Mogler Lukas (Orcid ID: 0000-0001-8987-2785)

Auwärter Volker (Orcid ID: 0000-0002-1883-2804)

Stove Christophe (Orcid ID: 0000-0001-9117-4759)

Thevis Mario (Orcid ID: 0000-0002-1535-6451)

Brandt Simon (Orcid ID: 0000-0001-8632-5372)

Functional evaluation of carboxy metabolites of synthetic cannabinoid receptor agonists featuring scaffolds based on L-valine or L-*tert*-leucine

Elise Wouters[1], Lukas Mogler[2], Annelies Cannaert[1], Volker Auwärter[2] and Christophe Stove[1*]

[1]Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Gent, Belgium

[2]Institute of Forensic Medicine, Forensic Toxicology, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Albertstr. 9, 79104 Freiburg, Germany

*Corresponding author: christophe.stove@ugent.be.
Tel.: +32 9 264 81 35. Fax. +32 9 264 81 83.

Short title: Functional evaluation of SCRA metabolites

Abstract

Indole and indazole-based synthetic cannabinoid receptor agonists (SCRAs), featuring valine or *tert*-leucine substituents, are commonly abused new psychoactive substances (NPS). A major metabolic pathway for these SCRAs is hydrolysis of the terminal amide or methylester functionalities. Although these hydrolysis products were already detected as main ingredients in some 'legal highs', these metabolites are often poorly characterized. Here, we report a systematic investigation of the activity of seven common hydrolysis metabolites of fifteen SCRAs featuring scaffolds based on L-valine or L-*tert*-leucine in direct comparison to their parent compounds. An activity-based cannabinoid receptor 1 (CB_1) bio-assay was used for activity profiling of SCRAs and their metabolites in a stable HEK293T cell system. The recruitment of β-arrestin2 to the activated CB_1 (each fused to one part of a split Nanoluciferase) was provoked by adding the (putative) SCRAs. Luminescence of the functionally complemented luciferase was monitored by a 96-well plate-reader. The major hydrolysis metabolites of 5F-AB-PINACA, ADB-CHMICA, ADB-CHMINACA, ADB-FUBICA, and their methyl- and ethylester derivatives showed no detectable CB_1 activation at concentrations

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/dta.2607

up to 1 µM. On the other hand, metabolites of 5F-ADB-PINACA, AB-CHMINACA and ADB-FUBINACA did retain activity, although significantly reduced as compared to the parent compounds (EC_{50} values > 100 nM). Activity-based characterization of SCRAs and their metabolites at CB_1 may not only allow a better insight into the complex interplay between SCRAs and their metabolites in intoxications, but may also allow to apply the concept of 'activity equivalents' present in biological fluids or, alternatively, in confiscated materials.

Keywords

New psychoactive substances, synthetic cannabinoid receptor agonists, carboxy metabolites, bio-assay, cannabinoid receptor 1

1. Introduction

During the last decade, new psychoactive substances (NPS) have emerged on the illicit drug market, with synthetic cannabinoid receptor agonists (SCRAs) being the most prominent class in Europe.[1] SCRAs are man-made substances that exert their effects through binding to two G-protein coupled receptors (GPCRs), namely cannabinoid receptors 1 (CB_1) and 2 (CB_2).[2,3] CB_1 receptors are primarily located in the central nervous system (the brain and spinal cord) and mediate the psychotropic effect of cannabis (alternatives), whereas CB_2 receptors are mostly expressed in the peripheral nervous system, the spleen and the immune system and are involved in immunosuppression and pain perception mediation.[4,5]

Synthetic cannabimimetics, which mimic the actions of endogenous and natural compounds acting on the cannabinoid receptors, are often far more potent than the main psychoactive substance found in cannabis, delta-9-tetrahydrocannabinol (Δ^9-THC). The intake of these highly potent drugs of abuse has caused considerable morbidity and mortality in the United States, Europe and Japan, as well as in many other countries.[6-8] Observed adverse effects include agitation, hypertension, acute kidney injury and tachycardia and may even result in fatalities.[9-16]

As xenobiotics, SCRAs undergo extensive metabolism and clearance, mainly by the liver, to remove these substances from the human body. The goal of drug metabolism is to detoxify potentially harmful compounds and excrete them from the body. In certain cases, bioactivation of these compounds, which might lead or contribute to toxicological effects, has been reported.

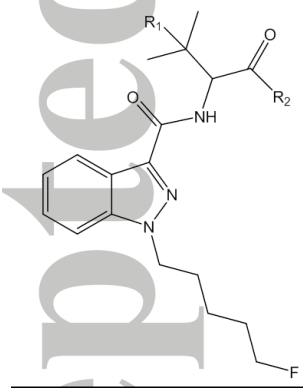
For example, major hydroxylated metabolites of JWH-018 and AM-2201 retained full agonist activity at nanomolar concentrations.[17,18]

Although hydroxylation of the pentyl side chain of SCRAs is a common metabolism pathway, the major pathway for SCRAs featuring scaffolds based on L-valine and L-*tert*-leucine involves amide hydrolysis of the L-valinamide or L-*tert*-leucinamide functional groups[19-21] or methyl- or ethylester hydrolysis of L-valinate or L-*tert*-leucinate groups.[22,23] Hydrolysis of the terminal functionality is more pronounced for compounds containing an L-valinate or L-*tert*-leucinate group compared to their amide analogs, due to the more metabolically labile characteristic of the methylester functionality.[24] The enzyme responsible for the biotransformation of the primary amide moiety, resulting in major carboxylic acid metabolites, was identified in human liver microsomes (HLMs) as carboxylesterase 1 (CES1).[23,24] In addition to a transformation catalyzed by enzymes, a pyrolytic formation of the carboxylic acids has to be considered when the drugs are smoked.[25,26]

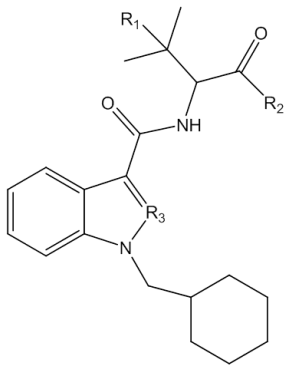
Further adding to the complexity of the SCRA problem is that, in addition to the rapid and constant emergence of new SCRAs, relatively little is known about the pharmacology of these SCRAs and their metabolites. More particularly, only few studies have focused on the pharmacological characteristics of hydrolysis metabolites.[17,27-34] Recently, there has been an increased interest, since knowledge about SCRA metabolite activity data might empower forensic laboratories to elaborate on the possible contributory role of these metabolites in human intoxications and in side-effects related to SCRAs. A concept of 'total cannabinoid activity', rather than merely the cannabinoid activity related to a main compound can be envisaged. When referring the activity in a biological matrix relative to that of a given concentration of a reference compound, this concept can further be defined as 'activity equivalents' being present in e.g. blood or serum of an intoxicated or deceased person.

