7-Hydroxymitragynine is an Active Metabolite of Mitragynine and a Key Mediator of its Analgesic Effects

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Supporting Information

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Opioid Functional Activity of Potential Desmethylmitragynine Metabolites

Table S1. Functional Activity of Potential Desmethylmitragynine Metabolites at hMOR.

Compound	EC_{50} ± SEM (E_{max}) (μM)
mitragynine	0.339 ± 0.178 (34%)[1]
1	0.681 ± 0.379 (29%)[1]
2	0.995 ± 0.344 (80%)
3	X

Agonist activity at human MOR (hMOR) indicated by EC_{50} values, maximal efficacy (E_{max}) relative to [D-Ala[2], *N*-Me-Phe[4], Gly-ol[5]]-enkephalin (DAMGO); X = no agonist activity up to 100 μM; All data points represent mean ± SEM (μM) of n ≥ 2.

Opioid Functional Activity of Desmethylmitragynine Metabolites. The functional activity of the three possible desmethylmitragynine metabolites, compounds **1**, **2**, and **3**, at the human mu-opioid receptor (hMOR) was assessed using a bioluminescence resonance energy transfer (BRET) functional assay measuring G protein activation. Compound **1** had previously been reported by us to be less potent and less efficacious than mitragynine for activation of hMOR.[1] For the present work, we also evaluated the activity of desmethyl compounds **2** and **3**. Compound **2** was found to be more efficacious than mitragynine for activation of hMOR but ~3-fold less potent, while compound **3** was found to be completely inactive as an agonist at concentrations up to 100 μM. Accordingly, none of the three possible desmethylmitragynine metabolites are likely to account for the MOR-dependent analgesic activity of mitragynine.

Microsome Stability of 7-Hydroxymitragynine

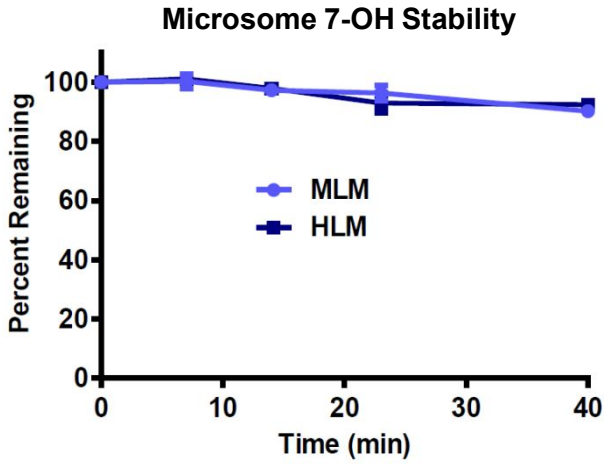


Figure S1. Relative percent remaining of 7-OH was quantified by LC-MS/MS during incubations with HLM and MLM. 7-OH was highly stable in both preparations, suggesting that it is not subject to significant hepatic metabolism through CYP-mediated Phase I oxidation reactions. All data points represent the means of two independent experiments with two incubations per experiment, with error bars representing ±SEM.

Plasma Stability of Mitragynine and 7-Hydroxymitragynine

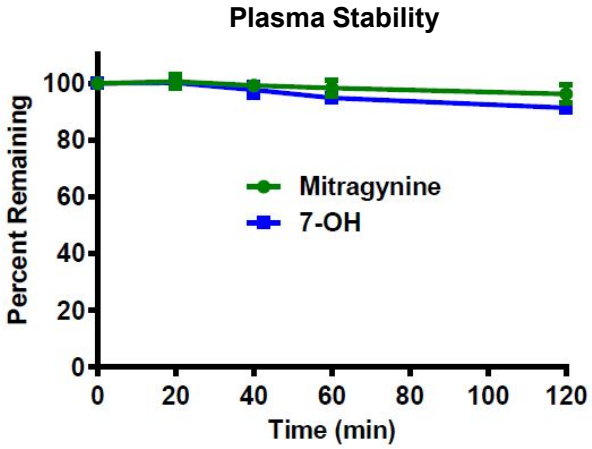


Figure S2. Relative percent remaining of mitragynine and 7-OH was quantified by LC-MS/MS during incubation of the compounds in mouse plasma. Both mitragynine and 7-OH were highly stable under the tested conditions. All data points represent the means of two independent experiments with two incubations per experiment, with error bars representing ±SEM.

Plasma Stability. To confirm that plasma metabolism does not play a role in mediating mitragynine's pharmacokinetics or analgesic activity, the stability of mitragynine and active metabolite 7-hydroxymitragynine was tested in mouse plasma. Both compounds were highly stable under the tested conditions.

