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Supplementary Materials for

A bifunctional nociceptin and mu opioid receptor agonist is analgesic without opioid side effects in nonhuman primates

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Other Supplementary Material for this manuscript includes the following:

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Supplementary Materials and Methods

Supplementary Methods

Chemical synthesis procedures for 3-substituted spiroisoquinolinones compounds

General experimental detail: Thin layer chromatography (TLC) was performed on Analtech silica gel GF 250 micron TLC plates. The plates were visualized with a 254 nm ultraviolet (UV) light and staining with iodine. Flash chromatography was carried out on F60 silica gel, 230-400 mesh, 60 Å (Silicycle SiliaFlash). Nuclear magnetic resonance (NMR) was recorded on a Varian Mercury Plus NMR (300 megahertz (MHz)), Varian Mercury 300 (300 MHz), or Varian 400 (400 MHz) using deuteriochloroform ($CDCl_3$), deuterated dimethylsulfoxide (DMSO-d_6) or deuteriomethanol (MeOD-d_4). Mass spectra were obtained on a LCQ Fleet Ion Trap liquid chromatography/mass spectrometer (LC/MS), a micromass ZMD 1000 or PE Sciex API 150EX LC/MS using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) mode. Elemental analyses were performed by Atlantic Microlabs, Norcross, GA. For all final compounds, high pressure liquid chromatography (HPLC) analysis was performed on a reverse phase Agilent Zorbax SB-Phenyl column (5 µm, 2.1 x150 mm), using a binary gradient of 95:5 solvent A (95/5 H_2O/ACN + 0.1% formic acid):solvent B (5/95 H_2O/ACN + 0.1% formic acid) (linear gradient 0% B to 100%B) for 10 minutes, at a flow rate of 0.4 mL/min. Eluted peaks were monitored at 254 nm with a Shimadzu SPD-10AVP ultraviolet-visible (UV-Vis) detector. All final compounds tested were confirmed to be of >95% purity by the HPLC method described above.

Syn-2-(1'-(4-isopropylcyclohexyl)-3-oxo-1H-spiro[isoquinoline-4,4'-piperidin]-2(3H)-yl)acetonitrile (1): To a solution of **I-1** (fig. S1)**,** prepared as described previously (*73*), (1.65 g,

4.84 mmol) in 40 ml of tetrahydrofuran (THF) under an argon atmosphere, was added, in portions 60% sodium hydride (NaH) in mineral oil (0.969 g, 24.2 mmol) and the mixture was stirred at room temperature for 0.5 h. The mixture was cooled in an ice bath and a solution of bromoacetonitrile (1.74 g, 14.5 mmol) in 20 ml of THF was added dropwise over 0.25 h and allowed to come to room temperature and stirred for 14 h. The mixture was treated with saturated sodium bicarbonate and extracted with ethyl acetate, dried over magnesium sulfate, and evaporated to dryness. Purification by chromatography on silica gel eluting with methanol/ethyl acetate/hexane/ammonium hydroxide (2:49:49:0.1) afforded 1.31 g of **1,** 71% yield. A portion of the free base was converted to the hydrochloride salt. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.2 (1H, m), 7.51 (1H, d, 6 Hz), 7.41 (1H, t, J = 6Hz), 7.35 (1H, t, 6 Hz), 7.34 (1H, d, J = 6Hz), 4.74 (2H, s), 4.56 (2H, s), 3.4-3.5 (4H, m), 3.2 (1H, m), 2.18 (2H, d, J = 11 Hz), 1.84 (4H, m), 1.68 (4H, m), 1.41 (2H, m), 1.14 (2H, m), 0.88 (6H, d, J = 5Hz). MS m/z 380 $(M+H)^+$. Anal. ($C_{24}H_{33}N_3O$•HCl•1.4H_2O) Calc: C, 65.33; H, 8.41; N, 9.52. Found: C, 65.09; H, 8.15; N, 9.26.