In this context, we investigated here the activity of fifteen parent compounds, featuring scaffolds based on L-valine and L-*tert*-leucine, and their respective carboxy metabolites, using a bio-assay that monitors activation of the CB_1 receptor. Compounds of this structural family, carrying carboxamide L-valine and L-*tert*-leucine moieties, are highly prevalent on the European market and show a high potency, resulting in numerous cases of severe intoxications.[26,35]

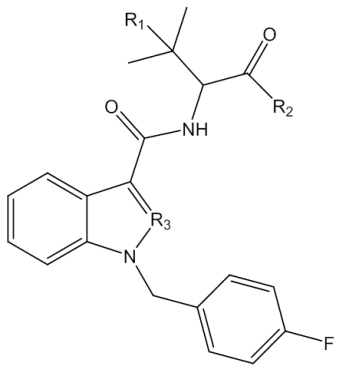
SCRAs and their structural analogues, derived from L-valinamide or L-valinate investigated in this study, include 5F-AB-PINACA, 5F-AMB-PINACA, 5F-AEB-PINACA, AB-CHMINACA and AMB-CHMINACA (Figure 1). SCRAs and their structural analogues, derived from L-*tert*-leucinamide or L-*tert*-leucinate, include 5F-ADB-PINACA, 5F-MDMB-PINACA, ADB-CHMICA, MDMB-CHMICA, ADB-CHMINACA, MDMB-CHMINACA, ADB-FUBICA, MDMB-FUBICA, ADB-FUBINACA and MDMB-FUBINACA (Figure 1). As the same hydrolysis metabolite featuring a carboxylic acid moiety might derive from more than one parent compound, hydrolysis of these fifteen SCRAs results in seven carboxy metabolites, i.e. 5F-AB-PINACA-COOH, AB-CHMINACA-COOH, 5F-ADB-PINACA-COOH, ADB-CHMICA-COOH, ADB-CHMINACA-COOH, ADB-FUBICA-COOH and ADB-FUBINACA-COOH. Pharmacological properties of the above-mentioned hydrolysis metabolites were compared with those of their parent compounds. The objective of this study was to clarify the possible impact of amide or ester hydrolysis across a range of SCRAs featuring scaffolds based on L-valine or L-*tert*-leucine, to allow a better insight into their potential contribution to the *in vivo* toxicity profile.



Drug/**metabolite**	R_1	R_2
5F-ADB-PINACA (1)	CH_3	NH_2
5F-MDMB-PINACA (2)	CH_3	OCH_3
5F-ADB-PINACA-COOH (3)	**CH_3**	**OH**
5F-AB-PINACA (4)	H	NH_2
5F-AMB-PINACA (5)	H	OCH_3
5F-AEB-PINACA (6)	H	OC_2H_5
5F-AB-PINACA-COOH (7)	**H**	**OH**



Drug/**metabolite**	R_1	R_2	R_3
ADB-CHMICA (8)	CH_3	NH_2	CH
MDMB-CHMICA (9)	CH_3	OCH_3	CH
ADB-CHMICA-COOH (10)	**CH_3**	**OH**	**CH**
ADB-CHMINACA (11)	CH_3	NH_2	N
MDMB-CHMINACA (12)	CH_3	OCH_3	N
ADB-CHMINACA-COOH (13)	**CH_3**	**OH**	**N**
AB-CHMINACA (14)	H	NH_2	N
AMB-CHMINACA (15)	H	OCH_3	N
AB-CHMINACA-COOH (16)	**H**	**OH**	**N**



Drug/**metabolite**	R_1	R_2	R_3
ADB-FUBICA (17)	CH_3	NH_2	CH
MDMB-FUBICA (18)	CH_3	OCH_3	CH
ADB-FUBICA-COOH (19)	**CH_3**	**OH**	**CH**
ADB-FUBINACA (20)	CH_3	NH_2	N
MDMB-FUBINACA (21)	CH_3	OCH_3	N
ADB-FUBINACA-COOH (22)	**CH_3**	**OH**	**N**

Figure 1 Structures of SCRAs featuring a scaffold based on L-valine (white) and L-*tert*-leucine (blue) and their respective most common carboxy hydrolysis metabolites.

2. Materials and methods

2.1. Chemicals and reagents

Reference standards of all parent compounds including 5F-ADB-PINACA (*N*-[(2*S*)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(5-fluoropentyl)-1*H*-indazole-3-carboxamide), 5F-MDMB-PINACA (methyl (2*S*)-2-{[1-(5-fluoropentyl-1*H*-indazole-3-carbonyl]amino}-3,3-dimethylbutanoate), 5F-AB-PINACA (*N*-[(2*S*)-1-(amino-3-methyl-1-oxobutan-2-yl]-1-(5-fluoropentyl-1*H*-indazole-3-carboxamide), 5F-AMB-PINACA (methyl (2*S*)-2-{[1-(5-fluoropentyl)-1*H*-indazole-3-carbonyl]amino}-3-methylbutanoate), 5F-AEB-PINACA (ethyl (2*S*)-2-{[1-(5-fluoropentyl)-1*H*-indazole-3-carbonyl]amino}-3-methylbutanoate), ADB-CHMINACA (*N*-[(2*S*)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide), MDMB-CHMICA (methyl (2*S*)-2-{[1-(cyclohexylmethyl)-1*H*-indole-3-carbonyl]amino}-3,3-dimethylbutanoate), ADB-CHMICA (*N*-[(2*S*)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)-1*H*-indole-3-carboxamide), MDMB-CHMINACA (methyl (2*S*)-2-{[1-(cyclohexylmethyl)-1*H*-indazole-3-carbonyl]amino}-3,3-dimethylbutanoate), AB-CHMINACA (*N*-[(2*S*)-1-amino-3-methyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide), AMB-CHMINACA (methyl (2*S*)-2-{[1-(cyclohexylmethyl)-1*H*-indazole-3-carbonyl]amino}-3-methylbytanoate), ADB-FUBICA (*N*-[(2*S*)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-[(4-fluorophenyl)methyl]-1*H*-indole-3-carboxamide), MDMB-FUBICA (methyl (2*S*)-2-({1-[(4-fluorophenyl)methyl]-1*H*-indole-3-carbonyl}amino)-3,3-dimethylbutanoate), ADB-FUBINACA (*N*-[(2*S*)-1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-[(4-fluoropheny1)methyl]-1*H*-indazole-3-carboxamide), MDMB-FUBINACA (methyl (2*S*)-2-({1-[(4-fluorophenyl)methyl]-1*H*-indazole-3-carbonyl}amino)-3,3-dimethylbutanoate) and their metabolites 5F-AB-PINACA-COOH ((2*S*)-3-methyl-2-{[1-(5-fluoropentyl)-1*H*-indazole-3-carbonyl]amino}butanoic acid), 5F-ADB-PINACA-COOH ((*S*)-2-{[1-(5-fluoropentyl)-1*H*-indazole-3-carbonyl]amino}-3,3-dimethylbutanoic acid), ADB-FUBICA-COOH ((*S*)-2-{[1-(4-fluorobenzyl)-1*H*-indole-3-carbonyl]amino}-3,3-dimethylbutanoic acid), ADB-CHMINACA-COOH ((2*S*)-2-{[1-(cyclohexylmethyl)-1*H*-indazole-3-carbonyl]amino}-3,3-dimethylbutanoic acid), AB-CHMINACA-COOH ((2*S*)-2-{[1-(cyclohexylmethyl)-1*H*-indazole-3-carbonyl]amino}-3-methylbutanoic acid), ADB-CHMICA-COOH ((*S*)-2-{[1-(5-fluoropentyl)-1*H*-indazole-3-carbonyl]amino}-3,3-dimethylbutanoic acid) were purchased from Cayman Chemical (Ann Arbor, MI, ISA).

JWH-018 ((naphthalen-1-yl)(1-pentyl-1*H*-indol-3-yl)methanone) was purchased from LGC (Wesel, Germany). Poly-D-lysine and fetal bovine serum (FBS) were procured from Sigma-Aldrich (Steinheim, Germany). Dulbecco's modified eagle medium (DMEM) + GlutaMAX, Opti-MEM I Reduced Serum, trypsine, penicillin and streptomycin were procured from Thermo Fisher Scientific (Pittsburg, PA, USA). Nano-Glo® Live Cell substrate furimazine and Nano-Glo® dilution buffer were purchased from Promega (Madison, WI, USA). Methanol and DMSO were purchased from Fisher Scientific (Leicestershire, UK).

