Buprenorphine Metabolites, Buprenorphine-3-glucuronide and Norbuprenorphine-3-glucuronide, Are Biologically Active

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ABSTRACT

Background: The long-lasting high-affinity opioid buprenorphine has complex pharmacology, including ceiling effects with respect to analgesia and respiratory depression. Plasma concentrations of the major buprenorphine metabolites norbuprenorphine, buprenorphine-3-glucuronide, and norbuprenorphine-3-glucuronide approximate or exceed those of the parent drug. Buprenorphine glucuronide metabolites pharmacology is undefined. This investigation determined binding and pharmacologic activity of the two glucuronide metabolites, and in comparison with buprenorphine and norbuprenorphine.

Methods: Competitive inhibition of radioligand binding to human μ, κ, and δ opioid and nociceptin receptors was used to determine glucuronide binding affinities for these receptors. Common opiate effects were assessed *in vivo* in Swiss-Webster mice. Antinociception was assessed using a tail-flick assay, respiratory effects were measured using unrestrained whole-body plethysmography, and sedation was assessed by inhibition of locomotion measured by open-field testing.

Results: Buprenorphine-3-glucuronide had high affinity for human μ (Ki [inhibition constant] = 4.9 ± 2.7 pM), δ (Ki = 270 ± 0.4 nM), and nociceptin (Ki = 36 ± 0.3 μM) but not κ receptors. Norbuprenorphine-3-glucuronide had affinity for human κ (Ki = 300 ± 0.5 nM) and nociceptin (Ki = 18 ± 0.2 μM) but not μ or δ receptors. At the dose tested, buprenorphine-3-glucuronide had a small antinociceptive effect. Neither glucuronide had significant effects on respiratory rate, but norbuprenorphine-3-glucuronide decreased tidal volume. Norbuprenorphine-3-glucuronide also caused sedation.

Conclusions: Both glucuronide metabolites of buprenorphine are biologically active at doses relevant to metabolite exposures, which occur after buprenorphine. Activity of the glucuronides may contribute to the overall pharmacology of buprenorphine.

What We Already Know about This Topic

- Buprenorphine is an opioid that has a complex pharmacology, including ceiling analgesic and respiratory depressant effects
- Relative exposure to buprenorphine metabolites exceeds exposure to buprenorphine in humans
- One metabolite, norbuprenorphine, causes dose-dependent full respiratory depression in rats

What This Article Tells Us That Is New

- Buprenorphine-3-glucuronide and norbuprenorphine-3-glucuronide are the first active opioid-3-glucuronide metabolites to be identified
- Buprenorphine-3-glucuronide had mild antinociceptive activity in a mouse acute pain model
- Norbuprenorphine-3-glucuronide had a sedative effect and decreased tidal volume in mice

BUPRENORPHINE is a long-lasting, high-affinity opioid, available for three decades for treating pain and opiate addiction.[1] Buprenorphine is marketed for addiction therapy in sublingual tablets or films, both alone and coformulated with naloxone (to discourage diversion and parenteral administration). Initially approved for treatment of pain, buprenorphine has more recently been used for opiate withdrawal therapy and is now being considered for other drug addictions such as cocaine and ethanol.[2] A transdermal formulation was recently approved for the treatment of moderate-severe chronic pain.[3] Buprenorphine displays unusual pharmacology.[4] It is a partial μ agonist, δ and κ antagonist,

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Received from the Departments of Anesthesiology, Biochemistry and Molecular Biophysics, Medicine, and Pathology and Immunology, Washington University in St. Louis, St. Louis, Missouri. Submitted for publication May 11, 2011. Accepted for publication August 24, 2011. Supported by National Institutes of Health (Bethesda, Maryland) grants R01 DA02931 and K24 DA00417 (to Dr. Kharasch). Additional support was provided by the National Institutes of Health Neuroscience Blueprint Interdisciplinary Center Core Grant P30 NS057105 (to Washington University in St. Louis).

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and nociceptin receptor (formerly termed the opioid receptor-like ORL1 receptor) agonist. It has ceiling effects with respect to both analgesia and respiratory depression.[5–9] Despite years of clinical use, the mechanisms by which buprenorphine exerts its pharmacologic effects remain undefined.

Buprenorphine is extensively metabolized in humans, with minimal parent drug excreted in urine.[10,11] The primary route is *N*-dealkylation to norbuprenorphine, catalyzed mainly (80–90%) by the cytochrome P450 enzymes CYP3A4/5, with contributions from CYP2C8 and CYP2C9.[12–14] Both buprenorphine and norbuprenorphine undergo glucuronidation by UDP-glucuronosyl transferases (UGT) to buprenorphine-3-glucuronide (B3G) and norbuprenorphine-3-glucuronide (N3G).[15] B3G formation is catalyzed mainly by UGT2B7 and UGT1A1, with some contribution from UGT1A3 and 2B17, and N3G formation is catalyzed predominantly by UGT1A3 and UGT1A1.[16,17] Based on molar area under the plasma concentration *versus* time curves, glucuronides constitute 70% of a buprenorphine dose. In humans, peak plasma norbuprenorphine concentrations equal or exceed those of buprenorphine, and relative exposures of norbuprenorphine, B3G, and N3G based on molar area under the concentration in plasma *versus* time curve are 200%, 100%, and 600% those of buprenorphine.[13,18–20] If buprenorphine metabolites are pharmacologically active, buprenorphine metabolism could constitute a bioactivation pathway.

