Comparison of the µ-opioid receptor antagonists methocinnamox (MCAM) and naloxone to reverse and prevent the ventilatory depressant effects of fentanyl, carfentanil, 3-methylfentanyl, and heroin in male rats

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ABSTRACT

The number of opioid overdose deaths has increased significantly over the past decade. The life-threatening effect of opioids is hypoventilation that can be reversed by the µ-opioid receptor (MOR) antagonist naloxone; however, because of the very short duration of action of naloxone, re-emergence of MOR agonist-induced hypoventilation can occur, requiring additional doses of naloxone. The MOR antagonist methocinnamox (MCAM) antagonizes hypoventilation by the non-morphinan fentanyl and the morphinan heroin in laboratory animals with an unusually long duration of action. Whole-body plethysmography was used to compare the potency and effectiveness of MCAM and naloxone for preventing and reversing hypoventilation by fentanyl, heroin, and the ultra-potent and longer-acting fentanyl analogs carfentanil and 3-methylfentanyl in male rats breathing normal air. Sessions comprised a 45-minute habituation period followed by intravenous (i.v.) administration of saline or an acute dose of MOR agonist. The rank order of potency to decrease ventilation was 3-methylfentanyl > carfentanil > fentanyl > heroin. MCAM (0.0001-0.1 mg/kg) and naloxone (0.0001-0.01 mg/kg) dose-dependently reversed hypoventilation by 3-methylfentanyl (0.01 mg/kg), carfentanil (0.01 mg/kg), fentanyl (0.1 mg/kg), or heroin (3.2 mg/kg). For prevention studies, MCAM, naloxone, or vehicle was administered i.v. 22, 46, or 70 hours prior to a MOR agonist. When administered 22 hours earlier, MCAM (0.1-1.0 mg/kg) but not naloxone (1.0 mg/kg) prevented hypoventilation by each MOR agonist. This study demonstrates the effectiveness of MCAM to reverse and prevent hypoventilation by MOR agonists including ultra-potent fentanyl analogs that have a long duration of action.
SIGNIFICANCE STATEMENT

The number of opioid overdose deaths increased over the past decade despite the availability of antagonists that can prevent and reverse the effects of opioids. This study demonstrates the effectiveness and long duration of action of the μ-opioid receptor (MOR) antagonist methocinnamox (MCAM) for reversing and preventing hypoventilation by MOR agonists including ultra-potent fentanyl analogs. These results provide support for the notion that MCAM has the potential to positively impact the ongoing opioid crisis by reversing and preventing opioid overdose.
INTRODUCTION

Among an estimated 106,699 drug overdose deaths in 2021 in the United States, three out of four deaths (80,411) were attributed to opioids (CDC, 2021) with three of four victims being male (CDC, 2021). Whereas the opioid epidemic began with the mis-use of prescription opioids and was further fueled by widely available inexpensive heroin, today the most commonly detected opioids in overdose victims are synthetic $\mu$-opioid receptor (MOR) agonists (e.g., non-morphinan phenylpiperidine fentanyl and its analogs; Jones et al., 2018; Hedegaard et al., 2021). Hypoventilation is the predominant life-threatening effect of MOR agonists (Stoeckel et al., 1982; Pattinson, 2008). Opioid-induced hypoventilation is characterized by decreased ventilatory frequency ($f$), decreased ventilatory volume (tidal volume [$V_T$]), often with upper airway dysfunction (Savilampi et al., 2013, 2014), and irregular breathing pattern (Bouillon et al., 2003).

The opioid receptor antagonist naloxone (Narcan®) reverses opioid agonist-induced ventilatory depression, although its effectiveness is limited by a short duration of action (Kassick et al., 2021; Kang et al., 2022; Voronkov et al., 2022) that can result in renarcanization. It is estimated that up to 90% of opioid overdose rescues required multiple doses of naloxone, possibly due to the increasing use and availability of fentanyl and its potent analogs (Bardsley, 2019; Abdelal et al., 2022). It has also been reported that naloxone is generally less effective at reversing ventilatory depression by fentanyl and its analogs (Kelly et al., 2023; Sutcliffe et al., 2022) compared with reversal of ventilatory depression by heroin and other morphinan opioids. A recent study (Coffin et al., 2022) estimated that naloxone was less effective at reducing the annual overdose death rate of fentanyl (12.0%) compared with heroin (26.4%). Under hypercapnic (i.e., 5% CO$_2$ in air) conditions in mice, naloxone was also less effective at reversing hypoventilation by fentanyl compared with morphine (Hill et al., 2020). Moreover, the ability of naloxone to reverse and protect against opioid-induced ventilatory depression can vary among fentanyl and its analogs. For example, naloxone was less effective to reverse hypoventilation by the ultra-potent fentanyl analog carfentanil ([4-methoxycarbonyl] fentanyl; 0.022 mg) than fentanyl (2.97 mg) in an in silico study (Mann et al., 2022). Moreover, a single dose of the opioid receptor antagonist naltrexone was 3-fold less effective in antagonizing the effects of carfentanil compared with the effects of fentanyl in rats discriminating fentanyl from vehicle (Flynn and France, 2021). An intranasal formulation of another opioid receptor antagonist, with a longer duration of action than naloxone (nalmefene), was recently approved by the Food and Drug Administration (FDA) for treating opioid overdose. In preclinical studies, the MOR antagonist methocinnamox (MCAM) attenuates the abuse-related and
adverse effects of MOR agonists including carfentanil and 3-methylfentanyl. For example, daily administration of MCAM significantly increased ED$_{50}$ values for carfentanil, 3-methylfentanyl, fentanyl, and heroin in monkeys responding under a drug versus food choice procedure (Gerak and France, 2023). MCAM also reversed and prevented fentanyl-induced hypoventilation in rats (Jimenez et al., 2021) and heroin-induced hypoventilation in rhesus monkeys (Gerak et al., 2019) with an unusually long duration of action. Thus, MCAM might improve clinical outcomes and save lives by reversing and providing extended protection from opioid overdose by fentanyl and other MOR agonists. Given that deaths due to re-aracorization after naloxone treatment have been reported (Rudolph et al., 2011; Rzasa Lynn and Galinkin, 2018), it is important to reverse as well as prevent opioid overdose. Currently, it is not demonstrated whether MCAM can reverse and prevent various effects including hypoventilation by the MOR agonists that are more potent than fentanyl.

This study used whole-body plethysmography to compare the potency and effectiveness of MCAM and naloxone for reversing and preventing hypoventilation by fentanyl, the ultra-potent fentanyl analogs carfentanil and 3-methylfentanyl, and heroin in male rats breathing normal air. Normocapnic conditions were employed to model a clinically relevant physiological environment. The present study compared the duration of action of MCAM to prevent hypoventilation, antinociception, and hyperthermia by four MOR agonists. The results of this study demonstrate the effectiveness of MCAM to reverse MOR-agonist induced hypoventilation and prevent hypoventilation, antinociception, and hyperthermia by MOR agonists, including ultra-potent fentanyl analogs.