Syn-2-(2-aminoethyl)-1'-(4- isopropylcyclohexyl)-1,2-dihydro-3H-spiro[isoquinoline-4,4'-piperidin]-3-one (2): To a solution of **1** (1.37 g, 3.61 mmol) dissolved in 30 ml of methanol and 3.3 ml of concentrated hydrochloric acid was added platinum oxide hydrate (178 mg) and stirred under an atmosphere of hydrogen gas at 50^{o}C for 3 h. The mixture was cooled to room temperature, filtered through Celite, and evaporated to dryness. The residue was purified by chromatography on silica gel eluting with methanol/dichloromethane/ammonium hydroxide (11:89:0.1) which afforded 1.37 g of **2**, in a 90% yield. A portion of the free base was converted to the hydrochloride salt. ^{1}H NMR (300 MHz, DMSO-d_6) δ 10.6 (1H, m), 8.06 (3H, m), 7.54 (1H, d, J = 6 Hz), 7.38 (1H, t, 6 Hz), 7.32 (1H, t, J = 6 Hz), 7.26 (1H, d, J = 6 Hz), 4.68 (2H, s),

3.68 (2H, m), 3.45 (3H, m), 3.18 (2H, m), 3.03 (2H, m), 2.23 (2H, d, J = 11 Hz), 1.87 (4H, d, J = 8 Hz), 1.67 (3H, m), 1.41 (2H, m), 1.15 (1H, m), 0.88 (6H, d, J = 5 Hz). MS m/z 384 $(M+H)^+$. Anal. ($C_{24}H_{37}N_3O$• 2HCl•1.9H_2O) Calc: C, 58.74; H, 8.79; N, 8.56. Found: C, 58.47; H, 8.48; N, 8.25.

Syn-phenyl-(N-(2-(1'-(4-isopropylcyclohexyl)-3-oxo-1H-spiro[isoquinoline-4,4'-piperidin]-2(3H)-yl)ethyl)sulfamoyl)carbamate (I-2): A solution of chlorosulfonyl isocyanate (0.76 g, 5.4 mmol) in 20 ml of dichloromethane was cooled in an ice bath under an argon atmosphere and treated with benzyl alcohol (0.58 g, 5.4 mmol). After stirring for 0.25 h, the mixture was added to a solution of **2** (1.29 g, 3.36 mmol) in 20 ml of dichloromethane containing triethylamine (0.68 g, 6.72 mmol) which was cooled in an ice bath under an argon atmosphere. The resultant mixture was stirred at 5^{o}C for 1 h and then at room temperature for 14 h. The mixture was treated with saturated sodium bicarbonate, extracted with dichloromethane, dried over magnesium sulfate, and evaporated to dryness. Purification by chromatography on silica gel eluting with methanol/dichloromethane/ammonium hydroxide (3:97:0.1) afforded 1.68 g of **I-2**, 84% yield. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.28-7.38 (6H, m), 7.11-7.25 (3H, m), 6.94 (1H, m), 5.27 (1H, m), 5.07 (2H, s), 4.31 (1H, m), 3.57 (3H, m), 3.2 (4H, m), 3.0 (1H, m), 2.35 (1H, m), 2.04 (2H, m), 1.87 (5H, m), 1.58 (3H, m), 1.31 (2H, m), 1.18 (1H, m), 0.89 (6H, d, J = 5 Hz). MS m/z 597 $(M+H)^+$.

