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Interactive Effects of μ-Opioid and Adrenergic-α_2 Receptor Agonists in Rats: Pharmacological Investigation of the Primary Kratom Alkaloid Mitragynine and Its Metabolite 7-Hydroxymitragynine

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Non-standard abbreviations: δ-opioid receptor (DOR), κ-opioid receptor (KOR), μ-opioid receptor (MOR), (+)-4-[(α*R*)-α-((2*S*,5*R*)-4-allyl-2,5-dimethyl-1-piperazinyl)-3-methoxybenzyl]-*N*,*N*-diethylbenzamide (SNC80), (+)-(5α,7α,8β)-*N*-methyl-*N*-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl]-benzeneacetamide (U-69,593), 2-(2,3-dihydro-2-methoxy-1,4-benzodioxin-2-yl)-4,5-dihydro-1*H*-imidazole (RX821002), 7-hydroxymitagynine (7-OH-MG), Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), analysis of variance (ANOVA), adrenergic-α_2 ($A\alpha_2R$), Chinese hamster ovary (CHO), [D-Ala^2, D-Leu^5]-Enkephalin (DADLE), [D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin (DAMGO), ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), fixed ratio (FR), human embryonic kidney (HEK), inhibition constant (Ki), Institutional Animal Care and Use Committee (IACUC), intraperitoneally (i.p.), intrathecally (i.t.), light-emitting diode (LED), mitragynine (MG), National Institutes of Health (NIH), orally by gavage (p.o.), percent maximum possible antinociceptive effect (% MPE), standard error of mean (SEM), subcutaneously (s.c.), Tris, $MgCl_2$, and ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (TME)

ABSTRACT

The primary kratom alkaloid mitragynine is proposed to act through multiple mechanisms, including actions at μ-opioid (MORs), adrenergic-α_2 receptors (Aα_2Rs), as well as conversion *in vivo* to a MOR agonist metabolite (i.e., 7-hydroxymitragynine). Aα_2R and MOR agonists can produce antinociceptive synergism. Here, contributions of both receptors to produce mitragynine-related effects were assessed by measuring receptor binding in cell membranes, and in rats, pharmacological behavioral effect antagonism studies. Mitragynine displayed binding affinity at both receptors, whereas 7-hydroxymitragynine only displayed MOR binding affinity. Compounds were tested for their capacity to decrease food-maintained responding and rectal temperature and to produce antinociception in the hotplate test. Prototypical MOR agonists and 7-hydroxymitragynine, but not mitragynine, produced antinociception. MOR agonist and 7-hydroxymitragynine rate-deceasing and antinociceptive effects were antagonized by the opioid antagonist naltrexone, but not by the Aα_2R antagonist yohimbine. Hypothermia only resulted from reference Aα_2R agonists. The rate-deceasing and hypothermic effects of reference Aα_2R agonists were antagonized by yohimbine but not naltrexone. Neither naltrexone nor yohimbine antagonized the rate-decreasing effects of mitragynine. Mitragynine and 7-hydroxymitragynine increased the potency of the antinociceptive effects of Aα_2R but not MOR reference agonists. Only mitragynine produced hypothermic effects. Isobolographic analyses for the rate-decreasing effects of the reference Aα_2R and MOR agonists was also conducted. These results suggest mitragynine and 7-hydroxymitragynine may produce antinociceptive synergism with Aα_2R and MOR agonists. When combined with Aα_2R agonists, mitragynine could also produce hypothermic synergism.

Significance Statement

Mitragynine is proposed to target the μ-opioid (MOR) and adrenergic-α_2 receptor (Aα_2R) and to produce behavioral effects through conversion to its MOR agonist metabolite 7-hydroxymitragynine. Isobolographic analyses indicated supra-additivity in some dose ratio combinations. This study suggests mitragynine and 7-hydroxymitragynine may produce antinociceptive synergism with Aα_2R and MOR agonists. When combined with Aα_2R agonists, mitragynine could also produce hypothermic synergism.

INTRODUCTION

Prescription μ-opioid receptor (MOR) agonists are a primary medication class to treat severe pain (Haq et al., 2021; Montgomery, 2022). However, due to the current high incidents of opioid overdose in the United States (Mattson et al., 2021), there is the need for novel analgesics that are equally effective as MOR agonists, but are safer. One of the adverse effects of MOR agonists is the development of dependence and withdrawal. The current medications to treat opioid dependence and withdrawal are either MOR or adrenergic-α_2 receptor (Aα_2R) agonists.

Mitragyna speciosa (kratom), a plant native to Southeast Asia, is used as a self-remedy to alleviate opioid withdrawal symptoms in countries such as Malaysia and Thailand (Singh et al., 2014). The use of kratom has increased significantly in the West where kratom products are used for pain reduction, opioid dependence, as well as recreationally (Lydecker et al., 2016; Sharma et al., 2019). Mitragynine (MG), the primary alkaloid in kratom, has received much attention due to its MOR activity (Matsumoto et al., 1996; Shamima et al., 2012; Harun et al., 2015; Varadi et al., 2016; Kruegel et al., 2019; Obeng et al., 2020; Obeng et al., 2021b; Chakraborty et al., 2021). However, MG appears to have a complex pharmacology that may include Aα_2R activity. For example, the antinociceptive effects of MG were reversed by both opioid (naloxone) and Aα_2R (yohimbine and idazoxan) antagonists (Matsumoto et al., 1996; Kruegel et al., 2019; Foss et al., 2020).

Decreased overreliance on prescription MOR agonists for pain management could be achieved by combining MOR agonists with non-opioid analgesics, thereby reducing the analgesic dose of the prescribed MOR agonist (i.e., opioid-sparing effect) (Wilkerson et al., 2016; Wilkerson et al., 2017; Wilkerson et al., 2019; Obeng et al., 2021a). Although the antinociceptive effectiveness of Aα_2R agonists is generally lower than that of MOR agonists, Aα_2R agonists have well-established opioid-sparing effects and have been safely used (Crassous et al., 2007; Giovannoni et al., 2009; Tonner, 2017; Valverde and Skelding, 2019). It has been hypothesized that the basis of Aα_2R agonist opioid-sparing effects is due to antinociceptive synergism (supra-additivity) between agonists at these receptors. For example, an inactive dose of the Aα_2R agonist clonidine (0.016 mg/kg) increased the antinociceptive potency of morphine four- to five-fold without producing

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tolerance in the mouse tail flick assay (Spaulding et al., 1979). The opioid-sparing effects of $A\alpha_2R$ agonists have been demonstrated regardless of rodent species (i.e. mouse and rat), antinociceptive assays (e.g. hotplate, tail pressure, and formalin), and combinations of agonists at these receptors (Drasner and Fields, 1988; Ossipov et al., 1990b; Plummer et al., 1992; Meert and Kock, 1994; Stone et al., 1997; Hao et al., 2000; Tajerian et al., 2012; Stone et al., 2014). Importantly, antinociceptive synergism was not accompanied with non-specific motor (rotarod and open field tests) or cardiovascular (pulse oximetry) disruptions (Tajerian et al., 2012; Stone et al., 2014). Additionally, the adverse effects of the $A\alpha_2R$ agonists are far less severe than those of the MOR agonists (Walker et al., 2002). In marked contrast to the MOR agonists, $A\alpha_2R$ agonists have low, if any, potential for development of abuse and dependence (Arnsten and Li, 2005; Clemow and Walker, 2014; Gowing et al., 2016) which suggests that $A\alpha_2R$ agonists may be ideal for reducing opioid use and overdose. Given the capacity of the $A\alpha_2R$ agonists to reduce opioid use as well as the agonistic activity of MG at MOR and $A\alpha_2R$ mentioned above (Matsumoto et al., 1996; Kruegel et al., 2019; Foss et al., 2020; Chakraborty et al., 2021), we hypothesized that MG mitigates opioid withdrawal through dual agonism at these receptors.

Herein, we first assessed preclinical interaction profiles of reference agonists at MOR (methadone and morphine) and $A\alpha_2R$ (lofexidine and clonidine) in rats by measuring effects of drugs on schedule-controlled responding for food, response latency in the hotplate test, and rectal temperature (Boxwalla et al., 2010). Interactions between agonists at the κ-opioid receptor (KOR, U69,593) and $A\alpha_2R$ were also investigated. The mechanism underlying the activity of these compounds was further investigated using antagonists at the MOR (naltrexone) and $A\alpha_2R$ (yohimbine). Isobolographic analyses were conducted to investigate synergism between MOR and $A\alpha_2R$ agonists. In addition, we compared the contribution of MOR and $A\alpha_2R$ to the activity of MG and 7-hydroxymitragynine (7-OH-MG), a MOR active metabolite of MG (Kruegel et al., 2019). A receptor binding assay was employed to assess affinity of test compounds at these receptors.

METHODS AND MATERIALS

Compounds. The following are sources of compounds: [^{3}H][D-Ala^2, D-Leu^5]-Enkephalin ([^{3}H]DADLE) (PerkinElmer, Boston, MA), [^{3}H][D-Ala^2, *N*-MePhe^4, Gly-ol]-enkephalin ([^{3}H]DAMGO) (PerkinElmer),

[^{3}H]RX821002 (PerkinElmer), [^{3}H]U69,593 (PerkinElmer), clonidine hydrochloride (XGen Pharmaceuticals DJB, Inc., Horseheads, NY), lofexidine hydrochloride (Sigma-Aldrich Co., St. Louis, MO), (-)-methadone hydrochloride (National Institute on Drug Abuse, Drug Supply Program, Rockville, MD), (-)-MG hydrochloride [extracted as described in Hiranita *et al* (Hiranita et al., 2019)], (-)-7-OH-MG [semi-synthesized from MG as in Obeng et al (Obeng et al., 2021b)], (-)-morphine sulfate pentahydrate (National Institute on Drug Abuse), (-)-naltrexone hydrochloride (Sigma-Aldrich Co.), U69,593 (Sigma-Aldrich Co.), and yohimbine hydrochloride (Sigma-Aldrich Co.). Dose/concentration is expressed as the weight of the salt form listed above, or as a base if no salt form is noted. For *in vitro* studies, compounds were dissolved in dimethyl sulfoxide (Sigma-Aldrich Co.) to form stock concentrations of 10 mM. For behavioral studies, a vehicle consisting of sterile water containing 5% Tween 80 (polyoxyethylenesorbitanmonooleate, Sigma-Aldrich Co.) and 5% propylene glycol (Sigma-Aldrich Co.) was used. Compounds and vehicle were administered intraperitoneally (i.p.) in a volume of 1.0—10 mL/kg per body weight. MG and vehicle were also administered subcutaneously (s.c.) and orally (p.o.) via gavage in volumes of 1.0—10 mL/kg.

In Vitro Receptor Binding Assay. [^{3}H]RX821002 (PerkinElmer) was used to label both the human adrenergic-α_{2A} and -α_{2C} receptors ($A\alpha_{2A}R$ and $A\alpha_{2C}R$) (O'Rourke et al., 1994). These two $A\alpha_2R$ subtypes were chosen because they are involved in antinociception (Brede et al., 2004). L-α-2A (ATCC® CRL11180 ™) and L-α-2C (ATCC®CRL-11181™) L-cells (American Type Culture Collection, Manassas, VA) were used for the $A\alpha_{2A}R$ and $A\alpha_{2C}R$, respectively. [^{3}H]DADLE, [^{3}H]U69,593, and [^{3}H]DAMGO were used to label the human δ-opioid receptor (DOR), KOR, MOR, respectively, as described previously (Obeng et al., 2021b). The binding assay at the opioid receptor subtypes was conducted using monoclonal opioid receptors expressed in Chinese hamster ovary (CHO) cell lines for the DOR (generous gift from Dr. Stephen J. Cutler, University of South Carolina) and MOR (PerkinElmer). The KORs (generous gift from Dr. Stephen J. Cutler, University of South Carolina) were expressed in human embryonic kidney (HEK) cells. The K_d and B_{max} values for the radioligands at each receptor subtype were first determined using a saturation assay (Table S1). The Bradford protein assay was utilized to determine and adjust the concentration of protein required for the assay (Tal et al., 1985). Ten µg of each membrane protein was separately incubated with one of the radioligands in the presence of different

concentrations of test compounds in TME [(50 mM Tris (Sigma-Aldrich), 3 mM $MgCl_2$ (Sigma-Aldrich), and 0.2 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N′*,*N′*-tetraacetic acid (EGTA, Sigma-Aldrich), pH 7.7)] buffer for 60 minutes at room temperature. The bound radioligand was separated by filtration using the Connectorate filtermat harvester for 96-well microplates (Inotech, Dietikon, Switzerland) and counted for radioactivity using a MicroBeta2 microplate counter (PerkinElmer). Specific binding at each $A\alpha_2R$ subtype was determined as the difference in binding obtained in the absence and presence of 10 μM lofexidine (Table S1). Specific binding at the DOR, KOR, and MOR was determined as the difference in binding obtained in the absence and presence of 10 μM SNC-80, U69,593, and naltrexone, respectively.

