay, in accordance with National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animals Resources, 2011).

**Antinociceptive Assays**

**Warm Water Tail Withdrawal (WWTW).** To determine the *in vivo* bioavailability and antagonist activity of BU10119 at MOPr and KOPr we examined the effects of BU10119 on morphine- and ethylketocyclazocine (EKC)-induced antinociception in C57BL/6 mice using the warm-water tail-withdrawal assay. Mice were given a single injection of saline (i.p. for morphine; s.c. for EKC) to determine baseline latency, followed by three to four cumulative doses of agonist at 30 min intervals. The distal tip of the mouse’s tail was placed in a 50 °C warm water bath following each injection. The latency to tail flick was measured with a cutoff time of 20 sec to prevent tissue damage (Lamberts et al., 2013). Mice received a pretreatment injection of 1 mg/kg or 10 mg/kg BU10119 (i.p.) 30 min before either agonist. Additionally, 1 mg/kg naltrexone (i.p.) served as a positive control against morphine- and EKC-induced antinociception.

**Acetic-Acid Writhing.** This test was used to evaluate *in vivo* effects of BU10119 at DOPr. The selective DOPr agonist SNC80 was administered s.c. 30 min prior to i.p. injection of 0.4 ml of 0.56% acetic acid. After five min, mice were placed into an acrylic test chamber for a 30-min observation period. The number of stretches was counted (Dripps et al., 2017). Writhing was operationally defined as a contraction of the abdomen followed by extension of the hind limbs. To determine the in vivo effects of BU10119, mice received a pretreatment injection of 1 mg/kg BU10119 (s.c.) 30 min before SNC80 administration.

**Von Frey Assay.** Mechanical allodynia was used to determine the pharmacological effects of BU10119 at NOPr. NOPr agonists have been shown to produce allodynia in the von Frey assay
(McDougall and Larson, 2006; present study). The “up-down” method was used to assess mechanical allodynia. The hind paw was stimulated using von Frey filaments (North Coast Medical, Morgan Hill, CA), starting with the 0.6 g filament and increased to a max filament of 6.0 g (Ignatowska-Jankowska et al., 2015; McDougall and Larson, 2006). A response was operationally defined as licking or lifting of the paw away from the stimulus. The NOPr agonist SCH221510 (i.p.) and BU10119 (i.p.) were given 30 min prior to the test. To determine if the allodynia effects produced by BU10119 were mediated by NOPr, either vehicle or the NOPr antagonist, J-113397 was administered (s.c.) 30 min prior to 1 mg/kg BU10119.

**Locomotor Activity Studies.** To determine if the observed antinociceptive or CPP effects of BU12004 were due to locomotor impairment or sedation, we employed an open field experiment. Experimental sessions were conducted in three standard Plexiglas open field arenas (29cm x 29cm x 20 cm) enclosed within sound attenuating cubicles (Med-Associates). Each chamber was equipped with three 16-beam IR arrays, which tracked distance traveled (cm) and rearing. Med-Associates Activity Monitor (version 7) was used for data collection. Mice received a 30 min acclimation session prior to the start of testing. A between subjects design was used in which the baseline activity (i.e., distance traveled) from the 30 min acclimation session was used to match groups to ensure there were no differences between groups (F (2, 29) = 0.25, P = 0.79). Mice were administered BU12004 (0-10 mg/kg; i.p.) 30 mins prior to the test session, then placed in the open field arena. Behavior was recorded for 30 mins.

**Reinstatement Studies**

**Apparatus.** Mice were trained in a standard two chamber CPP box (Med Associates Inc.). The CPP box consisted of a white side and black side that were separated by a neutral wall equipped with a manual guillotine style door. The white side was equipped with a mesh floor; whereas, the
black side was equipped with a steel bar floor. Illumination in both chambers was the same.

Locomotor activity was accessed with IR photobeam detectors.

**Acquisition of Cocaine Conditioned Place Preference.** CPP training, drug-primed reinstatement, and stress-primed reinstatement protocols were adapted from published methods (Brabant et al., 2005; Carey et al., 2007; McLaughlin et al., 2003; Szumlinski et al., 2002). Preliminary studies showed that a two-day conditioning procedure with 15 mg/kg cocaine produced a robust place preference that would extinguish using “natural extinction” training.

CPP experiments consisted of five phases: Bias test (day 1), conditioning training with either cocaine or saline (days 2 and 3), preference test day (day 4), extinction training (day 5+ until criteria were met), and reinstatement testing (the day following meeting extinction criteria). A bias test session was conducted on day one in which mice were placed in the box and allowed to freely explore both sides of the CPP box for 30 min. Mice that had a strong initial preference for one side (>70% of time spent on one side) were eliminated from the study (n = 7; <3% of all animals used in CPP experiments). An unbiased counterbalanced design was used to assign drug pairings such that approximately half the mice received cocaine conditioning on the black side, and the other half received cocaine conditioning on the white side. Cocaine pairing was assigned regardless of their bias test (i.e., some mice received cocaine on the side where they spent more time during the bias test day, and others received cocaine on the side where they spent less time). On conditioning days (days 2 and 3) mice received saline (s.c.) in the morning and were confined to the saline-paired side for 30 min. In the afternoon, mice received 15.0 mg/kg cocaine (s.c.) and were confined to the drug-paired side for 30 min. The same conditioning was repeated the next day (day 3). Mice were tested 24 h following the last conditioning session. On the test day, no injections were given prior to the preference test session and mice were free to explore both
sides of the CPP box for 30 min. Time spent on each side was recorded. Mice that did not
demonstrate a preference for the cocaine-paired side were eliminated from the re-instatement
study (n = 20; <10% of all animals used in CPP experiments). To determine the effect on
acquisition of cocaine CPP, BU10119 (1.0 and 10 mg/kg) was administered 30 min prior to
cocaine during the conditioning phase (days 2 and 3), and cocaine preference was determined on
day 4.