Formation of 7-Hydroxymitragynine in Mouse Liver Microsomes

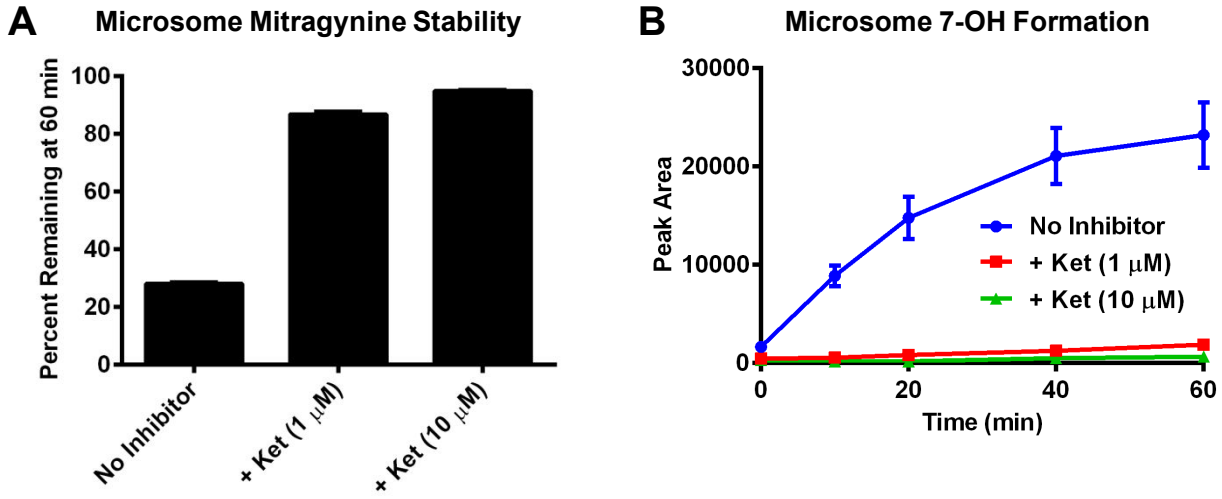
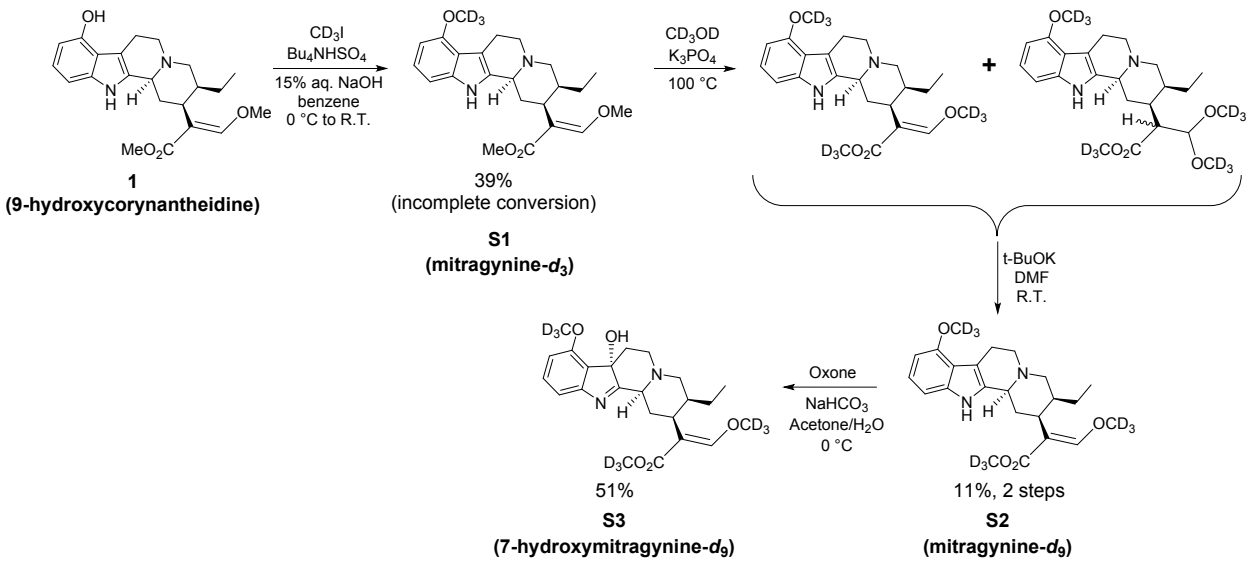


Figure S3. The CYP3A-dependence of 7-OH formation was confirmed in mouse liver microsomes (MLM). **(A)** In MLM, the CYP3A isoform inhibitor ketoconazole (Ket) attenuated metabolic decomposition of mitragynine. **(B)** During incubation of mitragynine with MLM, ketoconazole also inhibited formation of 7-OH. All data points represent the means of two independent experiments with two incubations per experiment, with error bars representing ±SEM.

Conversion of Mitragynine to 7-OH is Mediated by CYP3A in Mouse Liver Microsomes. We sought to confirm that 7-OH was also formed from mitragynine in mouse liver microsomes (MLM) and that this conversion was mediated by CYP3A. To that end, mitragynine was incubated with MLM alone and in the presence of the CYP3A inhibitor ketoconazole (1 or 10 μM). Ketoconazole robustly inhibited both decomposition of mitragynine (**Figure S3A**) and formation of 7-OH (**Figure S3B**).

Synthesis of Deuterated Mitragynine Analogs

Scheme S1. Synthesis of Deuterated Mitragynine Analogs



Synthesis of Deuterated Mitragynine Analogs. Mitragynine was demethylated as previously reported[1] to provide compound **1** (9-hydroxycorynantheidine), which served as the starting material for synthesis of deuterated analogs (**Scheme S1**). Phenol **1** was methylated with iodomethane-d_3 to give compound **2**. This material was then treated with methanol-d_4 under basic conditions to exchange the 2 methyl groups of the acrylate moiety with their fully deuterated counterparts. This procedure yielded a mixture of the desired compound **S2** and the corresponding dimethyl acetal, which both reduced yield and was difficult to separate. Accordingly, before purification, the crude mixture was treated with potassium tert-butoxide to induce elimination of the acetal byproduct and enrich the mixture in compound **S2**. Finally, **S2** was oxidized with Oxone monopersulfate to provide **S3**.

