



[biblio.ugent.be](https://biblio.ugent.be)

The UGent Institutional Repository is the electronic archiving and dissemination platform for all UGent research publications. Ghent University has implemented a mandate stipulating that all academic publications of UGent researchers should be deposited and archived in this repository. Except for items where current copyright restrictions apply, these papers are available in Open Access.

This item is the archived peer-reviewed author-version of:

Title: Activity-based Detection of Consumption of Synthetic Cannabinoids in Authentic Urine Samples Using a Stable Cannabinoid Reporter System

Authors: Cannaert, Annelies, Florian Franz, Volker Auwärter, and Christophe Stove

In: Analytical Chemistry 89 (17): 9527–9536, 2017

**To refer to or to cite this work, please use the citation to the published version:**

Cannaert, Annelies, Florian Franz, Volker Auwärter, and Christophe Stove. 2017. "Activity-based Detection of Consumption of Synthetic Cannabinoids in Authentic Urine Samples Using a Stable Cannabinoid Reporter System." Analytical Chemistry 89 (17): 9527–9536. DOI10.1021/acs.analchem.7b02552

# Activity-based detection of consumption of synthetic cannabinoids in authentic urine samples using a stable cannabinoid reporter system.

Annelies Cannaert<sup>1,§</sup>, Florian Franz<sup>2,§</sup>, Volker Auwärter<sup>2</sup>, Christophe P. Stove<sup>1,\*</sup>

<sup>1</sup>Laboratory of Toxicology, Department of Bioanalysis, Faculty of Pharmaceutical Sciences, Ghent University, Ottergemsesteenweg 460, 9000 Ghent, Belgium

<sup>2</sup>Institute of Forensic Medicine, Forensic Toxicology, Medical Center – University of Freiburg, Faculty of Medicine, University of Freiburg, Albertstr. 9, 79104 Freiburg, Germany

<sup>§</sup>equally contributed.

---

**ABSTRACT:** Synthetic cannabinoids (SCs) continue to be the largest group of new psychoactive substances (NPS) monitored by the European Monitoring Center of Drugs and Drugs of Abuse (EMCDDA). The identification and subsequent prohibition of single SCs has driven clandestine chemists to produce analogues of increasing structural diversity, intended to evade legislation. That structural diversity, combined with the mostly unknown metabolic profiles of these new SCs, poses a big challenge for the conventional targeted analytical assays, as it is difficult to screen for ‘unknown’ compounds. Therefore, an alternative screening method, not directly based on the structure but on the activity of the SC, may offer a solution for this problem. We generated stable CB1 and CB2 receptor activation assays based on functional complementation of a split NanoLuc luciferase and used these to test an expanded set of recent SCs (UR-144, XLR-11 and their thermal degradation products; AB-CHMINACA and ADB-CHMINACA) and their major phase I metabolites. By doing so, we demonstrate that several major metabolites of these SCs retain their activity at the cannabinoid receptors. These active metabolites may prolong the parent compound’s psychotropic and physiological effects and may contribute to the toxicity profile. Utility of the generated stable cell systems as a first-line screening tool for SCs in urine was also demonstrated using a relatively large set of authentic urine samples. Our data indicate that the stable CB reporter assays detect CB receptor activation by extracts of urine in which SCs (or their metabolites) are present at low- or subnanomolar (ng/ml) level. Hence, the developed assays do not only allow activity profiling of SCs and their metabolites, it may also serve as a screening tool, complementing targeted and untargeted analytical assays and/or preceding analytical (mass spectrometry based) confirmation.

---

Synthetic cannabinoids (SCs) continue to be the largest group of new psychoactive substances (NPS) monitored by the European Monitoring Center of Drugs and Drugs of Abuse (EMCDDA).<sup>1</sup> These “legal” alternatives for cannabis were first reported in 2008, at the time containing JWH-018 and CP 47,497-C8.<sup>2-3</sup> Many novel SCs were discovered since then, acting as agonists at the cannabinoid receptor 1 (CB1) and 2 (CB2). Although various products are labeled with warnings like ‘not for human consumption’, they are intended to mimic the psychoactive effects of cannabis. Many SCs are unknown prior to first detection by forensic chemists, and little to nothing is known about their activity in humans. The lack of data regarding the pharmacological and toxicological properties of emerging SCs poses worldwide a continuous challenge for scientists, healthcare workers, and lawmakers.<sup>4-6</sup>

The identification and subsequent prohibition of single SCs has driven clandestine chemists to produce analogues of increasing structural diversity, intended to evade legislation.<sup>5</sup> <sup>7-8</sup> Legislations based on individual structures are consequently stepping behind, but also the newer analogue laws in the US (2012)<sup>9</sup> and UK (2016)<sup>10</sup> controlling all “cannabimimetic” agents and substances with psychoactive properties (e.g. via the CB1 receptor) are challenged as the specific pharmacology of these new compounds is widely unknown.<sup>11</sup> This could be efficiently countered by applying these new compounds in biological assays to establish their cannabinoid activity and therefore their illegality. The structural diversity, combined with the mostly unknown metabolic profiles of these new SCs, also poses a big challenge for the conventional targeted analytical assays, as it is difficult to screen for ‘unknown’ compounds.<sup>5, 7, 12</sup> Although untargeted methods (e.g. high resolution mass spectrometry) are capable to screen for ‘unknown’ substances, these methods have limitations capacity- and sensitivity-wise. Immunoassays based on specific antibodies are of limited use because of missing cross-reactivity and insufficient sensitivity.<sup>13</sup> Therefore, alternative

screening methods not directly based on the structure of the SC may offer a solution for this problem. An activity-based assay may serve this purpose, by functioning as a first-line screening tool, complementing the conventional targeted and untargeted analytical methods. However, the detection of low concentrations of SCs in biological fluids requires high sensitivity bioassays, capable of monitoring low- or subnanomolar (ng/ml) concentrations of SCs. Also, the presence of active metabolites is a prerequisite if the screening tool is to be applied on urine samples, as SCs are extensively metabolized.<sup>14</sup> The presence of active metabolites was demonstrated following metabolism of JWH-018, JWH-073, XLR-11, JWH-122, MAM-2201, JWH-210, EAM-2201, PB-22 and 5F-PB-22.<sup>15-20</sup> We recently reported on novel cell based CB reporter bioassays for the activity-based detection of SCs and their metabolites, demonstrating cannabinoid activity in an authentic urine sample as a proof-of-concept.<sup>18</sup> The principle of this cell based bioassays is activity-based, where activation of the CB1 or CB2 receptor leads to  $\beta$ -arrestin 2 ( $\beta$ arr2) recruitment, which results in functional complementation of a split NanoLuc luciferase. This functional complementation restores the NanoLuc luciferase activity, resulting in a bioluminescent signal in the presence of the substrate furimazine, which can be read out with a standard luminometer. While the proof-of-concept of our CB reporter bioassays was successful, there were several limitations. First, the transient transfection used imposed a heavy workload and suffered from significant inter-experiment variability (depending on the transfection efficiency). Second, only a limited set of SCs (and metabolites) were tested. Third, only a proof-of-concept for one single user was demonstrated. To overcome these limitations, we generated stable cell systems and applied these on an expanded set of more recent SCs (UR-144, XLR-11 and their thermal degradation products; AB-CHMINACA and ADB-CHMINACA) and their major phase I metabolites.

UR-144 and its 5-fluoro analogue, XLR-11, belong to the tetramethylcyclopropyl indolyl ketone family (see Figure 1A and 1B). They were first reported to the EMCDDA in February 2012 by Latvian (XLR-11), Finnish and Polish (UR-144) authorities. The use of UR-144 and XLR-11 has been associated with acute kidney injury, acute ischemic events (upon inhalation) and death.<sup>21-24</sup> AB-CHMINACA and ADB-CHMINACA are part of a particularly prevalent class of SCs, first described in a Pfizer patent.<sup>25</sup> Their structure is comprised of an indazole core, modified by a cyclohexylmethyl group at the 1-position, and a valine- or tert-leucine-derived carboxamide moiety at the 3-position (see Figure 1C and 1D). AB-CHMINACA was formally reported to the EMCDDA in April 2014 following identification in Latvia,<sup>26</sup> and was later detected in various countries all over the world.<sup>27-28</sup> ADB-CHMINACA was first reported in September 2014 in Hungary.<sup>26</sup> The use of AB-CHMINACA and ADB-CHMINACA was implicated in clinical reports of acute delirium, agitation, seizures, respiratory failure and death.<sup>24, 29-32</sup> For most of the metabolites of these SCs, there is no information on their cannabinoid receptor activities. As it was demonstrated that several SCs are metabolized to a number of highly active metabolites,<sup>15-20</sup> activity-profiling of UR-144, XLR-11, their thermal degradation products, AB-CHMINACA and ADB-CHMINACA and their major phase I metabolites might help to explain the distinct adverse clinical manifestations that were observed with the use of these drugs. Finally, the generated stable cell systems were applied on a relatively large set of authentic urine samples to evaluate their potential as a screening tool for SCs in urine.

