JPET Fast Forward. Published on November 30, 2016 as DOI: 10.1124/jpet.116.235630
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Behavioral Characterization of *Kappa* Opioid Receptor Agonist Spiradoline and Cannabinoid Receptor Agonist CP55940 Mixtures in Rats

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Running Title Page

Running title: Spiradoline and CP55940 Interactions

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Manuscript statistics:

Text Pages: 15

Tables: 3

Figures: 2 (+1 supplemental figure)

References: 58

Abstract: 249 words

Introduction: 614 words

Discussion: 1158 words

Non-standard abbreviations:

CP55940, 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol; spiradoline, 2-(3,4-dichlorophenyl)- N-methyl- N-[(5R,7S,8S)- 7-pyrrolidin-1-yl- 1-oxaspiro[4.5]decan-8-yl] acetamide

Recommended section assignment: Behavioral Pharmacology

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ABSTRACT

Pain is a significant clinical problem and there is a need for more effective treatments with reduced adverse effects that currently limit the use of *mu* opioid receptor agonists. Synthetic *kappa* opioid receptor agonists have no abuse liability and well-documented antinociceptive effects; however, adverse effects (diuresis, dysphoria) preclude their use in the clinic. Combining *kappa* opioids with non-opioid drugs (cannabinoid receptor agonists) allows for smaller doses of each drug to produce antinociception. This study tested whether a potentially useful effect of the *kappa* opioid receptor agonist spiradoline (antinociception) is selectively enhanced by the cannabinoid receptor agonist CP55940. Cumulative dose-response functions were determined in 8 male Sprague Dawley rats for spiradoline (0.032-32.0 mg/kg, i.p.) and CP55940 (0.0032-1.0 mg/kg, i.p.) for antinociception, hypothermia, food-maintained responding, and diuresis. Alone, each drug dose-dependently increased tail withdrawal latencies from 50^{o}C water, decreased body temperature by ~4^{o}C, and eliminated food-maintained responding. Spiradoline but not CP55940 significantly increased urine output at doses that eliminated responding. Smaller doses of spiradoline and CP55940 in mixtures (3:1, 1:1, and 1:3 spiradoline:CP55940) had effects comparable to those observed with larger doses of either drug administered alone: the interaction was additive for antinociception and additive or greater than additive for hypothermia and food-maintained responding. Collectively these data fail to provide support for the use of these mixtures for treating acute pain; however, *kappa* opioid/cannabinoid mixtures might be useful for treating pain under other conditions (e.g., chronic pain), but only if the adverse effects of both drugs are not enhanced in mixtures.

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INTRODUCTION

Pain is the most common reason people seek medical care (St. Stauver et al., 2013); *mu* opioid receptor agonists remain the drugs of choice for treating moderate to severe pain despite well-documented adverse effects (e.g., respiratory depression, abuse, overdose). *Mu* opioid receptor agonists are the most widely abused of all prescription medications and fatal overdoses have reached epidemic levels (Centers for Disease Control and Prevention [CDC], 2016; Rosenblatt and Catlin, 2012; Volkow et al., 2014). There is a need for more effective treatments that have fewer of the deleterious effects that currently limit the clinical use of opioids. Unlike *mu* opioid receptor agonists, synthetic *kappa* opioid receptor agonists are not likely to be abused because they are devoid of positive reinforcing effects (Chavkin, 2011; Lalanne et al., 2014; Tejeda et al., 2013; Woods and Winger, 1987). The antinociceptive effects of *kappa* opioid receptor agonists are comparable to those of *mu* opioid receptor agonists in various animal models of pain (Binder et al., 2001; Desmeules et al., 1993; Gerak and France, 2016; Kivell and Prisinzano, 2010; Smith et al. 2008); however, doses producing antinociception also produce conditioned place aversion and diuresis (Leander, 1983; Shippenberg and Herz, 1988; Zhang et al., 2005). Significant adverse effects of *kappa* opioids that preclude their use in the clinic include dysphoria, hallucinations, and diuresis (Peters et al., 1987; Pfeiffer et al., 1986; Walsh et al., 2001). However, the therapeutic potential of *kappa* opioids might be realized if smaller doses produced antinociception without adverse effects, thereby creating a favorable therapeutic window.

One strategy for possibly avoiding adverse effects is to combine *kappa* opioids with drugs that produce antinociception through non-opioid mechanisms (e.g., cannabinoid receptor agonists). Mixtures of smaller doses of a *kappa* opioid and a cannabinoid have antinociceptive effects that are equivalent to the effects of larger doses of either drug given alone, and this interaction is additive (Maguire and France, 2016). The use of smaller doses to achieve the desired (therapeutic) effect might also reduce the likelihood of adverse effects; for example,

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cannabinoids do not enhance the discriminative stimulus or reinforcing effects of *mu* opioid receptor agonists (Maguire et al., 2013). If adverse effects are not apparent at smaller doses, then *kappa* opioid/cannabinoid mixtures could be useful for treating pain and might be preferred to *mu* opioid receptor agonists.

