

JPET#249839

***In Vitro* Pharmacological Characterization of Buprenorphine,  
Samidorphan, and Combinations Being Developed as an  
Adjunctive Treatment  
for Major Depressive Disorder**

Jean M. Bidlack, Brian I. Knapp, Daniel R. Deaver, Margarita Plotnikava,

Derrick Arnelle, Angela M. Wonsey, May Fern Toh,

Sokhom S. Pin, and Mark N. Namchuk

Department of Pharmacology and Physiology, University of Rochester, School of  
Medicine and Dentistry, Rochester, NY 14642 (J.M.B., B.I.K.)

and

Alkermes, Inc., Waltham, MA 02451 (D.R.D., M.P., D.A., A.M.W., M.F.T., S.S.P.,  
M.N.N.)

JPET#249839

**Running Title:** Buprenorphine and Samidorphan Pharmacological Properties

Corresponding Author: Jean M. Bidlack, Ph.D., Department of Pharmacology and Physiology,  
P.O. Box 711, University of Rochester, School of Medicine and Dentistry, Rochester, New York  
14642-8711, USA; Tel. (585) 275-5600; Fax (585) 273-2652; E-Mail:

[Jean\\_Bidlack@urmc.rochester.edu](mailto:Jean_Bidlack@urmc.rochester.edu)

Number of text pages: 24

Number of tables: 9

Number of figures: 7 and two supplementary figures

Number of references: 42

Number of words in Abstract: 255

Number of words in Introduction: 778 (including references)

Number of words in Discussion: 1492 (including references)

**Abbreviations:** ALKS 5461, the combination of buprenorphine and samidorphan at a 1:1 (mg/mg) dose ratio; Assay Buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.2 mM EGTA, pH 7.4); BRET, bioluminescence resonance energy transfer; BUP, buprenorphine, CHO, Chinese hamster ovary; DAMGO, [D-Ala<sup>2</sup>,NMePhe<sup>4</sup>-Gly-ol<sup>5</sup>]enkephalin; DMEM, Dulbecco's modified Eagle's medium; DOR, delta-opioid receptor; GP Buffer, 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, and 50 μM GDP; GRK2, G protein-coupled receptor kinase 2; HEK 293, human embryonic kidney 293; KOR, kappa-opioid receptor; MDD, major depressive disorder; Membrane Buffer (50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, pH 7.4); MOR, mu-opioid receptor; NOP, nociceptin/orphanin FQ; nor-BNI, nor-binaltorphimine; PEI, polyethyleneimine; PTX, pertussis toxin; SAM, samidorphan, (3-carboxyamido-4-hydroxy naltrexone); SNC-80, (+)-4-[(alpha R)-alpha-((2S,5R)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-N,N-diethylbenzamide; TRV 130, oliceridine; U50,488, 2-(3,4-dichlorophenyl)-N-methyl-N-[(1R,2R)-2-pyrrolidin-1-ylcyclohexyl]acetamide; U69,593, (+)-(5α,7α,8β)-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide.

**Section:** Drug Discovery and Translational Medicine

JPET#249839

## ABSTRACT

A combination of buprenorphine (BUP) and samidorphan (SAM) at a 1:1 fixed dose ratio (mg/mg) is being investigated as an adjunctive treatment for major depressive disorder (BUP/SAM, ALKS 5461). Both [<sup>3</sup>H]BUP and [<sup>3</sup>H]SAM bound to the mu, kappa and delta opioid receptors (MOR, KOR and DOR, respectively) with K<sub>d</sub> values of 3 nM or less. [<sup>3</sup>H]BUP dissociated slower from the MOR than [<sup>3</sup>H]SAM did. In the [<sup>35</sup>S]GTPγS assay, BUP was a partial agonist at the MOR, KOR and DOR. SAM was an antagonist at the MOR and a partial agonist at the KOR and DOR. The pharmacology of the combination of SAM and BUP was characterized at ratios similar to the molar ratios of both compounds at steady state in humans. In all assessments, SAM reduced the efficacy of BUP at the MOR without altering its potency. At the KOR, SAM had no significant effect on the activity of BUP. In bioluminescent resonance energy transfer assays, SAM, naltrexone, and naloxone were partial agonists when the MOR was coupled to the G<sub>α<sub>0B</sub></sub> and G<sub>α<sub>z</sub></sub>, and were antagonists when coupled to G<sub>α<sub>i</sub></sub>. At the KOR, SAM was a partial agonist activating G<sub>α<sub>0A</sub></sub> and G<sub>α<sub>0B</sub></sub> and a full agonist in stimulating G<sub>α<sub>z</sub></sub>. SAM inhibited BUP's recruitment of β-arrestin to the MOR, suggesting an attenuation of BUP's efficacy in activating G proteins correlated with an inhibition of β-arrestin recruitment. The collective data suggest that SAM attenuates the efficacy of BUP under all conditions tested at the MOR and DOR, but had little effect on BUP activity at the KOR.

## Introduction

BUP/SAM (ALKS 5461) (Fig. 1) consists of the partial  $\mu$ -opioid agonist buprenorphine (BUP) and the  $\mu$ -opioid antagonist samidorphan (SAM) at a 1:1 (mg/mg) dose ratio. The combination is being studied as adjunctive therapy in patients having an inadequate response to antidepressants (Ehrich et al., 2015; Fava et al., 2016) and represents a potential treatment for major depressive disorder (MDD) working through opioid receptor modulation.

Numerous lines of evidence suggest BUP's potential in treating depression. In humans, BUP has been shown to have antidepressive effects in treatment-resistant patients (Bodkin et al., 1995; Nyhuis et al., 2008; Kosten, 2016). Preclinical assessments support a role for the  $\mu$ ,  $\kappa$ , and  $\delta$  opioid receptors (MOR, KOR, and DOR, respectively) in the treatment of depression (Lutz and Kieffer, 2013) and a role for both the MOR and KOR has been postulated for BUP in producing anti-depressive and anti-anxiolytic effects (Falcon et al., 2015; Falcon et al., 2016; Robinson et al., 2017; Almatroudi et al., 2018). SAM was synthesized as a naltrexone derivative containing a 3-carboxamido group and was shown to have high affinity for the MOR (Wentland et al., 2005) and to act as an antagonist at the MOR and a partial agonist at the KOR and DOR (Wentland et al., 2009). In pre-clinical studies, appropriate titration of SAM attenuates the abuse potential of BUP, while having no effect on the antidepressant efficacy (Smith et al., 2017).

Despite BUP's clinical use, relatively little is known about the receptor binding and *in vitro* pharmacological properties of BUP at the MOR, KOR and DOR and the nociceptin/orphanin FQ (NOP) receptor. The few previous [ $^3$ H]BUP binding experiments were performed before the cloning of the multiple opioid receptors and used rodent brain and spinal cord membranes (Villiger and Taylor, 1981; Villiger and Taylor, 1982; Villiger, 1984; Boas and Villiger, 1985).

JPET#249839

[<sup>3</sup>H]BUP binding to rat spinal cord membranes gave a curvilinear Scatchard plot, indicative of multiple binding sites (Villiger and Taylor, 1982).

Activation of the MOR results in signaling through  $G_{\alpha_{i/o}}$  proteins to inhibit adenylyl cyclase activity, activate  $K^+$  channels, inhibit  $Ca^{2+}$  channels, and activate mitogen-activated protein kinases (Law et al., 2000; Lamberts et al., 2018). BUP is a partial agonist at the MOR (Huang et al., 2001). Based on animal and human studies, some investigators characterize BUP as a KOR antagonist (Leander, 1987) though, previous [<sup>35</sup>S]GTP $\gamma$ S binding studies have reported it is a partial agonist at the KOR (Huang et al., 2001; Wentland et al., 2009). BUP was a DOR antagonist in behavioral studies and in [<sup>35</sup>S]GTP $\gamma$ S binding studies using membranes from the NG108-15 cell line (Negus et al., 2002). There is general agreement that BUP is a partial agonist at the NOP receptor but it is less potent at the NOP receptor than at the opioid receptors (Huang et al., 2001; Cami-Kobeci et al., 2011; Khroyan et al., 2015).

Until recently, it has not been possible to study the signaling of an opioid receptor interacting with one specific G protein subunit. Using a bioluminescent resonance energy transfer (BRET) assay with the MOR expressed with a specific  $G_{\alpha}$  protein, endomorphin-1, a  $\mu$ -selective opioid agonist, produced maximal efficacy when the MOR was signaling through  $G_{\alpha_{oA}}$  and  $G_{\alpha_{oB}}$  but only partial agonist activity when the MOR was signaling through  $G_{\alpha_{i1}}$ ,  $G_{\alpha_{i2}}$ ,  $G_{\alpha_{i3}}$ , or  $G_{\alpha_z}$  (Masuho et al., 2015). Similarly at the KOR, the  $\kappa$ -endogenous peptide dynorphin A produced maximal efficacy when the KOR signaled through  $G_{\alpha_{oA}}$  and  $G_{\alpha_{oB}}$  and had partial agonist activity when the KOR signaled through  $G_{\alpha_{i1}}$ ,  $G_{\alpha_{i2}}$ ,  $G_{\alpha_{i3}}$ , or  $G_{\alpha_z}$ . Enkephalin produced a similar response at the DOR (Masuho et al., 2015). These data suggest opioids signal promiscuously, but with varying activity through the multiple G proteins.

The recruitment of  $\beta$ -arrestin by opioids has been associated with the deleterious effects associated with opioid use (Schmid et al., 2017). Hence, compounds that bias away from  $\beta$ -arrestin recruitment have garnered great interest. A number of compounds such as BUP

JPET#249839

(McPherson et al., 2010; Chen et al., 2013; Grinnell et al., 2016), oliceridine (TRV130) (Chen et al., 2013), mitragynine and 7-hydroxymitragynine (Kruegel et al., 2016), and PZM21 (Manglik et al., 2016) produce little or no  $\beta$ -arrestin recruitment to the MOR. However, these molecules are also partial agonists in activating  $G_{\alpha_{i/o}}$  signaling mediated by the MOR.

The goal of the current study was to understand the *in vitro* pharmacological properties of BUP and SAM alone as well as in combination at molar ratios similar to those observed in humans upon administration of BUP/SAM combination. Given their likely mechanistic role in MDD treatment, the MOR and KOR activity of the combination was characterized more extensively. Additionally, the diversity of techniques employed allowed an assessment of comparability of various assay systems.

JPET#249839

## Materials and Methods

**Compounds and Cells.** SAM (3-carboxyamido-4-hydroxy naltrexone) was synthesized as previously described (Wentland et al., 2005). BUP-HCl was obtained from Siegfried (Pennsville, NJ). DAMGO, U50,488-HCl hydrate and SNC-80 were obtained from Sigma-Aldrich Corp. (St. Louis, MO). [Tyrosyl-3,5-<sup>3</sup>H(N)] DAMGO (47.1 Ci/mmol), [phenyl-3,4-<sup>3</sup>H]U69,593 (44.6 Ci/mmol), [leucyl-3,4,5-<sup>3</sup>H]nociception (123.6 Ci/mmol) and [<sup>35</sup>S]GTPγS were obtained from Perkin Elmer, Inc. (Waltham, MA). [5,7-<sup>3</sup>H] Naltrindole (30 Ci/mmol) and [ring-<sup>3</sup>H]BUP-HCl (20 Ci/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [<sup>3</sup>H]SAM (20 Ci/mmol) was provided by Moravek Biochemicals (Brea, CA).

Chinese hamster ovary (CHO) cells stably expressing the human MOR, KOR, or DOR (hMOR-CHO, hKOR-CHO, or hDOR-CHO) and CHO cells expressing the NOP receptor were used in this study. hMOR-CHO cells were provided by Dr. George Uhl (NIDA Intramural Program, Baltimore, MD) and hKOR-CHO cells were provided by Dr. Lee-Yuan Liu-Chen (Temple University, Philadelphia, PA). hDOR-CHO and CHO cells stably expressing the human NOP receptor were obtained from Dr. Larry Toll (Florida Atlantic University, Boca Raton, FL).

**Membrane Preparations.** hMOR-CHO, hKOR-CHO, or hDOR-CHO cells and CHO cells stably expressing the NOP receptor, were cultured in 100-mm dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum and penicillin–streptomycin (10,000 U/mL) at 37°C in a 10% CO<sub>2</sub> atmosphere. When the cells were at confluence, the cells were gently scraped from the 100-mm dishes and were added to a 50-ml centrifuge tube. The cells were centrifuged at 1,000 x g for 10 min at 4°C. Cell pellets prepared for radioligand binding assays were resuspended in 50 mM Tris-HCl, pH 7.4. Resuspended cell pellets from all centrifuge tubes were combined and homogenized using a Polytron cell homogenizer. The

JPET#249839

homogenized preparation was centrifuged at 18,000 x g for 30 min at 4°C. The pellet was resuspended in 40 ml of cold 50 mM Tris-HCl, pH 7.4, and the centrifugation step was repeated. The final membrane pellet was resuspended in 50 mM Tris-HCl, pH 7.4 at a protein concentration between 3 to 5 mg/ml. These membranes were used in the radioligand receptor binding assays. Cell membranes prepared for [<sup>35</sup>S]GTP $\gamma$ S assays were resuspended in phosphate-buffered saline buffer containing 0.04% EDTA and centrifuged at 1,000 x g for 10 min at 4°C. The supernatant was removed and the cell pellet resuspended and homogenized in 50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 1 mM EGTA, pH 7.4 (Membrane Buffer). The homogenized preparation was centrifuged as described above and the final membrane pellet resuspended in 50 mM Tris-HCl, 3 mM MgCl<sub>2</sub>, 100 mM NaCl, 0.2 mM EGTA, pH 7.4 (Assay Buffer) at a membrane protein concentration between 3 to 5 mg/ml. Membrane protein concentrations were determined with bovine serum albumin as the control as previously described (Bradford, 1976). Membranes were stored at -80°C until use.