Synthetic Procedures

General Considerations. No unexpected or unusually high safety hazards were encountered. Reagents and solvents were obtained from commercial sources and were used

without further purification unless otherwise stated (including anhydrous solvents). All reactions were performed in flame-dried glassware under an argon atmosphere unless otherwise stated, and monitored by TLC using solvent mixtures appropriate to each reaction. All column chromatography was performed on silica gel (40-63μm). Preparative TLC was conducted on glass plates coated with a 1 mm silica layer. For compounds containing a basic nitrogen, Et_3N was often used in the mobile phase in order to provide better resolution. In these cases, TLC plates were pre-soaked in the Et_3N-containing solvent and then allowed to dry briefly before use in analysis (or separation in the case of preparative TLC), such that an accurate representation of R_f was obtained. Nuclear magnetic resonance spectra were recorded on 400 or 500 MHz instruments as indicated. Chemical shifts are reported as δ values in ppm referenced to $CDCl_3$ (^{1}H NMR = 7.26 and ^{13}C NMR = 77.16) or CD_3OD (^{1}H NMR = 3.31 and ^{13}C NMR = 49.00). Multiplicity is indicated as follows: s (singlet); d (doublet); t (triplet); q (quartet); dd (doublet of doublets); dt (doublet of triplets); td (triplet of doublets); m (multiplet); br (broad). All carbon peaks are rounded to one decimal place unless such rounding would cause two close peaks to become identical. In these cases, two decimal places are retained. Low-resolution mass spectra (LRMS) were recorded on a quadrupole mass spectrometer (ionization mode: APCI+). High-resolution mass spectra (HRMS) were recorded on a quadrupole-time-of-flight mass spectrometer (ionization mode: ESI+). The mass change due to loss of an electron is accounted for in calculated ion masses.

Mitragynine. Mitragynine free base was obtained by extraction from powdered *Mitragyna speciosa* leaves as previously described.[1] Spectral and physical properties of the material thus obtained were in agreement with those previously reported.[1]

7-Hydroxymitragynine (7-OH): PIFA Oxidation and Photooxidation. The procedures for [bis(trifluoroacetoxy)iodo]benzene (PIFA)-mediated and light-mediated oxidations of mitragynine to give 7-hydroxymitragynine have been previously reported.[1]

7-Hydroxymitragynine: Oxone Oxidation. To a solution of **mitragynine** (199 mg, 0.500 mmol) in acetone (15 mL), was added saturated aqueous $NaHCO_3$ (10 mL), and the mixture was cooled to 0 °C. A solution of Oxone monopersulfate ($2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$, MW = 615.5; 308 mg, 0.500 mmol) in water (5 mL) was then added dropwise over 25 minutes and the mixture left to stir at 0 °C for an additional 15 minutes. At this time, the reaction was diluted with water (60 mL) and extracted with EtOAc (3 x 30 mL). The combined organics were washed with brine (30 mL), dried over Na_2SO_4, and concentrated *in vacuo* to give the crude product as a pale-orange foam (170 mg). This material was purified by column chromatography (6:4 hexanes:EtOAc + 2% Et_3N) to provide pure **7-hydroxymitragynine** as an amorphous, pale-yellow solid (115 mg, 55%). Spectral and physical properties were in agreement with those previously reported.[1]

Methyl (*E*)-2-((2*S*,3*S*,12b*S*)-3-ethyl-8-hydroxy-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-2-yl)-3-methoxyacrylate (1 = 9-hydroxycorynantheindine). Compound **1** was prepared by demethylation of mitragynine according to previously described procedures.[1] Spectral and physical properties of the material thus obtained were in agreement with those previously reported.[1]

Methyl 2-((2*S*,3*S*,12b*S*)-3-ethyl-8-methoxy-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-2-yl)-3-hydroxyacrylate (2). Compound **2** was prepared by total synthesis according to previously described procedures.[1] Spectral and physical properties of the material thus obtained were in agreement with those previously reported.[1] **Note:** Compound **2** occurs as a complex mixture of the aldehyde tautomer and the *E* and *Z* enol tautomers.

(*E*)-2-((2*S*,3*S*,12b*S*)-3-Ethyl-8-methoxy-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-2-yl)-3-methoxyacrylic acid (3). To a solution of **mitragynine** (59.8 mg, 0.150 mmol) in MeOH (1.5 mL) was added water (1.5 mL) and K_3PO_4 (63.7 mg, 0.300 mmol) and the mixture was heated to 100 °C in a sealed vial for 23 h. At this time, the mixture was diluted with water (5 mL), extracted with Et_2O (3 x 5 mL), and the combined organics were washed with water (5 mL), dried over Na2SO4, and concentrated *in vacuo* to give a pale-yellow foam (43.4 mg). LRMS indicated that this crude material consisted of a mixture of the desired product, residual mitragynine, and mitragynine dimethylacetal. This material was purified by preparative TLC (9:1 acetone:MeOH, 20 x 20 cm plate) to give compound **3** as an amorphous, pale-tan solid (17.1 mg, 30%). **^{1}H NMR (500 MHz, CD_3OD)** δ 7.43 (s, 1H), 6.99 (t, *J* = 7.9 Hz, 1H), 6.93 (d, *J* = 7.9 Hz, 1H), 6.45 (d, *J* = 7.6 Hz, 1H), 3.84 (s, 3H), 3.79 (d, *J* = 11.8 Hz, 1H), 3.72 (s, 3H), 3.37 – 3.20 (m, 4H), 3.14 – 3.04 (m, 1H), 2.96 – 2.87 (m, 1H), 2.84 (dd, *J* = 12.3, 4.2 Hz, 1H), 2.56 – 2.45 (m, 1H), 2.39 – 2.28 (m, 1H), 1.94 – 1.84 (m, 1H), 1.68 – 1.55 (m, 1H), 1.50 – 1.38 (m, 1H), 0.91 (t, *J* = 7.4 Hz, 3H); **^{13}C NMR (126 MHz, CD_3OD)** δ 174.5, 160.8, 155.7, 139.6, 130.66, 123.6, 117.9, 114.2, 106.9, 105.8, 100.3, 62.7, 61.6, 57.6, 55.5, 54.5, 40.5, 39.1, 29.9, 23.0, 20.5, 12.9; HRMS (ESI+) calcd. for $C_{22}H_{29}N_2O_4^+$ $[M+H]^+$ 385.2122, found 385.2123.