**MATERIALS AND METHODS**

**Subjects.** Eight adult male Sprague-Dawley rats (weighing 338–356 g at the beginning of the study and 466–527 g nine months later at the end of the study) were purchased from Envigo RMS, LLC (Indianapolis, IN). Rats were housed individually in a vivarium maintained at 23.0 ± 1.5° C (mean ± standard deviation) and 40 ± 20% humidity under a 14/10-hour light/dark cycle (lights on at 0600 hours). Experiments were conducted at 0700-1130 hours during the light cycle. Rats had free access to food (7912 irradiated LM-485 Mouse/Rat Sterilizable Diet, Envigo RMS, LLC) and filtered reverse osmosis tap water (Edstrom BFS-675 Bottle Filling Station, Edstrom Industries, Inc., Waterford, WI) in the home cage. Subjects were maintained and experiments were conducted in accordance with the Institutional Animal Care and Use Committee at The University of Texas Health Science Center at San Antonio and the guidelines of the Committee on Care and Use of Laboratory Animal Resources and

**Surgery.** Following a minimum of 5 days in the vivarium, rats received a chronic indwelling intravenous (i.v.) catheter using aseptic techniques as described previously (Hiranita et al., 2023). Immediately after surgery, rats received subcutaneous injections of 5.0 mg/kg Baytril® (Bayer HealthCare LLC, Shawnee, KS) and 1.0 mg/kg meloxicam (Covetrus, Portland, ME). After surgery, rats were allowed at least 5 days to recover before experiments commenced. Additionally, 5.0 mg/kg Baytril® and 1.0 mg/kg meloxicam were administered 24 and 12 hours, respectively, after surgery. Catheters were flushed daily with 0.5 mL heparinized saline (100 U/mL; Mylan, Canonsburg, PA) using PinPort injectors (PNP3M50, Instech Laboratories, Inc.) immediately before experiments and also on nonexperimental days. The 22-G access ports were capped with protective aluminum caps (VABRC, Instech Laboratories, Inc.) when rats were returned to their home cage. If a catheter malfunctioned (e.g., blockage or leakage), a new catheter was implanted in the right (one rat, 217 days after initial catheter implantation) or left femoral (one rat, 246 days after initial catheter implantation) or left external jugular vein (one rat, 197 days after initial catheter implantation). Catheter patency was evaluated when manual flushing suggested blockage. Rats were handled and habituated to the experimental chamber and conditions for 5–7 days prior to beginning the study.

**Rectal Temperature.** Rectal temperature was measured as described previously (Hiranita et al., 2023).

**Warm Water Tail Withdrawal.** Two water baths (EW-14576-00; Cole-Parmer, Vernon Hills, IL) were maintained at constant temperatures (45 and 50°C) throughout the experiment. Each rat was manually restrained with its tail hanging freely, and the distal 5 to 10 cm of the tail was immersed in water with the two temperatures tested in a mixed order. A stopwatch was used to measure the latency for rats to remove their tails from water. A cut-off time of 15 seconds was imposed at each temperature. Tail withdrawal latencies were determined immediately each after rectal temperature measurement.

**Ventilation.** Ventilation was measured as described previously (Jimenez et al., 2021; Hiranita et al., 2023). Immediately after rectal temperature was measured and tail withdrawal latency was assessed, rats were placed in the whole-body plethysmography chambers and studied under normocapnic conditions (Supplemental Figure S1). Each session began with a 45-minute habituation period (~45 to 0 minutes) followed by administration of vehicle or drugs(s) and a 60-minute test period (0 to 60 minutes). Test compounds were administered i.v. at the beginning of the test period (time 0) and 5 minutes later using the infusion pumps for the reversal experiments. For prevention
studies, i.v. pretreatments were manually infused 22, 46, or 70 hours before a test period (Supplemental Figure S3A). At time 0, saline, fentanyl (0.0032-0.1 mg/kg), carfentanil (0.00032-0.01 mg/kg), 3-methylfentanyl (0.00032-0.01 mg/kg), or heroin (0.1-3.2 mg/kg) was administered i.v. using infusion pumps. β-Cyclodextrin vehicle (10%) was injected alone, with naloxone (0.0001-0.01 and 1.0 mg/kg for the reversal and prevention experiments, respectively), or with MCAM (0.0001-0.1 and 0.1-1.0 mg/kg for the reversal and prevention experiments, respectively) 22, 46, or 70 hours before or 5 minutes after time 0. Infusions were followed by a saline flush of 0.5 mL over five seconds. Immediately after the test period, rats were removed from chambers, and rectal temperature and tail withdrawal latency were again measured. Test sessions were separated by at least 4 days with the order of drugs and doses varying nonsystematically among subjects. A control session was conducted on the day prior to each drug test session; in control sessions rats received an infusion of saline (time 0) and an infusion of 10% β-cyclodextrin vehicle (22, 46, or 70 hours before or 5 minutes after time 0). There was no evidence of tolerance to opioid-induced hypoventilation (e.g., Supplemental Figure S6) and no signs of opioid withdrawal (e.g., diarrhea).

Data Analyses. The primary dependent variables were ventilatory frequency (f, breaths per minute), tidal volume (VT, mL/breath/kg), and the product of f and VT, minute volume (VE, mL/minute/kg), and time for ventilation to return to baseline VE (estimated by linear regression for individual subjects then averaged among subjects), tail withdrawal latencies at two temperatures, and change from baseline (°C) for rectal temperature. Ventilatory parameters were averaged in one-minute bins with VE normalized to individual averages during the ventilation test period under the saline/vehicle condition depending on when 10% β-cyclodextrin vehicle was administered. Data normalization reflects individual changes more precisely compared with presentation of group averages of absolute values. The baseline tail withdrawal latency and rectal temperature for individual subjects were the average of all baseline values immediately before ventilation experiments for the reversal experiments or immediately before administration of 10% β-cyclodextrin vehicle alone or with naloxone or MCAM for the prevention experiments. Tail-withdrawal latencies were converted to a percentage of the maximum possible effect (15 seconds) as follows: [(test latency - control latency)/(15 seconds – control latency)] × 100% and then averaged across eight rats. Changes from baseline for rectal temperature were the temperatures measured after ventilation experiments (°C) subtracted by the baseline temperature (°C). Figures were created with GraphPad Prism version 9 for Windows (GraphPad Software, La Jolla, CA). Data are expressed as the mean ± 1 standard error of mean (SEM) for 8 rats. Time- and dose-effect functions were statistically analyzed with SigmaPlot version 12.0 (Systat Software
Inc., San Jose, CA) by one- (dose) or two- (dose and time or treatment with naloxone or MCAM) way repeated-measures analyses of variance (ANOVA) followed by post-hoc Bonferroni t tests for pairwise comparisons. Using the GraphPad Prism software, the dose-effect functions were further analyzed with linear regression (Snedecor and Cochran, 1967) to calculate effective doses as follows: 1) ED₅₀, potency of the MOR agonists to decrease ventilation or increase tail withdrawal latency; 2) ED₈₀, potency for naloxone and MCAM to reverse hypoventilation by the MOR agonists; and 3) ED₁°C, potency for the MOR agonists to increase rectal temperature by 1°C. Also calculated were the slopes and their 95% confidence intervals (95% CI). Fisher’s exact test (two tails) was used to assess the correlation between in vivo potency (hypoventilation and hyperthermia) and MOR affinity (i.e., Ki values from the published literature) using GraphPad Prism software. The R² and P values are shown in Figure 2. Molecular mass values used were 372.9 for fentanyl, 431.0 for carfentanil, 387.0 for 3-methylfentanyl, and 405.9 for heroin. For the dose-effect analyses of MOR agonists alone and in the prevention experiments, area under the curve (AUC) for VE over the first 10 minutes of the test period (AUC₀₋₁₀) was quantified for individual subjects then averaged among the eight rats. For the dose-effect analyses of naloxone and MCAM reversal, the first 10-minute AUC for VE after administration of naloxone, MCAM, or vehicle (5-15-minutes, AUC₅₋₁₅) was quantified for individual subjects then averaged for eight rats. For all analyses, the criterion for statistical significance was p < 0.05.