Kappa opioid receptors are thought to mediate, in part, antinociceptive but not other (e.g., cataplexy) effects of cannabinoid receptor agonists (Smith et al., 1994) through spinal mechanisms and the release of endogenous opioids such as dynorphin B (Pugh et al., 1996; Pugh et al., 1997). Furthermore, although *kappa* opioids and cannabinoids can have shared adverse effects, those shared effects can be mediated by different mechanisms thereby allowing for smaller doses of each drug (in mixtures) to produce antinociception, compared with larger doses of either drug alone, and potentially avoiding adverse effects. This study characterized the effects of the *kappa* opioid receptor agonist spiradoline and the non-selective CB1/CB2 cannabinoid receptor agonist CP55940, alone and in mixtures, to test the hypothesis that the potency of spiradoline/CP55940 mixtures to produce antinociception is greater than the potency to produce adverse effects. A warm water tail withdrawal procedure was used to measure antinociceptive effects. Hypothermia, diuresis, and rate-decreasing effects (food-maintained responding) served as indices of adverse effects. Hypothermia and diuresis have been reported for both cannabinoids and *kappa* opioids (Adler and Geller, 1988; Chopda et al., 2013; Dykstra et al., 1987; McGregor et al., 1996; Paronis et al., 2012; Paronis et al., 2013; Rawls and Benamar, 2011; Wadenberg, 2003). Food-maintained responding was used to assess behavioral suppression. The nature of the interaction between spiradoline and CP55940 was examined quantitatively using dose-additivity analyses (Tallarida, 2006, 2011).

MATERIALS and METHODS

Subjects

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Eight male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighed 230-250 g upon arrival and were individually housed in 45 x 24 x 20 cm plastic cages with rodent bedding (Harlan Taklad, Madison WI). Rats had free access to standard rodent chow (Harlan Teklad) and water for two weeks while they were habituated to handling. Once experiments began, rats were fed 5-15 g daily allowing them to grow such that they weighted approximately 85% of the body weight of free-feeding rats. Thereafter, body weights were maintained at 350 $\pm$ 5 g by daily food rations provided after sessions. Water was continuously available outside of experimental sessions. A 14:10 light:dark cycle was in effect (lights on at 0630 hr) with sessions conducted during the light period (starting between 1100 and 1200 hr and lasting 3 hr). The same rats were used in all assays, with drug tests separated by at least one week. Operant (food) sessions were conducted 6-7 days per week, excluding days on which antinociception tests occurred (for detailed description of order of drug testing across assays see Drugs section below). The experimental protocol was approved by the Institutional Animal Care and Use Committee at the University of Texas Health Science Center at San Antonio and in accordance with guidelines set forth by the Guide for the Care and Use of Laboratory Animals (2011).

Apparatus and Procedure

Warm Water Tail Withdrawal and Body Temperature

Warm water tail withdrawal was used to measure antinociceptive effects and body temperature was assessed during the same sessions. Water baths (EW-14576-00, Cole-Parmer, Vernon Hills, IL) were maintained at 40, 50, and 55^{o}C. Sessions comprised 6 30-min cycles with an injection given at the start of each cycle. After each injection, the rat was returned to its home cage for 28 min. Next, the rat was positioned on the palm of the experimenter's hand and 5 cm of the tail was lowered into a water bath. Three water temperatures were tested in a randomized order and separated by approximately 20 s. Latency (sec) to completely remove the tail from the water was recorded by the experimenter using a stopwatch. To avoid

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any adverse effects, the maximum possible latency was 20 s. Body temperature was recorded at the end of each cycle with a rectal thermometer (PhysiTemp Instruments, Inc., Clifton, NJ), and the next injection was given before returning the rat to its home cage. Vehicle injections were given in the first cycle, and cumulative doses of drug were given in the remaining 5 cycles, with the stipulation that latencies in the first cycle were not more than 5 s for 50 and 55^{o}C water and were at least 15 s for 40^{o}C water. When the maximum (20-s) latency was observed for 50^{o}C water during a cycle, tail withdrawal latencies were not assessed in subsequent cycles; however, cumulative dosing continued for the remaining cycles in order to assess drug effects on body temperature and to standardize drug exposure among subjects.

Food-Maintained Responding and Urine Output

Sessions were conducted in commercially available operant conditioning chambers (31 × 24 × 21 cm; ENV-008CT; Med Associates, Inc.) enclosed in ventilated, sound-attenuating cubicles (ENV-022M; Med Associates, Inc., St Albans, VT). The side panels of the chamber were Plexiglas, and the rear and front panels were aluminum. The front panel was equipped with two response levers horizontally aligned 11.5 cm apart. Above each lever was a 2.5-cm diameter translucent disk that could be illuminated white with a 100 mA light (lever lights). A feeder dispensed 45-mg food pellets (PJAI-0045; Noyes Precision Pellets, Research Diets Inc., New Brunswick, NJ) to a 5 × 5 cm food aperture centrally located between the two levers. The rear panel was equipped with a 100 mA house light centered 2 cm from the top of the chamber. The chamber had a steel rod floor, below which a drop pan with bedding collected feces and urine. MED-PC IV software and a PC-compatible interface (Med Associates, Inc.) controlled stimulus events and recorded data.

As for antinociception studies, operant sessions consisted of 6 30-min cycles. After a 25-min timeout, when the chamber was dark and responses to either lever were recorded but had no programmed consequence, the house light and left lever light were illuminated and completion of 10 consecutive responses on the left lever (fixed-ratio 10 schedule) resulted in the

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delivery of a food pellet (signaled with a 0.1-s flash of the house light). When 10 pellets were delivered before 5 min elapsed, the house light and the lever light were extinguished for the remainder of the cycle. At the end of each session, feces were removed from the drop pan and the pan was weighed; the difference in the weight of the pan before and after the session provided a measure of urine output.

