Interactions of Cathinone NPS with Human Transporters and Receptors in Transfected Cells

Linda D. Simmler, PhD, & Matthias E. Liechti, MD

Division of Clinical Pharmacology and Toxicology, Department of Biomedicine and Department of Clinical Research, University Hospital Basel, Basel Switzerland

Abstract
Pharmacological assays carried out in transfected cells have been very useful for describing the mechanism of action of cathinone new psychoactive substances (NPS). These in vitro characterizations provide fast and reliable information on psychoactive substances soon after they emerge for recreational use. Well-investigated comparator compounds, such as methamphetamine, 3,4-methylenedioxymethamphetamine, cocaine, and lysergic acid diethylamide, should always be included in the characterization to enhance the translation of the in vitro data into clinically useful information. We classified cathinone NPS according to their pharmacology at monoamine transporters and receptors. Cathinone NPS are monoamine uptake inhibitors and most induce transporter-mediated monoamine efflux with weak to no activity at pre- or postsynaptic receptors. Cathinones with a nitrogen-containing pyrrolidine ring emerged as NPS that are extremely potent transporter inhibitors but not monoamine releasers. Cathinones exhibit clinically relevant differences in relative potencies at serotonin vs. dopamine transporters. Additionally, cathinone NPS have more dopaminergic vs. serotonergic properties compared with their non-β-keto amphetamine analogs, suggesting more stimulant and reinforcing properties. In conclusion, in vitro pharmacological assays in heterologous expression systems help to predict the psychoactive and toxicological effects of NPS.

Keywords: Cathinones, new psychoactive substances, pharmacology, in vitro, heterologous expression systems, transporters, uptake, efflux

Content
1 Introduction .................................................................................................................................. 2
2 Methods for Studying Transporter and Receptor Pharmacology in Transfected Cells ... 3
3 Effects on Cathinone Analogs on Transporter-Mediated Uptake ........................................ 10
4 Effects of Cathinones on Transporter-Mediated Efflux ........................................................ 12
5 Drug Interactions with G-Protein-Coupled Receptors ......................................................... 13
6 Summary .................................................................................................................................... 14
References ...................................................................................................................................... 14
1 Introduction

In 2014, the European Union Early Warning System (EMCDDA, 2015) reported the emergence of 101 new psychoactive substances (NPS). The variety of largely unknown NPS is still increasing compared with recent years. With this high number of new substances, rapid testing systems are needed to obtain an immediate understanding of the mechanism of action of these NPS. Animal studies that utilize behavioral paradigms (e.g., to test abuse liability) or neurochemical assessments (e.g., microdialysis and voltammetry) to investigate the pharmacology and toxicology of new compounds in vivo are relatively expensive and require weeks or months to conduct. Moreover, typically only a small number of substances can be tested. In contrast, rapid first characterizations of new compounds can be performed within days in a laboratory with a set of well-established in vitro assays and using reference data from well-known substances. Typically, relatively simple in vitro pharmacological assays with transfected cell lines have limited significance in neuroscientific research because more complex behavioral and circuit-wide conclusions are required for a comprehensive understanding of the mechanism of action of psychoactive substances in the brain. Transfected cell lines in heterologous expression systems only reveal the mechanism of action of drugs on specific targets that are expressed by the host cell. Therefore, any complex whole-brain interactions are lacking. However, to elucidate the pharmacology of a larger set of unknown compounds, in vitro assays are highly valuable as the first screening tools. Through decades of intensive animal and clinical experimental studies on various psychoactive substances (e.g., cocaine, methamphetamine, 3,4-methylenedioxymethamphetamine [MDMA], and lysergic acid diethylamide [LSD]), their mechanism of action in vitro and pharmacological effects in vivo are relatively well known, thus allowing translational interpretations of in vitro data on NPS (Liechti, 2015). Thus, the clinical pharmacology of NPS can be predicted based on similarities between the in vitro mechanisms of action of NPS and well-known and also clinically characterized comparator compounds.

Our in vitro characterization of cathinone NPS has allowed the rapid characterization of these newly emerging substances at known human targets of psychoactive compounds (Rickli et al., 2015a; Simmler et al., 2013; Simmler et al., 2014a). In the context of in vitro and in vivo studies in other laboratories (Baumann et al., 2012; Baumann et al., 2013; Iversen et al., 2013) and clinical reports, we found that in vitro characterizations are consistent with in vivo data but allow for the faster initial characterization of larger numbers of newly emerging compounds. Cathinone NPS have striking differences in pharmacological potencies to inhibit monoamine transporters, which are relevant to appraisals of the type of psychoactivity, abuse liability, and to some extent clinical toxicity. For example, in vitro testing has shown that 3,4-methylenedioxypyrvalerone (MDPV) inhibits the dopamine transporter (DAT) and norepinephrine transporter (NET) far more potently when compared with classic psychostimulants, such as cocaine and methamphetamine (Baumann et al., 2013; Simmler et al., 2013), suggesting that small doses may exert large clinical effects and enhance the risk of overdose. This information is essential for users of these compounds and clinicians who treat overdose cases. However, pharmacological properties, such as bioavailability and blood-brain barrier permeability, are also important for determining the potency of a substance in vivo. Additional pharmacological studies are thus needed.
for a more comprehensive characterization. Overall, *in vitro* profiling is particularly helpful for systematic comparative characterizations of a large number of substances, in which basic and rapid information on the compounds’ pharmacological characteristics is essential, such as with the current NPS problem.

In this article, we discuss the principles of *in vitro* pharmacological assays that are used to characterize the primary mechanisms of action of cathinone NPS. We discuss the advantages and limitations of such assays with regard to the rapid emergence of NPS in recent years. We also highlight methodological issues and discuss the main characteristics of cathinone NPS in these assays.