## MATERIALS AND METHODS

### *Chemical Reagents.*

All chemical reagents used are listed in [Supporting Information Data S-1](#). Blank urine samples were donated by volunteers and tested for the absence of SCs and their metabolites prior to use. Mobile phase A (1% acetonitrile, 0.1% formic acid and 2mM ammonium formate in water) and mobile phase B (0.1% formic acid, 2 mM ammonium formate in acetonitrile) were freshly prepared prior to LC-MS/MS analysis.

### *Retroviral Constructs.*

The CB1-LgBiT, CB2-SmBiT, SmBiT- $\beta$ arr2 and LgBiT- $\beta$ arr2 expression vectors were generated as previously described.<sup>18</sup> To generate the retroviral vectors, the coding sequences of interest, flanked by BamHI/EcoRI (for CB1-LgBiT and CB2-SmBiT) or BamHI/NotI restriction sites (SmBiT- $\beta$ arr2 and LgBiT- $\beta$ arr2) were PCR-amplified and cloned into corresponding digested retroviral vectors pLZRS-IRES-EGFP (CB-constructs) or pLZRS-IRES-dNGFR ( $\beta$ arr2-constructs), as described in [Supporting Information Data S-2](#). The integrity of all retroviral plasmids was confirmed by DNA sequencing. This yielded four retroviral vectors, each of which leads to co-expression of a gene of interest with either Enhanced Green Fluorescent Protein (EGFP) for the CB-constructs or truncated Nerve Growth Factor Receptor (dNGFR) for the  $\beta$ arr2-constructs. These

markers (EGFP and dNGFR) can be used for cell sorting and to check the stability of the cell lines by flow cytometry.

### *Production of Retrovirus and Retroviral Transduction.*

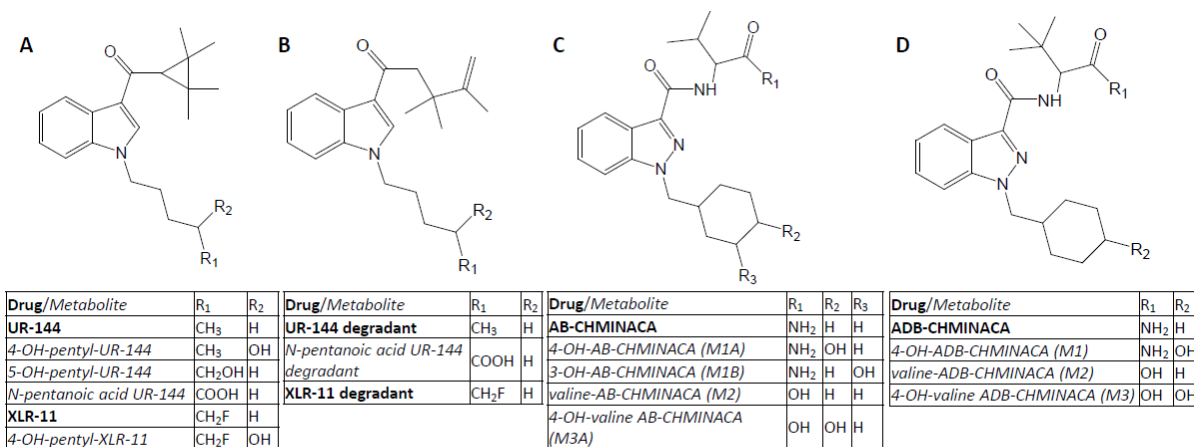
The Phoenix-Amphotropic packaging cell line<sup>33</sup> (a kind gift from prof. Bruno Verhasselt, Department of Clinical Chemistry, Microbiology, and Immunology, Ghent University, Belgium) was transfected with the LZRS-(CB-insert)-IRES-EGFP and the LZRS-( $\beta$ arr2-insert)-IRES-dNGFR plasmids, by using calcium phosphate precipitation (Invitrogen, San Diego, CA, USA). After two weeks of puromycin selection, the retroviral supernatant was harvested, spun (10 min at 350  $\times$  g) and aliquots of the supernatant were stored at -80°C until use. For transduction of human embryonic kidney (HEK) 293T, cells were seeded in a 96-well plate at 10<sup>4</sup> cells/well. After 24 h, the medium was changed for the retroviral supernatant, which had been preincubated for 10 minutes with Dotap (Roche Diagnostics). The cells were co-transduced with viruses containing both CB and  $\beta$ arr2 constructs by mixing the respective retrovirus containing supernatants. To increase transduction efficiency, cells were spun (90 minutes, 950  $\times$  g, 32°C). Transduction efficiency was evaluated by flow cytometry 48 hours after transduction, via assessment of expression of EGFP (for CB1-LgBiT and CB2-SmBiT) and dNGFR (for SmBiT- $\beta$ arr2 and LgBiT- $\beta$ arr2). For the latter, an APC-linked antibody against dNGFR was used (Chromaprobe, Inc.).

### *Cell Sorting and Cell Culture.*

Cell sorting was done on a BD FACSAria III, equipped with 405 nm, 488 nm, 561 nm, and 640 nm lasers (BD Biosciences, Erembodegem, Belgium). The cells needed to be positive for both EGFP and dNGFR, as they need to contain either the combination CB1-LgBiT/SmBiT- $\beta$ arr2 or CB2-SmBiT/LgBiT- $\beta$ arr2. All cells were routinely maintained at 37°C, 5% CO<sub>2</sub>, under humidified atmosphere in DMEM (high glucose) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM of glutamine, 100 IU/ml of penicillin, 100  $\mu$ g/ml of streptomycin and 0.25  $\mu$ g/ml of amphotericin B. Stability of the cell lines was followed up by flow cytometric analysis. For experiments, cells were plated on poly-D-lysine coated 96-well plates at 5 $\times$ 10<sup>4</sup> cells/well and incubated overnight.

### *Cannabinoid Reporter Assay.*

The cells were washed twice with Opti-MEM® I Reduced Serum Medium to remove any remaining FBS, and 100  $\mu$ L of Opti-MEM® I was added. The Nano-Glo Live Cell reagent (Promega), a nonlytic detection reagent containing the cell permeable furimazine substrate, was prepared by diluting the Nano-Glo Live Cell substrate 20 $\times$  using Nano-Glo LCS Dilution buffer, and 25  $\mu$ l was added to each well. Subsequently, the plate was placed in a luminometer, the GloMAX96 (Promega). Luminescence was monitored during the equilibration period until the signal stabilized (30–45 min). For agonist experiments, we added 10  $\mu$ l per well of test compounds, present as 13.5 $\times$  stocks in 50% methanol in Opti-MEM I. The luminescence was continuously detected for 120 min. Solvent controls were run in all experiments; the final concentration of methanol (3.7%) did not pose a problem given the short readout time of the assay.



**Figure 1.** Structures of SCs and metabolites. SCs belonging to the tetramethylcyclopropyl indolyl ketone family: UR-144 and XLR-11 (A) and thermal degradant products (B). AB-CHMINACA (C) and ADB-CHMINACA (D), which contain an indazole core modified at the 1-position with a cyclohexylmethyl group, and at the 3-position with a valine- or tert-leucine-derived carboxamide.

### Statistical Analysis.

Curve fitting and statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA). The results are represented as mean area under the curve (AUC)  $\pm$  standard error of mean (SEM) with at least three replicates for each data point (unless stated otherwise). Curve fitting of concentration–effect curves via nonlinear regression was employed to determine EC<sub>50</sub> (a measure of potency). To evaluate the activity of the different SCs and their metabolites, results are represented as the percentage (%) CB activation (relative to the maximum receptor activation of JWH-018)  $\pm$  SEM, with at least three replicates for each data point. Here, the absolute signals were baseline-corrected by subtracting the vehicle control samples and were corrected for the inter-well variability before the AUC calculations (see Supporting Information Data S-3). A one-way ANOVA, followed by Dunnett’s post hoc test, was used to determine statistical significance ( $P < 0.05$ ) (i) between all compounds and the reference compound JWH-018, (ii) within a group between a parent compound and the other compounds in that group (e.g., all compounds related to AB-CHMINACA vs. AB-CHMINACA itself), and (iii) between the signals obtained from the compounds and those from solvent controls.