Drug tests occurred after 3 consecutive non-drug sessions in which overall response rates were within 20% of the mean. For drug tests, vehicle was injected in the first cycle and cumulative doses of drug in the remaining 5 cycles. Additionally, there were 4 sessions in which saline was administered in all cycles to control for handling and injecting; the criteria for saline tests were the same as for drug tests.

Drugs

2-(3,4-dichlorophenyl)-N-methyl-N-[(5R,7S,8S)-7-pyrrolidin-1-yl-1-oxaspiro[4.5]decan-8-yl] (spiradoline; Upjohn, Kalamazoo, MI) was dissolved in sterile 0.9% saline solution; 2-[(1R,2R,5R)-5-hydroxy-2-(3-hydroxypropyl) cyclohexyl]-5-(2-methyloctan-2-yl)phenol (CP55940; Sigma-Aldrich, St. Louis, MO) and spiradoline/CP55940 mixtures were dissolved in 1:1:18 solution of ethanol, emulphor, and saline, respectively. Injections were given intraperitoneally (i.p.) in a volume of 1 ml/kg body weight. For all procedures, cumulative doses were administered with 30-min inter-injection intervals, and all drug tests (sessions) were separated by at least one week. Spiradoline and CP55940 dose-response curves were determined in an alternating order; rats were randomly assigned to initially receive either spiradoline or CP55940. The first drug test was conducted for food-maintained responding and urine output and the second for antinociception and body temperature; this order of testing alternated until dose-response curves for spiradoline and CP55940, each administered alone, were determined twice for each rat. The mean ED_{50} values for antinociception (50°C water) and response rates for food were used to determine the doses for spiradoline/CP55940 mixtures in

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ratios of 3:1, 1:1, and 1:3 (see Table 1, also Figure 1B). Dose-response curves for drug mixtures were singly determined for antinociception and body temperature (3:1, 1:3, and 1:1 spiradoline:CP55940 [order randomly selected but consistent across rats]) and then for food-maintained responding and urine output (1:1, 1:3, 3:1 spiradoline:CP55940).

Data Analyses

Tail withdrawal latency was expressed as a percentage of the maximum possible effect according the following formula: (Test Latency – Control Latency / 20 – Control Latency) * 100, where control latency corresponds to the effect of vehicle in the first cycle. Only data from 50^{o}C water are shown because latencies were nearly exclusively 20 s for 40^{o}C water and less than 5 s for 55^{o}C water. Rectal body temperature was expressed as percent maximum effect according to the following formula: (Test Body Temperature – Control Body Temperature / 33.5 – Control Body Temperature) * 100. A maximum of 33.5^{o}C was selected because the lowest body temperature observed for any individual rat was 33.3 and 33.4^{o}C, respectively, for spiradoline and CP55940 given alone. Response rates for food were expressed as a percentage of the control response rate according to the following formula: (Test Response Rate / Control Response Rate) *100. Urine output was expressed as an absolute change (g) from control. The mean from the 3 preceding sessions served as the control for response rate as well as urine output.

For each rat, data were averaged across two determinations for each drug test, then a linear regression was fit to those dose-response curves. A linear regression was fit to dose-response curves encompassing doses that ranged from ineffective to effective, using the largest dose that produced not more than 20% effect and the smallest dose that produced at least 80% effect to define the linear portion of the curve. No more than one dose producing less than 20% effect was included and no more than one dose producing greater than 80% was included. ED_{50} values for each rat were determined and those values were averaged across the group to provide the basis for the doses used in the mixtures. Dose-response data were not obtained for

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urine output because cumulative doses were administered in operant sessions and urine output was measured only at the end of each session. The interaction between spiradoline and CP55940 for antinociception, body temperature, and food-maintained responding was examined as previously described (Tallarida 2006, 2011). Based on the ED_{50}, E_{max}, and slope of each drug (given alone), the dose of CP55940 in the mixture was converted to spiradoline equivalence for individual rats according to the following equation described by Grabovsky and Tallarida (2004): $b\mathrm{eq}(a) = ED_{50}\,A / [(E_{max}\,A / E_{max}\,B)\,(1 + ED_{50}\,B^{q} / b^{q}) - 1]^{1/p}$ where ED_{50} A and ED_{50} B are the doses of drugs A and B estimated to produce a 50% effect, E_{max} A and E_{max} B are the maximum effect levels for drugs A and B, *a* is dose of drug A, and *q* and *p* are the slopes derived from the linear regression analyses of drugs A and B, respectively. The total additive dose (CP55940 in spiradoline equivalence plus spiradoline) was calculated by adding $b\mathrm{eq}(a) + a$, and that was used to determine predicted effects (additive interaction) for individual rats using the following equation described by Grabovsky and Tallarida (2004): Predicted Effect Level = $[E_{max}\,A\,(\mathrm{eq}A^{p})] / [(E_{max}\,A\,(\mathrm{eq}A^{p})) + (ED_{50}\,A^{p})]$. Next, a linear regression was fit to all data (i.e., from all rats, not averaged) between the largest dose that produced not more than 20% and the smallest dose that produced at least 80% of the predicted effects and the observed effects (empirically determined). No more than one dose producing less than 20% effect was included and no more than one dose producing greater than 80% was included for individual rats in order to obtain the most accurate estimate of the slope of the linear portion of the dose-response curve. The slopes and y-intercepts were compared using an F test for each ratio. Urine data were analyzed with a one-way repeated measures ANOVA and Bonferroni's post hoc test. ED_{50} values were analyzed with a two-way repeated measures ANOVA (drug x assay) and Bonferroni's post hoc test for multiple comparisons. Analyses were conducted using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA).

