ORIGINAL ARTICLE

Evidence of enzyme-mediated transesterification of synthetic cannabinoids with ethanol: potential toxicological impact

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Abstract

Purpose Synthetic cannabinoids (SCs) represent a large proportion of novel psychoactive substances on the black market and have caused a number of deaths. Polydrug use including combination of SCs and ethanol could further complicate the toxicological impact. To the best of our knowledge, there have been no reports presenting evidence of transesterification between SCs and ethanol in vitro.

Methods The in vitro metabolism of the four carboxylate SCs PB-22, NPB-22, 5-fluoro-PB-22 (5F-PB-22), and 5-fluoro-NPB-22 (5F-NPB-22) in the presence of ethanol using human liver microsomes with and without appropriate enzyme inhibitors was studied. Newly identified SC ethyl esters were chemically synthesised and fully characterised. The activity of these SCs and their ethanol transesterification products were assessed using cannabinoid receptor (CB_1 and CB_2) activation assays.

Results SCs/ethanol transesterification products were detected and studied using liquid chromatography–high-resolution mass spectrometry. We have shown that the SC ethyl ester formation is mediated by human carboxyl esterase enzymes. The ethyl esters exhibited a reduced activity for the CB receptors compared with their parent compounds.

Conclusions These novel ethyl esters may be useful additional markers of cannabinoid administration, and especially so if they prove to have longer half-lives than their parent compounds.

Keywords Carboxylate synthetic cannabinoids · In vitro drug metabolism · Transesterification · 5F-NPB-22 · Ethanol · Biomarkers

Introduction

The use of synthetic cannabinoid receptor agonists, or simply 'synthetic cannabinoids' (SCs), is a global toxicological conundrum. Their use appears to have escalated during 2008–2015 [1] under the pretence that these compounds are 'safe' and 'legal' [2], and it is assumed that their use cannot easily be detected [3]. Although there has been a decline in seized SCs recently, many of the recent substances are highly potent and harmful [1]. Originally, these compounds, which circumvented drug legislation in many countries, were sold over-the-counter in so-called headshops and on the Internet as vividly branded products such as 'Spice' in

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Europe and 'K2' in the United States. The weakness of the Misuse of Drugs Act [4] and incorporated schedules in the UK could not readily keep up with the rapidity of novel compounds reaching the market, but the more recent UK Psychoactive Substances Act (PSA) in 2016 [5] appears to be having an impact. While some data suggest that the use of novel psychoactive substances (NPS), including SCs, has decreased since the introduction of the PSA, other studies suggest little impact in the number of emergency department presentations, and their use remains prevalent and problematic [6]. As of the end of 2017, more than 670 NPS were monitored by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA), and 179 out of these were SCs [1]. In the UK, this is especially relevant in the prisoner population and amongst those facing homelessness [3, 7]. Not only are the clinical features of acute SC toxicity difficult to identify and treat, particularly for the more recent third-generation SCs [6, 8, 9], but the analysis of these compounds in bulk-drug seizures and in biological samples can also be challenging, even using the most modern analytical instrumentation. Analysis is complicated by (1) the sheer number of SCs that exist [10, 11], (2) their physicochemical diversity, (3) the frequent formation of multiple (pharmacologically active) metabolites [10, 12–14], (4) their (in)stability and chemical transformations when ingested via smoking (i.e., their pyrolysis products [15–18]), (5) their potency, which transpires to low concentrations in biological samples, and (6) the limited availability of reference materials. To aid the identification of SCs and their metabolites, in vitro models based on incubation with human liver microsomes (HLMs) and other enzyme fractions [19], coupled with in silico predictions, are commonly used to determine the most likely metabolic routes of 'new' SCs [20]. In silico models are also extremely useful at predicting the pharmacology and potential toxicology of SCs [21, 22]. Such literature resources are invaluable for building large screening libraries to maximise the chances of detecting toxicologically relevant SC use in samples.

However, in substance-using populations, polydrug use is common [23]. Considerations of drug-drug interactions with SCs are important clinically [24], but possible or unexpected biotransformation due to co-ingested compounds should also be considered. For example, transesterification of cocaine following concomitant use of ethanol and cocaine is well known to produce the pharmacologically active metabolite cocaethylene [25, 26]. Similarly, transesterification has also been demonstrated for methylphenidate, resulting in the production of ethylphenidate (itself an NPS with misuse potential) [27]. These reactions are primarily catalysed by human carboxylesterases (hCES) in the liver, which participate in phase I metabolism of xenobiotics. Transesterification of PB-22 can also be observed as a thermal degradation artefact when detecting by gas chromatography–mass spectrometry (GC–MS) in the presence of methanol or ethanol as a solvent [28].

There is increasing evidence confirming the use of SCs together with ethanol. A recent United Nations Office on Drugs and Crime (UNODC) report [29] highlighted ethanol as the third most frequent substance found in SC-related fatalities. In addition, ester-based SCs including 5F-PB-22 were detected, together with ethanol and other substances in postmortem cases [30, 31]. ADB-CHMINACA (also known as MAB-CHMINACA) and ethanol were both detected in a patient admitted to hospit [32]. A further case reported a fatal poisoning with the synthetic cannabinoid AB-CHMINACA and ethanol [33]. Finally, blood and urine ethanol concentrations were reported in three fatalities related to the use of 5F-ADB, 5F-PB-22 and AB-CHMINACA (5F-PB-22 = 2.60 and 3.58 g/kg, AB-CHMINACA = 1.45 and 2.57 g/kg, 5F-ADB = 0.09 and 0.12 g/kg for blood and urine ethanol concentrations, respectively) [34]. It is apparent that the discovery of novel biomarkers of concomitant SC-ethanol consumption should facilitate the interpretation of such analytical investigations and may allow both extending the detection time and assisting in the understanding of the patterns of toxicity associated with such polydrug abuse.

In this study, we report in vitro evidence from HLM incubation experiments for the transesterification of four ester-containing SCs, namely PB-22, NPB-22, 5F-PB-22, and 5F-NPB-22 (Fig. 1), in the presence of ethanol, giving rise to a further group of potential new indicators of combined SC/ethanol use. These compounds were chosen because of the presence of a labile ester bond in their structures, making them amenable to the described chemical reaction, as well as their prevalence [35].

To evaluate whether the transesterification products might be more biologically active than the parent compounds, we used an activity-based cannabinoid receptor assay [36–38] to investigate the in vitro receptor activity of the ethyl esters formed by transesterification of ethanol with these SCs.