Drugs. Carfentanil hydrochloride (HCl), fentanyl HCl, heroin HCl, 3-methylfentanyl HCl, and naloxone HCl were generously provided by the Drug Supply Program of the National Institute on Drug Abuse (Rockville, MD). MCAM HCl was purchased from Syncom (Groningen, NL). Carfentanil, fentanyl, heroin, and 3-methylfentanyl were dissolved in physiological saline (0.9% sodium chloride, 2B1323, Baxter Healthcare Corporation, Deerfield, IL). Naloxone and MCAM were dissolved in 10% w/v β-cyclodextrin (Janssen Research & Development, LLC, Raritan, NJ) in sterile water (B. Braun Medical Inc., Irvine, CA). All drugs and their vehicles were administered i.v. in a volume of 0.5-1.0 mL/kg body weight. Solutions were passed through a filter (CH2225-PES, polyethersulfone syringe filter, 0.22 µm, 25 mm, ThermoFisher Scientific, Waltham, MA) prior to i.v. infusion. Doses are expressed in mg/kg body weight as the salt forms.

RESULTS

Baseline parameters for the reversal experiments. Supplemental Figure S1B represents ventilation time-effect functions under baseline conditions when an injection of saline was followed by an injection of 10% β-
cycloextrin vehicle (saline/vehicle). During the habituation period (minutes -44 to 0), $V_E$ decreased by approximately 550 mL/minute/kg for the first 30 minutes then stabilized at approximately 440 mL/minute/kg for the reminder of the control session. Table 1 represents the mean ± 1 SEM of baseline parameters of ventilation for the test period, tail withdrawal response latency, and rectal temperature. Both tail withdrawal response latency and rectal temperature were also stable before and after ventilation sessions (filled symbols above Vehicle in Supplemental Figure S2C).

**Effects of MOR agonists and antagonists administered alone.** Figure 1 represents time-effect functions of the MOR agonists alone on ventilation. Values in each panel are normalized to the saline/vehicle conditions in Supplemental Figure S1B. Supplemental Table S1 summarizes results of statistical analyses of the time-effect functions and filled symbols represent significant differences from saline/vehicle.

Fentanyl altered $V_E$ in a dose- and time-related manner (Figure 1A). The smallest dose (0.0032 mg/kg) of fentanyl (squares) was inactive. A dose of 0.01 mg/kg fentanyl (triangles) significantly decreased $V_E$ to 76% of control for minutes 0 to 5 then increased $V_E$ to 167% of control for minutes 7 to 25. A dose of 0.032 mg/kg fentanyl (circles) had a more sustained decrease and subsequently shorter increase in $V_E$ although the magnitude of changes was similar across these two doses of fentanyl. A larger dose of 0.1 mg/kg fentanyl (diamonds) had a greater and more sustained effect in decreasing and subsequently increasing $V_E$. Increases in $V_E$ were similar for all three active doses of fentanyl.

Similar to fentanyl, carfentanil altered $V_E$ in a dose- and time-related manner although the duration of action of carfentanil was longer compared with fentanyl (Figure 1B). The smallest dose (0.00032 mg/kg) of carfentanil (squares) did not significantly affect $V_E$. A dose of 0.001 mg/kg carfentanil (triangles) significantly decreased $V_E$ to 66% of control for minutes 0 to 7 then increased $V_E$ to 151% of control from minute 12 to the end of session (minute 60). A dose of 0.0032 mg/kg carfentanil (circles) had a more sustained decrease and subsequently increase in $V_E$ and a still larger dose (0.01 mg/kg, diamonds) further decreased $V_E$ for nearly the entire 60-minute session.

Similar to fentanyl and carfentanil, 3-methylfentanyl altered $V_E$ in a dose- and time-related manner with the duration of action of 3-methylfentanyl being similar to that of carfentanil (Figure 1C). The smallest dose (0.00032 mg/kg) of 3-methylfentanyl (squares) did not significantly affect $V_E$. Intermediate doses (0.001 and 0.0032 mg/kg) of 3-methylfentanyl (triangles and circles, respectively) initially decreased and subsequently increased $V_E$. The
largest dose of 3-methylfentanyl (0.01 mg/kg, diamonds) further decreased $V_E$ for nearly the entire 60-minute session.

Similar to fentanyl and its analogs, heroin altered $V_E$ in a dose- and time-related manner although the duration of action of heroin was more similar to fentanyl than to carfentanil or 3-methylfentanyl (Figure 1D). The smallest dose (0.1 mg/kg) of heroin (squares) did not significantly affect $V_E$. A dose of 0.32 mg/kg heroin (triangles) only increased $V_E$, to a maximum of 150% of control for minutes 2 to 22. Doses of 0.56 and 1.0 mg/kg heroin initially decreased then increased $V_E$ in a dose- and time-related manner. The largest dose of heroin (3.2 mg/kg, diamonds) only decreased $V_E$. The magnitude of the initial decrease in $V_E$ was similar for the two larger doses of heroin. Although occurring at different times after drug administration, the magnitude and duration of increases in $V_E$ were also relatively similar across the three intermediate doses (0.32, 0.56 and 1.0 mg/kg) of heroin.

Panels A and B of Figure 2 show dose-effect functions for the MOR agonists alone on ventilation, plotted as AUC for minutes 0 to 10 and expressed as a percentage of the saline/vehicle control condition (calculated for individual subjects then averaged among 8 subjects). Table 2 and Supplemental Table S2 summarize results of statistical analyses of the dose-effect functions in Figure 2. Fentanyl significantly (denoted by filled symbols) decreased $V_E$ to 29% of control at a dose of 0.1 mg/kg (circles, Figure 2A; $ED_{50}$ value = 0.0703 mg/kg, Table 2). The average time for $V_E$ to return to baseline (control) was 28 minutes after a dose of 0.1 mg/kg fentanyl (circles, Figure 2B). Similarly, carfentanil and 3-methylfentanyl decreased $V_E$ to less than 30% of control at a dose of 0.01 mg/kg, with both fentanyl analogs being approximately 10-fold more potent than fentanyl to decrease $V_E$ (squares and inverted triangles, respectively; Figure 2A). The time for $V_E$ to return to baseline was greater than 60 minutes after administration of 0.01 mg/kg of carfentanil or 3-methylfentanyl (squares and inverted triangles, respectively; Figure 2B). Heroin significantly increased $V_E$ to 127% of control at a dose of 0.32 mg/kg and significantly decreased $V_E$ to 27.0% of control at a dose of 3.2 mg/kg (triangles; Figure 2A). Heroin was 28-fold less potent than fentanyl to decrease $V_E$. The average time for $V_E$ to return to baseline was 39.6 minutes after administration of 3.2 mg/kg heroin (triangles, Figure 2B). There was no significant difference in slope across the dose-effect functions for the four MOR agonists (Table 2).

Figure 2C shows dose-effect functions for the MOR agonists alone on tail withdrawal latency. Table 2 and Supplemental Table S2 summarize results of statistical analyses of the dose-effect functions in Figure 2C. There was no significant change in the percent maximum possible effect in saline/vehicle control sessions (filled
diamond). Fentanyl significantly increased tail withdrawal latency to an average of 34.7% of the maximum possible effect at a dose of 0.1 mg/kg (circles). Carfentanil and 3-methylfentanyl significantly increased tail withdrawal latency to 100% of the maximum possible effect at a dose of 0.01 mg/kg (squares and inverted triangles, respectively). Heroin significantly increased tail withdrawal latency to an average of 69.2% of the maximum possible effect at a dose of 3.2 mg/kg (triangles). Heroin was at least 478-fold less potent than carfentanil and 3-methylfentanyl to increase tail withdrawal latency (Tables 2).

There was no significant difference in slope among the carfentanil, 3-methylfentanyl, fentanyl, and heroin dose-effect functions (Table 2).

