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Activity-based detection of new psychoactive substances as an alternative screening approach in forensic toxicology.

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Ghent, 2018,

The promoters,

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Dankwoord

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Table of Contents

List of abbreviations

4-ANPP	despropionyl fentanyl
5F-ADB	N-[[1-(5-fluoropentyl)-1H-indazol-3-yl]carbonyl]-3-methyl-D-valine, methyl ester
5F-PB-22	1-(5-fluoropentyl)-8-quinolinyl ester-1H-indole-3-carboxylic acid
11-OH-THC	11-hydroxy-Δ^9-tetrahydrocannabinol
AB-CHMINACA	N-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide
ADB-CHMINACA	N-[1-(aminocarbonyl)-2,2-dimethylpropyl]-1-(cyclohexylmethyl)-1H-indazole-3-carboxamide
AB-FUBINACA	N-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-[(4-fluorophenyl)methyl]-1H-indazole-3-carboxamide
AH-7921	3,4-dichloro-N-[[1-(dimethylamino)cyclohexyl]methyl]-benzamide
ANOVA	analysis of variance
APC	allophycocyanin
ARE	androgen response element
AUC	area under the curve
βarr2	β-arrestin 2
CB	cannabinoid receptor
CALUX	chemical-activated luciferase gene expression
CP 47,497-C8	5-(1,1-dimethyloctyl)-2-[(1R,3S)-3-hydroxycyclohexyl]-phenol
DAMGO	[d-Ala^2-MePhe^4-Gly-ol]encephalin
dNGFR	truncated Nerve Growth Factor Receptor
DOR	δ-opioid receptor
EC_{50}	concentration giving a half maximum response
E. *coli*	Escherichia *coli*
EG-018	naphthalen-1-yl(9-pentyl-9H-carbazol-3-yl)methanone
EGFP	enhanced Green Fluorescent Protein
EMCDDA	European Monitoring Centre for Drugs and Drug Addiction
EAM-2201	(4-ethyl-1-naphthalenyl)[1-(5-fluoropentyl)-1H-indol-3-yl]-methanone
EU	European Union
FLIPR	Fluorescent Imaging Plate Reader
FUB-AMB	N-[[1-[(4-fluorophenyl)methyl]-1H-indazol-3-yl]carbonyl]-L-valine, methyl ester
Furanyl fentanyl	N-phenyl-N-[1-(2-phenylethyl)-4-piperidinyl]-2-furancarboxamide
GC	gas chromatography
GPCR	G-protein coupled receptor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRK2	G-protein coupled receptor kinase 2
GTP	guanosine triphosphate

h	hour
HEK	human embryonic kidney
HR	high resolution
HSP	Heat Shock Proteins
IC_{50}	half maximal inhibitory concentration
IRES	internal ribosomal entry site
JWH-018	(1-pentyl-1H-indol-3-yl)-1-naphthalenyl-methanone
JWH-073	(1-butyl-1H-indol-3-yl)-1-naphthalenyl-methanone
JWH-122	(4-methyl-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)-methanone
JWH-210	(4-ethyl-1-naphthalenyl)(1-pentyl-1H-indol-3-yl)-methanone
KOR	κ-opioid receptor
LC	liquid chromatography
LgBiT	Large BiT, subunit of NanoLuc luciferase
LLOQ	lower limit of quantification
LOD	limit of detection
LOQ	limit of quantification
LSD	Lysergic acid diethylamide
MAM-2201	[1-(5-fluoropentyl)-1H-indol-3-yl](4-methyl-1-naphthalenyl)-methanone
MDA	3,4-methylenedioxyamphetamine
MDMA	3,4-methylenedioxymethamphetamine
MDMB-CHMICA	N-[[1-(cyclohexylmethyl)-1H-indol-3-yl]carbonyl]-3-methyl-L-valine, methyl ester
MDMB-CHMINACA	N-[[1-(cyclohexylmethyl)-1H-indazol-3-yl]carbonyl]-3-methyl-L-valine, methyl ester
MeOH	methanol
mL	milliliter
MOR	μ-opioid receptor
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MT-45	1-cyclohexyl-4-(1,2-diphenylethyl)-piperazine
ng	nanogram
NPS	New psychoactive substances
PB-22	1-pentyl-8-quinolinyl ester-1H-indole-3-carboxylic acid
PCR	polymerase chain reaction
POI	protein of interest
PR	progesterone receptor
PRE	progesterone response element
Q-TOF	Quadrupole Time-Of-Flight

RLU	relative light unit
SCRA	synthetic cannabinoid receptor agonist
SEM	standard error of mean
SmBiT	Small BiT, subunit of NanoLuc luciferase
SR	steroid hormone receptor
SRE	steroid response element
THC	Δ^9-tetrahydrocannabinol
THCCOOH	tetrahydrocannabinol carboxylic acid
U-47700	*trans*-3,4-dichloro-N-[2-(dimethylamino)cyclohexyl]-N-methyl-benzamide
UK	United Kingdom
ULOQ	upper limit of quantification
UNODC	United Nations Office on Drugs and Crime
UR-144	(1-pentyl-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)-methanone
US	United States
WADA	World Anti-Doping Agency
XLR-11	(1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone

CHAPTER 1:
AIMS AND OUTLINE OF THE THESIS

1.1 Introduction to new psychoactive substances

New psychoactive substances (NPS) are defined by the UNODC as "substances of abuse, either in a pure form or a preparation, that are not controlled by the 1961 Single Convention on Narcotic Drugs or the 1971 Convention on Psychotropic Substances, but which may pose a public health threat"[1-2]. In the context of this definition, the term ''new'' does not necessarily refer to new chemical entities, but rather those compounds that have recently become available on the recreational drug market[2].

Many NPS are created by modifying the chemical structure of illegal drugs or prescribed medications to generate substances which circumvent existing drug control laws. They are usually intended to mimic the effects of controlled drugs, while others are aimed at small groups who wish to explore them for possible novel effects ("psychonauts"). As governments pass legislations to render specific NPS illegal, new replacement analogs are synthesized and marketed to stay one step ahead of regulators and law enforcement[3].

In recent years, there has been an explosive growth in the market for NPS. By the end of 2016, more than 620 NPS had been reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) (Figure 1.1)[3]. These substances are not covered by international drug controls and make up a broad range of drugs such as synthetic cannabinoids, stimulants, opioids and benzodiazepines. These new substances are often produced in bulk quantities by chemical and pharmaceutical companies in China, from where they are shipped to Europe, where they are processed into products, packaged and sold. They are openly sold on the internet as 'research chemicals' or on the deep web, through darknet markets, supported by technologies that hide buyer and seller identities. These substances can be sold under their own name or be falsely labelled as other illicit drugs such as heroin, cocaine, ecstasy and benzodiazepines[3].

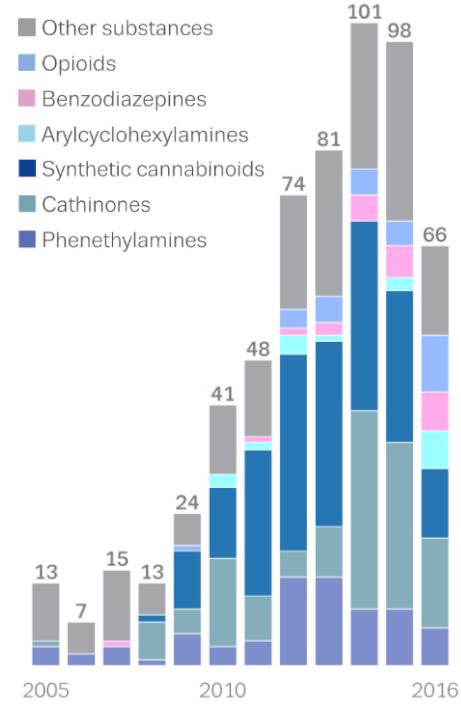

Figure 1.1. Number of NPS reported to the EU Early Warning System (2005-2016). Source: EMCDDA

In this thesis, the focus lies on two NPS subgroups: synthetic cannabinoids (also referred to as synthetic cannabinoid receptor agonists, SCRAs) and synthetic opioids.

1.1.1 Synthetic cannabinoids

Synthetic cannabinoid receptor agonists (SCRAs) are a group of substances that mimic the effects of Δ^9-tetrahydrocannabinol (THC), which is the substance that is responsible for the major psychoactive effects of cannabis. Like THC, SCRAs bind to the endogenous cannabinoid receptors 1 and 2 (CB1 and CB2) in the body. Stimulation of central CB1 receptors produces the desired euphoria and relaxation effects sought by natural and synthetic cannabinoids users, while CB2 receptors, primarily located in the periphery, are critical for immune functions and represent a potential therapeutic opportunity. Many SCRAs were first developed by scientists investigating how cannabinoids affect the body and to see if they could work as medicines to treat a number of diseases and their symptoms, such as neurodegenerative diseases, drug dependence, pain disorders and cancer (e.g. Laboratory of John W. Huffman (JWH compounds), Pfizer Inc. (CP compounds), Hebrew University (HU compounds), and Alexandros Makriyannis (AM compounds))[4-11].

However, so far it has proven difficult to separate the desired medicinal properties from unwanted psychoactive effects[12-13]. This is why SCRAs have found their way into the illicit drug market as (formerly) 'legal' replacements for cannabis. Since 2005 there were internet rumors of 'herbal smoking mixtures' sold as 'legal highs' that could produce 'strong' cannabis-like effects, but it wasn't until 2008 that forensic investigators in Germany and Austria first detected the synthetic cannabinoid JWH-018, in a product sold under the brand name 'Spice'[14]. Currently, they are the largest group of new psychoactive substances monitored by the EMCDDA, with 169 newly detected compounds from 2008-2016[3].

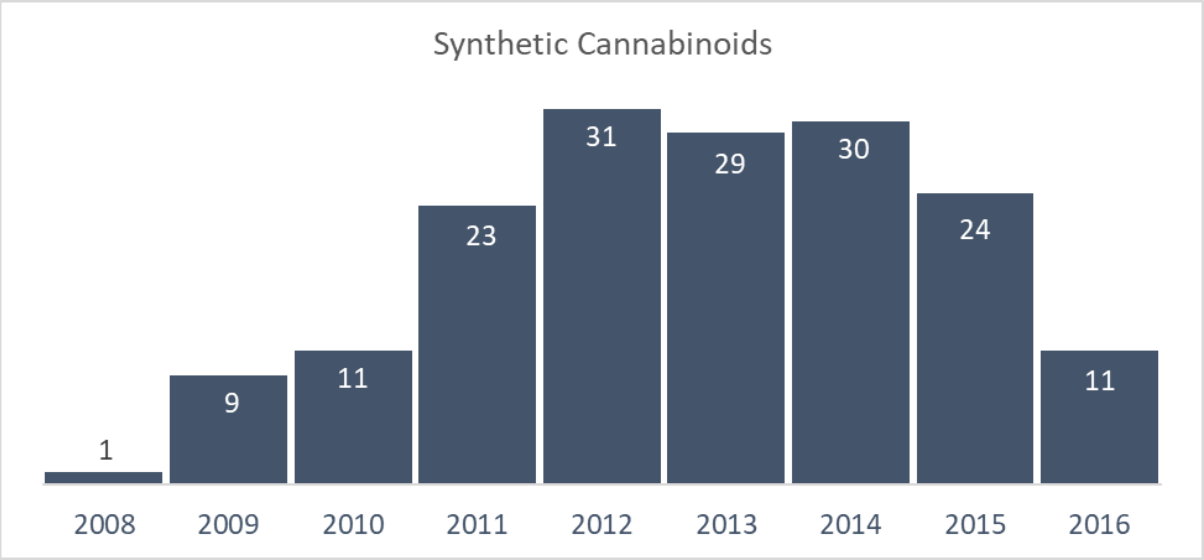

Figure 1.2. The emergence of novel SCRAs reported to the EMCDDA from 2008-2016. (Source: EMCDDA)

SCRAs are primarily sold as 'herbal blends' or 'incense' products under a variety of brand names (Spice and K2, as the most known ones). The products most commonly consist of inert dried plant material, on which one or more SCRAs are sprayed. They can also be purchased as research chemicals. SCRAs are most commonly smoked, although administration via oral consumption, anal insertion or nasal spray has also been reported[13].

Relatively little is known about how these substances work and how they exert their toxic effects in humans. Amongst the frequently reported adverse health effects associated with SCRAs use are agitation, nausea and an abnormally fast, racing heartbeat[15-16]. Serious adverse events, such as stroke, seizure, heart attack, breakdown of muscle tissue, kidney damage, psychosis and severe or prolonged vomiting, and associated deaths are less common[4, 17-20]. Symptoms suggestive of dependence and withdrawal have also been reported[21]. SCRA use has also manifested as outbreaks of mass poisonings (so-called "zombie outbreaks")[22]. It is possible that, along with being highly potent (SCRAs often act as full agonists at CB1 and CB2, in contrast to THC, which is a partial agonist), some may also have long half-lives and/or be converted to active metabolites[23-27], potentially leading to a prolonged psychoactive effect.

The number of SCRAs, their chemical diversity and the speed of their emergence make this group of compounds particularly challenging in terms of detection, monitoring, and responding. Suppliers simply aim to mimic the effects of THC. In essence, this makes each SCRA disposable. When one SCRA is (or is about to be) legally controlled, manufacturers quite often already have one or more replacement substances ready for sale. An elaborate description of the different subclasses of SCRAs is beyond the scope of this "Aims and Outline" Chapter. Several excellent reviews on this matter have been published[28-30].

The rapid proliferation of novel SCRAs makes the detection of these new derivatives challenging in different contexts, such as forensic, clinical and analytical chemistry[31-32]. The recent proliferation of SCRAs and other NPS has initiated considerable interest in the development of so-called 'untargeted' screening strategies in order to detect and identify novel compounds without the use of certified reference materials or mass spectral libraries. High-resolution mass spectrometry (HRMS) has been the method of choice for broad screening of NPS in a wide range of contexts because of its ability to measure accurate masses using both data-dependent and data-independent acquisition[32]. However, due to the time-consuming and expensive character of this technique, this method is not routinely implemented in most clinical and forensic laboratories. It should also be considered that the sensitivity of HRMS configurations, often requiring a threshold to be reached, may preclude detection of SCRAs, which are often present at low- or sub-nanogram per ml levels in biological fluids. Moreover, SCRAs are strongly metabolized and the metabolism of novel SCRAs is often poorly characterized, which again results in these compounds being missed by

HRMS. Therefore, alternative 'untargeted' screening methods, which are less expensive and more routinely applicable, may offer a solution for this problem.

The rapid proliferation of novel SCRAs poses problems for legislators as well. While laws based on individual structures are consequently one step behind, the newer analogue laws in the US (2012)[33] and the UK (2016)[34], controlling all "cannabimimetic" agents and substances with psychoactive properties (e.g., via the CB1 receptor), may also be challenged, as the specific pharmacology of these new compounds is widely unknown[35]. In other words, one would first need to apply these compounds in a biological assay to establish their cannabinoid activity and therefore their illegality. In Belgium, recently a generic structure law was introduced for SCRAs, illegalizing all derivatives from certain basic structures[36]. In essence, this legislation puts a lot of pressure on toxicological laboratories, as these should actually be capable of detecting all these substances (current and future ones), which is virtually an impossible task.

To tackle the above-mentioned problems, activity-based bioassays, capable of detecting compounds with cannabinoid activity, might help in the detection of the structurally diverse class of synthetic cannabinoids. The concept of **activity-based reporter bioassays for the screening of abused substances in biological matrices** is not entirely new. Besides the assays developed within the framework of this thesis, activity-based assays for screening biofluids for the presence of steroid hormones (androgens, estrogens, glucocorticoids) have been reported. An overview of these applications is given in **Chapter 2**.

The development of **activity-based bioassays, capable of monitoring cannabinoid activity**, is described in **Chapter 3**. The applicability of these assays to monitor cannabinoid activity was shown by evaluating the potency of several SCRAs and their major metabolites. The results confirmed earlier reports that several SCRAs retain their activity upon metabolization[23-27]. In this Chapter, a **first successful proof-of-concept** is also described, suggesting the potential to apply these cannabinoid reporter assays as a **screening assay on authentic urine samples**. We initially applied our bioassays on urine samples because of the anticipated higher concentrations in urine, the fact that many phase I SCRA metabolites apparently retain activity at CB receptors and the combined presence of distinct active metabolites is likely to be beneficial for the assays' sensitivity.

In **Chapter 4**, the **application of the cannabinoid reporter assays as a screening tool for SCRAs in urine was further explored**. First, **stable cell systems were generated,** as the transient transfection approach used in **Chapter 3** imposed a heavy workload and suffered from significant interexperiment variability (depending on the transfection efficiency). Next, the generated stable cell systems were evaluated on an expanded set of SCRAs and were applied on a relatively large set of authentic urine samples (n = 74) to evaluate their potential as a screening tool for SCRAs in urine.

In **Chapter 5**, **additional modifications were made to the cannabinoid reporter assays to improve the sensitivity**, as confirmed by re-analyzing authentic urine samples from **Chapter 4** with the newly developed cell systems. The new stable cell systems were successfully used to **screen for cannabinoid activity in a set of authentic serum** (n = 45) **and plasma** (n = 73) **samples**. Our results suggest that these new, stable cannabinoid reporter systems may serve as a first-line screening tool, complementing the conventional targeted and untargeted analytical methods.

1.1.2 Synthetic opioids

Synthetic opioids represent a group of narcotic analgesic drugs, with similar properties to opiates and opioids, which are posing a serious threat to the health of consumers[2, 12, 37-39]. In both Europe and North America, the recent emergence of highly potent new synthetic opioids is causing considerable morbidity and mortality. Many of these are derivatives of fentanyl, a therapeutically used drug. However, new synthetic opioids such as AH-7921, MT-45, and U-47700, with structures distinct from those of known therapeutic or recreational drugs, have also emerged[39]. The new opioids occur in various forms: mainly powders, tablets, capsules, and since 2014, also as liquids[3]. They are sold online, as well as via the conventional illicit drug market.

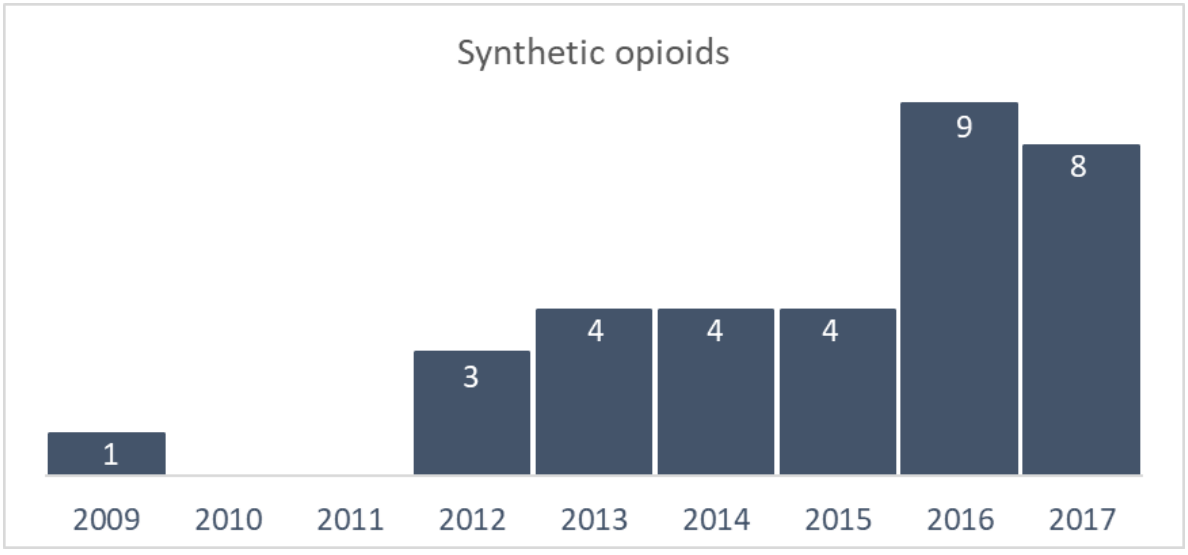

Figure 1.3. The emergence of novel synthetic opioids reported to the EMCDDA from 2009-2017 (data incomplete for 2017). (Source: EMCDDA)

Overall, 33 new opioids have been detected on the European drug market from 2009-2017 (Figure 1.3). Although currently the new synthetic opioids only play a smaller role in the European drug market, they are highly potent substances that pose a serious threat to individual and public health[3]. In the US, a recent surge in illicit opioid overdoses, driven by synthetic opioids, has been observed from 2013-2016 (6-fold increase) (Figure 1.4), with indications that there has been a further increase in 2017. Similarly in Europe, an increasing

number of deaths is attributed to synthetic opioids. Findings in our lab also confirm this alarming trend.

Synthetic opioids are substances that are synthesized to act as agonists for the opioid receptors (μ, δ, and κ subtypes), mainly found in the brain, spinal cord and digestive tract[2, 12, 37-39]. The major pharmacologic action that is strived for is analgesia. However, the synthetic opioids also depress the respiratory system, constrict the pupils, and produce drowsiness and euphoria. The most common side effects include nausea, dizziness, vomiting, fatigue, headache, and constipation. Repeated use leads to the development of tolerance and dependence[39]. Most of the novel synthetic opioids act as full agonists, with varying potencies, at the μ-opioid receptor. They were initially explored by research groups or pharmaceutical companies to investigate compounds that had potential for medicinal use, but have recently found their way to the illicit drug market.

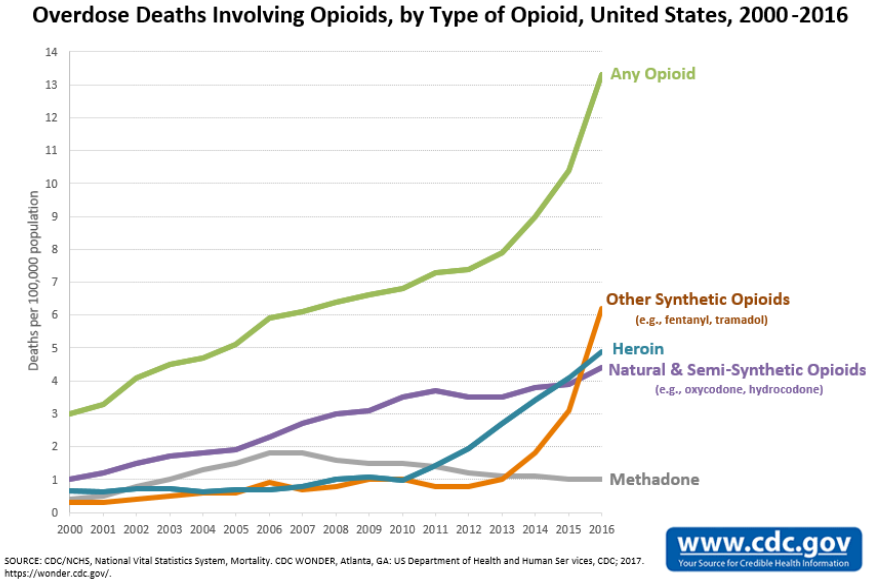

Figure 1.4. Overdose deaths involving opioids, by type of opioid (US, 2000-2016, Centers of Disease Control and Prevention, CDC). https://www.cdc.gov/drugoverdose/data/analysis.html

It is important to mention that these synthetic opioids are between 10 and 100 times more potent than morphine in their pharmacological action[40]. They are increasingly used as stand-alone products, as adulterants in heroin or as constituents of counterfeit prescription medications[2, 12, 37-39]. Due to the narrow therapeutic index, the use of synthetic opioids in the recreational drug scene is exceptionally dangerous, especially in opioid intolerant users. High doses might result in death due to respiratory arrest and pulmonary edema. Importantly, serious interactions can occur when fentanyls are mixed with heroin, cocaine, alcohol, and other CNS depressants, in particular benzodiazepines[39, 41-42].

When emergency medical care workers suspect that an individual is intoxicated with opioids the opioid antagonist naloxone is typically given. Naloxone is a semi-synthetic competitive opioid antagonist, which quickly reverses the effects of an opioid overdose and has been

used in clinical and hospital overdose management since the 1970s. With the rise of the opioid epidemic and increased mortality as a result of opioid overdoses, there have been many initiatives to combat this health crisis. The provision of naloxone kits to opioid users and others likely to witness opioid overdoses has emerged over the last 20 years as a novel harm reduction intervention to make the antidote available in situations of need[3]. Several countries in Europe have introduced take-home naloxone programs that combine provision of the antidote with training in overdose prevention and emergency management. In the US, health care providers, including pharmacists, have been allowed to prescribe, dispense, and/ or administer naloxone in an attempt to save lives. In addition, pharmacists play a role in counseling and educating patients, family members, caregivers, and bystanders on the safe administration of naloxone (via intramuscular, intravenous or subcutaneous injection or nasal formulation (e.g. NARCAN® Nasal Spray) in the event of an emergency[43].

Current evidence suggests that the availability of (new) synthetic opioids on the illicit drug market and related acute and lethal intoxications are underestimated because of the analytical challenges posed. There are inherent difficulties in identifying non-scheduled compounds, owing to wide variations in chemical structure, a lack of commercially available standards and a continuous change of the nature of the substances used in the drug scene.

In Belgium, a generic structure law was introduced which controls fentanyl and all its structural analogs[36]. Other synthetic opioids (non-fentanyl analogs) such as AH-7921, MT-45, and U-47700, are banned via their specific structure. The generic structure law forces the toxicological laboratories to be able to detect all fentanyl analogs, which is practically impossible. In addition, the (new) synthetic opioids, particularly fentanyl-related compounds, are active in very low doses, due to their high potency, resulting in very low concentrations (low to sub-ng/mL) of the parent compounds and their metabolites in biofluids. Moreover, many users are likely unknowingly consuming these compounds (e.g. as adulterants in products sold as heroin or other counterfeit pain killers). As a consequence, they may escape detection because many labs do not perform routine testing for these drugs and their detection requires dedicated analytical screening methods with sufficiently high sensitivity and specificity[2, 12, 37-39].

Therefore, an alternative **untargeted approach for the detection of opiates and (synthetic) opioids, not directly based on their structure, but on their μ-opioid receptor (MOR) activity**, **was developed**, as described in **Chapter 6**. The performance of the developed MOR reporter bioassay was **successfully evaluated on 107 authentic blood samples** from postmortem toxicology casework containing synthetic opioids. Such an approach may serve as a first-line screening tool, complementing the conventional analytical methods which are currently used.

An **application of the MOR reporter assay** is described in **Chapter 7**, where **a case report on a fatal carfentanil intoxication** is discussed. Carfentanil is an extremely potent opioid with ~10 000 times the potency of morphine in the tail withdrawal test in rats[44]. It has recently been reported as a contaminant in street heroin and cocaine in the USA and Europe and is associated with an increased number of life-threatening emergency department admissions and deaths[45-48].

1.2 Outline and aims of the thesis

The MOR reporter assay (described in **Chapters 6 and 7**) is a further expansion of alternative activity-based screening methods, following the bioassays developed for the detection of synthetic cannabinoid receptor agonists (described in **Chapters 3, 4 and 5**). This consolidates the novel concept of activity-based screening for a broad range of new psychoactive substances, which are posing substantial challenges to clinical and forensic toxicology laboratories.

An overview of **applications of *in vitro* activity-based reporter bioassays for the screening of abused substances in biological matrices** is given in **Chapter 2**. Here, the application of bioassays to screen for other abused substances such as steroid hormones (androgens, estrogens, glucocorticoids) is discussed.

In **Chapter 8** the broader international context, the relevance and the future perspectives are described. **Chapter 9** gives a summary an general conclusion. **Table 1.1** outlines the aims of each Chapter in this thesis.

Table 1.1. Overview of the aims of each Chapter.

Chapter	Aim
1	Aims and Outline of the thesis: Introduction to new psychoactive substances
2	Activity-based reporter assays for screening of abused substances in biological matrices : an overview.
3	Detection and activity profiling of SCRAs and their metabolites with an newly developed bioassay.
4	Activity-based detection of consumption of SCRAs in authentic urine samples using a stable cannabinoid reporter system.
5	An improved activity-based detection method of cannabinoids in serum and plasma samples.
6	A novel activity-based concept to screen biological matrices for the presence of opiates and (synthetic) opioids.
7	Activity-based detection and bioanalytical confirmation of a fatal carfentanil intoxication: a case report.
8	Broader international context, relevance and future perspectives.
9	Conclusion and summary

1.3 References

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CHAPTER 2: ACTIVITY-BASED REPORTER ASSAYS FOR THE SCREENING OF ABUSED SUBSTANCES IN BIOLOGICAL MATRICES: AN OVERVIEW

Based on

Annelies Cannaert, Marthe Vandeputte, Sarah Wille, Christophe P. Stove. Looks don't matter, it is what you do that counts: activity-based reporter assays as a new concept for abused substance screening in biological matrices. Manuscript in Preparation.

2.1 Introduction

The number of novel designer drugs that is abused is constantly growing. This increase can be seen by the sharp rise of new psychoactive substances (NPS) during the last decade. More than 620 NPS have appeared on the European drug market, as reported by the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA)[1]. These substances are characterized by a high market dynamics and are often not covered by international drug controls and make up a broad range of drugs such as synthetic cannabinoids, stimulants, opioids and benzodiazepines. These NPS are in many cases marketed as 'legal' replacements for illicit drugs (e.g. 'synthetic cannabinoids' for cannabis products)[1].

Steroid hormones are among the most popular performance enhancing drugs abused in both elite and amateur sports[2-3] and their use is prohibited by the World Anti-Doping Agency (WADA) at all times, in and out of competition[4]. Steroids are mainly associated with doping by elite athletes to enhance athletic performance, but since the 1980s, their use by male non-athlete weightlifters to improve appearance by building muscle mass has exceeded their use by competitive athletes[5]. Apart from their continued abuse in sports, steroid hormones are also found as illicitly used growth-promoting agents in meat-producing animals to increase meat production, resulting in higher earnings[6]. The use of such growth promoters in livestock production, however, falls under the European ban published in 1988 (EU directive 96/22/EC). Instead of providing a limitative list of forbidden hormones, the ban prohibits all substances having hormonal actions[7-9].

Previously, drug and doping control focused on 'conventional' drugs of abuse or approved therapeutics. However, drug users and athletes have started to misuse substances that were not tested for and/or were not clinically approved[1, 10-11]. Although steroid hormones have been studied for over 50 years and during that period numerous compounds with a variety of functional groups have been produced, only a small number has been introduced to the pharmaceutical market. In order to try to evade detection, some have resorted to the use of even more dangerous forms, the designer steroids. These steroids are manufactured to closely resemble existing known compounds, but with sufficient chemical diversity to ensure that their detection is more difficult[9, 12].

A worrying common feature of NPS and designer steroids is that no or limited data are available about the safety of these substances. The use of synthetic cannabinoids has been associated with agitation, nausea/vomiting, kidney failure, cardiovascular problems and psychological disorders as well as death[13-15]. The use of synthetic opioids, due to the high potency and small therapeutic window of these compounds, has been associated with opioid intoxications and numerous deaths[1]. Anabolic steroid use also causes a lot of side effects, such as cardiovascular disease, liver damage, virilisation and gynaecomasty.

Remainders of growth promoting agents in consumer products may have inadvertent effects as well. The health issues posed by these compounds often present serious problems for amateur bodybuilders and recreational athletes misusing steroids, even more so than for professional athletes, because of the insufficient medical attendance and supervision[16].

Amongst the reasons to (ab)use NPS or designer steroids is the lower chance of getting caught, as routine drug or sports doping tests may miss these compounds. Indeed, established gas chromatography mass spectrometry (GC-MS) and liquid chromatography tandem mass-spectrometry (LC-MS/MS) methods are typically set up for the monitoring of selected ions or of particular MS/MS transitions of known substances. Furthermore, other methods, such as immunoassay screenings, might fail to detect novel substances that are being abused, due to the lack of and sometimes unpredictable cross-reactivity[9, 11, 16-18]. In general, the combined immunoreactivity of all compounds that are structurally related to the immunogen will determine the endpoint of these methods[19]. Moreover, immunoassays have a cumbersome developing process and with the high dynamics of the market of new substances, the developed immunoassays might struggle to keep up.

The use of untargeted MS-based screening methods (e.g. GC-MS and especially high-resolution mass spectrometry (HRMS)) has gained considerable interest to detect and identify novel compounds, although also here the absence of certified reference materials or mass spectral libraries poses a challenge. In this context, HRMS has been the method of choice for the broad screening of substances because of its ability to measure a compound's or a fragment's mass with sufficiently high accuracy, allowing its elemental composition to be determined directly[20-22]. However, sensitivity constraints may be present, requiring an analyte to be present at sufficiently high concentration to trigger an acquisition. Moreover, due to the expensive and time-consuming character of this technique, this method is not ideal to function as a screening method and is not routinely implemented in most laboratories.

Given the above, it is clear that there lies potential in novel 'untargeted' screening approaches, which are less expensive, more high-throughput-amenable and more routinely applicable. Activity-based assays, capable of monitoring the biological activity of an abused substance in a biological matrix, have been proposed as such an alternative 'untargeted' screening approach. These biological assays do not require knowledge about a compound's structure and could be used as a screening tool to identify potentially positive samples. In this review, we focus on activity-based reporter bioassays for the detection of NPS -more specifically synthetic cannabinoids and opioids- and steroid hormones in biological matrices.

2.2 Ideal *in vitro* activity-based assay

An ideal *in vitro* bioassay for screening purposes should be rapid, simple, sensitive, selective, reproducible and inexpensive.

- Rapid: As bioassays are to be applied as a screening tool, analysis should be fast and/or multiple analyses should be possible in one run. Shorter test duration allows the analysis of more samples per time frame.
- Simple: The assay should not require a lot of technical experience or highly sophisticated equipment. For cell-based assays, the generation of stable cell lines or the availability of yeast cells improves the simplicity of the assay.
- Sensitivity: Assay sensitivity is primordial as the aim is to detect physiologically relevant concentrations of drugs or hormones in (extracts of) biofluids.
- Selectivity: Although the developed assays should be considered as a screening tool and hence (depending on the context) some level of false positives may be allowed, they should be as selective as possible.
- Reproducible: The results of the screening method should be robust. Independently performed assays should provide consistent (positive and negative) results.
- Inexpensive and high-throughput-amenable: The screening assay should ideally be applicable on large sample sets to identify suspicious samples, which can subsequently be tested with more advanced systems. In addition, as the purpose is to reduce the number of samples that needs to be tested further, the price per analysis should be limited. Automatability and low consumable cost are important in this respect.

2.3 New psychoactive substances: synthetic cannabinoids and opioids

Synthetic cannabinoid receptor agonists (SCRAs) continue to be the largest group of new psychoactive substances (NPS) monitored by the EMCDDA. SCRAs are often marketed as a "safe" and "legal" alternative to marijuana. However, recent reports indicate that many of these compounds may produce serious adverse health effects[13-15]. SCRAs were originally synthesized by research laboratories to investigate the endocannabinoid system or as potential therapeutic drugs because they interact with cannabinoid receptors CB1 and CB2. The last decade, however, they have reappeared via the Internet as designer drugs, being promoted as so-called "legal highs"[1, 23-25].

Although synthetic opioids represent a smaller segment of the illicit drug market, there is an increasing number of reports on the rise of these compounds and on the harms they cause,

including non-fatal intoxications and deaths. Synthetic opioids are substances that were initially synthesized to act as agonists for the opioid receptors (μ, δ, and κ subtypes), mainly found in the brain, spinal cord and digestive tract[18, 26-28]. Most act as full agonists, with varying potencies, at the μ-opioid receptor (MOR) and were initially explored by research groups or pharmaceutical companies for potential medicinal use. The last few years, they have found their way to the illicit drug market, being sold as such or in mixtures with other drugs, such as heroin or even cocaine [29]. Both the cannabinoid receptors, CB1 and CB2, and the μ-opioid receptor, MOR, are G-protein coupled receptors (GPCRs). Through the $G_{i/o}$ family of G-proteins, these are coupled to a wide variety of signal transduction pathways. GPCRs are rapidly desensitized by recruitment of the cytosolic protein β-arrestin 2 (βarr2)[30-31].

Our research group recently reported on live cell-based reporter assays for activity-based detection of SCRAs (as well as their metabolites) and (synthetic) opioids in biofluids (see following chapters)[32-35]. The developed assays utilize a structural complementation-based approach, designed to monitor protein interactions within living cells (NanoLuc Binary Technology)[36]. More particularly, fusion constructs were generated between one of two inactive subunits of NanoLuc luciferase and either a GPCR (CB1 or CB2 or MOR) or βarr2. Upon GPCR activation, the cytosolic βarr2 protein, fused to one part of NanoLuc, will interact with the GPCR, fused to the other part of NanoLuc, leading to structural complementation of the NanoLuc luciferase subunits. This results in a restoration of luciferase activity, which generates a bioluminescent signal in the presence of the furimazine substrate (Figure 2.1).

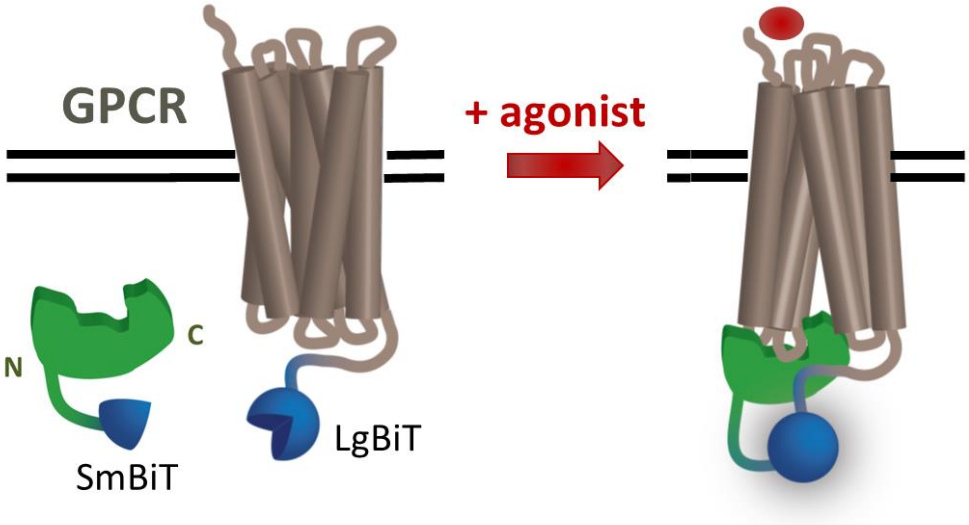

Figure 2.1. Example of the set-up of the G-protein coupled receptor activation assay (NanoBiT Technology).

The applicability of the cannabinoid reporter assay has been demonstrated in transient and stable mammalian cell systems for the detection of SCRAs (as well as their active metabolites) in authentic urine, serum and plasma samples[32-34]. The advantages of using

stable bioassays, as compared to a transient format, are a reduced workload and higher reproducibility within experiments. Moreover, in the stable cell line-based bioassays we designed, we built in the possibility to control the level of expression of the GPCR or βarr2. This was achieved by coupling the expression of the CB- and βarr2-constructs to that of co-expressed markers, which can be followed by flow cytometric analysis. Interestingly, as overexpression of the GPCR and βarr2 fusion proteins might lead to a counter selection of overexpressing cells, these co-expressed markers also allow to follow up the stability of the cell lines over time and, if needed, may allow cell sorting, to select for cells with a certain level of expression.

For the activity-based screening of SCRAs in urine samples, the sensitivity largely depends upon the presence of active (phase I) metabolites, as SCRAs are typically heavily metabolized, with hardly -if any- main compound being detectable in urine. Good sensitivities were obtained from urine samples for UR-144/XLR-11 users (94.4%; 17/18) and ADB-CHMINACA users (81.8%; 9/11). Surprisingly, in urine from users of the related AB-CHMINACA only a sensitivity of 33.3% (4/12) was found[33].

In contrast to urine, application of SCRA screening on extracts from blood (or plasma or serum derived thereof) will primarily rely on the presence of the parent compound. However, the highly potent nature of some compounds makes that in some instances active concentrations are in the low-to sub-ng/ml range, thus requiring highly sensitive detection. To improve the sensitivity of the existing SCRA bioassay, a modified assay was set up, in which a truncated rather than a full-length βarr2 protein was combined with either CB1 or CB2[34]. Application of this improved bioassay on a set of 45 serum samples resulted in a positive scoring of 18/22 SCRA positive samples, some with sub-ng/ml concentrations, corresponding with an analytical sensitivity of 82%. All SCRA negative samples were correctly scored negative in the CB1 and CB2 bioassays, leading to a specificity of 100% (21/21). The presence of other common drugs of abuse and/or low concentrations of Δ^9-tetrahydrocannabinol (THC; < 1 ng/ml) did not lead to a positive result. Only extracts from samples in which high concentrations of THC (> 12 ng/ml) were present gave rise to a positive result in 16/18 (89%) of cases, which is somewhat expected as the assay screens for cannabinoid activity[34].

A similar bioassay has been developed for the detection of opiates and synthetic opioids[35]. Here, in addition to expression of MOR- and βarr2-fusion constructs, overexpression of an additional protein, G-protein coupled receptor kinase 2, was necessary to achieve sufficient sensitivity. This protein promotes βarr2 recruitment to the activated MOR. Sensitivity and specificity of the MOR reporter assay were evaluated using 107 authentic postmortem blood samples with known presence or absence of the synthetic opioids U-47700 or furanyl fentanyl, as determined by LC-MS/MS and quadrupole-time of flight (Q-TOF) analysis[35]. A

first finding was that in 8 synthetic opioid positive samples no positive signal was obtained. In these samples, Q-TOF analysis revealed the MOR antagonist naloxone, which can obviously also prevent MOR activation induced by opioid agonists. Hence, evaluation was further based on non-naloxone containing samples. For U-47700 positive samples (74.5 – 547 ng/mL), sensitivity was 100% (8/8). For furanyl fentanyl containing samples (<1 – 38.8 ng/mL), 21 out of 22 samples (95%) were screened positive; it was not possible to test whether the missed sample contained naloxone. A specificity of 93% (55/59) was obtained for the opioid negatives. An additional 5 samples (found to contain opioids codeine, (nor)buprenorphine or loperamide) were correctly scored positive. In 5 negatively scored samples, Q-TOF analysis revealed the presence of alfentanil or sufentanil (both < 1 ng/ml) or dextromethorphan/levomethorphan or dextrorphan/levorphanol. For the latter two, as the LC-MS/MS method could not distinguish between the enantiomers (inactive dextro- and active levoform), it was not known what form was (mainly) present. The absence of detection of activity in these samples could be explained by the presence of the inactive enantiomer (dextroform)[35].

This MOR reporter bioassay was also applied on several biological matrices in a case report involving a fatal intoxication with carfentanil, an extremely potent opioid[37]. The extracts of the urine, vitreous and blood revealed a very potent opioid signal, even upon dilution of the sample. In this case, even the application of 1 µL of pure urine (without any sample preparation) in the bioassay generated a clearly positive signal.

There is a multitude of other GPCR activation assays available, several of which have been applied as research tools for studying CB and MOR receptor signaling.[38-50] However, as far as we are aware, only the reporter assays we developed have currently been applied on biological matrices as an untargeted screening strategy. It remains to be evaluated whether other, commercially available systems, can achieve the very high sensitivity that is required to screen biofluids for activity.

2.4 Steroid hormones

The parent compound from which all steroids are derived is cholesterol (Figure 2.2). During steroidogenesis different functional groups in varying orientations and oxidation states arise, resulting in a wide range of lipophilic, low-molecular weight, biologically active compounds. These serve as hormones, meaning that they may act as chemical messengers to regulate different cellular functions[51]. According to their biological activity and pharmacological effects, steroid hormones can be divided in two important groups. A first group includes the sex steroids, estrogens, progestogens and androgens, which produce sex differences and

support reproduction. The second group includes the glucocorticoids, which regulate many aspects of metabolism and immune function, and the mineralocorticoids, which regulate blood volume and electrolyte content[51].

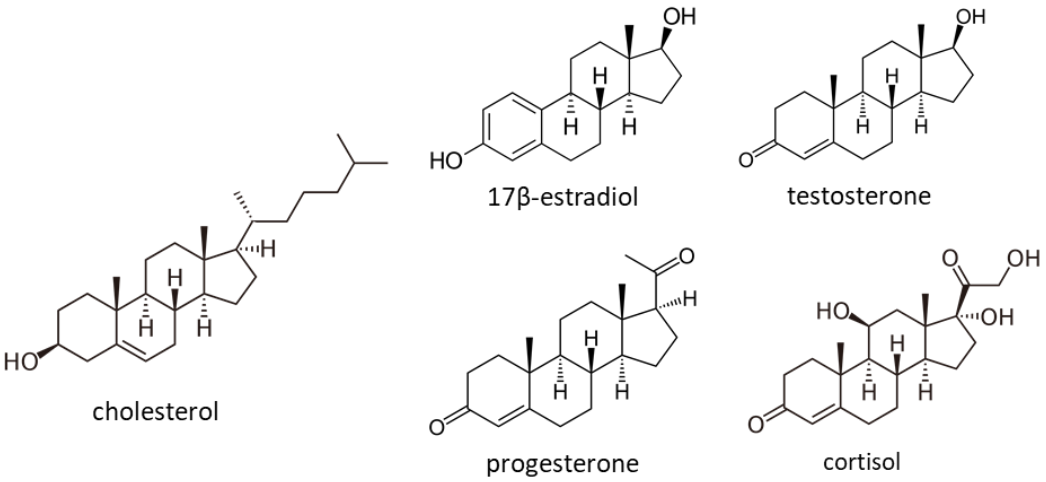

Figure 2.2. Chemical structures of cholesterol, 17β-estradiol (estrogen), testosterone (androgen), progesterone and cortisol (glucocorticoid).

The steroid hormone receptors (SR) are members of the large superfamily of ligand-activated transcription factors (Figure 2.3)[7, 52]. In its inactive form, the receptor is initially sequestered, in the cytosol of the target cell, under the form of large protein complexes containing Heat Shock Proteins (HSPs). Ligand binding induces dissociation of the receptor from these complexes. This allows the ligand-bound receptor to dimerize and, after phosphorylation, the now activated hormone-receptor complex translocates to the nucleus, where it recognizes a specific DNA sequence, the steroid response element (SRE), in the promoter of a steroid-regulated gene. The principle of this ligand-induced modulation of target genes forms the basis of test systems which can be used for steroid hormone screening (reporter gene bioassays, cfr. infra).

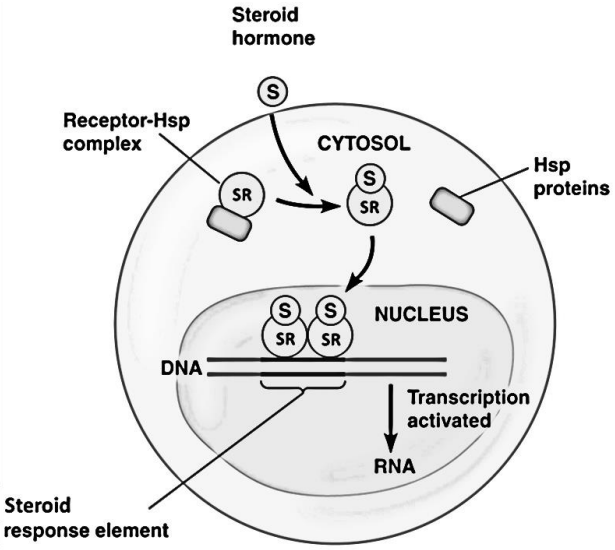

Figure 2.3. Signaling pathway of steroid hormones. Picture altered from Pearson Education Inc. 2012.

A major limitation of the methods that are routinely used in doping control is that they most often cannot identify compounds of unknown structure and rely on prior knowledge of the structure of a steroid[10, 20, 52-53]. The black market for hormones makes witty use of the 'loopholes' in detection methods by introducing so-called "designer steroids". These novel steroids are synthesized with the aim of being biologically active as hormones, while evading detection owing to their slightly modified chemical structure compared to known steroids[12]. The fact that they are often synthesized in clandestine laboratories without appropriate quality controls adds up to the potential health risks these substances pose to abusers and doped-meat consumers. In addition, both athletes and farmers attempt to evade detection by administering low-dose hormone cocktails. In this approach, each substance will be present at very low concentrations, challenging the limits of sensitivity of the screening assays used to detect hormonal substances (both immunoassays as MS-based methods). Yet, owing to their additive effect, considerable biological activity may be exerted[54].

In this context, also here, bioactivity-based tools (bioassays) may be applied for the detection of steroids, by focusing on common mechanisms of action. A wide range of *in vitro* bioassays to monitor the steroid activity of compounds, comprising receptor binding assays, cell proliferation assays and receptor-dependent gene-expression assays (the so-called reporter gene bioassays)[8]. As the focus of this review lies on activity-based reporter bioassays, the former two will only be addressed shortly.

2.4.1 Receptor binding assays

Receptor binding assays can be used to detect all compounds having affinity for a given receptor. The principle is based upon competition of a ligand in an unknown sample with a labeled (usually radiolabeled) ligand for binding to a receptor. The extent to which the unknown sample replaces receptor binding of the labeled hormone correlates with its bioactivity. The principle of these radio-receptor assays is similar to that of conventional radio-immunoassays, in which antibodies are used instead of receptors. These assays thus monitor one steroid feature, i.e. binding to the steroid receptor, but cannot distinguish between receptor agonists and antagonists because only the strength of the binding of a substance to the receptor is determined and not the activation or deactivation of the receptor[7, 52, 55].

2.4.2 Cell proliferation assays

Cell proliferation is a process further down the pathway than binding and transcription. The E-screen was one of the first *in vitro* bioassays used to determine the estrogenic activity of compounds and extracts. In parallel to the E-screen, the A-screen was developed for

detecting androgenic activity. Although the simplicity of these assays is attractive, many factors have been shown to affect the outcome of the assay, reducing the reproducibility of these assays. These factors include, but are not restricted to other compounds that may have an impact on cell growth, as well as differences in cell line clones, cell culture conditions and serum lots, thus complicating standardization of the assay to ensure inter-laboratory reproducibility[7, 56]. Furthermore, proliferative responses can only be determined after a number of days, resulting in a test that is not very rapid.