Materials and methods

Chemicals and reagents

PB-22, NPB-22, 5F-PB-22 and 5F-NPB-22 were kindly provided by TICTAC Communications (London, UK). Their identity and purity were confirmed by nuclear magnetic resonance (NMR) spectroscopy and high-performance liquid chromatography–ultraviolet spectroscopy (HPLC–UV) (% purity > 90%). Methanol, acetonitrile, formic acid (all LC–MS grade) were purchased from Fisher Scientific (Loughborough, UK). Trifluoroacetic acid (TFA), chloroform-d (CDCl$_3$, isotopic purity 99.8 atom %D) were from Sigma-Aldrich (Dorset, UK). Deionised water was purified

Fig. 1 Chemical structures of
5F-NPB-22, NPB-22, PB-22
and 5F-PB-22 (top panel), their
corresponding ethyl esters (mid-
dle panel) and their hydrolysis
product (bottom panel). *SCs*
synthetic cannabinoids
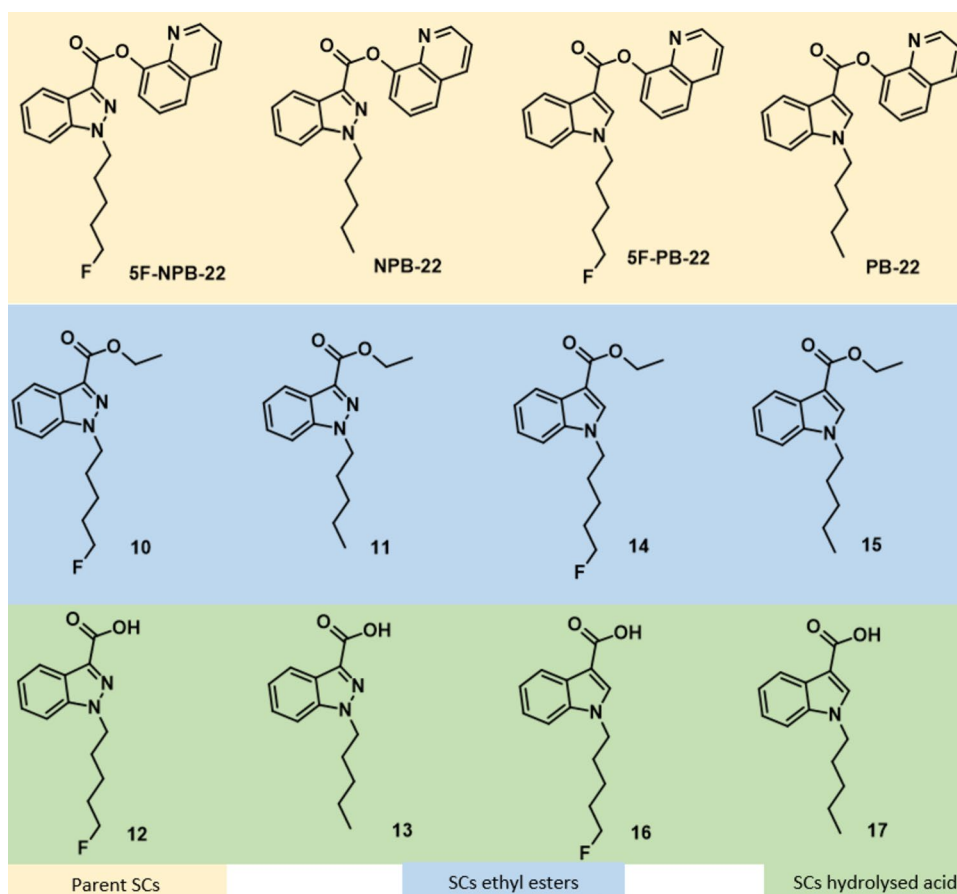
to 18.2 MΩ cm resistivity using a PURELAB Ultra sys-
tem (ELGA, Woodridge, UK). Aqueous phosphate buffer
(0.5 mol/L, pH 7.4), pooled HLMs (150 donors), and
reduced nicotinamide adenine dinucleotide phosphate
(NADPH) regenerating system solutions A and B were pur-
chased from Corning (Woburn, MA, USA); ethyl indazole-
3-carboxylate and 1-bromo-5-fluoropentane from Fluoro-
Chem (Hadfield, UK); 1,5-diiodopentane from Alfa Aesar
(Lancashire, UK); ethyl indole-3-carboxylate and potas-
sium *tert*-butoxide (*t*BuOK) from Aldrich (Dorset, UK);
potassium hydroxide (KOH), bis-*p*-nitrophenylphosphate
(BNPP), tetrahydrofuran (THF), hexane, ethyl acetate and
ethanol from Sigma-Aldrich (St. Louis, MO, USA); anhy-
drous THF from Acros Organics (Geel, Belgium).

Synthesis and characterisation of ethyl esters

Synthesis of the ethyl esters (compounds **10**, **11**, **14** and
15, Fig. 1) was carried out via nucleophilic substitutions of
the N1 nitrogen atoms of (1) ethyl indazole-3-carboxylate
and (2) ethyl indole-3-carboxylate, each with 1,5-diiodo-
pentane and 1-bromo-5-fluoropentane, to form the non-
fluorinated and fluorinated analytes, respectively. Ester

hydrolysis products (compounds **12**, **13**, **16** and **17**, Fig. 1)
were synthesised by alkaline hydrolysis of ethyl esters.
Summary experimental data are given in supplementary
material section S1. Ethyl esters were characterised using
NMR (^{1}H-NMR, ^{13}C-NMR, homonuclear correlation
spectroscopy—COSY, and heteronuclear single-quantum
correlation spectroscopy—HSQC) (NMR, Bruker Avance
DRX 400 MHz; Bruker BioSpin, Rheinstetten, Germany).

Further analysis was carried out using reversed-phase
high-performance liquid chromatography with diode-array
detection (HPLC–DAD, HP-1050) (Agilent Technologies,
Santa Clara, CA, USA) using a 100 × 2.1 mm i.d. C8 column
(5 μm, Kinetex®; Phenomenex, Torrance, CA, USA). Solu-
tions of each compound [100 μg/mL in 0.1% (*v/v*) TFA in
water] were eluted using a water/acetonitrile gradient (both
containing 0.1% (*v/v*) TFA, ambient temperature, 31 min
analysis time). Finally, solutions of each compound [300 ng/
mL in 10% (*v/v*) acetonitrile in deionised water containing
0.3% (*v/v*) formic acid] were analysed by liquid chromatog-
raphy–high-resolution mass spectrometry (LC–HRMS) to
generate reference retention times and product ion (MS^2)
spectra for the in vitro incubation experiments.

In vitro incubation

The HLM method was based on that previously described [19]. Briefly, solutions containing 10 μg of each substrate (1 mg/mL, 10 μL) were added to Eppendorf LoBind™ tubes (Stevenage, UK), and evaporated to dryness. Buffer solution (0.1 mol/L aqueous phosphate buffer, pH 7.4, 500 μL), NADPH-regenerating solutions [50 μL solution A: 26 mmol/L NADP$^+$, 66 mmol/L glucose-6-phosphate, and 66 mmol/L MgCl$_2$ (aq); 10 μL solution B: 40 U/mL glucose-6-phosphate dehydrogenase in 5 mmol/L sodium citrate], and deionised water (390 μL) were added. The tubes were capped, vortex-mixed, and preincubated (37 °C) with continuous gentle shaking for 10 min using an Eppendorf ThermoMixer® (Stevenage, UK). Reactions were initiated by the addition of HLM solution [protein content 20 mg/mL in 250 mmol/L sucrose (aq), 50 μL], gently mixed by hand and were incubated using an Eppendorf ThermoMixer® (2 h, total reaction volume 1000 μL). A negative control was prepared by incubating each of the compounds with buffer solution and water, omitting the HLM and NADPH solutions. For incubations with ethanol, the initial volume of deionised water added to the incubation mixture was reduced to 340 μL, and aqueous ethanol solution [5.86% (*v/v*), 50 μL] was added. The final concentration of ethanol was 50 mmol/L (0.23 g/dL). In addition, incubations were carried out in the presence of specific carboxylesterase (CES), and nonspecific esterase inhibitors (BNPP and saturated NaF solution, respectively). For both experiments, the initial volume of deionised water added to the incubation mixture was reduced to 290 μL, and aqueous ethanol solution [5.86% (*v/v*), 50 μL] and inhibitor solution [either 1 mmol/L BNPP, or 10% (*w/v*) NaF in water, 50 μL] was added. The final concentration of ethanol was maintained at 50 mmol/L.

