Designer drugs pose a challenge to control programs (such as anti-doping control), to forensic and clinical toxicology, and to legislative authorities. An enormous variety of chemically diverse drugs, requiring adequate monitoring in specific populations, hampers timely detection in the corresponding laboratories. The rapid emergence of new drugs requires continuous development or adaptation of methodologies and evaluation of these drugs' pharmacological and toxicological properties. This PhD dissertation includes the study of activity-based detection as a proactive strategy to cope with the challenges posed by designer drugs. Different aspects were evaluated, including (i) pharmacological characterization for different purposes, (ii) untargeted detection in human biological matrices, and (iii) understanding of drug intoxications.

Chapter I provides an overview of the scientific research on the use of activity-based assays for the detection of performance-enhancing drugs (PED) in biofluids, up till the writing of this dissertation. Significant scientific progress has been established for several classes of PED, with a pronounced focus on hormones such as (designer) steroids, stemming from the discovery of the first designer drug in doping in 2004. From this literature study, important characteristics of cell-based assays were identified that require consideration upon the design of an optimal assay format. Widespread implementation of such detection methods in routine practice has lagged, likely related to the atypical nature of these techniques and the variability that is inherent to cell-based assays. However, the excellent performance of cannabinoid reporter assays, the recent validation studies of glucocorticoid assays and the development of new hypoxia-inducible factor (HIF) bioassays led to the identification of several promising applications for bioassays in control programs: (i) untargeted detection to guide intelligent sample storage, sample retesting and athlete targeting, (ii) bioassay-guided sample fractionation to guide the identification of bioactive compounds, and (iii) pharmacological characterization to prioritize research endeavors.

Chapter II describes the development and evaluation of new bioassays to monitor HIF heterodimerization. Using the natural protein-protein interaction between HIF subunits as a mechanism for detection, coupling of the NanoBiT® system to HIFα and HIFβ resulted in a new assay format for the detection and characterization of HIF-related compounds, as discussed in **Chapter II.1**. Two different cell-based assays were designed, allowing the monitoring of two isoforms of the HIF transcription factor (HIF1 and HIF2). These assays were successfully used to characterize clinical HIF stabilizers as well as general hypoxia mimetics. Optimization to yield a stable cell format, as reported in **Chapter II.2**, simplified the assay protocol and reduced the variability of the bioassay. This resulted in an increased sensitivity compared to the transient assay format in **Chapter II.1**, which was favorable for the detection of relevant concentrations in doping samples. Thorough comparison between the HIF1 and HIF2 bioassay identified the HIF1 stable assay as the optimal format to investigate biological samples. Hence, **Chapter II.2** serves as proof-of-concept that HIF stabilizers can be detected in urine samples by this

newly developed future-proof bioassay. At its current performance level, the sensitivity of the assay is hampered by the impact of the biological matrix. Future studies can be dedicated to eliminating any influence of matrix effects. Notwithstanding, at this point the HIF bioassay can serve an important purpose for retrospective studies to verify whether doping control has or has not been lagging behind over the past few years on the emergence of various potential HIF-related doping substances. Furthermore, both the HIF1 and HIF2 bioassay were thoroughly evaluated in **Chapter II.3** to determine potential applications for pharmacological purposes, identifying several benefits related to the assay formats and recognizing important considerations such as interferences and non-specific effects. This last part of chapter II demonstrates the high versatility of the newly developed assays including diverse experimental set-ups and different assay protocols that allow to measure both increases and decreases in the biological readout and enable the detection of both indirect and direct effects on HIF heterodimerization. Therefore, these newly developed HIF bioassays can serve as pharmacological tools in multiple disciplines for the characterization of substances with divergent mode of actions.