2.4.3 Reporter gene bioassays

Reporter gene bioassays exploit the natural signaling pathway of steroid hormones (Figure 2.3). In contrast to receptor binding assays, they also include the transactivation step and, consequently, can distinguish between receptor agonists and antagonists. Moreover, they might suffer less from matrix effects, as not all of the non-specific compounds will be able to enter the cells and reach the steroid receptors, as is the case with receptor preparations when using receptor binding assays. Many reporter gene assays have been developed, using both yeast and mammalian cells[7]. These are capable of amplifying and measuring biological activity, can be sensitive and provide information on the presence of steroid receptor activating compounds, independently of knowing the structure[10]. Both types of host cells have several advantages and drawbacks and the choice will depend on the intended purpose of the assay. Either way, it is important to keep in mind the different limitations while interpreting the results from these bioassays. When assays with both cell types are run in parallel, complementary information can be obtained.

I. Yeast

The first bioassays were developed in yeast because they grow easily and are economical, due to their rapid growth and easy attainment of stable transformants (compared to mammalian cells)[11, 57]. Typically, yeast cells are transformed with steroid receptor cDNA and a reporter vector containing an SRE, driving expression of a reporter gene, such as luciferase, β-galactosidase or a fluorescent protein. Fluorescence is associated with typical limitations, such as potentially high background and photobleaching, resulting in a lower sensitivity. However, it offers the advantage that the signal can be followed as a function of incubation time. Moreover, measurement of fluorescence is easier, quicker and cheaper than the measurement of β-galactosidase or luciferase activity, which may require cell wall disruption and/or the addition of expensive substrates. An additional advantage associated with the use of fluorescent proteins is that their read-out is not hindered by possible enzyme-inhibiting compounds[7, 58].

Because yeast cells are steroid independent for their growth and lack endogenous steroid receptors, yeast-based assays have a high specificity for hormones[9, 59]. Especially for androgens, the lack of known endogenous receptors in yeast is a great advantage compared with mammalian cell lines, as androgen responsive elements (AREs) can also be activated by the progesterone and glucocorticoid receptor (PR and GR). Mammalian cells containing either of these receptors experience cross-talk between the different steroids[7]. Many efforts to construct an ARE that is specific and no longer inducible by the PR or GR have remained without success. It is doubtful whether a specific ARE can be found, as the consensus progesterone and glucocorticoid responsive elements (PRE/GRE) are equal to the consensus ARE[58, 60-62].

Yeast-based assays have been demonstrated to be robust, being more resistant to environmental contaminants than mammalian cells. This is an important advantage when measuring complex samples[63-64] and can probably be attributed to the presence of the yeast cell wall, making the cell more tolerant to dirty matrices or extracts. This cell wall, however, may pose a disadvantage for certain compounds that may be hampered to enter the cell or are pumped out via efflux pumps before reaching the receptor[7, 65].

Amongst the challenges when setting up new yeast systems is that expression of mammalian proteins may pose problems such as incorrect folding, phosphorylation, glycosylation or other post-translational modification. Additionally, yeast systems lack the appropriate chaperone and co-regulator proteins (e.g. HSPs) which are necessary for proper steroid mediated transactivation[10-11, 66].

Table 2.1 provides an overview of the yeast-based assays that have been used in the context of detecting abuse of steroid hormones in athletes and meat-producing animals. These assays are briefly described below.

A yeast-based androgen screening assay with a secreted form of β-galactosidase was developed[67] and applied on authentic human urine samples from anabolic steroid abusers[16] and following the administration of methyltestosterone (MT)[68]. The yeast androgen assay was able to detect MT use in urine of volunteers after a single ingestion of MT for up to 307 h. In contrast, the detection limit of the GC–MS method, which was used in comparison was about 118 h after exposure. This difference can be explained by the fact that detection of MT abuse by GC–MS is dependent on tracing specifically known metabolites, whereas the yeast androgen assays detects the sum of the remnants of primary substance as well as of all known and unknown metabolites via their combined activity[68].

Another yeast-based androgen screening assay, using luciferase as reporter protein, was developed[69] and evaluated on human serum by Michelini and coworkers[58, 70]. The utilized *P. pyralis* luciferase was truncated to abolish peroxisomal targeting, thus allowing

measurement of luciferase expression in intact living cells. An advantage of both systems is that they do not require cell lysis prior to read-out. A further improvement in robustness of the latter assay included the introduction of an internal viability control based on a constitutively expressed red-emitting *P. pyralis* mutant luciferase. Applicability of the improved bioassay was demonstrated using urine and serum samples[71-72]. A major limitation in the study of Cevenini *et al*. is that prior to spiking the urine was first pretreated with charcoal to remove any endogenous steroids[71]. This charcoal-treated urine, which also served as a blank, does not reflect the real situation. Ekstrom *et al*. successfully monitored androgen activity following administration of testosterone to healthy males for 4 days in urine (not deconjugated) and up to 15 days in serum[72].

Wolf *et al*. developed two androgen yeast-based assays with yeast Enhanced Green Fluorescence Protein (yEGFP) as a reporter[73]. As the two yeast strains that were used were phylogenetically very different, the combination of both assays allows detection of the activity of a wide range of androgenic substances, as some androgens do not respond in one yeast, but do in the other.

The most-sensitive yeast-based androgen screen (A-YAS; *Arxula*-Yeast Androgen Screen) is based on transgenic *Arxula adeninivorans* yeast cells, engineered to express the human androgen receptor (hAR) gene, which may induce expression of a phytase reporter gene, resulting in conversion of *p*-nitrophenylphosphate to *p*-nitrophenol, allowing colorimetric read-out. A (limited) assessment in (deconjugated) cattle urine showed a correlation in androgen equivalence compared to the results obtained by GC-MS analysis[74].

Of the different yeast-based assays that were developed by the group of M. Nielen[63, 75], yEGFP appeared to be best suited as a reporter protein for high-throughput screening and was used to evaluate spiked urine samples[63]. The developed yeast-based estrogen and androgen assays were validated for qualitative screening for the presence of estrogenic activity in calf urine in accordance with EC decision 2002/657/EC[61, 76]. Applicability of the estrogen bioassay was demonstrated using a panel of more than 120 authentic calf urine samples[9]. When compared with the results obtained by GC-MS/MS, yeast-based screening yielded 5.6% false positives and only 1 (0.8%) false negative[9]. The urine of 17β-estradiol-treated veal calves was also tested, resulting in a sensitivity of 94.3% and a specificity of 100%[77]. A further expansion of the estrogen and androgen bioassay applications involved the set-up of a system via which extracts of calf urine could be analyzed by gradient LC with, in parallel, bioactivity and mass spectrometric detection. This was achieved via effluent splitting toward a 96-well fraction collector and an electrospray quadrupole time-of-flight mass spectrometer (Q-TOF-MS). The result was an estrogen/androgen biogram (see Figure 2.4)[56, 75, 78]. Next to urine samples, hair was evaluated as a matrix for both androgens and estrogens, revealing that it was possible to detect the presence of androgens up to at least

14 days after treatment with 60 mg testosterone cypionate and 60 mg testosterone decanoate and to detect the presence of estrogens up to at least 56 days after a single pour-on treatment with 25 mg of estradiol benzoate[56].

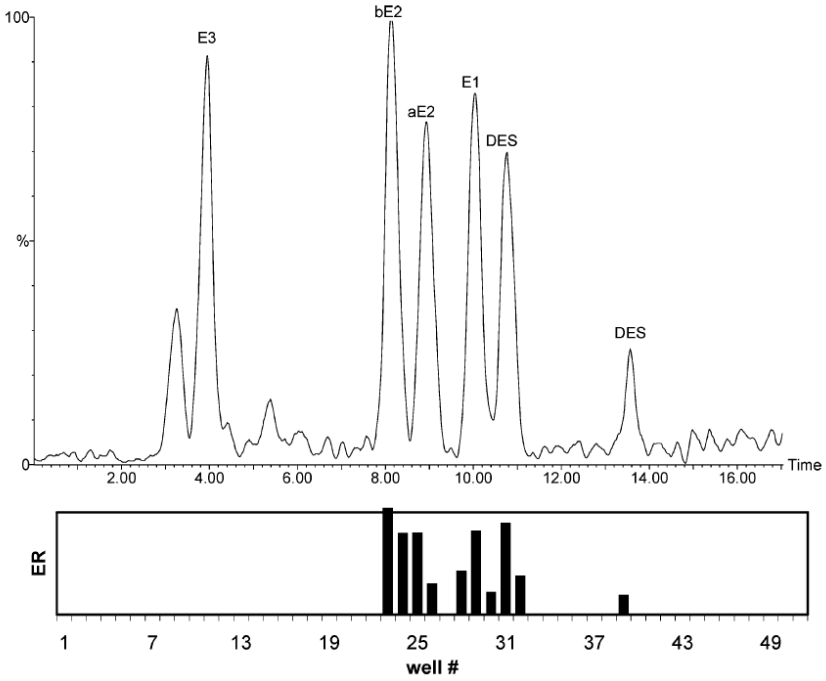

Figure 2.4. Example of a reconstructed LC/Q-TOF-MS chromatogram and reconstructed estrogen biogram of a standard mixture of estrogens (E3, estriol; bE2, 17β-estradiol; aE2, 17R-estradiol; E1, estrone and DES, diethylstilbestrol; 1 ng each). Reprinted (adapted) with permission from Nielen *et al*.[78]. Copyright 2004 American Chemical Society.

Burdge *et al*. used a yeast-based assay with β-galactosidose as a reporter (readily developed by Klein *et al*. in 1994[79]) to measure endogenous estrogen activity in bovine plasma[80]. Deconjugation was found to be a key step as estrogen conjugates (17β-oestradiol-3-glucuronide and 17β-oestradiol-3-sulfate) only produced a negligible response in the assay[79]. The authors demonstrated assay applicability by monitoring the signal generated by endogenous levels of estrogens during the reproductive cycle of females, suggesting potential application of this assay for surveillance of exogenous estrogens in cattle (especially in males, as in those the level of endogenous estrogens is very low)[80].

As a follow-up of the above-mentioned *Arxula adeninivorans* yeast assay, engineered to express a human steroid receptor that drives expression of a phytase reporter gene, Chamas *et al*. developed a yeast-based assay capable of detecting estrogens, progestogens, and androgens in a single step. This was achieved by combining three yeast strains, each expressing a different human receptor, driving the expression of different fluorescent reporter proteins. Application of the combined assay on marmoset (a monkey species) serum and comparison with an immunoassay for progestogens revealed a good correlation between both types of assays[81].

Table 2.1. Overview of yeast-based assays.

Matrix	Receptor	Reporter system	Incubation	EC_{50} value	Assay cells	Comments	References
AR assays: yeast-based							
Human urine[75]	hAR^{s}	β-galactosidase^{s}	O/N[57] 24h[75]	3.5 nM DHT[57] 4.73 nM T[57] 3.7 nM DHT[75] 5 nM T[75]	*S. cerevisiae* YPH500	Spiked urine samples only[75]. Screening limited to urine samples, not containing bioactive endogenous androgens. E_2 and P also respond to some extent.	Gaido *et al.* 1997[57] Nielen *et al.* 2006[75]
Human urine[16, 68]	hAR^{s}	secreted β-galactosidase^{s}	24h + O/N	-	*S. cerevisiae* PGKhAR	No deconjugation: steroid activity only reflects unconjugated androgens.	Sohoni *et al.* 1998[67] Zierau *et al.* 2008[16] Wolf *et al.* 2011[68]
Human serum[70]	hAR^{s}	*P. pyralis* luciferase^{s}	2.5h	10 nM T[69]	*S. cerevisiae* BMA64-1A	Preliminary screening of human serum samples. Potential application for detecting anabolic androgen abuse in athletes and cattle is mentioned.	Michelini *et al.* 2005[69] Michelini *et al.* 2005[70] Michelini *et al.* 2008[58]
Human urine[71-72] and serum[72]	hAR^{s}	*P. pyralis* luciferase^{s} *P. pyralis* luciferase^{s} (red emitting mutant)	2h	7.5 nM DHT[71] 15 nM T[71]	*S. cerevisiae* BMA64-1A	Spiked samples only[71]. Administration study with T[72]. Potential use for detection in athletes is suggested.	Cevenini *et al.* 2013[71] Ekstrom *et al.* 2013[72]
Human urine[75] Bovine urine[56, 61] and hair[56]	hAR^{s}	Yeast EGFP^{s}	24h[75] 24h[61] 21h[56]	 50 nM T[61] 76 nM T[56]	*S. cerevisiae* K20	Spiked samples only[75]. Screening limited to urine samples that do not contain endogenous androgens, such as calf urine and urine from preadolescents.	Nielen *et al.* 2006[75] Bovee *et al.* 2009[61] Becue *et al.* 2012[56]
Human urine	hAR^{s}	EGFP^{s}	24h	-	*S. cerevisiae* BY4741 *S. cerevisiae* KO110	No hydrolysis: steroid activity only reflects unconjugated androgens. Potential use for detecting abuse in athletes and cattle is suggested.	Wolf *et al.* 2010[73]
Bovine urine	hAR^{s}	*Klebsiella* sp. ASR1 phytase^{s} (A-YAS)	6-25h	0.95 nM DHT 0.98 nM T	*A. adeninivorans* G1212	Potential use for detecting abuse in athletes and cattle is suggested.	Gerlach *et al.* 2014[74]
ER assays: yeast-based							
Bovine plasma[80]	hER^{s}	β-galactosidase^{s}	18h	-	*S. cerevisiae* BJ3505	Potential use for detecting abuse in cattle is suggested.	Klein *et al.* 1994[79] Burdge *et al.* 1998[80]
Bovine urine and hair[56]	hERα^{s}	Yeast EGFP	4-24h[63] 24h[9, 76-78] 21h[56]	0.4 nM E_2[63] 0.7 nM E_2[56, 76] 0.5 nM E_2	*S. cerevisiae* K20	Spiked urine only[63, 76]. Screening limited to urine samples that do not contain bioactive endogenous estrogens.	Bovee *et al.* 2004[63] 2005[76] Nielen *et al.* 2004[78] 2006[9] Divari *et al.* 2010[77] Becue *et al.* 2012[56]
AR-ER-PR assay: yeast-based							
Marmoset serum	hAR^{s} hER^{s} hPR^{s}	hAR+GFP hER+DsRed2 hPR+CFP	18h	0.57 nM DHT 0.062 nM E_2 0.467 nM P	*A. adeninivorans* G1212	Simultaneous detection of estrogens, progestogens and androgens in one experiment.	Chamas *et al.* 2017[81]

Abbreviations: (h)AR, (human) androgen receptor; (h)ER, (human) estrogen receptor; CPP, cyan fluorescent protein; DsRed2, discosoma red fluorescent protein; DHT, dihydrotestosterone; E_2, 17β-estradiol; EC_{50}, concentration giving a half maximum response (i.e. sensitivity of the assay); (E)GFP, (enhanced) green fluorescent protein; O/N, overnight; P, progesterone; PGK, phosphoglycerate kinase; T, 17β-testosterone Superscript: ^{s} stable

II. Mammalian cells

Mammalian cell-based bioassays are typically developed in immortalized cell lines, which are relatively easy to culture. The SRE-reporter gene vectors are either transiently or stably introduced into these cells[10]. Stable cell lines have the possibility of vector loss and degradation over time, if they do not contain a selection marker. The use of transient transfection methods does not risk this vector loss and degradation, but brings along a heavier workload, as every new run requires a new transfection, and might lead to more variable results. Variation can be minimized, though, by using co-transfected control vectors that may serve as an internal control for transfection efficiency.

Cell choice is an important consideration for mammalian cell-based assays, because the cellular responsiveness will be determined by its environment, including the composition of cofactors and receptor expression levels, which varies between different types of mammalian cells[64].

Mammalian cell-based bioassays have been reported to have a higher sensitivity than yeast assays[7, 11, 53]. This can also be seen in Table 2.1 and 2.2, where the EC_{50} values from the androgen assays in mammalian cells are lower than those from the yeast-based assays. However, endogenous expression of steroid hormone receptors by mammalian cells (e.g. GRs in CHO cells[60]) can lead to nonspecific reactions[64]. More particularly, if a sample also contains other steroid hormones, as biological matrices typically do, these can bind to the endogenous receptors, also resulting in reporter gene transcription. Hence, this reduces the specificity of the measurement of steroid bioactivity. This makes the choice of the SRE particularly important[7]. Additionally, as the growth of mammalian cells requires the presence of serum, which contains small amounts of steroids and other growth factors, stripped serum should be used[7, 82]. For example, charcoal treatment of serum is an effective process for removing any interfering steroid compounds.

Below we discuss mammalian cell-based assays that have been used in the context of detecting abuse of steroid hormones in athletes and meat-producing animals. An overview is provided in Table 2.2.

Mammalian-cell based assays for the detection of androgens, using *P. pyralis* luciferase and yellow fluorescent protein as reporters, have been developed and evaluated on urine[2, 6, 83] and serum[84] samples. As mentioned above, a major drawback associated with the use of mammalian cells to screen for androgens is the cross-reactivity with other steroid hormones[2, 6], although Bailey *et al*. report this is less an issue with the androgen assay they developed.[83].

Table 2.2. Overview of mammalian cell-based assays.

Matrix	Receptor	Reporter	Incubation	EC_{50}	Assay cells	Comments	References
AR assays: mammalian-cell based							
Bovine urine	hAR[e]	*P. pyralis* luciferase[s] (AR-LUX)	24h	-	T47D	Endogenous expression of ER and PR might reduce specificity of the assay.	Blankvoort *et al.* 2003[6]
Human urine	hAR[s]	*P. pyralis* luciferase[s] (AR-CALUX)	24h	0.13 nM DHT[85] 0.63 nM T[85] 0.12 nM DHT[2] 0.87 nM T[2]	U2-OS	The AR-CALUX assay was first described by Sonneveld *et al.* and used to detect endogenous androgenic activity in human and fetal calf serum.[85] Houtman et al. evaluated spiked samples.[2] Cross reactivity with with dexamethasone, E_2 and some synthetic progestins.[2]	Sonneveld *et al.* 2004[85] Houtman *et al.* 2009[2]
Human serum	hAR[t] (partial)	*P. pyralis* luciferase[t]	O/N	-	COS-1	No strict doping abuse, but evaluation of DHT administration.	Raivio *et al.* 2002[84]
Human urine	hAR[s]	CFP-AR-YFP[s]	O/N	0.55 nM DHT 2.04 nM T	HeLa	Only detection of abuse if urine is collected soon after doping event.	Bailey *et al.* 2016[83]
GR assays: mammalian-cell based							
Human serum	hGR[t]	*P. pyralis* luciferase[t]	O/N	-	COS-1	No strict doping abuse, but evaluation of serum glucocorticoid bioactivity after inhalation of budesonide or fluticasone propionate in asthmatic children.	Raivio *et al.* 2002[86]
Bovine urine	hGRα[s]	*P. pyralis* luciferase[s] (GR CALUX)	24h	1.2 nM DM	U2-OS	The bioassays failed to detect the synthetic prohormone prednisone.	Pitardi *et al.* 2015[87]
Bovine liver	hGR[s]	*P. pyralis* luciferase[s] *R.* reniformis[s]	24h	13 nM DM	HeLa	Future experiments should assess if other biological matrices can be tested.	Schumacher *et al.* 2003[88]
Bovine urine[89-90] and liver[89, 91]	hGRα[s]	*P. pyralis* luciferase[s] (TGRM-Luc)	24h	6.2 nM DM[91] 7.1 nM DM[82] 7.9 nM DM[89] 2.0 nM DM[90]	T47D	Limited assessment of spiked urine samples. Partial validation on spiked liver samples[89]. All glucocorticoid treated animals were detected[90].	Willemsen *et al.* 2002[91], 2004[82], 2005[89] Connolly *et al.* 2009[90]
PR assays: mammalian-cell based							
Bovine urine[91] and liver[89, 91]	hPR[e]	*P. pyralis* luciferase[s] (TM-Luc)	24h	1.46 nM P[91] 1.5 nM P[82] 1.1 nM P[89]	T47D	Spiked samples only. Highly variable levels of endogenous natural hormones.	Willemsen *et al.* 2002[91], 2004[82] , 2005[89]

Abbreviations: (h)AR, (human) androgen receptor; (h)GR, (human) glucocorticoid receptor; (h)PR, (human) progesterone receptor; CALUX, Chemically Activated Luciferase eXpression; CFP, cyan fluorescent protein; DHT, dihydrotestosterone; DM, dexamethasone; E_2, 17β-estradiol; EC_{50}, concentration giving a half maximum response (i.e. sensitivity of the assay); EGFP, enhanced green fluorescent protein; O/N, overnight; P, progesterone; PGK, phosphoglycerate kinase; T, 17β -testosterone; YFP, yellow fluorescent protein. Superscripts: [s] stable; [t] transient; [e] endogenous

Pitardi *et al*. evaluated the GR-CALUX (Chemical-Activated LUciferase gene eXpression) bioassay on spiked and incurred bovine urine samples[87], while Schumacher *et al*. developed and applied a dual luciferase reporter screening assay for the detection of synthetic glucocorticoids in calf liver samples[88]. The latter assay used a second luciferase as an internal control to correct for assay variability and matrix effects. Oral administration of dexamethasone (0.4 mg/day for 20 days) was picked up by the bioassay from day 2 until day 20. In some samples, glucocorticoid activity could still be detected on day 21-23.[87] Analysis of liver samples from non-treated animals could be distinguished from those who had received an injection with dexamethasone or flumethasone[88]. Willemsen *et al*. developed mammalian-cell based assays for estrogens, androgens, glucocorticoids and progestogens[91], although only the glucocorticoid and progesterone reporter assay were (limitedly) assessed on spiked urine and liver samples[82, 89]. A partial validation (recovery, repeatability, capability of detection) for the detection of glucocorticoids on liver samples was performed, although the amount of samples was below 20, which is stated to be a minimum according to EU directive 2002/657/EC (concerning the performance of analytical methods and the interpretation of results)[89]. In the study of Connolly *et al*., all animals with glucocorticoids could be distinguished from those who did not receive treatment[90].

Apart from the above-described assays, many more reporter gene assays for steroid hormones are available. Several of these have been applied on a wide variety of matrices, such as feed, dietary supplements, wastewater etc. Discussion of these is beyond the scope of this review. For some clinical applications and for the determination of exposure to endocrine disrupting chemicals, steroid reporter assays have also been used. However, although potentially of use, the utility of these assays for detecting steroid abuse or misuse has not been formally demonstrated[19, 86, 92-95]. Only the assays listed in Table 2.1 and 2.2 have currently been applied on biological matrices for the purpose of screening for the (ab)use of steroid hormones.

2.5 Concluding Remarks

Bioactivity-based screening may be an effective tool to detect the presence in a biological matrix of unknown or new compounds (synthetic cannabinoids, opioids or steroids) that are not monitored via established mass spectrometry-based methods, which often work in MRM (multiple reaction monitoring) mode and/or apply (commercially) available libraries. Inherent to these activity-based assays is that they can only serve as a screening and eventually still require analytical methodology to establish a compound's presence or identity. When applied in large-scale screening programs, these bioassays may have the

potential to serve as a cost-effective tool to identify those samples that require further in-depth bioanalytical investigation[7].

An important aspect when considering the use of reporter assays for the detection of drugs and doping is whether the activity of endogenous or frequently encountered (legal or illegal) substances might interfere with the assay. In the case of monitoring cannabinoid activity in biological matrices, the presence of endogenous cannabinoids (endocannabinoids) is not expected to interfere with the read-out as these are only present at very low concentrations in blood (in the range of low pmol/mL). Although in some conditions (eating disorders, obesity, schizophrenia, post-exercise) the endocannabinoid concentrations can rise, this will never be to an extent that this would lead to interference (< 10 pmol/mL)[96-98]. On the other hand, the presence of natural cannabinoids (e.g. THC) may result in a positive read-out of the SCRA bioassay. This co-detection of the use of cannabis or cannabis-derived products is expected since the bioassays screen for all cannabinoid activity. It should be noted, though, that THC is overall only a weak agonist at the CB receptor, making it a less ideal target for the bioassays compared to the potent SCRAs. The observation that only a positive signal was obtained when high THC concentrations (> 12 ng/mL THC) were present in plasma - indicating recent or heavy cannabis use- is consistent with this[34]. For the opioid screening assay, not only the new synthetic opioids will be detected when using the activity-based assay, but also opiates (e.g. morphine) and opioids (e.g. fentanyl), which are clinically used as analgesic drugs. Positivity of these assays is quite easily picked up by routinely applied immunoassays or existing GC-MS or LC-MS/MS-based procedures. Yet, application of the bioassay in an early screening stage could be used to rule out a relevant presence of any (legal or illegal) opiate or opioid, which might render some 'targeted' immunoassay-based screening procedures superfluous. As mentioned above, the presence of naloxone might interfere with the read-out of the MOR bioassay. As we noted elsewhere, it remains to be evaluated whether the incorporation of a minimal amount of agonist already at the start of the assay may help to cope with this intrinsic limitation. If naloxone would be present, this would result in a decrease in the assay, which would also indicate that further testing of that sample is required.

For steroid screening purposes, activity-based screening assays are susceptible to substances that may be naturally present in the sample matrix, such as natural hormones: 17β-estradiol, testosterone, progesterone, cortisol and other endogenous analogues of these hormones[52]. This is an important and inherent limitation. As a consequence, the utility of activity-based screening for steroids is limited to those samples that do not (or only to a limited extent) contain bioactive endogenous hormones. Examples include urine from preadolescents or from calves for androgen activity assays[75]. Another way to cope with the presence of endogenous compounds in the sample matrix is to use biological passport programs, as has

been implemented for athletes. This allows comparison against an individual's prior personal measurements[99-101]. A potential additional problem with steroid screening is that there is an interplay between the commonly (ab)used cannabinoids and steroids. It has been reported that cannabis, and in particular its constituents THC and cannabinol, could interact with the AR in rat prostate cytosol[102] and that marijuana smokers show decreased fertility[103-104]. Cevenini *et al*. observed a strong anti-androgenic activity of the natural cannabinoids[71]. To what extent this might pose problems associated with steroid detection in real samples is unknown.

If activity-based bioassays are to be performed on urine samples, their capability to detect drug use depends upon the presence of active drug or drug metabolite in urine, and will often require deconjugation of inactive metabolite conjugates (via e.g. β-glucuronidase). E.g. for several SCRAs, it is known that almost no parent compound can be found in urine. However, the fact that many SCRA phase I metabolites are still active[32-33, 42-44, 46, 105] still allows the detection of SCRA use *via* urine. For synthetic opioids, urine-based screening does not pose a problem, as the active parent compounds are found in urine. For steroid hormones, however, it was reported that most of the known long-lasting androgen steroid phase I metabolites, known to be prevalent in urine following intake, are functionally inactive in an androgen bioassay[83]. Thus, the androgen assay would only detect abuse if a urine sample would be accidentally collected soon after the steroid doping[83]. Again, this is an important limitation.

Although the applicability of activity-based screening tools for steroid hormones has been evaluated since the early 2000s, it is clear from the above that these assays suffer from some inherent limitations, which may be one of the reasons why they are currently not routinely employed in doping control laboratories[106]. The cannabinoid and opioid reporter assays on the other hand do not seem to suffer from the problem of endogenous background. However, these were only very recently developed (the first report only dating from 2016)[32, 35] and, despite the fact that the first applications seem successful[33-35], there appears to be room for further improvement. Furthermore, it remains an open question whether broad dissemination will happen.

2.6 References

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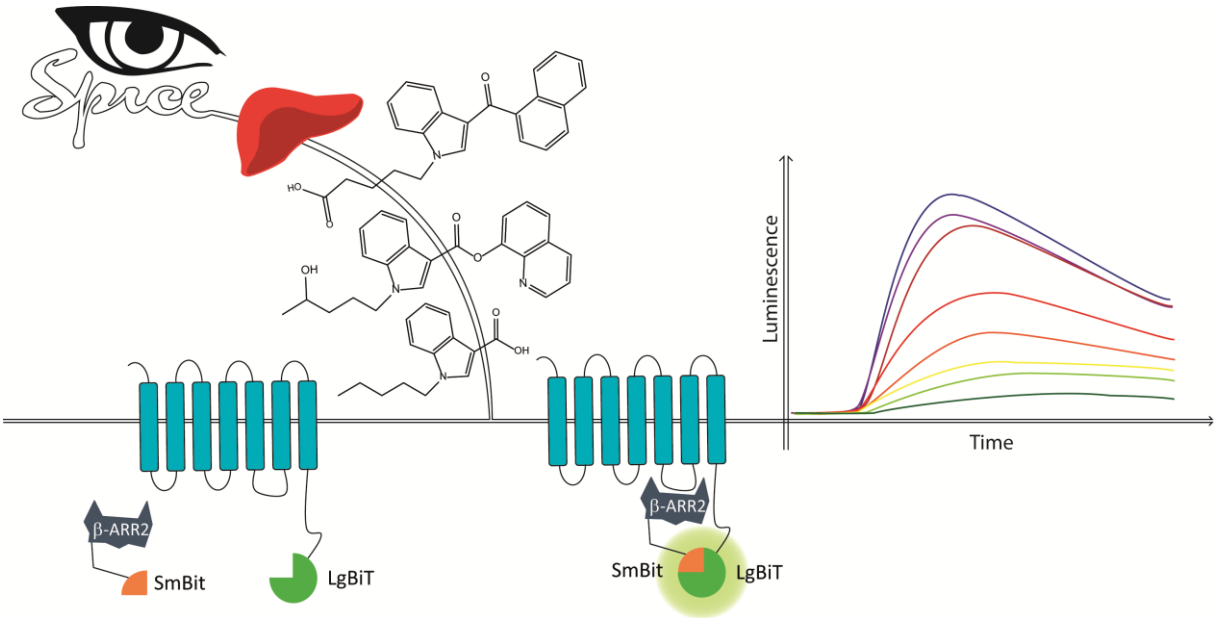
CHAPTER 3:
DETECTION AND ACTIVITY PROFILING OF SYNTHETIC CANNABINOID RECEPTOR AGONISTS AND THEIR METABOLITES WITH A NEWLY DEVELOPED BIOASSAY

Based on

Annelies Cannaert, Jolien Storme, Florian Franz, Volker Auwärter, Christophe P. Stove. Detection and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. *Analytical Chemistry* **2016** 88 (23), 11476-11485

Abstract

Synthetic cannabinoids receptor agonists (SCRAs) are the largest group of compounds currently monitored in Europe by the EU Early Warning System on new psychoactive substances. Emerging recreational use of these products has led to multiple cases of adverse health effects and even death. In contrast to marijuana, where Δ^9-tetrahydrocannabinol (Δ^9THC) is metabolized to only one major active metabolite, it has been reported that several major phase I metabolites of SCRAs remain biologically active, exerting cannabinoid (CB) receptor affinity, potency, and efficacy greater than those of Δ^9THC. It is therefore reasonable that more SCRAs can also be biotransformed into molecules with various levels of CB activity. Here, we developed and applied a new G-protein coupled receptor (GPCR) activation assay based on NanoLuc binary technology (Promega). More specifically, by demonstrating CB1 and CB2 receptor activation by JWH-018 and a selection of its metabolites, we are the first to show the suitability of the newly developed bioassay for monitoring GPCR-mediated activity. We also successfully applied this reporter system to evaluate the *in vitro* activity of JWH-122, JWH-210, and PB-22, their 5-fluoro analogues (MAM-2201, EAM-2201, and 5F-PB-22, respectively), and their main phase I metabolites. By doing so, we demonstrate that several major metabolites of these SCRAs retain their activity at cannabinoid receptors. All of these active metabolites may prolong the parent compound's psychotropic and physiological effects and may contribute to its toxicity profile. We also demonstrate a proof of concept of the applicability of the newly developed bioassay for screening urine for CB receptor activity exerted by SCRAs.



Graphical abstract of Chapter 3

3.1 Introduction

Synthetic cannabinoid (CB) receptor agonists, commonly referred to as synthetic cannabinoids (SCRAs), are the largest group of compounds currently monitored in Europe by the EU Early Warning System on new psychoactive substances (NPS)[1]. Although they are marketed as a "safe" and "legal" alternative to marijuana, recent reports indicate that many of these compounds may produce serious adverse health effects[2-3]. SCRAs were originally synthesized by research laboratories to investigate the endocannabinoid system or as potential therapeutic drugs because they interact with cannabinoid receptors CB1 and CB2. Currently, however, they have reappeared through the Internet as designer drugs, so-called "legal highs"[4-6]. In contrast to the major psychoactive constituent of marijuana, Δ^9-tetrahydrocannabinol (Δ^9THC), which is a partial agonist at both receptors, SCRAs may act as full agonists and may be selective for one receptor subtype.[7] The psychoactive effects derive from agonistic activity at CB1, predominantly found in the central nervous system. CB2 receptors are mainly associated with the immune system, but they are also expressed at a lower density in the brain[8-10]. CB1 and CB2 are G-protein coupled receptors (GPCRs). They are coupled through the $G_{i/o}$ family of G-proteins to signal transduction mechanisms that include inhibition of adenylyl cyclase, activation of mitogen-activated protein kinase, regulation of calcium and potassium channels (CB1 only), and other signal transduction pathways. GPCRs are rapidly desensitized by recruitment of the cytosolic protein β-arrestin 2 (βarr2)[11].

Unlike the widespread use of marijuana, which poses only relatively limited acute toxicity, serious adverse effects, often requiring medical attention, are not uncommon with SCRA consumption. Indeed, the relative risk of seeking emergency medical treatment following the use of SCRAs has been reported to be 30 times higher than that associated with the use of natural forms of cannabis[12]. Observed effects include central effects (psychosis, paranoia, agitation, seizures, and anxiety), cardiotoxic effects, acute kidney failure, respiratory depression, rhabdomyolysis, withdrawal symptoms, coma, and even death[6-7, 13-14]. The reason for the more profound adverse effects is not fully clear. It is known that the majority of SCRAs exhibits a higher affinity, potency, and efficacy at both the CB1 and CB2 receptors compared to those of Δ^9THC[7]. As SCRA products may be a combination of several compounds, it is possible that the resulting activation of CB1 and/or CB2 produces stronger physiological and psychotropic effects. Another difference between Δ^9THC and SCRAs is their metabolism. In contrast to marijuana, where Δ^9THC is metabolized to only one major active metabolite[15], it has been reported that several major metabolites of JWH-018, JWH-073, AM-2201, UR-144, and XLR-11 are still biologically active, exerting greater CB1 affinity, potency, and efficacy than Δ^9THC, both *in vitro* and *in vivo*[16-19]. The metabolites of JWH-018

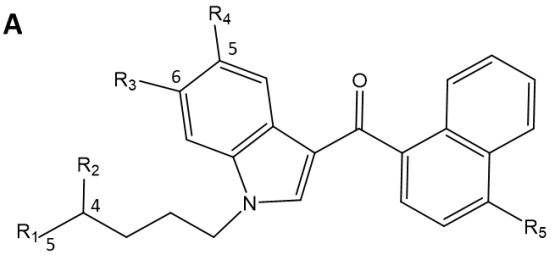
and JWH-073 also maintain their *in vitro* activity at CB2[20]. It is therefore reasonable to assume that other SCRAs are also biotransformed into molecules with various levels of activity at the CB receptors. These active metabolites may prolong the parent compound's psychotropic and physiological effects and may contribute to its toxicity profile. Greater knowledge of the activity of relevant metabolites of a wider set of SCRAs may allow us to gain better insight into the contribution of these active metabolites to the toxicity observed with SCRAs. Although there may be differences between *in vitro* and *in vivo* activities, these seem to be correlated; therefore, *in vitro* assays may serve this purpose.

Current methods used in the literature to determine the *in vitro* activity of SCRAs (and their metabolites) are the [^{35}S]GTPγS binding assay[17-26], a quantitative internalization assay[27-28], adenylyl cyclase assays[20, 29-30], and the commercial FLIPR membrane potential assay from Molecular Devices[16, 30-31]. There are also commercially available β-arrestin recruitment assays, which have been evaluated for CB1 and CB2 cannabinoid receptors. These include the imaging-based Redistribution and Transfluor assays (from Thermo Fisher Scientific and Molecular Devices, respectively) and the nonimaging-based Tango and PathHunter assays (from Thermo Fisher Scientific and DiscoveRx, respectively)[32-33].

The GTPγS binding assay directly measures the guanine nucleotide exchange of G-proteins, an early event after GPCR activation. Although this assay could be applied (because CB receptors are $G_{i/o}$-coupled), the radioactivity, high background, and requirement for a filtration step are important drawbacks[34-36]. The quantitative internalization assay, which evaluates the remaining percentage of cell surface receptors via antibody staining as a measurement of receptor activation, is not preferred as it is labor-intensive due to the multiple washing and binding steps. Adenylyl cyclase assays are based on the quantification of second messenger cAMP. However, in the case of $G_{i/o}$-coupled receptors, prestimulation is required (e.g., with forskolin, a direct activator of the adenylyl cyclase)[35-36]. The FLIPR membrane potential assay is designed to measure intracellular changes in calcium levels by using calcium-sensitive fluorescent dyes. The rapid and transient calcium flux makes the assay unsuitable for detecting slow binding agonists. The use of a fluorescent readout may also lead to false positive signals due to possible interference from other compounds[35-36]. The Redistribution and Transfluor assays evaluate receptor activation by monitoring receptor internalization via fusion proteins with green fluorescent protein (GFP). While a benefit of these assays is the real-time measurement and visualization of the GFP fusion protein during the internalization process, they require a dedicated imaging system and offer a relatively low throughput. In the Tango assay, GPCR activation is evaluated by the release of a transcription factor, which leads to expression of a reporter protein that can be quantified. Although reporter gene assays are sensitive, there are some concerns. These

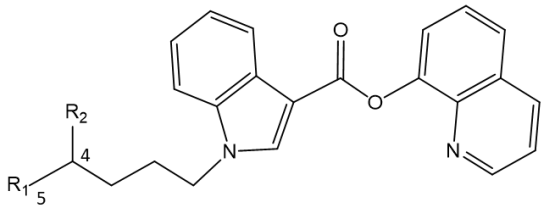
include the need for long incubation times, difficulties in antagonist detection due to reporter accumulation, and the high potential for false positives as reporter protein expression is a distal event to receptor activation. In the PathHunter assay, the β-arrestin–receptor interaction is measured via enzyme fragment complementation of β-galactosidase. The PathHunter assay, just as the Tango assay, can be read on a standard multimode reader and is easily adaptable for high-throughput screening, but its advantage over the Tango assay is that the detection is proximal to the receptor. The downsides of the PathHunter assay are its lack of flexibility for the end user and the limited time window for detection as the β-arrestin–receptor interaction is measured only 90–120 min after stimulation with the test compound[33, 35-37].

A

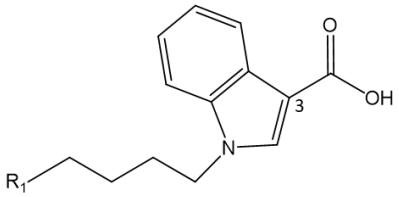


Drug/Metabolite	R_1	R_2	R_3	R_4	R_5
JWH-018	CH_3	H	H	H	H
4-OH-pentyl-JWH-018	CH_3	OH	H	H	H
5-OH-pentyl-JWH-018	CH_2OH	H	H	H	H
5-OH-indole-JWH-018	CH_3	H	H	OH	H
6-OH-indole-JWH-018	CH_3	H	OH	H	H
N-pentanoic acid JWH-018	COOH	H	H	H	H
JWH-122	CH_3	H	H	H	CH_3
4-OH-pentyl-JWH-122	CH_3	OH	H	H	CH_3
5-OH-pentyl-JWH-122	CH_2OH	H	H	H	CH_3
N-pentanoic acid JWH-122	COOH	H	H	H	CH_3
MAM-2201	CH_2F	H	H	H	CH_3
4-OH-pentyl-MAM-2201	CH_2F	OH	H	H	CH_3
JWH-210	CH_3	H	H	H	CH_2CH_3
4-OH-pentyl-JWH-210	CH_3	OH	H	H	CH_2CH_3
5-OH-pentyl-JWH-210	CH_2OH	H	H	H	CH_2CH_3
5-OH-indole-JWH-210	CH_3	H	H	OH	CH_2CH_3
N-pentanoic acid JWH-210	COOH	H	H	H	CH_2CH_3
EAM-2201	CH_2F	H	H	H	CH_2CH_3

B



Drug/Metabolite	R_1	R_2
PB-22	CH_3	H
4-OH-pentyl-PB-22	CH_3	OH
5-OH-pentyl-PB-22	CH_2OH	H
N-pentanoic acid PB-22	COOH	H
5F-PB-22	CH_2F	H



Drug/Metabolite	R_1
3-carboxyindole PB-22	CH_3
3-carboxyindole 5F-PB-22	CH_2F

Figure 3.1. Structures of SCRAs and metabolites. **(A)** SCRAs belonging to the aminoalkylindole family: JWH-018, JWH-122, MAM-2201, JWH-210, and EAM-2201. **(B)** PB-22 and 5F-PB-22, belonging to the indolecarboxylate family.

Here, we developed and applied a new GPCR activation assay based on NanoLuc binary technology (Promega). This technology has already successfully been applied to study protein–protein interactions[38]. We report on the application of this assay for the monitoring of GPCR activation, via ligand-induced interaction of βarr2 with a given GPCR. More specifically, by demonstrating activation of CB1 and CB2 cannabinoid receptors by JWH-018 and a selection of its metabolites, we are the first to show the suitability of the newly

developed bioassay for activity profiling of GPCR ligands. Next, we applied this reporter system to evaluate the *in vitro* activity of JWH-122, JWH-210, and PB-22, their 5-fluoro analogues (MAM-2201, EAM-2201, and 5F-PB-22, respectively), and their main phase I metabolites (Figure 3.1). By doing so, we demonstrate that several major metabolites of these SCRAs retain their activity at CB receptors. We also demonstrate a proof of concept of the applicability of the newly developed bioassay for detecting the presence of CB receptor activating compounds, notably SCRAs (and their metabolites), in urine.

3.2 Materials and Methods

3.2.1 Chemicals and Reagents

Dulbecco's modified eagle's medium (DMEM), Opti-MEM® I Reduced Serum Medium, penicillin/streptomycin (10.000 IU/ml and 10.000 µg/ml), amphotericin B (250 µg/ml), glutamine (200 mM), the restriction enzymes *Xho*I and *Eco*RI and the DNA polymerase (Phusion polymerase, a polymerase with proofreading activity) were purchased from Thermo Fisher Scientific (Pittsburg, PA, USA). The transfection reagent FuGENE® HD and the Nano-Glo Live Cell reagent were purchased from Promega (Madison, WI, USA). Primers were procured from Eurofins Genomics (Ebersberg, Germany). JWH-122 ((4-methyl-1-naphthyl)-(1-pentyl-1*H*-lindol-3-yl)methanone), JWH-210 ((4-ethyl-1-naphthyl)-(1-pentyl-1*H*-indol-3-yl)methanone), PB-22 (1-pentyl-1*H*-indole-3-carboxylic acid 8-quinolinyl ester), their 5-fluoro analogues MAM-2201, EAM-2201, 5F-PB-22, their metabolites and all deuterated standards were supplied by Cayman Chemical (Ann Arbor, MI, USA), except JWH-018 (naphthyl(1-pentyl-1*H*-indol-3-yl)methanone) was obtained from LGC (Wesel, Germany) and 5-OH-pentyl-JWH-018 and N-pentanoic acid JWH-018 were purchased from Sigma Aldrich. Fetal bovine serum (FBS), poly-D-lysine, formic acid (Rotipuran® ≥ 98 %, p.a.), potassium hydrogen phosphate (≥ 99 %, p.a.), 2-propanol (Rotisolv® ≥ 98 %, p.a.), acetonitrile (LC-MS grade), ammonium formate 10 M (99,995 %) and potassium hydroxide (puriss. p.a. ≥ 86 % (T) pellets) were from Sigma Aldrich (Steinheim, Germany). Roche Diagnostics (Mannheim, Germany) supplied the β-glucuronidase (*E. coli* K 12). Deionized water was prepared using a Medica® Pro deionizer from ELGA (Celle, Germany). Blank urine samples were donated by one volunteer and tested for the absence of synthetic cannabinoid metabolites prior to use. Mobile phase A (0.2 % formic acid and 2 mM ammonium formate in water) was freshly prepared prior to LC-MS/MS analysis. Mobile phase B was pure acetonitrile.

3.2.2 Plasmids and constructs

Plasmids containing the human *CNR1* (NM_016083) and *ARRB2* (NM_004313) coding sequences were purchased from Origene Technologies (Rockville, MD, USA). A plasmid

containing the human *CNR2* (NM_001841) coding sequence and the expression vectors, NB MCS-1, NB MCS-2, NB MCS-3 and NB MCS-4 were kindly provided by respectively, Atwood *et al*. and Promega (Madison, WI, USA). The expression vectors contain the sequences encoding the subunits of the NanoLuc® luciferase (LgBiT or SmBiT) and the flexible linker (GSSGGGGSGGGGSSG). All expression plasmids were constructed by cloning PCR products, flanked by a unique restriction site, into the respective vectors, as described below. All constructs were sequence-verified.

To generate the constructs, specific primers were used to PCR-amplify the coding sequence of interest, flanked by *Xho*I or *Eco*RI restriction sites (see Table 3.1). PCR was performed on 100 pg of plasmid DNA using the Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, Pittsburg, PA, USA) according to the manufacturer's instructions. Reactions were done in a Mastercycler™ Nexus Thermal Cycler (Eppendorf, Hamburg, Germany) with an initial denaturation at 98°C for 30s, 35 cycles of 98°C for 10s (denaturation), T_m for 30s (annealing), and 72°C for 1 min (elongation), followed by a final extension at 72°C for 5 min. The resulting amplification products were purified using E.Z.N.A.® MicroElute Cycle-Pure kit (VWR International, Radnor, PA, USA). Both the vector and the amplification products were digested with either *Xho*I or *Eco*RI restriction enzymes (Thermo Fisher Scientific) and purified using E.Z.N.A.® MicroElute Gel Extraction kit (VWR International). The digested PCR products were ligated into the corresponding dephosphorylated (TSAP Thermosensitive Alkaline Phosphatase, Promega), digested vector (see Table 3.2). After transformation of One Shot® Mach1™ T1 Phage-Resistant Chemically Competent E. coli (Thermo Fisher Scientific) with the ligated product, the ampicillin-resistant clones were screened by PCR using primers complementary to sequences within the insert and sequences of the vector surrounding the insert. The integrity of all constructs was confirmed by DNA sequencing. Positively screened colonies were grown and used for plasmid isolation, using E.Z.N.A.® Plasmid DNA Mini kit (VWR International).

Table 3.1. Primers used to clone the protein of interest (POI) in the expression plasmids. Six extra nucleotides precede the restriction site (underlined). In some primers, extra nucleotides were added to correct the reading frame. The Kozak sequence or stop codon (bold) were also added, if necessary. The nucleotides in italics are the coding sequences of the POI.

Vector	POI	Primers		T_m (°C)
NB MCS-1 NB MCS-2	CB1	Forward	ACTCAA CTCGAG ***ACC*** *ATGAAGTCGATCC*	69.6
		Reverse	ACTCAA CTCGAG CC *CAGAGCCTCGGC*	
	CB2	Forward	ACTCAA CTCGAG CC *CAGAGCCTCGGC*	71.0
		Reverse	ACTCAA CTCGAG CC *GCAATCAGAGAGG*	
	βarr2	Forward	ACTCAA GAATTC ***ACC*** *ATGGGGGAGAAACCCGGGACC*	71.1
		Reverse	ACTCAA GAATTC CC *GCAGAGTTGATCATCATAGTCG*	
NB MCS-3 NB MCS-4	βarr2	Forward	ACTCAA GAATTC A *ATGGGGGAGAAACCCGGGACC*	69.7
		Reverse	ACTCAA GAATTC ***TCA*** *GCAGAGTTGATCATCATAGTCG*	

Table 3.2. Lay-out of the expression vectors and restriction enzymes used for each protein of interest (POI).

Vector	Fusion protein	POI	Restriction enzyme
NB MCS-1	POI - Linker - LgBiT	CB1	*Xho*I
		CB2	*Xho*I
		βarr2	*Eco*RI
NB MCS-2	POI - Linker - SmBiT	CB1	*Xho*I
		CB2	*Xho*I
		βarr2	*Eco*RI
NB MCS-3	LgBiT - Linker - POI	βarr2	*Eco*RI
NB MCS-4	SmBiT - Linker - POI	βarr2	*Eco*RI

3.2.3 Cell culture and transfection

Human embryonic kidney (HEK) 293T cells were routinely maintained at 37°C, 5% CO_2, under humidified atmosphere in DMEM (high glucose) supplemented with 10% heat-inactivated FBS, 2 mM of glutamine, 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B. For experiments, HEK 293T cells were plated in 6-well plates at $5x10^5$ cells/well. The next day, cells were transiently transfected using FuGENE® HD reagent according to the manufacturer's protocol (optimal ratio of FuGENE:DNA 3:1). Transfection mixes contained 1.65 µg of each of the plasmids of interest. On the third day, cells were plated on poly-D-lysine-coated 96-well plates at $5x10^4$ cells/well and incubated overnight.

3.2.4 Cannabinoid Reporter Assay

Forty-eight hours after transfection, the cells were washed twice with Opti-MEM I reduced serum medium to remove any remaining FBS, and 100 µL of Opti-MEM I was added. The Nano-Glo Live Cell reagent, a nonlytic detection reagent containing the cell-permeable furimazine substrate, was prepared by diluting the Nano-Glo Live Cell substrate 20 × using Nano-Glo LCS Dilution buffer, and 25 µL was added to each well. Subsequently, the plate was placed in a GloMAX96 (Promega). Luminescence was monitored during the equilibration period until the signal was stabilized (30–45 min).

For agonist experiments, we added 10 µL per well of test compounds, present as 13.5× stocks in 50% methanol in Opti-MEM I. For antagonist experiments, 5 µL of the antagonist stock solution (26× stock solution in 50% methanol in Opti-MEM I) was incubated for 5 min before adding 5 µL of agonist (27× stock solution 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 advantage of the short readout time of the assay.

3.2.5 Statistical Analysis

Curve fitting and statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA). To select the optimal configuration for the CB reporter assay for both CB receptors, results are represented as mean area under the curve (AUC) ± standard error of mean (SEM) with six replicates for each data point (unless stated otherwise) and were statistically analyzed using Student's *t* test after *F*-test and Grubbs' outliers test ($\alpha = 0.05$). Curve fitting of concentration–effect curves via nonlinear regression was employed to determine EC/IC_{50} (a measure of potency).