### 2 Methods for Studying Transporter and Receptor Pharmacology in Transfected Cells

Stably transfected cells represent a heterologous expression system in which the protein of interest is expressed in a host cell that does not endogenously express the respective protein. For the pharmacological profiling of cathinone NPS, the respective monoamine transporter or pre- and postsynaptic receptor genes are introduced into neutral cell lines (Ramamoorthy et al., 1993; Revel et al., 2011; Tatsumi et al., 1997). Human embryonic kidney (HEK) 293 cells are very commonly used for stable transfections and subsequent pharmacological assays. For stable transfections, a plasmid with the cDNA sequence of the target protein from any species is introduced into the cells (Groskreutz & Schenborn, 1997). The co-introduction of a geneticin-resistance gene ensures that only transfected cells are maintained in culture (Chaudhary et al., 2012). The stable expression of a target protein is not necessarily required for *in vitro* pharmacological assays (Henry et al., 2006), but stable cell lines simplify the workflow because the step of transiently transfecting cells before each assay can be omitted. Transfected cell cultures are a standard procedure for molecular biology laboratories. With recent technological improvements (e.g., CRISPR/Cas9 technology), transfections are becoming even easier (Ran et al., 2013). Once stably transfected, the cells express the protein in high abundance both in the membrane, which is essential for functional assays, and in the cytoplasm (Chamba et al., 2008; Marazziti et al., 2007). For assays that are used for investigations of cathinone NPS, only one gene of interest is introduced per cell line, thus ensuring selectivity in the pharmacological assessment. Non-transfected cells can serve as a control for nonspecific drug action (i.e., nonspecific binding to the cell membrane; Ramamoorthy et al., 1993).

To comprehensively characterize psychoactive compounds at their typical neuronal target sites *in vitro*, the effects of these compounds on the different monoaminergic neurotransmitter uptake transporters and various neurotransmitter G-protein-coupled receptors need to be determined in a battery of assays. Therefore, individual cell lines that overexpress the respective target protein after transfection are used to determine binding affinity, uptake transport inhibition, and transporter-mediated efflux in separate assay setups. For transporters, uptake inhibition (e.g., in the case of cocaine) and the transport-mediated efflux of transmitter (e.g., in the case of most amphetamines) are determined in different assays. For the relevant receptors, functional assays are performed to determine agonistic or antagonistic properties, including information about full or partial agonist effects. Binding affinities at both transporters and
receptors are also frequently determined, but functional tests are considered more conclusive than binding affinities. The assay principles are described in more detail later in this chapter. Briefly, transport assays require a radiolabeled substrate of the transporters, usually endogenous neurotransmitters (Ramamoorthy et al., 1993). Through quantification of the transported radiolabeled substrates, the inhibition potencies or efflux characteristics of a specific substance can be determined. To determine binding affinities, a radioligand displacement principle is applied, in which the substance’s ability to compete with the radioligand for the binding site is quantified (Maguire et al., 2012). For receptor activity, cyclic adenosine monophosphate (cAMP) levels can be quantified (Zhang & Xie, 2012). This downstream factor indicates signaling that is induced by G-protein-coupled receptors, in which cAMP levels increase upon activation of the receptors or decrease upon inhibition of the receptors (Tate, 2012). For all of the assays, classic enzyme kinetics are the basis for calculating pharmacological determinants (i.e., \( IC_{50} \), \( EC_{50} \), and \( K_i \) values; Burlington & Widlanski, 2003; Cheng & Prusoff, 1973).

Heterologous expression systems for monoaminergic neurotransmitter transporters have been relevant in neuropsychopharmacology research since these transporters were first cloned. Transporter-expressing cell lines allow the characterization of psychoactive compounds (Tatsumi et al., 1997) and are also a useful tool for discovering psychoactive therapeutic drugs (Bang-Andersen et al., 2011). Furthermore, in vitro experiments with transfected cells formed the basis for many genetic mutations that were later engineered in mice, which now serve for in vivo investigations of psychoactive drugs or as preclinical models of mental disorders (Henry et al., 2006; Mazei-Robison et al., 2008; Prasad et al., 2005). For example, in vitro experiments allowed the construction of a transgenic mouse model with a 5-hydroxytryptamine (5-HT [serotonin]) transporter (SERT) mutation for the in vivo assessment of SERT-mediated effects of antidepressants or cocaine (Prosser et al., 2014; Thompson et al., 2011) or to shed light on functional abnormalities of the DAT variant Val559, which is being investigated as a potential mouse model of attention-deficit hyperactivity disorder (Mergy et al., 2014).

Today, heterologous expression systems are a relatively simple tool for use in any laboratory with basic cell culture and molecular biology setups. Furthermore, once cell lines stably express a specific receptor, these lines can be maintained by freezing stocks, and such stocks can then be used over decades. One of the greatest strengths of in vitro screening assays that use transfected cells is the high selectivity for the pharmacological targets of interest. For example, for DAT uptake inhibition, cells that overexpress DAT are used, while for SERT inhibition a different cell line overexpressing SERT is used. Due to separation of the targets in different runs no unspecific action at the second target can affect the result. Furthermore, human proteins can be overexpressed to assess pharmacological profiles directly with targets of the human species (Tatsumi et al., 1997). Species differences could be a concern in ex vivo or in vivo experiments because target proteins may exhibit distinct substance recognition between rodents/nonhuman primates and humans or show differential expression patterns. For example, the antidepressant imipramine is more potent at the human SERT than at the rat SERT, whereas cocaine inhibits both rat and human SERT with equal potencies (Barker & Blakely, 1996). The most common variant of the respective target is usually expressed in NPS screening, but its also feasible to generate cell lines with different variants of human transporters or receptors to specifically assess the pharmacological and toxicological
effects of psychoactive substances on less common gene variants. While many advantages are evident for the use of heterologous expression systems to screen NPS pharmacological profiles, there are also limitations and disadvantages compared to similar experimental approaches. Synaptosomes or brain slices are frequently used ex vivo-preparations to assess the pharmacology of psychoactive substances. In brain slices substantial cellular characteristics are still intact, and synaptosomes contain the full complement of synaptic proteins and synaptic vesicles (Wilhelm et al., 2014). Synaptosomes resemble the natural environment of the site of psychostimulant action more than transfected cell lines. Interpretations from experiments in transfected cells are limited since they lack elements of the protein machinery of intact neuronal membranes that could be critical for certain protein/substance interactions and consequences. However, for target-selective assays typically used for the determination of pharmacological constants unintended targets have to be pharmacologically blocked in synaptosomes (Rothman et al., 2001; Rothman et al., 1993). In this regard, both transfected cell lines and ex vivo preparations (e.g., synaptosomes) have their advantages and limitations for the screening of NPS pharmacology and should always be kept in mind when interpreting results. Nevertheless, pharmacological profiles of NPS assessed in transfected cells have largely been in accordance with data obtained from synaptosomes.