2.2. Cannabinoid reporter bio-assay

A live cell-based assay, based on the NanoLuc Binary Technology (NanoBiT®, Promega) was used to evaluate activity at CB_1. Upon activation of CB_1, an intracellular protein, β-arrestin2 (β-arr2), which participates in the agonist-mediated desensitization of GPCRs, will be recruited. The generation of a HEK293T cell line stably expressing both the CB_1 receptor (C-terminally fused to the large part of the NanoLuciferase; LgBiT) and β-arr2 (N-terminally fused to the small part of NanoLuciferase; SmBiT) has been described previously.[29] This cell line can be used for structure-activity relationship determination of reference compounds, as well as for the screening of biological matrices for cannabinoid activity.[32,36,37]

HEK293T cells stably expressing CB_1-LgBiT and SmBiT-β-arr2 were maintained in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 µg/mL streptomycin and 100 IU/l penicillin in a humidified environment (37°C, 5% CO_2). For the bio-assay, cells were seeded on a poly-D-lysine coated white 96-well plate at 5 x 10^4 cells/well. The next day, cells were washed twice with Opti-MEM I Reduced Serum Medium and 100 µl Opti-MEM I was added to the wells. First, the Nano-Glo Live Cell detection reagent (Promega) was prepared by a 20-fold dilution of the cell-permeable furimazine substrate in aqueous Nano-Glo LCS dilution buffer. Twenty-five µL of this reagent was added to each well and luminescence was monitored in a TriStar^2 LB 942 multimode microplate reader controlled by ICE software (Berthold Technologies GmbH & Co., Bad Wildbad, Germany) during an equilibration period of 15 minutes. Once the luminescent signal had stabilized, monitoring was shortly interrupted for the addition of 10 µL of the 13.5x concentrated agonist solutions in Opti-MEM I/methanol (50:50), ranging from 0.01 nM up to 10 µM end concentration in the 96-well. This resulted in a final volume percentage of 3.7% methanol in each 96-well, which does not pose a problem to the cells within the timeframe of the assay. Subsequently, luminescence was

detected for 120 minutes. A solvent control (blank), as well as a reference compound (JWH-018) was included in duplicate or triplicate on each plate.

2.4. Data processing

Relative Luminescence units (RLU) over time of all SCRAs were corrected for solvent control and inter-well variability. Concentration-responses (area under the curve; AUC) were calculated and normalized to the maximum response of the reference compound, JWH-018, arbitrarily set as 100%. The mean and standard error of the mean (SEM) were derived from three independent experiments (hence, n = 3), the results from each of these experiments stemming from the analysis of duplicates or triplicates (hence, each data point is the result of 7-9 determinations). EC_{50} values were determined using the GraphPad Prism software (San Diego, CA, USA), via non-linear regression analysis (variable slope, four parameters).

3. Results and discussion

The SCRAs investigated in this study can be divided into 3 main categories: (1) PINACA derivatives (1-pentyl-1*H*-indazole-3-carboxamide derivatives), (2) CHMICA and CHMINACA derivatives (1-(cyclohexylmethyl)-1*H*-indole-3-carboxamide and 1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide, respectively) and (3) FUBICA and FUBINACA derivatives (1-[(4-fluorophenyl)methyl]-1*H*-indole-3-carboxamide and 1-[(4-fluorophenyl)methyl]-1*H*-indazole-3-carboxamide, respectively). Each category is subdivided into scaffolds based on L-valine or L-*tert*-leucine, and are hereafter referred to as the numbers depicted in Figure 1. To quantify the potency and efficacy of the 15 SCRAs and their common metabolites, the EC_{50} and E_{max} values were determined. As JWH-018 was the first detected SCRA in the end of 2008[38] and the most commonly used standard SCRA in the field of toxicology, it was chosen as the reference compound. The observed EC_{50} value for JWH-018 in the bio-assay expressing CB_1 was 36.8 nM, with a 95% confidence interval (CI) of 28.6-50.4 nM, which is in agreement with earlier published data from our research group.[17,32]

3.1. 1-Pentyl-1*H*-indazole-3-carboxamide derivatives (PINACA)

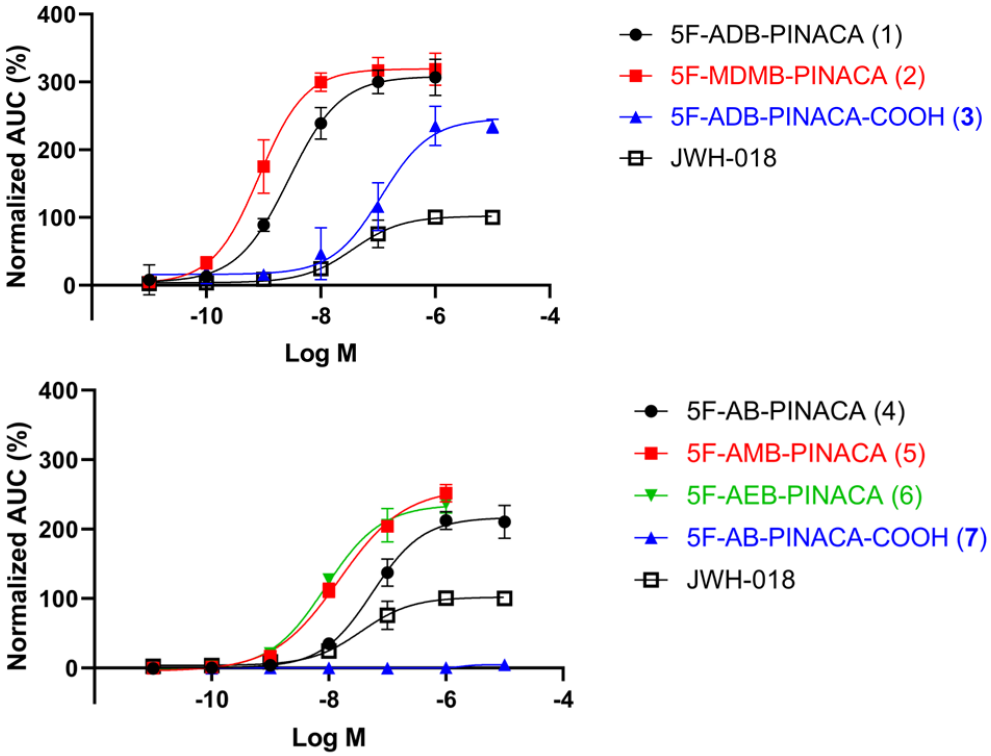


Figure 2 Sigmoidal concentration-response curves of PINACA-based SCRA derivatives for the CB_1 receptor. Each data point represents the mean value ± SEM of 3 experiments (n = 3).

All derivatives of the 1-Pentyl-1*H*-indazole-3-carboxamide family investigated in this study contain a 5-fluoropentyl group linked to N_1 of the indazole ring (Figure 1). The L-*tert*-leucinamide derivative 5F-ADB-PINACA (**1**) and the L-*tert*-leucinate derivatives 5F-MDMB-PINACA (**2**) showed a high potency at CB_1, with EC_{50} values of 2.76 nM (95% CI: 1.24-5.41 nM) and 0.84 nM (0.52-1.24 nM), respectively (Figure 2)(Table 1). A fatal intoxication in Japan due to intake of 5F-MDMB-PINACA (**2**), also referred to as 5F-ADB, resulted in postmortem blood concentrations of 0.19 ng/mL (0.5 nM) of the parent compound.[39] In that case, investigation of the urinary metabolites revealed the presence of the carboxy metabolite **3**, generated after ester hydrolysis by carboxylesterase. However, no blood or urinary concentration was reported for that metabolite.