Figure 2D shows dose-effect functions for the MOR agonists alone on rectal temperature. Table 2 and Supplemental Table S2 summarize results of statistical analyses of the dose-effect functions in Figure 2D. There was no significant change in rectal temperature in saline/vehicle control sessions (filled diamond). Fentanyl significantly increased rectal temperature to an average of 1.53 °C above baseline at a dose of 0.1 mg/kg (circles). Carfentanil and 3-methylfentanyl significantly increased rectal temperature to an average of 1.62 and 1.84 °C above baseline at doses of 0.01 and 0.0032 mg/kg, respectively (squares and inverted triangles, respectively). Carfentanil and 3-methylfentanyl were at least 11-fold more potent than fentanyl to increase rectal temperature (squares and inverted triangles, respectively). Heroin significantly increased rectal temperature to an average of 1.83 °C above baseline at a dose of 3.2 mg/kg (triangles). Heroin was 28-fold less potent than fentanyl to increase rectal temperature (Table 2).

There was no significant difference in slope among the MOR agonist dose-response curves for effects on rectal temperature (Table 2). The potency of MOR agonists to decrease ventilation (Figure 2E, Table 2) and increase body temperature (Figure 2F, Table 2) were positively correlated with the Ki values of these compounds at MOR.

Panels A, B, and C (left panel) of Supplemental Figure S2 show the effects of naloxone (0.0001-0.01 mg/kg) and MCAM (0.0001-0.1 mg/kg) alone on ventilation. Across a 100-fold or greater dose range, neither naloxone nor MCAM significantly altered ventilation (Supplemental Tables S3 and S4). In addition, neither naloxone nor MCAM significantly altered tail withdrawal latency or rectal temperature (Supplemental Figure S2C center and right panels; Supplemental Table S4).

Reversal of the effects of the MOR agonists by naloxone and MCAM. The effects of naloxone and MCAM on hypoventilation by 0.1 mg/kg fentanyl, 0.01 mg/kg carfentanil, 0.01 mg/kg 3-methylfentanyl, and 3.2 mg/kg heroin are shown in Figure 3 (gray symbols represent significant differences from solid diamonds; Supplemental
Table S5 summarizes results of statistical analyses of the time-effect functions. Naloxone and MCAM reversed hypoventilation by fentanyl (Figure 3 panels A and B, respectively), carfentanil (Figure 3 panels C and D, respectively), 3-methylfentanyl (Figure 3 panels E and F, respectively), and heroin (Figure 3 panels G and H, respectively) in a dose- and time-related manner. The smallest dose of naloxone (0.0001 mg/kg, triangles) had no significant effect whereas a 10-fold larger dose (0.001 mg/kg, inverted triangles) fully reversed the effects of all four MOR agonists on $V_E$ within 9 minutes after antagonist administration. A 10-fold larger dose of naloxone (0.01 mg/kg, circles) fully reversed hypoventilation by all four MOR agonists within 3 minutes after administration.

Similarly, the smallest dose of MCAM (0.0001 mg/kg, triangles) had no significant effect whereas a 10-fold larger dose (0.001 mg/kg, inverted triangles) fully reversed hypoventilation by all MOR agonists within 13 minutes after administration. Larger doses of MCAM, (0.01 and 0.1 mg/kg, circles and squares, respectively) fully reversed hypoventilation by all MOR agonists within 7 and 5 minutes, respectively, after administration. Reversal of fentanyl- (Figure 3A), carfentanil- (Figure 3C), and 3-methylfentanyl- (Figure 3E) induced hypoventilation by 0.01 mg/kg naloxone was no longer evident by minute 33, 38, and 38, respectively. In contrast, MCAM both reversed and prevented reemergence (i.e., “recarconization”) of hypoventilation by these opioid receptor agonists (Figure 3 panels B, D, F, and H).

Figure 4 show dose-effect functions for naloxone and MCAM in reversing the effects of MOR agonists (ventilation, antinociception, and hyperthermia). For ventilation, the AUC values in each panel (Figure 4 panels A-D) are expressed as a percentage of values obtained under saline/vehicle conditions for minutes 5 to 15. Gray symbols represent significant differences from vehicle (no naloxone or MCAM). Table 3 and Supplemental Table S6 summarize results of statistical analyses of the dose-effect functions. Naloxone and MCAM dose-dependently reversed decreases in $V_E$ by all four MOR agonists (Figure 4 panels A-H) with MCAM being slightly less potent than naloxone. There was no significant difference in slope of the MOR antagonist dose-effect functions among the four MOR agonists. Although MCAM was less potent than naloxone to reverse agonist-induced decreases in $V_E$, there was no significant difference in slope of the antagonist dose-effect functions. Naloxone and MCAM reversed the antinociceptive (tail withdrawal latency) and hyperthermic effects of MOR agonists in a dose-related manner (Figure 4 panels I-P). MCAM was slightly more potent than naloxone in reversing both the antinociceptive and hyperthermic effects of MOR agonists. A dose of 1.0 mg/kg naloxone fully reversed hypoventilation and nearly
fully reversed hyperthermia produced by each MOR agonist (Figure 4 panels A-H and M-P). The same dose of naloxone did not fully attenuate the antinociceptive effects of fentanyl, carfentanil, or heroin.

Baseline parameters for the prevention experiments. Supplemental Figure S3B shows baseline $V_E$ for administration of β-cyclodextrin vehicle administered 22, 46, or 70 hours before saline. $V_E$ was decreased slightly at minutes 1-6 when β-cyclodextrin was administered 22, 46, and 70 hours before administration of saline, compared with $V_E$ when β-cyclodextrin was administered 5 minutes after saline (Supplemental Table S7). Table 1 includes baseline parameters of ventilation, tail withdrawal latency, and rectal temperature for the prevention experiments; baseline values were not significantly different regardless of when β-cyclodextrin vehicle was administered.

Prevention of the effects of MOR agonists by naloxone and MCAM. Supplemental Figures S4 and S5 panels A-C show the effects of MCAM alone (0.1-1.0 mg/kg) and naloxone alone (1.0 mg/kg) on ventilation. Neither MCAM nor naloxone significantly altered ventilation regardless of when administered (Supplemental Tables S8 and S9). In addition, neither MCAM nor naloxone significantly altered tail withdrawal latency or rectal temperature regardless of when administered (Supplemental Figure S5 panels D-F; Supplemental Table S9).

Supplemental Figure S6 shows time-effect functions of β-cyclodextrin vehicle administered 22, 46, and 70 hours before or 5 minutes after administration of 0.1 mg/kg fentanyl, 0.01 mg/kg carfentanil, 0.01 mg/kg 3-methylfentanyl, or 3.2 mg/kg heroin. Absolute values of $V_E$ prior to data normalization are shown for comparison. Supplemental Table S10 summarizes results of statistical analyses of the time-effect functions. Gray symbols represent significant differences from β-cyclodextrin vehicle administered 5 minutes after administration of 0.1 mg/kg fentanyl, 0.01 mg/kg carfentanil, 0.01 mg/kg 3-methylfentanyl, or 3.2 mg/kg heroin. With the exception of only a few occasions when significant differences were obtained relative to β-cyclodextrin vehicle administered 5 minutes after administration of fentanyl, the time-effect functions of fentanyl were similar regardless of when β-cyclodextrin vehicle was administered (Supplemental Figure S6A). β-cyclodextrin vehicle did not significantly affect $V_E$ AUC for the entire 60-minute ventilation test period ($V_E$ AUC$_{0-60}$) or the time for $V_E$ to return to baseline (Supplemental Figure S7 panels A and E; Supplemental Table S11). Similarly, the time-effect functions of carfentanil, 3-methylfentanyl, and heroin were similar regardless of when β-cyclodextrin vehicle was administered (Supplemental Figures S6 panels B-D and S7 panels B and F-H). Nonetheless, $V_E$ AUC$_{0-60}$ values were greater when β-cyclodextrin vehicle was administered 22, 46, and 70 hours before administration of 3-methylfentanyl than when β-cyclodextrin vehicle was administered 5 minutes after administration of 3-methylfentanyl (Supplemental
Figure S7C). In contrast, $V_{E} AUC_{0-60}$ values were less when β-cyclodextrin vehicle was administered 22, 46, and 70 hours before administration of heroin compared with values when β-cyclodextrin vehicle was administered 5 minutes after administration of heroin (Supplemental Figure S7D). Treatment times of β-cyclodextrin vehicle did not have a significant effect on tail withdrawal latency or rectal temperature (Supplemental Figure S7 panels I-P).