It is self-evident that there are limitations to in vitro screenings with transfected cells or ex vivo preparations and various consequences of NPS use can only be assessed by in vivo testing, particularly behavior or long-term toxicity. With regards to pharmacological profiles, however, we would like to point out that the possibility of active metabolites should be considered. Heterologous cell lines for in vitro screenings of NPS pharmacology are largely unable to detect the possible contribution of active metabolites that could, however, be relevant in vivo. For example, 3,4-methylenedioxyamphetamine (MDA) is an active metabolite of MDMA and likely contributes to the subjective drug experience and toxicity associated with MDMA (de la Torre et al., 2000). Cathinone NPS may also have active metabolites that should be taken into account in more comprehensive pharmacological substance characterizations. For example, β-keto-MDA is a metabolite of methylone (Mueller & Rentsch, 2012) and interacts with monoamine transporters similarly to MDA in in vitro tests (Rickli et al., 2015b). In vitro testing for active metabolites requires knowledge of the metabolic pathway and synthesis of possibly active metabolites or the use of cell systems that contain metabolic enzymes. To elaborate the metabolites for every single NPS would be a very labor-intensive process. In vivo neurochemical studies that utilize microdialysis can be performed more easily and may include possible contributing effects of active metabolites on neurotransmission.

The specific assay setups for uptake and efflux transport assays vary considerably between laboratories. In the most widely used experimental setup for in vitro pharmacology, transfected cell lines are grown to adherence in well plates or small culture dishes. Adherence of the cells allows for the removal of uptake buffer and washing with ice-cold buffer to stop substrate transport. However, if timing is an essential factor in uptake experiments (which is usually more essential for substrate kinetics than for inhibition potencies [IC₅₀ values]), then the possibility of the rapid and timely termination of the uptake process is crucial. With suspended synaptosome
preparations, the use of a Brandel tissue harvester allows for the timely termination of 24-96 vials at once. It becomes more difficult when the assay is conducted on adherent cell cultures. Even with an automated wash station for cell culture plates, achieving satisfactory accuracy to terminate the uptake process can be either challenging or impossible. When we established the assay that is currently used in our laboratory, we chose to use a silicone-oil-centrifugation method. We perform the uptake assay in cell suspensions that are prepared from adherent cells. Centrifuging the cells through a silicone oil layer allows for rapid and precise termination of the uptake process and the cleaning of cells from the buffer (Torok et al., 1998). Silicone oil is used as a middle layer in a tube. In the centrifugation step, the cells but not radioactive uptake buffer transfer to the lower layer (consisting of 3 M KOH, which lyses the cells). We have found that this method is very reliable and precise, but handling can be more elaborate and more difficult than working with adherent cells or synaptosomes. No conclusive recommendation has been made for the ideal assay setup. In fact, every laboratory needs to establish and validate its own assay setup for transport assays. If the assay follows the rules of enzyme kinetics and if reproducibility within the laboratory can be demonstrated, then the specific details of the assay are of less concern.

Between uptake assays for different pharmacological targets (e.g., SERT vs. DAT uptake inhibition), direct comparisons even within a laboratory and setup cannot be guaranteed if only IC_{50} and not K_{i} values are determined. However, the inclusion of a set of comparator compounds (e.g., methamphetamine, MDMA, and cocaine) with widely reported pharmacological characteristics should serve to set the standard for comparisons of IC_{50} values between targets. For example, calculating the DAT/SERT ratio for well-known compounds like MDMA can be the reference for unknown compounds (Baumann et al., 2012; Rickli et al., 2015b). This again shows the importance of including well-known reference compounds in screening and that the value of a study increases according to the number of substances that are included.

Reproducibility within a laboratory is essential for the extensive characterization of multiple compounds. In general, for comparable IC_{50} values in large screenings within one laboratory requires strict adherence to the established protocol since IC_{50} values depend on substrate concentration, in addition to temperature and incubation times. We regularly test the reproducibility of IC_{50} values for our standard compounds and find that the values are very consistent across both time and experimenters. This regular validation ensures that the data for all substances that are reported from our laboratory can be directly compared with our previously reported data. To consider are also fluctuations in target protein expression in heterologous expression systems that could account for inconsistent IC_{50} values within one laboratory (Ukairo et al., 2007). However, if in vitro assays are set up with a targeted protein concentration within a linear range in a protein concentration vs. substrate transport relationship, moderate changes in cell number used for an individual assay or in target protein expression are usually tolerated and do not affect the reproducibility of IC_{50} values within laboratory, always given a linear relationship of target protein vs. substrate transport. As a side note, this is in contrast to transport kinetics (i.e., Michaelis-Menten kinetics), in which the maximal velocity is highly dependent on the expression levels of the transporter. With these considerations comparison of IC_{50} values within one laboratory is unproblematic. For direct comparison of pharmacological constants between different laboratories K_{i} values should be assessed,
since IC_{50} but not K_i values depend significantly on assay conditions (Burlingham & Widlanski, 2003). The determination of K_i values is more complex because it requires knowledge or assessment of the mode of inhibition (e.g., competitive, noncompetitive, or mixed; Burlingham & Widlanski, 2003). Although K_i values would be the best constants to determine, the rapid and extensive characterization of the effects of a large set of cathinone NPS on multiple targets usually does not allow the labor-intensive determination of K_i values. Given these limitations, in vitro screenings assessing IC_{50} values are only of value when a large of substances is assessed within one laboratory or if well-known comparator compounds are included as reference compounds that allow for an interpretation of pharmacological profiles relative to the reference compounds.