### Urine Sample Preparation.

Conjugate cleavage was conducted by adding 0.5 ml phosphate buffer (pH 6) and 30  $\mu$ l  $\beta$ -glucuronidase to 0.5 ml of urine, followed by 1 h incubation at 45 °C. Afterwards, 1.5 ml ice-cold acetonitrile and 0.5 ml 10 M ammonium formate were added. The mixture was shaken and centrifuged. One milliliter of the organic phase was transferred to a separate vial and evaporated to dryness under a stream of nitrogen at 40 °C. For analysis with the CB reporter assays, the evaporated extract was reconstituted in 100  $\mu$ l of Opti-MEM I/MeOH (50/50, v/v), of which 10  $\mu$ l was used per well (see the Cannabinoid Reporter Assay section). For LC-MS/MS analysis, another 0.5 ml aliquot was spiked with reference standards and internal standards (IS), if applicable, and processed as described above. The residue was reconstituted in 200  $\mu$ l of mobile phase A/B (50/50, v/v) prior to LC-MS/MS analysis. Fortified calibration samples (0.05, 0.1, 0.25, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0 ng/ml), control samples (0.07, 0.4, 4.0, 20.0, 40.0 ng/ml), as well as blank and zero (blank with IS) samples were used for quantification and method validation. The concentration of IS in the samples was 0.4 ng/ml for all IS, except for JWH-200-D5 (0.8 ng/ml).

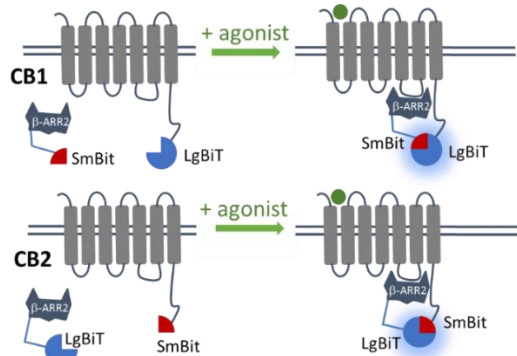
### LC-ESI-MS/MS Analysis of Urine Samples.

Quantification of SCs and their metabolites in authentic urine samples was performed by applying a fully validated LC-ESI-MS/MS method operating in positive MRM mode. Technical details concerning chromatographic and ionization conditions were reported elsewhere<sup>34</sup>, while the optimized MS parameters for each compound are listed in Supporting Information Data S-4. The method validation was conducted in accordance to the guidelines of the German speaking Society of Toxicological and Forensic Chemistry (GTFCh).<sup>35</sup> All validation data are summarized in Supporting Information Data S-5. In brief: selectivity was tested by analyzing different blank urine samples and no relevant interferences were observed. Linearity was achieved between 0.01 and 50.0 ng/ml, depending on the analyte. Calibration curves of UR-144 and XLR-11 as well as their degradation products and metabolites showed relatively steep slopes leading to rapid saturation of the detector and relatively narrow dynamic ranges. Since concentrations of the pentanoic acid metabolites of UR-144 and its degradation product are usually relatively high in authentic urine samples, additional quantification via quadratic regression was validated to extend the dynamic range for these two compounds. Limits of detection (LODs) ranged from 0.01 to 0.25 ng/ml. Limits of quantification (LOQs) ranged from 0.05 to 0.25 ng/ml. Accuracy of the method showed a bias between -9.4% and 13.1%, inter-day precision was below 11%, and intra-day precision below 10% over the analyzed control levels (0.07, 0.4, 4.0, 20.0, 40.0 ng/ml). Matrix effects and recoveries were evaluated according to the procedure suggested by Matuszewski *et al.*<sup>36</sup> While matrix effects were between 87 and 151% and showed standard deviations below 18% for most compounds and concentration levels, matrix effects were more pronounced at the lowest control level (0.07 ng/ml) and for ADB-CHMINACA M1 with a maximum enhancement of 213% and maximum standard deviation of 45%. In general, recoveries were between 81 and 94%, with small standard deviations (below 8%) for most compounds and concentration levels. Significantly lower recoveries were observed at the lowest concentration level (0.07 ng/ml) and for the compounds AB-CHMINACA M3A and ADB-CHMINACA M3 (the most polar substances covered by the method), with extreme values of 27%, but still sufficient reproducibility (standard deviations below 7%).

## RESULTS AND DISCUSSION

### Stable expression of the Cannabinoid Reporter Assay.

The cannabinoid reporter assays utilize a structural complementation-based approach to monitor protein interactions within living cells (NanoLuc Binary Technology). It makes use of inactive subunits of NanoLuc luciferase, Large BiT (LgBiT; 18 kDa) and Small BiT (SmBiT; 1 kDa), which are coupled to two proteins of interest, which are in our case the cannabinoid receptors, CB1 and CB2, and  $\beta$ -arrestin 2 ( $\beta$ arr2). Upon CB activation, the cytosolic  $\beta$ arr2 protein interacts with the receptor, leading to receptor desensitization and internalization. That interaction promotes structural complementation of the NanoLuc luciferase subunits, thereby restoring luciferase activity, which generates a bioluminescent signal in the presence of the furimazine substrate.

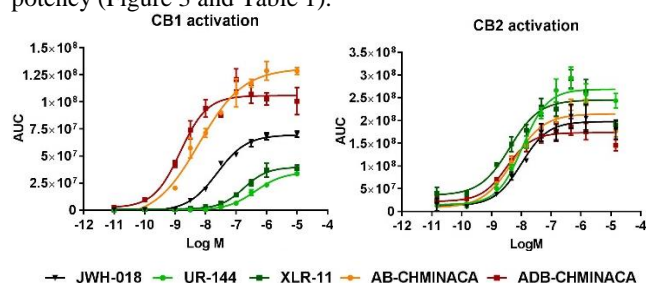


**Figure 2: Setup of the CB reporter assays for CB1 and CB2: CB1–LgBiT/SmBiT– $\beta$ arr2 and CB2–SmBiT/LgBiT– $\beta$ arr2.**

We previously set up and applied this reporter system in a transient format in which cells were transiently transfected, demonstrating applicability using a limited panel of SCs and providing a proof-of-principle for one authentic urine sample.<sup>18</sup> Here, we report on the establishment of two stable cell lines, either expressing the fusion proteins CB1–LgBiT/SmBiT– $\beta$ arr2 or CB2–SmBiT/LgBiT– $\beta$ arr2 (see Figure 2). These cell lines were obtained following retroviral transduction of HEK293T cells and flow cytometry-assisted cell sorting, to yield cell lines co-expressing the CB1 or CB2 construct with a  $\beta$ arr2 construct, with a purity of  $\geq 93\%$ . Via flow cytometric analysis of the co-expressed markers EGFP and dNGFR, the stability of these cell lines can be monitored in time (see Supporting Information Data S-6). This is important, since expression of these constructs may impose a negative effect on growth, which would jeopardize the cell line's utility in long term. We indeed observed some decrease in double positive (EGFP+ and dNGFR+) cells in time and utilized the cells until passage 20, in which double positivity remained  $\geq 70\%$ . Up to this point, we did not notice a measurable effect on our systems' performance. Yet, if deemed necessary, the stably co-expressed markers always offer the possibility to submit the cell lines to another round of cell sorting.

Upon stimulation of the stable systems with a known agonist, JWH-018, CB1–LgBiT and CB2–SmBiT showed a concentration-dependent interaction with SmBiT– $\beta$ arr2 and LgBiT– $\beta$ arr2, respectively, with  $EC_{50}$  values of 23.9 nM (95% CI: 18.3–31.6) and 6.8 nM (95% CI: 3.3–13.8). These values are in good correspondence with those determined using the transient system (CB1: 38.2 nM (95% CI: 27.1–55.7), CB2: 12.8 nM (95% CI: 5.6–26.0)).<sup>15</sup> The stable system was also applied on UR-144, XLR-11, AB-CHMINACA, and ADB-CHMINACA. Concentration-dependent curves were obtained

and  $EC_{50}$  values were determined as a measure of relative potency (Figure 3 and Table 1).



