Archives of Toxicology (2024) 98:3337–3350
https://doi.org/10.1007/s00204-024-03830-2

MOLECULAR TOXICOLOGY

Interpreting mono- and poly-SCRA intoxications from an activity-based point of view: JWH-018 equivalents in serum as a comparative measure

Liesl K. Janssens[1] · Michaela J. Sommer[2,3,4] · Katharina Elisabeth Grafinger[2,5] · Maren Hermanns-Clausen[3,6] · Volker Auwärter[2,3] · Christophe P. Stove[1]

Received: 1 May 2024 / Accepted: 25 July 2024 / Published online: 8 August 2024

Abstract

Synthetic cannabinoid receptor agonists (SCRAs) are a class of synthetic drugs that mimic and greatly surpass the effect of recreational cannabis. Acute SCRA intoxications are in general difficult to assess due to the large number of compounds involved, differing widely in both chemical structure and pharmacological properties. The rapid pace of emergence of unknown SCRAs hampers on one hand the timely availability of methods for identification and quantification to confirm and estimate the extent of the SCRA intoxication. On the other hand, lack of knowledge about the harm potential of emerging SCRAs hampers adequate interpretation of serum concentrations in intoxication cases. In the present study, a novel comparative measure for SCRA intoxications was evaluated, focusing on the cannabinoid activity (versus serum concentrations), which can be measured in serum extracts with an untargeted bioassay assessing ex vivo CB_1 activity. Application of this principle to a series of SCRA intoxication cases ($n=48$) allowed for the determination of activity equivalents, practically entailing a conversion from different SCRA serum concentrations to a JWH-018 equivalent. This allowed for the interpretation of both mono- ($n=34$) and poly-SCRA ($n=14$) intoxications, based on the intrinsic potential of the present serum levels to exert cannabinoid activity (cf. pharmacological/toxicological properties). A non-distinctive toxidrome was confirmed, showing no relation to CB_1 activity. The JWH-018 equivalent was partly related to the poison severity score (PSS) and causality of the clinical intoxication elicited by the SCRA. Altogether, this equivalent concept allows to comparatively and timely interpret (poly-)SCRA intoxications based on CB_1 activity.

Keywords Bioassay · Biological matrices · Serum · Patient · Cannabinoid · CB_1

✉ Christophe P. Stove
christophe.stove@ugent.be

1 Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ghent, Belgium

2 Institute of Forensic Medicine, Forensic Toxicology, Medical Center, University of Freiburg, Freiburg, Germany

3 Faculty of Medicine, University of Freiburg, Freiburg, Germany

4 Hermann Staudinger Graduate School, University of Freiburg, Freiburg, Germany

5 Institute of Chemistry and Bioanalytics, University of Applied Sciences and Arts Northwestern Switzerland, Muttenz, Switzerland

6 Department of General Pediatrics, Adolescent Medicine and Neonatology, Poisons Information Center, Center for Pediatrics, Medical Center, University of Freiburg, Freiburg, Germany

Introduction

SCRAs are chemically synthesized drugs that interact with the cannabinoid receptors CB_1 and CB_2, mimicking (and often greatly exceeding) the cannabimimetic effects of Δ^9-tetrahydrocannabinol (Δ^9-THC), the primary psychoactive component in cannabis (Winstock and Barratt 2013; Castaneto et al. 2014; De Oliveira et al. 2023). These synthetic variants were initially developed in the 1970s to study the endocannabinoid system and to explore potential therapeutics (Castaneto et al. 2014; De Luca and Fattore 2018; Sholler et al. 2020). However, many SCRAs have found their way into the recreational drug market, spearheaded by JWH-018, a preclinical compound which was confirmed in an unregulated recreational drug product in Germany in 2008 (Auwärter et al. 2009). JWH-018 and CP47,497-C8 became the first SCRAs identified in these herbal blends and

were soon classified as new psychoactive substances (NPS) (European Monitoring Centre for Drugs and Drug Addiction 2009; Auwärter et al. 2009). Over fifteen years later, SCRAs are the largest class of NPS, comprising of more than 245 substances monitored by the European Monitoring Centre for Drugs and Drugs Addiction (EMCDDA), creating a highly dynamic drug market with compounds disappearing and emerging continuously (European Monitoring Centre for Drugs and Drug Addiction 2023).

SCRAs are generally more active at the cannabinoid receptors than Δ^9-THC (Banister and Connor 2018). This entails a greater risk of toxicity by increasing the chance of side effects of SCRAs in comparison to Δ^9-THC (De Oliveira et al. 2023). SCRAs have appealed to both cannabis and polydrug users as they are perceived to be safer than other drugs of abuse, easily accessible over the internet and may avoid positive cannabinoid urine tests (Vandrey et al. 2012; Castaneto et al. 2014; De Oliveira et al. 2023). The worldwide use and the greater toxicity associated with SCRAs is evident from the numerous case reports and observational studies, reporting on various toxicities for different SCRAs, as recently reviewed by De Oliveira et al. (De Oliveira et al. 2023). Many different SCRAs have been listed in intoxication reports, including AB-CHMINACA, ADB-CHMINACA, AB-FUBINACA, MDMB-CHMICA, 5F-ADB, JWH-018 and 5F-AKB-48 (or 5F-APINACA), which have been associated with a wide range of toxicological effects (De Oliveira et al. 2023).

Importantly, SCRAs encompass a large number of highly diverse compounds, both in terms of chemical structure as well as in terms of pharmacological properties (Banister and Connor 2018; Banister et al. 2019; Cannaert et al. 2020b; Grafinger et al. 2021b). On one hand, the rapid pace of structural evolution hampers the effective monitoring, analysis, and characterization of SCRAs. On the other hand, the evident wide range in pharmacological properties of SCRAs, including highly potent and highly efficacious compounds, impedes clinical understanding of SCRA intoxications. Substantial efforts are being made with structure–activity relationship studies to understand (and predict) the corresponding changes in pharmacology induced by (small) differences in chemical structure (Wiley et al. 1998; Noble et al. 2019; Sparkes et al. 2021; Grafinger et al. 2021a, b; Pike et al. 2021; Janssens et al. 2023). Timely characterization is of utmost importance to identify harmful compounds, aid clinical interpretation and guide prioritization of legislative efforts. In addition, pro-active analysis (e.g., untargeted screening with activity-based assays/high resolution mass spectrometry (HRMS)) and timely development of identification and quantification methods (mass spectrometry) for SCRAs are invaluable to get a hold of upcoming/ongoing public health threats and aid clinical diagnosis (confirmation) (Cannaert et al. 2018, 2019; Janssens et al. 2022a). Smaller doses of SCRAs are linked to higher degrees of toxicity (as compared to Δ^9-THC), however, the danger (extent of toxicity and harmful dose) is hypothesized to depend highly on the individual SCRA and its pharmacological profile (De Oliveira et al. 2023). Acute SCRA intoxications are, therefore, difficult to identify/interpret for multiple reasons: (i) challenging clinical diagnosis because of unexpected effects and absence of a distinct toxidrome to identify SCRA intoxications, (ii) rapid pace of emergence of unknown SCRAs and the (possibly) delayed availability of identification/quantification methods to confirm and estimate the extent of the SCRA intoxication, (iii) (possible) lack of knowledge on the SCRA pharmacology and harm potential, including toxic doses, (iv) challenging interpretation of (frequent) poly-SCRA intoxications.

SCRA concentrations in blood/plasma/serum do not provide a full picture of the extent of a SCRA intoxication, since the latter also requires insight into the SCRAs' harm potential and the combined effect of multiple SCRAs. In a recent study involving mono-SCRA intoxications with 5F-MDMB-PICA, we linked the concentrations in serum to the ex vivo cannabinoid activity, as measured with a functional assay applied to serum sample extracts and predicted by pharmacological profiling of 5F-MDMB-PICA (Janssens et al. 2022b). In the present study, we took this one step further and aimed at comparatively evaluating recent SCRA intoxications involving different SCRAs and including both mono- and poly-SCRA intoxications.

To aid the (clinical) interpretation of individual intoxication cases, we propose in this study the concept of *activity equivalents* as a means to estimate and compare the extents of SCRA intoxications from a cannabinoid activity point of view. For the present study, a unique data set of SCRA intoxications from the Poisons Information Center Freiburg was used, combined with serum SCRA concentrations and assessment of ex vivo CB_1 activation in serum with an in vitro cell-based assay. JWH-018 was used as the 'reference SCRA' for normalization to provide a comparative and comprehensible measure to express the cannabinoid activity (i.e., *JWH-018 equivalents*), since this firstly identified SCRA is widely used as a reference compound in SCRA research. This activity equivalent concept considers a combination of contributing toxicity factors (SCRA concentrations, receptor activation potential, etc.) for the estimation of the extent of an intoxication. We validated the relationship between the *measured* JWH-018 equivalent$_{MEAS}$ and the SCRA concentrations and SCRAs' intrinsic pharmacology, using a mathematical approach to derive a *calculated* JWH-018 equivalent$_{CALC}$. We hypothesize that the use of this concept should facilitate the comparative evaluation of intoxications involving one or multiple SCRAs of various origins and with different pharmacological properties, without requiring prior pharmacology investigation.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM GlutaMAX™), Opti-MEM I Reduced Serum Medium (Opti-MEM I), penicillin–streptomycin (5 000 U/mL) and amphotericin B (250 µg/mL) were supplied by Thermo Fisher Scientific (Merelbeke, Belgium). JWH-018 (1-pentyl-3-(1-naphthoyl)-indole) was purchased from Cayman Chemical Company (Ann Arbor, MI, US). Deionized water was prepared in-house using a Medica® Pro deionizer from ELGA (Celle, Germany) in Freiburg (Germany). Fetal bovine serum (FBS), sodium bicarbonate and poly-D-lysine were obtained from Sigma Aldrich (Overijse, Belgium). The Nano-Glo® Live Cell reagent was procured from Promega (Madison, WI, US). For sample preparation in Belgium, hexane and ethyl acetate were purchased from CHEM-LAB NV (Zedelgem, Belgium) and sodium carbonate (≥ 99.5%, anhydrous) was purchased from Merck (Hoeilaart, Belgium). Methanol (MeOH) (HiPerSolv CHROMANORM®) and acetonitrile (ACN) (HiPerSolv CHROMANORM®) were purchased from VWR Chemicals (Leuven, Belgium). For the analytical analysis in Freiburg, ethyl acetate (p.a.) was obtained from Honeywell Riedel-de Häen® (Seelze, Germany), sodium hydrogen carbonate (≥ 99.5%, anhydrous) and formic acid (p.a.) were from Carl Roth GmbH (Karlsruhe, Germany) and ammonium formate was obtained from Sigma–Aldrich (Steinheim, Germany).