Despite having a strongly reduced potency when compared to the parent compounds **1** and **2**, hydrolyzed metabolite **3** still shows a higher E_{max} value than that of reference compound JWH-018 (± 2.5-fold) (Figure 2)(Table 1). Furthermore, the potency of metabolite **3** was 41-fold reduced when compared to parent compound **1**, resulting in an EC_{50} value of 113 nM for 5F-ADB-PINACA-COOH (**3**). Longworth *et al.*[31] investigated the activity of the non-fluorinated

structural analogue ADB-PINACA and its hydrolyzed metabolite ADB-PINACA-COOH, using a fluorometric assay of membrane potential. These authors reported a 256-fold reduction in potency of the metabolite ADB-PINACA-COOH compared to its parent compound (EC_{50} value of 1.3 nM for the parent compound *vs.* 333 nM for its metabolite). The fact that hydrolysis of parent compound **1** is apparently less detrimental for CB_1 activation than hydrolysis of its non-fluorinated structural analogue ADB-PINACA might be explained by the reported beneficial effect of fluorination of the aliphatic tail for the overall activity of the compound[14], attributable to the electronegativity of the halogen. Alternatively, we cannot exclude that assay differences underlie this dissimilarity.

Investigation of the L-valinamide derivative, 5F-AB-PINACA (**4**), as well as the methyl and ethyl L-valinate derivatives, 5F-AMB-PINACA (**5**) and 5F-AEB-PINACA (**6**), respectively, revealed notable structure activity relationships (SARs) in this study. The EC_{50} values for **4**, **5** and **6** at CB_1 were 55.4 nM (31.2-85.1), 15.1 nM (10.2-23.9) and 8.76 nM (5.5-17.0) (Figure 2)(Table 1). Substitution of the amide group by methyl or ethyl ester groups resulted in higher potency towards CB_1. However, this finding is somewhat discordant with the findings by Banister *et al.*,[38] who reported a lower potency at CB_1 for **5** than **4** (1.9 nM vs 0.48 nM) by the implementation of a Fluorometric Imaging Plate Reader (FLIPR®) assay. Whereas this assay measures the Gβγ-mediated activation of inwardly rectifying potassium channels, the NanoBiT®-based bio-assay measures the direct recruitment of β-arr2 to the CB_1 receptor. Therefore, one should keep in mind that findings might differ due to different experimental assay set-ups (canonical vs. non-canonical), which could even reflect - although still mostly unexplored for SCRAs - biased signaling. On the other hand, similar efficacies for compounds **4**, **5** and **6** were observed, namely an efficacy about 2.5-fold that of JWH-018 (Table 1).

The amide hydrolysis product of parent compound **4**, 5F-AB-PINACA-COOH (**7**), was the third most prevalent metabolite generated in human hepatocytes.[21] In the bio-assay, metabolite **7** of parent compounds **4**, **5** and **6** shows almost no activity at CB_1, which is in agreement with earlier observations by Noble *et al.* [32] for the hydrolyzed metabolite of the structural analogue AB-PINACA. As even the most 'inactive' metabolite within this study, i.e. metabolite **7**, still shows some minor activity at high concentration (10 µM), none of the screened metabolites are considered to be an antagonist.

Overall, consistent with earlier published data[38,40], the L-*tert*-leucine-functionalized SCRAs (R_1= CH_3) (Figure 1), like **1** and **2** (EC_{50} = 0.84-2.76 nM), are more potent than their L-valine-functionalized SCRA counterparts **4**, **5** and **6** (EC_{50} = 8.76-55.4 nM). This also holds true for the carboxy metabolite **3** of the L-*tert*-leucine-functionalized SCRAs, which has an EC_{50} value of 113 nM, whereas no activity could be observed for the hydrolyzed metabolite **7** of the L-valine-functionalized SCRA derivatives.

3.2. 1-(Cyclohexylmethyl)-1*H*-indole-3-carboxamide (CHMICA) and 1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide (CHMINACA)

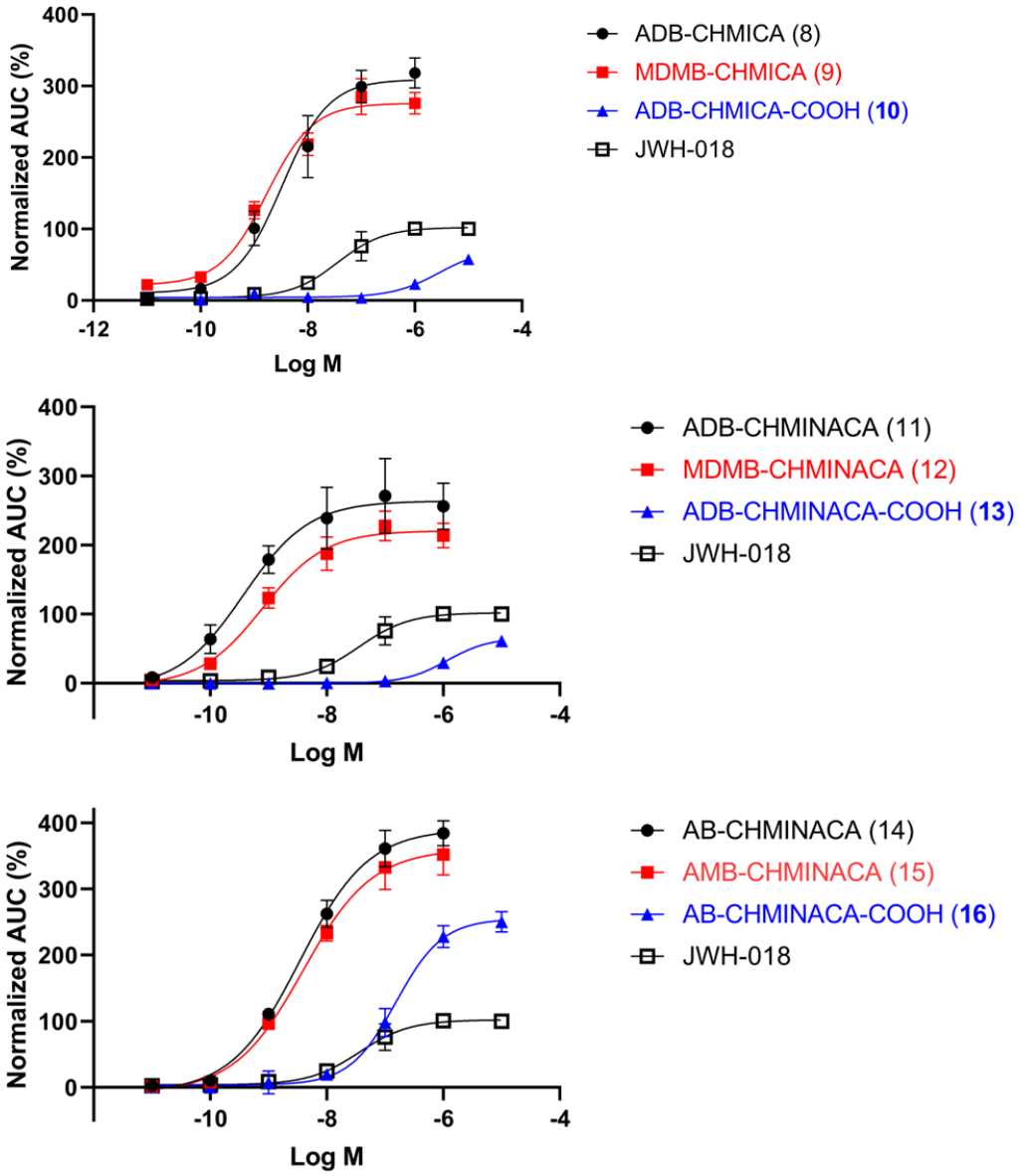


Figure 3 Sigmoidal concentration-response curves of CHMICA- and CHMINACA-based SCRA derivatives for the CB_1 receptor. Each data point represents the mean value ± SEM of 3 experiments (n = 3).