**Figure 3. The concentration-dependent interaction of CB1 (A) and CB2 (B) with  $\beta$ arr2 upon stimulation with different SCs. AUC, area under the curve. Data are given as mean AUC  $\pm$  SEM (n=5-6).**

Drug	CB1 $EC_{50}$ (nM)	CB2 $EC_{50}$ (nM)
JWH-018	23.9 (18.3-31.6)	6.8 (3.29-13.8)
UR-144	426 (312-635)	7.4 (4.5-12)
XLR-11	179 (113-285)	2.8 (1.0-6.6)
AB-CHMINACA	6.1 (3.1-11.4)	3.7 (2.1-6.3)
ADB-CHMINACA	1.49 (0.69-2.61)	2.2 (1.0-4.3)

**Table 1.  $EC_{50}$  values of different SCs.  $EC_{50}$  values are presented as a measure of potency. Data are given as  $EC_{50}$  values (95% CI profile likelihood).**

Although it is difficult to compare  $EC_{50}$  values from different assays (due to different experimental setups), our values are in line with those found in the literature. E.g. it is known that UR-144 and XLR-11 bind CB2 with a higher affinity than CB1.<sup>37-39</sup> This is not surprising, given that these compounds are structurally related to a series of indol-3-yl-cycloalkyl ketones that were originally synthesized by Abbott Laboratories as part of their effort to develop CB2-selective cannabinoids.<sup>37</sup> That CB2 selectivity is reflected in our in vitro functional data. Banister *et al.* also reported a clear CB2 preference for UR-144 (FLIPR membrane potential assay in AtT-20 cells), although for XLR-11 an equal level of activation of both CB receptors was found.<sup>12</sup> This may derive from the fact that the studies were done on different cell types, which may lead to different signaling pathways. Our in vitro functional data also confirm that AB-CHMINACA and ADB-CHMINACA are highly potent SCs, which is consistent with the low  $EC_{50}$  values reported in literature, varying from 0.278–7.8 nM and 21 nM (for AB-CHMINACA), for respectively CB1 and CB2.<sup>12, 25, 40-41</sup> Interestingly, our finding that ADB-CHMINACA is about 4 times more potent than AB-CHMINACA at CB1 confirms data from an earlier report by Buchler *et al.* (GTP $\gamma$ S binding assay in CHO cell membranes,  $EC_{50}$  values for CB1 of 2.55 nM and 0.620 nM for AB-CHMINACA and ADB-CHMINACA, respectively).<sup>22</sup> For the efficacy in terms of  $\beta$ arr2 recruitment, we observed that both AB-CHMINACA and ADB-CHMINACA showed a stronger  $\beta$ arr2 recruitment at CB1 than JWH-018, a known full agonist at CB1, an observation we also made for other SCs, such as JWH-122, JWH-210, PB-22 and their 5-fluoro-analogues.<sup>18</sup>

### Application of the CB Reporter Assays on SCs and Their Main Phase I Metabolites.

#### UR-144 and XLR-11

Biotransformation of UR-144 and XLR-11 (and their thermal degradant products, generated by smoking)<sup>38, 42-43</sup> leads to common phase I metabolites: the N-pentanoic acid UR-144 and N-pentanoic acid UR-144 degradant metabolites. UR-144 metabolism also results in trace amounts of the 4-OH-

pentyl-UR-144 metabolite, whereas for XLR-11, the 5-OH-pentyl-UR-144 and 4-OH-pentyl-XLR-11 metabolites are also found in authentic urine samples (unpublished observations).<sup>44</sup> The ring open degradants of XLR-11 and UR-144 were reported to possess a higher affinity than their parent compounds at both CB receptors.<sup>35</sup> Also for the 5-OH-pentyl-UR-144 metabolite, there is already some information on the binding and the functional activity (via FLIPR membrane potential assay) at the CB receptors. More specifically, it was reported to be CB2 selective.<sup>15,37</sup> Since, apart from the above-described limited and fragmented information, the activity of most UR-144 and XLR-11 metabolites at CB receptors is not known, we evaluated these with our CB reporter bioassays. For each of these compounds, we assessed CB1 and CB2 receptor activation, at a receptor saturating concentration (10  $\mu$ M), with JWH-018 as a reference (Figure 4, [Supporting Information Data S-7](#)).

UR-144, XLR-11, their degradant products, and their metabolites all showed significant CB1 receptor activation, although there were major differences between the different compounds (see Figure 4). UR-144, 4-OH-pentyl-UR-144, 5-OH-pentyl-UR-144, 4-OH-pentyl-XLR-144, and both N-pentanoic acid metabolites show a significantly lower level of CB1 activation relative to the reference JWH-018, whereas the degradant product of UR-144 shows a significantly higher level of receptor activation. XLR-11 and its degradant show a similar level of activation compared to JWH-018. For both degradants it was reported that they show an increase in  $E_{max}$  at CB1 compared to UR-144 and XLR-11 (GTP $\gamma$ S binding assay in HEK293 cell membranes)<sup>38</sup>, although we only observed this for the UR-144 degradant. At CB2, UR-144, its degradant product, and its metabolites all showed significant receptor activation, which was not significantly different from the reference compound JWH-018. Only UR-144 degradant pentanoic acid showed a slightly lower level of activation compared to its parent compound UR-144. For XLR-11, both the XLR-11 degradant product and the 4-OH-pentyl metabolite showed a lower level of CB2 activation relative to XLR-11, but they did not significantly differ from the reference JWH-018. Our findings are consistent with those reported in literature, in which a similar  $E_{max}$  at CB2 for UR-144 and XLR-11 was reported, although we observed a statistically significant difference when comparing the XLR-11 degradant with XLR-11.<sup>38</sup> This difference could be related to the different experimental setup.

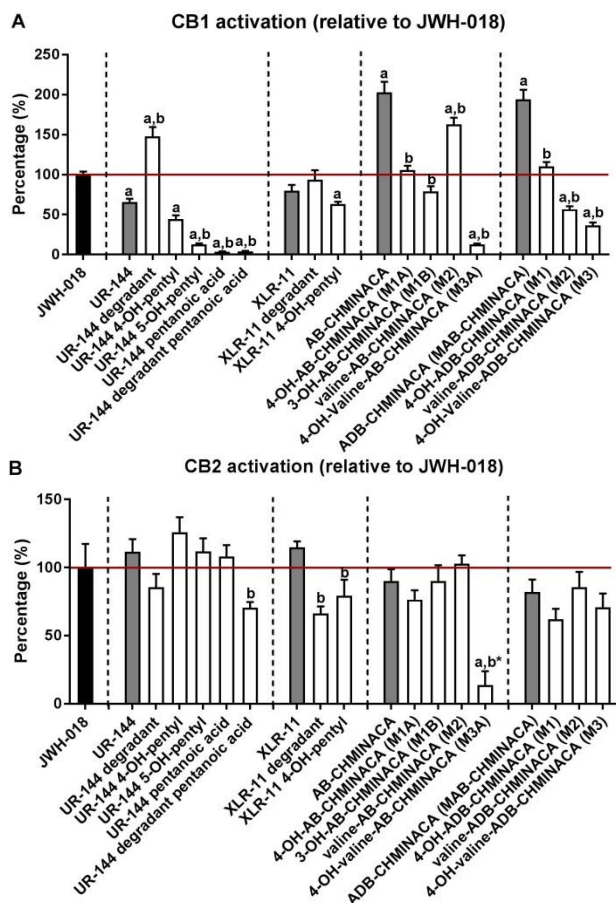
#### **AB-CHMINACA and ADB-CHMINACA.**

To select the major phase I metabolites of AB-CHMINACA and ADB-CHMINACA that were to be tested in our CB reporter bioassays, we first analyzed authentic urine samples via LC-ESI-MS/MS to identify these. For AB-CHMINACA, the major phase I metabolites identified are the 4-OH-AB-CHMINACA (M1A), valine-AB-CHMINACA (M2), 4-OH-valine-AB-CHMINACA (M3A), and two isomers of the M3A metabolite. The latter two could not be tested for activity as no reference standards were available. The 3-OH-AB-CHMINACA metabolite (M1B) was present to a lesser extent (unpublished observations). In previous studies on identification and quantification of metabolites of AB-CHMINACA in urine specimens from abusers, metabolites monohydroxylated on the cyclohexyl moiety (corresponding to M1A and M1B) and another metabolite carboxylated at the terminus of the amide linker (M2) were detected.<sup>32,45-48</sup> Also