Intoxication cases

This study included intoxication cases from a prospective observational study of patients treated in the emergency departments after the consumption of an NPS, as previously described (Hermanns-Clausen et al. 2018; Sommer et al. 2022). Roughly 250 German emergency departments (mainly in southern Germany) reported to the Poisons Information Center Freiburg about NPS intoxications. Study inclusion criteria for the observational study were the following: patients treated in an emergency department after reported or suspected NPS intake and whose treating physician contacted the Poisons Information Center. Patient recruitment was conducted with informed consent and for patients aged under 18, the consent of the caregivers was obtained. Physicians recorded clinical symptoms and follow-up information through a structured questionnaire. Collected serum samples and completed questionnaires were sent to the Poisons Information Center Freiburg. Serum samples were analyzed by the Institute of Forensic Medicine Freiburg via liquid chromatography coupled to tandem mass spectrometry (LC–MS/MS). The study was conducted in accordance with the Declaration of Helsinki and approved by the regional ethics committee of the University of Freiburg (No. 235/13_130683). Intoxication cases that were included in this specific study are the cases for which SCRA use was confirmed via LC–MS/MS and for which the sample volume allowed for additional investigation with the bioassay.

LC–MS/MS analysis of serum samples

Sample preparation of the serum samples was performed using liquid–liquid extraction (LLE). Briefly, 200 µL of serum or blood was extracted with 200 µL ammonium formate and 1 mL ACN. Part of the supernatant (800 µL) was evaporated to dryness (N_2, 40 °C) and reconstituted in mobile phase. Quantification of the samples was performed by LC–MS/MS analysis using an Ultimate 3000RS UHPLC (Dionex, Sunnyvale, USA) coupled to a QTRAP® 6500 triple quadrupole-linear ion trap instrument (SCIEX, Darmstadt, Germany) with positive electrospray ionization (ESI). A Kinetex® C18 column (2.6 µm, 100 Å, 100 × 2.1 mm; Phenomenex, Aschaffenburg, Germany) was used for chromatographic separation. The mobile phase consisted of 1% v/v ACN, 0.1% v/v HCOOH, 2 mM ammonium formate in water (mobile phase A) and 0.1% v/v HCOOH, 2 mM ammonium formate in ACN (mobile phase B), which were prepared freshly prior to analysis. The total LC run time was 12 min, using the following gradient: mobile phase B starting at 20%, linearly increased to 60% in 1.5 min, further increased to 65% in 1.5 min, held at 65% for 1.5 min, further increased to 90% in 2.5 min, held at 90% for 2 min, and then decreased to starting conditions in 0.1 min and held for 1.9 min for re-equilibration. The autosampler was constantly kept at 10 °C and the column oven temperature was 40 °C. The injection volume was 10 µL. The mass spectrometer was operated with positive electrospray ionization in multiple reaction monitoring (MRM) mode. The scheduled multiple reaction monitoring (sMRM) method included two transitions for each analyte and one transition for the internal standard. MRM transitions were recorded in a time window of ± 22.5s around the expected retention time. For each compound, the declustering potential (DP), the entrance potential (EP), the collision energy (CE), and cell exit potential (CXP) were optimized.

Sample preparation for bioassay

Serum samples (130 to 200 µL) were mixed with 500 µL of carbonate buffer (pH 10) in a glass tube (15.5 × 100 mm). Addition of 3 mL of *N*-hexane:ethyl acetate mixture (99:1 V/V), followed by extensive mixing (1 min)

and centrifugation (10 min at 2500 rpm), allowed LLE of the SCRAs. The organic phase was transferred to another glass tube (16 × 100 mm) and evaporated to dryness at 40 °C under a stream of nitrogen (Zymark Turbovap, Zymark Ltd., Chesire, UK).

Calibration standards were prepared from blank serum, extracted as described above and post-extraction spiked with stock solutions of JWH-018 (1:1 MeOH/Opti-MEM I) to yield extract concentrations of 5, 10, 25, 50, 100, 500, 1000, 5000, 10 000 ng/mL.

Cell culture and cannabinoid reporter assay

A previously reported live cell-based reporter assay that monitors protein − protein interactions (recruitment of truncated β-arrestin 2 to the cannabinoid 1 receptor) via the NanoLuc® Binary Technology was used to assess the cannabinoid activity in the biological samples (Cannaert et al. 2018). Cells, stably expressing the CB_1 reporter system, were routinely maintained at 37 °C, 5% CO_2, under humidified atmosphere in DMEM (high glucose) supplemented with 10% heat-inactivated FBS, 100 IU/mL of penicillin, 100 g/mL of streptomycin, and 0.25 g/mL of amphotericin B (Cannaert et al. 2018). For experiments, cells were plated on poly-D-lysine coated 96-well plates at 5×10^4 cells/well and incubated overnight. The cells were washed twice with Opti-MEM I to remove any remaining FBS, and 100 µL of Opti-MEM I was added. The Nano-Glo Live Cell reagent (Promega, Madison, USA), a nonlytic detection reagent containing the cell-permeable furimazine substrate, was prepared by diluting the Nano-Glo Live Cell substrate 1:20 in Nano-Glo LCS Dilution buffer and 25 µL was added to the wells. Subsequently, luminescence was measured in a Tristar^2 LB 942 Multimode Microplate Reader (Berthold, Technologies GmbH & Co., Bad Wildbad, Germany) to establish equilibration of the signal (10–15 min). For the evaluation of biological extracts, evaporated extracts were reconstituted in 100 µL of 1:1 MeOH/Opti-MEM I, of which 10 µL was added per well. Luminescence was monitored continuously over a period of 120 min. In every experiment, two calibration curves (JWH-018, 5–10,000 ng/mL) and reconstituted blank extracts were taken along. The final concentration of methanol (3.7%) did not interfere with the viability of the cells in the short term or with the readout of the bioassay.

Data analysis

All samples and calibrators were run in duplicate. Curve fitting of the calibration curves was performed using GraphPad Prism 9 software (San Diego, CA, US) via nonlinear regression (four parameter logistic fit; 4PL). The data are represented as mean areas under the curve (AUC) ± standard error of means (SEM) with two replicates for each data point. The AUC values are corrected for interwell variability and solvent control. Curve fitting allowed to use the JWH-018 calibration curve to calculate JWH-018 equivalent$_{MEAS}$ in the biological samples (including correction for the sample enrichment) based on the detected CB_1 activity, using the following formula (Eq. 1):

$$x = c\left(\frac{a-d}{y-d} - 1\right)^{1/b}. \quad (1)$$

In this formula a is the minimal value, b is the hill's slope, c is the point of inflection, d is the maximal value, x is the concentration, and y is the AUC. Kruskal–Wallis analysis was used for comparison of the hill's slope and the point of inflection over different runs. For the determination of JWH-018 equivalents$_{MEAS}$, numerical values were only assigned for signals within the range of the calibration curve. Samples with mean AUC values above or below the range of the fitted calibration curve were classified as either too high or too low to be calculated by the bioassay, respectively. Depending on the signal obtained, samples were assigned the label > 5000 ng/mL (expressed as serum concentration, calculated assuming a starting serum volume of 200 µL) when the signal exceeded that of the highest calibrator and as 'non-quantifiable' (NQ, i.e., activity detected, but below the lowest point of the calibration curve) if the signal lay below the bottom of the sigmoidal calibration curve or if the calculated concentration was below the lowest calibrator (5 ng/mL in extract), corresponding to a serum concentration of 2.5 ng/mL (assuming a starting serum volume of 200 µL). Those samples for which the signals were not distinguishable from blanks, were labeled as 'no activity detected' (ND). The determination 'ND' or 'NQ' was based on visual interpretation of the CB_1 activation profile, objectified by use of a decision tree (Janssens et al. 2022a).