**Extinction Training.** Mice received daily “natural extinction” training (Monday-Friday). Mice
were placed into the CPP box and were free to explore both sides of the CPP box for 30 min.
Mice did not receive an injection during extinction training. Extinction continued until the
preference for the cocaine-paired side was reduced by at least 50% of the initial test preference
(day 4). Extinction was determined for each mouse individually (not as a group); preference
times from the bias test day (day 1) were used to determine preference scores (see data analysis
section). Mice that did not extinguish their preference after 40 sessions were eliminated from the
experiment (n = 18; <10% of all animals used in CPP experiments).

**Cocaine-Primed Reinstatement.** Once mice met the extinction criteria, they underwent
cocaine-primed (15 mg/kg i.p.) reinstatement. Mice received an injection of BU10119 (0.1-10.0
mg/kg), buprenorphine (0.1-1.0 mg/kg), BU12004 (1.0 or 10.0 mg/kg), naloxone (10.0 mg/kg),
SCH221510 (1.0 and 10.0 mg/kg) or vehicle 30 min prior to cocaine and preference was
measured over 30 mins. Pretreatment times of the drugs were based on published literature.
Specifically, Maguire et al., (2020) showed that BU10119 reached a $T_{\text{max}}$ in 20 mins following
systemic administration in rats and BU10119 was effective at blocking remifentanil-prime
reinstatement in a nonhuman primate model of self-administration with a 15 min pretreatment
time. Additionally, a 30 min pretreatment time for buprenorphine has been shown to block cocaine-primed reinstatement in a model of rat self-administration (Comer et al., 1993).

**Stressed-Primed Reinstatement.** Mice received an injection of 1.0 mg/kg BU10119, 1.0 mg/kg buprenorphine, 1.0 mg/kg BU12004 or vehicle 30 min prior to the swim-stress. To generate a swim stress, mice were placed in a glass cylinder (18 cm tall x 14 cm in diameter) filled with 30°C water to a depth of 14 cm for 6 min. Following swim stress, mice were towel dried and placed in the CPP boxes for 30 min. Time spent in each side was recorded.

**Drugs**

[^3]H-Diprenorphine was used for binding experiments at MOPr, KOPr, and DOPr;[^3]H-Nociception for NOPr. DAMGO, U69.593, SNC80, and[^14]Arg,[^15]Lys]Nociception were used as standards for[^35]S[GTPγS experiments. BU10119 and BU12004 were synthesized as previously described and were >98% pure (Cueva et al., 2015). Morphine, naloxone, naltrexone, cocaine, and buprenorphine were obtained from the National Institute on Drug Abuse Research Resource Drug Supply Program (Bethesda, MD, USA). DAMGO, U69.593, Ethylketazocine (EKC), and SNC80 were purchased from Sigma Aldrich (St. Louis, MO, USA). (±) J-113397 and SCH221510 were purchased from Tocris Sciences (Minneapolis, MN, USA). All compounds were dissolved in 0.9% saline except for EKC and SNC80, which were dissolved in 3 % (v/v) 1 M HCl and brought to volume with sterile water and BU10119, BU12004, J-113397, and SCH221510, which were dissolved in a 1:1:8 ethanol: alkamuls: sterile water. Drugs were administered by subcutaneous (s.c.) or intraperitoneal (i.p.) injection at a volume of 10 mL/kg.

**Data Analysis.**

All data were analyzed using GraphPad Prism (7.0; San Diego, CA). Competition binding data were converted to binding affinities (Ki, nM). *In vitro*, functional data were
analyzed to provide potency values (EC$_{50}$, nM) and maximal effects compared to the standard agonists DAMGO (MOPr), SNC80 (DOPr), U69,593 (KOPr), and [$_{14}$Arg, $_{15}$Lys]nociceptin (NOPr) or antagonist affinity values (K$_B$) if no agonism was observed. For warm water tail withdrawal, data are expressed as a percentage of maximum possible effect (% MPE), where % MPE = (post-drug latency - baseline latency) / (cutoff latency - baseline latency) X 100. ED$_{50}$ value with 95% confidence intervals was calculated using the least-squares method of linear regression with the linear portion of the dose-effect curve. One-way analysis of variance (ANOVA) was conducted on all antinociceptive behavioral assays. For the initial cocaine preference, place preference score = time spent on the drug-paired side on test day (day 4) - time spent on the drug-paired side on the bias test day (day 1). For the extinction phase, place preference scores = time spent on the drug-paired side on an extinction day - time spent on the drug-paired side on the bias test day (day 1). A paired t-test was conducted to determine if there was a significant reduction in preference time from the first preference test and the extinction test. For the drug-primed and stress-primed reinstatement assays, an independent t-test or one-way ANOVA was conducted based on the number of treatment conditions. The criterion for significance was P < 0.05, and all significant ANOVAs were followed by a Tukey post hoc test.

**Results**

**In vitro Assays**

**Competition Radioligand Binding.** Table 1 shows the binding affinity of BU10119, BU12004, and buprenorphine at human MOPr, DOPr, KOPr, and NOPr in membrane preparations of CHO cells heterologously expressing each receptor individually. All three compounds have similar nanomolar binding affinity for MOPr, DOPr, and KOPr with no selectivity between these
receptors (Table 1). In contrast, there was a clear rank order of binding affinity at the NOPr:
BU10119 (Ki = 80 ± 30 nM) > BU12004 (Ki = 600 ± 120 nM) > buprenorphine (Ki = 3200 ± 700 nM) (Table 1).