Methyl (*E*)-2-((2*S*,3*S*,12b*S*)-3-ethyl-8-(methoxy-*d*$_3$)-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-2-yl)-3-methoxyacrylate (S1 = mitragynine-*d*$_3$). To a solution of **1** (1.54 g, 4.00 mmol) in benzene (145 mL) at room temperature was added 15% m/m aqueous NaOH (3.20 g of solution, 12.00 mmol), tetrabutylammonium hydrogensulfate (407 mg, 1.20 mmol), and iodomethane-*d*$_3$ (305 µL, 696 mg, 4.80 mmol) and the mixture was stirred for 2 h. Additional iodomethane-*d*$_3$ (76 µL, 174 mg, 1.20 mmol) was then added and stirring was continued for another 2 h. The mixture was then diluted with CH_2Cl_2 (150 mL), washed with water (2 x 100 mL) and brine (50 mL), dried over Na_2SO_4, and concentrated to give a foamy solid (1.71 g). This material was purified by column chromatography (7:3 hexanes:EtOAc + 2% Et_3N, 4 column volumes → 1:1 hexanes:EtOAc + 2% Et_3N, 4 column volumes) to provide both unconverted starting material (610 mg) and the pure product **S1 (mitragynine-*d*$_3$)** as a tan foam (629 mg, 39%). **^{1}H NMR (500 MHz, $CDCl_3$)** δ 7.66 (br s, 1H), 7.43 (s, 1H), 6.99 (t, *J* = 7.9 Hz, 1H), 6.90 (dd, *J* = 8.1, 0.7 Hz, 1H), 6.45 (dd, *J* = 7.7, 0.7 Hz, 1H), 3.73 (s, 3H), 3.71 (s, 3H), 3.19 – 3.08 (m, 2H), 3.07 – 2.99 (m, 2H), 2.99 – 2.94 (m, 1H), 2.94 – 2.89 (m, 1H), 2.57 – 2.48 (m, 2H), 2.45 (ddd, *J* = 11.5, 3.4, 1.3 Hz, 1H), 1.84 – 1.73 (m, 2H), 1.65 – 1.59 (m, 1H), 1.25 – 1.15 (m, 1H), 0.87 (t, *J* = 7.4 Hz, 3H); **^{13}C NMR (126 MHz, $CDCl_3$)** δ 169.4, 160.7, 154.6, 137.4, 133.8, 121.8, 117.7, 111.6, 107.8, 104.3, 99.7, 61.6, 61.4, 57.8, 54.6 (h, J_{CD} = 21 Hz), 53.9, 51.5, 40.8, 40.1, 30.0, 24.1, 19.2, 13.0; HRMS (ESI+) calcd. for $C_{23}H_{28}D_3N_2O_4^+$ $[M+H]^+$ 402.2467, found 402.2468.

Methyl-*d*$_3$ (*E*)-2-((2*S*,3*S*,12b*S*)-3-ethyl-8-(methoxy-*d*$_3$)-1,2,3,4,6,7,12,12b-octahydroindolo[2,3-*a*]quinolizin-2-yl)-3-(methoxy-*d*$_3$)acrylate (S2 = mitragynine-*d*$_9$). Compound **S1** (402 mg, 1.00 mmol) and K_3PO_4 (425 mg, 2.00 mmol) were combined in methanol-*d*$_4$ (8.0 mL) and the mixture was heated to 100 °C in a sealed vial and stirred for 25 h (NMR confirmed exchange of methyl groups for deuterated methyl groups). The reaction mixture was then diluted with CH_2Cl_2 (80 mL), washed with saturated aqueous $NaHCO_3$ (40 mL) and water (40 mL), dried over Na_2SO_4, and concentrated to provide the crude, deuterated acetal intermediate as a tan foam (299 mg). This material was combined with potassium tert-butoxide (302 mg, 2.69 mmol, ~4 equivalents), anhydrous DMF (42 mL) was added, and the mixture was left to stir at room temperature for 19 h (**Note:** conversion of acetal to product may be monitored by NMR, but conversion is incomplete). The reaction was then poured into water (250 mL) and extracted with Et_2O (200 mL, 2 x 100 mL, 50 mL). The combined organics were washed with water (2 x 50 mL) and brine (50 mL), dried over Na_2SO_4, and concentrated to give a pale-orange foam (159 mg). This material was split in two and each half purified by repeated preparative TLC

(7:3 hexanes:EtOAc + 2% Et_3N, 20 x 20 cm plates, for each plate solvent was run up 2x to provide additional resolution) to provide the pure product **S2** as a pale-yellow foam (44.6 mg, 11% over 2 steps). **^{1}H NMR (500 MHz, $CDCl_3$)** δ 7.77 (br s, 1H), 7.43 (s, 1H), 6.99 (t, *J* = 7.9 Hz, 1H), 6.90 (d, *J* = 8.0 Hz, 1H), 6.45 (d, *J* = 7.7 Hz, 1H), 3.19 – 3.08 (m, 2H), 3.07 – 3.00 (m, 2H), 3.00 – 2.95 (m, 1H), 2.92 (dd, *J* = 11.3, 5.7 Hz, 1H), 2.58 – 2.48 (m, 2H), 2.48 – 2.43 (m, 1H), 1.86 – 1.74 (m, 2H), 1.67 – 1.60 (m, 1H), 1.26 – 1.15 (m, 1H), 0.87 (t, *J* = 7.4 Hz, 3H); **^{13}C NMR (126 MHz, $CDCl_3$)** δ 169.4, 160.6, 154.7, 137.4, 133.9, 121.9, 117.8, 111.7, 108.0, 104.3, 99.9, 61.4, 60.8 (h, J_{CD} = 23 Hz), 57.9, 54.6 (h, J_{CD} = 23 Hz), 53.9, 50.7 (h, J_{CD} = 21 Hz), 40.9, 40.1, 30.1, 24.1, 19.3, 13.0; HRMS (ESI+) calcd. for $C_{23}H_{22}D_9N_2O_4^+$ $[M+H]^+$ 408.2843, found 408.2842.