Since 2014, numerous non-fatal and fatal intoxications with the L-*tert*-leucine derivative MDMB-CHMICA (**9**) have been reported.[9,12,41-46] Subsequently, the UK Government implemented the 'Psychoactive Substances Act' in 2016 to counteract MDMB-CHMICA-related intoxications. Nevertheless, only a minor reduction in the availability of MDMB-CHMICA from internet-based suppliers could be observed.[47] Although there is only limited data published concerning its structural analogue, ADB-CHMICA (**8**), which features a primary amide moiety, both parent compounds **8** and **9** are extremely potent at CB_1, with EC_{50} values of 3.31 nM for compound **8** and 1.77 nM for compound **9** (Figure 3)(Table 1). Their hydrolysis metabolite **10** was found to be one of the most abundant *in vivo* Phase I metabolites in urine samples.[26] Hydrolysis of the primary amide or methylester severely impairs the activity of the metabolite compared to the parent compounds **8** and **9** (Figure 3). Notwithstanding, an efficacy of 57.6% of the E_{max} value of JWH-018 at high concentrations (10 µM) was retained.

ADB-CHMINACA (**11**) is one of the most potent CB_1 agonists from the panel of SCRAs investigated in this study, with an EC_{50} value of 0.34 nM (Figure 3)(Table 1), which is in agreement with earlier published data.[29,32,34,48] ADB-CHMINACA (**11**), also referred to as MAB-CHMINACA, is sold over the Internet as a white powder and is mostly smoked in order to get 'high'.[49] Typical doses reported by users, who mix it with herbs, vary from 0.25 mg up to heavy doses of 2 mg. In a fatal case in 2015, the presence of ADB-CHMINACA (**11**) was shown for the first time in human specimens, with the highest concentration of the parent compound found in the liver (156 ng/g).[15] Subsequently, various intoxications with the highly potent ADB-CHMINACA have been reported, with blood concentrations of four individuals ranging from 5.2 up to 14.6 ng/mL (or 14 nM up to 39 nM).[48] The adverse effects observed in these individuals included vomiting, seizures, limb twisting, muscle tremors, aggression, agitation, slurred speech, among others. Amide hydrolysis was detected in ADB-CHMINACA (**11**) as well as in AB-CHMINACA (**14**) metabolism, but only as a minor transformation, since the cyclohexylmethyl tail appeared to be the preferred site of transformation.[20,50,51] Although no EC_{50} value could be determined for hydrolysis metabolite **13** of parent compounds **11** and **12**, it still showed significant activity at CB_1 at high concentrations, with an efficacy of 68.2% relative to JWH-018 (in agreement with findings of Cannaert *et al*., 2017[29]). While the human consumption of this metabolite has not been reported before, metabolite **13**, also referred to as

DMBA-CHMINACA, has recently been identified in a white powder-type product delivered in an airmail package to Korea.[52]

Besides MDMB-CHMICA (**9**), the chemically closely related L-valine derivative AB-CHMINACA (**14**) has also frequently been encountered in forensic case work. It was identified amongst prevalently consumed drugs of abuse in patients presenting to an emergency department in London in the first half of 2015.[9] The use of these drugs has been associated with adverse effects including cardiovascular effects, neurotoxicity and neuropsychiatric effects, potentially even more serious severe toxicity effects compared to the earlier generation SCRAs.[12] The L-*tert*-leucine derivatives **14** and **15** showed a lower potency compared to their L-valine counterparts **11** and **12** (Figure 3). An EC_{50} value of 3.45 nM (1.96-6.14) was determined for compound **14** (Table 1), which is in agreement with the low EC_{50} value of 7.4 ± 1.5 nM reported by Wiley *et al.* [53], which was determined by the binding of a slowly hydrolysable GTP analog to the Gα-subunit using [^{35}S]GTPγS turnover assay, instead of the β-arr2 recruitment assay used in this study. Hydrolysis metabolite **16** has been identified as a hydrolysis product of parent compounds **14** and **15** in a number of studies in urine and hair of abusers.[54-56] Metabolite **16** clearly retains activity (EC_{50} = 155 nM), although with a 45-fold impaired potency when compared to the parent compounds (Table **1**). It has been reported by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) that the hydrolysis product of **14** and **15**, also listed under the name MBA-CHMINACA, has been sold as a 'research chemical' under the name 'AB-CHMINACA'.[35] This research chemical was purchased via the Internet from online vendors and analyzed at the Institute of Forensic Medicine, Freiburg, Germany.

3.3. 1-[4-Fluorophenyl)methyl]-1*H*-indole-3-carboxamide (FUBICA) and 1-[(4-fluorophenyl)methyl]-1*H*-indazole-3-carboxamide derivatives (FUBINACA)

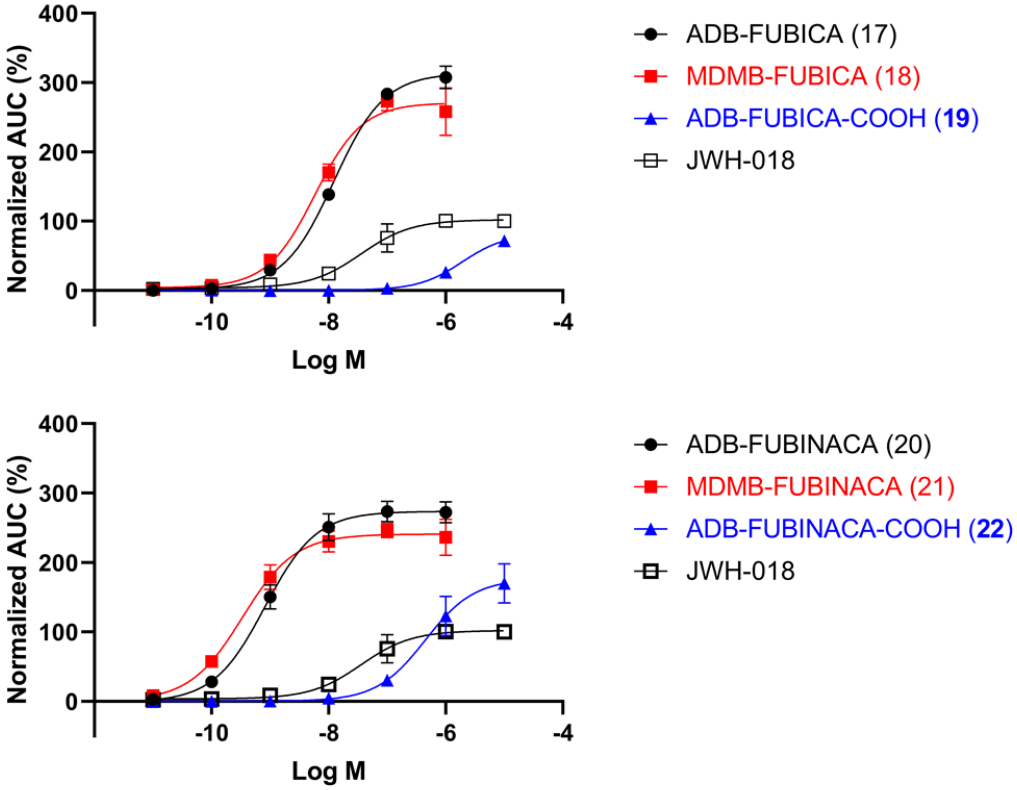


Figure 4 Sigmoidal concentration-response curves of FUBICA- and FUBINACA-based SCRA derivatives for the CB_1 receptor. Each data point represents the mean value ± SEM of 3 experiments (n = 3).