Syn-N-(2-{3-oxo-1'-[4-(propan-2-yl)cyclohexyl]-2,3-dihydro-1H-spiro[isoquinoline-4,4'-piperidine]-2-yl}ethyl)aminosulfamide (5): To a solution of **I-2** (1.51 g, 2.53 mmol) dissolved in 80 ml of methanol and 10 ml of 7N ammonia in methanol was added 10% Pd/C (150 mg) and

stirred under an atmosphere of hydrogen gas for 4 h. The mixture was filtered through Celite and evaporated to dryness. The residue was purified by chromatography eluting with methanol/ethyl acetate/hexane/ammonium hydroxide (14:43:43:0.1) afforded 0.625 g of **5**, 40% yield **.** ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.51 (1H, d, J = 6 Hz), 7.33 (1H, t, J = 6Hz), 7.25 (1H, t, J = 6Hz), 7.18 (1H, d, J = 6 Hz), 5.2 (1H, m), 4.57 (2H, s), 3.74 (2H, t, J = 4 Hz), 3.38 (2H, t, J = 4 Hz), 2.81 (3H, m), 2.33 (2H, m), 2.23 (2H, m), 2.04 (2H, m), 1.71 (2H, m), 1.59 (6H, m), 1.36 (2H, m), 1.12 (1H, m), 0.87 (6H, d, 5 Hz). MS m/z 463 $(M+H)^+$. A portion of the base was converted to the hydrochloride salt. Anal. ($C_{24}H_{38}N_4O_3S$•HCl•0.6H_2O) Calc: C, 56.53; H, 7.95; N, 10.99. Found: C, 56.24; H, 7.73; N, 10.87.

In vitro pharmacological characterization methods

Cells. Human NOP, MOP, DOP, and KOP receptors were individually expressed in Chinese hamster ovary cells stably transfected with the human receptor cDNA, as we have described previously (*27*, *40*). The hNOP, hDOP, hKOP-FLAG19 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.5% penicillin/streptomycin, in 150-mm tissue culture dishes. The hKOP-CN cells were grown in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, in the presence of 0.5% penicillin/streptomycin and no G418. The hMOP cells in 50% F12/DMEM with 10% fetal bovine serum, in the presence of 0.4 mg/ml G418 and 0.5% penicillin/streptomycin. Kappa-CN cells were used for KOP radioligand binding assays, while Kappa-FLG19 cells were used in KOP [^{35}S]GTPγS functional assays.

Membrane preparation. The cell lines were grown to confluency, then harvested for membrane preparation. The membranes were prepared in 50 mM Tris buffer (pH 7.4). Cells were scraped and centrifuged at 500 × g for 12 mins. The cell pellet was homogenized in 50 mM Tris with a Fisher Scientific PowerGen 125 rotor-stator type homogenizer, centrifuged at 20,000 × g for 25 mins, washed and recentrifuged once more at 20,000 × g for 25 mins, and aliquoted at a concentration of 3 mg/ml protein per vial and stored in a -80 ˚C freezer till further use.

Receptor binding. Compounds were dissolved at 10 mM stock in 100% dimethylsulfoxide (DMSO). The assay was performed in a 96–well polystyrene plate by adding 100 µl of compound, triplicates of six concentrations of each test compound (1µM – 0.01 nM), and 100 µl of tritiated ligands [^{3}H]DAMGO ([D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin) (51.0 Ci/mmole, 0.59 nM for MOP), [^{3}H]DPDPE ((D-Penicillamine^2,D-Penicillamine^5)-Enkephalin) (42.0 Ci/mmole, 1.11 nM for DOP), [^{3}H]U69593 (41.7 Ci/mmole, 1.05 nM for KOP), and [^{3}H]N/OFQ (130 Ci/mmole, 0.12 nM for NOP). Finally, 800 µl of membrane was added to the wells. Nonspecific binding was determined by using 1.0 µM of the unlabeled nociceptin for NOP, 10 µM unlabeled DAMGO for MOP, 1.0 µM unlabeled DPDPE for DOP, and 10 µM unlabeled U69,593 for KOP. Assays were initiated by addition of 800 µl of membrane per well (approximately 3 mg membrane homogenate per plate). Samples were incubated for 60 min at 25˚C in a total volume of 1.0 ml. In NOP receptor experiments, 1 mg/ml BSA was added to the compound dilution buffer. The incubation was terminated by rapid filtration through 0.5% PEI-soaked glass fiber filter mats (GF/C Filtermat A, Perkin-Elmer) on a Tomtec Mach III cell harvester and washed 5 times with 0.5 ml of ice-cold 50 nM Tris-HCl, pH 7.4 buffer. The filters were dried overnight and soaked with scintillation cocktail before counting on a Wallac Beta plate 1205 liquid

