BU08073 a Buprenorphine Analog with Partial Agonist Activity at mu Receptors *in vitro* but Long-Lasting Opioid Antagonist Activity *in vivo* in Mice

T. V. Khroyan[1], J. Wu[2], W. E. Polgar[1], G. Cami-Kobeci[3], N. Fotaki[3], S. M. Husbands[3], L. Toll[2]

1. SRI International 333 Ravenswood Ave, Menlo Park, CA 94025, USA, 2. Torrey Pines Institute for Molecular Studies, 11350 SW Village Parkway, Port St. Lucie, FL 34990, USA, 3. Department of Pharmacy and Pharmacology, University of Bath, Claverton Down, Bath, BA2 7AY, UK

Running Title: BU08073 a long lasting opiate antagonist

Corresponding authors: For correspondence concerning pharmacology please contact Dr. Lawrence Toll, ltoll@tpims.org. For correspondence concerning chemistry, please contact Dr. Stephen Husbands, s.m.husbands@bath.ac.uk

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1111/bph.12796

Background and purpose. Buprenorphine is potent analgesic with high affinity at mu, delta, and kappa and moderate affinity at NOP receptors. Nevertheless, NOP receptor activation modulates the *in vivo* activity of buprenorphine. Structure activity studies were conducted to design buprenorphine analogs with high affinity at each of these receptors and to characterize them in *in vitro* and *in vivo* assays.

Experimental approach. Compounds were tested for binding affinity and functional activity using [^{35}S]GTPγS binding at each receptor and a whole cell fluorescent assay at mu receptors. BU08073 was evaluated for antinociceptive agonist and antagonist activity and for its effects on anxiety in mice.

Key results. BU08073 binds with high affinity to all opioid receptors. It has virtually no efficacy at delta, kappa, and NOP receptors, whereas at mu receptors, BU08073 has similar efficacy as buprenorphine in both functional assays. Alone, BU08073 has anxiogenic activity and produces very little antinociception. However, BU08073 blocks morphine and U50,488-mediated antinociception. This blockade is not evident at 1 h post-treatment, but is present at 6 h and remains for up to 3-6 days.

Conclusions and Implications. These studies provide structural requirements for synthesis of "universal" opioid ligands. BU08073 has high affinity at all the opioid receptors, with moderate efficacy at mu receptors and reduced efficacy at NOP receptors, a profile suggesting potential analgesic activity. On the contrary, BU08073 has long lasting antagonist activity, indicating that the pharmacokinetics of a compound dictates not only the time course of the behavior but also what receptor-mediated behavior will be observed.

Keywords. Antinociception, Mu opioid receptor, kappa opioid receptor, NOP receptor, binding, tail flick assay, zero maze, BU08073, buprenorphine.

Abbreviations. Beta-FNA, beta-Funaltrexamine; CHO cells, Chinese Hamster Ovary cells; CYP-2C8, cytochrome P450 -2-C8; DAMGO, [D-Ala2, N-MePhe4, Gly-ol]-enkephalin); DMEM, Dulbecco's Modified Eagle Medium;; DMSO, dimethylsulfoxide; DPDPE, [D-Pen2,D-Pen5]Enkephalin), HBSS, Hanks Balanced Salt Solution; JDTic, (3R)-7-Hydroxy-N-[(2S)-1-[(3R,4R)-4-(3-hydroxyphenyl)-3,4-dimethylpiperidin-1-yl]-3-methylbutan-2-yl]-1,2,3,4-tetrahydroisoquinoline-3-carboxamide; N/OFQ, nociceptin/orphanin FQ; NOP receptor Nociceptin OPioid receptor; PEG, polyelthylene glycol, SB612111, (5S,7S)-7-{[4-(2,6-dichlorophenyl)piperidin-1-yl]methyl}-1-methyl-6,7,8,9-tetrahydro-5H-benzo[7]annulen-5-ol; U50,488, 2-(3,4-dichlorophenyl)-N-methyl-N-[(1R,2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide; U69,593, N-methyl-2-phenyl-N-[(5R,7S,8S)-7-(pyrrolidin-1-yl)-1-oxaspiro[4.5]dec-8-yl]acetamide.

INTRODUCTION

The affinity and efficacy of opioid compounds generally can explain their ultimate physiological activity. Based upon original observations, a compound with high efficacy at mu receptors was likely to be analgesic with the concomitant side effects of constipation, respiratory depression, and euphoria leading to abuse liability. Conversely, a compound with high efficacy at kappa receptors was likely to be analgesic, with reduced constipation and respiratory depression, but dysphoria rather than euphoria (Martin, 1983; Martin *et al.*, 1976).

If high efficacy mu receptor opiates, such as morphine and fentanyl are plagued by dangerous side effects such as abuse liability and respiratory depression, in theory lower efficacy at the opioid receptors would potentially lead to an analgesic with a lower mu receptor-mediated side effect profile. This concept was epitomized by buprenorphine, a high affinity partial mu receptor agonist and high affinity delta and kappa antagonist (Cowan *et al.,* 1977; Lutfy *et al.,* 2004; Toll *et al.,* 1998). More recently it was determined that buprenorphine also has moderate affinity at NOP receptors, the fourth member of the opioid receptor family (Huang *et al.,* 2001; Spagnolo *et al.,* 2007). Buprenorphine has been used successfully as an analgesic since the 1980s. Although it is a potent analgesic, the antinociceptive activity is dependent upon the stimulus intensity and it can have an inverted U shaped dose response curve, with reduced antinociception at high doses (Cowan *et al.*, 1977; Lutfy *et al.*, 2003). Buprenorphine also has reduced abuse liability and reduced respiratory depression, compared to morphine, a feature attributed to the partial agonist component of its profile (Dahan *et al.*, 2005; Mello *et al.*, 1993). Furthermore, because it is a lipophilic, long lasting compound, buprenorphine has demonstrated great success as an opioid abuse maintenance medication, as an alternative to methadone. Buprenorphine also blocks self-administration of both cocaine and alcohol in various species, including

people (Mello *et al.*, 1989; Montoya *et al.*, 2004). It has been suggested that the downward portion of the inverted U shaped dose response for antinociception, as well as the ability of buprenorphine to attenuate drug seeking is due to activation of NOP receptors (Ciccocioppo *et al.*, 2007; Lutfy *et al.*, 2003). This hypothesis is consistent with many studies demonstrating: 1. other mixed NOP/mu compounds, in addition to buprenorphine, also have antinociception that can be potentiated by the NOP antagonist SB612111, indicating that mu receptor-mediated antinociception can be reduced by the NOP agonist activity residing in the same molecule (Khroyan *et al.*, 2009; Spagnolo *et al.*, 2008); and 2. N/OFQ as well as selective NOP receptor agonists have been demonstrated to attenuate both conditioned place preference and self-administration of a variety of abused drugs (Ciccocioppo *et al.,* 2004; Kotlinska *et al.,* 2003; Sakoori *et al.,* 2004; Shoblock *et al.,* 2005).

