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Antinociceptive and Discriminative Stimulus Effects of Six Novel Psychoactive Opioid Substances in Male Rats

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ABSTRACT

Compounds with novel or fentanyl-like structures continue to appear on the illicit drug market and have been responsible for fatalities, yet there are limited preclinical pharmacological data available to evaluate the risk of these compounds to public health. The purpose of the present study was to examine acetyl fentanyl, butyryl fentanyl, 3,4-dichloro-*N*-[[1-(dimethylamino)cyclohexyl]methyl]benzamide (AH-7921), 1-cyclohexyl-4-(1,2-diphenylethyl)piperazine (MT-45), 4-chloro-*N*-[1-(2-phenylethyl)-2-piperidinylidene]-benzenesulfonamide (W-15), and 4-chloro-*N*-[1-[2-(4-nitrophenyl)ethyl]-2-piperidinylidene]-benzenesulfonamide (W-18) for their relative potency to reference opioids and their susceptibility to naltrexone antagonism using the 55°C warm-water, tail-withdrawal assay of antinociception and a morphine drug discrimination assay in male, Sprague-Dawley rats. In the antinociception assay, groups of 8 rats per drug were placed into restraining tubes, their tails were immersed into 40° or 55°C water, and the latency for tail withdrawal was measured with a cutoff time of 15 seconds. In the drug discrimination assay, rats ($n = 11$) were trained to discriminate between 3.2 mg/kg morphine and saline, subcutaneously, in a two-choice, drug discrimination procedure under a fixed ratio-5 schedule of sucrose pellet delivery. Morphine, fentanyl, and four of the synthetic opioids dose dependently produced antinociception and fully substituted for morphine in the drug discrimination assay with the following rank order of potency: fentanyl > butyryl fentanyl > acetyl fentanyl > AH-7921 > MT45 > morphine. All drugs that produced antinociception or morphine-like discriminative stimulus effects were blocked by naltrexone. W-15 and W-18 did not show antinociceptive or morphine-like discriminative stimulus effects at the doses tested supporting a lack of opioid activity for these two compounds. These findings suggest that butyryl fentanyl, acetyl fentanyl, AH-7941, and MT-45 have abuse liability like other opioid agonists.

SIGNIFICANCE STATEMENT

As novel psychoactive substances appear on the illicit drug market, preclinical pharmacological testing is required to assist law enforcement, medical professionals, and legal regulators with decisions about potential public health risks. In this study, four synthetic opioids, acetyl fentanyl, butyryl fentanyl, AH-7921, and MT-45 produced effects similar to fentanyl and morphine and were blocked by naltrexone. These data suggest the four synthetic opioids possess similar abuse liability risks as typical opioid agonists.

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Introduction

Novel psychoactive substances continue to be a growing global health concern, and synthetic opioids have been increasingly implicated in deaths due to overdose [e.g., (UNODC, 2020)]. In the United States, the National Forensic Laboratory Information System systematically collects results from drug chemistry analyses conducted by State and local forensic laboratories. The National Forensic Laboratory Information System reported law enforcement encounters of numerous synthetic opioids of various structural classes including fentanyl-like and other substances. In response, national and international agencies have reviewed or placed regulatory controls on many opioids including substances that are structurally related to fentanyl under Schedule I restriction (e.g., US-DEA,

ABBREVIATIONS: AH-7921, (3,4-dichloro-*N*-[[1-(dimethylamino)cyclohexyl]methyl]benzamide); C.L., Confidence Limits; MOR, μ opioid receptor; MT-45, (1-cyclohexyl-4-(1,2-diphenylethyl)piperazine); W-15, (4-chloro-*N*-[1-(2-phenylethyl)-2-piperidinylidene]-benzenesulfonamide); W-18, (4-chloro-*N*-[1-[2-(4-nitrophenyl)ethyl]-2-piperidinylidene]-benzenesulfonamide).

2008; US-DEA, 2017; US-DEA, 2018) or international regulation (WHO-ECDD, 2019; US-DEA, 2020).

Different opioids activate μ opioid receptor (MOR), κ opioid receptor, and δ opioid receptor to various degrees to produce such effects as analgesia, constipation, respiratory depression, and euphoria, although the predominant mechanism for these effects is activation through the MOR (Williams et al., 2013). In radioligand binding studies, the opioid fentanyl has high affinity and selectivity for MOR (Costa et al., 1992; Eshleman et al., 2020). Fentanyl has high lipophilicity, which allows rapid diffusion through the blood-brain barrier for a fast onset of action. Fentanyl is approximately 50–100 times more potent than morphine and requires repeated dosing with naloxone to reverse fentanyl overdose (Armenian et al., 2018). Between 2012 and 2014, two fentanyl analogs, acetyl fentanyl and butyryl fentanyl, appeared on the global illicit drug market and were implicated in toxicities and overdoses (EMCDDA, 2016; US-DEA, 2017; US-DEA, 2018). W-15 and W-18, two other analogs of fentanyl from a published patent (Knauss et al., 1984), appeared briefly on the illicit market in 2013–2016, causing significant alarm due to their reported high potency in a single antinociception assay. Such instances of rapid appearance on the illicit market reinforce the need for both in vitro and in vivo pharmacological testing to inform law enforcement, medical professionals, and regulators when dealing with these novel synthetic opioids.

Other synthetic opioids, such as AH-7921 and MT-45, come from a broad class of chemical compounds with a wide range of chemical structures (Zawilska, 2017; Solimini et al., 2018). These other opioid compounds originate from past drug discovery analgesic programs, and the structures have been resurrected for illegal purposes. For example, AH-7921 was originally developed by Allen and Hanburys in the mid-1970s as a possible analgesic (Harper et al., 1974), but concerns of potential abuse liability limited its development (Solimini et al., 2018). More recently, AH-7941 appeared in the United States, the United Kingdom, Sweden, Norway, and Japan, resulting in toxicities and overdoses (EMCDDA, 2014b; Kronstrand et al., 2014; Rambaran et al., 2018). Similarly, MT-45 was initially developed and later abandoned by Dainippon Pharmaceutical Company in Japan as an alternate analgesic chemically distinct from other opioids (Natsuka et al., 1978, 1987). MT-45 also has appeared in illicit global drug markets resulting in toxicities and overdoses (EMCDDA, 2014a; Fels et al., 2017; Solimini et al., 2018).

The use of a competitive antagonist is a key component to pharmacologically characterize novel compounds. In the present set of experiments, we examined six novel synthetic opioids in rats using two common assays for determining potential opioid-like abuse liability. One test, the 55°C warm-water, tail-withdrawal assay, screens for opioid antinociceptive effects and preferentially identifies MOR opioid intermediate to full agonists, especially when opioid receptor antagonists are used (Woods et al., 1992; Walker et al., 1994). A second assay, the drug discrimination assay, determines whether novel compounds produce an interoceptive cue that generalizes to an interoceptive cue of a well characterized training drug such as morphine. If the novel compound fully substitutes for the training drug, it is highly likely they share similar mechanisms of action (Solinas et al., 2006; Rocha et al., 2008). To assess the potency of acetyl fentanyl, butyryl fentanyl, AH-7921, MT-45, W-15, and W-18, these substances were examined alone and in combination with naltrexone. The effectiveness and potencies of the novel test substances to produce antinociceptive, discriminative stimulus, and rate-decreasing effects were compared with morphine and fentanyl to assess the contribution of MOR activity in the behavioral actions of the novel test substances. As multiple doses of naltrexone were used in the warm-water, tail-withdrawal assay, a Schild regression analysis was performed to estimate in vivo pA_2 values and determine whether multiple receptors or nonequilibrium conditions may have contributed to the behavioral effects of the opioids tested in the present experiments (Dykstra et al., 1988; Walker et al., 1994).

Materials and Methods

Subjects. For the warm-water, tail-withdrawal procedure, 64 male, Sprague-Dawley rats (Taconic Laboratories, Cranbury, NJ) were pair-housed in polycarbonate cages in a colony room maintained under a reverse 12-hour light/dark cycle. Water and food were freely available in the home cage. Rats were acclimated for a period of one week in the housing facility prior to the studies. As this was a within-subject design, each group of rats (n = 8) was tested once per week with an agonist or the same agonist in combination with a dose of naltrexone.

