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# The piperazine analogue para-fluorophenylpiperazine alters timing of the physiological effects of the Synthetic Cannabinoid Receptor Agonist AMB-FUBINACA, without changing its discriminative stimulus, signalling effects, or metabolism

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## Abstract

AMB-FUBINACA is a synthetic cannabinoid receptor agonist (SCRA), which has been associated with substantial abuse and health harm since 2016 in many countries including New Zealand. A characteristic of AMB-FUBINACA use in New Zealand has included the observation that forensic samples (from autopsies) and drugs seized by police have often been found to contain *para*-fluorophenylpiperazine (pFPP), a relatively little-characterised piperazine analogue that has been suggested to act through 5HT1a serotonin receptors. In the current study, we aimed to characterise the interactions of these two agents in rat physiological endpoints using plethysmography and telemetry, and to examine whether pFPP altered the subjective effects of AMB-FUBINACA in mice trained to differentiate a cannabinoid (THC) from vehicle. Though pFPP did not alter the ability of AMB-FUBINACA to substitute for THC, it did appear to abate some of the physiological effects of AMB-FUBINACA in rats by delaying the onset of AMB-FUBINACA-mediated hypothermia and shortening duration of bradycardia. In HEK cells stably expressing the CB1 cannabinoid receptor, 5HT1a, or both CB1 and 5HT1a, cAMP signalling was recorded using a BRET biosensor (CAMYEL) to assess possible direct receptor interactions. Although low potency pFPP agonism at 5HT1a was confirmed, little evidence for signalling interactions was detected in these assays: additive or synergistic effects on potency or efficacy were not detected between pFPP and AMB-FUBINACA-mediated cAMP inhibition. Experiments utilising higher potency, classical 5HT1a ligands (agonist 8OH-DPAT and antagonist WAY100635) also failed to reveal evidence for mutual CB1/5HT1a interactions or cross-antagonism. Finally, the ability of pFPP to alter the metabolism of AMB-FUBINACA in rat and human liver microsomes into its primary carboxylic acid metabolite via carboxylesterase-1 was assessed by HPLC; no inhibition was detected. Overall, the effects we have observed do not suggest that increased harm/toxicity would result from the combination of pFPP and AMB-FUBINACA.

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synthetic cannabinoid receptor agonists; party pills; polypharmacology; cardiovascular effects; plethysmography; telemetry

## 1. Introduction:

The emergence of synthetic cannabinoid receptor agonists (SCRAs) as drugs of abuse has received significant attention over the last 10 years (reviewed in Banister and Connor, 2018; Patel et al., 2021; Wouters et al., 2019), with a particular upsurge in awareness following the well-known New York City "zombie outbreak" involving the SCRA AMB-FUBINACA (Adams et al., 2017). SCRAs exert psychoactive effects through the CB1 cannabinoid receptor, but with substantially greater efficacy and potency than $\Delta^9$-tetrahydrocannabinol (THC), the primary psychoactive constituent of plant cannabis. The route of SCRA administration is typically smoking or vaping – a fact which has substantial implications on the pharmacokinetics of these compounds (Wiley et al., 2019).

New Zealand appears to have suffered a particularly heavy toll from SCRA-related toxicities, including at least 64 deaths in Auckland alone between May 2017 and February 2019 (Morrow et al., 2020) as well as nearly 1200 ambulance callouts being attributed to SCRA incidents between January and October 2018, mainly in the Auckland region (Bennett, n.d.). AMB-FUBINACA dominated this outbreak. In drug samples seized by police in 2017, AMB-FUBINACA concentrations ranged from 5-417 grams of AMB-FUBINACA per kilogram of plant material (with a mean of 81 g/kg) (Somerville et al., 2019) – greatly higher than the New York City "zombie outbreak" drug samples, which averaged 16 g/kg (Adams et al., 2017). It is likely that dose was a major factor in the level of harm associated with SCRAs during this outbreak. However, despite increasing research attention, the mechanisms of SCRA toxicity remain an open question – it is unclear as to whether on-target (CB1 and/or CB2 cannabinoid receptor-mediated) effects are sufficient to account for the adverse clinical indications observed, particularly because many have not been classically cannabimimetic (Robson et al., 2020).

We have performed comparative molecular pharmacology characterisations of AMB-FUBINACA (Finlay et al., 2019), including an agonist bias study of a panel of SCRAs at CB1 (Patel et al., 2020). To date, these data have shown that AMB-FUBINACA has similar effects to other SCRAs and displays characteristically high efficacy and potency in most CB1-mediated signalling and regulatory endpoints (Finlay et al., 2019; Patel et al., 2020, and reviewed in Patel et al., 2021). These observations are consistent with other *in vitro* and *in vivo* (behavioural) studies (e.g., Banister et al., 2016; Gamage et al., 2018). We have also recently shown that AMB-FUBINACA undergoes metabolic transformation via carboxylesterase 1 (CES1) into its dominant carboxylic acid metabolite, which is significantly less active at CB1 (Webb et al., 2023).

A characteristic of illicit AMB-FUBINACA use in the 2017-2019 Auckland outbreak was that the piperazine analogue *para*-fluorophenylpiperazine (pFPP) was often found added to drug preparations. For example, 35% of AMB-FUBINACA-containing samples analysed in

one study also contained pFPP (Johnson et al., 2020), while in another study, pFPP was detected in up to 43% of AMB-FUBINACA fatalities (Morrow et al., 2020). Piperazines, particularly benzylpiperazine (BZP), have a complex history in the recreational drug market in New Zealand, where they were legally marketed as “Party Pills” between 1999 and 2008. They were then scheduled under the Misuse of Drugs Act as Class C Controlled Drugs (Johnson et al., 2020; Kerr and Davis, 2011; Russell and Bogun, 2011) and are no longer legally available. These compounds produce amphetamine-like effects in humans by a complex mechanism involving changes to neurotransmitter (noradrenaline/dopamine/serotonin) transport and processing, in addition to some direct receptor effects (Baumann et al., 2004; Kerr and Davis, 2011; Sheridan et al., 2007). The first reports of pFPP described it as a metabolite of the sedative hypnotic drug niaprazine and it was shown to significantly reduce levels of the serotonin metabolite 5-hydroxyindoleacetic acid (5HIAA) in rat brain (Keane et al., 1982; Sanjuan et al., 1983). In a subsequent study, pFPP was shown to bind to 5HT1-class receptors and was hypothesised to act as a 5HT1a receptor agonist (though this was not directly investigated), on the basis that a downstream consequence of 5HT1a activity is a decrease in 5HIAA levels (Scherman et al., 1988).

The possible contribution of pFPP to SCRA toxicity and negative health outcomes has not previously been investigated and was the basis for the current study. One complexity in considering this question is that xenobiotics may interact at several possible levels to give rise to biological changes. Three such contexts were considered in this study: system/physiological interactions, signalling interactions, and metabolic interactions. We have therefore investigated the effects of AMB-FUBINACA and pFPP (alone and in combination) in rat telemetry and plethysmography, mouse drug discrimination, CB1/5HT1a receptor signalling interactions (using cAMP inhibition in HEK cells as a readout), and rat and human liver microsome metabolism of AMB-FUBINACA into its dominant, largely inactive acid metabolite.

## 2. Methods and Materials:

### 2.1 Part One – Animal studies

**2.1.1 Subjects—**For the plethysmography and telemetry experiment, adult male and female Long Evans rats (420-430 g and 250-320 g for males and females, respectively, at the beginning of the experiment; Envigo, Indianapolis, IN) were surgically implanted with HD-S1-F0 telemetry probes at DSI (Data Sciences International, Inc., St. Paul, MN) and allowed to recover before shipment to RTI. For the drug discrimination experiment, adult male and female C57Bl6J mice (23-26 g and 15-21 g for males and females, respectively, at the beginning of the experiment; Envigo, Indianapolis, IN) were used. Upon arrival, all rodents were individually housed in polycarbonate cages with hardwood bedding in a temperature-controlled (20-22°C) environment with a 12 h light-dark cycle (lights on at 7am). After at least 7 days of acclimation with unrestricted access to rodent chow (Purina® Certified 5002 Rodent Chow, Barnes Supply, Durham, NC, USA), mice were maintained at ~90% of free-feeding body weight for the duration of the study. Food was available freely in the home cages of the rats, and water was available *ad libitum* in the home cages of all rodents. All studies were carried out in accordance with guidelines published in the Guide

for the Care and Use of Laboratory Animals (National Research Council, 2011) and were approved by our Institutional Animal Care and Use Committee. All efforts were made to minimize animal suffering, to reduce the number of animals used, and to utilize alternatives to *in vivo* techniques, if available.