the combination of both metabolites (monohydroxylation at cyclohexyl moiety and carboxylation at the outer amide) were reported to be found in urine specimens (M3A and isomers).<sup>31,45</sup> For ADB-CHMINACA, major metabolites in the authentic urine samples were 4-OH-ADB-CHMINACA (M1), an M1 isomer, 4-OH-valine-ADB-CHMINACA (M3), and four M3 isomer metabolites. Also the valine-ADB-CHMINACA (M2) metabolite was found in authentic urine samples (unpublished observations). The M1 and M3 metabolite isomers of ADB-CHMINACA were not available as reference standards and could therefore not be tested. Using human hepatocyte cultures, Carlier *et al.* also recently found M1 and its isomer to be important ADB-CHMINACA metabolites. These authors did not identify any carboxylated metabolites (M2 and M3), which may be owing to the limitation of using in vitro systems for mimicking human metabolism.<sup>47</sup> Very recently, Hasegawa *et al.* reported on the identification and quantification of 2 predominant metabolites of ADB-CHMINACA in an authentic post-mortem human urine specimen: the M1 metabolite and the M11 metabolite (corresponding to the M1 metabolite, with additional hydroxylation at the tert-butyl moiety in the amide linker).<sup>48</sup> The latter was only reported to be a minor metabolite by Carlier *et al.* and was not present in the authentic urine samples we examined from different living individuals. Hence, we did not include M11 as a test compound in our assay.<sup>47</sup> For both AB- and ADB-CHMINACA also the parent compound was present in urine samples containing high concentrations of metabolites (unpublished observations). Each of these compounds was evaluated with our bioassays at a receptor saturating concentration (10  $\mu$ M), with JWH-018 as a reference (Figure 4, [Supporting Information Data S-7](#)).

AB-CHMINACA, ADB-CHMINACA, and all evaluated metabolites, except 4-OH-valine-AB-CHMINACA (M3A), showed significant CB1 and CB2 activation, although there were major differences between the different compounds. The highest signals were obtained for the parent compounds, AB-CHMINACA and ADB-CHMINACA, which showed a significantly higher level of CB1 activation relative to JWH-018. While, as compared to the parent compounds, all metabolites showed a reduced level of CB1 activation, the valine-AB-CHMINACA metabolite (M2) still displayed a significantly stronger level of CB1 activation than JWH-018. For 4-OH-AB-CHMINACA (M1A) and 3-OH-AB-CHMINACA (M1B), as well as for the monohydroxylated metabolite of ADB-CHMINACA (M1), there was no significant difference compared to the reference JWH-018. 4-OH-valine-AB-CHMINACA (M3A) showed the lowest level of CB1 activation. Also CB1 activation by valine-ADB-CHMINACA (M2) and 4-OH-valine-ADB-CHMINACA (M3) was significantly lower than that induced by JWH-018. At CB2, all compounds, except 4-OH-valine-AB-CHMINACA (M3A), yielded a signal that was not significantly different from that of the reference JWH-018. The finding that the valine metabolites of AB-CHMINACA and ADB-CHMINACA (M2 metabolites) still showed CB1 activation was surprising, because these metabolites were

reported to have little, if any, affinity to the CB1 receptor ( $K_i = 380$  nM and  $K_i > 4010$  nM, respectively).<sup>25</sup> Overall, these data demonstrate that, although metabolization results in a reduced activity in all instances, the vast majority of metabolites still has considerable activity at CB1 and CB2 (in many cases comparable with the reference JWH-018). Only when the valine metabolite is additionally hydroxylated (or, vice versa, when in the hydroxylated metabolite the outer amide group is oxidized to a carbonyl group), most activity is lost.



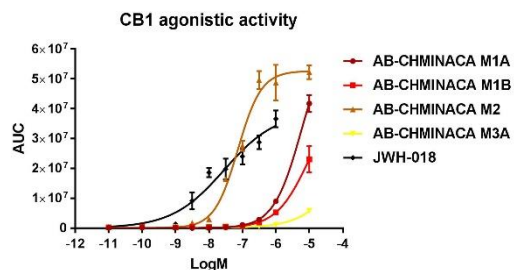
**Figure 4. Activation of CB1 receptor (A) and CB2 receptor (B).** Values designated with (a) above error bars denote a significant difference from the reference compound, JWH-018 ( $P \leq 0.05$ , one-way ANOVA with Dunnett's multiple comparison post hoc test). Values designated with (b) are significantly different from the reference compound within a group (groups are separated via vertical dotted lines). Bars assigned with an (\*) are not significantly different from basal levels. Data are given as the mean percentage CB receptor activation (in comparison to the receptor activation of the reference, JWH-018)  $\pm$  SEM ( $n = 4$ ).

#### Application of the CB Reporter Assays on authentic urine samples from SC users.

Two batches of urine samples were analyzed. Samples of the first batch mainly comprised urine samples positive for metabolites of UR-144, XLR-11, AB-CHMINACA, or ADB-CHMINACA as confirmed via LC-ESI-MS/MS analysis. Analysis of this batch served to evaluate the sensitivity of the bioassays. The second batch of authentic urine samples included a higher proportion of SC negative samples and was

used to score the specificity. Both CB reporter assays were used to score urinary extracts from both batches. The scoring (positive/negative) of randomized samples was done blind-coded by two individuals independently, who were unaware of the number of positives per batch. If the final scoring of the sample differed between the two individuals, which was eventually only the case for one sample, we conservatively decided to consider the sample negative.

The first batch contained 42 urine samples (41 positives and 1 negative) and was analyzed along with 4 known blanks (see Table 2). From the 18 urine samples from users who had consumed either UR-144 or XLR-11, 17 were scored positive (94.4%) (Table 2A). The extract of the one sample that was missed, was strongly colored and contained low levels of XLR-11 metabolites (see Table 2A). In general, a pronounced coloration of the extract was found to influence the signal obtained in the CB reporter assays (more specifically resulting in a drop of signal), which makes the scoring of such samples difficult (see Supporting Information Data S-8). The pronounced coloration is not linked to the creatinine content of the urine sample, as can be seen in Table 2. From the 12 samples positive for AB-CHMINACA metabolites, only 4 were scored positive (33.3%) (Table 2B). This low detection rate was unexpected, as the activity profiling of the AB-CHMINACA metabolites (performed at 10  $\mu$ M) had revealed activity at both CB1 and CB2. Further evaluation of the activity of the AB-CHMINACA metabolites demonstrated that the M1 metabolites had strongly reduced potency (see Figure 5, right shift of the curves). This was less the case for the M2 metabolite, although also here, the curve only started to rise at higher concentrations, compared to the JWH-018 reference (Figure 5). For the two negatively scored samples with M2 metabolite  $> 50$  ng/ml coloration of the extract may explain the false negative result. The false negative results of the other samples can likely be explained by the fact that the concentrations of the metabolites were too low to give rise to a signal that could clearly be distinguished from background. As some samples with relatively low metabolite concentrations were scored positive (though weakly), the metabolite concentrations in these samples might lie at the current assays tipping point.



**Figure 5. The concentration-dependent interaction of CB1 with  $\beta$ arr2 upon stimulation with the major phase I metabolites of AB-CHMINACA.** AUC, area under the curve. Data are given as mean AUC  $\pm$  SEM ( $n = 4-6$ ).

Nine out of 11 (81.8%) urine samples from users who had consumed ADB-CHMINACA were scored positive (Table 2C). The two missed cases both contained lower

concentrations of ADB-CHMINACA metabolites (approx. 2.5 ng/mL of the major metabolite M1), one also being strongly colored, resulting in a drop of signal (see [Supporting Information Data S-8](#)). The unknown blank was scored correctly negative (not shown in Table 2). Overall, this leads to a sensitivity of 73.2% (30/41) for the first batch of urine samples.