Results

LC–MS/MS analysis

Identified compounds and serum concentrations are listed for the individual samples in Supplementary Information. In total, twelve different SCRAs were quantified: 5F-ADB ($n = 14$, 0.4–9.7 ng/mL), MDMB-CHMICA ($n = 12$, 0.04–10.0 ng/mL), MDMB-4en-PINACA ($n = 10$, 0.1–0.56 ng/mL), ADB-CHMINACA ($n = 8$, 0.23–7.80 ng/mL), 5F-MDMB-PICA ($n = 6$, 0.1–45 ng/mL), AB-CHMINACA ($n = 5$, 7.3–23 ng/mL), AB-FUBINACA ($n = 3$, 0.63–4.5 ng/mL), AMB-CHMICA ($n = 1$, 0.41 ng/mL), 5F-AKB-48 ($n = 1$, 2.48 ng/mL), CUMYL-PEGACLONE ($n = 1$, 1.2 ng/mL), 4F-MDMB-BICA ($n = 1$, 5.2 ng/mL) and AB-PINACA

($n = 1$, 0.33 ng/mL). MDMB-4en-PINACA and 5F-MDMB-PICA were also detected below their lower limit of quantification (LLOQ: < 0.1 ng/mL, $n = 7$ and $n = 1$, respectively). In these cases, the LLOQ was used as quantitative result for further data analysis.

Determination of JWH-018 equivalents

An in vitro, cell-based bioassay was used to assess CB_1 activation by the SCRAs in the extracts from serum samples from individual intoxication cases (n = 48) alongside calibration curves of a reference SCRA, JWH-018 (Cannaert et al. 2020a). This bioassay measures cannabinoid activity via monitoring the recruitment of β-arrestin2 (coupled to one part of a split-nanoluciferase) to activated CB_1 (coupled to the complementing part of this split-nanoluciferase) (Cannaert et al. 2016, 2018). Functional complementation of the coupled split-nanoluciferase enables the generation of a bioluminescent signal in the presence of a substrate (furimazine), which is a direct measurement of the extent of receptor activation during the 2 h assay (Dixon et al. 2016; Cannaert et al. 2016, 2018). The intoxication samples were measured in different runs and in every run two calibration curves were included for the reference SCRA JWH-018 (extract concentrations ranging from 5 to 10,000 ng/mL). Via normalization of the AUC signals (cumulative luminescence over 2 h) to the maximal receptor activation of JWH-018 in each range, confirmation of overlap between calibration curves was established (Fig. 1). Statistical analysis did not reveal any significant difference between the hill's slopes (b, Eq. 1) and the points of inflection (c, Eq. 1) of the calibration curves from the different runs. This confirms the robustness of the bioassay towards detection of cannabinoid activity in serum extracts. As reported before, this sigmoidal calibration model enables estimation of drug concentrations with the applied bioassay (Cannaert et al. 2020a).

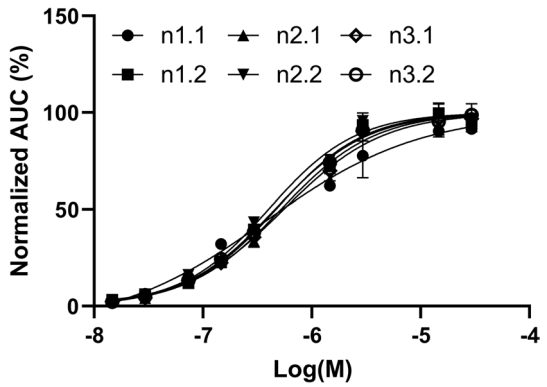

Fig. 1 Overlap of all JWH-018 calibration curves from three independent experiments, each encompassing two independently prepared sets of calibrators. Data are normalized to the maximal JWH-018 receptor activation within each separate calibration curve

The calibration model allows to derive a novel comparative measure for cannabinoid activity in the biological samples, through the determination of *activity equivalents*. Based on the bioassay signal that was obtained for the extracts of the serum samples (involving various SCRAs and metabolites) we derived "*JWH-018 equivalents*$_{MEAS}$" (Table 1), using the sigmoidal calibration model (Eq. 1). Essentially, this JWH-018 equivalent$_{MEAS}$ entails that the cannabinoid activity, which was measured in the serum extracts, relates to a certain serum concentration (x ng/mL) if the present SCRA would have been JWH-018. In other words, this JWH-018 equivalent$_{MEAS}$ would result in the same cannabinoid activity in the bioassay as the (concentration of) SCRA(s) present in the serum extract. Such JWH-018 equivalents$_{MEAS}$ could be determined in 30 samples, covering a theoretical JWH-018 range of 3.6–1048 ng/mL. Two samples showing pronounced CB_1 activity, with signals exceeding that of the fitted calibration curve, were semi-quantified as > 5000 ng/mL. In 11 samples CB_1 activity was detected, yet no numerical value could be derived as the obtained signals, though distinguishable from blanks, were below the lowest point of the JWH-018 calibration curve, with 2.5 ng/mL as corresponding JWH-018 equivalent$_{MEAS}$ (assuming a serum volume of 200 µL) (Janssens et al.

Table 1 Measured JWH-018 equivalents$_{MEAS}$ (equiv., ng/mL), in samples for individual intoxication cases. Intoxication cases were assigned an arbitrary number (#) based on increasing JWH-018 equivalent (NQ, not quantified; ND, no activity detected)

#	Equiv., ng/mL	#	Equiv., ng/mL	#	Equiv., ng/mL	#	Equiv., ng/mL	#	Equiv., ng/mL	#	Equiv., ng/mL
1	ND	**9**	NQ	**17**	3.6	**25**	7.6	**33**	16.8	**41**	37.3
2	ND	**10**	NQ	**18**	3.8	**26**	7.8	**34**	17.3	**42**	37.5
3	ND	**11**	NQ	**19**	4.6	**27**	8.3	**35**	23.0	**43**	47.2
4	ND	**12**	NQ	**20**	4.7	**28**	10.9	**36**	23.7	**44**	69.3
5	ND	**13**	NQ	**21**	4.9	**29**	11.4	**37**	24.5	**45**	78.3
6	NQ	**14**	NQ	**22**	5.8	**30**	11.5	**38**	25.1	**46**	1048
7	NQ	**15**	NQ	**23**	6.3	**31**	14.8	**39**	33.0	**47**	> 5000
8	NQ	**16**	NQ	**24**	7.2	**32**	16.2	**40**	36.1	**48**	> 5000

2022a). These samples were assigned an arbitrary JWH-018 equivalent$_{MEAS}$ of 1.25 ng/mL (0.5 times the lowest calibration point). In five samples no activity was detected—these were arbitrarily assigned 0 ng/mL (Janssens et al. 2022a). It should be noted that the same extraction efficiency (i.e., 100%) was assumed for all SCRAs in the determination of the JWH-018 equivalents$_{MEAS}$—inherently no internal standards can be taken along in the bioassays, as these would also activate CB_1. Hence, the assigned values are to be considered as 'minimal equivalents', since recoveries will most likely not be 100% for all analytes. In addition, the activity that was measured in the extracts represent the *combined activities* of all analytes—essentially (different) main compounds and metabolites, which likely also entail different extraction efficiencies.

Measured JWH-018 equivalent$_{MEAS}$—what is it related to?

While the JWH-018 equivalents$_{MEAS}$ represents an equivalent concentration of one (reference) SCRA as a comparative measure from an activity-based point of view (also referred to as activity equivalent), the measured ex vivo activity in the sample extracts represents the *combined activity* of all active compounds in the bioassay. In multiple intoxication cases, the samples included multiple/different SCRAs (n = 14) and corresponding (or other) SCRA hydrolysis products (n = 48) (Supplementary Information, (other) SCRA metabolites were not targeted by the MS-method). Depending on the receptor activation potential of each individual compound present in the sample extract, these can all contribute to the ex vivo activity that is detected with the bioassay. This study included intoxications involving 12 different SCRAs that inherently attain various capacities of activating CB_1. Pharmacological characterization of SCRAs is the subject of published and (ever-)ongoing research, given the constant emergence of new compounds and their—at the time—unknown harm potential (Noble et al. 2019; Cannaert et al. 2020b; Grafinger et al. 2021b; Deventer et al. 2022; European Monitoring Centre for Drugs and Drug Addiction 2023; Janssens et al. 2023). The resulting pharmacological profile is typically expressed in literature as potency (EC_{50}) and efficacy (E_{max}) values, obtained with a certain bioassay. To link the measured JWH-018 equivalent$_{MEAS}$ with the pharmacological profiles of the individual SCRAs, we used previously published potency and efficacy estimates, determined with the same assay format (CB_1-β-arr2 recruitment assay) as applied in this study (compiled in Supplementary Table 2, including references). Of note, these values may show some biological—and other—variation over different studies, performed at different moments in time with different batches of cell freezings and by different operators. In case of multiple studies reporting data on the same SCRA in the CB_1-β-arr2 recruitment assay, the data compiled in Supplementary Table 2 and used for calculations in this study, were (randomly) chosen, with a preference for those studies with a direct comparison to JWH-018 as a reference SCRA.