scintillation counter. Radioactivity was determined as counts per minutes (cpm). Full characterization of compounds included analysis of the data for IC_{50} values and Hill coefficients using GraphPad Prism (ISI, San Diego, CA). Ki values were determined by the method of Cheng and Prusoff (*74*).

[^{35}S]GTPγS functional assay. The efficacy of the compounds were determined by the ability to stimulate [^{35}S]GTPγS binding to cell membranes, and compared to standard agonists. The functional assay was conducted in Buffer A, containing 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 mM magnesium chloride ($MgCl_2$) 100 mM sodium chloride (NaCl) at pH 7.4. Membrane prepared as described above was incubated with [^{35}S]GTPγS (150,000 cpm/well), guanosine diphosphate (GDP) (10 μM), and the test compound, in a total volume of 1 ml for 60 minutes at 25˚C. Samples were filtered over Filtermat A and counted as described for the binding assays. A dose response curve with a prototypical full agonist at the respective receptor was conducted in each experiment to identify full and partial agonist compounds. Typically, the standard full agonists (N/OFQ for NOP, DAMGO for MOP, U69593 for KOP, and DPDPE DOP) showed at least 2-fold to 5-fold stimulation over basal. The stimulation by the standard full agonists was taken as 100% when comparing stimulation by the test compound (Table 1 in Main Text).

Details for molecular docking and computational modeling

The details of the active-state NOP homology model used for docking can be obtained from our published paper (*75*). For the MOP receptor docking, the active-state MOP crystal structure Protein Data Bank (PDB) ID: 5C1M was used. Compounds were sketched and minimized using

MMFF94 force field and charges in SybylX 1.2. Molecular docking was carried out using the Surflex-dock module in SybylX 1.2. Docking was performed using the Geom protocol in Surflex-dock. The protomol was defined using the existing ligand (Ro 64-6198 for NOP and BU-74 for MOP) inside the receptor binding site. A total of 20 poses were retained for each molecule. The top scoring poses were analyzed and used for structure-based design.

In vivo pharmacokinetics and brain penetration of AT-121 in rats

Male Sprague-Dawley rats (250–300 g) were administered a single dose of AT-121 in the formulated vehicle via subcutaneous injection. Dosing volume and dose were 3 ml/kg and 3 mg/kg, respectively. AT-121 was formulated as a 1 mg/ml solution in 5% dimethylacetamide / 95% of 30% SBECD (betacyclodextrin sulfobutyl ether). The following sampling times were used (n = 3/time point): 0.5, 1, 2, 4, 8 h after dosing. Rats were lightly anesthetized with CO_2, blood was collected via cardiac puncture, and brains were removed from the cranium. Blood samples were collected into vials containing K2-EDTA, and vials were capped and temporarily stored on ice. Brain samples were temporarily put on dry ice. Plasma was separated from blood by centrifugation and frozen at -80°C. Brains were rinsed with ice-cold phosphate buffered saline, blotted dry, weighed, and frozen. Plasma and brain samples were stored at -80°C before bioanalytical analysis.