Different setups for monoamine efflux assays have been described, all resulting in similar qualitative characterizations of compounds. Although different setups are valid, establishing an efflux assay can be difficult. Efflux can be measured using electrophysiological methods (Hilber et al., 2005; Khoshbouei et al., 2003), which allow the very reliable determination of transporter-mediated monoamine release and its associated currents that are induced by compounds. However, because patch-clamp electrophysiology requires specialized recording equipment, we only discuss radiolabeled substrate transport assays herein. Rothman et al. (Rothman et al., 2001) reported the use of efflux assays with rat synaptosomes, in which synaptosomes were first preloaded to steady-state with the radioactive substrate via transporter-mediated uptake. Release was then induced without removing the radioactive uptake buffer. Using this method, a high signal-to-noise ratio was reported, but efflux potency values could be determined. Verrico et al. adapted this protocol for transfected HEK293 cells in suspension (Verrico et al., 2007). We initially followed this protocol (Hysek et al., 2012c) but later adapted it according to the principles reported by Scholze et al. (Scholze et al., 2000), who used a superfusion system. The superfusion system is preferentially used for rodent tissue slices that are preloaded with radioactive transporter substrates (Mergy et al., 2014), but it can also be adapted for transfected cells (Pifl et al., 1995; Scholze et al., 2000). Transfected cells are grown on coverslips and loaded with radioactive substrates. They are then moved to superfusion chambers where the cells are constantly superfused with non-radioactive buffer (Scholze et al., 2000). The advantage of this method is that the radioactive substrates that are released are transported away from the cells or tissue (Raiteri et al., 1974) so that the reuptake of released substrate should not occur. We adapted this principle to our laboratory but used well plates instead of a superfusion system. To achieve a similar effect as superfusion with regard to the immediate removal of released substrate, we took advantage of the dilution effect. Using a high buffer-to-cell ratio, the monoamine substrate that is released by the cells is distributed in a large volume of buffer, resulting in negligible extracellular substrate concentrations. To achieve a high buffer-to-cell ratio, we used special 24-well plates (XF24, Seahorse Biosciences, North Billerica, MA, USA), which fit 1 ml of buffer per well, but the area for cell growth is as small as the one from a regular 96-well plate. Therefore, the buffer-to-cell ratio is much higher than the one in a standard cell culture 96-well plate or 24-well plate, thus providing an optimal assay setup for testing substance-induced monoamine efflux. Release is quantified by assessing the monoamine radioactivity that remains in the cells after incubation with the test substance and compared with a vehicle control. Additionally, radioactivity that is associated with the released monoamine can be
measured in the supernatant. In transfected cells, an apparent release of approximately 20% for pure uptake inhibitors is observed even with the superfusion method, most likely because of the high expression levels of transporters that transport nonspecifically released monoamines back into the cells (Scholze et al., 2000). Thus, uptake inhibitors need to be included as a negative control condition to account for apparent release. Apparent release can be lowered if a substance is a substrate and thus removed from the binding site of the radioligand displacement assay (Simmler et al., 2013; Tatsumi et al., 1997). However, for both receptors and transporters, the functional assays are considered to have higher predictive validity with regard to in vivo effects. For the transporter, functional information is derived from the uptake and efflux assays. Specifically for substances that are releasers and thus substrates of the transporters, the binding properties or even the binding sites can differ from the radioligand that is to be displaced. Additionally, the substrates are transported and thus removed from competition with the radioligand. Binding affinity values do not necessarily reflect the functional uptake inhibition potency (Simmler et al., 2013). This is a common phenomenon for binding studies that use ligands that are also transporter substrates because transport of the substrate can alter the apparent binding affinity (Marcusson et al., 1986; Nelson & Rudnick, 1979; Talvenheimo et al., 1979). Thus, if a substance is a substrate-type releaser, then its binding affinity, when assessed by the described displacement assay, is not representative. This discrepancy between binding affinities and uptake inhibition potencies can even be used to characterize a substance as substrate-type release or pure uptake blocker (Eshleman et al., 1999; Rothman et al., 1999).

The determination of binding affinity is more common for receptors than for transporters. However, it is also important for receptor pharmacology to distinguish between functional activity and binding affinity (Zhang & Xie, 2012). The concepts for assessing activity and affinity in heterologous expression systems are different. To determine binding affinity, only the target protein from the expression is required. Therefore, isolated membrane preparations that can be stored in a frozen state are usually made from transfected cells. In radioligand displacement assays, the binding affinities of compounds at the binding site of the radioligand are determined. Functional information with regard to activation or inactivation of a G-protein-coupled receptor can be gained
from cAMP measurements in living transfected cells using convenient, commercially available kits that do not require radioactivity. The activation of G-protein-coupled receptors results in a concentration-dependent increase in cAMP levels, the activation potency of which can be determined (EC\textsubscript{50} value). Similarly, the activation of G-protein-coupled receptors can be assessed by measuring intracellular calcium changes (Rickli et al., 2015c). With the inclusion of a known full agonist (typically an endogenous ligand) in the assay, the maximal efficacy can be determined. Full agonists induce maximal efficacy, whereas partial agonists induce only partial efficacy compared to endogenous ligands.

With regard to the translational relevance of in vitro screenings, setting the data in an informative clinical context is essential. Comparisons with well-known psychoactive substances inform about the similarity of NPS to these substances with known subjective effects, toxicity, and abuse liability. Furthermore, data on the link between pharmacological targets and subjective/physiological effects are needed. Several rodent and human studies have contributed to our understanding of the roles of DAT, SERT, and NET inhibition in the mode of action of psychoactive drugs. In rodents, particularly mice, genetic modification allows the elimination of a specific target and assessment of the behavioral and molecular impacts of the knockout. Constitutive knockout mouse models generally have the limitation of compensatory alterations that can occur, thus resulting in distinct phenotypes that are not ideal for finding explicit target-mediated effects (Kalueff et al., 2010; Viggiano et al., 2003; Xu et al., 2000). Nevertheless, several knockout studies have implicated the DAT and SERT in the actions of psychostimulants. For example, SERT knockout mice exhibit greater rewarding effects of cocaine in the conditioned place preference paradigm compared with wildtype mice (Sora et al., 2001). More sophisticated genetic models with a triple amino acid mutation in the DAT gene showed that DAT inhibition is necessary for cocaine-induced conditioned place preference (O'Neill et al., 2014) and cocaine-evoked synaptic plasticity (Brown et al., 2010). Clinical studies that assess pharmacological interactions between a psychostimulant and receptor-selective antagonists or well-characterized transporter ligands shed light on specific molecular target mediating subjective effects and acute toxicity in humans. For example, our laboratory investigated the mode of action of MDMA in humans by blocking the NET, SERT, or DAT or combinations thereof (Hysek et al., 2011; Hysek et al., 2012c; Hysek et al., 2014; Liechti et al., 2000; Schmid et al., 2014; Schmid et al., 2015). These studies showed that NET and α\textsubscript{1}-adrenergic stimulation are crucially involved in MDMA-induced sympathomimetic activation, including elevations of blood pressure and body temperature (Hysek et al., 2012a; Hysek et al., 2013; Hysek et al., 2011; Schmid et al., 2015) and that the SERT-mediated release of 5-HT is involved in the subjective entactogenic/empathogenic effects of MDMA (Hysek et al., 2012b; Hysek et al., 2012c; Liechti et al., 2000). Interactions with the DAT and activation of the DA system are generally considered responsible for the reinforcing and addictive properties of a substance (Howell & Wilcox, 2002). Accordingly, NPS that mostly interact with the SERT can be expected to produce more empathogenic MDMA-like effects, in contrast to NPS that mostly interact with the NET and DAT and are thus expected to produce more stimulant-type effects and addiction similar to methamphetamine (Liechti, 2015; Simmler et al., 2013). Additionally, we noted that substances, such as MDMA, that primarily release endogenous monoamines via the
transporter may have a shorter duration of action despite having a long plasma half-life (Hysek et al., 2012c) than substances that only inhibit a transporter (e.g., pyrovalerone cathinones; Derungs et al., 2011) or interact with postsynaptic receptors (e.g., hallucinogens; Dolder et al., 2015; Rickli et al., 2015c).