RESULTS

There were no significant differences between the two control conditions (i.e., 1:1:18 vehicle and saline) for any of the assays. Mean (±1 SEM) control tail withdrawal latencies from 50°C water were 3.1 (0.3) s for the 1:1:18 vehicle (administered prior to CP55940) and 3.5 (0.2) s for saline (administered prior to spiradoline); mean control body temperatures were 37.4 (0.1) °C for 1:1:18 vehicle and 37.4 (0.1) °C for saline. Control response rates were 0.86 (0.05) and 0.82 (0.05) responses per second for 1:1:18 vehicle and saline, respectively. Urine output was 1.39 (0.08) g for 1:1:18 vehicle and 1.42 (0.07) g for saline. Rates of responding were stable across 6 saline cycles as follows: 0.87 (0.04), 0.87 (0.05), 0.94 (0.04), 0.94 (0.05), 0.89 (0.04), and 0.85 (0.06).

Cumulative doses of CP55940 alone and spiradoline alone increased tail withdrawal latencies from 50°C water to greater than 80% of the maximum possible effect, decreased responding for food to less than 20% of control response rates, and decreased body temperature to at least 80% of the maximum observed effect (Figure 1A). Individual ED_{50} values for the two drugs are shown in Figure 1B. The rank order potency of CP55940 across the three measures was response rate > antinociception > body temperature, and the rank order potency of spiradoline was response rate > body temperature > antinociception. Potency differences across assays were statistically significant for spiradoline ($t=8.36$, $p<0.0001$ for response rate versus antinociception; $t=3.34$, $p<0.05$ for body temperature versus response rate; $t=5.01$, $p<0.001$ for body temperature versus antinociception) but not for CP55940 ($t=1.07$, $p>0.05$ for response rate versus. antinociception; $t=0.25$, $p>0.05$ for body temperature versus response rate; and $t=0.82$, $p>0.05$ for body temperature versus antinociception). At doses that markedly affected both responding for food (decreasing rates to less than 20% of control) and body temperature (decreasing temperature to at least 50% of the maximum observed effect), spiradoline (3.2 mg/kg), but not CP55940 (0.32 mg/kg), significantly increased urine output ($t=4.54$, $p<0.0001$; Table 2).

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In mixtures, CP55940 enhanced the potency of spiradoline, and spiradoline enhanced the potency of CP55940. As the ratio of spiradoline:CP55940 in the mixture decreased, the dose-effect functions shifted to the left (see Supplemental Figure 1). The nature of this interaction was determined using the quantitative methods of dose-equivalence and dose-additivity and is presented in Figure 2. Mixtures of spiradoline and CP55940 increased tail withdrawal latency from 50^{o}C water to at least 80% of the maximum possible effect (filled squares, Figure 2A), decreased body temperature to at least 80% of the maximum observed effect (filled circles, Figure 2B), and decreased responding for food to at least 20% of control rates (filled diamonds, Figure 2C) in a dose-related manner.

For antinociception, the observed effects for all three ratios were not significantly different from the predicted effects (open squares, Figure 2A; additive interaction, see Table 3 for statistical analyses of slopes and y-intercepts). For body temperature, the observed effects for all three ratios were significantly different from the predicted effects (open circles, Figure 2B). For the 3:1 spiradoline/CP55940 mixture (Figure 2B, left panel), the observed effects were greater than the predicted effects. The y-intercepts (Table 3; [$F(1,67)=23.70$, $p<0.001$]), but not the slopes (Table 3; [$F(1,66)=3.91$, $p=0.052$]) were significantly different, indicating a greater than additive interaction for the 3:1 mixture. For the 1:1 and 1:3 spiradoline/CP55940 mixtures (Figure 2B, center and right panels), the slopes were significantly different for the observed effects (Table 3; [$F(1,62)=20.14$, $p=<0.001$ for 1:1 and $F(1,63)=10.5$, $p=0.0019$ for 1:3]). Although the y-intercepts could not be compared statistically, the observed effects for the 1:3 mixture were shifted to the left (approximately 1/2 log unit smaller doses) of the predicted effects (Figure 2B, right panel), and the y-intercept was 2-fold greater than the predicted effects (Table 3). Thus, the interaction for the 1:3 mixture appeared to be greater than additive.

For response rate, the observed effects for the 3:1 and 1:1 spiradoline/CP55940 mixtures (Figure 2C, left and center panels) were not significantly different from the predicted effects (open diamonds, Figure 2C; see Table 3 for statistical analyses of slopes and y-

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intercepts). For the 1:3 spiradoline/CP55940 mixture (Figure 2C, right panel), the observed effects were shifted to the left of the predicted effects. The y-intercept (Table 3; [$F(1,41)=6.02$, $p=0.018$]), but not the slope (Table 3; [$F(1,40)=2.88$, $p=0.98$]), was significantly greater for the observed effects compared with the predicted effects, indicating a greater than additive interaction for the 1:3 mixture.