Of the FUBICA and FUBINACA derivatives, featuring a fluorinated benzene ring coupled to N_1 of the indole or indazole ring, only L-*tert*-leucine derivatives were investigated in this study (Figure 1). For ADB-FUBICA (**17**) an EC_{50} value of 12.3 nM (9.68-16.1) and an E_{max} value 3-fold higher than that of JWH-018 was determined (Figure 4)(Table 1). MDMB-FUBICA (**18**), which has been reported to be more selective towards CB_2 compared to the CB_1 receptor, shows an efficacy of 5.79 nM for CB_1, in agreement with GTPγS binding results by Doi *et al.* (2018) [57] (EC_{50} = 9.72 nM) and FLIPR® assay results by Banister *et al.* (2016) [40] (EC_{50} = 2.7 nM). HLM incubation studies yielded the Phase I metabolite **19** from the parent compound ADB-FUBICA (**17**) following carboxamide hydrolysis.[58] At high concentrations, metabolite **19** reached an E_{max} value comparable with 81.8% of the efficacy of the reference compound JWH-018.

ADB-FUBINACA (**20**) intake has led to multiple severe intoxications.[59-61] In a fatal case reported in the USA, related to the intake of compound **20**, a concentration of 7.3 ng/mL (19.1 nM) was detected in postmortem blood.[61] In a nonfatal case, an even higher serum concentration of 15.6 ng/mL (41 nM) was determined after intake of ADB-FUBINACA (**20**) by ingesting two drops of e-cigarette fluid.[59] Its structural analogue, MDMB-FUBINACA (**21**) has similarly been linked to more than 600 poisonings, including 15 deaths over 2 weeks in Russia in 2014.[62] Consequently, compound **21** has been designated as one of the deadliest cannabimimetics sold to date.[63] Interestingly, the structure of this highly potent agonist bound to the CB_1-$G\alpha_i$ complex has recently been unraveled.[63] Delineating the structural basis of this complex revealed a C-shape binding mode of **21**, which stabilizes the active conformation of CB_1 to a greater extent compared to the partial agonist Δ^9-THC. Also in this study, both compounds **20** and **21** show extremely high potency as CB_1 agonists, with EC_{50} values of 0.82 nM and 0.36 nM, respectively (Figure 4)(Table 1). Whereas the estimated EC_{50} value of compound **20** is in agreement with the low EC_{50} value of 1.2 nM determined by Banister *et al.*[38] using a FLIPR® membrane potential assay, the EC_{50} value of compound **21** estimated here (0.36 nM) deviates to a larger extent from the result obtained by Banister *et al*. (3.9 nM) but is in good agreement with the EC_{50} value of 0.26 nM, as determined by [^{35}S]GTPγS assay by Gamage *et al.*[30] As already mentioned earlier, different EC_{50} value outcomes can be explained by the implementation of different functional signaling assays, different cell lines (CHO vs HEK293T cells) or even biased signaling. Hydrolysis of the terminal moieties (primary amide and methylester for ADB-FUBINACA (**20**) and MDMB-FUBINACA (**21**), respectively) was found to be a common major metabolic pathway for both parent compounds.[64,65] Metabolite **22** was the most abundant metabolite in 9 postmortem urine samples, associated with MDMB-FUBINACA (**21**) intake.[65] This metabolite **22** still showed significant, albeit impaired, activity at CB_1 at high concentrations, with an EC_{50} value of 450 nM and an E_{max} of 176.6 %, relative to the efficacy of the reference compound JWH-018.

4. Conclusion

The present study provides critical and missing data related to the potential toxicological characteristics of common hydrolysis metabolites of 15 SCRAs featuring scaffolds based on L-valine and L-*tert*-leucine. Our results indicate an overall severely impaired activity of these metabolites at CB_1. Nevertheless, a broad variety in metabolite activity could be detected in the chemically closely related SCRA panel in this study, ranging from no activity detected to EC_{50} values around 150 nM. Comparison of our data to already published activity data on the investigated compounds once more revealed that EC_{50} values may markedly vary among different assays, possibly due to differences in functional signaling and choice of cell lines. Nevertheless, our data highlight that the hydrolysis metabolites of closely related SCRAs can possess markedly distinct pharmacological characteristics. Although we observed a strong reduction in efficacy (E_{max}) of the carboxy metabolites of L-valine and L-*tert*-leucine SCRAs for CB_1, when compared to the parent compounds, the efficacy of certain metabolites is still higher than that of JWH-018. This might imply that metabolites from ADB-FUBINACA, 5F-ADB-PINACA and AB-CHMINACA and their methyl- and ethylester functionalities could potentially contribute to the overall pharmacological or toxicological response *in vivo*.

ACKNOWLEDGMENTS

E. Wouters was supported by BRAIN project grant (NPSSAY) from The Belgian Science Policy Office, A. Cannaert was granted by Bijzonder Onderzoeksfonds (BOF) with grant number PDO026-18 and C. Stove was supported by Bijzonder Onderzoeksfonds (BOF) with grant numbers 01J15517 and 01N00814.

5. References

1. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). Perspectives on drugs: Synthetic cannabinoids in Europe. 2017. http://www.emcdda.europa.eu/system/files/publications/2753/POD_Synthetic%20cannabinoids_0.pdf. Accessed 21 December 2018.
2. Matsuda LA, Lolait SJ, Brownstein MJ, Young AC, Bonner TI. Structure of a cannabinoid receptor and functional expression of the cloned cDNA. *Nature.* 1990;346(6284):561-564.
3. Munro S, Thomas KL, Abu-Shaar M. Molecular characterization of a peripheral receptor for cannabinoids. *Nature.* 1993;365(6441):61-65.
4. Mechoulam R, Parker LA. The endocannabinoid system and the brain. *Annu Rev Psychol.* 2013;64:21-47.
5. Pertwee RG. Pharmacology of cannabinoid CB1 and CB2 receptors. *Pharmacol Ther.* 1997;74(2):129-180.
6. Kikura-Hanajiri R, Kawamura NU, Goda Y. Changes in the prevalence of new psychoactive substances before and after the introduction of the generic scheduling of synthetic cannabinoids in Japan. *Drug Test Anal.* 2014;6(7-8):832-839.
7. Spiller HA, Ryan ML, Weston RG, Jansen J. Clinical experience with and analytical confirmation of "bath salts" and "legal highs" (synthetic cathinones) in the United States. *Clin Toxicol.* 2011;49(6):499-505.
8. Zawilska JB, Andrzejczak D. Next generation of novel psychoactive substances on the horizon - A complex problem to face. *Drug Alcohol Depend.* 2015;157:1-17.
9. Abouchedid R, Hudson S, Thurtle N, et al. Analytical confirmation of synthetic cannabinoids in a cohort of 179 presentations with acute recreational drug toxicity to an Emergency Department in London, UK in the first half of 2015. *Clin Toxicol.* 2017;55(5):338-345.
10. Hermanns-Clausen M, Kithinji J, Spehl M, et al. Adverse effects after the use of JWH-210 - a case series from the EU Spice II plus project. *Drug Test Anal.* 2016;8(10):1030-1038.
11. Hermanns-Clausen M, Kneisel S, Szabo B, Auwarter V. Acute toxicity due to the confirmed consumption of synthetic cannabinoids: clinical and laboratory findings. *Addiction.* 2013;108(3):534-544.
12. Hermanns-Clausen M, Muller D, Kithinji J, et al. Acute side effects after consumption of the new synthetic cannabinoids AB-CHMINACA and MDMB-CHMICA. *Clin Toxicol.* 2018;56(6):404-411.
13. Behonick G, Shanks KG, Firchau DJ, et al. Four postmortem case reports with quantitative detection of the synthetic cannabinoid, 5F-PB-22. *J Anal Toxicol.* 2014;38(8):559-562.
14. Gurney SM, Scott KS, Kacinko SL, Presley BC, Logan BK. Pharmacology, toxicology, and adverse effects of synthetic cannabinoid drugs. *Forensic Sci Rev.* 2014;26(1):53-78.
15. Hasegawa K, Wurita A, Minakata K, et al. Postmortem distribution of AB-CHMINACA, 5-fluoro-AMB, and diphenidine in body fluids and solid tissues in a fatal poisoning case: usefulness of adipose tissue for detection of the drugs in unchanged forms. *Forensic Toxicol.* 2015;33(1):45-53.
16. Thornton SL, Wood C, Friesen MW, Gerona RR. Synthetic cannabinoid use associated with acute kidney injury. *Clin Toxicol.* 2013;51(3):189-190.
17. Cannaert A, Storme J, Franz F, Auwarter V, Stove CP. Detection and activity profiling of synthetic cannabinoids and their metabolites with a newly developed bioassay. *Anal Chem.* 2016;88(23):11476-11485.
18. Chimalakonda KC, Seely KA, Bratton SM, et al. Cytochrome P450-mediated oxidative metabolism of abused synthetic cannabinoids found in K2/Spice: identification of novel cannabinoid receptor ligands. *Drug Metab Dispos.* 2012;40(11):2174-2184.
19. Castaneto MS, Wohlfarth A, Pang SK, et al. Identification of AB-FUBINACA metabolites in human hepatocytes and urine using high-resolution mass spectrometry. *Forensic Toxicol.* 2015;33(2):295-310.
20. Erratico C, Negreira N, Norouzizadeh H, et al. In vitro and in vivo human metabolism of the synthetic cannabinoid AB-CHMINACA. *Drug Test Anal.* 2015;7(10):866-876.