Metabolism of buprenorphine to norbuprenorphine was initially considered to be an inactivation pathway, because norbuprenorphine in rats had 1/50th the analgesic potency of buprenorphine based on intravenous dose and one fourth the potency based on intracerebroventricular dose.[21] Evidence now suggests that dealkylation of buprenorphine to norbuprenorphine is actually a bioactivation pathway. Norbuprenorphine is a potent opioid agonist, with high affinities for μ, δ, and κ opioid receptors.[22] In rats, norbuprenorphine caused dose-dependent respiratory depression and was 10 times more potent than buprenorphine.[8,23] Norbuprenorphine respiratory depression was opioid receptor-mediated, and also antagonized by buprenorphine.[8] In sheep, norbuprenorphine also had respiratory depressant effects.[24] Unlike buprenorphine, which is a partial μ receptor agonist with slow receptor dissociation rates, norbuprenorphine in rats has rapid μ receptor binding and is a full agonist, causing full respiratory depression.[8,25] Because clinical plasma norbuprenorphine concentrations equal or exceed those of buprenorphine, norbuprenorphine formation may be a bioactivation rather than inactivation pathway in humans.

No information is available about the pharmacology of the buprenorphine and norbuprenorphine glucuronides. Although drug glucuronidation is generally considered a detoxification and inactivation pathway, there is precedence for active 6-glucuronide metabolites of drugs.[26,27] Opioids are a particularly noteworthy and clinically important example, best exemplified by morphine-6-glucuronide.[28,29] Morphine-6-glucuronide has μ and δ receptor affinity similar to that of morphine, and is 300 times more potent than morphine when administered intracerebroventricularly. Clinically, approximately 10% of morphine is metabolized to morphine-6-glucuronide. Although initial studies of morphine-6-glucuronide at doses (0.04–0.1 mg/kg) approximating concentrations resulting from *in vivo* morphine glucuronidation showed little effect, higher doses produced effective and long-lasting analgesia, and morphine-6-glucuronide has been used clinically.[29] Glucuronides of dihydromorphine and codeine have also been implicated in the biologic effects of their parent drugs.[28,30] Therefore, glucuronidation may theoretically be a buprenorphine bioactivation pathway, and the pharmacologic activity of buprenorphine or norbuprenorphine glucuronides could have significant clinical effects. This would also be the first example of active 3-glucuronides. Nonetheless, pharmacologic effects of buprenorphine and norbuprenorphine glucuronides are unknown. This investigation tested the hypothesis that these glucuronide metabolites are pharmacologically active. In addition, this work pertains to the US Food and Drug Administration guidance on drug metabolites, which defines a major metabolite as comprising more than 10% of parent drug systemic exposure (area under the curve) at steady state, and suggests it be considered for safety assessment.‖

Materials and Methods

Reagents

Unless otherwise noted, reagents were from Sigma-Aldrich Chemical Company (St. Louis, MO). ^{3}H-diprenorphine and ^{3}H-nociceptin were from Perkin Elmer (Waltham, MA). Buprenorphine, norbuprenorphine, and B3G were from the National Institute on Drug Abuse (Bethesda, MD). N3G was synthesized according to Fan *et al.*[31] Naloxone was from Cerillant (Round Rock, TX). Membrane preparations from Chinese hamster ovary cells expressing human μ or δ receptors and from human embryonic kidney cells expressing the human nociceptin receptor were purchased from Perkin Elmer. Chinese hamster ovary cells stably expressing the human κ opioid receptor were obtained from the laboratory of Dr. Richard Rothman (National Institutes of Health, Bethesda, MD).[32]

Preparation of Cell Membranes

Membranes from Chinese hamster ovary cells stably expressing the human κ opioid receptor were prepared for ligand binding assays as described by Zhu *et al.*,[33] with modifications. Adherent cells were washed three times in ice-cold phosphate-buffered saline, harvested in hypotonic lysis buf-

‖ Guidance for Industry, Safety Testing of Drug Metabolites; Food and Drug Administration; Center for Drug Evaluation and Research, February 2008; http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm079266.pdf. Accessed August 12, 2011.

fer (20 mM Tris HCl pH 7.5 with 5 mM $MgCl_2$), and incubated in lysis buffer on ice for 1 h. After incubation, the cells were further disrupted by sonication using a probe sonicator three times for 18 s each. The sonicated samples were centrifuged at 40,000 g for 30 min. The pellet was resuspended in binding assay buffer plus 5% protease inhibitors (Complete protease inhibitor cocktail tablets, EDTA free, Santa Cruz Biotechnology, Santa Cruz, CA) and passed through a 26½ gauge needle for homogenization. Protein content was determined by protein assay (Bio-Rad Protein Assay, Bio-Rad Laboratories, Hercules, CA), using bovine serum albumin as the standard. Presence of the κ receptor was confirmed by Western blot. Aliquots were flash frozen in liquid nitrogen and stored at −80°C.

Opioid Receptor Affinity Assays

The μ and δ opioid receptor and nociceptin receptor membrane preparations (Perkin Elmer) were diluted according to manufacturer's recommendations. Competitive displacement of radioligand binding was performed using a method modified from Huang *et al.*[22] Competitive displacement of ^{3}H-diprenorphine (0.4 nM) binding to μ, δ, and κ receptors by buprenorphine, norbuprenorphine, B3G, and N3G was performed in the absence or presence of at least seven concentrations of each test compound. Nonspecific binding was determined by the addition of the specific inhibitor naloxone (10 μM). Binding was carried out in binding assay buffer (50 mM Tris HCl with 1 mM EGTA). Bound and unbound ^{3}H were separated by vacuum filtration over Whatman grade GF/C filters (GE Healthcare, Piscataway, NJ) presoaked in buffer (50 mM Tris HCl, pH 4.0, with 1 mM EGTA, 0.4% bovine serum albumin, 0.01% polylysine). Filters were rinsed four times with 4 ml ice-cold 50 mM Tris HCl pH 4.0 and dried overnight at room temperature.

Competitive displacement of ^{3}H-nociceptin (0.1 nM) binding to nociceptin receptors was determined in the absence or presence of at least seven various concentrations of each test compound. Nonspecific binding was determined by the addition of the specific inhibitor dynorphin A (20 μM). Binding was carried out in assay buffer (50 mM HEPES pH 7.4, 10 mM $MgCl_2$, 1 mM EDTA). Bound ^{3}H was separated from unbound by vacuum filtration over Whatman GF/C filters presoaked in assay buffer with 0.5% polylysine. Filters were rinsed four times with 4 ml ice-cold assay buffer and dried overnight at room temperature.

