0022-3565/08/3243-1073–1083$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
JPET 324:1073–1083, 2008

Vol. 324, No. 3
132142/3311241
Printed in U.S.A.

2-Methoxymethyl-Salvinorin B Is a Potent κ Opioid Receptor Agonist with Longer Lasting Action in Vivo Than Salvinorin A

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Received September 30, 2007; accepted December 17, 2007

ABSTRACT

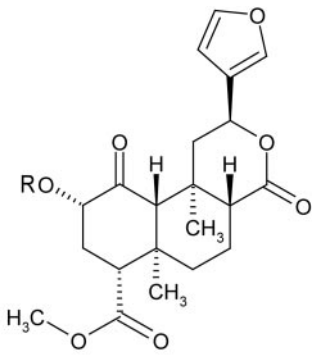
Salvinorin (Sal) A is a naturally occurring, selective κ opioid receptor (KOPR) agonist with a short duration of action in vivo. Pharmacological properties of a C(2) derivative, 2-methoxymethyl (MOM)-Sal B, were characterized. MOM-Sal B bound to KOPR with high selectivity and displayed ~3-fold higher affinity than U50,488H [(*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate] and Sal A. It acted as a full agonist at KOPR in guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate binding and was ~5- and ~7-fold more potent than U50,488H and Sal A, respectively. In Chinese hamster ovary cells stably expressing KOPR, all three κ agonists internalized or down-regulated KOPR to similar extents, with MOM-Sal B being the most potent. In mice, MOM-Sal B (0.05–1 mg/kg s.c.) caused immediate and dose-dependent immobility lasting ~3 h, which was blocked by norbinaltorphimine. In contrast, ambulation in a Y-maze was increased when rats received MOM-Sal B (1–5 mg/kg s.c.). In addition, MOM-Sal B (0.5–5 mg/kg i.p.) produced antinociception (hot-plate test) and hypothermia in a dose-dependent manner in rats. MOM-Sal B was more potent than U50,488H in both tests and more efficacious than U50,488H in the hot-plate test. These latter two in vivo effects were blocked by norbinaltorphimine, indicating KOPR-mediated actions. Sal A at 10 mg/kg elicited neither antinociception nor hypothermia 30 min after administration to rats. In summary, MOM-Sal B is a potent and efficacious KOPR agonist with longer lasting in vivo effects than Sal A.

Activation of the κ opioid receptor (KOPR), one of three major types of opioid receptors, produces many effects including analgesia, dysphoria, antipruritis, water diuresis, and hypothermia (Liu-Chen, 2004). Many selective nonpeptide KOPR agonists have been synthesized; most are arylacetamide compounds, including U50,488H, U69,593, ICI 204,448, and asimadoline. Nalfurafine is an exception, being an epoxymorphinan.

Chewing or smoking the leaves of *Salvia divinorum* or drinking juice of crushed leaves causes hallucinations in humans (Siebert, 1994). Salvinorin (Sal) A (Fig. 1), a neoclerodane diterpene, was isolated and identified as the main active ingredient (Valdés, 1994). In a large scale screening of G protein-coupled receptors, transporters, and ion channels by radioligand binding assays, Roth et al. (2002) found that Sal A was a highly potent and selective agonist for the KOPR. Since then, several pharmacological studies on Sal A have been performed. Sal A binds to KOPR with a high affinity similar to U50,488H, a prototypic selective KOPR agonist. It does not show significant affinities to μ and δ opioid receptors or the nociceptin/orphanin FQ receptor (Wang et al., 2005). Sal A is reported to be more selective for KOPR than U50,488H or U69,593 (Beguin et al., 2008). Sal A acts as a very potent full agonist at KOPR in [^{35}S]GTPγS binding, intracellular calcium mobilization, and potassium conductance assays (Chavkin et al., 2004; Wang et al., 2005). Sal A

This study was supported by National Institutes of Health Grants R01-DA019688 and R01-DA019688 (to D.Y.W.L.), R01-DA022694 (to S.M.R.), and R01-DA17302 to (L.-Y.L.-C.).
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.107.132142.

ABBREVIATIONS: KOPR, κ opioid receptor; (−)U50,488H, (*trans*)-3,4-dichloro-*N*-methyl-*N*-[2-(1-pyrrolidinyl)-cyclohexyl]benzeneacetamide methanesulfonate; U69,593, (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]benzeneacetamide; ICI 204,448, (*RS*)-[3-[1-[[(3,4-dichlorophenyl) acetyl]methylamino]-2-(1-pyrrolidinyl)ethyl]phenoxy]acetic acid; asimadoline, *N*-[(1*S*)-2-[(3*S*)-3-hydroxypyrrolidin-1-yl]-1-phenylethyl]-*N*-methyl-2,2-di(phenyl)acetamide; nalfurafine, 17-cyclopropylmethyl-3,14β-dihydroxy-4,5α-epoxy-6β-[*N*-methyl-*trans*-3-(3-furyl) acrylamido]morphinan hydrochloride; Sal, salvinorin; norBNI, norbinaltorphimine; MOM, 2-methoxymethyl; [^{35}S]GTPγS, guanosine 5′-*O*-(3-[^{35}S]thio)triphosphate; CHO, Chinese hamster ovary; CHO-FLAG-hKOPR, CHO cells with stable expression of FLAG-tagged human κ opioid receptor; ANOVA, analysis of variance; %MPA, percentage of maximum possible analgesia; enadoline, 2-(1-benzofuran-4-yl)-*N*-methyl-*N*-[(5*R*,7*S*,8*S*)-7-pyrrolidin-1-yl-1-oxaspiro[4.5]decan-8-yl]acetamide; ΔTb, change in body temperature.



Salvinorin A: R = CH_3CO
Salvinorin B: R = H
MOM-Sal B: R = CH_3OCH_2

Fig. 1. Chemical structures of Sal A, Sal B, and MOM-Sal B.

also promotes internalization and down-regulation of the human KOPR in cultured cells (Wang et al., 2005).

Sal A produces many in vivo effects characteristically mediated by KOPR, such as sedation (Fantegrossi et al., 2005), antinociception (Ansonoff et al., 2006; John et al., 2006; McCurdy et al., 2006), hypothermia (Ansonoff et al., 2006), and depression-like effects (Carlezon et al., 2006). It decreases dopamine levels in caudate putamen that can be blocked by norbinaltorphimine (norBNI), a selective KOPR antagonist (Zhang et al., 2005). In mice, Sal A causes conditioned place aversion (Zhang et al., 2005), decreased locomotor activity (Zhang et al., 2005), and impaired climbing behavior on an inverted screen task (Fantegrossi et al., 2005).

It is noteworthy that the duration of in vivo effects of Sal A is short regardless of species, behavioral endpoint, or administration route. The hallucinogenic effect of Sal A in humans is brief when inhaled, reaching full effect in ~30 s, lasting 5 to 10 min, and subsiding in 20 to 30 min (Siebert, 1994). In mice, the antinociceptive effect of Sal A diminished within 20 min after i.p. injection in the acetic acid abdominal constriction test (Wang et al., 2005; McCurdy et al., 2006). However, in the mouse tail-flick test, its antinociceptive effect was undetectable 20 min after i.t. injection or 30 min after i.c.v. injection (John et al., 2006). Intravenous administration of Sal A increased prolactin levels in rhesus monkeys, but the duration was only 30 min, shorter than that of U69,593 (90 min) at the same dose (Butelman et al., 2007). A pharmacokinetic study in nonhuman primates showed that the elimination $t_{1/2}$ of Sal A in the serum was less than 1 h (Schmidt et al., 2005a).

The chemical structure of Sal A suggests that it may be hydrolyzed at the C(2) position by esterase to Sal B (Fig. 1), which has a much lower affinity for the KOPR than Sal A (Chavkin et al., 2004; Béguin et al., 2005; Lee et al., 2005b). Sal B has been determined as the principal metabolite of Sal A ex vivo (Schmidt et al., 2005b). However, Sal B was not detected in the blood after i.v. injection of Sal A in rhesus monkeys (Schmidt et al., 2005a). Sal B may be accumulated in the extravascular compartment or be excreted via other routes.

Sal A represents a new class of KOPR ligand with a unique non-nitrogen structure. Efforts have been devoted to characterize its structure-activity relationship and to develop more metabolically stable agonists and antagonists for use as pharmacological tools and perhaps as therapeutic agents. We have synthesized a series of C(2) derivatives of Sal A (Béguin et al., 2005; Lee et al., 2005a), one of which, 2-methoxymethyl (MOM)-Sal B (Fig. 1), is a selective KOPR full agonist more potent than Sal A or U50,488H (Lee et al., 2005a). In this study, we further characterized in vitro and in vivo pharmacological properties of this compound.

Materials and Methods

Materials. Sal A was isolated from leaves of *S. divinorum*, and MOM-Sal B was synthesized in Dr. David Y.W. Lee's laboratory as described previously (Lee et al., 2005a). U50,488H was obtained from the National Institute on Drug Abuse (Bethesda, MD). [15,16-^{3}H]Diprenorphine (56 Ci/mmol) and [^{35}S]GTPγS (1250 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). GDP and anti-FLAG M_1 monoclonal or polyclonal (F7425) antibodies were purchased from Sigma-Aldrich (St. Louis, MO). The following reagents were obtained from the indicated companies: GTPγS (Roche Diagnostics, Indianapolis, IN); geneticin (G418; Cellgro Mediatech, Inc., Herndon, VA); goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR); SuperSignal West Pico Chemiluminescent Substrate kit, Restore Western Blot Stripping Buffer (Pierce Chemical, Rockford, IL); and Opti-MEMI reduced serum (Invitrogen, Carlsbad, CA).

Animals. Male Sprague-Dawley rats (Zivic-Miller, Pittsburgh, PA) weighing 200 to 250 g (age, 60–80 days) were housed two per cage. Male Swiss-Webster mice (Ace Laboratories, Boyertown, PA) weighing 25 to 30 g were housed five per cage. The animals were maintained on a 12-h light/dark cycle with free access to food and water. Experimental procedures were approved by the Temple University Institutional Animal Care and Use Committee.

Cell Lines. CHO cells with stable expression of the human KOPR (CHO-FLAG-hKOPR) were established previously (Zhu et al., 1997). Cells were cultured in 100-mm culture dishes in Dulbecco's modified Eagle's medium/Ham's F-12 medium supplemented with 10% fetal calf serum, 0.4 mg/ml geneticin, 100 units/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere consisting of 5% CO_2 and 95% air at 37°C.

Cell Membrane Preparation. Cells were washed twice and harvested in Versene solution (0.54 mM EDTA, 140 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, and 1 mM glucose) and centrifuged at 500*g* for 3 min. The cell pellet was suspended in lysis buffer (5 mM Tris, pH 7.4, 5 mM EDTA, 5 mM EGTA, and 0.1 mM phenylmethylsulfonyl fluoride), passed through a 26× three-eighths-gauge needle 10 times, and then centrifuged at 46,000*g* for 30 min. The pellet was resuspended in lysis buffer and centrifuged again. The membrane pellet was resuspended in 50 mM Tris-HCl buffer (50 mM Tris, pH 7.4, and 1 mM EGTA), aliquoted and frozen in dry ice/ethanol, and stored at −80°C. All procedures were performed at 4°C.

Receptor Binding. Competition inhibition by MOM-Sal B, Sal A, and U50,488H of [^{3}H]diprenorphine (0.4 nM) binding to opioid receptors was performed in the absence or presence of various concentrations of each compound. Binding was carried out in binding buffer (50 mM Tris, 1 mM EDTA, pH 7.4) at room temperature for 1 h in duplicate in a final volume of 0.5 ml with ~10 μg of membrane protein. Naloxone (10 μM) was used to define nonspecific binding. Bound and free [^{3}H]diprenorphine were separated by filtration under reduced pressure with GF/B filters presoaked with 50 mM Tris, pH 7.4, 0.1 mg/ml bovine serum albumin, and 0.2% polyethylenimine. Radioactivity on filters was determined by liquid scintillation counting. Each experiment was performed in duplicate and repeated at least three times. Binding data and K_i values of each drug were analyzed and determined with the Prism 3.0 program (GraphPad Software Inc., San Diego, CA).

[^{35}S]GTPγS Binding. Membranes (~10 μg) were incubated in [^{35}S]GTPγS binding buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM $MgCl_2$, and 1 mM EDTA with 10 μM GDP freshly added) containing [^{35}S]GTPγS (~80 pM) with or without a compound in a total volume of 0.5 ml for 60 min at 30°C. Nonspecific binding was defined by incubation in the presence of 10 μM GTPγS. Bound and free [^{35}S]GTPγS were separated by filtration with GF/B filters under reduced pressure. Radioactivity on filters was determined by liquid scintillation counting. EC_{50} values and maximal responses (E_{max}) of compounds were determined by curve fitting to the equation for a sigmoidal curve: Y = bottom + (top − bottom)/(1 + 10^(LogEC$_{50}$ − X)). X is the logarithm of concentration, and Y is the response.

Fluorescence Flow Cytometry. CHO-FLAG-hKOPR grown on 100-mm Petri dishes was left untreated or was treated for 30 min at 37°C with MOM-Sal B, Sal A, or U50,488H at the indicated concentration. After harvesting, 1.5 × 10^6 cells were used for the flow cytometric assay without permeabilization of cell membranes. Because Ca^{2+} is required for M_1 monoclonal anti-FLAG antibody binding to the antigen, all solutions used in the subsequent steps contain 1 mM $CaCl_2$. Cells were washed once with Opti-MEM I and then incubated with M_1 antibody (1 μg/ml, 1:2000) in 1 ml of Opti-MEM I for 1 h. After another three washes with phosphate-buffered saline, cells were incubated with Alexa Fluor 488-conjugated goat anti-mouse IgG (1 μg/ml, 1:2000) in 1 ml of Opti-MEM I for 1 h, washed three times, and then resuspended with 1 ml of phosphate-buffered saline. All solutions used were ice-cold, and all of the above procedures were performed in a cold room. Surface FLAG-hKOPR immunofluorescence of 1 × 10^4 cells was quantified using a FACScan (BD Biosciences, San Jose, CA), and the mean fluorescence intensity of a single cell was calculated. The mean fluorescence intensity of cells stained only with the second antibody was also measured and subtracted from the mean intensity. Drug-induced percent change of cell surface FLAG-hKOPR = 100 × (fluorescence intensity in treatment group − fluorescence intensity in vehicle group)/(fluorescence intensity in vehicle group).

Receptor Down-Regulation. CHO-FLAG-hKOPR cells were treated with MOM-Sal B, Sal A, U50,488H, or vehicle at 37°C for 4 h. The cells were harvested with Versene buffer, and cell number was determined by a Z1 cell and particle counter (Beckman Coulter, Fullerton, CA). One million cells were solubilized in 200 μl of 2× Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 4% SDS, 100 mM dithiothreitol, 10% glycerol, and 0.1% bromphenol blue). Cells (2 × 10^5; 40 μl of sample buffer) per lane were subjected to Tricine-SDS-polyacrylamide gel electrophoresis on 8% separating gel. The proteins were transferred to Immobilon-P polyvinylidene difluoride transfer membranes, which were then incubated with blocking solution [5% nonfat dry milk in Tris-buffered saline/Tween 20 buffer (25 mM Tris, 150 mM NaCl, and 0.1% Tween 20, pH 7.6)] for at least 1 h and then overnight with rabbit polyclonal anti-FLAG (F7425) antibody (0.8 mg/ml, 1:5000) in blocking solution at 4°C on a shaker. The polyvinylidene difluoride membranes were washed three times with the Tris-buffered saline/Tween 20 buffer and incubated at room temperature for 2 h with horseradish peroxidase-linked goat anti-rabbit IgG (1:1000) in blocking solution. The protein bands were visualized by applying SuperSignal West Pico Chemiluminescent substrate and then digitalized with the Fuji LAS-1000 Plus Gel Documentation System (Fuji Film, Tokyo, Japan). The densitometric analyses of FLAG-hKOPR bands were performed using ImageGauge 4.1 software (Fuji Film). Quantitative comparison of optical densities of FLAG-hKOPR between drug- and vehicle-treated cells was carried out to assess agonist-promoted regulation of FLAG-hKOPR. Percent change of FLAG-hKOPR equals 100 × (optical density in drug-treated group − optical density in vehicle-treated group)/(optical density in vehicle-treated group).

Exploratory Activities of Rats in a Y-Maze. The Y-maze was made of opaque gray acrylic boards with three equally sized arms. Each arm of the symmetrical maze was 48 cm in length, 13 cm in width, and 33 cm in height. The apparatus was illuminated by a 100-W bulb positioned above the center of the Y. The rats were separated into four groups of six rats each, and they received injections s.c. with either vehicle (ethanol/1% Tween 80/water in 1:1:8 ratio) or MOM-Sal B (1, 2.5, or 5 mg/kg) at 5-min intervals. Thirty minutes later, each rat was placed in the center of the Y-maze, and spontaneous activity was observed for 3 min. Horizontal and vertical movements were quantified by counting each arm entry and each vertical rear, respectively. The number of rats showing at least one body grooming episode was also counted. The Y-maze was cleaned with water and then wiped dry between rats. The data were analyzed with one-way analysis of variance (ANOVA) to determine whether there were significant differences among groups. If so, Dunnett's post hoc test was performed to determine whether there was a significant difference between the control and each treatment group.

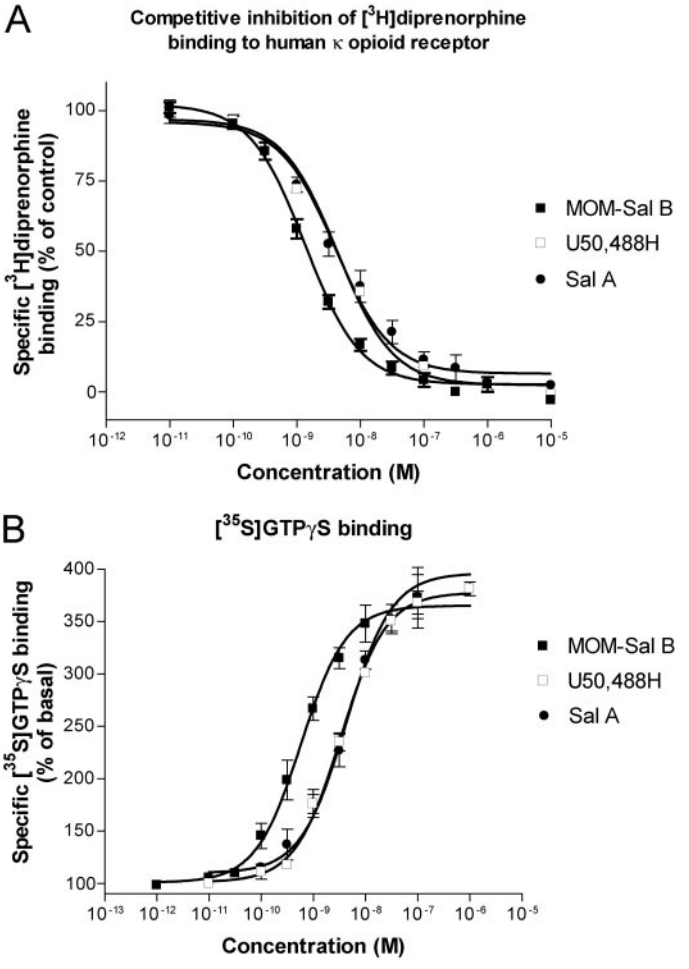


Fig. 2. A, competitive inhibition by MOM-Sal B, Sal A, or U50,488H of [^{3}H]diprenorphine binding to KOPR. Membranes were prepared from CHO-FLAG-hKOPR cells. Binding was carried out with ~0.4 nM [^{3}H]diprenorphine in the presence or absence of various concentrations of MOM-Sal B, Sal A, or U50,488H as described under *Materials and Methods*. Data were normalized to the percentage of specific binding. Each value represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. Apparent K_i values are shown in Table 1. B, stimulation of [^{35}S]GTPγS binding to membranes of CHO-FLAG-hKOPR cells by MOM-Sal B, Sal A, or U50,488H. [^{35}S]GTPγS binding to membranes was performed with various concentrations of each compound as described under *Materials and Methods*. Nonspecific binding was determined in the presence of 10 μM cold GTPγS. Basal [^{35}S]GTPγS binding in the absence of added compounds was ~2000 dpm. Data were normalized to percentage of the basal [^{35}S]GTPγS binding. Each value represents the mean ± S.E.M. of at least three independent experiments performed in duplicate. EC_{50} values and maximal responses are shown in Table 1.

TABLE 1
Pharmacological characterization of MOM-Sal B

	Competitive Inhibition of [^{3}H]Diprenorphine Binding	[^{35}S]GTPγS Binding		Internalization of FLAG-hKOPR		Down-Regulation of FLAG-hKOPR	
	K_i	EC_{50}	E_{max}*	EC_{50}	E_{max}*	EC_{50}	E_{max}*
	nM	*nM*		*nM*		*nM*	
U50,488H	1.4 ± 0.3	3.4 ± 0.5	100	97.1 ± 19.2	100	125.1 ± 42.0	100
Sal A	1.3 ± 0.5	4.5 ± 1.2	100.2 ± 5.1	522.2 ± 45.1	96.1 ± 1.5	142.1 ± 10.7	94.3 ± 2.8
MOM-Sal B	0.4 ± 0.02	0.6 ± 0.2	97.8 ± 6.4	7.4 ± 0.5	109.2 ± 4.2	6.3 ± 1.2	89.0 ± 3.9

* Efficacy determined as the percentage of maximal response produced by U50,488H.

Body Temperature Experiments with Rats. Experiments were started between 8:00 and 9:00 AM. Rats were placed into an environmental room maintained at a constant temperature of 21 ± 0.3°C and relative humidity of 52 ± 2% on the morning of the experiment. Animals were then allowed to acclimate for 90 min before taking the first temperature reading. Before drug administration, baseline temperatures were taken every 30 min for 90 min using a thermistor probe (YSI series 400; Yellow Springs Instrument Co., Yellow Springs, OH), which was lubricated and inserted approximately 7 cm into the colon. A digital thermometer (model 49TA; YSI) was used to record the body temperatures of unrestrained rats. Rats received injections with vehicle (50% dimethyl sulfoxide-saline solution), MOM-Sal B (0.5, 1, 2.5, or 5 mg/kg i.p.), or Sal A (10 mg/kg i.p.) following the baseline interval, and body temperatures were recorded at 30, 60, 90, and 120 min after injection. Doses of MOM-Sal B were based on previous in vivo data generated in mice (Ansonoff et al., 2006). For comparative purposes, a second set of experiments tested the effects of the prototypical κ opioid agonist, U50,488H, on body temperature. After the baseline interval, rats received injections with vehicle (sterile, pyrogen-free saline) or graded doses of U50,488H (5, 10, or 20 mg/kg i.p.), and body temperatures were recorded at 30, 60, 90, and 120 min after injection. Each rat was used once and immediately euthanized after the experiments.

Antinociception Test with Rats. Antinociceptive responses were measured on a hot-plate set at 52.5°C. The response was defined by the animal either licking the forepaws or hindpaws or flicking the hindpaws. In these studies, the most prominent response was forepaw licking. To avoid tissue damage, the animals were exposed to the hot-plate (Ugo Basile model 7280; Ugo Basile, Comerio, Italy) for a maximum of 30 s. In all experiments, rats were initially tested for baseline latency, and the latency was tested again at 30, 60, 90, and 120 min after drug injection. The dosing schedule for rats in the antinociceptive experiments was identical to the paradigm described for the body temperature experiments.

Statistical Analysis. Values of $P < 0.05$ were considered to be statistically significant. For body temperature experiments, three consecutive temperature readings were taken and averaged to establish a baseline temperature before drug injection. Data were calculated as the mean ± S.E.M. of the change in body temperature. Before analysis, all body temperature data were transformed into "normalized ranks" to address non-normality. Transformed data were analyzed using a two-way (group, time) mixed-model ANOVA with repeated measures on time followed by pair-wise multiple comparisons incorporating the Bonferroni correction. For antinociception experiments, data were calculated as the percentage of maximum possible analgesia (%MPA) ± S.E.M. Data were analyzed using the two-way ANOVA described above.

Results

MOM-Sal B Selectively Bound to hKOPR with High Affinity and Selectivity. MOM-Sal B (Fig. 1) exhibited a high affinity to hKOPR with K_i of 0.4 nM, which was ~3-fold higher than that of U50,488H (K_i, 1.4 nM) or Sal A (K_i, 1.3 nM) (Fig. 2A; Table 1). MOM-Sal B did not display significant affinities to μ or δ opioid receptors (K_i > 1000 nM).

MOM-Sal B Was a Potent Full Agonist at hKOPR. Activation of KOPR enhances binding of [^{35}S]GTPγS to G proteins in membranes, which has been used to determine the efficacies and potencies of test compounds (Zhu et al., 1997). MOM-Sal B, Sal A, and U50,488H enhance [^{35}S]GTPγS binding to membranes prepared from CHO-FLAG-hKOPR cells. Maximal [^{35}S]GTPγS binding induced by MOM-Sal B was similar to that by U50,488H or Sal A. In accordance with its affinity, MOM-Sal B was more potent in activating G proteins with an EC_{50} of 0.6 nM, which was 5- to 7-fold lower than U50,488H (EC_{50}, 3.4 nM) and Sal A (EC_{50}, 4.5 nM) (Fig. 2B; Table 1).

MOM-Sal B Promoted Internalization of hKOPR with High Potency. hKOPR undergoes internalization upon activation, which is thought to be related to desensitization and resensitization of the receptors (Li et al., 1999). CHO-FLAG-hKOPR cells were treated with various concentrations of MOM-Sal B, U50,488H, Sal A, or vehicle for 30 min. Cell surface receptors were labeled with immunofluorescence and quanti-

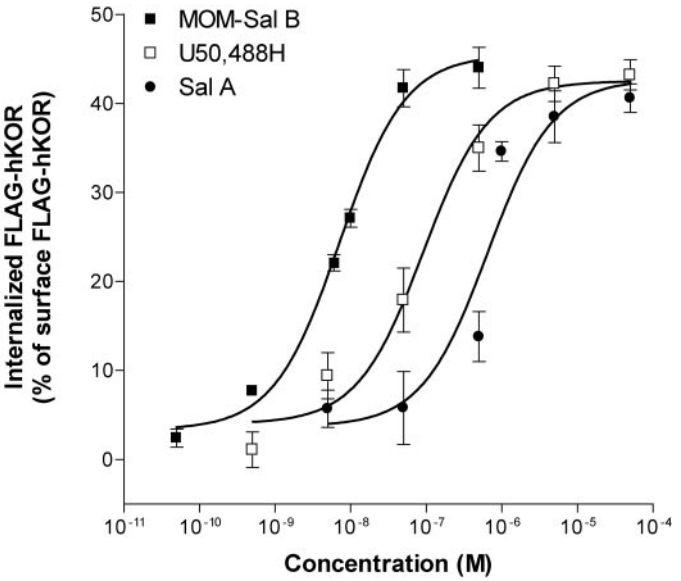


Fig. 3. Dose-response relationship of MOM-Sal B-, Sal A-, and U50,488H-induced internalization of FLAG-hKOPR expressed in CHO cells. CHO-FLAG-hKOPR cells grown on 12-well plates were left untreated or treated with different concentrations of MOM-Sal B, Sal A, or U50,488H at 37°C for 30 min. Cells were then suspended and incubated with M_1 anti-FLAG mouse monoclonal antibody followed by goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 at 4°C. Cell surface immunofluorescence was measured by fluorescence flow cytometry on a FACScan, and internalized receptor was determined as described under *Materials and Methods*. Each value represents the mean ± S.E.M. of three independent experiments.

fied with flow cytometry. Internalization of hKOPR was determined by the decrease of cell surface fluorescence intensities. All three compounds caused internalization of hKOPR to similar extents (~40% of total surface receptors) (Fig. 3). It is interesting to note that, although MOM-Sal B, U50,488H, and Sal A showed comparable binding affinities to hKOPR and potencies in activating G proteins, MOM-Sal B (EC_{50}, 7.4 nM) was ~13- and ~70-fold more potent than U50,488H (EC_{50}, 97.1 nM) and Sal A (EC_{50}, 522 nM), respectively, in inducing internalization of hKOPR (Fig. 3; Table 1).

MOM-Sal B Induced Down-Regulation of hKOPR with High Potency. CHO-FLAG-hKOPR cells were treated with various concentrations of MOM-Sal B, U50,488H, Sal A, or vehicle for 4 h. Changes in mature receptor levels were determined using Western immunoblotting. We have shown previously that FLAG-hKOPR expressed in CHO cells migrated as two bands with molecular masses of 45 and 55 kDa (Chen et al., 2006). In addition, the 55-kDa band represents the fully glycosylated FLAG-hKOPRs (mature form), most of which locates in plasma membranes, whereas the 45-kDa band represents *N*-linked glycosylated high-mannose intermediates (immature form), which mainly locate in endothelium reticulum and *cis*-Golgi (Chen et al., 2006). A decrease in the 55-kDa band was used as an indication of down-regulation. All three compounds caused down-regulation of hKOPR in a dose-dependent manner by similar maximal extents (~25% of total mature receptors). However, MOM-Sal B (EC_{50}, 6.3 nM) was much more potent than U50,488H (EC_{50}, 125 nM) and Sal A (EC_{50}, 142 nM) (Fig. 4; Table 1).

MOM-Sal B Caused Rapid-Onset and Long-Lasting Immobility in Mice. When Swiss-Webster mice received injections with MOM-Sal B (0.05–1 mg/kg s.c.), locomotor activity in the home cage was immediately and profoundly decreased compared with those animals receiving vehicle. The mice receiving 0.30 and 1 mg/kg MOM-Sal B became immobile in ~1 min and remained static for ~3 h; however, the animals retained an intact righting reflex. The dramatic inhibitory effect of MOM-Sal B on motor activities in mice was completely blocked if the animals were pretreated with norBNI (10 mg/kg i.p., −16 h).

Antinociceptive assays were attempted with MOM-Sal B at lower doses (0.005–0.03 mg/kg) that did not cause significant behavioral depression. In this dose range, MOM-Sal B had no marked antinociceptive effect in mouse formalin and abdominal constriction tests (data not shown).

Effects of MOM-Sal B on the Exploratory Activities of Rats in the Y-Maze. MOM-Sal B influenced the spontaneous behavior of rats during their 3-min exposure to the novel Y-maze. Ambulation/exploration (as reflected by arm entries) was increased. Vertical rearing was decreased with all three doses tested (1–5 mg/kg, $P < 0.01$), relative to vehicle-treated animals (Fig. 5). All six rats receiving vehicle explored the maze during the first 2 min, then displayed body grooming episodes during the 3rd min. Only one of the 18 rats treated with MOM-Sal B groomed and this animal received the lowest dose (1 mg/kg).

