Summary and conclusion

Some 15 years ago, new psychoactive substances (NPS) started appearing on recreational drug markets in Europe and elsewhere. Fueled by globalization and attempts to circumvent legislation, the NPS phenomenon grew quickly, both in number of substances as well as in complexity. Of the different classes of NPS, the growing group of new synthetic opioids (NSOs) is of particular concern owing to the high risk of overdose linked with opioid misuse. NSOs can be broadly categorized into analogues of the potent analgesic fentanyl and more structurally diverse, non-fentanyl-related substances.

Between 2012 and 2018, the majority of emerging NSOs were fentanyl analogues. By 2019, this balance had shifted towards the appearance of non-fentanyl NSOs, shaping what is considered to be the current 'post-fentanyl analogue era'. Many of these compounds were once pursued by the pharmaceutical industry for their potential as narcotic analgesics, but were never marketed as such. Today, several of these *old* drugs are being diverted to the recreational drug market as NSOs. At the same time, substances that have never been studied before are being manufactured and distributed as truly *new* synthetic opioids. The seemingly endless list of possible chemical scaffolds makes it difficult to anticipate which compound may emerge next.

Whether old, new, or (un)expected - insight into the biological effects of emerging NSOs is crucial to inform stakeholders of the potential harm that is linked with the presence of these substances on recreational drug markets. The primary goal of the work collated in this thesis is to assist in the risk assessment of non-fentanyl NSOs by means of *in vitro* and *in vivo* pharmacological characterization. As a secondary goal, this thesis illustrates the versatility of an *in vitro* NanoBiT®-based bioassay in the context of clinical and forensic toxicology.

Chapter 1 explains why the use of opioids is a double-edged sword, associated with both powerful analgesia as well as potentially lethal respiratory depression. The reader is introduced to the μ-opioid receptor (MOR), the primary molecular target responsible for the main effects of clinically applied as well as abused opioids. To gain insight into the pharmacology of NSOs, different *in vitro* assays monitoring MOR activation have been applied. **Chapter 2** provides an overview of the potential and pitfalls of commonly applied assays, and includes a compilation of published *in vitro* potencies and efficacies, as well as bias assessments of NSOs.

Chapters 3-8 provide a pharmacological perspective on NSOs of the post-fentanyl analogue era. Starting with U-type opioids, **Chapter 3** demonstrates the difference in MOR activation potential between two positional isomers of naphthyl U-47700. These findings illustrate how small structural changes to a molecule may greatly impact opioid activity. **Chapter 4** centers around the class of cinnamylpiperazine 'AP'-opioids, including 2-methyl AP-237 and AP-238. *In vitro* characterization indicates that cinnamylpiperazines are generally less active than fentanyl, which could be linked to the relatively higher concentrations of the former in a series of postmortem toxicology cases. **Chapter 5** concentrates on the characterization of dipyanone, desmethylmoramide, and acetoxymethylketobemidone - NSOs that are structurally related to the prescription opioids methadone and ketobemidone. Dipyanone, which shows a comparable *in vitro* opioid activity to methadone, was additionally identified in a seized powder and in a postmortem sample from a polydrug intoxication.

Chapter 6 is dedicated to the pharmacological characterization of 2-benzylbenzimidazole 'nitazene' opioids. Over the course of the research period described in this thesis (2019-2023), this group of NSOs started gaining particular attention: from the first identification of isotonitazene in 2019, to the successive emergence of 14 analogues, and their rapidly growing involvement in drug-related fatalities – in just 4 years' time, 2-benzylbenzimidazole 'nitazene' opioids became the predominant class of non-fentanyl NSOs in many parts of the world. ***Chapter 6.A*** sets the stage by detailing the 'rise and fall' of isotonitazene on recreational drug markets, exemplifying the characteristic dynamic life cycle of NSOs. The following chapters are organized according to the different 'waves' in which differentially substituted nitazenes entered the recreational drug markets. ***Chapter 6.B.1*** introduces the main structure-activity relationships of nitazenes of the 'first wave', such as isotonitazene, metonitazene, and etodesnitazene. In ***Chapter 6.B.2***, an adapted protocol of the standard NanoBiT® MOR activation assay is successfully applied for the activity-based screening of isotonitazene and other NSOs in authentic samples. The transition to the 'second wave' of nitazenes is documented in ***Chapter 6.C***. This chapter includes the *in vitro* and *in vivo* characterization of *N*-pyrrolidino etonitazene (***Chapter 6.C.1***) and *N*-piperidinyl etonitazene (***Chapter 6.C.2***), the results of which indicate important opioid activity. The harm potential of other 'second wave' nitazenes is evaluated in ***Chapter 6.C.3***, which combines *in vitro* pharmacology with the largest case series involving nitazenes described to date. Finally, ***Chapter 6.D*** reports the *in vitro* and

in vivo evaluation of nitazenes that may be expected to emerge in a potential third 'future wave'. The relevance of this predictive approach was confirmed when ethyleneoxynitazene, one of the proposed 'prophetic' nitazenes, was identified for the first time on the recreational drug market early 2023.

In **Chapter 7**, the focus is shifted from NSOs with a benzimidazole core (i.e., nitazenes) to related benzimidazolone-containing NSOs. ***Chapter 7.A*** details the first identification and characterization of brorphine, a potent NSO that was detected in a powder and in serum samples of a patient seeking help for detoxification. In ***Chapter 7.B***, more insight is gained into the *in vitro* and *in vivo* pharmacology of brorphine and 4 'prophetic' analogues. The results demonstrate that brorphine-like compounds have the potential to cause harm in users should they emerge as NSOs. Finally, in **Chapter 8** ***(A-C)***, the opioid activity of structurally diverse non-fentanyl NSOs (including selected NSOs discussed in separate chapters) is compared directly using a selection of *in vitro* and *in vivo* assays.

Chapter 9 explores the broader international context of this thesis, including the different stages of the opioid overdose epidemic in the United States and insights into the changing landscape of the recreational opioid market in Europe. It portrays the growing public health risk posed by nitazenes, exacerbated by their presence at the street level, and explores the combined role of legislation, treatment, and harm reduction strategies in mitigating opioid-related harms.

Synthetic opioids – old, new, and (un)expected – will continue to emerge on recreational drug markets in the future. While some will cause no more than a ripple, others may form entirely new waves. The work described in this thesis demonstrates how pharmacological research may help to navigate this complex landscape. New insights into the biological effects of emerging NSOs were gained, allowing evidence-based risk assessments needed to direct and prioritize legislation and harm reduction strategies. The applied *in vitro* NanoBiT® assay proved useful for a variety of purposes, including evaluation of the opioid activity present in drug material or biological samples – assessments that will become increasingly important if NSOs continue to integrate with the established illicit drug market in the future. This delicate situation requires a coordinated, multidisciplinary response. Pharmacological characterization is just one piece of that puzzle – however, it is fundamental to help to turn the tide on the synthetic opioid problem.