21. Wohlfarth A, Castaneto MS, Zhu M, et al. Pentylindole/Pentylindazole synthetic cannabinoids and their 5-Fluoro analogs produce different primary metabolites: metabolite profiling for AB-PINACA and 5F-AB-PINACA. *The AAPS journal.* 2015;17(3):660-677.
22. Andersson M, Diao X, Wohlfarth A, Scheidweiler KB, Huestis MA. Metabolic profiling of new synthetic cannabinoids AMB and 5F-AMB by human hepatocyte and liver microsome incubations and high-resolution mass spectrometry. *RCM.* 2016;30(8):1067-1078.
23. Thomsen R, Nielsen LM, Holm NB, Rasmussen HB, Linnet K, Consortium I. Synthetic cannabimimetic agents metabolized by carboxylesterases. *Drug Test Anal.* 2015;7(7):565-576.
24. Franz F, Jechle H, Wilde M, et al. Structure-metabolism relationships of valine and tert-leucine-derived synthetic cannabinoid receptor agonists: a systematic comparison of the in vitro phase I metabolism using pooled human liver microsomes and high-resolution mass spectrometry. *Forensic Toxicol.* 2019.
25. Franz F, Angerer V, Hermanns-Clausen M, Auwarter V, Moosmann B. Metabolites of synthetic cannabinoids in hair--proof of consumption or false friends for interpretation? *Anal Bioanal Chem.* 2016;408(13):3445-3452.
26. Franz F, Angerer V, Moosmann B, Auwarter V. Phase I metabolism of the highly potent synthetic cannabinoid MDMB-CHMICA and detection in human urine samples. *Drug Test Anal.* 2017;9(5):744-753.
27. Brents LK, Gallus-Zawada A, Radominska-Pandya A, et al. Monohydroxylated metabolites of the K2 synthetic cannabinoid JWH-073 retain intermediate to high cannabinoid 1 receptor (CB1R) affinity and exhibit neutral antagonist to partial agonist activity. *Biochem Pharmacol.* 2012;83(7):952-961.
28. Brents LK, Reichard EE, Zimmerman SM, Moran JH, Fantegrossi WE, Prather PL. Phase I hydroxylated metabolites of the K2 synthetic cannabinoid JWH-018 retain in vitro and in vivo cannabinoid 1 receptor affinity and activity. *PLoS One.* 2011;6(7):e21917.
29. Cannaert A, Franz F, Auwarter V, Stove CP. Activity-based detection of consumption of synthetic cannabinoids in authentic urine samples using a stable cannabinoid reporter system. *Anal Chem.* 2017;89(17):9527-9536.
30. Gamage TF, Farquhar CE, Lefever TW, et al. Molecular and behavioral pharmacological characterization of abused synthetic cannabinoids MMB- and MDMB-FUBINACA, MN-18, NNEI, CUMYL-PICA, and 5-Fluoro-CUMYL-PICA. *J Pharmacol Exp Ther.* 2018;365(2):437-446.
31. Longworth M, Connor M, Banister SD, Kassiou M. Synthesis and pharmacological profiling of the metabolites of synthetic cannabinoid drugs APICA, STS-135, ADB-PINACA, and 5F-ADB-PINACA. *ACS Chem Neurosci.* 2017;8(8):1673-1680.
32. Noble C, Cannaert A, Linnet K, Stove CP. 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.* 2018.
33. Rajasekaran M, Brents LK, Franks LN, Moran JH, Prather PL. Human metabolites of synthetic cannabinoids JWH-018 and JWH-073 bind with high affinity and act as potent agonists at cannabinoid type-2 receptors. *Toxicol Appl Pharmacol.* 2013;269(2):100-108.
34. Buchler IP, Hayes MJ, S.G. H, Inventors. Indazole derivatives. 2009.
35. European Monitoring Centre for Drugs and Drug Addiction (EMCDDA). . https://ednd.emcdda.europa.eu/html.cfm/index6555EN.html. Accessed 13 December 2018.
36. Cannaert A, Storme J, Hess C, Auwarter V, Wille SMR, Stove CP. Activity-based detection of cannabinoids in serum and plasma samples. *Clin Chem.* 2018;64(6):918-926.
37. Cannaert A, Vandeputte M, Hudson S, Wood DM, Dargan PI, Stove CP. Validation of activity-based screening for synthetic cannabinoid receptor agonists in a large set of serum samples. *Clin Chem.* 2019;65(2):347-349.
38. Banister SD, Connor M. The chemistry and pharmacology of synthetic cannabinoid receptor agonist new psychoactive substances: evolution. *Handb Exp Pharmacol.* 2018.
39. Kusano M, Zaitsu K, Taki K, et al. Fatal intoxication by 5F-ADB and diphenidine: Detection, quantification, and investigation of their main metabolic pathways in humans by LC/MS/MS and LC/Q-TOFMS. *Drug Test Anal.* 2018;10(2):284-293.