Methyl-*d*$_3$ (*E*)-2-((2*S*,3*S*,7a*S*,12b*S*)-3-ethyl-7a-hydroxy-8-(methoxy-*d*$_3$)-1,2,3,4,6,7,7a,12b-octahydroindolo[2,3-*a*]quinolizin-2-yl)-3-(methoxy-*d*$_3$)acrylate (S3 = 7-hydroxymitragynine-*d*$_9$). To a solution of compound **S2** (53.0 mg, 0.130 mmol) in acetone (3.9 mL) was added saturated aqueous $NaHCO_3$ (2.6 mL) and the cloudy mixture was cooled to 0 °C. A solution of Oxone monopersulfate ($2KHSO_5 \cdot KHSO_4 \cdot K_2SO_4$, MW = 615.5; 80.0 mg, 0.130 mmol) in water (1.3 mL) was then added dropwise over 15 min. and the reaction was stirred for an additional 30 min. at 0 °C. The mixture was then diluted with water (20 mL) and extracted with EtOAc (3 x 10 mL). The combined organics were washed with brine (5 mL), dried over Na_2SO_4, and concentrated to give a pale-yellow foam (40.7 mg). This material was purified by preparative TLC (1:1 hexanes:EtOAc + 2% Et_3N, 20 x 20 cm plate) to give the pure product **S3** as a pale-yellow foam (28.2 mg, 51%). **^{1}H NMR (500 MHz, $CDCl_3$)** δ 7.43 (s, 1H), 7.27 (t, *J* = 7.9 Hz, 1H), 7.19 (d, *J* = 7.6 Hz, 1H), 6.70 (d, *J* = 8.2 Hz, 1H), 3.12 (dd, *J* = 11.1, 2.5 Hz, 1H), 3.07 – 2.96 (m, 2H), 2.85 – 2.74 (m, 2H), 2.68 – 2.59 (m, 2H), 2.52 – 2.45 (m, 1H), 1.87 (dt, *J* = 13.6, 3.1 Hz, 1H), 1.77 – 1.55 (m, 3H), 1.30 – 1.18 (m, 1H), 0.82 (t, *J* = 7.3 Hz, 3H); **^{13}C NMR (126 MHz, $CDCl_3$)** δ 184.4, 169.4, 160.8, 156.0, 155.2, 130.8, 126.7, 114.3, 111.4, 109.0, 81.1, 61.6, 61.0 (h, J_{CD} = 21 Hz), 58.3, 54.8 (h, J_{CD} = 23 Hz), 50.5 (h, J_{CD} = 23 Hz), 50.2, 40.7, 39.4, 35.9, 26.2, 19.1, 13.0; HRMS (ESI+) calcd. for $C_{23}H_{22}D_9N_2O_5^+$ $[M+H]^+$ 424.2792, found 424.2791.

Biological Procedures

No unexpected or unusually high safety hazards were encountered.

BRET Functional Assays

Materials. HEK-293T cells were obtained from the American Type Culture Collection (Rockville, MD) and were cultured in a 5% CO_2 atmosphere at 37°C in Dulbecco's Modified Eagle Medium (high glucose #11965; Life Technologies Corp.; Grand Island, NY) supplemented with 10% FBS (Premium Select, Atlanta Biologicals; Atlanta, GA), 100 U/mL penicillin, and 100 µg/mL streptomycin (#15140, Life Technologies).

DNA Constructs. The human MOR (hMOR) was obtained from the Missouri S&T Resource Center. The human G protein constructs used here have been previously described and were provided by C. Galés or were obtained from the Missouri S&T Resource Center unless otherwise noted.[2,3] The G proteins used included untagged $G\alpha_{oB}$ with Renilla luciferase 8 (RLuc8) inserted at position 91 ($G\alpha_{oB}$-RLuc8); $G\beta_1$ (β_1); $G\gamma_2$ which we fused to the full-length mVenus at its *N*-terminus via the amino acid linker GSAGT (mVenus-γ2). All constructs were sequence-confirmed prior to use in experiments.

Transfection. The following cDNA amounts were transfected into HEK-293T cells (5 x 10^6 cells/plate) in 10-cm dishes using polyethylenimine (PEI) in a 1:1 ratio (diluted in Opti-MEM,

Life Technologies): 2.5 μg hMOR, 0.125 μg $G\alpha_{oB}$-RLuc8, 6.25 μg β_1, 6.25 μg mVenus-γ2. Cells were maintained in the HEK-293T media described above. After 24 hours the media was changed, and the experiment was performed 24 hours later (48 hours after transfection).

BRET. Transfected cells were dissociated and re-suspended in phosphate-buffered saline (PBS). Approximately 200,000 cells/well were added to a black-framed, white-well, 96-well plate (#60050; Perkin Elmer; Waltham, MA). The microplate was centrifuged and the cells were re-suspended in PBS. After 5 minutes, 5 μM of the luciferase substrate coelenterazine H was added to each well. After 5 minutes, ligands were added and the BRET signal was measured 5 minutes later on a PHERAstar FS plate reader. The BRET signal was quantified by calculating the ratio of the light emitted by the energy acceptor, mVenus (510-540 nm), over the light emitted by the energy donor, RLuc8 (485 nm). This drug-induced BRET signal was normalized using the E_{max} of [D-Ala^2, *N*-Me-Phe^4, Gly-ol^5]-enkephalin (DAMGO) as the maximal response at hMOR. Dose response curves were fit using a three-parameter logistic equation in GraphPad Prism 6.