Remaining bound radioactivity was determined by liquid scintillation counting. Each experiment was performed in triplicate and repeated at least twice. Specific and nonspecific binding of ^{3}H-diprenorphine to the μ, κ, and δ receptors was approximately 2,500 counts per minute and 300 counts per minute, respectively. Specific and nonspecific binding of ^{3}H-nociceptin to the nociceptin receptor was approximately 3,000 counts per minute and 700 counts per minute, respectively. Inhibition constant (Ki) values of each compound were determined by nonlinear regression analysis and the Cheng-Prusoff equation[34] (SigmaPlot 11.2, Systat Corp, San Jose, CA) after subtracting nonspecific binding. Dissociation constant (Kd) values of the nonspecific inhibitors used in the calculations were from the manufacturer of the cell membrane preparations.

Animals

Experiments were performed in accordance with the guidelines of the National Institutes of Health and were approved by the Animal Care and Use Committee of Washington University (St. Louis, MO). Male Swiss Webster mice (Taconic Farms, Germantown, NY), age 7–9 weeks (35–45 g), were used in all experiments. All mice were group-housed on a 12:12-h light/dark schedule with *ad libitum* access to food and water. Doses of norbuprenorphine, buprenorphine-3-glucuronide, and norbuprenorphine-3-glucuronide were chosen to reflect the relative exposure of each metabolite in humans after a buprenorphine dose.[13,17–19] An initial tail-flick nociception experiment was performed to determine the dose of buprenorphine (0.1, 0.3, 1, 10, or 100 mg/kg) that produced the maximum possible effect. Results showed that 0.3 mg/kg (0.6 μmol/kg) had the greatest analgesic response, with a maximum possible effect of 100% at 30 min. Therefore, norbuprenorphine, B3G, and N3G were dosed at two, one, and six times 0.6 μmol/kg, respectively.

Tail-flick Assay

A tail-flick assay was used to test the antinociceptive effect of the glucuronides.[5,35] Tail-flick latency, defined by the time in seconds for tail withdrawal from a warm water bath (52°C) was measured using an IITC 500 warm water tail immersion test analgesia meter (IITC Life Science, Woodland Hills, CA). Mice (10/group) were dosed subcutaneously with either saline vehicle (control) or drug (0.1–100 mg/kg buprenorphine, 1 mg/kg B3G, 1 mg/kg norbuprenorphine, and 2.22 and 22.2 mg/kg N3G). Each animal was injected only once. Tail-flick latency was measured every 15 min for 90 min after drug administration. A separate experiment (no drug) was performed to determine the baseline tail- flick latency for each mouse. A cutoff of 10 s was used to prevent tissue damage. Animals not responding within 3 s were excluded from the assay. Maximum possible effect was calculated as: $[(T1 - T0)/(T2 - T0)] \times 100$, where $T0$ and $T1$ represent latencies before and after drug administration, and $T2$ is the cutoff time.

Unrestrained Whole-body Plethysmography

Measurements of ventilation parameters were obtained using unrestrained whole-body plethysmography (Buxco Research Systems, Wilmington, NC). The plethysmograph consisted of eight animal chambers with orifices for entry and exit of breathing air, and a 1-ml syringe permitting calibrations, connected to a differential pressure transducer. The air entry orifice was connected to a source of compressed breathing air. Each chamber was calibrated with 1 ml room air immediately before each experiment. Each awake mouse was placed in a chamber. Ventilation parameters were recorded for 20 min predosing. Each animal was removed from the

chamber, received the drug subcutaneously, and was replaced in the chamber. Postdosing ventilation parameters were recorded for 1 h. Four animals were studied in each group. Respiratory values were calculated by Biosystems XA software (Buxco Research Systems).

Open-field Locomotor Testing

Locomotor activity was measured in an open field using a VersaMax Animal Activity Monitor (Accuscan Instruments, Inc. Columbus, OH) as previously described.[36] After habituation to the test room, test compound was administered subcutaneously to a single mouse, which was immediately placed in the test chamber. Locomotor activity was assessed by recording photobeam breaks for 60 min. Total distance traveled, time spent moving, and the numbers of beam breaks (horizontal activity) were calculated for the entire chamber. Data were combined and reported as total activity/time. Four mice were tested in each group.

Disposition of Norbuprenorphine Glucuronides

To test the hypothesis that pharmacologic activity of B3G and N3G could be due to hydrolysis of the glucuronides back to the aglycones, plasma and brain concentrations of buprenorphine, norbuprenophine, B3G, and N3G were determined after subcutaneous injection of either B3G or N3G. Drug-naïve Swiss Webster mice (4 per group) were administered B3G (1 mg/kg) or N3G (2.22 mg/kg). After 60 min, mice were anesthetized with sevoflurane and blood was collected by cardiac puncture into heparinized microtainers (BD Biosciences, Franklin Lakes, NJ) and then centrifuged at 14,000 rpm for 1 min to separate plasma. After exsanguination, whole brains were collected and flash frozen. Plasma and brain were stored at −80°C until analysis.