**Agonist and Antagonist Activity.** The agonist activities of BU10119, BU12004, and buprenorphine at the opioid receptors were evaluated using the [35S]-GTPγS functional assay (Table 2). As expected, buprenorphine was a potent partial agonist at MOPr (EC50 = 0.8 ± 0.3 nM) with a maximal stimulation of 35 ± 6% of the standard MOPr agonist DAMGO. In contrast, BU10119 and BU12004 did not stimulate [35S]-GTPγS binding, suggesting they act as MOPr antagonists in this assay. Accordingly, both compounds shifted the concentration-response curve of DAMGO to the right, demonstrating antagonist activity. Antagonist affinity values (KB) of the compounds calculated from these data were comparable to their binding affinities (Ki) from competition binding assays (Table 1). To confirm the buprenorphine analogues are antagonists at MOPr, we examined their activity in two other functional assays, inhibition of forskolin-stimulated cAMP accumulation and recruitment of β-arrestin2. Inhibition of forskolin-stimulated cAMP accumulation is an assay with higher sensitivity enabling better detection of low efficacy partial agonists. In this assay, BU10119 had no effect, corroborating antagonist activity at MOPr; whereas BU12004 (EC50 = 200 ± 68 nM), with a maximal effect of 23 ± 2% of that seen by DAMGO (EC50 = 11 ± 2 nM) showed partial MOPr agonist activity, albeit less then buprenorphine (maximal effect = 34 ± 5%). Neither BU10119 nor BU12004 recruited β-arrestin2 to MOPr, which, like the [35S]-GTPγS assay, involves less signal amplification than cAMP accumulation. The discrepancy in the MOPr agonist activity of BU12004 across the assays is consistent with partial agonists producing antagonist or agonist activity depending on the efficacy requirements of the assay and system used (Kenakin, 2018).
At NOPr, BU10119 (EC$_{50} = 120 \pm 20$ nM, with a maximal stimulation of $38 \pm 5\%$ compared to the standard $^{14}$Arg,$^{15}$Lys-nociceptin) was 18-fold more potent than buprenorphine (EC$_{50} = 2100 \pm 300$ nM; maximal effect $44 \pm 4\%$) at stimulating $[^{35}]$S-GTP$_{y}$S binding (Table 2). BU12004 did not significantly stimulate $[^{35}]$S-GTP$_{y}$S binding but instead, shifted the concentration-response curve for the NOPr agonist $[^{14}]$Arg,$^{15}$Lys]-nociceptin to the right, exhibiting antagonist activity ($K_B = 190 \pm 30$ nM).

BU10119, BU12004, or buprenorphine failed to stimulate $[^{35}]$S-GTP$_{y}$S binding at DOPr or KOPr and accordingly acted as DOPr and KOPr antagonists, producing a rightward shifts in the concentration-response curves for SNC80 and EKC, respectively. All three compounds produce similar $K_B$ values at DOPr and KOPr (Table 2).

Taken together, the varying in vitro profiles of buprenorphine, BU10119, and BU12004 across opioid receptors provide tools to evaluate the role of these receptors in in vivo animal models.

**In Silico Docking Studies.** To better understand why BU10119, BU12004, and buprenorphine have different activities at NOPr, each compound was docked into the inactive NOPr structure obtained from co-crystallization with the NOPr peptide-like antagonist C-24 (PDB code 4EA3.A) using Molecular Operating Environment software (MOE; Chemcomp.com). Simulations at other receptors were not performed because the small differences in affinity of the three compounds across MOPr, DOPr and KOPr suggest minimal differences in ligand conformation at these receptors. Moreover, orvinols are far less studied at NOPr than the traditional opioid receptors (Husbands, 2013). The docking procedures were first validated by re-docking the co-crystallized NOP antagonist (C-24) into the NOPr orthosteric site. The docked structure accurately reproduced the co-crystalized ligand conformation with an RMSD = 0.73 Å.
between the co-crystallized C-24 conformation and C-24 docked pose. NOPr binding pocket residues within 4.5 Å of C-24 matched those in the NOPr crystal structures (Thompson et al., 2012; Miller et al., 2015) and retained the critical Asp130 salt bridge (Fig. 2).

Overlay of BU12004 with C-24 (Fig. 2A) in the binding site shows common residue contacts for these two antagonists. However, comparison of the docked poses of buprenorphine, BU10119, and BU12004 predicts that the C20 (R₂) substituents may interact with different NOPr residues (Fig. 2B-E). The aryl methyl of BU12004 fits into a sub-pocket populated by Ile110, Asp110, and Gln107, forming several van der Waals interactions, in addition to a π-hydrogen bond interaction between the 2-Me-benzyl ring of BU12004 and Arg302. In contrast, the benzyl moiety of BU10119, lacking the 2-Me substituent, forms van der Waals’s contacts with a larger pocket that includes residues Val126, Cys200, and Asp110 and does not exhibit a π-H with Arg302. The NOPr ligand co-crystallized with C-24 shows an H-bond between the amide nitrogen of C-24 and Gln 107. The docking poses of BU10119 and BU12004 predict that these molecules interact with this same Gln via their C20-OH substituent. On the other hand, the C20-hydroxyl of buprenorphine appeared directed away from this residue. A search for the lowest energy conformations revealed the rotation of R₂ substituent could be due to the presence of the larger C7 R₁ substituent (-CH₃) of BU10119 and BU12004, compared to the R₁ substituent (H) in buprenorphine (Fig. 1).

**In Vivo Studies**

**Antagonist Activity of BU10119 at MOPr, DOPr and KOPr.** To determine the *in vivo* bioavailability and antagonist activity of BU10119 at MOPr, KOPr, and DOPr, we examined the effects of BU10119 on morphine-, EKC-, and SNC80-induced antinociceptive activity in C57BL/6 mice using the warm-water tail-withdrawal and acetic-acid writhing assays (Fig. 3).
When administered alone, morphine (ED$_{50}$ = 3.1 mg/kg [C.I. = 2.7-3.4]) and EKC (ED$_{50}$ = 1.2 mg/kg [C.I. = 0.8-1.7]) produced a dose-dependent antinociceptive effect (Fig. 3A, B). Pretreatment with 1.0 mg/kg BU10119 produced a 7-fold rightward shift in the morphine dose-response curve (ED$_{50}$ = 20.5 mg/kg [C.I. = 15.3-27.6]); whereas 1.0 mg/kg naltrexone pretreatment produced a 4-fold shift of the morphine curve (ED$_{50}$ = 11.5 mg/kg [C.I. = 9.7-13.6]). Pretreatment with 1.0 mg/kg BU10119 or naltrexone produced a similar 5-fold rightward shift of the EKC dose response curve (ED$_{50}$ = 5.7 mg/kg [C.I. = 4.2-7.8]) or naltrexone (ED$_{50}$ = 5.4 mg/kg [C.I. = 3.3-8.8], respectively). BU10119 at 10 mg/kg completely blocked the antinociceptive effects of EKC (Fig. 3B).