**2.1.2 Apparatus—**Rat respiration was assessed in a Buxco FinePointe Whole Body Plethysmography System (DSI). During measurement, rats were confined to cylindrical Plexiglas chambers (17.78 cm diameter X 9.65 cm height) located in a laboratory with 12-h light-dark cycle (lights on at 7 am). Each plethysmography chamber rested on a RPC-1 Physiotel telemetry receiver platform (DSI), with associated Matrix 2.0 transducer (DSI) interface with the data acquisition computer, to allow simultaneous measurement of respiratory and cardiac parameters. Measurement of cardiac functioning was accomplished by means of surgically implanted HD-S1-F0 rat telemetry probes. Respiratory and cardiac data collection was controlled by FinePointe (version 2.7.0.11788; DSI) and Ponemah (version 6.50; DSI) software, respectively.

Mice in the drug discrimination procedure were trained and tested in mouse operant chambers (Coulbourn Instruments, Whitehall, PA) housed within light- and sound-attenuating cubicles. Each chamber was outfitted with a house light, white noise generator, and two nose-poke apertures with stimulus lights over each aperture. A pellet feeder delivered 20 mg food pellets (Bioserv Inc., Frenchtown, NJ) into an illuminated pellet trough centred between the two apertures. Chamber operations (i.e., illumination of lights, generation of white noise, delivery of food pellets, and recording of responses) were controlled by a computer system (Graphic State Software, v3.03; Coulbourn Instruments).

**2.1.3 Drugs and reagents—**AMB-FUBINACA (National Institute on Drug Abuse, NIDA, Rockville, MD) and $\Delta^9$-tetrahydrocannabinol (THC; NIDA) were suspended in a 7.8% polysorbate 80 (Fisher Scientific, Pittsburgh, PA) and 92.2% saline (Patterson Vet Supply, Blythewood, SC) mixture for systemic administration. The vehicle for *para*-fluorophenylpiperazine (pFPP; Cayman Chemical, Ann Arbor, MI) was saline (Patterson Vet Supply). All compounds were administered via intraperitoneal (*i.p.*) injection at a volume of 1 ml/kg for rats and 10 ml/kg for mice.

**2.1.4 Plethysmography and telemetry in rats—**The overall study design involved repeated sessions of simultaneous and automated measurement of respiratory and cardiac parameters in unrestrained male and female rats (n=3 and 2, respectively) after administration of vehicle or AMB-FUBINACA, alone and in combination with 10 mg/kg pFPP, with a minimum of two days between each dose or dose combination. For a separate study completed at least one month prior to the present experiment, THC dose-effect curves had been determined in all rats via four routes of administration: *i.p.*, oral, *s.c.*, and aerosol exposure. Procedural details for plethysmography and telemetry measurement varied from those used in the present study, complicating inclusion of the data from the prior study herein. During the current study, rats were weighed before each session and placed into the plethysmography chambers for 60 min and their temperature and cardiac and pulmonary functioning was monitored. After this 60-min adaptation period, each rat was removed from the chamber and injected *i.p.* twice: the first time with a dose of AMB-FUBINACA or

vehicle followed by a dose of 10 mg/kg pFPP or vehicle. Immediately after the second injection, the rat was placed back into the plethysmography chamber. After 30 min in the chamber, measurement of temperature, cardiac and pulmonary functioning occurred for an additional 240 min. Upon removal from the plethysmography chambers, rats were returned to their home cages. During each session, temperature and measures of respiratory and cardiac function were recorded in 1-min bins.

**2.1.5 Drug discrimination procedure in mice—**As described previously (Gamage et al., 2018; Vann et al., 2009), male and female C57BL/6J mice (n=10 and 6, respectively) were trained to respond on one of the two nose-poke apertures in operant chambers following *i.p.* administration of the THC training dose (5.6 mg/kg) and to respond on the other aperture following *i.p.* vehicle administration according to a fixed ratio 10 (FR10) schedule of food reinforcement, under which 10 consecutive responses on the correct (injection-appropriate) aperture resulted in delivery of a food pellet. Responses on the incorrect aperture reset the ratio requirement on the correct aperture. Prior to each daily training session, mice received a single injection of 5.6 mg/kg THC or vehicle in a double alternation schedule (e.g., two sessions with THC pre-injection followed by two sessions with vehicle pre-injection). These single daily 15 min training sessions were held on weekdays until the mice consistently met three criteria: (1) the first completed FR10 was on the correct aperture, (2) ≥ 80% of the total responding occurred on the correct aperture, and (3) response rate was ≥ 0.1 responses/s. When these criteria had been met for the most recent THC training dose and vehicle sessions and 8 of the 10 most recent sessions, reliable discrimination had been established and stimulus substitution testing began.

During 15 min stimulus substitution tests, 10 consecutive responses on either aperture delivered reinforcement. If a mouse responded on the other aperture prior to completing 10 responses on an aperture, the ratio requirement on the original aperture was reset. To be eligible for a stimulus substitution test, a mouse needed to have completed a training session the previous day in which the criteria for reliable discrimination (i.e., ≥ 0.1 responses/s with the first completed FR10 registered on the injection-appropriate aperture and ≥ 80% of responses on this aperture) had been met. In addition, the mouse must have met these same criteria during the most recent training session with the alternate training compound (i.e., THC training dose or vehicle). Baseline discrimination training sessions continued other non-test weekdays. After passing stimulus substitution tests for the training drug and vehicle (i.e., responses consistent with reliable discrimination criteria), an initial substitution dose-response curve was determined for THC in each sex.

After acquisition of the discrimination and determination of the initial THC dose-response curve, all mice were tested with other synthetic cannabinoids for another project (Marusich et al., 2022). At the end of these studies, the current study was started. For this study, a dose-effect curve for THC was re-determined. Subsequently, a dose-effect curve for AMB-FUBINACA, in combination with vehicle or 3 mg/kg pFPP, was evaluated followed by a dose-effect curve with THC in combination with 3 mg/kg pFPP. This pFPP dose was chosen after probe tests with 10 mg/kg pFPP produced substantial decreases in response rates when administered alone (data not shown). [The 10 mg/kg pFPP dose was retained for the physiological experiment where the purpose was to screen for potential adverse effects

of the AMB-FUBINACA/pFPP combinations.] All compounds were administered *i.p.*, with pFPP or vehicle administered immediately after the cannabinoid injection at 30 min prior to the start of the session.

**2.1.6 Data analysis—**In rats (see section 2.1.4 above), minute volume (ml/min) and heart rate (beats/min) were chosen as the respiratory and cardiac variables of interest. Minute volume, the amount of air intake per min, is a function of tidal volume (amount of air intake per breath) and frequency (breaths/min) and represents a measure of overall respiratory functioning. Temperature (°C) was also measured via telemetry probes. For the purposes of analysis, all physiological data were collated into 15-min averages. The first 60 min consisted of a pre-injection acclimation period, which was followed by removal from the plethysmography chamber, injection, and placement back into the chamber. Data measurement was re-started 30 min after the injection and continued for 240 min. Post-injection data for each measure were analyzed using three separate two-factor (dose X time) repeated analyses of variance (ANOVA) for both sexes combined. The first ANOVA for each measure compared the saline and pFPP 10 mg/kg conditions over time. The second ANOVA compared saline and all doses of AMB-FUBINACA alone, while the third ANOVA compared 10 mg/kg pFPP and all doses of AMB-FUBINACA in combination with this pFPP dose. When significant main effects or interactions were revealed by ANOVA, Tukey post hoc tests ($\alpha = 0.05$) were used to determine differences between means and baseline (i.e., value of the measure at the final pre-drug timepoint). NCSS 11 Statistical Software (2016; NCSS, LLC. Kaysville, Utah, USA, ncss.com/software/ncss) was used for all analyses.

For each drug discrimination session in mice (see section 2.1.5 above), responses on the drug aperture as a percentage of all responses during the session and response rate (all responses/s) were calculated. As appropriate, $ED_{50}$ values were calculated using linear least squares regression analysis. Data from sessions in which a mouse did not earn food reinforcement (i.e., fewer than 10 consecutive responses were registered on either aperture) were excluded from analysis of percentage of drug response selection, but response rate data were included. Response rate data were analysed with separate repeated measures (cannabinoid dose X pFPP dose) ANOVAs for each sex, followed by Tukey post hoc tests ($\alpha=0.05$) to determine differences between means.

## 2.2 Part Two – *In vitro* studies

**2.2.1 Molecular Biology and Cell culture—**All cell culture-based experiments were performed using HEK293 cell lines stably expressing receptors of interest. Cells were grown in vented polystyrene cell culture flasks in high glucose DMEM (ThermoFisher Scientific) supplemented with 10% NZ-origin foetal bovine serum (FBS), at 37°C in a humidified incubator in appropriate selection antibiotics (250 μg/ml Zeocin for cell lines with transfected with pEF4 vectors, and 400 μg/ml G418 for pcDNA3.1+ vectors). The cell line stably expressing N-terminally 3x HA tagged human (h-)CB1 has been previously described (Cawston et al., 2013).