Subjects. Adult female and male Sprague Dawley rats at ten weeks old upon arrival (Taconics, Germantown, NY, N=4 per sex) were housed individually and acclimated for at least three days to a temperature- (21.9°C ± 1.9°C) and humidity-controlled (53% ± 14%) vivarium with a 12-hour light/dark cycle (lights on at 07:00 hours E.S.T. in the daylight saving time period) during which food (2918 Teklad global 18% protein rodent diets, Envigo, Frenchtown, NJ) and reverse osmosis water were available at all times. After the acclimation period, individual body weights were maintained at no less than 85% of free feeding body weight as well as no less than 2.5 of Body Conditioning Score (Ullman-Culleré and Foltz, 1999), by adjusting daily food rations. The free feeding body weight was redetermined as requested by the veterinary staff at University of Florida. Access to chow (Dustless Precision Pellets Grain-Based Rodent Diet, Bio-Serv, Frenchtown, NJ) was provided in the rats' home cages approximately 30 minutes following daily experimental sessions. In addition to chow consumption, rats consumed a maximum of fifty 45-mg sucrose pellets (Dustless Precision Pellets® 45 mg, Sucrose, Bio-Serv) available during experimental sessions for schedule-controlled responding as described below. The animal protocol was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Florida and was in accordance with the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Apparatus. The apparatus and procedures for the operant-conditioning and hotplate experiments below were as previously described (Hiranita et al., 2019; Wilkerson et al., 2019; Obeng et al., 2021b).

1) Operant Conditioning Apparatus. Eight operant-conditioning chambers (Model ENV-008; Med Associates Inc., Fairfax, VT) were used, each enclosed within a sound-attenuating cubicle equipped with a fan for ventilation and white noise to mask extraneous sounds. On the front wall of each chamber were two retractable, 5-cm-long response levers, 5 cm from the midline and 9 cm above the grid floor. A response was defined as a downward displacement of the right lever with a force approximating 0.20 N whereas the left lever was not used. Two amber light-emitting diodes (LEDs) were aligned horizontally above two levers (one LED/lever); however, only the right LED and lever were activated for the correct study. A receptacle for the delivery of 45-mg sucrose pellets (Dustless Precision Pellets® 45 mg, Sucrose, Bio-Serv) via a pellet dispenser (Model ENV-203-20; Med Associates Inc.) was mounted on the midline of the front wall between the levers and 2 cm above the floor. Each operant conditioning chamber was connected to a Dell desktop computer (Intel® Core™ i7-7700 3.60 GHz processor, 16.0 GB of RAM, Microsoft® Windows 10) through an interface (MED-SYST-8, Med Associates Inc.). Med-PC software version V (Med Associates Inc.) controlled experimental events and recorded responses. The chamber assignments remained the same for each subject throughout the study.

2) Hotplate. A square plate (Hot Plate Analgesia Meter, 1440 Analgesia Hot Plate with RS-232 Port and Software, Columbus Instruments, Columbus, OH) was surrounded by a clear acrylic cubicle with a lid. The stability of temperature on the plate surface was verified at 52°C ± 0.1°C 30 minutes prior to each use.

3) Rectal Thermometer. An uninsulated microprobe (50313 Rat Rectal Probe, Stoelting, Wood Dale, IL) and a digital thermometer (50315 Body Temperature Thermometer, Stoelting) were used to measure rectal temperature. Veterinary ophthalmic ointment (Puralube®, Dechra Veterinary Products, Overland Park, KS) was applied to the tip of the microprobe prior to each use.

In vivo procedures. The temperature, humidity, and light/dark cycle in the experimental room were equivalent to those in the vivarium. After the acclimation period to the vivarium, schedule-controlled responding experiments were conducted in the light cycle (08:00 to 11:00 hours E.S.T. in the daylight-saving time period) at the same time each day seven days per week. On drug test days, temperature and hotplate experiments were also conducted in that order (Figure 1). Prior to the start of each daily experiment, body weight was measured.

The sample size of each experimental group per treatment was eight using a within-subject design (N=4 per sex). The doses of each test compound per injection were incremented sequentially at approximately 20-minute intervals (Figure 1).

1) Within-Session, Six-Cycle Schedule-Controlled Responding. A) Lever-response shaping. Each experimental session commenced by placing an experimental subject in an individually assigned chamber daily up to 120 minutes. Each session started with the presentation of the right retractable lever and the illumination of the LED above the right lever. Each downward deflection of the right lever turned off the LEDs and activated the pellet dispenser for 0.1 seconds [fixed-ratio (FR) 1 schedule] followed by a 0.1-second time-out period during which LEDs were turned off and responding had no scheduled consequences; the retractable lever remained presented during this time-out time. After 50 reinforcers per session were presented within 20 minutes for two consecutive sessions under the terminal FR10 schedule of reinforcement, and daily sessions were divided into multiple, discrete cycles.

B) Training. Each session consisted of six, 20-minute cycles with each cycle consisting of a 15-minute pretest phase and a five-minute test phase in the operant-conditioning chambers (Figure 1). Immediately prior to each cycle, vehicle was injected i.p. and each animal was placed in the assigned chamber. Upon commencement of each session and at the beginning of each pretest phase, the right response lever was extended into the chamber but the stimulus light remained off. Responses on the lever had no scheduled consequences. Upon commencement of each test phase of the cycle, the stimulus light was illuminated. Thereafter, all the experimental variables for the stimulus changes and response timeout conditions under the FR10 schedule of reinforcement were identical to those for response shaping except that the maximal number of food reinforcers delivered was fixed at ten per cycle. When 10 food reinforcers were delivered during each test phase, the stimulus light was turned off and lever responding had no scheduled consequences. Upon completion of the last test phase, the lever was retracted and the stimulus light was turned off. Then, each animal was placed back to their home cages. Training continued until overall response rates (responses per second) across six

cycles for two consecutive sessions were stably maintained with less than 25% variation, as determined per individual subject.

C) Testing. All the experimental variables were identical to those for the training period. However, a dose of a test compound was also injected per cycle other than vehicle. The first injection received was either vehicle or the pretreatment compound (i.e. antagonists naltrexone or yohimbine). The subsequent five injections were either vehicle or test compound. Each test compound was dosed cumulatively such that each dose per cycle was a subtraction from a summation of all the previous doses administered to achieve the target dose. The doses of the compounds administered (mg/kg) increased by either quarter or half log unit increments. Each test session was separated by a minimum of 72 hours and was studied with a non-systematic order of compounds and doses. During the inter-test maintenance sessions, all the experimental variables were identical to those for the training period, without any determination of the hotplate latency and rectal temperature as described immediately below. Vehicle was injected at the beginning of each pretest phase.

Among food-maintained behavior, hotplate response latency, and rectal temperature, only analyses of food-maintained behavior allowed to determine ED_{50} values of all the reference agonists at MOR, KOR, and $A\alpha_2R$ (see *Data analysis*). For the combinations of reference agonists, the cumulative doses in quarter log units in the mixtures per animal were determined based on the ED_{50} values of the rate-decreasing effects of reference agonist alone (Table 1), (Wilkerson et al., 2019). To determine the pharmacological influence of each drug on the observed effects, three ED_{50} ratios of drug mixtures were used. The order of testing was determined randomly. All dose-effect functions for drug mixtures were singly determined.

2) Hotplate and Rectal Temperature. On drug test days, the microprobe tip was inserted approximately 2.0-cm into each subject's rectum and individual baseline temperature was measured within 10 seconds. Immediately after the baseline measurement of rectal temperature, each subject was manually placed on the heated plate and baseline hotplate response latency was determined manually using a stopwatch (Martin Stopwatch, Martin Sports, Carlstadt, NJ) by trained and experimentally blinded raters. Hotplate response latency was measured until the subject jumped, licked or shook the back paws, or up to 60 seconds to avoid tissue damage, whichever occurred first.

Immediately following the determination of the baseline values, each subject underwent an injection of a dose of a test compound or vehicle and was placed in their respective operant conditioning chamber. Immediately after each cycle of the schedule-controlled responding experiment (cycles 1 to 6), rectal temperature and hotplate response latency were measured followed by an injection of a dose of the test compound or vehicle in this order.

Data analysis. The dependent variables in each figure are shown as mean values ± standard error of the mean (SEM). Mean and SEM values per group of eight subjects were calculated as a function of compound doses, cycles, or dose ratios of combined compounds. Statistical analyses were conducted using GraphPad Prism version 9 for Windows (GraphPad Software, Inc., San Diego, CA), SigmaPlot version 14.0 (Systat Software Inc., San Jose, CA), or R-4.1/RStudio Desktop (R Core Team, 2017). Comparisons were considered significant when a *P* value was less than 0.05. A one-, two-, or three-way (repeated-measures) analysis of variance (ANOVA) followed by *post hoc* Bonferroni *t* tests was used as appropriate to analyze the effects of the compound dose, cycle, sex, dose ratio, or tolerance (assessment order: first or last dose-effect assessment for morphine, U69,593, and lofexidine; Figure S2 and Table S5-S7). For the three-way repeated measures ANOVA, GraphPad Prism software was used for all 2 by 2 by X design and the RStudio Desktop software was used for all others.

For rectal temperature and hotplate latency, each mean baseline value was determined per animal from all the baseline values determined on the drug sessions used in the following analyses. Hotplate latency values were converted to percent maximum possible antinociceptive effect (% MPE) with the following equation: (100 × [(experimental test latency value – the averaged baseline latency value) / (60 seconds – the averaged baseline latency value)]). Changes in rectal temperature were calculated individually as the test value subtracted from the averaged baseline value. Rates of responding maintained by presentations of food pellets (responses/second) were expressed as a percentage of control, defined as the mean baseline rates across six daily cycles during all sessions one day prior to each test session. There was no increased or decreased trend for either hotplate latency, rectal temperature, or response rate baseline values (P values > 0.05). The dose-

effect functions of morphine, U69,593, and lofexidine were determined twice, once at the start and once at the end of the within-subjects drug assessments. Only when the mean effect of a compound to reduce schedule-controlled responding or to increase MPE was greater than 50% of maximum effects were the ED_{50} and slope values calculated using multiple linear regression (Snedecor and Cochran, 1967) and GraphPad Prism version 9 for Windows (GraphPad Software), where slopes were allowed to vary (Tallarida, 2000). Because only α_2R agonists produced 2°C or greater hypothermia, $ED_{-2°C}$ values were also individually calculated to compare the hypothermic potency. Only points on the linear part of the ascending (%MPE) and descending (response rate and rectal temperature) limbs of the dose–effect functions were used. If the 95% confidence intervals (CIs) of the ED_{50}, $ED_{-2°C}$, and slope values did not overlap, or the potency or slope ratio of the compound alone or in combination with another compound did not include 1, potencies or slopes of the compounds were deemed statistically different. Among food-maintained behavior, hotplate response latency, and rectal temperature, only analyses of food-maintained behavior allowed to determine ED_{50} values of all the reference agonists at MOR, KOR, and Aα_2R. For the mixture studies, the cumulative doses in quarter log units in the mixtures per animal were determined based on the ED_{50} values of the rate-decreasing effects of reference agonist alone (Wilkerson et al., 2019). That is, a within-subjects design was used, and each subject received dose combinations that were equivalent to the dose ratio based upon the individual ED_{50} of a drug to decrease response rates in that subject. The theoretical additive ED50 value of the combined drugs was calculated from the individual dose-effect functions to determine synergistic, additive, or subadditive interactions as previously described (Wilkerson et al., 2016; Wilkerson et al., 2017; Wilkerson et al., 2019). The combination was assumed to equal the sum of the effects of each drug. The experimentally derived ED50 values (Zmix) from the dose-effect functions of the ratios were compared to the predicted additive ED50 values (Zadd) via a Fisher's exact test (Wilkerson et al., 2016; Wilkerson et al., 2017; Wilkerson et al., 2019). If the empirically derived value and the theoretical value did not significantly differ, the interaction was considered additive (Tallarida, 2001; 2006). For the *in vitro* studies, the assays were conducted in triplicate and repeated at least three times and the IC_{50} values were determined using a nonlinear, least-squares regression analysis (Prism 9;

GraphPad Software, Inc.) and then converted to K_i values using the Cheng–Prusoff equation (Cheng and Prusoff, 1973). The 95%CI (asymptotic) was calculated using Prism 9.

RESULTS

Only the primary findings are shown here. Full details are described in the supplemental materials.

Receptor Binding. The Ki (nM) values of reference Aα_2R ligands clonidine, lofexidine, and yohimbine were 5.97, 1.21, and 8.24 at the Aα_{2A}R, and 60.8, 7.62, and 7.77 at the Aα_{2C}R, respectively (Table 1). The Ki values of reference Aα_2R ligands at opioid receptor subtypes and of reference opioid receptor ligands (methadone, morphine, naltrexone, and U69,593) at Aα_2R subtypes were not determined due to lack of inhibition up to 10 μM (Table 1). The Ki values of MG were 4,420 and 4,040 nM at the Aα_{2A}R and Aα_{2C}R, respectively, whereas those of 7-OH-MG at these receptors were not determined due to lack of inhibition up to 10 μM. Both MG and 7-OH-MG had higher affinities at the MOR than at the DOR and KOR; however, 7-OH-MG had a 9-fold higher affinity at the MOR than MG (Figure 2 and Table 1). A summary of scintillation counting conditions employed for assessing affinity at various binding sites in competition for the radioligands labeling human Aα_2R and opioid receptor subtypes can be found in Supp. Table 1.