The effects of MOR agonists on ventilation 22 hours after administration of naloxone or MCAM are shown in Figures 5 and 6 (gray symbols represent significant differences from solid diamonds; Supplemental Tables S12 and S13 summarize results of statistical analyses of the time- and dose-effect functions, respectively). Up to a dose (1.0 mg/kg) 100-fold larger than the dose that was effective in the reversal experiments (circles in Figure 3 panels A, C, E, and G), 24-hour pretreatment with naloxone did not attenuate hypoventilation by fentanyl (Figure 5A and diamonds in Figure 6 panels A and E), carfentanil (Figure 5C and diamonds in Figure 6 panels B and F), 3-methylfentanyl (Figure 5E and diamonds in Figure 6 panels C and G), or heroin (Figure 5G and diamonds in Figure 6 panels D and H). In contrast, when administered 22 hours earlier, MCAM attenuated hypoventilation by fentanyl (Figures 5B and 6 panels A and E), carfentanil (Figures 5D and 6 panels B and F), 3-methylfentanyl (Figures 5F and 6 panels C and G), and heroin (Figures 5H and 6 panels D and H) in a dose- and time-related manner. The smallest dose of MCAM (0.1 mg/kg, triangles) that was effective in reversing hypoventilation by MOR agonists (squares in Figure 3 panels B, D, F, and H), had no significant effect whereas a 3-fold larger dose of MCAM (0.32 mg/kg, circles) significantly decreased both the magnitude and the duration of hypoventilation by MOR agonists. A 3-fold larger dose of MCAM (1.0 mg/kg, squares) fully prevented hypoventilation by all MOR agonists. Similarly, 22 hours after administration, MCAM but not naloxone (1.0 mg/kg) prevented the antinociceptive and hyperthermic effects of MOR agonists (Figure 6 panels I-P; Supplemental Table S13).

The duration of action of MCAM in preventing the effects of the MOR agonists was further assessed with pretreatments of 46 and 70 hours (Figures 7 and 8; Supplemental Tables S12 and S13). A dose of 1.0 mg/kg MCAM administered 46 hours earlier significantly attenuated hypoventilation, antinociception, and hyperthermia by the MOR agonists. However, the same dose of MCAM was not effective when administered 70 hours before a MOR agonist (Figures 7 and 8).

DISCUSSION
The major findings in this study are as follows. First, all four MOR agonists decreased ventilation, increased tail withdrawal latency (antinociception), and increased body temperature (hyperthermia), with carfentanil and 3-methylfentanyl being approximately 10-fold more potent than fentanyl and 100-fold more potent than heroin. Moreover, the duration of hypoventilation after carfentanil and 3-methylfentanyl was nearly twice as long than the duration of hypoventilation of an equi-effective dose of fentanyl. Second, the duration of action of the MOR agonists varied among the three primary dependent variables, with antinociception and hyperthermia still evident after the effects of MOR agonists on ventilation we no longer evident. Over the doses studied, antinociceptive effects were larger with carfentanil and 3-methylfentanyl than with fentanyl or heroin, whereas the magnitude of hyperthermia was similar among the four agonists. Third, both MCAM and naloxone reversed hypoventilation by fentanyl, fentanyl analogs, and the morphinan MOR agonist heroin, with MCAM being slightly less potent than naloxone. However, MCAM and naloxone were equi-effective as measured by the time required for ventilation to return to baseline values, after administration of an antagonist. Fourth, confirming early studies on MCAM (e.g., Jimenez et al., 2021), MCAM had a much longer duration of antagonist action compared with naloxone. In the reversal experiment, hypoventilation reemerged for fentanyl, carfentanil, and 3-methylfentanyl after reversal by naloxone but not after reversal by MCAM. MCAM but not naloxone prevented the ventilatory depressant, antinociceptive, and hyperthermic effects of MOR agonists for at least 46 hours after acute administration of 1.0 mg/kg. Thus, the present study extended the ability of MCAM to reverse and prevent various effects including hypoventilation by the MOR agonists that are more potent than fentanyl.

In this study, MCAM was slightly less potent than naloxone to reverse hypoventilation by MOR agonists and consistent with a prior study (Jimenez et al., 2021) the slopes for MCAM to reverse fentanyl-induced hypoventilation were shallower than those of naloxone. However, MCAM and naloxone were equally effective at reversing hypoventilation by fentanyl, ultra-potent fentanyl analogs, and heroin. A previous study reported naloxone to be less effective in reversing hypoventilation by fentanyl compared with hypoventilation by the morphinan morphine in mice (Hill et al., 2020). The apparent difference in the effectiveness of naloxone to reverse fentanyl-induced hypoventilation might result from non-pharmacological factors. In the current study naloxone effectively reversed hypoventilation by all four MOR agonists. For example, the environmental conditions under which breathing is studied might impact drug effects. The prior study (Hill et al., 2020) used hypercapnic conditions whereas the current study used normal air. Similarly, methamphetamine attenuated fentanyl-induced hypoventilation
in rats breathing normal air (Hiranita et al., 2023) whereas methamphetamine modestly exacerbated fentanyl hypoventilation under hypercapnic conditions in mice (Elder et al., 2023). Other experimental details could also contribute to differences among studies. For example, the current study was conducted in the light cycle whereas the prior study (Hill et al., 2020) was conducted in the dark cycle. Housing conditions can also impact drug effects. For example, (±)-amphetamine was 12-fold more potent in causing lethality in group-housed mice (five mice per cage; LD₅₀ = 21 mg/kg, orally) compared with individually housed mice (LD₅₀ = 248 mg/kg ([Fanelli, 1973])). The prior study with naloxone used group-housed mice whereas the current study used individually housed rats. It remains to be determined which of these or possibly other factors (environmental conditions, housing conditions, light/dark cycle, species) might contribute to the observed differences in the effectiveness of naloxone. Importantly in the current study, the delay from administration of antagonist to the normalization of breathing was similar for naloxone and MCAM, further supporting the potential utility of MCAM as a rescue medication for opioid overdose.

Acute administration of 1.0 mg/kg MCAM, but not naloxone, prevented hypoventilation by each of the MOR agonists. Moreover, prevention of hypoventilation by MCAM was not significantly different among the four MOR agonists. In contrast, the opioid receptor antagonist naltrexone was 3-fold less potent in antagonizing the fentanyl-like discriminative-stimulus effects of carfentanil compared with antagonism of the discriminative stimulus effects of fentanyl (Flynn and France, 2021). This difference might be related to the specific antagonist (naloxone versus naltrexone), although MCAM and naltrexone increased ED₅₀ values similarly for carfentanil, 3-methylfentanyl, fentanyl, and heroin to choose infusions over food reinforcers in rhesus monkeys responding under a drug versus food choice procedure (Gerak and France, 2023), or to the particular measure of MOR agonist activity (discrimination versus ventilation versus self-administration).