An overview of the intoxication cases is presented in Fig. 2 (analytical investigation on the top side, bioassay investigation on the lower side), showing which SCRA(s) was (were) present in each sample, and at what (molar) concentration(s). The samples are ordered based on increasing JWH-018 equivalents, illustrated by the heatmap below. This increase in JWH-018 equivalent$_{MEAS}$ corresponds with a general trend of increasing concentrations of SCRAs that were found. Additionally, the SCRAs were ordered based on increasing potency (color-coded in the legend) as this is hypothesized to be a (first) important intrinsic characteristic of SCRAs that influences the estimation of a JWH-018 equivalent$_{MEAS}$. The potency of a compound will typically show a strong inverse correlation with a typical recreational dose of that compound. Compounds with a high potency only require low doses (and, hence, corresponding low blood concentrations) to already result in a pronounced activity (and, hence, high JWH-018 equivalents$_{MEAS}$). Based on different studies in literature, the potency decreases in the following order: CUMYL-PEGACLONE (Janssens et al. 2020) > ADB-CHMINACA (Wouters et al. 2019) > MAB-CHMINACA (Wouters et al. 2019) > 5F-ADB (Wouters et al. 2019) > MDMB-4en-PINACA (Grafinger et al. 2021b) > MDMB-CHMICA (Wouters et al. 2020) > AMB-CHMICA (Wouters et al. 2020) > 5F-MDMB-PICA (Noble et al. 2019) > 5F-AKB-48 (Wouters et al. 2020) > AB-CHMINACA (Wouters et al. 2019) > AB-FUBINACA (Noble et al. 2019) > JWH-018 (Grafinger et al. 2021b) > AB-PINACA (Noble et al. 2019) > 4F-MDMB-BICA (Cannaert et al. 2020b) (Supplementary Table 2). Indeed, in Fig. 2 increasing JWH-018 equivalents$_{MEAS}$ mostly correspond to (i) higher concentrations of present SCRAs (e.g., sample 20 *versus* sample 30); or (ii) the presence of (lower concentrations of) more potent SCRAs (e.g., sample 40 versus sample 44). However, these patterns are not ever-present throughout the entire dataset, e.g., sample 45 represents a lower concentration of a less potent SCRA compared to sample 44, whereas the JWH-018 equivalent$_{MEAS}$ was somewhat higher (78 ng/mL *versus* 69 ng/mL).

The pharmacology of SCRAs is characterized by on the one hand the potency, which is related to the concentrations at which SCRAs can exert effects, and on the other hand, the efficacy, which corresponds to the extent of cannabinoid activity that can intrinsically be achieved by the SCRA. This intrinsic maximal activation potential will also influence the estimation of JWH-018 equivalents$_{MEAS}$, as many SCRAs have higher efficacies than JWH-018. A higher efficacy entails greater cannabinoid activity at concentrations equal to the EC_{50}. Generally, the estimated JWH-018 equivalents

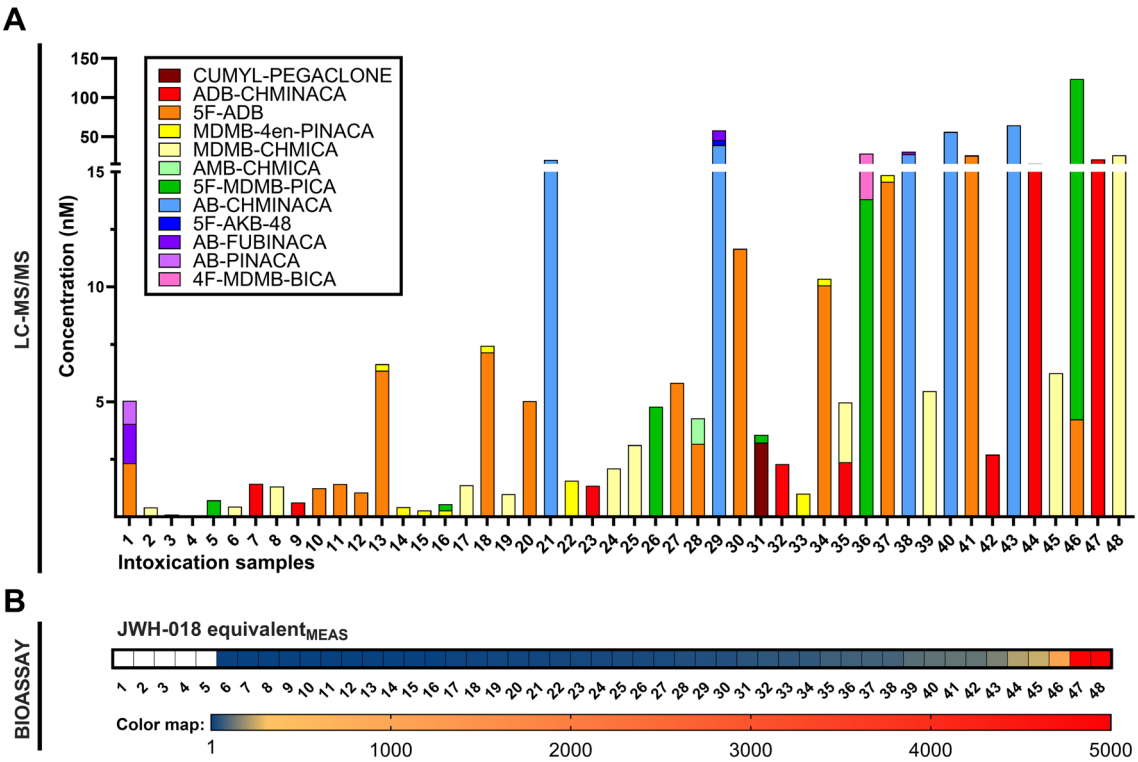

Fig. 2 Comparison of the molar serum SCRA concentrations and the JWH-018 equivalent$_{MEAS}$. **A** Molar concentrations of parent SCRAs in the intoxication serum samples. The legend and the color code are ordered based on (reported) potencies: dark red is most potent (CUMYL-PEGACLONE), light purple is least potent (4F-MDMB-BICA). **B** Heatmap of the JWH-018 equivalents$_{MEAS}$. The lower bar shows the legend for interpretation of the colors in the heatmap, covering a JWH-018 equivalent$_{MEAS}$ range of 1–5000 ng/mL

are related to the concentration of the SCRA parent compound, which was detected, and its intrinsic relative activity (Ra_i) compared to JWH-018 (Fig. 3; Supplementary Table 2, determined by Eq. 2 based on data of individual studies). This Ra_i-value represents both the potency and the efficacy of the compounds relative to a reference compound—in this case JWH-018—that is used to provide a comparative measure. A Ra_i value > 1, entails that the present SCRA is intrinsically more active than JWH-018, while a Ra_i value < 1 means that the SCRA is less active than JWH-018.

$$Ra_i = \frac{Emax_i.EC50_{REF}}{EC50_i.Emax_{REF}}. \quad (2)$$

When focusing on mono-SCRA intoxications, mostly higher JWH-018 equivalents$_{MEAS}$ are estimated in comparison to the concentrations of the parent SCRAs found in serum (Fig. 3). These concentrations are expressed as molar concentrations for adequate comparison (calculated by conversion with the corresponding molecular weight). The former observation corresponds to the fact that all SCRAs involved in these mono-SCRA intoxications are characterized by a $Ra_i > 1$, relative to JWH-018. Generally, for compounds with a $Ra_i > 1$, a lower concentration is needed to yield the same activity at CB_1 as JWH-018, while compounds with a $Ra_i < 1$ would require higher concentrations than JWH-018 to yield the same effect. For the mono-SCRA intoxications in this study, higher JWH-018 equivalents were related to either high concentrations of a present SCRA, to a high Ra_i value, or the combination of both.

Calculated JWH-018 equivalent$_{CALC}$ – validation of relationship to pharmacological parameters

Figure 3 suggests that the JWH-018 equivalent$_{MEAS}$ is related to the concentrations of the SCRA and the intrinsic relative activity. To verify this relationship, we have calculated the JWH-018 equivalent$_{CALC}$ based on the analytically determined serum concentrations and the pharmacological parameters described in literature (potency and efficacy). In case of poly-SCRA intoxications, these multiple SCRAs are present at different concentrations and will all entail a different (relative) pharmacology (Ra_i). To estimate the intrinsic contribution of the individual SCRAs to the overall ex vivo cannabinoid activity (and estimated JWH-018 equivalent$_{MEAS}$), convergence of the relative intrinsic activity and the present (molar) concentrations of SCRAs is required. We, therefore, introduce a new concept of

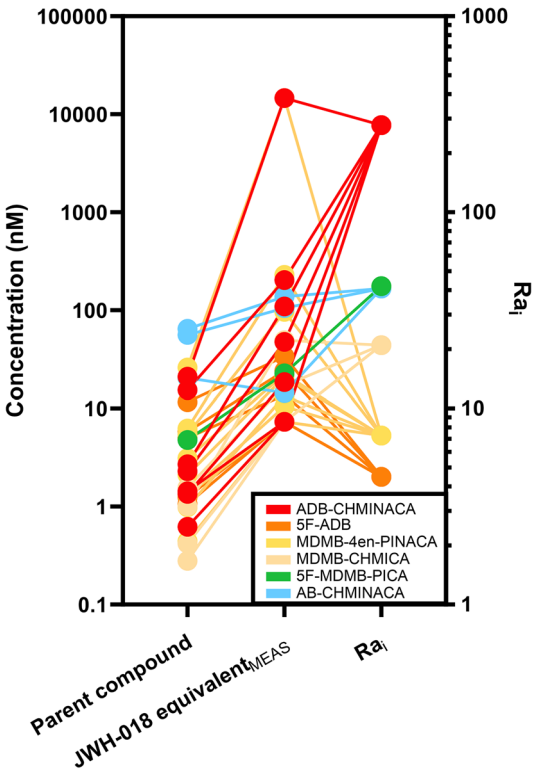

Fig. 3 Correlation of the JWH-018 equivalent$_{MEAS}$ to the serum concentration and the intrinsic relative activity (Ra_i) of the parent compound in mono-SCRA intoxications. All SCRAs involved in mono-SCRA intoxications show a higher intrinsic activity ($Ra_i > 1$) relative to reference compound JWH-018. Samples are color-coded for the SCRA detected in serum