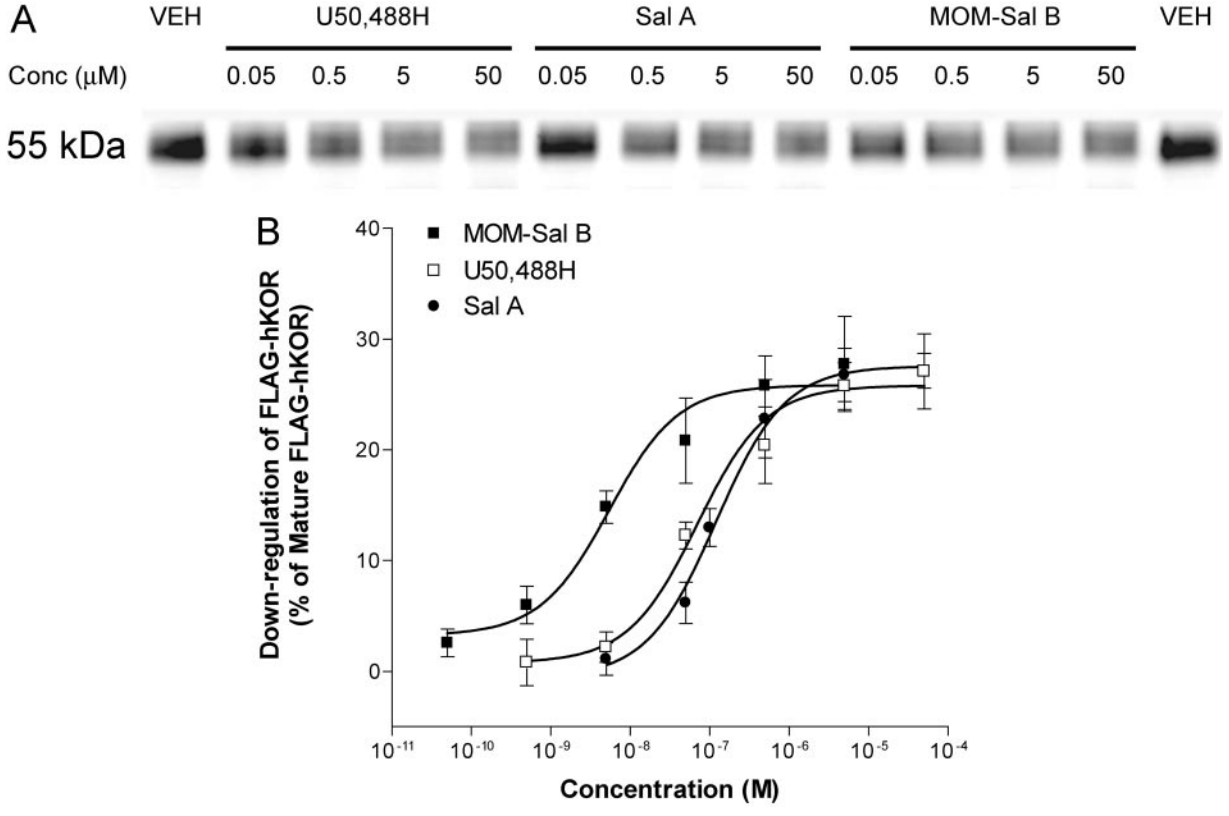


Fig. 4. Dose-response relationship of MOM-Sal B-, Sal A-, and U50,488H-induced down-regulation of FLAG-hKOPR expressed in CHO cells. CHO-FLAG-HKOPR cells were treated with vehicle (VEH) or various concentrations of MOM-Sal B, Sal A, or U50,488H at 37°C for 4 h. Cells were harvested, solubilized with 2× Laemmli sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis. The receptors were detected by sequential incubation with rabbit anti-FLAG antibodies and horseradish peroxidase-conjugated goat anti-rabbit antibody followed by enhanced chemiluminescence. A, one representative immunoblotting result is shown. B, immunoblot results were quantitated by ImageGauge software and plotted relative to the amount of receptors detected in control cells. Receptor down-regulation was determined as described under *Materials and Methods*. Each value represents mean ± S.E.M. of at least three independent experiments.

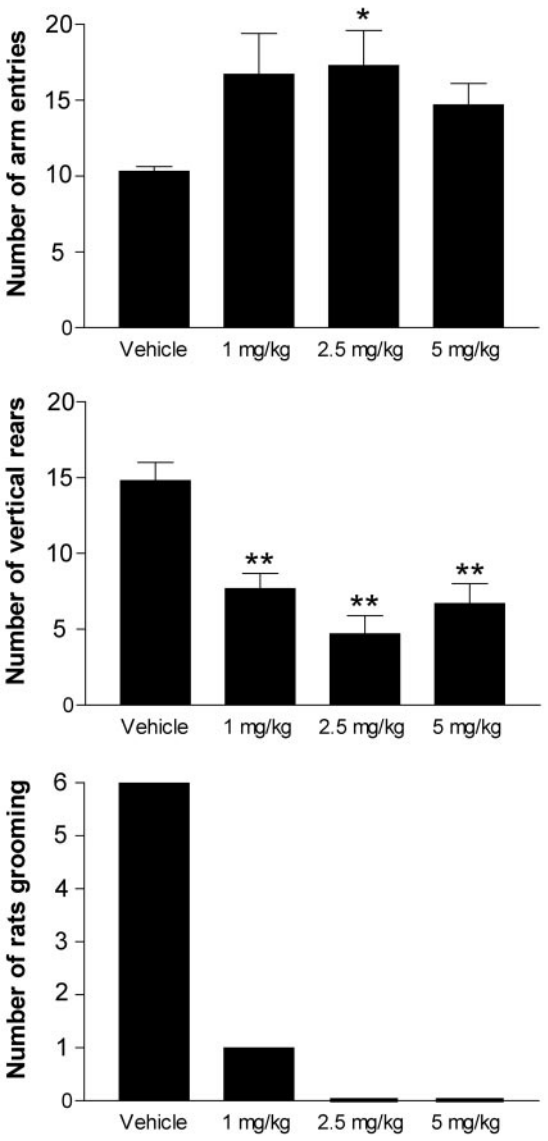


Fig. 5. Effects of MOM-Sal B on exploratory activities of rats in a Y-maze. Rats were divided into four groups of six animals and administered MOM-Sal B (1, 2.5, and 5 mg/kg s.c.) or vehicle. The incidence of arm entries, vertical rears, and grooming episodes by the rats was counted for 3 min. *, $P < 0.05$; **, $P < 0.01$, compared with vehicle.

MOM-Sal B Produced Antinociceptive Effects with Higher Potency and Efficacy Than U50,488H. The antinociceptive effect of MOM-Sal B was examined using the hot-plate test and compared against U50,488H and Sal A. Rats were divided into five groups of 6 to 11 animals and received an i.p. injection of MOM-Sal B (0.5, 1, 2.5, or 5 mg/kg) or vehicle. The hot-plate test was performed every 30 min for 2 h (Fig. 6A). The average baseline response latencies ranged from 3.2 to 3.8 s, and there was no difference among groups. Two-way ANOVA revealed a significant drug interaction [$F(4,44) = 48.56$, $P < 0.0001$], time interaction [$F(3,132) = 31.39$, $P < 0.0001$], and drug × time interaction [$F(12,132) = 4.993$, $P < 0.0001$]. A Bonferroni multiple comparisons test revealed that 2.5 and 5 mg/kg MOM-Sal B produced significant increases in response latency occurring 30, 60, 90, and 120 min postinjection compared with rats receiving vehicle ($P < 0.05$; Fig. 6A). Rats receiving 1 mg/kg MOM-Sal B displayed significant antinociception 30, 60, and 90 min postinjection ($P < 0.05$; Fig. 6A). The effect of MOM-Sal B was rapid in onset, with the peak antinociceptive response occurring 30 min after injection. The lowest dose (0.5 mg/kg) of MOM-Sal B did not elicit significant antinociception compared with vehicle ($P > 0.05$; Fig. 6A).

For the U50,488H experiments, rats were divided into four groups of 9 to 11 animals and received an i.p. injection of U50,488H (5, 10, or 20 mg/kg) or vehicle. Two-way ANOVA revealed a significant drug interaction [$F(3,35) = 43.47$, $P < 0.0001$], time interaction [$F(3,105) = 13.67$, $P < 0.0001$], and drug × time interaction [$F(9,105) = 3.621$, $P < 0.0001$]. A Bonferroni multiple comparisons test revealed that 20 mg/kg U50,488H produced significant antinociception compared with rats receiving vehicle, with significant increases in response latency occurring 30, 60, and 90 min postinjection ($P < 0.05$; Fig. 6B). Rats receiving 10 mg/kg U50,488H displayed significant antinociception 30 and 60 min postinjection ($P < 0.05$; Fig. 6B). The lowest dose (5 mg/kg) did not elicit significant antinociception compared with vehicle ($P > 0.05$; Fig. 6B).

MOM-Sal B produced significant antinociception at 10-fold lower dose (1 mg/kg) than U50,488H (10 mg/kg). MOM-Sal B at 5 mg/kg increased response latencies of the rats to 26.1 ± 1.8 s 30 min after administration, whereas U50,488H at 20 mg/kg increased latencies to 9.2 ± 1.1 s. Therefore, MOM-Sal B is more potent and efficacious than U50,488H in producing antinociception (Fig. 6C). Sal A at 10 mg/kg did not exhibit significant antinociception 30 min after injection (Fig. 6A).

MOM-Sal B Produced Hypothermic Effects with Higher Potency Than U50,488H. Activation of KOPR decreases body temperature (Adler and Geller, 1993). The hypothermic effects of MOM-Sal B, U50,488H, and Sal A were compared. In the MOM-Sal B experiments, rats were again divided into five groups of six animals and received an i.p. injection of MOM-Sal B (0.5, 1, 2.5, or 5 mg/kg) or vehicle (Fig. 7A). The average baseline body temperatures of the rats used in this experiment were 37.2 to 37.3°C, and there were no differences among groups. Two-way ANOVA revealed a significant drug interaction [$F(4,44) = 23.81$, $P < 0.0001$], time interaction [$F(3,132) = 17.73$, $P < 0.0001$], and drug × time interaction [$F(12,132) = 3.203$, $P = 0.0001$]. A Bonferroni multiple comparisons test revealed that, 2.5 and 5 mg/kg MOM-Sal B produced significant hypothermia at 30, 60, and 90 min postinjection ($P < 0.05$, compared with vehicle) (Fig. 7A). Rats receiving 1 mg/kg MOM-Sal B displayed significant hypothermia 30 and 60 min postinjection ($P < 0.05$, compared with vehicle). Similar to the antinociceptive results, the hypothermic effect of MOM-Sal B was rapid, with peak hypothermia occurring 30 to 60 min after injection. The lowest dose (0.5 mg/kg) of MOM-Sal B did not elicit significant hypothermia compared with vehicle ($P > 0.05$).

In the U50,488H experiments, rats were again divided into four groups of six to nine animals and received an i.p. injection of U50,488H (5, 10, or 20 mg/kg) or vehicle. Two-way ANOVA revealed a significant drug interaction [$F(3,22) = 15.12$, $P < 0.0001$] and time interaction [$F(3,66) = 7.387$, $P < 0.0001$]. The drug × time interaction [$F(9,132) = 1.286$, $P = 0.2368$] did not attain statistical significance. A Bonferroni multiple comparisons test revealed that 10 and 20 mg/kg U50,488H produced significant hypothermia at 30, 60, and 90 min postinjection ($P < 0.05$ compared with vehicle) (Fig. 7B). Rats receiving 5 mg/kg U50,488H did not display signif-

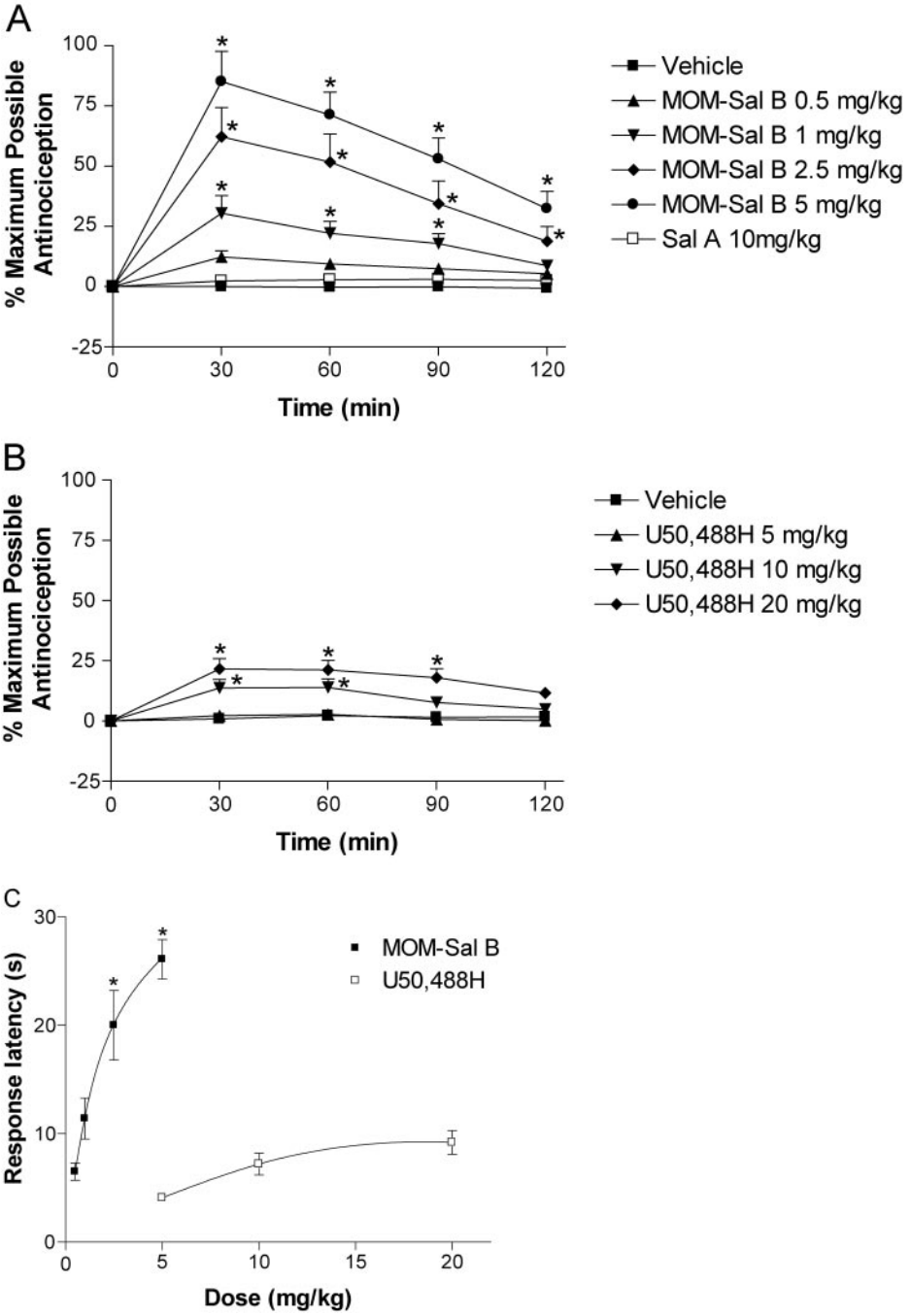


Fig. 6. Dose-related antinociception effects of MOM-Sal B in the rat hot-plate test. Rats were divided into 10 groups of 6 to 11 animals, and basal response latencies were determined. Rats were administered MOM-Sal B (A; 0.5, 1, 2.5, or 5 mg/kg i.p.), Sal A (10 mg/kg i.p.), U50,488H (B; 5, 10, or 20 mg/kg i.p.), or an equivalent volume of vehicle. The vehicle for MOM-Sal B and Sal A was 50% dimethyl sulfoxide/saline, whereas that for U50,488H was sterile, pyrogen-free saline. Response latencies were measured at 30-min intervals for 2 h. The cut-off was 30 s. Data are expressed as the mean ± S.E.M. of the %MPA from baseline (time 0). *, $P < 0.05$, compared with vehicle. C, dose-related relationship of antinociception effects of MOM-Sal B and U50,488H 30 min after administration in the rat hot-plate test. Data are expressed as the mean ± S.E.M. of response latencies (seconds). *, $P < 0.05$ by Student's t test, compared with response latency induced by U50,488H at 20 mg/kg.

icant hypothermia compared with vehicle-treated rats ($P > 0.05$). No significant hypothermic effect was observed for 10 mg/kg Sal A at 30 min postinjection (Fig. 7A).

MOM-Sal B was more potent than U50,488H in decreasing body temperature and caused significant hypothermia at a 10-fold lower dose (1 mg/kg) than U50,488H (10 mg/kg). The maximal decrease in body temperature elicited by MOM-Sal B (1.8 ± 0.3°C at 5 mg/kg) was not different from that by U50,488H (1.5 ± 0.3°C at 20 mg/kg) ($P > 0.05$) (Fig. 7C).

norBNI Blocked Antinociception and Hypothermia Caused by MOM-Sal B. Twenty-four hours after receiving an injection of the selective KOPR antagonist norBNI (10 mg/kg s.c.), rats were administered MOM-Sal B (2.5 mg/kg i.p.) or vehicle. norBNI by itself had no effect on response latency in the hot-plate test or on body temperature. norBNI inhibited both antinociceptive and hypothermic effects of MOM-Sal B observed 30 min after injection (Fig. 8). The complete blockade by norBNI indicates that the effects of MOM-Sal B are mediated by KOPR.

Discussion

We have demonstrated that MOM-Sal B is a selective full agonist at KOPR with higher affinity for the hKOPR than U50,488H and Sal A. It is more potent than U50,488H and Sal A in hKOPR-mediated G protein activation and in promoting internalization and down-regulation of the hKOPR in vitro. MOM-Sal B has antinociceptive and hypothermic ef-

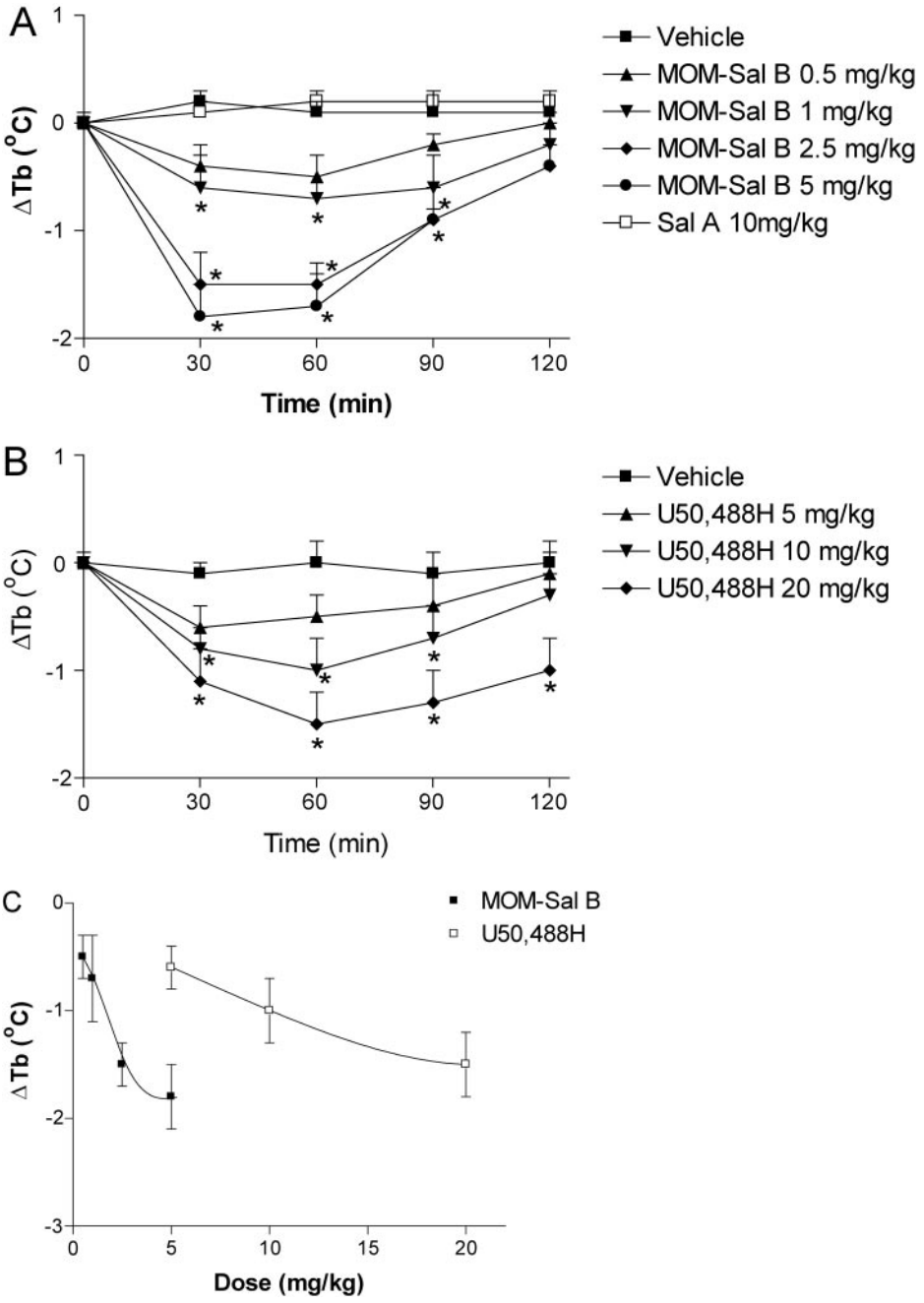


Fig. 7. Dose-related hypothermic effects of MOM-Sal B in rats. Rats were divided into 10 groups of six to nine animals, and body temperatures were measured. Rats were administered MOM-Sal B (A; 0.5, 1, 2.5, or 5 mg/kg i.p.), Sal A (10 mg/kg i.p.), U50,488H (B; 5, 10, or 20 mg/kg i.p.), or an equivalent volume of vehicle (50% dimethyl sulfoxide/saline for MOM-Sal B and Sal A and sterile, pyrogen-free saline for U50,488H). Rectal temperature was measured at 30-min intervals for 2 h. Data are expressed as the mean ± S.E.M. of the change in body temperature (ΔTb) from baseline (time 0). *, $P < 0.05$, compared with vehicle. C, dose-related relationship of MOM-Sal B- and U50,488H-induced hypothermic effects. Data are expressed as the mean ± S.E.M. of the ΔTb from baseline.

fects with high potency and longer lasting in vivo effects than Sal A. MOM-Sal B is more efficacious than U50,488H in the rat hot-plate assay, and its effect is blocked by pretreatment with norBNI, indicating that it is mediated by KOPR. These results demonstrate that KOPR agonists are more effective in antithermal nociception in the rat than previously realized. MOM-Sal B caused rapid-onset inhibition of motor activity in the mouse that lasted approximately 3 h and altered the spontaneous behavior of rats when exposed to an unfamiliar environment.

MOM-Sal B Is Longer Acting Than Sal A in Vivo

Sal A (4 mg/kg) was previously shown to have an antinociceptive effect lasting less than 20 min (McCurdy et al., 2006). In contrast, the antinociceptive and hypothermic effects of MOM-Sal B (2.5 mg/kg) last 120 and 90 min, respectively. Sal A is suggested to be hydrolyzed by esterases in body fluid to form salvinorin B, which has a much lower affinity to KOPR (Béguin et al., 2005; Lee et al., 2005b). MOM-Sal B has an ether bond at C(2), whereas Sal A has an ester bond. The ether bond has a much lower chemical reactivity than the ester bond in vivo. Therefore, it is possible that MOM-Sal B is more metabolically stable than Sal A in vivo.

MOM-Sal B Alters Motor Activities in Mice and Rats

In Swiss-Webster mice, MOM-Sal B (0.05–1 mg/kg) caused a dose-dependent rapid-onset immobility, which lasted approximately 3 h. U50,488H and Sal A caused behavioral depression in mice as well, but their effects were not as profound as MOM-Sal B. It may be attributed to the higher efficacy of MOM-Sal B on KOPR in vivo. In addition, MOM-