To evaluate the activity of the different SCRAs and their metabolites, results are represented as the percentage (%) CB activation (relative to the receptor activation of JWH-018) ± 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 (Figure 3.2). First, the absolute raw signals (colored lines) were baseline-corrected by subtracting the average of the signals from vehicle control samples (black lines) (A to B). Next the signals were corrected for the inter-well variability by forcing the curve through 0 (B to C). The AUC were calculated and normalized by the AUC of the reference compound, JWH-018 (Figure 3.2).

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 JWH-122 vs JWH-122), and (iii) between the signals obtained from the compounds and those from solvent controls. To plot the activity profiles of the natural cannabinoids and urinary samples, the normalized raw data are shown (see Figure 3.3). Here, the absolute signals were corrected by forcing all the curves through the same starting point at time point 0 (Figure 3.3 from A to B).

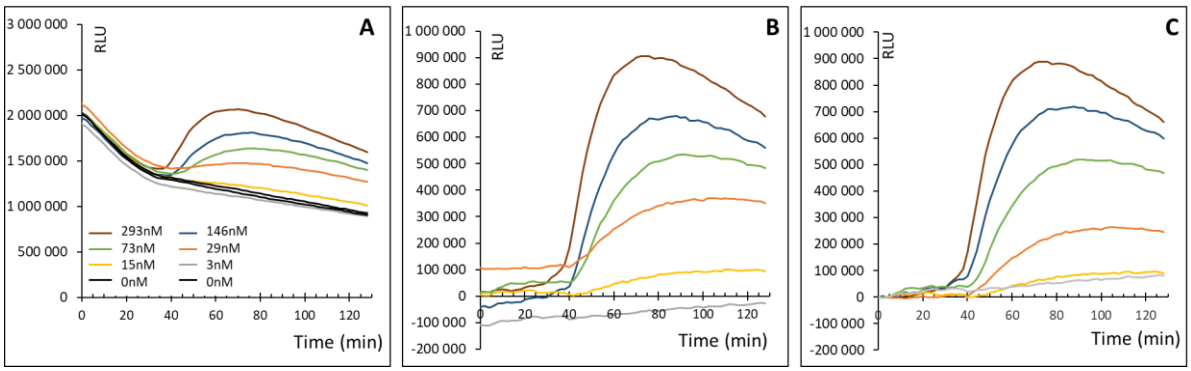


Figure 3.2. Example of solvent control and inter-well correction. **(A)** Absolute raw signals (colored lines) were baseline-corrected by subtracting the average of the signals from vehicle control samples (black lines) **(B)**. Next, the signals were corrected for the inter-well variability by forcing the curve through 0 **(C)**.

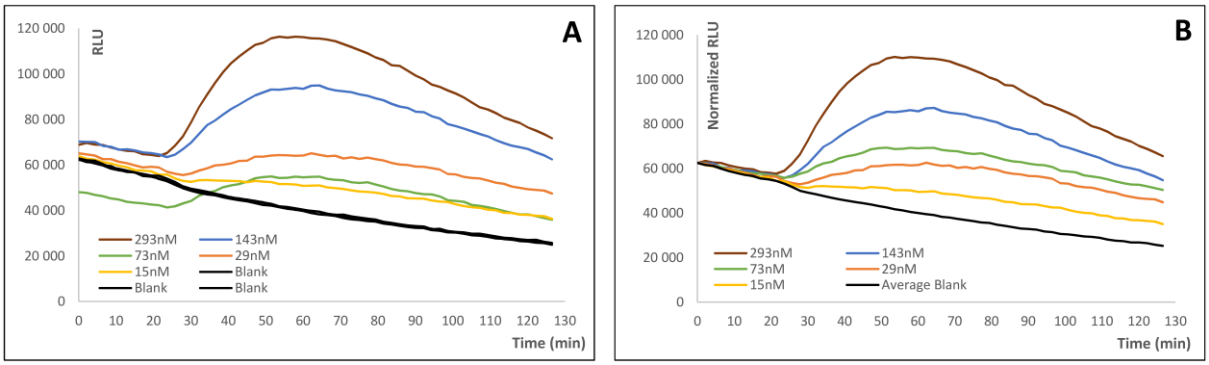


Figure 3.3. Example of inter-well correction. The absolute signals **(A)** were corrected by forcing all the curves through the same starting point at time point 0 **(B)**.

3.2.6 Urine Sample Preparation

For conjugate cleavage, 0.5 mL of phosphate buffer (pH 6) and 30 µL of β-glucuronidase were added to 0.5 mL of urine, followed by a 1 h incubation at 45 °C. Then, 1.5 mL of ice-cold acetonitrile and 0.5 mL of 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. For analysis with the applied CB reporter assay, the evaporated extract was reconstituted in 100 µL of Opti-MEM I/MeOH (50/50, v/v), of which 10 µL was used per well (see the Cannabinoid Reporter Assay section). For LC-MS/MS analysis, another 0.5 mL aliquot was spiked with internal standards (2 ng/mL; see Table 3.3) and processed as described above. The residue was reconstituted in 200 µL of mobile phase A/B (50/50, v/v) prior to LC-MS/MS analysis.

3.2.7 LC-ESI-MS/MS Analysis of Urine Samples

Quantification of SCRA metabolites in a genuine urine sample was performed by applying a semi-quantitative LC-MS/MS method. Selectivity and specificity were tested by analyzing six blank samples. Linearity was given for all analytes from 0.01 to 10.0 ng/mL. The lowest calibrator level was defined as the lower limit of quantification (LLOQ). Precision (< 15%) and accuracy (< ±15%) were assessed by analysis of control samples. Concentrations below the calibration range were extrapolated using the peak area ratio of the lowest calibrator and were reported only if the identification criteria were fulfilled (retention time, signal-to-noise ratio > 3:1, qualifier ion/quantifier ion ratio). Matrix effects were not assessed since semi-quantitative values were considered acceptable for the proof-of-concept comparison with the qualitative results of the applied reporter assay. Settings used for the chromatographic separation and the tandem mass spectrometry analysis are described elsewhere[39]. Table 3.3 gives details on the optimized MS settings of the quantified analytes and internal standards. All LC-MS/MS analyses were performed by Florian Franz at the University of Freiburg.

Table 3.3. Supporting table showing MRM transitions and optimized MS parameters for the analytes detected in the urine samples and the used internal standards.

Analyte	Q1 [amu]	Q3 [amu]	DP [V]	EP [V]	CE [V]	CXP [V]	Internal standard
JWH-018 4-OH-pentyl	358	155	150	5	30	15	D5-JWH-018 4-OH-pentyl
	358	127	150	5	65	15	
JWH-018 5-OH-pentyl	358	155	150	5	30	15	D5-JWH-018 5-OH-pentyl
	358	127	150	5	65	15	
JWH-122 4-OH-pentyl	372	169	160	7	30	12	D5-JWH-122 5-OH-pentyl
	372	141	160	7	57	16	
JWH-122 5-OH-pentyl	372	169	160	7	30	12	D5-JWH-122 5-OH-pentyl
	372	141	160	7	57	16	
JWH-122 *N*-pentanoic acid	386	169	190	4	35	13	D4-JWH-018 *N*-pentanoic acid
	386	141	190	4	61	11	
MAM-2201 4-OH-pentyl	390	169	160	11	35	20	D5-JWH-018 5-OH-pentyl
	390	141	160	11	65	22	
JWH-210 4-OH-pentyl	386	183	160	5	31	15	D7-JWH-018 6-OH-indole
	386	153	160	5	50	13	
JWH-210 5-OH-indole	386	183	180	4	32	16	D5-JWH-250
	386	230	180	4	32	18	
JWH-210 5-OH-pentyl	386	183	160	5	31	15	D7-JWH-018 6-OH-indole
	386	153	160	5	50	13	
JWH-210 *N*-pentanoic acid	400	183	160	11	35	14	D7-JWH-073 6-OH-indole
	400	155	160	11	55	20	
PB-22 3-carboxyindole	232	132	130	3	29	16	D7-JWH-073 6-OH-indole
	232	118	130	3	30	14	
PB-22 4-OH-pentyl	375	144	110	5	35	23	D5-JWH-073 *N*-butanoic acid
	375	69	110	5	55	15	
PB-22 5-OH-pentyl	375	230	110	6	45	15	D5-JWH-073 *N*-butanoic acid
	375	144	110	5	35	23	
PB-22 *N*-pentanoic acid	389	244	140	4	30	16	D5-JWH-073 *N*-butanoic acid
	389	144	140	4	48	16	
5F-PB-22 3-carboxyindole	250	206	110	3	22	16	D5-JWH-250 4-OH-pentyl
	250	118	110	3	26	13	
D5-JWH-018 4-OH-pentyl	363	155	180	4	25	11	---
D5-JWH-018 5-OH-pentyl	363	155	165	10	33	12	---
D7-JWH-018 6-OH-indole	367	155	180	4	35	12	---
D4-JWH-018 *N*-pentanoic acid	376	155	170	10	35	7	---
D7-JWH-073 6-OH-indole	351	155	170	6	35	12	---
D5-JWH-073 *N*-butanoic acid	363	155	190	5	35	11	---
D5-JWH-122 5-OH-pentyl	377	169	185	5	29	14	---
D5-JWH-250	341	121	175	10	30	8	---
D5-JWH-250 4-OH-pentyl	357	121	150	8	31	15	---

Q1 *m/z* of the precursor ion, Q2 *m/z* of the fragment ion, DP declustering potential, EP entrance potential, CE collision energy, CXP collision cell exit potential.

3.3 Results and Discussion

3.3.1 Design of the CB Reporter Assay

NanoLuc binary technology utilizes a structural complementation-based approach to monitor protein interactions within living cells. 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. Protein interaction promotes structural complementation of the subunits, thereby restoring NanoLuc luciferase activity, which generates a bioluminescent signal in the presence of the furimazine substrate. To monitor GPCR activation, we made use of its stimulation-dependent interaction with the cytosolic adaptor protein βarr2, which mediates receptor desensitization and internalization in a widely distributed manner throughout the GPCR family[40-42]. Here, we aimed at establishing assays capable of monitoring activation of CB1 and CB2 receptors using a panel of SCRAs and their metabolites. To this end, we designed constructs in which the LgBiT or SmBiT subunit is coupled to the CB1 or CB2 C-terminus and to the N- or C-terminus of βarr2. To assess the functional complementation of the LgBiT and SmBiT fusion proteins upon GPCR activation, all possible combinations were tested by stimulation with a known agonist, JWH-018 (Figure 3.4).

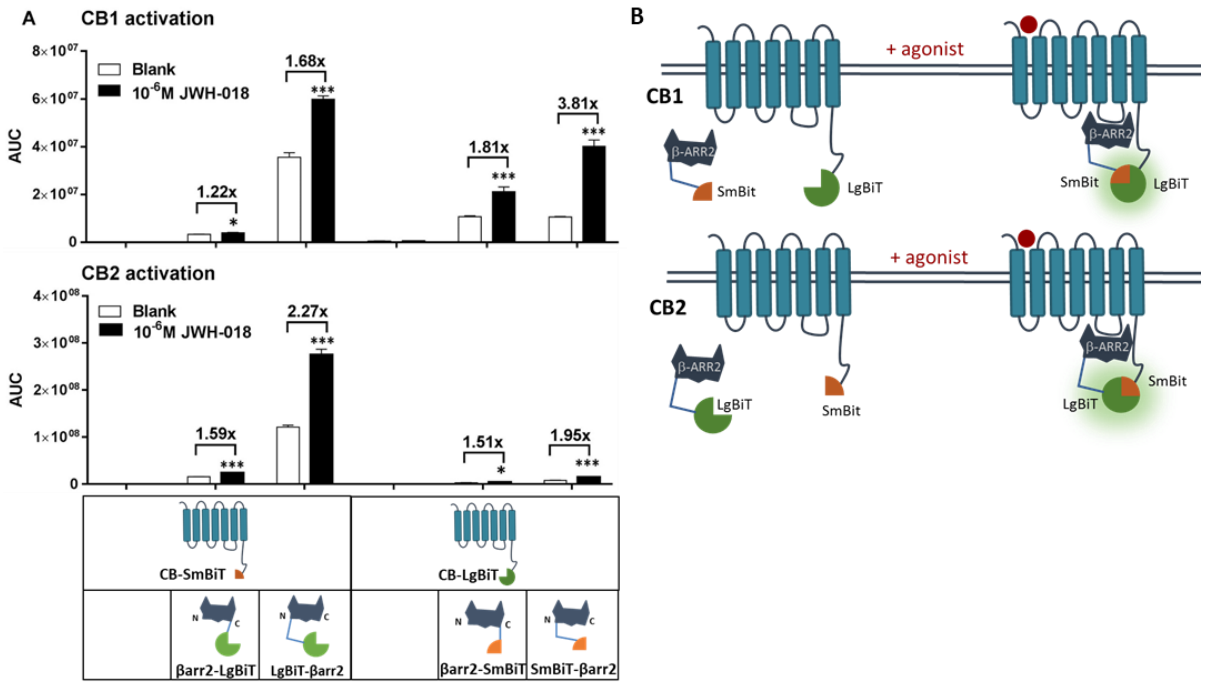


Figure 3.4. Comparison of the different combinations at both CB receptors. **(A)** CB reporter assays for CB1 (upper graph) and CB2 (lower graph). A stimulation-dependent interaction of βarr2 with both CB receptors was consistently observed. No ligand-dependent effects could be detected for the single CB fusion protein. Data are given as the mean AUC ± SEM (n = 5–6); *$P \leq 0.01$, **$P \leq 0.001$, ***$P \leq 0.0001$ (two-sided t-test). **(B)** Optimal design of the CB reporter assays for CB1 and CB2: CB1–LgBiT/SmBiT–βarr2 and CB2–SmBiT/LgBiT–βarr2.

Whenever both the CB and βarr2 fusion proteins were present together, unstimulated cells readily showed a signal above background (i.e., the signal when only the CB fusion protein was present), pointing at some level of constitutive CB−βarr2 interaction (open bars in Figure 3.4A). Regardless of the combination of CB and βarr2 fusion proteins used, a significant increase in signal was observed upon agonist stimulation (closed bars in Figure 3.4A). The highest signals were observed for the CB–SmBiT/LgBiT−βarr2 combinations. For CB2, this combination also yielded the largest increase (2.27-fold) when comparing stimulated vs nonstimulated cells. For CB1, however, the combination of CB1–LgBiT/SmBiT−βarr2, although it gave somewhat lower absolute signals, yielded the largest increase (3.81-fold) following activation. Hence, further experiments were performed with cells that were transiently transfected with either the CB1–LgBiT/SmBiT−βarr2 combination or the CB2–SmBiT/LgBiT−βarr2 combination (Figure 3.4B).

3.3.2 Concentration Dependence of the CB Reporter Assays

Upon stimulation with a known agonist, JWH-018, CB1–LgBiT and CB2–SmBiT showed a concentration-dependent interaction with SmBiT−βarr2 and LgBiT−βarr2, respectively, with EC_{50} values of 38.2 and 12.8 nM (Figure 3.5A and B; Table 3.4).

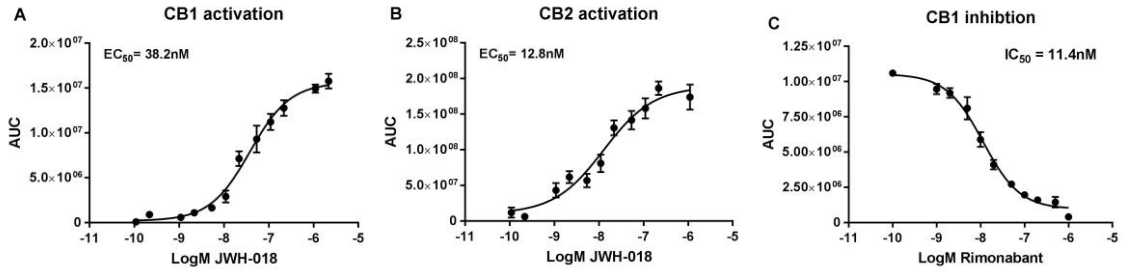


Figure 3.5. Concentration-dependent interaction of CB1 **(A)** and CB2 **(B)** with βarr2 upon stimulation with JWH-018. **(C)** Interaction of βarr2 with the CB1 receptor, induced by JWH-018 at its ED_{80} concentration, was blocked by Rimonabant, a selective CB1 antagonist, in a concentration-dependent manner. AUC, area under the curve (luminescence over time). Data are given as the mean AUC ± SEM (n = 5–6).

Table 3.4. EC_{50} values (as a measure of potency) of different SCRAs, determined via curve fitting of concentration-effect curves via non-linear regression. Data are given as EC_{50} values (95% CI profile likelihood).

Drug	CB1 EC_{50} (nM)	CB2 EC_{50} (nM)
JWH-018	38.2 (27.1-55.7)	12.8 (5.6-26.0)
JWH-122	71.7 (52.3-104.4)	9.2 (5.1-15.9)
MAM-2201	60.5 (44.3-87.5)	2.7 (1.1-5.1)
JWH-210	25.3 (18.3-34.7)	17.5 (10.1-29.1)
EAM-2201	4.8 (3.2-7.2)	3.7 (1.9-6.5)
PB-22	0.86 (0.53-1.33)	0.82 (0.30-1.46)
5F-PB-22	0.84 (0.51-1.40)	0.70 (0.47-0.97)

Similarly, for other SCRAs, concentration-dependence was obtained and EC_{50} values were determined as a measure of relative potency (Table 3.4; Figure 3.6). 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. More specifically, reported EC_{50} values for CB1 of JWH-018, JWH-122, and JWH-210 ([^{35}S]GTPγS binding assay: 36, 32.9, and 20.4 nM, respectively)[24] are in line with the EC_{50} values we obtained, ranging from 25.3 to 71.7 nM (Table 3.3; Figure 3.6). For PB-22 and 5F-PB-22, which are known to have an even higher potency[16], we obtained subnanomolar EC_{50} values using the newly developed assay. Again, these EC_{50} values match the order of magnitude of those found in the literature (FLIPR membrane potential assay: CB1 5.1 and 2.8 nM vs 102 nM for JWH-018; CB2 37 and 11 nM vs 133 nM for JWH-018)[16]. Our data are also in line with the observation by Banister *et al.* that terminal fluorination of the N-pentyl results in increased CB receptor potency[16].

For CB1, the parent compounds JWH-122, JWH-210, and PB-22 and their 5-fluoro analogues (MAM-2201, EAM-2201 and 5F-PB-22, respectively) showed CB1 activation that was significantly higher than the reference JWH-018 (Figure 3.6A). This may point at an intrinsic high efficacy of these compounds or, alternatively, to more efficient recruitment of βarr2 upon receptor activation, or both. The JWH-018-induced recruitment of βarr2 to CB1 was blocked by Rimonabant, a selective CB1 antagonist, in a concentration-dependent manner (Figure 3.5C), demonstrating the specificity of the assay. Curve fitting of concentration–effect curves via nonlinear regression was employed to determine the IC_{50} of Rimonabant, which was 11.4 nM (95% CI profile likelihood: 8.6–15.1 nM) and is in line with the IC_{50} value of 17.6 nM (Eu-GTPγS binding assay) found in the literature[43].

3.3.3 Application of the CB Reporter Assays on SCRAs and Their Main Phase I Metabolites

I. JWH-018

JWH-018 was the first SCRA reported in Germany in December 2008 as one of the active components of the herbal blend "Spice"[4-5]. It is a naphthoylindole, belonging to the aminoalkylindole family (Figure 3.1). Although its chemical structure differs substantially from that of Δ^9THC, it produces similar effects and has been reported to be more potent than Δ^9THC (Table 3.5). Importantly, several metabolites of JWH-018 have been reported to have partial to full agonist activity at CB1 and CB2[18-20]. To further validate our newly developed CB reporter assay, we applied it to JWH-018 and a selection of its metabolites, reported as the major phase I metabolites occurring in urine, i.e., the 4- and 5-OH-pentyl, 5- and 6-OH-indole, and the N-pentanoic acid metabolites[44-46].

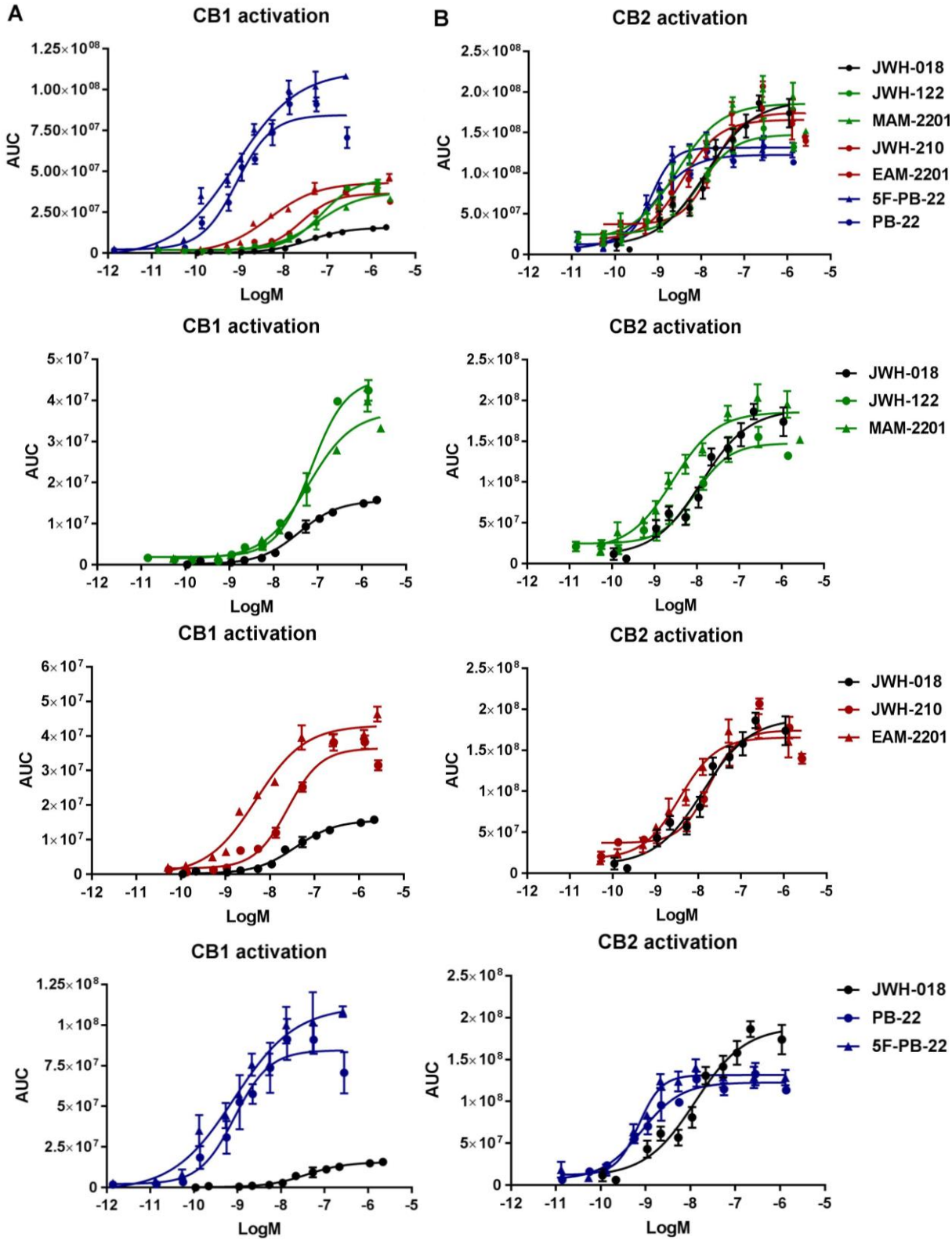


Figure 3.6. The concentration-dependent interaction of CB1 **(A)** and CB2 **(B)** with βarr2 upon stimulation with different SCRAs. AUC, area under the curve. Data are given as mean AUC ± SEM (n=5-6).

Table 3.5. Potency of Δ^9THC and JWH-018.

Drug	Functional assay	CB1 EC_{50} (nM)	CB2 EC_{50} (nM)	Reference
Δ^9THC	GTPγS binding	81 ± 34 167 ± 84.7 77.0 ± 29.9	- - -	Breivogel *et al.* (2001)[21] Brents *et al.* (2011)[18] Brents *et al*. (2012)[17]
	FLIPR membrane potential assay	250 172	1157 -	Banister *et al.* (2015a)[16] Banister *et al*. (2015b)[31]
	Adenylate cyclase inhibition	-	57.9 ± 19.8	Rajaskaran *et al.* (2013)[20]
JWH-018	GTPγS binding	36 6.8 ± 2.5 20.2 ± 1.3	- - -	Nakajima *et al.* (2011)[24] Brents *et al.* (2011)[18] De Luca *et al.* (2013)[22]
	FLIPR membrane potential assay	102 18	133 22	Banister *et al.* (2015a)[16] Banister *et al.* (2015b)[31]
	Adenylate cyclase inhibition	-	3.6 ± 2.2	Rajaskaran *et al.* (2013)[20]
	Internalization assay	2.8 10.1	-	Atwood *et al.* (2010)[27] Atwood *et al.* (2011)[28]

For each of these compounds, we assessed βarr2 recruitment to either the CB1 or CB2 receptor at an arbitrarily chosen concentration of 1 µM, corresponding to a receptor saturating concentration of JWH-018. Unlike JWH-018 and all of the monohydroxylated metabolites, which activated both CB receptors (Figure 3.7, Table 3.6), the N-pentanoic acid metabolite did not induce a significant difference from basal levels at both receptors, which is in line with its reported lack of affinity for CB1 and CB2 receptors (K_i for CB1 and CB2 ≥ 10 000 nM)[18-20].

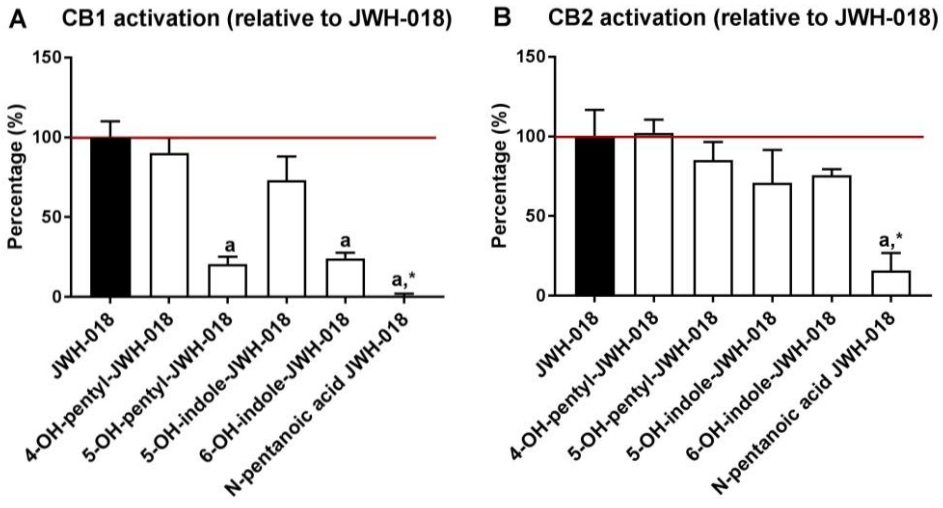


Figure 3.7. Activation of CB1 receptor **(A)** and CB2 receptor **(B)** by JWH-018 and its major phase I metabolites at 1 µM. Bars assigned with (a) above the error bars are significantly different from the reference compound JWH-018 ($P \leq 0.05$, one-way ANOVA with Dunnett's multiple comparison post hoc test). Bars assigned with an (*) are not significantly different from basal levels. Data are given as mean % CB receptor activation (in comparison to the receptor activation of the reference, JWH-018) ± SEM (*n* = at least 3 replicates).

The extent of CB1 and CB2 activation varied for individual metabolites. For CB1, there was no statistical difference in the level of receptor activation by the 4-OH-pentyl and 5-OH-indole metabolites when compared to the JWH-018 parent compound. The two other hydroxylated metabolites (5-OH-pentyl and 6-OH-indole metabolites) produced significantly less CB1 activation, 20.7 and 24.1% relative to JWH-018, respectively (Figure 3.7A and Table 3.6). For CB2, all hydroxylated metabolites yielded a signal that was not significantly different from that obtained after stimulation with JWH-018, indicating that these metabolites also retain their activity at CB2 (Figure 3.7B), which is consistent with the literature[18-20]. It is worth noting that, when compared to the literature, there is no perfect overlay in the relative activity of the metabolites. Several reasons may account for these more subtle differences, most notably the different experimental setups that have been used (e.g., [^{35}S]GTPγS binding assay and adenylyl cyclase assay vs βarr2 recruitment)[18-20].

Table 3.6. Comparison of relative potential to activate CB1 and CB2 at 1 µM. The data are presented as the % CB activation (relative to the receptor activation of JWH-018) ± SEM (number of replicates).

Drug/metabolite	**Relative potential of CB1 activation at 1 µM**	**Relative potential of CB2 activation at 1 µM**
JWH-018	100.0 ± 10.6 (20)	100 ± 17.6 (20)
4-OH-pentyl JWH-018	90.3 ± 10.9 (4)	102.3 ± 9.5 (4)
5-OH-pentyl-JWH-018	20.7 ± 5.3 (4)	85.1 ± 13.2 (4)
5-OH-indole-JWH-018	73.1 ± 18.3 (3)	70.8 ± 24.0 (4)
6-OH-indole-JWH-018	24.1 ± 4.1 (4)	75.7 ± 4.3 (4)
N-pentanoic acid JWH-018	0.53 ± 1.77 (4)	15.9 ± 12.6 (4)
JWH-122	173.4 ± 17.6 (4)	94.0 ± 7.5 (4)
4-OH-pentyl-JWH-122	165.2 ± 13.0 (3)	99.4 ± 4.1 (4)
MAM-2201	174.7 ± 31.2 (3)	97.4 ± 23.0 (3)
5-OH-pentyl-JWH-122	99.5 ± 5.9 (4)	96.3 ± 10.9 (4)
4-OH-pentyl-MAM-2201	173.0 ± 20.2 (4)	87.6 ± 13.5 (4)
N-pentanoic acid JWH-122	1.4 ± 3.7 (4)	3.6 ± 6.2 (3)
JWH-210	197.9 ± 13.3 (4)	84.5 ± 12.0 (4)
4-OH-pentyl-JWH-210	179.3 ± 19.0 (3)	115.4 ± 22.2 (4)
5-OH-pentyl-JWH-210	157.8 ± 12.3 (4)	101.4 ± 12.7 (4)
5-OH-indole-JWH-210	100.3 ± 13.4 (3)	90.9 ± 18.9 (4)
EAM-2201	239.7 ± 10.5 (4)	92.4 ± 25.3 (4)
N-pentanoic acid JWH-210	1.9 ± 1.6 (3)	54.8 ± 7.8 (4)
PB-22	287.0 ± 29.6 (4)	137.6 ± 13.8 (3)
3-carboxyindole PB-22	1.3 ± 3.6 (4)	1.6 ± 4.3 (3)
4-OH-pentyl PB-22	208.0 ± 21.2 (4)	144.6 ± 33.0 (4)
5F-PB-22	278.8 ± 9.2 (3)	131.9 ± 9.3 (4)
3-carboxyindole 5F-PB-22	3.1 ± 5.0 (4)	4.6 ± 13.1 (4)
5-OH-pentyl PB-22	171.0 ± 6.9 (3)	142.3 ± 13.3 (4)
N-pentanoic acid PB-22	25.8 ± 3.4 (4)	43.5 ± 5.3 (4)

II. JWH-122, JWH-210, MAM-2201, and EAM-2201

JWH-122 and JWH-210 and their 5-fluoro analogues, MAM-2201 and EAM-2201, respectively, belong to the naphthoylindole family. They only differ from JWH-018 by the addition of a methyl/ethyl on the naphthyl moiety (Figure 3.1). These SCRAs became popular in 2010–2011 after the prohibition of the "first generation" of SCRAs (e.g., JWH-018, JWH-073)[47-48]. The shift to these agonists, some of which are more potent than JWH-018 (Table 3.4), has led to more cases with serious symptoms, including a reported fatality of MAM-2201 poisoning[49-52].

Biotransformation of JWH-122 and MAM-2201 leads to common metabolites: the 5-OH-pentyl and N-pentanoic acid JWH-122 metabolites. MAM-2201 also produces trace amounts of the 4-OH-pentyl-MAM-2201 metabolite[53]. Both *in vitro* metabolism studies[53] and analyses of authentic urine samples from users (unpublished observations)[53] demonstrated that the 5-OH-pentyl-JWH-122 metabolite was the primary phase I metabolite of MAM-2201, whereas the 4-OH-pentyl-JWH-122 metabolite was predominant and exclusive in JWH-122 metabolism[53]. From the available reference standards for metabolites of JWH-210, the 4-OH-pentyl metabolite was the most prevalent phase I metabolite in urine. The other phase I metabolites that were present in decreasing abundance are the 5-OH-indole and 5-OH-pentyl metabolites. For EAM-2201, the 5-OH-pentyl-JWH-210 metabolite was the most abundant phase I metabolite in the urine of users, followed by the N-pentanoic acid JWH-210 metabolite (unpublished observations). Since the activity of these metabolites at CB receptors is not known, we evaluated these with our new bioassay. Again, all compounds were tested at 1 μM, with JWH-018 as a reference.

JWH-122, MAM-2201, JWH-210, EAM-2201, and all of the monohydroxylated metabolites showed significant activation of both receptors. The N-pentanoic acid JWH-122 metabolite did not induce a significant difference from basal levels at both receptors, but, unexpectedly, the N-pentanoic acid JWH-210 metabolite did show CB2 receptor activation, which, although somewhat lower, was not significantly different from the parent compound, JWH-210. (Figure 3.8A and B; Table 3.6). The signal obtained for almost all hydroxylated metabolites was not significantly different from that induced by the corresponding parent compounds, JWH-122 and JWH-210. Only 5-OH-pentyl-JWH-122 and 5-OH-indole-JWH-210 yielded signals that were significantly lower, but they still induced levels of CB1 activation that were not statistically different from our reference compound, JWH-018. For CB2, there was no statistical difference in the level of receptor activation produced by the reference compound (JWH-018), JWH-122, MAM-2201, JWH-210, EAM-2201, and their monohydroxylated metabolites.

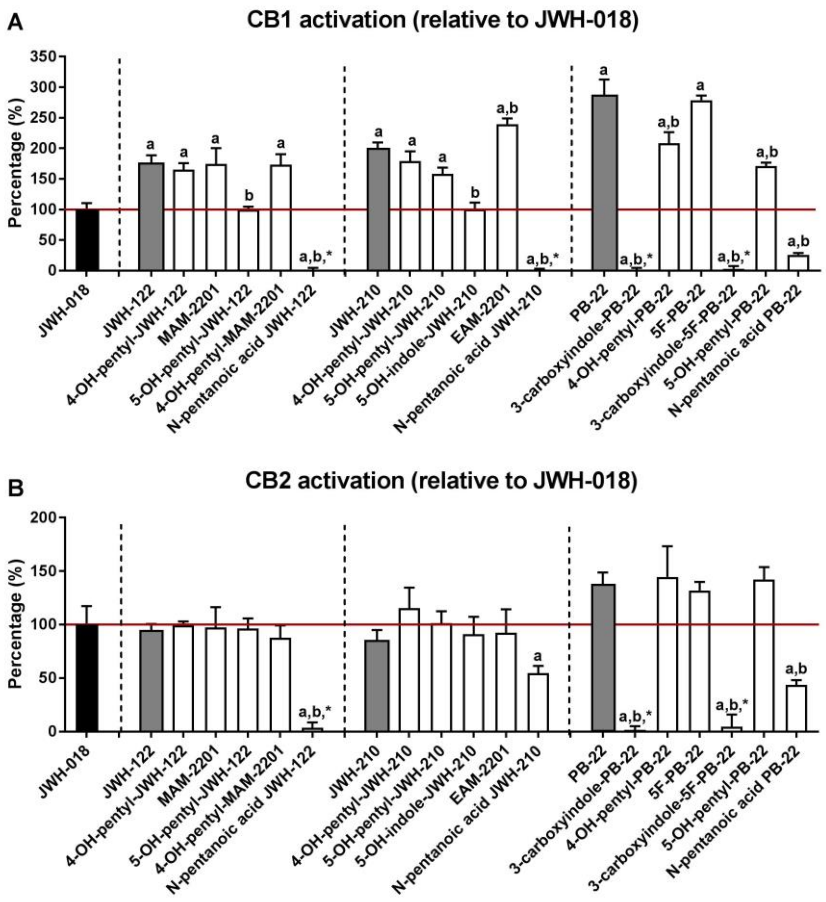


Figure 3.8. Activation of CB1 receptor **(A)** and CB2 receptor **(B)**. Values designated with (a) above error bars denote a significant difference from the reference compound, JWH-018 ($P \leq 0.05$, one-way ANOVA with Dunnett's multiple comparison post hoc test). Values designated with (b) are significantly 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) ± SEM (*n* = at least 3 replicates).

III. PB-22 and 5F-PB-22

PB-22 (also named QUPIC) and 5F-PB-22 belong to the quinolin-8-yl indolecarboxylate family, differing from the earlier generation naphthoylindoles by the replacement of the naphthalene group with an ester-linked quinolin-8-yl moiety (Figure 3.1). These compounds were first reported to the EMCDDA in November 2012 following their seizure by Finnish customs authorities[5]. PB-22 and 5F-PB-22 have been implicated in clinical reports of seizure[54-55], with 5F-PB-22 having been associated with several adverse reactions, comprising anxiety, paranoia, headache, vomiting, sweating, and nausea[56]. 5F-PB-22 has also been detected in several fatal intoxications in the USA[57]. PB-22 and 5F-PB-22 are known to be very potent SCRAs, both possessing subnanomolar potency at CB receptors (Table 3.4), but nothing is known about the activities of their metabolites. The metabolism of PB-22 and 5F-PB-22 has primarily been investigated via *in vitro* metabolism studies[58-60]. After examining

authentic urine samples from users who consumed PB-22 and 5F-PB-22, we selected some metabolites to assess their activity at CB receptors. For PB-22, the 3-carboxyindole was the major phase I metabolite, with the 4-OH-pentyl metabolite usually being the second most prevalent. A similar metabolic profile was seen for 5F-PB-22, with the 3-carboxyindole as most prominent and the 5-OH-pentyl as second most abundant phase I metabolite.

The reporter assay was used to evaluate the intrinsic activity at the CB1 and CB2 receptors of PB-22, 5F-PB-22, and all of the above-mentioned metabolites, as well as that of the N-pentanoic acid metabolite, which is also found in most urine samples (unpublished observations), all at a fixed concentration of 1 µM. PB-22 and 5F-PB-22 showed significantly stronger receptor activation at both CB receptors in comparison to the reference, JWH-018: 2.87- and 2.79-fold increases at the CB1 receptor and 1.38- and 1.32-fold increases at the CB2 receptor, respectively (Figure 3.8A and B; Table 3.6). The capability of the major phase I metabolites 3-carboxyindole-PB-22 and 3-carboxyindole-5F-PB-22 to activate the CB receptors did not differ significantly from basal levels. There was also no antagonistic activity observed for these two compounds (Figure 3.9), indicating that the 3-carboxyindole metabolites do not induce effects at the CB receptors. This suggests that the quinolin-8-yl moiety is crucial for CB receptor binding, just as the naphthoyl group is important for the naphthoylindoles through its aromatic stacking with the receptor[61].

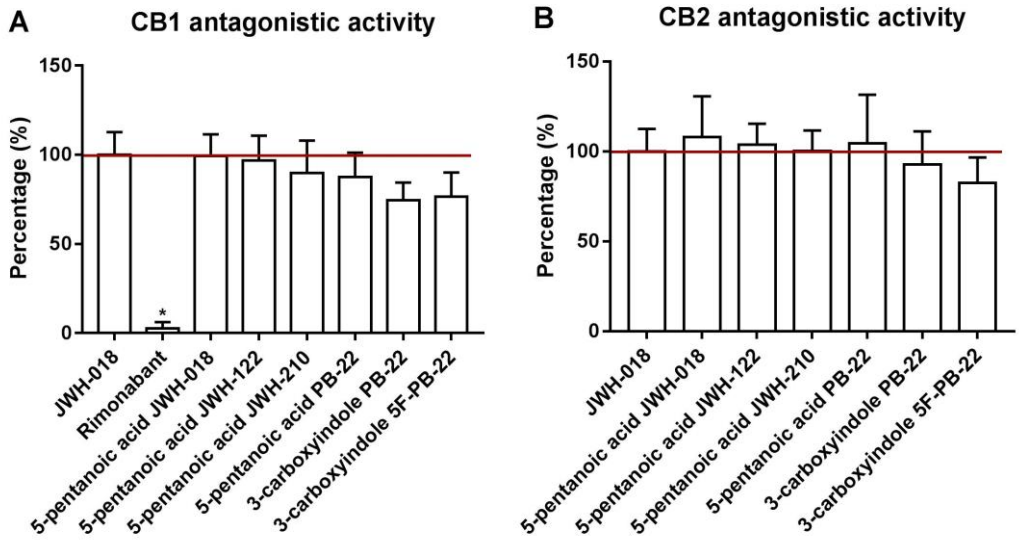


Figure 3.9. Evaluation of antagonistic properties of several SCRA metabolites. The cells were stimulated with 1 µM of the test compound and were incubated for 5 min before adding a fixed concentration of JWH-018 (ED_{80} concentration). The bar 'JWH-018' marks the response when no antagonist is present. The bar 'Rimonabant' was taken as a positive control for CB1 antagonism. Bars assigned with an (*) are not significantly different from basal levels. All test compounds did not show antagonistic activity.

The second most abundant metabolites (the 4- and 5-OH-pentyl-PB-22 metabolites) showed CB1 receptor activation, which was significantly lower in comparison to that of PB-22, but it was still significantly higher than that induced by our reference compound, JWH-018 (208.0 and 171.0%, respectively). Although PB-22, 5F-PB-22, and the hydroxylated metabolites yielded higher levels of CB2 receptor activation, this activation did not significantly differ from the reference compound, JWH-018. The N-pentanoic acid PB-22 metabolite did show a significant activation at the CB2 receptor (43.5%), just as N-pentanoic acid JWH-210, but unexpectedly, it also showed a significant activation of the CB1 receptor compared to basal levels (25.8%), although this activation remained significantly lower than that induced by JWH-018 and PB-22.

In conclusion, at the evaluated concentration, all hydroxylated metabolites of JWH-122, JWH-210, and PB-22 and their 5-fluoro analogues yielded a similar or sometimes even a significantly higher signal at the CB receptors than the reference agonist, JWH-018, suggesting a functional relevance for these compounds. The two N-pentanoic acid metabolites of JWH-210 and PB-22 consistently showed activity at one or both CB receptors. This was not expected based on literature data for the N-pentanoic acid metabolites of JWH-018 and JWH-073[17-18, 20]. It is known that SCRAs quickly reach maximum concentrations in blood upon use. A high serum concentration of JWH-018 was reached upon smoking[62]. These compounds can have very long terminal half-lives (in the range of several days), as has been demonstrated in heavy, chronic users, due to extensive distribution in deeper tissue[63]. A small oral single-dose self-administration pharmacokinetic study showed that the serum concentration of the metabolites exceeded that of the parent compound (AM-2201 in that case) at all-time points (1.5–21 h), suggesting a combination of slow resorption and a fast metabolic transformation upon oral uptake[64]. Although little is known about the concentrations and half-lives of the metabolites in the blood of users, the presence of active metabolites may prolong a compound's psychotropic and physiological effects, thereby contributing to its toxicity profile.

3.3.4 Application of the CB Reporter Assays on Natural Cannabinoids (Δ^9THC and CBD) and the Main Phase I Metabolites of Δ^9THC

In addition to SCRAs, the natural cannabinoids Δ^9THC and cannabidiol (CBD), as well as the major phase I metabolites of Δ^9THC, 11-OH-THC, and THCCOOH, were evaluated in the CB reporter assay, all at 1 µM concentration (corresponding to 314.5, 314.5, 330.5, and 344.4 ng/mL, respectively). Δ^9THC levels in urine are typically quite low (with 30 ng/mL corresponding to a very high concentration in recent or heavy users). Urinary concentrations of 11-OH-THC and THCCOOH (free + conjugated) can reach up to a few hundred or a few thousand ng/mL, respectively, in cases with heavy or recent use of cannabis[65-67].

Taking as reference the signal obtained with 1 µM JWH-018, no or only low-level activation was seen with the natural cannabinoids (Figure 3.10). THCCOOH shows no activation at both receptors, either at 1 (Figure 3.10) or 10 µM (3144 ng/mL; data not shown), in line with expectations. Δ^9THC shows some activation at CB1 at 1 µM, but it does not show any activation at CB2. 11-OH-THC shows a low level of activation at 1 µM at both receptors, which is more pronounced for CB1 compared to that for CB2. For CBD, which is found only at low concentrations in the urine of cannabis users (typically below 10 ng/mL)[65], we found no activity at both receptors at a concentration of 1 µM (data not shown). However, if oral/oromucosal CBD is administered, (very) high concentrations may be obtained in the urine[68-69], which may influence the signal obtained in our reporter assay.

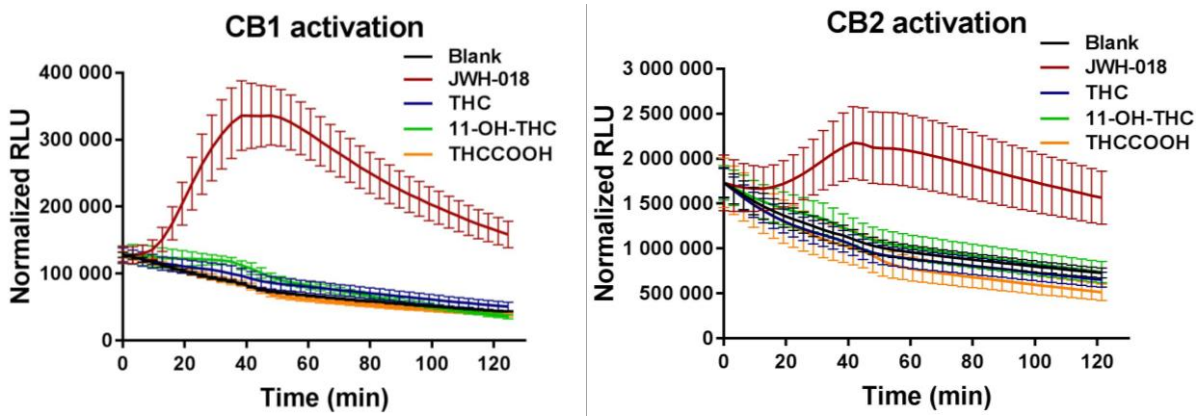


Figure 3.10. Activation profiles at CB1 **(A)** and CB2 **(B)** for JWH-018 (red), Δ^9THC (blue), 11-OH-THC (green), and THCCOOH (orange) at a concentration of 1 µM. Data are given as normalized relative light units (RLU) ± SEM (*n* = 4, except for JWH-018 for CB2 where *n* = 2).

3.3.5 Application of the CB Reporter Assay as a First-Line Screening Tool in Urine: Proof of Concept

A promising future application of the newly developed CB reporter assay may be its deployment as a first-line screening tool, complementing targeted and untargeted analytical assays and/or preceding analytical (mass spectrometry based) confirmation. However, the low concentrations of SCRAs in biological fluids require high-sensitivity bioassays capable of monitoring low-nanomolar or subnanomolar (ng/mL) levels of SCRAs. We therefore analyzed urine samples of two separate individuals, spiked with one of the two major metabolites of JWH-210, 4- and 5-OH-pentyl-JWH-210, at a concentration of 2 ng/mL (5.2 nM). The signals obtained for the spiked urine samples could be distinguished from the blanks in both the CB1 and CB2 bioassays (Figure 3.11A–D).

Next, we analyzed three separate blank urine samples and a genuine urine sample from a user who had consumed a mixture of JWH-018, JWH-122, and JWH-210 (Figure 3.11E and F). These urine samples were split in two parts. One part was subjected to a semi-quantitative

LC-MS/MS method, and the other part was evaluated in our newly developed bioassay. LC-MS/MS analysis confirmed the presence of metabolites of JWH-018, JWH-122, and JWH-210 at low- or subnanomolar (ng/mL) levels, thereby confirming the intake of the aforementioned SCRAs (Table 3.7). Evaluation of the extract with our bioassay resulted in a signal that could clearly be distinguished from the three control urine extracts, both for CB1 and CB2 receptors (Figure 3.11E and F). Although this result clearly needs to be extended using a larger panel of authentic samples (which is beyond the scope of the current study), it indicates the applicability of the CB reporter assay in the context of screening biological matrices.

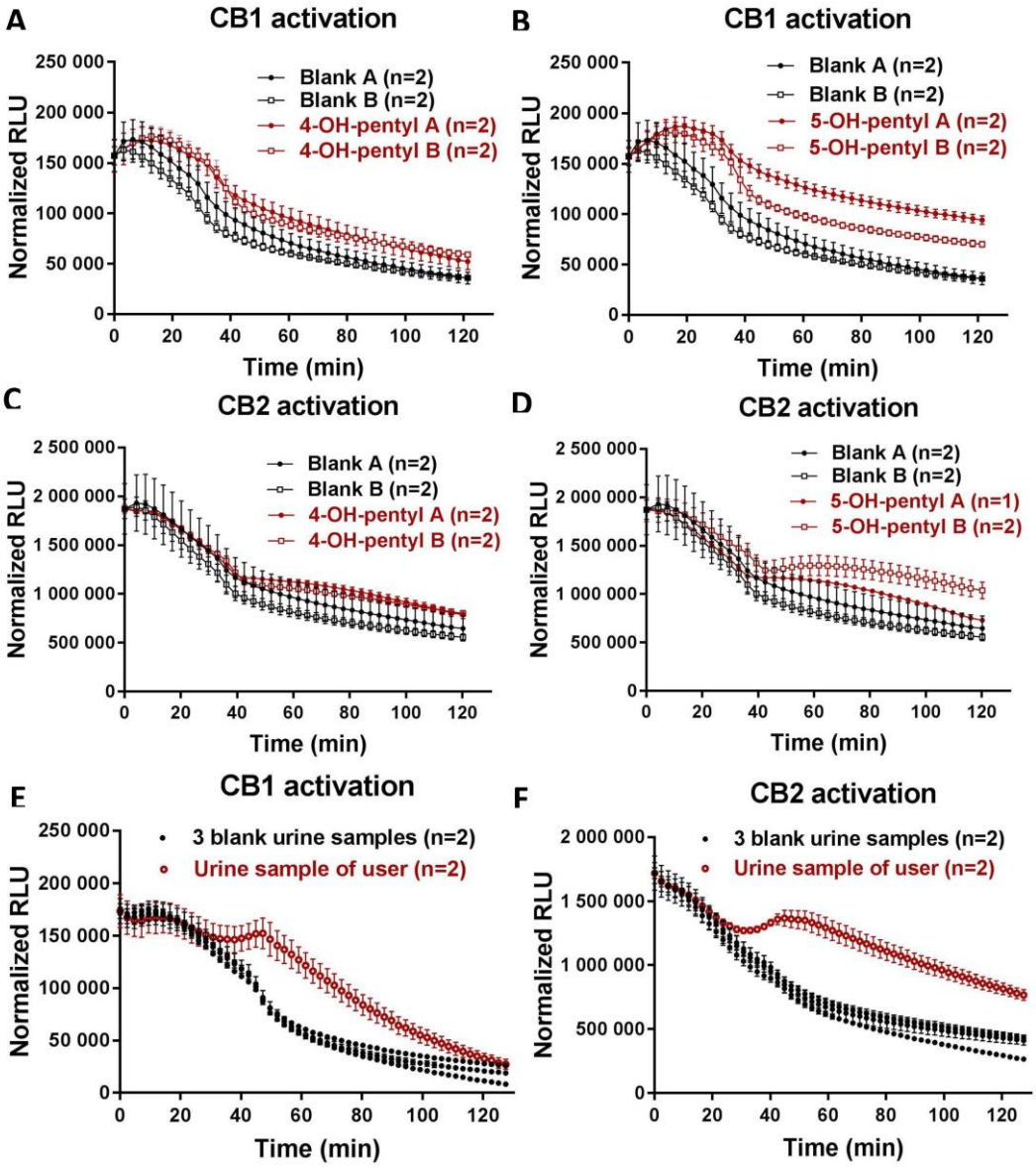


Figure 3.11. (A–D) Analyses of blank and spiked urine samples (2 ng/mL) of two separate individuals. Shown are the normalized raw data of the activation profiles obtained for CB1 (A, B) and CB2 (C, D) for 4-OH-pentyl-JWH-210 (A, C) and 5-OH-pentyl-JWH-210 (B, D). **(E, F)** Analyses of three separate blank urine samples and a urine sample from a SCRA user via the CB reporter assay for CB1 (E) and CB2 (F). Data are given as normalized relative light units (RLU) ± SEM.

Table 3.7. Semi-quantitative analysis of urine sample of a SCRA user via LC-ESI-MS/MS analysis.

Drug	Metabolite	Semi-quantitative result
JWH-018	4-OH-pentyl	1.96 nM (0.7 ng/ml)
	5-OH-pentyl	1.12 nM (0.4 ng/ml)
JWH-122	4-OH-pentyl	0.19 nM (0.07 ng/ml)
	5-OH-pentyl	<0.03 nM (<0.01 ng/ml)
JWH-210	4-OH-pentyl	3.46 nM (1.4 ng/ml)
	5-OH-pentyl	0.26 nM (0.1 ng/ml)
	5-OH-indole	0.16 nM (0.06 ng/ml)

3.4 Conclusion

We successfully developed a CB receptor activation assay based on NanoLuc binary technology. This assay combines several advantages over other CB receptor activation assays as it is a relatively simple, nonradioactive, sensitive, and homogeneous method, only requiring basic cell culture equipment and a standard luminometer. CB receptor activation is measured proximal to the receptor, which is known to reduce the incidence of false positives. The developed assay allows real-time monitoring of receptor activation as the measurement starts from the moment the test compound is added (which is in contrast to the commercially available PathHunter assay). The system is also flexible, as the CB receptors can be easily replaced by another GPCR. The newly developed bioassay was applied to determine the *in vitro* activity of several SCRAs and their metabolites. We observed that several major metabolites retain their activity at CB receptors. The high potency and efficacy of SCRAs, coupled with their metabolism to a number of highly active metabolites, might help to explain the distinct adverse clinical manifestations that have been observed with SCRA use.

When considering CB receptors, the developed bioassay may be used for structure–activity relationship (SAR) studies, but importantly, it may also be useful as newer NPS legislations start to implement “activity” (possibly expressed as potency and/or efficacy) rather than the identity of the drug or its chemical structure. For example, in 2012, the USA implemented a legislation that essentially illegalized all SCRAs as the new law broadly covers any material or mixture that contains any amount of “cannabimimetic” agents, their salts, isomers, or salts of isomers[70]. Similarly, in the UK, a new law on “legal highs” has been implemented since May 2016[71]. The Psychoactive Substances Act differs from the established approach to drug control under the Misuse of Drugs Act 1971 as it covers substances by virtue of their psychoactive properties, as defined by the Advisory Council on the Misuse of Drugs. Herein, the definition of a substance producing a psychoactive effect includes *“...(a substance) which produces a response in in vitro tests qualitatively identical to substances controlled under the*

Misuse of Drugs Act 1971..."[72]. One of the receptors included in these *in vitro* tests, to demonstrate "psychoactivity" for the purposes of the Psychoactive Substances Act, is the CB1 receptor[70]. It is clear that solely deducing activity from SAR studies will inevitably lead to discussions that can be efficiently countered by the developed bioassay.

Our data indicate that the newly developed CB reporter assay detects CB receptor activation by extracts of biological matrices in which SCRAs (or metabolites) are present at low- or subnanomolar levels of SCRAs. In the future, this may allow its deployment as a first-line screening tool, complementing targeted and untargeted analytical assays and/or preceding analytical (mass spectrometry based) confirmation. Although the SCRAs (and metabolites) tested here, as well as other SCRAs (and corresponding metabolites, unpublished observations) were found to be active, we cannot exclude at this point that there may be SCRAs for which the major phase I metabolites are inactive. Moreover, in cases where there is a considerable delay between use and sampling, with only trace levels remaining present, these trace levels may not be sufficient to generate a signal in our bioassay. However, it should be mentioned that the latter also holds true for analytical assays. For natural cannabinoids, we cannot exclude that very high urinary levels of Δ^9THC and/or 11-OH-THC may give rise to a positive result after recent or heavy cannabis use, which is not surprising as we screen for CB activity. In addition, oral or oromucosal use of products containing high CBD levels may influence the signal obtained in our CB reporter assay. However, in both of the above-mentioned scenarios, we expect that this will not pose a problem, as recent or heavy use of natural cannabinoids will be easily picked up by conventional screening assays.

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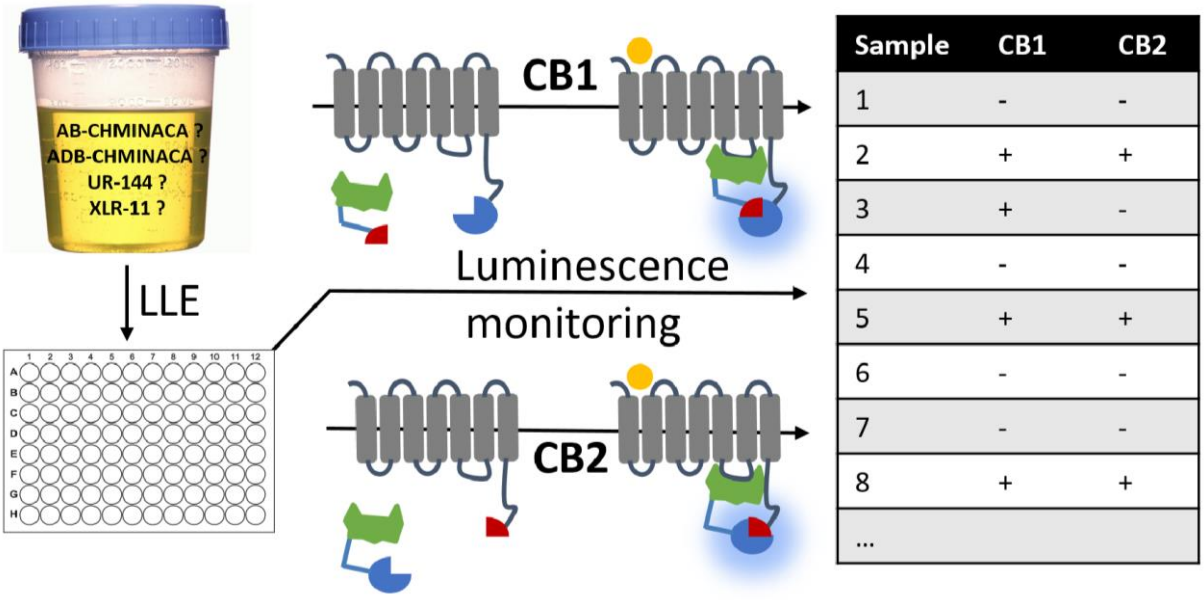
CHAPTER 4: ACTIVITY-BASED DETECTION OF CONSUMPTION OF SYNTHETIC CANNABINOID RECEPTOR AGONISTS IN AUTHENTIC URINE SAMPLES USING A STABLE CANNABINOID REPORTER SYSTEM

Based on

Annelies Cannaert, Florian Franz, Volker Auwärter, Christophe P. Stove. Activity-based Detection of Consumption of Synthetic Cannabinoids in Authentic Urine Samples using a Stable Cannabinoid Reporter System. *Analytical Chemistry* **2017** 89 (17), 9527-9536

Abstract

Synthetic cannabinoids receptor agonists (SCRAs) 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 SCRAs 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 SCRAs, 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 SCRA, 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 SCRAs (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 SCRAs 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 SCRAs 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 SCRAs (or their metabolites) are present at low- or subnanomolar (ng/mL) level. Hence, the developed assays do not only allow activity profiling of SCRAs and their metabolites, it may also serve as a screening tool, complementing targeted and untargeted analytical assays and preceding analytical (mass spectrometry based) confirmation.



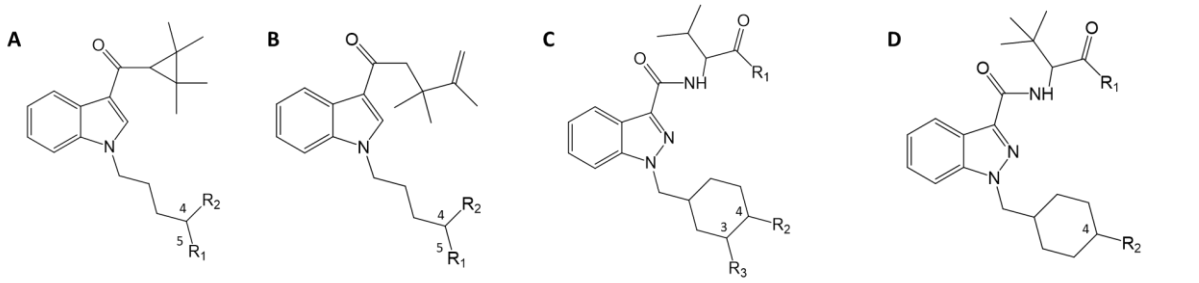
Graphical abstract of Chapter 4

4.1 Introduction

Synthetic cannabinoids receptor agonists (SCRAs) continue to be the largest group of new psychoactive substances (NPS) monitored by the European Monitoring Center of Drugs and Drugs of Abuse (EMCDDA)[1]. These "legal" alternatives for cannabis were first reported in 2008, at the time containing JWH-018 and CP 47,497-C8[2-3]. Many novel SCRAs 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 SCRAs 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 SCRAs poses worldwide a continuous challenge for scientists, healthcare workers, and lawmakers[4-6].

The identification and subsequent prohibition of single SCRAs has driven clandestine chemists to produce analogues of increasing structural diversity, intended to evade legislation[6-8]. Legislations based on individual structures are consequently stepping behind, but the newer analogue laws in the US (2012)[9] and UK (2016)[10] controlling all "cannabimimetic" agents and substances with psychoactive properties (e.g., via the CB1 receptor) are also challenged by the specific pharmacology of these new compounds being widely unknown[11]. 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 SCRAs, also poses a big challenge for the conventional targeted analytical assays, as it is difficult to screen for "unknown" compounds[6, 8, 12]. Although untargeted methods (e.g., high resolution mass spectrometry and GC-MS) are capable to screen for unknown substances, these methods have limitations in capacity and sensitivity. Immunoassays based on specific antibodies are of limited use because of missing cross-reactivity and insufficient sensitivity[13]. Therefore, alternative screening methods not directly based on the structure of the SCRA 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 SCRAs in biological fluids requires high sensitivity bioassays, capable of monitoring low- or subnanomolar (ng/mL) concentrations of SCRAs. Moreover, the presence of active metabolites is a prerequisite if the screening tool is to be applied on urine samples, as SCRAs are extensively metabolized[14]. 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 (see Chapter 3)[15-20]. In Chapter 3, we also reported on novel cell

based CB reporter bioassays for the activity-based detection of SCRAs and their metabolites, demonstrating cannabinoid activity in an authentic urine sample as a proof-of-concept[18]. The principle of this cell based bioassays is activity-based, where activation of the CB1 or CB2 receptor leads to β-arrestin 2 (βarr2) 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 interexperiment variability (depending on the transfection efficiency). Second, only a limited set of SCRAs (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 SCRAs (UR-144, XLR-11, and their thermal degradation products; AB-CHMINACA and ADB-CHMINACA) and their major phase I metabolites.



Drug/*Metabolite*	R_1	R_2
UR-144	CH_3	H
4-OH-pentyl-UR-144	CH_3	OH
5-OH-pentyl-UR-144	CH_2OH	H
N-pentanoic acid UR-144	COOH	H
XLR-11	CH_2F	H
4-OH-pentyl-XLR-11	CH_2F	OH

Drug/*Metabolite*	R_1	R_2
UR-144 degradant	CH_3	H
N-pentanoic acid UR-144 degradant	COOH	H
XLR-11 degradant	CH_2F	H

Drug/*Metabolite*	R_1	R_2	R_3
AB-CHMINACA	NH_2	H	H
4-OH-AB-CHMINACA (M1A)	NH_2	OH	H
3-OH-AB-CHMINACA (M1B)	NH_2	H	OH
valine-AB-CHMINACA (M2)	OH	H	H
4-OH-valine AB-CHMINACA (M3A)	OH	OH	H

Drug/*Metabolite*	R_1	R_2
ADB-CHMINACA	NH_2	H
4-OH-ADB-CHMINACA (M1)	NH_2	OH
valine-ADB-CHMINACA (M2)	OH	H
4-OH-valine ADB-CHMINACA (M3)	OH	OH

Figure 4.1. Structures of SCRAs and metabolites. SCRAs 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.

UR-144 and its 5-fluoro analogue, XLR-11, belong to the tetramethylcyclopropyl indolyl ketone family (see Figure 4.1A and B). 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[21-24]. AB-CHMINACA and ADB-CHMINACA are part of a particularly prevalent class of SCRAs, first described in a Pfizer patent[25]. Their structure comprises 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 4.1C and D). AB-CHMINACA was formally reported to the EMCDDA in April 2014, following identification in

Latvia[26], and was later detected in various countries all over the world[27-28]. ADB-CHMINACA was first reported in September 2014 in Hungary[26]. The use of AB-CHMINACA and ADB-CHMINACA was implicated in clinical reports of acute delirium, agitation, seizures, respiratory failure, and death[24, 29-32]. For most of the metabolites of these SCRAs, there is no information on their cannabinoid receptor activities. As it was demonstrated that several SCRAs are metabolized to a number of highly active metabolites[15-20], 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 SCRAs in urine.

4.2 Materials and Methods

4.2.1 Chemicals and Reagents

Dulbecco's modified eagle's medium (DMEM), Opti-MEM® I Reduced Serum Medium, penicillin/streptomycin (10,000 IU/ml and 10,000 µg/ml), amphotericin B (250 µg/ml), glutamine (200 mM), the restriction enzymes *Bam*HI, *Eco*RI and *Not*I, and the DNA polymerase (Phusion polymerase, a polymerase with proofreading activity) were purchased from Thermo Fisher Scientific (Pittsburg, PA, USA). Primers were procured from Eurofins Genomics (Ebersberg, Germany). UR-144 ((1-pentyl-1H-indol-3-yl(2,2,3,3-tetramethylcyclopropyl)-methanone), XLR-11 ((1-(5-fluoropentyl)-1H-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)-methanone), AB-CHMINACA (N-[(2S)-1-amino-3-methyl-1-oxobutan-2-yl]-1-(cyclohexyl-methyl)indazole-3-carboxamide), ADB-CHMINACA (N-[1-amino-3,3-dimethyl-1-oxobutan-2-yl]-1-(cyclohexylmethyl)indazole-3-carboxamide), and their metabolites as well as all internal standards (5F-AMB, FUB-PB-22, JWH-007-D9, JWH-018_4-OH-pentyl-D5, JWH-081_5-OH-pentyl-D5, JWH-122_5-OH-pentyl-D5, JWH-200-D5, MAM-2201-D5, UR-144_5-OH-pentyl-D5, UR-144_pentanoic acid-D5, UR-144-D5, XLR-11-D5) were supplied by Cayman Chemical (Ann Arbor, MI, USA). Formic acid (Rotipuran® ≥ 98%, p.a.), potassium hydrogen phosphate (≥ 99%, p.a.), and 2-propanol (Rotisolv® ≥ 99.95%, LC-MS-Grade) were obtained from Carl Roth (Karlsruhe, Germany). Acetonitrile (HPLC Gradient Grade) was from J.T. Baker (Deventer, The Netherlands). Fetal bovine serum (FBS), poly-D-lysine, ammonium formate 10 M (BioUltra) and potassium hydroxide (puriss. p.a. ≥ 86% (T) pellets) were from Sigma Aldrich (Steinheim, Germany). Roche Diagnostics (Mannheim, Germany) supplied the β-glucuronidase (E. coli K 12). Deionized water was prepared using a Medica® Pro deionizer from ELGA (Celle, Germany). Blank urine samples were donated by volunteers and tested for the absence of SCRAs and their metabolites prior to use. Mobile

phase A (1% acetonitrile, 0.1% formic acid, and 2 mM 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.

4.2.2 Retroviral Constructs

The CB1–LgBiT, CB2–SmBiT, SmBiT−βarr2, and LgBiT−βarr2 expression vectors were generated as described in Chapter 3[18]. To generate the retroviral vectors, specific primers were used to PCR-amplify the coding sequence of interest (see Table 4.1), flanked by *Bam*HI/*Eco*RI (for CB1−LgBiT and CB2−SmBiT) or *Bam*HI/*Not*I restriction sites (SmBiT−βarr2 and LgBiT−βarr2). PCR was performed on 100 pg of plasmid DNA using the Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, Pittsburg, PA, USA) according to the manufacturer's instructions. Reactions were done in a Mastercycler™ Nexus Thermal Cycler (Eppendorf, Hamburg, Germany) with an initial denaturation at 98°C for 30s, 35 cycles of 98°C for 10s (denaturation), T_m for 30s (annealing), and 72°C for 1 min (elongation), followed by a final extension at 72°C for 5 min. The resulting amplification products were purified using E.Z.N.A.® MicroElute Cycle-Pure kit (VWR International, Radnor, PA, USA).

Table 4.1. Primers used to clone the insert in the retroviral vector. Six extra nucleotides precede the restriction site (underlined). The Kozak sequence or stop codon (bold) were also marked. The nucleotides in italics are the coding sequences of the insert.

Retroviral vector	**Insert**	**Primers**		**T_m (°C)**
pLZRS-IRES-EGFP	CB1−LgBiT	Forward	ACTCAA GGATCC ***ACC*** *ATGAAGTCGATCC*	69.6
		Reverse	ACTCAA GAATTC **TTA** GCTGTTGATGGTTACTCGG	
	CB2−SmBiT	Forward	ACTCAA GGATCC ***ACC*** *ATGGAGGAATGCTG*	71.1
		Reverse	ACTCAA GAATTC **TTA** CAGAATCTCCTCGAACAGCC	
pLZRS-IRES-dNGFR	SmBiT-βarr2	Forward	ACTCAA *GGATCC* **ACC** ATGGTCTTCACACTCG	72.5
		Reverse	ACTCAA GCGGCCGC ***TCA*** *GCAGAGTTGATCATCATAGTCG*	
	LgBiT−βarr2	Forward	ACTCAA *GGATCC* ***ACC*** ATGGTGACCGGCTACCGGC	76.1
		Reverse	ACTCAA GCGGCCGC ***TCA*** *GCAGAGTTGATCATCATAGTCG*	

Both the retroviral vector and the amplification products were digested with either *Bam*HI/*Eco*RI or *Bam*HI/*Not*I restriction enzymes (Thermo Fisher Scientific) and purified using E.Z.N.A.® MicroElute Gel Extraction kit (VWR International). The digested PCR products were ligated into the corresponding dephosphorylated (TSAP Thermosensitive Alkaline Phosphatase, Promega), digested vector (see Table 4.1). After transformation of One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli* (Thermo Fisher Scientific) with the ligated product, the ampicillin-resistant clones were screened by PCR using primers complementary to sequences within the insert and sequences of the vector surrounding the

insert. Positively screened colonies were grown and used for plasmid isolation, using E.Z.N.A.® Plasmid DNA Mini kit (VWR International). The integrity of all retroviral plasmids was confirmed by DNA sequencing. This yielded four retroviral vectors, each of which leads to coexpression 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 βarr2-constructs. These markers (EGFP and dNGFR) can be used for cell sorting and to check the stability of the cell lines by flow cytometry.

4.2.3 Production of Retrovirus and Retroviral Transduction

The Phoenix-Amphotropic packaging cell line[33] (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-(βarr2-insert)-IRES-dNGFR plasmids, by using calcium phosphate precipitation (Invitrogen, San Diego, CA, USA). After 2 weeks of puromycin selection, the retroviral supernatant was harvested, spun (10 min at 350 × *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 10^4 cells/well. After 24 h, the medium was changed for the retroviral supernatant, which had been preincubated for 10 min with Dotap (Roche Diagnostics). The cells were cotransduced with viruses containing both CB and βarr2 constructs by mixing the respective retrovirus containing supernatants. To increase transduction efficiency, cells were spun (90 min, 950 × g, 32 °C). Transduction efficiency was evaluated by flow cytometry 48 h after transduction, via assessment of expression of EGFP (for CB1-LgBiT and CB2-SmBiT) and dNGFR (for SmBiT-βarr2 and LgBiT-βarr2). For the latter, an allophycocyanin (APC)-linked antibody against dNGFR was used (Chromaprobe, Inc.).

4.2.4 Cell Sorting and Cell Culture

Cell sorting was done on a BD FACSAria III, equipped with 405, 488, 561, 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−βarr2 or CB2–SmBiT/LgBiT−βarr2. All cells were routinely maintained at 37 °C, 5% CO_2, 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 µg/mL of streptomycin, and 0.25 µg/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 × 10^4 cells/well and incubated overnight.

3.2.5 Cannabinoid Reporter Assay

The cells were washed twice with Opti-MEM I Reduced Serum Medium to remove any remaining FBS, and 100 µL 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× using Nano-Glo LCS Dilution buffer, and 25 µL 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 µL per well of test compounds, present as 13.5× 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.

4.2.6 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) ± 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_{50} (a measure of potency). To evaluate the activity of the different SCRAs and their metabolites, results are represented as the percentage (%) CB activation (relative to the maximum receptor activation of JWH-018) ± 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 Figure 4.2). 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.

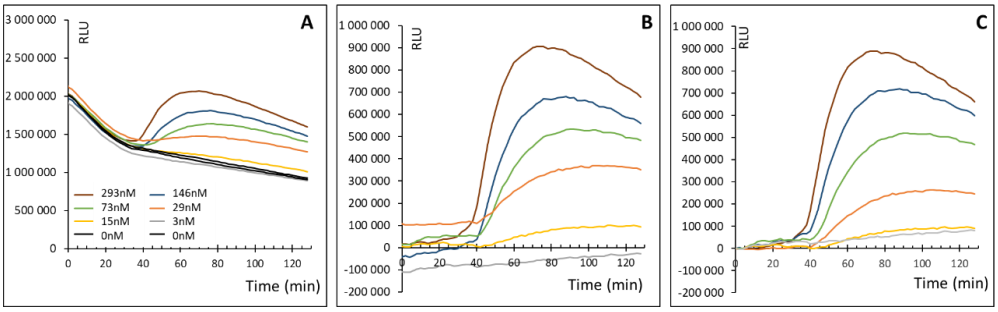


Figure 4.2. (A) Absolute raw signals (colored lines) were baseline-corrected by subtracting the average of the signals from vehicle control samples (black lines) **(B)**. Next, the signals were corrected for the inter-well variability by forcing the curve through 0 **(C)**.

4.2.7 Urine Sample Preparation

Conjugate cleavage was conducted by adding 0.5 mL of phosphate buffer (pH 6) and 30 µL of β-glucuronidase to 0.5 mL of urine, followed by 1 h incubation at 45 °C. Afterward, 1.5 mL of ice-cold acetonitrile and 0.5 mL of 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 µL of Opti-MEM I/MeOH (50/50, v/v), of which 10 µL 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 µL 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).

4.2.8 LC-ESI-MS/MS Analysis of Urine Samples

Quantification of SCRAs and their metabolites in authentic urine samples was performed by Florian Franz at the University of Freiburg 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[34], while the optimized MS parameters for each compound are listed in Table 4.2. The method validation was conducted in accordance to the guidelines of the German speaking Society of Toxicological and Forensic Chemistry (GTFCh)[35]. All validation data are summarized in Addendum (at the end of this Chapter). 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%, interday precision was below 11%, and intraday 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*.[36]

Table 4.2. Mass spectrometric parameters of the LC-ESI-MS/MS confirmation method and assignment of the internal standards used for quantification.

ID	Dwell time [msec]	Q1 [*m/z*]	Q3 [*m/z*]	DP [V]	EP [V]	CE [V]	CXP [V]	Internal Standard
AB-CHMINACA	20	357	241	75	10	37	8	JWH-122 5-OH-pentyl-D_5
	20	357	312	75	10	23	12	
AB-CHMINACA M1A	20	373	328	75	10	23	12	JWH-200-D_5
	20	373	257	75	10	35	10	
AB-CHMINACA M1B	20	373	328	75	10	23	12	JWH-200-D_5
	20	373	356	75	10	35	14	
AB-CHMINACA M2	20	358	241	75	10	31	10	FUB-PB-22
	20	358	145	75	10	49	16	
AB-CHMINACA M3A	20	374	239	70	10	33	10	JWH-200-D_5
	20	374	145	70	10	55	18	
ADB-CHMINACA	20	371	241	70	10	39	4	5F-AMB
	20	371	326	70	10	25	12	
ADB-CHMINACA M1	20	387	257	70	10	37	10	JWH-200-D_5
	20	387	342	70	10	25	12	
ADB-CHMINACA M2	20	372	241	70	10	33	10	FUB-PB-22
	20	372	145	70	10	51	4	
ADB-CHMINACA M3	20	388	257	70	10	33	10	JWH-200-D_5
	20	388	342	70	10	23	12	
UR-144	20	312	125	90	10	29	4	UR-144-D_5
	20	312	97	90	10	39	15	
UR-144 degradant	20	312	214	90	10	29	8	JWH-007-D_9
	20	312	144	90	10	49	18	
UR-144 degradant pentanoic acid	20	342	244	90	10	31	10	JWH-018 4-OH-pentyl-D_5
	20	342	144	90	10	47	18	
UR-144 4-OH-pentyl	20	328	125	90	10	27	4	UR-144 5-OH-pentyl-D_5
	20	328	97	90	10	39	15	
UR-144 5-OH-pentyl	20	328	125	90	10	27	4	UR-144 5-OH-pentyl-D_5
	20	328	97	90	10	39	15	
UR-144 pentanoic acid	20	342	125	90	10	29	4	UR-144 pentanoic acid-D_5
	20	342	97	90	10	39	15	
XLR-11	20	330	125	90	10	31	16	XLR-11-D_5
	20	330	232	90	10	37	10	
XLR-11 degradant	20	330	232	90	10	33	8	MAM-2201-D_5
	20	330	144	90	10	51	16	
XLR-11 4-OH-pentyl	20	346	248	90	10	31	10	JWH-081 5-OH-pentyl-D_5
	20	346	144	90	10	47	18	
XLR-11 6-OH-indole	20	346	248	90	10	31	4	5F-AMB
	20	346	160	90	10	50	18	
5F-AMB	20	364	233	70	10	31	8	
FUB-PB-22	20	397	252	70	10	23	10	
JWH-007-D_9	20	365	155	70	10	35	20	
JWH-018 4-OH-pentyl-D_5	20	363	155	70	10	25	11	
JWH-081 5-OH-pentyl-D_5	20	393	185	70	10	37	24	
JWH-122 5-OH-pentyl-D_5	20	377	169	70	10	31	22	
JWH-200-D_5	20	390	155	70	10	25	16	
MAM-2201-D_5	20	379	169	70	10	37	20	
UR-144 5-OH-pentyl-D_5	20	333	125	90	10	27	14	
UR-144 pentanoic acid-D_5	20	347	125	90	10	29	14	
UR-144-D_5	20	317	125	90	10	31	16	
XLR-11-D_5	20	335	125	90	10	31	16	

Q1 *m/z* of the precursor ion, Q2 *m/z* of the fragment ion, DP declustering potential, EP entrance potential, CE collision energy, CXP collision cell exit potential.

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%).

4.3 Results and Discussion

4.3.1 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 β-arrestin 2 (βarr2). Upon CB activation, the cytosolic βarr2 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.

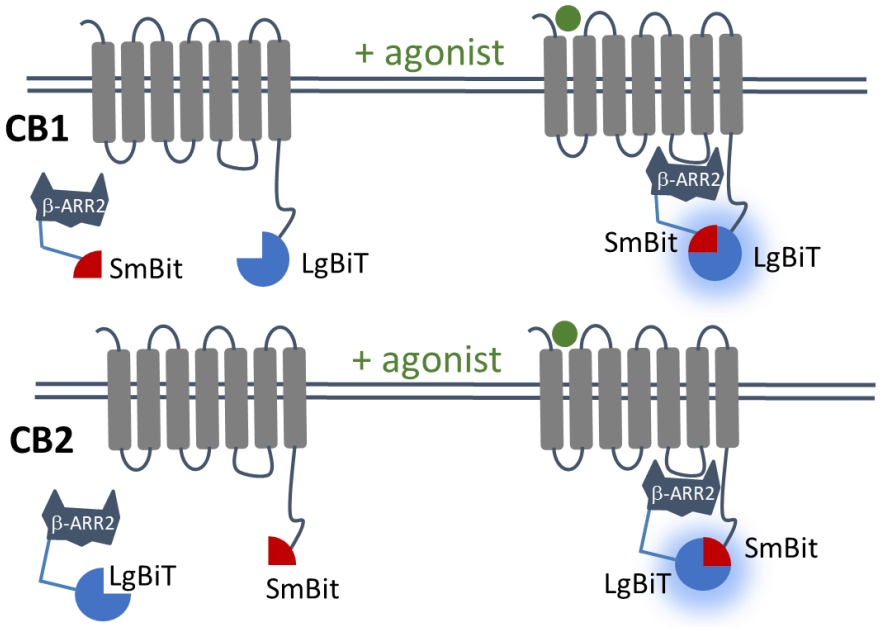


Figure 4.3. Setup of the CB reporter assays for CB1 and CB2: CB1–LgBiT/SmBiT–βarr2 and CB2–SmBiT/LgBiT–βarr2.

In Chapter 3, we set up and applied this reporter system in a transient format in which cells were transiently transfected, demonstrating applicability using a limited panel of SCRAs and providing a proof-of-principle for one authentic urine sample[18]. Here, we report on the establishment of two stable cell lines, either expressing the fusion proteins CB1–LgBiT/SmBiT−βarr2 or CB2–SmBiT/LgBiT−βarr2 (see Figure 4.3). These cell lines were obtained following retroviral transduction of HEK293T cells and flow cytometry-assisted cell sorting, to yield cell lines coexpressing the CB1 or CB2 construct with a βarr2 construct, with a purity of ≥ 93%. Via flow cytometric analysis of the coexpressed markers EGFP and dNGFR, the stability of these cell lines can be monitored in time (see Figure 4.5 and 4.6). 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 ≥70% (Figure 4.5 and 4.6). Up to this point, we did not notice a measurable effect on our systems' performance. Yet, if deemed necessary, the stably coexpressed markers always offer the possibility to submit the cell lines to another round of cell sorting.

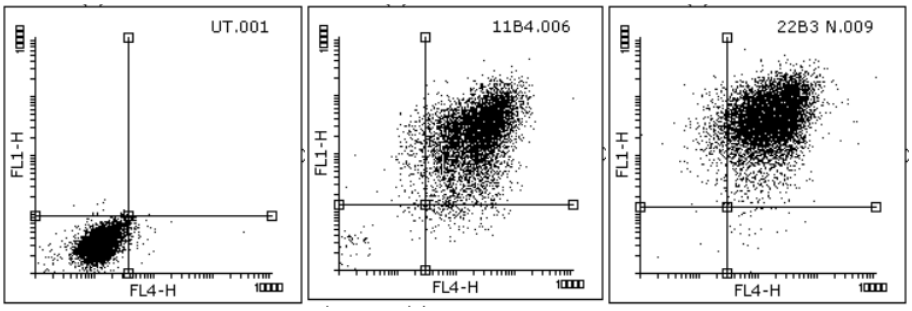


Figure 4.4. From left to right: untransduced cells, CB1 stable cell line and CB2 stable cell line. In the Y-axis, the level of EGFP (FL1-H) is shown. This fluorescent protein is a marker for the expression of the receptor construct. The X-axis (FL4-H) shows the level of the fluorophore APC (allophycocyanin), which is a marker for the expression of the βarr2 construct. The βarr2-construct is co-expressed with dNGFR, which can be visualized with an APC-linked antibody against dNGFR. Untransduced cells are double negative (bottom left quadrant). After retroviral transduction and cell sorting, ≥ 93% of the cells is double positive (upper right quadrant), meaning they express the receptor construct, as well as the βarr2 construct.

<u>Evolution over time for CB1-LgBiT/SmBiT-βarr2 stable cell line:</u>

93% at first passage. 87% at 12th passage. 81% at 15th passage. 70% at 20th passage.

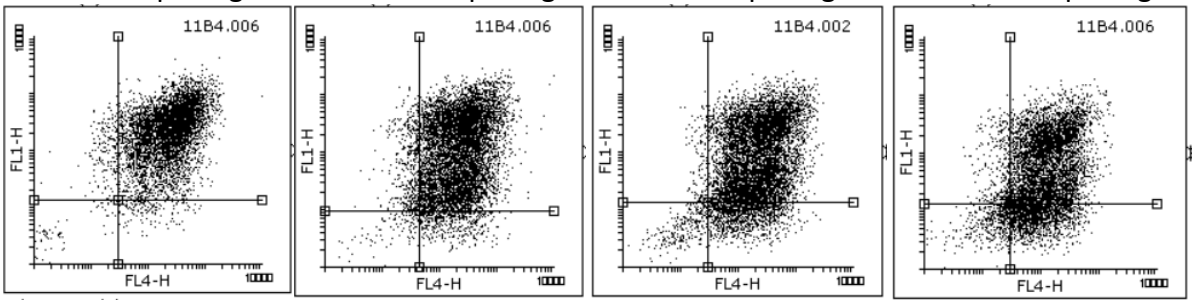


Figure 4.5. Evolution of stability of the cell line over time.

Evolution over time for CB2-SmBiT/LgBiT-βarr2 stable cell line

95% at first passage. 89% at 12th passage. 88% at 15th passage. 81% at 20th passage.

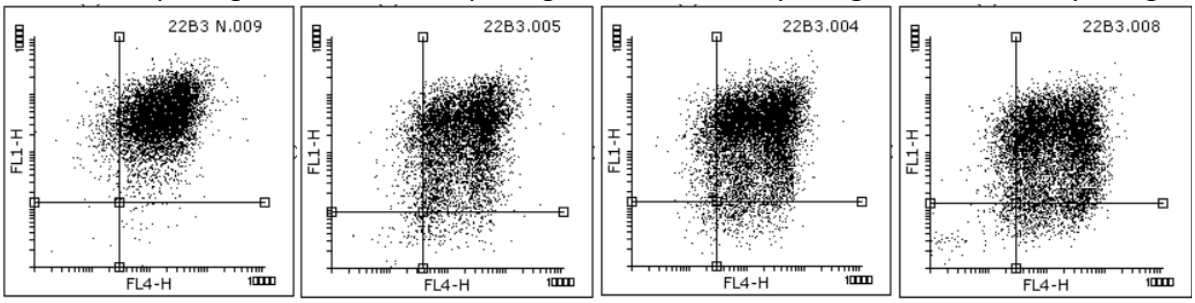


Figure 4.6. Evolution of stability of the cell line over time.

Upon stimulation of the stable systems with a known agonist, JWH-018, CB1–LgBiT, and CB2–SmBiT showed a concentration-dependent interaction with SmBiT−βarr2 and LgBiT−βarr2, 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 in Chapter 3 (CB1, 38.2 nM (95% CI = 27.1–55.7); CB2, 12.8 nM (95% CI = 5.6–26.0))[15]. 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 4.7 and Table 4.3).

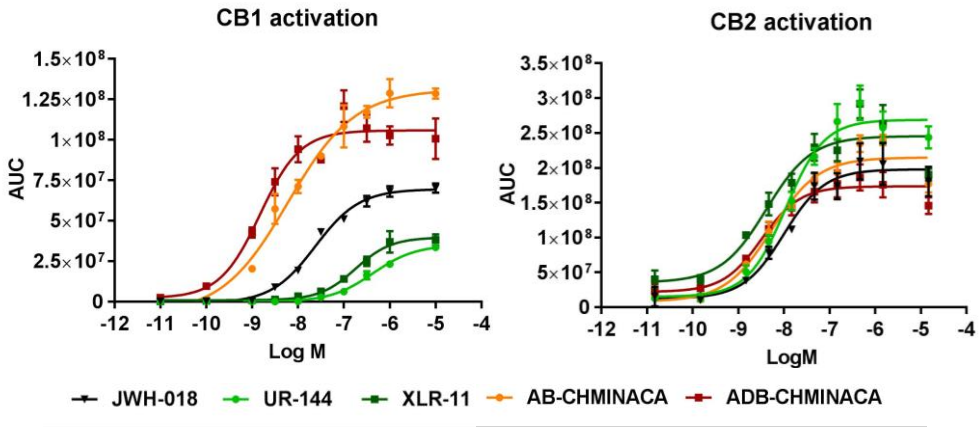


Figure 4.7. Concentration-dependent interaction of CB1 **(A)** and CB2 **(B)** with βarr2 upon stimulation with different SCRAs. AUC, area under the curve. Data are given as mean AUC ± SEM (*n* = 5–6).

Table 4.3. EC_{50} values of different SCRAs. EC_{50} values are presented as a measure of potency. Data are given as EC50 values (95% CI profile likelihood).

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)

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. For example, it is known that UR-144 and XLR-11 bind CB2 with a higher affinity than CB1[37-39]. 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[37]. 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[12]. 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 SCRAs, which is consistent with the low EC_{50} values reported in literature, varying from 0.278 to 7.8 nM and 21 nM (for AB-CHMINACA), for respectively CB1 and CB2[12, 25, 40-41]. 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.* (GTPγS 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)[22]. For the efficacy in terms of βarr2 recruitment, we observed that both AB-CHMINACA and ADB-CHMINACA showed a stronger βarr2 recruitment at CB1 than JWH-018, a known full agonist at CB1, an observation we also made for other SCRAs in Chapter 3, such as JWH-122, JWH-210, PB-22, and their 5-fluoro-analogues[18].

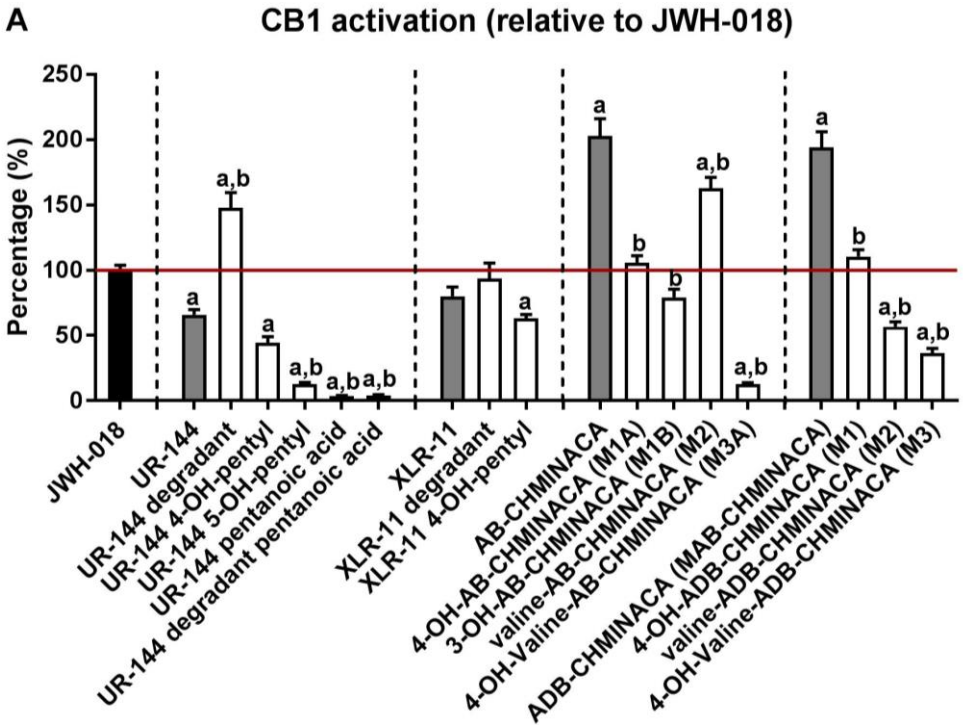
4.3.2 Application of the CB Reporter Assays on SCRAs and Their Main Phase I Metabolites

I. UR-144 and XLR-11

Biotransformation of UR-144 and XLR-11 (and their thermal degradant products, generated by smoking)[38, 42-43] 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)[44]. 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[38]. For the 5-OH-pentyl-UR-144 metabolite, there is also 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[15, 37]. 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 µM), with JWH-018 as a reference (Figure 4.8 and Table 4.4).

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 4.8). 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 (GTPγS binding assay in HEK293 cell membranes)[38], 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[38]. This difference could be related to the different experimental setup.



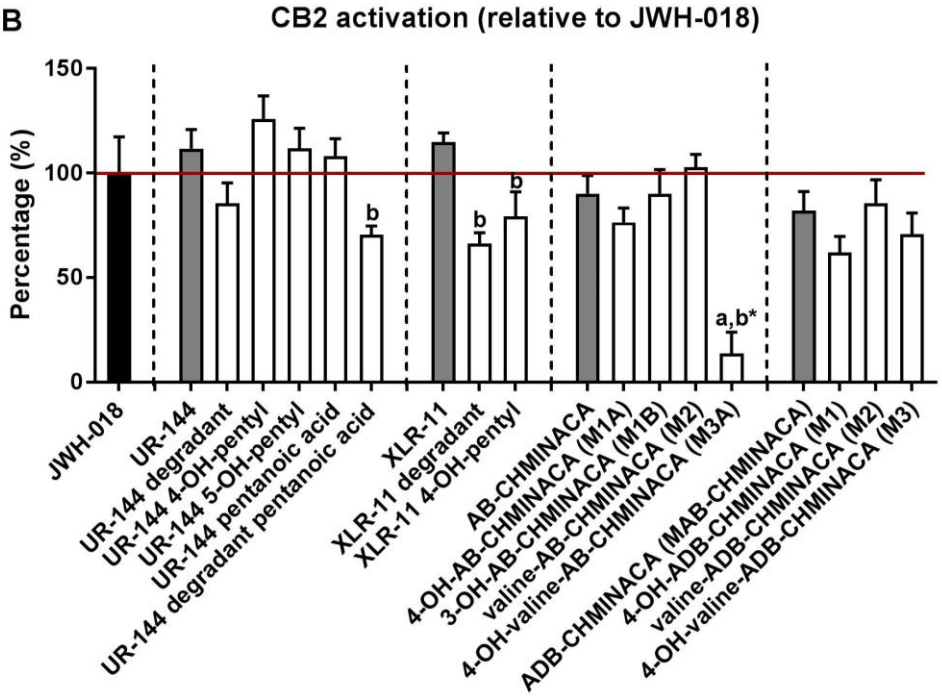


Figure 4.8. Activation of CB1 receptor **(A)** and CB2 receptor **(B)**. Values designated with (a) above error bars denote a significant difference from the reference compound, JWH-018 ($P \leq 0.05$, one-way ANOVA with Dunnett's multiple comparison post hoc test). Values designated with (b) are significantly 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) ± SEM (n = 4).

Table 4.4. Comparison of efficacy to activate CB1 and CB2 at 10 µM. The data are presented as the % CB activation (relative to the receptor activation of JWH-018) ± SEM (number of replicates).

Drug/metabolite	Relative efficacy of CB1 activation at 10 µM	Relative efficacy of CB2 activation at 10 µM
JWH-018	100 ± 4.6 (4)	100 ± 19.9 (4)
UR-144	65.9 ± 4.7 (4)	111.7 ± 10.5 (4)
UR-144 degradant	147.8 ± 13.4 (4)	85.6 ± 11.2 (4)
UR-144 4-OH-pentyl	44.5 ± 5.2 (4)	125.9 ± 12.7 (4)
UR-144 5-OH-pentyl	12.7 ± 1.3 (4)	111.8 ± 11.2 (4)
UR-144 pentanoic acid	3.58 ± 0.4 (4)	108.2 ± 9.6 (4)
UR-144 degradant pentanoic acid	4.01 ± 0.67 (4)	70.4 ± 4.9 (4)
XLR-11	80.1 ± 8.3 (4)	114.8 ± 5.0 (4)
XLR-11 degradant	93.7 ± 13.7 (4)	66.3 ± 6.0 (4)
XLR-11 4-OH-pentyl	63.2 ± 3.5 (4)	79.3 ± 13.6 (4)
AB-CHMINACA	202.9 ± 15.4 (4)	90.2 ± 10.0 (4)
4-OH-AB-CHMINACA (M1a)	105.8 ± 6.3 (4)	76.4 ± 8.0 (4)
3-OH-AB-CHMINACA (M1b)	79.2 ± 7.1 (4)	90.2 ± 13.3 (4)

valine-AB-CHMINACA (M2)	162.9 ± 9.5 (4)	102.9 ± 7.1 (4)
4-OH-valine-AB-CHMINACA (M3A)	12.9 ± 1.19 (4)	13.6 ± 10.3 (4)
ADB-CHMINACA	194.3 ± 13.7 (4)	82.1 ± 10.6 (4)
4-OH-AB-CHMINACA (M1)	110.5 ± 6.0 (4)	62.1 ± 8.67 (4)
valine-ADB-CHMINACA (M2)	56.9 ± 4.3 (4)	85.7 ± 12.8 (4)
4-OH-valine-ADB-CHMINACA (M3)	36.4 ± 4.3 (4)	70.9 ± 11.6 (4)

II. 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[32, 45-48]. The combination of both metabolites (monohydroxylation at cyclohexyl moiety and carboxylation at the outer amide) was also reported to be found in urine specimens (M3A and isomers)[31, 46]. 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. The valine-ADB-CHMINACA (M2) metabolite was also 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 metabolization[45]. 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)[48]. 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[45]. 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 µM), with JWH-018 as a reference (Figure 4.8, Table 4.4).

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. 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)[25]. 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.

4.3.3 Application of the CB Reporter Assays on authentic urine samples from SCRA 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 SCRA 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 4.5). From the 18 urine samples from users who had consumed either UR-144 or XLR-11, 17 were scored positive (94.4%) (Table 4.5A). The extract of the one sample that was missed, was strongly colored and contained low levels of XLR-11 metabolites (see Table 4.5A). 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. The pronounced coloration is not linked to the creatinine content of the urine sample, as can be seen in Table 4.5. From the 12 samples positive for AB-CHMINACA metabolites, only 4 were scored positive (33.3%) (Table 4.5B). This low detection rate was unexpected, as the activity profiling of the AB-CHMINACA metabolites (performed at 10 µM) 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 4.9, 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 4.9). 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.

Table 4.5. 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 level (*) is determined by most potent metabolite: +, <1 ng/mL; ++, 1–10 ng/mL; +++, 10–50 ng/mL, ++++, >50 ng/mL.

A

			UR-144 [ng/ml]				XLR-11 [ng/ml]	
SCRA	**Level***	**Creatinine [mg/dl]**	**4-OH-pentyl**	**5-OH-pentyl**	**pentanoic acid**	**Degradant pentanoic acid**	**4-OH-pentyl**	**Score in bioassays**
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		**8.14**	29.80	> 50	0.21	+
XLR-11	++	38		**5.14**	23.40	> 50	0.17	+
XLR-11	++	300		**4.64**	33.10	> 50	0.38	+
XLR-11	++	160		**4.27**	10.80	> 50	0.81	+
XLR-11	++	360		**4.11**	15.90	> 50	0.12	+
XLR-11	++	34		**2.92**	10.20	> 50	0.13	+
XLR-11	++	190		**2.26**	8.40	> 50	0.11	+
XLR-11	++	230		**1.28**	0.40	16.70		+
XLR-11	+	320		**0.99**	7.56	> 50		+
XLR-11	+	53		**0.86**	6.69	44.50		+
XLR-11	+	230		**0.36**	2.31	36.20		-
XLR-11	+	130		**0.31**	1.80	24.10		+
XLR-11	+	180		**0.21**	0.64	11.90		+

B

			AB-CHMINACA [ng/ml]					
SCRA	**Level***	**Creatinine [mg/dl]**	**Parent**	**M1A**	**M1B**	**M2**	**M3A**	**Score in bioassays**
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	**16.40**	8.95	-
AB-CHMINACA	+++	140		18.40	1.28	**16.00**	4.86	-
AB-CHMINACA	++	40		13.30	1.50	**8.41**	4.16	+
AB-CHMINACA	++	230		4.68	0.46	**6.32**	3.46	-
AB-CHMINACA	++	110		1.37	0.43	**1.45**	1.32	-
AB-CHMINACA	++	50		1.27	0.10	**1.37**	0.42	-
AB-CHMINACA	++	54		1.54	0.15	**1.06**	0.62	+
AB-CHMINACA	+	150		4.42	0.37	**0.95**	0.53	-

C

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

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. Nine out of 11 (81.8%) urine samples from users who had consumed ADB-CHMINACA were scored positive (Table 4.5C). The two missed cases both contained lower concentrations of ADB-CHMINACA metabolites (approximately 2.5 ng/mL of the major metabolite M1), one also being strongly colored, resulting in a drop of signal. The unknown blank was scored correctly negative (not shown in Table 4.5). Overall, this leads to a sensitivity of 73.2% (30/41) for the first batch of urine samples.

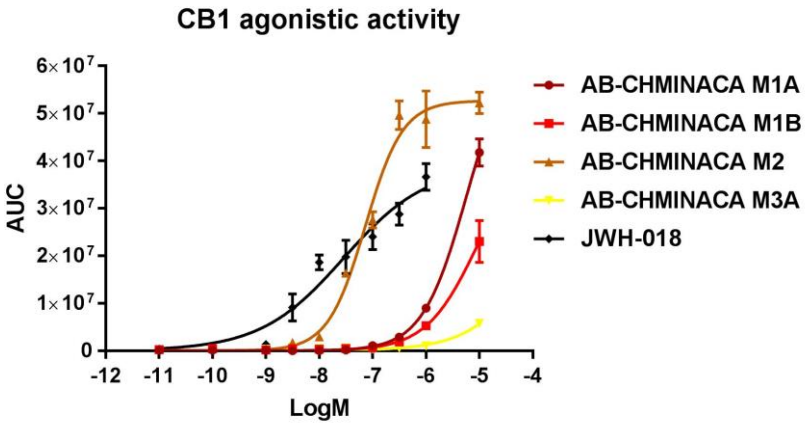


Figure 4.9. Concentration-dependent interaction of CB1 with βarr2 upon stimulation with the major phase I metabolites of AB-CHMINACA. AUC, area under the curve. Data are given as mean AUC ± SEM (*n* = 4–6).

The second batch contained 32 urine samples (8 SCRA positive and 24 SCRA negative samples) and was analyzed along with 4 known blanks (Table 4.6). The SCRA 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 4.6B). 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 4.6A). The sensitivity results are linked to the type of SCRAs included in the batch of analyzed urine samples. Other SCRAs can give different sensitivity rates. From the 24 SCRA negative urine samples, 19 were scored negative. Among the 5 positively scored SCRA negative samples, three authentic urine samples contained THC–COOH (concentrations of other cannabis-related substances unknown), demonstrating use of natural cannabinoids. Although we confirmed that THC–COOH does not possess any detectable cannabinoid activity[18] (see also spiked THC–COOH sample 15 in Table 4.6B), 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 SCRA negative samples, no explanation could be found for the positive scoring. Additional screening with the ToxTyper approach[49] did also 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.

Table 4.6. List of authentic urine samples from users of UR-144/XLR-11, AB-CHMINACA, or ADB-CHMINACA **(A)** and the SCRA negative urine samples **(B)**. The intensity of the color of the extract is shown by the different shades of gray. In **(A)** the level (*) is determined by most potent metabolite: +, <1 ng/mL; ++, 1–10 ng/mL; +++, 10–50 ng/mL, ++++, >50 ng/mL. In **(B)** the asterix (*) indicates that the concentrations of other cannabis-related substances are unknown.

A

			UR-144 [ng/ml]				XLR-11 [ng/ml]	
SCRA	**Level***	**Creatinine [mg/dl]**	**4-OH-pentyl**	**5-OH-pentyl**	**pentanoic acid**	**Degradant pentanoic acid**	**4-OH-pentyl**	**Score in bioassays**
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		**4.64**	33.10	> 50	0.38	+

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

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

B	**SCRA 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)*, benzoylecgonine (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 (+)	-

4.4 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 in Chapter 3, which were in a transient format[18], 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 coexpressed markers. The CB reporter assays were applied to determine the *in vitro* activity of a new set of SCRAs (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 SCRAs, 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 SCRAs. 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 SCRAs on a relatively large set of authentic urine samples. Given the continuous modifications to the SCRAs' 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 SCRAs in a panel of authentic urine samples, therefore circumventing the need to know the specific structure of the SCRA. Our data indicate that the stable CB reporter assays detect CB receptor activation by extracts of urine in which SCRAs (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 SCRAs 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 (see Chapter 5). 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[48]. 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. This is both an obvious and important limitation, which, according to our preliminary data, may be less an issue in serum/plasma samples, which contain primarily the parent compounds (see Chapter 5). 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 SCRA screening strategies have been demonstrated to have limited value, recognizing only clearly related structures, which is not surprising[13]. Therefore, we believe that our data do support the potential of deploying CB receptor activation assays as a first-line screening tool to detect SCRA use in urine samples, complementing targeted and untargeted analytical assays or preceding analytical (mass spectrometry based) confirmation.

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4.6 Addendum

Validation data of the LC-ESI-MS/MS confirmation method.

LOD	Limit of Detection
LOQ	Limit of Quantification
LLOQ	Lower Limit of Quantification
ULOQ	Upper Limit of Quantification

Analyte	LOD [ng/ml]	LLOQ = LOQ [ng/ml]	ULOQ [ng/ml]	Weighting	Regression	Accuracy [%] 0.07 [ng/ml]	0.40 [ng/ml]	4.00 [ng/ml]	20.00 [ng/ml]	40.00 [ng/ml]
AB-CHMINACA	0.01	0.05	10.00	$1/x^2$	linear	-2.20	-6.48	-6.20	x	x
AB-CHMINACA M1A	0.10	0.10	50.00	$1/x^2$	linear	x	-4.00	-2.86	2.44	-2.30
AB-CHMINACA M1B	0.10	0.10	50.00	$1/x^2$	linear	x	-0.30	-0.11	1.69	-6.50
AB-CHMINACA M2	0.01	0.05	50.00	$1/x^2$	linear	-1.30	-3.67	0.22	-5.50	0.05
AB-CHMINACA M3A	0.25	0.25	50.00	$1/x^2$	linear	x	3.20	2.41	6.72	8.30
ADB-CHMINACA	0.01	0.05	25.00	$1/x^2$	linear	-0.10	0.87	0.46	-3.79	x
ADB-CHMINACA M1	0.05	0.05	50.00	$1/x^2$	linear	-3.80	-7.48	-6.58	-3.70	-6.41
ADB-CHMINACA M2	0.01	0.05	25.00	$1/x^2$	linear	3.70	1.79	3.25	2.20	x
ADB-CHMINACA M3	0.25	0.25	50.00	$1/x^2$	linear	x	-4.60	-9.44	-4.38	-1.50
UR-144	0.05	0.05	10.00	$1/x$	linear	5.00	3.81	6.00	x	x
UR-144 degradant	0.01	0.05	5.00	$1/x^2$	linear	10.22	9.77	-8.02	x	x
UR-144 degradant pentanoic acid	0.05	0.05	10.00	$1/x^2$	linear	3.10	-1.38	2.30	x	x
UR-144 degradant pentanoic acid	0.05	0.05	50.00	$1/x^2$	quadratic	2.70	-2.84	2.78	4.50	-1.00
UR-144 4-OH-pentyl	0.01	0.05	10.00	$1/x^2$	linear	5.60	5.60	6.58	x	x
UR-144 5-OH-pentyl	0.05	0.05	10.00	$1/x^2$	linear	8.90	8.03	7.58	x	x
UR-144 pentanoic acid	0.10	0.10	25.00	$1/x^2$	linear	x	9.20	13.09	4.31	x
UR-144 pentanoic acid	0.10	0.10	50.00	$1/x^2$	quadratic	x	6.70	13.03	12.39	7.00
XLR-11	0.01	0.05	5.00	$1/x$	linear	0.60	-3.06	-2.58	x	x
XLR-11 degradant	0.01	0.05	5.00	$1/x^2$	linear	7.10	6.12	-7.61	x	x
XLR-11 4-OH-pentyl	0.01	0.05	5.00	$1/x^2$	linear	10.12	5.66	5.96	x	x

Analyte	Precision, inter-day [%]					Precision, intra-day [%]				
	0.07 [ng/ml]	0.40 [ng/ml]	4.00 [ng/ml]	20.00 [ng/ml]	40.00 [ng/ml]	0.07 [ng/ml]	0.40 [ng/ml]	4.00 [ng/ml]	20.00 [ng/ml]	40.00 [ng/ml]
AB-CHMINACA	9.11	6.38	6.11	x	x	8.63	3.50	2.26	x	x
AB-CHMINACA M1A	x	8.26	5.65	7.36	7.89	x	8.26	3.60	7.19	6.18
AB-CHMINACA M1B	x	6.68	6.21	6.50	6.61	x	6.68	4.10	6.50	6.61
AB-CHMINACA M2	7.72	5.96	7.60	8.77	6.50	2.36	5.85	2.24	8.68	4.10
AB-CHMINACA M3A	x	6.41	4.51	7.11	4.40	x	6.32	4.50	7.06	3.79
ADB-CHMINACA	10.03	7.68	7.59	8.08	x	6.31	7.46	2.75	6.15	x
ADB-CHMINACA M1	9.66	6.53	6.53	8.85	9.97	9.66	6.50	4.46	6.77	6.10
ADB-CHMINACA M2	5.74	6.48	7.13	10.48	x	5.72	6.10	3.32	5.37	x
ADB-CHMINACA M3	x	7.06	4.61	6.53	6.32	x	5.90	3.20	5.54	6.32
UR-144	8.06	6.14	6.53	x	x	8.06	5.00	3.37	x	X
UR-144 degradant	8.66	8.20	5.32	x	x	8.66	5.70	2.68	x	X
UR-144 degradant pentanoic acid	7.84	7.11	7.27	x	x	6.42	3.70	2.85	x	X
UR-144 degradant pentanoic acid	7.38	7.07	7.20	5.80	5.62	6.36	3.70	2.95	5.80	5.60
UR-144 4-OH-pentyl	7.64	6.74	5.52	x	x	3.14	4.10	2.66	x	X
UR-144 5-OH-pentyl	10.80	5.90	7.21	x	x	5.12	4.10	2.45	x	X
UR-144 pentanoic acid	x	5.69	5.43	6.44	x	x	4.29	2.60	4.33	X
UR-144 pentanoic acid	x	5.51	6.93	6.52	7.62	x	4.25	5.40	4.77	6.73
XLR-11	8.07	5.76	6.78	x	x	3.23	4.80	1.94	x	X
XLR-11 degradant	6.78	7.72	7.20	x	x	4.48	4.10	2.10	x	X
XLR-11 4-OH-pentyl	9.43	7.22	8.48	x	x	9.16	3.20	1.90	x	X

Analyte	Matrix effects [%]					Recovery [%]				
	0.07 [ng/ml]	0.40 [ng/ml]	4.00 [ng/ml]	20.00 [ng/ml]	40.00 [ng/ml]	0.07 [ng/ml]	0.40 [ng/ml]	4.00 [ng/ml]	20.00 [ng/ml]	40.00 [ng/ml]
AB-CHMINACA	213±35	147±17	151±18	x	x	49±6	94±3	91±2	x	X
AB-CHMINACA M1A	x	138±13	132±18	108±15	119±15	x	83±5	83±3	85±2	88±4
AB-CHMINACA M1B	x	128±16	133±16	109±13	119±9	x	84±3	81±2	85±3	82±3
AB-CHMINACA M2	176±30	100±3	104±3	88±4	101±2	27±4	83±3	83±3	87±2	91±1
AB-CHMINACA M3A	x	132±5	133±3	110±7	126±5	x	44±2	43±1	43±1	46±1
ADB-CHMINACA	147±17	102±6	106±6	89±4	x	43±5	89±3	87±4	93±5	X
ADB-CHMINACA M1	188±45	124±23	128±24	103±18	113±17	45±5	84±2	83±2	85±2	86±2
ADB-CHMINACA M2	151±18	98±4	107±6	89±6	x	41±5	86±4	83±2	85±3	X
ADB-CHMINACA M3	x	117±7	119±7	99±6	113±5	x	52±3	52±2	52±1	55±2
UR-144	144±14	101±4	106±4	x	x	44±5	87±7	86±4	x	X
UR-144 degradant	142±16	101±4	102±2	x	x	44±5	88±4	92±2	x	X
UR-144 degradant pentanoic acid	150±18	98±5	106±5	89±4	101±2	43±6	91±3	89±2	92±3	93±1
UR-144 4-OH-pentyl	148±18	102±3	103±3	x	x	44±6	88±2	89±3	x	X
UR-144 5-OH-pentyl	155±15	103±3	107±3	x	x	45±5	92±5	91±1	x	X
UR-144 pentanoic acid	x	97±4	100±3	87±5	100±1	x	90±2	89±1	91±3	93±1
XLR-11	141±11	97±6	104±2	x	x	45±5	90±6	90±2	x	X
XLR-11 degradant	144±15	97±4	102±2	x	x	44±4	91±3	93±1	x	X
XLR-11 4-OH-pentyl	162±23	106±8	112±9	x	x	43±4	93±3	91±1	x	X

CHAPTER 5:
AN IMPROVED ACTIVITY-BASED DETECTION METHOD OF CANNABINOIDS IN SERUM AND PLASMA SAMPLES

Based on

Annelies Cannaert, Jolien Storme, Cornelius Hess, Volker Auwärter, Sarah M.R Wille, Christophe P. Stove. Activity-based detection of cannabinoids in serum and plasma samples. *Clinical Chemistry* **2018**. In press. DOI: 10.1373/clinchem.2017.285361

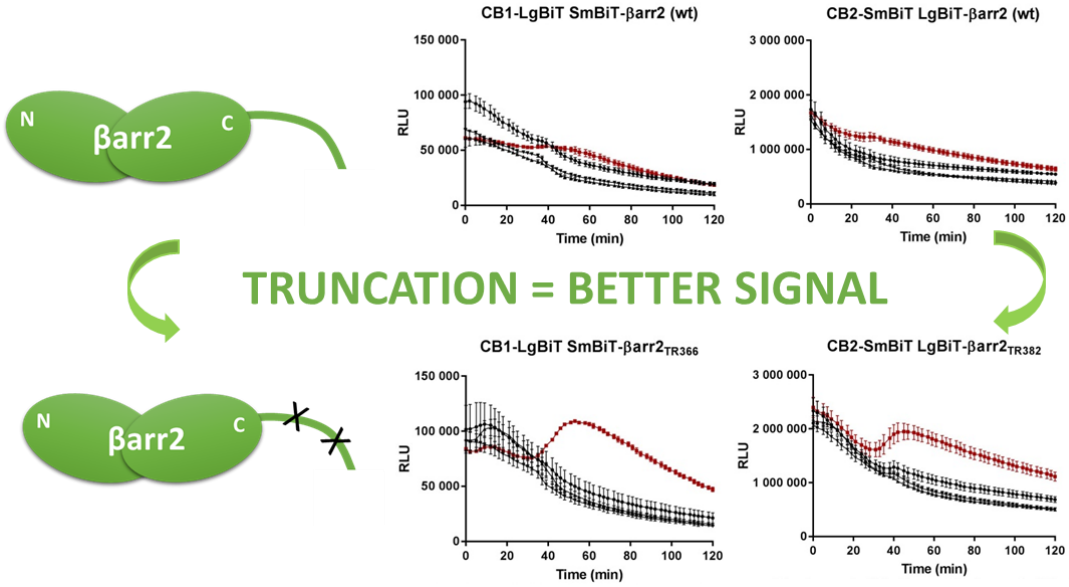
Abstract

Synthetic cannabinoids continue to be the largest group of new psychoactive substances monitored by the European Monitoring Centre of Drugs and Drug Addiction. The rapid proliferation of novel analogues makes the detection of these new derivatives challenging and has initiated considerable interest in the development of so-called 'untargeted' screening strategies to detect these compounds.

We developed new, stable bioassays in which cannabinoid receptor activation by cannabinoids lead to recruitment of truncated β-arrestin 2 (βarr2) to the cannabinoid receptors, resulting in functional complementation of a split luciferase, allowing read-out via bioluminescence. Aliquots (500 µl) of authentic serum (n = 45) and plasma (n = 73) samples were used for simple liquid-liquid extraction using hexane:ethyl acetate (99:1 v/v). Following evaporation and reconstitution in 100 µl of Opti-MEM® I/methanol (50/50 v/v), 10 µl of these extracts was analyzed in the bioassays.

Truncation of βarr2 significantly (P = 0.0034 and 0.0427 for both cannabinoid receptors, respectively, unpaired student's t-test) improved the analytical sensitivity over the previously published bioassays, applied on urine samples. The new bioassays detected cannabinoid receptor activation by authentic serum or plasma extracts, in which synthetic cannabinoids were present at low- or sub-ng/ml level or in which Δ^9-tetrahydrocannabinol was present at concentrations above 12 ng/ml. For synthetic cannabinoid detection, analytical sensitivity was 82%, with an analytical specificity of 100%.

The bioassays have the potential to serve as a first-line screening tool for (synthetic) cannabinoid activity in serum or plasma and may complement conventional analytical assays and/or precede analytical (mass spectrometry based) confirmation.



Graphical abstract of Chapter 5

5.1 Introduction

According to the latest European Drug Report, more than 620 new psychoactive substances (NPS) were reported to the Early Warning System of the European Monitoring Centre of Drugs and Drug Addiction, with the number of new analogs reported in the last 5 years comprising approximately 70% of the total number. The NPS market has been traditionally dominated by synthetic cannabinoid receptor agonists (SCRAs), with 169 analogs detected since 2008[1]. The SCRAs are designed to exert similar pharmacological effects as the traditional recreational drug cannabis but are intended to circumvent legislation[2-3]. The rapid proliferation of novel analogs makes the detection of these new derivatives challenging in different contexts, such as forensic, clinical and analytical chemistry[3-4]. Furthermore, the activity-based analog laws of the US[5] and UK[6] are challenged since the pharmacology of these new derivatives is often unknown. This could be efficiently countered by applying these new substances in activity-based biological assays to establish their cannabinoid activity and therefore their illegality.

The recent proliferation of NPS has initiated considerable interest in the development of so-called 'untargeted' screening strategies to detect and identify novel compounds without the use of certified reference materials or mass spectral libraries. High-resolution mass spectrometry (HRMS) has been the method of choice for broad screening of NPS in a wide range of contexts because of its ability to measure accurate masses using both data-dependent and data-independent acquisition[4]. However, due to the time-consuming and expensive character of this technique, this method is not routinely implemented in many clinical and forensic laboratories. Furthermore, the analytical sensitivity of HRMS configurations, often requiring a threshold to be reached, may preclude detection of SCRAs, which are often present at low- or sub-ng/ml concentrations in biological fluids. Moreover, SCRAs are strongly metabolized and the metabolism of novel SCRAs is often poorly characterized, which again may lead to these compounds being missed by HRMS. Therefore, alternative 'untargeted' screening methods, which are less expensive and more routinely applicable, may offer a solution for this problem. An activity-based bioassay may serve this purpose, by functioning as a first-line screening tool, complementing the conventional targeted and untargeted analytical methods.

In Chapter 3 and 4, we reported on novel cell-based cannabinoid (CB) reporter bioassays for the detection of SCRAs and their metabolites, demonstrating cannabinoid activity in authentic urine samples[7-8]. The principle of these bioassays is activity-based, where activation of the CB1 or CB2 receptor leads to β-arrestin 2 (βarr2) recruitment, which results in functional complementation of a split NanoLuc luciferase, thereby restoring luciferase

activity. In the presence of the substrate furimazine, this results in a bioluminescent signal, which can be read out with a standard luminometer. We initially applied our bioassays on urine samples because of *i*) the anticipated higher concentrations in urine, *ii*) the fact that many phase I SCRA metabolites retain activity at CB receptors, and *iii*) the combined presence of distinct active metabolites is likely to be beneficial for the assays' sensitivity. However, in some cases the analytical sensitivity in urine was not sufficient. For its application with serum or plasma samples even lower concentrations than in urine are expected, therefore we aimed at improving the analytical sensitivity of the bioassays.

Here, we present modified bioassays in which new stable cell lines were generated to evaluate whether the use of βarr2 C-terminal truncated mutants would improve the analytical sensitivity of the bioassays. The idea to truncate βarr2 was based upon its prominent role in G-protein coupled receptor (GPCR) desensitization and signaling. More specifically, the C-terminus was liberated following a conformational change upon βarr2-GPCR interaction and could subsequently interact with proteins of the endocytic machinery, driving GPCR internalization. Truncation of this C-terminus promotes the stability of the GPCR-βarr2 interaction (more details in Discussion). This approach resulted in new, improved stable cell systems, which were used to screen for cannabinoid activity in a set of authentic serum (n = 45) and plasma (n = 73) samples.

5.2 Materials and Methods

5.2.1 Chemicals and Reagents

Dulbecco's modified eagle's medium, Opti-MEM® I Reduced Serum Medium, penicillin/streptomycin (10,000 IU/ml and 10,000 µg/ml), amphotericin B (250 µg/ml), glutamine (200 mM), the restriction enzymes *Bam*HI, *Eco*RI and *Not*I, and the DNA polymerase (Phusion polymerase, a polymerase with proofreading activity) were purchased from Thermo Fisher Scientific (Pittsburg, PA, USA). Primers were procured from Eurofins Genomics (Ebersberg, Germany). Fetal bovine serum (FBS) and poly-D-lysine were from Sigma Aldrich (Overijse, Belgium). Roche Diagnostics (Mannheim, Germany) supplied the *β*-glucuronidase (*E. coli* K 12). Detailed information on chemical reagents used for processing urine[8], serum[9] and plasma[10] samples are described elsewhere.

5.2.2 Plasmids and constructs

The CB1−Large BiT (LgBiT), CB2−Small BiT (SmBiT), LgBiT−βarr2 and SmBiT−βarr2 expression vectors and the retroviral vectors pLZRS-CB1−LgBiT-IRES(Internal Ribosome Entry Site)-EGFP(Enhanced Green Fluorescent Protein), pLZRS-CB2−SmBiT-IRES-EGFP, pLZRS-LgBiT−βarr2-IRES-dNGFR(truncated Nerve Growth Factor Receptor) and pLZRS-SmBiT−βarr2-

IRES-dNGFR have been described in Chapter 3 and 4[8]. To generate the expression plasmids containing the truncated βarr2 forms and the retroviral vectors containing the truncated βarr2 fusion constructs, specific primers were used to PCR-amplify the coding sequence of interest, flanked by unique restriction sites (see Table 5.1 and Table 5.2), as described below. PCR was performed on 100 pg of plasmid DNA using the Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, Pittsburg, PA, USA) according to the manufacturer's instructions. Reactions were done in a Mastercycler™ Nexus Thermal Cycler (Eppendorf, Hamburg, Germany) with an initial denaturation at 98°C for 30s, 35 cycles of 98°C for 10s (denaturation), T_m for 30s (annealing), and 72°C for 1 min (elongation), followed by a final extension at 72°C for 5 min. The resulting amplification products were purified using E.Z.N.A.® MicroElute Cycle-Pure kit (VWR International, Radnor, PA, USA).

Table 5.1. Primers used to clone the protein of interest (POI) in the expression plasmids and to generate the retroviral vectors (pLZRS-(insert)-IRES-dNGFR). Six extra nucleotides precede the restriction site (underlined). In some primers, extra nucleotides were added to correct the reading frame. The Kozak sequence or stop codon (bold) were also added, if necessary. The nucleotides in italics are the coding sequences of the POI.

Vector	POI	Primers		T_m (°C)
NB MCS-3 NB MCS-4	βarr2Δ382	Forward	ACTCAA GAATTC A *ATGGGGGAGAAACCCGGGACC*	70.4
		Reverse	ACTCAA GAATTC **TCA** *TGTGGCATAGTTGGTATCAAATTC*	
	βarr2Δ366	Forward	ACTCAA GAATTC A *ATGGGGGAGAAACCCGGGACC*	73.7
		Reverse	ACTCAA GAATTC **TCA** *ATCTGTCTCCGGAGCGGC*	
pLZRS-IRES-dNGFR	LgBiT−βarr2 Δ382	Forward	ACTCAA *GGATCC* ***ACC*** ATGGTGACCGGCTACCGGC	75.1
		Reverse	ACTCAA *GCGGCCGC* **TCA** TGTGGCATAGTTGG	
	LgBiT−βarr2 Δ366	Forward	ACTCAA *GGATCC* ***ACC*** ATGGTGACCGGCTACCGGC	77.6
		Reverse	ACTCAA *GCGGCCGC* **TCA** ATCTGTCTCCGGAGC	
	SmBiT−βarr2 Δ382	Forward	ACTCAA *GGATCC* **ACC** ATGGTCTTCACACTCG	72.5
		Reverse	ACTCAA *GCGGCCGC* **TCA** TGTGGCATAGTTGG	
	SmBiT−βarr2 Δ366	Forward	ACTCAA *GGATCC* **ACC** ATGGTCTTCACACTCG	72.5
		Reverse	ACTCAA *GCGGCCGC* **TCA** ATCTGTCTCCGGAGC	

Both the vector and the amplification products were digested with *Eco*RI (for the expression plasmids) and *Bam*HI/*Not*I (for the retroviral vectors) restriction enzymes (Thermo Fisher Scientific) and purified using E.Z.N.A.® MicroElute Gel Extraction kit (VWR International). The digested PCR products were ligated into the corresponding dephosphorylated (TSAP Thermosensitive Alkaline Phosphatase, Promega), digested vector (see Table 5.2). After transformation of One Shot® Mach1™ T1 Phage-Resistant Chemically Competent E. coli (Thermo Fisher Scientific) with the ligated product, the ampicillin-resistant clones were screened by PCR using primers complementary to sequences within the insert and sequences of the vector surrounding the insert. The integrity of all constructs was confirmed by DNA sequencing. Positively screened colonies were grown and used for plasmid isolation,

using E.Z.N.A.® Plasmid DNA Mini kit (VWR International). This yielded retroviral vectors, each of which lead to co-expression of a gene of interest with either EGFP for the CB-constructs or truncated dNGFR for the βarr2-constructs. These markers (EGFP and dNGFR) could be used for cell sorting and to check the stability of the cell lines by flow cytometry. Production of retroviruses, retroviral transduction of HEK293T cells for stable cell line generation and cell sorting was performed as previously described[8].

Table 5.2. Lay-out of the expression and retroviral vectors and restriction enzymes used for each protein of interest (POI) or fusion protein.

Vector	Fusion protein	POI	Restriction enzyme
NB MCS-3	LgBiT - Linker - POI	βarr2Δ382	*Eco*RI
		βarr2Δ366	*Eco*RI
NB MCS-4	SmBiT - Linker - POI	βarr2Δ382	*Eco*RI
		βarr2Δ366	*Eco*RI
pLZRS-IRES-EGFP	LgBiT - βarr2Δ382	-	*Bam*HI/*Not*I
	LgBiT - βarr2Δ366	-	*Bam*HI/*Not*I
	SmBiT - βarr2Δ382	-	*Bam*HI/*Not*I
	SmBiT - βarr2Δ366	-	*Bam*HI/*Not*I

5.2.3 Cell Culture and Cannabinoid Reporter Assay

Cells were routinely maintained at 37°C, 5% CO_2, under humidified atmosphere in Dulbecco's modified eagle's medium (high glucose) supplemented with 10% heat-inactivated FBS, 2 mM of glutamine, 100 IU/ml of penicillin, 100 µg/ml of streptomycin and 0.25 µg/ml of amphotericin B. Stability of the cell lines was followed by flow cytometric analysis. For experiments, cells were plated on poly-D-lysine coated 96-well plates at 5×10^4 cells/well and incubated overnight. The cells were washed twice with Opti-MEM® I Reduced Serum Medium to remove any remaining FBS, and 100 µl of Opti-MEM® I was added. The Nano-Glo Live Cell reagent (Promega, Madison, WI, USA), a nonlytic detection reagent containing the cell permeable furimazine substrate, was prepared by diluting the Nano-Glo Live Cell substrate by 20-fold using Nano-Glo LCS Dilution buffer, and 25 µl was added to each well. Subsequently, the plate was placed in a GloMAX96 (Promega) luminometer. Luminescence was monitored during the equilibration period until the signal stabilized (30−45 min). For agonist experiments, we added 10 µl per well of test compounds, present as 13.5× stocks in 50% methanol in Opti-MEM® I. For analysis of biological extracts, evaporated extracts (prepared as described below) were reconstituted in 100 µl of Opti-MEM® I/methanol (50/50 v/v), of which 10 µl was added per well. The luminescence was continuously detected for 120 min. Solvent controls were run in all experiments; the final concentration of methanol (3.7%) did not interfere with the viability of the cells at short term or with the read-out of the bioassay.

5.2.4 Urine Sample Preparation and Analysis

Sample preparation of urine samples (500 µl) was performed as described before[8]. Briefly, after β-glucuronidase treatment, urine samples were extracted with ammonium formate and acetonitrile (salting-out assisted liquid-liquid extraction). After shaking and centrifugation, the organic layer was transferred into a separate vial and evaporated. A previously validated LC-MS/MS method[8] was applied for quantification of SCRAs and their metabolites by Florian Franz for the University of Freiburg.

5.2.5 Serum and Plasma Sample Preparation

To serum/plasma samples (500 µl, if available), 500 µl of carbonate buffer (pH 10) was added in a glass tube (16 x 100 mm). After adding 4 ml of n-hexane/ethyl acetate mixture (99/1 v/v), vortexing (1 min) and centrifugation (10 min at 2900 × g), the organic phase was transferred to another glass tube (16 x 100 mm) and evaporated at room temperature under a gentle stream of nitrogen[9-10].

5.2.6 Analysis of Serum Samples

The samples were analyzed using a previously published method for the determination of 93 synthetic cannabinoids in serum[9] by Cornelius Hess from the University of Bonn. A systematic toxicological analysis by immunochemical methods for cocaine and its metabolites, opioids, cannabinoids, amphetamine, methamphetamine, 3,4-methyleendioxymethamphetamine (MDMA) and derivatives, methadone, benzodiazepines, and tricyclic antidepressants followed by an unknown screening for pharmaceuticals by LC-MS/MS and an analysis for ethanol was conducted besides analysis of SCRAs[9].

5.2.7 Analysis of Plasma Samples

Sodium fluoride anticoagulated blood samples (4 ml; n = 76) were obtained from drivers that had a positive on-site oral fluid test (Drugwipe-5S®, Securetec, Germany), either during roadside controls or after minor accidents. The samples were transferred to the laboratory within 24 h. After centrifugation at 2200 × g to obtain plasma, the obtained samples were stored in Greiner Bio-One tubes (Frickengrasen, Germany) at −20°C until analysis. The target quantification of Δ^9-tetrahydrocannabinol (THC), cocaine, benzoylecgonine, amphetamine, MDMA and morphine was performed via published LC-MS/MS (positive electrospray ionization, multi reaction monitoring) methods[11-13]. In one case, the presence of the synthetic cannabinoid AB-FUBINACA (*N*-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-[(4-fluorophenyl)methyl]-1*H*-indazole-3-carboxamide) was confirmed via HRMS, as published recently[10] by Camille Richeval, Melodie Nachon-Phanithavong and Jean-michel Gaulier (University of Lille).

5.2.8 Statistical Analysis

Curve fitting and statistical analyses were performed using GraphPad Prism software (San Diego, CA, USA). To select the optimal configuration for the cannabinoid reporter assay for both CB receptors, the absolute signals were baseline-corrected by subtracting the blank samples. The results in Figure 5.3 are represented as mean area under the curve between 60-120 min ± the standard error of the mean (n = 2-3).

5.3 Results

5.3.1 Development of bioassays with improved sensitivity by using truncated βarr2 forms

The principle of the cannabinoid reporter assays is based on the interaction of the cannabinoid receptors, CB1 and CB2, with the cytosolic adapter protein β-arrestin 2 (βarr2). One of the two inactive subunits of NanoLuc luciferase, Large BiT (LgBiT; 18 kDa) and Small BiT (SmBiT; 1 kDa), is coupled to either βarr2 or to CB1 or CB2. Upon CB activation, the cytosolic βarr2 protein interacts with the receptor, thereby promoting structural complementation of the NanoLuc luciferase and restoring the luciferase activity, which generates a bioluminescent signal in the presence of the furimazine substrate (NanoLuc Binary Technology). For the CB1 receptor, the optimal combination with the wild type βarr2 was established to be CB1-LgBiT/SmBiT-βarr2. For the CB2 receptor, the optimal combination with the wild type βarr2 was found to be CB2-SmBiT/LgBiT-βarr2 (Figure 5.1)[7].

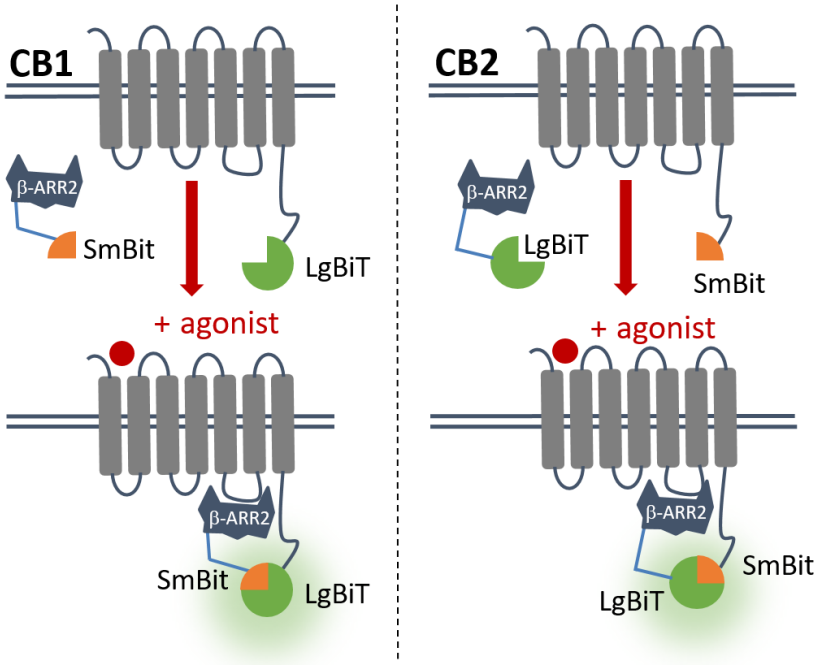


Figure 5.1. Setup of the CB reporter assays for CB1 and CB2: CB1−LgBiT/SmBiT−βarr2 and CB2−SmBiT/LgBiT−βarr2[8].

Two truncated βarr2 forms (βarr2$_{TR382}$ and βarr2$_{TR366}$) were compared to wild type to see if βarr2 recruitment improved upon addition of low concentrations of a known agonist, JWH-018 (Figure 5.2 and 5.3). For CB1, both SmBiT-βarr2$_{TR382}$ and SmBiT-βarr2$_{TR366}$ yielded higher signals, the signal for SmBiT-βarr2$_{TR366}$ being significantly higher than for wild type βarr2 (P = 0.0034, unpaired student's *t*-test, Figure 5.3). For CB2, LgBiT-βarr2$_{TR382}$ gave a significantly higher signal (P = 0.0427, unpaired student's *t*-test, Figure 5.3). Therefore, we selected the CB1-LgBiT SmBiT-βarr2$_{TR366}$ and CB2-SmBiT LgBiT-βarr2$_{TR382}$ combinations for further evaluation and application.

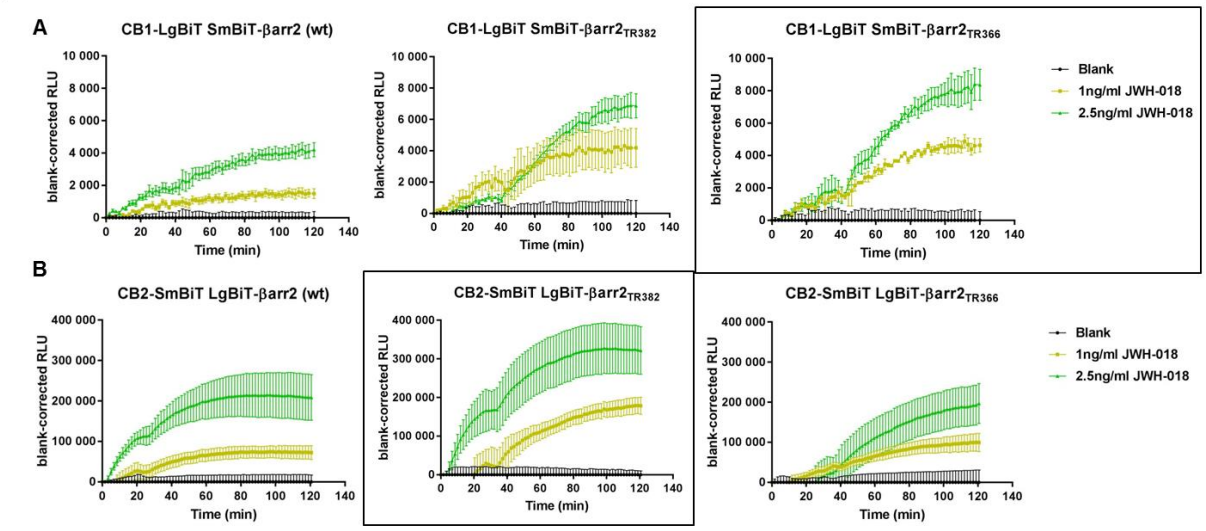


Figure 5.2. Evaluation of CB reporter assays for CB1 **(A)** and CB2 **(B)** with different βarr2 isoforms. A representative experiment is shown (n = 4). The chosen systems are boxed. RLU = Relative Light Units, mean ± the standard error of mean (n = 2-3).

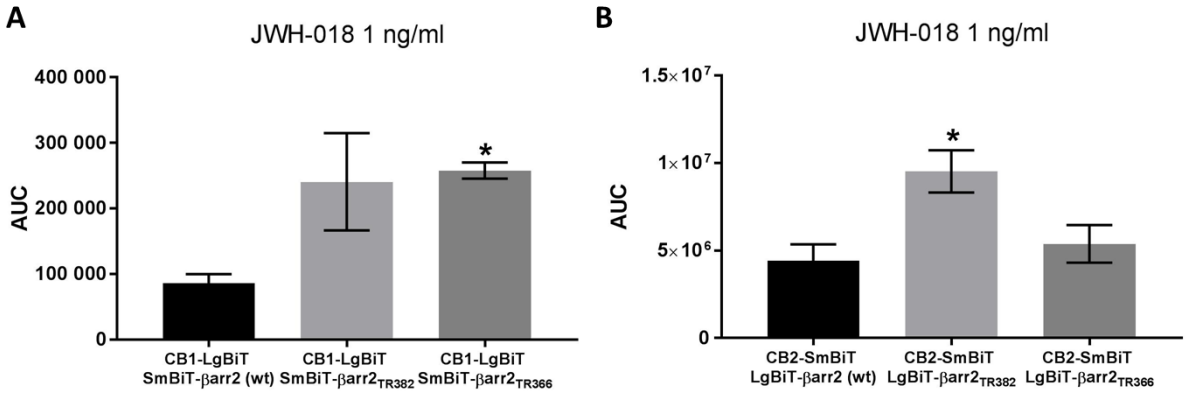


Figure 5.3. Evaluation of CB reporter assays for CB1 **(A)** and CB2 **(B)** with different βarr2 isoforms. The results are represented as mean area under the curve (AUC) between 60-120 min ± standard error of the mean (n = 2-3). Bars assigned with an (*) are significantly different from wild type (P = 0.0034 and 0.0427 for CB1 and CB2, respectively, unpaired student's *t*-test).

5.3.2 Application of CB bioassays with truncated βarr2 forms on batch of authentic urine samples

Extracts from a batch of 42 authentic urine samples were analyzed along with 4 known blanks on the previously established stable cell lines containing wild type βarr2[8] and the newly generated cell lines containing selected truncated forms of βarr2. Forty-one out of 42 urine samples was positive for SCRAs (at least one metabolite of UR-144 (1-pentyl-1*H*-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)-methanone), XLR-11 (1-(5-fluoropentyl)-1*H*-indol-3-yl)(2,2,3,3-tetramethylcyclopropyl)methanone), AB-CHMINACA (*N*-[(1S)-1-(aminocarbonyl)-2-methylpropyl]-1-(cyclohexylmethyl)-1*H*-indazole-3-carboxamide) or ADB-CHMINACA (*N*-(1-amino-3,3-dimethyl-1-oxobutan-2-yl)-1-(cyclohexylmethyl)-1*H*-indole-3-carboxamide)), analytically confirmed via LC-MS/MS (Table 5.3)[8].

Analysis of the urine samples with both the conventional (wild type βarr2) and the new (truncated βarr2) cell lines showed that the read-out of the truncated βarr2 cell lines allowed for an easier interpretation of the obtained signal (Figure 5.4). Importantly, in one case, a sample that was falsely scored negative in the assays using wild type βarr2 was correctly scored positive in the assays using the truncated βarr2 (Table 5.3).

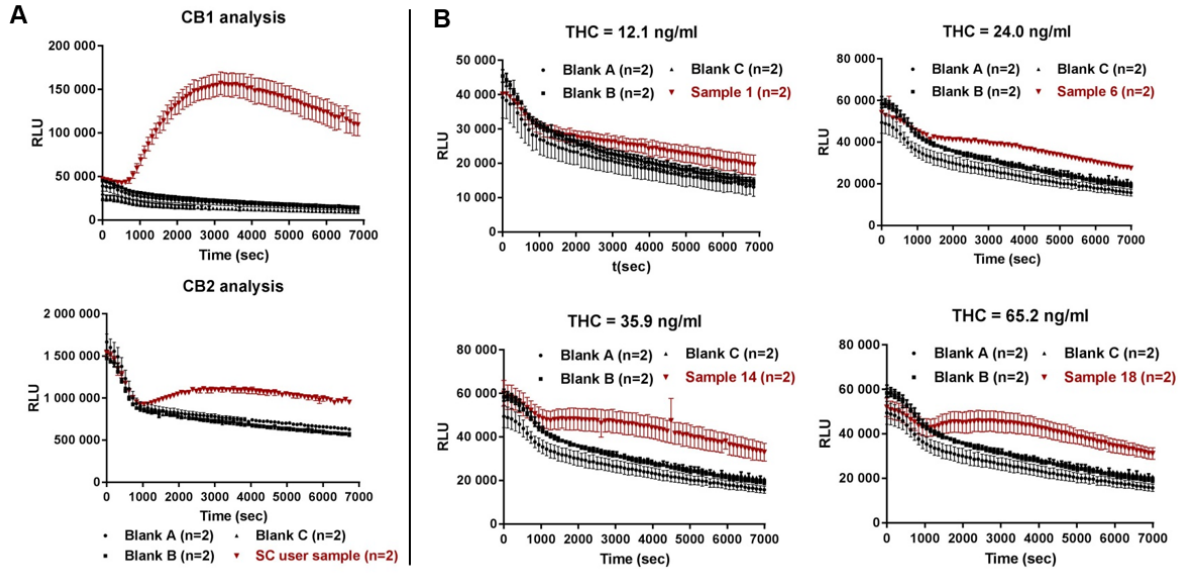


Figure 5.4. Examples of improved read out of the CB1 and CB2 assays in the truncated βarr2 cell lines **(B)**, as compared to the wild type βarr2 cell lines **(A)** as reference. Extracts of authentic user samples (contents outlined in Table 5.3) were tested. RLU = Relative Light Units, mean ± the standard error of the mean (n = 2).

Table 5.3. List of authentic urine samples from users of UR-144/XLR-11 **(A)**, AB-CHMINACA **(B)**, or ADB-CHMINACA **(C)**. The bioassays scored the samples as either positive (1) or negative (0). The sample where the classification of a negative sample changed to a positively scored sample in the improved bioassays is marked bold red. Urinary extracts used as example in Figure 5.4 are marked with 'sample 1-4'. The level is determined by most potent metabolite (bold): + < 1 ng/mL, ++ 1-10 ng/mL, +++ 10-50 ng/mL, ++++ > 50 ng/mL. The intensity of the color of the extract is shown by the different shades of grey (darker if extract was more colored). Strong coloration of the urine extract had a negative impact on the analysis of the bioassay, as reported earlier (in Chapter 4).

A

		UR-144 (ng/mL)				XLR-11 (ng/mL)			
SCRA	**Level**	**4-OH-pentyl (ng/mL)**	**5-OH-pentyl (ng/mL)**	**Pentanoic acid (ng/mL)**	**Degradant pentanoic acid (ng/ml)**	**4-OH-pentyl (ng/mL)**	**Score in bioassay**	**Score in improved bioassay**	
UR-144	+	0.68	**0.18**	2.02	35.30		1	1	
UR-144	+	0.42	**0.32**	0.73	3.65		1	1	
XLR-11	+++		**> 10**	> 50	> 50	3.13	1	1	
XLR-11	+++		**> 10**	44.80	> 50	1.39	1	1	
XLR-11	+++		**> 10**	46.80	> 50	0.44	1	1	
XLR-11	++		**8.14**	29.80	> 50	0.21	1	1	
XLR-11	++		**5.14**	23.40	> 50	0.17	1	1	
XLR-11	++		**4.64**	33.10	> 50	0.38	1	1	
XLR-11	++		**4.27**	10.80	> 50	0.81	1	1	
XLR-11	++		**4.11**	15.90	> 50	0.12	1	1	
XLR-11	++		**2.92**	10.20	> 50	0.13	1	1	
XLR-11	++		**2.26**	8.40	> 50	0.11	1	1	
XLR-11	++		**1.28**	0.40	16.70		1	1	Sample 4
XLR-11	+		**0.99**	7.56	> 50		1	1	
XLR-11	+		**0.86**	6.69	44.50		1	1	
XLR-11	+		**0.36**	2.31	36.20		0	0	
XLR-11	+		**0.31**	1.80	24.10		1	1	
XLR-11	+		**0.21**	0.64	11.90		1	1	

B

		AB-CHMINACA						
SCRA	**Level**	**Parent (ng/mL)**	**M1A (ng/mL)**	**M1B (ng/mL)**	**M2 (ng/mL)**	**M3A (ng/mL)**	**Score in bioassay**	**Score in improved bioassay**
AB-CHMINACA	++++	0.61	> 50	20.00	**> 50**	> 50	1	1
AB-CHMINACA	++++	0.26	> 50	6.55	**> 50**	34.80	1	1
AB-CHMINACA	++++		> 50	12.70	**> 50**	> 50	0	0
AB-CHMINACA	++++		> 50	3.24	**> 50**	35.70	0	0
AB-CHMINACA	+++		22.70	1.46	**16.40**	8.95	0	0
AB-CHMINACA	+++		18.40	1.28	**16.00**	4.86	0	0
AB-CHMINACA	++		13.30	1.50	**8.41**	4.16	1	1
AB-CHMINACA	++		4.68	0.46	**6.32**	3.46	0	0
AB-CHMINACA	++		1.37	0.43	**1.45**	1.32	0	0
AB-CHMINACA	++		1.27	0.10	**1.37**	0.42	0	0
AB-CHMINACA	++		1.54	0.15	**1.06**	0.62	1	1
AB-CHMINACA	+		4.42	0.37	**0.95**	0.53	0	0

C		ADB-CHMINACA						
SCRA	Level	Parent (ng/mL)	M1 (ng/mL)	M2 (ng/mL)	M3 (ng/mL)	Score in bioassay	Score in improved bioassay	
ADB-CHMINACA	++++	0.19	**> 50**	4.33	> 50	1	1	
ADB-CHMINACA	+++		**43.90**	3.79	38.80	1	1	
ADB-CHMINACA	+++		**30.80**	2.90	32.60	1	1	
ADB-CHMINACA	+++		**16.20**	1.00	16.10	1	1	
ADB-CHMINACA	+++		**15.90**	2.20	25.10	1	1	
ADB-CHMINACA	++	0.14	**9.99**	0.22	4.64	1	1	Sample 3
ADB-CHMINACA	++		**5.01**	0.15	1.31	1	1	Sample 1
ADB-CHMINACA	++		**3.81**	0.31	2.50	1	1	Sample 2
ADB-CHMINACA	++		**2.57**	0.30	2.08	1	1	
ADB-CHMINACA	++		**2.55**	0.47	3.04	0	0	
ADB-CHMINACA	**++**		**2.44**	**0.32**	**3.68**	**0**	**1**	

5.3.3 Application of CB bioassays with truncated βarr2 forms on batches of authentic serum and plasma samples

Extracts of 45 authentic serum samples (including 2 known blanks) were analyzed using the new stable cell lines (CB1-LgBiT/SmBiT-βarr2$_{TR366}$ and CB2-SmBiT/LgBiT-βarr2$_{TR382}$). Although several of these serum samples were strongly hemolyzed, clear extracts were obtained and no interference with the analysis was observed.

Both the CB1 and CB2 reporter assays were used to score serum extracts. 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. The profile obtained from an unknown sample was compared to the ones obtained from blanks. A clearly positive sample resulted in a strong rise in RLU. Less clearly positive samples had a less prominent rise in RLU, but either showed a small increase in the beginning of the profile or had an upward profile at the end, compared to the blank signal.

From the 22 positive samples, 18 were scored positive in the bioassays (82%; Table 5.4A). Four positive samples containing low concentrations of MDMB-CHMICA (≤ 0.54 ng/ml), AB-CHMINACA (0.2 ng/ml) and EG-018 (1.07 ng/ml) were missed (Table 5.4B). All SCRA negative samples were correctly scored negative in the bioassays, leading to a specificity of 100% (Table 5.4C).

Table 5.4. Results of the analysis of authentic serum samples with the new bioassays. **(A)** The correctly scored SCRA positive samples, **(B)** the missed SCRA positive samples and **(C)** the correctly scored SCRA negative samples. (*) not quantified.

A	**Detected SCRAs by LC-MS/MS**	**Other findings**
1	MDMB-CHMICA < 0.2 ng/mL	-
2	MDMB-CHMICA < 0.2 ng/mL	2.60 ‰ ethanol
3	MDMB-CHMICA 0.4 ng/mL	-
4	MDMB-CHMICA 1.68 ng/mL	0.41 ‰ ethanol, diphenhydramine (*)
5	AB-CHMINACA 1.1 ng/mL	-
6	5F-PB-22 3.1 ng/mL	-
7	5F-ADB 3.5 ng/mL	-
8	MDMB-CHMICA 15.0 ng/mL	-
9	MDMB-CHMICA < 0.2 ng/mL, 5F-ADB < 0.2 ng/mL	-
10	MDMB-CHMINACA positive (*), ADB-CHMINACA positive (*)	THCCOOH 3.4 ng/mL
11	MDMB-CHMICA positive (*), 5F-APINACA positive (*)	-
12	EG-018 0.23 g/mL, PB-22 < 0.2 ng/mL, MDMB-CHMICA 4.2 ng/mL	-
13	5F-ADB 0.45 ng/mL, FUB-AMB < 0.2 ng/mL	-
14	ADB-FUBINACA 0.1 ng/mL, EG-018 8.8 ng/mL	THC 2.0 ng/mL, 11-OH-THC 0.7 ng/mL, THCCOOH 8.4 ng/mL, sertraline 12 ng/mL, norsertraline (*), olanzapine 12.4 ng/mL
15	5F-ADB 1.0 ng/mL, MDMB-CHMICA 0.38 ng/mL	midazolam 59.0 ng/mL
16	EG-018 1 ng/mL, 5F-ADB < 0.2 ng/mL, MDMB-CHMICA 0.8 ng/mL, 5F-APINACA < 0.2 ng/mL	THCCOOH 3.6 ng/mL, quetiapine < 10 ng/mL, 7-OH-quetiapine 10.2 ng/mL, haloperidol 15.6 ng/mL, midazolam 150 ng/mL, α-OH-midazolam 27.0 ng/mL
17	MDMB-CHMICA 2.76 ng/mL, AB-CHMINACA 1.1 ng/mL	-
18	MDMB-CHMICA 6.4 g/mL, EG-018 0.7 ng/mL, PB-22 < 0.2 ng/mL	doxepin 14.2 ng/Ml, nordoxepin 16.2 ng/mL

B	**Detected SCRAs by LC-MS/MS**	**Other findings**
1	MDMB-CHMICA 0.54 ng/mL	THCCOOH 3.4 ng/mL
2	MDMB-CHMICA < 0.2 ng/mL	-
3	AB-CHMINACA 0.2 ng/mL	amitriptylin 24.2 ng/mL, nortriptylin 48.8 ng/mL, citalopram 53.0 ng/mL, N-desmethylcitalopram 26.4 ng/mL, metoprolol (*)
4	EG-018 1.07 ng/mL	-

C	SCRA negative samples: other findings
1	amphetamine 69.6 ng/mL, methamphetamine 71.7 ng/mL
2	1.43 ‰ ethanol
3	2.28 ‰ ethanol
4	-
5	-
6	-
7	-
8	amphetamine 40.2 ng/mL, methamphetamine 210 ng/mL, MDMA 105 ng/mL, MDA 11.8 ng/mL
9	-
10	amphetamine 7.1 ng/mL, methamphetamine 96.9 ng/mL
11	-
12	-
13	amphetamine 27.2 ng/mL, methamphetamine 280 ng/mL
14	-
15	amphetamine 129 ng/mL
16	2.50 ‰ ethanol, citalopram 141 ng/mL, N-desmethylcitalopram 11.5 ng/mL, diazepam < 2.5 ng/mL, nordiazepam < 2.5 ng/mL
17	-
18	doxepine 66.7 ng/mL, nordoxepine 58.9 ng/mL, buprenorphine 0.9 ng/mL, norbuprenorphine 1.7 ng/mL, diazepam 709 ng/mL, nordiazepam 1,330 ng/mL, oxazepam 30.0 ng/mL, temazepam 34.6 ng/mL
19	-
20	-
21	-

To further test the applicability of the bioassays, as well as their propensity to score THC positive samples, we went on to apply the assays on a set of extracts from 73 authentic plasma samples, obtained from drivers that had an on-site positive drug oral fluid test (positivity was not necessarily for cannabinoids). Again, both the CB1 and CB2 reporter assays were used to score plasma extracts. The batch included one sample positive for AB-FUBINACA, 18 samples containing a high THC concentration (range 12.1 - 64.2 ng/ml) and 54 samples potentially containing several drugs of abuse and/or THC not exceeding 1 ng/ml. These samples were run with 3 known blank plasma samples. Scoring was done as described above.

The SCRA positive sample (AB-FUBINACA 7.8 ng/ml, Figure 5.5A and Table 5.5) was clearly identified with both bioassays. The CB1 analyses also showed a positive result for 16/18 (89%) of the samples containing a high (≥ 12 ng/ml) THC concentration (Figure 5.5B and Table 5.5), whereas this was not the case for CB2 analyses. All other samples containing other drugs of abuse, therapeutic drugs and/or low concentrations of THC (< 1 ng/ml), came back negative in both bioassays (Table 5.5). As there was no basis to believe these samples were SCRA positive, no further MS analysis was done.

Table 5.5. Analysis of the plasma samples. **(A)** The 'SCRA positive' sample, **(B)** the samples containing a high level of THC and **(C)** the samples potentially containing several drugs (of abuse) and/or a low level of THC (< 1 ng/ml).

A	**SCRAs by LC-MS/MS**	**Other findings**
1	AB-FUBINACA (7.8 ng/mL)	THC (1.9 ng/ml), THCCOOH (15.1 ng/ml), methiopopramine (not quantified)

B	**Samples containing high concentration of THC**
1	THC (12.1 ng/mL)
2	THC (19.8 ng/mL)
3	THC (21.2 ng/mL)
4	THC (21.5 ng/mL)
5	THC (21.9 ng/mL)
6	THC (24.0 ng/mL)
7	THC (27.7 ng/mL)
8	THC (29.6 ng/mL)
9	THC (30.1 ng/mL)
10	THC (32.6 ng/mL)
11	THC (32.7 ng/mL)
12	THC (33.6 ng/mL)
13	THC (34.9 ng/mL)
14	THC (35.9 ng/mL)
15	THC (37.9 ng/mL)
16	THC (39.9 ng/mL)
17	THC (57.0 ng/mL)
18	THC (64.2 ng/mL)

C	**Samples containing several drugs (of abuse) and/or low level of THC (< 1 ng/mL)**
1-20	-
21	fentanyl (2 ng/mL)
22	tramadol (> 300 ng/mL)
23	tramadol (> 300 ng/mL), fentanyl (0.9 ng/mL)
24	benzoylecgonine (10 ng/mL)
25	benzoylecgonine (37 ng/mL), methylecgonine (11 ng/mL)
26	amphetamine (6 ng/mL)
27	amphetamine (29 ng/mL), GHB 215 µg/mL
28	THC (0.1 ng/mL)
29	THC (0.1 ng/mL)
30	THC (0.1 ng/mL)
31	THC (0.2 ng/mL)
32	THC (0.2 ng/mL), amphetamine (306 ng/mL)
33	THC (0.2 ng/mL), benzoylecgonine (26 ng/mL)
34	THC (0.2 ng/mL)
35	THC (0.2 ng/mL), cocaine (24 ng/mL), benzoylecgonine (255 ng/mL), methylecgonine (31 ng/mL), cocaethylene (24 ng/mL)
36	THC (0.2 ng/mL)

37	THC (0.3 ng/mL)
38	THC (0.3 ng/mL)
39	THC (0.3 ng/mL)
40	THC (0.3 ng/mL), benzoylecgonine (12 ng/mL)
41	THC (0.3 ng/mL)
42	THC (0.4 ng/mL), amphetamine (283 ng/mL)
43	THC (0.4 ng/mL)
44	THC (0.5 ng/mL), amphetamine (477 ng/mL)
45	THC (0.5 ng/mL), cocaine (37 ng/mL), benzoylecgonine (82 ng/mL)
46	THC (0.5 ng/mL), amphetamine (19 ng/mL), MDMA (669 ng/mL), MDA (23 ng/mL), cocaine (7 ng/mL), benzoylecgonine (157 ng/mL), methylecgonine (11 ng/mL)
47	THC (0.6 ng/mL), amphetamine (178 ng/mL), GHB (150 µg/mL), cocaine (34 ng/mL), benzoylecgonine (171 ng/mL), methylecgonine (25 ng/mL)
48	THC (0.6 ng/mL), amphetamine (2.2 ng/mL), MDMA (57 ng/mL), MDA (4.4 ng/mL)
49	THC (0.6 ng/mL), cocaine (311 ng/mL), benzoylecgonine (10 000 ng/mL), methylecgonine (188 ng/mL), cocaethylene (76 ng/mL)
50	THC (0.7 ng/mL), cocaine (2 ng/mL), benzyolecgonine (333 ng/mL)
51	THC (0.8 ng/mL), amphetamine (51 ng/mL), cocaine (387 ng/mL), benzoylecgonine (2,295 ng/mL), methylecgonine (216 ng/mL), cocaethylene (8 ng/mL)
52	THC (0.8 ng/ml), cocaine (83 ng/mL), benzoylecgonine (1 188 ng/mL), methylecgonine (121 ng/mL)
53	THC (0.9 ng/mL), amphetamine (2.2 ng/mL), cocaine (14 ng/mL), benzoylecgonine (105 ng/mL), methylecgonine (13 ng/mL), cocaethylene (7ng/mL)
54	THC (0.9 ng/mL), amphetamine (222 ng/mL), cocaine (202 ng/mL), benzoylecgonine (422 ng/mL), methylecgonine (34 ng/mL), cocaethylene (14 ng/mL)

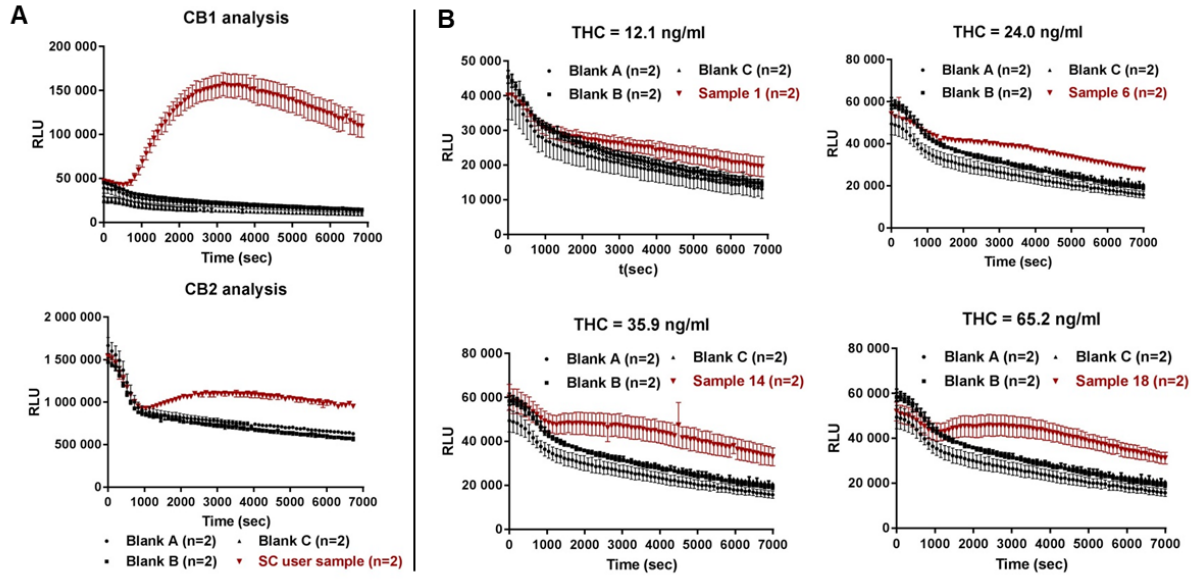


Figure 5.5. (A) CB1 and CB2 analysis of the SCRA positive sample. **(B)** Examples of CB1 analyses of samples containing a high concentration of THC.

5.4 Discussion

Currently, conventional 'targeted' and 'untargeted' analytical methods struggle to screen for SCRAs in biological matrices because structures are continuously altered to evade legislation on controlled substances and due to the low concentration of SCRAs in biological fluids[14-15]. We recently reported on a novel activity-based screening method for the detection of SCRAs and their metabolites, thereby avoiding the need to know the specific structure of the SCRA, demonstrating cannabinoid activity in authentic urine samples[7-8]. Since the sensitivity was not sufficient for the application to the lower concentrations in serum or plasma compared to urine, we aimed at improving the analytical sensitivity of our bioassays using truncated βarr2 forms. Because we reasoned that stabilizing βarr2-GPCR interaction may lead to increased sensitivity of the assays, we constructed two C-terminal βarr2 truncation mutants. The first truncation mutant, $\beta arr2_{TR382}$, comprises amino acids 1-382 of the 410 amino acid long wild type βarr2 molecule. Because the C-terminus of βarr2 is involved in an intramolecular interaction, keeping it in its basal, non-active state, removal of the C-terminus gives a constitutively active $\beta arr2_{TR382}$ truncation mutant. This mutant is recruited to the activated GPCR independent of GPCR phosphorylation[16], which has been shown to result in an increased signal in another type of assay, scoring the activation of the vasopressin, dopamine and β-adrenergic receptors[17]. In the second mutant $\beta arr2_{TR366}$, we eliminated the predominant binding site (involving residues 367-385) for clathrins, which are proteins of the endocytic machinery, thereby essentially eliminating clathrin binding (≈90% reduced)[18-19]. In this way, internalization of the CB receptor is reduced, which may provide a more pronounced response in the assay. Upon selection of the best truncated βarr2 isoform for each bioassay with the known agonist JWH-018 (Figure 5.2 and 5.3), the newly generated cell lines were evaluated on authentic urine samples, showing that the truncated βarr2 cell lines gave a stronger signal for βarr2 recruitment, allowing for an easier interpretation of the read out compared to the wild type βarr2 cell lines (Figure 5.4).

Next, the applicability of the stable CB1 and CB2 receptor activation assays was evaluated for the detection of SCRAs in a panel of authentic serum and plasma samples. Our data indicate that the stable CB reporter assays detect CB receptor activation by serum and plasma extracts in which SCRAs are present at low- or sub-ng/mL concentration (Table 5.4, Table 5.5, Figure 5.5A). In contrast to the urine analysis, where CB receptor activation mainly relies upon the presence of active SCRA metabolites, the activation of the bioassays by blood extracts primarily stems from the presence of the SCRA parent compound. Hence, the analysis of blood derived samples is less susceptible to potential SCRA metabolization to inactive metabolites. In a set of 45 serum samples, 18/22 SCRA positive samples, some with sub-ng/mL concentrations, were scored positive via the new bioassays, yielding an analytical

sensitivity of 82%. The presence of other drugs of abuse and/or low concentrations of THC (< 1 ng/mL), tested here, did not influence the CB reporter bioassays. Only samples in which high concentrations of THC (> 12 ng/mL) were present gave rise to a positive result in 16/18 (89%) of cases, which is somewhat expected since we screen for CB activity (Figure 5.5B). We cannot fully explain why two samples with a high THC concentration (21.5 and 32.7 ng/mL) were missed. The fact that THC is overall only a weak agonist at the CB receptor makes it not an ideal target for our bioassays. In addition, the signal obtained in our bioassays is the result of the activities of all cannabinoids that are present. Because we only quantified THC, it remains unknown to what extent other natural cannabinoids did or did not contribute to a signal in the investigated samples.

High concentrations of THC are needed to generate a signal in the bioassays, as THC only acts as a partial agonist at the CB receptor, in contrast to SCRAs, that typically act as full agonists[2]. This difference in level of activation can also be seen by comparing the relative light units level from the SCRA positive (7.8 ng/ml AB-FUBINACA) sample (Figure 5.5A, upper panel) with those of the highly THC positive (> 12 ng/ml) samples (Figure 5.5B). Hence, detection of cannabis use is limited to those samples with a high THC concentration. In practice, we envisage use of the bioassays in combination with a conventional immunoassay for natural cannabinoids. Importantly, in none of the evaluated serum samples (n = 45) a false positive result was obtained, yielding a specificity of 100%. An interference study, readily performed in the framework of Chapter 4[8], resulted in a list of 288 compounds that do not interfere with the read-out of these bioassays (see Addendum at the end of this Chapter). The presence of endocannabinoids is also not expected to interfere with the bioassays as these are only present at very low concentrations in blood (in the range of low pmol/mL). Although in some conditions (eating disorders, obesity, schizophrenia, post-exercise) the endocannabinoid concentrations can rise, this will never be to an extent that would lead to interference (< 10 pmol/mL)[20-23]. For a small subset of SCRAs, the limit of activity detection, i.e. the lowest concentration that gives an activity-based signal that is clearly distinguishable from blank, was determined (Table 5.6 and Figure 5.6). These values are rather theoretical since in authentic SCRA positive samples a mixture of parent compound(s) and (active) metabolites is present. The signal obtained from the activity-based analysis of a sample will thus be a result of all these compounds together. The obtained signal in authentic samples also depends on the extraction efficiencies of the diverse compounds and their metabolites. In practice, in a real sample, a main compound may be present at a concentration below the limit of detection and yet a signal may be picked up, because of the co-presence of active metabolites. This is actually a key advantage of this activity-based approach.

Table 5.6. Limits of activity detection of several SCRAs at CB1 and CB2 receptors.

	CB1 (ng/mL)	CB2 (ng/mL)
JWH-018	0.92	2.92
UR-144	2.66	0.84
XLR-11	2.81	0.89
AB-CHMINACA	0.10	0.96
ADB-CHMINACA	0.01	1.00

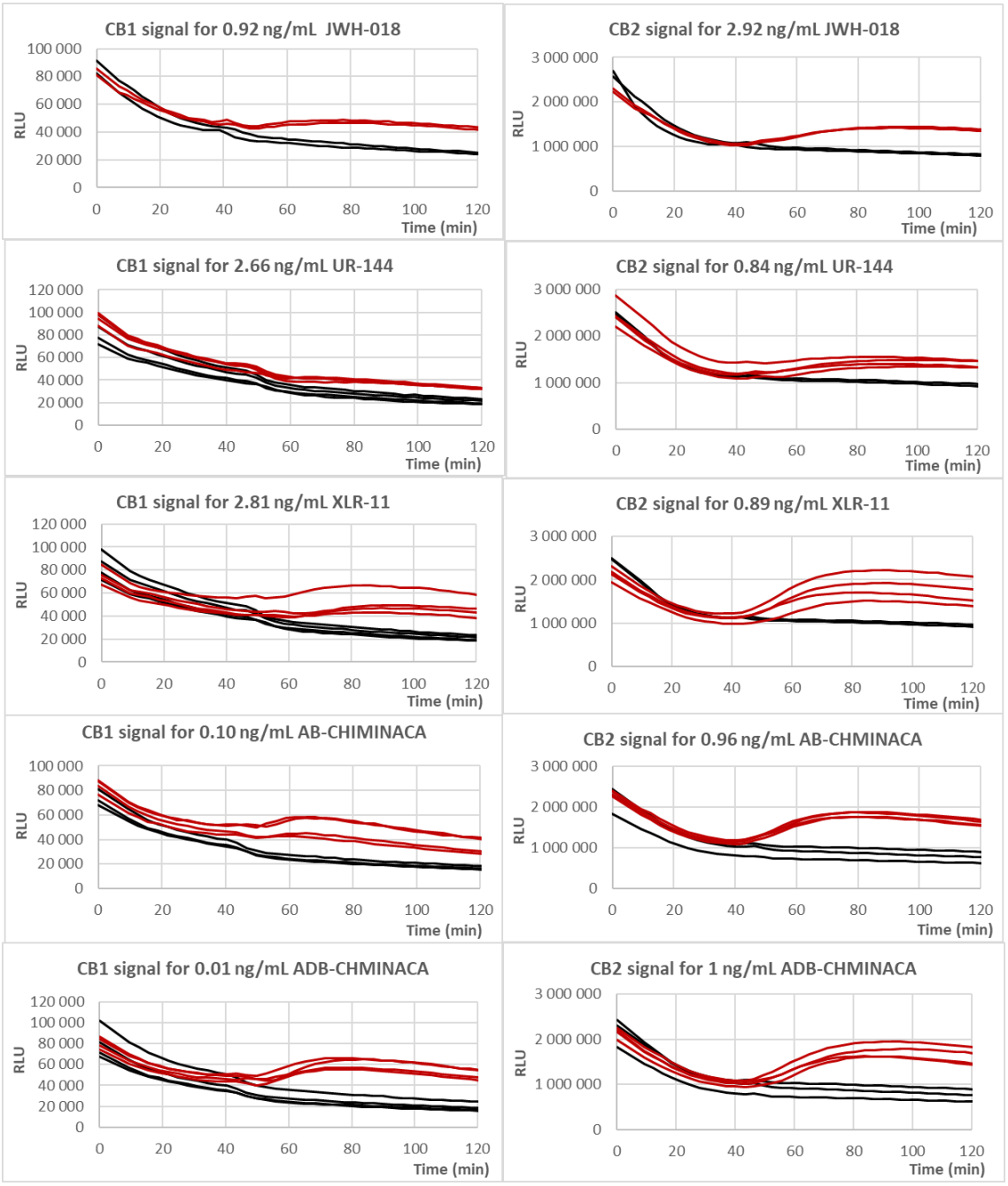


Figure 5.6. Limits of activity detection of several SCRAs at CB1 and CB2 receptors. RLU= Relative Light Units (n = 2-4)

Because the detection is activity-based, these bioassays intrinsically detect current and potentially future SCRAs, in contrast to MS-based methods, which require updated libraries. Inherent to the use of cannabinoid receptor-based bioassays is that also serum or plasma from strong cannabis users may inevitably test positive, although at limited extent. This can efficiently be countered by combining the bioassays proposed here with a simple immunoassay-based screening for natural cannabinoids, as is now already often routinely applied in a clinical laboratory setting. These immunoassays are relatively cheap and offer good analytical sensitivities for determining the presence of natural cannabinoids in various biological fluids. A low signal in the absence of natural cannabinoid positivity points at the presence of SCRAs. A high signal in our bioassay clearly indicates the presence of SCRAs, as even high THC concentrations do not result in a high signal. Low concentrations of THC are not able to generate a sufficiently high signal in the bioassays to result in a positive scoring.

Although application of the CB1 and CB2 bioassays reported here is relatively simple, it does require the presence of a basic cell culture facility and some basic skills. Therefore, we do not envisage a global implementation in all clinical laboratories but see this more in a centralized setting or in larger, specialized laboratories, where samples can be analyzed at a higher throughput. Establishing computer-based learning to allow automated scoring of the samples as well as robotized pipetting steps may help to achieve high-throughput. A future step to allow a more general use is the generation of kits consisting of frozen cells, plates to seed the cells, media, reagents, and positive and negative controls. In this format, there could be as little as 24 h between thawing of the cells and read-out of the result.

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5.5 Addendum

List of 288 compounds that did not interfere with the read-out of the bioassay. These compounds were spiked into urine at the concentration mentioned.

Substance	**Concentration spiked in urine sample (ng/mL)**
Benzodiazepines	
3-Hydroxybromazepam	500
3-Hydroxyflubromazepam	100
3-Hydroxyphenazepam	100
7-Aminoclonazepam	500
7-Aminoflunitrazepam	200
Adinazolam	100
α-Hydroxyalprazolam	200
α-Hydroxymidazolam	2000
Alprazolam	200
Bromazepam	500
Clonazepam	500
Clonazolam	100
Delorazepam	100
Deschloroetizolam	100
Diazepam	2000
Diclazepam	100
Etizolam	100
Flubromazepam	100
Flubromazolam	100
Flunitrazepam	200
Flunitrazepam	100
Lorazepam	2000
Lorazepam	100
Lormetazepam	200
Lormetazepam	100
Meclonazepam	100
Metizolam	100
Midazolam	2000
N-Desmethylflunitrazepam	200
N-Desmethylflunitrazepam	100
Nifoxipam	100
Nitrazepam	500
Nitrazolam	100
Nordazepam	2000
Oxazepam	2000
Phenazepam	100
Pyrazolam	100
Ro5-4864 (4'-chlorodiazepam)	100
Temazepam	2000
Tetrazepam	2000
Zolpidem	500
Zopiclone	200

(Designer) Hallucinogens	
1P-LSD	100
25B-NBF	100
25B-NBOMe	100
25C-NBOMe	100
25D-NBOMe	100
25E-NBOMe	100
25G-NBOMe	100
25H-NBOMe	100
25I-NBF	100
25I-NBMD	100
25I-NBOH	100
25I-NBOMe	100
25P-NBOMe	100
25N-NBOMe	100
25T2-NBOMe	100
25T4-NBOMe	100
25T7-NBOMe	100
2C-B	100
2C-B-fly	100
2C-C	100
2C-D	100
2C-E	100
2C-H	100
2C-I	100
2C-N	100
2C-P	100
2C-T-2	100
2C-T-4	100
2C-T-7	100
3C-B-fly	100
3C-E	100
4-AcO-DET (4-Acetoxy-N,N-diethyltryptamine)	100
4-OH-DET (4-Hydroxy-N,N-diethyltryptamine)	100
4-OH-MET (4-hydroxy-N-methyl-N-ethyltryptamine)	100
5-APB-NBOMe	100
5-MeO-AMT (5-methoxy-α-methyltryptamine)	100
5-MeO-DALT (5-Methoxy-N,N-diallyl-tryptamine)	100
5-MeO-DMT (5-methoxy-N,N-dimethyltryptamine)	100
5-MeO-TMT	100
Allylescaline	100
α-ET (α-Ethyltryptamine)	100
AMT (α-Methyltryptamine)	100
bk-2C-B	100
Bromo-Dragonfly	100
DET (Diethyltryptamine)	100
DiPT (N,N-Diisopropyltryptamine)	100
DOB (2,5-Dimethoxy-4-bromoamphetamine)	100
DOC (2,5-Dimethoxy-4-chloroamphetamine)	100
DOET (2,5-Dimethoxy-4-ethylamphetamine)	100
DOF (2,5-Dimethoxy-4-fluoroamphetamine)	100

DOI (2,5-Dimethoxy-4-iodoamphetamine)	100
DOIP (2,5-Dimethoxy-4-isopropylamphetamine)	100
DOM (2,5-Dimethoxy-4-methylamphetamine)	100
DMT (Dimethyltryptamine)	100
DPT (Dipropyltryptamine)	100
Escaline	100
Psilocine	100
PCP	100

Opioids/opiates	
4-Chlor-Isobutyrfentanyl	100
4-Methoxybutyrfentanyl	100
4-fluorobutyrfentanyl	100
6-Monoacetylcodeine (MAC)	100
6-Monoacetylmorphine (MAM)	100
7-Hydroxymitragynine	100
Acryloylfantanyl	100
AH-7921	100
Alfentanil	100
Acetylfentanyl	100
Benzodioxole fentanyl	100
Benzylfentanyl	100
Butyrylfentanyl	100
Carfentanil	100
Cyclopentyl fentanyl	100
Desomorphine	100
Dextromethorphan	100
Dihydrocodeine	100
Dihydromorphine	100
Furanylfentanyl	100
Hydrocodone	100
Hydromorphone	100
Meptazinol	100
Mitragynine	100
MT-45 (IC-6)	100
Nalbuphine	100
Naloxone	100
Naltrexone	100
Noroxycodone	100
Noscapine	100
Ocfentanil	100
Oxycodone	100
Oxymorphone	100
Papaverine	100
Pentazocine	100
Pethidine	100
Pholcodine	100
Piritramide	100
Remifentanil	100
Sufentanil	100
U-47700	100

(Designer) Stimulants	
2,5-DMA (Dimethoxyamphetamine)	100
2-AI (2-Aminoindane)	100
2-F-Amphetamine	100
2-F-Methamphetamine	100
2-FMC (2-Fluoromethcathinone)	100
2-MAPB (2-(2-Methylaminopropyl)benzofuran)	100
2-MMC (2-Methylmethcathinone)	100
3,4-CTMP (3,4-Dichloromethylphenidate)	100
3,4-DMA (3,4-Dimethoxyamphetamine)	100
3,4-DMMC (3,4-Dimethylmethcathinone)	100
3-Cl-Methcathinone	100
3-F-Amphetamine	100
3-F-Methamphetamine	100
3-FMC (3-Fluoromethcathinone)	100
3-F-Phenetrazine	100
3-FPM (3-Fluorophenmetrazine)	100
3-Me-Buphedrone	100
3-MeO-MC (3-Methoxymethcathinone)	100
4-Br-Methcathinone	100
4-APDB	100
4-CAB (4-Chlorophenylisobutylamine)	100
4-CMC (4-Chloromethcathinone)	100
4-Cl-Methamphetamine	100
4-Cl-PVP (4-Chloropyrrolidinopentiophenone)	100
4-EAPB (4-(2-Ethylaminopropyl)benzofuran)	100
4-Ethylethcathinone	100
4-Ethylmethcathinone	100
4-F-Ethylphenidate	100
4-F-IPV (4-fluoro-N-isopropyl-pentedrone)	100
4-F-Amphetamine	100
4-F-Methamphetamine	100
4-F-Buphedrone	100
4-FMC (4-fluoromethcathinone)	100
4-F-Methylphenidate	100
4-F-PV8	100
4-F-PV9	100
4-F-α-PBP (4-fluoro-α-pyrrolidinobutyrophenone)	100
4-F-α-PVP (4-fluoro-α-pyrrolidinopentiophenone)	100
4-MAPB (4-(2-Methylaminopropyl)benzofuran)	100
4-Me-Buphedrone	100
4-Me-Methamphetamine	100
4-MeO-PV9	100
3,4-MeO-α-PHP (3,4-MeO-alpha-pyrrolidinohexiophenone)	100
4-MeO-PVP (4-methoxypyrrolidinopentiophenone)	100
4-Me-Pentedrone	100
4-Me-α-PHP (4-MeO-alpha-pyrrolidinohexiophenone)	100
4-MMC (4-Methylmethcathinone, mephedrone)	100
4-MPM (4-Methylphenmetrazine)	100
4-MTA (4-Methylthioamfetamine)	100
5-APB (5-(2-aminopropyl)benzofuran)	100

5-APDI (5-(2-Aminopropyl)-2,3-dihydro-1H-indene)	100
5-BPDi (Indanyl-α-Pyrrolidinohexanophenone)	100
5-DBFPV (benzofuran analogue of α-PVP)	100
5-EAPB (5-(2-Ethylaminopropyl)benzofuran)	100
5-IT (5-(2-Aminopropyl)indole)	100
5-MAPB (5-(2-Methylaminopropyl)benzofuran)	100
5-MAPDB	100
5-MBPB	100
5-PPDi (3',4'-trimethylene-α-Pyrrolidinobutiophenone)	100
6-APB (6-(2-aminopropyl)benzofuran)	100
6-APDB	100
6-EAPB (6-(2-Ethylaminopropyl)benzofuran)	100
6-MAPB (6-(2-Methylaminopropyl)benzofuran)	100
7-APDB	100
ALEPH-2	100
ALEPH-4	100
Amphetamine	100
α-Naphyrone	100
α-PHP (α-Pyrrolidinohexiophenone)	100
α-PNP (α-Pyrrolidinononanophenone)	100
α-PVP (α-Pyrrolidinovalerophenone)	100
Benzylpiperazine	100
bk-MDDMA (Dimethylone)	100
Buphedrone	100
Butylone	100
Cathine	100
Cathinone	100
Chloropseudoephedrine	100
DBZP (Dibenzylpiperazine)	100
Desoxypipradrol (2-diphenylmethylpiperidine (2-DPMP))	100
Dimethylcathinone	100
Ephedrine	100
Ethylcathinone	100
Ethylamphetamine	100
Ethylnaphtidate	100
Ethylone	100
Isopentedrone	100
Isophenmetrazine	100
mCPP	100
MDA (3,4-methyleendioxyamfetamine)	100
MDAI (5,6-methylenedioxy-2-aminoindane)	100
MDAT (6,7-Methylenedioxy-2-aminotetralin)	100
MDMA (3,4-Methylenedioxymethamphetamine)	100
MDPBP (3',4'-Methylenedioxy-α-pyrrolidinobutyrophenone)	100
MDPHP (3,4-Methylenedioxypyrrolidinohexiophenone)	100
MDPPP (3',4'-Methylenedioxy-α-pyrrolidinopropiophenone)	100
MDPV (Methylendioxypyrovaleron)	100
MEAI (5-Methoxy-2-indanamine)	100
Methamnetamine	100

Methamphetamine	100
Methcathinone	100
Methylnaphtidate	100
Methylone	100
Methylphenidate	100
Mexedrone	100
N-Ethylpentylone	100
Nitracaine	100
N-Methyl-2-AI (N-methyl-2-Aminoindane)	100
Norephedrine	100
NRG-3	100
Pentedrone	100
Pentylone	100
Phenetrazine	100
PMA (para-Methoxyamphetamine)	100
PMMA (4-methoxymethamfetamine)	100
PV9	100
Pyrovalerone	100
Ritalinic acid	100
TMA (3,4,5) (Trimethoxyamphetamine)	100
TMA-2 (Trimethoxyamphetamine)	100
TMA-6 (Trimethoxyamphetamine)	100

Other	
2-MeO-Ketamine	100
5-IAI (5-Iodo-2-aminoindane)	100
Ambroxol	100
a-Me-AHP	100
a-PAVP	100
Atropine	100
DB-MDBP	100
Dimethocaine	100
Diphenidine	100
Etaqualone	100
Fl-Modafinil	100
Ketamine	100
Lidocaine	100
Loperamide	100
Mebroqualone	100
Mephtetramine	100
Methiopropamine	100
Methoxetamine	100
Methoxphenidine	100
Metoclopramide	100
Modafiendz (N-Methylbisfluoromodafinil)	100
N-Ethylnorketamine	100
Norketamine	100
Phenibut	100
TFMPP (Trifluormethylphenylpiperazine)	100
W-18	100

CHAPTER 6:
ACTIVITY-BASED CONCEPT TO SCREEN BIOLOGICAL MATRICES FOR OPIATES AND (SYNTHETIC) OPIOIDS

Based on

Annelies Cannaert, Lakshmi Vasudevan, Melissa Friscia, Amanda L. A. Mohr, Sarah M.R. Wille, Christophe P. Stove. Activity-based concept to screen biological matrices for opiates and (synthetic) opioids. *Clinical Chemistry* **2018**. In press.

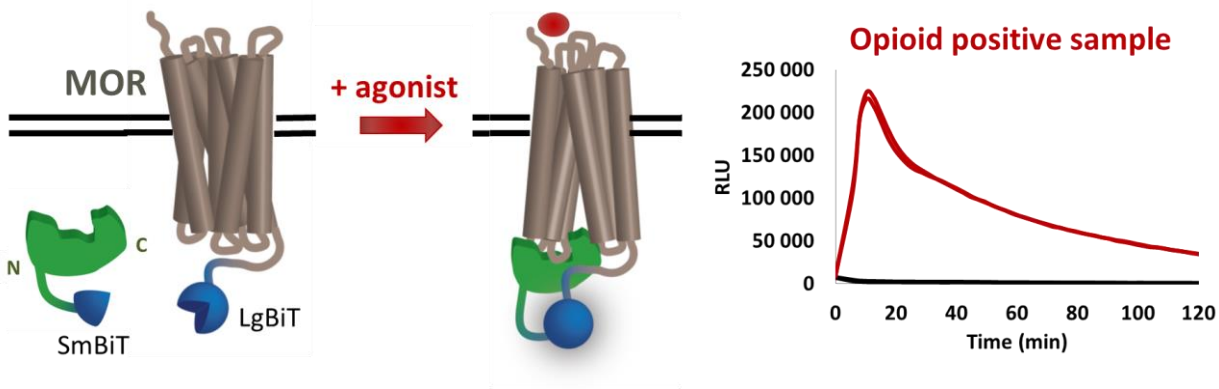
Abstract

Detection of new highly potent synthetic opioids is challenging as new compounds enter the market. Here we present a novel screening method for the detection of opiates and (synthetic) opioids based on their activity.

A cell-based system was set up in which activation of the μ-opioid receptor (MOR) led to recruitment of β-arrestin 2, resulting in functional complementation of a split NanoLuc luciferase and allowing read-out via bioluminescence. Assay performance was evaluated on 107 postmortem blood samples. Blood (500 µL) was extracted via solid phase extraction. Following evaporation and reconstitution in 100 µL of Opti-MEM®I, 20 µL was analyzed in the bioassay.

In eight samples containing synthetic opioids, where no positive signal was obtained in the bioassay, Quadrupole Time-Of-Flight mass spectrometry revealed the MOR antagonist naloxone, which can prevent receptor activation. Hence, further evaluation did not include these samples. For U-47700 (74.5-547 ng/mL) and furanyl fentanyl (<1-38.8 ng/mL), detection was 100% (8/8) for U-47700 and 95% (21/22) for furanyl fentanyl. An analytical specificity of 93% (55/59) was obtained for the opioid negatives. From an additional 10 samples found to contain other opioids, 5 were correctly scored positive. Non-detection in 5 cases could be explained by very low concentrations (<1 ng/mL alfentanil/sufentanil) or presence of inactive enantiomers.

The MOR reporter assay allows rapid identification of opioid activity in blood. Although the co-occurrence of opioid antagonists is currently a limitation, the bioassay's high detection capability, specificity and untargeted nature may render it a useful first-line screening tool to investigate potential opioid intoxications.



Graphical abstract of Chapter 6

6.1 Introduction

In Europe and North America, highly potent synthetic opioids, which mimic the effects of heroin and morphine, are a growing health threat[1-4]. While representing only a relatively small segment of the illicit market, there is an increasing number of reports on the rise of these compounds and on the harm they cause. Thirty-three new synthetic opioids were detected in Europe between 2009 and 2017 [5]. In the US, a recent surge (6-fold increase) in illicit opioid overdoses, driven by synthetic opioids, was observed from 2013-2016[6]. Synthetic opioids are substances created to act as agonists for the opioid receptors (μ, δ, and κ subtypes), mainly found in the brain, spinal cord and digestive tract[2]. The major pharmacologic actions of morphine (e.g., euphoria, analgesia, sedation, respiratory depression, decreased gastrointestinal motility and physical dependence) are all due to agonistic actions at the μ-opioid receptor (MOR)[7-8]. Most of the novel synthetic opioids act as full agonists, with varying potencies, at the MOR. These synthetic opioids were initially explored by research groups or pharmaceutical companies for their potential medicinal use, but have recently found their way to the illicit drug market[1-4].

These new synthetic opioids are a major public health concern due to their high potency, ease of accessibility over the internet and distribution into the regular street heroin supply, where they are often mixed with or substituted for heroin, leading to life-threatening respiratory depression and death[1-4]. The high potency and the low dose required to produce the desired effects, in addition to the continuous change in chemical structure, makes it challenging for clinical and forensic toxicologists to investigate intoxication and death cases caused by these novel opioids[1-3].

The true extent of the synthetic opioid epidemic is likely underestimated due to these compounds not being included in routine drug detection[1]. Standard opiate immunoassays fail to detect synthetic opioids since they have little structural homology to morphine. There are immunoassays available for fentanyl and its analogs, but due to difference in cross-reactivity, multiple assays are needed to detect all analogs since some immunoassays feature broad cross-reactivity, while others are highly specific[1, 9]. And although immunoassays for detecting non-fentanyl analogs such as AH-7921, U-47700 and MT-45 have recently become commercially available, the cumbersome process of developing immunoassays and the rapid increase in the number and variety of new synthetic opioids remains a challenge[3]. Furthermore, the need to continuously add novel fentanyl and non-fentanyl analogs to existing mass spectral libraries used in targeted drug screening methods is an issue, because certified reference materials for the main compounds and/or metabolites are not always available.

In Chapter 3, 4 and 5, we reported on the successful development of cell-based cannabinoid reporter assays for the activity-based detection of synthetic cannabinoids and their metabolites, capable of demonstrating cannabinoid activity in authentic urine and blood samples[10-12]. The bioassay reported here consolidates activity-based screening as a general principle in toxicological screening and uses the MOR to screen for opioid activity in biological samples. The principle of the bioassay is based on activation of MOR, which leads to the recruitment of the β-arrestin 2 (βarr2) protein, which results in functional complementation of a split NanoLuc luciferase, thereby restoring luciferase activity. In the presence of the substrate furimazine, this results in a bioluminescent signal that can be read out with a standard luminometer. Here, we report on a new alternative untargeted screening method for the detection of opiates and (synthetic) opioids, not directly based on their structure, but on their opioid activity. This activity-based assay may serve as a first-line screening tool, complementing the conventional analytical methods currently used.

6.2 Materials and Methods

6.2.1 Chemicals and Reagents

U-47700 and furanyl fentanyl were procured from Cayman Chemical (Ann Arbor, Michigan, USA). Hydromorphone hydrochloride was obtained from Fagron (Nazareth, Belgium). Buprenorphine, norbuprenorphine, fentanyl, loperamide, carfentanil and naloxone were obtained from LGC Standards (Wesel, Germany). Dulbecco's modified eagle's medium, Opti-MEM®I Reduced Serum Medium, penicillin/streptomycin (5.000 IU/mL and 5.000 µg/mL), amphotericin B (250 µg/mL), glutamine (200 mM), the restriction enzyme *Sac*I, and the DNA polymerase (Phusion polymerase) were purchased from Thermo Fisher Scientific (Pittsburg, PA, USA). Primers were procured from Eurofins Genomics (Ebersberg, Germany). [d-Ala^2-MePhe^4-Gly-ol]encephalin (DAMGO), fetal bovine serum (FBS) and poly-D-lysine were from Sigma Aldrich (Overijse, Belgium). Detailed information on chemical reagents used for processing of blood samples can be found elsewhere[13].

6.2.2 Plasmids and constructs

The plasmid containing the human *ARRB2* (NM_004313) coding sequence was purchased from Origene Technologies (Rockville, MD, USA). The human *OPRM1* (NM_000914) coding sequence was kindly provided by Prof. K. Van Craenbroeck. The expression vectors, NB MCS-1, NB MCS-2, NB MCS-3 and NB MCS-4 were kindly provided by Promega (Madison, WI, USA). The expression vectors contain the sequences encoding the subunits of the NanoLuc® luciferase (LgBiT or SmBiT) and the flexible linker (GSSGGGGSGGGGSSG). The βarr2−LgBiT, βarr2−SmBiT, LgBiT−βarr2 and SmBiT−βarr2 expression vectors were described in Chapter

3[11]. Expression plasmids containing MOR constructs (MOR−LgBiT and MOR−SmBiT) were constructed by cloning PCR products, flanked by a *Sac*I site, into the respective vectors, NB MCS-1 and NB MSC-2 using the primers described in Table 6.1.

Table 6.1. Primers used to clone the protein of interest (POI) in the expression plasmids. Six extra nucleotides precede the restriction site *Sac*I (underlined). In the reverse primer, an extra nucleotide was added to correct the reading frame. The Kozak sequence (bold) was also added. The nucleotides in italics are the coding sequences of the POI.

Vector	POI	Primers (5' → 3')		T_m (°C)
NB MCS-1	MOR	Forward	ACTCAA GAGCTC **ACC** *ATGGACAGCAGCGCTGCCC*	77.3
NB MCS-2		Reverse	ACTCAA GAGCTC C *GGGCAACGGAGCAGTTTCTGC*	

PCR was performed on 100 pg of plasmid DNA using the Phusion High-Fidelity PCR Master Mix with HF Buffer (Thermo Fisher Scientific, Pittsburg, PA, USA) according to the manufacturer's instructions. Reactions were done in a Mastercycler™ Nexus Thermal Cycler (Eppendorf, Hamburg, Germany) with an initial denaturation at 98°C for 30s, 35 cycles of 98°C for 10s (denaturation), Tm for 30s (annealing), and 72°C for 90s (elongation), followed by a final extension at 72°C for 5min. The resulting amplification products were purified using E.Z.N.A.® MicroElute Cycle-Pure kit (VWR International, Radnor, PA, USA).

Both the expression vectors and the amplification products were digested with *Sac*I restriction enzyme (Thermo Fisher Scientific) and purified using E.Z.N.A.® MicroElute Gel Extraction kit (VWR International). The digested PCR products were ligated into the corresponding dephosphorylated (TSAP Thermosensitive Alkaline Phosphatase, Promega), digested vector. After transformation of One Shot® Mach1™ T1 Phage-Resistant Chemically Competent E. coli (Thermo Fisher Scientific) with the ligated product, the ampicillin-resistant clones were screened by PCR using primers complementary to sequences within the insert and sequences of the vector surrounding the insert. Positively screened colonies were grown and used for plasmid isolation, using E.Z.N.A.® Plasmid DNA Mini kit (VWR International). The integrity of all inserts was confirmed by DNA sequencing. The GRK2 plasmid was a kind gift from Laura Bohn[14].

6.2.3 Cell Culture and MOR Reporter Assay

Human embryonic kidney (HEK) 293T cells were routinely maintained at 37°C, 5% CO_2, under humidified atmosphere in Dulbecco's modified eagle's medium (high glucose) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 0.25 µg/mL amphotericin B. For experiments, HEK 293T cells were plated in 6-well plates at $5x10^5$ cells/well. The next day, cells were transiently transfected using FuGENE® HD reagent according to the manufacturer's protocol (optimal ratio of FuGENE:DNA 3:1). Transfection mixes contained 3.3 µg of the plasmids of interest. On the

third day, cells were plated on poly-D-lysine-coated 96-well plates at $5x10^4$ cells/well and incubated overnight. The cells were washed twice with Opti-MEM® I Reduced Serum Medium to remove any remaining FBS, and 90 µl of Opti-MEM® I was added. The Nano-Glo Live Cell reagent (Promega, Madison, WI, USA), a nonlytic detection reagent containing the cell permeable furimazine substrate, was prepared by diluting the Nano-Glo Live Cell substrate 20-fold using Nano-Glo LCS Dilution buffer, and 25 µl 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 min).

For agonist experiments, we added 20 µl per well of test compounds, present as 6.75× stocks in Opti-MEM®I. For antagonist experiments, 10 µL of the antagonist stock solution (12.5× stock solution in Opti-MEM® I) was incubated for 5 min before adding 10 µL of agonist (13.5× stock solution in Opti-MEM® I). For analysis of biological extracts, evaporated extracts (see further, Blood Sample Preparation) were reconstituted in 100 µL of Opti-MEM® I, of which 20 µL was added per well. The luminescence was continuously detected for 120 min. Solvent controls were run in all experiments.

6.2.4 Statistical Analysis

Curve fitting and statistical analyses were performed using GraphPad Prism (San Diego, CA, USA). To select the optimal configuration for the MOR reporter assay, results are represented as mean area under the curve (AUC) ± standard error of mean (SEM) with five-six replicates for each data point and were statistically analyzed using Student's *t* test after *F*-test and Grubbs' outliers test ($\alpha = 0.05$). Curve fitting of concentration−effect curves via nonlinear regression was employed to determine EC/IC_{50} (a measure of potency).

6.2.5 Blood Sample Preparation and Analysis

Samples were extracted by solid phase extraction using 130 mg Clean Screen® DAU extraction columns (UCT, Bristol, PA, USA). To an aliquot of 500 µL of blood, 50 µL of internal standard solution was added (in case of liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis). Samples were pretreated with 2 mL of phosphate buffer (pH 6), mixed and centrifuged for 5 min. The columns were conditioned with 3 mL of methanol, followed by 3 mL of deionized water and 1 mL of phosphate buffer. After application of the sample, the columns were washed with 1.5 mL deionized water, 0.5 mL 0.1 M acetic acid and 1.5 mL methanol and dried under full vacuum for 5 min. Samples were eluted with 2 mL of ethyl acetate/acetonitrile/ammonium hydroxide (78/20/2). Finally, the eluent was evaporated to dryness at 40°C.

For the MOR reporter assay, the residue was redissolved in 100 µL Opti-MEM®I. For the LC-MS/MS analysis, the residue was reconstituted in 60/40 mobile phase (0.1% formic acid in water: 0.1% formic acid in methanol). The blood samples were analyzed using a previously published validated method for the determination of synthetic opioids U-47700, U-50488 and furanyl fentanyl in blood[13] by Melissa Friscia and Amanda Mohr. The method was updated to included despropionyl fentanyl (4-ANPP), butyrylfentanyl and α-methylfentanyl. All authentic samples were also routinely evaluated for common drugs of abuse and therapeutic compounds, including other MOR agonists, using gas chromatography–mass spectrometry (GC-MS) and LC-Quadrupole Time-of-Flight (Q-TOF, Xevo G2-S, Waters Corporation).

6.3 Results

6.3.1 Design of the MOR Reporter Assay

NanoLuc binary technology utilizes a structural complementation-based approach to monitor protein interactions within living cells. 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. Here, the aim was to establish an assay capable of monitoring activation of MOR by authentic biological extracts. Constructs were designed in which the LgBiT or SmBiT subunit was coupled to the MOR C-terminus and to the N- or C-terminus of βarr2. Upon MOR activation, βarr2 interacted with the receptor, promoting structural complementation of the NanoLuc luciferase subunits. This restored luciferase activity, which generated a bioluminescent signal in the presence of the furimazine substrate. To assess functional complementation of the LgBiT and SmBiT fusion proteins upon MOR activation, all possible combinations were tested by stimulation with a known MOR agonist, DAMGO (Figure 6.1).

Whenever MOR and βarr2 fusion proteins were present together, unstimulated cells readily showed a signal above background (i.e., the signal when only the MOR fusion protein was present), pointing at some level of constitutive MOR−βarr2 interaction (grey bars in Figure 6.1A). Regardless of the combination of MOR and βarr2 fusion proteins used, a significant increase in signal was observed upon agonist stimulation (black bars in Figure 6.1A). Although the highest signals were observed for the MOR−SmBiT/LgBiT−βarr2 combination, the MOR−LgBiT/SmBiT−βarr2 combination yielded the largest increase (5.69-fold) following activation. Hence, further experiments were conducted with the MOR−LgBiT/SmBiT−βarr2 combination (Figure 6.1B).