'Estimated Intrinsic Relative activity' (EIRa, calculated by Eq. 3, Fig. 4) for straightforward prediction of the individual contributions of each SCRA. This concept is to be understood as the conversion of one SCRA concentration to the concentration of a reference SCRA based on their relative intrinsic potential to exert cannabinoid activity. In this study, the EIRa corresponds to the molar concentration (nM) of an individual SCRA that is converted to a JWH-018 concentration based on its CB_1 activation potential (using the bioassay employed here), relative to JWH-018, allowing the estimation of a theoretical contribution to the combined CB_1 activity that is measured in serum.

$$\mathrm{EIRa} = \mathrm{Ra}_i \times \mathrm{Concentration}_{\mathrm{molar.}} \quad (3)$$

A comparison of the predicted contributions (EIRa) and the measured JWH-018 equivalents$_{MEAS}$ is presented in Fig. 5. For mono-SCRA intoxications, the EIRa can be considered a *calculated* activity equivalent (JWH-018 equivalent$_{CALC}$). For poly-SCRA intoxications the calculated JWH-018 equivalent$_{CALC}$ consists of a combination of the individual contributions (EIRa). For simplicity, an additive effect is assumed in this study and contributions of co-present SCRAs are depicted as stacked bars for visual interpretation (SCRA1, SCRA2, SCRA3, ordered based on decreasing estimated EIRa). In most cases the (sum of the) predicted contribution(s) (EIRa) was higher than the corresponding JWH-018 equivalent$_{MEAS}$, as measured in the extract (Fig. 5). For intoxication case 4, no EIRa could be calculated due to the lack of parent compound that was analytically found in the confirmation analysis. For nine mono-SCRA intoxication cases (6, 17, 19, 24, 33, 39, 45, 47 and 48), the JWH-018 equivalent$_{MEAS}$ exceeded the predicted EIRa. These cases included intoxication with MDMB-CHMICA (6, 17, 19, 24, 39, 45, 48), MDMB-4en-PINACA (33) and ADB-CHMINACA (47). Whereas calculation of the EIRa allows prediction of a JWH-018 equivalent$_{CALC}$ based on the SCRA concentration and the SCRA's potency and efficacy, relative to the reference that was used to provide activity

Intrinsic relative activity: *Take into account pharmacodynamic parameters (potency & efficacy)*	$\boldsymbol{Ra_i = \frac{Emax_i \,.\, EC50_{REF}}{EC50_i \,.\, Emax_{REF}}}$	(Equation 2)
Contribution to activity in serum: *Converge pharmacodynamic properties with present serum concentrations*	$\boldsymbol{EIRa = Ra_i \; x \; Concentration_{molar}}$	(Equation 3)
Calculated JWH-018 equivalent Mono-SCRA intoxications	$\boldsymbol{JWH-018\ equivalent_{CALC} = EIRa}$	(For each SCRA in serum)
Calculated JWH-018 equivalent Poly-SCRA intoxications *Assumption of additive effects*	$\boldsymbol{JWH-018\ equivalent_{CALC} = EIRa\ SCRA\ 1 + EIRa\ SCRA\ 2 + \ \ldots}$	

Fig. 4 Method to calculate the JWH-018 equivalent$_{CALC}$ as validation approach for the correlation with serum concentrations and intrinsic pharmacology. This method includes the intrinsic relative activity (Ra_i) of each present SCRA in the intoxication sample to account for the SCRA pharmacology. Convergence of the Ra_i with the serum concentrations leads to the estimated intrinsic relative activity (EIRa). This EIRa is used as the JWH-018 equivalent$_{CALC}$ in case of mono-SCRA intoxications. For poly-SCRA intoxications, additive effects are assumed to derive the JWH-018 equivalent$_{CALC}$ by adding the individual contributions (EIRa) of the different SCRAs

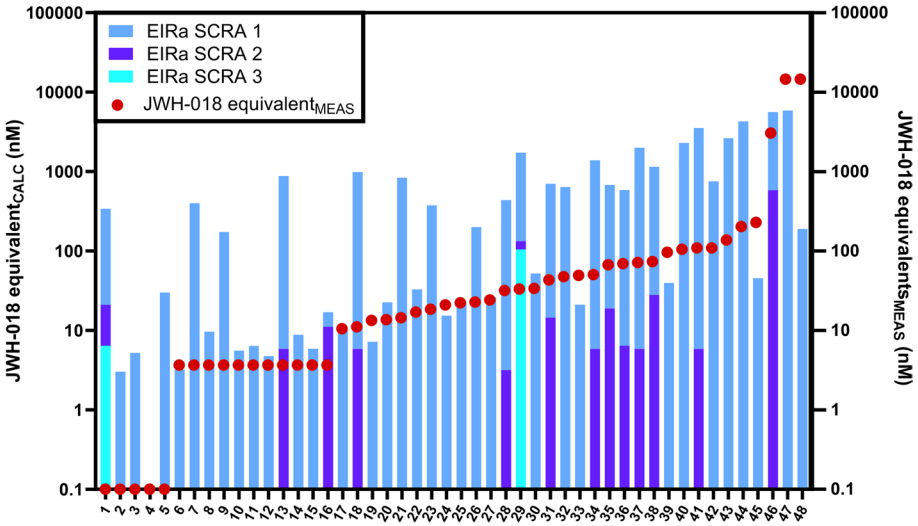

Fig. 5 Comparison of the JWH-018 equivalent$_{CALC}$ and the JWH-018 equivalent$_{MEAS}$ in mono- and poly-SCRA intoxications. The contribution of each SCRA is calculated as the estimated intrinsic relative activity (EIRa), which is plotted for the different SCRAs. This EIRa (or the sum of the EIRa of different SCRAs, in case of poly-SCRA intoxications) forms the JWH-018 equivalent$_{CALC}$. For poly-SCRA intoxications, the SCRAs were ordered based on estimated contribution (EIRa SCRA 1 > EIRa SCRA 2 > EIRa SCRA 3; do note the logarithmic scale). JWH-018 equivalents$_{MEAS}$ (otherwise reported as ng/mL) are presented in nanomolar concentrations for comparison

equivalents, it does not account for differential hill-slopes in sigmoidal pharmacological profiling, differential recovery, differential binding affinity, possible competition and/or matrix effects. Hence, these factors, in addition to metabolite activity, might explain the discrepancies.

Clinical intoxication in relation to JWH-018 equivalents$_{MEAS}$

A variety of toxicological effects was observed in the intoxicated patients at the hospital, including effects on cardiovascular functions, the central nervous system, motoric functioning, respiratory functions, glycemic status, etc. Interestingly, opposite effects were observed in different patients such as breathing pauses (temporary apnoea) or bradypnoea *versus* hyperventilation, mydriasis *versus* miosis, hyperglycemia *versus* hypoglycemia, tachycardia *versus* bradycardia and hypertension *versus* hypotension. Symptoms that were most observed (≥ 20% of the cases) were tachycardia ($n = 16$), nausea and/or vomiting ($n = 14$), epileptic seizures ($n = 12$), agitation ($n = 10$) and disorientation ($n = 10$).

The clinical presentation of the intoxicated patients was not clear-cut, as was observed before for mono-SCRA intoxications with 5F-MDMB-PICA (Janssens et al. 2022b). No patterns could be observed for frequently occurring symptoms in relation to increasing JWH-018 equivalents$_{MEAS}$ in serum (Fig. 6). However, in all but four cases polydrug use was observed, which obscures interpretation and hampers the causality assessment of presented symptoms to the specific intoxication with SCRAs. Other drugs that were detected include THC and its metabolites ($n = 18$), various first-aid drugs ($n = 8$; e.g., lidocaine, paracetamol, rocuronium), antipsychotics ($n = 9$; e.g., quetiapine, risperidone), antidepressants (n = 4; e.g., venlafaxine, paroxetine, citalopram), benzodiazepines ($n = 12$; e.g., midazolam, temazepam, oxazepam), anti-epileptics ($n = 3$; e.g., levetiracetam, pregabalin, gabapentin), cathinones ($n = 9$; e.g., 3-MMC, pentylone), opioids ($n = 3$; e.g., morphine, fentanyl), MDMA ($n = 6$), and amphetamine ($n = 5$). Additionally, a withdrawal syndrome was the suspected diagnosis in five cases, which also hampers the assignment to toxicity of SCRAs. Some symptoms were only documented in single cases. In Fig. 6 these symptoms were ordered within each category (i.e., clinical or laboratory) based on the (increasing) corresponding JWH-018 equivalent$_{MEAS}$ that was measured in serum: epistaxis, cyanosis, somnolence, euphoria, aphasia, rhabdomyolysis, hypoglycemia, miosis (pinpoint pupils), leukocytosis, exsiccosis, double vision and (initially) increased potassium levels. The latter two observations were only noted in two intoxicated patients with very high serum activity equivalents (> 5000 ng/mL JWH-018). These latter two cases also had the highest number of documented symptoms (8 different symptoms). Only for one other intoxication case (sample 30, corresponding with an activity of 11.5 ng/mL JWH-018) seven different toxicological effects were observed.