For the drug discrimination experiments, 11 male, Sprague-Dawley rats (Taconic Laboratories, Cranbury, NJ) were singly housed in polycarbonate cages with extra enrichment in a colony room maintained under a reverse 12-hour light/dark cycle. Water and food were freely available during the one-week acclimation period in the housing facility. Thereafter, rats were weighed Monday through Friday and placed on a daily maintenance diet which consisted of approximately 14–16 g of Purina rat chow. This amount of food in combination with the sucrose pellets earned in the experimental session allowed a slow but steady rate of growth while motivating the rats to respond under the schedule of reinforcement described below.

All rats were maintained in accordance with the guidelines of the Institutional Animal Care and Use Committee of Temple University and the *Guide for the Care and Use of Laboratory Animals* (National Research Council, Washington, DC, National Academies Press; 8th edition, 2011).

Apparatus. For the tail-withdrawal assay, eight rodent restraint tubes were used to restrain the rats. A Precision model 181 water bath maintained the temperature of the water at approximately 75°C; desired water temperatures of 40° or 55°C were obtained using a Thermos brand thermos (diameter = 8 cm) by mixing appropriately with tap water. A Sensortek model GAT-12 with Bailey/Sensortek Type T thermocouple was used to measure the temperature of the water. Tail-withdrawal latencies were measured by visual observation and recorded manually through a hand-operated digital stopwatch with a time resolution of 1/100 second.

The drug discrimination experiments were conducted in six operant experimental chambers (model ENV-008CT, Med Associates, Inc., St. Albans, VT) located within ventilated sound attenuating enclosures (ENV-018MD). On the front wall of each chamber were two stimulus lights (model ENV-221M) located directly above two levers (model ENV-110M), a center receptacle located between the two levers, and a pellet feeder (model ENV-200R2M) positioned above a stainless steel grid floor. A house light and ventilator fan were located on the opposite wall. Experimental contingencies were controlled, and data were collected through a computer-driven interface (model SG-503) programmed using MED-PC IV software (Med Associates, Inc.).

Warm-Water, Tail-Withdrawal Procedure. Rats (n = 8 per group) were placed into restraining tubes under a blue disposable cover and habituated to the tubes and the testing room for 30 minutes on two days the week prior to testing. On the test day, the rats were placed into the restraining tubes with their tails hanging freely. The

last 5–10 cm of their tails were immersed into the Thermos containing 40° or 55°C water, and the latency for tail withdrawal was measured. A cutoff time of 15 seconds was imposed so that if the rat did not remove its tail within 15 seconds, the experimenter removed the stimulus. The first three stimulus presentations during a test were 40°C, as a control for a rat that might remove its tail from the water independent of water temperature. If the rat kept its tail in the 40°C water for 15 seconds during two of the three presentations, the rat remained in the experiment (all rats kept their tails in the 40°C water for 15 seconds during two of the three presentations; Supplemental Table 1). A 2-minute interval occurred between each stimulus temperature presentation. Next, one control latency value for 55°C was obtained for each rat. Once all baseline measures were obtained, the first dose of test compound was injected subcutaneously in the dorsal flank. After a 15-minute pretreatment period, the tail-withdrawal latencies for 40°C and 55°C were redetermined in each rat with a 2-minute interval of time between the temperature presentations for the group. The order of presentation each stimulus of 40° and 55°C water was varied unsystematically from trial to trial. As a cumulative-dosing strategy was used, at the conclusion of the 10-minute testing period, the rat was removed from the restrainer, and another acute injection of test compound was administered so that the total cumulative dose was increased 0.25–0.5 $\log_{10}$ unit. After another 15-minute pretreatment period, the latency values for 40°C and 55°C were taken again during the 10-minute testing period. The total trial time (pretreatment and testing period) for each dose was therefore 25 minutes (see Diagram 1). During antagonism studies, the first trial was a test of the antinociceptive effects of the antagonist alone. The test procedure continued until the rat's tail remained in the 55°C water for 15 seconds, the solubility limits of a compound were reached, or another behavior interfered with the measurements (i.e., respiratory depression).

As this was a within-subjects design, rats were assigned to a drug group and were tested once a week with either the compound alone or a dose of antagonist prior to the full dose-response curve for the compound. Therefore, each set of dose-response curves for a particular drug were obtained in the same group of rats. The dose-response curves for each compound alone were tested first in each group of rats, and the next test was generally 0.1 mg/kg naltrexone versus compound to assess the general susceptibility of the novel compound to opioid antagonism. Thereafter, naltrexone doses and the accompanying compound doses were chosen based on the results of the previous week's tests. The entire test was limited to four to six trials to control for the time course of naltrexone and the amount of time the rats were in the restrainers (Walker et al., 1994).

Two-Choice Drug Discrimination Procedure. Rats were trained to discriminate 3.2 mg/kg morphine from saline in a two-choice procedure under a fixed ratio-5 schedule of banana-flavored, sucrose pellet delivery using modifications of a previously published training and testing methodology (Walker et al., 1994). After rats were trained to lever press on both levers, the fixed ratio was increased from 1 to 5 until responding stabilized after approximately 2 to 3 weeks. Thereafter, the discrimination training sessions begun. Morphine or saline were injected subcutaneously in the dorsal flank 15 minutes prior to the start of the training sessions. After 15 minutes, the house and stimulus lights illuminated, and the trial lasted for 50 reinforcers or 15 minutes, whichever occurred first. Responding on the inappropriate lever was not reinforced and reset the ratio requirement. Morphine and saline injections occurred in a semirandom single or double alternating fashion until rats emitted 80% of responses on the injection-correct lever before both the first reinforcer (i.e., the first fixed ratio-5 emitted) and 85% for the total session for seven consecutive training sessions. Further, rats were required to meet testing criteria on four training sessions of double alteration and three training sessions of single alteration within the seven consecutive training sessions. Thereafter, rats were required to meet the testing criteria for at least one saline and one morphine training session between tests (Supplemental Table 2). Single doses of morphine, fentanyl, acetyl fentanyl, butyryl fentanyl, AH-7921, MT-45, W-15, or W-18 were tested for their ability to substitute for the discriminative stimulus effects of morphine and to decrease response rates. Test trials were identical to the training trials except a completed ratio on either lever was reinforced. All compounds were administered 15 minutes prior to the test session. To evaluate the susceptibility of each compound to opioid antagonism, a dose of 0.03 or 0.1 mg/kg naltrexone was administered 15 minutes prior to the dose of test compound. As W-15 and W-18 failed to produce morphine-lever responding at the doses tested, these compounds were not tested with naltrexone.

Data Analysis. Latency measures obtained in tail-withdrawal experiments were transformed into the percentage of maximum effect by the formula:

$$\%\ \text{maximum effect} = \frac{(\text{test latency} - \text{control latency}) \times 100}{(15\ \text{second} - \text{control latency})}$$

Each rat served as its own control. If the rat removed its tail faster than the control latency, a value of zero (percent effect) was assigned. Once a full effect of 15 seconds was obtained after a given dose of the agonist, the rat was returned to its home cage, and additional doses were not tested for that rat. This reduced the likelihood of overdose and fatality in the rats when examining high doses of the opioids.

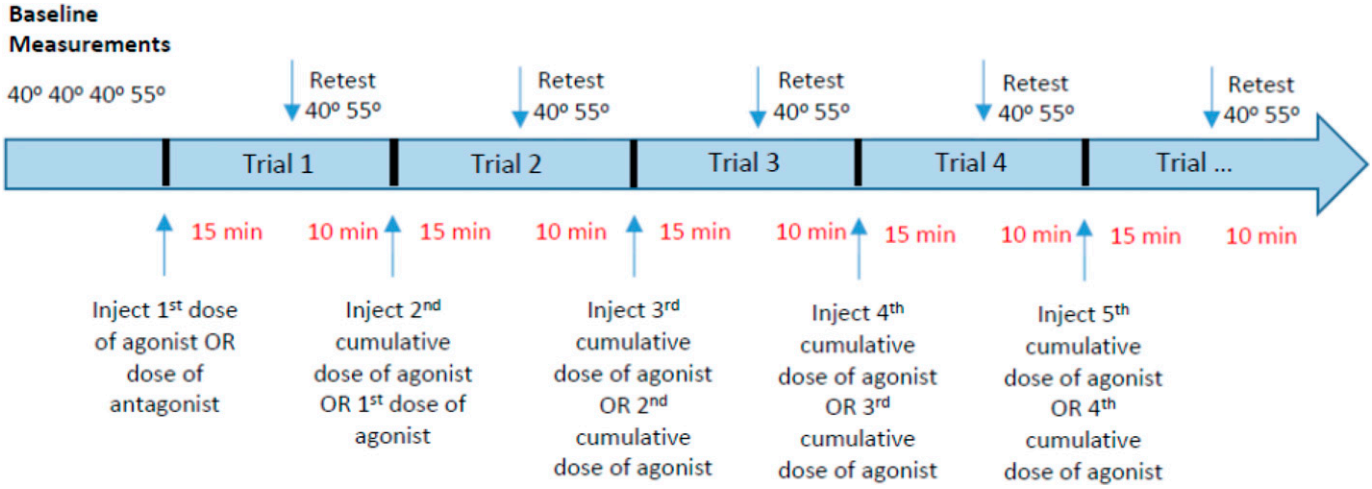

Diagram. 1. Warm-water tail-withdrawal procedure with cumulative dosing.

Discriminative performance is presented as the percentage of responses emitted on the morphine-appropriate lever by the total number of responses emitted on both levers during the trial. These data were analyzed only if 5 or more responses (one fixed ratio) were emitted during the trial. Rates of responding were calculated by dividing the total number of responses on both levers by the total seconds for the trial. Data for the discriminative performance or response rates for all rats tested at a given dose were averaged and plotted as a function of dose. For individual as well as grouped data, a full substitution was defined as ≥85% responding on the morphine-appropriate lever, partial substitution was defined as between 20% and 85% responding on the morphine-appropriate lever, and responding below 20% was considered as no substitution for the training dose of morphine. Effects at each dose of drug were expressed as a group mean, along with the S.E.M. The data from rats that failed to complete one full ratio (i.e., 5 responses) were included in the response rate but not the discrimination data and are noted in the figure legends.

The dose that produced a 50% maximum effect or a 50% morphine-lever responding was taken as the ED_{50} for each dose-response curve. ED_{50} values and ±95% confidence limits (C.L.) were calculated using linear curve fitting analysis with fitting constraints of minimal and maximal effects set as 0% and 100% using the linear portions of the dose-response curves. Analysis of covariance of multiple lines was used to determine whether the slopes of the dose-response curves for a given agonist alone and the agonist in combination with naltrexone were parallel. Most compounds were not tested with doses to produce full rate-decreasing effects in the drug discrimination assay. Therefore, a common value was determined for each compound to reduce responses rates to 0.8 responses per second to estimate potency for this behavioral effect. To examine agonist dose dependence and the effect of naltrexone treatment in both assays, two-way ANOVAs followed by Tukey's Multiple Comparison tests were used with dose and treatment as the factors. In the ANOVAs, sphericity, or equal variability of differences, was not assumed and was adjusted for using the Geisser-Greenhouse correction. The data from the rates of responding were not subjected to these analyses. Significance was reported when the analyses reached a significance level of at least $P < 0.05$. All graphics, linear regression fitting, and statistical analyses were performed using GraphPad Prism version 9.1 for Windows, GraphPad Software, La Jolla, California, www.graphpad.com.

To perform a Schild analysis from the data collected using the warm-water, tail-withdrawal procedure, the linear portions of the dose-response curves were subjected to linear regression as described above. To perform a Schild regression and determine the potency of the naltrexone for each agonist in the warm-water, tail-withdrawal assay, in vivo pA_2 values and Hill slopes were determined as previously described (Arunlakshana and Schild, 1959) with drug doses substituted for drug concentrations (Takemori et al., 1972; Walker et al., 1994). An in vivo pA_2 value is the negative logarithm of the molar dose of antagonist producing a 2-fold shift to the right in the agonist dose-response curve (Arunlakshana and Schild, 1959). As the 95% C.L. on all the slope values of the Schild regression included −1, the slopes were constrained to −1, and the in vivo pA_2 value recalculated. In vivo pA_2 values, slope values, 95% C.L., linear regressions, and analyses of covariance were determined using GraphPad Prism Version 9.1 for Windows.

Drugs. Morphine sulfate, fentanyl hydrochloride, and naltrexone hydrochloride were obtained from the National Institute on Drug Abuse Drug Supply Program (Research Triangle Park, NC). Acetyl fentanyl, butyryl fentanyl, AH-7921, MT-45, W-15, and W-18 were obtained from the Reference Materials Program, Drug Enforcement Administration's Special Testing and Research Laboratory (Dulles, VA). Morphine, fentanyl, acetyl fentanyl, butyryl fentanyl, and naltrexone were dissolved in sterile, physiologic saline. Low concentrations of AH-7921 and MT-45 were dissolved in sterile, physiologic saline, and for higher concentrations of AH-7921 and MT-45, a few drops of 8% lactic acid were added. AH-7921 and MT-45 solutions were then sonicated for 1–4 hours. Solutions were prepared to administer each injection in a volume of 1.0 ml/kg s.c. into the dorsal flank. Occasionally, solubility of the solutions limited the testing of higher doses. W-15 and W-18 required extensive preparation in several different vehicles. The best suspensions were achieved for W-15 in a 1:1:18 ethanol, Cremophor, and saline vehicle and for W-18 in a 20% DMSO and sterile water vehicle.

Results

Warm-Water, Tail-Withdrawal Procedure. Across the 25 different tests in the 8 groups of rats, the average baseline latency per group to withdrawal the tail from 55°C water was 3.63 seconds (range from 1.87 to 5.15 seconds). All baseline latencies from 40°C and 55°C water are listed in Supplemental Table 1. Morphine and fentanyl produced full antinociceptive effects (Fig. 1), although fentanyl was approximately 120-fold more potent than morphine in this assay. Two-way ANOVA revealed dose dependence for both morphine [$F(6, 118) = 35.07$; $P < 0.0001$] and fentanyl [$F(4, 94) = 40.41$; $P < 0.0001$]. Both butyryl fentanyl and acetyl fentanyl produced full antinociceptive effects with potencies between fentanyl and morphine. Butyryl fentanyl was approximately 3-fold more potent than acetyl fentanyl. Two-way ANOVA revealed dose dependence for both butyryl fentanyl [$F(4, 97) = 70.98$; $P < 0.0001$] and acetyl fentanyl [$F(6, 134) = 62.53$; $P < 0.0001$]. AH-7921 produced full antinociceptive effects with a similar potency as acetyl fentanyl, and MT-45 produced full antinociceptive effects with a similar potency to morphine. Two-way ANOVA revealed dose dependence for both AH-7921 [$F(6, 142) = 45.50$, $P < 0.0001$] and MT-45 [$F(6, 114) = 34.12$; $P < 0.0001$]. Higher doses of AH-7921 produced Straub tail in three of the rats tested. No significant toxicity was observed for the drugs tested alone, except one rat died after injection with 0.0032 mg/kg fentanyl. The ED_{50} values (±95% confidence limits) for all drugs to produce antinociceptive effects are listed in Table 1. After multiple attempts to dissolve or suspend W-15 in solution, a Cremophor vehicle produced the best solution. Nevertheless, W-15 failed to produce antinociceptive effects at any dose tested including the largest dose tested, 10 mg/kg W-15. After multiple attempts to dissolve or suspend W-18 in solution, 20% DMSO produced the best solution. However, W-18 failed to produce antinociception at the highest dose that

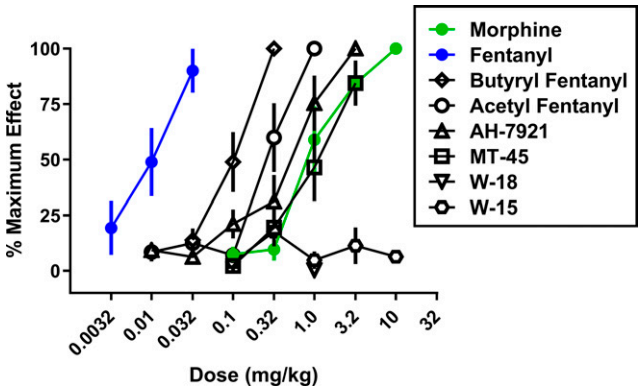

Fig. 1. Antinociceptive effects of morphine, fentanyl, and six test compounds in the warm-water, tail-withdrawal assay. Ordinate: the percentage of maximal antinociceptive response (15 seconds). Abscissa: dose of drug, in milligrams per kilogram. Each point is the average of 8 rats except the point for W18, which is the average of 7 rats. Vertical lines represent S.E.M. unless the S.E.M. is smaller than the size of the symbol.

TABLE 1
Potency comparisons for test compounds relative to morphine and fentanyl

	Tail Withdrawal ED_{50} (±95% C.L.[a])	Drug Discrimination ED_{50} (±95% C.L.)	Dose in mg/kg to Reduce Rates to 0.8 resp/s (±95% C.L.)[b]
	mg/kg	*mg/kg*	
Morphine	1.1 (0.67 to1.5)	1.1 (0.76 to 1.5)	1.2 (ND)[c]
MT-45	0.93 (0.60 to 1.6)	0.50 (0.36 to 0.78)	0.80 (ND)
AH-7921	0.37 (0.25 to 0.54)	0.37 (0.29 to 0.48)	0.39 (0.16 to 1.5)
Acetyl fentanyl	0.28 (0.21 to 0.36)	0.12 (0.094 to 0.15)	0.13 (0.074 to 0.18)
Butyryl fentanyl	0.089 (0.067 to 0.12)	0.029 (0.021 to 0.039)	0.016 (0.0074 to 0.024)
Fentanyl	0.0091 (0.0050 to 0.016)	0.0065 (0.0044 to 0.0089)	0.0043 (0.0016 to 0.0070)

[a]C.L., confidence limits.
[b]Compounds were not tested to full rate-decreasing effects so a common estimate from a give value was determined for each compound.
[c]Confidence limits were unable to be calculated. ND, not determined.

could be put into solution, 1.0 mg/kg W-18. No other antinociceptive effects were observed for W-15 or W-18 in any other vehicles tested (data not shown).

Antagonism studies were completed with three doses of naltrexone in combination with full dose-response curves of morphine, fentanyl, butyryl fentanyl, acetyl fentanyl, AH-7921, and MT-45 (Fig. 2). In general, naltrexone produced dose-dependent antagonism of the antinociceptive effects of all the agonists tested. Two-way ANOVAs revealed an effect of naltrexone treatment on the antinociceptive effects of morphine [$F(3, 118) = 19.21$; $P < 0.0001$], fentanyl [$F(3, 94) = 19.59$; $P < 0.0001$], butyryl fentanyl [$F(3, 97) = 26.84$; $P < 0.001$], acetyl fentanyl [$F(3, 134) = 27.77$; $P < 0.0001$], AH-7941 [$F(3, 142) = 20.92$; $P < 0.0001$], and MT-45 [$F(3, 114) = 41.37$; $P < 0.0001$]. During the antagonism studies, two rats died after receiving 56 mg/kg morphine in combination with 0.1 mg/kg naltrexone approximately 6 hours after the test, one rat died after receiving 3.2 mg/kg butyryl fentanyl in combination with 0.1 mg/kg naltrexone during one test, and another rat died after 1.0 mg/kg butyryl fentanyl in combination with 0.01 mg/kg naltrexone in another test. One rat died after receiving 32 mg/kg MT-45 in combination with 0.032 mg/kg naltrexone, three additional rats died after receiving 56 mg/kg MT-45 in combination with 0.1 mg/kg naltrexone.

To compare the potency of naltrexone to block the antinociceptive effects of the different agonists, a Schild analysis was performed (Fig. 3). As all morphine, fentanyl, butyryl fentanyl, acetyl fentanyl, AH-7921, and MT45 dose-response curves in combination with naltrexone were parallel to the initial control dose-response curves as determined by analysis of covariance, the ED_{50} values were used to construct Schild regressions. Naltrexone was approximately equipotent as an antagonist of all agonists (Table 2), although there were some notable observations. The lowest doses of naltrexone were more potent in combination with morphine and fentanyl resulting in shallow regression slopes of -0.42 and -0.46 and higher in vivo pA_2 values of 9.1 and 8.8, respectively, than for naltrexone with the other agonists butyryl fentanyl, acetyl fentanyl, AH-7921, and MT-45. However, the confidence limits on all the naltrexone regressions included unity and were constrained to a common slope of -1 to estimate the final in vivo pA_2 values for all six agonists. The constrained in vivo pA_2 values for naltrexone with all six agonists were within the same range between 7.5 and 8.1 with overlapping 95% C.L. suggesting that overall, naltrexone was equipotent as an antagonist of the antinociceptive effects of these opioids.

Drug Discrimination. There were eleven rats that acquired the discrimination between 3.2 mg/kg morphine versus saline and met initial testing criteria in 34 days (range 12–110 days). The morphine and saline training data and the rates of responding for the days prior to test days are listed in Supplemental Table 2. Saline injections, administered under test conditions, produced less than 1% morphine-lever responding, i.e., 99% saline-appropriate responding (Fig. 4, top panel). A dose-dependent substitution [$F(3, 30) = 68.41$; $P < 0.0001$] was obtained for increasing doses of morphine until a full effect was obtained after the training dose of 3.2 mg/kg morphine. Rates of responding for the training dose of morphine under test conditions were slightly reduced by approximately 35% of saline control response rates for the highest morphine dose tested (Fig. 4, bottom panel). Fentanyl produced a dose-dependent effect [$F(3, 31) = 42.32$, $P < 0.0001$] with full substitution for the training dose of morphine occurring at a dose of 0.032 mg/kg fentanyl. This dose of 0.032 mg/kg fentanyl was accompanied by approximately a 75% decrease in response rates for the highest fentanyl dose tested compared with vehicle control response rates. All rats except one responded to complete at least one ratio. In the drug discrimination assay, fentanyl was approximately 170-fold more potent than morphine (Table 1).

Butyryl fentanyl [$F(3, 32) = 30.17$; $P < 0.0001$] and acetyl fentanyl [$F(3, 31) = 72.81$; $P < 0.0001$] produced significant dose-dependent morphine-like discriminative stimulus effects (Fig. 4, top panel). Potencies of butyryl fentanyl and acetyl fentanyl to substitute for the morphine discriminative stimulus fell between fentanyl and morphine, with butyryl fentanyl being approximately 4-fold more potent than acetyl fentanyl (Table 1). Butyryl fentanyl and acetyl fentanyl reduced response rates by approximately 60% and 45%, respectively, at the highest doses tested compared with saline test response rates (Fig. 4, bottom panel). AH-7921 [$F(3, 32) = 45.84$; $P < 0.0001$] and MT-45 [$F(3, 32) = 61.19$; $P < 0.0001$] produced dose-dependent, full morphine-like discriminative stimulus effects with potencies slightly less than morphine (Fig. 4, top panel). These compounds reduced response rates by 30% and 20% compared with saline test response rates at the highest doses tested (Fig. 4, bottom panel). The highest single doses that could be tested for W-15 (6.8 mg/kg) and W-18 (1.0 mg/kg) failed to produce significant morphine-like discriminative stimulus or response rate-decreasing effects (Fig. 4). The ED_{50} values (±95% confidence limits) for drugs to substitute for morphine's discriminative stimulus effects are presented in Table 1. As not all drugs were tested up to doses that significantly decreased response rates, the doses of drug to produce 0.8 responses/s were calculated and presented in Table 1 to compare relative potencies for these six drugs.

Antagonism studies were completed with naltrexone in combination with morphine, fentanyl, butyryl fentanyl, acetyl fentanyl, AH-7921, or MT-45. Naltrexone dose dependently

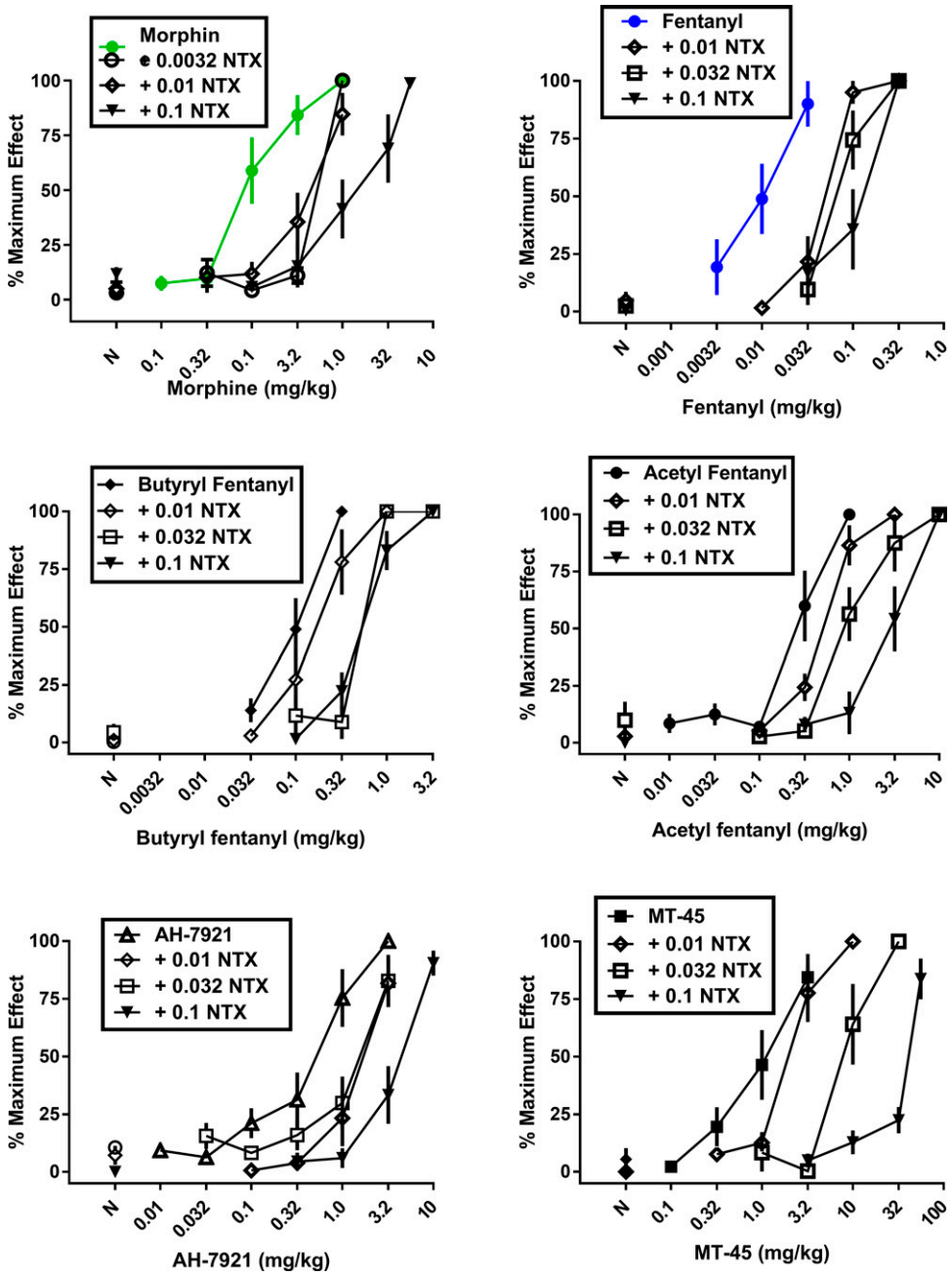

Fig. 2. Naltrexone (N) antagonism of the antinociceptive effects of morphine, fentanyl, butyryl fentanyl, acetyl fentanyl, AH-7921, and MT-45. Ordinate: the percentage of maximal antinociceptive response (15 seconds). Abscissa: dose of agonist in milligrams per kilogram. Naltrexone was administered 25 minutes prior to the first dose of agonist. Each point is the average of one observation in 6–8 rats. Points above N are the effects of naltrexone alone prior to the test. Other details as in Fig. 1.

shifted the morphine discriminative stimulus effects to the right (Fig. 5, upper, left panels). Two-way ANOVA revealed an effect for naltrexone treatment [$F(2, 74) = 25.21$; $P < 0.001$] and morphine dose [$F(4, 74) = 15.30$; $P < 0.001$]. The dose-response curves obtained for morphine and morphine with naltrexone were parallel allowing a comparison of the ED_{50} values across dose-response curves. Pretreatment with 0.03 and 0.1 mg/kg naltrexone produced an 8.4-fold and an 18-fold shift of the control ED_{50} value of morphine, respectively (Table 3). Naltrexone blocked the rate-decreasing effects of morphine; however, the incomplete dose-response curves make the potency differences difficult. Naltrexone shifted the potencies of fentanyl to substitute for the discriminative stimulus effects of morphine to the right (Fig. 5, upper, right panels). Two-way ANOVA revealed an effect for naltrexone treatment [$F(2, 80) = 22.35$; $P < 0.001$] and fentanyl dose [$F(5, 80) = 10.02$; $P < 0.001$]. The slopes of all the dose-response curves for fentanyl and fentanyl in combination with naltrexone were not parallel [$F(2,84) = 5.081$; $P < 0.008$]. The dose of 0.03 mg/kg naltrexone shifted the potency of fentanyl 6-fold to the right, but the shallow slope of the 0.1 mg/kg naltrexone and fentanyl dose-response curve overestimates the potency shift as

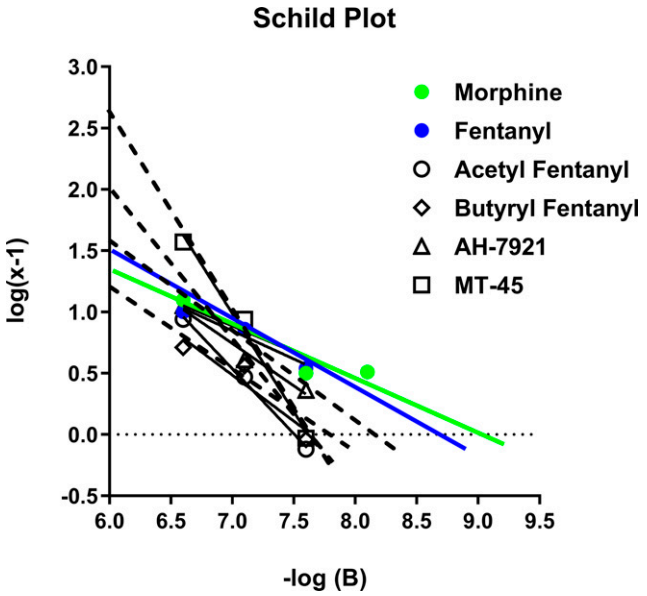

Fig. 3. Schild plot for naltrexone as an antagonist of the antinociceptive effects of six compounds. Ordinate: logarithm of the quantity (ED_{50} value of the agonist in the presence of antagonist divided by the ED_{50} value of the agonist alone) minus 1. Abscissa: negative logarithm of the molar dose of naltrexone. The data for the Schild plot were obtained from Fig. 2.

approximately 20-fold. A dose of 0.03 mg/kg naltrexone produced approximately a 10-fold shift for the rate-decreasing effects of fentanyl. However, testing of the fentanyl doses in combination with 0.1 mg/kg naltrexone was limited due to concerns of toxicity as one rat required additional naltrexone due to observed respiratory depression.

Naltrexone shifted the potency of butyryl fentanyl to substitute for the discriminative stimulus of morphine to the right (Fig. 6, left panels). Two-way ANOVA revealed an effect for naltrexone treatment [F (2, 74) = 42.00; $P < 0.001$] and butyryl fentanyl dose [F (4, 74) = 35.88; $P < 0.001$]. All dose-response curves for butyryl fentanyl alone and in combination with naltrexone were parallel allowing for a comparison of ED_{50} values. Doses of 0.03 and 0.1 mg/kg naltrexone produced a 3.1- and 12-fold shift of the butyryl fentanyl dose-response curve, respectively (Table 3). A dose of 0.03 mg/kg naltrexone shifted the butyryl fentanyl rate-decreasing effects by approximately 6-fold. Potency estimates for 0.1 mg/kg naltrexone to block butyryl fentanyl rate-decreasing effects could not be determined, because higher butyryl fentanyl doses were not tested due to concerns for toxicity.

Naltrexone shifted the potency of acetyl fentanyl to substitute for the discriminative stimulus of morphine to the right (Fig. 6, middle left panel). Two-way ANOVA revealed an effect for naltrexone treatment [F (2, 73) = 38.04; $P < 0.001$] and acetyl fentanyl dose [F (5, 73) = 14.71; $P < 0.001$]. The slopes of the dose-response curves for acetyl fentanyl and acetyl fentanyl in combination with naltrexone were not parallel [F (2,79) = 4.879; $P < 0.01$], therefore, the ED_{50} values cannot be strictly compared. The shallow slope of the 0.1 mg/kg naltrexone and acetyl fentanyl dose-response curve suggests a potency shift to approximately 38-fold. In addition, it appears that 0.1 mg/kg naltrexone produced a large shift to the right for the potency of acetyl fentanyl to decrease response rates, however, the lack of a full control dose-response curve for acetyl fentanyl makes this estimate of potency ambiguous.

Naltrexone shifted the potency of AH-7921 to substitute for the discriminative stimulus of morphine to the right (Fig. 6, middle right panel). Two-way ANOVA revealed an effect for naltrexone treatment [F (2, 65) = 31.32; $P < 0.001$] and AH-7921 dose [F (4, 65) = 15.82; $P < 0.001$]. However, the slopes of the dose-response curves for AH-7921 and AH-7921 in combination with naltrexone were not parallel [F (2, 66) = 6.638; $P < 0.0024$], therefore, the ED_{50} values cannot be fully compared. The dose of 0.03 mg/kg naltrexone shifted the potency of AH-7921 5.5-fold to the right, but the shallow slope of the 0.1 mg/kg naltrexone and AH-7921 dose-response curve overestimates the potency shift as approximately 20-fold. Given the AH-7921 doses tested, the potency of 0.1 mg/kg naltrexone to block the rate-decreasing effects of AH-7921 is unclear. Naltrexone shifted the potency of MT-45 to substitute for the discriminative stimulus of morphine to the right (Fig. 6, right panels). Two-way ANOVA revealed an effect for naltrexone treatment [F (2, 66) = 46.07; $P < 0.001$] and MT-45 dose [F (4, 66) = 24.11; $P < 0.001$]. The slopes of the dose-response curves for MT-45 and MT-45 in combination with naltrexone were not parallel [F (2, 67) = 5.97; $P < 0.0041$], therefore, the ED_{50} values cannot be fully compared. The potency of naltrexone to block the rate-decreasing effects of MT-45 could not be determined based on the doses tested. Higher doses of AH-7921 were not tested due to solubility issues.

Discussion

In two tests commonly used to assess in vivo MOR activity, the warm-water, tail-withdrawal, and drug discrimination assays, four novel synthetic substances produced effects like morphine and fentanyl with the relative potencies: fentanyl > butyryl fentanyl > acetyl fentanyl > AH-7921 > MT-45 >

TABLE 2
Apparent pA2 values for naltrexone as an antagonist of six compounds in the warm-water, tail-withdrawal assay Values are moles per kg and slope of the Schild plot.

Agonist	Apparent pA^2 Estimate	Slope (± 95% C.L.)[a]	Apparent pA2 Estimate (±95% C.L.) [Slope Constrained][b]
Morphine	9.1	−0.42 (−2.4 to 1.5)	8.1 (7.0 to 9.3)
Fentanyl	8.8	−0.46 (−1.8 to 0.86)	7.9 (7.2 to 8.6)
Butyryl fentanyl	7.7	−0.75 (−4.1 to 2.6)	7.5 (7.1 to 8.0)
Acetyl fentanyl	7.5	−1.1 (−1.9 to −0.18)	7.5 (7.4 to 7.6)
AH-7921	8.1	−0.69 (−2.1 to 0.70)	7.8 (7.4 to 8.2)
MT-45	7.6	−1.6 (−4.1 to 0.89)	7.9 (7.1 to 8.7)

[a]C.L., confidence limits.
[b]Slopes included −1 therefore were constrained to −1 for comparisons.

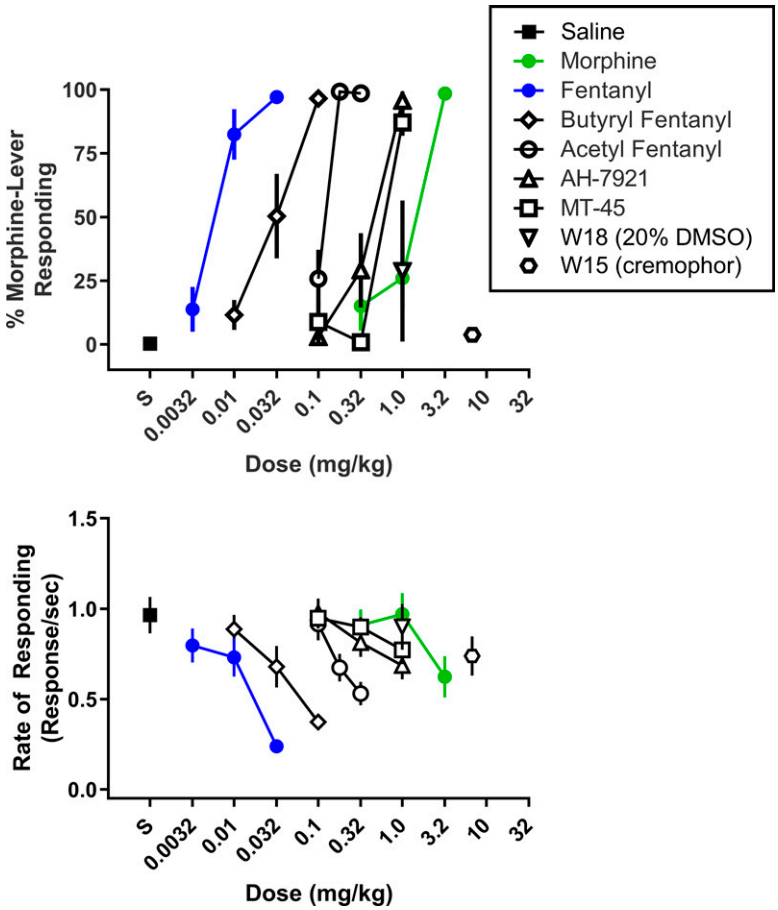

Fig. 4. Discriminative stimulus and rate-decreasing effects of morphine, fentanyl, and six test compounds in rats trained to discriminate 3.2 mg/kg morphine from saline (S). Ordinate, upper panel: percentage of total responses made on the morphine-appropriate lever. Ordinate, lower panel: rates of responding during the 15-minute test period calculated as responses per second. Abscissa: doses of drugs in mg/kg. Points above S indicate effects of a saline injection administered under test conditions. Each data point is the average of one test in 8–11 rats. Vertical lines represent S.E.M. unless the S.E.M. is smaller than the size of the symbol.

morphine. In addition, all six compounds maintained approximately the same relative potency to decrease ongoing response rates to the best that could be determined based on the highest doses tested in the drug discrimination assay. In general, dosing generally stopped out of potential toxicity concerns in the well trained drug discrimination rats once greater than 85% morphine-lever responding was obtained. Indeed, even a low dose of fentanyl alone and higher doses of morphine, butyryl fentanyl, and MT-45 in combination with naltrexone produced lethality in a few rats in the antinociception assay. Solubility also limited testing higher doses of AH-7921 especially in combination with naltrexone. Nevertheless, the orderliness of the potency relationships for the six agonists in these well established assays suggests that these agonists are likely to be producing their effects through MOR.

The observation that fentanyl produced antinociception and substituted for the discriminative stimulus effects of morphine in rats supports findings from previous studies (Walker et al., 1994; Zhang et al., 2000; Schwienteck et al., 2019). As an opioid, fentanyl produces most of the same effects as morphine such as euphoria, sedation, respiratory depression, constipation, and miosis, albeit with higher potency (Vardanyan and Hruby, 2014; Armenian et al., 2018; Pichini et al., 2018). As fentanyl was fully effective in both assays, the observations that acetyl fentanyl and butyryl fentanyl produced full antinociception and morphine-like stimulus effects were anticipated. In [^{3}H]DAMGO radioligand binding assays, Ki values for acetyl fentanyl and butyryl fentanyl were 4.28 and 0.405 nM, respectively, compared with 0.252 nM for morphine and 0.135 nM for fentanyl. Acetyl fentanyl and butyryl fentanyl were less potent than fentanyl to stimulate [^{35}S]GTPγS binding at MOR (Eshleman et al., 2020). Interestingly, both acetyl fentanyl and butyryl fentanyl were only partially effective (~60%) at stimulating [^{35}S]GTPγ binding at MOR, suggesting potential partial agonism for these two compounds in the in vitro assay. However, when tested as inhibitors of [^{3}H]DAMGO in the MOR-mediated [^{35}S]GTPγ binding assay, no evidence of antagonistic activity in the nanomolar range was observed for acetyl fentanyl and butyryl fentanyl, which would be expected of partial agonists (Eshleman et al., 2020). In vivo, acetyl fentanyl and butyryl fentanyl were less potent than fentanyl but fully effective in the acetic acid writhing test in mice (Higashikawa and Suzuki, 2008), similar to the results observed in the present study using the warm-water, tail-withdrawal procedure in rats. These results for acetyl fentanyl and butyryl fentanyl taken together support the notion that

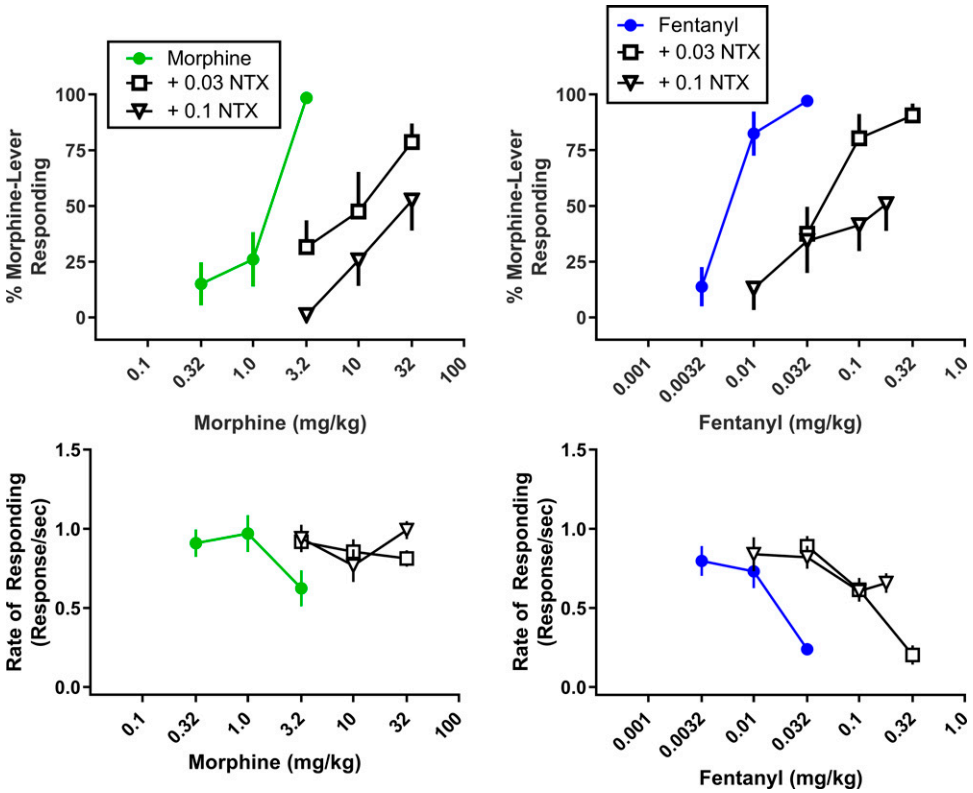

Fig. 5. Naltrexone antagonism of the discriminative and rate-decreasing effects of morphine and fentanyl. Ordinate, upper panels: percentage of total responses made on the morphine-appropriate lever. Ordinate, lower panels: rates of responding during the 15-minute test period calculated as responses per sec. Abscissa: doses of morphine or fentanyl in mg/kg. Each data point is the average of one test in 8–11 rats. Naltrexone was administered 15 minutes prior to the dose of agonist in the single-trial procedure. NTX, naltrexone. Other details as in Fig. 4.

acetyl fentanyl and butyryl fentanyl are behaviorally similar to fentanyl, and that both compounds would likely have abuse liability similar to fentanyl.

Despite being structurally different than morphine or fentanyl, AH-7921 and MT-45 produced full antinociception and substituted for the discriminative stimulus effects of morphine in the present study. These results are not surprising based on limited previous studies. Radioligand binding studies indicate that both AH-7921 (Loew et al., 1988; Rafique et al., 2017) and MT-45 (Baumann et al., 2018; Vandeputte et al., 2020) have either a similar or slightly lower affinity for MOR than morphine. In vivo, preclinical studies in rodents revealed that AH-7921 and MT-45 produced effects in common to other MOR agonists, namely antinociception, hypothermia, Straub tail, inhibition of gut motility, and decreased respiratory rate (Nakamura and Shimizu, 1976; Hayes and Tyers, 1983; Baumann et al., 2018; Bilel et al., 2020). In humans, AH-7921 and MT-45 produce toxicological effects like those of other abused opioids, namely sedation, miosis, nausea, respiratory depression, cyanosis, and reduced oxygen saturation (EMCDDA, 2014a; Katselou et al., 2015; Solimini et al., 2018). In the rat warm-water tail-withdrawal assay described here, MT-45 was approximately equipotent to morphine and blocked by naltrexone, similar to studies in which MT-45 produced naloxone-reversible antinociception in the mouse tail-flick and tail-pinch tests (Baumann et al., 2018; Bilel et al., 2020). Although the discriminative stimulus effects of AH-7921 or MT-45 have not been previously studied, AH-7921 produced conditioned place preference and self-administration in rodents (Cha et al., 2018) similar to other MOR agonists. In the present studies, both AH-7921 and MT-45 produced morphine-like discriminative stimulus effects that were blocked by naltrexone. However, the potency estimates for naltrexone as an antagonist in this assay cannot be fully determined, because higher dose testing was limited by concerns of toxicity observed in the antinociception study as well as insoluble high concentration solutions of AH-7921. Nevertheless, the control dose-response curves for the AH-7921 and MT-45 were shifted at least 10-fold rightward after pretreatment with 0.1 mg/kg naltrexone, indicating that these behavioral effects were sensitive to a MOR antagonist.

Two compounds that did not produce either antinociception or morphine-like discriminative stimulus effects in the present

TABLE 3
Relative potency changes for agonists after administration of either 0.03 or 0.1 mg/kg naltrexone in the drug discrimination assay

	Naltrexone + Agonist ED_{50} value/ Control Agonist ED_{50} value	Value/Control ED_{50} Values
Agonist	0.03 mg/kg Naltrexone	0.1 mg/kg Naltrexone
Morphine	8.4	18
Fentanyl	6.0	[20][a]
Butyryl fentanyl	3.1	12
Acetyl fentanyl	ND[b]	[38][a]
AH-7921	5.5	ND[b]
MT-45	ND[b]	ND[b]

[a]Slope of the dose-response curve not parallel to the control dose-response curves. The ED_{50} value change is an estimate.
[b]ND, not determined because the dose-response curve did not reach 50% at the doses that were tested.

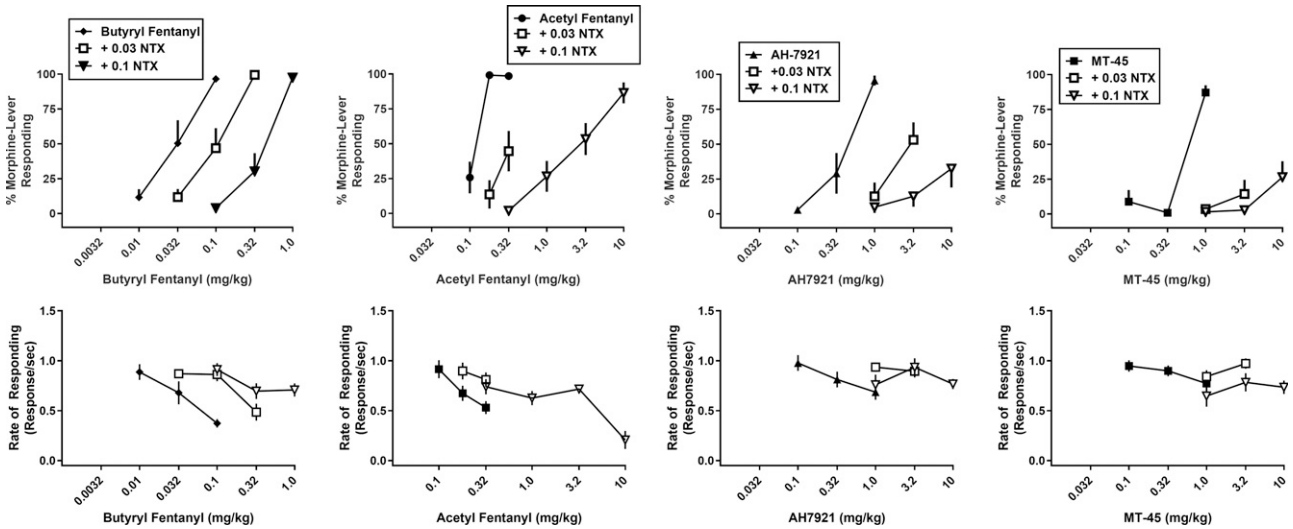

Fig. 6. Naltrexone antagonism of the discriminative and rate-decreasing effects of butyryl fentanyl, acetyl fentanyl, AH-7921, and MT-45. Ordinate, upper panels: percentage of total responses made on the morphine-appropriate lever. Ordinate, lower panels: rates of responding during the 15-minute test period calculated as responses per sec. Five rats did not emit 5 or more responses after a combination of 0.1 mg/kg naltrexone and 10 mg/kg acetyl fentanyl, so their data were only included in the response rate panel. Abscissa: doses of drug in mg/kg. Each point is the average of one test in 8–11 rats. Naltrexone was administered 15 minutes prior to the first dose of agonist. Other details as in Fig. 4.

study were W-15 and W-18. The original patent for these compounds reported that 0.007 mg/kg W-15 and 0.0000037 mg/kg W-18 produced the same inhibition of phenylquinone-induced writhing in mice as that of 0.037 mg/kg of morphine (Knauss et al., 1984). When W-15 and W-18 appeared in Canada in 2013–2016 on the illicit drug market and in toxicological reports, emergency scheduling was instated to protect the public health, as literally no pharmacological data were available to authorities outside of the patents at that time (Gonçalves, 2016). However, an extensive characterization of W-15 and W-18 found no opioid activity in tail-flick, acetic acid writhing, hyperlocomotion, or Straub tail assays in mice and no significant MOR, κ opioid receptor, or δ opioid receptor radioligand binding, no allosteric activity, or Gi-dependent inhibition of cAMP of cloned human opioid receptors (Huang et al., 2017). Similarly, in the current study, neither compound produced antinociception, morphine-like discriminative stimulus effects, or rate-decreasing effects in rats. The observation that W-15 and W-18 produced antinociception in the phenylquinone-induced writhing test in mice but not acetic acid stretching test in mice, or tail-flick in mice or rats might be related to a physiochemical property of these compounds interacting with phenylquinone. Or, as suggested by Huang et al. (2017), as the inhibition of phenylquinone-induced writhing is not selective for opioids, W-15 and W-18 might have decreased the behavioral response through some other mechanisms (Huang et al., 2017). These negative findings with W-15 and W-18 reinforce the need for in vitro and in vivo pharmacological testing in both mice and rats to inform law enforcement, medical professionals, and regulators when a compound does or does not pose public health risks to properly allocate limited resources.

In the warm-water, tail-withdrawal assay, full agonist dose-response curves for six compounds were examined with three naltrexone doses using established multiple-trial, cumulative-dosing protocols. In vivo Schild regression analysis with a single antagonist can be used to characterize the receptor through which an agonist can produce different behavioral effects (Takemori et al., 1972; Dykstra et al., 1988). The naltrexone in vivo pA_2 values with morphine and fentanyl of 8.1 and 7.9 moles/kg agree with those reported previously (Dykstra et al., 1988; Walker et al., 1994; Steinmiller and Young, 2008) as well as in other antinociception assays in other species (Pitts et al., 1996; Li et al., 2008). The in vivo pA_2 values of 7.5–7.9 moles/kg for naltrexone in combination with butyryl fentanyl, acetyl fentanyl, AH-7921, and MT-45 were also within the range of values for naltrexone with MOR agonists in this assay. Although the in vivo pA_2 values and slopes of the regressions have wide 95% C.L. in some cases, the fact that multiple doses of naltrexone were tested in combination with all six agonists provides a more confident total estimate of potency for naltrexone in an antinociceptive assay with a certain degree of inherent variability.

Naltrexone was also studied in combination with morphine, fentanyl, butyryl fentanyl, acetyl fentanyl, AH-7921, and MT-45 in the drug discrimination assay. Two doses of naltrexone were studied in combination with each agonist although full dose-response curves were not obtained in all experiments for a few reasons. First, the drug discrimination assay was conducted in a single-trial, single-dosing design as opposed to the multiple-trial, cumulative-dosing procedure used in the tail-withdrawal assay. This design limits the total number of doses to one or two that can be tested per week. A second limitation was the concern over potential toxicities of high doses of these relatively unknown opioid agonists in the drug discrimination rats. To train all eleven rats to discriminate 3.2 mg/kg morphine from saline took approximately four weeks, so the accidental loss of these subjects can impact the completion of the study, especially when the pharmacological data needs to be collected rapidly for potential scheduling decisions. In the current study, the antinociceptive tests with any given agonist were performed prior to the drug discrimination tests, which allowed a more precise estimate of doses to test and to avoid in the drug discrimination assay. Nevertheless, when comparing 0.03 or 0.1 mg/kg naltrexone across the two assays for morphine, fentanyl, butyryl fentanyl, and AH-7921, the

agonists with parallel shifts after a naltrexone pretreatment, the potencies of naltrexone were the same. The observation that naltrexone is equally potent as an antagonist of the agonists across the two assays using single dosing and multiple doses and that in vivo pA_2 values are similar in the tail-withdrawal assay is further confirmation that both behavioral effects are mediated through actions predominantly at the MOR as demonstrated in a previous study using more traditional opioid agonists (Walker et al., 1994; Steinmiller and Young, 2008).

Authorship Contributions

Participated in research design: Walker, Tella, Prioleau, Fang.

Conducted experiments: Walker, Chambers, Korber.

Performed data analysis: Walker, Chambers, Korber.

Wrote or contributed to the writing of the manuscript: Walker, Tella, Prioleau, Fang.

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