To evaluate DOPr activity in vivo, we employed the acetic-acid writhing assay as DOPr agonists are less responsive in assays using thermal nociception (Broom et al., 2002; Mosberg et al., 2014). SNC80 (32.0 mg/kg) significantly decreased writhing following the 0.6% acetic acid injection (F (3, 20) = 5.02, P = 0.009; Fig. 3C). Pretreatment with 1.0 mg/kg BU10119 completely reversed the antinociceptive effect of 32.0 mg/kg SNC80. Additionally, BU10119 (10.0 mg/kg) did not alter writhing behavior when given alone, indicating that it does not have agonist activity, even in this low efficacy-requiring assay (Fig. 3C).

**Partial Agonist Activity of BU10119 at NOPr.** BU10119 exhibited partial agonist activation at the NOPr in the $[^{35}\text{S}]$-GTP$\gamma$S binding assay (Table 1). In vivo, NOPr agonists produce mechanical allodynia (McDougall and Larson, 2006). Thus, the Von Frey assay was used to determine if BU10119 induces allodynia, as determined by a more sensitive paw withdrawal threshold. The NOPr agonist SCH221510 (1.0 mg/kg, i.p.), used as a positive control, significantly reduced paw withdrawal threshold in mice, producing an allodynic response (t (10) = 8.56, P < 0.001, Fig. 3D). Treatment with 1.0 mg/kg BU10119 also significantly reduced paw
withdrawal threshold that was reversed by the selective NOPr antagonist J-113397 (3.2 mg/kg) (F (2,28) = 18.16, P < 0.001, Fig. 3E).

**Partial Agonist Activity of BU12004 at MOPr.** BU12004 produced a partial agonist effect at MOPr in vitro in the forskolin-stimulated cAMP inhibition assay. To determine if the MOPr partial agonist activity is physiologically relevant after systemic administration, we examined the effect of BU12004 in the acetic-acid writhing assay assay. Treatment with 10 mg/kg BU12004 or 1 mg/kg morphine reduced the number of writhes observed (F (3, 22) = 20.57, p < 0.001; Fig. 4A). In the higher efficacy requiring warm-water tail withdrawal assay, BU12004 failed to alter withdrawal latency (Fig. 4B; see pretreatment data point); however, BU12004 (10 mg/kg) potentiated the antinociception effects produced by small morphine doses (1 mg/kg) and attenuated the antinociceptive effects of large morphine doses (10 mg/kg) (Interaction: F (12, 64) = 2.09, P = 0.029; Fig. 4B). Open-field locomotor activity was measured to determine if BU12004-induced motor impairment was a confounding factor in the antinociception or CPP assays. However, BU12004 (1 or 10 mg/kg) did not significantly alter distance traveled (F (2,29) = 1.53, P = 0.23; Fig. 4C) or rearing (F (2,29) = 0.33, P = 0.72; Fig. 4D). Together, these results suggest BU12004 is a partial agonist at MOPr.

**CPP: cocaine-primed reinstatement experiments.** The above results suggest that in vivo, as in vitro BU10119 behaves as a MOPr, DOPr and KOPr antagonist and a NOPr partial agonist, whereas BU12004 behaves as a DOPr, KOPr, NOPr antagonist, and a MOPr partial agonist. We then asked if this compendium of activities would prevent reinstatement of cocaine seeking behavior as measured in a CPP paradigm. We used a two-day cocaine conditioning protocol that, in our hands, produced a robust preference for the drug-paired side with a mean ± SEM of place preference score (sec) of 407.4 ± 13.0 across all 158 mice used in the study. Moreover, this two-
day conditioning procedure permitted a reliable extinction of the drug preference that was significantly different from the preference test day with a mean ± SEM place preference score (sec) of 64.9 ± 11.4 across all mice (t (193) = 31.51, P < 0.001). The mean ± SEM number of days to extinction for all mice was 3.5 ± 0.2 (a breakdown of days to extinction for each treatment condition can be found in Table 3).

Figure 5 shows the effects BU10119, buprenorphine, and BU12004 on cocaine-primed reinstatement. Pretreatment with BU10119 (1.0 and 10.0 mg/kg) 30 min prior to cocaine dose-dependently decreased this cocaine-primed reinstatement (F (3, 27) = 6.11, P = 0.002; Fig. 5A). In contrast, BU10119 failed to alter cocaine-induced hyperactivity during reinstatement (F (3, 27) = 2.38, P > 0.05; Fig 5B). Buprenorphine (1.0 mg/kg) also attenuated cocaine-primed reinstatement without significantly altering cocaine-induced hyperactivity (Reinstatement: F (3, 32) = 3.19, P = 0.038; Activity: F (3, 32) = 0.12, P = 0.95; Fig. 5C, D). BU12004 (1 and 10 mg/kg) failed to significantly alter cocaine-primed reinstatement (F (2, 27) = 1.23, P = 0.31; Fig. 5E), however, 10 mg/kg BU12004 significantly decreased cocaine-induced hyperactivity (F (2, 27) = 7.50, P = 0.002; Fig. 5F). On the other hand, as stated above, 10mg/kg BU12004 did not alter locomotor activity in an open field test when administered alone.

To test which components of the pharmacological activity of BU10119 might be responsible for inhibiting cocaine-reinstatement, we determined the effects of the non-selective DOPr, KOPr, and MOPr antagonist naloxone, which lacks activity at NOPr, and the selective full NOPr agonist SCH215510 on cocaine-primed reinstatement. Pretreatment with 10.0 mg/kg naloxone did not significantly change cocaine-primed reinstatement or cocaine-induced hyperactivity (Reinstatement: t (13) = 0.17, P = 0.86; Activity: t (13) = 0.76, P = 0.46; Fig. 6A, B). On the other hand, SCH215510 (10.0 mg/kg) significantly reduced cocaine-primed
reinstatement without significantly altering cocaine-induced hyperactivity at this dose
(Reinstatement: F (2, 31) = 5.32, P = 0.01 Fig. 6C; Activity: F (2, 31) = 3.81, P = 0.03 Fig. 6D).