In the current study, two ultra-potent analogs of fentanyl were compared to fentanyl and heroin for their effects on measures of ventilation, antinociception, and body temperature. Carfentanil and 3-methylfentanyl were equipotent across all measures and at least 10-fold more potent than fentanyl to produce hypoventilation and hyperthermia. The duration of action of the fentanyl analogs to produce hypoventilation, antinociception, and hyperthermia was longer than the duration of action of fentanyl and heroin. These differences in potency and duration of action among fentanyl and its analogs are consistent with results of previous studies in rats and mice that studied ventilation (Huang et al., 1984), antinociception (Huang et al., 1984; Bi-Yi et al., 1999), i.v. self-
administration (Wei et al., 2023), and pharmacokinetics (Jin et al., 1986; Alburges et al., 1992). Additionally, the 10-fold greater potency of 3-methyl fentanyl relative to fentanyl was also observed in a study using nonhuman primates. For example, 3-methylfentanyl was approximately 10-fold more potent than fentanyl in rhesus monkeys choosing between i.v. infusions and food (Gerak and France, 2023). In the same study, carfentanil was 100-fold more potent than fentanyl (Gerak and France, 2023). The apparently different relative potency of fentanyl analogs among species warrants further investigation so as to strengthen and provide a empirically validated context for the translatability of results from preclinical studies to human patients.

The potency and dose-dependency of the hypothermic effects of MOR in this study are very similar to the effects of fentanyl and heroin reported in a previous study that was conducted under similar experimental conditions (Hiranita et al., 2023). A dose of 0.01 mg/kg carfentanil i.v. increased body temperature by an average of 1.6°C measured with a rectal probe in male Sprague-Dawley rats. However, a subcutaneous injection of the same dose of carfentanil decreased body temperature by ~3°C measured with a subcutaneous temperature probe in male Sprague-Dawley rats (Bergh et al., 2019). This marked difference in the effects of carfentanil on body temperature (i.e., as much as 4.6°C) is not likely due to stress-induced hyperthermia by insertion of a rectal probe or by measuring antinociception with warm water (Adriaan Bouwknecht et al., 2007; Bae et al., 2007) because there was no significant change in rectal temperature in control experiments (e.g., saline before or after administration of cyclodextrin vehicle). However, the whole-body plethysmography chamber used in the current study is a relatively small space that precludes ranging locomotion. Moreover, hyperthermia by smaller doses of fentanyl (0.04 mg/kg, i.v.) and heroin (0.4 mg/kg, i.v.), compared with doses used in the present study (0.1 and 3.2 mg/kg, respectively), was approximately 0.5°C greater in muscle than in skin of rats 60 minutes after administration of a MOR agonist (Solis et al., 2017). Thus, the particular environment in which rats were studies (i.e., in the current study rats were in plethysmograph chambers for more than an hour before other measures occurred) and the different location of thermo-recording might have impacted measures of drug effects on body temperature between the current and previous (Bergh et al., 2019) studies.

A dose of 1.0 mg/kg of naloxone fully reversed hypoventilation and nearly fully reversed hyperthermia by each MOR agonist. The same dose of naloxone was less effective in reversing the antinociceptive effects of these agonists. The effectiveness of naloxone across these different measures might be related different efficacy
requirements among these assays. For example, a lower efficacy requiring condition (i.e., lower temperature) in the antinociception procedure might have resulted in full reversal of agonist effects by a dose of 1.0 mg/kg naloxone.

In summary, the current study demonstrates the effectiveness of MCAM to reverse and prevent hypoventilation by various MOR agonists including ultra-potent fentanyl analogs that have a longer duration of action compared with fentanyl. These results support the view that MCAM has the potential to improve clinical outcomes to treat and prevent opioid overdose.

ACKNOWLEDGEMENTS

Portion of these data were presented as follows:


2. Ho NP, Hiranita T, and France CP (2022) Effects of fentanyl alone and in combination with methamphetamine on ventilation and rectal temperature in rat. Florida Chapter American College of Physicians 2022; 2022 October 22; Ft. Lauderdale, FL.

3. Hiranita T, Ho NP, and France CP (2023) Comparison of the mu-opioid receptor antagonists methocinnamox (MCAM) and naloxone to reverse the ventilatory-depressant effects of fentanyl, heroin, and carfentanil in rats. 33rd Annual Texas Research Society on Alcoholism Scientific Meeting; 2023 February 17; College Station, TX.

4. Hiranita T, Ho NP, and France CP (2023) Comparison of methocinnamox (MCAM) and naloxone to reverse and prevent the ventilatory depressant effects of fentanyl, heroin, and carfentanil in rats. 15th Annual Behavior, Biology, and Chemistry Translational Research in Addiction meeting 2023; 2023 March 25-26; San Antonio, TX.

5. Hiranita T, Ho NP, and France CP (2023) Comparison of the mu-opioid receptor antagonists methocinnamox (MCAM) and naloxone to reverse the ventilatory depressant effects of fentanyl, heroin, carfentanil, and 3-methylfentanyl in male rats. 20th Annual Center for Biomedical Neuroscience Retreat 2023; 2023 May 12, 2023; San Antonio, TX.
6. Hiranita T, Ho NP, and France CP (2023) Comparison of the \textit{mu}-opioid receptor antagonists methocinnamox (MCAM) and naloxone to reverse the ventilatory-depressant effects of fentanyl and heroin in male rats. \textit{Annual American Society for Pharmacology and Experimental Therapeutics (ASPET)} 2023; 2023 May 18-21; St. Louis, MO.

7. Hiranita T, Ho NP, and France CP (2023) Ventilatory effects of fentanyl, heroin, and \textit{d}-methamphetamine, alone and in mixtures, in male rats. \textit{Annual College on Problems of Drug Dependence (CPDD)} 2023; 2023 June 17-21; Denver, CO.
DATA AVAILABILITY STATEMENT

The authors declare that all the data supporting the findings of this study are available within the paper and its Supplemental Data.

AUTHOR CONTRIBUTIONS

Participated in research design: Hiranita and France

Conducted experiments: Hiranita and Ho

Contributed new reagents or analytic tools: N/A

Performed data analysis: Hiranita

Wrote or contributed to the writing of the manuscript: Hiranita, Ho, and France
REFERENCES.
Hiranita T, Ho NP and France CP (2023) Ventilatory effects of fentanyl, heroin, and d-methamphetamine , alone and in mixtures, in male rats breathing normal air. *J Pharmacol Exp Ther*.


**FOOTNOTES**

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FIGURE LEGENDS

Figure 1. Effects of the MOR agonists alone on ventilation in rats breathing normal air. Abscissae: time in minutes. Ordinates: minute volume ($V_E$). Data are expressed as a percentage of $V_E$ under baseline (control) conditions (horizontal dashed lines) when rats received an infusion of saline followed 5 minutes later by an infusion of 10% β-cyclodextrin (saline/vehicle; see Supplemental Figure S1B). Symbols represent the mean ± 1 SEM for 8 rats. Fentanyl (A), carfentanil (B), 3-methylfentanyl (C), and heroin (D) were administered i.v. at time 0 and 10% β-cyclodextrin vehicle was administered i.v. 5 minutes later (indicated by arrows on abscissae and vertical dashed lines). Gray symbols indicate a significant difference from saline/vehicle values (see Supplemental Table S1 for details of statistical analyses).