A pcDNA3.1+ construct containing the human (h-)5HT1a receptor was purchased from the cDNA Resource Center (cat# HTR01A0000, Bloomsberg, PA, USA) and was cloned into pcDNA3.1(+) and pEF4/V5-HisA vectors with the addition of a myc-tag at the 5' end of the open reading frame (receptor N-terminus). Primers encoding the myc-tag were used to generate a myc-tagged 5HT1a wild type clone by PCR (KAPA HiFI, KAPA Biosystems). The PCR product was ligated into pGEM-T Easy Vector (Promega), according to the manufacturer's instructions, then subcloned into both expression vectors with EcoRI-HF (NEB). The resulting constructs were transfected both into low passage wildtype HEK293 cells (pEF4 construct; Zeocin-resistant), and the previously described 3HA-hCB1 HEK cell line (pcDNA3.1+ G418-resistant, to create a dual-expressing 3HA-hCB1/myc-h5HT1a HEK line) with Lipofectamine 2000 (ThermoFisher Scientific) according to the manufacturer's instructions. New cell lines were then clonally isolated prior to use in assays.

**2.2.2 Drugs—**AMB-FUBINACA was a generous gift from Dr Samuel Banister (University of Sydney, Australia), and *para*-fluorophenylpiperazine (4-fluorophenylpiperazine, CAS 2252-63-3) was purchased from Indofine Chemical Company (Hillsborough, NJ). Both were prepared in dimethyl sulfoxide (DMSO anhydrous, Sigma Aldrich, St Louis, MO) at 10 mM and 31.6 mM, respectively, and stored in single-use aliquots at −80 °C. Forskolin (Cayman Chemical Company, Ann Arbor, MI) was prepared at 31.6 mM in DMSO and stored in large multi-use aliquots. 8-hydroxy DPAT (8OH-DPAT) and WAY100635 were also purchased from Cayman Chemical Company, were prepared at 10 mM or 31.6 mM in DMSO, and stored as single use aliquots at −80°C.

**2.2.3 Cyclic-AMP signalling assays—**A real-time bioluminescence resonance energy transfer (BRET) assay, CAMYEL (Jiang et al., 2007), was used to monitor intracellular cyclic-AMP (cAMP) signalling, as previously described (Cawston et al., 2013; Finlay et al., 2017; Hunter et al., 2017). In brief, stable cell lines were seeded at either 6 or 3 million cells per 10 cm polystyrene cell culture dish, in regular culture medium (high glucose DMEM supplemented with 10% FBS), and grown for 24 h or 48 h, respectively (wildtype HEK cells were seeded at 3 or 2 million per dish and grown for the same period). Cells were then approximately 50% confluent for transfection. A transfection mixture was prepared, comprising 5 μg DNA and 30 μg of high molecular weight, linear polyethyleneimine (PEI, Polysciences) in 500 μl of sterile isotonic saline (150 mM NaCl). After a 10 minute incubation, culture medium was replaced, transfection mix was dispensed dropwise, and dishes were returned to the incubator for a further 24 h. Cells were then lifted with 0.05% trypsin/EDTA, counted, and seeded in normal growth medium at a density of 40-60,000 cells per well in white 96-well CulturPlates (PerkinElmer), pre-coated with 0.05 mg/ml high molecular weight poly-D-lysine (PDL, Sigma). Plates were then incubator overnight prior to assay. On assay day, growth medium was aspirated, wells were washed with PBS to remove phenol red, and assay medium (phenol-free high glucose medium, 25 mM HEPES, supplemented with 1 mg/ml low fatty acid bovine serum albumin, BSA, MPBiomedicals, Auckland, NZ) was dispensed per well in an appropriate volume to result in a final stimulation volume of 100 μl after additional of coelenterazine-H and drugs. Prior to each assay run, cells were equilibrated for 30 minutes, and then coelenterazine-H (5 mM in absolute ethanol, Nanolight Technologies/Prolume, Pinetop, AZ, USA) was

prepared in assay medium and dispensed to each well (10 µl at 10x concentration, for a final concentration of 5 µM in 100 µl) and pre-incubated for 5 minutes in a 37°C plate reader. Forskolin or vehicle, and other drugs (or their vehicles) were prepared at 10x concentration in assay medium (vehicle-controlled in all wells) and dispensed/mixed in polypropylene, low-binding V-well dispensing plate (Hangzhou Gene Era Biotech Co Ltd) in equal volumes. At the conclusion of the coelenterazine-H preincubation, forskolin/vehicle and drugs/vehicles were then dispensed into the assay plate with a multichannel pipette (10 µl per drug component) and the plate reader (LUMIStar Omega, BMG Labtech, Ortenberg, Germany) was immediately started. BRET signals were detected simultaneously with BRET1 filters (475-30 and 535-30 nm), continuously for 20-25 minutes with an interval time of 0.5 s/well.

**2.2.4 Rat and human liver microsome metabolism and HPLC assays—**HPLC-grade methanol and acetonitrile were purchased from VWR (Radnor, PA, USA). Human liver microsomes (pooled from 50 donors) were purchased from ThermoFisher Scientific (Waltham, MA, USA); male Wistar rat (150-200 g) liver microsomes that had been previously prepared from untreated rats according to standard protocols (Guengerich, 1989) and approved by the University of Otago animal ethics committee (AEC# 20/132) were gifted.

The metabolism protocol used was derived from Apirakkan et al. (2020) and Menzies et al. (2014) and optimised as previously described (Webb et al., 2023). The experiments combined AMB-FUBINACA and pFPP; concentrations of the combination treatment were derived from Johnson et al. (2020) where the mean mass ratio of AMB-FUBINACA:pFPP within seized plant material in NZ was 80:41 (molar ratio = 1:1.09; n = 55). The final reaction concentration of AMB-FUBINACA remained constant at 1.15 µg/ml (3 µM). The combination treatment included AMB-FUBINACA in combination with three final concentrations of pFPP: low, 0.589 µg/ml (3.27 µM); medium, 1.179 µg/ml (6.54 µM); and high, 2.358 µg/ml (13.38 µM). Medium and high concentrations of pFPP represent a 2- and 4- fold increase in the mass ratio of pFPP when compared to AMB-FUBINACA (1.15 µg/ml), respectively. AMB-FUBINACA and pFPP were chosen to be introduced simultaneously into the incubation as this would best mimic acute administration of the dried plant material to a subject containing both compounds, as described in Johnson et al. (2020). All chromatographic runs were performed on a Prominence high performance liquid chromatography (HPLC) system (Shimadzu Corporation, Kyoto, Japan), comprising a CBM-20A HPLC modular system controller, a DGU-20A3R degassing unit, dual LC-20AR solvent delivery modules, a SIL-20ACHT autosampler and a CTO-20AC column oven. An SPD-M20A UV/vis photodiode array detector (DAD) was used for detection, and full spectra were recorded in the range of 190-800 nm. Chromatographic separations were achieved using a Phenomenex® Gemini® (5 µm, C18, 110 Å, 4.6 x 150 mm) 714145-14 column. LabSolutions software was used for equipment control and data acquisition. All run conditions were used as previously described (Webb et al., 2023).

**2.2.5 Data analysis—**BRET data were exported from the plate reader into Excel 2019, and inverse BRET ratios (460/535 nm) were plotted in GraphPad Prism v8 or v9

(GraphPad, La Jolla, CA). Area-under-the-curve analysis and subsequent normalizations were performed in GraphPad Prism, to create concentration–response plots. Statistical testing was performed in SigmaPlot (Systat Software, v14.5) and entailed normality (Shapiro-Wilk) and equal variance (Brown-Forsythe) tests for parametric test assumptions, followed by ANOVA as described in-text. Where a significant ANOVA result was determined ($p<0.05$, see metabolism assays only), a Holm-Šídák post-test was used to identify which groups differed from a control (identified in-text, indicated by *).

## 3. Results:

### 3.1 Telemetry and plethysmography

As shown in the top panels of Figure 1, temperature (Fig. 1A), heart rate (Fig. 1D), and minute volume (Fig. 1G) remained constant in vehicle-treated rats over the entire post-injection measurement period, as compared to the 60-min time point ($p>0.05$), with the exception that temperature and minute volume exhibited slight (but statistically significant) decreases at later time points post-injection: 225-240 min (temperature) and 240 min (heart rate) [$F(15,60)=2.65$, $p=0.004$ and $F(15,60)=4.13$, $p<0.0001$, respectively, for the drug X time interactions; Fig. 1A and D, respectively]. Similarly, all three measures remained constant over time after injection with 10 mg/kg pFPP alone. This pFPP dose produced only early and transient decreases in temperature or heart rate, in comparison to vehicle treatment (Fig. 1A and D, respectively), but produced a significant decrease in respiratory minute volume across the post-injection session [main effect of drug: $F(1,4)=9.35$, $p=0.04$; Fig. 1G].