We found that the vehicle groups for BU10119 and BU12004 produced reinstatement
scores that were ~200 sec greater than the initial cocaine preference (Fig. 5A, C); however, this
same trend was not present in the saline control group for the buprenorphine experiments (Fig.
5B). To determine if the vehicle (10% ethanol, 10% alkamuls, 80% water) would produce
reinstatement in the absence of cocaine, mice were injected with vehicle or saline prior to the
reinstatement test in the absence of cocaine. Additionally, there is concern over the development
of opioid compounds that have any activity at MOPr due to the potential abuse liability.
Therefore, we determined if these opioid ligands would produce a reinstatement response in the
absence of cocaine. Neither BU10119 (10.0 mg/kg) nor its vehicle elicited reinstatement in the
absence of cocaine as compared to the extinction day (Fig. 7A). In contrast, 10.0 mg/kg
BU12004 produced a robust reinstatement that was consistent with the initial test day (F (3, 32)
= 4.62, P = 0.0086; Fig. 7A). Neither saline nor buprenorphine (1.0 mg/kg) significantly elicited
reinstatement in the absence of cocaine (F (2, 27) = 0.74, P = 0.48; Fig. 7B).

**CPP: Swim stress-primed reinstatement experiments.** To determine if the effects of BU10119
and buprenorphine on reinstatement extended beyond drug-induced reinstatement, we tested the
effects of BU10119, buprenorphine, and BU12004 on swim stress-induced reinstatement of
cocaine conditioned place preference (Fig. 8). BU10119 (1.0 mg/kg), buprenorphine (1.0
mg/kg), and BU12004 (1.0 mg/kg) all blocked swim stress-primed reinstatement as compared
with vehicle control without significantly altering locomotor activity (Reinstatement: F (3, 21) =
7.20, P = 0.0017; Activity: F (3, 21) = 2.49, P = 0.088; Fig. 8A, B). There were no significant
differences between these compounds in their ability to block swim stress-induced reinstatement.
**Effects of BU10119 on the acquisition of cocaine CPP.** Lastly, we determined if BU10119 would attenuate the acquisition of cocaine place preference. For these studies, BU10119 was administered 30 min prior to cocaine administration during the conditioning phase (days 2 and 3), then cocaine preference was determined on day 4. Pretreatment of 1.0 or 10.0 mg/kg BU10119 failed to attenuate cocaine-induced place preference (F (2, 12) = 0.46, P = 0.64; Fig. 9).

**Discussion**

The present study demonstrates that the buprenorphine derivative, BU10119, inhibits drug-primed and stress-primed reinstatement of cocaine reward behavior in mice in a manner consistent with its NOPr partial agonist and KOPr antagonist profile. Additionally, BU10119 produced a greater rightward shift in morphine-induced antinociception as compared with naltrexone at the same doses, indicating that BU10119 is an effective MOPr antagonist. Therefore, BU10119 should have little to no abuse potential. Thus, the study indicates BU10119 mimics the *in vivo* effects of buprenorphine/naltrexone combinations, previously reported to attenuate cocaine-primed and stress-primed reinstatement in rodent models (Cordery et al., 2012), without the concerns of the MOPr agonist:antagonist ratio or pharmacokinetic problems of the drug combination. This is consistent with previous reports that BU10119 neither produces a CPP in mice (Almatroudi et al., 2018) nor maintains responding in rhesus monkeys trained to self-administer remifentanil (Maguire et al., 2020).

The pharmacological mechanisms responsible for blocking cocaine- and stress-primed reinstatement are likely different, making non-selective opioid ligands more valuable than selective ligands. Since buprenorphine, BU10119 and BU12004 have varying degrees of affinity
and efficacy for MOPr and NOPr, we considered the receptor mechanism underlying the inhibition of reinstatement of cocaine CPP. First, neither MOPr agonism nor antagonism appears required to block cocaine-primed reinstatement, as neither BU12004 (MOPr partial agonist) nor naloxone (MOPr antagonist) attenuated cocaine-primed reinstatement. Also, buprenorphine has been shown to block cocaine-primed reinstatement in both the presence and absence of naltrexone (Comer et al., 1993; Cordery et al., 2012; Sorge et al., 2015). The fact that the rank order of maximal MOPr-mediated inhibition of adenylyl cyclase of the compounds (BUP > BU12004 with BU10119 showing no agonist response), did not correspond to their ability to block cocaine reinstatement is consistent with a non-MOPr mediated mechanism.

On the other hand, both BU10119 and buprenorphine blocked cocaine-primed reinstatement and have some degree of agonist activity at NOPr, supported by the finding that the NOPr agonist SCH215510 also blocked cocaine-primed reinstatement. Indeed, the NOPr system has been shown to block or reduce drug seeking behaviors for several classes of abused drugs, including cocaine and opioids (Ciccocioppo et al., 2019). For example, studies have found that intracerebroventricular (i.c.v.) administration of the endogenous NOPr agonist nociceptin attenuates the acquisition of CPP to cocaine (Sakoori and Murphy 2004). In addition, NOPr knockout mice or mice pretreated with the NOPr antagonist J113397 show an enhanced effect of cocaine in CPP experiments, suggesting that endogenous NOPr activation may blunt the rewarding effects of cocaine (Marquez et al., 2008b). Finally, the NOPr agonist activity of buprenorphine has been shown to blunt its rewarding properties as measured using CPP (Marquez et al., 2008a). Administration of nociceptin via the i.c.v route attenuates the increase in extracellular dopamine in the nucleus accumbens following cocaine administration, providing a mechanistic explanation for the role of the NOPr system (Lufty et al., 2001). Taken together,
these data suggest that NOPr agonism likely contributes to the ability of BU10119 and buprenorphine to attenuate cocaine seeking in rodents. Since we only used male mice for the CPP studies, future work should compare responses in female mice which are more sensitive to cocaine CPP (Carroll et al., 2004; Feltenstein et al., 2011; Hilderbrand and Lasek, 2014). In addition, it would be valuable to evaluate the role of KOPr and DOPr antagonists in preventing cocaine-induced reinstatement and other models of cocaine-seeking behavior. KOPr antagonism or DOPr antagonism alone would not seem to be responsible for our observations as BU12004 and naloxone, both of which display KOPr and DOPr antagonism, failed to block cocaine-primed reinstatement. On the other hand, we cannot rule out that the antagonist activity of BU10119 and buprenorphine at these receptors works together with NOPr agonism. Unlike BU10119 and buprenorphine, BU12004 at 10 mg/kg, somewhat reduced cocaine-mediated locomotor activity. However, this does not explain its lack of effectiveness since the compound did not alter locomotion in an open field assay and was effective in blocking stress-induced reinstatement. It is feasible that BU12004 and naloxone could be effective at blocking cocaine-primed reinstatement under different dose and/or time conditions as they do have some opioid receptor actions in common with BU10119 and buprenorphine. Follow-up studies are necessary to fully confirm our findings.