A		UR-144 [ng/ml]				XLR-11 [ng/ml]	Score in bioassays	
SC	Level*	Creatinine [mg/dl]	4-OH-pentyl	5-OH-pentyl	pentanoic acid	Degradant pentanoic acid		
UR-144	+	420	0.68	0.18	2.02	35.30	+	
UR-144	+	99	0.42	0.32	0.73	3.65	+	
XLR-11	+++	260		> 10	> 50	> 50	3.13	+
XLR-11	+++	240		> 10	44.80	> 50	1.39	+
XLR-11	+++	190		> 10	46.80	> 50	0.44	+
XLR-11	++	390		<b>8.14</b>	29.80	> 50	0.21	+
XLR-11	++	38		<b>5.14</b>	23.40	> 50	0.17	+
XLR-11	++	300		<b>4.64</b>	33.10	> 50	0.38	+
XLR-11	++	160		<b>4.27</b>	10.80	> 50	0.81	+
XLR-11	++	360		<b>4.11</b>	15.90	> 50	0.12	+
XLR-11	++	34		<b>2.92</b>	10.20	> 50	0.13	+
XLR-11	++	190		<b>2.26</b>	8.40	> 50	0.11	+
XLR-11	++	230		<b>1.28</b>	0.40	16.70	+	+
XLR-11	+	320		<b>0.99</b>	7.56	> 50	+	+
XLR-11	+	53		<b>0.86</b>	6.69	44.50	+	+
XLR-11	+	230		<b>0.36</b>	2.31	36.20	-	-
XLR-11	+	130		<b>0.31</b>	1.80	24.10	+	+
XLR-11	+	180		<b>0.21</b>	0.64	11.90	+	+

B		AB-CHMINACA [ng/ml]					Score in bioassays	
SC	Level*	Creatinine [mg/dl]	Parent	M1A	M1B	M2		M3A
AB-CHMINACA	++++	31	0.61	> 50	20.00	> 50	> 50	+
AB-CHMINACA	++++	260	0.26	> 50	6.55	> 50	34.80	+
AB-CHMINACA	++++	260		> 50	12.70	> 50	> 50	-
AB-CHMINACA	++++	310		> 50	3.24	> 50	35.70	-
AB-CHMINACA	+++	89		22.70	1.46	<b>16.40</b>	8.95	-
AB-CHMINACA	+++	140		18.40	1.28	<b>16.00</b>	4.86	-
AB-CHMINACA	++	40		13.30	1.50	<b>8.41</b>	4.16	+
AB-CHMINACA	++	230		4.68	0.46	<b>6.32</b>	3.46	-
AB-CHMINACA	++	110		1.37	0.43	<b>1.45</b>	1.32	-
AB-CHMINACA	++	50		1.27	0.10	<b>1.37</b>	0.42	-
AB-CHMINACA	++	54		1.54	0.15	<b>1.06</b>	0.62	+
AB-CHMINACA	+	150		4.42	0.37	<b>0.95</b>	0.53	-

C		ADB-CHMINACA [ng/ml]				Score in bioassays	
SC	Level*	Creatinine [mg/dl]	Parent	M1	M2		M3
ADB-CHMINACA	++++	350	0.19	> 50	4.33	> 50	+
ADB-CHMINACA	+++	94		<b>43.90</b>	3.79	38.80	+
ADB-CHMINACA	+++	240		<b>30.80</b>	2.90	32.60	+
ADB-CHMINACA	+++	210		<b>16.20</b>	1.00	16.10	+
ADB-CHMINACA	+++	310		<b>15.90</b>	2.20	25.10	+
ADB-CHMINACA	++	100	0.14	<b>9.99</b>	0.22	4.64	+
ADB-CHMINACA	++	79		<b>5.01</b>	0.15	1.31	+
ADB-CHMINACA	++	45		<b>3.81</b>	0.31	2.50	+
ADB-CHMINACA	++	110		<b>2.57</b>	0.30	2.08	+
ADB-CHMINACA	++	320		<b>2.55</b>	0.47	3.04	-
ADB-CHMINACA	++	220		<b>2.44</b>	0.32	3.68	-

\*level is determined by most potent metabolite (bold): + <1 ng/ml, ++ 1-10 ng/ml, +++ 10-50 ng/ml, ++++ > 50 ng/ml.

**Table 2. List of authentic urine samples from users of UR-144/XLR-11 (A), AB-CHMINACA (B), or ADB-CHMINACA (C). The intensity of the color of the extract is shown by the different shades of gray.**

The second batch contained 32 urine samples (8 SC positive and 24 SC negative samples) and was analyzed along with 4 known blanks (Table 3). The SC negative samples were full blanks (n=14), authentic urine samples containing (metabolites of) drugs of abuse (cocaine, diverse stimulants, THC, and opiates) and also a urine sample spiked with 1 µg/ml THC-COOH (Table 3B). From the 8 samples from users who consumed either UR-144, XLR-11, AB-CHMINACA, and ADB-CHMINACA, 6 were scored positive, leading to a sensitivity of 75% (6/8), which aligns with the overall sensitivity of the first batch of urine samples (73.2% - 30/41). The extracts of the two samples that were missed contained

AB-CHMINACA or ADB-CHMINACA metabolites at relatively low (AB-CHMINACA) or very low (ADB-CHMINACA) concentrations (Table 3A). The sensitivity results are linked to the type of SCs included in the batch of analyzed urine samples. Other SCs can give different sensitivity rates. From the 24 SC negative urine samples, 19 were scored negative. Amongst the 5 positively scored SC negative samples, three authentic urine samples contained THC-COOH (levels of other cannabis-related substances unknown), demonstrating use of natural cannabinoids. Although we confirmed that THC-COOH does not possess any detectable cannabinoid activity<sup>18</sup> (see also spiked THC-COOH sample 15 in Table 3B and [Supporting Information Data S-8](#)), the presence of other cannabinoids, such as THC and 11-OH-THC, may result in a (genuine) positive result in natural cannabis users. This does not pose a problem as these positive samples are also easily picked up by conventional (natural) cannabinoid screening methods. For two out of the 5 positively scored SC negative samples, no explanation could be found for the positive scoring.

A		UR-144 [ng/ml]				XLR-11 [ng/ml]	Score in bioassays	
SC	Level*	Creatinine [mg/dl]	4-OH-pentyl	5-OH-pentyl	pentanoic acid	Degradant pentanoic acid		
UR-144	+	99	0.42	0.32	0.73	3.65	+	
UR-144	+	420	0.68	0.18	2.02	35.30	+	
XLR-11	+++	300		> 10	44.80	> 50	1.39	+
XLR-11	++	240		<b>4.64</b>	33.10	> 50	0.38	+

B		AB-CHMINACA [ng/ml]					Score in bioassays	
SC	Level*	Creatinine [mg/dl]	Parent	M1A	M1B	M2		M3A
AB-CHMINACA	+++	180	0.90	> 50	32.90	<b>11.80</b>	6.33	+
AB-CHMINACA	++	180		13.10	3.83	<b>2.69</b>	0.84	-

C		ADB-CHMINACA [ng/ml]				Score in bioassays	
SC	Level*	Creatinine [mg/dl]	Parent	M1	M2		M3
ADB-CHMINACA	+++	220		<b>39.80</b>	3.87	39.20	+
ADB-CHMINACA	+	89		<b>0.6</b>	0.06	0.58	-

\*level is determined by most potent metabolite (+ <1 ng/ml, ++ 1-10 ng/ml, +++ 10-50 ng/ml, ++++ > 50 ng/ml)

B		SC negative samples	Score in bioassays
1	Blank urine		-
2	Blank urine		-
3	Blank urine		+
4	Blank urine		-
5	Blank urine		-
6	Blank urine		+
7	Blank urine		-
8	Blank urine		-
9	Blank urine		-
10	Blank urine		-
11	Blank urine		-
12	Blank urine		-
13	Blank urine		-
14	Blank urine		-
15	Blank spiked with THC-COOH (1 µg/ml)		-
16	THC-COOH (21 ng/ml)*		+
17	THC-COOH (43 ng/ml)*		+
18	THC-COOH (95 ng/ml)*		-
19	THC-COOH (230 ng/ml)*		-
20	THC-COOH (910 ng/ml)*		+
21	THC-COOH (120 ng/ml)*, benzylecgonine (210 ng/ml), ecgonine methylester (31 ng/ml)		-
22	THC-COOH (450 ng/ml)*, Methamphetamine (approx. 7.5 ng/ml), MDMA (> 5,000 ng/ml), MDA (approx. 3,500 ng/ml), Methylone (approx. 5.4 ng/ml)		-
23	Amphetamine (approx. 4.4 ng/ml)		-
24	Morphine (280 ng/ml), Codeine (35 ng/ml), Noscapine (+)		-

\*level is determined by most potent metabolite (bold): + <1 ng/ml, ++ 1-10 ng/ml, +++ 10-50 ng/ml, ++++ > 50 ng/ml.