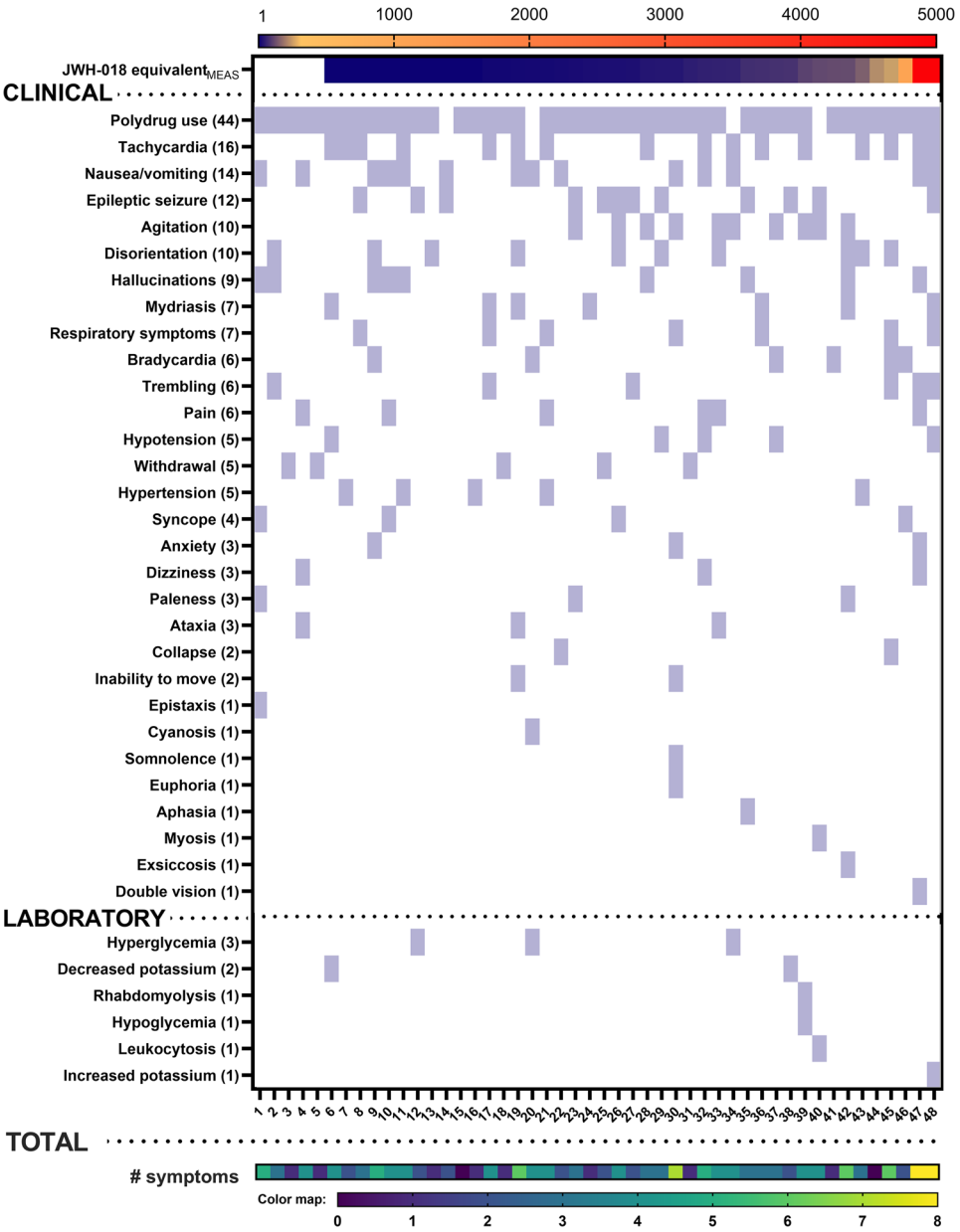

Fig. 6 Symptoms (or observations) in intoxicated patients with confirmed SCRA use, with the patients ranked based on the corresponding JWH-018 equivalents$_{MEAS}$ in serum. The JWH-018 equivalents$_{MEAS}$ are indicated in the upper heatmap. Clinical and laboratory-based symptoms and observations are ordered from top to bottom by the frequency of occurrence (indicated by the boxes). The total number of observed symptoms per patient is indicated in the lower heatmap. The term 'respiratory symptoms' subsumes breathlessness ($n=4$), bradypnoea ($n=1$), short episodes of apnoea ($n=1$) and hyperventilation ($n=1$)

Toxicity assessment in relation to JWH-018 equivalents$_{MEAS}$

Even though polydrug use was frequently observed in this case series, causality of the intoxication symptoms and SCRA intake was in most cases probable or certain. The severity of the poisoning was also evaluated by assigning a poisoning severity score (PSS) to every intoxication, ranging from 1 (minor), 2 (moderate) to 3 (severe) (Persson et al. 1998). No fatal intoxications (PSS = 4) were included in this study. The correlation of the PSS grade with the JWH-018 equivalents$_{MEAS}$ was analyzed by plotting the JWH-018 equivalents$_{MEAS}$ of the intoxication cases per PSS score (Fig. 7). In addition, we indicated the causality (not assessable, probable or certain) on the plot. On one hand, this allowed us to derive tentative thresholds: a JWH-018 equivalent$_{MEAS}$ > 24 ng/mL always resulted in PSS > 1, whereas a JWH-018 equivalent$_{MEAS}$ > 47 ng/mL always corresponded to certain causality. On the other hand, a wide range of JWH-018 equivalents$_{MEAS}$, including both low and high equivalents (1.25–5000 ng/mL), corresponded to PSS of 2 or 3 and certain causality assessment for the present SCRAs. Hence, whereas a low JWH-018 equivalent$_{MEAS}$ wasn't necessarily associated with a low PSS, a high JWH-018 equivalent$_{MEAS}$ (above the observed thresholds) always resulted in more severe poisoning and it was associated with a certain causality of the SCRA to the intoxication. The tentative thresholds could, therefore, be seen

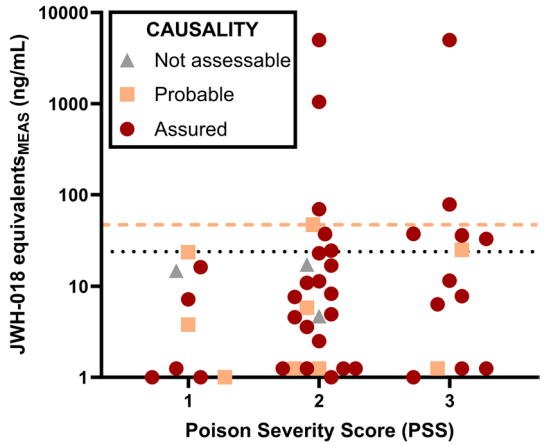

Fig. 7 Toxicity appraisal of SCRAs in intoxication cases (PSS, causality), in relation to the JWH-018 activity equivalents$_{MEAS}$ in serum. The determined thresholds of JWH-018 equivalents$_{MEAS}$ corresponding to the upper limits for PSS = 1 (black, 24 ng/mL) or probable causality (orange, 47 ng/mL) are illustrated by a dotted and a dashed line, respectively

as upper limits for, respectively, minor poison severity and the SCRA being associated to the symptoms with probable causality. It is important though to remark that the JWH-018 equivalents$_{MEAS}$ were not homogenously distributed throughout the dataset, with 12 samples exceeding the JWH-018 equivalent$_{MEAS}$ cut-off of 24 ng/mL (3 of which were above 1000 ng/mL), 11 samples with an arbitrarily assigned JWH-018 equivalent$_{MEAS}$ of 1.25 ng/mL and 5 samples for which no ex vivo cannabinoid activity was detected (arbitrarily assigned as 0 ng/mL). While this imbalance might reflect the real-life situation (i.e., less intoxications with a very high ex vivo cannabinoid activity), it could also skew the data, rendering the proposed thresholds as 'tentative'.

Discussion

Forty-eight SCRA intoxication cases were investigated from three points of view: (i) serum levels of SCRAs were analytically determined with LC–MS/MS; (ii) the ex vivo cannabinoid activity of serum extracts was assessed via a CB_1 bioassay to determine JWH-018 equivalents$_{MEAS}$; and (iii) the clinical presentation of the patients reported by the treating physicians, followed by a grading of the reported symptoms according to PSS as well as a causality assessment, was evaluated by the consulted physicians in the Poisons Information Center. In total, 12 different SCRAs were detected, at concentrations that were generally in line with those reported in literature for non-fatal intoxications (Adamowicz and Gieroń 2016; Allibe et al. 2017; Mohr et al. 2022; Janssens et al. 2022b; De Oliveira et al. 2023). A variety of symptoms were observed in the patients, corresponding to a non-distinctive toxidrome of SCRAs, as reported before (De Oliveira et al. 2023). Opposing symptoms were observed amongst patients and similarities with the clinical syndrome of other drug classes could be observed, such as reduced respiratory rate (*cf.* opioid intoxication) (De Oliveira et al. 2023). Importantly, polydrug use was largely present amongst the patient cohort and should be considered as a confounder in the clinical assessment.

The concept of activity equivalents was put forward after demonstrating the ability of the bioassay used here to (semi-)quantify concentrations based on sigmoidal calibration models (Cannaert et al. 2020a; Janssens et al. 2022b). In this study, we used the prototypical SCRA JWH-018 as a reference to express the CB_1 activating potential of serum extracts (containing a variety of SCRAs) in a comprehensible and uniform manner, by referring to the extent of cannabinoid activity as JWH-018 equivalents$_{MEAS}$. Hence, all ex vivo cannabinoid activities that were measured in the serum extracts could be expressed as a JWH-018 equivalent$_{MEAS}$, as long as they were within the sigmoidal window of the calibration model.