The behavioral differences between buprenorphine, BU10119, and BU12004 are likely derived from the differences in NOPr affinity and efficacy. Consequently, we used in silico docking experiments to determine potential ligand and receptor interactions to explain these differences. BU12004 is a moderate affinity antagonist at NOPr, whereas BU10119 is a partial agonist and buprenorphine a low potency partial agonist. In silico docking of buprenorphine and its analogues into the NOPr provided some insight into these differences. The docked poses of all
molecules retained the critical Asp130 salt bridge which is important for binding to all opioid receptors (e.g. Granier et al., 2012; Perez-Aguilar et al., 2012; Thompson et al., 2012; Fenalti et al., 2014; Che et al., 2018). These docked poses predict a hydrogen bond between the NOPr Gln107 residue and the C20-hydroxyl of BU10119 and BU12004, or the amide nitrogen of the NOP antagonist, C-24; in contrast, buprenorphine did not form an H-bond with Gln107. A Gln107Ala mutation in the NOPr reduces the affinity of C-24 by 10-fold and the potency of nociceptin by > 300-fold (Thompson et al., 2012). Therefore, the inability of buprenorphine to form this bond may explain its weaker NOPr affinity and potency. In addition, docking of BU12004 identified a π-hydrogen bond between the 2-MePh moiety on the ligand and Arg302 located on TM7, which could inhibit the movement of this TM domain, required for agonist activation, thereby stabilizing the inactive NOPr conformation (Manglik & Kruse, 2017); this interaction was not seen with BU10119 which may explain the agonist activity in this molecule.

An important caveat to the above conclusions is that we used an antagonist bound structure of NOPr since an agonist bound structure is not available. Differences in the binding pocket between agonist and antagonist structures in the opioid receptors are subtle (Huang et al., 2015), but even so the above comments can only be viewed as a guide for the design of future experiments.

In contrast to its lack of effect on drug primed reinstatement of cocaine CPP, BU12004 did attenuate stress-primed reinstatement, along with BU10119 and buprenorphine. Although the three molecules have differing NOPr activities, all are KOPr antagonists, at least at the doses tested, suggesting the latter activity might be responsible for preventing stress-induced reinstatement. Several studies have found that KOPr antagonists block stress-primed but not
drug-primed reinstatement (Carey et al., 2007; Husbands, 2013; Jackson et al., 2013; Redila et al., 2008). Moreover, endogenous dynorphins and subsequent activation of KOPr contribute to overall stress responses (Chavkin, 2011; Carroll and Carlezon, 2013; Carlezon and Krystal, 2016; Mague et al., 2003) and KOPr activation is required for swim-stress and social defeat stress enhancement of cocaine and ethanol place preferences scores (McLaughlin et al., 2003; McLaughlin et al., 2006; Sperling et al., 2010) as well as stress-primed reinstatement of nicotine (Jackson et al., 2013).

The current findings that pretreatment with BU10119, like the buprenorphine/naltrexone combination (Cordery et al., 2012), attenuated cocaine-primed and stress-primed reinstatement is in contrast to a study in rhesus monkeys that found BU10119 blocked heroin-primed reinstatement but failed to alter cocaine-plus-cue-primed reinstatement (Maguire et al., 2020). The different effectiveness of BU10119 to block cocaine-primed reinstatement in these two studies might be attributed to the different behavioral assays employed (self-administration vs. CPP) and/or species differences, for example, differences in the localization and level of expression of NOP system components (Bridge et al., 2003). Even though these data suggest more work is needed to understand the differences between rodent and monkey studies, the current findings warrant the further investigation of compounds like BU10119 with antagonist activity at MOPr and KOPr and agonist activity at NOPr as potential treatments for cocaine-use disorder. A potential bonus of this approach is the combined NOPr agonist and KOPr antagonist activity could protect against co-morbid anxiety and depression (Almatroudi et al., 2018; Lambert, 2008).

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**Author Contributions**

Participated in research design: Traynor, Jutkiewicz, Hillhouse, and Olson

Conducted experiments: Hillhouse, Olson, Hallahan, Meurice, Rysztak, Sears, Koppenhaver, and West

Contributed new reagents or analytic tools: Ostovar and Husbands

Performed data analysis: Hillhouse, Olson, Rysztak, and Sears

Wrote or contributed to the writing of the manuscript: Hillhouse, Olson, Jutkiewicz and Traynor
Footnotes

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Financial Disclosure

Stephen M Husbands is an inventor on the patent that describes OREX-1019/BU10119 and was a consultant to Orexigen Therapeutics during this project.
References


Figure 1. Structures of Buprenorphine, BU10119 and BU12004.

Figure 2. Theoretical docked poses of BU10119, buprenorphine, and BU12004 in the orthosteric pocket of NOPr (PDB, 4EA3) using Molecular Operating Environment (MOE). (A) BU12004 (pink) overlaid with the co-crystallized NOPr antagonist C-24 (cyan). (B) Buprenorphine (orange), BU10119 (green), and BU12004 (pink) poses show different orientations of the R₂ substituent (see Fig 1). R₂ groups of buprenorphine and BU10119 point towards Gln107, whereas the R₂ group of BU12004 is predicted to form an π-H bond with Arg302. In (A) and (B) molecular surfaces are shown in dark grey, van der Waals surfaces in transparent blue (hydrophilic), green (lipophilic), and gray (neutral). Amino-acid residues are shown as grey sticks and ligands as the ball and stick representations. (C-E) 2D representation of buprenorphine, BU10119, BU12004 poses and interactions with NOPr residues within 4 Å of the ligand atoms. Residues in mauve are polar and acidic (red outline) or basic (blue outline); those in green are hydrophobic. Interactions are shown by dotted lines (blue for backbone atoms, green for side-chain atoms) with the arrowhead towards the acceptor atom.