Analytical Methods

Analysis of buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide in brain and plasma was performed on an API 4000 QTRAP mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA)-Agilent 1100 series HPLC system (Agilent, Wilmington, DE). The mass spectrometer was equipped with a Turbo Ion Spray ionization source operating in positive ionization mode. Chromatographic separation was performed on a Waters XBridge C8 column (150 × 2.1 mm, 3.5 μm) (Waters Corp, Milford, MA). The injection volume was 30 μl and the oven temperature was 25°C. The HPLC mobile phase (0.25 ml/min) was 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). The gradient program was 5% B for 0 min, linear gradient to 40% B between 0 and 0.5 min, held at 40% B until 2.5 min, linear gradient to 90% between 2.5 and 5 min, held at 90% B until 8 min, then reequilibrated to initial conditions (5% B) between 8.01 and 15.0 min. Under these conditions, retention times were 7.62, 6.73, 6.52, and 6.00 min, respectively, for buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide. Both Q1 and Q3 quadrupoles were optimized to unit mass resolution, and the mass spectrometer conditions were optimized for each analyte. The instrument was operated in positive-ion mode with an ion spray voltage of 5,200 V. The curtain gas was set at 15, ion source gas 1 at 40, ion source gas 2 at 50, and collision gas set at the high setting. Multiple reaction monitoring transitions for each analyte and internal standard were m/z 468.5 > 55.2 for buprenorphine, m/z 414.3 > 82.9 for norbuprenorphine, m/z 644.3 > 468.5 for buprenorphine glucuronide, m/z 590.4 > 414.3 for norbuprenorphine glucuronide, m/z 472.5 > 59.2 for buprenorphine d4, and m/z 417.3 > 82.9 for norbuprenorphine d3. Analytes were quantified using area ratios and standard curves prepared using calibration standards in blank media.

Brain samples were prepared immediately before analysis, by dounce homogenization with 4 ml Hanks buffered salt solution to 1 g mouse brain. Mouse brain calibration standards and quality control samples were prepared by similarly homogenizing mouse brain, and 500 ul of buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide solution (mixture at 50 mg/ml each in methanol) were added to 9.5 ml mouse brain homogenate to prepare 2.5 mg/ml working stock solution. Calibration standards for buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide in brain homogenate were prepared at 0.12, 0.62, 1.25, 6.25, 12.5, and 25 ng/ml. Quality-control samples were prepared at 0.1, 1.0, and 10 ng/ml. Mouse plasma calibration standards and quality control samples were prepared by adding 500 ul of buprenorphine, norbuprenorphine, buprenorphine glucuronide, and norbuprenorphine glucuronide solution (mixture at 50 mg/ml each in methanol) to 9.5 ml mouse plasma to prepare 2.5 mg/ml working stock solution. Calibration standards in mouse plasma were prepared at 0.62, 1.25, 2.5, 5, 10, 25, 50, 100, 250, and 500 ng/ml whereas quality control samples were prepared at 1, 10, and 100 ng/ml. Sample preparation steps for both mouse brain and plasma were as follows: Experimental, quality control, and calibration samples (100 μl) were mixed with 400 ul of acetonitrile containing buprenorphine d4 and norbuprenorphine d3 (10 ng/ml each) and vortexed for 2 min. The samples were centrifuged at 3,000 rpm for 5 min. The supernatant (250 μl) were removed and evaporated to dryness and reconstituted in 100 μl 0.1% acetic acid for brain samples and 500 μl 0.1% acetic acid for plasma samples.

Statistical Analysis

The results are expressed as the mean ± SD. Two-way ANOVA (time, drug group) was used, followed by the Student-Newman-Keuls test, to test for significant differences between groups (SigmaPlot 11.2). Statistical significance was assigned at $P < 0.05$. Nonnormal data were log-transformed for ANOVA.

Table 1. Receptor Affinity of Buprenorphine and Buprenorphine Metabolites

	μ	δ	κ	Nociceptin
Buprenorphine	2.7 ± 0.4 pM	33 ± 1.6 nM	2.1 ± 0.2 pM	25 ± 0.3 μM
Norbuprenorphine	1.8 ± 0.4 pM	1.3 ± 0.2 μM	1.3 ± 0.3 pM	N.B.
Buprenorphine-3-glucuronide	4.9 ± 2.7 pM	270 ± 0.4 nM	N.B.	36 ± 0.3 μM
Norbuprenorphine-3-glucuronide	N.B.	N.B.	300 ± 0.5 nM	18 ± 0.2 μM

Results are shown as apparent Ki (inhibition constant) values of buprenorphine, norbuprenorphine, buprenorphine-3-glucuronide, and norbuprenorphine-3-glucuronide for human μ, δ, and κ opioid receptors and the human nociceptin receptor. Apparent Ki values were calculated from the equation $Ki = IC_{50}/(1 + ([L]/Kd))$. IC_{50} values were derived from the competition curves shown in fig. 1.
N.B. = no binding.

Results

Binding Affinity for Opioid Receptors

Competitive inhibition of ^{3}H-diprenorphine to opiate receptors was used to determine the binding affinities of buprenorphine, norbuprenorphine, B3G, and N3G for the μ, κ, and δ opioid receptors (table 1, fig. 1). B3G inhibited ^{3}H-diprenorphine μ receptor binding with high affinity, with a Ki in the picomolar range. N3G did not inhibit ^{3}H-diprenorphine μ receptor binding even at concentrations as high as 2.5 mM. B3G also inhibited ^{3}H diprenorphine binding to the δ opioid receptor with a Ki in the nanomolar range. N3G did not inhibit ^{3}H-diprenorphine binding to the δ opioid receptor even at concentrations as high as 2.5 mM. N3G but not B3G inhibited radioligand binding to the κ opioid receptor. Buprenorphine and norbuprenorphine had affinities for the receptors in the subnanomolar and nanomolar ranges, which is consistent with reports from other laboratories.[22]