After incubation, all reactions were quenched by the addition of ice-cold acetonitrile (250 μL). Quenched solutions were vortex-mixed and centrifuged (13,000 *g*, 15 min). Supernatants were transferred to glass autosampler vials for analysis by LC–HRMS (5 μL injection volume).

LC–HRMS

An UltiMate 3000™ HPLC system coupled to a Q Exactive™ high-resolution mass spectrometer (both Thermo Scientific, San Jose, CA, USA) was used. Xcalibur™ software (version 14.0) was used for instrument control and data analysis. Chromatographic separation was performed using an Acquity™ BEH C18 column (50×2.1 mm i.d., 1.7 μm) (Waters, Borehamwood, UK). Eluents consisted of 0.3% (*v/v*) formic acid in deionised water (eluent A) and in acetonitrile (eluent B). Column temperature was maintained at 40 °C. The elution gradient started at 10% eluent B (2 min), ramped to 95% (over 5 min), and then decreased

immediately to 10% (over 1 min) prior to re-equilibration. The total analysis time was 10 min.

Heated electrospray ionisation (HESI-II) settings were: sheath gas flow-rate 70; auxiliary gas flow-rate 10; spray voltage 3.75 kV; capillary temperature 320 °C; S-lens radio frequency level 55.0; auxiliary gas temperature 350 °C. Full-scan acquisition parameters were: positive ionisation mode; scan range *m/z* 100–1000; resolution 70,000 full width at half maximum (FWHM); automatic gain control (AGC) target 1×10^6. MS^2 acquisition parameters were: parallel reaction monitoring mode (based on an inclusion list containing the appropriate precursor ions); scan range *m/z* 100–750; resolution 35,000 FWHM; AGC target 1×10^6. The isolation width was 1.2 amu. The normalised collision energy (NCE) was optimised manually using available standards and in-house synthesised products. For data review, total ion chromatograms were extracted using a ± 2.5 ppm extraction window based on the exact *m/z* values.

Cannabinoid receptor assay

To assess the biological activities of the compounds, live cell-based reporter assays that monitor protein-protein interactions via the NanoLuc Binary Technology were used. Here, the receptor activation is evaluated via the interaction between β-arrestin 2 (βarr2), a cytosolic protein, and the cannabinoid receptor CB$_1$ or CB$_2$. Both βarr2 and CB$_1$/CB$_2$ are fused to an inactive part of nanoluciferase. When CB$_1$ or CB$_2$ are activated by a ligand, βarr2 is recruited to the receptor, allowing interaction of the complementary nanoluciferase subunits, yielding a functional enzyme that generates a bioluminescent signal in the presence of the substrate furimazine. Details regarding the development of the stable CB$_1$ and CB$_2$ cell lines used here have been reported elsewhere [36]. The original human embryonic kidney (HEK) T293 cell line was provided by Prof. O. De Wever (Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University Hospital, Belgium).

The cells were routinely maintained at 37 °C, 5% CO$_2$, under humidified atmosphere in Dulbecco's modified Eagle's medium (GlutaMAX™; Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 IU/mL penicillin, 100 mg/L streptomycin and 0.25 mg/L amphotericin B. Stability of the cell lines was followed by flow cytometric analysis. For experiments, cells were deposited on poly-D-lysine coated 96-well plates (Thermo Fisher Scientific) at 5×10^4 cells/well and incubated overnight. The cells were washed twice with Opti-MEM® I Reduced Serum Medium (Thermo Fisher Scientific) to remove any remaining FBS, and 100 μL Opti-MEM® I was added. The Nano-Glo Live Cell reagent (Promega, Madison, WI, USA), a nonlytic detection reagent containing

the cell permeable furimazine substrate, was prepared by 20-fold dilution of the Nano-Glo Live Cell substrate using Nano-Glo LCS Dilution buffer, and 25 µL were added to each well. Subsequently, the plate was placed into a TriStar[2] LB 942 multimode microplate reader (Berthold Technologies GmbH & Co., Bad Wildbad, Germany). Luminescence was monitored during the equilibration period until the signal stabilised (15 min). We added 10 µL per well of test compounds, present as concentrated (13.5-fold) stock solutions in 50% (*v/v*) methanol in Opti-MEM® I. The luminescence was continuously detected for 120 min. Solvent controls were analysed with all experiments. The final concentration of methanol (3.7% *v/v*) has previously been verified to be well-tolerated by the cells [36].

Curve fitting and statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA). The results are represented as mean area under the curve (AUC) ± standard deviation (SD) with at least five replicates for each data point (obtained in three independent experiments). All results were normalised to the E_{max} of JWH-018 (= 100%), our reference compound. Curve fitting of concentration-effect curves via nonlinear regression (four parameters logistic fit) was employed to determine EC_{50} (a measure of potency) and the E_{max} (a measure of efficacy). The maximal effect of all compounds was determined by either the E_{max} (for the parent compounds) or the maximal effect that was achieved for the compound at high concentrations.

Results and discussion

Characterisation of compounds

Data from the characterisation experiments (NMR and HPLC–UV) for the synthesised compounds (**10-17**) are presented in supplementary material section S1. Retention times, precursor ion *m/z* values (all [M+H]$^+$), and major product ion *m/z* values for the parent compounds and the eight synthesised compounds generated using the LC–HRMS method are summarised in Table 1.

For the indoles PB-22 and 5F-PB-22, the characteristic major product ions observed were in agreement with previously published data [39], resulting from successive losses of the hydroxyquinolinyl substructures (*m/z* 214.1228 and 232.1121 for PB-22 and 5F-PB-22, respectively) and the (fluoro-) alkyl chains (*m/z* 144.0445 and 144.0435 for both compounds).

Product ions spectra for the two indazoles, NPB-22 and 5F-NPB-22, showed analogous product ions (*m/z* 215.1183 and 233.1074 for NPB-22 and 5F-NPB-22, respectively, plus 145.0398 and 145.0389 for both compounds). In addition, the indazoles gave rise to fragment ions corresponding to losses of the quinolinyl substructures, i.e., of the same *m/z* as the precursor ions for compounds **13** and **12**, the ester hydrolysis products (*m/z* 233.1288 and

Table 1 Summary LC–HRMS data for parent and synthesised compounds PB-22, NPB-22, 5F-PB-22, 5F-NPB-22, and metabolites **10-17**

Compound (formula)	RT (min)	Precursor ion accurate *m/z* (Δ ppm)	Major product ion(s) accurate *m/z* (Δ ppm)
PB-22 ($C_{23}H_{22}N_2O_2$)	6.21	359.1759 (1.39)	214.1228 (0.93), 144.0445 (0.69)
NPB-22 ($C_{22}H_{21}N_3O_2$)	6.29	360.1714 (1.94)	233.1288 (1.29), 215.1183 (1.86), 145.0398 (1.38)
5F-PB-22 ($C_{23}H_{21}N_2O_2F$)	6.05	377.1652 (−2.12)	232.1121 (−4.74), 144.0435 (−6.25)
5F-NPB-22 ($C_{22}H_{20}N_3O_2F$)	6.03	378.1603 (−2.38)	251.1178 (−4.78), 233.1074 (−4.72), 213.1015 (−3.28), 145.0389 (−4.83)
PB-22 ethyl ester (**15**) ($C_{16}H_{21}NO_2$)	6.52	260.1651 (2.31)	232.1336 (1.72), 214.1231 (2.34), 188.1439 (2.66), 132.0811 (2.27), 118.0654 (2.54)
NPB-22 ethyl ester (**11**) ($C_{15}H_{20}N_2O_2$)	6.34	261.1598 (0.00)	233.1282 (−1.29), 215.1178 (−0.46), 145.0395 (−0.70)
5F-PB-22 ethyl ester (**14**) ($C_{16}H_{20}NO_2F$)	5.98	278.1556 (1.80)	250.1241 (1.20), 232.1136 (1.72), 206.1345 (2.43), 132.0811 (2.27)
5F-NPB-22 ethyl ester (**10**) ($C_{15}H_{19}N_2O_2F$)	5.82	279.1503 (0.00)	251.1188 (−0.80), 233.1083 (−0.86), 213.1021 (−0.47), 145.0396 (0.00)
PB-22 ester hydrolysis product (**17**) ($C_{14}H_{17}NO_2$)	5.61	232.1334 (0.86)	214.1225 (−0.47), 188.1433 (−0.53), 132.0807 (−0.76), 118.0651 (0.00)
NPB-22 ester hydrolysis product (**13**) ($C_{13}H_{16}N_2O_2$)	5.21	233.1291 (2.57)	215.1183 (1.86), 163.0505 (1.84), 145.0399 (2.07)
5F-PB-22 ester hydrolysis product (**16**) ($C_{14}H_{16}NO_2F$)	5.03	250.1244 (2.40)	232.1135 (1.29), 206.1344 (1.94), 132.0811 (2.27)
5F-NPB-22 ester hydrolysis product (**12**) ($C_{13}H_{15}N_2O_2F$)	4.85	251.1192 (2.80)	233.1084 (−0.43), 213.1022 (0.00), 145.0397 (0.69)
5F-NPB-22 methyl ester ($C_{14}H_{17}N_2O_2F$)	5.48	265.1354 (2.64)	251.1195 (1.99), 233.1089 (1.72), 213.1029 (3.28)

LC–HRMS liquid chromatography–high-resolution mass spectrometry, *RT* retention time

251.1178 for NPB-22 and 5F-NPB-22, respectively). For 5F-NPB-22, a defluorinated product ion was also observed (*m/z* 213.1015) which was not observed in the case of 5F-PB-22.

For the ethyl ester and ester hydrolysis products of PB-22 (compounds **15** and **17**), the major protonated carbonyl-indole core fragment ion (*m/z* 144.0444) was not observed at any significant relative abundance. Instead, the proposed alkyl-indole and alkyl-indole carboxylic acid product ions (*m/z* 188.1439 and 232.1336, respectively) were the major product ions for PB-22 ethyl ester, compound **15** (Fig. 2), in addition to a small amount of the ion at *m/z* 214.1231 also observed for both compounds **15** and **17**. The ethyl ester of PB-22 gave rise to a product ion at *m/z* 132.0811 and 118.0654, which were observed as major product ions of PB-22 ester hydrolysis, compound **17**.

With respect to the ethyl esters and hydrolysis products of 5F-PB-22 (compounds **14** and **16**), similar fragments as PB-22 ethyl ester and ester hydrolysis were observed. The major product ions were the alkyl-indole, *m/z* 206.1337; and product ions at *m/z* 250.1234, 232.1129 and 118.0650 being the fragments of alkyl-indole carboxylic acid, alkyl-indole carbaldehyde and indole group, respectively. The ethyl ester of 5F-PB-22 also gave rise to a product ion at *m/z* 132.0806.

Observed product ions of NPB-22 ethyl ester and 5F-NPB-22 ethyl ester included the alkyl-indazole carboxylic acid, alkyl-indazole carbaldehyde, and indazole carbaldehyde (*m/z* 233.1276, 215.1171, and 145.0391 for NPB-22, *m/z* 251.1187, 233.1082, and 145.0395 for 5F-NPB-22, respectively). The 5F-NPB-22 ethyl ester also yielded a defluorinated fragment (*m/z* 213.1020) (Fig. 3) as observed in MS^2 result of 5F-NPB-22 parent compound. The ester

hydrolysis product ions of NPB-22 and 5F-NPB-22 gave similar results to those of their corresponding ethyl esters.

HLM incubation experiments

PB-22 and 5F-PB-22

In the absence of ethanol, metabolism of PB-22 proceeded as previously described to produce a series of phase I metabolites [39]. The major metabolite (by relative abundance) was the ester hydrolysis product (compound **17**). Of note, this compound was also observed at a lower abundance (approximately 1% of the parent compound) in the negative control samples, in which no HLMs were included in the incubation (see supplementary material S3.4). In addition to the ester hydrolysis product, a series of hydroxylated metabolites were observed, with characteristic MS^2 fragments suggesting monohydroxylation on (1) the pentyl chain, (2) the indole core and (3) the quinolinyl substructure, carbonylation of the pentyl chain, and successive hydroxylation products following ester hydrolysis and *N*-dealkylation. When ethanol was present, in addition to these metabolites, the previously synthesised and characterised PB-22 ethyl ester was also detected along with further peaks suggesting monohydroxylation of the ethyl ester (Fig. 4). No ethyl ester was observed when PB-22 was incubated with ethanol in the presence of either saturated NaF (nonspecific CES inhibitor) or 1 mmol/L BNPP (specific CES inhibitor) (see supplementary material S3.2-S3.3). The observed LC–HRMS analyses and the MS^2 spectra of ethyl esters when SCs were incubated with HLM and ethanol matched with the synthetic standards (see supplementary material S2.1-2.8 and S1.28-1.35).

Fig. 2 Product ion (MS^2) spectrum (annotated with proposed fragment ion structures) for the ethyl ester of PB-22 (compound **15**, exact *m/z* for protonated molecular ion 260.1645) with normalised collision energy (NCE) at 25
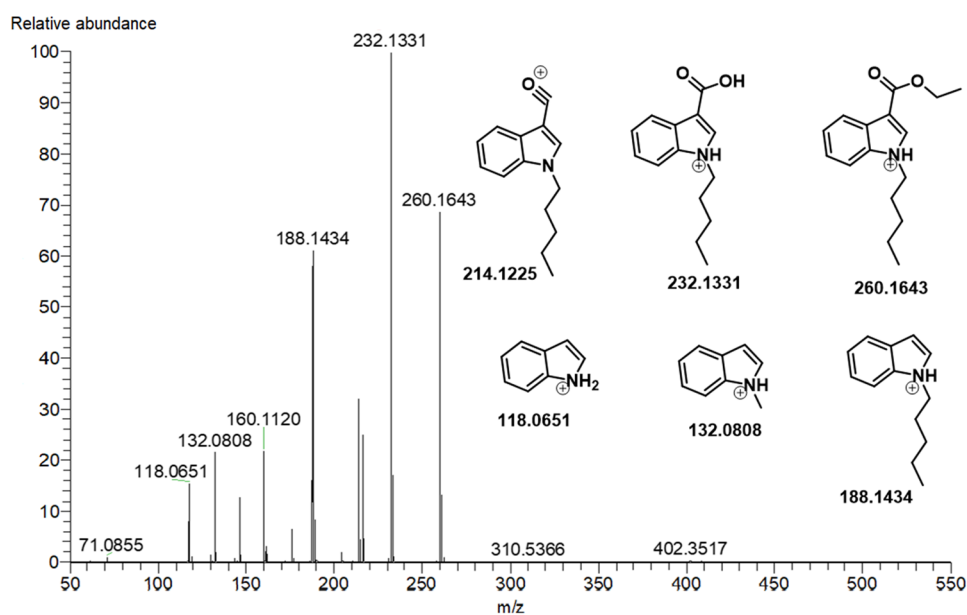
Fig. 3 MS^2 spectrum (annotated with proposed fragment ion structures) for the ethyl ester of 5F-NPB-22 (compound **10**, exact *m/z* for protonated molecular ion 279.1503) with NCE at 35
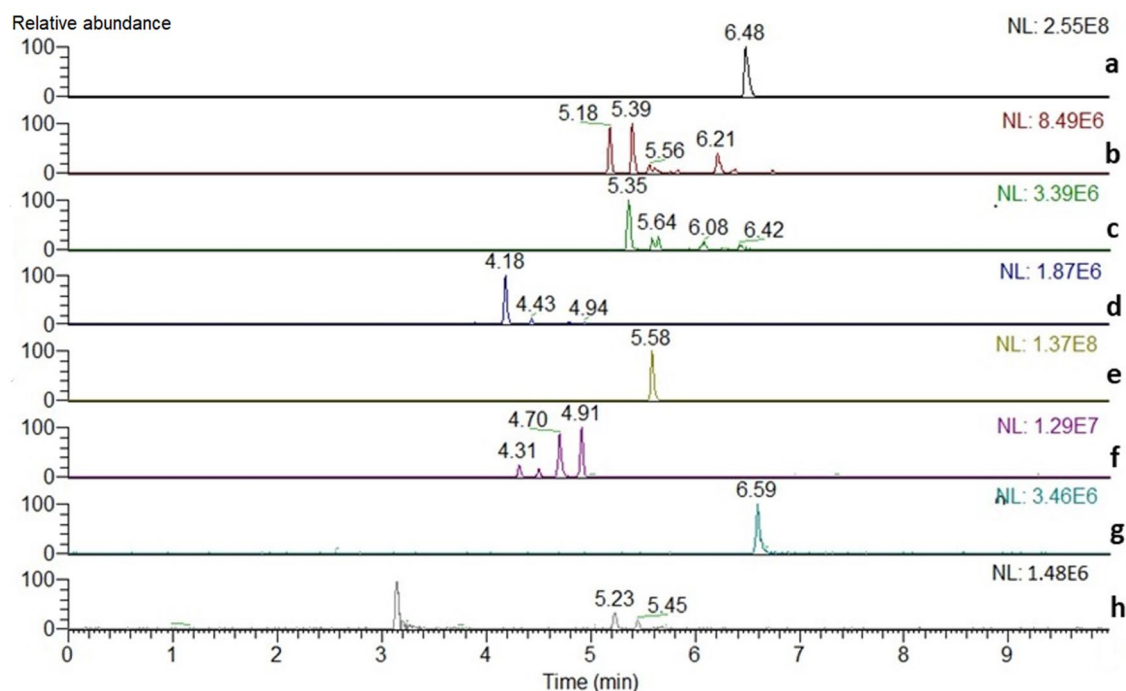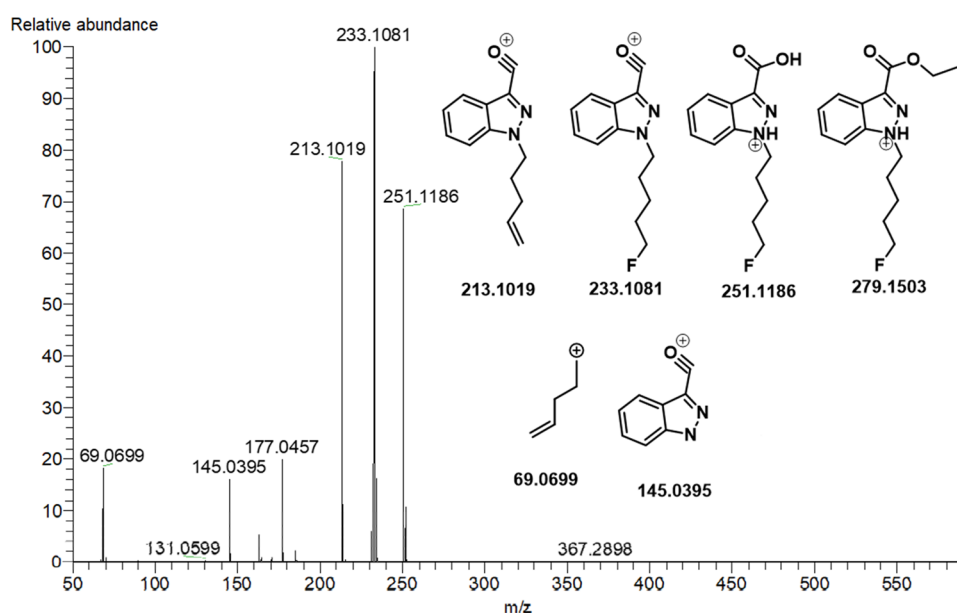
Fig. 4 Overlaid extracted ion chromatograms (exact mass ± 2.5 ppm) of the major detected PB-22 human liver microsome (HLM)-derived metabolites in the presence of ethanol: **a** = parent drug PB-22; **b** = monohydroxylation; **c** = carbonylation; **d** = monohydroxylation with *N*-dealkylation; **e** = ester hydrolysis (**17**); **f** = ester hydrolysis with monohydroxylation; **g** = PB-22 ethyl ester (**15**); **h** = PB-22 ethyl ester with monohydroxylation

A summary of the observed in vitro phase I metabolism, including transesterification in the presence of ethanol, for PB-22 is shown in Fig. 5.

For the fluorinated indole analogue 5F-PB-22, oxidative, reductive defluorination and oxidative defluorination to carboxylic acid products (see supplementary material

S3.8) were observed in addition to the metabolites seen for PB-22. In the presence of ethanol, and ethanol plus CES-inhibitor, similar results to its non-fluorinated analogue were observed.

These data demonstrate for the first-time evidence of hCES-mediated transesterification of selected

Fig. 5 Proposed metabolic pathway for PB-22, inclusive of ethyl ester formation in the presence of ethanol. (**a**–**h**: refer to Fig. 4)
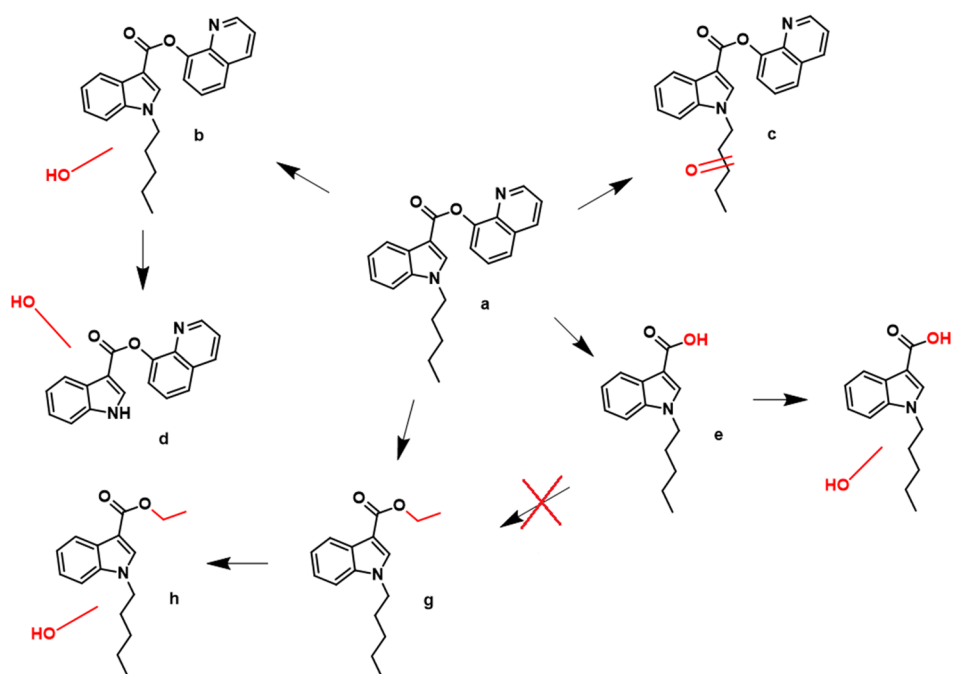
ester-containing SCs in the presence of ethanol, which gives rise to a new family of potential markers for SC and ethanol concomitant use. In the case of PB-22 and 5F-PB-22, our findings suggested that the ethyl ester formation was catalysed by hCES (most likely hCES-1 subtype as in the case of cocaine metabolism), because no ethyl ester formation was observed in the absence of the HLMs, nor when CES activity was inhibited (by either nonspecific or specific inhibitors). Furthermore, multiple hydroxylated metabolites of the ethyl esters were also observed, which may constitute additional useful (likely urinary) biomarkers of combined SC/ethanol use in toxicological analyses. However, PB-22 contains a labile ester bond in the structure. Thus, when using GC–MS with an elevated temperature in the presence of ethanol as solvent, transesterification to produce the ethyl ester of PB-22 can be observed [28].

NPB-22 and 5F-NPB-22

As for the two indazoles, NPB-22 produced similar in vitro metabolites to 5F-NPB-22. Hydrolysis of the ester linkage was the major metabolic pathway for both indazole analogues, NPB-22 and 5F-NPB-22. A series of monohydroxylated ester hydrolysis products were observed. In addition, ester hydrolysis followed by oxidative defluorination was detected for 5F-NPB-22. Extracted ion chromatograms for the metabolites of 5F-NPB-22 from in vitro HLM experiments are presented in Fig. 6 and the proposed metabolic pathway of 5F-NPB-22 (without ethanol added) is presented in Fig. 7. To the best of our knowledge, this is the first report of the in vitro metabolism of 5F-NPB22.

In the presence of ethanol, the ethyl esters of NPB-22 and 5F-NPB-22 were both detected. Interestingly, a small amount of the ethyl ester was observed (approximately 0.5% of the parent compound by abundance) in the presence of only buffer and ethanol (i.e., without any HLMs, see supplementary material S3.11 and S3.14). Indazole SCs clearly display more reactivity towards nucleophilic agents than their indole counterparts. Indeed, the NPB-22 family was more rapidly prone to hydrolysis, methanolysis and, therefore, ethanolysis (Fig. 8), even in the absence of any biocatalyst.

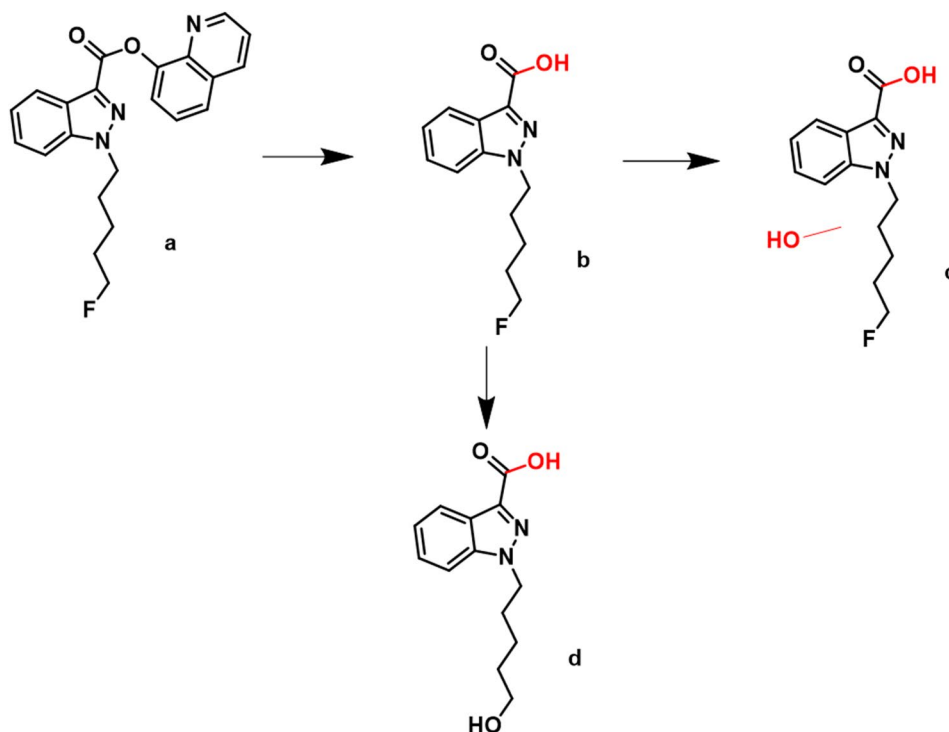### Incubation of ester hydrolysis products with HLM

To further elucidate the mechanism of formation of the newly detected SC ethyl esters, hydrolysed SC esters (compounds **12**, **13**, **16** and **17**) were separately incubated with HLMs to monitor any ethyl ester product formation. This only yielded hydroxylated metabolites (data not shown). Incubation with HLMs and ethanol did not result in the formation of ethyl esters (illustrated in supplementary material S4). We concluded that the investigated biocatalysed reactions were highly dependent on the nature of the substrate: SC-ethanol transesterification occurred; however, esterification of the hydrolysed esters with ethanol was not observed. Indeed, hCES is well known to accommodate ester, thioester, carbamate, and amide bonds in its active site [40–42], suggesting that the hydrolysed esters, bearing a free carboxylic acid, are not good substrates for this enzyme family.
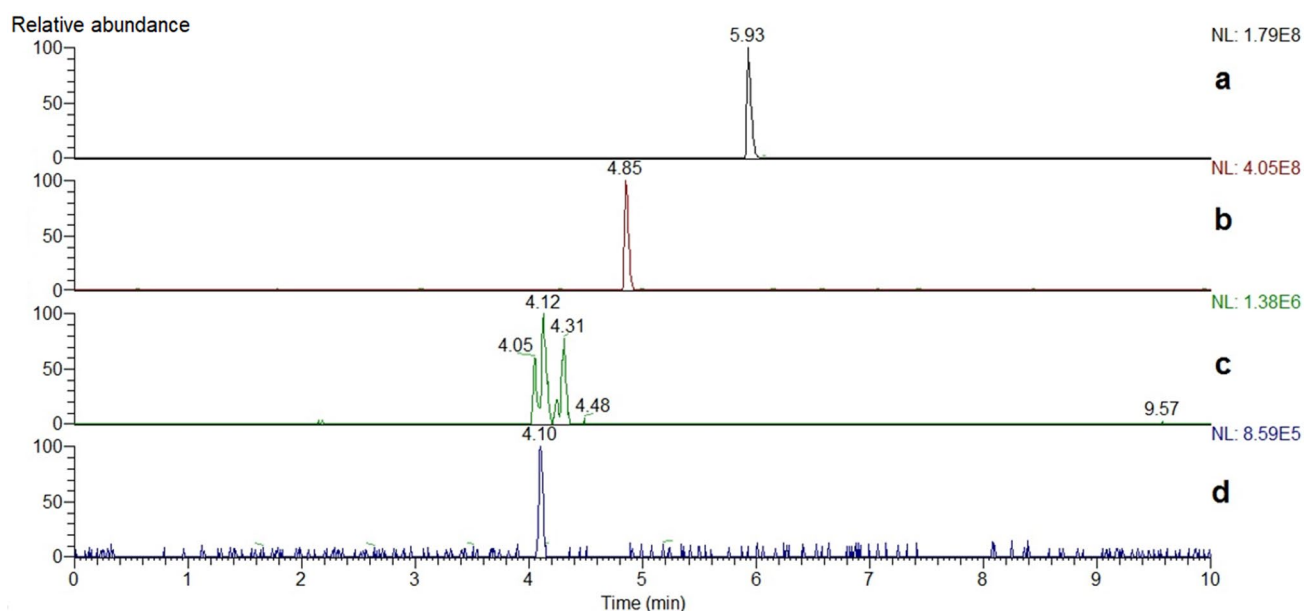
Fig. 6 Overlaid extracted ion chromatograms (exact mass ± 2.5 ppm) of 5F-NPB-22 HLM-derived metabolites (without ethanol): **a** = parent drug 5F-NPB-22; **b** = ester hydrolysis (**12**); **c** = ester hydrolysis with monohydroxylation; **d** = ester hydrolysis with oxidative defluorination


**Fig. 7** Proposed metabolic pathways for 5F-NPB-22 (**a–d** refer to Fig. 6)

Biological evaluation of PB-22, NPB-22, 5F-PB-22, 5F-NPB-22, their ethyl esters and their ester hydrolysis products

All parent compounds (PB-22, NPB-22, 5F-PB-22, 5F-NPB-22) showed a concentration dependent response in both the CB_1 and CB_2 receptor activation assays (Fig. 9). The EC_{50} and E_{max} values were determined as a measure of relative potency and efficacy, respectively (see supplementary material S5.1). Both the EC_{50} and E_{max} values of PB-22
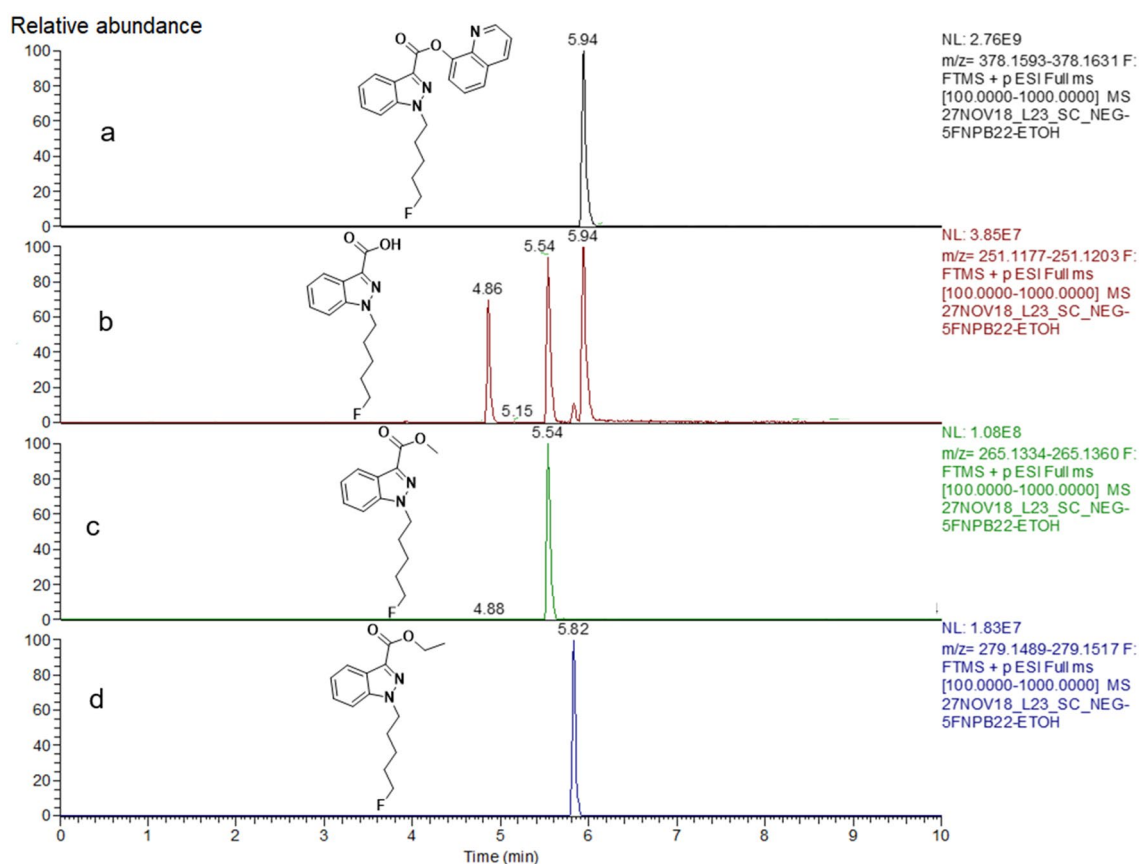
Fig. 8 Overlaid extracted ion chromatograms (exact mass ± 2.5 ppm) of 5F-NPB-22 in the presence of methanol and ethanol (without HLM); **a** = 5F-NPB-22; **b** = hydrolysis of 5F NPB-22; **c** = methanolysis of 5F-NPB-22; **d** = ethanolysis of 5F-NPB-22
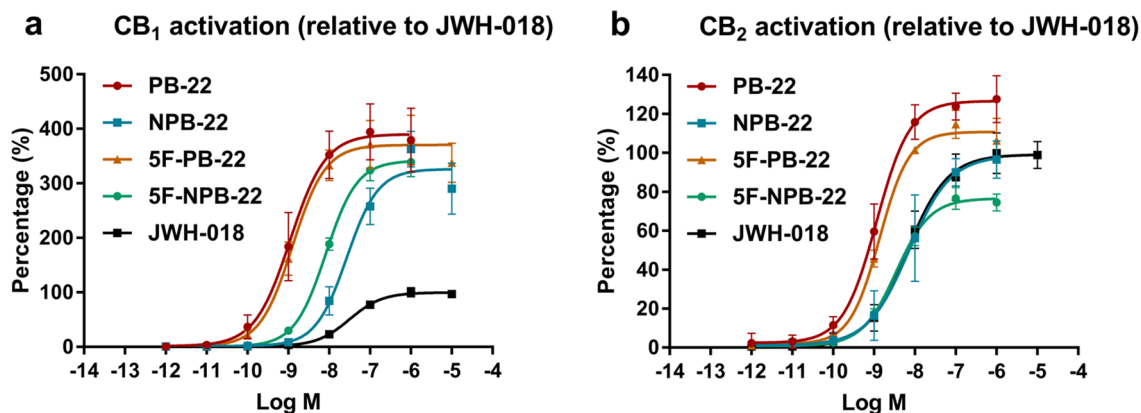

Fig. 9 Concentration-dependent response at CB_1 (**a**) and CB_2 (**b**) upon stimulation with different SCs. Data are given as mean receptor activation (%) ± standard deviation (SD) (n = 5–8), normalised to the E_{max} of JWH-018

and 5F-PB-22 were in good correspondence with the previously reported result using the same reporter assays [43].

The ester hydrolysis products and ethyl esters were also evaluated at both CB receptors (except the 5F-PB-22 hydrolysis product as this was not available for analysis, but has previously been reported to have no activity at CB_1 and CB_2 [43]). At CB_1 receptors, hydrolysis resulted in a strong reduction of the activity (to < 1% that of JWH-018) (Fig. 10a, exact values in supplementary material S5.2). The ethyl esters of PB-22 and 5F-PB-22 also showed a strongly

Fig. 10 The maximal effect obtained at CB_1 (**a**) and CB_2 (**b**). Bars assigned with an asterisk are not significantly different from basal levels. Data are given as the mean percentage CB receptor activation (in comparison to the E_{max} of JWH-018) $\pm$ SD ($n = 5$–8). *EtOH* ethanol
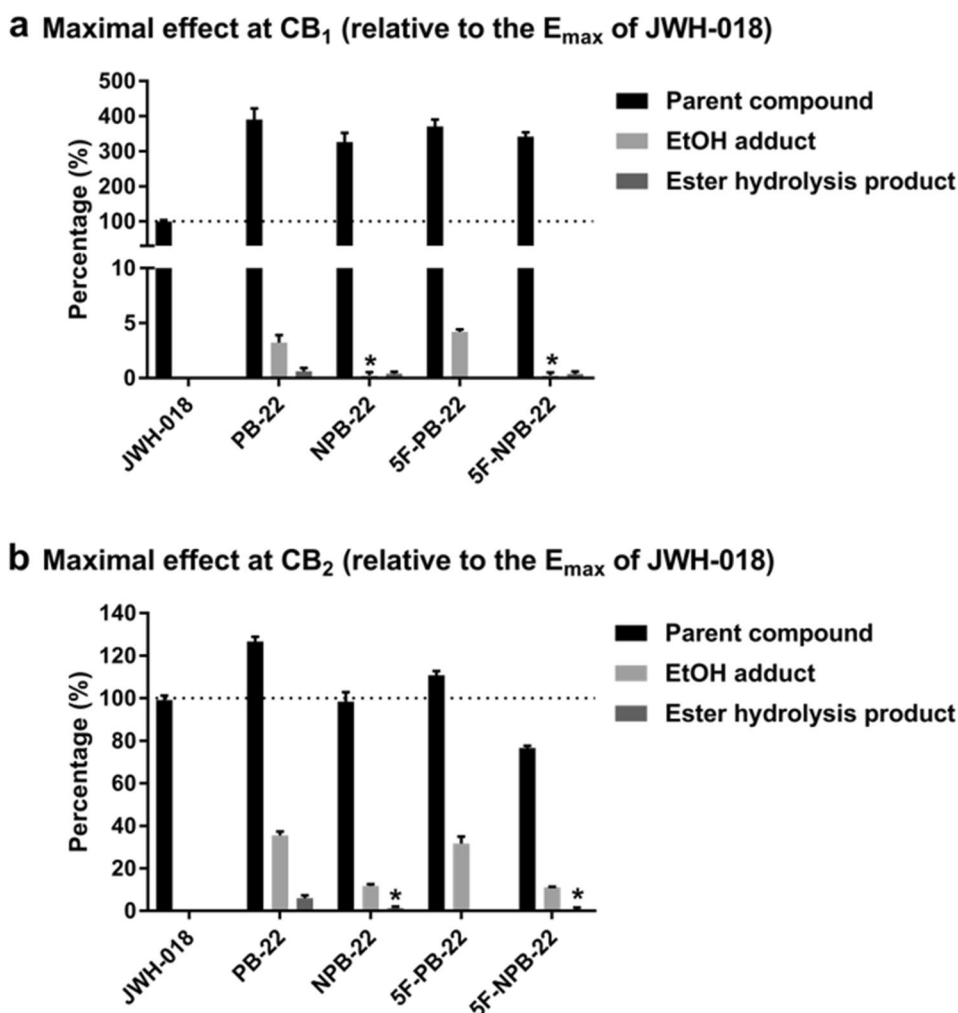
reduced CB_1 activation (3.2 and 4.2% for the PB-22 and 5F-PB-22 ethyl esters, respectively). No significant CB_1 receptor activation was found for the indazole (NPB) derivatives. When compared with the E_{max} of the parent compounds, the relative activity of the ester hydrolysis products did not exceed 0.16%, while that of the ethyl esters did not exceed 1.1%.

With respect to CB_2 activity, hydrolysis of the ester in the parent compound resulted in a strong reduction of the activity: only 6% CB_2 receptor activation for the ester hydrolysis product of PB-22 was found and an almost complete loss in CB_2 receptor activation was found for the ester hydrolysis products of NPB-22 and 5F-NPB-22 (Fig. 10b, exact values in supplementary material S5.3). For PB-22, the ethyl esters resulted in a reduced CB_2 activation compared to the parent compound, although still a 35.5% CB_2 receptor activation (relative to JWH-018) was found at high concentrations (1 μM). The ethyl ester of 5F-PB-22 resulted in a 31.7% CB_2 activation. For the ethyl esters of the NPB-derivatives, 11.7 and 11% CB_2 receptor activation was found. When

compared to the E_{max} of the parent compounds, the activity of the ester hydrolysis products did not exceed 4.73%, while that of the ethyl esters did not exceed 28.6%. Because of the limited receptor activation at high concentrations, the contribution of these ethyl esters to the pharmacological profile of SCs is expected to be minimal. This is distinct from the CB_1 and CB_2 receptor activation by the hydroxyl-metabolites of (5F)-PB-22 (hydroxy-group on the pentyl side chain), where much higher activities (> 100% E_{max} JWH-018) were found [43].

Conclusions

Polydrug use is very common amongst illicit and recreational drug users. Here, we have shown in vitro evidence of the formation of SC-ethyl esters through an hCES-catalysed transesterification. Given that there are a number of reports which describe fatal and nonfatal cases in which toxicological findings are indicative of combined use of SCs and

ethanol, it is suggested that clinical and forensic laboratories should monitor the potential appearance of the reported ethyl esters and/or their hydroxylated metabolites to facilitate interpretation of such cases. It is worth mentioning that detection of the described ethyl esters may be associated with several ester-containing SCs (common metabolites to a number of SCs); therefore, their detection should be accompanied by the concomitant presence of parent drugs (often difficult to be detected in urine) or appropriate metabolites that are unique to a certain SC to avoid any ambiguous reporting. The elimination profiles are needed to understand their potential contribution to toxicity in humans using SCs, especially if they prove to have a longer half-life than the parent compound. However, the ethics of administration of these unlicensed substances to humans makes this difficult, and the relevance of animal studies is questionable.

Compliance with ethical standards

Conflict of interest Paul Dargan is a member of the Scientific Committee of the European Monitoring Centre for Drugs and Drug Addiction and Chair of the Scientific Committee of the European Association of Poisons Control Centres and Clinical Toxicologists. All other authors have no conflict of interest to declare.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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