Figure 3. In vivo activity of BU10119 in assays of antinociception. Effects of BU10119 on (A) morphine-induced and (B) EKC-induced antinociception in the 50°C warm water tail withdrawal assay. (C) Effects of BU10119 on SNC80-induced antinociception in acetic acid-induce stretching. Effects of 1.0 mg/kg SCH221510 (D) and BU10119 (E) on von Frey paw withdrawal threshold. Significant ANOVAs were followed by a Tukey post hoc test. All data show mean ± SEM. Group sizes: (A) Morphine WWTW: morphine alone (N = 14), BU10119/naloxone pretreatment (N = 6-7); (B) EKC WWTW: EKC alone (N = 17), BU10119/naloxone pretreatment (N = 8), 10 mg/kg BU10119 pretreatment (N = 4); (C) Acetic acid-induce writhing
(N = 6); (D) SCH221510 von Frey (N = 6); (E) BU10119 von Frey (N = 10-11). *p < 0.05, ***p < 0.001, vs. Vehicle; +P < 0.05 vs 32.0 mg/kg SNC80 or 1.0 mg/kg BU10119.

**Figure 4.** Effects of BU12004 on acetic acid-induced writhing (A), morphine-induced antinociception in the 50°C warm water tail withdrawal assay (B), and locomotor activity (C, D). In (B) vehicle or BU12004 was given as a pretreatment 30 mins before the first dose of morphine. Significant ANOVAs were followed by a Tukey post hoc test. All data show mean ± SEM. Group sizes: (A) Morphine WWTW: morphine alone and 1 mg/kg BU12004 (N = 6), 10 mg/kg BU12004 (N = 4); (B) Acetic acid-induce writhing: Vehicle (N = 8), 1.0 and 10 mg/kg BU12004 (N = 6), 1.0 mg/kg morphine (N = 6). (C, D) Locomotor activity and rearing: Vehicle (N = 10), 1.0 and 10.0 mg/kg BU12004 (N = 11). ***p < 0.001, vs. Vehicle.

**Figure 5.** Effects of BU10119 (A, B), buprenorphine (C, D), and BU12004 (E, F) on cocaine-primed reinstatement in mice. (Left Panels) Bars on left of dashed line show the initial cocaine preference and extinction scores. Bars on right of dashed line show the effects of BU10119, buprenorphine, and BU12004 on cocaine-primed reinstatement. (Right Panels) Show cocaine-induced hyperactivity following cocaine-primed reinstatement. Significant ANOVAs were followed by a Tukey post hoc test. All data show mean ± SEM. Group sizes: (A) Vehicle (N = 12), BU10119 0.1 mg/kg (N = 7), 1.0 mg/kg (N = 6), 10 mg/kg (N = 7); (B) Vehicle (N = 12), BU12004 1.0 mg/kg (N = 6), 10 mg/kg (N = 12); (C) Saline (N = 8), Buprenorphine 0.1 mg/kg (N = 9), 0.3 mg/kg (N = 11), 1.0 mg/kg (N = 8). *p < 0.05, **p < 0.01 vs. Vehicle. +P < 0.05 vs 1.0 mg/kg BU12004.

**Figure 6.** Effects of naloxone (A, B) and SCH221510 (C, D) on cocaine-primed reinstatement in mice. Details as in Figure 5. Significant ANOVAs were followed by a Tukey post hoc test. All
data show mean ± SEM. Group sizes: (A) Saline (N = 7), 10.0 mg/kg naloxone (N = 8); (B) Vehicle (N = 19), SCH221510 1.0 mg/kg (N = 8), 10.0 mg/kg (N = 10). *p < 0.05 vs. Vehicle. +P < 0.05 vs 1.0 mg/kg SCH221510.

**Figure 7.** (A) Effects of BU vehicle (10% ethanol, 10% alkamuls, 80% water), BU10119 (10 mg/kg), and BU12004 (10 mg/kg) on reinstatement in the absence of cocaine-prime injection. (B) Effects of saline and buprenorphine on reinstatement in the absence of cocaine-prime injection. Details as in Figure 5. Significant ANOVAs were followed by a Tukey post hoc test. All data show mean ± SEM. Group sizes: (A) N = 6 for all groups; (B) N = 6 for all groups. **p < 0.01 vs. Vehicle.

**Figure 8.** Effects of BU10119, buprenorphine, and BU12004 on swim stress-primed reinstatement in mice. Details as in Figure 5. Significant ANOVAs were followed by a Tukey post hoc test. All data show mean ± SEM. Group sizes: Vehicle (N = 9), 1.0 mg/kg BU10119 (N = 6), 1.0 mg/kg BU12004 (N = 7), 1.0 mg/kg buprenorphine (N = 4). *p < 0.05, **p < 0.01 vs. Vehicle.

**Figure 9.** Effects of BU10119 on the acquisition of cocaine place preference. Details as in Figure 5. All data show mean ± SEM. Group sizes: Vehicle (N = 7), 1.0 and 10 mg/kg BU10119 (N = 4) each group.
Table 1. *In vitro* binding affinity of buprenorphine, BU10119, and BU12004 at MOPr, KOPr, DOPr, and NOPr

<table>
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<th>MOPr</th>
<th>KOPr</th>
<th>DOPr</th>
<th>NOPr</th>
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<tr>
<td>Buprenorphine</td>
<td>0.53 ± 0.17</td>
<td>0.87 ± 0.43</td>
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<td>BU10119</td>
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<td>BU12004</td>
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<td>Naloxone</td>
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<td>1.2 ± 0.2</td>
<td>46 ± 10</td>
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<tr>
<td>[1^4 Arg, 1^5 Lys] Nociceptin</td>
<td>ND</td>
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<td>ND</td>
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Affinity determined using membrane preparations of CHO cells expressing MOPr, KOPr, DOPr, or NOPr. \(^1\) [3H]-Diprenorphine was used as the radioligand at MOPr, KOPr, and DOPr; \(^2\) [3H]-Nociception was used at NOPr. Each experiment was conducted in duplicate, on independent days for n=3-6. Data presented as mean ± SEM.
Table 2. *In vitro* stimulation of $[^{35}S]$GTPγS binding by Buprenorphine, BU10119, and BU12004 at MOPr, KOPr, DOPr, and NOPr.