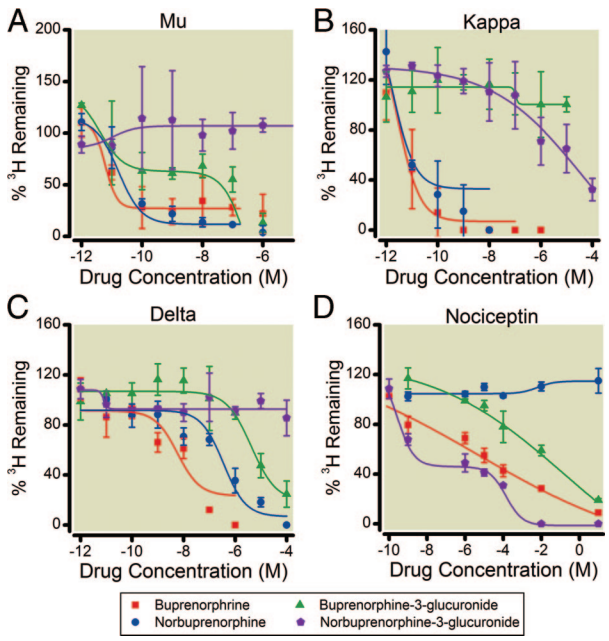

Fig. 1. Competitive inhibition by buprenorphine, norbuprenorphine, buprenorphine-3-glucuronide (B3G), and norbuprenorphine-3-glucuronide (N3G) of ^{3}H-diprenorphine to human μ (*A*), κ (*B*), and δ (*C*) opioid receptors, and ^{3}H nociceptin binding to the nociceptin receptor (*D*). Binding to the opioid receptors and the nociceptin receptor was carried out with 0.4 nM ^{3}H diprenorphine and 0.1 nM ^{3}H-nociceptin, respectively, in the presence or absence of varying concentrations of buprenorphine (*red square*), norbuprenorphine (*blue circle*), B3G (*green triangle*), and N3G (*purple pentagon*). Data were normalized to percentage of specific binding. Each data point represents the mean ± SD (n = 9). Lines are predicted results based on the Ki values obtained by nonlinear regression analysis of the observed data, using the equation shown in table 1. Apparent Ki values are shown in table 1.

Competitive inhibition of ^{3}H-nociceptin was used to determine the binding affinities of the metabolites for the nociceptin receptor. Both glucuronide metabolites displayed Ki values for the nociceptin receptor in the micromolar range. Consistent with previous reports,[22] buprenorphine had low affinity for this receptor; however, norbuprenorphine did not displace the radioligand.

Antinociceptive Activity of B3G and N3G

The antinociceptive effect of each glucuronide was tested using a hot water tail-flick latency assay. At the doses tested, both glucuronides had antinociceptive effects (fig. 2). The response to B3G was brief, with onset, time to peak, and return to baseline within 60 min. N3G had a small but statistically significant analgesic effect lasting approximately 45 min with a peak at 45 min. A slight decrease in tail-flick latency compared with that of control samples was seen with the starting dose of N3G tested. To test whether this was a submaximal, dose-dependent response, a tenfold greater dose was also tested. A similar slight decrease in the tail-flick latency was noticed, but was not statistically different from the lower dose, and neither was different from control samples (data not shown). The antinociceptive effect of 1 mg/kg norbuprenorphine was approximately one-fifth that of 0.3 mg/kg buprenorphine. As shown in previous reports,[21] buprenorphine and norbuprenorphine effects followed the same time course, with the same time to onset and time to peak. The time to onset for both compounds was 15 min, with peak effects at 45 min.

Respiratory Effects of B3G and N3G

The effect of B3G, N3G, buprenorphine, and norbuprenorphine on respiratory rate was measured using unrestrained whole-body plethysmography. Neither B3G nor N3G had a

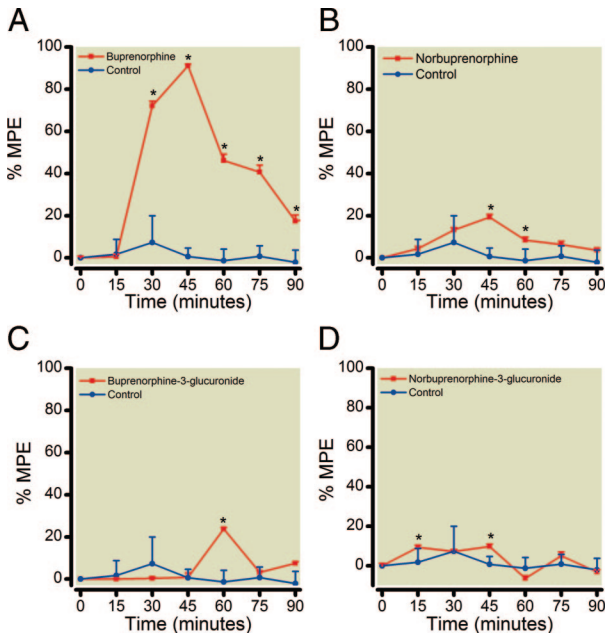

Fig. 2. Antinociceptive effects of buprenorphine (*A*), norbuprenorphine (*B*), buprenorphine-3-glucuronide (*C*), and norbuprenorphine-3-glucuronide (*D*) in an acute pain model. Time to withdrawal of the tail from a hot water (52°C) bath, or tail-flick latency, was measured after subcutaneous injection of vehicle or buprenorphine (0.3 mg/kg; 0.6 μmol/kg), norbuprenorphine (1 mg/kg; 1.2 μmol/kg), buprenorphine-3-glucuronide (1 mg/kg; 0.6 μmol/kg), or norbuprenorphine-3-glucuronide (2.2 mg/kg, 3.8 μmol/kg). The % maximum possible effect (MPE) was calculated every 15 min for 90 min. Each data point is the mean ± SD (n = 10 per group). *Asterisk,* significantly different from control sample ($P < 0.05$) by two-way ANOVA.