The middle panels of Figure 1 illustrate the effects of AMB-FUBINACA alone across time for each of the three measures whereas the bottom panels show the effects of AMB-FUBINACA and pFPP combination across time. In addition, to facilitate visual comparisons of the effects of pFPP vs. vehicle pre-treatment across AMB-FUBINACA doses, side-by-side dose-effect curves for AMB-FUBINACA with and without 10 mg/kg pFPP at selected time points are provided in Supplemental Figure 1.

Compared to the saline/vehicle condition, AMB-FUBINACA dose-dependently decreased temperature, regardless of whether it was administered alone (Fig. 1B) or in combination with 10 mg/kg pFPP (Fig. 1C). Although the 0.03 and 0.1 mg/kg doses of AMB-FUBINACA had no significant effect on temperature, the 0.3 and 1 mg/kg doses produced hypothermia at one or more post-injection time points as compared to the vehicle/saline condition [$F(60,240)=3.69$, $p<0.0001$; Fig. 1B]. For 1 mg/kg AMB-FUBINACA alone, this hypothermic effect endured for the entire 240-min post-injection measurement period. Although the magnitude of the maximal decrease was similar for the 0.3 mg/kg dose, the effect dissipated by 135 min after injection. When combined with 10 mg/kg pFPP, AMB-FUBINACA (0.3 and 1 mg/kg) again decreased temperature as compared to 10 mg/kg pFPP alone [$F(60,240)=6.45$, $p<0.0001$; Fig. 1C]; however, onset of a significant hypothermic effect did not occur until 75 min post-injection for either AMB-FUBINACA dose. Significant hypothermia continued throughout the session for the 1 mg/kg dose and until 165 min for the 0.3 mg/kg dose.

Concomitant with its hypothermic effect, AMB-FUBINACA decreased heart rate at the 0.3 and 1 mg/kg doses when it was administered alone [dose X time interaction: $F(60,240)=4.02$, $p<0.0001$; Fig. 1E] or in combination with 10 mg/kg pFPP [dose X time interaction: $F(60,240)=6.17$, $p<0.0001$; Fig. 1F]. Significant decreases in heart rate were observed during most of the post-injection session when 1 mg/kg AMB-FUBINACA was administered alone, whereas decreases produced by 0.3 mg/kg AMB-FUBINACA lasted approximately half of the post-injection session (Fig. 1E). Co-administration of 10 mg/kg pFPP shortened the duration over which these decreases were observed for both AMB-FUBINACA doses (Fig. 1F).

Figure 1 also shows the effects of AMB-FUBINACA alone (panel H) and in combination with 10 mg/kg pFPP (panel I) on respiratory minute volume. As for the other two measures, 1 mg/kg AMB-FUBINACA alone and with pFPP significantly decreased minute volume for part or all of the post-injection session [dose X time interaction: $F(60,240)=2.71$, $p<0.0001$ and main effect of dose: $F(4,16)=18.38$, $p<0.0001$, respectively; Fig. 1H and I, respectively]. In addition, a transient decrease in minute volume was observed early in the session following injection with 0.3 mg/kg AMB-FUBINACA alone (Fig. 1H).

### 3.2 Drug discrimination results

When administered alone, THC and AMB-FUBINACA produced dose-dependent increases in responding on the THC-associated aperture in both sexes, with the bulk of responses at higher doses of each substance occurring on this aperture (Fig. 2, left and right top panels for males and females, respectively). While both compounds exhibited similar potencies across sex, AMB-FUBINACA was 49-70-fold more potent than THC (Table 1). Concomitant injection with 3 mg/kg pFPP did not meaningfully alter this overall pattern of results in either sex (Fig. 2; Table 1). Compared to vehicle, neither cannabinoid (alone or in combination with pFPP) significantly affected response rates in either sex (Fig. 2; left and right bottom panels for males and females, respectively).

### 3.3 5HT1a and CB1 receptor signalling interactions

In HEK cells expressing either myc-h5HT1a, 3HA-hCB1, or both receptors, each receptor signalled as expected in the cAMP pathway when stimulated with cognate ligands: the 5HT1a specific agonist 8OH-DPAT induced Gαi-mediated inhibition of forskolin-induced cAMP levels with similar potency in both the 5HT1a-alone and 5HT1a/CB1-expressing cell lines without showing activity in the cell line that expressed only CB1 (Fig. 3A, Table 2). Likewise, AMB-FUBINACA inhibited forskolin-induced cAMP levels in CB1- and 5HT1a/CB1-expressing cells with similar potencies, and was without effect in 5HT1a-expressing cells (Fig. 3B, Table 2). The putative agonism of pFPP at 5HT1a (Scherman et al., 1988) was confirmed, with cAMP inhibition occurring in both HEK lines that expressed 5HT1a, but with very low potency (Fig. 3C, Table 2). The lack of differences in potency or efficacy for any drug in cells expressing either receptor-alone *cf.* dual-expressing 5HT1a/CB1 cell lines establishes that responses can be meaningfully compared between the cell lines to help determine whether multi-drug stimulations result in functional changes in receptor function. In addition, these data suggest that basal functional interactions between 5HT1a and CB1 (under conditions of stimulation of only one receptor) probably do not occur.

Cells were stimulated with AMB-FUBINACA and a high but non-saturating concentration of pFPP (31.6 μM). AMB-FUBINACA potency and efficacy (Emax) did not significantly differ in the presence or absence of pFPP in either the 5HT1a/CB1 or CB1-expressing cell lines (Fig. 3D, Table 2, 1-way ANOVAs). As expected, no AMB-FUBINACA response was detected in the 5HT1a-alone cell line, and no pFPP response was observed in the CB1-alone cell line (Fig. 3B–C).

The potency of pFPP at 5HT1a was lower than expected (Table 2, pEC50 4.83 ± 0.13, $n$=3) on the basis of its previously reported affinity (pKi 6.52, Scherman et al., 1988). To probe possible pFPP interactions with 5HT1a at concentrations below those at which agonism was observed, experiments were performed to identify possible antagonist activity (in comparison to the widely-report 5HT1a antagonist, WAY100635). Alone, WAY100635 induced negligible activity through 5HT1a (Fig. 4A), though limited evidence of very low efficacy agonism was seen in the dual expressing 5HT1a/CB1 cell line. In an antagonism paradigm, however, this effect was not apparent – in the presence of an approximately EC50 concentration of 8OH-DPAT (31.6 nM), cAMP inhibition in both 5HT1a-expressing cell lines was inhibited with high potency (Fig. 4B). pFPP was not observed to have any antagonist effect on cAMP state induced by 8OH-DPAT in any cell line (Fig. 4C). Finally, a “cross-antagonism” paradigm was utilised to determine if 5HT1a blockade with WAY100635 would unmask altered CB1 function and reveal evidence for 5HT1a/CB1 receptor-level interaction (as has been previously described for GPCRs, Moreno et al., 2014, 2011, including CB1, Viñals et al., 2015). No evidence for a receptor-level functional interaction was observed, as no shift in AMB-FUBINACA potency or efficacy was detected in the presence of 1 μM WAY100635 (Fig. 4D, Table 2, 1-way ANOVAs [with pFPP, see above]).

### 3.4 Metabolic interactions

Carboxylesterase 1 (CES1)-mediated metabolism of AMB-FUBINACA occurs robustly in liver microsome preparations, in the absence of NADPH (an essential cofactor for cytochrome P450-mediated metabolic reactions) (Webb et al., 2023). To determine whether pFPP had an inhibitory effect on the conversion of AMB-FUBINACA into its primary acid metabolite, three concentrations of pFPP (0.59, 1.18, 2.36 μg/ml) were co-incubated with a fixed amount of 1.15 μg/ml AMB-FUBINACA as described above. Following a 20 second (human) or 12.5 minute (rat) incubation, significant (approximately 50%) AMB-FUBINACA metabolism occurred in both rat and human liver microsomes compared to the matched no-microsome condition (“-Control”, 1-way ANOVA, $p<0.05$), and this was not altered by any concentration of pFPP (Fig. 5) in either microsome preparation.

## 4. Discussion:

Co-use of AMB-FUBINACA and pFPP is a unique characteristic of the SCRA outbreak in New Zealand, but how these compounds interact (at the physiological level, as well as mechanisms thereof) had not been considered to date. Interactions between exogenous pharmacological agents are complex and may occur at many levels. Novel effects produced by two drugs acting independently but modulating the same physiological endpoint(s) are

common features in system physiology, though the mechanisms by which these effects occur may be highly divergent. Physical or functional interactions between the affected receptor molecules themselves (for example, heterodimerisation, functional synergism/antagonism), and metabolic interactions due to shared enzymatic processing (in the form of altered clearance time, or different metabolites) are merely two of many possible mechanisms that underpin these system physiology interactions. In the present study, we examined the effects of AMB-FUBINACA and pFPP alone and in combination on physiological and behavioural endpoints and investigated several possible mechanisms through which these two compounds may interact.