Figure 2. Dose-effect functions for the effects of the MOR agonists alone on ventilation (from time-effect functions shown in Figure 1; panels A and B), tail withdrawal latency (C), and rectal body temperature (D). Abscissae: A-D, dose in mg/kg (i.v.); E and F, ex vivo $Ki$ values at MOR (see Table 2). Ordinates: A, area under the curve (AUC) from 0 to 10 minutes after drug administration expressed as a percent of control (saline/vehicle; horizontal dashed lines); B, the time in minutes for $V_E$ to return to baseline (control) values; C, tail withdrawal latency expressed as a percentage of maximum possible effects in water at 50°C; D, rectal temperature expressed as a change from baseline in degrees C; E, dose of MOR agonists in µmol/kg to produce hypoventilation; F, dose of MOR to increase body temperature by 1°C ($ED_{10}^\circ C$). Symbols represent the mean ± 1 SEM for 8 rats. Gray symbols represent a significant difference from saline/vehicle values (see Table 2 and Supplemental Table S2 for details of statistical analyses).

Figure 3. Reversal by naloxone (left panels) and MCAM (right panels) of hypoventilation by 0.1 mg/kg fentanyl (A and B), 0.01 mg/kg carfentanil (C and D), 0.01 mg/kg 3-methylfentanyl (E and F), and 3.2 mg/kg heroin (G and H). Naloxone and MCAM were administered 5 minutes after administration of a MOR agonist. Effects of the MOR agonists before vehicle (solid diamonds) are replotted from Figure 1. See Figure 1 for other details (see Supplemental Table S5 for details of statistical analyses).

Figure 4. Dose-effect functions for reversal by naloxone and MCAM of the ventilatory depressant (from time-effect functions shown in Figure 3; panels A-H), antinociceptive (I-L), and hyperthermic (M-P) effects of 0.1 mg/kg.
fentanyl (A, E, I and M), 0.01 mg/kg carfentanil (B, F, J and N), 0.01 mg/kg 3-methylfentanyl (C, G, K and O), and 3.2 mg/kg heroin (D, H, L and P). AUC data (A-D) are expressed as a percentage of AUC from 5 to 15 minutes after administration of a MOR agonist expressed as a percent of control (saline/vehicle; horizontal dashed lines). See Figure 2 for other details (see Table 3 and Supplemental Table S6 for details of statistical analyses).

Figure 5. Effects of 0.1 mg/kg fentanyl (A and B), 0.01 mg/kg carfentanil (C and D), 0.01 mg/kg 3-methylfentanyl (E and F), and 3.2 mg/kg heroin (G and H) on $V_E$ 22 hours after administration of naloxone (left panels) or MCAM (right panels). See Figure 3 for other details (see Supplemental Table S12 for details of statistical analyses).

Figure 6. Dose-effect functions for MCAM and naloxone administered 22 hours before 0.1 mg/kg fentanyl (A, E, I and M), 0.01 mg/kg carfentanil (B, F, J and N), 0.01 mg/kg 3-methylfentanyl (C, G, K and O), and 3.2 mg/kg heroin (D, H, L and P) for ventilation (from time-effect functions shown in Figure 5), tail withdrawal latency (I-L), and rectal temperature (M-P). AUC data (A-D) are the percentage of AUC from 0 to 10 minutes after administration of a MOR agonist and expressed as a percent of saline control (horizontal dashed lines). See Figure 4 for other details (see Table 3 and Supplemental Table S13 for details of statistical analyses).

Figure 7. Effects of 0.1 mg/kg fentanyl (A and B), 0.01 mg/kg carfentanil (C and D), 0.01 mg/kg 3-methylfentanyl (E and F), and 3.2 mg/kg heroin (G and H) on ventilation ($V_E$) 46 (left panels) and 70 (right panels) hours after administration of vehicle, 0.32 mg/kg MCAM, or 1.0 mg/kg MCAM. See Figure 5 for other details (see Supplemental Table S12 for details of statistical analyses).

Figure 8. Dose-effect functions for MCAM administered 22, 46, and 70 hours before 0.1 mg/kg fentanyl (A, E, I and M), 0.01 mg/kg carfentanil (B, F, J and N), 0.01 mg/kg 3-methylfentanyl (C, G, K and O), and 3.2 mg/kg heroin (D, H, L and P) for ventilation (from time-effect functions shown in Figures 5 and 7), tail withdrawal latency (I-L), and rectal temperature (M-P). Effects of MCAM administered 22 hours before administration of a MOR agonist (squares) are replotted from Figure 6. See Figure 6 for other details (see Table 3 and Supplemental Table S13 for details of statistical analyses).
**TABLES**

**Table 1.** Baseline values for ventilation [frequency ($f$), tidal volume ($V_T$), and minute volume ($V_E$)], tail withdrawal latency, and rectal temperature. Ventilatory parameters ($f$, $V_T$, and $V_E$) are from control test sessions (i.e., minutes 0-60); control values from all tail withdrawal latency and rectal temperature assessments prior to daily sessions are used, including test and control sessions. Each value represents the mean ± 1 SEM of 8 male rats. There was no SEM for tail withdrawal latency at 45°C because tail withdrawal latency at that temperature was the maximum possible effect in all subjects (15 seconds). For each measure, there were no significant differences in treatment time of β-cyclodextrin vehicle.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>β-cyclodextrin vehicle 5 minutes after saline</th>
<th>β-cyclodextrin vehicle 22 hours before saline</th>
<th>β-cyclodextrin vehicle 46 hours before saline</th>
<th>β-cyclodextrin vehicle 70 hours before saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_E$ (mL/minute/kg)</td>
<td>442.6 ± 4.2</td>
<td>430.8 ± 17.0</td>
<td>434.1 ± 17.1</td>
<td>432.5 ± 24.7</td>
</tr>
<tr>
<td>$f$ (breaths/minute)</td>
<td>105.4 ± 1.9</td>
<td>104.9 ± 1.5</td>
<td>103.9 ± 2.1</td>
<td>102.9 ± 1.3</td>
</tr>
<tr>
<td>$V_T$ (mL/breath/kg)</td>
<td>4.96 ± 0.03</td>
<td>4.93 ± 0.07</td>
<td>4.92 ± 0.09</td>
<td>4.91 ± 0.10</td>
</tr>
<tr>
<td>Tail withdrawal latency at 45°C (seconds)</td>
<td>15.00 ± 0.00</td>
<td>15.00 ± 0.00</td>
<td>15.00 ± 0.00</td>
<td>15.00 ± 0.00</td>
</tr>
<tr>
<td>Tail withdrawal latency at 50°C (seconds)</td>
<td>3.07 ± 0.08</td>
<td>2.98 ± 0.05</td>
<td>3.05 ± 0.09</td>
<td>3.01 ± 0.07</td>
</tr>
<tr>
<td>Rectal temperature (°C)</td>
<td>37.07 ± 0.03</td>
<td>37.07 ± 0.03</td>
<td>37.07 ± 0.03</td>
<td>37.07 ± 0.03</td>
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</table>
Table 2. Effective doses in mg/kg (i.v.) and slope values of the MOR agonists alone for hypoventilation, antinociception, and hyperthermia. All values (95% CIs in brackets) are calculated from Figure 2 from 8 male rats. For hypoventilation, the V̇E AUC values for 10 minutes after administration of the MOR agonists (i.e., minutes 0-10 during the test period) are normalized to those of the corresponding saline/vehicle control conditions. Ki values from the literature were used for a correlational analysis (see Figure 2 panels E and F).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hypoventilation</th>
<th>Antinociception</th>
<th>Hyperthermia</th>
<th>Ki values at MOR</th>
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</thead>
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<tr>
<td></td>
<td>ED₅₀ or ED₁-C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fentanyl alone</td>
<td>0.0703 (0.0637, 0.0782)</td>
<td>Not determined (up to 34.7% maximum possible effect)</td>
<td>0.0583 (0.0433, 0.0774)</td>
<td>3.3 ± 0.45ᵃ</td>
</tr>
<tr>
<td>Slope</td>
<td>-75.5 (-83.9, -67.2)</td>
<td>Not applicable</td>
<td>1.60 (1.13, 1.24)</td>
<td></td>
</tr>
<tr>
<td>Carfentanil alone</td>
<td>0.00676 (0.00608, 0.00758)</td>
<td>0.00438 (0.00393, 0.00484)</td>
<td>0.00510 (0.00349, 0.00685)</td>
<td>0.42 ± 0.04ᵇ</td>
</tr>
<tr>
<td>Slope</td>
<td>-46.2 (-51.3, -41.1)</td>
<td>67.1 (60.3, 73.9)</td>
<td>1.56 (1.11, 2.02)</td>
<td></td>
</tr>
<tr>
<td>3-Methylfentanyl alone</td>
<td>0.00613 (0.00501, 0.00764)</td>
<td>0.00447 (0.00402, 0.00494)</td>
<td>0.00288 (0.00240, 0.00366)</td>
<td>0.49 ± 0.23ᶜ</td>
</tr>
<tr>
<td>Slope</td>
<td>-49.4 (-60.3, -38.5)</td>
<td>67.1 (60.3, 73.9)</td>
<td>2.23 (1.30, 3.15)</td>
<td></td>
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<tr>
<td>Heroin alone</td>
<td>1.98 (1.58, 2.57)</td>
<td>2.14 (1.92, 2.41)</td>
<td>1.61 (1.18, 2.23)</td>
<td>18.6 ± 2.78ᵈ</td>
</tr>
<tr>
<td>Slope</td>
<td>-104 (-116, -91.9)</td>
<td>37.7 (28.5, 46.9)</td>
<td>1.02 (0.620, 1.42)</td>
<td></td>
</tr>
</tbody>
</table>