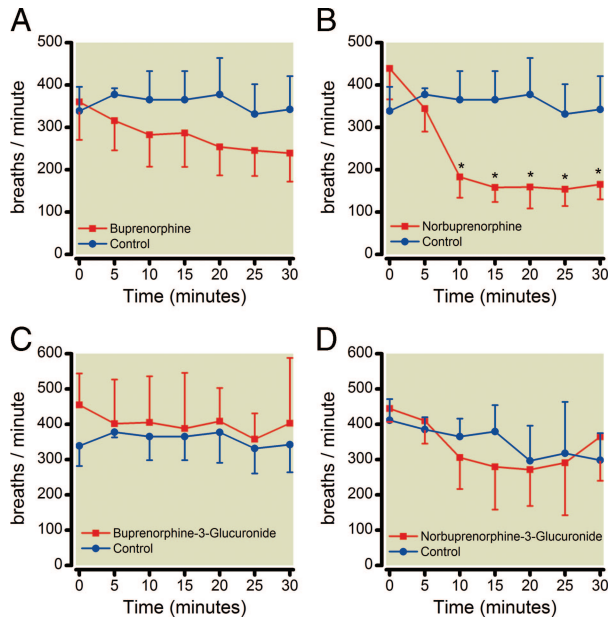

Fig. 3. Effect of buprenorphine (*A*), norbuprenorphine (*B*), buprenorphine-3-glucuronide (*C*), and norbuprenorphine-3-glucuronide (*D*) on respiratory rate. Unrestrained whole-body plethysmography was used to study the effect on respiratory rate. Data were recorded for 30 min after subcutaneous injection of vehicle or buprenorphine (0.3 mg/kg; 0.6 μmol/kg), norbuprenorphine (1 mg/kg; 1.2 μmol/kg), buprenorphine-3-glucuronide (1 mg/kg; 0.6 μmol/kg), or norbuprenorphine-3-glucuronide (2.2 mg/kg, 3.8 μmol/kg). Norbuprenorphine caused a marked decrease in respiratory rate 10 min after drug dose. Results are the mean ± SD (n = 4 per group). *Asterisk*, significantly different from control sample ($P < 0.05$) by two-way ANOVA.

significant effect on respiratory rate at the dose tested (fig. 3); however, N3G did significantly decrease tidal volume (fig. 4). Buprenorphine (0.3 mg/kg) did not have a significant effect on respiratory rate, whereas 1 mg/kg norbuprenorphine elicited a pronounced reduction in respiratory rate, with an onset within 10 min of drug administration. Buprenorphine and norbuprenorphine effects on respiratory rate were similar to those previously reported,[23,25] and neither compound had an effect on tidal volume. Neither buprenorphine nor B3G affected minute ventilation, whereas both norbuprenorphine and N3G decreased minute ventilation in an equivalent manner.

Effects of Buprenorphine, Norbuprenorphine, B3G, and N3G on Locomotor Activity

Open-field testing was performed to identify and quantify effects of buprenorphine, norbuprenorphine, B3G, and N3G on locomotor activity (fig. 5). This test measures the exploratory activity of animals in a novel environment. Drug-induced sedation will override the desire to explore a novel environment, resulting in a reduced number of movements per unit time. Each animal was monitored for 1 h immediately after subcutaneous drug administration. Both norbuprenorphine and N3G caused a significant decrease in total activity compared with control samples. Buprenorphine and B3G did not cause a decrease in activity compared with control samples.

Brain and Plasma Concentrations of Buprenorphine, Norbuprenorphine, B3G, and N3G

The extent of hydrolysis of each glucuronide to either of the aglycones was investigated by analysis of plasma and brain homogenate 60 min after subcutaneous injection of either B3G or N3G (table 2). There was minimal aglycone detected in plasma or brain after administration of either glucuronide. In brains of mice administered B3G, there was no buprenorphine detected, and norbuprenorphine was 2% of the glucuronide concentration. In plasma, no buprenorphine was detected, and norbuprenorphine was 1% of the glucuronide concentration. In mice administered N3G, brain norbuprenorphine concentration was 9% of the glucuronide, and in plasma it was less than 1%. There was no buprenorphine detected in brain or plasma of these mice.

Discussion

Buprenorphine has unusual and complex pharmacology. Like other μ agonists, it causes analgesia, respiratory depres-

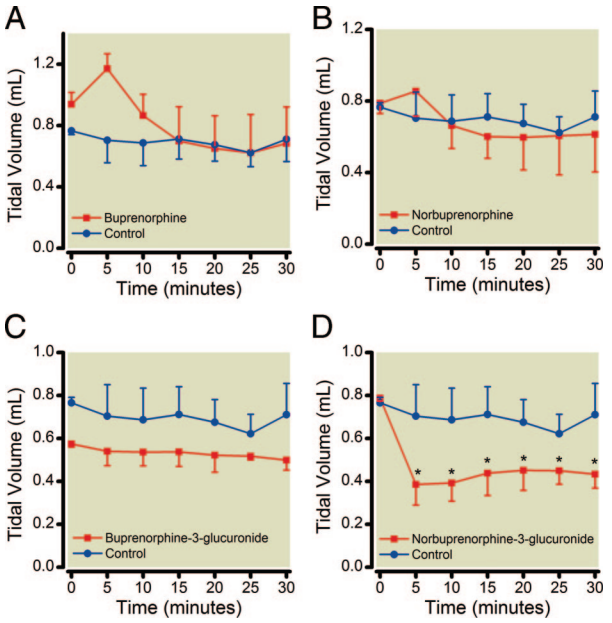

Fig. 4. Effect of buprenorphine (*A*), norbuprenorphine (*B*), buprenorphine-3-glucuronide (*C*), and norbuprenorphine-3-glucuronide (*D*) on tidal volume. Unrestrained whole-body plethysmography was used to study the effect on tidal volume. Data were recorded for 30 min after subcutaneous injection of vehicle or buprenorphine (0.3 mg/kg; 0.6 μmol/kg), norbuprenorphine (1 mg/kg; 1.2 μmol/kg), buprenorphine-3-glucuronide (1 mg/kg; 0.6 μmol/kg), or norbuprenorphine-3-glucuronide (2.2 mg/kg, 3.8 μmol/kg). Norbuprenorphine-3-glucuronide caused a marked decrease in tidal volume 5 min after drug dose. Results are the mean ± SD (n = 4 per group). *Asterisk*, significantly different from control sample ($P < 0.05$) by two-way ANOVA.