Synthetic cannabinoid receptor agonists (SCRAs) as recreational drugs are the protagonists of **Chapter III**. Readily developed bioassays for the monitoring of these cannabinoid designer drugs, classified as new psychoactive substances (NPS), are employed to manifest the versatility of cell-based assays. The three different aspects mentioned at the beginning of this summary also featured in **Chapter III**. *Pharmacological characterization* served the purpose of profiling the activity of SCRAs, including the assessment of so-called biased signaling, in **Chapter III.1**. Activity-profiling gives insight into a compound's intrinsic receptor activation potential, which is (amongst other aspects) related to its pharmacological and/or toxicological effects. *In vitro* characterization is highly relevant in drug discovery, in the preclinical development phase of therapeutics, in the assessment of harm potential of emerging drugs, and in the prioritization of research endeavors, legislative efforts and control measures. The latter purpose is particularly applicable to **Chapter III.6**, where a large panel of both naturally and synthetic variants of Δ^9-tetrahydrocannabinol (Δ^9-THC) were screened for cannabinoid activity to estimate their potential for abuse, based on their potential to activate the CB_1 cannabinoid receptor. This resulted in the identification of multiple compounds with higher cannabinoid activity than the primary psychoactive constituent in cannabis (Δ^9-THC) – and may be next in line to appear on the recreational drug market. This information was passed on to the European (EMCDDA) and national authorities (Sciensano) to support the decision-making regarding legislative adaptations and prioritization of control measures. Pharmacological characterization also serves the purpose of enabling structure-activity relationship studies, involving structurally related compounds, as discussed in **Chapter III.2** and **Chapter III.6**. These studies are important directories for the design of new drugs in the field of medicinal chemistry and for the prediction of pharmacological effects of structural analogs of designer drugs before they appear on drug markets. **Chapter III.2** also demonstrated differences

between the characterization by different assay formats. The changes in kinetics upon truncation of assay proteins and the ceiling effect of assays involving signal amplification, emphasize the importance of considering what signaling event the bioassay is monitoring. The selection of the ideal bioassay format depends on the questions that need to be answered (e.g., therapeutic potential, harm potential), which on its turn depends on the purpose that is to be served (respectively, drug design or public health).

The feasibility of *untargeted screening* with activity-based assays is highlighted in **Chapter III.3**. A screening assay is supposed to be universal, rapid, simple, sensitive, selective, reproducible and inexpensive. Universality is confirmed in this study by the continued excellent performance of the cannabinoid reporter bioassay upon detection of an entirely different panel of drugs as compared to 3 years prior. Sensitivity is greatly emphasized by the low concentrations of SCRAs and SCRA metabolites in serum that resulted in a positive screening outcome. Furthermore, this chapter demonstrated the possibility of computer-assisted scoring by means of a machine learning model, allowing to speed up data analysis and reduce the time and workload for the laboratory staff.

Lastly, **Chapter III.4** and **Chapter III.5** study the added-value of using activity-based assays for the evaluation and understanding of recreational *drug intoxications*. Both chapters detect *ex vivo* cannabinoid activity with the cannabinoid reporter bioassay in serum samples and interpret this measure by means of pharmacological characterization. **Chapter III.4** focuses on mono-SCRA intoxications with one particular SCRA, showing the possibility to predict the cannabinoid activity in serum extracts based on pharmacological characterization of the SCRA (and its metabolites). This study thereby demonstrates the achievability of correcting the bioassay readout for matrix effects and reveals a link of cannabinoid activity in serum to the level of consciousness of intoxicated patients. **Chapter III.5** explores the use of a reference SCRA to comprehensively approach mono- and poly-SCRA intoxications by using 'activity equivalents'. This approach allows simultaneous untargeted screening and evaluation of the extent of the intoxication, without prior knowledge on the drug that was taken.

In conclusion, this dissertation contributes to the scientific progress envisaging the complementary value of activity-based methods in routine testing strategies. A new untargeted cell-based assay was developed for the proactive detection of (unknown) doping substances. Feasibility and added-value of activity-based detection as a screening approach was demonstrated and discussed as a future-proof method for the detection of designer drugs. Insights were gained into recreational drug intoxications, providing a novel strategy to interpret the intoxication of patients with unknown designer drugs. The different purposes of pharmacological characterization were exhibited, having implications on different levels and in different fields. Hence, the various applications, benefits, experimental protocols and fields of interest, illustrate the high versatility of cell-based research, with significance for both science and society.