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<td>Buprenorphine</td>
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<td>44 ± 4%</td>
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<td>2100 ± 300</td>
<td>44 ± 4%</td>
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</table>

$[^{35}S]$GTPγS activity assays were performed in membrane preparations of CHO cells expressing MOPr, KOPr, DOPr, or NOPr. EC<sub>50</sub> and Emax% calculated using DAMGO<sup>1</sup>, U69,593<sup>2</sup>, SNC80<sup>3</sup>, and $[^{14}\text{Arg, 15\text{Lys}]}$Nociception<sup>4</sup> at MOPr, KOPr, DOPr, and NOPr, respectively. Potency of the standards (nM) were: DAMGO, 33 ± 0.31; U69593, 0.93 ± 0.4, SNC80, 1.1 ± 0.31; $[^{14}\text{Arg, 15\text{Lys}]}$Nociception, 0.52 ± 0.06. Antagonist affinity (K<sub>B</sub>) was determined if Emax < 15%. Each experiment was conducted in duplicate, on independent days for n=3-6. Data presented as mean ± SEM.
Table 3. Number of extinction days until mice met extinction criteria of at least 50% reduction in preference for drug paired side (Mean [± SEM])

<table>
<thead>
<tr>
<th>Figure 5</th>
<th>BU10119</th>
<th>Vehicle</th>
<th>0.1</th>
<th>1.0</th>
<th>10.0</th>
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<tbody>
<tr>
<td></td>
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<td></td>
<td>2.58 (± 0.34)</td>
<td>4.43 (± 1.34)</td>
<td>4.5 (± 1.77)</td>
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<tr>
<td>BU12004</td>
<td>Vehicle</td>
<td>1.0</td>
<td>2.58 (± 0.34)</td>
<td>2.33 (± 0.33)</td>
<td>2.5 (± 0.5)</td>
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<tr>
<td>Buprenorphine</td>
<td>Saline</td>
<td>0.1</td>
<td>2.88 (± 0.74)</td>
<td>4.56 (± 1.29)</td>
<td>4.46 (± 0.91)</td>
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<table>
<thead>
<tr>
<th>Figure 6</th>
<th>Naloxone</th>
<th>Saline</th>
<th>10.0</th>
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<tr>
<td></td>
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<td>2 (± 0)</td>
<td>2.88 (± 0.58)</td>
</tr>
<tr>
<td>SCH221510</td>
<td>Vehicle</td>
<td>1.0</td>
<td>4.16 (± 0.48)</td>
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<table>
<thead>
<tr>
<th>Figure 7</th>
<th>Saline reinstatement</th>
<th>BU Veh</th>
<th>BU10119</th>
<th>BU12004</th>
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<tbody>
<tr>
<td></td>
<td>2.5 (± 0.34)</td>
<td>2.5 (± 0.34)</td>
<td>4.5 (± 1.91)</td>
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<tr>
<td>Saline reinstatement</td>
<td>Saline</td>
<td>Buprenorphine</td>
<td>1.83 (± 0.30)</td>
<td>4.17 (± 1.276)</td>
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<table>
<thead>
<tr>
<th>Figure 8</th>
<th>Swim Stress</th>
<th>Vehicle</th>
<th>BU10119</th>
<th>BU12004</th>
<th>Buprenorphine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.33 (± 0.97)</td>
<td>2.86 (± 0.86)</td>
<td>2.63 (± 0.32)</td>
<td>3 (± 0)</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1

Buprenorphine

\[ R_1 = H \]
\[ R_2 = \]

BU10119

\[ R_1 = \text{CH}_3 \]
\[ R_2 = \]

BU12004

\[ R_1 = \text{CH}_3 \]
\[ R_2 = \]
Fig. 2

A. Side view
B. Top view
C. Buprenorphine
D. BU10119
E. BU12004
Fig. 3

(A) Morphine alone
- + 1 mg/kg NTX
- + 1 mg/kg BU10119

(B) EKC alone
- + 1 mg/kg NTX
- + 1 mg/kg BU10119
- + 10 mg/kg BU10119

(C) # of Winites

(D) SCH221510 (mg/kg)

(E) BU10119 (mg/kg)

- **
- ***
- ++
- *
Fig. 4

(A) Number of Writhe

- Bars represent mean ± SEM
- 0: Vehicle
- 1: 1 mg/kg BU12004
- 10: 10 mg/kg BU12004

*** p < 0.001

(B) Withdrawal Latency (sec)

- Vehicle
- 1 mg/kg BU12004
- 10 mg/kg BU12004

* p < 0.05

(C) Distance Traveled (cm)

- Bars represent mean ± SEM
- 0: Vehicle
- 1: 1 mg/kg BU12004
- 10: 10 mg/kg BU12004

(D) Rearing (Sum)

- Bars represent mean ± SEM
- 0: Vehicle
- 1: 1 mg/kg BU12004
- 10: 10 mg/kg BU12004
**Fig. 7**

(A) Vehicle or BU compound Primed Reinstatement

(B) Saline or opioid drug Primed Reinstatement

Mean Place Preference Score (sec)

Test  Ext  Veh  BU10119  BU12004

Treatment Condition

Test  Ext  Saline  BUP

Treatment Condition
Fig. 8

(A) Swim Stress-induced Reinstatement

(B) Locomotor Activity Counts
Fig. 9

Acquisition of cocaine CPP

Mean Place Preference Score (sec)

<table>
<thead>
<tr>
<th>BU10119 (mg/kg)</th>
<th>0</th>
<th>1.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- 0 mg/kg
- 1.0 mg/kg
- 10.0 mg/kg