**Radioligand Competition Binding Assays.** To determine the binding affinities (K<sub>i</sub> values) of BUP and SAM for MOR, KOR, DOR and NOP receptor, 12 different concentrations of each compound were incubated with a specific radioligand and CHO membranes expressing a single type of receptor in 50 mM Tris-HCl, pH 7.4 at a final volume of 1 ml. The radioligand concentrations used were 0.25 nM [<sup>3</sup>H]DAMGO, 0.4 nM [<sup>3</sup>H]U69,593, 0.2 nM [<sup>3</sup>H]naltrindole, and 0.1 nM [<sup>3</sup>H]nociceptin, for measuring MOR, KOR, DOR and NOP receptor binding, respectively. Nonspecific binding was measured using 10 μM naloxone in experiments with the μ-selective peptide [<sup>3</sup>H]DAMGO and the κ-selective alkaloid [<sup>3</sup>H]U69,593, and 100 μM naloxone in experiments with the δ antagonist [<sup>3</sup>H]naltrindole. Unlabeled nociceptin, at 1 μM, was used to measure nonspecific binding in assays with [<sup>3</sup>H]nociceptin. Binding was initiated with the addition of CHO membranes (50 μg of membrane protein for MOR, 25 μg for KOR, 20 μg for DOR, and 2 μg for NOP receptor). Assays were incubated for 60 min except assays with



JPET#249839

[<sup>3</sup>H]naltrindole, which required a 3-hr incubation. All assays were performed at 25°C and terminated by rapid vacuum filtration through Whatman #32 glass fiber filters using a Brandel cell harvester (Biomedical Research and Development Laboratories Inc., Gaithersburg, MD) followed by three ice-cold washes with 50 mM Tris-HCl, pH 7.4. Filters were presoaked for 60 min in 0.25% polyethyleneimine (PEI) solution to reduce free radioligand binding to the filter. Binding assays with [<sup>3</sup>H]DAMGO did not require PEI-soaked filters. Samples were collected and counted in 2 ml of Ecoscint A scintillation fluid (National Diagnostics, Atlanta, GA) for 2 min each using a LS 6500 scintillation counter (Beckman Coulter Inc., Fullerton, CA).

To determine the affinity of BUP, SAM and other opioid full and partial agonists and antagonists under high- and low- affinity binding conditions at the MOR, binding experiments were performed as previously described with some modifications (Emmerson et al., 1996). High affinity binding was measured in 50 mM Tris-HCl buffer, pH 7.4. Low affinity binding was measured in 50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM DTT, and 50 μM GDP (GP Buffer) as previously described (Emmerson et al., 1996). To measure high-affinity binding, we used the μ-selective agonist [<sup>3</sup>H]DAMGO (0.25 nM) instead of [<sup>3</sup>H]sufentanil, which had been used previously (Emmerson et al., 1996). To measure low affinity binding, [<sup>3</sup>H]naloxone (1.0 nM) was used instead of [<sup>3</sup>H]naltrexone (Emmerson et al., 1996). In a final volume of 1 ml, hMOR-CHO membranes, 50 μg of protein, were incubated either in 50 mM Tris-HCl, pH 7.4, or GP buffer containing eight different concentrations of the compound and either [<sup>3</sup>H]DAMGO to measure high affinity binding or [<sup>3</sup>H]naloxone to measure low affinity binding. Unlabeled naloxone at a final concentration of 10 μM was used to measure nonspecific binding. Membranes were added last to the incubation tube. After a 60-min incubation at 25°C, membranes were filtered through Whatman #32 glass fiber filters, followed by washing with cold 50 mM Tris-HCl, pH 7.4, and counting of the filters as described above.

JPET#249839

For competition binding experiments, the specific binding of the radioligand in the absence of other competing compounds was set at 100%. The percent of control binding was calculated for the increasing concentrations of the competing compounds. A non-linear regression curve was fit to the data using SigmaPlot. The  $IC_{50}$  values, the concentration of the opioid needed to inhibit 50% of the control binding, were calculated from the curves.  $K_i$  values were calculated as described previously (Cheng and Prusoff, 1973). Data are reported as the mean  $K_i$  value  $\pm$  S.E.M. from three separate experiments performed in triplicate.

**Radioligand Saturation Binding Assays and Competition Binding Experiments.** We characterized the binding affinity ( $K_d$ ) and maximum binding ( $B_{max}$ ) of [ $^3$ H]BUP and [ $^3$ H]SAM under high and low affinity binding conditions to hMOR-, hKOR-, and hDOR- CHO membranes. High affinity binding was measured in 50 mM Tris-HCl buffer, pH 7.4. Low affinity binding was measured in GP buffer as described above. Eight concentrations of either [ $^3$ H]BUP or [ $^3$ H]SAM were incubated with CHO membranes expressing either the MOR, KOR, or DOR in 50 mM Tris-HCl, pH 7.4 or GP buffer at a final volume of 1 ml. Nonspecific binding was measured with 10  $\mu$ M naloxone. CHO membranes were added last. The amount of membrane used was chosen to maximize the specific signal while keeping specific binding below 10% of total binding. Saturation binding assays with [ $^3$ H]BUP used 100  $\mu$ g hMOR-CHO, 15  $\mu$ g hKOR-CHO, and 10  $\mu$ g hDOR-CHO membrane protein; those with [ $^3$ H]SAM used 20  $\mu$ g hMOR-CHO, 25  $\mu$ g hKOR-CHO, and 20  $\mu$ g hDOR-CHO membrane protein. Binding was terminated by rapid vacuum filtration through PEI-soaked glass fiber filters after a 90-min incubation for [ $^3$ H]BUP and after 60-min incubation at 25°C for [ $^3$ H]SAM. Samples were washed and counted as described above.

SAM, BUP, naltrexone, and naloxone were tested to determine the  $K_i$  values for these compounds in inhibiting [ $^3$ H]BUP and [ $^3$ H]SAM binding to the MOR in Tris-HCl and GP buffers.

JPET#249839

Membrane protein, 100  $\mu\text{g}$ , from hMOR-CHO cells was incubated with 0.4 nM [ $^3\text{H}$ ]BUP for 90 min or 0.05 nM [ $^3\text{H}$ ]SAM for 60 min at 25  $^\circ\text{C}$  in a final volume of 1 ml in either Tris-HCl, pH 7.4 or GP buffers. Nonspecific binding was measured by the inclusion of 10  $\mu\text{M}$  naloxone. The compounds were tested at 12 different concentrations and membranes were added last to the sample. A non-linear regression curve was fit to the data using SigmaPlot. The  $\text{IC}_{50}$  values were calculated from the curves. The  $K_d$  values for [ $^3\text{H}$ ]BUP and [ $^3\text{H}$ ]SAM in both buffers were used to calculate the  $K_i$  values.  $K_i$  values were calculated as described previously (Cheng and Prusoff, 1973). Data are reported as the mean  $K_i$  value  $\pm$  S.E.M. from three separate experiments performed in triplicate.

**Radioligand Association and Dissociation Assays.** We investigated the kinetic binding properties of [ $^3\text{H}$ ]BUP and [ $^3\text{H}$ ]SAM at MOR, KOR and DOR under high- and low- affinity binding conditions. Association assays were performed by incubating the radioligands with hMOR-CHO, hKOR-CHO and hDOR-CHO cell membranes for various times in 50 mM Tris-HCl, pH 7.4 or GP buffer. Nonspecific binding was measured at each time point by addition of 10  $\mu\text{M}$  naloxone. Dissociation assays were performed by incubating the radioligands with membranes (90 min for [ $^3\text{H}$ ]BUP and 60 min for [ $^3\text{H}$ ]SAM) to reach equilibrium at 25 $^\circ\text{C}$  and then, adding unlabeled BUP (10  $\mu\text{M}$ ) or SAM (1  $\mu\text{M}$ ) at different times to displace the bound radioligand. Concentrations of [ $^3\text{H}$ ]BUP and [ $^3\text{H}$ ]SAM near their respective  $K_d$  values under high and low affinity binding conditions were used in both the association and dissociation assays. [ $^3\text{H}$ ]BUP concentrations were 0.4 nM, 0.2 nM, and 1.5 nM for high-affinity binding assays, and 0.4 nM, 0.1 nM and 0.5 nM for low-affinity binding assays at MOR, KOR and DOR, respectively. [ $^3\text{H}$ ]SAM concentrations were 0.05 nM, 0.2 nM, and 1.0 nM for high-affinity binding assays, and 0.05 nM, 2.0 nM, and 1.5 nM for low-affinity binding assays at MOR, KOR and DOR, respectively. Membrane protein amounts used for association and dissociation assays at each receptor under high- and low- affinity binding conditions were the same as those used for the

JPET#249839

saturation binding assays. All assays were terminated by rapid vacuum filtration through PEI-soaked filters and filters were washed and counted as described above.

Association binding data were analyzed with SigmaPlot, using a non-linear regression through the origin. The association  $T_{1/2}$  value, the time required to reach 50% of maximal binding, was calculated from the curve. Dissociation binding data were expressed as percent of control binding. Control binding is the specific binding measured at equilibrium (90 min for [<sup>3</sup>H]BUP and 60 min for [<sup>3</sup>H]SAM) and was set at 100%. Non-linear regression curves were fit to the data using SigmaPlot. The time required for dissociation of 50% of the control binding (dissociation  $T_{1/2}$ ) was determined from the curve.  $K_{on}$  and  $K_{off}$  rates and  $K_d$  values were calculated using GraphPad Prism. The data are reported as mean  $\pm$  S.E.M. from three separate experiments, performed in triplicate.

**[<sup>35</sup>S]GTP $\gamma$ S Binding Assays.** Agonist and antagonist properties of BUP and SAM were investigated using the [<sup>35</sup>S]GTP $\gamma$ S binding assay to measure G-protein activation in response to ligand-receptor binding. Various concentrations of either BUP or SAM were incubated with hMOR-, hKOR- or hDOR- CHO membranes for 60 min at 30 °C in Assay Buffer with 3  $\mu$ M GDP and 0.08 nM [<sup>35</sup>S]GTP $\gamma$ S in a final volume of 0.5 ml. Unlabeled GTP $\gamma$ S, at 10  $\mu$ M, was used to measure nonspecific binding. As controls, maximal stimulation was measured with 10  $\mu$ M of either DAMGO, U50,488 or SNC80 for the MOR, KOR, and DOR, respectively, and was set at 100%. The agonist effects of 1:1, 1:5, and 1:50 molar concentrations of BUP to SAM in [<sup>35</sup>S]GTP $\gamma$ S binding assays at MOR and KOR were also determined. The data are expressed as percent stimulation of [<sup>35</sup>S]GTP $\gamma$ S binding. Non-linear regression curves were fit to the data using SigmaPlot software. Maximal stimulation ( $E_{max}$ ) values and the concentrations producing 50% maximal stimulation ( $EC_{50}$  values) were calculated from the curves. Data are expressed as mean  $\pm$  S.E.M. from three separate experiments, performed in triplicate.

JPET#249839

Antagonist activity of BUP or SAM was determined in the presence of a concentration of full agonist that produced approximately 80% of a maximal response alone. The concentrations of the full agonists were, 200 nM DAMGO ( $\mu$ ), 30 nM U50,488 ( $\kappa$ ), or 10 nM SNC-80 ( $\delta$ ), and the maximum agonist stimulation was set at 100%. Maximum antagonism was determined using a pure antagonist in the presence of an agonist represents the lower limit, 0% stimulation. Naloxone, at 10  $\mu$ M, was used for experiments at MOR and DOR, whereas 1  $\mu$ M nor-binaltorphimine (nor-BNI) was used for experiments at the KOR. All assays were initiated by the final addition of CHO membranes and incubated for 60 min in a 30°C water bath. The assays were terminated using rapid vacuum filtration through glass fiber filters. The samples were washed three times with ice-cold 50 mM Tris-HCl, pH 7.4, collected, and counted as described above.  $I_{\max}$  values, the maximal inhibition of the agonist stimulation, and  $IC_{50}$  values, the concentration of compound producing 50% of the maximal inhibition, were calculated from non-linear regression curves generated in SigmaPlot. Data are expressed as mean  $\pm$  S.E.M. from three experiments performed in triplicate.

**Bioluminescence Resonance Energy Transfer (BRET) Assays.** Human embryonic kidney 293 (HEK 293) cells and BRET biosensor constructs (Namkung et al., 2016) were provided by Domain Therapeutics, Inc. (Montreal, Canada). Cells were grown in DMEM, supplemented with 10% fetal bovine serum, 1X penicillin and streptomycin, and were maintained at 37°C in a humidified incubator containing 5% CO<sub>2</sub>. All cell culture reagents were purchased from Thermo Fisher (Waltham, MA). Cells were detached, counted, and re-suspended into cell culture medium. Transfection with receptor and biosensor plasmids, were performed using with 3  $\mu$ g/ml of PEI as the transfection reagent, and  $1.8 \times 10^6$  cells in T-75 cell culture flask. Serial dilutions of test compounds were prepared in 100% DMSO using the HP D300 digital dispenser, and 384-well low volume plate (white opaque, PerkinElmer). Forty-eight hr after transfection, cells were washed once with PBS, removed with trypsin, centrifuged at

JPET#249839

1000 x g, and the pellet was resuspended in Tyrode-HEPES buffer (Boston BioProducts, Ashland, MA). Cells (10,000 cells per well) were dispensed into pre-made compound plates containing an opioid using the automated BioTek Multi-Flo reagent dispenser, and incubated at room temperature for 50 min. Coelenterazine (NanoLight Technologies, Pinetop, AZ) was subsequently added at a final concentration of 2  $\mu$ M. Cells were incubated for an additional 10 min at room temperature, and BRET readings were captured using the Envision plate reader (PerkinElmer; filters: 400nm/70nm, 515nm/20nm). BRET signals were determined by calculating the ratio of light emitted by GFP-acceptor (515 nm) over light emitted by luciferase-donor (400 nm). For each test compound/biosensor pair, the test compound-induced BRET signals were normalized to the BRET signal obtained from DAMGO, U50,488, and SNC-80 for the MOR, KOR, and DOR, respectively. Maximal and minimal BRET signals were defined by 20  $\mu$ M DAMGO/20  $\mu$ M U50,488/20  $\mu$ M SNC-80, or cells in the absence of agonists, respectively.

JPET#249839

## Results

**Radioligand Competition Binding Experiments.** Using membranes from hMOR-, hKOR- and hDOR- CHO cells, competition binding experiments showed that BUP had the highest affinity for the human KOR with a  $K_i$  value of  $0.23 \pm 0.0067$  nM, with 2- and 10- fold lower affinity for the MOR and DOR, respectively (Table 1). SAM had the highest affinity for the MOR with a  $K_i$  value of  $0.052 \pm 0.0044$  nM, 8-fold higher than BUP's affinity for the MOR. SAM had essentially the same affinity as BUP for the KOR and DOR. BUP bound to the NOP receptor with a  $K_i$  value that was more than 400-fold greater than observed at the KOR and MOR, and SAM did not inhibit [ $^3$ H]nociceptin binding at concentrations up to 10  $\mu$ M.

$K_i$  values obtained for BUP and SAM were next determined for the MOR under conditions favoring high- and low- affinity binding states. High-affinity binding was performed in 50 mM Tris-HCl, pH 7.4 buffer with [ $^3$ H]DAMGO as the radioligand. Low-affinity binding was measured in GP buffer with [ $^3$ H]naloxone as the radioligand. BUP and SAM were compared with a series of full agonists, partial agonists and antagonists (Table 2). The ratio of the  $K_i$  value in the low-affinity receptor state divided by the  $K_i$  value obtained for the compound with the receptor in the high-affinity state was indicative of the preference for one binding condition over the other. Full MOR agonists demonstrated up to a 530-fold preference for the high-affinity binding state over the low-affinity state, and a rank order of preference of morphine > methadone > fentanyl  $\geq$  DAMGO. These findings agree with previous results (Emmerson et al., 1996). Partial agonists also demonstrated greater affinity for the MOR when the binding experiment was performed with the high-affinity 50 mM Tris-HCl, pH 7.4, buffer instead of the low-affinity GP buffer, though the shifts were smaller than with full agonists (Table 2). The exception was BUP,

JPET#249839

which had a 2.5-fold higher affinity for the MOR in the GP buffer. Indeed, the pattern of activity with BUP was similar to MOR antagonists which all showed less than a 2-fold shift in binding affinity between the assay conditions. SAM had a 2-fold higher affinity for the MOR under low-affinity binding condition. Hence both SAM and BUP showed little change in  $K_i$  values upon shifting from low- to high- affinity binding conditions. The fact that BUP had a higher affinity for the MOR in the low-affinity binding condition suggests that this unique property may contribute to physiological effects observed with BUP.