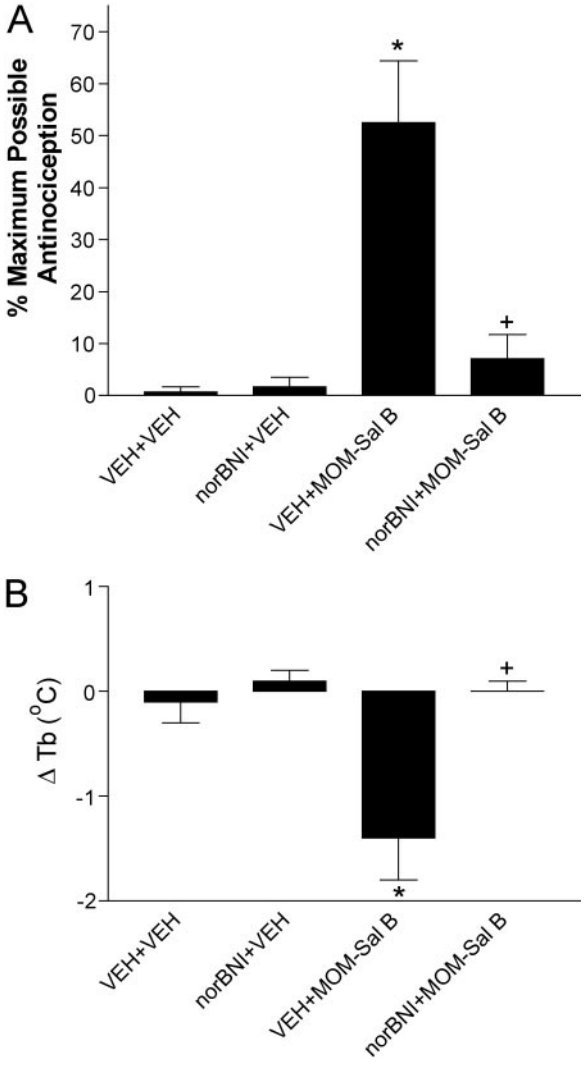


Fig. 8. Effects of norBNI on the antinociception and hypothermia caused by MOM-Sal B. Twenty-four hours after receiving norBNI (10 mg/kg s.c.) or vehicle (VEH), rats were given MOM-Sal B (2.5 mg/kg i.p.) or VEH. Response latency in the hot-plate or body temperature tests was measured 30 min after MOM-Sal B injection. Data are expressed as the mean ± S.E.M. of the %MPA (A) or ΔTb (B) from baseline. $n = 6$ rats per group. *, $P < 0.05$ compared with VEH + VEH; +, $P < 0.05$ compared with VEH + MOM-Sal B.

Sal B had a much more rapid onset and a much longer duration of action.

It is interesting to note that this sedative effect of MOM-Sal B was not overtly apparent in Sprague-Dawley rats, where ambulation was increased in the Y-maze experiment. The marked decrease in vertical rearing and body grooming may perhaps be interpreted as a subtle indication of behavioral depression. The absence of body grooming in rats after treatment with κ agonists has been noted previously (Jackson and Cooper, 1988) and points to this class of compounds suppressing spontaneous tactile reflexes in addition to a range of chemically diverse scratch-inducing agents (Inan and Cowan, 2006).

Antinociceptive and Thermal Actions of MOM-Sal B

KOPR-mediated antinociception has been demonstrated to be stimulus-specific in rats and mice. KOPR agonists, including U50,488H, U69,593, and enadoline, are effective against noxious mechanical stimuli (the paw pressure test) or chemical stimuli (the acetic acid-induced abdominal constriction and formalin tests) but only weakly effective against noxious heat stimuli (hot-plate and tail-flick tests) in mice and rats (Porreca et al., 1984; Hayes et al., 1987; Schmauss, 1987; Millan, 1989; McLaughlin et al., 1995). Antinociceptive effects of KOPR agonists against noxious heat are intensity-dependent, being greatest against low-intensity and weakest against high-intensity heat (Millan, 1989). Our results that U50,488H is not efficacious in the hot-plate test in the rat is consistent with these reports.

Interestingly, we found that MOM-Sal B was much more efficacious than U50,488H for thermal antinociception in the rat. The antinociceptive effect of MOM-Sal B was antagonized by norBNI, indicating that it is KOPR-mediated.

Our findings suggest that U50,488H may be a partial agonist compared with MOM-Sal B when using thermal antinociception as the endpoint. In addition, KOPR may play a more significant role in thermal antinociception in the rat than previously demonstrated if it can be fully activated by agonists such as MOM-Sal B. These results also suggest that there are few or no spare KOPRs in the neuronal circuitry for thermal antinociception in the rat. It remains to be determined whether this is true in mice, guinea pigs, and even humans.

In contrast, MOM-Sal B and U50,488H seem to cause similar maximal decreases in body temperature in the rat, suggesting that there may be spare KOPRs in the neuronal circuitry for hypothermia. In addition, MOM-Sal B and U50,488H exhibited similar efficacy in stimulating [^{35}S]GTPγS binding, suggesting a receptor reserve in CHO-hKOPR cells.

Structure-Activation Relationship of MOM-Sal B at the C(2) Position

By comparing binding affinities of various analogs of Sal A with the hKOPR, Roth et al. demonstrated that the methyl ester and furan ring were critical for its binding to KOPR, whereas the lactone and ketone groups were not (Munro et al., 2005). It has been reported that the size of ester or ether substituents at C(2) affects the binding affinity significantly (Béguin et al., 2005). In general, there is a quite limited space tolerance at C(2). For instance, the affinity is decreased when the ether substituents become longer. However, if the size is too small, such as with salvinorin B or 2-methyl-salvinorin B, affinity to KOPR is lost as well. Therefore, 2-3 and unbranched atom chains appear to be optimal for binding to KOPR. The length of the C(2) side chain of MOM-Sal B fell in this range.

Regulation of KOPR

MOM-Sal B, U50,488H, and Sal A Promote Internalization and Down-Regulation of KOPR in Vitro. The finding that the EC_{50} values of MOM-Sal B, U50,488H, and Sal A for promoting internalization and down-regulation were much higher than those for activating G proteins is consistent with our previous results (Li et al., 2003). They are in accordance with the notion that there are multiple activated receptor conformations, and those required for receptor internalization and down-regulation represent a subset of those resulting in G protein activation (Li et al., 2003). We have previously reported that U50,488H, dynorphin A, and etorphine are full agonists at KOPR in activating G proteins, but only U50,488H and dynor-

phin A promoted KOPR phosphorylation and internalization (Li et al., 1999; Li et al., 2003). It is interesting to note that MOM-Sal B was ~7-fold more potent than Sal A in activating G protein, but it was ~70-fold more potent than Sal A in inducing internalization of KOPR. MOM-Sal B may induce more of the conformations of the KOPRs that greatly facilitate internalization and down-regulation, whereas the conformation of KOPRs stabilized by Sal A is least prone to internalization. The possibility that Sal A is less stable in the cell medium cannot be excluded.

The relative potencies of U50,488H and Sal A in inducing internalization are consistent with our previous findings (Wang et al., 2005). MOM-Sal B, Sal A, and U50,488H caused similar levels of down-regulation of KOPR that were ~25% of total mature receptors; however, Sal A induced less down-regulation of KOPR than U50,488H in our previous study (Wang et al., 2005). It may be due to different stabilities of Sal A in different batches of cell culture medium. Sal A is kept in the medium for 4 h at 37°C, and its degradation rate may vary depending on what and how many active enzymes, especially esterases, are present in each batch of cell culture serum.

Drug Development Using Sal A as a Lead Compound. KOPR agonists have therapeutic potentials as analgesics, water diuretics, antipruritics, and anticraving agents for cocaine addicts; however, the characteristic dysphoric effects have hindered clinical utility (Liu-Chen, 2004). Nalfurafine is the only selective KOPR agonist used clinically, specifically to treat uremic pruritus in kidney dialysis patients (Wikstrom et al., 2005).

The recent identification of Sal A as a selective KOPR full agonist paved a new avenue for the development of novel KOPR compounds. Sal A has a unique chemical structure and is the only non-nitrogenous KOPR agonist. A KOPR antagonist has been shown to have antidepressant-like effects preclinically (Todtenkopf et al., 2004). In a recent study, two derivatives of Sal A, oxadiazole and salvidivin A, have been reported to act as KOPR antagonists in the [^{35}S]GTPγS binding assay (Simpson et al., 2007). It is encouraging that Sal A may be used as a lead compound to develop KOPR antagonists with therapeutic potential in, for example, clinical depression.

In summary, MOM-Sal B, a potent and selective KOPR agonist, is a Sal A derivative that showed longer lasting antinociceptive and hypothermic effects and higher potency than Sal A. Interestingly, to the best of our knowledge, MOM-Sal B is the first KOPR-selective compound to have higher efficacy than U50,488H in a rat thermal antinociceptive test. MOM-Sal B affects the overt behavior of mice and rats. It represents an excellent molecular tool for pharmacological studies of KOPR both in vitro and in vivo.

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