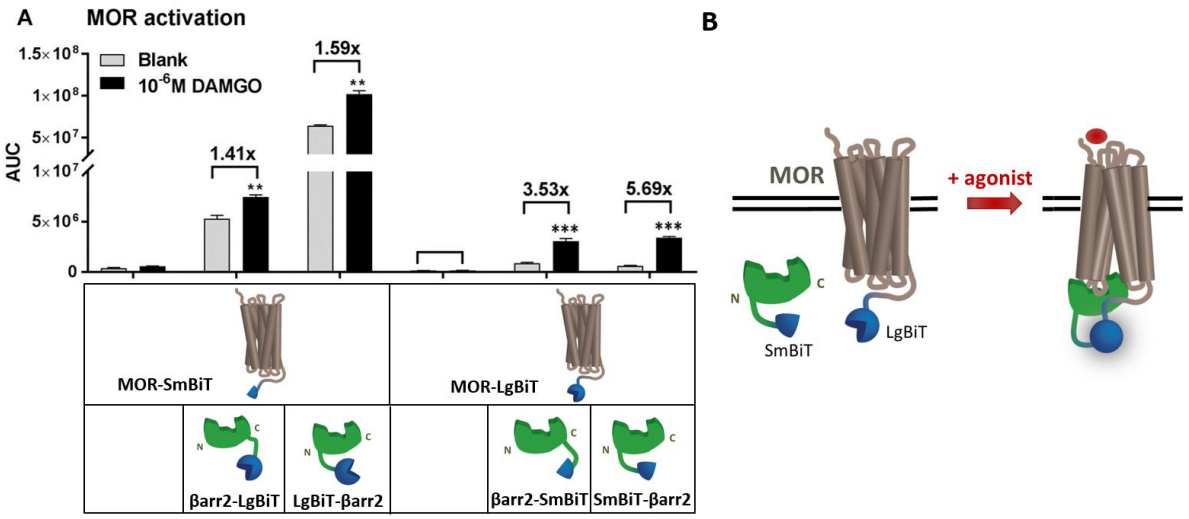


Figure 6.1. (A) Comparison of the different configurations of the MOR reporter assay. HEK293T cells were transiently transfected with equal amounts of the fusion constructs (total DNA amount 3.3 µg). Upon stimulation with 1µM of DAMGO, the luminescence of each combination was assessed. Data are given as the mean area under the curve (AUC) ± SEM (n = 6); *P ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001 (two-sided t-test). **(B)** Optimal design of the MOR reporter assay: MOR−LgBiT/SmBiT−βarr2.

6.3.2 G-protein coupled receptor kinase confers increased sensitivity to the MOR Reporter Assay

When hydromorphone, a more soluble morphine analog, was applied in the bioassay, a concentration dependent effect was observed, although at low concentrations (1 - 10 nM [0.285 - 2.85 ng/mL] hydromorphone) no signal was obtained (Figure 6.2A, inset). Because our aim was to use this MOR reporter assay to screen for opioid activity in biological matrices, the ability to generate a signal at lower concentrations was a prerequisite. Morphine-like opioids differ profoundly from opioids such as DAMGO in their propensity to induce MOR phosphorylation and internalization[14-15]. Only upon overexpression of G-protein coupled receptor kinase 2 (GRK2), morphine-like opioids have been reported to gain the capacity to induce MOR phosphorylation and internalization[14, 16].

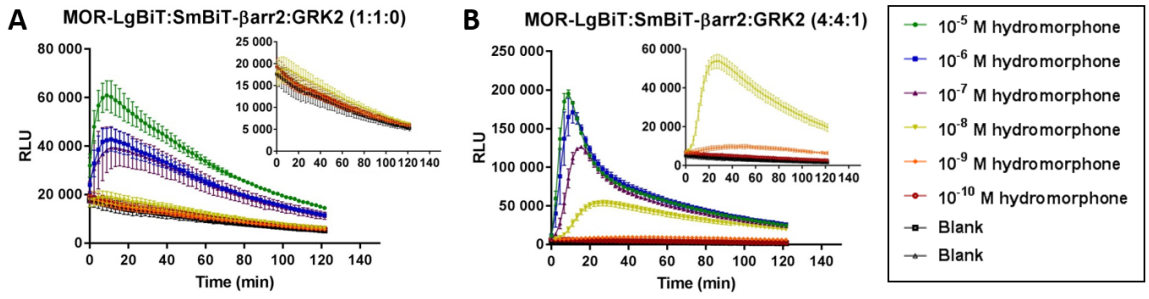


Figure 6.2. Influence of GRK2 on the performance of the MOR reporter assay. HEK293T cells were transiently transfected with MOR−LgBiT, SmBiT−βarr2 and GRK2 in different ratios **(A)** 1:1:0 and **(B)** 4:4:1 (total DNA amount 3.3 µg). A concentration gradient with hydromorphone was applied and the bioluminescence was measured for 120 minutes. Data are given as the mean relative light units (RLU) ± SEM (n = 2).

Since the functionality of our bioassay relied on βarr2 recruitment, which is known to depend on MOR phosphorylation, we evaluated the influence of adding GRK2 to our cell system. Co-expression of GRK2 resulted in a stronger recruitment of SmBiT-βarr2 to MOR-LgBiT (Figure 6.2B, inset). The result was an increased analytical sensitivity of the MOR reporter assay, with detectable signals at concentrations as low as 1 nM (= 0.285 ng/mL) of hydromorphone. Further experiments were performed with cells that were transiently transfected with MOR−LgBiT, SmBiT−βarr2 and GRK2 (ratio 4:4:1).

6.3.3 Application of the MOR Reporter Assay on different opioids

Concentration-dependent curves were obtained for a panel of opioid agonists/antagonist and EC_{50}/IC_{50} values were determined as a measure of relative potency (Table 6.2).

Table 6.2. EC/IC_{50} values (measure of potency) of different opioids. Curve fitting of concentration−effect curves via nonlinear regression was employed to determine EC/IC_{50}. Data are given as EC/IC_{50} values (95% CI profile likelihood).

Opioid agonist	**MOR EC_{50} (in nM)**
DAMGO	2.14 (1.35-4.63)
Hydromorphone	9.37 (5.60-14.8)
Buprenorphine	8.56 (6.52-11.3)
Norbuprenorphine	1.28 (0.93-2.48)
Fentanyl	4.32 (2.43-7.83)
U-47700	6.52 (3.83-11.4)
Furanyl fentanyl	2.98 (0.57-11.4)
Carfentanil	0.027 (0.021-0.035)
Opioid antagonist	**MOR IC_{50} (in nM)**
Naloxone	0.61 (0.32-1.22)

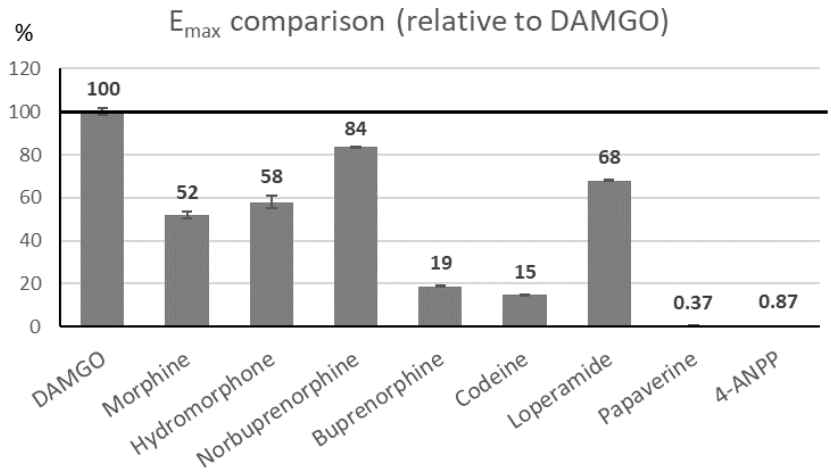


Figure 6.3. Comparison of E_{max} of different opioids. HEK293T cells were transiently transfected with MOR−LgBiT, SmBiT−βarr2 and GRK2 in a ratio of 4:4:1 (total DNA amount 3.3 µg). Upon stimulation with 1000 ng/mL of each compound, the luminescence of each combination was assessed. Results were normalized to DAMGO. Data are given as the normalized E_{max} ± SEM (n = 3).

Although it was difficult to compare EC_{50} values from different assays, due to different experimental setups, our values were in line with those found in the literature, with most compounds having EC_{50} values in the low nanomolar range. Only carfentanil, an ultra-potent

synthetic opioid, has an EC_{50} in the subnanomolar range. Feasel[17] reported an EC_{50} of 0.006 nM for carfentanil and 0.511 nM for fentanyl (PerkinElmer® LANCE Ultra cAMP Assay)[17], supporting the substantially stronger potency of the former also found here (carfentanil EC_{50} = 0.027 nM; fentanyl EC_{50} = 4.32 nM). For buprenorphine and its active metabolite norbuprenorphine, the bioassay's results (Figure 6.3; DAMGO E_{max} = 100±7%, buprenorphine E_{max} = 19±2%; norbuprenorphine E_{max} = 84±6%) were also consistent with literature (E_{max} = 38±8% and 81±4% for buprenorphine and norbuprenorphine respectively, compared to reference agonist DAMGO (E_{max} = 100%); GTPγS binding assay in CHO cell membranes)[18].

6.3.4 Application of the MOR Reporter Assay on authentic blood samples

Hundred and seven authentic unique postmortem blood samples were analyzed in two batches in the opioid reporter assay (Table 6.3). For the positive samples, selection was based upon the presence of U-47700 (9 samples) or furanyl fentanyl (29 samples). Both opioids have been implied in intoxications and fatalities in Europe and the US[13, 19-37] and have been placed into temporary Schedule I status under the Controlled Substances Act since November 2016[38-39]. In April 2018, U-47700 has been placed definitively under Schedule I of Controlled Substances Act[39]. 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. There were also 50 intra- and inter-batch replicates (both positives and negatives), resulting in a total of 157 samples that were scored (Table 6.4). The presence and number of these replicates were not known by the two scoring individuals. As a result, some samples were unknowingly analyzed in duplicate/triplicate. All but 3 out of 50 blind-scored replicates generated the same result, supporting the consistency of the MOR reporter assay. Interestingly, these three cases with discrepant scoring were samples that eventually turned out to be false positives (see below), with the first scoring yielding only a weak positivity.

Table 6.3. Results from the bioassay of all 107 authentic blood samples (* estimated concentration).

Positively scored samples	**Negatively scored samples**
8 x U-47700 (74.5 – 547 ng/mL)	1 x U-47700 (252 ng/mL) **+ naloxone**
21 x furanyl fentanyl (< 1 – 38.8 ng/mL)	7 x furanyl fentanyl (< 1 – 42.9 ng/mL) **+ naloxone**
	1 x furanyl fentanyl (< 1 ng/mL), unknown if naloxone was present
4 'opioid negative' samples	55 'opioid negative' samples
	1 x **alfentanil** (< 0.8 ng/mL*)
3 x **(nor)buprenorphine**	1 x **sufentanil** (< 0.4 ng/mL*)
1 x **loperamide/desmethylloperamide**	2 x dextrorphan/**levorphanol**
1 x **codeine/**4-ANPP/papaverine	1 x dextromethorphan/**levomethorphan**

From the nine samples containing U-47700 (at 74.5 – 547 ng/mL), eight were correctly scored positive (Table 6.3, Table 6.4). From the 29 samples containing furanyl fentanyl, 21 were correctly scored positive (<1 – 38.8 ng/mL). In the 8 missed samples (1 containing 252 ng/mL U-47700 and 7 containing <1 to 42.9 ng/mL furanyl fentanyl), several contained relatively high levels of opioids, an additional Q-TOF screening was performed to find an explanation for the non-detection. In one sample, containing <1 ng/mL furanyl fentanyl, there was no sample left to do the additional Q-TOF analysis. In all other samples that had been missed (n = 8), Q-TOF analysis revealed the presence of naloxone, a known MOR antagonist. Naloxone present in the extract can inhibit MOR activation, ultimately preventing the formation of an opioid signal. Therefore, the presence of naloxone in a biological extract is an intrinsic limitation when using the MOR reporter assay, as applied here, for screening, as detection is based on the agonistic properties of opioids. Hence, a negative result obtained from samples containing naloxone (or other MOR antagonists) should be considered inconclusive. Overall, when not considering the naloxone containing samples, for the U-47700 samples all eight samples were scored positive, leading to a sensitivity of 100% (8/8). For furanyl fentanyl, where one sample could not be retested for the presence of naloxone, a sensitivity of 95% (21/22) was achieved.

Interestingly, besides the samples that had been selected because they contained U-47700 or furanyl fentanyl, nine other samples were scored as positive (Table 6.3, Table 6.4). In three samples of these, the screening results revealed the presence of norbuprenorphine and buprenorphine, which are known MOR agonists (Table 6.2, Figure 6.3). In fourth positively scored sample, an additional Q-TOF analysis revealed the presence of the MOR agonist loperamide and its active metabolite desmethylloperamide (Figure 6.3)[40]. The additional Q-TOF analysis revealed the presence of 4-ANPP (8.7 ng/mL), codeine and papavarine (both not quantified) in a fifth positively scored sample. 4-ANPP is an intermediate in the synthesis of fentanyl and can be found as an impurity in fentanyl preparations. However, 4-ANPP is not able to generate a signal in the MOR reporter assay at high concentrations (1 000 ng/mL, Figure 6.3). Papaverine also does not show any opioid activity (Figure 6.3). Codeine, on the other hand, is capable of generating a signal, although to a lesser extent than loperamide and norbuprenorphine, but at a similar level as buprenorphine (Figure 6.3). In four positively scored samples, the Q-TOF analysis did not reveal any opioid compounds. These samples did give a profile distinct from the blank that was run in the same batch, although the positivity was relatively weak compared to the samples containing opioids (Figure 6.4). Interestingly, three of these samples were unknowingly scored again in another batch, there being scored negative. Hence, during the first scoring these were false positives.

Table 6.4. List of 107 authentic blood samples (part A).

Sample number	Number of replicates analyzed	Content	Bioassay results	Screened Via	Screen findings
U-47700 positive samples					
1	2	U-47700 (74.8)	+	QTOF	**Naloxone,** Etizolam, 3-Fluorophenmetrazine
2	1	U-47700 (103)	+	QTOF	Diphenhydramine 694 ng/mL
3	2	U-47700 (242)	+	QTOF	Delta-9 carboxy THC 5.3 ng/mL
4	1	U-47700 (252)	-	QTOF	Caffeine, Desmethylcitalopram, Citalopram, N,N-didesmethyl U-47700, Nalorphine, **Naloxone,** Nicotine, Trazodone
5	1	U-47700 (337)	+	Not Screened-NMS	
6	1	U-47700 (374.6)	+	QTOF	Amphetamine, Caffeine, Cotinine, Paroxetine
7	1	U-47700 (382)	+	QTOF	Amphetamine 12 ng/mL, caffeine
8	1	U-47700 (453)	+	Not Screened-NMS	
9	2	U-47700 (547)	+	QTOF	Nicotine, Cotinine, Caffeine, Etizolam, Clonazepam, Diclazepam
Furanyl fentanyl (Fu-F) positive samples					
10	1	Fu-F (Positive)/4-ANPP (Positive)	- (FN)	GC/MS	Nicotine, Cotinine, Diphenhydramine, Caffeine, Mirtazapine, Norhydroxyzine, Desmethylsertraline, Amiodipine
11	1	Fu-F (Positive)/4-ANPP (Positive)	-	QTOF	4-ANPP, Caffeine, Codeine, Cotinine, Diazepam, Furanylfentanyl, Morphine, Nalorphine, **Naloxone,** Nicotinamide, Nicotine, Nordiazepam, Quinine/Quinidine, Hydroquinidine, Temazepam
12	1	Fu-F (Positive)/4-ANPP (7.3)	+	GC/MS	No positive findings, Screened for fentanyl
13	1	Fu-F (Positive)/4-ANPP (5.3)	+	GC/MS	No positive findings, Screened for fentanyl
14	1	Fu-F (1)	+	GC/MS	4-ANPP-Positive, Nicotine, Amphetamine, Cotinine, Diphenhydramine, Doxylamine, Caffeine, Nordiphenhydramine, Norfentanyl, Quinine, Temazepam
15	2	Fu-F (1.0)/4-ANPP (1.6)	-	QTOF	Nicotine, Cotinine, Diphenhydramine, Caffeine, Mirtazapine, Nordiphenhydramine, Desmethylsertraline, Desmethylmirtazapine, Sertraline, Furanylfentanyl, 8-hydroxymirtazapine, Nalorphine, **Naloxone,** Prazosine, Theobromine
16	1	Fu-F(2.1)/4-ANPP (6.4)	+	GC/MS	No positive findings, Screened for fentanyl
17	1	Fu-F (2.2)/4-ANPP (1.6)	-	QTOF	Acetaminophen, Caffeine, Cocaine, Cotinine, Diphenhydramine, Furanylfentanyl, Hydromorphone, **Naloxone,** Nicotinamide, Nicotine, Noscapine
18	1	Fu-F(2.2)/4-ANPP (23.3)	+	GC/MS	Nicotine, Cotinine, Methylecgonine, Diphenhydramine, Doxylamine, Dextromethorphan, Cocaine, Nordiphenhydramine, Quinine
19	2	Fu-F (2.5)/4-ANPP (Positive)	+	GC/MS	No positive findings, Screened for fentanyl
20	1	Fu-F (2.7)/4-ANPP (15.9)	+	GC/MS	No positive findings, Screened for fentanyl
21	1	Fu-F (2.8)/4-ANPP (10)	+	GC/MS	No positive findings, Screened for fentanyl
22	2	Fu-F (4.6)/4-ANPP (18.2)	+	GC/MS	No positive findings, Screened for fentanyl
23	1	Fu-F (5.8)	+	GC/MS	4-ANPP-Positive, Nicotine, Amphetamine, Methamphetamine,Cotinine, Diphenhydramine, Caffeine, Cathine, Ephedrine, Nordiphenhydramine, Quinine
24	2	Fu-F (6.0)/4-ANPP (14.0)	+	GC/MS	No positive findings, Screened for fentanyl
25	2	Fu-F (6.1)/4-ANPP (11.5)	+	GC/MS	No positive findings, Screened for fentanyl
26	1	Fu-F (6.1)/4-ANPP (7.0)	+	GC/MS	No positive findings
27	1	Fu-F (6.6)/4-ANP (2.2)	-	QTOF	Benzoylecgonine, Caffeine, Cocaethylene, Cocaine, Diphenhydramine, Ecgonine methyl ester, Furanylfentanyl, Metoprolol, Hydroxymetoprolol, **Naloxone,** Nicotine, Quinine/Quinidine
28	1	Fu-F (7.0)/4-ANPP (15.8)	+	GC/MS	4-ANPP-Positive, Nicotine, cotinine, diphenhydramine, caffeine, 4-EEC, amitriptylline, nordiphenhydramine, nortriptylline, norcyclobenzaprine
29	2	Fu-F (7.3)/4-ANPP (7.6)	-	QTOF	4-ANPP, Caffeine, Cotinine, Diphenhydramine, Ecgonine methyl ester, Furanylfentanyl, Hydroquinidine, Morphine, Nalorphine, **Naloxone,** Nicotine, Quinine/Quinidine
30	1	Fu-F (7.5)	+	GC/MS	4-ANPP-Positive, Nicotine, Cotinine, Diphenhydramine, Nordiphenhydramine, Quinine
31	1	Fu-F (11.8)	+	GC/MS	4-ANPP-Positive, Nicotine, MPBP, Cotinine, Diphenhydramine, EMDP, EDDP, Methadone, Nordiphenhydramine, Quinine, Alprazolam
32	1	Fu-F (12.4)	+	QTOF	THC (0.84 ng/mL), **Naloxone**, 4-ANPP
33	2	Fu-F (13)/4-ANPP (5.5)	+	GC/MS	Diphenhydramine, Caffeine, Nordiphenhydramine
34	1	Fu-F (15.5)	+	GC/MS	4-ANPP-Positive, Nicotine, Cotinine, Ecgonine Methyl Ester, Ethylecgonine, Diphenhydramine, Caffeine, Ropivacaine, Cocaine, Nordiphenhydramine, Levamisole, Quinine
35	1	Fu-F (29.7)	-	QTOF	4-ANPP-Positive, Nicotine, MPBP, Cotinine, Diphenhydramine, Caffeine, Nordiphenhydramine, Alprazolam, **Naloxone**
36	1	Fu-F (35.2)	+	GC/MS	4-ANPP-Positive, Nicotine, Methylecgonine, Diphenhydramine, Caffeine, Methadone, Nordiphenhydramine, Alprazolam
37	1	Fu-F (38.8)	+	GC/MS	4-ANPP-Positive, Nicotine, MPBP, Cotinine, Ecgonine methyl ester, Diphenhydramine, Amitriptylline, Cocaine, Cyclobenzaprine, Cocaethylene, Mirtazipine, Nordiphenhydramine, Levamisole, Nortryptylline, norcyclobenzaprine, desmethylmirtazipine
38	1	Fu-F (42.9)/4-ANPP (>100)	-	QTOF	Ethanol, Caffeine, **Naloxone,** 7-aminoclonazepam, Paroxetine
Other positive samples from bioassay					
39	2	(Nor)buprenorphine	+	QTOF	Nicotine, Cotinine, **Naloxone,** Theophylline, Caffeine, **Norbuprenorphine, Burprenorphrine**, Alpha-OH-Alprazolam, Alprazolam
40	2	(nor)buprenorphine	+	QTOF	Cotinine, Acetaminophen, Caffeine, 7-Aminoclonazepam, 9-Hydroxyrisperidone, **Norbuprenorphine, Buprenorphine**, Amitriptylline, Nortriptylline, Clonazepam, Oxazepam, Temazepam, Nordiazepam, Diazepam
41	1	(Nor)buprenorphine	+	QTOF	Nicotine, Cotinine, Caffeine, **Norbuprenorphine, Buprenorphine**, Desmethylsertraline, Lorazepam
42	2	(Desmethyl)loperamide	+	QTOF	Acetminophne, Atropine, Caffeine, Diphenhydramine, Lamotrigine, **Loperamide**, Metoprolol, **Desmethylloperamide,** Hydroxymetoprolol, Nicotine, mCPP, Trazodone
43	1	4-ANPP (8.7), codeine	+	QTOF	4-ANPP, Caffeine, **Codeine,** Diphenhydramine, Nicotine and Papaverine

Table 6.4. List of 107 authentic blood samples (part B).

False positives					
44	2	No Opioids (Blank)	-/+ (FP)	QTOF	7-aminoclonazepam, Clonazepam, Tempazepam, Diazepam, Caffeine
45	2	No Opioids (Blank)	-/+ (FP)	QTOF	Cotinine, Amphetamine, Caffeine, Paroxetine, Fluoxetine, Norfluoxetine, THC
46	2	No Opioids (Blank)	-/+ (FP)	QTOF	Caffeine, Diphendramine, Theobromine
47	1	No Opioids (Blank)	+ (FP)	QTOF	Glipizide
True negatives					
48	2	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Acetaminophen, Caffeine, Diphenhydramine
49	1	No Opioids (Blank)	-	QTOF	Blank
50	3	No Opioids (Blank)	-	GC/MS	Caffeine, Theobromine
51	3	No Opioids (Blank)	-	QTOF	Benzoylecgonine, Cocaine
52	2	No Opioids (Blank)	-	QTOF	Caffeine, Mirtazapine, Hydroxybupropion, Bupropion, Norclozapine, Quetiapine, Cyclobenzaprine
53	2	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Caffeine, Diphenhydramine, Alprazolam
54	2	No Opioids (Blank)	-	QTOF	Caffeine, Diphenhydramine
55	2	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Atropine, Lidocaine
56	2	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Diphenhydramine, Nordiazepam
57	2	No Opioids (Blank)	-	QTOF	Caffeine, Cyclobenzaprine, Duloxetine, Amitriptylline, Nortriptylline, Alprazolam
58	1	No Opioids (Blank)	-	QTOF	Monoethylglycinexylidide (MEGX), Lidocaine, Phentermine, Zolpidem
59	1	No Opioids (Blank)	-	QTOF	Lamotrigine, Quetiapine, Alprazolam
60	1	No Opioids (Blank)	-	QTOF	Alprazolam
61	1	No Opioids (Blank)	-	QTOF	Alpha-OH-Alprazolam, Alprazolam
62	3	No Opioids (Blank)	-	QTOF	Blank
63	2	No Opioids (Blank)	-	QTOF	Caffeine
64	2	No Opioids (Blank)	-	QTOF	Amphetamine, Methamphetamine, Caffeine
65	2	No Opioids (Blank)	-	QTOF	Caffeine, Olanzapine, Mirtazapine, Fluoxetine, Norfluoxetine, Lorazepam
66	2	No Opioids (Blank)	-	QTOF	Caffeine, Guaifenesin, Duloxetine
67	1	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Amphetamine, Caffeine
68	3	No Opioids (Blank)	-	QTOF	Caffeine
69	2	No Opioids (Blank)	-	QTOF	Caffeine, Glyburide
70	1	No Opioids (Blank)	-	QTOF	Atropine, Caffeine
71	2	No Opioids (Blank)	-	QTOF	Theophylline, Caffeine, Warfarin, Trimethoprim
72	2	No Opioids (Blank)	-	QTOF	Cotinine, Olazapine, Caffeine, Haloperidol, Quetiapine
73	3	No Opioids (Blank)	-	QTOF	Caffeine
74	1	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Caffeine
75	1	No Opioids (Blank)	-	QTOF	Citalopram/Escitalopram, Alpha-OH-Alprazolam
76	1	No Opioids (Blank)	-	QTOF	Ziprasidone
77	1	No Opioids (Blank)	-	QTOF	Blank
78	1	No Opioids (Blank)	-	QTOF	Caffeine
79	1	No Opioids (Blank)	-	QTOF	Blank
80	1	No Opioids (Blank)	-	QTOF	Caffeine
81	1	Naloxone	-	QTOF	Naloxone Only
82	1	No Opioids (Blank)	-	QTOF	Blank
83	1	No Opioids (Blank)	-	QTOF	Caffeine, Trazodone
84	1	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Caffeine
85	1	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Caffeine, Nordiazepam
86	1	No Opioids (Blank)	-	QTOF	Blank
87	1	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Methamphetamine, Caffeine, Etomidate
88	1	No Opioids (Blank)	-	QTOF	Nitotine, Cotinine, Phendimetrazine
89	2	No Opioids (Blank)	-	QTOF	Caffeine
90	1	No Opioids (Blank)	-	QTOF	Caffeine
91	1	No Opioids (Blank)	-	QTOF	Caffeine
92	2	No Opioids (Blank)	-	QTOF	Blank
93	1	No Opioids (Blank)	-	QTOF	Caffeine, Diphenhydramine, Duloxetine
94	1	No Opioids (Blank)	-	QTOF	Theophylline, Caffeine, 7-Aminoclonazepam, Citalopram/Escitalopram, Promethazine, Cyclobenzaprine
95	1	No Opioids (Blank)	-	QTOF	Nicotine, Cotinine, Quetiapine, Sertraline, Desmethylsertraline
96	1	No Opioids (Blank)	-	QTOF	Cotinine, Caffeine, Desmethylsertraline, Alprazolam
97	2	No Opioids (Blank)	-	QTOF	Amphetamine, Topiramate, Paroxetine
98	2	No Opioids (Blank)	-	QTOF	Caffeine, Carbamazepine-epoxide, Maprotiline, Amitryptyline, Carbamazepine, Nortriptyline, Lorazepam, Nifedipine
99	2	No Opioids (Blank)	-	QTOF	Cotinine, Phenylpropanolamine, Norpseudoephedrine, Pseudoephedrine, Caffeine
100	1	No Opioids (Blank)	-	QTOF	Norpseudoephedrine, Pseudoephedrine, Caffeine
101	1	No Opioids (Blank)	-	QTOF	Amitriptyline, Nortriptyline, Ibuprofen
102	2	No Opioids (Blank)	-	QTOF	Caffeine, Sertraline, Desmethysertraline
Negative in bioassay, but presence of opioids via Q-TOF					
103	2	Sufentanil	-	QTOF	Caffeine, Haloperidol, **Sufentanil,** Nordiazepam
104	2	Alfentanil	-	QTOF	Acetaminophen, Caffeine, Lidocaine, Diphenhydramine, **Alfentanil,** Midazolam, 1-hydroxymidazolam, Prochlorperazine
105	2	Dextrorphan/Levorphanol	-	QTOF	**Dextrorphan/Levorphanol,** Chlorpheniramine, Sertraline, Desmethylsertraline
106	1	Dextro/Levomethorphan	-	QTOF	Caffeine, Doxylamine, **Dextro/Levomethorphan,** Paroxetine, Desmethylsertraline
107	2	Dextrorphan/Levorphanol, sufentanil	-	QTOF	Acetaminophen, Theophylline, Amphetamine, Methamphetamine, Caffeine, **Dextrorphan/Levorphanol**

Next to the negatively scored samples described above, another 60 samples were scored negative in our bioassay (Table 6.3, Table 6.4). The Q-TOF analysis revealed the presence of alfentanil and sufentanil, both synthetic opioids, in two samples. These samples were not picked up by the MOR reporter assay, presumably due to the very low concentrations that were present (not quantifiable, but estimated below 1 ng/mL). Three samples contained dextromethorphan/levomethorphan or dextrorphan/levorphanol. Levomethorphan and levorphanol are known MOR agonists, whereas their isomeric counterparts do not have any opioid activity. As the LC-MS/MS method cannot distinguish between the enantiomers (dextro- and levoform), it is not known what form is (mainly) present. As no activity is found in these samples, this could be explained by the presence of the inactive enantiomer (dextroform). From the 59 opioid negative samples, 55 samples were correctly scored negative (minus the 4 false positive samples, see above), leading to an analytical specificity of 93% (55/59). When considering the fact that 3 out of the 4 false positives were unknowingly scored once more, then yielding a negative result, specificity could be up to 98% (58/59).

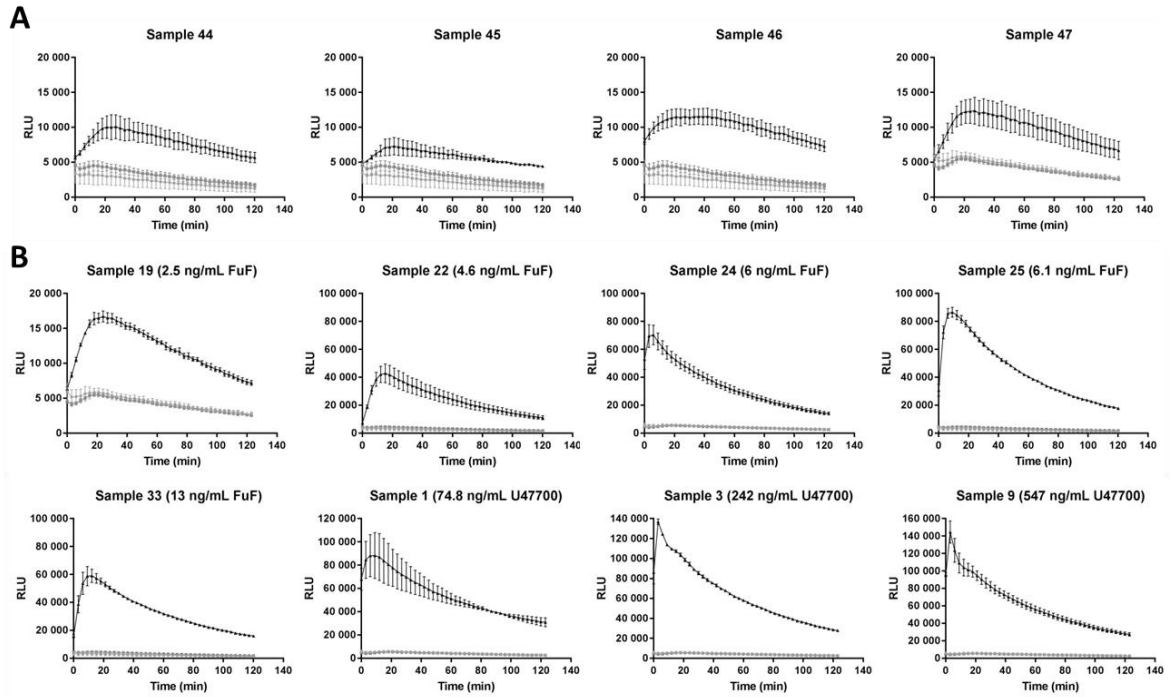


Figure 6.4. Examples of the read out of the MOR reporter assay. **(A)** The four false positives. **(B)** Positive samples run in same batch. All graphs have two blanks (gray) and the sample (black). Data are given as the mean relative light units (RLU) ± SEM (n = 2).

6.4 Conclusion

Here, we developed an alternative untargeted screening method for the detection of opiates and (synthetic) opioids, not directly based on their structure, but on their opioid activity. The MOR reporter assay allowed a rapid identification of opioid activity in blood samples.

Although we measured opioid activity for 120 min, strong opioid intoxications had their maximal signal within 15-20 min, allowing a quick confirmation of a suspected opioid overdose when having the assays ready for use. In all cases the positive outcome would not have changed if the analysis would have stopped at 20 min. In the negative cases, though, the full 120 min allowed a better comparison with the blanks. The read-out of the bioassay happens in a 96-well plate, allowing the analysis of multiple samples in the same run. This high-throughput capability is especially important when screening of large patient cohorts is needed, e.g. in the context of chemical warfare or attacks or for centralized labs that need to analyze large batches of clinical and/or forensic samples. Mass spectrometry is still required for definitive identification of the opioid but is often not routinely available in hospital laboratories for real time testing and is less relevant in acute intoxications[1].

An intrinsic limitation of the MOR reporter assay, as applied here, is that the presence of opioid antagonists, such as naloxone, interfere with the read-out. As the developed bioassay works via the agonistic properties of opioids, the presence of an antagonist might prevent the formation of an opioid signal. Likely in the naloxone positive cases in this study, the naloxone administration came too late to counteract the central nervous system depression in the patient. However, in these samples naloxone may still be present in concentrations that are high enough to counteract the opioid activity in the bioassay. In a clinical context, in the vast majority of patients where naloxone is administered, this will be known by the physician. As a consequence, it will be important that this information is passed on to the laboratory deploying the assay or, alternatively, the bioassay should be performed together with a naloxone assay. If naloxone is present in the sample, a negative result in the MOR reporter assay should at this stage be considered inconclusive, as naloxone might have prevented formation of a signal. A positive result in the bioassay in the presence of naloxone, should be considered positive. In this case the amount of naloxone was not enough to hamper formation of the opioid signal (which is the case in sample 1, 32 and 39 in Table 6.4). Potential solution to cope with this limitation imposed by the presence of opioid antagonists might be to include a minimal concentration of a MOR agonist readily at the start of the bioassay. When naloxone is present, a decrease in signal will be seen in that case. Again, that would suggest the involvement of an opioid, as naloxone will likely have been administrated for a reason.

The MOR reporter assay reported here consolidates the novel principle of activity-based screening for a broad range of new psychoactive substances, which are posing substantial challenges to clinical and forensic toxicology laboratories. It will be important to extend the application of this bioassay to even larger cohorts of patient samples to further establish the assay's performance.

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CHAPTER 7: ACTIVITY-BASED DETECTION AND BIOANALYTICAL CONFIRMATION OF A FATAL CARFENTANIL INTOXICATION: A CASE REPORT

Based on

Annelies Cannaert, Lars Ambach, Peter Blanckaert, Christophe P. Stove. Activity-based detection and bioanalytical confirmation of a fatal carfentanil intoxication. *Frontiers in Pharmacology* **2018**, 9, 486. DOI: 10.3389/fphar.2018.00486.

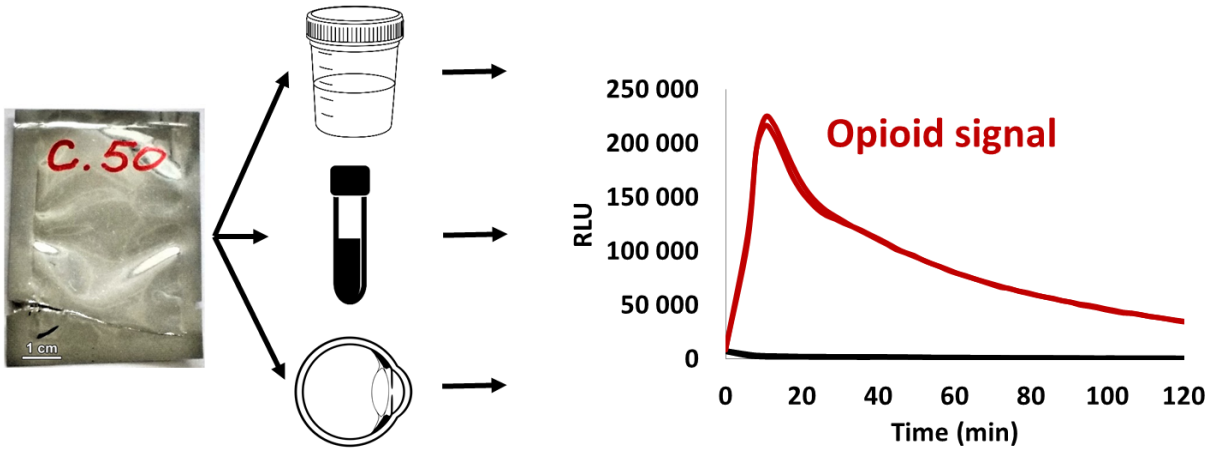
Abstract

Carfentanil, one of the most potent opioids known, has recently been reported as a contaminant in street heroin in the United States and Europe, and is associated with an increased number of life-threatening emergency department admissions and deaths. Here, we report on the application of a novel in vitro opioid activity reporter assay and a sensitive bioanalytical assay in the context of a fatal carfentanil intoxication, revealing the highest carfentanil concentrations reported until now.

A 21-year-old male was found dead at home with a note stating that he had taken carfentanil with suicidal intentions. A foil bag and plastic bag labeled “C.50” were found at the scene. These bags were similar to a sample obtained by the Belgian Early Warning System on Drugs from a German darknet shop and to those found in the context of a fatality in Norway.

Blood, urine and vitreous, obtained during autopsy, were screened with a newly developed in vitro opioid activity reporter assay able to detect compounds based on their m-opioid receptor activity rather than their chemical structure. All extracts showed strong opioid activity. Results were confirmed by a bioanalytical assay, which revealed extremely high concentrations for carfentanil and norcarfentanil. It should be noted that carfentanil concentrations are typically in pg/mL, but here they were 92 ng/mL in blood, 2.8 ng/mL in urine, and 23 ng/mL in vitreous. The blood and vitreous contained 0.532 and 0.300 ng/mL norcarfentanil, respectively. No norcarfentanil was detected in urine.

This is the first report where a novel activity-based opioid screening assay was successfully deployed in a forensic case. Confirmation and quantification using a validated bioanalytical procedure revealed the, to our knowledge, highest carfentanil concentrations reported in humans so far.



Graphical abstract of Chapter 7

7.1 Introduction

Carfentanil, a very potent derivative of the pharmaceutical opioid fentanyl, was developed in 1974 by Janssen Pharmaceutica[1]. It is one of the most potent opioids known at ~10 000 times the potency of morphine and ~30-100 times the potency of fentanyl in the tail withdrawal test in rats[1]. Commercially, it is always sold in combination with the μ-opioid antagonist naloxone due to its extreme toxicity in humans. Carfentanil is used to immobilize large exotic wildlife and has been implicated in the 2002 Moscow theatre hostage crisis[2-3]. Recently, carfentanil and other synthetic opioids have been reported as a contaminant in street heroin in the USA and Europe, and have been associated with an increased number of life-threatening emergency department admissions and deaths[4-7]. Here, we report on the application of a novel cell-based bioassay and a sensitive bioanalytical assay using analytical equiment in the context of a fatal carfentanil intoxication, in which we found the highest carfentanil concentrations reported until now.

7.2 Case Presentation

A 21-year-old male was found dead at home along with a note stating that he had taken carfentanil with suicidal intentions, in addition notifying first responders that care should be taken, given the potency of the compound. A foil bag and plastic bag labelled “C.50” were found at the scene (Figure 7.1A), suggesting that up to 50 mg of carfentanil may have been insufflated by the decedent. Remarkably, during routine monitoring of new psychoactive substances (NPS) present on darknet websites by the Belgian Early Warning System on Drugs, a carfentanil sample was obtained with strikingly similar packaging and handwriting as the packaging found on the scene of death in this toxicological case (Figure 7.1B). A similar bag with identical labelling in similar handwriting has also been reported in the context of a fatality in Norway (Figure 7.1C), where the powder was apparently ordered from a German darknet shop[8]. Based on this information, the vendor (or primary source) is most probably the same vendor as mentioned in other publications[8-9].

A swab of the plastic bag tested positive for carfentanil via GC-MS analysis. Biological matrices available were blood, urine and vitreous. Routine toxicological analyses were performed on peripheral blood and urine. This involved, in addition to immunological screening by EMIT and ELISA, the use of HPLC-diode-array detection (DAD) and GC–MS for screening and quantification of drugs and headspace-GC-FID for the determination of ethanol and other volatile compounds, essentially following procedures described before[10]. GC-MS screening of blood and urine revealed the presence of caffeine, theobromine, propranolol, sertraline and cannabinoids in nontoxic doses. Immuno-assay based screening for fentanyl (Fentanyl Direct Elisa Kit, Immunalysis, Pomona, CA, USA) was negative.

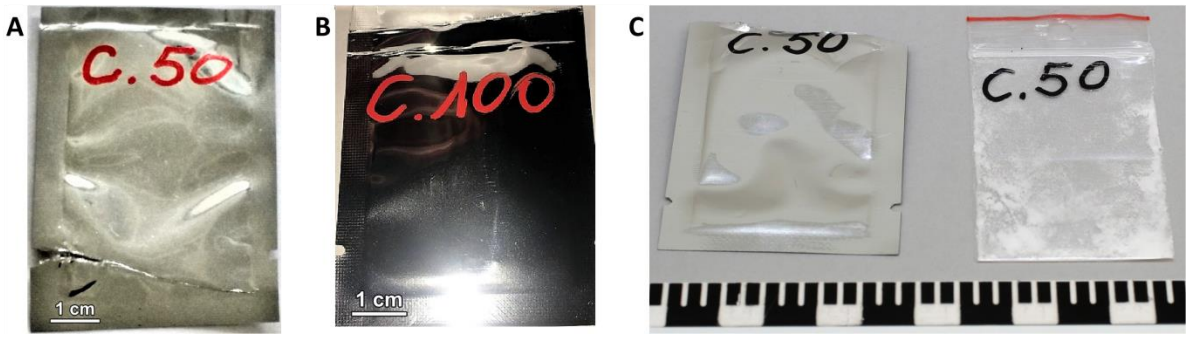


Figure 7.1. (A) foil bag found at the scene, **(B)** foil bag obtained by the Belgian Early Warning System on Drugs, **(C)** foil bag and plastic bag found in a fatality in Norway (image used with kind permission of the National Criminal Investigation Service/Photo (Norway)[8].

An additional opioid screening of the biological matrices was done with a new in-house developed μ-opioid receptor (MOR) activity reporter assay. We recently reported on cell-based cannabinoid reporter assays for the activity-based detection of synthetic cannabinoids and their metabolites, demonstrating cannabinoid activity in authentic urine and blood samples[11-12]. A similar bioassay was set up by using the μ-opioid receptor to screen for opioid activity in bulk materials and biological samples (Chapter 6)[13]. The principle of the bioassay is activity-based, using an *in vitro* cell system, in which activation of the μ-opioid receptor leads to the recruitment of the cytosolic β-arrestin 2 (βarr2) protein, which results in functional complementation of a split NanoLuc luciferase, thereby restoring luciferase activity. In the presence of the substrate furimazine, this results in a bioluminescent signal, which can be read out with a standard luminometer.

In practice, expression vectors encoding human MOR or βarr2, fused via a flexible linker to the subunits of NanoLuc luciferase (LgBiT or SmBiT), were generated using standard molecular biology techniques, similar as in Chapter 3[14]. These constructs, with addition of a G-protein coupled receptor kinase 2, were used to transiently transfect human embryonic kidney (HEK) 293T cells, which were seeded in poly-D-lysine-coated 96-well plates at 5×10^4 cells/well and incubated overnight before performing the assay. On the day of the assay, the cells were washed twice with Opti-MEM® I reduced serum medium to remove any remaining fetal bovine serum, and 90 µL of Opti-MEM® I was added. The Nano-Glo Live Cell reagent, a nonlytic detection reagent containing the cell-permeable furimazine substrate, was prepared by diluting the Nano-Glo Live Cell substrate 20× using Nano-Glo LCS Dilution buffer, and 25 µL was added to each well. Subsequently, the plate was placed in a GloMAX96 plate reader (Promega, Madison, WI, USA). Luminescence was monitored during the equilibration period until the signal stabilized (30 min). For agonist experiments, we added 20 µl per well of test compounds, present as 6.75× stocks in Opti-MEM® I. Also for the analysis of biological extracts, 20 µL was added per well. These extracts were generated from 250 µL of matrix (blood, urine or vitreous), which was added to 1000 µL of ice-cold acetonitrile, followed by

shaking for 5 min at 1400 RPM and centrifuging for 20 min at 20 000 *g*. After evaporation of 1 mL of supernatant under nitrogen at 40 °C, the extract was reconstituted in 100 µl of Opti-MEM® I. The luminescence was continuously detected (105 or 120 min).

Application of carfentanil and fentanyl solutions on the MOR reporter assay resulted in concentration-dependent curves and EC_{50} (95% confidence interval profile likelihood) values were determined for carfentanil (EC_{50} = 0.027 nM [0.021-0.035]) and fentanyl (EC_{50} = 4.32 nM [2.43-7.83]) as a measure of relative potency (Figure 7.2A). 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 literature. Feasel (2017) stated in his dissertation an EC_{50} of 0.006 nM for carfentanil and 0.511 nM for fentanyl (PerkinElmer® LANCE Ultra cAMP Assay)[15], which supports the significantly stronger potency, which is also found here. Norcarfentanil, the major metabolite of carfentanil, was only able to generate low opioid activity at a high concentration (1 µM / 326 ng/mL) (Figure 7.2A). All extracts from the three matrices (blood, urine and vitreous) showed very strong opioid activity. Even application of 1 µL of urine sample from the presented case (without any sample preparation) on the bioassay was able to generate a clear positive signal in the MOR reporter assay (Figure 7.2B).

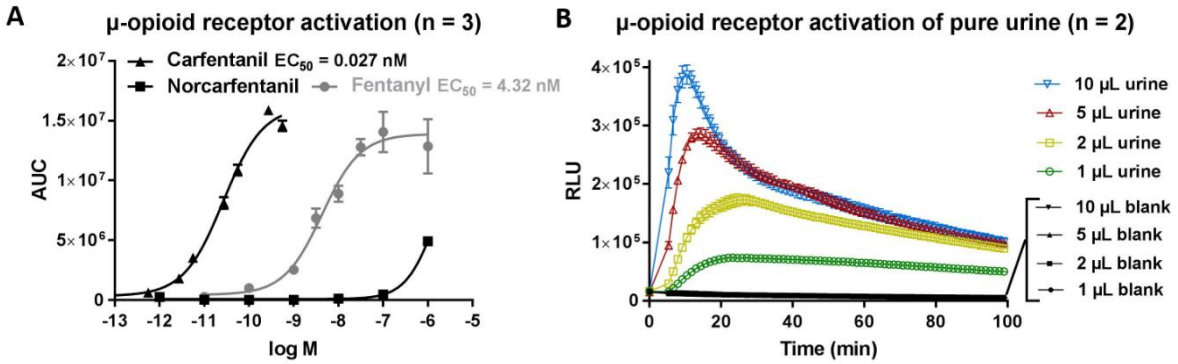


Figure 7.2. (A) µ-opioid receptor activation by fentanyl, carfentanil and norcarfentanil. **(B)** µ-opioid receptor activation of pure urine without sample preparation. AUC = area under curve. RLU = relative light units.

The screening results from the opioid activity reporter assay were confirmed by Lars Ambach with an LC-MS/MS method for carfentanil and norcarfentanil. To 250 µL sample (blood, urine or vitreous), 10 µL of internal standard solution containing fentanyl-D_5 and norcarfentanil-D_5 (0.25 ng/mL and 12.5 ng/mL, respectively) in methanol were added. Sample processing was as described above, except that reconstitution was with 55 µL acetonitrile, of which 50 µL were then mixed with 50 µL of mobile phase A (H_2O + 0.1% HCOOH) in an autosampler vial with 100 µL insert. For the analysis of carfentanil, the injection volume was 20 µL, whereas for the determination of norcarfentanil, 10 µL were injected. Chromatographic separation was achieved on a Kinetex Biphenyl column (50 × 2.1 mm, 2.6 µm) (Phenomenex, Utrecht,

The Netherlands) in a 3.7 min gradient using H_2O + 0.1% HCOOH and methanol + 0.1% HCOOH as mobile phases, at a flow rate of 0.6 mL/min. The following gradient was used: 0-0.2 min: 5%B, 0.25-0.35 min: 5-30% B, 0.35-1.5 min: 30-95% B, 1.5-2.5 min: 95% B, 2.5-2.51 min: 95-5% B, 2.51-3.7 min: 5% B. A QTRAP 5500 mass spectrometer (SCIEX, Nieuwerkerk aan den Ijssel, The Netherlands) with positive electrospray ionization in multiple reaction monitoring mode was used for detection.

For carfentanil, the following transitions were used: 395.2 > 246.1 (quantifier, declustering potential (DP): 70 V, collision energy (CE): 27 eV, collision cell exit potential (CXP): 12 V) and 395.2 > 146.2 (qualifier, DP: 70 V, CE: 37 eV, CXP: 9 V). For norcarfentanil, the transitions were 291.1 > 142.2 (quantifier, DP: 74 V, CE: 22 eV, CXP: 7 V) and 291.1 > 146.2 (qualifier, DP: 74 V, CE: 37 eV, CXP: 10 V). For fentanyl-D_5, 342.2 > 188.2 (DP: 110 V, CE: 32 eV, CXP: 10 V) was used. For norcarfentanil-D_5, the transition was 296.1 > 151.1 (DP: 75 V, CE: 38 eV, CXP: 8 V). The entrance potential was 10 V for all transitions; source temperature was set to 600 °C, ion spray voltage to 2000 V, curtain gas to 35 psi, gas 1 to 40 psi and gas 2 to 50 psi.

The method was validated in whole blood. Eight-point calibration curves were set up for carfentanil (range: 0.0025–2.5 ng/mL, linear regression with $1/x^2$ weighting) and norcarfentanil (range: 0.025–25 ng/mL, linear regression with $1/x^2$ weighting). Quality control samples at 0.015/0.25 ng/mL for carfentanil and at 0.15/2.5 ng/mL for norcarfentanil were run in sixplicate on 4 days, yielding acceptable intra- and inter-run imprecision (intra-run: <8.8%, inter-run: <14%) and bias (< ±8.7%, n = 24 at two different concentrations). Matrix effects were assessed at the two above-mentioned concentrations by comparing the signal ratios of analyte to internal standard of post-extraction-spiked samples with those of standards spiked in neat injection solvent (n = 6). Matrix effects were 78% for carfentanil and 118% for norcarfentanil. Extraction efficiency, assessed by comparing the signal ratios of analyte to internal standard of pre- versus post-extraction-spiked samples, was 66% for carfentanil and 24% for norcarfentanil (n = 6, at the two above-mentioned concentrations). Also, autosampler stability (change in concentration <9% for at least 3 days, n = 6, two different concentrations), specificity and carry-over (none within calibration range) were successfully evaluated. Dilution integrity was checked by spiking blood and aqueous samples with 100 ng/mL carfentanil and norcarfentanil, then diluting 1:1000 with blank matrix (n = 6) and comparing relative peak areas to control samples with 0.1 ng/mL (n = 6). Differences were ≤ ±13.5%.