40. Banister SD, Longworth M, Kevin R, et al. Pharmacology of valinate and tert-leucinate synthetic cannabinoids 5F-AMBICA, 5F-AMB, 5F-ADB, AMB-FUBINACA, MDMB-FUBINACA, MDMB-CHMICA, and their analogues. *ACS Chem Neurosci.* 2016;7(9):1241-1254.
41. Adamowicz P. Fatal intoxication with synthetic cannabinoid MDMB-CHMICA. *Forensic Sci Int.* 2016;261:e5-10.
42. Angerer V, Franz F, Schwarze B, Moosmann B, Auwarter V. Reply to 'sudden cardiac death following use of the synthetic cannabinoid MDMB-CHMICA'. *J Anal Toxicol.* 2016;40(3):240-242.
43. Gaunitz F, Lehmann S, Thomas A, Thevis M, Rothschild MA, Mercer-Chalmers-Bender K. Post-mortem distribution of the synthetic cannabinoid MDMB-CHMICA and its metabolites in a case of combined drug intoxication. *Int J Legal Med.* 2018;132(6):1645-1657.
44. Hill SL, Najafi J, Dunn M, et al. Clinical toxicity following analytically confirmed use of the synthetic cannabinoid receptor agonist MDMB-CHMICA. A report from the Identification Of Novel psychoActive substances (IONA) study. *Clin Toxicol.* 2016;54(8):638-643.
45. Seywright A, Torrance HJ, Wylie FM, McKeown DA, Lowe DJ, Stevenson R. Analysis and clinical findings of cases positive for the novel synthetic cannabinoid receptor agonist MDMB-CHMICA. *Clin Toxicol.* 2016;54(8):632-637.
46. Westin AA, Frost J, Brede WR, et al. Sudden cardiac death following use of the synthetic cannabinoid MDMB-CHMICA. *J Anal Toxicol.* 2016;40(1):86-87.
47. Haden M, Wood DM, Dargan PI. The impact of the Psychoactive Substances Act 2016 on the online availability of MDMB-CHMICA. *QJM.* 2017;110(10):619-622.
48. Adamowicz P, Gieron J. Acute intoxication of four individuals following use of the synthetic cannabinoid MAB-CHMINACA. *Clin Toxicol.* 2016;54(8):650-654.
49. Wurita A, Hasegawa K, Minakata K, et al. Identification and quantitation of 5-fluoro-ADB-PINACA and MAB-CHMINACA in dubious herbal products. *Forensic Toxicol.* 2015;33(2):213-220.
50. Carlier J, Diao X, Sempio C, Huestis MA. Identification of new synthetic cannabinoid ADB-CHMINACA (MAB-CHMINACA) metabolites in human hepatocytes. *The AAPS journal.* 2017;19(2):568-577.
51. Hasegawa K, Minakata K, Gonmori K, et al. Identification and quantification of predominant metabolites of synthetic cannabinoid MAB-CHMINACA in an authentic human urine specimen. *Drug Test Anal.* 2018;10(2):365-371.
52. Lee JH, Jung A, Park HN, et al. Identification and characterization of an indazole-3-carboxamide class synthetic cannabinoid: 2-[1-(cyclohexylmethyl)-1H-indazole-3-carboxamido]-3,3-dimethylbutanoic acid (DMBA-CHMINACA). *Forensic Sci Int.* 2018;291:167-174.
53. Wiley JL, Marusich JA, Lefever TW, et al. AB-CHMINACA, AB-PINACA, and FUBIMINA: Affinity and potency of novel synthetic cannabinoids in producing delta9-tetrahydrocannabinol-like effects in mice. *J Pharmacol Exp Ther.* 2015;354(3):328-339.
54. Sim J, Cho HS, Lee J, In S, Kim E. Determination of AB-CHMINACA and its metabolites in human hair and their deposition in hair of abusers. *J Pharm Biomed Anal.* 2017;140:162-168.
55. Tyndall JA, Gerona R, De Portu G, et al. An outbreak of acute delirium from exposure to the synthetic cannabinoid AB-CHMINACA. *Clin Toxicol.* 2015;53(10):950-956.
56. Wurita A, Hasegawa K, Minakata K, et al. Identification and quantification of metabolites of AB-CHMINACA in a urine specimen of an abuser. *Leg Med (Tokyo).* 2016;19:113-118.
57. Doi T, Tagami T, Takeda A, Asada A, Sawabe Y. Evaluation of carboxamide-type synthetic cannabinoids as CB1/CB2 receptor agonists: difference between the enantiomers. *Forensic Toxicol.* 2018;36(1):51-60.
58. Li J, Liu C, Li T, Hua Z. UPLC-HR-MS/MS-based determination study on the metabolism of four synthetic cannabinoids, ADB-FUBICA, AB-FUBICA, AB-BICA and ADB-BICA, by human liver microsomes. *BMC.* 2018;32(3).
59. Lam RPK, Tang MHY, Leung SC, Chong YK, Tsui MSH, Mak TWL. Supraventricular tachycardia and acute confusion following ingestion of e-cigarette fluid containing AB-

FUBINACA and ADB-FUBINACA: a case report with quantitative analysis of serum drug concentrations. *Clin Toxicol.* 2017;55(7):662-667.
60. Nacca N, Schult R, Loflin R, et al. Coma, seizures, atrioventricular block, and hypoglycemia in an ADB-FUBINACA body-packer. *J Emerg Med.* 2018;55(6):788-791.
61. Shanks KG, Clark W, Behonick G. Death associated with the use of the synthetic cannabinoid ADB-FUBINACA. *J Anal Toxicol.* 2016;40(3):236-239.
62. Shevyrin V, Melkozerov V, Nevero A, et al. Identification and analytical characteristics of synthetic cannabinoids with an indazole-3-carboxamide structure bearing a N-1-methoxycarbonylalkyl group. *Anal Bioanal Chem.* 2015;407(21):6301-6315.
63. Krishna Kumar K, Shalev-Benami M, Robertson MJ, et al. Structure of a signaling cannabinoid receptor 1-G protein complex. *Cell.* 2019;176(3):448-458 e412.
64. Carlier J, Diao X, Wohlfarth A, Scheidweiler K, Huestis MA. In vitro metabolite profiling of ADB-FUBINACA, a new synthetic cannabinoid. *Curr Neuropharmacol.* 2017;15(5):682-691.
65. Kavanagh P, Grigoryev A, Krupina N. Detection of metabolites of two synthetic cannabimimetics, MDMB-FUBINACA and ADB-FUBINACA, in authentic human urine specimens by accurate mass LC-MS: a comparison of intersecting metabolic patterns. *Forensic Toxicol.* 2017;35(2):284-300.

Table 1 Potency (EC_{50}), efficacy ($E_{max,}$ relative to JWH-018) of the selected SCRAs and metabolites (n = 3).

Compound	EC_{50} (nM) (95 % CI)	E_{max} (%) (95 % CI)	Metabolite/Parent EC_{50}
5F-ADB-PINACA (1)	2.76 (1.24-5.41)	308.4 (277.8-346.4)	
5F-MDMB-PINACA (2)	0.84 (0.52-1.24)	319.3 (291.0-353.6)	
5F-ADB-PINACA-COOH (3)	113 (51.3-242)	245.6 (204.2-349.6)	40.9
5F-AB-PINACA (4)	55.4 (31.2-85.1)	216.8 (196.4-241.5)	
5F-AMB-PINACA (5)	15.1 (10.2-23.9)	258.6 (238.2-287.0)	
5F-AEB-PINACA (6)	8.76 (5.5-17.0)	235.4 (211.3-273.3)	
5F-AB-PINACA-COOH (7)	ND	ND	
ADB-CHMICA (8)	3.31 (0.94-12.4)	327.2 (280.4-459.4)	
MDMB-CHMICA (9)	1.77 (0.79-3.83)	285.1 (258.5-321.5)	
ADB-CHMICA-COOH (10)	ND	57.6 (35.7-92.4)*	
ADB-CHMINACA (11)	0.34 (0.02-0.91)	262.6 (236.7-301.1)	
MDMB-CHMINACA (12)	0.78 (0.22-1.90)	226.7 (202.9-261.2)	
ADB-CHMINACA-COOH (13)	ND	68.2 (63.5-73.0)*	
AB-CHMINACA (14)	3.45 (1.96-6.14)	390.5 (358.4-435.0)	
AMB-CHMINACA (15)	3.91 (1.86-8.44)	360.1 (322.2-421.2)	
AB-CHMINACA-COOH (16)	155 (97.4-277.6)	254.8 (227.3-292.3)	44.9
ADB-FUBICA (17)	12.3 (9.68-16.1)	313.6 (297.0-333.2)	
MDMB-FUBICA (18)	5.79 (2.97-10.25)	270.6 (243.7-303.0)	
ADB-FUBICA-COOH (19)	ND	81.8 (65.4-99.6)*	
ADB-FUBINACA (20)	0.82 (0.46-1.34)	273.6 (254.7-295.6)	
MDMB-FUBINACA (21)	0.36 (0.17-0.69)	240.9 (221.4-263.3)	
ADB-FUBINACA-COOH (22)	450 (176-749)	176.6 (141.0-314.7)	548.8
JWH-018	36.8 (28.6-50.4)	102.0 (97.6-107.5)	

*Maximum effect seen at 10 µM
ND: not determined since saturation has not been reached