Reference MOR Agonists Alone. Repeated vehicle injections did not alter response rates, rectal temperature, or nociceptive responding (Supp. Fig. 1, Supp. Table 2, Supp, Table 3). Morphine dose-dependently and significantly decreased response rates and rectal temperature, as well as produced antinociception (Figure 3, upper panels, upward triangles; Supp. Table 4). The ED_{50} values of morphine to decrease response rates and to produce antinociception are shown in Table 2. The potency of morphine to produce the rate-decreasing effects was 4-fold more potent than that for antinociception (Table 2).

Methadone significantly decreased response rates and rectal temperature, and produced antinociception (Figure 3, upper panels, downward triangles; Supp. Table 4, Supp. Table 5). Relative to morphine, methadone was 7- and 5-fold more potent to produce rate-decreasing and antinociceptive effects, respectively (Table 2).

Reference KOR Agonist Alone. U69,593 significantly decreased response rates and rectal temperature, and produced antinociception (Figure 3, upper panels, circles; Supp. Table 6). Relative to morphine, U69,593 was 2- and 4-fold more potent to produce the rate-decreasing and antinociceptive effects, respectively (Table 2). U69,593 was equipotent to decrease response rates and produce antinociception, as measured by increased %MPE (Table 2). There was no significant change in potency across the rates of responding, antinociception, or rectal temperature (Supp. Figure 2; Table 2 and Supp. Table 6).

Reference Aα_2R Agonists Alone. Lofexidine significantly decreased response rates and rectal temperature, and significantly increased %MPE; the antinociceptive effects of lofexidine reached statistical significance but the maximum effects of lofexidine were a mean of 17.3% and significantly less than those of reference MOR agonists ($F_{1,6}$=361, P<0.001, two-way repeated measures ANOVA; Figure 3, upper panels, diamonds; Supp. Table 7). In contrast, as compared to the reference MOR agonists, the hypothermic effects of lofexidine were significantly greater, e.g., 4.1°C decrease in rectal temperature at 0.56 mg/kg (Figure 3). Lofexidine was 38-fold more potent than morphine to produce the rate-decreasing effects (Table 2). The potency of lofexidine to reduce response rates was 3-fold greater than its potency to decrease rectal temperature (Table 2).

Clonidine significantly decreased response rates and rectal temperature; however, statistically significant antinociception was not obtained (Figure 3, upper panels, squares; Supp. Table 7). Clonidine was 4- and 3-fold more potent than lofexidine to produce the rate-decreasing and hypothermic effects, respectively (Table 2). The potency of clonidine to produce the rate-decreasing effects was 4-fold more potent than that for the hypothermic effects (Table 2).

MG and 7-OH-MG Alone. When administered i.p., MG significantly decreased response rates; however, neither statistically significant antinociception nor altered rectal temperature was obtained (Figure 3, lower panels, circles; Supp. Table 8). MG (i.p.) was 4-fold more potent than i.p. morphine to produce the rate-decreasing effects (Table 2). MG had been expected to produce antinociceptive and hypothermic effects because other effects produced by MG are antagonized by MOR and A_2R antagonists (Foss et al., 2020; Obeng

et al., 2021b). Thus, the route of administration of MG was varied and the effects of 7-OH-MG, an active metabolite of MG at the MOR, were assessed.

Both p.o. and s.c. MG significantly decreased rates of responding, and no significant antinociception was observed; there were relatively small yet significant increases in rectal temperature (Figure 3, lower panels, downward and upward triangles, respectively; Supp. Table 8). MG administered p.o. and s.c. was 3- and 6-fold less potent, respectively, than i.p. MG to produce the rate-decreasing effects (Table 2).

In contrast to MG, i.p. 7-OH-MG significantly decreased response rates and produced hot plate antinociception; however, no significant effects on rectal temperature were obtained (Figure 3, lower panels, squares; Supp. Table 8). The potency of 7-OH-MG to reduce response rates was approximately 4-fold more potent than its potency to produce antinociception (Table 2).

Reference MOR Agonists in Combination with Naltrexone or Yohimbine. By themselves, naltrexone (0.032, 1 mg/kg, i.p.) and yohimbine (1, 3.2 mg/kg, i.p.), did not alter food-maintained behavior, antinociception, or rectal temperature (Supp. Fig. 3, and Supp. Table 9). Naltrexone dose-dependently and significantly shifted to the right the dose-effect functions of the rate-decreasing and antinociceptive effects of morphine (Figure 4; Table 2, and Supp. Table 4). The lower dose of naltrexone (0.032 mg/kg) produced significant antagonism of the rate-decreasing and antinociceptive effects of morphine (Table 2). Yohimbine (3.2 mg/kg) did not significantly change the effects of morphine on rates of responding, antinociception, or changes in rectal temperature (Figure 4; Table 2, and Supp. Table 4).

Naltrexone (0.032 mg/kg) produced a 5-fold rightward shift of the methadone rate-decreasing dose-effect function (Figure 4; Tables 2, and Supp, Table 4). Yohimbine (3.2 mg/kg) did not significantly modify the effects of methadone on rates of responding, antinociception, or changes in rectal temperature (Figure 4; Table 2, and Supp. Table 4).

U69,593 in Combination with Naltrexone or Yohimbine. Naltrexone (0.032 mg/kg) produced a small but statistically significant leftward shift of the U69,593 rate-decreasing dose-effect function, but did not modify U69,593 antinociceptive or hypothermic effects (Figure 4; Table 2, and Supp. Table 6). Naltrexone (1.0

mg/kg) significantly antagonized the rate-decreasing, antinociceptive, and hypothermic effects of U69,593 (Figure 4; Table 2, and Supp. Table 6). Naltrexone produced a 5- and 3-fold, respectively, rightward shift of the U69,593 rate-decreasing and antinociceptive dose-effect function (Table 2). Yohimbine (3.2 mg/kg) did not modify U69,593-related rates of responding, antinociception, or rectal temperature (Figure 4; Table 2, and Supp. Table 6).

Reference Aα_2R Agonists in Combination with Naltrexone or Yohimbine. Naltrexone did not modify the effects of lofexidine on rates of responding, hot plate antinociception, or rectal temperature (Figure 4; Table 2 and Supp. Table 7). Yohimbine dose-dependently and significantly shifted to the right the dose-effect functions of the rate-decreasing and hypothermic effects of lofexidine (Figure 4; Table 2 and Supp. Table 7). The lower dose of yohimbine (1.0 mg/kg) produced a 4-fold shift to the right of the lofexidine dose-effect functions to decrease response rates and rectal temperature (Supp. Table 7).

Naltrexone did not modify the effects of clonidine on rates of responding, antinociception, or rectal temperature (Figure 4; Table 2, and Supp. Table 7). Yohimbine (1.0 mg/kg) produced an 8- and 4-fold, respectively, rightward shift of the clonidine rate-decreasing and hypothermic dose-effect function (Figure 4; Table 2 and Supp.Table 7).

MG (i.p.) and 7-OH-MG in Combination with Naltrexone or Yohimbine. Because the i.p. route was most potent among the three routes of administration tested in decreasing the response rates, the i.p. route was used to assess the pharmacological impact of naltrexone (1.0 mg/kg) or yohimbine (3.2 mg/kg) on MG-related behaviors and physiology. Neither naltrexone nor yohimbine significantly modified the dose-effect function of MG to decrease responding (Figure 5; Table 2 and Supp. Table 8). Naltrexone (0.032 mg/kg) significantly shifted the dose-effect functions of 7-OH-MG 3-fold rightward for both rate-decreasing and antinociceptive effects (Figure 5; Table 2 and Supp. Table 8). In contrast, yohimbine (3.2 mg/kg) did not significantly modify the rate-decreasing or antinociceptive 7-OH-MG dose-effect functions (Figure 5, Table 2, and Supp. Table 8).

Reference Agonists in Combination with MG or 7-OH-MG. By themselves, MG (17.8 mg/kg, i.p.) and 7-OH-MG (0.32 mg/kg, i.p.), did not alter food-maintained behavior, antinociception, or rectal temperature (Supp. Fig. 3, and Supp. Table 9). Pretreatment effects of behaviorally inactive doses of MG (17.8 mg/kg) or 7-OH-

MG (0.32 mg/kg) were assessed on the effects of reference agonists tested above in order to understand the interaction of MG or its metabolite with the reference agonists (Figure 6). Neither MG nor 7-OH-MG significantly modified the rate-decreasing and antinociceptive dose-effect functions of morphine and methadone (Figure 6, Table 2, and Supp. Table 4).

MG pretreatment did not significantly modify the rate-decreasing, antinociceptive, and hypothermic dose-effect functions of U69,593 (Figure 6; Table 2 and Supp. Table 6). 7-OH-MG did not significantly alter the dose-effect functions of rates of responding or rectal temperature for U69,593 whereas 7-OH-MG produced a significant 4-fold rightward shift in the U69,593 hotplate antinociception dose-effect function (Figure 6, Table 2, and Supp. Table 6).

MG produced a leftward shift in both lofexidine and clonidine rate-decreasing and hypothermic effect dose-effect functions (Figure 6; Table 2 and Supp. Table 7). When combined with MG, lofexidine and clonidine produced significantly greater hotplate antinociception than either lofexidine alone or clonidine alone (Figure 6; Table 2 and Supp. Table 7). The mean hotplate antinociceptive values, expressed as %MPE, of lofexidine alone and clonidine alone were less than 20% (Figure 6). As with MG, 7-OH-MG shifted to the left the dose-effect functions of the rate-decreasing effects of lofexidine and clonidine and rendered lofexidine and clonidine antinociceptive (Figure 6; Table 2 and Supp. Table 7). However, and in contrast to MG, 7-OH-MG did not significantly modify either lofexidine or clonidine hypothermic dose-effect functions (Figure 6; Table 2 and Supp. Table 7).

Combinations of the Reference Agonists. Among food-maintained behavior, hotplate response latency, and rectal temperature, only analyses of food-maintained behavior were used to determine the ED_{50} values of all the reference agonists at MOR, KOR, and Aα_2R (Table 3). Based on the calculated rate decreasing ED_{50} values of each reference compound alone, doses for the mixtures in ED_{50} ratios of 3:1, 1:1, and 1:2 parts morphine to lofexidine, were administered cumulatively in quarter log units (Table 3). Each drug combination produced dose-related decreases in response rates (Supp. Fig. 4; Supp. Table 10). Hotplate antinociception and hypothermia were also assessed. All morphine dose ratios produced similar leftward antinociceptive morphine dose-effect function shifts. As the morphine dose ratio increased (i.e., 1:2, 1:1, 3:1 morphine to lofexidine) the

hypothermia dose-effect functions shifted further to the left (Supp. Fig. 4; Supp. Table 10). As the lofexidine dose ratio *decreased* (i.e., 1:2, 1:1, 3:1 morphine to lofexidine) the antinociception dose-effect functions shifted further to the left (Supp. Fig. S4; Supp. Table 10). All lofexidine dose ratios produced similar leftward lofexidine hyperthermic dose effect function shifts.

We also examined, based upon the ED_{50} doses to decrease response rates, 2:1, 1:2, and 3:1 morphine to clonidine dose mixtures. Each drug combination produced dose-related decreases in response rates. We found similar shifts as seen with morphine and lofexidine, in the morphine and clonidine antinociceptive and hypothermia dose-effect relationships (Supp. Fig. 5; Supp. Table 10). A similar trend for inverse opioid and adrenergic receptor agonist antinociceptive and hypothermic dose-effect function shifts, based on the relative opioid to adrenergic receptor agonist dose ratio were also consistently observed with 1:2, 1:1, 3:1 methadone to lofexidine (Supp. Fig. 6; Supp. Table 10), 4:1, 2:1, 1:1 methadone to clonidine (Supp. Fig. 7; Supp. Table 10), 1:2, 1:1, 2:1 U69,593 to lofexidine (Supp. Fig. 8; Supp. Table 10) and 1:2, 2:1, 3:1 U69,593 to clonidine (Supp. Fig. 9; Supp. Table 10) ED_{50} ratios.

Interactive Effects of Reference Compounds. Sub-additivity for drug combination rate decreasing effects was not observed in any of the above discussed morphine to lofexidine, morphine to clonidine, methadone to lofexidine, methadone to clonidine, U69,593 to lofexidine, or U69,593 to clonidine drug combinations (Figure 7; Table 4). Additive effects were generally observed, with a few exceptions where supra-additivity was found. Supra-additivity was observed under the following dose ratios 1:1 and 1:2 morphine to lofexidine, 2:1 and 1:2 morphine to clonidine, 2:1 methadone to clonidine, 1:1, 1:2, 2:1 U69,593 to lofexidine, 2:1, 1:2 U69,593 to clonidine (Figure 7; Table 4).

DISCUSSION

In this study we observed several novel findings. MG had comparable binding affinities at $A\alpha_2R$ and MOR whereas 7-OH-MG, an active metabolite of MG, had relatively high affinity at MOR and negligible affinity at $A\alpha_2R$. Among three experimental assays employed in this study, we examined drug-drug schedule-controlled responding interactions via isobolar analysis. MG and 7-OH-MG potentiated the rate-decreasing effects of $A\alpha_2R$ agonists, but not MOR agonists, and increased the potency of $A\alpha_2R$ agonists to produce

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antinociception. MG but not 7-OH-MG potentiated the hypothermic effects of the $A\alpha_2R$ agonists. Neither naltrexone nor yohimbine antagonized the rate-decreasing effects of MG, whereas naltrexone, but not yohimbine, antagonized the rate-deceasing effects of 7-OH-MG. Thus, these isobolar analyses suggest that to produce the opioid-sparing effects of $A\alpha_2R$ agonists a specific dose combination is required. In addition, these results suggest that MG and 7-OH-MG may produce antinociceptive synergism with both $A\alpha_2R$ and MOR agonists. Furthermore, MG but not 7-OH-MG when combined with $A\alpha_2R$ agonists may produce hypothermic synergism.

The supra-additive interactions between MOR and $A\alpha_2R$ on schedule-controlled responding was observed at various dose ratios (i.e., 2:1, 1:1, 1:2) and these interactive effects may be specific to schedule-controlled responding. For example, in several mouse and rat antinociception studies others have found supra-additive interactions between MOR and $A\alpha_2R$ only when mixtures included low proportions of the MOR agonist relative to an $A\alpha_2R$ agonist based on their individual potencies (Spaulding et al., 1979; Drasner and Fields, 1988; Tajerian et al., 2012; Stone et al., 2014). Additionally, our findings demonstrate that schedule-controlled responding supra-additive interactions at $A\alpha_2R$ were not pharmacologically specific for MOR, as supra-additive interactions with $A\alpha_2R$ agonists were observed with the KOR agonist U69,593. These results highlight the importance of the proportions of MOR agonists in complex drug mixtures on observed behavior. An additional consideration for these studies is that here we only examine schedule-controlled responding drug-drug interactions via isobolar analysis. Although we additionally studied hotplate antinociception and hypothermia in these animals, we are unable to determine if these observed dose-response function shifts were subadditive, additive or supra-additive. Additional experiments beyond the scope of the current study would identify antinociceptive and hypothermic drug-drug additivity interactions.

Although not explicitly examined in the present study, supra-additive antinociception resulting from combinations of $A\alpha_2R$ and KOR agonists has been reported (Ossipov et al., 1990a; Roerig, 1994). Specifically, supra-additive antinociception was produced in rats using a tail withdrawal assay when three parts of clonidine and one part of U69,593 were administered intrathecally (i.t.) (Ossipov et al., 1990a). Further, supra-additive

antinociception was produced in mice using the tail withdrawal assay when one part of clonidine and one part of the KOR agonist U50-488H were administered intrathecally (i.t.) (Roerig, 1994). When compared to our additive KOR and Aα_2R schedule-controlled responding behavioral findings in rats, there are a number of differences across the present and previous studies that may contribute to the observed differences in additive vs. supra-additive drug effects (Ossipov et al., 1990a; Roerig, 1994); assays employed (i.e., antinociception vs. schedule-controlled responding), the routes of administration of compounds (i.e., i.p. versus i.t.), and drug history (i.e. a complex drug history versus naive). These differences may individually and combined yield different receptor densities and receptor pools that mediate the underlying observed behavioral results.

The affinities of MG at both MOR and Aα_2R were approximately equal whereas the affinity of 7-OH-MG was high at the MOR (77.9 nM) and negligible at the Aα_2R. In our studies, MG failed to mimic the antinociceptive effects of MOR agonists or the hypothermic effects of Aα_2R agonists. These findings are in contrast to previously reported results which demonstrated that MG produced antinociceptive effects in C57BL/6J mice (Chakraborty et al., 2021). Additionally, neither naltrexone nor yohimbine antagonized mitragynine-induced decreases in food-maintained behavior. Under the same experimental conditions, naltrexone antagonized the effects of MOR agonists, and yohimbine antagonized the effects of Aα_2R agonists. In contrast to MG, 7-OH-MG mimicked the effects of morphine and methadone. Superficially, these MG results suggest no contribution of the MOR or Aα_2R to the pharmacological effects of MG in rats. However, as the discriminative-stimulus effects of MG in rats were antagonized by naltrexone, our current results do not broadly apply to all in vivo pharmacological assessments (Obeng et al., 2021b). Additionally, in a neuropathic pain model, the anti-allodynic effects of MG in rats were antagonized by yohimbine (Foss et al., 2020). The inability of naltrexone to antagonize the rate-decreasing effects of MG has previously been reported (Hiranita et al., 2019; Obeng et al., 2021b). Naltrexone was 3.2-fold less potent in antagonizing the rate-decreasing effects of morphine than in antagonizing the discriminative-stimulus effects of morphine in rats (Obeng et al., 2021b). Thus, the sensitivity to the pharmacological activity of interest differs across experimental assays employed.

Both MG and 7-OH-MG potentiated the rate-decreasing effects of lofexidine and clonidine, but not those of morphine and methadone, and increased the maximum antinociceptive effects of the $A\alpha_2R$ agonists. However, MG, but not 7-OH-MG, potentiated the hypothermic effects of the reference $A\alpha_2R$ agonists. The MG-induced potentiation of the hypothermic and antinociceptive effects of the reference $A\alpha_2R$ agonists might suggest positive allosteric effects of MG at the $A\alpha_2R$; however, there is currently no such published report or supportive evidence. Nonetheless, there are clinical implications, in that, MG can be used to enhance the clinical effects of $A\alpha_2R$ agonists; such as pain relief as well as the ability to block the acute withdrawal symptoms in chronic opioid users. Additionally, the in vivo "apparent" positive allosteric effects of MG at the $A\alpha_2R$ might indicate a challenging hypothesis that MG could mitigate opioid withdrawal (Wilson et al., 2020; Wilson et al., 2021) primarily due to allosteric agonism at the $A\alpha_2R$ rather than dual agonism at the MOR and $A\alpha_2R$ (Chakraborty et al., 2021). It is worth noting that MG is metabolized by CYP3A4 to 7-OH-MG (Kamble et al., 2019; Basiliere and Kerrigan, 2020; Chakraborty et al., 2021). It was recently reported that metabolic conversion of 7-OH-MG does not contribute to MG pharmacological activity (Berthold et al., 2022). However, other studies showed that 7-OH MG does contribute to the analgesic and respiratory depressive effects of MG, albeit its contribution was found to be limited by metabolic saturation (Kruegel et al., 2019; Chakraborty et al., 2021; Hill et al., 2022). In the study by Berthold and colleagues it was demonstrated that in mice treated with MG doses which produced significant hotplate antinociception, 7-OH-MG brain levels remained significantly below the observed 7-OH-MG brain levels found in 7-OH-MG treated mice that were dosed sufficiently to produce acute antinociception (Berthold et al., 2022). In this study, the pharmacological activity of 7-OH-MG was quite different from that of MG, which contradicts the hypothesis that 7-OH-MG is responsible for the "apparent" antinociceptive effects of MG in mice (Kruegel et al., 2019). The inconsistency between the present and previous (Kruegel et al., 2019) studies might simply be due to a difference in species (i.e., rat vs. mouse, respectively).

To assess the therapeutic utility of these kratom alkaloids, future studies should examine the sub-additive, additive vs. supra-additive effects of MG, 7-OH-MG, and MOR as well as $A\alpha_2R$ agonists in relevant pathological pain and drug dependence models. In conclusion, supra-additive interaction between agonism at

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the MOR and Aα_2R depend on the dose combination ratio and MOR agonist used. Affinity of MG at these receptors was approximately equal whereas no considerable affinity of 7-OH-MG was found at the Aα_2R.

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Contributed new reagents or analytic tools: Leon and McCurdy.

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Wrote or contributed to the writing of the manuscript: Obeng, Leon, Zuarth Gonzalez, Da Silva, Shiomitsu, Soto, McCurdy, McMahon, Wilkerson, and Hiranita.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Schematic presentation of experimental timelines on test and inter-test sessions. The rate-decreasing, hypothermic, and antinociceptive effects of test compounds were repeatedly assessed in eight rats (four rats per sex) by measuring schedule-controlled responding (SCR) for presentation of food pellets, rectal temperature (RT), and hotplate (HP) response latency, respectively. RT and HP response latency were measured manually in this order only on test days. RT was measured using a microprobe. HP response latency was measured by placing each rat on a heated hotplate at 52°C and using a stopwatch. The experimental session consisted of six 20-minute experimental cycles and lasted for 120 minutes. On the test days, baseline values of RT and HP response latency were measured before the experimental session. After each rat received an injection (i.p., p.o., or s.c.) (T=0 minutes), the first experimental cycle commenced by placing the rat in the operant-conditioning chamber. Each experimental cycle consisted of the 15-minute timeout period and then 5-minute period for data collection of lever-pressing responses for presentations of food pellets using an automated system. Immediately following each 20-minute cycle, RT and HP response latency were measured in this order. Then, each rat received an injection of a dose of test compound and the second cycle commenced by placing the rat in the operant-conditioning chamber. Doses of each test compound was administered cumulatively. The experimental procedures on inter-test days were basically identical to those on test sessions.

However, RT and HP response latency were not measured on inter-test days. In addition, only vehicle was administered on inter-test days. The inter-test sessions were conducted consecutively at least twice. See Methods section for more details.

Figure 2. Displacement of radioligands at opioid receptor and Aα_2R subtypes. Ordinates: percentage of specific radiotracer bound to membrane preparations. Abscissae: concentrations of each competing compound (log scale). Each data point represents the mean results of three repeated experiments; vertical bars represent S.E.M. (N = 3) from at least three independent triplicate replications per sample. Ki and 95% CI values from curve-fitting analyses of these data are shown in Table 1. Note that affinity of MG at the MOR and Aα_2R was approximately equal whereas no considerable affinity of 7-OH-MG was found at the Aα_2R.

Figure 3. The rate-decreasing, antinociceptive, and hypothermic effects of various compounds alone in rats. Abscissae: Vehicle and cumulative dose of compound in mg/kg (log scale). Ordinates: *Left panels*, percentage of mean rates of responding after repeated administration of vehicle during inter-test sessions; *middle panels*, percentage of maximum possible effect (%MPE) in the hotplate assay; *right panels*, changes in rectal temperature from mean baselines. Each point represents the mean ± SEM (N=4 per sex per data point). All compounds were administered i.p. 15 minutes before each 5-minute period for data collection for food-maintained behavior and MG was also administered p.o. and s.c. (lower panels). The data for morphine, U69,593, and lofexidine on the first assessment were plotted. *Upper left*: The rate-decreasing effects of vehicle, the reference MOR agonists (morphine and methadone), reference Aα_2R agonists (lofexidine and clonidine), and reference KOR agonist U69,593. Filled circles represent repeated vehicle (i.p.) administration. Morphine dose (i.p., upward triangles); vehicle, 5.6, 10, 17.8, 32, and 56 mg/kg. Methadone dose (i.p., downward triangles); vehicle, 0.32, 0.56, 1.0, 1.78, and 3.2 mg/kg. Lofexidine doses (i.p., diamonds); vehicle, 0.056, 0.1, 0.178, 0.32, and 0.56 mg/kg. Clonidine doses (i.p., squares); vehicle, 0.0178, 0.032, 0.056, 0.1, and 0.178 mg/kg. U69,593 doses (i.p., open circles); 0.56, 1.0, 1.78, 3.2, and 5.6 mg/kg. *Upper middle*: The antinociceptive effects of reference compounds. *Upper right*: The hypothermic effects of reference compounds. *Lower left*: The rate-decreasing effects of MG and 7-OH-MG. MG dose (i.p., circles); vehicle, and 5.6, 10, 17.8, 32, and 56 mg/kg. MG dose (p.o., circles); vehicle, 17.8, 32, 56, 100, and 178 mg/kg. MG dose

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(s.c., triangles); vehicle, 17.8, 32, 56, 100, and 178 mg/kg. 7-OH-MG dose (i.p., squares); vehicle, 0.32, 1.0, 3.2, 10, and 32 mg/kg. *Lower middle*: The antinociceptive effects of MG and 7-OH-MG. *Lower right*: The hypothermic effects of MG and 7-OH-MG. Each gray symbol indicates a significant difference from vehicle per corresponding cycle. Note that all test compounds decreased food-maintained behavior. Robust antinociception was produced by the reference MOR agonists but not by the reference Aα_2R agonists whereas robust hypothermia was produced by the reference Aα_2R agonists but not by the reference MOR agonists. Regardless of the route of administration, MG did not produce robust antinociception or hypothermia. As with the reference MOR agonists, 7-OH-MG produced robust antinociception but did not produce significant hypothermia.

Figure 4. The rate-decreasing, antinociceptive, and hypothermic effects of reference agonists in the presence of naltrexone (NLT; opioid receptor antagonist) or yohimbine (YHM; Aα_2R antagonist). Abscissae: Vehicle and cumulative dose of reference agonist in mg/kg (i.p., log scale). Ordinates: *Top row*, percentage of mean rates of responding after repeated administration of vehicle during inter-test sessions; *middle row*, percentage of maximum possible effects in the hotplate assay; *bottom row*, changes in rectal temperature from mean baselines. Each point represents the mean ± SEM (N=4 per sex per data point). Naltrexone and yohimbine were administered i.p. immediately before each session and all reference agonists were administered i.p. 15 minutes before each 5-minute period for data collection for food-maintained behavior. Each data of compound alone (i.e. "None" in each figure key) was replotted from Figure 3. *Leftmost panels*: The effects of morphine. Morphine dose alone (filled circles) and in the presence of 3.2 mg/kg yohimbine (open squares); vehicle, 5.6, 10, 17.8, 32, and 56 mg/kg. Morphine dose in the presence of 0.032 mg/kg naltrexone (open upward triangles); vehicle, 17.8, 32, 56, 100, and 178 mg/kg. Morphine dose in the presence of 1.0 mg/kg naltrexone (open downward triangles); vehicle, 56, 100, 178, 320, and 560 mg/kg. *Second leftmost panels*: The effects of methadone. Methadone dose alone (filled circles) and in the presence of 3.2 mg/kg yohimbine (open squares); vehicle, 0.32, 0.56, 1.0, 1.78, and 3.2 mg/kg. Methadone dose in the presence of 1.0 mg/kg naltrexone (open downward triangles); vehicle, 1.0, 1.78, 3.2, 5.6, and 10 mg/kg. *Third leftmost panels*: The effects of U69,593. U69,593 dose alone (filled circles) and in the presence of 0.032 mg/kg naltrexone (open upward triangles) or

3.2 mg/kg yohimbine (open squares); vehicle, 0.56, 1.0, 1.78, 3.2, and 5.6 mg/kg. U69,593 dose in the presence of 1.0 mg/kg naltrexone (open downward triangles); vehicle, 1.78, 3.2, 5.6, 10, and 17.8 mg/kg. *Forth leftmost panels*: The effects of lofexidine. Lofexidine dose alone (filled circles) and in the presence of 1.0 mg/kg naltrexone (open downward triangles); vehicle, 0.056, 0.1, 0.178, 0.32, and 0.56 mg/kg. Lofexidine dose in the presence of 1.0 mg/kg yohimbine (diamonds); vehicle, and 0.178, 0.32, 0.56, 1.0, and 1.78 mg/kg. Lofexidine dose in the presence of 3.2 mg/kg yohimbine (open squares); vehicle, 0.56, 1.0, 1.78, 3.2, and 5.6 mg/kg. *Rightmost panels*: The effects of clonidine. Clonidine alone and in the presence of 1.0 mg/kg naltrexone (open downward triangles); vehicle, 0.0178, 0.032, 0.056, 0.1, and 0.178 mg/kg. Clonidine dose in the presence of 3.2 mg/kg yohimbine (open squares); vehicle, 0.056, 0.1, 0.178, 0.32, and 0.56 mg/kg. Each gray symbol indicates a significant difference from vehicle per corresponding cycle as shown in **Figure 3**. Note that the lower dose of naltrexone antagonized the rate-decreasing and antinociceptive effects of the reference MOR agonists. The higher dose of naltrexone antagonized the rate-decreasing and antinociceptive effects of morphine and U69,593. The lower dose of yohimbine antagonized the rate-decreasing and hypothermic effects of the reference Aα_2R agonists.

Figure 5. The rate-decreasing, antinociceptive, and hypothermic effects of MG and 7-OH-MG in the presence of naltrexone (NLT: opioid receptor antagonist) or yohimbine (YHM; Aα_2R antagonist). Abscissae: Vehicle and cumulative dose of test compound in mg/kg (i.p., log scale). Ordinates: *Top row*, percentage of mean rates of responding after repeated administration of vehicle during inter-test sessions; *middle row*, percentage of maximum possible effects in the hotplate assay; *bottom row*, changes in rectal temperature from mean baselines. Each point represents the mean ± SEM (N=4 per sex per data point). Naltrexone and yohimbine were administered i.p. immediately before each session and all other compounds were administered i.p. 15 minutes before each 5-minute period for data collection for food-maintained behavior. Each data of test compound alone (i.e. "None" in each figure key) was replotted from Figure 3. *Left panels*: The effects of MG. MG dose alone (filled circles) and in the presence of 1.0 mg/kg naltrexone (open downward triangles) or 3.2 mg/kg yohimbine (open squares); vehicle, 5.6, 10, 17.8, 32, and 56 mg/kg. *Right panels*: The effects of 7-OH-MG. 7-OH-MG dose alone (filled circles) and in the presence of 3.2 mg/kg yohimbine (open squares); vehicle,

0.32, 1.0, 3.2, 10, and 32 mg/kg. 7-OH-MG dose in the presence of .032 mg/kg naltrexone (open upward triangles); vehicle, 1.0, 3.2, 10, 32, and 56 mg/kg. Each gray symbol indicates a significant difference from vehicle per corresponding cycle as shown in **Figure 3**. Note that each high dose of naltrexone and yohimbine did not significantly antagonize the rate-decreasing effects of MG. The lower dose of naltrexone antagonized the rate-decreasing and antinociceptive effects of 7-OH-MG.

Figure 6. The rate-decreasing, antinociceptive, and hypothermic effects of reference agonists in the presence of MG and 7-OH-MG. Abscissae: Vehicle and cumulative dose of reference agonist in mg/kg (i.p., log scale). Ordinates: *Top row*, percentage of mean rates of responding after repeated administration of vehicle during inter-test sessions; *middle row*, percentage of maximum possible effects in the hotplate assay; *bottom row*, changes in rectal temperature from mean baselines. Each point represents the mean ± SEM (N=4 per sex per data point). MG and 7-OH-MG were administered i.p. immediately before each session and all reference agonists were administered i.p. 15 minutes before each 5-minute period for data collection for food-maintained behavior. Each data of reference agonists alone (i.e. "None" in each figure key) was replotted from **Figure 3**. *Leftmost panels*: The effects of morphine. Morphine dose alone (filled circles) and in the presence of 17.8 mg/kg MG (open squares) or 0.32 mg/kg 7-OH-MG (open diamonds); vehicle, 5.6, 10, 17.8, 32, and 56 mg/kg. *Second leftmost panels*: The effects of methadone. Methadone dose alone (filled circles) and in the presence of 17.8 mg/kg MG (open squares) or 0.32 mg/kg 7-OH-MG (open diamonds); vehicle, 0.32, 0.56, 1.0, 1.78, and 3.2 mg/kg. *Third leftmost panels*: The effects of U69,593. U69,593 dose alone (filled circles) and in the presence of 17.8 mg/kg MG (open squares) or 0.32 mg/kg 7-OH-MG (open diamonds); vehicle, 0.56, 1.0, 1.78, 3.2, and 5.6 mg/kg. *Forth leftmost panels*: The effects of lofexidine. Lofexidine dose alone (filled circles) and in the presence of 0.32 mg/kg 7-OH-MG (open diamonds); vehicle, 0.056, 0.1, 0.178, 0.32, and 0.56 mg/kg. Lofexidine dose in the presence of 17.8 mg/kg MG (open squares); vehicle, 0.0178, 0.032, 0.056, 0.1, and 0.178 mg/kg. *Rightmost panels*: The effects of clonidine. Clonidine alone and in the presence of 0.32 mg/kg 7-OH-MG (open diamonds); vehicle, 0.0178, 0.032, 0.056, 0.1, and 0.178 mg/kg. Clonidine dose in the presence of 17.8 mg/kg MG (open squares); vehicle, 0.0056, 0.01, 0.0178, 0.032, and 0.056 mg/kg. Each gray symbol indicates a significant difference from vehicle per corresponding cycle as shown in **Figure 3**.

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Note that MG potentiated the rate-decreasing and hypothermic effects of the reference $A\alpha_2R$ agonists. In the presence of MG and 7-OH-MG, the reference $A\alpha_2R$ agonists also produced relatively robust antinociception.

Figure 7. Isobolographic analysis of reference $A\alpha_2R$ agonists combined with MOR or KOR reference agonists. Ordinates, ED_{50} values of morphine (*left panels*), methadone (*middle panels*), and U69,593 (*right panels*) in mg/kg. Abscissae, ED_{50} values of lofexidine (*upper panels*) and clonidine (*lower panels*) in mg/kg. Each point represents the ED_{50} value and error bars represent 95% CIs. The points at which the line of additivity crosses the ordinates and abscissae represent the ED_{50} values of each compound alone. The line of additivity (dashed line) represents combinations of doses that would be predicted to produce a 50% effect if the compounds were strictly dose-additive. The vertical and horizontal lines around each data point represent the 95% CIs. * Indicates at least $p < 0.05$ difference between Zmix and Zadd for a respective dose combination, denoting supra-additivity.

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Table 1 Inhibition of binding of the radioligands labeling $A\alpha_2R$ and opioid receptor subtypes. Values are Ki values for displacement of the radioligands (see Table S1). Values in parentheses are 95% CIs unless noted. Values listed from previous studies were also added as reference.

Compound	$A\alpha_{2A}R$ K_i Value (nM)	$A\alpha_{2C}R$ K_i Value (nM)	DOR K_i Value (nM)	KOR K_i Value (nM)	MOR K_i Value (nM)	$A\alpha_{2C}$ /$A\alpha_{2A}$	$A\alpha_{2A}$/MOR	$A\alpha_{2C}$/MOR
Clonidine	5.97 (3.66, 10.4)	60.8 (33.7, 115)	No inhibition up to 10 μM	No inhibition up to 10 μM	No inhibition up to 10 μM	10.2	NA	NA
7-OH-MG	No inhibition up to 10 μM	No inhibition up to 10 μM	243 (168, 355)	220 (162, 302)	77.9 (45.8, 152)	NA	NA	NA
Lofexidine	1.21 (0.60, 2.43)	7.62 (3.96, 14.8)	No inhibition up to 10 μM	No inhibition up to 10 μM	No inhibition up to 10 μM	6.30	NA	NA
Methadone	No inhibition up to 10 μM	No inhibition up to 10 μM	No inhibition up to 10 μM	481 (294, 816)	6.61 (5.27, 8.32)	NA	NA	NA
MG	4,420 (2,720,	4,040 (1,880,	6,800 (2,980,	1,700 (1,090,	709 (451, 1,130)[a]	0.914	6.23	5.70

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	7,670)[a] 4,720 (SEM: 120)[b] 2.3 μM[c]	6,820)[a] 2,320 (SEM: 140)[b] 3.5 μM[c]	15,900)[a]	2,710)[a]				
Morphine	No inhibition up to 10 μM	No inhibition up to 10 μM	250 (177, 346)[a]	40.4 (23.7, 70.9)[a]	4.19 (2.03, 11.1)[a]	NA	NA	NA
Naltrexone	No inhibition up to 10 μM	No inhibition up to 10 μM	37.2 (26.3, 53.0)[a]	1.19 (0.803, 1.79)[a]	1.84 (1.14, 3.03)[a]	NA	NA	NA
U69,593	No inhibition up to 10 μM	No inhibition up to 10 μM	6,700 (2,160, 28,000)[a]	1.62[a] (1.02, 2.64)[a]	3,180 (1,050, 11,600)[a]	NA	NA	NA
Yohimbine	8.24 (5.40, 12.8)	7.77 (4.76, 12.8)	No inhibition up to 10 μM	No inhibition up to 10 μM	No inhibition up to 10 μM	0.943	NA	NA

Ki: Inhibition constant.

NA: Not applicable

[a] Human recombinant CHO cells using [^{3}H]RX821002 conducted at Eurofins Cerep (Celle l'Evescault, France) (Obeng et al., 2020).

[b] Binding at human opioid receptor cell lines (Obeng et al., 2021b).

[c] Binding at adrenergic receptors ($A\alpha_{2A}$ and $A\alpha_{2C}$) conducted at the National Institute of Mental Health Psychoactive Drug Screening Program (NIMH, PDSP) (Ellis et al., 2020).

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Table 2 ED_{50} and E_{-2C° values in mg/kg for the rate-decreasing, antinociceptive, hypothermic effects of various compounds as shown in **Figures 3-6, S2-4**. The sample sizes are described in each figure legend. Each value is a combination of females and males unless otherwise noted. Potency ratios (SEMs) are calculated by dividing the ED_{50} or E_{-2C° values for producing the antinociceptive or hypothermic effects, respectively, by the ED_{50} values for producing the rate-decreasing effects. Values in parentheses are 95% CIs. Significant differences are bold.

Morphine Dose					
Combination	ED_{50} or E_{-2C° (SEM)			Potency Ratio	
	Decrease in Response Rate (ED_{50})	Antinociception (ED_{50})	Hypothermia (E_{-2C°)	Antinociception / Decrease in Response Rate	Hypothermia / Decrease in Response Rate
Morphine Alone	9.81 (7.32, 12.30)	39.30 (37.18, 41.43)	Not Applicable	**4.00 (3.02, 5.66)**	Not Applicable
Morphine + 0.032 mg/kg Naltrexone	43.8 (41.6, 46.0)	210 (188, 232)	Not Applicable	**4.79 (4.09, 5.58)**	Not Applicable
Morphine + 1.0 mg/kg Naltrexone	309 (257, 361)	Not Applicable	Not Applicable	Not Applicable	Not Applicable
Morphine + 3.2 mg/kg Yohimbine	13.5 (11.1, 15.9)	24.3 (19.0, 29.6)	Not Applicable	**1.80 (1.20, 2.67)**	Not Applicable
Morphine + 17.8 mg/kg MG	9.29 (6.55, 12.03)	35.9 (33.8, 38.0)	Not Applicable	**3.86 (2.81, 5.80)**	Not Applicable
Morphine + 0.32 mg/kg	19.5 (15.8, 23.2)	33.1 (29.6, 36.6)	Not	**1.70 (1.28, 2.32)**	Not

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7-OH-MG			Applicable		Applicable
Methadone Dose					
Combination	ED_{50} or $E_{-2C^{\circ}}$ (SEM)			Potency Ratio	
	Decrease in Response Rate (ED_{50})	Antinociception (ED_{50})	Hypothermia ($E_{-2C^{\circ}}$)	Antinociception / Decrease in Response Rate	Hypothermia / Decrease in Response Rate
Methadone Alone	0.70 (0.48, 0.92)	2.22 (1.74, 2.70)	Not Applicable	**3.17 (1.89, 5.63)**	Not Applicable
Methadone + 0.032 mg/kg Naltrexone	2.87 (2.65, 3.09)	25.3 (24.3, 26.3)	Not Applicable	**8.81 (7.86, 9.92)**	Not Applicable
Methadone + 3.2 mg/kg Yohimbine	1.19 (1.00, 1.40)	2.28 (1.80, 2.76)	Not Applicable	**1.91 (1.29, 2.76)**	Not Applicable
Methadone + 17.8 mg/kg MG	1.04 (0.92, 1.16)	2.25 (2.05, 2.45)	Not Applicable	**2.16 (1.77, 2.66)**	Not Applicable
Methadone + 0.32 mg/kg 7-OH-MG	1.16 (0.86, 1.50)	1.93 (1.86, 2.00)	Not Applicable	**1.66 (1.24, 2.33)**	Not Applicable
U69,593 Dose					
Combination	ED_{50} or $E_{-2C^{\circ}}$ (SEM)			Potency Ratio	
	Decrease in Response Rate (ED_{50})	Antinociception (ED_{50})	Hypothermia ($E_{-2C^{\circ}}$)	Antinociception / Decrease in Response Rate	Hypothermia / Decrease in Response Rate
U69,593 Alone	2.17 (1.70, 2.65)	3.17 (2.39, 3.95)	Not	1.46 (0.90, 2.32)	Not

			Applicable		Applicable
U69,593 + 0.032 mg/kg Naltrexone	Not Applicable	1.86 (1.52, 2.20)	Not Applicable	Not Applicable	Not Applicable
U69,593 + 1.0 mg/kg Naltrexone	14.58 (11.87, 17.29)	49.07 (47.0, 51.20)	Not Applicable	**3.36 (2.72, 4.31)**	Not Applicable
U69,593 + 3.2 mg/kg Yohimbine	2.28 (1.80, 2.80)	2.62 (1.84, 3.40)	Not Applicable	1.15 (0.657, 1.89)	Not Applicable
U69,593 + 17.8 mg/kg MG	3.10 (2.74, 3.46)	4.66 (4.15, 5.17)	Not Applicable	**1.50 (1.19, 1.89)**	Not Applicable
U69,593 + 0.32 mg/kg 7-OH-MG	5.61 (4.67, 6.55)	16.0 (15.0, 16.9)	Not Applicable	2.85 (2.29, 3.62)	Not Applicable
Lofexidine Dose					
Combination	ED_{50} or $E_{-2C°}$ (SEM)			Potency Ratio	
	Decrease in Response Rate (ED_{50})	Antinociception (ED_{50})	Hypothermia ($E_{-2C°}$)	Antinociception / Decrease in Response Rate	Hypothermia / Decrease in Response Rate
Lofexidine Alone	0.153 (0.121, 0.185)	Not Applicable	0.294 (0.267, 0.321)	Not Applicable	**1.92 (1.44, 2.65)**
Lofexidine + 1.0 mg/kg Naltrexone	0.107 (0.085, 0.129)	Not Applicable	0.395 (0.332, 0.458)	Not Applicable	**3.69 (2.57, 5.39)**
Lofexidine + 1.0 mg/kg Yohimbine	0.788 (0.683, 0.893)	Not Applicable	1.06 (0.887, 1.23)	Not Applicable	1.35 (0.993, 1.80)

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Lofexidine + 3.2 mg/kg Yohimbine	1.89 (1.60, 2.18)	Not Applicable	3.69 (2.84, 4.54)	Not Applicable	**1.95 (1.30, 2.84)**
Lofexidine + 17.8 mg/kg MG	0.019 (0.014, 0.024)	0.168 (0.161, 0.175)	0.037 (0.027, 0.046)	**8.84 (6.71, 12.5)**	**1.95 (1.13, 3.29)**
Lofexidine + 0.32 mg/kg 7-OH-MG	Not Applicable	0.472 (0.457, 0.487)	0.208 (0.181, 0.235)	Not Applicable	Not Applicable
Clonidine Dose					
Combination	ED_{50} or $E_{-2C°}$ (SEM)			Potency Ratio	
	Decrease in Response Rate (ED_{50})	Antinociception (ED_{50})	Hypothermia ($E_{-2C°}$)	Antinociception / Decrease in Response Rate	Hypothermia / Decrease in Response Rate
Clonidine Alone	0.048 (0.038, 0.058)	Not Applicable	0.094 (0.088, 0.100)	Not Applicable	**1.96 (1.52, 2.63)**
Clonidine + 1.0 mg/kg Naltrexone	0.054 (0.044, 0.064)	Not Applicable	0.105 (0.087, 0.123)	Not Applicable	1.94 (1.36, 2.80)
Clonidine + 1.0 mg/kg Yohimbine	0.186 (0.159, 0.213)	Not Applicable	0.544 (0.474, 0.614)	Not Applicable	2.92 (2.23, 3.86)
Clonidine + 17.8 mg/kg MG	Not Applicable (no more than 50% data point)	0.042 (0.039, 0.186)	0.0633 (0.0501, 0.045)	Not Applicable	Not Applicable
Clonidine + 0.32 mg/kg 7-OH-MG	Not Applicable (no more than 50% data point)	Not Applicable (up to 47.5% MPE)	0.093 (0.089, 0.097)	Not Applicable	Not Applicable

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MG Dose					
Combination	ED_{50} or $E_{-2C^{\circ}}$ (SEM)			Potency Ratio	
	Decrease in Response Rate (ED_{50})	Antinociception (ED_{50})	Hypothermia ($E_{-2C^{\circ}}$)	Antinociception / Decrease in Response Rate	Hypothermia / Decrease in Response Rate
MG Alone (i.p.)	27.2 (21.0, 33.4)	Not Applicable	Not Applicable	Not Applicable	Not Applicable
MG (i.p.) + 1.0 mg/kg Naltrexone	33.8 (22.7, 45.0)	Not Applicable	Not Applicable	Not Applicable	Not Applicable
MG (i.p.) + 3.2 mg/kg Yohimbine	32.0 (27.0, 37.0)	Not Applicable	Not Applicable	Not Applicable	Not Applicable
MG Alone (p.o.)	89.3 (69.8, 108)	Not Applicable	Not Applicable	Not Applicable	Not Applicable
MG Alone (s.c.)	161 (118, 204)	Not Applicable	Not Applicable	Not Applicable	Not Applicable
7-OH-MG Dose					
Combination	ED_{50} or $E_{-2C^{\circ}}$ (SEM)			Potency Ratio	
	Decrease in Response Rate (ED_{50})	Antinociception (ED_{50})	Hypothermia ($E_{-2C^{\circ}}$)	Antinociception / Decrease in Response Rate	Hypothermia / Decrease in Response Rate
7-OH-MG Alone	1.82 (1.22, 2.42)	9.13 (7.41, 10.9)	Not Applicable	**5.02 (3.06, 8.93)**	Not Applicable

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7-OH-MG + 0.032 mg/kg Naltrexone	17.5 (14.4, 20.7)	41.8 (38.2, 45.5)	Not Applicable	**2.39 (1.85, 3.16)**	Not Applicable
7-OH-MG + 3.2 mg/kg Yohimbine	3.07 (2.53, 3.61)	15.7 (14.1, 17.3)	Not Applicable	**5.11 (3.91, 6.84)**	Not Applicable

Table 3 Cumulative doses of test compounds (mg/kg) studied in compound mixtures. Values in parentheses are S.E.M.

1 Morphine : 1 Lofexidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Morphine	Vehicle	1.79 (0.447)	3.19 (0.795)	5.69 (1.42)	10.1 (2.52)	18.0 (4.48)
Lofexidine	Vehicle	0.0196 (0.00433)	0.0348 (0.00771)	0.0620 (0.0137)	0.110 (0.0244)	0.196 (0.0435)
1 Morphine : 2 Lofexidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Morphine	Vehicle	0.897 (0.223)	1.60 (0.398)	2.84 (0.708)	5.06 (1.26)	9.01 (2.24)
Lofexidine	Vehicle	0.0293 (0.00650)	0.0522 (0.0116)	0.0930 (0.0206)	0.165 (0.0367)	0.295 (0.0652)
3 Morphine : 1 Lofexidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Morphine	Vehicle	2.69 (0.670)	4.79 (1.19)	8.53 (2.12)	15.2 (3.78)	27.0 (6.73)
Lofexidine	Vehicle	0.00978 (0.00217)	0.0174 (0.00386)	0.0310 (0.00686)	0.0552 (0.0122)	0.0982 (0.0217)
2 Morphine : 1 Clonidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Morphine	Vehicle	1.79 (0.447)	3.19 (0.795)	5.69 (1.42)	10.1 (2.52)	18.0 (4.48)
Clonidine	Vehicle	0.00379 (0.000994)	0.00675 (0.00177)	0.0120 (0.00315)	0.0214 (0.00561)	0.0381 (0.00998)

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1 Morphine : 2 Clonidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Morphine	Vehicle	0.897 (0.223)	1.60 (0.398)	2.84 (0.708)	5.06 (1.26)	9.01 (2.24)
Clonidine	Vehicle	0.00569 (0.00149)	0.0101 (0.00266)	0.0180 (0.00473)	0.0321 (0.00841)	0.0571 (0.0150)
3 Morphine : 1 Clonidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Morphine	Vehicle	2.69 (0.670)	4.79 (1.19)	8.53 (2.12)	15.2 (3.78)	27.0 (6.73)
Clonidine	Vehicle	0.00190 (0.000497)	0.00338 (0.000885)	0.00601 (0.00158)	0.0107 (0.00280)	0.0190 (0.00499)
1 Methadone : 1 Lofexidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Methadone	Vehicle	0.144 (0.0429)	0.257 (0.0764)	0.457 (0.136)	0.813 (0.242)	1.45 (0.431)
Lofexidine	Vehicle	0.0196 (0.00433)	0.0348 (0.00771)	0.0620 (0.0137)	0.110 (0.0244)	0.196 (0.0435)
1 Methadone : 2 Lofexidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Methadone	Vehicle	0.0721 (0.0215)	0.128 (0.0382)	0.228 (0.0680)	0.407 (0.121)	0.724 (0.215)
Lofexidine	Vehicle	0.0293 (0.00650)	0.0522 (0.0166)	0.0930 (0.0206)	0.165 (0.0367)	0.295 (0.0652)
3 Methadone : 1 Lofexidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Methadone	Vehicle	0.216 (0.0644)	0.385 (0.115)	0.685 (0.204)	1.22 (0.363)	2.17 (0.646)
Lofexidine	Vehicle	0.00978 (0.00217)	0.0174 (0.00386)	0.0310 (0.00686)	0.0552 (0.0122)	0.0982 (0.0217)
2 Methadone : 1 Clonidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6

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Methadone	Vehicle	0.144 (0.0429)	0.257 (0.0764)	0.457 (0.136)	0.813 (0.242)	1.45 (0.431)
Clonidine	Vehicle	0.00379 (0.000994)	0.00675 (0.00177)	0.0120 (0.00315)	0.0214 (0.00561)	0.0381 (0.00998)
1 Methadone : 1 Clonidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Methadone	Vehicle	0.0721 (0.0215)	0.128 (0.0382)	0.228 (0.0680)	0.406 (0.121)	0.724 (0.215)
Clonidine	Vehicle	0.00569 (0.00149)	0.0101 (0.00266)	0.0180 (0.00473)	0.0321 (0.00841)	0.0571 (0.0150)
4 Methadone : 1 Clonidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
Methadone	Vehicle	0.216 (0.0644)	0.385 (0.115)	0.685 (0.204)	1.22 (0.363)	2.17 (0.646)
Clonidine	Vehicle	0.00190 (0.000467)	0.00338 (0.000885)	0.00601 (0.00158)	0.0107 (0.00280)	0.0190 (0.00499)
1 U69,593 : 1 Lofexidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
U69,593	Vehicle	0.346 (0.0471)	0.616 (0.0839)	1.10 (0.149)	1.95 (0.266)	3.47 (0.473)
Lofexidine	Vehicle	0.0177 (0.00346)	0.0316 (0.00616)	0.0562 (0.0110)	0.100 (0.0195)	0.178 (0.0348)
1 U69,593 : 2 Lofexidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
U69,593	Vehicle	0.173 (0.0236)	0.308 (0.0420)	0.548 (0.0747)	0.975 (0.133)	1.74 (0.237)
Lofexidine	Vehicle	0.0339 (0.00757)	0.0603 (0.135)	0.107 (0.0240)	0.191 (0.0427)	0.340 (0.760)
2 U69,593 : 1 Lofexidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
U69,593	Vehicle	0.519 (0.0707)	0.923 (0.126)	1.64 (0.224)	2.93 (0.399)	5.21 (0.710)