**Table 3. List of authentic urine samples from users of UR-144/XLR-11, AB-CHMINACA, or ADB-CHMINACA (A) and**



**the SC negative urine samples (B). The intensity of the color of the extract is shown by the different shades of gray.**

Also additional screening with the ToxTyper approach<sup>49</sup> did not reveal any relevant compounds. Hence these samples should be considered as genuine false positives. Therefore, we can conclude that our CB reporter bioassays yielded a false positive result in 2/21 cases, resulting in a specificity of 90.5%. Application on an even larger scale – which is beyond the scope of this study – is warranted to confirm these percentages.

## CONCLUSION

We successfully developed stable CB1 and CB2 receptor activation assays based on the principle of functional complementation of a split NanoLuc luciferase. In contrast to the initially developed assays, which were in a transient format<sup>18</sup>, the newly developed assays are in a stable cell format, offering a reduced workload, a higher reproducibility within experiments, and a control on stability, via co-expressed markers. The CB reporter assays were applied to determine the *in vitro* activity of a new set of SCs (UR-144, XLR-11 and their thermal degradation products; AB-CHMINACA and ADB-CHMINACA) and their metabolites at CB1 and CB2 receptors, revealing for the first time that several of their major phase I metabolites retain activity at the CB receptors. The high potency of SCs, in combination with their metabolism to a number of highly active metabolites, might help to explain the distinct adverse clinical manifestations that were observed with the use of these SCs. Interestingly, AB-CHMINACA and ADB-CHMINACA were more efficacious at CB1, compared to the known full agonist JWH-018, but whether this relates to more toxicity is unknown.

Finally, we evaluated the utility of the bioassays as a screening method for SCs on a relatively large set of authentic urine samples. Given the continuous modifications to the SCs' structure to circumvent laws on controlled substances, conventional targeted analytical methods struggle as it is difficult to continuously update 'in-house' libraries and to screen for 'unknown' compounds. Another critical problem is that these high-potency drugs often result in very low drug concentrations. Here, we are the first to apply an activity-based screening method for the detection of SCs in a panel of authentic urine samples, therefore circumventing the need to know the specific structure of the SC. Our data indicate that the stable CB reporter assays detect CB receptor activation by extracts of urine in which SCs (or their metabolites) are present at low- or subnanomolar (ng/ml) level. The presence of other drugs (of abuse), tested here, did not influence the CB reporter bioassays. The presence of natural cannabinoids may give rise to a positive result though, which is not surprising as we screen for CB activity. Confirmation of these cannabis positive samples can be done via conventional THC assays and, if positive, actually does not require further testing for SCs as the person readily is considered positive. Two genuine blanks (9.5%) were falsely scored positive. Evaluation on large sample numbers –which is beyond the scope of the

current study – is needed to further substantiate this. Application of colored extracts in our bioassays yielded false negative results in several instances. Optimization of extraction could possibly solve this issue. On the other hand, the data obtained for AB-CHMINACA – with a rather low detection rate of positive samples – indicate that there is still room for improvement of the CB receptor activation assays (something we are actively pursuing). The low detection rate with AB-CHMINACA is in contrast with the good sensitivity we obtained for the ADB-CHMINACA positive samples (81.8%). This may be related to subtle differences in metabolic pathways between AB- and ADB-CHMINACA, despite the minor structural difference (i.e. the propyl and tert-butyl moiety for AB- and ADB-CHMINACA, respectively), as well as to a difference in potency of metabolites of ADB- vs AB-CHMINACA.<sup>48</sup> Notably, while the AB-CHMINACA metabolites appear to have a reduced potency, several of these metabolites demonstrated high efficacy at both CB receptors. Hence, not surprisingly, the application of our bioassays on urine specimens relies on the presence of sufficiently high concentrations of sufficiently potent metabolites. Anyway, it should be kept in mind that these CB1/CB2 bioassays are meant to serve as a screening tool, complementing existing assays, with as unique advantages the independence of mass-based information, as well as the fact that no antibody recognition is required. Indeed, immunoassay-based SC screening strategies have been demonstrated to have limited value, recognizing only clearly related structures, which is not surprising.<sup>13</sup> Therefore, we believe that our data do support the potential of deploying CB receptor activation assays as a first-line screening tool to detect SC use in urine samples, complementing targeted and untargeted analytical assays and/or preceding analytical (mass spectrometry based) confirmation.

## ASSOCIATED CONTENT

### Supporting Information

Additional information as noted in text. The Supporting Information is available free of charge on the ACS Publications website.

S-1: Chemical Reagents; S-2: Generation of Retroviral Constructs; S-3: Explanation of correction for solvent controls and for inter-well variability; S-4: Mass spectrometric parameters of the LC-ESI-MS/MS confirmation method; S-5: Validation data of the LC-ESI-MS/MS confirmation method; S-6: Evaluation of the stability of the created cell lines via flow cytometry; S-7: Comparison of relative efficacy to activate CB1 and CB2 at 10  $\mu$ M; S-8: Activation profiles of several authentic urine samples.

## AUTHOR INFORMATION

### Corresponding Author

\* Email: [christophe.stove@ugent.be](mailto:christophe.stove@ugent.be). Tel.: +32 9 264 81 35. Fax: +32 9 264 81 83.

### Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

## ACKNOWLEDGMENT

The authors wish to acknowledge Dr. Ann Baeyens, Sophie Vermaut and prof. Bruno Verhasselt for their help with the generation of the stable cell lines. A. Cannart would like to acknowledge the Agency for Innovation by Science and Technology (IWT-Flanders) for granting her a Grant for Strategic Basic Research (application number: 121504). The research activities of the Institute of Forensic Medicine Freiburg were financially supported by the 'Prevention of and Fight against Crime' program of the European Commission (JUST/2013/ISEC/DRUGS/AG/6421).