The effects of 3:1, 1:1, and 1:3 spiradoline/CP55940 mixtures on urine output are shown in Table 2. For all mixtures, urine output was increased significantly ($t=5.63$, $p<0.0001$ for 3:1; $t=3.29$, $p<0.05$ for 1:1; $t=5.03$, $p<0.001$) compared with saline although these increases were not significantly different from the effect of spiradoline alone.

DISCUSSION

Pain remains a significant clinical problem (Elman et al., 2013; Gaskin and Richard, 2012) and *mu* opioid receptor agonists remain the drugs of choice for treating moderate to severe pain, despite the high abuse liability of these drugs and a current epidemic of fatal drug overdoses (Volkow et al., 2014; Wightman et al., 2012). Consequently, there is a pressing need to identify alternative pharmacotherapies with reduced adverse effects that currently limit the clinical use of *mu* opioid receptor agonists. Previous work showed that mixtures comprising small doses of the *kappa* opioid receptor agonist spiradoline and the cannabinoid receptor agonist CP55940 had antinociceptive effects that were equivalent to the effects of larger doses of either drug given alone (Maguire and France, 2016). In that study, dose-additivity analyses revealed an additive interaction for antinociception, suggesting that spiradoline/CP55940 mixtures might be useful for treating pain, but only if adverse effects of mixtures were less than additive. The purpose of the present experiment was to examine the therapeutic potential of spiradoline/CP55940 mixtures by characterizing effects on antinociception, body temperature, urine output, and food-maintained responding. The hypothesis was that smaller doses in mixtures have antinociceptive effects in the absence of adverse effects.

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CP55940 alone and spiradoline alone dose-dependently increased tail withdrawal latency (antinociception), decreased body temperature (hypothermia), and decreased responding for food, and these results were consistent with previous studies (Brandt and France, 1996; Craft et al., 2012; Deng et al., 2015; De Vry and Jentzsch, 2004; Leander, 1983; Mello and Negus, 1998; Smith et al., 2003; Terner et al., 2003). Hypothermic effects have been reported for the *kappa* opioid receptor agonists U50488, U69593, and salvinorin A (Baker and Meert, 2002; Cavicchini et al., 1989; Nemmani et al., 2001; Srinivas et al., 2002) and there is a report of spiradoline producing hypothermia after intracerebroventricular administration (Adler and Geller, 1993). The present study generated full dose-response functions for the hypothermic effects of spiradoline after i.p. administration. Cannabinoid receptor agonists have well-documented diuretic effects (Paronis et al., 2013); up to doses affecting food-maintained responding, spiradoline alone but not CP55940 alone significantly increased urine output. Despite the lack of statistical significance, CP55940 (0.32 mg/kg) modestly increased urine output (1.2 g above control levels); however, there was considerable variability among subjects with marked diuretic effects in some but not all rats. Urine output was assessed in operant sessions when 0.32 mg/kg CP55940 was the largest cumulative dose tested; it is possible that larger doses of CP55940 significantly increase urine output in all rats.

ED_{50} values for each drug administered alone were used to determine the doses for CP55940/spiradoline mixtures in ratios of 3:1, 1:1 and 1:3. CP55940 and spiradoline have similar onsets and durations of action when administered i.p., with onset occurring within 30 min and offset occurring within 3 hr (Barret et al., 2002; Briggs et al., 1998; Chang et al., 2011; Hamamtoto et al., 2007; Tseng and Craft, 2001); this similarity in time course was the basis for using cumulative dosing` for drugs alone and in mixtures. Mixtures comprising smaller doses of CP55940 and spiradoline had effects that were equivalent to or greater than the effects observed with larger doses of either drug administered alone. For example, the ED_{50} values of each drug alone (7.74 mg/kg spiradoline and 0.25 mg/kg CP55940) were the largest cumulative

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doses administered of the 1:1 spiradoline/CP55940 mixture and produced at least 80% of the maximum possible effect for antinociception, consistent with a previous study (Maguire and France, 2016). Similarly, when 1.1 mg/kg spiradoline and 0.13 mg/kg CP55940 (ED_{50} values for decreasing food-maintained responding) were administered as the largest cumulative dose of the 1:1 mixture, responding for food was nearly eliminated.

Interactions for the mixtures across assays were examined quantitatively using dose-additivity analyses (Tallarida, 2006, 2011). The nature of the interaction between spiradoline and CP55940 was additive for antinociceptive effects with all mixtures; in contrast, interactions were greater than additive with some mixtures for hypothermia (3:1 and 1:3) and food-maintained responding (1:3). The slopes of the observed effects were steeper than the slopes of the predicted effects in two conditions (hypothermia, 1:1 and 1:3 mixtures), indicating that the nature of the interaction varied depending on dose; the interaction was additive or less than additive for small doses of mixtures and greater than additive for larger doses of mixtures. Thus, at doses of mixtures producing antinociception, the interaction for hypothermia was either additive (1:1) or greater than additive (1:3).

Dose-additivity analyses were not conducted for diuresis because total urine output was measured only at the end of the session (i.e., dose-response curves were not determined); however, it was evident that all three spiradoline/CP55940 mixtures increased urine output to levels that were comparable to 3.2 mg/kg spiradoline administered alone. There was more variability in urine output among rats with the mixtures compared with saline or either drug administered alone. Thus, at least for some rats and some mixtures, the magnitude of the diuretic effect of spiradoline was diminished by the addition of a dose of CP55940 that was without effect alone. While diuresis can be a desired effect under some conditions, frequent and high volume urination might pose a significant impediment during the treatment of pain, particularly in patients with restricted mobility or other medical complications.

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In summary, the primary findings of this study are that interactions between CP55940 and spiradoline were additive for antinociception and were additive or greater than additive (synergistic) for hypothermia and food-maintained responding. Additionally, doses that eliminated responding for food and increased urine output were without antinociceptive effects. Collectively, these findings fail to provide support for the notion that an acceptable therapeutic window (i.e., for treating pain) can be established for a *kappa* opioid receptor agonist by combining it with a cannabinoid receptor agonist because other effects of spiradoline were also enhanced by CP55940. Significant adverse effects of *kappa* opioids that preclude their use in the clinic include hallucinations and dysphoria (Pfeiffer et al., 1986) and it is unknown whether cannabinoids enhance those effects in humans. Although cannabinoids are widely abused (Rahn and Hohmann, 2009; Pertwee, 2009; Wang et al., 2008), they can also produce aversive effects (McGregor et al., 1996; Tzschentke, 2007). Δ^9-Tetrahydrocannabinol-induced conditioned place avoidance is diminished in knockout mice lacking either *kappa* opioid receptors or the endogenous *kappa* opioid peptide dynorphin (Ghozland et al., 2002; Zimmer et al., 2001), suggesting an interaction between cannabinoid receptor agonists and *kappa* opioid receptor agonists for aversive effects. In rats, the aversive effects, but not the antinociceptive effects, of *kappa* opioid receptor agonists are attenuated during a state of chronic inflammatory pain (Shippenberg et al., 1988), raising the possibility those kappa opioid/cannabinoid mixtures might interact differently under chronic pain conditions, compared with the acute pain model used in the current study. Thus, mixtures of *kappa* opioids and cannabinoids might have therapeutic potential for treating chronic pain or other types of pain (not caused by a thermal stimulus) but only if adverse effects can be avoided and an acceptable therapeutic window can be established.

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ACKNOWLEDGMENTS

The authors thank Lisa Gerak for helpful comments on the data set and previous drafts of this manuscript.

AUTHORSHIP CONTRIBUTIONS

Participated in research design: Minervini, France

Conducted experiments: Minervini, Dahal

Contributed new reagents or analytic tools: n/a

Performed data analysis: Minervini, Dahal

Wrote or contributed to the writing of the manuscript: Minervini, France

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FOOTNOTES

This work was supported by the National Institutes of Health National Institute on Drug Abuse [Grants K05DA017918, T32DA031115] and the Welch Foundation [Grant AQ-0039]. The content expressed here is solely the responsibly of the authors and does not necessarily represent the views of the NIH.

Portions of this manuscript were presented at the 2016 annual meetings Experimental Biology and American Society for Pharmacology and Experimental Therapeutics in San Diego, CA and Behavior, Biology, and Chemistry: Translational Research in Addiction in San Antonio, TX.

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FIGURE LEGENDS

Figure 1. (A) Effects of CP55940 (upper) and spiradoline (lower) alone on tail withdrawal latency from 50^{o}C water (squares), responding for food (diamonds), and body temperature (circles) in 8 rats. Abscissae: cumulative dose in mg per kg body weight. Ordinate: % maximum possible effect ± 1 SEM. **(B)** Dot plot showing individual ED_{50} values for CP55940 and spiradoline alone for the data presented in **A**, except that open symbols are used to avoid concealing the mean (horizontal lines) and error (± 1 SEM). Abscissae: assays (rate of responding for food, body temperature, and antinociception) for both drugs. Ordinate: ED_{50} values in mg per kg body weight.

Figure 2. Predicted effects for an additive interaction (open symbols, dashed lines) and observed effects (filled symbols, solid lines) of the spiradoline:CP55940 mixtures for each dose ratio (columns) across three assays (rows) in 8 rats. Abscissae: spiradoline equivalent dose (total additive dose; see “Data Analyses” for details) in the mixture (± 1 SEM). See Table 3 for statistical analyses. **(A)** Comparison of predicted and observed antinociceptive effects with 50^{o}C water (squares). Ordinate: % maximum possible effect (± 1 SEM). **(B)** Comparison of predicted and observed effects on body temperature (circles). Ordinate: % maximum effect (± 1 SEM). **(C)** Comparison of predicted and observed effects on responding for food (diamonds). Ordinate: % control response rate (± 1 SEM).

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Table 1. Doses (mg/kg) of spiradoline and CP55940 in drug mixtures.

	Spiradoline:CP55940 (mg/kg)					
	3:1		**1:1**		**1:3**	
Cycle	**Spiradoline**	**CP55940**	**Spiradoline**	**CP55940**	**Spiradoline**	**CP55940**
Antinociception and Body Temperature						
1	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle
2	1.45	0.0156	0.48	0.016	0.48	0.0469
3	2.90	0.031	0.97	0.031	0.97	0.094
4	5.81	0.063	1.94	0.063	1.94	0.188
5	11.61	0.125	3.87	0.125	3.87	0.375
6	23.22	0.250	7.74[1]	0.250[1]	7.74	0.750
Response Rate and Urine Output						
1	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle	Vehicle
2	0.21	0.008	0.07	0.008	0.07	0.024
3	0.41	0.016	0.14	0.016	0.14	0.049
4	0.83	0.033	0.28	0.033	0.28	0.098
5	1.65	0.065	0.55	0.067	0.55	0.2195
6	3.30	0.130	1.10[2]	0.130[2]	1.10	0.390

[1] ED_{50} of drugs given alone for antinociceptive effects (50°C water).

[2] ED_{50} of drugs given alone for effects on food-maintained responding.

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Table 2. Statistical analyses of the effects of saline and drug treatments on urine output, expressed as an absolute change (g) from control. The control was the mean urine output from 3 sessions preceding each treatment.

Treatment	**Dose (mg/kg)**	**Urine (Δ Control, g)**	**SEM**	**Post-hoc Test (Drug versus Saline)**
Saline	-	-0.251	0.115	-
CP55940	0.32	1.185	0.348	t=1.02, n.s.
Spiradoline	3.2	6.127	0.211	t=4.54, ***P<0.0001
3:1 Spiradoline:CP55940	3.3 Spiradoline + 0.13 CP55940	7.658	1.370	t=5.63, ***P<0.0001
1:1 Spiradoline:CP55940	1.1 Spiradoline + 0.13 CP55940	4.362	1.512	t=3.29, *P<0.05
1:3 Spiradoline:CP55940	1.1 Spiradoline + 0.39 CP55940	6.813	0.994	t=5.03, ***P<0.001

n.s. = not significant

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Table 3. Statistical analyses of the predicted (additive) versus observed (empirically determined) effects of spiradoline:CP55940 mixtures for antinociception, body temperature, and responding for food as shown in Figure 2. Note that y-intercepts are the values of y when log(x) = 0.

		Predicted	**Observed**	**F Test**	**P**
Antinociception					
3:1 Spiradoline:CP55940	Slope	74.76	70.22	F(1,66)=0.12	0.733
	y-Intercept	-13.31	-12.14	F(1,67)=0.32	0.574
1:1 Spiradoline:CP55940	Slope	73.18	78.44	F(1,66)=0.25	0.620
	y-Intercept	-11.04	-10.09	F(1,67)=1.60	0.210
1:3 Spiradoline:CP55940	Slope	69.59	80.72	F(1,65)=0.76	0.388
	y-Intercept	-8.75	-16.27	F(1,66)=0.16	0.695
Body Temperature					
3:1 Spiradoline:CP55940	Slope	52.98	63.80	F(1,66)=3.91	0.052
	y-Intercept	14.48	17.51	F(1,67)=23.70	***<0.001
1:1 Spiradoline:CP55940	Slope	50.14	76.63	F(1,62)=20.14	***<0.001
	y-Intercept	16.55	2.52	-	-
1:3 Spiradoline:CP55940	Slope	49.68	65.97	F(1,63)=10.50	**0.0019
	y-Intercept	16.57	32.47	-	-
Response Rate					
3:1 Spiradoline:CP55940	Slope	-77.12	-81.45	F(1,42)=0.02	0.881
	y-Intercept	43.34	43.67	F(1,43)=0.01	0.946
1:1 Spiradoline:CP55940	Slope	-93.95	-160.20	F(1,39)=3.52	0.068
	y-Intercept	40.65	43.30	F(1,40)=0.64	0.427
1:3 Spiradoline:CP55940	Slope	-103.40	-151.60	F(1,40)=2.88	0.098
	y-Intercept	44.15	17.54	F(1,41)=6.02	*0.018

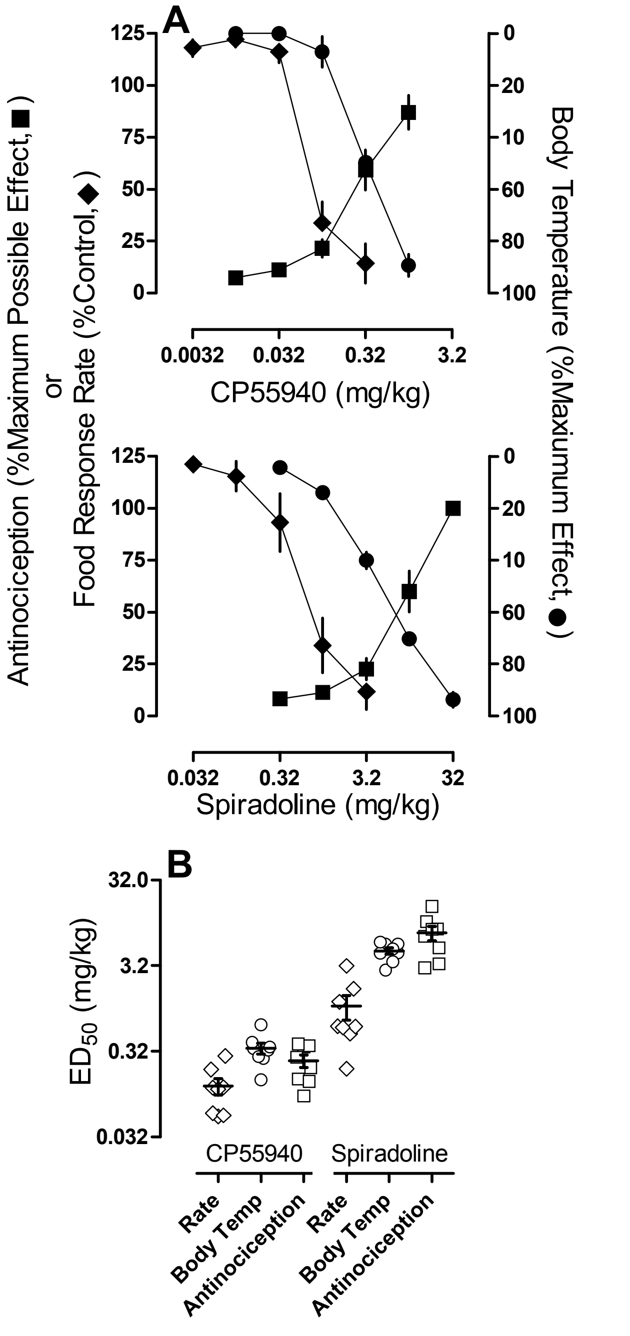
A
Antinociception (%Maximum Possible Effect, ■)
or
Food Response Rate (%Control, ◆)
Body Temperature (%Maxiumum Effect, ●)
125
100
75
50
25
0
0
20
10
60
80
100
0.0032
0.032
0.32
3.2
CP55940 (mg/kg)
0.032
0.32
3.2
32
Spiradoline (mg/kg)
B
32.0
3.2
0.32
0.032
ED_{50} (mg/kg)
CP55940
Spiradoline
Rate
Body Temp
Antinociception
Rate
Body Temp
Antinociception

Figure 1

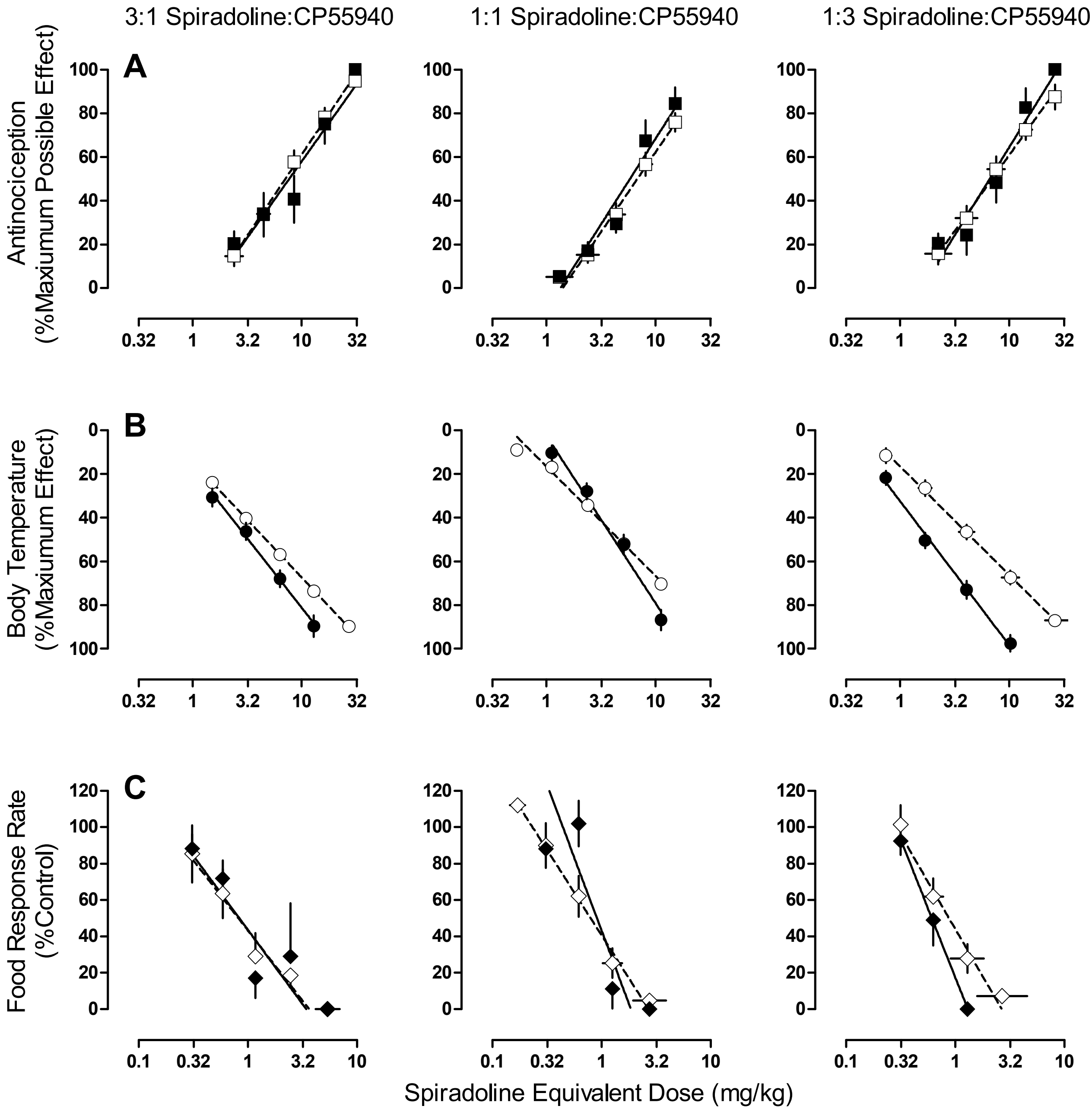
3:1 Spiradoline:CP55940
1:1 Spiradoline:CP55940
1:3 Spiradoline:CP55940
A
B
C
Antinociception
(%Maxiumum Possible Effect)
Body Temperature
(%Maxiumum Effect)
Food Response Rate
(%Control)
Spiradoline Equivalent Dose (mg/kg)

Figure 2