To examine polypharmacy effects on physiology (particularly endpoints relevant for SCRA adverse effects, Morrow et al., 2020), rat plethysmography and telemetry were used to monitor temperature, heart rate, and respiration under various *i.p.* drug administration conditions. AMB-FUBINACA alone caused a significant, dose-dependent reduction in temperature at doses of 0.3 and 1 mg/kg; a reduction mirrored in decreased heart rate and decreased respiratory minute volume. At the 1 mg/kg dose, hypothermia and bradycardia were sustained across the 240-min session, with decreased respiratory minute volume co-occurring during most of this time. Previous studies have demonstrated that hypothermia is a CB1-mediated effect of both phytocannabinoids and SCRAs (Tai and Fantegrossi, 2014) . Similarly, respiratory depression as a consequence of SCRA use is relatively well-documented (Alon and Saint-Fleur, 2017; Manini et al., 2022; Morrow et al., 2020), including confirmation of CB1 involvement in this effect (Wiese et al., 2021), as is cannabinoid-induced bradycardia in rodents (Lake et al., 1997; Pacher et al., 2005). This correspondence between hypothermia and decreases in heart rate and minute volume is not unexpected. Compared to normothermia, heart rate and respiratory minute volume significantly decrease as rectal temperature decreases below 35°C in rats (Torbati et al., 2000). It is therefore possible that bradycardia and respiratory depression occur following AMB-FUBINACA administration by two independent mechanisms: firstly, as a direct downstream effect of central or peripheral CB1 activation and secondly, as a physiological consequence of hypothermia in rodents.

In contrast with AMB-FUBINACA, 10 mg/kg pFPP alone produced only transient decreases in temperature and heart rate, but like AMB-FUBINACA, it induced a sustained decrease in respiratory minute volume. While not completely characterized, pFPP's pharmacological effects are presumed to be related to its effects on monoamine transport (Baumann et al., 2004; Kerr and Davis, 2011; Sheridan et al., 2007) and its direct interaction with the serotonin system (Keane et al., 1982; Sanjuan et al., 1983; Scherman et al., 1988); namely, 5HT1a agonism. 5HT1a agonism is generally thought to stimulate respiration; the 5HT1a partial agonist buspirone (10 and 20 mg/kg, *i.p.*) increases respiratory rate, tidal volume, and minute ventilation in rats (Mendelson et al., 1990), while other 5HT1a agonists attenuate opioid-induced respiratory depression of minute ventilation (Kimura et al., 2013; Ren et al., 2015; Sahibzada et al., 2000). Interestingly, however, other research suggests that activation of 5HT1a autoreceptors in serotonergic neurons may produce opposite effects to agonism of 5HT1a heteroreceptors in the respiratory network of the nervous system: in mice, the latter potentiated respiratory output, while the former depressed breathing activity (Corcoran et al., 2014). If the pFPP-mediated decrease in respiratory minute volume (Fig. 1G) was wholly

5HT1a-mediated, the data in this study suggest that the depressant effects of serotonergic neuron autoreceptors dominate.

Co-administration of AMB-FUBINACA and pFPP in rats resulted in a delay in the onset of AMB-FUBINACA-mediated hypothermia (Fig. 1C). This finding is consistent with a previous report of CB1/5HT1a co-stimulation, in which midbrain raphe nuclei stimulation of 5HT1a autoreceptors attenuated CB1/THC-mediated hypothermia (Malone and Taylor, 2001). However, this interaction is also surprising given that 5HT1a agonism is itself pro-hypothermic (Naumenko et al., 2011). This apparent incompatibility is likely explained again by the effects of distinct 5HT1a populations; those expressed on serotonergic neurons (autoreceptors, as in the raphe nuclei, Malone and Taylor, 2001) which produce hyperthermic effects (via inputs to the hypothalamus, Moore et al., 1978), and heteroreceptors expressed postsynaptically, which are responsible for the hypothermic effects of 5HT1a (Bagdy and To, 1997).

In the current study, co-administration of AMB-FUBINACA and pFPP had similar effects on heart rate to the moderating effects observed for SCRA-associated hypothermia: AMB-FUBINACA-mediated bradycardia was of shorter duration in the presence of 10 mg/kg pFPP (Fig. 1E/F). The mechanism underpinning this attenuation is not clear, particularly in the light of literature that suggests that 5HT1a agonism results in bradycardia in rodents (Dreteler et al., 1990; Youn et al., 2013).

Although the primary focus of this study is on investigation of the effects of possible interactions of pFPP and AMB-FUBINACA on physiology, it is also worth considering whether co-administration of the two substances may be related to user attempts to modulate negative psychological effects that may accompany higher doses of cannabinoids (Hunault et al., 2014), particularly since product samples seized in New Zealand had much higher AMB-FUBINACA concentrations compared to those reported in the United States of America. Drug discrimination, an animal model of the subjective effects of cannabinoids (Balster and Prescott, 1992), was used to examine this issue. Results showed that AMB-FUBINACA potently substituted for THC in mice trained to discriminate THC from vehicle (consistent with previous reports, Gamage et al., 2018). However, the presence of 3 mg/kg pFPP did not produce cannabimimetic effects by itself, nor did it alter the potency of THC-like discriminative stimulus effects for either THC or AMB-FUBINACA (Fig. 2, Table 1), suggesting that pFPP does not alter the psychoactive effects of cannabinoids. Consequently, modulation of the subjective effects of AMB-FUBINACA by co-use of pFPP in humans would not be predicted to be effective, albeit it is possible that pFPP may alter the time course of cannabinoid psychoactivity as this issue was not evaluated in this study.

While divergent receptor activities based on nervous system expression/localisation (such as those described for the AMB-FUBINACA/pFPP co-administration effects on temperature) appear to offer a reasonable mechanistic explanation for at least some of the non-additive co-administration physiological effects observed, it also raises the possibility of other types of polypharmacy effect such as functional or physical CB1/5HT1a receptor-level interactions. Previous studies have demonstrated that pFPP has affinity for the 5HT1a receptor (Scherman et al., 1988), but to the best of our knowledge, this is the first report that has characterised

its activity; cAMP signalling assays show that pFPP is a low potency 5HT1a agonist (Fig. 3C, Table 2). CB1 is well-known to be promiscuous in its G protein coupling, and is also believed to form functional heteromers with other GPCRs such that classical signalling is disrupted (e.g., Glass and Felder, 1997; Kearn et al., 2005; Navarro et al., 2008). Studies have already shown that the CB1 and 5HT1a receptors are co-expressed in the brain, including on serotonergic neurons (Lau and Schloss, 2008) which express 5HT1a autoreceptors. This mechanism may be a means by which CB1 agonism could amplify or otherwise modulate the inhibitory effects on serotonergic tone mediated by 5HT1a autoreceptors. In addition, some recent data have suggested that SCRAs or their metabolites may have “off-target” activity at 5HT1a – and that piperazines may also exhibit similar polypharmacological characteristics via other receptors (namely the mu-opioid receptor) (Åstrand et al., 2020).

In the current study, we examined CB1/5HT1a signalling interactions in response to these two compounds. No cross-receptor activity was detected for either AMB-FUBINACA or pFPP, and each agent alone acted similarly in cells expressing its cognate receptor alone or in a dual CB1/5HT1a-expressing cell line (Fig. 3, Table 2). Cross-antagonism was also not observed (Fig. 4), further suggesting a lack of direct molecular CB1/5HT1a interaction. In addition to its 5HT1a effects, pFPP has demonstrated affinity for 5HT2-class receptors with low affinity (Scherman et al., 1988). While potential pFPP interactions with the cannabinoid system through 5HT2 receptors were not considered in the current study, it is worth noting that there is increasing interest in potential bi-directional interactions between the CB1 and 5HT2a receptor systems (reviewed in Ibarra-Lecue et al., 2021), and these receptors are known to heterodimerise (Viñals et al., 2015). This study showed that stimulation of HEK293T cells expressing 5HT2a and CB1 with the 5HT2a agonist DOI switched the dominant G protein signal from Gq (the classical 5HT2a second messenger pathway) to a pertussis toxin sensitive (Gi)-linked pathway. CB1/5HT2a cross-antagonism was also observed (Viñals et al., 2015). Together, these observations would justify investigations into whether pFPP activity at 5HT2a might offer a mechanism for the AMB-FUBINACA/pFPP effects observed in the current study.