## REFERENCES

- EMCDDA, *European Drug Report: Trends and Developments 2017*.
- Auwärter, V.; Dresen, S.; Weinmann, W.; Müller, M.; Pütz, M.; Ferreiros, N., *J. Mass. Spectrom.* **2009**, 44 (5), 832-7.
- Uchiyama, N.; Kikura-Hanajiri, R.; Kawahara, N.; Haishima, Y.; Goda, Y., *Chem. Pharm. Bull. (Tokyo)* **2009**, 57 (4), 439-41.
- Castaneto, M. S.; Gorelick, D. A.; Desrosiers, N. A.; Hartman, R. L.; Pirard, S.; Huestis, M. A., *Drug Alcohol Depend.* **2014**, 144, 12-41.
- Zawilska, J. B.; Andrzejczak, D., *Drug Alcohol Depend.* **2015**, 157, 1-17.
- Banister, S. D.; Longworth, M.; Kevin, R.; Sachdev, S.; Santiago, M.; Stuart, J.; Mack, J. B.; Glass, M.; McGregor, I. S.; Connor, M.; Kassiou, M., *ACS Chem. Neurosci.* **2016**, 7 (9), 1241-54.
- Kikura-Hanajiri, R.; Kawamura, N. U.; Goda, Y., *Drug Test. Anal.* **2014**, 6 (7-8), 832-9.
- Bijlsma, L.; Ibáñez, M.; Miserez, B.; Ma, S. T. F.; Shine, T.; Ramsey, J.; Hernández, F., *Forensic Toxicol.* **2017**, 1-13.
- USA, *Food and Drug Administration Safety and Innovation Act (FDASIA): Synthetic Drug Abuse Prevention Act of 2012* **2012**, Section 1151-1153, 126 STAT. 1130-32.
- UK, *Psychoactive Substances Act 2016* **2016**, Chapter 2, 1-57.
- Huestis, M. A.; Tyndale, R. F., *Clin. Pharmacol. Ther.* **2017**, 101 (2), 152-7.
- Longworth, M.; Banister, S. D.; Mack, J. B.; Glass, M.; Connor, M.; Kassiou, M., *Forensic Toxicol.* **2016**, 34, 286-303.
- Franz, F.; Angerer, V.; Jechle, H.; Pegoro, M.; Ertl, H.; Weinfurter, G.; Janele, D.; Schlogl, C.; Friedl, M.; Gerl, S.; Mielke, R.; Zehnle, R.; Wagner, M.; Moosmann, B.; Auwarter, V., *Clin. Chem. Lab. Med.* **2017**. DOI: 10.1515/cclm-2016-0831
- Diao, X.; Huestis, M. A., *Clin. Pharmacol. Ther.* **2017**, 101 (2), 239-53.
- Banister, S. D.; Stuart, J.; Kevin, R. C.; Edington, A.; Longworth, M.; Wilkinson, S. M.; Beinart, C.; Buchanan, A. S.; Hibbs, D. E.; Glass, M.; Connor, M.; McGregor, I. S.; Kassiou, M., *ACS Chem. Neurosci.* **2015**, 6 (8), 1445-58.
- Brents, L. K.; Gallus-Zawada, A.; Radominska-Pandya, A.; Vasiljevik, T.; Prinszano, T. E.; Fantegrossi, W. E.; Moran, J. H.; Prather, P. L., *Biochem. Pharmacol.* **2012**, 83 (7), 952-61.
- Brents, L. K.; Reichard, E. E.; Zimmerman, S. M.; Moran, J. H.; Fantegrossi, W. E.; Prather, P. L., *PLoS One* **2011**, 6 (7), e21917.
- Cannaert, A.; Storme, J.; Franz, F.; Auwarter, V.; Stove, C. P., *Anal. Chem.* **2016**, 88 (23), 11476-85.
- Chimalakonda, K. C.; Seely, K. A.; Bratton, S. M.; Brents, L. K.; Moran, C. L.; Endres, G. W.; James, L. P.; Hollenberg, P. F.; Prather, P. L.; Radominska-Pandya, A.; Moran, J. H., *Drug Metab. Dispos.* **2012**, 40 (11), 2174-84.
- Rajasekaran, M.; Brents, L. K.; Franks, L. N.; Moran, J. H.; Prather, P. L., *Toxicol. Appl. Pharmacol.* **2013**, 269 (2), 100-8.
- Adamowicz, P.; Gieron, J.; Gil, D.; Lechowicz, W.; Skulska, A.; Tokarczyk, B., *Forensic Sci. Int.* **2017**, 273, e18-e21.
- Shanks, K. G.; Winston, D.; Heidingsfelder, J.; Behonick, G., *Forensic Sci. Int.* **2015**, 252, e6-9.
- Takematsu, M.; Hoffman, R. S.; Nelson, L. S.; Schechter, J. M.; Moran, J. H.; Wiener, S. W., *Clin Toxicol (Phila)* **2014**, 52 (9), 973-5.
- Trecki, J.; Gerona, R. R.; Schwartz, M. D., *N. Engl. J. Med.* **2015**, 373 (2), 103-7.
- Buchler, I. P.; Hayes, M. J.; Hedge, S. G.; Hockerman, S. L.; Jones, D. E.; Kortum, S. W.; Rico, J. G.; Tenbrink, R. E.; Wu, K. K., *Int. Pat. Appl. WO 2009/106980 A2* **2009**.
- EMCDDA, *New psychoactive substances in Europe: An update of the EU Early Warning System* **2015**.
- Langer, N.; Lindigkeit, R.; Schiebel, H. M.; Papke, U.; Ernst, L.; Beuerle, T., *Forensic Sci. Int.* **2016**, 269, 31-41.
- Uchiyama, N.; Shimokawa, Y.; Kawamura, M.; Kikura-Hanajiri, R.; Hakamatsuka, T., *Forensic Toxicol.* **2014**, 32 (2), 266-81.
- Abouchedid, R.; Hudson, S.; Thurtle, N.; Yamamoto, T.; Ho, J. H.; Bailey, G.; Wood, M.; Sadones, N.; Stove, C. P.; Dines, A.; Archer, J. R. H.; Wood, D. M.; Dargan, P. I., *Clin. Toxicol. (Phila)* **2017**, 55 (5), 338-45.
- Adamowicz, P.; Gieron, J., *Clin. Toxicol. (Phila)* **2016**, 54 (8), 650-4.
- Katz, K. D.; Leonetti, A. L.; Bailey, B. C.; Surmaitis, R. M.; Eustice, E. R.; Kacinko, S.; Wheatley, S. M., *West. J. Emerg. Med.* **2016**, 17 (3), 290-4.
- Tyndall, J. A.; Gerona, R.; De Portu, G.; Trecki, J.; Elie, M. C.; Lucas, J.; Shish, J.; Rand, K.; Bazydlo, L.; Holder, M.; Ryan, M. F.; Myers, P.; Iovine, N.; Plourde, M.; Weeks, E.; Hanley, J. R.; Endres, G.; St Germaine, D.; Dobrowolski, P. J.; Schwartz, M., *Clin. Toxicol. (Phila)* **2015**, 53 (10), 950-6.
- Swift, S.; Lorens, J.; Achacoso, P.; Nolan, G. P., *Curr. Protoc. Immunol.* **2001**, Chapter 10, Unit 10 17C.
- Franz, F.; Angerer, V.; Hermanns-Clausen, M.; Auwarter, V.; Moosmann, B., *Anal. Bioanal. Chem.* **2016**, 408 (13), 3445-52.
- Peters, F. T.; Hartung, M.; Herbold, M.; Schmitt, G.; Daldrup, T.; Mußhoff, F., *Toxicchem. Krimtech.* **2009**, 76 (GTFCh validation guidelines for forensic toxicological analysis), 185-208.
- Matuszewski, B. K.; Constanzer, M. L.; Chavez-Eng, C. M., *Anal. Chem.* **2003**, 75 (13), 3019-30.
- Frost, J. M.; Dart, M. J.; Tietje, K. R.; Garrison, T. R.; Grayson, G. K.; Daza, A. V.; El-Kouhen, O. F.; Yao, B. B.; Hsieh, G. C.; Pai, M.; Zhu, C. Z.; Chandran, P.; Meyer, M. D., *J. Med. Chem.* **2010**, 53 (1), 295-315.
- Thomas, B. F.; Lefever, T. W.; Cortes, R. A.; Grabenauer, M.; Kovach, A. L.; Cox, A. O.; Patel, P. R.; Pollard, G. T.; Marusich, J. A.; Kevin, R. C.; Gamate, T. F.; Wiley, J. L., *J. Pharmacol. Exp. Ther.* **2017**, 361 (1), 162-71.
- Wiley, J. L.; Marusich, J. A.; Lefever, T. W.; Grabenauer, M.; Moore, K. N.; Thomas, B. F., *Neuropharmacology* **2013**, 75, 145-54.
- Franz, F.; Angerer, V.; Moosmann, B.; Auwarter, V., *Drug Test. Anal.* **2017**, 9 (5), 744-53.
- Wiley, J. L.; Marusich, J. A.; Lefever, T. W.; Antonazzo, K. R.; Wallgren, M. T.; Cortes, R. A.; Patel, P. R.; Grabenauer, M.; Moore, K. N.; Thomas, B. F., *J. Pharmacol. Exp. Ther.* **2015**, 354 (3), 328-39.
- Adamowicz, P.; Zuba, D.; Sekula, K., *Forensic Sci. Int.* **2013**, 233 (1-3), 320-7.
- Grigoryev, A.; Kavanagh, P.; Melnik, A.; Savchuk, S.; Simonov, A., *J. Anal. Toxicol.* **2013**, 37 (5), 265-76.
- Jang, M.; Kim, I. S.; Park, Y. N.; Kim, J.; Han, I.; Baeck, S.; Yang, W.; Yoo, H. H., *Anal. Bioanal. Chem.* **2016**, 408 (2), 503-16.
- Erratico, C.; Negreira, N.; Norouzizadeh, H.; Covaci, A.; Neels, H.; Maudens, K.; van Nuijs, A. L., *Drug Test. Anal.* **2015**, 7 (10), 866-76.
- Wurita, A.; Hasegawa, K.; Minakata, K.; Gonmori, K.; Nozawa, H.; Yamagishi, I.; Suzuki, O.; Watanabe, K., *Leg. Med. (Tokyo)* **2016**, 19, 113-8.
- Carlier, J.; Diao, X.; Sempio, C.; Huestis, M. A., *AAPS J.* **2017**, 19 (2), 568-77.
- Hasegawa, K.; Minakata, K.; Gonmori, K.; Nozawa, H.; Yamagishi, I.; Watanabe, K.; Suzuki, O., *Drug Test. Anal.* **2017** DOI: 10.1002/dta.2220
- Kempf, J.; Traber, J.; Auwarter, V.; Huppertz, L. M., *Forensic Sci. Int.* **2014**, 243, 84-9.

Insert Table of Content here:

