Characterization of the discriminative stimulus effects of binary mixtures of *mu* opioid receptor agonists in rats discriminating fentanyl

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Abstract

Drug overdose deaths involving synthetic opioids, primarily fentanyl, have risen dramatically over the past decade and are currently the driving force of the opioid epidemic in the United States. Fentanyl analogs with greater potency than fentanyl (e.g., carfentanil) pose serious risk to public health. While fentanyl analogs are primarily encountered by humans as constituents of a mixture of drugs, research has primarily evaluated the effects of these drugs alone. The present study characterized interactions between *mu* opioid receptor agonists in seven male Sprague-Dawley rats trained to discriminate 10 µg/kg fentanyl from saline while responding under a fixed-ratio 10 schedule of food presentation. Dose-effect curves were determined for each drug alone and in binary mixtures (fentanyl:heroin, fentanyl:carfentanil, and heroin:carfentanil) at fixed-dose ratios of 3:1, 1:1, and 1:3 relative to the ED₅₀ for each drug when given alone. Dose addition analyses were used to determine the nature of the drug-drug interaction for each mixture. Additive interactions were observed for all binary mixtures at each fixed dose ratio, except the 1:3 fentanyl:carfentanil mixture which exhibited supra-additive effects at the 80% effect level. These results suggest a lack of a significant interaction between the discriminative stimulus effects of these *mu* opioid receptor agonists at the doses tested in this study. Future studies expanding these findings to the respiratory depressant effects of these drugs are of significant importance to rule out possible interactions directly relevant to opioid overdose that occur at doses much larger than those tested in this study.

Significance Statement

In the United States, drug overdose deaths involving synthetic opioids, primarily fentanyls including superpotent fentanyl analogs (e.g., carfentanil), have increased 12-fold over the past decade. While previous studies have evaluated the effects of carfentanil alone, fentanyl analogs are encountered by humans as a mixture with other drugs; this study determined the effects of mixtures of carfentanil and other opioids (fentanyl and heroin) to characterize interactions between these drugs that might contribute to their apparent increased lethality in humans.

Introduction

Provisional reports from the Centers for Disease Control and Prevention estimate that over 93,000 drug overdose deaths occurred in 2020, a 29.4% increase from 2019, with over 69,000 of these deaths involving opioids (Ahmad *et al.*, 2021). While overdoses involving heroin or prescription opioids have stabilized or are decreasing, overdoses involving synthetics opioids such as fentanyl have risen rapidly over the past 6 years, accounting for 82.6% of opioid overdose deaths in 2020 (61.7% of all overdose deaths; Ahmad *et al.*, 2021). The emergence of fentanyl analogs, some of which are much more potent than fentanyl (e.g., carfentanil), pose serious risk to public health. While the majority of research on fentanyl analogs has evaluated the effects of individual drugs, illicit preparations of opioids and other drugs frequently contain more than one pharmacologically active compound, often two or more opioids (Singh *et al.*, 2019).

Data on the involvement of fentanyl analogs in overdose deaths are difficult to obtain because they are not detected in standard drug screens (Solbeck *et al.*, 2021). However, one study evaluating urine samples in Chicago from a five month period spanning 2018-2019 discovered at least one fentanyl analogue in 65.3% of opioid positive urine samples, highlighting the prevalence of exposure to these drugs (Chhabra *et al.*, 2021). A three-year study of urine samples in New York City discovered that from 2016-2019 fentanyl-positive urine screens more than tripled from ~15% to over 50%. Interestingly, 67% of individuals with fentanyl-positive urine reported being unaware that they had taken fentanyl (Martinez *et al.*, 2021), and a similar study reported that seven individuals from a single methadone clinic had urine test positive for carfentanil despite being unaware they had taken the drug (Szczesniak *et al.*, 2021). Taken together these studies illustrate the prevalence of exposures, often incidental, to fentanyl analogs among drug users.

Carfentanil is a fentanyl analog reported to be >1000 times more potent than morphine and 20-190 times more potent than fentanyl (Van Bever *et al.*, 1976; Maguire *et al.*, 1992). Due to its potency, adulteration of regional drug supplies with carfentanil can have grave consequences; one study determined that in 10 states that carfentanil was present in 11% of opioid overdose deaths, and another study in Florida attributed 1100 deaths to carfentanil over a 20-month period in 2016-2017 (O'Donnell *et al.*, 2018; Delcher *et al.*, 2020). Some clinical reports suggest that larger doses of naloxone, the only drug currently approved to treat opioid overdose, may be required to reverse overdose involving carfentanil or other fentanyl analogs (Bardsley, 2019; Leen and Juurlink, 2019; Moss and Carlo, 2019), and some preclinical studies have shown reduced effectiveness of the opioid antagonists naloxone and naltrexone to antagonize the effects of carfentanil compared with other opioid receptor agonists (Wong *et al.*, 2017; Langston *et al.*, 2020; Flynn and France, 2021). While these studies evaluated carfentanil alone, human exposure to carfentanil primarily occurs incidentally alongside other opioids such as heroin or fentanyl (Krotulski *et al.*, 2021; Solbeck *et al.*, 2021).

Exposure to carfentanil occurring primarily as a constituent of a mixture with other drugs, and evidence of diminished antagonism of the effects of carfentanil relative to other opioid agonists, underscore the need for understanding potential interactions between carfentanil and other opioid receptor agonists. Understanding drug interactions that might contribute to the apparent increased lethality of drug overdoses involving carfentanil will provide important new knowledge relevant to opioid overdose in humans. The goal of this study was to characterize interactions between *mu* opioid receptor agonists using drug discrimination and dose-addition analysis. Dose-addition analyses provide a powerful quantitative approach for characterizing interactions between drugs that produce the same pharmacologic effect based on the concept of dose equivalence (Tallarida and Raffa, 2010). The pharmacological selectivity of drug discrimination and the stability of behavior maintained by this procedure is ideal for *in vivo* determination of the

potency or effectiveness of drugs mixtures that share pharmacological properties with the training drug and, thus, amenable to quantitative analyses of drug mixtures. The current studies evaluated the discriminative stimulus effects of fentanyl, heroin, and carfentanil alone and as binary mixtures in rats trained to discriminate 10 $\mu\text{g/kg}$ fentanyl from saline. Dose-addition analyses were then conducted to determine whether the effects of each mixture differed from those predicted for a simply additive interaction. Given that the primary site of action for all three constituent drugs in the mixtures studied is the *mu* opioid receptor and no anticipation of any non-competitive interaction, it was expected that all three binary mixtures of these drugs would have strictly additive interactions.

Materials and methods

Subjects

Seven male Sprague Dawley rats (8 weeks old; 240-260 g; Envigo Inc., Chicago, IL) were individually housed in an environmentally controlled vivarium with a 14:10 hour light/dark cycle maintained at $22.2 \pm 0.5^{\circ}\text{C}$. Experiments were conducted daily during the light cycle. Shortly after beginning training the study was interrupted by the COVID-19 pandemic and rats were free-fed for a period of 5 weeks. When experiments resumed rats weighed $341.3 \pm 7.5\text{g}$ (mean $\pm$ 1 standard deviation). During the study rats were fed 11-15 grams of standard rat chow per day to maintain body weight ($372.4 \pm 1.9\text{ g}$; mean over the testing period of the study $\pm$ 1 standard deviation) and had free access to water. This study was approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio and the Guide for Care and Use of Laboratory Animals.

Apparatus

Operant conditioning chambers (26 x 32 x 21.5 cm, Med Associates, Inc., St. Albans, VT) enclosed in ventilated, sound-attenuated enclosures were equipped with two levers with a white stimulus light above each lever. Sucrose pellets (45 mg; Bio-Serv, Flemington, NJ) could be dispensed by a pellet feeder into a trough located between the levers. Experimental procedures were programmed, and data were collected using MED-PC IV software and interface (Med Associates, Inc.).

Drugs

Fentanyl hydrochloride, carfentanil hydrochloride, heroin hydrochloride, (Drug Supply Program, National Institute on Drug Abuse, Rockville, MD) and spiradoline mesylate (Upjohn, Kalamazoo, MI) were dissolved in sterile saline. Doses of all drugs are expressed as the salt. Drugs were administered i.p., at a volume of 1 mL/kg of body weight. *Training*

All rats were trained to discriminate fentanyl (10.0 $\mu\text{g/kg}$; i.p.) from saline in a two-lever discrimination procedure where responding was maintained by delivery of sucrose pellets under a fixed-ratio 10 schedule of reinforcement. The lever designated correct for fentanyl-appropriate responding was counterbalanced across rats (4 right, 3 left). Training sessions began immediately following an i.p. injection of fentanyl or saline with a 10-minute blackout period during which the chamber was dark and neither lever was active, followed by a 15-minute response period during which the white lights above both levers were illuminated and 10 consecutive responses on the injection-appropriate lever resulted in the delivery of a single sucrose pellet. Rats could earn a maximum of 20 food pellets during the session. Responses on the injection-inappropriate lever were counted and reset the fixed-ratio requirement on the injection-appropriate lever. Testing began once a rat satisfied the following criteria for five consecutive sessions: at least 90% of responses occurring on the injection-appropriate lever; and fewer than 10 responses occurring on the injection-inappropriate lever prior to the delivery of the first pellet. At no point in the study were rats exposed to the same training condition for more than two consecutive sessions.

Testing

Test sessions were identical to training sessions except that both levers were active (10 consecutive responses on either lever delivered a pellet, and a response on one lever reset the response requirement on the other lever). Test sessions occurred following two consecutive days of meeting the training criteria. Immediately before test sessions the rats received an i.p. injection of saline, fentanyl (1-10 $\mu\text{g/kg}$), carfentanil (0.1-1 $\mu\text{g/kg}$), or heroin (32-320 $\mu\text{g/kg}$), the *kappa* opioid receptor agonist spiradoline (100-320 $\mu\text{g/kg}$), or one of three binary drug mixtures (fentanyl:heroin, fentanyl:carfentanil, heroin:carfentanil). Spiradoline was studied to test the selectivity of the discrimination assay for *mu* opioid receptor agonists. Binary mixtures of drugs were given in one of three fixed dose ratios: 3:1, 1:1, 1:3, relative to the ED_{50} of each drug when

given alone. The day following a test session rats received saline and the session was conducted under test conditions to allow for detection of any residual behavioral effects of the preceding test day. No dose of any drug studied had a significant effect on responding the day following a test session.

Dose-effect curves for each drug alone were doubly determined for each subject and are reported as the average across all subjects. For two animals, there was a significant (~10-fold) difference in potency between the first and second determination of the carfentanil dose-effect curve. Carfentanil dose-effect curves were redetermined a third time in all animals; the second and third determinations were not significantly different and were used for the described analyses.

For each fixed dose ratio of each binary mixture three dose pairs predicted to span the 20% and 80% effect levels were determined to be tested *a priori*. Animals were randomly assigned the order in which each binary mixture was tested, and the order in which the three fixed dose ratios (3:1, 1:1, 1:3) within each binary mixture were tested. All three fixed dose ratios within a binary mixture were tested before moving on to the next mixture. Within each fixed dose ratio the individual dose pairs were tested in order of ascending dose, to allow exclusion of a dose pair from testing if a smaller dose pair suppressed responding such that a single fixed-ratio was not completed. No dose pairs were excluded based on this criterion, though the largest dose pairs tested in some mixtures did suppress response rates to that degree. If after testing the three predetermined dose pairs within a given fixed dose ratio there was not one data point with more than 80% fentanyl-appropriate responding and one data point with less than 20% fentanyl-appropriate responding, another dose pair of that dose ratio was tested – where the dose of both constituent drugs in the mixture was increased or decreased by a half-log unit based on whether a larger or smaller dose was needed. For simplicity data from these tests are not shown.

To test for potential changes in sensitivity to the discriminative stimulus effects of each drug alone over time, dose-effect curves for each constituent drug of a particular mixture were redetermined following the completion of each dose ratio within that mixture, e.g., dose-effect curves for fentanyl and carfentanil alone were redetermined once an animal had completed testing of the 3:1, 1:1, and 1:3 fixed dose ratios of the fentanyl:carfentanil mixture.

Analysis of individual drugs A linear regression was fitted to the linear portion (spanning the 20% and 80% effect levels) of the average log dose-effect function (from two determinations) for each *mu* opioid receptor agonist alone, within each animal. This regression was used to calculate ED₅₀ values in individual subjects for each *mu* opioid receptor agonist. The slopes of the regression lines and calculated ED₅₀ values for each *mu* opioid receptor agonist were compared using a one-way repeated measures ANOVA and post-hoc Tukey's multiple comparisons test. For each drug given alone, ED₅₀ values were calculated for each redetermination and compared with the ED₅₀ values of the initial doubly determined dose-effect curve for each drug using a one-way repeated measures ANOVA. Data for fentanyl-appropriate responding and rate of lever pressing are represented as the group mean $\pm$ 1 standard error of the mean (SEM). Data for fentanyl-appropriate responding were not included in the analyses and are not plotted in the figures if the rat did not complete at least one fixed-ratio requirement on either lever within a test session; response rates from such test sessions are still included. Data points representing less than all 7 animals are indicated with the true *n* in parentheses.

Analysis of drug mixtures

Predicted effects for each mixture were calculated based on the concept of dose equivalence (Tallarida and Raffa, 2010), using the slope and intercept of the linear regression determined for each drug in each animal. To calculate the predicted effect for a particular dose pair, the dose of heroin or carfentanil (drug B) present in each mixture was first converted to fentanyl (drug A) equivalents using the following equation (Gannon *et al.*, 2018):

$$\text{Dose } B_{eq}A = [(\text{Slope}^B \times \text{Dose}^B) + (\text{int}^B - \text{int}^A)]/\text{Slope}^A$$

where Slope^A and Slope^B , and int^A and int^B are the slope and y-intercept, respectively, of the linear regression fitted to log dose-effect curves for drug A (fentanyl) and drug B (heroin or carfentanil) in each animal. The calculated fentanyl equivalents of heroin or carfentanil were then summed with each other (heroin:carfentanil mixture) or the dose of fentanyl present in each mixture (fentanyl:heroin and fentanyl:carfentanil mixtures) to determine the total fentanyl equivalents present in each fixed dose pair. Total fentanyl equivalents were then used to calculate the predicted effect for each dose pair.

$$\text{Predicted additive effect} = (\text{Slope}^A \times \text{Total dose}_{eq}A) + \text{int}^A$$

Predicted and experimentally determined (observed) dose-effect curves for each mixture represent the group mean $\pm$ 1 SEM for total fentanyl equivalents (mg/kg), and group mean $\pm$ 1 SEM predicted effect or observed fentanyl-appropriate responding. Group means for the dose of each constituent drug in each dose pair are shown in Table 2, and the total calculated fentanyl equivalents for each dose pair are shown in Table 3. Linear regressions were fitted to the predicted and observed log dose-effect curves for each mixture as described above, and the regression line was used to calculate the predicted and observed ED_{20} , ED_{50} , and ED_{80} for each mixture. Calculated ED_{20} , ED_{50} , and ED_{80} values for predicted and observed dose-effect curves were used to calculate potency ratios (e.g., $\log ED_{50}^{\text{observed}}/\log ED_{50}^{\text{predicted}}$) for each mixture in individual rats. Differences between predicted additive and observed effects at each effect level were considered statistically significant if the 95% confidence interval of the group potency ratio did not include 1. Potency ratios for each mixture at each effect level are summarized in Table 4. Effects of each drug mixture on the rate of lever pressing were also determined and are shown in Figure 3 as a function of fentanyl equivalents. The limited range of doses studied

precluded further quantitative analysis of these data (i.e., determination of dose-equivalence and predicted effects on rate of responding).

All regressions and statistical tests were performed using GraphPad Prism version 9 and $\alpha=0.05$.

Results

Discriminative stimulus effects of individual drugs

Rats satisfied the training criteria in an average (± 1 SEM) of 30.4 ± 12.5 training sessions. The μ opioid receptor agonists fentanyl, heroin, and carfentanil dose-dependently occasioned responding on the fentanyl-appropriate lever, while the κ opioid receptor agonist spiradoline occasioned less than 20% fentanyl-appropriate responding at both doses tested (Figure 1, upper panel). All drugs tested dose-dependently reduced the rate of lever pressing (Figure 1, lower panel). Analysis of ED_{50} values (the dose theoretically expected to occasion 50% responding on the fentanyl-appropriate lever) revealed a main effect of drug ($F_{2,12}=9.98$, $p=0.02$) where heroin was approximately 40-fold less potent ($p=0.0467$), and carfentanil was 8-fold more potent ($p=0.0009$) than fentanyl. The slopes of the dose-effect curves for heroin and carfentanil were not significantly different from that of fentanyl ($F_{2,12}=2.31$, $p=0.15$). Analysis of ED_{50} values revealed that there was no statistically significant difference between the initial determination of ED_{50} values and the redetermination of ED_{50} values for each drug alone when redetermined following each mixture in which it was a constituent (Table 1).

Discriminative stimulus effects of binary mixtures of fentanyl and heroin

Predicted additive and observed dose-effect curves for mixtures of fentanyl and heroin are shown in Figure 2. Each fixed dose ratio tested (3:1, 1:1, and 1:3) dose-dependently occasioned responding on the fentanyl-associated lever (Figure 2; upper row) and reduced the rate of lever pressing (Figure 3; upper row). Responding occurred almost exclusively on the fentanyl-associated lever at the largest dose pair tested. Analysis of potency ratios revealed no departure from predicted additivity at the 20%, 50%, or 80% effect level (Figure 4; upper row). There was moderate inter-subject variability with potency ratios determined in most animals being approximately 1, excepting rats 1 and 4 for the 1:1 and 3:1 mixtures, respectively.

Discriminative stimulus effects of binary mixtures of fentanyl and carfentanil

Dose-effect curves for the predicted additive and experimentally observed discriminative stimulus effects of mixtures of fentanyl and carfentanil are shown in Figure 2. A dose-dependent increase in fentanyl-appropriate responding (Figure 2; middle row) and decrease in rate of responding (Figure 3; middle row) was observed following administration of each fixed dose ratio (3:1, 1:1, and 1:3). All animals responded near-exclusively on the fentanyl-appropriate lever at the largest dose pair tested for each dose ratio. However, for the 3:1 and 1:3 fentanyl:carfentanil mixtures, the middle dose pair tested was sufficient to occasion >95% fentanyl-appropriate responding in six out of seven, and all seven animals, respectively. In general potency ratios for each fixed dose ratios at the 20% effect level were ~1 (Figure 4; middle row, left panel). However, at the 50% and 80% effect levels potency ratios were on average >1 for all three fixed dose ratios (Figure 4; middle row, center and right panels). At both the 50% and 80% effect levels six, three, and seven out of eight rats had a potency ratio of >1 for the 3:1, 1:1, and 1:3 fixed dose ratios, respectively, suggesting a departure from additivity. This departure from additivity was only significant at the 80% effect level for the 1:3 fixed dose ratio. While the majority of rats had potency ratios of >1 for some other dose ratios and at other effect levels, these deviations from additivity were small in magnitude resulting in a confidence interval spanning the line of additivity (1).

Discriminative stimulus effects of binary mixtures of heroin and carfentanil

Predicted additive and observed dose-effect curves for mixtures of heroin and carfentanil are shown in Figure 2. Similar to the other mixtures, all three fixed dose ratios dose-dependently increased fentanyl-appropriate responding (Figure 2; lower row) and reduced rate of responding (Figure 3; lower row). The observed ED₂₀, ED₅₀, and ED₈₀ for each fixed dose ratio did not significantly differ from the predicted values based on the 95% confidence interval including 1. However, for the 3:1 and 1:3 fixed dose ratios, there was marked inter-subject variability in the

potency of the mixtures tested. The smallest dose pair tested in both of these mixtures occasioned >80% fentanyl-appropriate responding in three out of seven rats. Rats 2 and 4 responded >80% on the fentanyl-paired lever at the smallest dose pair for both the 3:1 and 1:3 fixed dose ratios, while rat 6 only did so at the 3:1 fixed dose ratio and rat 8 only did so at the 1:3 fixed dose ratio. When the dose for each constituent drug was reduced by one half-log unit from the smallest dose pair of the 1:3 fixed dose ratio mixture planned to be tested *a priori*, the resulting dose pair was still sufficient to maintain at least 80% fentanyl-appropriate responding in rats 2 and 4, resulting in potency ratios far exceeding 1 (Figure 4; bottom row).

Discussion

The *mu* opioid receptor agonists fentanyl, heroin, and carfentanil all dose-dependently occasioned responding on the fentanyl-associated lever, while the *kappa* opioid receptor agonist spiradoline did not. These results are consistent with the well-established pharmacological selectivity of drug discrimination procedures (Holtzman, 1985), and studies showing that rats trained to discriminate fentanyl will only respond on the fentanyl-paired lever after receiving a sufficiently large dose of a *mu* opioid receptor agonist (Colpaert *et al.*, 1975; Shannon and Holtzman, 1976). The relative potencies of *mu* opioid receptor agonists in this study were as follows: carfentanil>fentanyl>heroin, where carfentanil was 8-fold more potent than fentanyl and 335-fold more potent than heroin. These data are consistent with previous studies in rats discriminating fentanyl, in which carfentanil was 15-fold more potent than fentanyl and 250-fold more potent than heroin (Flynn and France, 2021). Dose-effect curves for constituent drugs did not differ over the duration (~12 months) of the study, highlighting the stability of behavior maintained by drug discrimination procedures and thus their utility for quantitative studies of drug interactions.

Three binary mixtures (fentanyl:heroin, fentanyl:carfentanil, and heroin:carfentanil) were evaluated at three fixed dose ratios (3:1, 1:1, and 1:3), and potency ratios were compared at three effect levels (20%, 50%, 80%). All three binary mixtures dose-dependently occasioned fentanyl-appropriate responding and reduced the rate of lever pressing at each of the fixed dose ratios. Using dose-addition analysis to compare the predicted additive effect for each mixture to the experimentally determined effects allowed for detection of subadditive (potency ratios <1) or supra-additive (potency ratios >1) interactions. Of the conditions studied, only the 1:3 heroin:carfentanil mixture showed significant deviation from additivity, and only at the 80% effect level. In general, potency ratios at larger effect levels for individual animals for fentanyl:carfentanil mixtures tended to be <1, suggesting supra-additivity however, at the group

level the potency ratios did not significantly differ from additivity as determined by the 95% confidence interval spanning 1. Intersubject variability was greatest for heroin:carfentanil mixtures, with some animals responding on the fentanyl-appropriate lever at the smallest dose pair determined to be tested *a priori*, though this too was not sufficient to result in supra-additive effects at the group level. Interestingly, the fixed dose pair conditions in which carfentanil was the more prevalent constituent (fentanyl:carfentanil 1:3 and heroin:carfentanil 1:3) were two of the conditions that resulted in the largest number of animals with potency ratios >1 at the 50% and 80% effect levels (7 out of 7 animals and 6 out of 7 animals, respectively).

Overall, the results of this study suggest that interactions between the discriminative stimulus effects of the *mu* opioid receptor agonists heroin, fentanyl, and carfentanil are strictly additive in nature at the doses examined in these studies. This does not, however, rule out the possibility of interactions at larger doses that may be of particular relevance to the respiratory depressant (i.e., potentially lethal) effects of these drugs, compared with the relatively small doses that are studied using drug discrimination. Given the lack of an interaction deviating from additivity in this study, the most likely interaction that might occur at larger doses would be between fentanyl and carfentanil, as both drugs are metabolized in part by the same cytochrome P-450 enzymes (e.g., CYP3A4; Feierman and Lasker, 1996; Tateishi *et al.*, 1996; Kong and Walz, 2021). Some reports suggest that the pharmacokinetics of carfentanil are non-linear at large doses, likely related to the high lipophilicity of carfentanil and resulting differences in absorption and distribution (Bergh *et al.*, 2019). Potential pharmacokinetic interactions with fentanyl might result in the non-linear accumulation of carfentanil at lower doses, increasing the risk of adverse events.

Drug overdose deaths involving synthetic opioids have risen dramatically over the past decade, highlighting the need to evaluate the effects of these drugs and the effectiveness of opioid receptor antagonists to block/reverse those effects, relative to other opioid receptor agonists.

With almost all exposures to superpotent fentanyl analogs (e.g., carfentanil) occurring when the fentanyl analog is present in a mixture with other drugs, it is imperative to study these drugs in drug mixtures. The present study found that interactions between the *mu* opioid receptor agonists heroin, fentanyl, and carfentanil are additive at small doses, although individual differences in some mixtures tended towards supra-additivity. Future studies will characterize interactions between the respiratory depressant effects of these drugs, as well as potential differences in antagonism of the effects of opioid mixtures containing carfentanil relative to the constituent drugs alone.

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Authorship contributions

Participated in research design: Flynn and France

Conducted experiments: Flynn

Performed data analysis: Flynn

Wrote or contributed to the writing of the manuscript: Flynn and France

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Conflict

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Footnotes

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- b) Part of this work was presented as a poster or oral presentation at the following conferences:

Shawn M. Flynn and Charles P. France. Discriminative stimulus effects of mu opioid receptor agonist mixtures. Presented at 13th Annual Behavior, Biology, and Chemistry: Translational Research in Addiction; 2021 March 5-7; virtual.

Shawn M. Flynn and Charles P. France. Interactions between mu opioid receptor agonists in rats discriminating fentanyl. Presented at American Society for Pharmacology and Experimental Therapeutics at Experimental Biology; 2021 April 27-20; virtual.

- c) Reprint requests: Charles P. France, Department of Pharmacology, University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive (Mail Code 7764), San Antonio, TX, 78229, USA. Email: france@uthscsa.edu

Figure Legends

Figure 1. Discriminative stimulus and rate decreasing effects of fentanyl (circles), heroin (triangles), carfentanil (squares) and spiradoline (inverted triangles) in rats trained to discriminate fentanyl. S indicates data collected when saline was administered at the start of the session. Dose-effect curves for each drug were doubly determined in each animal, excepting carfentanil, for which a third determination was necessary and the average of the second and third determinations are shown here (see: *Testing*). Group mean $\pm$ 1 SEM fentanyl-appropriate responding (top) and response rate in lever presses per second (bottom) plotted as a function of dose in μg per kilogram of body weight; $n=7$ except for points indicated with parentheses (n).

Figure 2. Observed (solid black line, black symbols) and predicted additive (broken gray line) dose-effect curves for binary mixtures of fentanyl and heroin (top row), fentanyl and carfentanil (middle row), and heroin and carfentanil (bottom row). Each mixture was tested at three fixed dose ratios: 3:1 (left column), 1:1 (center column), and 1:3 (right column), relative to the ED_{50} for each constituent drug when given alone. Group mean $\pm$ 1 SEM percent observed or predicted fentanyl-appropriate responding is plotted as a function of group mean $\pm$ 1 SEM total fentanyl equivalents in $\mu\text{g}/\text{kg}$ of body weight; $n=7$. Horizontal error bars represent variance in fentanyl equivalents as this parameter was calculated for individual rats.

Figure 3. The effects of binary mixtures of fentanyl and heroin (top row), fentanyl and carfentanil (middle row), and heroin and carfentanil (bottom row) on rate of lever pressing. Data are from the same sessions shown in Figure 2. Each mixture was tested at three fixed dose ratios: 3:1 (left column), 1:1 (center column), and 1:3 (right column), relative to the ED_{50} for fentanyl-appropriate responding for each constituent drug when given alone. Group mean $\pm$ 1

SEM rate of responding is plotted as a function of mean $\pm$ 1 SEM total fentanyl equivalents in $\mu\text{g/kg}$ of body weight; $n=7$. Horizontal error bars represent variance in fentanyl equivalents as this parameter was calculated for individual rats.

Figure 4. Potency ratios (observed/predicted) for binary mixtures of fentanyl and heroin (top row), fentanyl and carfentanil (middle row), and heroin and carfentanil (bottom row) at three effect levels: 20% (left column), 50% (center column), and 80% (right column). Group mean and 95% confidence interval are represented by the vertical black lines and error bars, respectively. Potency ratios for individual animals are represented by animal numbers. Potency ratios <1 (left of the vertical dotted line) suggest sub-additive effects, while potency ratios >1 (right of the vertical dotted line) suggest supra-additive effects.

Table 1. Initial and redetermined ED₅₀ values and 95% confidence intervals for the discriminative stimulus effects of fentanyl, heroin, and carfentanil. Values for individual drugs were redetermined following completion of testing for a mixture in which each drug was a constituent.

Drug	Initial determination		Fentanyl:Heroin			Fentanyl:Carfentanil			Heroin:Carfentanil		
	ED50 (µg/kg)					ED50 (µg/kg)					
	Mean	95% CI	Mean	95% CI	<i>p</i>	Mean	95% CI	<i>p</i>	Mean	95% CI	<i>p</i>
Fentanyl	4.8	3.3-6.2	4.6	2.8-6.4	0.97	4.0	2.1-5.9	0.77	-	-	-
Heroin	200.3 ^a	46.8-353.7	219.3	64.8-373.9	0.47	-	-	-	176.3	97.0-255.5	0.92
Carfentanil	0.6 ^a	0.1-1.2	-	-	-	0.3	0.1-0.5	0.4	0.4	0.2-0.6	0.48

p-values for each redetermination represent comparison with initial determination using one-way repeated measures ANOVA

^a Initial determinations significantly different from fentanyl; *p*<0.05

Table 2. Composition of binary mixtures of fentanyl and heroin, fentanyl and carfentanil, and heroin and carfentanil.

Fentanyl $\mu\text{g/kg}$:Heroin $\mu\text{g/kg}$ <i>mean (SEM)</i>	Fentanyl $\mu\text{g/kg}$:Carfentanil $\mu\text{g/kg}$ <i>mean (SEM)</i>	Heroin $\mu\text{g/kg}$:Carfentanil $\mu\text{g/kg}$ <i>mean (SEM)</i>
3:1	3:1	3:1
1.4 (0.2) : 20.0 (5.9)	1.43 (0.2) : 0.1 (0.0)	60.1 (17.6) : 0.1 (0.0)
4.5 (0.5) : 63.3 (18.6)	4.51 (0.5) : 0.2 (0.2)	190.0 (55.6) : 0.2 (0.1)
14.3 (1.7) : 200.3 (58.7)	14.3 (1.7) : 0.6 (0.2)	600.8 (176.0) : 0.6 (0.2)
1:1	1:1	1:1
0.5 (0.1) : 20.0 (5.9)	0.5 (0.1) : 0.1 (0.0)	20.0 (5.9) : 0.1 (0.0)
1.5 (0.2) : 63.3 (18.6)	1.5 (0.2) : 0.2 (0.1)	63.3 (18.6) : 0.2 (0.1)
4.8 (0.6) : 200.3 (58.7)	4.8 (0.6) : 0.6 (0.2)	200.3 (58.7) : 0.6 (0.2)
1:3	1:3	1:3
0.5 (0.1) : 60.1 (17.6)	0.5 (0.1) : 0.2 (0.1)	20.0 (5.9) : 0.2 (0.1)
1.5 (0.2) : 190.0 (55.6)	1.5 (0.2) : 0.6 (0.2)	63.3 (18.6) : 0.6 (0.2)
4.8 (0.6) : 600.8 (178)	4.8 (0.6) : 1.9 (0.6)	200.3 (58.7) : 1.9 (0.6)

Table 3. Total calculated fentanyl equivalents for fixed dose pairs of binary mixtures of fentanyl and heroin, fentanyl and carfentanil, and heroin and carfentanil.

Total fentanyl equivalents $\mu\text{g/kg}$; <i>mean (SEM)</i>		
Fentanyl:Heroin	Fentanyl:Carfentanil	Heroin:Carfentanil
3:1	3:1	3:1
2.3 (0.4)	2.3 (0.4)	2.8 (0.5)
6.4 (0.9)	6.5 (0.9)	6.6 (0.9)
19.0 (2.4)	19.0 (2.4)	16.9 (2.3)
1:1	1:1	1:1
1.3 (0.3)	1.4 (0.3)	1.8 (0.4)
3.4 (0.6)	3.5 (0.5)	3.9 (0.7)
9.5 (1.1)	9.5 (1.2)	9.5 (1.2)
1:3	1:3	1:3
2.3 (0.4)	2.4 (0.4)	2.8 (0.5)
6.1 (0.8)	6.1 (0.8)	6.5 (0.9)
16.9 (2.3)	16.4 (2.5)	16.4 (2.5)

Table 4. Predicted and observed discriminative stimulus effects of binary mixtures of fentanyl and heroin, fentanyl and carfentanil, and heroin and carfentanil.

Mixture	ED ₂₀ (µg/kg; fentanyl equivalents)			ED ₅₀ (µg/kg; fentanyl equivalents)			ED ₈₀ (µg/kg; fentanyl equivalents)		
	Predicted mean (95% CI)	Observed mean (95% CI)	Potency ratio mean (95% CI)	Predicted mean (95% CI)	Observed mean (95% CI)	Potency ratio mean (95% CI)	Predicted mean (95% CI)	Observed mean (95% CI)	Potency ratio mean (95% CI)
Fentanyl:Heroin									
3:1	3.26 (2.09-4.42)	4.16 (1.76-6.55)	0.98 (0.93-1.04)	4.75 (3.27-6.24)	5.76 (2.40-9.11)	1.00 (0.922-1.07)	7.02 (5.09-8.94)	7.99 (3.28-12.70)	1.02 (0.91-1.12)
1:1	3.26 (2.09-4.42)	3.91 (2.04-5.78)	0.98 (0.83-1.14)	4.75 (3.27-6.24)	5.32 (2.89-7.76)	0.99 (0.84-1.14)	7.02 (5.09-8.94)	7.26 (4.04-10.48)	1.00 (0.85-1.15)
1:3	3.26 (2.09-4.42)	4.45 (1.96-6.33)	0.97 (0.86-1.07)	4.75 (3.27-6.24)	5.53 (2.29-8.76)	0.99 (0.88-1.09)	7.02 (5.09-8.94)	7.44 (2.67-12.22)	1.01 (0.91-1.12)
Fentanyl:Carfentanil									
3:1	3.26 (2.09-4.42)	2.84 (1.78-3.91)	1.01 (0.90-1.12)	4.75 (3.27-6.24)	3.87 (2.49-5.24)	1.03 (0.91-1.15)	7.02 (5.09-8.94)	5.27 (3.48-7.05)	1.06 (0.92-1.19)
1:1	3.26 (2.09-4.42)	3.45 (1.73-5.16)	1.03 (0.92-1.13)	4.75 (3.27-6.24)	4.56 (2.30-6.82)	1.05 (0.94-1.16)	7.02 (5.09-8.94)	6.06 (3.04-9.09)	1.07 (0.95-1.18)
1:3	3.26 (2.09-4.42)	2.60 (1.35-3.85)	1.05 (0.96-1.14)	4.75 (3.27-6.24)	3.41 (1.88-4.94)	1.07 (0.98-1.16)	7.02 (5.09-8.94)	4.49 (2.57-6.41)	1.10 (1.01-1.19) ^a
Heroin:Carfentanil									
3:1	3.26 (2.09-4.42)	3.70 (0.49-6.90)	1.01 (0.92-1.10)	4.75 (3.27-6.24)	4.76 (0.84-8.69)	1.03 (0.93-1.14)	7.02 (5.09-8.94)	6.15 (1.34-10.96)	1.06 (0.95-1.18)
1:1	3.26 (2.09-4.42)	3.53 (1.30-5.77)	1.03 (0.86-1.21)	4.75 (3.27-6.24)	4.55 (1.84-7.27)	1.06 (0.88-1.23)	7.02 (5.09-8.94)	5.89 (2.53-9.25)	1.08 (0.90-1.26)
1:3	3.26 (2.09-4.42)	3.03 (0.00-6.25)	1.08 (0.90-1.26)	4.75 (3.27-6.24)	4.02 (0.00-8.42)	1.11 (0.92-1.30)	7.02 (5.09-8.94)	5.36 (0.00-11.39)	1.14 (0.94-1.34)

^a Significant difference between predicted and observed ED_{20,50,80} value as defined by a 95% confidence interval that does not include 1.

Figure 1

Figure 2

Figure 3

Figure 4