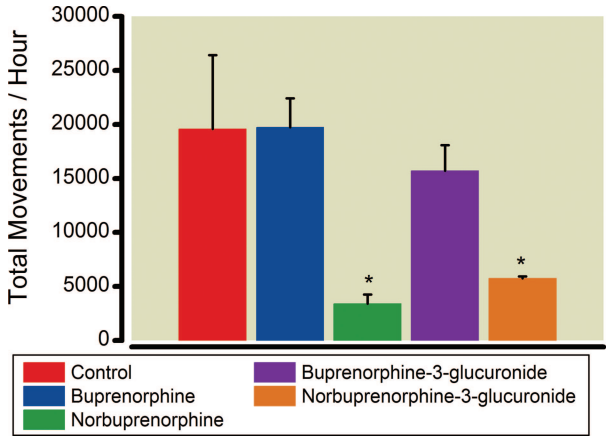

Fig. 5. Sedative effects of buprenorphine, norbuprenorphine, buprenorphine-3-glucuronide, and norbuprenorphine-3-glucuronide. The effect of buprenorphine, norbuprenorphine, buprenorphine-3-glucuronide, and norbuprenorphine-3-glucuronide on locomotion was quantified in an open-field test. A single mouse was habituated to the test room, administered vehicle or buprenorphine (0.3 mg/kg; 0.6 μmol/kg), norbuprenorphine (1 mg/kg; 1.2 μmol/kg), buprenorphine-3-glucuronide (1 mg/kg; 0.6 μmol/kg), or nor buprenorphine-3-glucuronide (2.2 mg/kg, 3.8 μmol/kg) and placed in a test chamber. Total activity was recorded by photobeam breaks for 1 h. Each result is the mean ± SD (n = 4 per group). *Asterisk*, significantly different from control sample ($P < 0.05$) by one-way ANOVA.

sion, miosis, and mood changes, but there is a ceiling effect at higher intravenous and sublingual doses.[6,37,38] Although buprenorphine ceiling effects have been attributed to partial μ antagonism, and to differential opioid receptor-mediated actions at different concentrations, buprenorphine pharmacology remains a mechanistic conundrum. Particularly, the contribution(s) of buprenorphine metabolite(s) toward the clinical effects of buprenorphine remain undefined. Only the *N*-demethylated metabolite, norbuprenorphine, has been evaluated in animals. For the first time, this investigation provides evidence that two glucuronide metabolites of buprenorphine are pharmacologically active. In addition, it is the first example of active opioid-3-glucuronides.

The activity profile of B3G included μ, δ, and nociceptin receptor binding, and an antinociceptive effect in an acute pain model. At the clinically relevant dose tested, the magnitude of B3G antinociception was approximately one-fourth that of buprenorphine. In addition, the onset and peak of antinociception occurred at 60 min, compared with 30 and 45 min with buprenorphine. There are several potential explanations for both the lesser antinociception and the slower onset of B3G compared with buprenorphine. The low response could reflect lower potency and/or efficacy. In support of this hypothesis, the affinity of B3G for the μ receptor was half that of buprenorphine. Similarly, the μ receptor affinity of morphine-6-glucuronide was less than that of the parent drug morphine.[39] Nevertheless, morphine-6-glucuronide elicited an analgesic response similar to morphine when the dose of morphine-6-glucuronide reflected plasma concentrations that occur after a morphine dose.[29] Further studies are warranted to determine the full dose-response effect of B3G and its potency and efficacy relative to buprenorphine. Whether B3G exhibits an inverted U-shaped dose–response curve, analogous to that of buprenorphine, is still unknown. Although this investigation shows that B3G has high affinity for the μ and δ receptors and moderate affinity for the nociceptin receptor, it does not address whether B3G binding activates or antagonizes these receptor pathways. However, all known active opioid glucuronides are receptor agonists. The later onset of analgesic effect of B3G compared with buprenorphine could be due to differences in brain access between the more hydrophilic glucuronide and the highly lipophilic aglycone, and/or to differences in receptor kinetics between the two compounds. Given that there was no buprenorphine detected in the brains of mice administered B3G, and that the minimal norbuprenophine detected in brain was much less than has been shown to elicit any physiologic response,[21] it can be concluded that the observed pharmacologic effect of B3G was due to the glucuronide itself, rather than hydrolysis to and activity of the aglycone (buprenorphine).

Table 2. Plasma and Brain Concentrations of Buprenorphine and Buprenorphine Metabolites after Administration of Buprenorphine Glucuronides

Concentration (ng/ml)	Compound Dosed	
	Buprenorphine-3-glucuronide (1 mg/kg)	Norbuprenorphine-3-glucuronide (2.2 mg/kg)
Plasma		
Buprenorphine-3-glucuronide	133 ± 44	ND
Norbuprenorphine-3-glucuronide	1.5 ± 0.8	360 ± 111
Buprenorphine	ND	ND
Norbuprenorphine	ND	0.65 ± 0.20
Brain Homogenate		
Buprenorphine-3-glucuronide	1.4 ± 0.7	ND
Norbuprenorphine-3-glucuronide	ND	2.1 ± 0.7
Buprenorphine	ND	ND
Norbuprenorphine	0.03 ± 0.05	0.19 ± 0.14

Results are the mean ± SD (n = 4).
ND = not detected.

The activity profile of N3G included κ and nociceptin receptor binding, reduction of tidal volume, and marked reduction of locomotor activity. The significant decrease in tidal volume but not respiratory rate suggests that N3G may have activity at receptors other than the opioid receptors. The lack of effect on respiratory rate may also be attributable to the lack of N3G binding to the μ opioid receptor. The sedative effect of N3G was comparable to that of norbuprenorphine and could be mediated through either the κ or the nociceptin receptor, because activation of either receptor acts on dopamine neurotransmission and can result in decreased locomotor activity.[40–42] Despite not having binding affinity for the μ receptor, N3G did have a small antinociceptive effect, much less than that of the other three compounds tested. The antinociceptive effect of N3G may be the result of κ receptor activation. κ receptor-mediated analgesia has been shown, generally in animal models.[42] Activation of the nociceptin receptor has not been associated with antinociception; conversely, it has been shown to elicit a hyperalgesic or antianalgesic response in rodent models of acute pain.[40,43] Although synergy and/or opposition of nociceptin and other opioid receptor-associated pathways is not yet fully understood, evidence suggests that nociceptin activation may at least partially antagonize μ receptor-mediated analgesia.[41,44] If the same is true for κ-mediated analgesia, then the limited analgesic response to N3G may be due to intrinsically low potency or to opposition of the nociceptin and κ receptors with respect to analgesia. Whether N3G is a κ-receptor agonist or antagonist remains to be fully defined. Norbuprenorphine was detected in the brains of mice administered N3G; however, the amount was less than 10% of the total glucuronide present and less than the concentration shown to elicit a pharmacologic effect.[21] Moreover, whereas norbuprenorphine decreased respiratory rate, N3G did not. Therefore, it can be concluded that the effect of N3G was due to the glucuronide itself, rather than hydrolysis to and activity of the aglycone (norbuprenorphine).