Bioanalytical method. Plasma samples were thawed on ice, and a 50-µl aliquot was transferred to a 1-ml plastic tube. 5 µl methanol (MeOH) was added to the samples. 200 µl of 50 ng/ml internal standard (terfenadine) in MeOH / acetonitrile (1:1, v/v) was added to the plasma, vortexed, and centrifuged (15 mins at 4000 rpm). The resultant supernatants were diluted 10x with

MeOH/water (1:1, v/v, containing 0.1% formic acid) for injection and analyzed for AT-121. Brain samples were thawed on ice, placed into plastic tubes, and 4 volumes of purified water per gram of brain was added (1:4, w/v). Brains were homogenized using a mechanical high speed tissue homogenizer; 50 μl of brain homogenate was transferred to a 1-ml plastic tube. 5 μl MeOH was added to the samples. 200 μl of 50 ng/ml internal standard (terfenadine) in MeOH / acetonitrile (1:1, v/v) was added, vortexed, and centrifuged (15 mins at 4000 rpm). The resultant supernatants were diluted 10x with MeOH/water (1:1, v/v, containing 0.1% formic acid) for injection and analyzed for AT-121.

Processed plasma and brain samples were analyzed for AT-121 and internal standard by liquid chromatography-tandem mass spectrometry (LC/MS/MS) on an AB Sciex API 4000 (Applied Biosystems). The LC separation used a binary gradient and C18 reverse phase column [Kinetex C18 100A, 30 × 3 mm). The column was maintained at room temperature. Mobile phase A consisted of 0.05% formic acid in 5 mM aqueous NH_4OAc and mobile phase B consisted of 0.1% formic acid in acetonitrile. The flow rate was held constant at 0.7 ml/min. Detection of AT-121 and internal standard was using electrospray ionization operating in positive ion mode. Multiple reaction monitoring (MRM) of the mass transition 463.34 atomic mass units to 260.30 atomic mass units was used for detection of AT-121. No additional peaks were observed in the same MRM channel as AT-121 (no metabolite peaks observed) AT-121 and internal standard eluted within 2.50 min after injection.

Pharmacokinetic data analysis. The AT-121 noncompartmental plasma and brain pharmacokinetics were determined using the program WinNonlin version 4.0.1 (Pharsight,). The area under the curve plasma concentration-time profile was established by linear trapezoidal rule.

The terminal phase half-life was calculated using at least three data points in the terminal phase excluding the Cmax. Standard deviations are represented by error bars on pharmacokinetic figures.

In vitro stability of AT-121 in primate plasma

Monkey plasma was prepared from whole blood by centrifugation at 4000 rpm at 4˚C for 15 mins and stored at -80˚C for future use. AT-121 was dissolved in DMSO to make a 10 mM stock solution, from which a 100 μM working solution was prepared by adding 1 μl stock solution to 99 μl of DMSO. The plasma was pre-warmed in a 37˚C waterbath for 15 mins. 0.5 μl of compound was added to 49.5 μl of plasma per well and mixed by vortexing. The reaction was incubated for 0, 5, 15, 30, 60 and 120 mins, and reaction stopped by adding 300 μl of MeOH/acetonitrile (1:1 v/v) containing an internal standard, and analyzed by LC-MS/MS, using reverse-phase HPLC, as described above for the pharmacokinetic analysis. The data was analyzed by linear regression of % remaining versus time plot to obtain $t_{1/2}$.

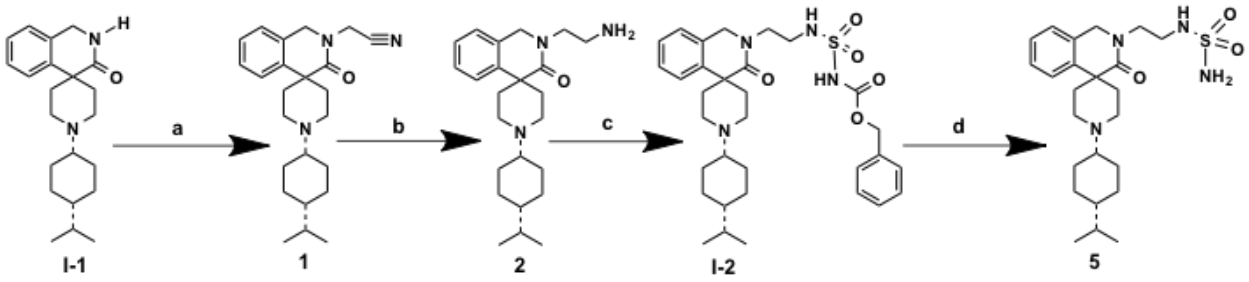

Fig. S1. Chemical synthesis scheme for compounds 1 to 6. *Reagents and conditions.* a) NaH, $BrCH_2CN$, THF, 14 h, rt; b) H_2, PtO_2 hydrate, MeOH, conc HCl, 50˚C, 3 h; c) Chlorosulfonyl isocyanate, benzyl alcohol, CH_2Cl_2, 5˚C, then Et_3N, CH_2Cl_2, amine, 14 h, rt; d) H_2, 10% Pd/C, MeOH, NH_3, 4 h.

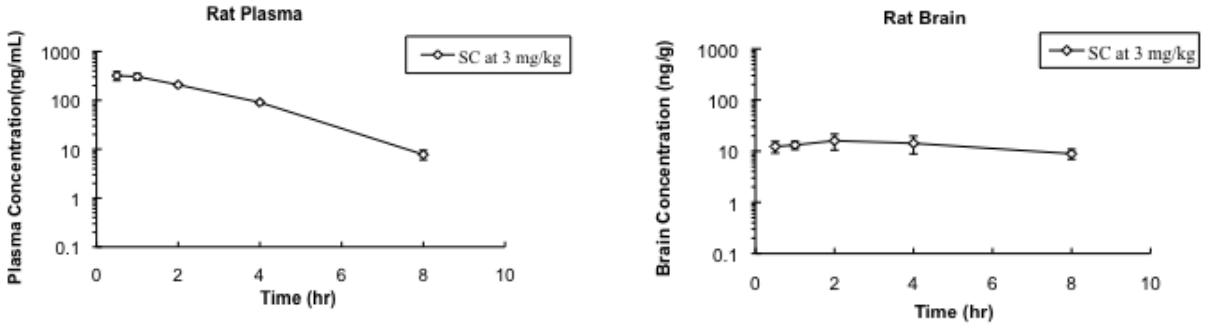

Fig. S2. AT-121 plasma and brain concentrations after a single subcutaneous administration of 3 mg/kg to male Sprague-Dawley rats. The concentrations were calculated per milliliter of plasma or per gram of brain, respectively. Each data point represents Mean ± standard deviation (S.D.); n is 3 per time point.

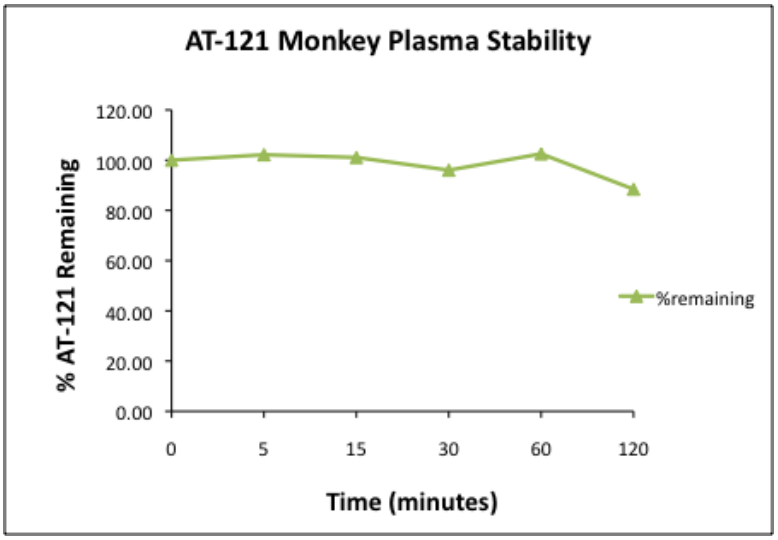

Fig. S3. In vitro plasma stability assessment of AT-121 in nonhuman primate plasma. AT-121 at a final concentration of 1 μM was incubated with monkey plasma and analyzed at 0, 5, 15, 30, 60 and 120 mins.

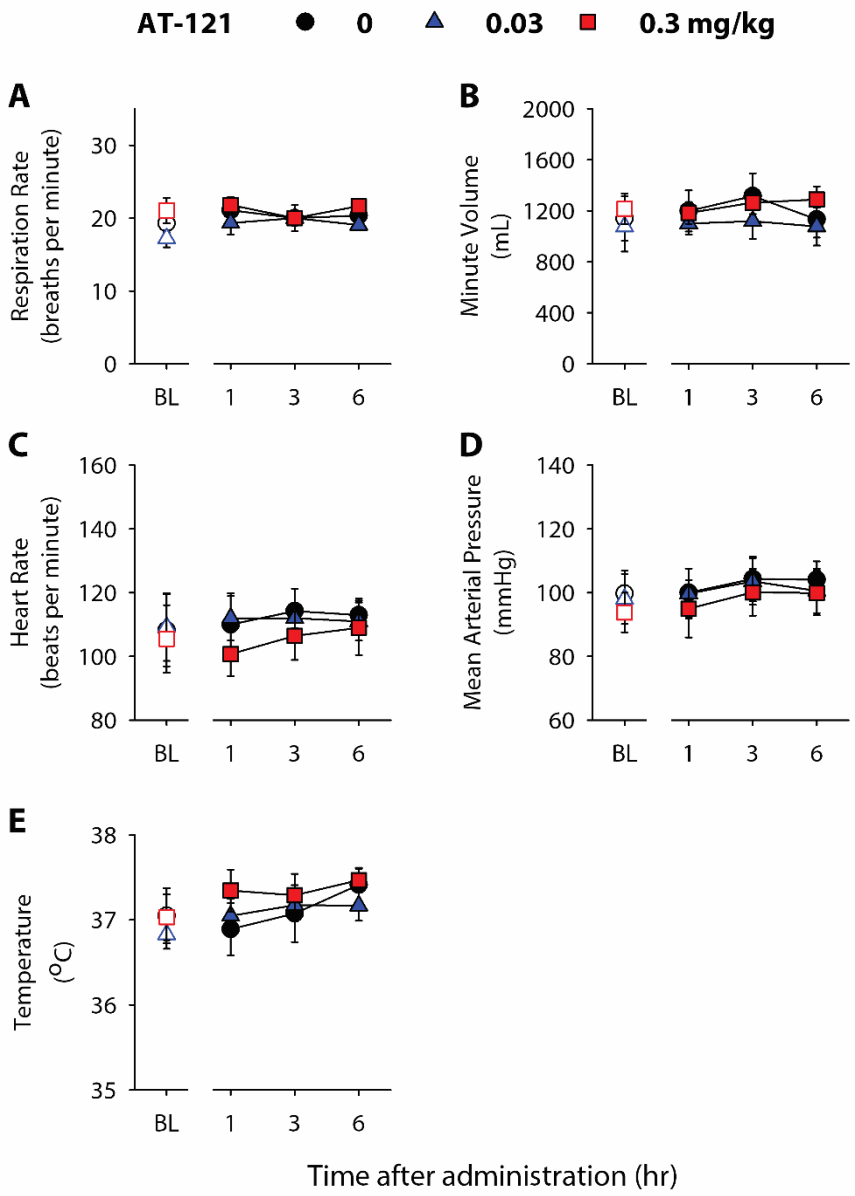

Fig. S4. Effects of systemic administration of AT-121 on physiologic functions of freely moving nonhuman primates implanted with telemetric probes. (A) Respiration rate. (B) Minute volume. (C) Heart rate. (D) Mean arterial pressure. (E) Body temperature. Each data point represents mean ± SEM (n = 4) from each individual data averaged from a 15-min time block. All compounds were delivered intramuscularly. Open symbols represent baselines of different dosing conditions for the same monkeys before administration.

Table S1. Brain sample records for AT-121 brain penetration after subcutaneous administration of 3 mg/kg to male Sprague-Dawley rats.

Brain Records:						
Rat #	**Time (hr)**	**Brain Weight (g)**	**Volume of Water Added (mL)**	**Tested Brain Conc. (ng/mL)**	**Calculated Brain Conc. (ng/g)**	**Average Brain Conc. (ng/g)**
30 min-1	0.5	1.615	6.460	2.73	13.7	**12.3**
30 min-2		1.576	6.304	1.75	8.75	
30 min-3		1.688	6.752	2.91	14.6	
1 hr-4	1	1.405	5.620	2.15	10.8	**13.2**
1 hr-5		1.576	6.304	2.68	13.4	
1 hr-6		1.532	6.128	3.06	15.3	
2 hr-7	2	1.509	6.036	4.02	20.1	**16.0**
2 hr-8		1.698	6.792	3.61	18.1	
2 hr-9		1.544	6.176	1.96	9.80	
4 hr-10	4	1.420	5.680	1.61	8.05	**14.2**
4 hr-11		1.708	6.832	3.56	17.8	
4 hr-12		1.684	6.736	3.37	16.9	
8 hr-13	8	1.730	6.920	2.08	10.4	**8.90**
8 hr-14		1.577	6.308	1.92	9.60	
8 hr-15		1.550	6.200	1.34	6.70	

Table S2. Pharmacokinetic parameters (plasma and brain) for AT-121 after subcutaneous administration in male Sprague-Dawley rats.

	$t_{1/2}$ (h)	t_{max} (h)	C_{max} (ng/mL)	AUC_{last} (h*ng/mL)	AUC_{inf} (h*ng/mL)	Mean residence time MRT (h)	B/P ratio (AUC)
Plasma	1.24	0.5	320	988	1002	2.28	
Brain	6.90	2.0	16.0	100	189	10.4	0.18

Table S3. Plasma stability assessment of AT-121 in nonhuman primate plasma.

AT-121 concentration (1 μM), incubation time 120 mins.

Compound ID	$t_{1/2}$ mins
AT-121	>500 mins
Control-Tetracaine	<0.5 mins

Table S4. Baseline values for the nonhuman primate tail-withdrawal assay that are normalized across different dosing conditions.

Figure 2A. Baseline values of tail-withdrawal latencies

Dose (mg/kg)	0	0.003	0.01	0.03
Mean ± SEM (sec)	2.62 ± 0.18	2.14 ± 0.18	2.79 ± 0.19	2.57 ± 0.21

Figure 2B. Baseline values of tail-withdrawal latencies

	Capsaicin only
Mean ± SEM (sec)	2.41 ± 0.16

Figure 2C. Baseline values of tail-withdrawal latencies

Pretreatment	Vehicle	Naltrexone	J-113397	Naltrexone + J-11397
Mean ± SEM (sec)	2.58 ± 0.1	2.33 ± 0.08	2.41 ± 0.34	2.28 ± 0.14

Figure 2D. Baseline values of tail-withdrawal latencies

Drug	AT-121	Morphine
Mean ± SEM (sec)	2.08 ± 0.23	2.55 ± 0.22

Figure 6C. Baseline values of tail-withdrawal latencies

Day	BL	Day 30
Mean ± SEM (sec)	2.26 ± 0.18	3.67 ± 0.5

Figure 6D. Baseline values of tail-withdrawal latencies

Day	BL	Day 30
Mean ± SEM (sec)	2.35 ± 0.16	2.38 ± 0.13