The vitreous sample was quantified using a calibration curve in ultra-pure water. The urine sample was quantified by standard addition. To quantify carfentanil concentrations, blood and vitreous samples had to be diluted 1:1000 with blank blood and water, respectively,

while the urine sample was diluted 1:100 with blank urine. For norcarfentanil, undiluted samples were analyzed. Carfentanil concentrations were 92 ng/mL in blood, 2.8 ng/mL in urine, and 23 ng/mL in vitreous. The blood and vitreous contained 0.532 and 0.300 ng/mL norcarfentanil, respectively. No norcarfentanil was detected in urine. It should be noted that carfentanil concentrations are typically in the pg/mL range (Papsun *et al.*, 2017: 0.1–14 ng/mL, median: 0.38 ng/mL; Shanks and Behonick, 2017: 0.0102–2 ng/mL, median: 0.0984 ng/mL; Hikin *et al.*, 2018: 0.09–4 ng/mL, median: 0.234 ng/mL)[5-6, 16].

7.3 Discussion

Given the continued emergence of novel synthetic opioids, the major disadvantage for their detection via immunoassays, GC-MS and LC-MS/MS analysis is that the methods are often targeted in nature or, for the latter two, limited by the availability of pre-established mass spectral libraries. Here in this case, the immunoassay for fentanyl did not pick up carfentanil, a fentanyl analog, due to the lack of cross-reactivity. Therefore, an alternative untargeted approach for the detection of (synthetic) opioids, not directly based on the structure of the opioids, but on their opioid activity, was applied. Such an approach may serve as a first-line screening tool, complementing the conventional analytical methods which are currently used.

The high ratio of carfentanil/norcarfentanil in blood and vitreous and the absence of norcarfentanil in urine can be explained by the presumably sudden death of the victim caused by the massive overdose. The detected concentrations of carfentanil are, to the best of our knowledge, the highest ever reported in a human being. Other intoxications always state sub-ng to low ng/mL levels of carfentanil[5-6, 16-19]. In conclusion, this is the first report in which a novel activity-based opioid screening assay was successfully deployed in a forensic case, where confirmation and quantification using a validated bioanalytical procedure revealed very high carfentanil concentrations.

7.4 References

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CHAPTER 8:
BROADER INTERNATIONAL CONTEXT, RELEVANCE AND FUTURE PERSPECTIVES

The human taste for addictive substances capable of changing the functions of the central nervous system can be traced back to the earliest human records[1]. These psychoactive substances are widely distributed in the plant kingdom, and many of them were discovered already early on[2]. They have been used by priests in religious ceremonies (e.g. *Amanita muscaria*), by healers for medicinal purposes (e.g. opium), or have found their way into the general population (e.g. alcohol, nicotine, caffeine).

In the course of history, efficient methods of purification of these natural products were developed and these compounds were administrated through new routes, allowing faster access to the brain in higher concentrations. For alcohol, distillation made it possible to obtain beverages with a higher alcohol content, making it easier for people to become drunk. Similarly, cigarettes promote deep inhalation into the lungs, which allows nicotine to be rapidly absorbed into the bloodstream and to reach the brain in a few seconds, compared to alternatives such as snuffing, smoking cigars and chewing tobacco. Opium is also an example of a substance whose pattern of use changed in the last centuries, from a medication used for pain relief and anesthesia to a substance associated with abuse and dependence. Opium's capacity to induce dependence was probably reinforced by the purification of morphine and the synthesis of heroin and other potent compounds, that were available for injection[1].

Substance abuse has been described since antiquity, where Alexander the Great's death was linked to severe alcohol abuse[3]. Substance abuse has been a longstanding public health problem and is associated with substantial societal costs. Historically, drug use has been confined to a relatively limited number of drugs that were mostly well known, such as alcohol, amphetamines, cannabis, cocaine, heroin and LSD[4]. The popularity of certain drugs of abuse or drug classes changed over time and new formulations of known drugs resulted in a peak of drug use (e.g. "crack" cocaine in the 80s). However, in the last few years, the number of new drugs being introduced to the illicit drug market has drastically increased[4]. This is clearly illustrated by the fact that between 2005-2016 over 620 new psychoactive substances (NPS) were reported for the first time in Europe[5].

In addition to the rate at which new illicit drugs are being introduced, the manner in which they are being produced and sold also marks a dramatic shift from the past. In prior generations, new drugs were introduced through established illegal drug supply networks and/or were medications diverted from medical use due to their reinforcing/intoxicating properties. Many of the current generation of drugs, however, are being sold over the Internet. These drugs can be sold as "research chemicals" or are disguised in packages that do not list the drug contents, and suggest that the products are to be used as e.g. "incense" or "bath salts," and are "not for human consumption", as a means of circumventing drug laws and regulation[5]. These synthetic drugs generate the perception to be relatively safe 'legal' alternatives to established illicit drugs. Their use as intoxicants is openly described on Internet

forums and chat rooms dedicated to drug use, where consumers in many cases refer to brand names and labeling (e.g. Spice, K2)[4].

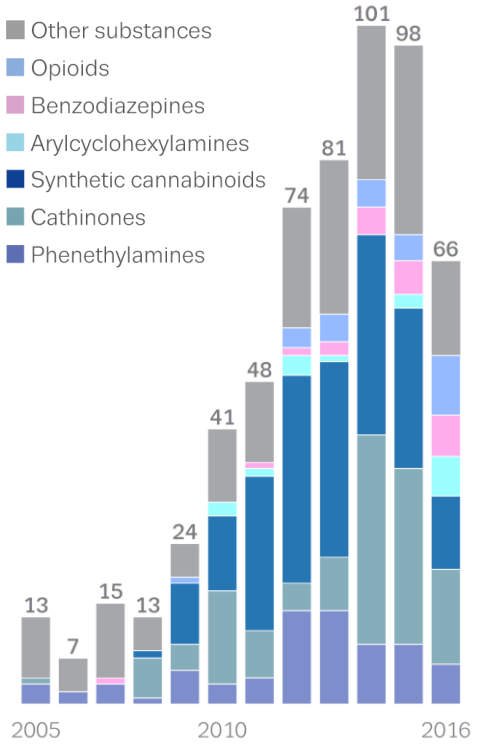


Figure 8.1. Number of NPS reported to the EU Early Warning System (2005-2016). Source: EMCDDA

From 2005 until 2014, the number of new drugs that was reported to the EU Early Warning System raised drastically (from 13 to 101 new compounds that had entered the market). In 2015, the number of new detections stabilized, while a decrease was observed in 2016, with 'only' 66 new compounds entering the European market (Figure 8.1)[5]. The causes of this decrease are unclear, but may in part be due to measures taken by national governments in Europe to prohibit new substances, particularly their open sale as 'legal highs'. An additional factor may be control measures and law enforcement operations in China, targeting laboratories producing these new substances. This decrease is a positive sign, especially if it would turn out to be sustained. However, the overall availability of NPS has not reduced. Moreover, even if the pace at which new substances are being introduced may be slowing down, the overall number of substances available on the market continues to grow[5]. There are also signs that some classes of NPS, notably synthetic cathinones and synthetic cannabinoid receptor agonists (SCRAs), are now establishing a foothold in the illicit drug market[5]. The reasons behind the (ab)use of NPS are variable and complex. Nevertheless a few common factors can be identified, including reduced availability of illicit drugs, competitive prices, their 'legal' status, specific qualities of the substances themselves ("psychonauts") and the fact that NPS are hard to detect in routine drug tests[6].

Approaches to detect NPS in biological matrices encompass immunoassays, as well as targeted and untargeted (high resolution) mass spectrometry-based methods. Although at the moment some known NPS can be detected via rapid immunological tests, these tests are quickly outdated as they target a chemical structure and cannot cope with the continuous evolution in NPS structure (Figure 8.2 and 8.3). Mass spectrometry-based techniques, on the other hand, can be time-consuming, tedious and expensive. Therefore, the detection of these new substances remains challenging in different contexts, such as forensic, clinical and analytical chemistry.

The recent proliferation of NPS has initiated considerable interest in the development of so-called 'untargeted' screening strategies (e.g. full-scan liquid or gas chromatography (high resolution) mass spectrometry) in order to detect and identify novel compounds without the use of certified reference materials or mass spectral libraries[7-8]. As a complement to existing screening strategies, we developed a novel concept for screening biological matrices for the presence of NPS, not relying on antibody-based or mass spectrometry-based recognition of the structure of these compounds, but based on their receptor activity. In this thesis, the focus lay on two groups within the NPS: the synthetic cannabinoid receptor agonists (SCRAs) and the synthetic opioids. Both classes exert their activity through G-protein coupled receptors (GPCRs).

- **SCRAs**: Currently, SCRAs are the largest group of new psychoactive substances monitored by the EMCDDA, with 169 compounds from 2008-2016. The occurrence of different SCRAs changes over time (Figure 8.2). While the number of new SCRAs entering the market might be decreasing, the amount that is seized on yearly basis is still very high[5].

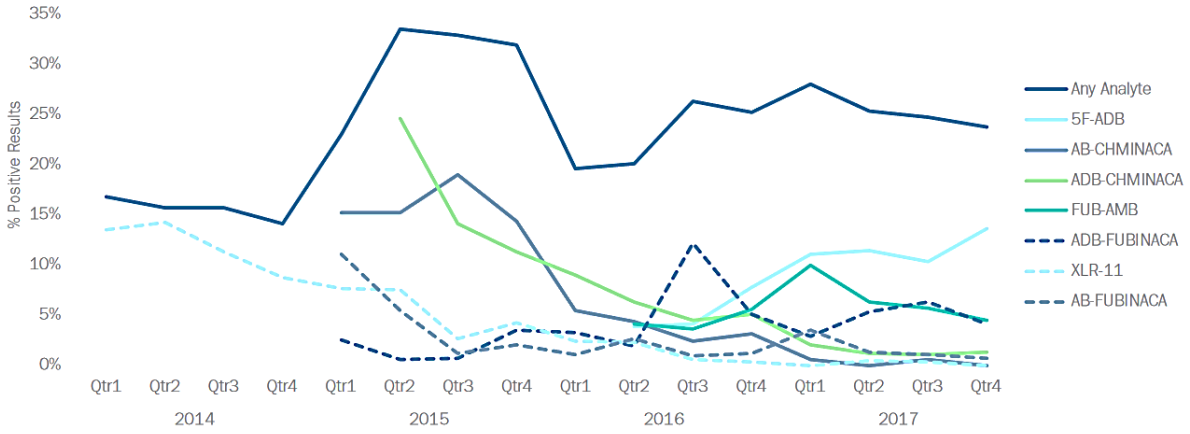


Figure 8.2. Detection of SCRAs in blood by NMS Labs (US), from 2014 to 2017[9].

- **Synthetic opioids**: The rise of synthetic opioids on the drug market started in 2012. Although in absolute figures the new synthetic opioids only play a smaller role in the European drug market, they are highly potent substances that pose a serious threat to individual and public health[5]. In Europe and especially North America, their recent emergence is causing considerable morbidity and mortality. Similar to the SCRAs, the occurrence of the synthetic opioids on the drug market changes over time (Figure 8.3).

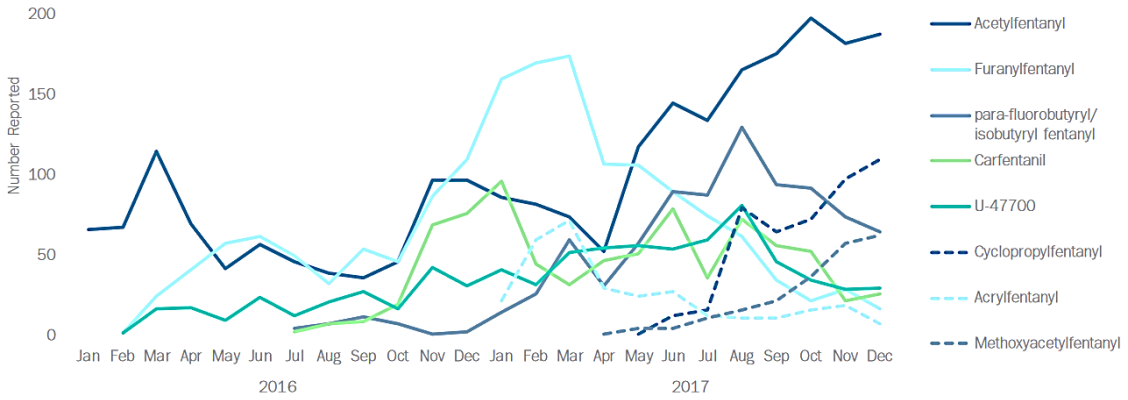


Figure 8.3. Detection of fentanyl analogs in blood by NMS Labs (US), from 2016 to 2017[10].

In **Chapter 2**, the characteristics of an ideal *in vitro* bioassay for screening purposes are discussed. These included the following characteristics: rapid, simple, sensitive, selective, reproducible and inexpensive. Here, we evaluated these characteristics for the developed bioassays for the detection of SCRAs and synthetic opioids.

- **Rapid:** As bioassays are to be applied as a screening tool, analysis should be fast and/or multiple analyses should be possible in one run. The bioassays developed in this thesis are performed in a 96-well plate, allowing multiple analyses at the same time. Although the total analysis time of 120 min can be seen as long, in the case of the opioid reporter assay strong opioid intoxications have their maximal signal within 15-20 minutes, allowing a quick confirmation of a suspected opioid overdose when having the assays ready for use. In the negative cases, though, the full 120 minutes allows a better comparison with the blanks.
- **Simple:** The bioassays do not require a lot of technical experience. In a clinical setting, lab technicians working in microbiology already have the expertise to work in a sterile environment. Additionally, the generation of stable cell lines improved the simplicity of the assays. Furthermore, the read out of the bioassay does not require highly sophisticated equipment, a standard luminometer suffices.
- **Sensitivity:** Assay sensitivity is primordial as the aim is to detect physiologically relevant concentrations of SCRAs and synthetic opioids in (extracts of) biofluids. We initially applied our SCRA bioassays on urine samples because of *i)* the anticipated higher concentrations in urine, *ii)* the fact that many phase I SCRA metabolites retain activity at CB receptors, and *iii)* the combined presence of distinct active metabolites is likely to be beneficial for the assays' sensitivity. In urine, the sensitivity of the bioassays depends on the type of SCRA (96% for UR-144/XLR-11 vs. 36% for AB-CHMINACA) as the metabolism between different SCRAs may vary. In contrast to the urine analysis, where CB receptor

activation mainly relies upon the presence of active SCRA metabolites, the activation of the bioassays by blood extracts primarily stems from the presence of the SCRA parent compound. Hence, the analysis of blood derived samples is less susceptible to potential SCRA metabolization to inactive metabolites. The results of the SCRA analysis of serum samples (n = 43) resulted in a sensitivity of 82%. A newer study (not included in this thesis) confirms the good detection rate of the bioassays for SCRAs (sensitivity of 100% (52/52) obtained in large study of 395 serum samples). For the opioid analysis, only the blood has been evaluated as a matrix, achieving good sensitivities.

- **Selectivity:** Although the developed assays should be considered as a screening tool and hence (depending on the context) some level of false positives may be allowed, they should be as selective as possible. In all bioassays, the specificity lay above 91%. In the SCRA bioassays, also samples from recent or heavy cannabis users, where natural cannabinoids are present in the extract, might result in a positive signal, as can be expected. For the opioid analysis, (ab)use of classic opiates/opioids (e.g. morphine, heroin) will also yield a positive signal. Additionally opioid antagonists (e.g. naloxone), used as an antidote in the case of overdoses (see more below), will interfere with the read out of the bioassay as an antagonist will hamper the activation of the μ-opioid receptor.
- **Reproducible:** The results of the screening method should be robust. For the SCRA bioassay, all extracts were run twice and gave the same outcome. In the evaluation of the opioid bioassay (described in Chapter 6), we had 50 intra- and inter-run replicate samples (both positives and negatives). The presence and number of these replicate samples were not known by the two scoring individuals. As a result, some samples were unknowingly analyzed in duplicate/triplicate. All but 3 out of 50 blind-scored replicate samples generated the same result, supporting the consistency of the opioid reporter assay. Interestingly, these three cases with discrepant scoring were samples that eventually turned out to be false positives.
- **Inexpensive and high-throughput-amenable:** The bioassays developed in this thesis are performed in a 96-well plate, allowing large samples sets to be run to identify suspicious samples, which can subsequently be tested with more advanced systems. The price for the bioassays is around 1 euro per well, being competitive with or lower than the price for conventional immune assays.

In **Chapter 3, 4 and 5**, the development and application of the SCRA screening assays on several biological matrices were discussed. While applying the MOR reporter assay on a set of authentic blood samples (as discussed in **Chapter 6**), an inherent limitation of this opioid activity-based screening approach readily became apparent: the activity-based assay fails in the presence of opioid antagonists, such as naloxone, which are used as antidotes for opioid

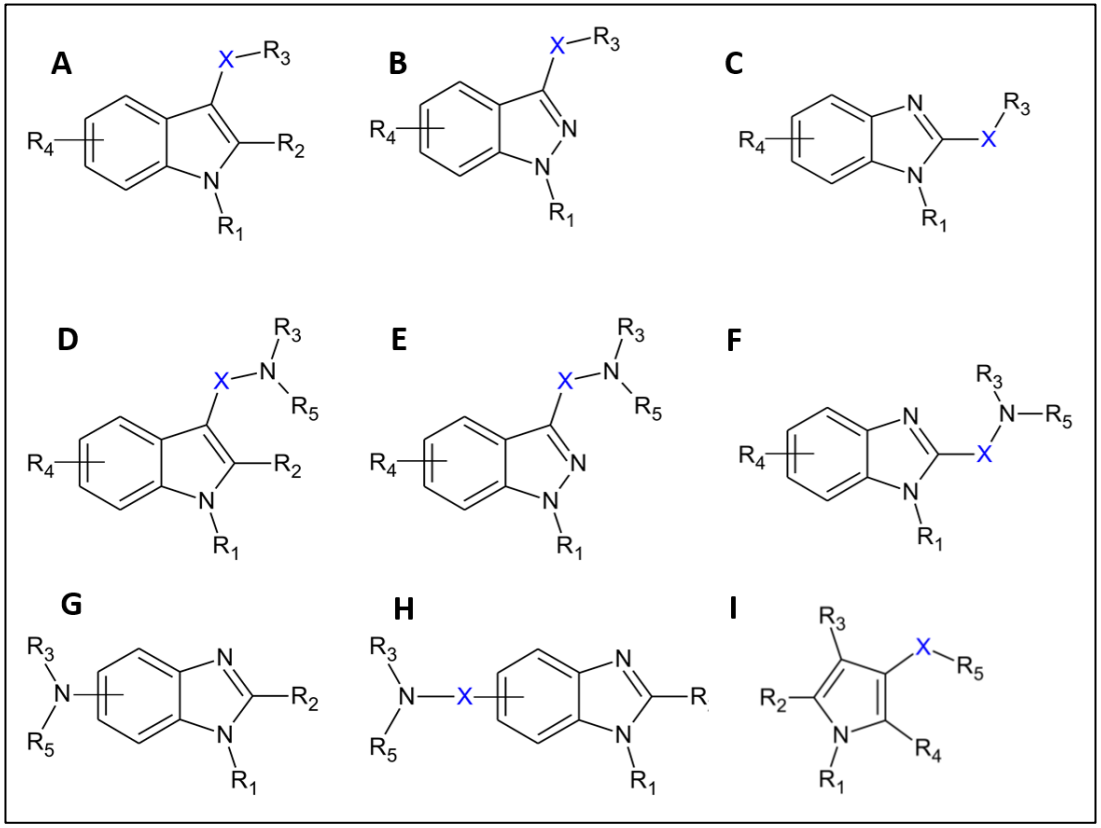
overdoses. Until recently, this administration only took place in a controlled setting (hospital, in the presence of a doctor), but now also a self-administration form has entered the market. Narcan® Nasal Spray is the first and only FDA-approved nasal form of naloxone for the emergency treatment of a known or suspected opioid overdose. This spray counteracts the life-threatening effects of opioid overdose. Since most accidental overdoses occur in a home setting, it was developed for first responders, as well as family, friends, and caregivers. Obviously, the presence of an opioid antagonist in bodily fluids poses a potential problem for the principle onto which the developed activity-based bioassay is based: when dealing with a sample where both an opioid agonist and an opioid antagonist are present, the latter can inhibit the effect of the former. The result is a false negative, i.e. a sample where an opioid may be present at a high concentration, but which is falsely scored negative. One way to deal with this in the future is to optimize the screening system in such a way that a basal level of activation is present**.** This level should be minimal, in order not to lose sensitivity for detecting other opioids. On the other hand, the level of activation should be high enough in order to allow visualization of a decrease when an antagonist is present. Different ligands at different (minimal) concentrations will need to be tested to come to a robust, optimized system. Either way, a deviation from blank points at the possible involvement of an opioid: a decrease in signal points at the presence of an antagonist, an increase will indicate the presence of opioids.

Next to the application of the bioassays as a screening tool, it can also help with legislative issues. Legislations based on individual structures are consequently lagging behind, as the identification and subsequent prohibition of single SCRAs drives clandestine chemists to produce analogues of increasing structural diversity, intended to evade legislation[8, 11-12]. Alternatively, the newer analogue laws in the US (2012)[13] and UK (2016)[14], controlling all "cannabimimetic" agents and substances with psychoactive properties (e.g., via the CB1 receptor), are also challenged as the specific pharmacology of new compounds is mostly unknown[15]. This could be efficiently countered by applying these new compounds in biological assays to establish their cannabinoid activity and therefore their illegality.

In more and more countries, including Belgium, analog laws or generic structure laws are introduced, aiming at rendering all current and future analogs of a given structure illegal. On the next two pages, the current Belgian structure legislation is displayed for SCRAs and the fentanyl derivatives[16]. On top of this, also some SCRAs, fentanyl analogs and non-fentanyl opioids are illegalized via a nominative legislation. This generic structure law is only part of the solution, as this implies that toxicological labs should be able to detect (use of) all these substances, which is currently an utopia.

BELGIAN GENERIC STRUCTURE LAW FOR SYNTHETIC CANNABINOID RECEPTOR AGONISTS (ROYAL DECREE. 06.09.2017)

For the indoles (A, D), indazoles (B,E) and benzodiazoles (C,F,G,H)



X = -CH2, -C(=O)-, -CH2O-, -C(=O)O- or –C(=O)NH-.

R_1: C_nH2_{n+1}, C_nH2_{n-1}, C_nH2_{2n-3} (n = 1-7), phenyl, benzyl, cyclohexylmethyl; with potential extra substitutions with one of the following groups or combination thereof: OH, C(=O)OH, halogen, CN, tetrahydropyranyl, morpholinyl, N-methylpyrrolidinyl, N-methylpiperidinyl or another functional group with maximum 7 C-atoms.

R_2: H, C_nH2_{n+1}, C_nH2_{n-1}, C_nH2_{2n-3} (n = 1-7).

R_3: phenyl, benzyl, phenylethyl, naphthalenyl, adamantanyl, quinolinyl, tetracyclopropyl or another functional group with maximum 7 C-atoms; with potential extra substitutions with one of the following groups or combination thereof: halogen, OH, CH_2OH, C(=O)OH, azide, dimethylamino, CN, NO_2 or another functional group with maximum 7 C-atoms.

R_4: H, halogen, methyl, OH, OCH_3, NO_2, CN (on any position the 6-ring of the indole-, indazole- or beznodiazole-group).

R_5: H, phenyl, benzyl, phenylethyl, naphthalenyl, adamantanyl, quinolinyl, tetracyclopropyl or another functional group with maximum 7 C-atoms; with potential extra substitutions with one of the following groups or combination thereof: halogen, OH, CH_2OH, C(=O)OH, azide, dimethylamino, CN, NO_2 or anoher functional group with maximum 7 C-atoms.

For the pyrroles (I)

X = -CH2, -C(=O)-, -CH2O-, -C(=O)O- or –C(=O)NH-.

R_1: C_nH2_{n+1}, C_nH2_{n-1}, C_nH2_{2n-3} (n = 1-7), phenyl, benzyl, cyclohexylmethyl; with potential extra substitutions with one of the following groups or combination thereof: OH, C(=O)OH, halogen, CN, tetrahydropyranyl, morpholinyl, N-methylpyrrolidinyl, N-methylpiperidinyl or another functional group with maximum 7 C-atoms.

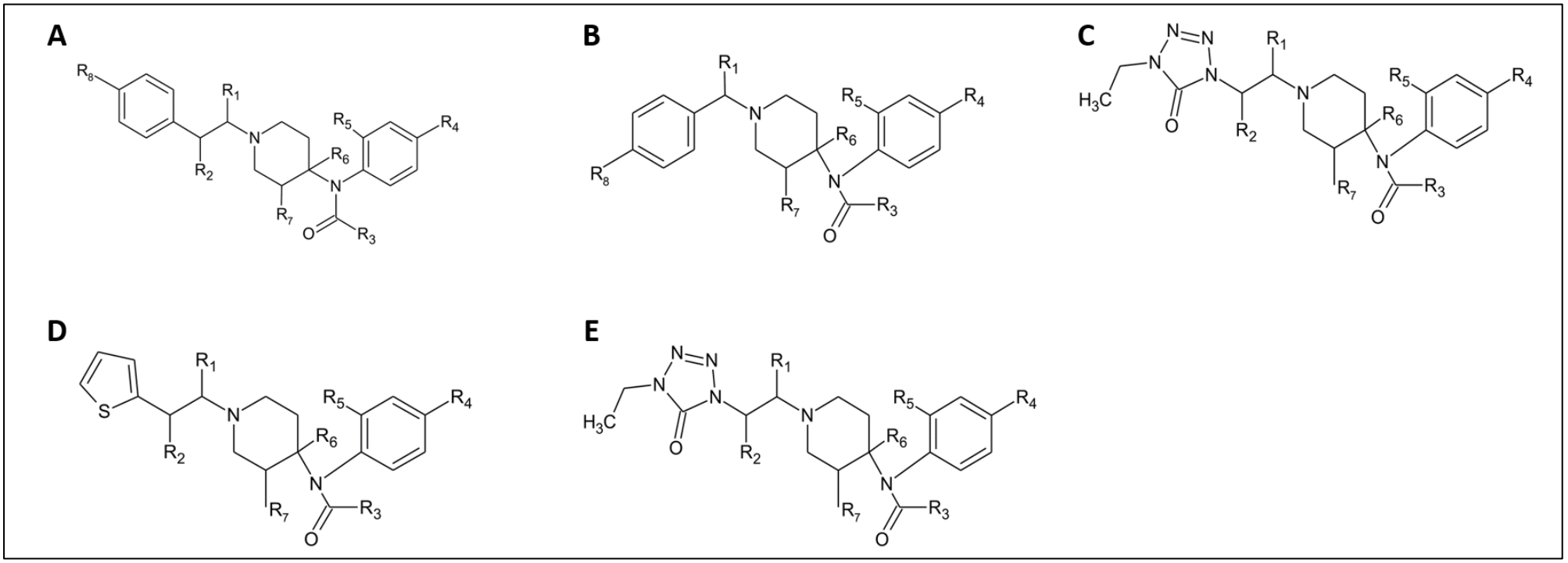
R_2: H, halogen, phenyl, halogenphenyl, naphtyl, or another functional group with maximum 7 C-atoms.

R_3: H, halogen, phenyl, halogenphenyl, naphtyl, or another functional group with maximum 7 C-atoms.

R_4: H, halogen, phenyl, halogenphenyl, naphtyl, or another functional group with maximum 7 C-atoms.

R_5: naphtylgroup, or one or more mono- or polycycloalkylgroups (maximum 7 C-atoms), potentially with extra halogen substitutions.

BELGIAN GENERIC STRUCTURE LAW FOR FENTANYL DERIVATIVES (ROYAL DECREE. 06.09.2017)



***N*-phenyl-1-(2-phenylethyl)piperidin-4-amine (A), 1-benzyl-*N*-phenylpiperidin-4-amine (B), 1-ethyl-4-{[4-(phenylamino)piperidin-1-yl]methyl}[1]-1,4-dihydro-5*H*-tetrazol-5-one (C), *N*-phenyl-1-[2-(thiophen-2-yl)ethyl]piperidin-4-amine (D), 1-ethyl-*N*-phenylpiperin-4-amine (E).**

R_1: H, CH_3

R_2: H, OH

R_3: C_2H_5, $CH(CH_3)_2$, CH_2-O-CH_3 or another functional group with maximum 7 C-atoms.

R_4: H, halogen, OCH_3

R_5: H, halogen, OCH_3

R_6: H, CH_3, $C(O)OCH_3$, CH_2-O-CH_3

R_7: H, CH_3

R_8: H, halogen, OCH_3

In future, further expansion for opioid detection could be the inclusion of the other opioid receptors, the κ-opioid receptor (KOR) and the δ-opioid receptor (DOR), as some synthetic opioids are (more) selective for these receptors. Another class of drugs, that can be targeted with a similar activity-based approach can be psychedelics, more specific tryptamines and phenethylamines (e.g. NBOMes), which act as agonists at the serotonin receptor (5-HT_{2A}), also a GPCR. Here, it will be important to evaluate if common drugs (e.g. serotonin reuptake inhibitors, commonly used as antidepressants) will influence the read-out of the bioassay.

Although application of the activity-based bioassays reported in this thesis is relatively simple, it does require the presence of a basic cell culture facility and some basic skills. Therefore, we do not envisage a global implementation in all clinical laboratories but see this more in a centralized setting or in larger, specialized laboratories, where samples can be analyzed at a higher throughput. Establishing computer-based learning to allow automated scoring of the samples, reducing the format from 96-well to 384-well plates, as well as robotized pipetting steps may help to achieve high-throughput. A future step to allow a more general use is the generation of kits consisting of frozen cells, plates to seed the cells, media, reagents, and positive and negative controls. In this format, there could be as little as 24 h between thawing of the cells and read-out of the result. In addition, this format would only require minimal technical experience for the technician.

The bioassay-based detection, presented in this thesis, also has some limitations. To determine if a sample contains cannabinoid and/or opioid activity, multiple assays need to be performed (CB1, CB2 and MOR bioassay). The current set-up does not allow simultaneous detection of cannabinoids and opioids within the same bioassay. In addition, although we presented a case report on an opioid intoxication (**Chapter 7**) in which we showed that the application of pure urine (without sample preparation) was able to generate a positive result in the MOR reporter assay, we do believe some sample preparation will be necessary for the bioassay's sensitivity. The carfentanil intoxication was an exceptional case in which very high levels of carfentanil, a super potent opioid, were found. In that case, even 1 µL of urine was enough to generate an opioid signal. Moreover, application of pure biofluids is only possible for watery fluids, such as urine, serum, plasma and vitreous, but not for (lysed) blood, as addition of the latter in the bioassays interferes with the read-out. For analysis in the bioassay the same sample preparation as the one used for other screening methods (e.g. LC-MS) can be used, with as a difference that no (labeled) internal standard (with cannabinoid/opioid activity) can be added, as this may interfere. The extracts of the biofluids do not need to be fully clear. Although dirty extracts might interfere with the read-out of the bioassay and may reduce its sensitivity (as we found for the SCRA assays; **Chapter 4**), there is no risk of damaging (expensive) equipment, which is the case when e.g. using LC-MS(/MS).

While the activity-based bioassays can discriminate between positive and negative samples, they remain screening assays, not capable of identifying the substance that was used. They can only indicate in which direction should be (not) looked. In the case of the cannabinoid bioassay, it is possible to discriminate between the use of SCRAs and cannabis (with active compound THC, a partial agonist), as the signal obtained for SCRAs is much higher. Therefore a high signal in the cannabinoid bioassay does point to the use of SCRAs. Combining CB1 and CB2 bioassays can even indicate whether the SCRA that was used has a CB1 or CB2 preference. For opioids, the activity-based assay will not be able to discriminate between different opioids, as most of them are potent compounds. If a person consumed pure heroin or heroin, mixed with fentanyl analogs, in both cases an opioid signal will be obtained and further testing will be necessary to identify the specific substance. In addition, in the absence of opioid antagonists, a negative result in the opioid bioassay can rule out the involvement of an opioid in an intoxication.

To the best of our knowledge, the Laboratory of Toxicology at Ghent University is currently the only lab worldwide that has successfully applied activity-based bioassays to screen biological samples from SCRA and synthetic opioid users. In a workshop on SCRA detection in biofluids during the January 2018 meeting of *The International Association of Forensic Toxicologists* (TIAFT), globally the leading scientific organization for forensic toxicologists, a small survey amongst the attendants learned that colleague-forensic toxicologists did agree with the following statement: “There may be a role for bio-activity-based screening of SCRA in forensic toxicology”. Although at this stage we are far from widespread implementation, this apparent acceptance grade is promising. It remains to be seen whether this concept will be picked up by other laboratories or by companies or may serve as a basis for other bioassays.

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CHAPTER 9:
CONCLUSION AND SUMMARY

In this work we focus on a novel concept in the field of forensic toxicology: the activity-based detection of new psychoactive substances (NPS) as an alternative screening approach. The emergence of NPS in recent years has brought along an explosive growth in a new segment of the illegal drug market. NPS are typically created by modifying the chemical structure of illegal drugs or prescribed medications, to generate substances that are not covered by international drug controls. They are characterized by a high market dynamics and make up a broad range of drugs. In this work the main focus lay on synthetic cannabinoid receptor agonists (SCRAs) and synthetic opioids.

The rapid proliferation of NPS has sparked considerable interest in the development of so-called 'untargeted' screening strategies, employing e.g. high-resolution mass spectrometry. However, due to the expensive and time-consuming character of this technique, this method is not routinely implemented in most clinical and forensic laboratories. Therefore, **alternative 'untargeted' activity-based screening methods** may offer a solution for this problem, by functioning as a first-line screening tool, complementing the conventional targeted and untargeted analytical methods.

In **Chapter 1**, a short introduction on NPS, more specifically on SCRAs and synthetic opioids, is given. **Chapter 2** provides an overview of activity-based reporter assays for the screening of abused substances in biological matrices. These include, next to the SCRAs and synthetic opioids, also the steroid hormones.

In the activity-based bioassays for SCRAs and synthetic opioids, that were developed within the framework of this thesis, the activity is measured through the cannabinoid receptors, CB1 and CB2, and the μ-opioid receptor, MOR, respectively. These are G-protein coupled receptors (GPCRs). Through the $G_{i/o}$ family of G-proteins, these couple to several signal transduction mechanisms and are rapidly desensitized by recruitment of the cytosolic protein β-arrestin 2 (βarr2). The latter forms the basis of the developed bioassays in this thesis. The reporter assays we developed utilize a structural complementation-based approach to monitor protein interactions within living cells (NanoLuc Binary Technology). The concept makes use of inactive subunits of the enzyme NanoLuc luciferase. These subunits, large BiT (LgBiT, 18 kDa), and small BiT (SmBiT, 1 kDa), are each coupled to a protein of interest, in our case the receptor (CB1 or CB2 or MOR) and βarr2. Upon GPCR activation, the cytosolic βarr2 protein is recruited to the receptor. This interaction promotes structural complementation of the two NanoLuc luciferase subunits, thereby restoring luciferase activity, which generates a bioluminescent signal in the presence of the substrate.

In this thesis we report on the set-up of live cell-based cannabinoid reporter assays for the activity-based detection of SCRAs and their metabolites, demonstrating cannabinoid activity in authentic urine and blood samples[1-3]. The work preceded in several stages. Initially, transient mammalian cell systems were set up expressing one of both cannabinoid receptors (CB1 or CB2) together with βarr2 (**Chapter 3**)[1]. The suitability of these newly developed CB1

and CB2 bioassays for monitoring cannabinoid activity was evaluated by successfully applying several SCRAs and their main phase I metabolites to these reporter systems. By doing so, we demonstrated that several major metabolites of these SCRAs retain their activity at cannabinoid receptors[1], which is consistent with reports by others. This is an important finding as SCRAs are strongly metabolized and almost no parent compound is found in the urine. The presence of these active metabolites in urine allows for a longer detection window for detecting SCRA use. The SCRA reporter assays (for CB1 and CB2) were used as a proof-of-concept to demonstrate cannabinoid activity in an authentic urine sample[1].

In a second step, the transient cannabinoid reporter assays were improved by generating stable cell systems (**Chapter 4**)[2]. Advantages of the improved stable bioassays as compared to the initial transient format include (i) a reduced workload, (ii) higher reproducibility within experiments and (iii) a control on stability via co-expressed markers. The utility of the stable bioassays as a screening method for SCRAs was evaluated on a relatively large set (n = 74) of authentic urine samples[2]. In a next step we aimed at improving the sensitivity of the SCRA reporter assays, as lower concentrations of SCRAs can be expected in serum or plasma samples. For this purpose, two C-terminal βarr2 truncated mutants were evaluated (**Chapter 5**). The idea to truncate βarr2 was based upon its prominent role in GPCR desensitization and signaling. This approach resulted in improved stable cell systems, which were successfully used to screen for cannabinoid activity in a set of authentic serum (n = 45) and plasma (n = 73) samples[3].

Strengthened by the promising results we obtained for activity-based detection of SCRAs in biofluids, we set-up a similar concept for the activity-based screening of biofluids for the presence of opiates and synthetic opioids (**Chapter 6**)[4]. Here, an extra addition of G-protein coupled receptor kinase 2 was necessary to promote βarr2 recruitment to the MOR. Utility of the MOR reporter bioassay was demonstrated using a set of 107 authentic blood samples. In a case report involving a fatal intoxication with the extremely potent opioid carfentanil, the MOR bioassay was successfully applied (**Chapter 7**)[5].

Whilst there is a multitude of commercially available assays for monitoring GPCR activation, the reporter assays we developed are currently the only ones that have been applied on biological matrices as an untargeted screening strategy. Whether the success of the first applications[1-5], described here, will lead to a broad dissemination and further establishment of the concept of activity-based screening in forensic toxicology, only future can tell.

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SAMENVATTING

In deze thesis ontwikkelden we een nieuw concept in het gebied van forensische toxicologie: het opsporen van nieuwe psychoactieve substanties (NPS) op basis van hun activiteit in plaats van hun structuur als een alternatieve screeningsmethode. De opkomst van NPS in de afgelopen jaren heeft een explosieve groei meegebracht op de illegale drugsmarkt. NPS worden meestal gecreëerd door de chemische structuur van illegale drugs of voorgeschreven geneesmiddelen te veranderen, om zodoende nieuwe stoffen te genereren die niet onder de internationale drugscontrole vallen. De klasse van NPS wordt gekenmerkt door een hoge marktdynamiek en vormen een breed scala aan drugs. In dit werk lag de nadruk op synthetische cannabinoïd receptor agonisten (SCRAs) en synthetische opioïden.

De snelle proliferatie van NPS heeft geleid tot een aanzienlijke interesse in de ontwikkeling van zogenaamde '*untargeted*' screeningsstrategieën, b.v. hoge-resolutie massa spectrometrie. Vanwege het dure en tijdrovende karakter van deze techniek is deze methode echter niet routinematig geïmplementeerd in de meeste klinische en forensische laboratoria. Daarom kunnen alternatieve '*untargeted*' methoden, zoals op activiteit-gebaseerde technieken een oplossing bieden voor dit probleem, door te functioneren als eerstelijns screeningsmethodiek die de conventionele analysemethoden aanvult.

In **Hoofdstuk 1** wordt een korte introductie gegeven over NPS, meer specifiek over SCRAs en synthetische opioïden. **Hoofdstuk 2** biedt een overzicht van op activiteit-gebaseerde testen voor het screenen van misbruikte stoffen in biologische matrices. Deze omvatten, naast de SCRAs en synthetische opioïden, ook de steroïde hormonen.

In de op activiteit-gebaseerde testen voor SCRAs en synthetische opioïden, die in het kader van deze thesis werden ontwikkeld, wordt de activiteit gemeten via de cannabinoïd receptoren, respectievelijk CB1 en CB2, en de μ-opioïd receptor, MOR. Dit zijn G-proteïne gekoppelde receptoren (GPCRs). Via de $G_{i/o}$-familie van G-proteïnen koppelen deze aan verschillende signaaltransductiemechanismen. Ze worden snel geïnactiveerd door rekrutering van het cytosolische eiwit β-arrestin 2 (βarr2). Dit laatste vormt de basis van de in deze thesis ontwikkelde testen. De ontwikkelde testen maken gebruik van een structurele complementatie techniek om eiwitinteracties binnen levende cellen te volgen (NanoLuc Binary Technology). Het concept maakt gebruik van inactieve subeenheden van het enzym NanoLuc luciferase. Deze subeenheden, Large BiT (LgBiT, 18 kDa) en Small BiT (SmBiT, 1 kDa), zijn elk gekoppeld aan een proteïne van interesse, in ons geval de receptor (CB1 of CB2 of MOR) en βarr2. Na GPCR activatie wordt het βarr2 eiwit gerekruteerd naar de receptor. Deze interactie resulteert in de structurele complementatie van de twee NanoLuc luciferase subeenheden, waardoor de luciferase activiteit wordt hersteld. Na toevoeging van het NanoLuc substraat furimazine, kan het resulterend lichtsignaal vervolgens gemeten worden.

In deze thesis beschrijven we de ontwikkeling van cel-gebaseerde cannabinoïd testen voor de op activiteit-gebaseerde detectie van SCRAs en hun metabolieten, waarbij cannabinoïd activiteit wordt aangetoond in authentieke urine- en bloedstalen. Het werk ging in

verschillende stappen. Aanvankelijk werden er tijdelijke celsystemen opgezet die één van beide cannabinoïd receptoren (CB1 of CB2) samen met βarr2 tot expressie brachten **(Hoofdstuk 3)**. De geschiktheid van deze nieuw ontwikkelde CB1 en CB2 testen voor het detecteren van cannabinoïd activiteit werd met succes geëvalueerd door verschillende SCRAs en hun belangrijkste metabolieten op deze celsystemen toe te passen. Hiermee toonden we dat verschillende metabolieten van deze SCRAs hun activiteit behouden op cannabinoïd receptoren, wat consistent is met wat anderen rapporteren. Dit is een belangrijke bevinding omdat SCRAs sterk worden gemetaboliseerd en bijna geen oorspronkelijke drug wordt gevonden in urine. De aanwezigheid van deze actieve metabolieten in urine zorgt voor een langer detectievenster voor het opsporen van SCRA gebruik. De SCRA testen (voor CB1 en CB2) werden gebruikt als een *proof-of-concept* om cannabinoïd activiteit aan te tonen in een authentiek urinestaal.

In een tweede stap werden de transiënte SCRA testen verbeterd door stabiele celsystemen te genereren (**Hoofdstuk 4**). Voordelen van de stabiele celsystemen in vergelijking met de initiële transiënte testen omvatten (i) een verminderde werkbelasting, (ii) een hogere reproduceerbaarheid tussen experimenten en (iii) de mogelijkheid tot controle op stabiliteit via co-geëxpresseerde merkers. Het gebruik van stabiele celsystemen als screeningsmethode voor SCRAs werd geëvalueerd op een relatief groot aantal authentieke urinestalen (n = 74). In een volgende stap wilden we de gevoeligheid van de SCRA testen verbeteren, aangezien er lagere concentraties van SCRAs te verwachten zijn in serum- of plasmastalen. Daarom werden twee C-terminaal verkorte βarr2 mutanten gegenereerd en geëvalueerd (**Hoofdstuk 5**). Het idee om βarr2 te verkorten was gebaseerd op diens prominente rol in GPCR signalisatie. Deze aanpak resulteerde in verbeterde stabiele cel systemen, die met succes werden gebruikt om cannabinoïd activiteit te detecteren in authentieke serum (n = 45) en plasma (n = 73) stalen. Gesterkt door de veelbelovende resultaten die we behaalden met de op activiteit-gebaseerde detectie van SCRAs in biologische vloeistoffen, werd een soortgelijk concept opgezet voor het opsporen van opiaten en (synthetische) opioïden in biologische matrices (**Hoofdstuk 6**). Hier was een extra toevoeging van een eiwit, G-proteïne gekoppeld receptorkinase 2, noodzakelijk om de rekrutering van βarr2 naar de MOR te bevorderen. De bruikbaarheid van de MOR test werd aangetoond met behulp van een set van 107 authentieke bloedstalen. Ook in een zaak met een fatale intoxicatie met het extreem krachtige opioïde carfentanil werd de MOR test met succes toegepast (**Hoofdstuk 7**).

Hoewel er veel commercieel verkrijgbare testen beschikbaar zijn voor het monitoren van GPCR activatie, zijn de testen die hier ontwikkeld werden momenteel de enige die op biologische matrices toegepast zijn als een *'untargeted'* screeningsstrategie. Of het succes van de eerste applicaties, die hier worden beschreven, zal leiden tot een verdere verspreiding en gebruik van het concept van activiteit-gebaseerde screening in forensische toxicologie, kan alleen de toekomst vertellen.

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2. Discover Glo Tour 2016, Promega, Leuven, Belgium, April 18th 2016. Evaluation of GPCR activation using the NanoBiT technology. (Cannaert A).

3. 54th International conference of The International Association of Forensic Toxicology (TIAFT), Brisbane, Australia, August 28th-September 1st 2016. Application of a new activity-based assay that allows activity profiling of synthetic cannabinoids (and metabolites) and their detection in urine. (Cannaert A, Storme J, Franz F, Auwärter V, Stove C).

4. Scientific session of The Toxicological society of Belgium and Luxembourg (BLT), Antwerp, Belgium, March 30th 2017. An activity-based screening method for synthetic cannabinoids: from concept to application. (Cannaert A, , Franz F, Hess C, Auwärter V, Stove C).

5. Research Day 2017, Ghent, Belgium, April 4th 2017. Application of a new activity-based assay that allows activity profiling of synthetic cannabinoids (and metabolites) and their detection in urine. (Cannaert A, Storme J, Franz F, Auwärter V, Stove C).

6. Knowledge for Growth, Ghent, Belgium, May 21st 2017. New bioassay for detection and activity profiling of synthetic cannabinoids & metabolites. (Cannaert A, Storme J, Franz F, Auwärter V, Stove C).

7. 15th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology (IATDMCT), Kyoto, Japan, September 24-27th 2017. New bioassay for detection and activity profiling of synthetic opioids. (Cannaert A, Vasudevan L, Wilde M, Auwärter V, Van Craenenbroeck K, Wille S, Stove C)

8. 6e Journées Internationales de Toxicologie, Liège, Belgium, October 19-20th 2017. An activity-based screening method for synthetic cannabinoids: From concept to application. (Cannaert A, Franz F, Hess C, Wille S, Auwärter V, Stove C)

9. Lisbon Addictions, 2nd European Conference on Addictive Behaviors and Dependencies, Lisbon, Portugal, October 24-26th 2017. Development and Activity Profiling of Synthetic Cannabinoids and Their Metabolites with a Newly Developed Bioassay. (Cannaert A, Storme J, Franz F, Auwärter V, Stove C).

10. 55th International conference of The International Association of Forensic Toxicology (TIAFT), joint meeting with the Society of Forensic Toxicologists (SOFT), Boca Raton, Florida, US, January 7-11th 2018. An activity-based screening method for synthetic cannabinoids: From concept to application. (Cannaert A, Franz F, Hess C, Wille S, Auwärter V, Stove C)

11. 55th International conference of The International Association of Forensic Toxicology (TIAFT), joint meeting with the Society of Forensic Toxicologists (SOFT), Boca Raton, Florida, US, January 7-11th 2018. New bioassay for detection and activity profiling of synthetic opioids. (Cannaert A, Vasudevan L, Wilde M, Auwärter V, Van Craenenbroeck K, Wille S, Stove C)

12. 55th International conference of The International Association of Forensic Toxicology (TIAFT), joint meeting with the Society of Forensic Toxicologists (SOFT), Boca Raton, Florida, US, January 7-11th 2018. Bioassay-based screening of Synthetic cannabinoids: adding a new spice to the toxicologist's palette. (Cannaert A) Part of a workshop: Strategies for the Detection of Synthetic Cannabinoids in Biological Specimens.

13. Young Scientist Symposium at the 55th International conference of The International Association of Forensic Toxicology (TIAFT), joint meeting with the Society of Forensic Toxicologists (SOFT), Boca Raton, Florida, US, January 7-11th 2018. An alternative detection strategy for alternative drugs: the potential of bioassays to screen for new psychoactive substances. (Cannaert A)

Posters

1. 28th Conference of European Comparative Endocrinologists (CECE), Leuven, Belgium, August 25-26th 2016. Application of a new activity-based assay that allows activity profiling of synthetic cannabinoids (and metabolites) and their detection in urine. (Cannaert A, Storme J, Franz F, Auwärter V, Stove C).

2. 9th International conference of European Bioanalysis Forum (EBF), Barcelona, Spain, November 16-18th 2016. Detection and Activity Profiling of Synthetic Cannabinoids and Metabolites with a Newly Developed Bioassay. (Cannaert A, Storme J, Franz F, Auwärter V, Stove C)

3. 15th International Congress of Therapeutic Drug Monitoring & Clinical Toxicology (IATDMCT), Kyoto, Japan, September 24-27th 2017. An activity-based screening method for synthetic cannabinoids: From concept to application. (Cannaert A, Franz F, Hess C, Wille S, Auwärter V, Stove C)

4. Research Day & Student Research Symposium, Ghent, Belgium, April 19th, 2018. It doesn't matter what you look like, it's what you do that counts: Activity-based Bioassays for the Detection of Synthetic Cannabinoid Receptor Agonists in Serum. (Vandeputte M, Cannaert A, Stove C)

Scientific prizes

EMCDDA Scientific Award from the European Monitoring Centre for Drugs and Drug Addiction during the Lisbon Addiction 2017 conference held in Lisbon, Portugal from October 24-26th 2017.

TIAFT Young Scientist Award for Best Paper in 2016-2017, 11th of January 2018, from The International Association of Forensic Toxicology (TIAFT) during the 55th Annual meeting in Boca Raton, Florida, USA from January 7-11th 2018.

Memberships of scientific organizations

TIAFT (The International Association of Forensic Toxicologists)

BLT (Belgium Luxembourg Toxicological Society)

IATDMCT (International Association of Therapeutic Drug Monitoring and Clinical Toxicology)