In Vitro **Metabolism Studies**

Microsomes. Human microsomes were from pooled donors (XenoTech H0630, Lot N1210097 or Bioreclamation IVT #X008069, Lot HPH). Mouse liver microsomes were isolated from pooled (50), perfused livers of BALB/c male mice according to the standard protocol.[4]

Microsomal Stability. Stability studies in liver microsomes were performed by Bienta, Kiev, Ukraine. Microsomal incubations (human or mouse) were carried out in 96-well plates in 5 aliquots of 40 μL each (one for each time point). Liver microsomal incubation medium consisted of PBS (100 mM, pH 7.4), MgCl2 (3.3 mM), NADPH (3 mM), glucose-6-phosphate (5.3 mM), and glucose-6-phosphate dehydrogenase (0.67 units/mL), with 0.42 mg of liver microsomal protein per mL. Control incubations were performed by replacing the NADPH-cofactor system with PBS. Test compounds (2 μM, final solvent concentration 1.6 %) were incubated with microsomes at 37 °C, shaking at 100 rpm. Five time points over 40 minutes were analyzed. The reactions were stopped by adding 12 volumes of 90% acetonitrile-water to incubation aliquots, followed by protein sedimentation by centrifugation at 5500 rpm for 3 minutes. Supernatants were analyzed for parent compound remaining and metabolites as indicated using a fit-for-purpose liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, with authentic samples of each analyte used for calibration and identification. The relative percent remaining of parent compound in each sample was calculated by considering the raw peak area of the analyte in the 0-minute sample as 100%. The concentration of the metabolite 7-hydroxymitragynine (7-OH) in mitragynine incubations was calculated using analyte peak area in conjunction with a calibration curve created using reference samples of the analyte. Two independent experiments were performed with each incubation performed in duplicate for each time point. Control incubations with imipramine and propranolol were performed in each experiment to confirm microsome activity.

Plasma Stability. Stability studies in plasma were performed by Bienta, Kiev, Ukraine. Mouse plasma (with Na-EDTA, Lampire Biological Labs, USA, #7304309 or Li heparin, Bienta/Enamine Ltd.) incubations were carried out in 5 aliquots of 70 μL each (one for each time point). Test compounds (1 μM, final DMSO concentration 1%) were incubated at 37 °C with shaking at 100 rpm. Five time points over 120 minutes were analyzed. The reactions were stopped by adding 420 μL of acetonitrile-water mixture (90:10) with subsequent plasma protein sedimentation by centrifugation at 5500 rpm for 5 minutes. Supernatants were analyzed for parent compound remaining using a fit-for-purpose liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, with authentic samples of the analytes used for calibration and identification. The relative percent remaining of parent compound in each sample was calculated

by considering the raw peak area of the analyte in the 0-minute sample as 100%. Two independent experiments were performed with each incubation performed in duplicate for each time point. Control incubations with propantheline and verapamil were performed in each experiment to confirm plasma metabolic activity.

CYP Reaction Phenotyping. Cytochrome P450 (CYP) reaction phenotyping was performed by Bienta, Kiev, Ukraine. Mitragynine was incubated with recombinant human CYP enzymes and disappearance of the parent and formation of the metabolite 7-OH were monitored. The following Corning Supersomes™ preparations were used: Human CYP3A4 + Oxidoreductase + b5 (#456202), Human CYP2D6*1 (Val374) + Oxidoreductase (Corning, #456217), Human CYP2C9*1 (Arg144) + Oxidoreductase (Corning, #456218), Human CYP1A2 + Oxidoreductase (Corning, #456203), Human CYP2C19 + Oxidoreductase (Corning, #456219).

Incubations with individual human CYPs (1A2, 3A4, 2C9, 2C19, 2D6) were carried out in 96-well plates in 4 aliquots of 40 µL each (one for each time point). The incubation medium consisted of PBS (100 mM, pH 7.4), MgCl2 (3.3 mM), NADPH (3 mM), glucose-6-phosphate (5.3 mM), and glucose-6-phosphate dehydrogenase (0.67 units/mL) with 30 pmol of each enzyme per mL. Control incubations were performed by replacing the NADPH-cofactor system with PBS. Mitragynine or reference specific substrates (2 µM, final solvent concentration 1.6 %; 1A2 – phenacetin, 3A4 – testosterone, 2C9 – diclofenac, 2C19 – omeprazole, 2D6 – dextromethorphan) were incubated with each cytochrome at 37°C, shaking at 100 rpm. Four time points over 60 minutes were analyzed. The reactions were stopped by adding 3 volumes of acetonitrile to the incubation aliquots, followed by protein sedimentation by centrifugation at 5500 rpm for 3 minutes. Supernatants were analyzed for mitragynine remaining and 7-OH formed using a fit-for-purpose liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, with authentic samples of the analytes used for calibration and identification. The relative percent remaining of parent compound in each sample was calculated by considering the raw peak area of the analyte in the 0-minute sample as 100%. Formation of 7-OH was determined and reported as raw peak area of the analyte. Two independent experiments were performed with each incubation performed in duplicate for each time point.

CYP Reaction Phenotyping in Microsomes. CYP phenotyping was confirmed in human and mouse liver microsomes using selective CYP inhibitors. Human microsomes were from pooled donors (XenoTech H0630, Lot N1210097 or 1610016). Mouse liver microsomes were isolated from pooled (50), perfused livers of BALB/c male mice according to the standard protocol (Hill, J.R. in *Current Protocols in Pharmacology* 7.8.1-7.8.11, Wiley Interscience, 2003).

Microsomal incubations (human or mouse) were carried out in 96-well plates in 5 aliquots of 40 µL each (one for each time point). Liver microsomal incubation medium consisted of PBS (100 mM, pH 7.4), MgCl2 (3.3 mM), NADPH (3 mM), glucose-6-phosphate (5.3 mM), and glucose-6-phosphate dehydrogenase (0.67 units/mL), with 0.42 mg of liver microsomal protein per mL. Control incubations were performed by replacing the NADPH-cofactor system with PBS. Mitragynine alone (2 µM, final solvent concentration 1.6 %) or in the presence of selective CYP inhibitors (**Human:** ketoconazole – 1, 10 µM, ticlopidine – 20 µM, ketoconazole + ticlopidine – 1 + 20 µM; **Mouse:** ketoconazole – 1, 10 µM) was incubated with microsomes at 37 °C, shaking at 100 rpm. Five time points over 60 minutes were analyzed. The reactions were stopped by adding 3 volumes of acetonitrile (human) or 6 volumes of 90% acetonitrile-water (mouse) to the incubation aliquots, followed by protein sedimentation by centrifugation at 5500 rpm for 3 minutes. Supernatants were analyzed for mitragynine remaining and 7-OH formed using a fit-for-purpose liquid chromatography-tandem mass spectrometry (LC-MS/MS) method, with authentic samples of the analytes used for calibration and identification. The relative percent remaining of parent compound in each sample was calculated by considering the raw peak area of the analyte in the 0-minute sample as 100%. Formation of 7-OH was determined and reported as raw peak area of

the analyte. Two independent experiments were performed with each incubation performed in duplicate for each time point.

Analgesia and Pharmacokinetic (PK) Studies

Note on Mouse Strains. 129S mice of two different sub-strains were used – for analgesic dose-response studies and *in vivo* metabolic studies, we used 129S1 mice; for analgesic studies in knockout animals, 129S6 mice were used as the wild-type to exactly match the genetic background of the knockout animals.

Animals. For analgesic dose-response experiments and PK studies, male 129S1 mice, 7 weeks of age, were purchased from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in groups of five and allowed to acclimate for 1 week prior to testing. Mice had *ad libitum* access to food and water and were maintained on a 12-hour light/dark cycle. All testing was done in the light cycle.

For analgesic testing in knockout animals, wild-type, male 129S6 mice (24−38 g) were purchased from the Jackson Laboratory (Bar Harbor, ME). Exon-1/Exon-11 MOR-1 KO mice[5] were bred in the Pasternak laboratory at Memorial Sloan Kettering, while DOR-1 and KOR-1 KO mice on a 129 background were bred in the Pintar laboratory at Rutgers University. All mice used were opioid naïve. All mice were maintained on a 12-hour light/dark cycle with food and water available *ad libitum*, and housed in groups of five until testing. All testing was done in the light cycle.

All study protocols were approved by the Institutional Animal Care and Use Committees of Columbia University and the New York State Psychiatric Institution, Memorial Sloan Kettering Cancer Center, or Rutgers University, and were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Care was taken to minimize the number and suffering of animals.

Drugs. Mitraygnine, mitragynine-d_3, 7-hydroxymitragynine (7-OH), and 7-hydroxymitragynine-d_9 were obtained as described above. Selective opioid antagonists were purchased from Tocris Bioscience. Mitragynine and 7-OH were dissolved in ddH_2O, 2 molar equivalents of glacial acetic acid, and 1.25% *N*-methyl-2-pyrrolidone as a vehicle. Mitraygnine and 7-OH were administered at the indicated doses subcutaneously (s.c.) or by oral gavage (p.o.) at a volume of 10 mL/kg body weight. In tail-flick dose-response experiments, mitragynine and 7-OH were administered by the indicated route and in an ascending, cumulative dose fashion, with 1 hour between injections for both compounds.

Tail-Flick (Dose-Response). Analgesic activity was assessed in the tail-flick test 15 minutes (peak effect) after each dose of drug, followed by a 1-hour break before administration of the next higher dose. In this test, aversive heat is focused on the tail of a lightly restrained animal using a Tail-Flick Analgesia Meter (IITC Life Science Inc., Woodland Hills, CA). A cutoff time of 10 seconds was used to prevent tissue damage. Flicks were measured automatically at the time of tail withdrawal and heat intensity was set such that basal tail flicks were produced at 1.5-3 seconds. Baseline tail flicks were collected 1 hour before injection time. Data were analyzed as percent maximal effect, %MPE, which was calculated according to the formula: % MPE [(observed latency − baseline latency)/(maximal latency − baseline latency)] x 100. Dose–response curves were fit with a nonlinear regression (GraphPad Prism, La Jolla, CA), then compared using an extra-sum-of-squares F test to find the ED_{50}.

Tail-Flick (KO Animals and Naloxone Inhibition). Analgesia was tested in wild-type and KO animals by the radiant heat tail-flick technique using an Ugo Basile model 37360 instrument,

as previously described.[6,7] The intensity was set to achieve a baseline between 2 and 3 seconds. Baseline latencies were determined before experimental treatments for all mice. Tail-flick analgesia was assessed quantally as a doubling or greater of the baseline latency, with a maximal 10 second latency to minimize tissue damage to the tail. Data were analyzed as percent maximal effect, %MPE, which was calculated according to the formula: % MPE [(observed latency − baseline latency)/(maximal latency − baseline latency)] x 100. Compounds were administered subcutaneously (s.c.) or by oral gavage (p.o.), as indicated in the figures, and analgesia was assessed at the peak effect (15 minutes). For the antagonism studies, naloxone (1 mg/kg, s.c.) was administered 15 minutes prior to test drug administration.

Tail-Flick (PK Experiment). Equianalgesic doses (~1.3-fold ED_{50}) of mitragynine (140 mg/kg, s.c.) and 7-OH (0.7 mg/kg, s.c.) in 129S1 mice were selected base on the previously determined dose-response of each compound. On the day of PK sample collection, the selected doses were administered to independent groups of mice and equianalgesic activity was confirmed in the tail-flick test 15 minutes after drug administration. In this test, aversive heat is focused on the tail of a lightly restrained animal using a Tail-Flick Analgesia Meter (IITC Life Science Inc., Woodland Hills, CA). A cutoff time of 10 seconds was used to prevent tissue damage. Flicks were measured automatically at the time of tail withdrawal and heat intensity was set such that basal tail flicks were produced at 1.5-3 seconds. Baseline tail flicks were collected 1 hour before injection time.

PK Sample Collection. Animals were euthanized using cervical dislocation at 15 minutes or 60 minutes after drug administration, or immediately after recording the tail-flick response of each animal (15 minutes post drug administration), as indicated in the figures. Immediately after sacrifice, trunk blood was collected (~100 µl) into EDTA tubes (Greiner Bio-One, Monroe, NC) and whole brains were dissected out and stored at -80 °C for later analysis.

PK Sample Preparation. Mouse brain tissue was weighed and homogenized in LC-MS grade water. Samples (25 uL) of plasma or brain homogenate were spiked with internal standards (mitragynine-d_3 and 7-hydroxymitragynine-d_9) and the protein was precipitated by mixing with acetonitrile: methanol (3:1) followed by centrifugation at 13,500 x g for 8 minutes. The supernatant was used for LC-MS analysis.

LC-MS/MS. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of samples was conducted using an Agilent 6410 triple quadrupole mass spectrometer connected to an Agilent 1290 Infinity UHPLC (Agilent Technologies, Santa Clara, CA). Data acquisition and peak integration was performed using MassHunter software v 3.1. Five microliters of each sample were injected onto an Agilent Poroshell C18 column (50x2.1mm, 2.7u, 100A) maintained at 40 °C and a flow rate of 0.5 mL/min. Initial gradient conditions were 90% mobile phase A (0.1% formic acid in water) and 10% mobile phase B (0.1% formic acid in acetonitrile). Solvent B was increased linearly to 50% by 4.75 minutes and to 95% by 5 minutes, held for 1 minute and brought back to initial conditions by 6.25 minutes, with a total run time of 7.50 minutes. MS/MS analysis was performed using positive electrospray ionization under MRM mode with the following optimized parameters: capillary voltage 2 kV, gas temperature 350 °C; gas flow 13 L/min; nebulizer 60 psi. MRM transitions were as follows: mitragynine: 399.2>235.1; 7-OH: 415.2>190.1. Concentrations were quantitated by comparing integrated peak areas of each species against those of known amounts of purified standards. Lower limit of quantitation (LLOQ) in plasma was 0.5 ng/mL for both mitragynine and 7-OH. LLOQ in brain was 5 ng/g for mitragynine and 1 ng/g for 7-OH.

References

(1) Kruegel, A. C.; Gassaway, M. M.; Kapoor, A.; Váradi, A.; Majumdar, S.; Filizola, M.; Javitch, J. A.; Sames, D. Synthetic and Receptor Signaling Explorations of the *Mitragyna* Alkaloids: Mitragynine as an Atypical Molecular Framework for Opioid Receptor Modulators. *J. Am. Chem. Soc.* **2016**, *138* (21), 6754–6764 DOI: 10.1021/jacs.6b00360.

(2) Rives, M.-L.; Rossillo, M.; Liu-Chen, L.-Y.; Javitch, J. A. 6'-Guanidinonaltrindole (6'-GNTI) Is a G Protein-Biased κ-Opioid Receptor Agonist That Inhibits Arrestin Recruitment. *J. Biol. Chem.* **2012**, *287* (32), 27050–27054 DOI: 10.1074/jbc.C112.387332.

(3) Negri, A.; Rives, M.-L.; Caspers, M. J.; Prisinzano, T. E.; Javitch, J. A.; Filizola, M. Discovery of a Novel Selective Kappa-Opioid Receptor Agonist Using Crystal Structure-Based Virtual Screening. *J. Chem. Inf. Model.* **2013**, *53* (3), 521–526 DOI: 10.1021/ci400019t.

(4) Hill, J. R. In Vitro Drug Metabolism Using Liver Microsomes. In *Current Protocols in Pharmacology*; John Wiley & Sons, Inc.: Hoboken, NJ, USA, 2004; Vol. 23, p 7.8.1-7.8.11.

(5) Lu, Z.; Xu, J.; Rossi, G. C.; Majumdar, S.; Pasternak, G. W.; Pan, Y.-X. Mediation of Opioid Analgesia by a Truncated 6-Transmembrane GPCR. *J. Clin. Invest.* **2015**, *125* (7), 2626–2630 DOI: 10.1172/JCI81070.

(6) Váradi, A.; Hosztafi, S.; Le Rouzic, V.; Tóth, G.; Urai, Á.; Noszál, B.; Pasternak, G. W.; Grinnell, S. G.; Majumdar, S. Novel 6β-Acylaminomorphinans with Analgesic Activity. *Eur. J. Med. Chem.* **2013**, *69*, 786–789 DOI: 10.1016/j.ejmech.2013.09.031.

(7) Váradi, A.; Marrone, G. F.; Eans, S. O.; Ganno, M. L.; Subrath, J. J.; Le Rouzic, V.; Hunkele, A.; Pasternak, G. W.; McLaughlin, J. P.; Majumdar, S. Synthesis and Characterization of a Dual Kappa-Delta Opioid Receptor Agonist Analgesic Blocking Cocaine Reward Behavior. *ACS Chem. Neurosci.* **2015**, *6* (11), 1813–1824 DOI: 10.1021/acschemneuro.5b00153.