In *in vivo* studies in rodents and humans increase our knowledge of the effects and toxicity that are related to individual targets that mediate the complex actions of psychostimulants and help predict the toxicity of NPS. Dissecting the clinical roles of different neurotransmitter systems and attributing specific effects to specific targets or pharmacological profiles (e.g., DAT/SERT ratio; Liechti, 2015; Simmler et al., 2013) support the meaningful translation of *in vitro* NPS pharmacology to expected subjective effects and toxicity in humans. Newer techniques, such as optogenetic approaches, for dissecting brain circuitry or sophisticated transgenic animal models without compensatory alterations that can isolate target-mediated effects *in vivo* will continue to shape our understanding of psychoactive drug actions with regard to specific targets, which will also impact interpretations of the *in vitro* pharmacology of NPS.

### 3 Effects on Cathinone Analogs on Transporter-Mediated Uptake

All cathinone NPS inhibit transporter-mediated monoaminergic uptake but with different selectivity and relative potencies. The precise profile of relative DAT, SERT, and NET inhibition potencies likely determines the different experiences that are described by drug users. In the screening from our laboratory, most cathinone NPS are potent NET inhibitors, with uptake inhibition potencies in the submicromolar range (Table 1). *N,N*-dimethylcathinone, ethylone, methedrone, and 4-methylethcathinone are the exceptions with NET inhibition IC$_{50}$ in the low micromolar range. High potency for NET inhibition relative to DAT and SERT were also reported from other laboratories (Eshleman et al., 2013; Iversen et al., 2013; Rosenauer et al., 2013), but with less prominent fold-shifts compared to DAT inhibition. This likely arises from different assay conditions that determine the IC$_{50}$ values. However, the general high inhibition potency of NET for most cathinones NPS are consistent across laboratories. Drug-induced increases in NE markedly contribute to the psychostimulation of a drug and sympathomimetic toxicity (Hysek et al., 2011; Hysek et al., 2012c). We compared the common recreational doses that are taken in a single drug session and uptake inhibition potencies at the NET, SERT, or DAT and found that the recreational doses correlated mainly with NET inhibition potencies (Simmler et al., 2013). This is in agreement with Rothman *et al.* (2001) who found a linear correlation between release-induction potency in synaptosomes and oral doses producing Therefore, the *in vitro* inhibition potency at NET best predicts clinical potency and the doses that are likely to be used recreationally.

Significant differences in DAT and SERT inhibition potencies among cathinone NPS are evident (Iversen et al., 2013; Simmler et al., 2013; Simmler et al., 2014a). Many cathinone NPS are potent DAT inhibitors that are comparable to methamphetamine or cocaine, and some cathinone NPS are weak DAT inhibitors that are more comparable to MDMA. In our assays, methamphetamine and cocaine, which are well-known psychostimulants that act on the DAT, exhibit DAT inhibition potencies (IC$_{50}$ values) around 1 µM. Many pyrovalerone cathinones are extremely potent DAT inhibitors. The most popular pyrovalerone cathinone, MDPV, is 30-times more potent in inhibiting the
DAT in heterologous expression systems than cocaine (Eshleman et al., 2013; Simmler et al., 2013). Similarly in synaptosomes 40 – 50-fold differences in DAT inhibition potency between MDPV and cocaine were reported (Baumann et al., 2013). MDPV is also called “super coke,” and small doses may have strong and long-lasting effects because of its high potency and pure uptake inhibition (Ross et al., 2012). Severe toxicity and even deaths have resulted from the recreational use of this substance (Borek & Holstege, 2012; Murray et al., 2012). To avoid such cases, warnings could be issued for extremely potent substances like MDPV as soon as they emerge as recreationally used substances. Therefore, testing newly emerged NPS in *in vitro* pharmacological screenings as fast as possible is highly important to detect substances with high potencies at monoaminergic targets that are relevant to stimulant or other psychotropic actions.

Inhibition of the SERT is generally less represented among the cathinone derivatives but is characteristic for such substances as benzofuranes (Rickli et al., 2015b), aminoindanes, benzylpiperazines (Simmler et al., 2014b), and ring/para-substituted amphetamines (Rickli et al., 2015a), which have MDMA-like psychoactive properties. Compared with the serotonergic drug MDMA, only naphyrone among the cathinone NPS is equally potent in inhibiting the SERT (Iversen et al., 2013; Simmler et al., 2013). However, methedrone has a similar DAT/SERT inhibition ratio to MDMA, thus predicting a similar effect profile to MDMA, in addition to predicting high risk of hyperthermia because of its similarity to para-methoxy-amphetamine (Liechti, 2015; Simmler et al., 2014a). Other cathinone NPS inhibit the SERT with lower potencies, resulting in relatively more dopaminergic properties, or their SERT inhibition is negligible.

Ideally, the SERT inhibition potency of substances is set relative to their DAT inhibition. Relative activity at the DAT vs. SERT can serve as an indicator of the abuse liability of a psychoactive substance because potent SERT activity relative to DAT activity can be protective against the abuse of a drug (Bauer et al., 2013; Schindler et al., 2015; Wee et al., 2005). Substances with potent SERT inhibition are less reinforcing than substances with low SERT vs. DAT activity (Bauer et al., 2013; Rothman & Baumann, 2006; Wee et al., 2005). Using uptake inhibition potencies, we calculated DAT/SERT ratios (IC$_{50,SERT}$/IC$_{50,DAT}$). Note that the calculation with the reciprocal formula IC$_{50,SERT}$/IC$_{50,DAT}$ results in high DAT/SERT ratios for substances that inhibit DAT more potently (lower IC$_{50}$ value) than SERT (higher IC$_{50}$ value) and vice versa. In our hands, where cocaine has a DAT/SERT ratio of ~1, substances with a DAT/SERT ratio > 1 can be considered to have high abuse liability. Substances with a DAT/SERT ratio close to that of MDMA (0.1) likely have lower abuse liability. For example, we predicted particularly high abuse potential for MDPV based on its high DAT/SERT inhibition ratio (Simmler et al., 2013). Animal studies and clinical observations confirmed the potent reinforcing and rewarding properties of MDPV, confirming *in vitro* study-based predictions of abuse potential (Watterson et al., 2014; Watterson & Olive, 2014).

For some cathinone NPS in our screening studies, we determined the profile of respective structural amphetamine analogs that lack the β-keto group (Rickli et al., 2015b; Simmler et al., 2013; Simmler et al., 2014a). Adding a β-keto group to MDMA to form methylone resulted in a higher DAT/SERT ratio and thus higher predicted abuse liability. The shift in the DAT/SERT inhibition ratio that results from the addition of a β-keto group was less pronounced for amphetamines with an already high DAT/SERT
inhibition ratio, such as methamphetamine. Notably, a small change in the molecular structure of some amphetamines can result in a significantly different pharmacological profile.

4 Effects of Cathinones on Transporter-Mediated Efflux

Substances that inhibit monoamine transporters are either pure uptake inhibitors or releasers (Rothman et al., 2001). If they are monoamine releasers, then they induce transporter-mediated efflux, which should not be confused with exocytotic calcium-dependent vesicular monoamine release. Transporter-mediated efflux occurs when drugs act as substrates of the transporters (Sulzer et al., 2005). As substrates, the substances are transported into the cell. Because amphetamine analogs, such as MDMA and methamphetamine, are releasers (Rothman et al., 2001; Rudnick & Wall, 1992), it is of interest to characterize cathinone NPS as releasers or pure uptake inhibitors. All releasers or substrates, including the endogenous substrates (i.e., DA, NE, and 5-HT), present uptake inhibition properties because of competition for transport (Rothman et al., 2001). Therefore, uptake assays cannot determine whether a substance is an inhibitor or a substrate releaser, but separate efflux assays can determine whether a drug is a releaser or pure uptake inhibitor. Interestingly, pyrovalerone cathinones are pure uptake inhibitors (Table 2), although they are amphetamine-type substances. Most other cathinone NPS are releasers like their amphetamine analogs (Table 2).

We distinguish monoamine-releasing substances from pure monoamine uptake inhibitors, but the impact of release vs. pure uptake inhibition on psychoactive effects is unclear and likely less relevant than the DAT/SERT inhibition ratio (Liechti, 2015). This distinction is less relevant for subjective and stimulant effects than for cellular toxicity. Because release-inducing substances enter nerve terminals via transporters, they are more likely to exert intracellular effects and toxicity compared with pure uptake inhibitors (Sulzer et al., 2005). Typically, releasers act on vesicular monoamine transporters and deplete vesicles, which can have short- or long-term toxic consequences (Steinkellner et al., 2011).

With the large numbers of NPS reported in the recent years, there is need for a classification of NPS. NPS can be classified by their chemical structures. For example, Hill et al. (2011) classified MDMA as ring-substituted methylenedioxyphenethylamine, mephedrone as beta-ketonated amphetamine, and MDPV as beta-ketonated substituted methylenedioxyphenylethylamine. A structural classification is very useful for an audience with an interest in the chemical structure of NPS. An audience with a clinical focus might mainly be interested in anticipated subjective effects and toxicology. A classification according to pharmacological profiles are likely more meaningful for clinicians than chemical structures, particularly also since structural similarities not necessarily result in comparable pharmacological profiles. In our NPS screenings, we classify cathinone derivatives according to the similarity of their in vitro profile to methamphetamine, cocaine, and MDMA (Liechti, 2015; Simmler et al., 2013). DAT/NIT-selective pyrovalerone cathinones represent a separate group since they are extremely potent inhibitors. Importantly, small structural changes can markedly alter the pharmacological profile of substances, sometimes in an unpredicted manner, resulting in different psychoactive and toxicological effects. For example methylene, the β-keto
analog of MDMA, presents a prominent increase in DAT/SERT ratio, suggesting a higher abuse potential of methylone compared to MDMA (Baumann et al., 2012; Simmler et al., 2013). Classification according to pharmacology may thus be more conclusive as a reference for clinical applications than structural analogies.

5 Drug Interactions with G-Protein-Coupled Receptors

In addition to transporter pharmacology, assessing receptor interactions is necessary for a comprehensive pharmacological characterization of psychoactive substances. The major implications would be for the assessment of any hallucinogenic properties of NPS. LSD has high affinity for the 5-HT$_{2A}$ receptor (Nichols, 2004; Rickli et al., 2015c), which is associated with its hallucinogenic properties. Other drugs with potent 5-HT$_{2A}$ activity have been shown to substitute for LSD in drug-discrimination studies (Eshleman et al., 2014). In vitro activity at the 5-HT$_{2A}$ receptor is a good predictor of possible hallucinogenic effects and is likely the most relevant receptor/NPS interaction that is assessed in in vitro screening, particularly for potentially hallucinogenic compounds (Rickli et al., 2015c). The activation of DA D$_1$ receptors but not D$_2$ receptors might be sufficient for a substance to be rewarding (Caine et al., 2007). Noradrenergic receptors are involved in sympathomimetic toxicity, leading to vasoconstriction, hyperthermia, increased blood pressure, and increased heart rate (Hysek et al., 2012a; Hysek et al., 2013).

The main targets of amphetamine analogs are typically monoamine transporters, but some substances have weak affinity for monoamine receptors. However, it is questionable if direct receptor affinity contributes markedly to the overall drug effect of substances that foremost are transporter inhibitors. The rise in extracellular monoamine concentration that is evoked by a drug’s effects at the transporters results in neurotransmitter binding to postsynaptic receptors, which might cause that direct agonism has only negligible contribution to the overall drug effect. Direct antagonistic receptor activation might, to some extent, counteract neurotransmitter binding at postsynaptic receptors. We and others did not find any cathinones or amphetamines with relevant affinity at D$_1$, D$_2$, or D$_3$ receptors (Iversen et al., 2013; Rickli et al., 2015a; Simmler et al., 2013; Simmler et al., 2014a). However, some cathinone analogs exhibit weak affinity for 5-HT$_{2A}$ or 5-HT$_{2C}$ receptors and are low-potency 5-HT$_{2A}$ antagonists (Eshleman et al., 2013). Compared with hallucinogens that exert their psychoactive effects mainly via 5-HT receptors (e.g., the NPS benzodifuran 2C-B-Fly or novel N-2-methoxybenzyl-derivatives), with receptor binding values in the submicromolar range (Rickli et al., 2015b; Rickli et al., 2015c), the weak binding affinities of cathinones at these targets are likely irrelevant.