If activity at the NOP receptor can modulate buprenorphine's activity, it seems reasonable that a buprenorphine analog with high affinity at NOP receptors would further mediate opiate behaviors. There are two directions in which NOP binding could modulate the activity of buprenorphine. If NOP affinity and efficacy were increased, the compound might have reduced abuse liability compared to buprenorphine itself or act as a better drug abuse medication. Conversely, a buprenorphine analog with higher affinity at NOP receptors but with reduced efficacy at this receptor might have greater antinociceptive activity.

Accordingly, we have begun to conduct structure activity studies to identify buprenorphine analogs with increased affinity at NOP receptors. To this end we produced a series of buprenorphine analogs with high affinity at all four receptors in the opioid receptor family with variable efficacies at NOP receptors (Cami-Kobeci *et al.*, 2011). The first compound tested from this series was BU08028, a partial agonist at both NOP and mu receptors which proved to have mu-mediated antinociception and

reward that overpowered NOP-mediated inhibition (Khroyan *et al.*, 2011). Here we discuss BU08073, which has high affinity at all four receptors, exhibits *in vitro* partial agonist activity at mu, similar to buprenorphine, but has virtually no agonist activity at kappa, delta, and NOP receptors. However, in vivo, BU08073 produces an unusual profile in that it has virtually no antinociceptive activity in mice when tested alone, but has long-lasting antagonist activity at both mu and kappa receptors using the tail flick assay.

Materials and Methods

Animals. Male ICR mice weighing 25-30g at the start of the experiment were used. Animals were group-housed (N=10/cage) under standard laboratory conditions using nestlets as environmental enrichment in their cages and were kept on a 12:12-hr day/night cycle (lights on at 7:00am). Testing was conducted during the animals' light cycle between 9am-2pm. Animals were handled for 3-4 days before the experiments were conducted. On behavioral test days, animals were transported to the testing room and acclimated to the environment for 1 hr. Mice were maintained in accordance with the guidelines of SRI International and of the Guidelines for the Care and Use of Mammals in Neuroscience and Behavioral Research (National Research Council, 2003). Prior to any in vivo testing, approval for the behavioral protocols was obtained from the institutional ACUC of SRI International.

Drugs. The new buprenorphine analogues were synthesized using methods we have reported recently (Cami-Kobeci *et al.*, 2011; Greedy *et al.*, 2013) for other orvinols, but using the appropriate phenethyl magnesium bromide reagent in the Grignard addition step (see Supporting Information). For binding studies, compounds were dissolved in DMSO and diluted into Tris buffer, pH 7.5. For behavioral studies, BU08073 and U50,488 (Sigma Aldrich, St. Louis, MO) were dissolved in 1-2%

DMSO and 30% PEG 400 solution. Morphine hydrochloride (Eli Lilly & Co., Indianapolis, IN) was dissolved in water. Drugs were injected in a volume of 0.1 ml/30g s.c. Controls received 0.1 ml/30 g of the appropriate vehicle.

In vitro Characterization

Cell Culture. All receptors were individually expressed in CHO cells stably transfected with human receptor cDNA, The cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.1% penicillin/streptomycin, in 100-mm polystyrene culture dishes. For binding assays, the cells were scraped off the plate at confluence. Receptor expression levels were 1.2, 1.6, 1.8, and 3.7 pmol per mg protein for the NOP, mu, kappa, and delta opioid receptors respectively.

Receptor Binding. Binding to cell membranes was conducted in a 96-well format, as described previously (Dooley *et al.*, 1997; Toll *et al.*, 1998). Briefly, cells were removed from the plates, homogenized in 50 mM Tris pH 7.5, using a Polytron homogenizer, then centrifuged once and washed by an additional centrifugation at 27,000 x *g* for 15 min. The final pellet was re suspended in Tris, and the suspension incubated with [^{3}H]DAMGO (51 Ci/mmol, 1.6 nM), [^{3}H]Cl-DPDPE (42 Ci/mmol, 1.4 nM), [^{3}H]U69593 (41.7 Ci/mmol, 1.9 nM), or [^{3}H]N/OFQ (120 Ci/mmol, 0.2 nM) for binding to, mu, delta, kappa and NOP receptors respectively. Non-specific binding was determined with 1 μM of unlabeled DAMGO ([D-Ala2, N-MePhe4, Gly-ol]-enkephalin), DPDPE([D-Pen2,D-Pen5]Enkephalin), ethylketocyclazocine, and N/OFQ respectively. Samples were incubated for 60 min at 25˚C in a total volume of 1.0 ml, with 15 μg protein per well. The reaction was terminated by filtration using a Tomtec 96 harvester (Orange, CT) through glass fiber filters and radioactivity was counted on a Pharmacia

Biotech beta-plate liquid scintillation counter (Piscataway, NJ). IC_{50} values were calculated using Graphpad/Prism (ISI, San Diego, CA) and K_i values were determined by the method of Cheng and Prusoff (Cheng *et al.*, 1973)**.**

[^{35}S]GTPγS binding. [^{35}S]GTPγS binding was conducted basically as described by Traynor and Nahorski (Traynor *et al.,* 1995). Cells were scraped from tissue culture dishes into 20 mM Hepes, 1 mM EDTA, then centrifuged at 500 x *g* for 10 min. Cells were resuspended in this buffer and homogenized using a Polytron Homogenizer. The homogenate was centrifuged at 27,000 x *g* for 15 min, and the pellet re suspended in Buffer A, containing: 20 mM Hepes, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.4. The suspension was re centrifuged at 27,000 x *g* and suspended once more in Buffer A. For the binding assay, membranes (8-15 μg protein) were incubated with [^{35}S]GTPγS (50 pM), GDP (10 μM), and the appropriate compound, in a total volume of 1.0 ml, for 60 min at 25˚C. Samples were filtered over glass fiber filters and counted as described for the binding assays. Statistical analysis was conducted using the program Prism. For the antagonist assay, various concentrations of BU08073 were incubated in the presence of 100 nM N/OFQ to determine antagonist potency. Kb was determined by a modification of Cheng and Prusoff such that $Kb = IC_{50}/(1+[L]/EC_{50})$, where [L] is the concentration of N/OFQ and the EC_{50} of N/OFQ was 1.1 nM.

Membrane potential assay.