The activity profile of norbuprenorphine included affinity for the μ, δ, and κ receptors (but not the nociceptin receptor), respiratory depression, inhibition of locomotion, and an analgesic effect approximately one-fourth that of a lower dose (0.3 mg/kg) of buprenorphine (compared with 1 mg/kg norbuprenorphine). The onset and peak of analgesia after norbuprenorphine administration was at 45 and 60 min, respectively, mirroring the analgesic response to buprenorphine. The respiratory and sedative effects, however, occurred at 15 min. The activity profile of buprenorphine included affinity for all four receptors, and an analgesic effect of approximately 100% maximum possible effect. The antinoceptive effect of buprenorphine was the greatest of the four compounds tested in this experiment, both in magnitude and duration. Unlike norbuprenorphine, buprenorphine did not cause respiratory depression nor did it have an effect on locomotion/sedation.

Comparison of buprenorphine and the three metabolites is shown in table 3. Each compound has distinct pharmacologic effects, with B3G effects most similar to those of the parent drug. The effects of norbuprenorphine and N3G are pronounced and strikingly different from those of the parent drug. However, buprenorphine respiratory depression and sedation are not typically reported in animals, raising the question of whether buprenorphine antagonizes the effects of its metabolites, possibly through nociceptin receptor agonism or through differences in affinities for receptor subtypes. Indeed, buprenorphine could both protect against and reverse norbuprenorphine-induced respiratory depression in rats.[8] In rats, induction of CYP3A by dexamethasone increased plasma norbuprenorphine concentrations but did not result in respiratory depression after administration of high-dose buprenorphine.[45] Because the central nervous system activity of drug metabolites relies both on their formation (metabolism) and their accessibility to the brain (diffusion or transport across the blood-brain barrier), genetic variants or drug interactions with metabolizing enzymes

Table 3. Major Pharmacologic Effects of Buprenorphine and Buprenorphine Metabolites

—	Receptor Affinity				Effect		
—	μ	δ	κ	Nociceptin	Analgesia	Respiratory Depression	Sedation
Buprenorphine	+	+	+	+	+	−	−
Norbuprenorphine	+	+	+	−	+	+	+
Buprenorphine-3-glucuronide	+	+	−	+	+	−	−
Norbuprenorphine-3-glucuronide	−	−	+	+	−	+	+

and/or transport proteins could potentially affect the clinical response to buprenorphine *via* its metabolites. For example, norbuprenorphine but not buprenorphine is a substrate for the brain efflux transporter P-glycoprotein.[46,47]

Notably, of the four compounds tested, norbuprenorphine is the only one that causes respiratory depression and also does not have affinity for the nociceptin receptor. This may suggest a role for nociceptin activation in attenuation of μ receptor mediated respiratory depression. This hypothesis is supported by recent work with experimental compounds having activity at both μ and nociceptin receptors.[2] That inhibition of locomotion was observed only with norbuprenorphine and N3G, and yet these two compounds had very different receptor affinity profiles, is intriguing. As mentioned previously, inhibition of locomotion could be mediated by activation of the nociceptin receptor, yet N3G and not norbuprenorphine has affinity for that receptor. This suggests that either the same effect is mediated through different pathways activated by the different receptors, or that the effect is mediated through a receptor for which the two compounds both have affinity, such as the κ receptor. Conversely, it is also intriguing that B3G and buprenorphine, both with moderate affinity for the nociceptin receptor, do not elicit sedative effects. Experimental compounds with mixed nociceptin/μ receptor activation are sedative, suggesting that a mechanism other than activation of these receptors is preventing or antagonizing a sedative effect after a dose of buprenorphine or B3G.[48]

In conclusion, both B3G and N3G have receptor binding and pharmacologic activity. This is the first example of active opioid-3-glucuronides. Buprenorphine and its three major metabolites, norbuprenorphine, B3G, and N3G, have distinct pharmacologic profiles. Potential contribution of these metabolites to the biologic effects of buprenorphine adds to the complexity of buprenorphine pharmacology. Further investigation of B3G and N3G is warranted. B3G might ultimately merit exploration as a potential clinical analgesic, particularly if further studies confirm that it does not have adverse respiratory side effects.

The authors thank Richard B. Rothman, M.D., Ph.D., Clinical Psychopharmacology Section, Intramural Research Program, National Institute on Drug Abuse, National Institutes of Health, Baltimore, Maryland, for the generous gift of CHO cells expressing human kappa opioid receptors; Brad Manion, B.A., Research Assistant, Department of Anesthesiology, Washington University School of Medicine, St. Louis, Missouri, and Zi-wei Chen, Ph.D., Research Instructor in Anesthesiology, Department of Anesthesiology, Washington University School of Medicine, for their assistance with the radioligand binding experiments; and Rolley (Ed) Johnson, Pharm.D., Vice President for Scientific and Regulatory Affairs, Reckitt Benckiser Pharmaceuticals, Richmond, Virginia, and Moo Kwang Park, Ph.D., Program Director for Pharmaceutics and Clinical Supplies, Chemistry and Pharmaceutics Branch (CPB), Division of Pharmacotherapies and Medical Consequences of Drug Abuse, National Institute on Drug Abuse, Bethesda, Maryland, for their assistance in obtaining norbuprenorphine.

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