A final mechanism of xenobiotic interaction considered in the current study was that of enzymatic metabolism. The well-known example of grapefruit inhibiting CYP3A4 illustrates how inhibition of (or competition for) a metabolic process can alter compound half-life by preventing first pass metabolism (Bailey et al., 2013). We have recently demonstrated that AMB-FUBINACA is predominantly metabolised by carboxylesterase-1 in liver (Webb et al., 2023). In the current study, we used both rat and human liver microsome preparations to determine whether increasing concentrations of pFPP would alter the metabolism of pFPP into its acid metabolite over a fixed reaction time (approximately $t_{1/2}$ for each microsome preparation). No inhibition was observed at any concentration of pFPP. While this appears to rule out pFPP as a modulator of AMB-FUBINACA clearance time, pFPP has previously been implicated as an inhibitor of several CYP450 isoforms (particularly CYP1A2, 3A4, and 2C19, Antia et al., 2009), and any future studies that consider pFPP in polypharmacy contexts must therefore considers its effects on the activity of the vast array of xenobiotics that are processed by these CYP isoforms. Other potential metabolic factors that might be considered in future studies include by-products of metabolism: e.g., esterase activity

liberates methanol, a known toxin, and there are some published examples of methanol poisoning occurring as a result of esterase metabolism of a primary xenobiotic (Ghatak and Samanta, 2013). Pyrolytic degradation of AMB-FUBINACA and similar carboxamide SCRAs (which are usually recreationally consumed by smoking) is already known to liberate cyanide (>25 μg/mg) among other known toxicants (Kevin et al., 2019), though to our knowledge the clinical toxicology implications of exposure to these secondary compounds has not yet been investigated. Together, it appears likely that recreational users of AMB-FUBINACA/pFPP may be exposed to a milieu of toxic compounds additional to the parent compounds themselves, which must be considered in attempts to create a cohesive understanding of the factors that determine the effects of illicit drugs on physiological parameters.

This study makes clear that the physiological interactions of AMB-FUBINACA and pFPP are complex. While our data do not provide an obvious mechanism by which the presence of pFPP would contribute to the high level of death associated with the use of AMB-FUBINACA in New Zealand, a major limitation of the in vivo experiments is the limited range of pFPP doses assessed. Hence, these results cannot refute the possibility that a wider range of dose combinations would have revealed additional interactive physiological or behavioural effects of the two substances. Nevertheless, the minimal interactions observed in vivo combined with our in vitro data suggest that interactions of AMB-FUBINACA and pFPP are unlikely to be fully explained by molecular/signalling interactions of the CB1 and 5HT1a receptors. Our data also indicate that pFPP does not alter the primary CES1-mediated AMB-FUBINACA metabolism. Although other types of potential interaction remain untested, the present results indirectly offer tentative support for the hypothesis that the increased morbidity associated with AMB-FUBINACA use in New Zealand as compared to the United States is most likely related to increased concentrations of the substance observed in the products available in New Zealand.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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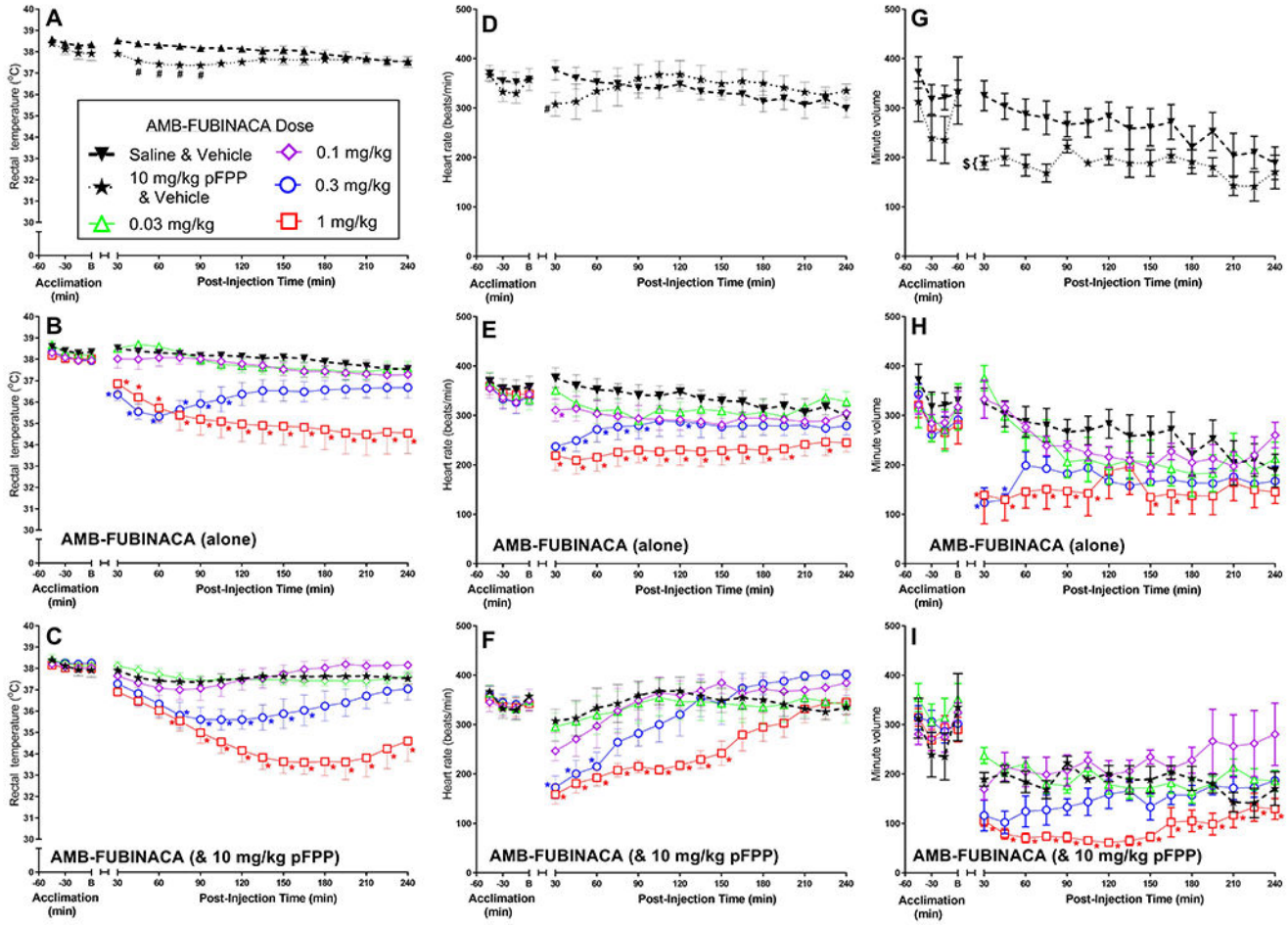


**Figure 1, Rat plethysmography and telemetry.**

Top panels show the effects of treatments of 10 mg/kg pFPP/vehicle and saline/vehicle on temperature **(A)**, heart rate **(D)**, and respiratory minute volume **(G)** during a 60 min acclimation period (left side of each panel) and during a 240-min post-injection schedule (right side of each panel). In the middle row of panels, the time-effect lines for saline/vehicle are duplicated from the respective top panels. In the bottom row of panels, the time-effect lines for pFPP/vehicle are duplicated from the top panels. Also shown in the middle and bottom rows of panels are the effects of AMB-FUBINACA alone **(B, E, H)** and in combination with 10 mg/kg pFPP **(C, F, I)** on temperature, heart rate, and respiratory minute volume, respectively. Each point shows the mean (± SEM) value for Sprague-Dawley rats (n=3 males and 2 females). X-axis 'B' annotation indicates the pre-injection baseline value at the end of the 60-min acclimation period. In panels A, D, and G: Hash sign '#' indicates a significant difference between vehicle and 10 mg/kg pFPP at a given time (dose*time interaction; $p<0.05$). Dollar sign '$' indicates a significant main effect ($p<0.05$) for pFPP compared to vehicle. For the remaining panels (B, C, E, F, H, I) which show the effects of AMB-FUBINACA, '*' asterisks indicate significant differences between the saline/vehicle condition **(B, E, H)** or 10 mg/kg pFPP/vehicle condition **(C, F, I)** at a given time point (dose*time interaction; $p<0.05$).

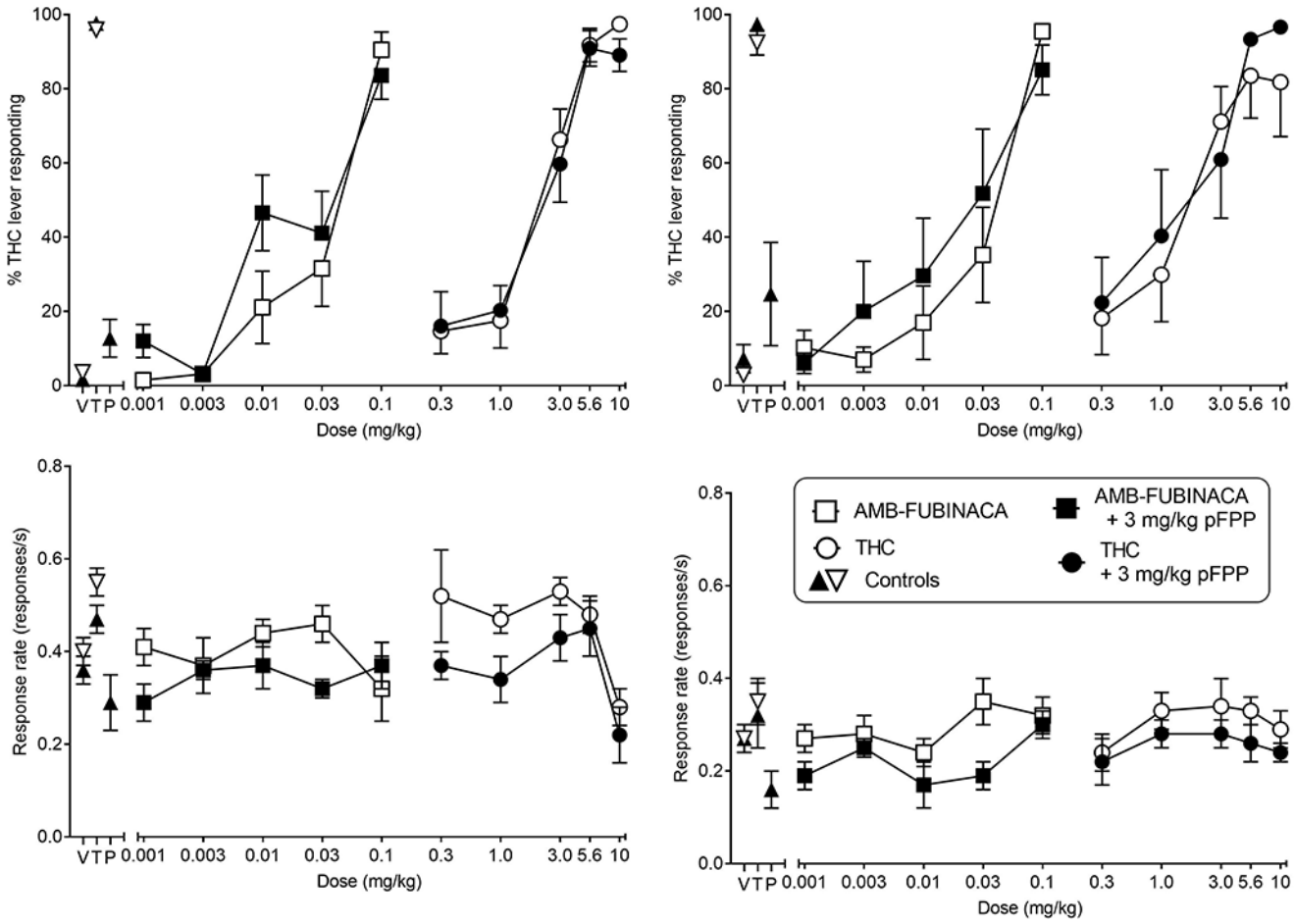


**Figure 2, Mouse drug discrimination.**

Effects of AMB-FUBINACA and THC alone (unfilled squares and circles, respectively) and in combination with 3 mg/kg pFPP (filled squares and circles, respectively) on percentage of responding on the THC-associated aperture (top panels) and response rates (responses/s; bottom panels) in adult male and female C57BL/6J mice (left and right panels, respectively) trained to discriminate 5.6 mg/kg THC from vehicle. Control points description: filled triangles (AMB-FUBINACA) and unfilled inverted triangles (THC). Each point represents the mean (± SEM) of data from 9-10 male mice (left panels) or 5-6 female mice (right panels). Asterisks (*) indicate a significant difference in response rate between vehicle and the indicated dose ($p<0.05$).

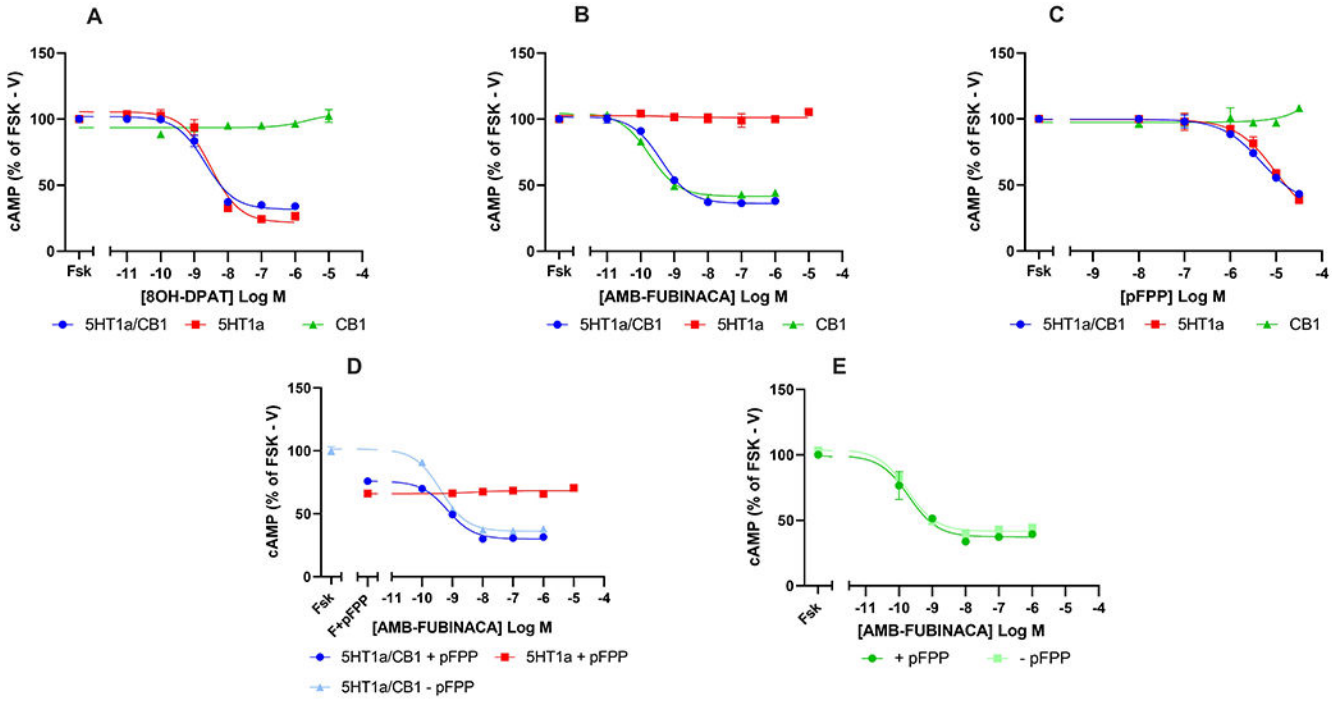


**Figure 3, hCB1 and h5HT1a cAMP signalling in HEK cells.**

cAMP signalling responses of HEK cells expressing both myc-h5HT1a and 3HA-hCB1 (blue), myc-h5HT1a alone (red), or 3HA-hCB1 alone (green) on stimulation with 5 μM forskolin (Fsk/F) and 8OH-DPAT **(A)**, AMB-FUBINACA **(B)**, or pFPP **(C)**. Responses to AMB-FUBINACA in the presence and absence of Fsk and 31.6 μM pFPP are also shown for the dual-expressing 5HT1a/CB1 HEK and 5HT1a-alone HEK lines **(D)**, and for the CB1-alone HEK line **(E)**. Data are taken from a representative experiment performed in technical duplicate (mean ± SD).