ᵃ[^3H]DAMGO (mean ± SEM) was used in rat brain (Yeadon and Kitchen, 1988).

ᵇ[^3H]cis-Ohmefentanyl was used in mouse brain (Bi-Yi et al., 1999).

c[^3H]DAMGO (mean ± SEM) was used in rat whole brain excluding the cerebellum (Yue et al., 2018).
Table 3. ED$_{80}$ values in mg/kg (i.v.) and slope values of the MOR antagonists to reverse and prevent hypoventilation by MOR agonists. Each value (95% CIs) is calculated from Figures 4 and 6 using 8 male rats. For the reversal experiments, data from minutes 5-15 were normalized to those of the corresponding saline control conditions to calculate ED$_{80}$ and slope values. For the prevention experiments when MCAM was administered 22 hours prior to administration of a MOR agonist, data from minutes 0-10 were used to calculate ED$_{80}$ and slope values.

<table>
<thead>
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<th>Treatment</th>
<th>Reversal</th>
<th>Prevention</th>
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</thead>
<tbody>
<tr>
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<td>Naloxone</td>
<td>MCAM</td>
</tr>
<tr>
<td>0.1 mg/kg fentanyl</td>
<td>ED$_{80}$ values for $V_E$ AUC</td>
<td>0.00483 (0.00396, 0.00578)</td>
</tr>
<tr>
<td></td>
<td>Slope values for $V_E$ AUC</td>
<td>45.7 (39.7, 51.7)</td>
</tr>
<tr>
<td></td>
<td>Slope values for return time</td>
<td>-9.12 (-9.95, -8.28)</td>
</tr>
<tr>
<td>0.01 mg/kg carfentanil</td>
<td>ED$_{80}$ values for $V_E$ AUC</td>
<td>0.00482 (0.00395, 0.00577)</td>
</tr>
<tr>
<td></td>
<td>Slope values for $V_E$ AUC</td>
<td>46.2 (41.5, 51.0)</td>
</tr>
<tr>
<td></td>
<td>Slope values for return time</td>
<td>-28.7 (-33.4, -24.1)</td>
</tr>
<tr>
<td>0.01 mg/kg 3-methylfentanyl</td>
<td>ED$_{80}$ values for $V_E$ AUC</td>
<td>0.00470 (0.00394, 0.00551)</td>
</tr>
<tr>
<td></td>
<td>Slope values for $V_E$ AUC</td>
<td>46.5 (40.4, 52.6)</td>
</tr>
<tr>
<td></td>
<td>Slope values for return time</td>
<td>-31.1 (-38.4, -24.1)</td>
</tr>
<tr>
<td>3.2 mg/kg heroin</td>
<td>ED$_{80}$ values for $V_E$ AUC</td>
<td>0.00466 (0.00369, 0.00573)</td>
</tr>
<tr>
<td></td>
<td>Slope values for $V_E$ AUC</td>
<td>47.8 (41.9, 53.7)</td>
</tr>
<tr>
<td></td>
<td>Slope values for return time</td>
<td>-15.1 (-18.5, -11.7)</td>
</tr>
</tbody>
</table>
Figure 1

A. Fentanyl alone

B. Carfentanil alone

C. 3-Methylfentanyl alone

D. Heroin alone
Figure 2

A

B

\[ V_{E} \text{ AUC}_{0-10} (\% \text{ saline/vehicle}) \]

Dose (mg/kg, i.v.)

C

D

% maximum possible effects at 50°C

Dose (mg/kg, i.v.)

\[ R^2 = 0.9855, p < 0.05 \]

E

F

\[ R^2 = 0.9858, p < 0.05 \]
Figure 3

Naloxone reversal

A. 0.1 mg/kg fentanyl

B. 0.1 mg/kg fentanyl

C. 0.01 mg/kg carfentanil

D. 0.01 mg/kg carfentanil

E. 0.01 mg/kg 3-methylfentanyl

F. 0.01 mg/kg 3-methylfentanyl

G. 3.2 mg/kg heroin

H. 3.2 mg/kg heroin
Figure 4

- **Vehicle**
- **Naloxone**
- **MCAM**

<table>
<thead>
<tr>
<th>Dose (mg/kg, i.v.)</th>
<th>0.1 mg/kg fentanyl</th>
<th>0.01 mg/kg carfentanil</th>
<th>0.01 mg/kg 3-methylfentanyl</th>
<th>3.2 mg/kg heroin</th>
</tr>
</thead>
</table>

- **A** $V_{E_{4AUC}}$ (saline/vehicle) %
- **B**
- **C**
- **D**

- **E** Minutes returning to $V_{E_{4}}$ baseline
- **F**
- **G**
- **H**

- **I** % Maximum Possible Effects at 50°C
- **J**
- **K**
- **L**

- **M** Changes from baseline (°C)
- **N**
- **O**
- **P**
Figure 5

**Naloxone 22 hours before**

- **A**: 0.1 mg/kg fentanyl

**MCAM 22 hours before**

- **B**: 0.1 mg/kg fentanyl

**C**: 0.01 mg/kg carfentanil

**D**: 0.01 mg/kg carfentanil

**E**: 0.01 mg/kg 3-methylfentanyl

**F**: 0.01 mg/kg 3-methylfentanyl

**G**: 3.2 mg/kg heroin

**H**: 3.2 mg/kg heroin
Figure 6

- **Vehicle**
- **MCAM**
- **Naloxone**

A. 0.1 mg/kg fentanyl
B. 0.01 mg/kg carfentanil
C. 0.01 mg/kg 3-methylfentanyl
D. 3.2 mg/kg heroin

E. Minutes returning to V_e baseline
F. % Maximum Possible Effects at 50°C
G. Change from baseline (°C)

Dose (mg/kg, i.v., 22 hours before)