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Lofexidine	Vehicle	0.0113 (0.00252)	0.0201 (0.00449)	0.0358 (0.00800)	0.0637 (0.0142)	0.113 (0.0253)
2 U69,593 : 1 Clonidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
U69,593	Vehicle	0.346 (0.0471)	0.616 (0.0839)	1.10 (0.149)	1.95 (0.266)	3.47 (0.473)
Clonidine	Vehicle	0.00382 (0.000956)	0.00680 (0.00170)	0.0121 (0.00303)	0.0215 (0.00539)	0.0384 (0.00960)
1 U69,593 : 2 Clonidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
U69,593	Vehicle	0.173 (0.0236)	0.308 (0.0420)	0.548 (0.0747)	0.975 (0.133)	1.74 (0.237)
Clonidine	Vehicle	0.00761 (0.00215)	0.0135 (0.00383)	0.0241 (0.00681)	0.0429 (0.0121)	0.0764 (0.0216)
3 U69,593 : 1 Clonidine						
Compound	Cycle 1	Cycle 2	Cycle 3	Cycle 4	Cycle 5	Cycle 6
U69,593	Vehicle	0.519 (0.0707)	0.923 (0.126)	1.64 (0.224)	2.93 (0.399)	5.21 (0.710)
Clonidine	Vehicle	0.00254 (0.000717)	0.00451 (0.00128)	0.00803 (0.00227)	0.0143 (0.00404)	0.0255 (0.00720)

Table 4 Theoretical Zadd (mg/kg), Experimental Zmix (mg/kg), their confidence intervals, and observed interactive effects of studied compound mixtures.

1 Morphine : 1 Lofexidine		
Zadd	Zmix	Interactive Effect
9.13 (6.55 - 11.71)	2.88 (1.94 - 3.81)	Supra-Additive
1 Morphine : 2 Lofexidine		
Zadd	Zmix	Interactive Effect
9.00 (6.45 – 11.54)	1.42 (0.932 – 1.91)	Supra-Additive

3 Morphine : 1 Lofexidine		
Zadd	Zmix	Interactive Effect
9.22 (3.14 – 15.31)	4.00 (2.23 – 6.23)	Additive
2 Morphine : 1 Clonidine		
Zadd	Zmix	Interactive Effect
9.26 (6.64 – 11.84)	2.90 (1.15 – 4.66)	Supra-Additive
1 Morphine : 2 Clonidine		
Zadd	Zmix	Interactive Effect
9.24 (6.63– 11.86)	1.57 (1.06 – 2.08)	Supra-Additive
3 Morphine : 1 Clonidine		
Zadd	Zmix	Interactive Effect
9.26 (6.65 – 11.88)	5.73 (3.50 – 7.95)	Additive
1 Methadone : 1 Lofexidine		
Zadd	Zmix	Interactive Effect
0.604 (0.541 – 0.668)	0.514 (0.084 – 0.944)	Additive
1 Methadone : 2 Lofexidine		
Zadd	Zmix	Interactive Effect
0.540 (0.159 - 0.922)	0.237 (0.170 – 0.304)	Additive
3 Methadone : 1 Lofexidine		
Zadd	Zmix	Interactive Effect
0.660 (0.279 – 1.04)	0.767 (0.488 – 1.05)	Additive
2 Methadone : 1 Clonidine		
Zadd	Zmix	Interactive Effect
0.683 (0.608 – 0.757)	0.280 (0.137 – 0.424)	Supra-Additive
1 Methadone : 1 Clonidine		

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Zadd	Zmix	Interactive Effect
0.672 (0.415 – 0.930)	0.183 (0.0261 – 0.340)	Supra-Additive
4 Methadone : 1 Clonidine		
Zadd	Zmix	Interactive Effect
0.688 (0.590 – 0.786)	0.680 (0.224 – 1.14)	Additive
1 U69,593 : 1 Lofexidine		
Zadd	Zmix	Interactive Effect
2.033 (1.71 – 2.35)	1.01 (0.907 – 1.108)	Supra-Additive
1 U69,593 : 2 Lofexidine		
Zadd	Zmix	Interactive Effect
1.91 (1.25 – 2.57)	0.484 (0.395 – 0.573)	Supra-Additive
2 U69,593 : 1 Lofexidine		
Zadd	Zmix	Interactive Effect
2.102 (1.73 – 2.47)	1.23 (0.903 – 1.56)	Supra-Additive
2 U69,593 : 1 Clonidine		
Zadd	Zmix	Interactive Effect
2.16 (1.82 – 2.50)	0.735 (0.189 – 1.28)	Supra-Additive
1 U69,593 : 2 Clonidine		
Zadd	Zmix	Interactive Effect
2.12 (1.73 – 2.51)	0.567 (0.462 – 0.672)	Supra-Additive
3 U69,593 : 1 Clonidine		
Zadd	Zmix	Interactive Effect
2.17 (1.82 – 2.51)	1.63 (1.38 – 1.87)	Additive