Mu opioid receptor functional activity in intact cells was determined by measuring receptor-induced membrane potential change, which can be directly read by Molecular Devices Membrane Potential Assay Kit (Blue Dye) using the FlexStation 3® microplate reader (Molecular Devices). This experiment is similar to a recently published assay in which forskolin induced changes in cAMP levels

which ultimately induced hyperpolarization of CHO cells (Knapman *et al.,* 2014). In this experiment, CHO cells transfected with human mu opioid receptor were seeded in a 96-well plate (30,000 cells per well) one day prior to the experiments. For agonist assays, after brief washing, the cells were loaded with 225 µl of HBSS assay buffer (Hank's Balanced Salt Solution with 20 mM of HEPES, pH7.4), containing the blue dye, and incubated at 37°C. After 30 minutes, 25 µl of the appropriate compounds were automatically dispensed into the wells by the FlexStation and receptor stimulation-mediated membrane potential change is recorded every 3s for 60s by reading 550–565 nm fluorescence excited at 530 nm wavelength. For the antagonist assay, the cells are loaded with 200 µl HBSS buffer containing the blue dye and incubated at 37°C. After 15 minutes, 25 µl of naloxone was added into corresponding wells, and after another 15 minutes, 25 µl of DAMGO or test compounds was added into wells by the FlexStateion, with fluorescence measured as described above. The change in fluorescence represents the maximum response, minus the minimum response for each well. Graphpad PRISM was used to determine the EC_{50} and IC_{50} values.

In vivo Characterization

Assessment of Thermal Nociception

Tail-Flick Assay. Acute nociception was assessed using the tail flick assay with an analgesia instrument (Stoelting) that uses radiant heat. This instrument is equipped with an automatic quantification of tail flick latency, and a 15 sec cutoff to prevent damage to the animal's tail. During testing, the focused beam of light was applied to the lower half of the animal's tail, and tail flick latency was recorded. Baseline values for tail flick latency were determined before drug administration in each animal. The mean basal tail flick latency was 4.34 ± 0.10 SEM.

Drug Regimen. In the first experiment, the effects of BU08073 alone were tested. Animals received a s.c. injection of BU08073 (0.3-10 mg/kg, N=6-9/group; total N=48) and were tested ½-, 1-, and 4-hr post-injection. Controls received an injection of vehicle prior to testing. Because BU08073 alone did not produce a high level of antinociception the effect of 3 mg/kg BU08073 on morphine (mu-mediated) antinociception was examined. In addition, given that BU08073 seems to be a potent antagonist at kappa receptors, its effects on U50,488 (kappa agonist) antinociception was also tested. For these experiments animals received a s.c. injection of BU08073 and 30-min prior to testing they received a s.c. injection of morphine (10 mg/kg) or U50,488 (30 mg/kg). The pretreatment time for BU08073 was altered such that tail-flick latencies were measured 1-hr, 6-hr, 1-day, 3-day, 6-day, and 10-day following the injection of BU08073. Different groups of animals (N=8/time point) were used at each post-injection time point so animals received 1 injection of BU08073 and 30-min prior to their test time they received an injection of either morphine or U50,488 (N=128 total for interaction experiments).

Statistical Analyses. Data from all animals tested were used in the statistical analyses. Antinociception (% maximum potential effect; % MPE) was quantified by the following formula: % MPE = 100 * [(test latency - baseline latency)/(15 - baseline latency)]. If the animal did not respond before the 15-s cutoff, the animal was assigned a score of 100%. Results examining the effects of BU08073 alone were analyzed by using repeated measures ANOVAs with dose (0, 0.3-10mg/kg) as the between group variable and post-injection time point (½-, 1-, and 4-hr) as the repeated measure followed by one way ANOVAs and Bonferonni post hoc tests where appropriate. For the effects of BU08073 on morphine or U50,488-induced antinociception, given that one dose of each drug were used and that different animals were used for each time point, a one way ANOVA with BU08073 post-injection time point (1hr, 6 hr, 1-day, 3-day, 6-day, and 10-day) was used as the between group variable. Significant effects were further analyzed by Bonferonni post hoc tests. The level of significance was set at $P<0.05$.

Assessment of Anxiety

Elevated Zero-Maze. This maze is an elevated annular platform with two opposite quadrants enclosed with clear Plexiglas and two open quadrants connecting the two enclosed quadrants (Shepherd *et al.*, 1994). The elevated zero maze similar to the elevated plus maze has been used as a model to measure anxiety in rodents repeatedly (Braun *et al.*, 2011; Khroyan *et al.*, 2012; Shepherd *et al.*, 1994). In our laboratory we have shown that diazepam significantly increased the amount of time spent in the open arms relative to vehicle controls indicative of its anxiolytic properties (Khroyan *et al.*, 2012). Similar lighting conditions and experimental set-up were used as before (Khroyan *et al.*, 2012) and animals were placed on the edge of the enclosed quadrant and behavior was assessed for 5 min. The amount of time spent in the open quadrants, latency to enter the open quadrant, and frequency of head dips over the edge of the platform were measured by an observer unaware of the animal's treatment group.

Animals received a s.c. injection of BU08073 (3 mg/kg) and were tested at the 6-hr, 1-day and 6-day post-injection time points. Different groups of animals were used at each time point tested (N=10). Separate groups of animals served as controls and received vehicle injection and were tested at the various time points indicated above. From previous experiments in our laboratory given that the data from animals given vehicle injection at different 'pretreatment' time points and testing on the zero maze has not shown a difference we used an N=5 at each time point (unpublished data). Because all of the vehicle animals at each time point did not differ ($F_{2,12}$=0.006; no differences observed with scatterplot data), the vehicle data were combined resulting in N=15 in the vehicle group. A total number of 45 animals were used for these experiments.

Statistical Analyses. The amount of time spent in the open quadrants, latency to enter the open quadrant, and the frequency of head dips were analyzed with one way ANOVAs with BU08073 post-injection time point (6-hr, 1-day, and 6-day) as the between group variable. Significant effects were further analyzed by Bonferonni post hoc tests. The level of significance was set at $P<0.05$.

Nomenclature. Receptor nomenclature in this manuscript conforms to BJP's Guide to Receptors and Channels (Alexander *et al.*, 2011; Cox *et al.*, 2014).

Results

In vitro Characterization: Receptor Binding and Functional Activity. A series of analogs of buprenorphine were designed and constructed for the purpose of increasing affinity at NOP receptors but maintaining affinity at the opioid receptor (Figure 1). Similar to our previously described compound BU08028, several compounds had high affinity, with Ki values of less than 10 nM, at all four opioid receptors (Table 1). The new ligands bound with high affinity to mu, kappa, and delta receptors with little difference between ligands. Only the somewhat lower affinity of the meta methyl-substituted analogue, BU09054, at delta fell outside of an otherwise narrow range. At NOP receptors the ligands displayed far higher affinity than buprenorphine, to the extent that a number of compounds had affinity for this receptor almost equivalent to that at the mu, kappa, and delta receptors. In general a chloro substituent appeared to be better tolerated than a methyl substituent for binding to NOP receptors.

Each compound was tested for functional activity using the [^{35}S]GTPγS binding assay. As seen in Table 2, generally, the compounds have moderate to low partial agonist activity at each receptor. Low partial agonist activity, at the mu receptor, in this assay has been reported for buprenorphine many

times, and the even lower agonist activity at NOP receptors is consistent with what we have reported previously (Khroyan *et al.,* 2009; Spagnolo *et al.,* 2008). However, in some publications the potency of buprenorphine is somewhat higher at mu receptors (Huang *et al.,* 2001) and efficacy considerably higher at NOP receptors (Bloms-Funke *et al.,* 2000; Huang *et al.,* 2001). In this assay five of the new compounds had efficacy at NOP receptors equivalent to or greater than buprenorphine. The three compounds with the highest efficacies (the unsubstituted and the ortho-substituted analogues) also proved to be more potent than buprenorphine at this receptor, but not to the extent predicted by their binding affinities. At the NOP receptor an ortho-substituent on the phenyl ring was associated with higher efficacy than a meta substituent with para-substitution leading to the lowest efficacy. A very similar SAR was seen at the mu receptor with para-substitution again giving the lowest efficacy compound; at delta and kappa receptors, the effect was at its most pronounced. Of the 7 new compounds, only the 2 para-substituted analogues BU08073 and BU08074 had very low, or no efficacy at the kappa receptor; each of the other compounds was a partial agonist with substantial (50 – 60%) efficacy. Because BU08073 had low partial agonist activity at NOP receptors it was tested for antagonist activity. As seen in Figure 2, BU08073 blocked N/OFQ-induced [^{35}S]GTPγS binding in a concentration dependent way, with an IC_{50} of 697 ± 20 nM (Kb = 14.4 nM).

To examine functional activity in an intact cell assay, we measured real time stimulation of membrane potential using the FlexStation. As seen in Figure 3, DAMGO hyperpolarizes CHO cells transfected with mu receptors (EC_{50} 1.96 ± 0.56 nM), and this is blocked by low concentrations of naloxone. In contrast to the [^{35}S]GTPγS binding assay, both buprenorphine and BU08073 are full agonists in this intact cell assay, with EC_{50} values of 45.5 ± 19 nM and 323 ± 140 nM respectively, indicating significant amplification of the signal with this assay.

Behavioral Activity:

The effects of BU08073 alone on nociception. Because of its moderate efficacy at mu receptors in the [^{35}S]GTPγS binding assay and full agonist activity in the whole cell assay, we tested BU08073 to determine potential antinociceptive activity. The effect of BU08073 on thermal antinociception is shown in Figure 4. The overall ANOVA indicated a significant interaction effect ($F_{10,84}$=4.8, P<0.05). Morphine produced a significant increase in %MPE relative to vehicle controls at each time points (P<0.05). However, BU08073 produced a very small but significant increase in %MPE relative to controls that was evident only at the 1-hr test point and following administration of the highest dose tested (10 mg/kg; P<0.05).

The effects of BU08073 on morphine or U50,488-induced antinociception. Because BU08073 did not produce a high level of antinociception we tested it as an antagonist to morphine antinociception (Figure 5A). The overall ANOVA indicated a significant effect ($F_{7,66}$=17.8, P<0.05). As expected, 10 mg/kg morphine produced an increase in %MPE 30-min post-injection, relative to vehicle controls (P<0.05). When 3 mg/kg of BU08073 was administered 30-min prior to morphine and animals were tested 30-min later, morphine antinociception was still evident. However, when BU08073 was given as a 6-hr, 1-Day, and 3-Day pretreatment prior to morphine administration, morphine antinociception was blocked and these groups of animals produced a similar level of %MPE as vehicle controls. BU08073 still attenuated morphine antinociception 6 days after a single administration of BU08073, (P<0.05), although this group also showed a significant increase in %MPE relative to vehicle controls (P<0.05), indicating mu receptor inhibition was finally subsiding. By day 10, pretreatment with BU08073 did not alter morphine induced antinociception and these animals produced a significant increase in %MPE relative to vehicle controls (P<0.05).

BU08073 is devoid of agonist activity at kappa receptors in the [^{35}S]GTPγS binding assay. Experiments were conducted to determine if this compound had long-lasting antagonist activity at kappa receptors as well, by determining the time course for inhibition of U50,488-mediated antinociceptive activity. As seen in Figure 5B, the ability of BU08073 to antagonize U50,488 was quite similar to its ability to antagonize morphine antinociception. The overall ANOVA indicated a significant effect ($F_{7,60}$=21.6, P<0.05). U50,488 (30 mg/kg) alone produced an increase in %MPE 30-min post-injection relative to vehicle controls (P<0.05). When BU08073 was given 30-min prior to U50,488 and animals were tested 1hr following administration of 3 mg/kg BU08073, U50,488 antinociception was still evident. However, when BU08073 was given as a 6-hr, 1-Day, and 3-Day pretreatment prior to U50,488 administration, U50,488-induced antinociception was no longer evident and these groups of animals were significantly different compared to U50,488 alone (P<0.05) and produced a similar level of %MPE as vehicle controls. By Day 6 and 10 after a single treatment with BU08073 the animals produced a significant increase in %MPE relative to vehicle controls (P<0.05) that were comparable with animals that received U50,488 alone.

The effects of BU08073 alone on anxiety. The effect of BU08073 on anxiety-related behaviors as captured by zero-maze activity is shown in Figure 6. The overall ANOVA indicated a significant effect looking at all the parameters tested ($F_{3,38}$=6.0, P<0.05, amount of time spent in the open quadrants; $F_{3,38}$=3.1, P<0.05, latency to enter the open quadrant; $F_{3,38}$=3.0, P<0.05, frequency of head dips). Animals that were tested 6-hr and 1-day following BU08073 spent less time in the open quadrants, had a greater latency to enter the open quadrants, and had a lower incidence of head dips compared to vehicle controls. Animals that received BU08073 and were tested 6-day post injection were no different than vehicle controls.

In silico pharmacokinetic characterization. BU08073 was examined in silico (ADMET Predictor 6.5, Simulations Plus Inc.), along with buprenorphine as a control, to predict its ADMET properties. BU08073 was predicted to be a better substrate than buprenorphine for CYP-2C8, a P450 enzyme thought to be responsible for some of the N-dealkylation of buprenorphine to norbuprenorphine. (Picard *et al.*, 2005). However, for buprenorphine, N-dealkylation results in an increase in efficacy as well as limiting access to the brain and this would not explain the lack of efficacy and delayed antagonism displayed by BU08073. No other substantial differences in metabolism were predicted between the two ligands. BU08073 has a higher clogP than buprenorphine, lower water solubility and is predicted to be more highly bound to plasma proteins (Table 3). Since both BU08073 and buprenorphine are predicted to have good blood-brain barrier (BBB) penetration, it is likely that the apparent slow kinetics of BU08073 relate to its high protein binding, resulting in a depot-like effect and slow release of the unbound form.

Discussion

As predicted by our earlier studies in the orvinols (Cami-Kobeci *et al.*, 2011), having a lipophilic group separated from C20 by a short chain results in increased NOP activity compared to buprenorphine which has the lipophilic t-butyl group attached directly to C20. This also results in higher efficacy at the other opioid receptors, again as would be predicted from earlier work. (Greedy *et al.*, 2013; Lewis *et al.*, 2004). The high affinity of the new ligands at NOPr relative to buprenorphine is in agreement with the finding of Yu et al. that TH-030418 (a thienylethyl orvinol analogue) had as high affinity at NOP receptors as at the other opioid receptors (Yu *et al.*, 2011). No indication of the efficacy of this compound at NOP receptors was given. Also recently reported was the dimethylphenethyl analogue that has similar affinity for NOP receptors as for the other opioid receptors (Cami-Kobeci *et al.*, 2011). In

[^{35}S]GTPγS assays, this analogue is a lower efficacy partial agonist at the mu receptor than BU08073 (17% vs 32%) but is also a partial agonist at each of the other receptors; this is different than BU08073, which is largely devoid of agonist activity at opioid receptors other than mu.

Buprenorphine, which has only approximately 28% maximal activity in the [^{35}S]GTPγS binding assay, as well as other partial mu opioid agonists, have potent antinociceptive activity in rodents and humans. However, efficacy of partial agonists is completely dependent upon the test being administered. This can be demonstrated both in vitro and in vivo. In the present study we have shown that both buprenorphine and BU0873 are partial agonists at mu receptors in the [^{35}S]GTPγS binding assay while in a whole cell *in vitro* assay in which we measured membrane potential changes, both buprenorphine and BU08073 demonstrate full agonist activity. Buprenorphine also has full agonist activity in an intact cell reporter gene assay at NOP receptors (Wnendt *et al.,* 1999) although it is very weak in the [^{35}S]GTPγS binding assay (Khroyan *et al.,* 2009; Spagnolo *et al.,* 2008). Presumably these differences are due to signal amplification and a high receptor reserve for the intact cells assays. In fact it is very common that compounds with partial agonist, or even antagonist activity in *in vitro* assays can have agonist activity when tested *in vivo*. Buprenorphine provides an example for this phenomenon. Buprenorphine has very low efficacy in the [^{35}S]GTPγS binding assay at NOP receptors (Spagnolo *et al.*, 2008), but has clear agonist activity at NOP receptors when measuring antinociception (Khroyan *et al.*, 2009; Lutfy *et al.*, 2003). As another example, the peptide [Phe1psi(CH2-NH)Gly2]-NC(1-13)-NH2 was described as the first NOP receptor antagonist when tested in the mouse vas deferens assay (Guerrini *et al.,* 1998), while further studies demonstrated that this compound has full agonist activity in transfected cells and is a potent as N/OFQ in blocking opiate analgesia in mice (Calo *et al.,* 1998; Okawa *et al.,* 1999). Due to agonist activity comparable to buprenorphine at mu receptors *in*

vitro, we expected BU08073 to have antinociceptive activity in vivo. Furthermore, high affinity at NOP receptors, with no apparent efficacy should act to potentiate the antinociceptive activity of this compound. However, BU08073 displays only very limited antinociception at a single dose and single time point, in the tail flick. Even more surprising was the observation that although BU08073 antagonized both mu- and kappa-mediated antinociception, antagonist activity of this compound was not evident at 1 h, a time point that is normally associated with maximal activity for many opiates, including its close analog buprenorphine. BU08073 also has high affinity and antagonist activity at the other receptors in the opiate receptor family. But it seems very unlikely that antagonism of these other receptors would block the expected mu-mediated antinociception.

When the antagonist activity of BU08073 was observed over longer periods of time it was determined that this compound has antagonist activity at both mu and kappa receptors that can last for 6 or more days after a single injection. Long lasting antagonists are not unknown. β-FNA is an opiate that has high affinity for mu, delta, and kappa receptors and acutely activates kappa but inhibits mu and delta receptors (Portoghese *et al.*, 1980). However, β-FNA has a reactive electrophilic functional group (a Michael acceptor) and once bound to the receptor, it forms a covalent bond with the mu receptor and thereby acts as an irreversible (and long lasting) antagonist, at this receptor only (Manglik *et al.,* 2012; Takemori *et al.,* 1981). It is often use as a mu receptor antagonist for in vivo studies since 24 h after administration it can block mu receptors without inhibiting delta or kappa to any significant extent (Liu-Chen *et al.*, 1991).

Several compounds can also antagonize kappa receptors for extended periods of time both *in vitro* and *in vivo*. Both nor-BNI and JDTic are very high affinity and selective kappa antagonists that

can block U50,488-mediated antinociception for up to 6 weeks after a single injection (Carroll *et al.*, 2004; Horan *et al.*, 1992; Jones *et al.*, 1992; Metcalf *et al.*, 2005). The mechanism of this extremely long duration of action is not completely clear. These compounds do not bind covalently, so it is not due to functionally removing the receptors. One hypothesis is that these two compounds, and a few other analogs, activate the c-Jun-N-terminal kinase (JNK) family of mitogen-activated protein kinases (MAPK), leading to prolonged inactivation of kappa receptor signaling persisting for weeks after a single exposure (Bruchas *et al.*, 2007; Melief *et al.*, 2010; Melief *et al.*, 2011). Another observation that is not incompatible with the JNK hypothesis is that nor-BNI can be found in the brain of mice, at quantities sufficient to antagonize kappa receptors for at least 3 weeks after a single i.p. administration, suggesting that the long-lasting effect of nor-BNI could be simply due to its pharmacokinetic parameters (Patkar *et al.*, 2013).

Similar to nor-BNI and JDTic, BU08073 does not have a reactive functional group and almost certainly does not form a covalent bond with opioid receptors. Unlike both the mu antagonist β-FNA and the kappa antagonists nor-BNI and JDTic, the long-lasting antagonist activity of BU08073 has a similar time course for both mu and kappa receptors, with antagonist activity most potent at 24 h and decreasing gradually over the next 6-10 days. Most likely, this is a pharmacokinetic phenomenon due to slow entry into the CNS.

Metabolism to an active, but antagonist metabolite, could potentially explain the behavioural results. The metabolism of buprenorphine, which is structurally very closely related, has been well characterised; thus N-dealkylation to give the equivalent nor-compound and glucuronidation at the C3 oxygen of both the parent and the nor compound (Husbands, 2013). Norbuprenorphine may play a role in the pharmacology of buprenorphine, but its profile is as a high affinity, highly efficacious compound

but with very limited access to the brain. Thus metabolism seems an unlikely explanation for the unusual profile of BU08073, although it cannot be ruled out completely.

Slow kinetics could account for the lack of effect of the compound 1 h after administration. What is unclear is how kinetics can affect the efficacy of the compound *in vivo*. *In vitro*, BU08073 has clear partial agonist activity in [^{35}S]GTPγS binding and greater agonist activity in an intact cell assay. Perhaps the slow increase in receptor occupation leads to desensitization prior to generation of a significant antinociceptive signal. Another possibility that may contribute to the actions of this drug is its binding sensitivity to pH. If dissociation is much more rapid at low pH, perhaps the receptor and ligand are internalized but not recycled appropriately. This would reduce receptor availability and thereby functionally antagonize agonist activity at the receptors. Alternately, it could be a function of some ligand directed signaling. It has been hypothesized that biased agonists at the mu receptor could have antinociceptive activity without some of the unwanted side effects (Law *et al.,* 2013). This could work the other direction as well. Perhaps the BU08073/mu receptor interaction does not activate an underlying pathway required for the expression of antinociception.

BU08073 also has long-lasting anxiogenic activity. This is different than the reported anxiolytic activity of systemically or locally administered morphine (Anseloni *et al.*, 1999; Zhang *et al.*, 2008) but consistent with the reported anxiogenic activity of buprenorphine (Lelong-Boulouard *et al.*, 2006). The reason why buprenorphine and BU08073 would have different effects on anxiety behavior than morphine is not clear. Morphine is quite selective for the mu receptor, so anxiogenic activity of buprenorphine and BU08073 might have to do with agonist or antagonist activity at one of the other opioid receptors or NOP. Since the kappa agonist U50488 has anxiogenic activity and these compounds are antagonists at kappa receptors, it seems unlikely that the anxiogenic activity of BU08073 is due to

kappa receptor inhibition. However, several delta receptor agonists have been demonstrated to have anxiolytic activity, so it is possible that delta receptor antagonism could lead to the observed anxiogenic activity (Perrine *et al.,* 2006; Saitoh *et al.,* 2004; Vergura *et al.,* 2008).

In conclusion, BU08073 is a buprenorphine analog with high affinity at each of the receptors in the opioid receptor family. Although it has moderate efficacy at mu receptors, this compound displays very weak antinociceptive activity, and instead exhibits a rather delayed and long lasting antagonism to both mu- and kappa receptor-mediated antinociceptive activity. This unusual property is probably due to very delayed pharmacokinetics. Studies are continuing to determine how pharmacokinetics can modulate opioid receptor mediated actions in vivo.

Acknowledgements

We would like to thank Simulations Plus Inc. for an evaluation license of the ADMET Predictor software. The work was supported by NIH grant DA023281 to LT and DA20469 to SMH.

List of Author Contributions

Khroyan, Wu, Polgar, and Fotaki conducted in vitro, in vivo or in silico experiments

Cami-Kobeci, and Husbands provided materials

Khroyan, Wu, Husbands, and Toll contributed to writing the manuscript

Statement of conflicts of interest

None

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Figure Captions

Figure 1. Structures of buprenorphine and novel compounds tested. Full synthetic details and analysis, including microanalysis are provided in Supporting Information.

Figure 2. BU08073 inhibits N/OFQ-stimulated [^{35}S]GTPγS binding in CHO cells transfected with NOP receptors. Experiments were conducted as described in Materials and Methods. Data in the figure are represented as mean ± SEM for a single representative experiment conducted in triplicate. The EC_{50} value for stimulation of [^{35}S]GTPγS binding by N/OFQ was 12.7 ± 0.56 nM (mean ± SEM from 3 individual experiments). To test as an antagonist the BU08073 dose response was conducted in the presence of 100 nM N/OFQ and the IC_{50} for BU08073 was 697 ± 20 nM (mean ± SEM from 3 individual experiments).

Figure 3. The effect of DAMGO, Buprenorphine, and BU08073 in a whole cell assay measuring membrane potential changes in CHO cells transfected with mu opioid receptors. Experiments were conducted using the FlexStation as described in Materials and Methods. Data shown in the figure are represented as mean ± SEM from a single representative experiment conducted in quadruplicates (A) DAMGO-induced membrane potential fluorescence change tracked for 50 seconds. (B) BU08073-induced membrane potential fluorescence change tracked for 50 seconds. (C) Agonist dose response curve for each compound. EC_{50} values were 1.96 ± 0.56, 45.5 ± 19 and 323 ± 140 (mean ± SEM from 4 individual experiments) for DAMGO, Buprenorphine and BU08073 respectively. (D) Antagonist effect of naloxone demonstrating that all compounds are acting through the opioid receptor. IC_{50} values of naloxone were 63 ± 11, 34 ± 20, and 101 ± 43 140 (mean ± SEM from 4 individual experiments) when inhibiting 100 nM DAMGO, 500 nM buprenorphine, and 2 μM BU08073 respectively.

Figure 4. The effects of a range of doses of BU08073 on tail flick latency compared to morphine (10 mg/kg) and vehicle controls at various post-injection time points (s.c., N=6-9/group). Data are mean %MPE ± SEM. *, P<0.05, significant difference from vehicle controls using repeated ANOVA.

Figure 5. The effects of 3 mg/kg BU08073 on morphine- (**A**) and U50,488-induced antinociception (**B**). All drugs were given via s.c. route of administration. Values on the x-axis indicate time elapsed from the BU 08073 injection. Vehicle, morphine and U 50,488 were given 30min prior to testing. Different groups of animals were used for each BU08073 pretreatment time point (N=8/group). Data are mean %MPE ± SEM. *, P<0.05, significant difference from vehicle controls. †, significant difference from morphine or U50,488 alone.

Figure 6. The effects of 3 mg/kg BU08073 (s.c.) on anxiety as measured using the elevated zero-maze examining time in the open quadrants (**A**), latency to enter an open quadrant (**B**), and number of dips over the side (**C**). Different groups of animals were used to assess the effects of BU08073 (N=10/time point) following different post-injection time points. Data are mean ± SEM. *, P<0.05, significant difference from vehicle controls.

Table 1. Binding affinities K_i of buprenorphine analogs compared with buprenorphine and other prototypical agonists at NOP and other opioid receptors. Data shown are mean ± SEM for at least two experiments conducted in triplicate. *Some of the data for the standard compounds are from (Khroyan *et al.*, 2009)

	Receptor Binding K_i (nM)			
Compound	**Mu ± SEM**	**Delta ± SEM**	**Kappa ± SEM**	**NOP ± SEM**
Buprenorphine	1.5 ± 0.8	6.1 ± 0.4	2.5 ± 1.21	77.4 ± 16.1
DAMGO	1.59 ± 0.17	300 ± 58.6	305 ± 46	>10,000
DPDPE	503 ± 10	1.24 ± 0.09	>10,000	>10,000
U69,593	>10,000	>10,000	1.6 ± 0.26	>10,000
N/OFQ	133 ± 30	>10,000	247 ± 3.4	0.08 ± 0.03
BU08069	2.31 ± 0.27	0.87 ± 0.11	1.53 ± 0.23	3.47 ± 0.54
BU08071	6.60 ± 2.72	2.05 ± 0.64	4.73 ± 0.78	19.8 ± 0.1
BU08072	3.91 ± 0.69	1.22 ± 0.46	4.63 ± 0.80	8.54 ± 0.42
BU08073	2.26 ± 0.33	3.20 ± 0.44	2.65 ± 0.87	7.60 ± 0.78
BU08074	3.90 ± 1.22	2.48 ± 0.06	2.88 ± 0.86	14.2 ± 0.62
BU09037	3.99 ± 0.36	6.01 ± 2.71	0.90 ± 0.35	5.38 ± 0.24
BU09054	5.70 ± 2.35	44.77 ± 14.8	6.48 ± 2.96	24.7 ± 2.19

Table 2. Stimulation of [^{35}S]GTPγS binding by buprenorphine analogs, compared with buprenorphine and other prototypical agonists at NOP and other opioid receptors. Data shown are mean ± SEM for at least two experiments conducted in triplicate.

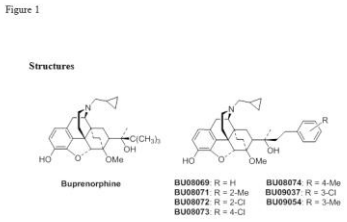
	Mu		**Delta**		**Kappa**		**NOP**	
Compound	**EC_{50}**	**% Stim**	**EC_{50}**	**% Stim**	**EC_{50}**	**% Stim**	**EC_{50}**	**% Stim**
Buprenorphine	10.2 ± 2.2	28.7 ± 1.0	>10,000		>10,000		251 ± 94	15.5 ± 5.8
DAMGO	35.3 ± 0.53	100	-----	-----	-----	-----	----	-----
DPDPE	-----	-----	6.86 ± 0.41	100	----	-----	-----	-----
U69,593	-----	-----	-----	-----	78.4 ± 8.8	100	-----	-----
N/OFQ	-----	-----	-----	-----	-----	-----	8.1 ± 1.38	100
BU08069	0.60 ± 0.34	37.0 ± 0.1	3.16 ± 1.68	24.8 ± 5.4	0.53 ± 0.19	52.6 ± 13	20.8 ± 4.2	24.6 ± 1.9
BU08071	4.90 ± 0.01	56.3 ± 8.3	7.35 ± 0.79	37.7 ± 4.9	1.35 ± 0.6	58.9 ± 2.6	66.3 ± 22	20.5 ± 2.7
BU08072	1.60 ± 0.17	58.7 ± 0.4	1.78 ± .76	72.9 ± 13	0.77 ± 0.3	49.6 ± 16	20.8 ± 8.2	33.5 ± 3.5
BU08073	2.90 ± 0.98	31.9 ± 1.0	*	9.70 ± 4.8	*	--------	*	8.2 ± 1.8
BU08074	*	10.9 ± 7.4	*	4.70 ± 0.2	*	9.20 ± 2.7	*	6.10 ± 0.75
BU09037	9.80 ± 4.54	65.2 ± 6.1	15.34 ± 0.6	58.2 ± 1.2	4.13 ± 2.77	55.2 ± 7.3	*	17.1 ± 1.5
BU09054	7.10 ± 0.90	38.5 ± 6.6	10.29 ± 3.9	27.7 ± 4.5	146 ± 53	50.9 ± 10	*	14.9 ± 1.4

*Stimulation was too low to accurately determine EC_{50} value.

Table 3. ADMET predictions for BU08073 compared to parent compound buprenorphine

	logP (simulation Plus model)	**Intrinsic water solubility (mg/mL)**	**Likelihood of BBB penetration**	**Log of Blood-brain partition coefficient**	**%unbound to plasma proteins**
BU08073	6	7.92E-03	High	0.58	12.47
Buprenorphine	4.72	5.64E-02	High	0.26	29.03

* Stimulation was too low to accurately determine EC_{50} value.



bph_12796_f1

Figure 2

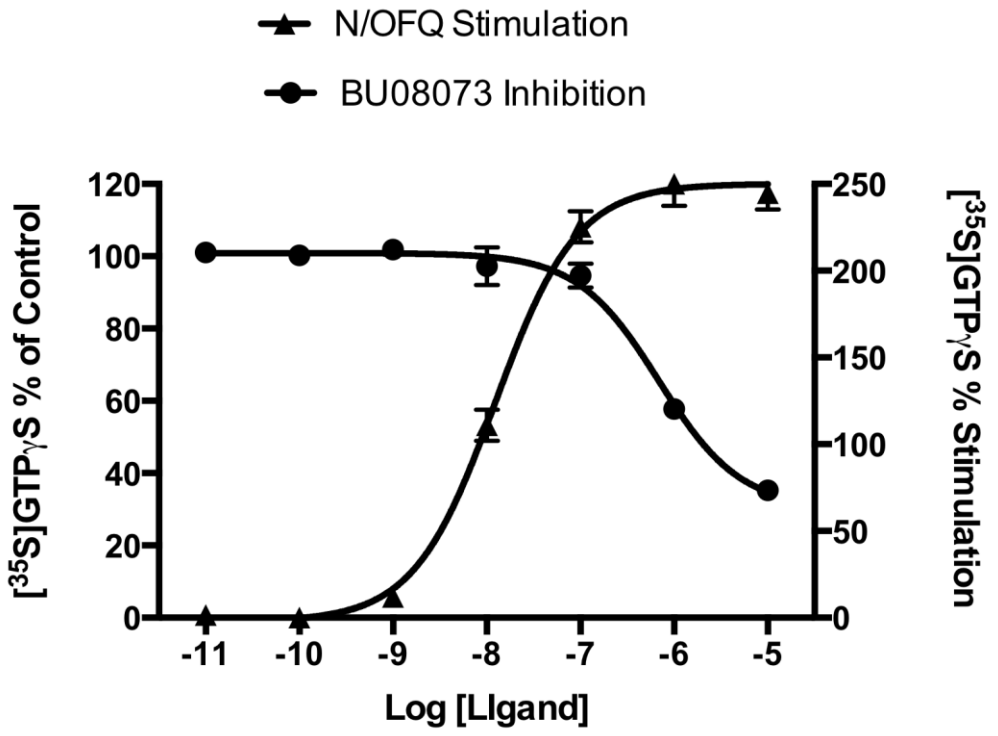


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Figure 3

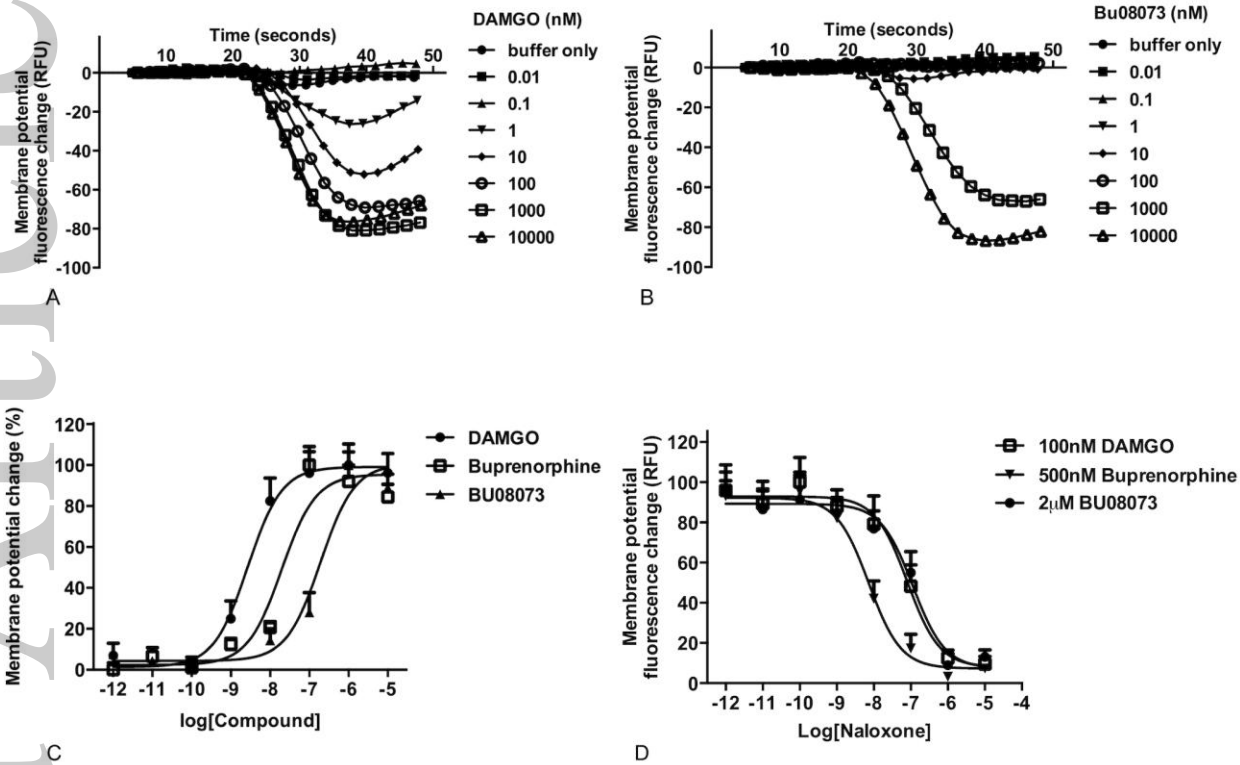

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Figure 4

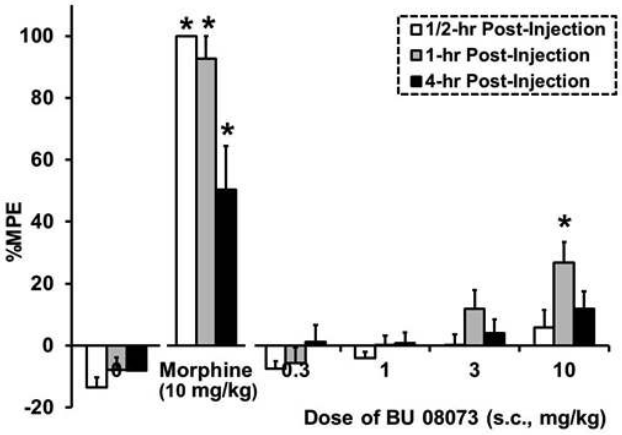

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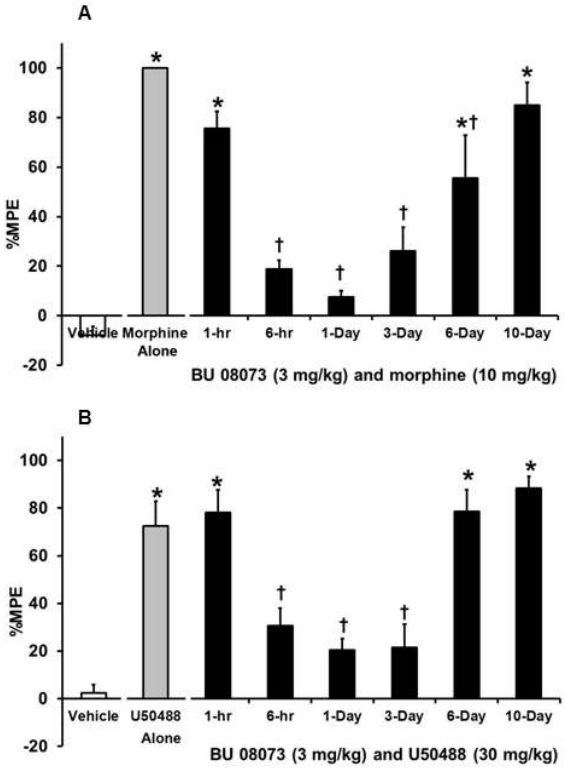
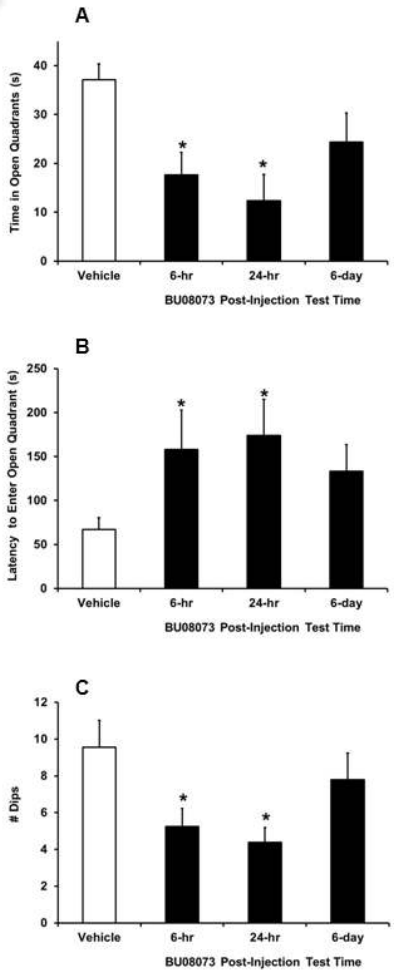
Figure 5



bph_12796_f5

Figure 6



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