In our pharmacological characterization of NPS, we also include the trace amine-associated receptor 1 (TAAR1; Rickli et al., 2015a; Simmler et al., 2013; Simmler et al., 2016; Simmler et al., 2014a). Methamphetamine and other amphetamine-type drugs have been shown to activate the TAAR1, and the TAAR1 could be a target for the pharmacological treatment of addiction (Jing & Li, 2015). Substance-mediated agonist effects at the TAAR1 may reduce the stimulant properties of MDMA and methamphetamine (Achat-Mendes et al., 2012; Di Cara et al., 2011). In contrast, cathinone NPS do not present affinity for the TAAR1 and may thus have more stimulant-
like effects and be more addictive than their amphetamine analogs because of the lack of this TAAR1-mediated “auto-inhibition,” in addition to their greater dopaminergic properties. This could be relevant for experiments conducted in rodents. In humans, however, direct affinity of psychoactive substances is probably negligible since in general no or only weak activation of the human TAAR1 by psychostimulants is evident from in vitro screenings (Simmler et al., 2016). Nevertheless, TAAR1 presents a promising target that could be highly relevant for psychostimulant treatment.

6 Summary

NPS continue to emerge and are recreationally used without much knowledge about their pharmacology or toxicology. In vitro characterizations of psychoactive compounds that utilize transfected cell lines are useful for gaining fast and translationally important information on cathinone NPS. The in vitro pharmacological profiles of cathinone NPS have predicted considerable abuse liability of these drugs and identified pyrovalerone cathinones with extremely high potencies for DAT inhibition. Small structural changes, such as the β-keto group in the amphetamine-basic structure, can substantially change the pharmacological profile of substances with regard to their potency and relative activity at different monoaminergic targets.

References


Cheng Y, & Prusoff WH (1973). Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. Biochem Pharmacol 22: 3099-3108.


### Table 1: Uptake inhibition potencies of cathinone NPS and the respective non-β-keto analogues.

<table>
<thead>
<tr>
<th>Cathinone-analogs</th>
<th>NET (IC_{50}) (µM) (95% CI)</th>
<th>DAT (IC_{50}) (µM) (95% CI)</th>
<th>SERT (IC_{50}) (µM) (95% CI)</th>
<th>Amphetamine-analogs</th>
<th>NET (IC_{50}) (µM) (95% CI)</th>
<th>DAT (IC_{50}) (µM) (95% CI)</th>
<th>SERT (IC_{50}) (µM) (95% CI)</th>
<th>Values published in</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Bromomethcathinone</td>
<td>0.41 (0.30-0.57)</td>
<td>5.6 (2.7-12)</td>
<td>2.2 (1.7-2.8)</td>
<td>Amphetamine</td>
<td>N,N-Dimethylcathinone</td>
<td>7.71 (5.1-12)</td>
<td>27 (21-36)</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Buphedrone</td>
<td>0.65 (0.51-0.85)</td>
<td>6.24 (3.3-13.5)</td>
<td>75 (23-270)</td>
<td>MBDB</td>
<td>Buphedrone</td>
<td>0.65 (0.51-0.85)</td>
<td>4.24 (3.3-5.5)</td>
<td>70 (2-270)</td>
</tr>
<tr>
<td>Cathinone</td>
<td>0.193 (0.15-0.25)</td>
<td>14.0 (10-20)</td>
<td>&gt;100</td>
<td>N,N-Dimethylcathinone</td>
<td>7.71 (5.1-12)</td>
<td>27 (21-36)</td>
<td>&gt;500</td>
<td></td>
</tr>
<tr>
<td>IV/N-Dimethylcathinone</td>
<td>7.71 (5-12)</td>
<td>27 (21-36)</td>
<td>&gt;500</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethcathinone</td>
<td>0.44 (0.34-0.56)</td>
<td>5.00 (3.7-6.8)</td>
<td>48 (4-529)</td>
<td>N-Ethylamphetamine</td>
<td>0.20 (0.15-0.27)</td>
<td>5.86 (4.8-7.1)</td>
<td>8.77 (4-13)</td>
<td></td>
</tr>
<tr>
<td>4-Ethylmethcathinone</td>
<td>2.5 (1.7-3.7)</td>
<td>31 (15-72)</td>
<td>4.3 (2.3-5.8)</td>
<td>MBDB</td>
<td>2.80 (1.9-4.1)</td>
<td>22 (20-26)</td>
<td>2.04 (1.4-3.0)</td>
<td></td>
</tr>
<tr>
<td>Ethlyone</td>
<td>2.54 (2-3.2)</td>
<td>5.68 (4.6-6.5)</td>
<td>4.46 (3.8-5.2)</td>
<td>MICA</td>
<td>1.02 (0.78-1.3)</td>
<td>9.3 (6.0-11)</td>
<td>1.27 (0.93-1.7)</td>
<td></td>
</tr>
<tr>
<td>Filloxidone</td>
<td>0.246 (0.16-0.37)</td>
<td>6.35 (4.2-9.5)</td>
<td>&gt;10</td>
<td></td>
<td>4-Fluoromethamphetamine</td>
<td>0.22 (0.14-0.35)</td>
<td>7.7 (5.2-14.5)</td>
<td>8.7 (5.5-20)</td>
</tr>
<tr>
<td>3-Fluoromethcathinone</td>
<td>0.19 (0.13-0.29)</td>
<td>1.71 (1.0-3.0)</td>
<td>56 (17-172)</td>
<td>MICA</td>
<td>0.42 (0.3-0.6)</td>
<td>20.5 (20.3-20.8)</td>
<td>4.9 (3.5-6.6)</td>
<td></td>
</tr>
<tr>
<td>β-keto MDA</td>
<td>1.6 (1.1-2.3)</td>
<td>14.1 (10-19)</td>
<td>21 (15-28)</td>
<td>MICA</td>
<td>0.42 (0.3-0.6)</td>
<td>20.5 (20.3-20.8)</td>
<td>4.9 (3.5-6.6)</td>
<td></td>
</tr>
<tr>
<td>MCPPB</td>
<td>0.15 (0.11-0.24)</td>
<td>0.11 (0.07-0.16)</td>
<td>15 (5-50)</td>
<td>MICA</td>
<td>0.42 (0.3-0.6)</td>
<td>20.5 (20.3-20.8)</td>
<td>4.9 (3.5-6.6)</td>
<td></td>
</tr>
<tr>
<td>MCPPP</td>
<td>0.67 (0.42-1.1)</td>
<td>0.53 (0.27-1.1)</td>
<td>75 (49-114)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPBV</td>
<td>0.54 (0.33-0.77)</td>
<td>0.301 (0.23-0.4)</td>
<td>9.30 (6.8-12.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mephedrone</td>
<td>0.254 (0.20-0.30)</td>
<td>3.31 (2.6-4.2)</td>
<td>4.84 (3.7-5.9)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methcathinone</td>
<td>0.185 (0.15-0.22)</td>
<td>1.12 (0.8-1.5)</td>
<td>&gt;10</td>
<td>Methamphetamine</td>
<td>0.164 (0.13-0.20)</td>
<td>1.05 (0.74-1.5)</td>
<td>&gt;10</td>
<td></td>
</tr>
<tr>
<td>Methedrone</td>
<td>2.34 (1.4-3.3)</td>
<td>35 (15-75)</td>
<td>4.73 (2.2-9.8)</td>
<td>PMA</td>
<td>1.30 (0.75-1.8)</td>
<td>49 (18-135)</td>
<td>1.27 (1.1-2.6)</td>
<td></td>
</tr>
<tr>
<td>4-Methylmethcathinone</td>
<td>2.23 (1.5-3.2)</td>
<td>4.28 (3.4-5.4)</td>
<td>4.00 (3.5-4.5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methylone</td>
<td>0.542 (0.39-0.75)</td>
<td>0.42 (0.3-0.6)</td>
<td>15.5 (10-26)</td>
<td>MDM</td>
<td>0.447 (0.33-0.60)</td>
<td>17 (12-24)</td>
<td>1.36 (1.0-2.0)</td>
<td></td>
</tr>
<tr>
<td>Naphyrone</td>
<td>0.25 (0.20-0.32)</td>
<td>0.47 (0.30-0.55)</td>
<td>0.86 (0.6-1.3)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentedrone</td>
<td>0.61 (0.52-0.70)</td>
<td>2.50 (2.1-3.2)</td>
<td>135 (5-270)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentylone</td>
<td>2.39 (1.5-3.4)</td>
<td>35 (15-75)</td>
<td>4.73 (2.2-9.8)</td>
<td>PMA</td>
<td>1.30 (0.75-1.8)</td>
<td>49 (18-135)</td>
<td>1.27 (1.1-2.6)</td>
<td></td>
</tr>
<tr>
<td>Pynvalonone</td>
<td>0.543 (0.33-0.80)</td>
<td>0.035 (0.02-0.04)</td>
<td>13.0 (10-15.8)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-PVP</td>
<td>0.02 (0.01-0.03)</td>
<td>0.04 (0.01-0.1)</td>
<td>&gt;100</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(1) Simmler et al., 2013, Br J Pharmacol (3) Rickli et al., 2015, Eur Neuropsychopharmacol
(2) Simmler et al., 2014, Neupharmacology (4) Rickli et al., 2015, Br J Pharmacol
Table 2: Qualitative characterization of cathinone NPS and the respective non-β-keto analogues as releasers at NET, DAT, and SERT.