**Association and Dissociation Rates for [<sup>3</sup>H]BUP and [<sup>3</sup>H]SAM at the Multiple Opioid Receptors.** [<sup>3</sup>H]BUP bound specifically to the MOR with specific binding being  $64 \pm 3.5\%$  and  $73 \pm 1.9\%$  of total binding in Tris-HCl and GP buffers, respectively. Fig. 2 shows the [<sup>3</sup>H]BUP association and dissociation time courses for the interaction of [<sup>3</sup>H]BUP with the MOR, KOR and DOR performed in both the high-affinity Tris-HCl buffer and the low-affinity GP buffer. As reported in Table 3, the association  $T_{1/2}$  value for [<sup>3</sup>H]BUP with the multiple opioid receptors was approximately 2-fold faster in the GP buffer than in the Tris-HCl buffer. The dissociation rate for [<sup>3</sup>H]BUP was much faster in the GP buffer than in the Tris-HCl buffer. After reaching equilibrium, the dissociation of [<sup>3</sup>H]BUP from the receptor was initiated by the addition of 10  $\mu$ M cold BUP. The dissociation  $T_{1/2}$  value for [<sup>3</sup>H]BUP was much longer at the MOR than at the KOR and DOR. In 50 mM Tris-HCl, pH 7.4, more than 23 hr was needed for 50% of the [<sup>3</sup>H]BUP to dissociate, while in the GP buffer, 200 min resulted in half of the [<sup>3</sup>H]BUP being dissociated from the MOR. While greater than 23 hr was needed for 50% dissociation from the MOR, 390 and 77 min were required for 50% dissociation from the KOR and DOR, respectively, in Tris-HCl buffer. In the GP buffer, the same rank order of rate of dissociation followed the results obtained with the Tris-HCl buffer with the DOR having the fastest rate of dissociation, followed by the KOR, and then the MOR which was 5 times slower than the KOR. Because of the slow dissociation of [<sup>3</sup>H]BUP from the MOR in the Tris-HCl buffer, it was not possible to measure the  $K_{off}$ ,  $K_{on}$ , and



JPET#249839

$K_d$  values. The rapid dissociation of [ $^3$ H]BUP from the DOR in the GP buffer prohibited the accurate determination of the  $K_{on}$  and  $K_d$  values.

[ $^3$ H]SAM had nonspecific binding of less than 1% when binding to the MOR in either Tris-HCl or GP buffers. Fig. 3 shows that the association rate for [ $^3$ H]SAM at the MOR was similar regardless of whether the Tris-HCl or GP buffer was used. In the GP buffer, the association rate for [ $^3$ H]SAM at the KOR and DOR was fast with the  $T_{1/2}$  values being less than 2 min (Table 3). Like [ $^3$ H]BUP, [ $^3$ H]SAM had the slowest dissociation from the MOR with dissociation  $T_{1/2}$  values of 260 and 44 min in the Tris-HCl and GP buffers, respectively, after the addition of 1  $\mu$ M cold SAM. Like the association rates at the KOR and DOR, the dissociation rates for [ $^3$ H]SAM at these receptors were fast with  $T_{1/2}$  values of less than 2 min in the GP buffers, while 47 and 20 min, respectively, were needed in the high-affinity Tris-HCl buffer. For equilibrium binding experiments, a 90-min incubation was used with [ $^3$ H]BUP and a 60-min incubation was used with [ $^3$ H]SAM.

**[ $^3$ H]BUP and [ $^3$ H]SAM Saturation Binding Experiments.** Fig. 4 shows the results from saturation binding experiments for both [ $^3$ H]BUP and [ $^3$ H]SAM binding to the MOR in Tris-HCl and GP buffers. For both radioligands and binding conditions, a linear Scatchard plot was obtained. Similar results were obtained with the KOR and DOR as summarized in Table 4. [ $^3$ H]BUP had the highest affinity for the KOR with  $K_d$  values of  $0.14 \pm 0.015$  nM and  $0.074 \pm 0.011$  nM in Tris-HCl and GP buffers, respectively. At the three opioid receptors, [ $^3$ H]BUP had higher affinity when the binding assay was performed in GP buffer than in Tris-HCl buffer, which is similar to the results obtained in competition binding experiments (Table 2). [ $^3$ H]SAM had the highest affinity for the MOR with  $K_d$  values of  $0.046 \pm 0.0027$  nM and  $0.044 \pm 0.0051$  nM in Tris-HCl and GP buffers, respectively. [ $^3$ H]SAM had good affinity for both the KOR and DOR in addition to having a high affinity for the MOR (Table 4). Notably,  $K_d$  values did not show large

JPET#249839

shifts between the GP and Tris-HCl buffers, with the exception of a 16-fold with the exception of a 16-fold decrease in [<sup>3</sup>H]SAM affinity for the KOR in the GP buffer. This decrease in the  $K_d$  value for [<sup>3</sup>H]SAM at the KOR agreed with the  $K_d$  value calculated from the association and dissociation data (Table 3).

$B_{max}$  values for both [<sup>3</sup>H]BUP and [<sup>3</sup>H]SAM were a reflection of the number of receptors expressed in the CHO cell lines. It is not clear why more moles of [<sup>3</sup>H]BUP were bound to all three opioid receptors than was observed with [<sup>3</sup>H]SAM since the same membrane preparations were used with both <sup>3</sup>H-labeled compounds.

**Competition Binding Experiments with [<sup>3</sup>H]BUP and [<sup>3</sup>H]SAM at the MOR.** Table 5 shows that SAM, naloxone, and naltrexone competed with 0.4 nM [<sup>3</sup>H]BUP for binding to the MOR. SAM had the highest affinity with  $K_i$  values of  $0.26 \pm 0.014$  nM and  $0.24 \pm 0.026$  nM when the binding experiments were performed in Tris-HCl and GP buffers, respectively. While SAM had a 5-fold higher affinity for the MOR when [<sup>3</sup>H]DAMGO was used as radioligand (Table 1), the  $K_i$  values for SAM in competing with [<sup>3</sup>H]BUP may be more representative of the pharmacology when the molecules are dosed as a combination *in vivo*. BUP, naloxone, and naltrexone were able to compete with 0.05 nM [<sup>3</sup>H]SAM for binding to the MOR. Naltrexone and BUP had  $K_i$  values of less than 0.4 nM regardless of which buffer was used in the binding experiments.

**[<sup>35</sup>S]GTP $\gamma$ S Binding Mediated by the MOR and KOR in the Presence of BUP and SAM Alone and in Combination.** BUP stimulated [<sup>35</sup>S]GTP $\gamma$ S binding at the MOR with an  $EC_{50}$  value of  $0.63 \pm 0.40$  nM and an  $E_{max}$  value of  $57 \pm 5.5\%$ , indicating that BUP was a potent partial agonist at the MOR (Fig. 5A and Table 6). BUP partially inhibited DAMGO-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding (Fig. 5C). SAM did not stimulate [<sup>35</sup>S]GTP $\gamma$ S binding for the MOR (Fig. 5A) but, SAM completely inhibited DAMGO-stimulated [<sup>35</sup>S]GTP $\gamma$ S binding (Fig. 5C and Table 6).

JPET#249839

SAM did not act as an inverse agonist at the MOR because SAM alone did not decrease basal [<sup>35</sup>S]GTP $\gamma$ S binding (Fig. 5A). An initial assessment of the pharmacology of the combination of BUP and SAM was conducted at a 1:1, 1:5 and 1:50 molar BUP:SAM ratio by assessing [<sup>35</sup>S]GTP $\gamma$ S binding using membranes from hMOR- and hKOR- CHO cells. As shown in Fig. 5A and Table 6, increasing the relative amount of SAM in the ratio caused the expected decrease in the E<sub>max</sub> value for the combination on the MOR with virtually no measureable activity present at the 1:50 ratio.

At the KOR, both BUP and SAM were partial agonists (Fig. 5B and D and Table 6). SAM was more efficacious than BUP at the KOR with an E<sub>max</sub> value of 56 ± 0.59%, while BUP had an E<sub>max</sub> value of 25 ± 1.3%. Interestingly, both the 1:1 and 1:5 BUP:SAM ratio showed similar E<sub>max</sub> values to BUP alone (25%, 29% and 29% for BUP alone, 1:1, 1:5 BUP:SAM respectively), while an increase in E<sub>max</sub> values was not observed until the 1:50 BUP:SAM (E<sub>max</sub> = 40%). These data suggest that SAM was less effective competing for binding with BUP on the KOR, despite having similar EC<sub>50</sub> values. Notably, a decrease in affinity at the KOR for SAM was observed under the GP binding conditions (Table 4) which was performed under similar buffer conditions to the [<sup>35</sup>S]GTP $\gamma$ S assay.

**BRET Assays to Study BUP and SAM Mediating the Coupling of the MOR, KOR, and DOR to Specific G Proteins.** Previous studies demonstrated the opioid receptors are coupled to the G $\alpha_{i/o}$  protein because opioids inhibit cyclic AMP production in a pertussis toxin (PTX)-sensitive manner (Law et al., 2000). A series of cellular assays were conducted where the signaling of BUP and SAM was studied for a specific opioid receptor coupled to a specific G $\alpha$  proteins using BRET (Audet et al., 2008). Fig. 6 shows the MOR, KOR, and DOR signaling through the G $\alpha_{i1}$ , G $\alpha_{i2}$ , and G $\alpha_{i3}$  proteins. BUP was a partial agonist for MOR signaling through all the G $\alpha_i$  proteins, with greater efficacy in signaling through G $\alpha_{i1}$  and G $\alpha_{i3}$  than in signaling

JPET#249839

through  $G\alpha_{i2}$  (Fig. 6A and Table 7). SAM was an antagonist when the MOR was signaling through the  $G\alpha_i$  proteins (Fig. 6B and Table 7). The combination of BUP and SAM was assessed at a 1:3 molar ratio, which approximates the steady-state concentrations of both molecules in humans for the BUP:SAM combination in development for MDD. The efficacy of BUP at the three  $G\alpha_i$  proteins was reduced by the inclusion of SAM, consistent with the previous [ $^{35}$ S]GTP $\gamma$ S activity assessments. Fig. 6 shows that both BUP and SAM mediated the activation of  $G\alpha_{oA}$ ,  $G\alpha_{oB}$ , and  $G\alpha_z$  by the MOR. At both  $G\alpha_{oA}$  and  $G\alpha_{oB}$ , BUP had  $E_{max}$  values of 87% and 89%, respectively, suggesting that BUP was almost a full agonist when the MOR was signaling through  $G\alpha_o$  proteins (Fig. 6A and Table 8). When signaling through the  $G\alpha_z$  protein, BUP had an  $E_{max}$  value of 92%, demonstrating that BUP was very efficacious when the MOR was signaling through the  $G\alpha_z$  protein (Fig. 6A and Table 8). SAM was a partial agonist at the MOR when signaling through  $G\alpha_{oA}$ ,  $G\alpha_{oB}$ , and  $G\alpha_z$  proteins (Fig. 6B and Table 8). Spider plots of the  $pEC_{50}$  and  $E_{max}$  values for BUP, SAM, and the 1:3 molar ratio of BUP:SAM in activating the  $G\alpha_i$ ,  $G\alpha_o$ , and  $G\alpha_z$  proteins allow for the easy comparison of potency and efficacy of BUP and SAM signaling through the MOR, KOR, and DOR interacting with one  $G\alpha$  subunit (Supplemental Figure 1). These findings show that both BUP and SAM were able to activate the MOR to signal through  $G\alpha_o$  and  $G\alpha_z$  proteins and that the concentrations of BUP needed to signal through  $G\alpha_o$  and  $G\alpha_z$  proteins were similar to the concentrations needed to activate  $G\alpha_i$  signaling (Table 7). In contrast, while SAM was an antagonist when the MOR was signaling through  $G\alpha_i$  proteins, it was a partial agonist when the MOR was signaling through  $G\alpha_o$  and  $G\alpha_z$  proteins (Fig. 6 and 7). In all cases assessed at the MOR, the  $E_{max}$  value observed with SAM was significantly lower than that observed with BUP, consistent with the intermediate  $E_{max}$  values observed in the 1:3 combination. Interestingly, a similar pattern of activity was noted for all of the MOR antagonists we assessed. Naltrexone was a partial agonist at the MOR when

JPET#249839

the MOR was activating the  $G_{\alpha_z}$  and to a lesser degree the  $G_{\alpha_{0B}}$  protein (Supplemental Figure 2 and Table 8). Naloxone had a similar profile as naltrexone (Table 8).

Both BUP and SAM were partial agonists when the KOR activated the  $G_{\alpha_i}$  proteins (Fig. 6D,E, , Table 7, Supplemental Figure 1). SAM had higher efficacy than BUP with  $E_{max}$  values ranging from 55-60% for all three  $G_{\alpha_i}$  proteins. When BUP and SAM were combined at a 1:3 molar ratio, the efficacy and potency of the combination closely matched that of BUP alone, suggesting SAM did not compete effectively with BUP as was observed in the [ $^{35}$ S]GTP $\gamma$ S activity assessment (Table 6). At the KOR, both BUP and SAM were partial agonists when the KOR signaled through the  $G_{\alpha_{0A}}$  and  $G_{\alpha_{0B}}$  proteins (Fig. 6D, E, Table 8). Similar to  $G_{\alpha_i}$  signaling, SAM had a greater efficacy at stimulating signaling through  $G_{\alpha_{0A}}$  and  $G_{\alpha_{0B}}$  than BUP did. Both compounds had higher efficacy when signaling through  $G_{\alpha_{0B}}$  than  $G_{\alpha_{0A}}$ . Similar to observations with  $G_{\alpha_i}$  signaling, the combination of BUP and SAM at a 1:3 molar ratio was virtually identical to BUP alone when signaling through  $G_{\alpha_o}$  proteins. (Fig. 6F and Table 8). When the KOR was signaling through  $G_{\alpha_z}$  proteins, both BUP and SAM were full agonists (Fig. 6D, E and Table 8).

At the DOR, BUP was a partial agonist with  $E_{max}$  values ranging from 36% for  $G_{\alpha_{i1}}$  to 18% for  $G_{\alpha_{i2}}$  (Fig. 6G and Table 7). SAM had  $E_{max}$  values of slightly less than 20% for  $G_{\alpha_{i1}}$  and  $G_{\alpha_{i3}}$  (Fig. 6H and Table 7). The efficacy of SAM at  $G_{\alpha_{i2}}$  was too low to be measured. Both BUP and SAM were less potent at the DOR than they were at the MOR and KOR. The 1:3 ratio of BUP:SAM showed  $E_{max}$  values intermediate to the two molecules alone suggesting they effectively compete with each other, though it should be noted given their similar potency and efficacy on the DOR, it was difficult to provide a definitive assessment (Fig. 6 I, and Table 8).

JPET#249839

At the DOR, both BUP and SAM were partial agonists when the DOR was signaling through  $G_{\alpha_{0A}}$ ,  $G_{\alpha_{0B}}$ , and  $G_{\alpha_z}$  proteins (Fig. 6 G and H, and Table 8).

The activation of the inward-rectifying  $K^+$  channel (Kir) in response to the opioid receptor activating  $G_{\alpha_{i2}}$  and  $G_{\alpha_{0A}}$  was studied and the results are reported in Table 9. The  $\beta$ - $\gamma$  subunits from the G protein interact with the  $K^+$  channel to activate the channel. BUP was a partial agonist in activating Kir when signaling was through either  $G_{\alpha_{i2}}$  or  $G_{\alpha_{0A}}$  and the MOR or DOR. These results are consistent with the results obtained for BUP activating the  $G_{\alpha_{i2}}$  and  $G_{\alpha_{0A}}$  proteins (Fig. 6 and Tables 7 and 8). At the KOR, both BUP and SAM stimulated  $G_{\alpha_{i2}}$ -Kir activity with  $E_{max}$  values of 14% and 32%, respectively. SAM did not activate Kir channels mediated by MOR signaling through  $G_{\alpha_{i2}}$  or  $G_{\alpha_{0A}}$  proteins. Because BUP had  $E_{max}$  values of 30% or less, the 1:3 combination of BUP:SAM reduced the  $E_{max}$  values to levels that were too low to measure. At the KOR, SAM activated  $G_{\alpha_{i2}}$ -Kir and  $G_{\alpha_{0A}}$ -Kir with  $E_{max}$  values of 32% and 21%, respectively (Table 9). BUP was less efficacious than SAM at the KOR in signaling through  $G_{\alpha_{i2}}$ -Kir and  $G_{\alpha_{0A}}$ -Kir (Table 9). BUP was able to signal through the DOR and both  $G_{\alpha_{i2}}$  and  $G_{\alpha_{0A}}$  to activate the Kir channel (Table 9). At the DOR, SAM did not signal through either  $G_{\alpha_{i2}}$  or  $G_{\alpha_{0A}}$  to activate the Kir channel. The results obtained with the activation of the Kir channel by the  $\beta$ - $\gamma$  subunits from either  $G_{\alpha_{i2}}$  or  $G_{\alpha_{0A}}$  paralleled the results obtained with the measurements of directly activating  $G_{\alpha_{i2}}$  and  $G_{\alpha_{0A}}$ .

Upon activating the MOR, BUP caused a partial recruitment of  $\beta$ -arrestin when the G protein-coupled receptor kinase 2 (GRK2) was included in the  $\beta$ -arrestin assay. BUP had an  $E_{max}$  value of 33%, while SAM showed no activity in the same assay (Fig. 7A and Table 9). The addition of SAM blocked  $\beta$ -arrestin recruitment by BUP rendering the 1:3 BUP:SAM combination inactive (Fig. 7A and Table 9), suggesting that this approach may block the deleterious effects

JPET#249839

associated with  $\beta$ -arrestin signaling. There was very little  $\beta$ -arrestin recruitment by either BUP or SAM at the KOR or DOR (Fig. 7B, C).

JPET#249839

## Discussion

A combination of BUP and SAM at a 1:1 dose ratio is currently in development as an adjunctive treatment for MDD (Ehrich et al., 2015; Fava et al., 2016). The studies herein were undertaken to describe the opioid pharmacology of both components individually as well as in combination.  $K_i$  and  $K_d$  determinations for BUP and SAM were conducted in both low and high affinity conditions. In aggregate, the data demonstrate SAM and BUP had the highest affinity for the MOR and KOR, followed by the DOR and relatively weak affinity for the NOP receptor. The findings with BUP agree with previous studies (Huang et al., 2001; Cami-Kobeci et al., 2011; Khroyan et al., 2015). Full and partial MOR agonists showed the expected decrease in affinity under low affinity binding conditions in accord with previous studies (Simon and Groth, 1975; Emmerson et al., 1996; Selley et al., 2000). However, BUP had higher affinity in the low affinity buffer, a behavior to date only observed with inverse agonists (Emmerson et al., 2004). Indeed, there was little difference in binding affinity between the low- and high- affinity binding conditions with either molecule with the exception of a 16-fold decrease in the  $K_d$  value for [ $^3$ H]SAM on the KOR under the low-affinity binding conditions. This finding was consistent with SAM acting as a partial agonist at the KOR in the [ $^{35}$ S]GTP $\gamma$ S binding and BRET assays. Based on kinetic studies, the difference in affinity could largely be attributed to an increase in the off rate. It is plausible that the addition of GDP promotes an inactive conformation(s) of the receptor (Selley et al., 2000; Chabre et al., 2009) suggesting BUP binds with equal affinity to both active and inactive conformations of the receptor.

Our studies also demonstrated that the  $K_i$  value obtained for SAM varied based on the radioligand used for the assessment.  $K_i$  values for SAM were in general higher when competing with [ $^3$ H]BUP, versus other radioligands across all assays and conditions. While such shifts



JPET#249839

won't affect rank order potency, they are important to consider when the two agents are administered in combination.

The [<sup>35</sup>S]GTP $\gamma$ S binding assays were conducted for BUP and SAM, paralleled the data obtained with the binding assessment, in particular under low affinity conditions and replicated previous studies conducted with BUP and SAM (Huang et al., 2001; Wentland et al., 2009). BUP showed similar potency as both an agonist and antagonist for both the MOR and KOR, again supporting that its binding affinity is not dramatically changed when binding the active or inactive form(s) of both receptors. Combination studies conducted with decreasing ratios of BUP:SAM demonstrated a decrease in the E<sub>max</sub> value on the MOR at all ratios tested versus BUP, while the E<sub>max</sub> value for BUP was not affected except at the 1:50 BUP:SAM ratio on the KOR. These data suggested that SAM effectively competed for binding with BUP on the MOR, but not on KOR. This relative decrease in affinity is consistent with the shift in affinity observed with SAM when assessed in low-affinity buffer conditions. Finally, signaling downstream of the MOR, KOR and DOR was assessed in intact HEK 293 cells using BRET. This technique allowed the activity BUP, SAM and the 1:3 BUP:SAM ratio to be assessed on individual G proteins as well as  $\beta$ -arrestin. The data suggest remarkable promiscuity for agonist signaling downstream of the opioid receptors. In general for both BUP and SAM, E<sub>max</sub> values were observed to shift across different signaling partners, while EC<sub>50</sub> values remained relatively unchanged. For example, BUP had E<sub>max</sub> values as low as 12% for G $\alpha_{i2}$  and as high as 92% signaling through G $\alpha_z$ . An increase in E<sub>max</sub> values for BUP, on the MOR signaling through the G $\alpha_{oA}$  over the G $\alpha_{i1}$ , had been reported previously (Saidak et al., 2006).

To estimate the *in vitro* pharmacology of clinical administration of BUP:SAM, a molar ratio of 1:3 BUP:SAM was examined in the same panel. These concentrations approximate the ratio of the two molecules observed in clinical studies at steady-state exposure. SAM attenuated BUP's

JPET#249839

$E_{max}$  values when signaling through all the tested G proteins. Across all assessments conducted on the MOR, the combination showed a decreased in  $E_{max}$  values compared to BUP alone, suggesting SAM effectively competes with, and antagonizes the activity of BUP, regardless if SAM functioned as an antagonist of partial agonist for the assay in question.

Notably a similar pattern of activity across the G proteins was observed for other MOR antagonists besides SAM. Naltrexone and naloxone were antagonists when the MOR was coupled to  $G\alpha_i$  proteins but, they were partial agonists when the MOR was signaling through the  $G\alpha_z$  and the  $G\alpha_{oB}$  proteins. Both compounds were most efficacious when the MOR was signaling through the  $G\alpha_z$  protein. A previous BRET study showed that naloxone activated  $G\alpha_{oA}$ ,  $G\alpha_{oB}$ , and  $G\alpha_z$  proteins, but not  $G\alpha_i$  proteins, when signaling through the MOR (Masuho et al., 2015). Given that neither naltrexone, naloxone nor SAM appear to be abused or have analgesic activity mediated by the MOR, the *in vivo* pharmacologic activity of the observed  $G\alpha_o$  and  $G\alpha_z$  will require further study.

In our studies, BUP was a partial agonist at the KOR when the receptor was signaling through  $G\alpha_i$  and  $G\alpha_o$  proteins and, a full agonist when the KOR was signaling through the  $G\alpha_z$  protein. Previous BUP studies using [ $^{35}$ S]GTP $\gamma$ S binding at the KOR provided highly variable results characterizing BUP as a partial agonist at the KOR (Huang et al., 2001; Wentland et al., 2009), or an inverse agonist (Grinnell et al., 2016). SAM was a partial agonist at the KOR, when signaling through  $G\alpha_i$  and  $G\alpha_o$ , and a full agonist signaling through  $G\alpha_z$  proteins. The 1:3 BUP:SAM combination showed activity that was virtually indistinguishable from BUP alone (see Fig. 7), suggesting that at concentrations of BUP/SAM observed in blood clinically, SAM does not effectively compete for binding with BUP at the KOR regardless of the signaling pathway analyzed. These results are fully consistent with the [ $^{35}$ S]GTP $\gamma$ S experiments in this study.

JPET#249839

Previous studies found no recruitment of  $\beta$ -arrestin to the MOR by BUP (McPherson et al., 2010; Chen et al., 2013; Grinnell et al., 2016). In this study, BUP had an  $E_{\max}$  value of 33% for  $\beta$ -arrestin recruitment to the MOR, in HEK 293 cells transfected with GRK2. Similar results have been reported for PMZ21 and TRV130 with GRK2-transfected cells (Manglik et al., 2016). Both SAM and the 1:3 molar combination of BUP:SAM showed no measureable activity in the  $\beta$ -arrestin recruitment assay. It is not clear whether this lack of  $\beta$ -arrestin recruitment is the result of the partial reduction of the efficacy of BUP or if the combination specifically blocks  $\beta$ -arrestin recruitment to the MOR. Notably, the only biased MOR ligands reported to date are partial agonists (Madariaga-Mazon et al., 2017).

In summary, our studies provide the most complete characterization of the *in vitro* pharmacology of BUP, SAM and combinations thereof published to date. The data demonstrate the unique property of BUP to retain similar binding potency across a myriad of assessments, showing similar potency when acting as an agonist or antagonist. Distinctive properties of BUP in comparison to other  $\mu$  partial agonists include its slow dissociation from the MOR, and its slightly higher affinity for the MOR in the presence of NaCl and GDP. These distinguishing pharmacological properties of BUP may account for its ability to retain similar potency across many signaling assessments. SAM largely functioned as an antagonist at the MOR, and partial agonist at the KOR. In all systems tested, SAM effectively decreased BUP's efficacy on the MOR, but was ineffective in modulating the KOR efficacy of BUP. Interestingly, the loss of potency at the KOR was not observed under standard high-affinity buffer systems, but was predicted when studies were conducted in low-affinity buffer conditions. The *in vivo* implications of the differing levels of signaling observed with different G proteins in our studies are unclear and warrant further study.

JPET#249839

Assessment of the 1:3 BUP:SAM indicate the combination retains low level of efficacy at the MOR, but functions like BUP at the KOR. In addition, the combination demonstrates a functional bias versus  $\beta$ -arrestin signaling, whereas BUP alone does not. Modulation of the opioid system through the MOR and KOR would be consistent with published studies demonstrating these targets may be of value in the treatment of MDD. For example, recent pre-clinical data suggest both MOR agonism and KOR antagonism could be of benefit depending on the behavioral domain assessed (Robinson et al., 2017). The current data does not address any contribution from potential metabolites of BUP or SAM though the relative potency of nor-BUP versus SAM and BUP at the MOR, a contribution of this metabolite seems unlikely (Huang et al., 2001). Additional studies are currently underway to examine the effects of BUP/SAM in numerous behavioral models to verify the contributions of MOR and KOR *in vivo* and to better understand the interaction of opioid modulation with other neurotransmitter systems related to depression.

JPET#249839

## **Acknowledgements**

We thank Bruce Roth and Kelly Nichols for technical assistance and Ingo Mugge for his help in generating the spider plots.

## **Authorship Contributions**

*Participated in research design:* Bidlack, Deaver Knapp, Pin, Namchuk

*Conducted experiments:* Knapp, Plotnikava, Arnelle, Wonsey, Toh

*Performed data analysis:* Bidlack, Knapp, Plotnikava, Arnelle, Wonsey, Toh, Pin

*Wrote or contributed to the writing of the manuscript:* Bidlack, Knapp, Pin, Toh, Namchuk

JPET#249839

## References

- Almatroudi A, Ostovar M, Bailey CP, Husbands SM and Bailey SJ (2018) Antidepressant-like effects of BU10119, a novel buprenorphine analogue with mixed kappa/mu receptor antagonist properties, in mice. *Br J Pharmacol* **175**:2869-2880.
- Audet N, Gales C, Archer-Lahlou E, Vallieres M, Schiller PW, Bouvier M and Pineyro G (2008) Bioluminescence resonance energy transfer assays reveal ligand-specific conformational changes within preformed signaling complexes containing delta-opioid receptors and heterotrimeric G proteins. *J Biol Chem* **283**:15078-15088.
- Boas RA and Villiger JW (1985) Clinical actions of fentanyl and buprenorphine. The significance of receptor binding. *Br J Anaesth* **57**:192-196.
- Bodkin JA, Zornberg GL, Lukas SE and Cole JO (1995) Buprenorphine treatment of refractory depression. *J Clin Psychopharmacol* **15**:49-57.
- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-254.
- Cami-Kobeci G, Polgar WE, Khroyan TV, Toll L and Husbands SM (2011) Structural determinants of opioid and NOP receptor activity in derivatives of buprenorphine. *J Med Chem* **54**:6531-6537.
- Chabre M, Deterre P and Antonny B (2009) The apparent cooperativity of some GPCRs does not necessarily imply dimerization. *Trends Pharmacol Sci* **30**:182-187.
- Chen XT, Pitis P, Liu G, Yuan C, Gotchev D, Cowan CL, Rominger DH, Koblish M, Dewire SM, Crombie AL, Violin JD and Yamashita DS (2013) Structure-activity relationships and discovery of a G protein biased mu opioid receptor ligand, [(3-methoxythiophen-2-yl)methyl]({2-[(9R)-9-(pyridin-2-yl)-6-oxaspiro-[4.5]decan-9-yl]ethyl})amine (TRV130), for the treatment of acute severe pain. *J Med Chem* **56**:8019-8031.

JPET#249839

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K<sub>1</sub>) and the concentration of inhibitor which causes 50 per cent inhibition (I<sub>50</sub>) of an enzymatic reaction. *Biochem Pharmacol* **22**:3099-3108.

Ehrich E, Turncliff R, Du Y, Leigh-Pemberton R, Fernandez E, Jones R and Fava M (2015) Evaluation of opioid modulation in major depressive disorder. *Neuropsychopharmacology* **40**:1448-1455.

Emmerson PJ, Clark MJ, Mansour A, Akil H, Woods JH and Medzihradsky F (1996) Characterization of opioid agonist efficacy in a C6 glioma cell line expressing the mu opioid receptor. *J Pharmacol Exp Ther* **278**:1121-1127.

Emmerson PJ, McKinzie JH, Surface PL, Suter TM, Mitch CH and Statnick MA (2004) Na<sup>+</sup> modulation, inverse agonism, and anorectic potency of 4-phenylpiperidine opioid antagonists. *Eur J Pharmacol* **494**:121-130.

Falcon E, Browne CA, Leon RM, Fleites VC, Sweeney R, Kirby LG and Lucki I (2016) Antidepressant-like effects of buprenorphine are mediated by kappa opioid receptors. *Neuropsychopharmacology* **41**:2344-2351.

Falcon E, Maier K, Robinson SA, Hill-Smith TE and Lucki I (2015) Effects of buprenorphine on behavioral tests for antidepressant and anxiolytic drugs in mice. *Psychopharmacology* **232**:907-915.

Fava M, Memisoglu A, Thase ME, Bodkin JA, Trivedi MH, de Somer M, Du Y, Leigh-Pemberton R, DiPetrillo L, Silverman B and Ehrich E (2016) Opioid modulation with buprenorphine/samidorphan as adjunctive treatment for inadequate response to antidepressants: a randomized double-blind placebo-controlled trial. *Am J Psychiatry* **173**:499-508.

Grinnell SG, Ansonoff M, Marrone GF, Lu Z, Narayan A, Xu J, Rossi G, Majumdar S, Pan YX, Bassoni DL, Pintar J and Pasternak GW (2016) Mediation of buprenorphine analgesia

JPET#249839

by a combination of traditional and truncated mu opioid receptor splice variants.

*Synapse* **70**:395-407.

Huang P, Kehner GB, Cowan A and Liu-Chen LY (2001) Comparison of pharmacological activities of buprenorphine and norbuprenorphine: norbuprenorphine is a potent opioid agonist. *J Pharmacol Exp Ther* **297**:688-695.

Khroyan TV, Wu J, Polgar WE, Cami-Kobeci G, Fotaki N, Husbands SM and Toll L (2015) BU08073 a buprenorphine analogue with partial agonist activity at mu-receptors in vitro but long-lasting opioid antagonist activity in vivo in mice. *Br J Pharmacol* **172**:668-680.

Kosten TR (2016) An Opioid for Depression? *Am J Psychiatry* **173**:446-447.

Kruegel AC, Gassaway MM, Kapoor A, Varadi A, Majumdar S, Filizola M, Javitch JA and Sames D (2016) Synthetic and Receptor Signaling Explorations of the Mitragyna Alkaloids: Mitragynine as an Atypical Molecular Framework for Opioid Receptor Modulators. *J Am Chem Soc* **138**:6754-6764.

Lamberts JT, Rosenthal LD, Jutkiewicz EM and Traynor JR (2018) Role of the guanine nucleotide binding protein, Galphao, in the development of morphine tolerance and dependence. *Psychopharmacology* **235**:71-82.

Law PY, Wong YH and Loh HH (2000) Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* **40**:389-430.

Leander JD (1987) Buprenorphine has potent kappa opioid receptor antagonist activity. *Neuropharmacology* **26**:1445-1447.

Lutz PE and Kieffer BL (2013) Opioid receptors: distinct roles in mood disorders. *Trends Neurosci* **36**:195-206.

Madariaga-Mazon A, Marmolejo-Valencia AF, Li Y, Toll L, Houghten RA and Martinez-Mayorga K (2017) Mu-Opioid receptor biased ligands: A safer and painless discovery of analgesics? *Drug Discov Today* **22**:1719-1729.



JPET#249839

- Manglik A, Lin H, Aryal DK, McCorvy JD, Dengler D, Corder G, Levit A, Kling RC, Bernat V, Hubner H, Huang XP, Sassano MF, Giguere PM, Lober S, Da D, Scherrer G, Kobilka BK, Gmeiner P, Roth BL and Shoichet BK (2016) Structure-based discovery of opioid analgesics with reduced side effects. *Nature* **537**:185-190.
- Masuho I, Ostrovskaya O, Kramer GM, Jones CD, Xie K and Martemyanov KA (2015) Distinct profiles of functional discrimination among G proteins determine the actions of G protein-coupled receptors. *Science Signal* **8**:ra123. doi: 10.1126/scisignal.aab4068
- McPherson J, Rivero G, Baptist M, Llorente J, Al-Sabah S, Krasel C, Dewey WL, Bailey CP, Rosethorne EM, Charlton SJ, Henderson G and Kelly E (2010) mu-Opioid receptors: correlation of agonist efficacy for signalling with ability to activate internalization. *Mol Pharmacol* **78**:756-766.
- Namkung Y, Le Gouill C, Lukashova V, Kobayashi H, Hogue M, Khoury E, Song M, Bouvier M and Laporte SA (2016) Monitoring G protein-coupled receptor and beta-arrestin trafficking in live cells using enhanced bystander BRET. *Nat Commun* **7**:12178 doi: 10.1038/ncomms12178.
- Negus SS, Bidlack JM, Mello NK, Furness MS, Rice KC and Brandt MR (2002) Delta opioid antagonist effects of buprenorphine in rhesus monkeys. *Behav Pharmacol* **13**:557-570.
- Nyhuis PW, Gastpar M and Scherbaum N (2008) Opiate treatment in depression refractory to antidepressants and electroconvulsive therapy. *J Clin Psychopharmacol* **28**:593-595.
- Robinson SA, Erickson RL, Browne CA and Lucki I (2017) A role for the mu opioid receptor in the antidepressant effects of buprenorphine. *Behav Brain Res* **319**:96-103.
- Saidak Z, Blake-Palmer K, Hay DL, Northup JK and Glass M (2006) Differential activation of G-proteins by mu-opioid receptor agonists. *Br J Pharmacol* **147**:671-680.
- Schmid CL, Kennedy NM, Ross NC, Lovell KM, Yue Z, Morgenweck J, Cameron MD, Bannister TD and Bohn LM (2017) Bias factor and therapeutic window correlate to predict safer opioid analgesics. *Cell* **171**:1165-1175.e1113.

JPET#249839

Selley DE, Cao CC, Liu Q and Childers SR (2000) Effects of sodium on agonist efficacy for G-protein activation in mu-opioid receptor-transfected CHO cells and rat thalamus. *Br J Pharmacol* **130**:987-996.

Simon EJ and Groth J (1975) Kinetics of opiate receptor inactivation by sulfhydryl reagents: evidence for conformational change in presence of sodium ions. *Proc Natl Acad Sci U S A* **72**:2404-2407.

Smith KL, Deaver DL, Dean III RL, Cunningham JI, Sanchez C and Eyerman DJ (2017) mu-Dependent effects of buprenorphine in combination with samidorphan on immobility behavior in Wistar-Kyoto rats. *Neuropsychopharmacology* **42**:S550-551.

Villiger JW (1984) Binding of buprenorphine to opiate receptors. Regulation by guanyl nucleotides and metal ions. *Neuropharmacology* **23**:373-375.

Villiger JW and Taylor KM (1981) Buprenorphine: characteristics of binding sites in the rat central nervous system. *Life Sci* **29**:2699-2708.

Villiger JW and Taylor KM (1982) Buprenorphine: high-affinity binding to dorsal spinal cord. *J Neurochem* **38**:1771-1773.

Wentland MP, Lou R, Lu Q, Bu Y, Denhardt C, Jin J, Ganorkar R, VanAlstine MA, Guo C, Cohen DJ and Bidlack JM (2009) Syntheses of novel high affinity ligands for opioid receptors. *Bioorg Med Chem Lett* **19**:2289-2294.

Wentland MP, Lu Q, Lou R, Bu Y, Knapp BI and Bidlack JM (2005) Synthesis and opioid receptor binding properties of a highly potent 4-hydroxy analogue of naltrexone. *Bioorg Med Chem Lett* **15**:2107-2110.

JPET#249839

## Legends for Figures

**Fig. 1.** Structures of SAM and BUP.

**Fig. 2.** Association and dissociation of [<sup>3</sup>H]BUP with MOR, KOR, and DOR. Association assays were performed by incubating [<sup>3</sup>H]BUP with hMOR-CHO, hKOR-CHO and hDOR-CHO cell membranes for various times in 50 mM Tris-HCl, pH 7.4 or GP buffer. Nonspecific binding was measured at each time point by inclusion of 10 μM naloxone. Dissociation assays were performed by incubating [<sup>3</sup>H]BUP with membranes for 90 min to reach equilibrium at 25°C and then, adding 10 μM BUP at different times to displace the bound [<sup>3</sup>H]BUP. The [<sup>3</sup>H]BUP concentrations used are listed in the Materials and Methods.

**Fig. 3.** Association and dissociation of [<sup>3</sup>H]SAM with MOR, KOR, and DOR. Association assays were performed by incubating [<sup>3</sup>H]SAM with hMOR-CHO, hKOR-CHO and hDOR-CHO cell membranes for various times in 50 mM Tris-HCl, pH 7.4 or GP buffer. Nonspecific binding was measured at each time point by inclusion of 10 μM naloxone. Dissociation assays were performed by incubating [<sup>3</sup>H]SAM with membranes for 60 min to reach equilibrium at 25°C and then, adding unlabeled 1 μM SAM at different times to displace the bound [<sup>3</sup>H]SAM.

**Fig. 4.** Saturation binding and representative Scatchard plots for [<sup>3</sup>H]SAM and [<sup>3</sup>H]BUP at the MOR in Tris-HCl or GP buffer. The binding affinity ( $K_d$ ) and maximum binding ( $B_{max}$ ) of [<sup>3</sup>H]SAM (A and B) and [<sup>3</sup>H]BUP (C and D) under high- (A and C) and low- affinity binding conditions (B and D) to hMOR-CHO membranes. High affinity binding was measured in 50 mM Tris-HCl buffer, pH 7.4. Low affinity binding was measured in GP buffer. Eight concentrations of either

JPET#249839

[<sup>3</sup>H]BUP or [<sup>3</sup>H]SAM were incubated with hMOR-CHO membranes in 50 mM Tris-HCl, pH 7.4 or GP buffer at a final volume of 1 ml. Nonspecific binding was measured with 10 μM naloxone. hMOR-CHO membranes were added last. Data are the mean fmol bound/mg of membrane protein ± S.E.M. A representative Scatchard plot is included for each condition.

**Fig. 5.** Stimulation and inhibition of [<sup>35</sup>S]GTP<sub>γ</sub>S binding to the MOR and KOR by BUP and SAM alone and in combination. A) Stimulation of [<sup>35</sup>S]GTP<sub>γ</sub>S binding mediated by the MOR by BUP and SAM alone or various molar ratios of the compounds together. B) Stimulation of [<sup>35</sup>S]GTP<sub>γ</sub>S binding at the KOR by BUP, SAM, or various molar ratios of the compounds together. C) Inhibition of agonist-stimulated [<sup>35</sup>S]GTP<sub>γ</sub>S binding at the MOR by BUP or SAM. D) Inhibition of agonist-stimulated [<sup>35</sup>S]GTP<sub>γ</sub>S binding at the KOR by BUP and SAM. When SAM and BUP were tested together, the total molarity is plotted.

**Fig. 6.** BRET studies showing BUP and SAM alone and in combination activating MOR (A-C), KOR (D-F), and DOR (G-I) signaling through G<sub>α1</sub>, G<sub>α2</sub>, G<sub>α3</sub>, G<sub>α<sub>oA</sub></sub>, G<sub>α<sub>oB</sub></sub> and G<sub>α<sub>z</sub></sub>.

Transfection of HEK 293 cells with receptor plasmids was performed with 3 μg/mL of PEI as the transfection reagent, and 1.8 x 10<sup>6</sup> cells in T-75 cell culture flask. Forty-eight hr after transfection, cells were washed once with PBS, removed with trypsin, centrifuged at 1,000 x g, and the pellet was re-suspended in Tyrode-HEPES buffer. Cells (10,000 cells per well) were dispensed into plates using the automated BioTek Multi-Flo reagent dispenser, and incubated at room temperature for 50 min with varying concentrations of BUP and SAM alone and in combination or control opioids. Coelenterazine was added subsequently at a final concentration of 2 μM. Cells were incubated for an additional 10 min at room temperature, and

JPET#249839

BRET readings were captured using the Envision plate reader (PerkinElmer; filters: 400nm/70nm, 515nm/20nm). BRET signals were determined by calculating the ratio of light emitted by GFP-acceptor (515 nm) over light emitted by luciferase-donor (400 nm). For each compound/biosensor pair, the compound-induced BRET signals were normalized to the BRET signal obtained from DAMGO, U50,488, and SNC-80 for the MOR, KOR and DOR, respectively. Maximal and minimal BRET signal is defined by 20  $\mu$ M DAMGO/20  $\mu$ M U50,488/20  $\mu$ M SNC-80, or cells in the absence of agonists, respectively.

**Fig. 7.** Recruitment of  $\beta$ -arrestin to the MOR (A), KOR (B), and DOR (C) by BUP and SAM alone and in combination. Transfection with receptor, GRK2, and biosensor plasmids was performed as described in Materials and Methods. Cells (10,000 cells per well) were dispensed into plates using the automated BioTek Multi-Flo reagent dispenser, and incubated with at room temperature for 50 min with varying concentrations of BUP and SAM alone and in combination or with control opioids. Coelenterazine was added subsequently at a final concentration of 2  $\mu$ M. Cells were incubated for an additional 10 min at room temperature, and BRET readings were captured using the Envision plate reader (PerkinElmer; filters: 400nm/70nm, 515nm/20nm). BRET signals were determined by calculating the ratio of light emitted by GFP-acceptor (515 nm) over light emitted by luciferase-donor (400 nm). For each compound/biosensor pair, the compound-induced BRET signals were normalized to the BRET signal obtained from DAMGO, U50,488, and SNC80 for the MOR, KOR, and DOR, respectively. Maximal and minimal BRET signal is defined by 20  $\mu$ M DAMGO/20  $\mu$ M U50,488/20  $\mu$ M SNC80, or cells in the absence of agonists, respectively. Morphine was included as a control.

JPET#249839

## Tables

TABLE 1

$K_i$  values for BUP and SAM in inhibiting binding to the MOR, KOR, DOR and NOP receptor

Membranes from CHO cells expressing one type of human opioid receptor or the nociceptin/FQ receptor were incubated with 12 different concentrations of BUP or SAM.  $IC_{50}$  values were determined and  $K_i$  values were calculated. Data are the mean  $K_i$  value  $\pm$  S.E.M. from three independent experiments performed in triplicate. ND, no detectable binding.

	MOR	KOR	DOR	NOP
	[ <sup>3</sup> H]DAMGO	[ <sup>3</sup> H]U69,593	[ <sup>3</sup> H]Naltrindole	[ <sup>3</sup> H]Nociceptin
	<i>K<sub>i</sub> (nM <math>\pm</math> SEM)</i>			
BUP	0.41 $\pm$ 0.0079	0.23 $\pm$ 0.0067	2.5 $\pm$ 0.15	150 $\pm$ 14
SAM	0.052 $\pm$ 0.0044	0.23 $\pm$ 0.018	2.7 $\pm$ 0.36	ND

JPET#249839

TABLE 2

Affinities of full and partial opioid agonists, and antagonists for binding to the MOR under high- and low- affinity binding conditions

$K_{low}/K_{high}$  is the  $K_i$  value obtained under low-affinity binding conditions in GP buffer divided by the  $K_i$  value obtained under high affinity Tris-HCl buffer conditions. Data are the mean  $K_i$  values  $\pm$  S.E.M. from three separate experiments performed in triplicate.

Compound	$K_{low}/K_{high}$	High Affinity Buffer	Low Affinity Buffer
		[ <sup>3</sup> H]DAMGO	[ <sup>3</sup> H]Naloxone
<u>Full Agonists</u>		$K_i$ (nM) $\pm$ SEM	
Morphine	530	0.32 $\pm$ 0.028	170 $\pm$ 7.5
Methadone	480	1.3 $\pm$ 0.14	630 $\pm$ 35
DAMGO	390	0.72 $\pm$ 0.084	280 $\pm$ 8.2
Fentanyl	380	0.47 $\pm$ 0.048	180 $\pm$ 7.8
<u>Partial Agonists</u>			
(-)-Pentazocine	37	2.1 $\pm$ 0.088	78 $\pm$ 5.1
Nalbuphine	19	0.70 $\pm$ 0.023	13 $\pm$ 0.18
Butorphanol	13	0.24 $\pm$ 0.015	3.2 $\pm$ 0.054
Nalfurafine	5.5	0.69 $\pm$ 0.053	3.8 $\pm$ 0.42
BUP	0.40	0.35 $\pm$ 0.064	0.14 $\pm$ 0.0071
<u>Antagonists</u>			
Naloxone	2.2	0.87 $\pm$ 0.12	1.9 $\pm$ 0.14
Naltrexone	1.4	0.31 $\pm$ 0.012	0.42 $\pm$ 0.014
Nalmefene	0.92	0.24 $\pm$ 0.022	0.22 $\pm$ 0.010
SAM	0.49	0.19 $\pm$ 0.020	0.094 $\pm$ 0.0073

JPET#249839

TABLE 3

Association and dissociation  $T_{1/2}$  times for [ $^3\text{H}$ ]BUP and [ $^3\text{H}$ ]SAM binding at the MOR, KOR and DOR in Tris-HCl and GP buffers

Data are the mean  $T_{1/2}$  values  $\pm$  S.E.M. from three experiments performed in triplicate. NA, not applicable.

<i>Tris-HCl Buffer</i>	$[^3\text{H}]\text{BUP}$			$[^3\text{H}]\text{SAM}$		
	MOR	KOR	DOR	MOR	KOR	DOR
<b>Association</b>						
$T_{1/2}$ (min)	25 $\pm$ 0.68	32 $\pm$ 1.9	32 $\pm$ 3.4	18 $\pm$ 0.46	7.6 $\pm$ 0.20	4.7 $\pm$ 0.42
$K_{\text{on}}$ ( $\text{nM}^{-1}\text{min}^{-1}$ )	NA	0.088 $\pm$ 0.011	0.0072 $\pm$ 0.0019	0.68 $\pm$ 0.022	0.35 $\pm$ 0.011	0.13 $\pm$ 0.014
<b>Dissociation</b>						
$T_{1/2}$ (min)	>1380	390 $\pm$ 47	77 $\pm$ 4.5	260 $\pm$ 14	47 $\pm$ 7.4	20 $\pm$ 1.8
$K_{\text{off}}$ ( $\text{min}^{-1}$ )	NA	0.0028 $\pm$ 0.00018	0.011 $\pm$ 0.0035	0.0030 $\pm$ 0.00075	0.021 $\pm$ 0.0051	0.021 $\pm$ 0.0017
$K_{\text{d}}$ (nM)	NA	0.033 $\pm$ 0.0044	1.7 $\pm$ 0.37	0.0044 $\pm$ 0.00014	0.059 $\pm$ 0.0019	0.17 $\pm$ 0.017
<i>GP Buffer</i>						
<b>Association</b>						
$T_{1/2}$ (min)	13 $\pm$ 0.83	13 $\pm$ 1.1	15 $\pm$ 1.5	13 $\pm$ 0.52	0.36 $\pm$ 0.027	1.4 $\pm$ 0.24
$K_{\text{on}}$ ( $\text{nM}^{-1}\text{min}^{-1}$ )	0.13 $\pm$ 0.0089	0.35 $\pm$ 0.048	NA	0.71 $\pm$ 0.045	0.50 $\pm$ 0.069	0.16 $\pm$ 0.13
<b>Dissociation</b>						
$T_{1/2}$ (min)	200 $\pm$ 7.0	41 $\pm$ 2.4	7.8 $\pm$ 0.75	44 $\pm$ 1.7	1.0 $\pm$ 0.055	1.5 $\pm$ 0.022
$K_{\text{off}}$ ( $\text{min}^{-1}$ )	0.0029 $\pm$ 0.00071	0.018 $\pm$ 0.0013	0.088 $\pm$ 0.0044	0.018 $\pm$ 0.00054	0.96 $\pm$ 0.044	0.39 $\pm$ 0.047
$K_{\text{d}}$ (nM)	0.023 $\pm$ 0.0016	0.054 $\pm$ 0.0070	NA	0.025 $\pm$ 0.0017	2.0 $\pm$ 0.31	3.9 $\pm$ 3.3



JPET#249839

TABLE 4

$K_d$  and  $B_{max}$  values for [ $^3$ H]BUP and [ $^3$ H]SAM binding to the MOR, KOR and DOR in Tris-HCl and GP buffers

Data are the mean  $K_d$  and  $B_{max}$  values  $\pm$  S.E.M. from at least three experiments performed in triplicate.

	[ $^3$ H]BUP			[ $^3$ H]SAM		
	MOR	KOR	DOR	MOR	KOR	DOR
<b>Tris-HCl Buffer</b>						
$K_d$ (nM)	0.31 $\pm$ 0.037	0.14 $\pm$ 0.015	1.4 $\pm$ 0.13	0.046 $\pm$ 0.0027	0.19 $\pm$ 0.018	0.94 $\pm$ 0.10
$B_{max}$ (fmol/mg of protein)	740 $\pm$ 49	1500 $\pm$ 230	5100 $\pm$ 230	460 $\pm$ 37	860 $\pm$ 110	1300 $\pm$ 48
<b>GP Buffer</b>						
$K_d$ (nM)	0.24 $\pm$ 0.036	0.074 $\pm$ 0.011	0.57 $\pm$ 0.052	0.044 $\pm$ 0.0051	3.0 $\pm$ 0.10	1.9 $\pm$ 0.059
$B_{max}$ (fmol/mg of protein)	620 $\pm$ 30	1600 $\pm$ 150	5500 $\pm$ 640	460 $\pm$ 50	700 $\pm$ 120	1800 $\pm$ 64

JPET#249839

TABLE 5

$K_i$  values for the inhibition of [ $^3$ H]BUP and [ $^3$ H]SAM binding to the MOR by SAM, BUP, naltrexone, and naloxone in Tris-HCl and GP buffers

Data are the mean  $K_i$  values  $\pm$  S.E.M. from three separate experiments performed in triplicate.

Compound	Tris-HCl Buffer	GP Buffer
<i>K<sub>i</sub> (nM <math>\pm</math> SEM)</i>		
<b>[<math>^3</math>H]BUP Binding</b>		
SAM	0.26 $\pm$ 0.014	0.24 $\pm$ 0.026
BUP	0.66 $\pm$ 0.13	0.64 $\pm$ 0.10
Naltrexone	0.81 $\pm$ 0.058	1.2 $\pm$ 0.12
Naloxone	4.5 $\pm$ 0.45	4.7 $\pm$ 0.47
<b>[<math>^3</math>H]SAM Binding</b>		
SAM	0.11 $\pm$ 0.0073	0.092 $\pm$ 0.016
BUP	0.34 $\pm$ 0.012	0.24 $\pm$ 0.018
Naltrexone	0.19 $\pm$ 0.020	0.29 $\pm$ 0.022
Naloxone	1.2 $\pm$ 0.073	1.5 $\pm$ 0.10

JPET#249839

TABLE 6

Efficacy and potency of BUP and SAM alone and in combination in stimulating [<sup>35</sup>S]GTP<sub>γ</sub>S binding mediated by the MOR, KOR, and DOR

Data are the mean values ± S.E.M. from three experiments performed in triplicate except where noted. The E<sub>max</sub> value is the % of maximal stimulation and I<sub>max</sub> is the % of maximal inhibition. NA, not applicable. ND, not determined.

Agonist Activity			Antagonist Activity	
<b>MOR</b>	<i>EC</i> <sub>50</sub> (nM)	<i>E</i> <sub>max</sub> (%)	<i>IC</i> <sub>50</sub> (nM)	<i>I</i> <sub>max</sub> (%)
BUP	0.63 ± 0.40	57 ± 5.5	0.42 ± 0.014	49 ± 2.0
SAM	NA	3.8 ± 0.67	0.73 ± 0.052	92 ± 2.4
1:1 BUP:SAM*	0.73 ± 0.72	32 ± 2.5	ND	ND
1:5 BUP:SAM	2.1 ± 0.32	16 ± 0.78	ND	ND
1:50 BUP:SAM	NA	7.2 ± 0.69	ND	ND
<b>KOR</b>				
BUP	0.46 ± 0.14	25 ± 1.3	0.71 ± 0.18	74 ± 0.98
SAM	1.4 ± 0.095	56 ± 0.59	15 ± 0.30	47 ± 3.3
1:1 BUP:SAM	0.74 ± 0.16	29 ± 1.8	ND	ND
1:5 BUP:SAM	1.2 ± 0.059	29 ± 0.19	ND	ND
1:50 BUP:SAM	1.1 ± 0.23	40 ± 2.2	ND	ND
<b>DOR</b>				
BUP	1.3 ± 0.041	19 ± 1.2	4.2 ± 0.74	78 ± 3.6
SAM	1.9 ± 0.47	21 ± 1.6	14 ± 2.8	72 ± 1.4

\* n = 2 independent experiments performed in triplicate with mean ± S.D. reported. Total molarity of BUP and SAM in combination was used to calculate the EC<sub>50</sub> values.

JPET#249839

TABLE 7

$EC_{50}$  and  $E_{max}$  values for BUP and SAM alone and in combination in stimulating the activation of  $G\alpha_{i1}$ ,  $G\alpha_{i2}$ , and  $G\alpha_{i3}$  mediated by the MOR, KOR, and DOR

$EC_{50}$  values are expressed in total molarity when BUP and SAM were combined. Data are the mean values  $\pm$  S.E.M. from six determinations. NA, not applicable

	$G\alpha_{i1}$		$G\alpha_{i2}$		$G\alpha_{i3}$	
	$EC_{50}$ (nM)	$E_{max}$ (%)	$EC_{50}$ (nM)	$E_{max}$ (%)	$EC_{50}$ (nM)	$E_{max}$ (%)
<b>MOR</b>						
DAMGO	28 $\pm$ 4.0	100	94 $\pm$ 17	100	15 $\pm$ 0.050	100
Morphine	62 $\pm$ 6.0	95 $\pm$ 1.0	260 $\pm$ 73	76 $\pm$ 4.0	31 $\pm$ 1.0	93 $\pm$ 5.0
BUP	0.70 $\pm$ 0.10	42 $\pm$ 1.0	0.40 $\pm$ 0.020	12 $\pm$ 0.25	0.72 $\pm$ 0.20	57 $\pm$ 4.0
SAM	No activity		No activity		0.35 $\pm$ 0.040	9.0 $\pm$ 1.0
1:3 BUP:SAM	0.80 $\pm$ 0.30	11 $\pm$ 1.0	No activity		0.98 $\pm$ 0.030	21 $\pm$ 4.0
<b>KOR</b>						
U50,488	2.2 $\pm$ 0.60	100	7.6 $\pm$ 2.0	100	1.3 $\pm$ 0.020	100
Morphine	560 $\pm$ 15	95 $\pm$ 6.0	760 $\pm$ 110	85 $\pm$ 7.0	540 $\pm$ 120	93 $\pm$ 1.5
BUP	1.2 $\pm$ 0.050	31 $\pm$ 5.0	1.4 $\pm$ 0.25	19 $\pm$ 3.0	1.5 $\pm$ 0.50	43 $\pm$ 3.0
SAM	1.9 $\pm$ 0.50	60 $\pm$ 8.0	3.4 $\pm$ 0.10	55 $\pm$ 0.50	1.9 $\pm$ 0.30	58 $\pm$ 4.0
1:3 BUP:SAM	1.4 $\pm$ 0.050	37 $\pm$ 5.0	1.2 $\pm$ 0.10	22 $\pm$ 3.0	1.2 $\pm$ 0.30	40 $\pm$ 3.0
<b>DOR</b>						
SNC-80	1.1 $\pm$ 0.20	100	6.0 $\pm$ 1.0	100	2.7 $\pm$ 0.80	100
Morphine	> 1 $\mu$ M	~50	> 10 $\mu$ M	~30	> 10 $\mu$ M	~50
BUP	2.6 $\pm$ 1.4	36 $\pm$ 4.0	3.0 $\pm$ 0.30	18 $\pm$ 2.0	2.8 $\pm$ 0.30	20 $\pm$ 2.0
SAM	7.6 $\pm$ 1.2	17 $\pm$ 2.0	NA	~5	13 $\pm$ 5.0	16 $\pm$ 0.30
1:3 BUP:SAM	5.8 $\pm$ 1.2	31 $\pm$ 3.0	7.6 $\pm$ 1.7	14 $\pm$ 2.0	4.3 $\pm$ 4.0	17 $\pm$ 2.0

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 19, 2024

JPET#249839

TABLE 8

EC<sub>50</sub> and E<sub>max</sub> values for BUP and SAM alone and in combination in stimulating the activation of G<sub>α<sub>oA</sub></sub>, G<sub>α<sub>oB</sub></sub>, and G<sub>α<sub>z</sub></sub> mediated by the MOR, KOR, and DOR. EC<sub>50</sub> values are expressed in total molarity when BUP and SAM were combined. Data are the mean values ± S.E.M. from six determinations. NA, not applicable

	G <sub>α<sub>oA</sub></sub>		G <sub>α<sub>oB</sub></sub>		G <sub>α<sub>z</sub></sub>	
	EC <sub>50</sub> (nM)	E <sub>max</sub> (%)	EC <sub>50</sub> (nM)	E <sub>max</sub> (%)	EC <sub>50</sub> (nM)	E <sub>max</sub> (%)
<b>MOR</b>						
DAMGO	8.0 ± 0.50	100	7.7 ± 2.0	100	4.2 ± 0.25	100
Morphine	12 ± 0.50	100 ± 4.0	11 ± 1.0	100 ± 2.0	6.0 ± 1.0	100 ± 1.0
BUP	0.40 ± 0.10	87 ± 2.0	0.30 ± 0.080	89 ± 2.5	0.90 ± 0.020	92 ± 3.5
SAM	1.1 ± 0.40	29 ± 2.0	0.26 ± 0.040	27 ± 7.0	0.060 ± 0.030	33 ± 2.0
1:3 BUP:SAM	0.80 ± 0.20	42 ± 2.0	0.15 ± 0.010	43 ± 7.0	0.10 ± 0.0050	52 ± 3.0
Naloxone	> 20,000	NA	19 ± 6.3	13 ± 0.57	27 ± 11	33 ± 3.0
Naltrexone	>20,000	NA	1.6 ± 0.52	15 ± 1.2	0.40 ± 0.043	40 ± 1.5
<b>KOR</b>						
U50,488	2.1 ± 0.050	100	0.80 ± 0.070	100	0.45 ± 0.14	100
Morphine	610 ± 29	98 ± 2.0	200 ± 44	98 ± 3.0	18 ± 1.5	94 ± 6.0
BUP	0.90 ± 0.40	35 ± 3.0	0.61 ± 0.19	66 ± 4.0	0.76 ± 0.080	104 ± 6.0
SAM	2.4 ± 0.60	61 ± 2.5	1.0 ± 0.10	86 ± 4.0	0.22 ± 0.050	95 ± 1.0
1:3 BUP:SAM	0.90 ± 0.14	35 ± 2.0	0.63 ± 0.090	64 ± 5.0	0.16 ± 0.010	85 ± 2.0
<b>DOR</b>						
SNC-80	0.70 ± 0.020	100	1.1 ± 0.10	100	0.30 ± 0.10	100
Morphine	> 1 μM	~80	3300 ± 1700	93 ± 5.0	340 ± 29	87 ± 1.0
BUP	2.7 ± 0.60	56 ± 4.0	1.5 ± 0.40	65 ± 3.0	0.40 ± 0.10	79 ± 4.0
SAM	3.4 ± 0.50	27 ± 2.0	5.4 ± 0.30	48 ± 2.0	0.90 ± 0.10	69 ± 6.0
1:3 BUP:SAM	5.7 ± 2.6	44 ± 4.0	2.9 ± 0.60	60 ± 2.0	0.60 ± 0.10	73 ± 5.0

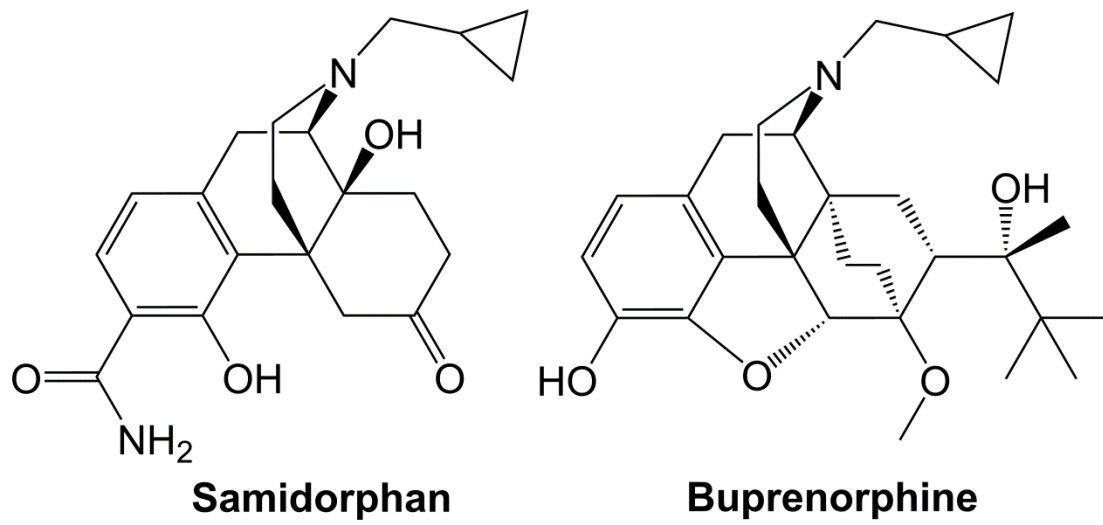
JPET#249839

TABLE 9

EC<sub>50</sub> and E<sub>max</sub> values for BUP and SAM alone and in combination in stimulating the activation of G<sub>α<sub>i2</sub></sub>-Kir, G<sub>α<sub>oA</sub></sub>-Kir, and β-arrestin recruitment mediated by the MOR, KOR, and DOR

EC<sub>50</sub> values are expressed in total molarity when buprenorphine and samidorphan were combined. Data are the mean values ± S.E.M. from six determinations. NA, not applicable

	G <sub>α<sub>i2</sub></sub> -Kir		G <sub>α<sub>oA</sub></sub> -Kir		β-Arrestin-GRK2	
	EC <sub>50</sub> (nM)	E <sub>max</sub> (%)	EC <sub>50</sub> (nM)	E <sub>max</sub> (%)	EC <sub>50</sub> (nM)	E <sub>max</sub> (%)
<b>MOR</b>						
DAMGO	30.0 ± 7.0	100	24 ± 1.0	100	32 ± 1.0	100
Morphine	142 ± 32	92 ± 2.0	89 ± 17	90 ± 4.0	71 ± 4.5	84 ± 2.0
BUP	0.40 ± 0.10	27 ± 0.70	0.30 ± 0.020	30 ± 0.020	0.40 ± 0.020	33 ± 3.5
SAM	No activity	NA	No activity	NA	No activity	NA
1:3 BUP:SAM	>10,000	~20	>100	~15	No activity	NA
<b>KOR</b>						
U50,488	3.6 ± 0.070	100	6.4 ± 1.1	100	58 ± 51	100
Morphine	960 ± 110	55 ± 9.0	> 1,000	~50	> 1,000	~50
BUP	0.80 ± 0.10	14 ± 4.0	NA	~7	NA	~15
SAM	2.5 ± 0.20	32 ± 4.0	5.0 ± 1.0	21 ± 2.0	NA	~12
1:3 BUP:SAM	0.60 ± 0.20	14 ± 3.0	NA	~7	NA	~13
<b>DOR</b>						
SNC-80	1.2 ± 0.40	100	1.0 ± 0.30	100	6.2 ± 2.1	100
Morphine	>10,000	~40	>10,000	~50	>10,000	~25
BUP	0.80 ± 0.10	56 ± 4.0	0.70 ± 0.020	37 ± 2.0	1.8 ± 1.0	16 ± 5.0
SAM	NA	~10	NA	~10	NA	~12
1:3 BUP:SAM	1.9 ± 1.3	20 ± 2.0	2.9 ± 0.10	31 ± 1.0	6.8 ± 2.0	14 ± 5.0



**Fig. 1**

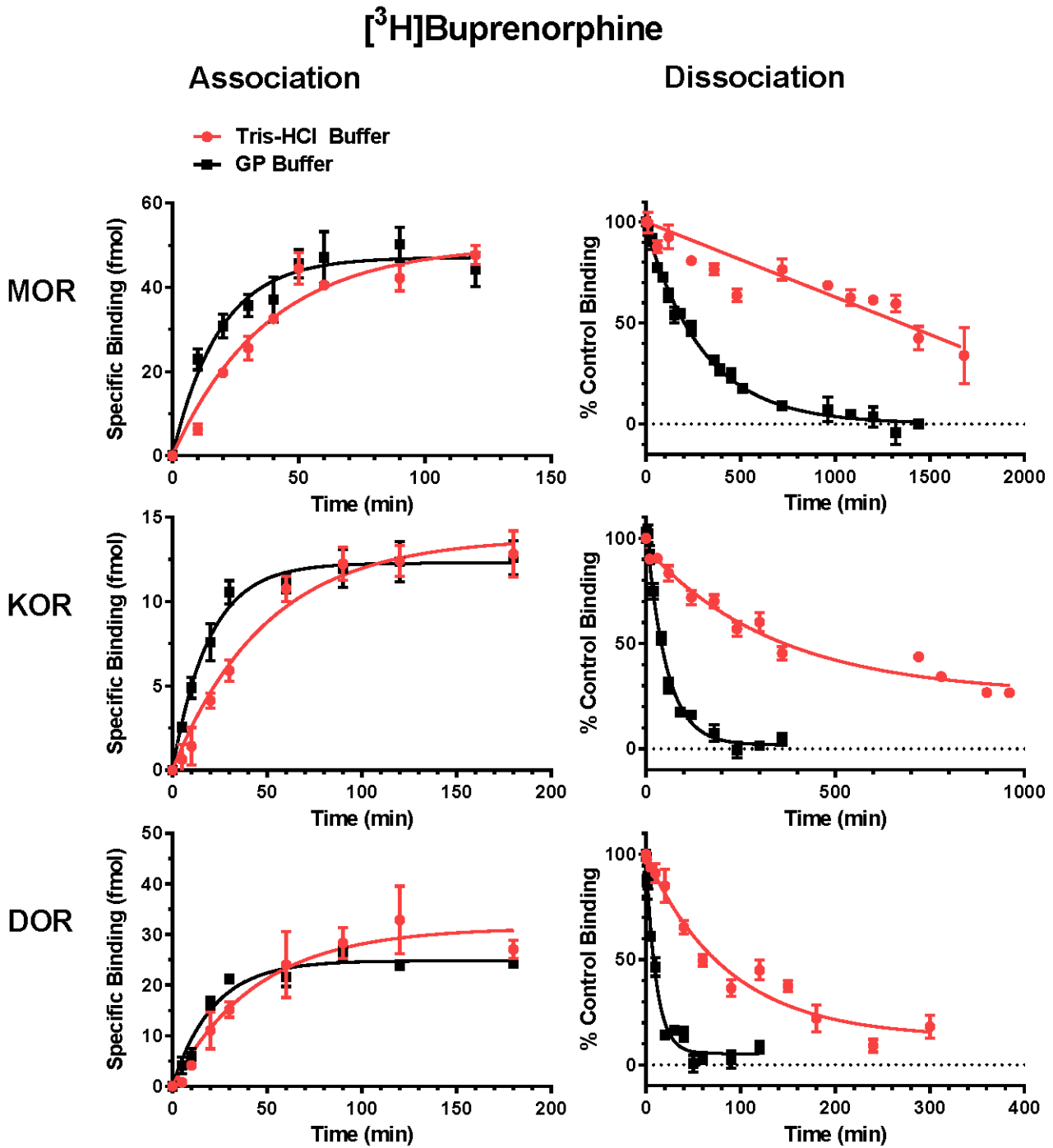


Fig. 2



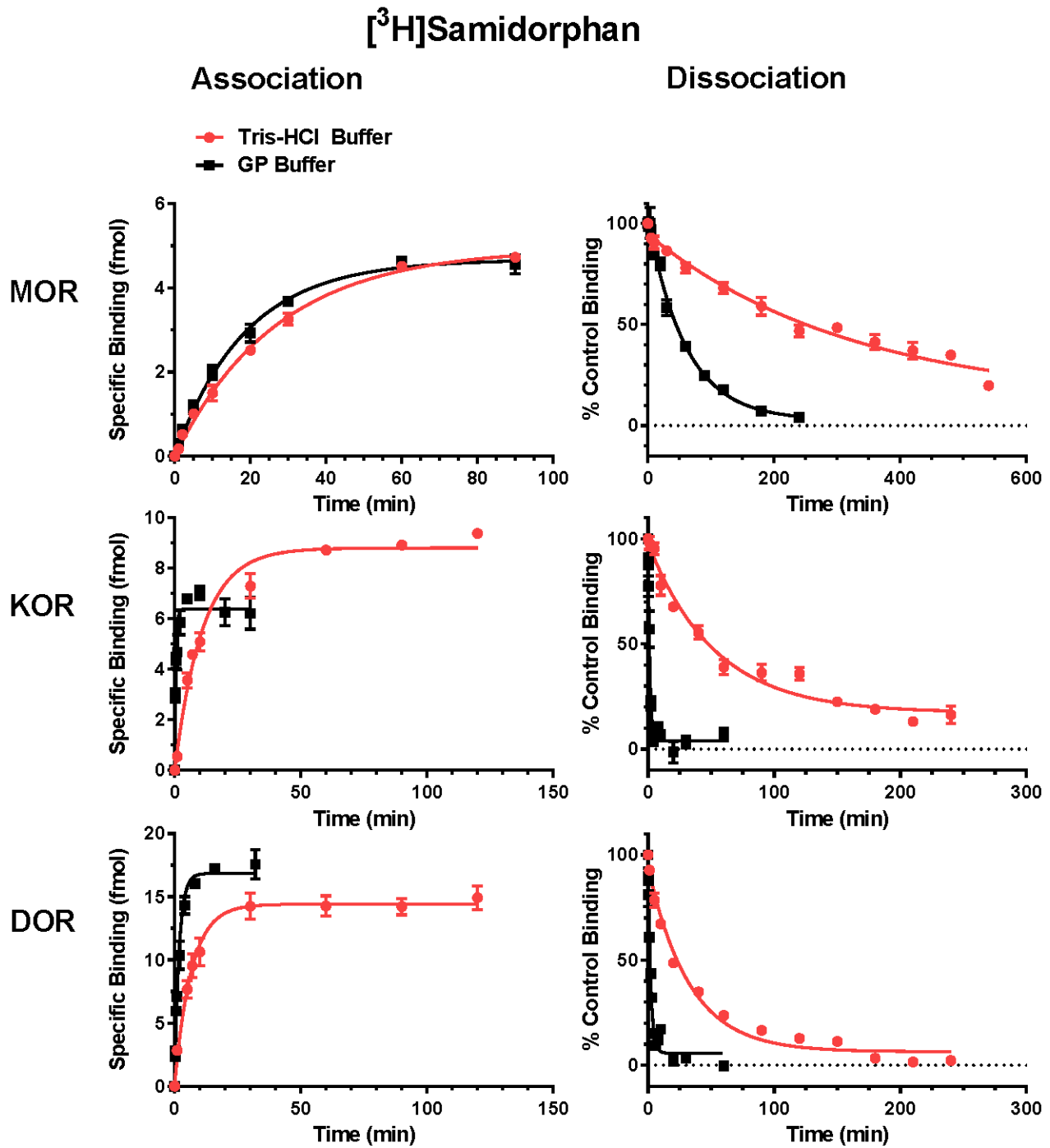


Fig. 3

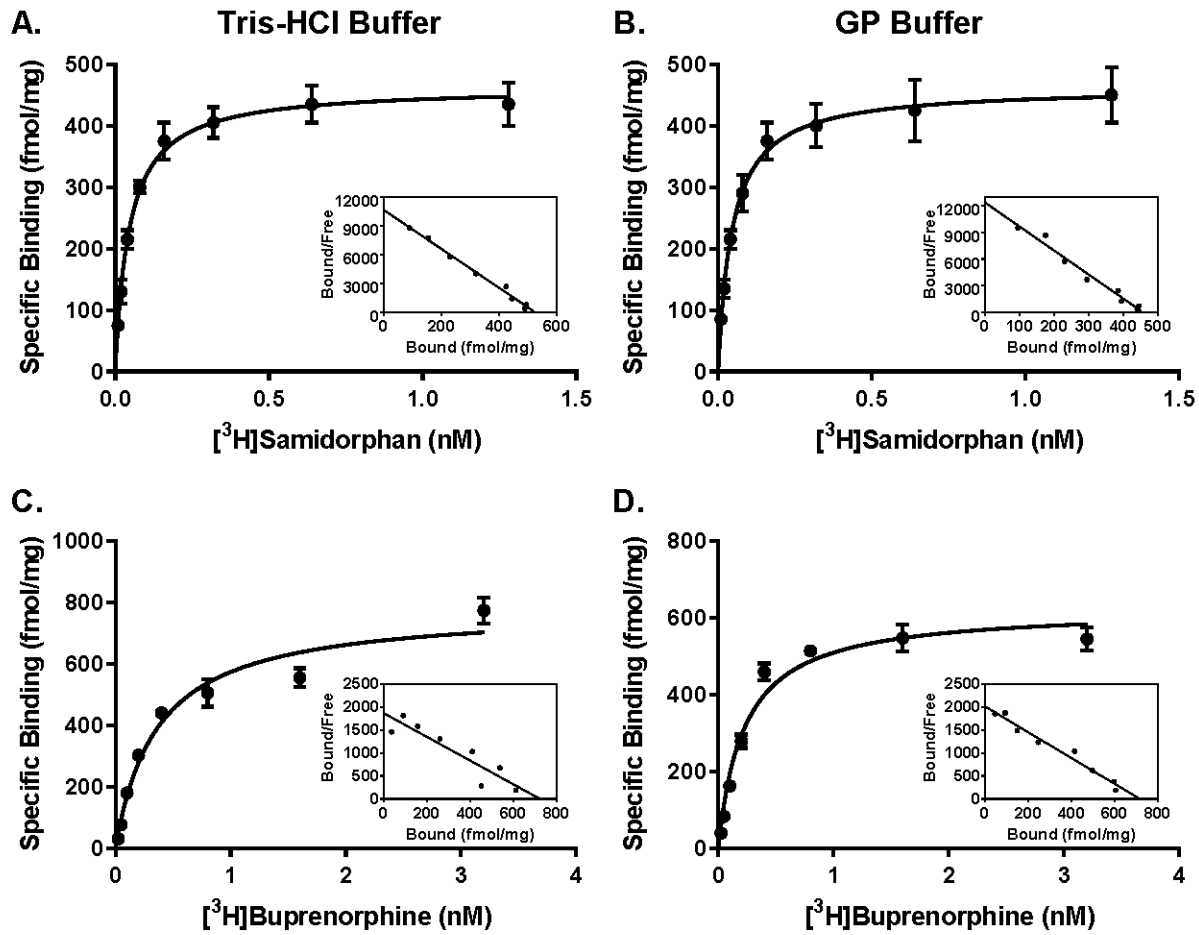


Fig. 4

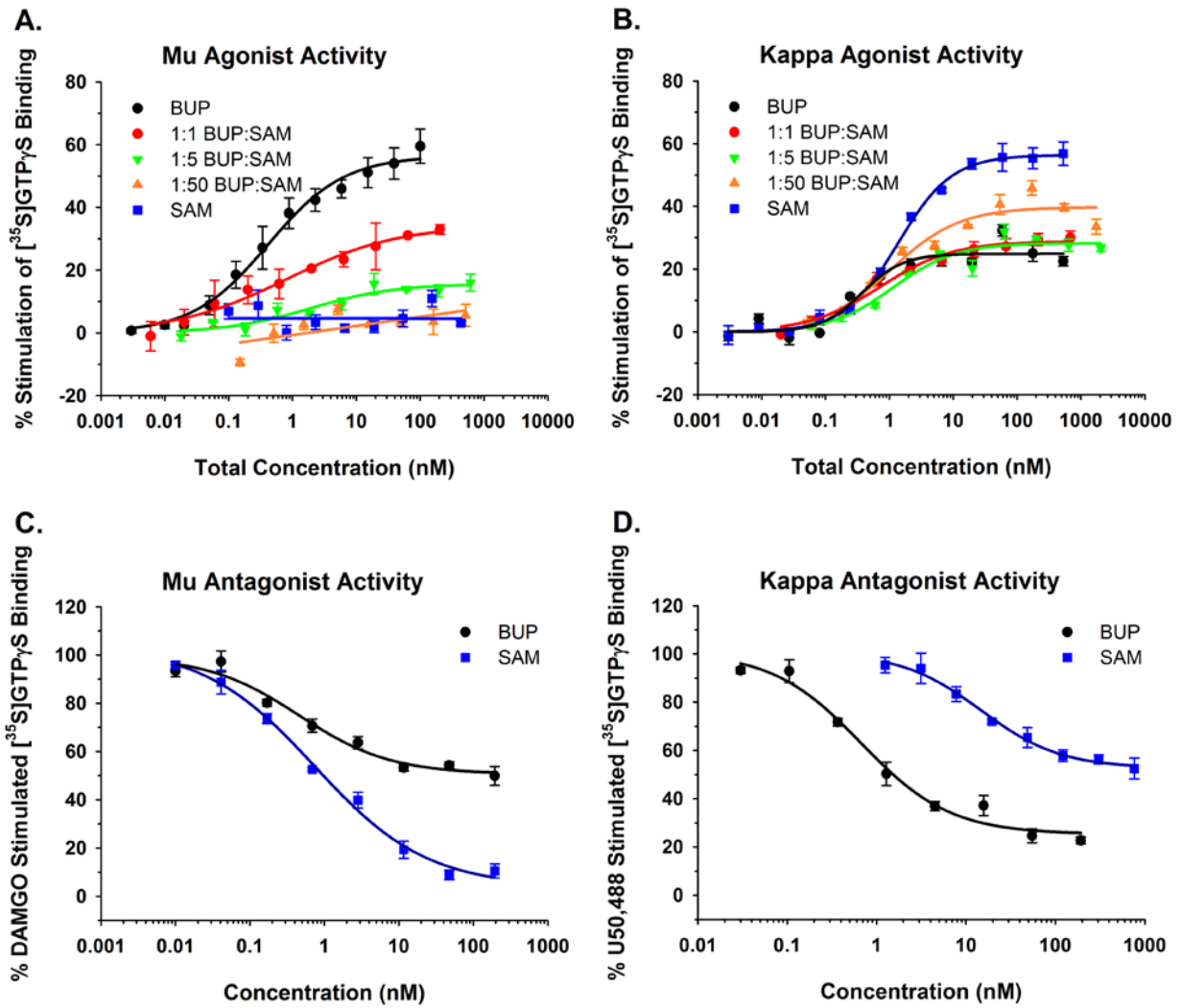
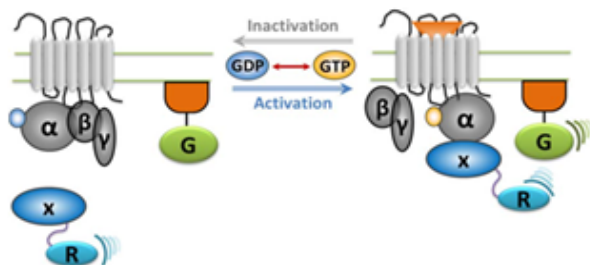


Fig. 5



HEK 293 cells were transfected transiently with one type of human opioid receptor and one type of G protein. For each compound/biosensor pair, the compound-induced BRET signals were normalized to the BRET signal obtained from 20  $\mu$ M of DAMGO, U50,488, and SNC-80 for the MOR, KOR and DOR, respectively.

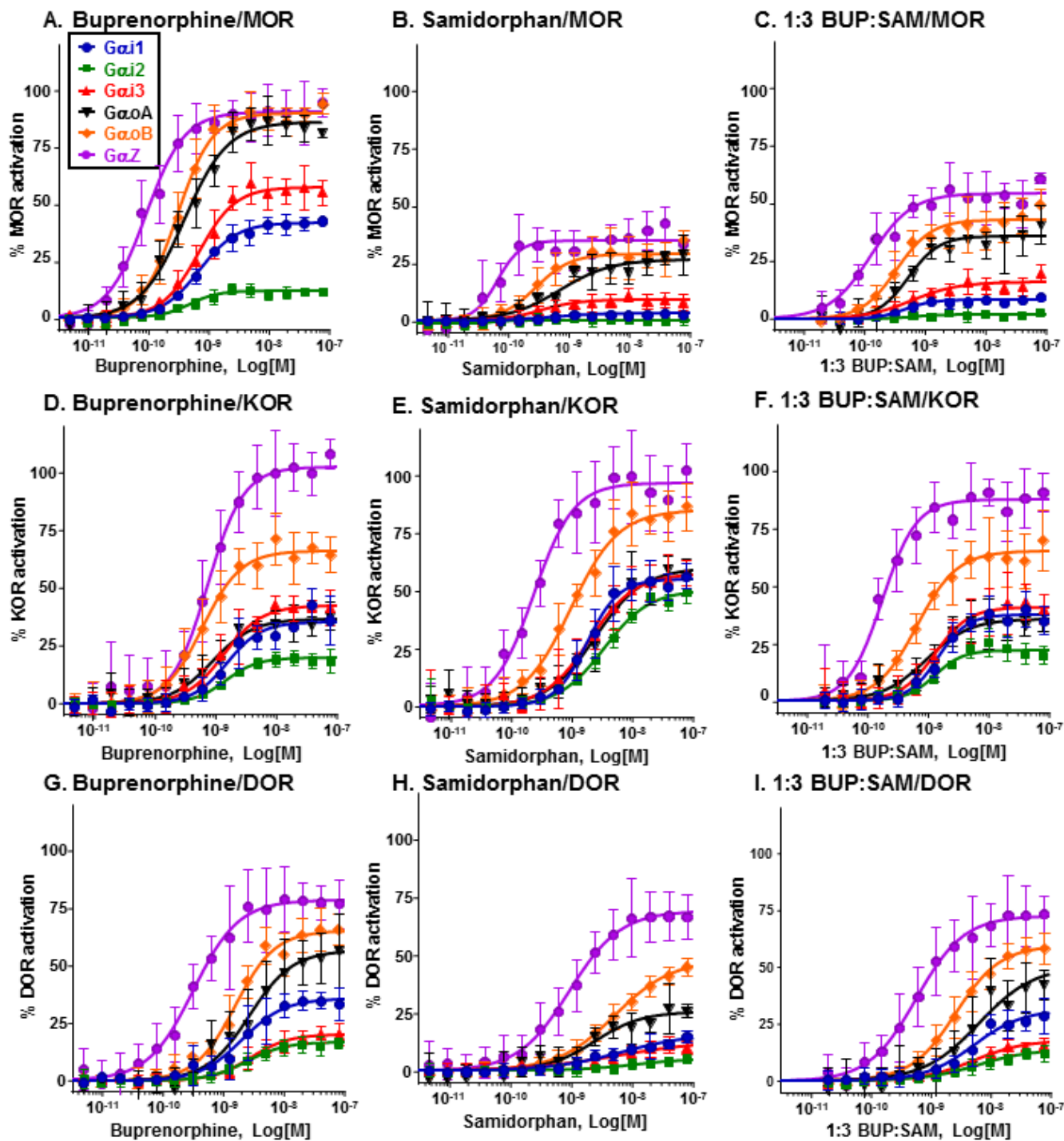


Fig. 6



HEK 293 cells were transfected transiently with one type of human opioid receptor, GRK2, and biosensor plasmids. For each compound/biosensor pair, the compound-induced BRET signals were normalized to the BRET signal obtained from 20  $\mu$ M of DAMGO, U50,488, and SNC-80 for the MOR, KOR and DOR, respectively.

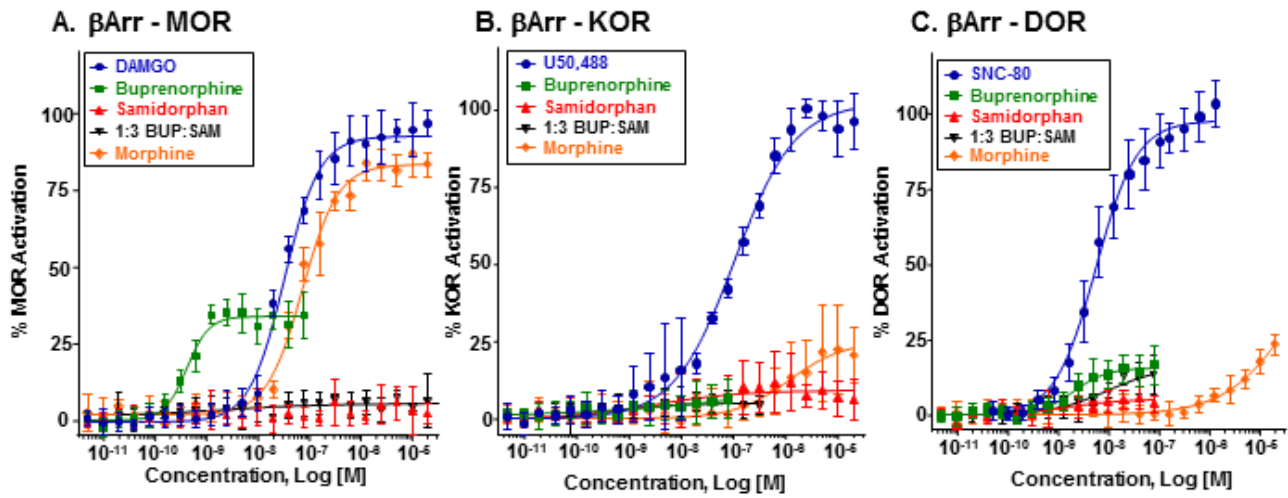


Fig. 7