The ex vivo cannabinoid activity measured in the bioassay is hypothesized to depend on (i) the concentrations of individual compounds present in the samples, (ii) the intrinsic CB_1 receptor activation potential of these compounds, and (iii) possibly competition between co-present compounds based on differential binding affinity. Whereas the Ra_i concept is usually used as a means to evaluate and express signaling bias, it allowed us to put the pharmacological profile (potency and efficacy combined) of the SCRAs in perspective relative to JWH-018, the reference compound used in this study (Wouters et al. 2020). Interestingly, all but one (4F-MDMB-BICA) of the SCRAs involved in the intoxication series of the current study are reported to be more active than JWH-018 (i.e., $Ra_i > 1$) (Noble et al. 2019; Wouters et al. 2019, 2020; Janssens et al. 2020; Cannaert et al. 2020b; Grafinger et al. 2021b). This is an important explanation as to why the JWH-018 equivalents$_{MEAS}$ are higher molar concentrations than the molar SCRA concentrations as derived through LC–MS/MS analysis of the serum samples. We next combined the SCRAs' calculated relative activity with their (molar) concentrations, to establish the new concept of Estimated Intrinsic Relative Activity (EIRa). By doing this for the individual SCRAs and combining the calculated theoretical contributions, a preliminary prediction of the *combined activity* of all SCRAs contained in a sample could be obtained, expressed as a JWH-018 equivalent$_{CALC}$. This newly introduced EIRa is related to the Estimated Intrinsic Potency/Efficacy (EIP/EIE) as proposed by Antonides et al., which was previously used to comparatively evaluate the CB_1 activity contained in seized drug samples and infused paper involving different SCRAs at different concentrations (Antonides et al. 2019, 2021).

The EIRa is essentially a combination of both EIP and EIE as it combines information on a substance's potency and efficacy. Using this EIRa to estimate the contributions of the quantified SCRAs and mathematically derive a JWH-018 equivalent$_{CALC}$ largely led to an overestimation (i.e., calculated JWH-018 equivalent$_{CALC}$ > measured JWH-018 equivalent$_{MEAS}$). A logical explanation for this lies in an incomplete recovery during the sample preparation and a matrix effect of the serum in the bioassay, as was previously shown when assessing the ex vivo cannabinoid activity in serum from mono-intoxications involving 5F-MDMB-PICA (Janssens et al. 2022b). In poly-SCRA intoxications, the presence of multiple SCRAs can additionally lead to competition between compounds with potentially different binding affinities, hence not leading to additive effects, as assumed here (Janssens et al. 2022b). In 9 cases the measured JWH-018 equivalent$_{MEAS}$ exceeded the EIRa. Besides limitations of the utilized concept, this could also be related to the co-presence of high concentrations of active metabolites that were not quantified in this study or to the co-presence of other SCRAs, which were not contained in the applied method (Cannaert et al. 2016; Gamage et al. 2019; Cabanlong et al. 2022). Of note, the identified metabolites and/or degradation products in this study were hydrolysis products, which have been shown to generally retain much lower CB_1 activation potential in comparison to the parent compound (Noble et al. 2019; Wouters et al. 2019). It is hypothesized that correction for incomplete recovery and for matrix effects could improve the concurrence between the predicted SCRA contributions as EIRa and the JWH-018 equivalent$_{MEAS}$, as was shown before (Janssens et al. 2022b). However, determination of the recovery of each individual SCRA in this study for the bioassay sample preparation method was outside the scope of the analytical investigation and not enough sample volume was available to study the matrix effects in the bioassay.

The JWH-018 equivalent$_{MEAS}$ is determined by running sample extracts alongside a calibration model. In essence, this allows to kill two birds with one stone to keep up with the dynamic NPS market. First, the application of serum on the bioassay allows a universal screening for the involvement of SCRAs in the intoxication, irrespective of the compound structure and mass spectral libraries (Cannaert et al. 2019; Janssens et al. 2022a). Secondly, the co-presence of the calibration model allows to interpret the extent of the intoxication from a CB_1 activity-based point of view, potentially even prior to identifying the SCRA involved and without any knowledge on its harm potential—presuming the latter is linked to CB_1 activation. The JWH-018 equivalent$_{MEAS}$ showed no clear relationship with the clinical presentation in terms of symptoms that were observed, apart from the fact that those intoxications with the highest JWH-018 equivalents$_{MEAS}$ presented with the highest number of symptoms. This lack of relationship between the JWH-018 equivalent$_{MEAS}$—determined based on CB_1 activity—and clinical features, could be related to CB_1-unrelated pharmacological effects of the different SCRAs. Further insight into the pharmacology of SCRAs at other molecular targets could shed further light on this. The toxicity assessment was partly related to the measured JWH-018 equivalent$_{MEAS}$, as upper limits for probable causality and minor intoxication symptoms could be observed, yet no lower limit for severe intoxications with a certain causality could be concluded. While these tentative thresholds support the link of high JWH-018 equivalents$_{MEAS}$ being linked to more severe intoxications, the inverse cannot be concluded, i.e., low JWH-018 equivalents$_{MEAS}$ were not necessarily associated to minor symptoms in intoxications—obviously, the concomitant presence of other drugs in most intoxication cases, as well as a possible delay between clinical presentation and sample collection are only some of the obfuscating factors that may explain this apparent discrepancy.

In conclusion, JWH-018 equivalents are proposed as a comparative measure to study mono-SCRA and poly-SCRA intoxications from a cannabinoid activity point of view. The measured JWH-018 equivalents$_{MEAS}$ were shown to be linked to both the concentration of the parent compound and the intrinsic CB_1 activity (in terms of potency and efficacy) relative to the reference JWH-018. This correlation was verified by comparing a mathematically derived JWH-018 equivalent$_{CALC}$ with the measured JWH-018 equivalent$_{MEAS}$. The JWH-018 equivalent$_{MEAS}$ showed to be related (in part) to the severity of symptoms according to PSS and the causal relationship of the SCRA to the acute drug intoxication. The clinical presentation of intoxicated patients confirmed a non-distinctive toxidrome of SCRAs with a high variety of toxicological symptoms and no evident relationship to the CB_1 activity in serum. Activity equivalent determination can aid in the identification of SCRA intoxications (untargeted screening), while simultaneously allowing evaluation of the extent of an intoxication (based on cannabinoid activity), without prior knowledge on the individual SCRA's pharmacological profile or harm potential. In those cases, exceeding a certain activity threshold it may guide the interpretation. In addition, the proposed concept allows (better) comparison of different SCRA intoxications potentially involving newly emerging SCRAs using a known reference SCRA as a comparative measure.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00204-024-03830-2.

Acknowledgements L.K. Janssens is supported by The Special Research Fund (BOF) of Ghent University (grant number BOF20/DOC/051). K. E. Grafinger gratefully acknowledges the Swiss National Science Foundation (Fund No. SNF_P2BEP3_191780) for her postdoctoral fellowship. Daria Morozova is acknowledged for the practical work she performed during her Master's thesis.

author contributions Conceptualization: LKJ and CPS; methodology: LKJ, MJS, VA and CS; investigation: LKJ and MJS; Resources: VA, MHC, CS; writing—original Draft: LKJ; writing—review and editing: LKJ, KEG, CPS, VA, MJS and MHC.

Data availability Data can be made available upon request.

Declarations

Conflict of interest The authors have no conflict of interest to declare.

References

Adamowicz P, Gieroń J (2016) Acute intoxication of four individuals following use of the synthetic cannabinoid MAB-CHMINACA. Clin Toxicol 54:650–654. https://doi.org/10.1080/15563650.2016.1190016

Allibe N, Richeval C, Willeman T et al (2017) Case reports: Four concomitant non-fatal intoxications with AB-FUBINACA and MDMA. Toxicol Anal Clin 29:101–110. https://doi.org/10.1016/j.toxac.2016.12.006

Antonides LH, Cannaert A, Norman C et al (2019) Enantiospecific synthesis, chiral separation, and biological activity of four indazole-3-carboxamide-type synthetic cannabinoid receptor agonists and their detection in seized drug samples. Front Chem 7:321. https://doi.org/10.3389/fchem.2019.00321

Antonides LH, Cannaert A, Norman C et al (2021) Shape matters: the application of activity-based *in vitro* bioassays and chiral profiling to the pharmacological evaluation of synthetic cannabinoid receptor agonists in drug-infused papers seized in prisons. Drug Test Anal 13:628–643. https://doi.org/10.1002/dta.2965

Auwärter V, Dresen S, Weinmann W et al (2009) 'Spice' and other herbal blends: harmless incense or cannabinoid designer drugs? J Mass Spectrom 44:832–837. https://doi.org/10.1002/jms.1558

Banister SD, Connor M (2018) The chemistry and pharmacology of synthetic cannabinoid receptor agonist new psychoactive substances: evolution. In: Maurer HH, Brandt SD (eds) New psychoactive substances. Springer International Publishing, Cham, pp 191–226

Banister SD, Adams A, Kevin RC et al (2019) Synthesis and pharmacology of new psychoactive substance 5F-CUMYL-P7AICA, a scaffold- hopping analog of synthetic cannabinoid receptor agonists 5F-CUMYL-PICA and 5F-CUMYL-PINACA. Drug Test Anal 11:279–291. https://doi.org/10.1002/dta.2491

Cabanlong CV, Russell LN, Fantegrossi WE, Prather PL (2022) Metabolites of synthetic cannabinoid 5F-MDMB-PINACA retain affinity, act as high efficacy agonists and exhibit atypical pharmacodynamic properties at CB1 receptors. Toxicol Sci. https://doi.org/10.1093/toxsci/kfac024

Cannaert A, Storme J, Franz F et al (2016) Detection and activity profiling of synthetic cannabinoids and their metabolites with a newly developed bioassay. Anal Chem 88:11476–11485. https://doi.org/10.1021/acs.analchem.6b02600

Cannaert A, Storme J, Hess C et al (2018) Activity-based detection of cannabinoids in serum and plasma samples. Clin Chem 64:918–926. https://doi.org/10.1373/clinchem.2017.285361

Cannaert A, Vandeputte M, Hudson S et al (2019) Validation of activity-based screening for Synthetic Cannabinoid Receptor Agonists in a large set of serum samples. Clin Chem 65:347–349. https://doi.org/10.1373/clinchem.2018.296905

Cannaert A, Ramírez Fernández MDM, Theunissen EL et al (2020a) Semiquantitative activity-based detection of JWH-018, a synthetic cannabinoid receptor agonist, in oral fluid after vaping. Anal Chem 92:6065–6071. https://doi.org/10.1021/acs.analchem.0c00484

Cannaert A, Sparkes E, Pike E et al (2020b) Synthesis and *in Vitro* cannabinoid receptor 1 activity of recently detected synthetic cannabinoids 4F-MDMB-BICA, 5F-MPP-PICA, MMB-4en-PICA, CUMYL-CBMICA, ADB-BINACA, APP-BINACA, 4F-MDMB-BINACA, MDMB-4en-PINACA, A-CHMINACA, 5F-AB-P7AICA, 5F-MDMB-P7AICA, and 5F-AP7AICA. ACS Chem Neurosci 11:4434–4446. https://doi.org/10.1021/acschemneuro.0c00644

Castaneto MS, Gorelick DA, Desrosiers NA et al (2014) Synthetic cannabinoids: Epidemiology, pharmacodynamics, and clinical implications. Drug Alcohol Depend 144:12–41. https://doi.org/10.1016/j.drugalcdep.2014.08.005

De Luca MA, Fattore L (2018) Therapeutic use of synthetic cannabinoids: still an open issue? Clin Ther 40:1457–1466. https://doi.org/10.1016/j.clinthera.2018.08.002

De Oliveira MC, Vides MC, Lassi DLS et al (2023) Toxicity of Synthetic Cannabinoids in K2/Spice: A Systematic Review. Brain Sci 13:990. https://doi.org/10.3390/brainsci13070990

Deventer MH, Van Uytfanghe K, Vinckier IMJ et al (2022) Cannabinoid receptor activation potential of the next generation, generic ban evading OXIZID synthetic cannabinoid receptor agonists. Drug Test Anal. https://doi.org/10.1002/dta.3283

Dixon AS, Schwinn MK, Hall MP et al (2016) NanoLuc complementation reporter optimized for accurate measurement of protein interactions in cells. ACS Chem Biol 11:400–408. https://doi.org/10.1021/acschembio.5b00753

European Monitoring Centre for Drugs and Drug Addiction (2009) 2009 Annual report on the state of the drugs problem in Europe. Publications Office, Luxembourg

European Monitoring Centre for Drugs and Drug Addiction (2023) European drug report 2023. Publications Office, LU

Gamage TF, Farquhar CE, McKinnie RJ et al (2019) Synthetic cannabinoid hydroxypentyl metabolites retain efficacy at human cannabinoid receptors. J Pharmacol Exp Ther 368:414–422. https://doi.org/10.1124/jpet.118.254425

Grafinger KE, Cannaert A, Ametovski A et al (2021a) Systematic evaluation of a panel of 30 synthetic cannabinoid receptor agonists structurally related to MMB-4en-PICA, MDMB-4en-PINACA, ADB-4en-PINACA, and MMB-4CN-BUTINACA using a combination of binding and different CB_1 receptor activation assays—part II: structure activity relationship assessment via a β-arrestin recruitment assay. Drug Test Anal 13:1402–1411. https://doi.org/10.1002/dta.3035

Grafinger KE, Vandeputte MM, Cannaert A et al (2021b) Systematic evaluation of a panel of 30 synthetic cannabinoid receptor agonists structurally related to MMB-4en-PICA, MDMB-4en-PINACA, ADB-4en-PINACA, and MMB-4CN-BUTINACA using a combination of binding and different CB1 receptor activation assays. Part III: The G protein pathway and critical comparison of different assays. Drug Test Anal 13:1412–1429. https://doi.org/10.1002/dta.3054

Hermanns-Clausen M, Müller D, Kithinji J et al (2018) Acute side effects after consumption of the new synthetic cannabinoids AB-CHMINACA and MDMB-CHMICA. Clin Toxicol 56:404–411. https://doi.org/10.1080/15563650.2017.1393082

Janssens L, Cannaert A, Connolly MJ et al (2020) *In vitro* activity profiling of Cumyl-PEGACLONE variants at the CB_1 receptor: Fluorination *versus* isomer exploration. Drug Test Anal 12:1336–1343. https://doi.org/10.1002/dta.2870

Janssens LK, Boeckaerts D, Hudson S et al (2022a) Machine learning to assist in large-scale, activity-based synthetic cannabinoid receptor agonist screening of serum samples. Clin Chem. https://doi.org/10.1093/clinchem/hvac027

Janssens LK, Hudson S, Wood DM et al (2022b) Linking in vitro and ex vivo CB1 activity with serum concentrations and clinical features in 5F-MDMB-PICA users to better understand SCRAs and their metabolites. Arch Toxicol 96:2935–2945. https://doi.org/10.1007/s00204-022-03355-6

Janssens LK, Ametovski A, Sparkes E et al (2023) Comprehensive characterization of a systematic library of alkyl and alicyclic synthetic cannabinoids related to CUMYL-PICA, CUMYL-BUTICA, CUMYL-CBMICA, and CUMYL-PINACA. ACS Chem Neurosci 14:35–52. https://doi.org/10.1021/acschemneuro.2c00408

Mohr ALA, Logan BK, Fogarty MF et al (2022) Reports of adverse events associated with use of novel psychoactive substances, 2017–2020: a review. J Anal Toxicol 46:e116–e185. https://doi.org/10.1093/jat/bkac023

Noble C, Cannaert A, Linnet K, Stove CP (2019) Application of an activity-based receptor bioassay to investigate the in vitro activity of selected indole- and indazole-3-carboxamide-based synthetic cannabinoids at CB1 and CB2 receptors. Drug Test Anal 11:501–511. https://doi.org/10.1002/dta.2517

Persson HE, Sjöberg GK, Haines JA, De Garbino JP (1998) Poisoning severity score. Grading of acute poisoning. J Toxicol Clin Toxicol 36:205–213. https://doi.org/10.3109/15563659809028940

Pike E, Grafinger KE, Cannaert A et al (2021) Systematic evaluation of a panel of 30 synthetic cannabinoid receptor agonists structurally related to MMB-4en-PICA, MDMB-4en-PINACA, ADB-4en-PINACA, and MMB-4CN-BUTINACA using a combination of binding and different CB_1 receptor activation assays: Part I—Synthesis, analytical characterization, and binding affinity for human CB_1 receptors. Drug Test Anal 13:1383–1401. https://doi.org/10.1002/dta.3037

Sholler DJ, Huestis MA, Amendolara B et al (2020) Therapeutic potential and safety considerations for the clinical use of synthetic cannabinoids. Pharmacol Biochem Behav 199:173059. https://doi.org/10.1016/j.pbb.2020.173059

Sommer MJ, Halter S, Angerer V et al (2022) Effect of new legislation in Germany on prevalence and harm of synthetic cannabinoids. Clin Toxicol 60:1130–1138. https://doi.org/10.1080/15563650.2022.2095282

Sparkes E, Cairns E, Kevin R et al (2021) Structure-activity relationships of valine-, tert-leucine-, and phenylalanine amino acid-derived synthetic cannabinoid receptor agonists related to ADB-BUTINACA, APP-BUTINACA, and ADB-P7AICA. RSC Med Chem. https://doi.org/10.1039/D1MD00242B

Vandrey R, Dunn KE, Fry JA, Girling ER (2012) A survey study to characterize use of Spice products (synthetic cannabinoids). Drug Alcohol Depend 120:238–241. https://doi.org/10.1016/j.drugalcdep.2011.07.011

Wiley JL, Compton DR, Dai D et al (1998) Structure-activity relationships of indole- and pyrrole-derived cannabinoids. J Pharmacol Exp Ther 285:995

Winstock AR, Barratt MJ (2013) Synthetic cannabis: a comparison of patterns of use and effect profile with natural cannabis in a large global sample. Drug Alcohol Depend 131:106–111. https://doi.org/10.1016/j.drugalcdep.2012.12.011

Wouters E, Mogler L, Cannaert A et al (2019) Functional evaluation of carboxy metabolites of synthetic cannabinoid receptor agonists featuring scaffolds based on L-valine or L-*tert*-leucine. Drug Test Anal 11:1183–1191. https://doi.org/10.1002/dta.2607

Wouters E, Walraed J, Robertson MJ et al (2020) Assessment of biased agonism among distinct synthetic cannabinoid receptor agonist scaffolds. ACS Pharmacol Transl Sci 3:285–295. https://doi.org/10.1021/acsptsci.9b00069