<table>
<thead>
<tr>
<th>Cathinone-analogs</th>
<th>NE efflux</th>
<th>DA efflux</th>
<th>5-HT efflux</th>
<th>Amphetamine-analogs</th>
<th>NE efflux</th>
<th>DA efflux</th>
<th>5-HT efflux</th>
<th>Values published in</th>
</tr>
</thead>
<tbody>
<tr>
<td>4-Bromomethcathinone</td>
<td>yes</td>
<td>yes</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td>Buphedrone</td>
<td>yes</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Buthylone</td>
<td>NA</td>
<td>no</td>
<td>yes</td>
<td>MBDB</td>
<td>NA</td>
<td>no</td>
<td>yes</td>
<td>(1)</td>
</tr>
<tr>
<td>Cathinone</td>
<td>NA</td>
<td>yes</td>
<td>no</td>
<td>Amphetamine</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>(1),(3)</td>
</tr>
<tr>
<td>N,N-Dimethcathinone</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Ethcathinone</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>N-Ethylamphetamine</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>(2)</td>
</tr>
<tr>
<td>4-Ethylmethcathinone</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td>Ethylone</td>
<td>NA</td>
<td>no</td>
<td>yes</td>
<td>MDEA</td>
<td>NA</td>
<td>no</td>
<td>yes</td>
<td>(1)</td>
</tr>
<tr>
<td>Flephedrone</td>
<td>yes</td>
<td>yes</td>
<td>yes*</td>
<td>4-Fluoromethamphetamine</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>(1),(3)</td>
</tr>
<tr>
<td>3-Fluoromethcathinone</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>β-keto MDA</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>MDA</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>(4)</td>
</tr>
<tr>
<td>MDPBP</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td>MDPPP</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(3)</td>
</tr>
<tr>
<td>MDPV</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1),(3)</td>
</tr>
<tr>
<td>Methedrone</td>
<td>yes</td>
<td>yes</td>
<td>yes*</td>
<td>Methamphetamine</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>(1),(3)</td>
</tr>
<tr>
<td>Methcathinone</td>
<td>yes</td>
<td>no</td>
<td>yes</td>
<td>PMMA</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>(2)</td>
</tr>
<tr>
<td>4-Methylethcathinone</td>
<td>no</td>
<td>no</td>
<td>yes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Methylone</td>
<td>NA</td>
<td>no</td>
<td>yes</td>
<td>MDMA</td>
<td>yes</td>
<td>yes</td>
<td>yes</td>
<td>(1),(3)</td>
</tr>
<tr>
<td>Naphryone</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1),(3)</td>
</tr>
<tr>
<td>Pentylone</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>Pyrovalerone</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(2)</td>
</tr>
<tr>
<td>α-PVP</td>
<td>no</td>
<td>no</td>
<td>no</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(1),(3)</td>
</tr>
</tbody>
</table>

NA) not assessed
*) Not significant in Rickli et al., 2015, Eur Neuropsychopharmacol
(1) Simmler et al., 2013, Br J Pharmacol
(2) Simmler et al., 2014, Neuropharmacology
(3) Rickli et al., 2015, Eur Neuropsychopharmacol
(4) Rickli et al., 2015, Br J Pharmacol

24
Figure 1

Mephedrone 4-Methylmethcathinone 4-Bromomethcathinone 3-Fluoromethcathinone

4-Ethylmethcathinone N,N-Dimethylmethcathinone Buphedrone Pentedrone

MDPPP MDPBP MDPV

Naphyrone α-PVP Pyrovalerone
Figure 2