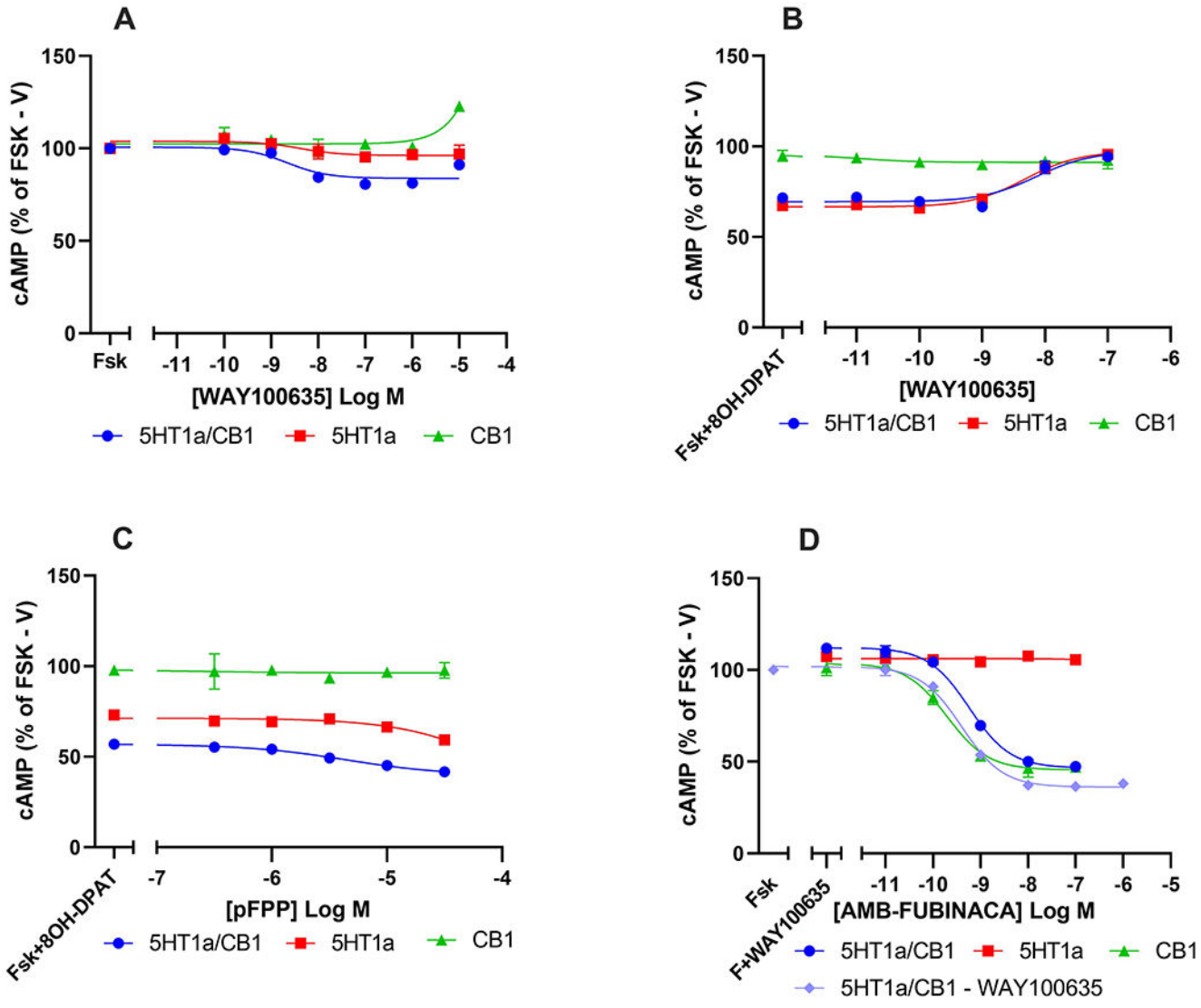


**Figure 4, cAMP signalling in 5HT1a antagonism paradigms.**

cAMP signalling responses of HEK cells expressing both myc-h5HT1a and 3HA-hCB1 (blue), myc-h5HT1a alone (red), or 3HA-hCB1 alone (green). Cells were stimulated with 5 μM forskolin (Fsk) and a concentration series of the 5HT1a antagonist WAY100635 alone **(A)** and in the presence of 31.6 nM 8OH-DPAT **(B)**, or with a concentration series of pFPP in the presence of 31.6 nM 8OH-DPAT **(C)**. All cell lines were also stimulated with 5 μM forskolin and a concentration series of AMB-FUBINACA in the presence of 1 μM WAY100635 (and in the absence of WAY, for the response of the dual-expressing 5HT1a/CB1 cell line only, light blue) **(D)**. Data are taken from a representative experiment performed in technical duplicate (mean ± SD).

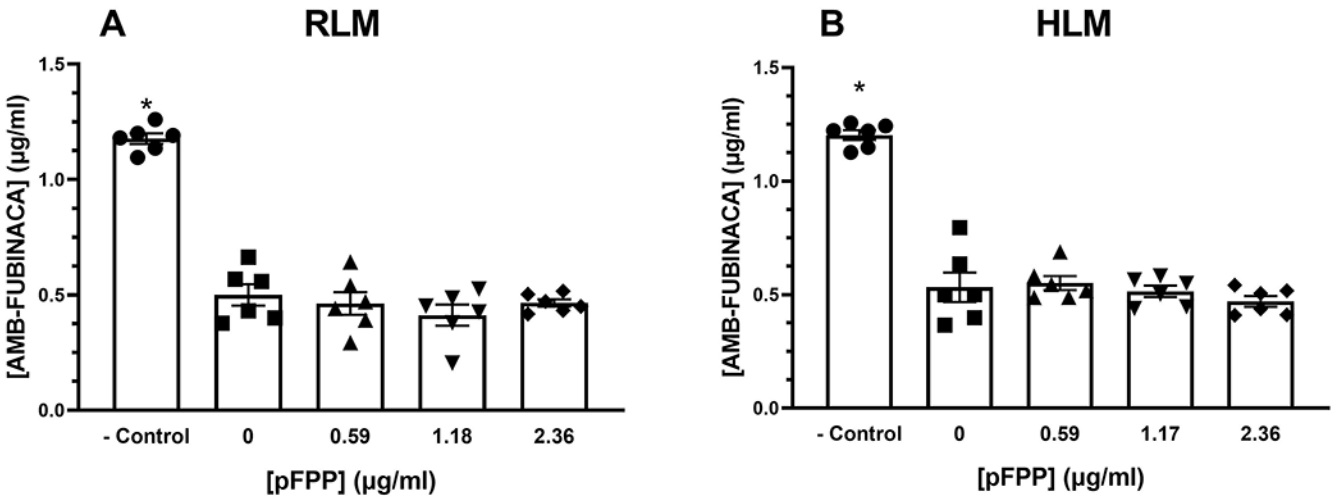


**Figure 5, Effect of pFPP on metabolism of AMB-FUBINACA in rat and human liver microsomes.**

Rat (1 mg/ml), RLM **(A)**, and human (0.25 mg/ml) liver microsomes, HLM **(B)**, were incubated with AMB-FUBINACA (1.15 µg/ml) in the absence of NADPH. Concentrations of AMB-FUBINACA following metabolism were determined using HPLC-DAD analysis. Negative controls contained no microsomes. Data is expressed as individual values and bars representing the mean ± SEM of six biological repeats, each conducted in duplicate. Significance was determined by a one-way ANOVA followed by Holm-Šídák post-test; * represents $p<0.05$ in comparison to 0 µg/ml pFPP.

**Table 1,**

Potency in THC drug discrimination in female and male C57BL/6 mice

| Sex | THC $ED_{50}$ (95% confidence limits) | | AMB-FUBINACA $ED_{50}$ (95% confidence limits) | |
|---|---|---|---|---|
| | Alone | + pFPP (3 mg/kg) | + Saline | + pFPP (3 mg/kg) |
| **Female**[a] | 1.46 mg/kg (0.62-3.40) | 1.24 mg/kg (0.46-3.34) | 0.03 mg/kg (0.006-0.14) | 0.02 mg/kg (0.01-0.04) |
| **Male**[b] | 2.10 mg/kg (1.73-2.56) | 2.10 mg/kg (1.06-4.17) | 0.03 mg/kg (0.007-0.14) | 0.02 mg/kg (0.006-0.08) |

[a] n=6;

[b] n=10

**Table 2,**

cAMP signalling potencies and efficacies of ligands investigated in HEK cells expressing 5HT1a/CB1, or 5HT1a-alone and CB1-alone

| Drug condition | Cell line | pEC50 ± SEM | Emax (% inhib of Fsk), ± SEM | *n* |
|---|---|---|---|---|
| 8OH-DPAT | 5HT1a/CB1 | 8.39 ± 0.21 | 66.6 ± 1.3 | 5 |
| | 5HT1a | 8.54 ± 0.03 | 71.5 ± 3.6 | 3 |
| AMB-FUBINACA | 5HT1a/CB1 | 9.21 ± 0.14 | 61.0 ± 1.7 | 4 |
| | CB1 | 9.56 ± 0.09 | 60.8 ± 1.7 | 3 |
| pFPP | 5HT1a/CB1 | ~5.20 ± 0.18$ | ND | 3 |
| | 5HT1a | ~4.83 ± 0.13$ | ND | 3 |
| AMB-FUBINACA + pFPP (31.6 μM) | 5HT1a/CB1 | 9.39 ± 0.11 | 64.9 ± 3.5 | 3 |
| | CB1 | 9.70 ± 0.16 | 57.9 ± 2.33 | 3 |
| AMB-FUBINACA + WAY100635 (1 μM) | 5HT1a/CB1 | 9.11 ± 0.16 | 59.4 ± 9.17 | 3 |
| | CB1 | 9.65 ± 0.14 | 55.7 ± 5.78 | 3 |

$Emax of pFPP concentration-response curves were not fully defined due to low potency. To allow approximation of potency values, curves were constrained to the maximum effect produced by 8OH-DPAT in each cell line.