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Figure 1

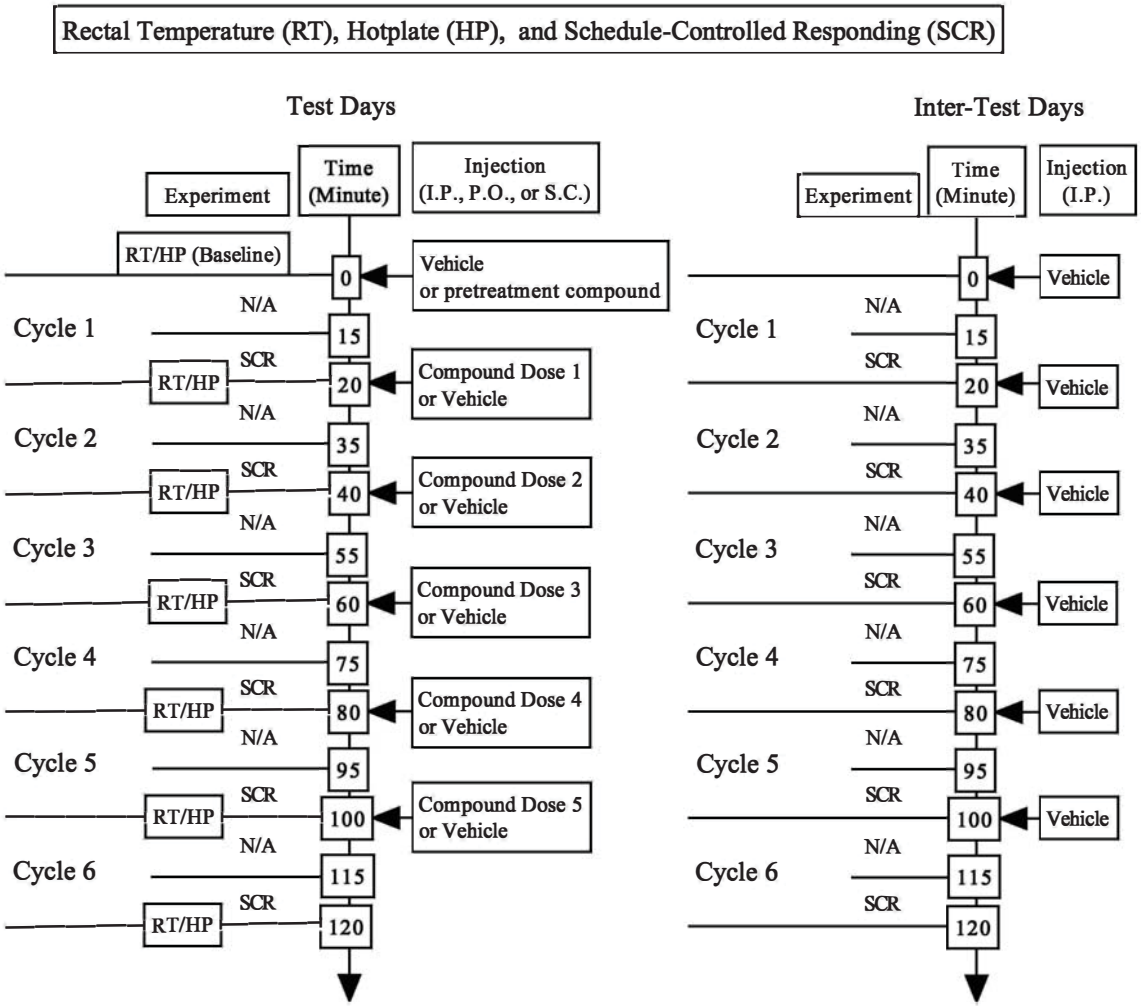

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Figure 2

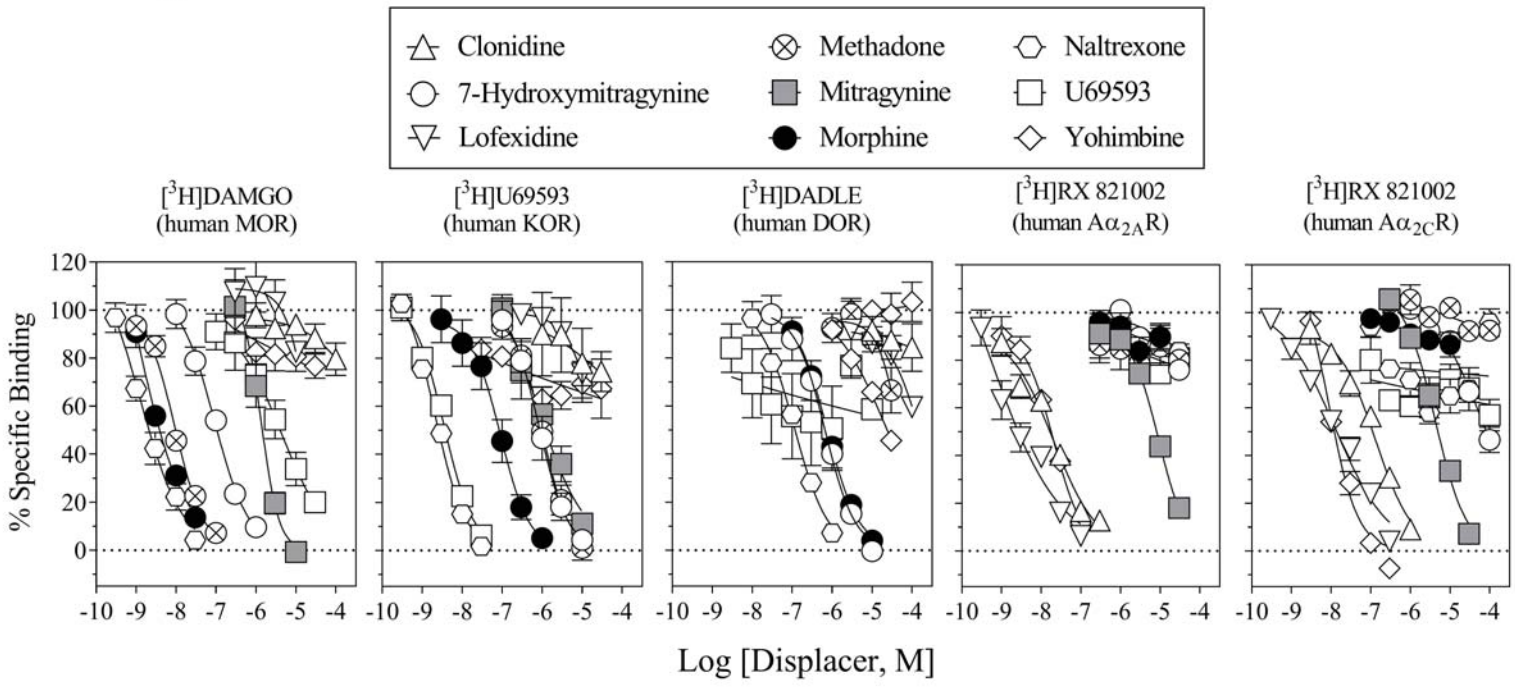

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Figure 3

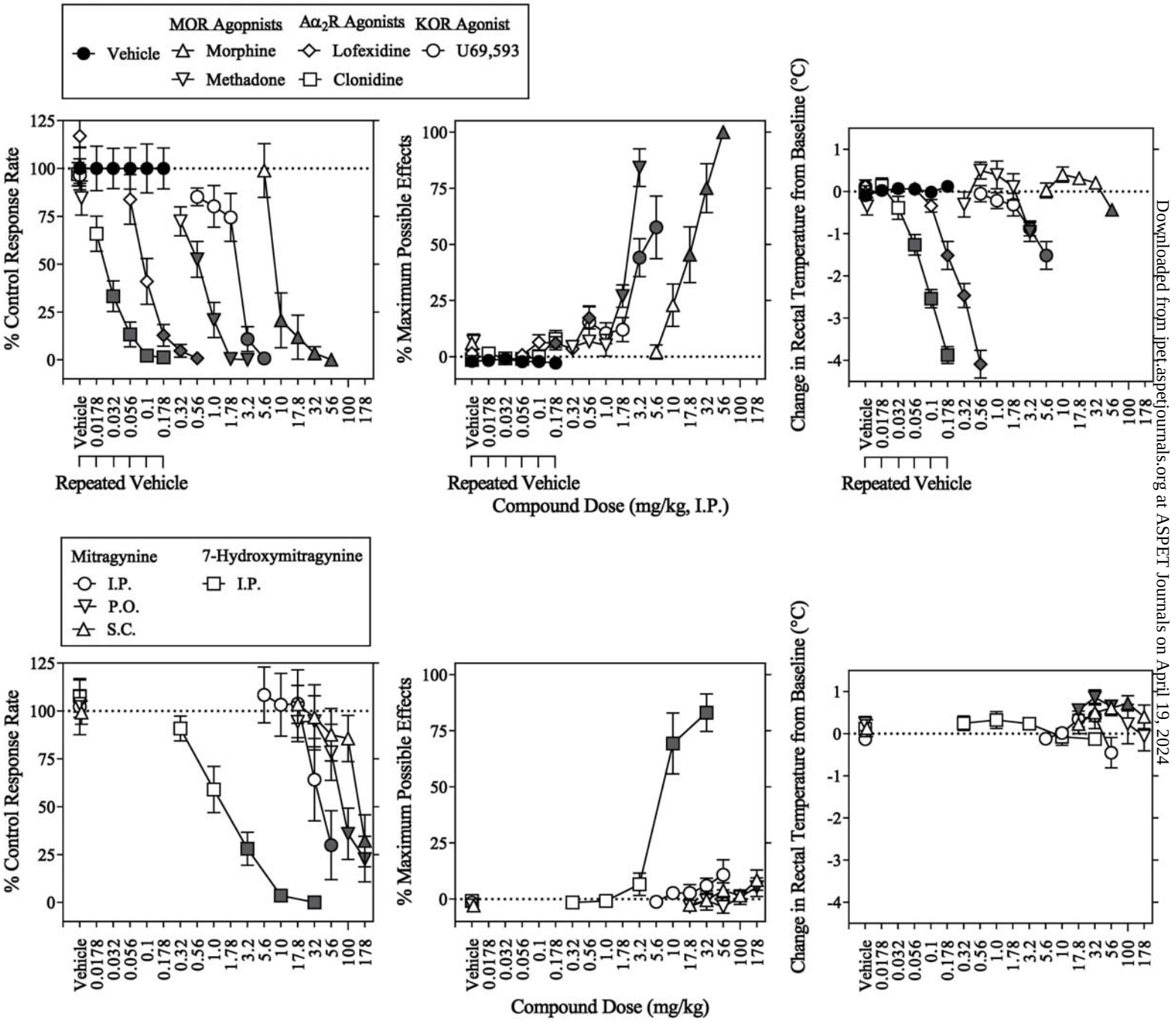

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Figure 4

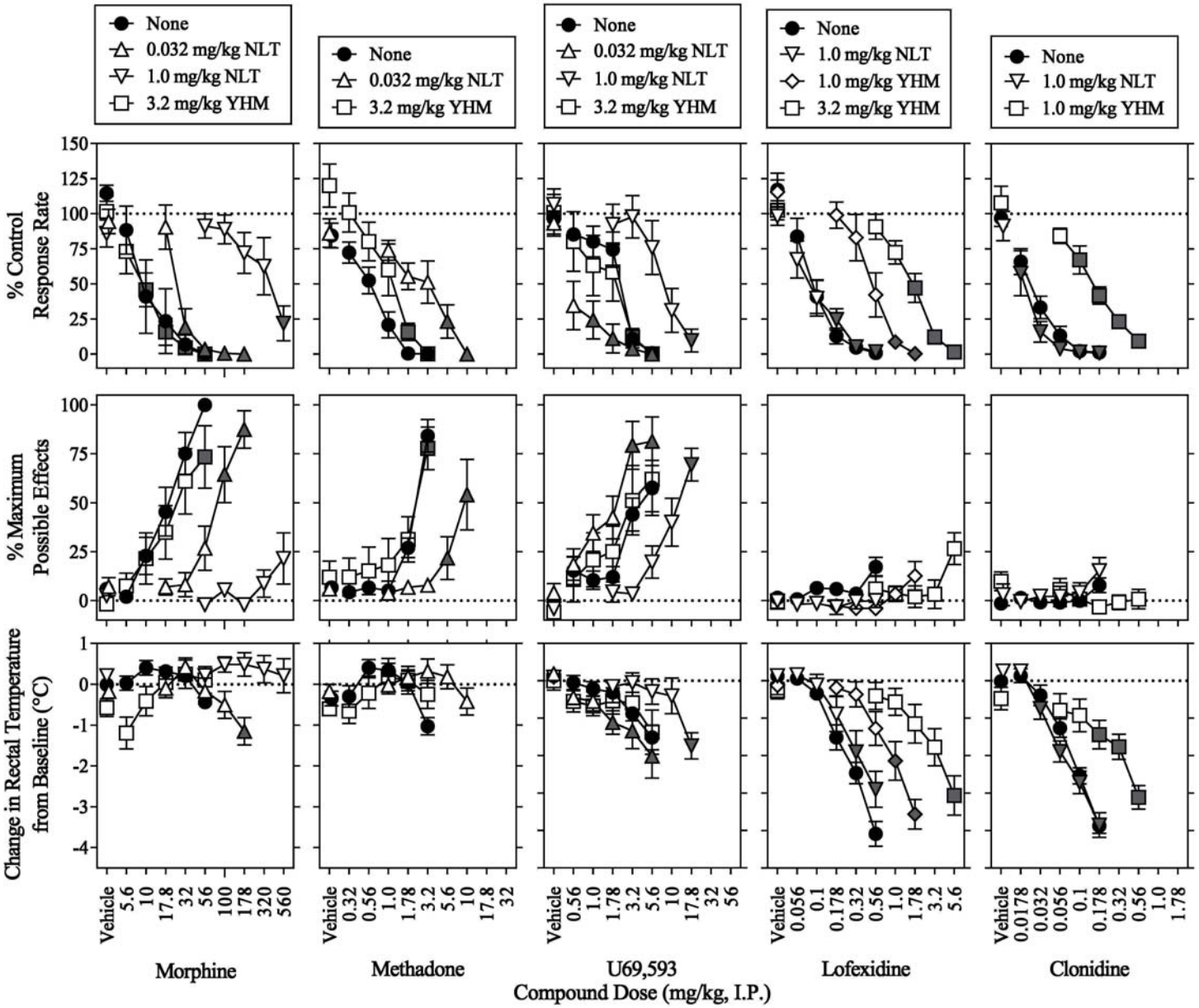

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Figure 5

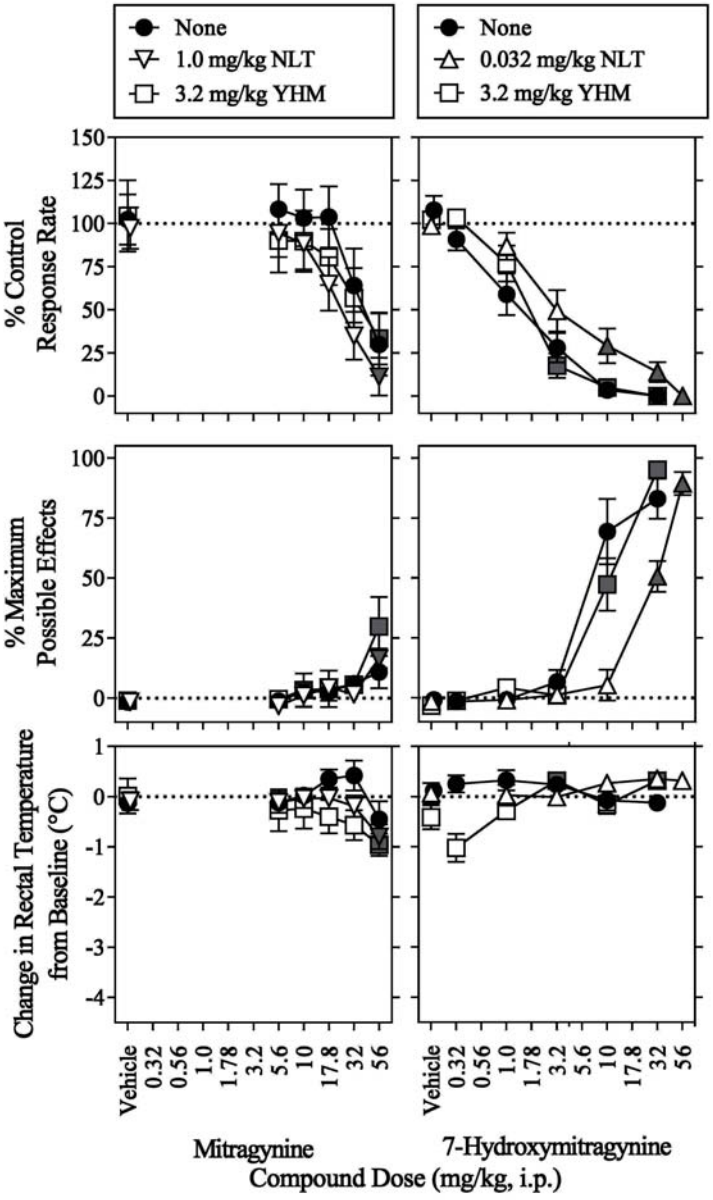

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Figure 6

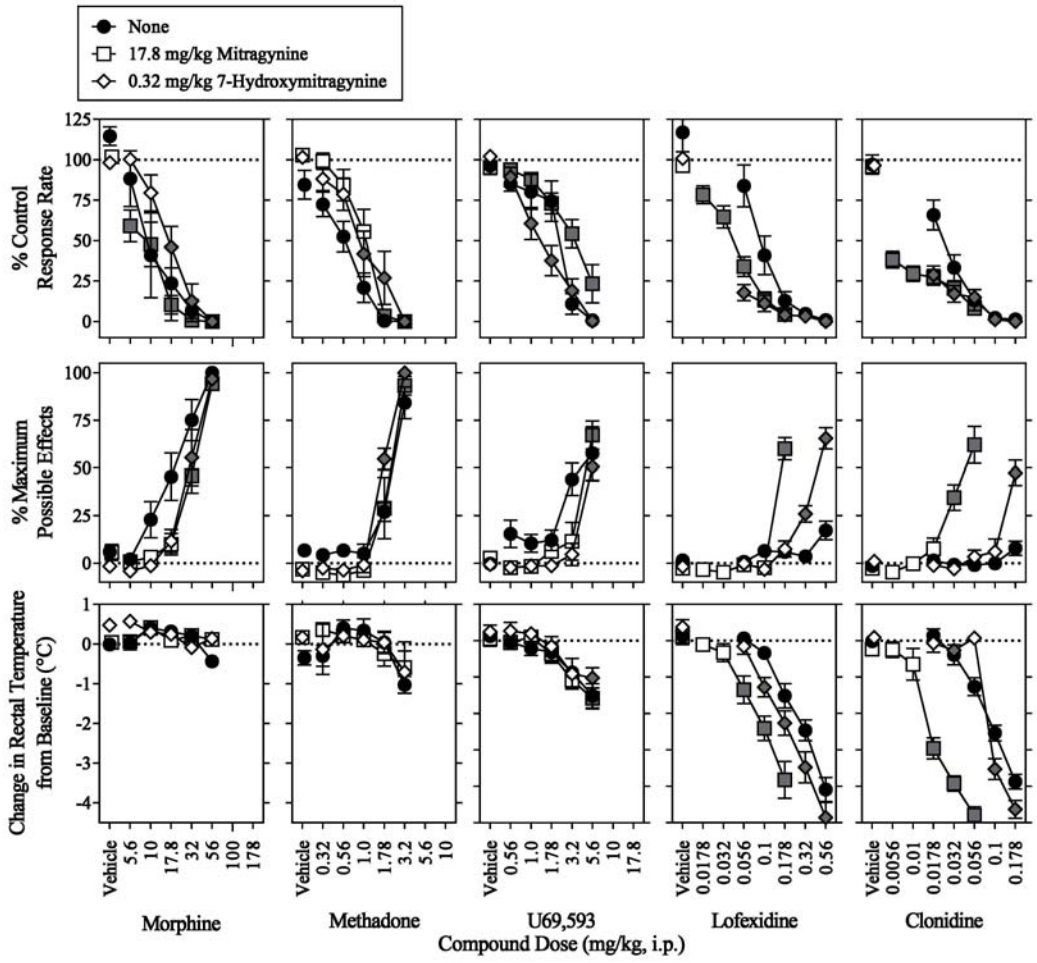

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Figure 7