PHARMACOLOGICAL AND TOXICOLOGICAL INVESTIGATIONS OF NEW PSYCHOACTIVE SUBSTANCES

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“An adult must make his own decision as to whether or not he should expose himself to a specific drug, be it available by prescription or proscribed by law, by measuring the potential good and bad with his own personal yardstick.”

— Alexander Shulgin, Pihkal: A Chemical Love Story.

PREFACE

This thesis is split into a pharmacology part and a toxicology part. The pharmacology part consists of investigations on the monoamine transporter and receptor interactions of traditional and newly emerged drugs, mainly stimulants and psychedelics; the toxicology part consists of investigations on mechanisms of hepatocellular toxicity of synthetic cathinones. All research described in this thesis has been published in peer-reviewed journals, and was performed between October 2014 and June 2018 in the Division of Clinical Pharmacology and Toxicology at the Department of Biomedicine of the University Hospital Basel and University of Basel, and partly at the pRED Roche Innovation Center Basel at F. Hoffmann-La Roche.

SUMMARY

Since the mid-2000s, an unprecedented amount of new psychoactive substances (NPSs) have emerged on the recreational drug market and have since then gained popularity as alternatives to traditional drugs of abuse. Besides clinical case reports, the pharmacological profiling and assessment of toxicity *in vitro* provide valuable information on the mechanism of action and possible risks associated with NPSs use.

Within the scope of the pharmacology part of this thesis, focus was laid on the potential of NPSs to inhibit norepinephrine (NE), dopamine (DA), and serotonin (5-HT) transporters (NET, DAT, and SERT, respectively) in transporter-transfected human embryonic kidney (HEK) 293 cells. In addition, monoamine transporter and receptor affinities were determined.

Analogs of the popular NPS 4-methylmethcathinone (mephedrone) potently inhibited NET and, with the exception of 3-methylmethcathinone (3-MMC), inhibited SERT more potently than DAT. Mephedrone and its analogs were substrate-type releasers of NE, DA, and 5-HT. The indole NPS 5-(2-aminopropyl)indole (5-IT) potently inhibited NE, DA, and 5-HT uptake and mediated an efflux of DA and 5-HT. Like amphetamine, its 4-methylated analog 4-methylamphetamine (4-MA) was a transporter substrate but in contrast to amphetamine, 4-MA had a higher selectivity for SERT *vs*. DAT. The two indane NPSs *N*-methyl-2-aminoindane (*N*-methyl-2-AI) and 5-methoxy-6-methyl-2-aminoindane (MMAI) were selective inhibitors of NE and 5-HT uptake, respectively, and selective releasers of the respective neurotransmitters. The predominant actions on SERT *vs.* DAT suggests that dimethylmethcathinones, 4-MA, and MMAI may mediate certain entactogenic effects similar to 3,4-methylenedioxymethamphetamine (MDMA), whereas 3-MMC, 5-IT, and *N*-methyl-2-AI are stimulants similar to amphetamine. Analogs of the prescription drug methylphenidate (MPH) mainly inhibited NET and DAT and showed only weak inhibition of SERT. Like MPH and cocaine, MPH-based NPSs did not elicit transporter-mediated efflux of any monoamines. The predominant actions on DAT *vs.* SERT indicates that theses compounds are associated with an increased abuse liability. Diclofensine, a NPS that was originally developed as antidepressant, was a triple monoamine inhibitor without releasing properties. The dissociative NPS diphenidine was an inhibitor of NET and DAT with moderate potency, and its methoxylated derivative methoxphenidine was a selective but weak NET inhibitor. Diphenidine and methoxphenidine were both devoid of any monoamine releasing properties.

2,5-Dimethoxy-4-substituted phenethylamines (2C drugs) and their *N*-2-methoxybenzyl ("NBOMe") analogs potently interacted with serotonergic 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors. The *N*-2-methoxybenzyl substitution of 2C drugs increased the binding affinity at serotonergic 5-HT_{2A} and 5-HT_{2C} receptors and monoamine transporters but reduced binding to the 5-HT_{1A} receptor. NBOMes and 2C drugs were mostly potent partial agonists at the 5-HT_{2A} and 5-HT_{2B} receptors. However, drugs with a bulky and lipophilic 4-substituent had a decreased or absent activation potential or efficacy at these receptors.

A correlation analysis revealed that the assessed *in vitro* data can help to predict human effective doses of stimulants and psychedelics. For stimulants, DAT and NET inhibition potency positively, and SERT inhibition potency inversely correlated with human doses reported on the Internet. For psychedelics, serotonin 5-HT_{2A} and 5-HT_{2C} but not 5-HT_{1A} receptor affinity significantly correlated with human effective doses. Serotonin receptor activation data did not correlate with human doses. However, it is a necessity to determine whether a drug activates the 5-HT_{2A} receptor in order to predict its potential to induce psychedelic effects in humans.

For the toxicological part of the thesis, focus was laid on hepatotoxic mechanisms of six synthetic cathinones in two hepatocellular cell lines. For bupropion, 3,4-methylenedioxypyrovalerone (MDPV), mephedrone, and naphyrone the depletion of cellular ATP content preceded cytotoxicity, suggesting mitochondrial toxicity. In contrast, methedrone and methylone depleted the cellular ATP pool and induced cytotoxicity at similar concentrations. Bupropion, MDPV, and naphyrone additionally decreased the mitochondrial membrane potential, confirming mitochondrial toxicity. Bupropion was the only compounds that uncoupled oxidative phosphorylation. Bupropion, MDPV, mephedrone, and naphyrone inhibited complex I and II of the electron transport chain, naphyrone also complex III. The cathinones associated with mitochondrial toxicity were shown to increase mitochondrial reactive oxygen species (ROS) and lactate production, and naphyrone and MDPV additionally depleted the cellular total glutathione (GSH) pool. Liver injury associated with these drugs is rare and affected persons likely have susceptibility factors rendering them more sensitive for the hepatotoxicity of these drugs.

ACKNOWLEDGEMENTS

The research presented in this thesis is a product of many people's contributions. It started with Stephan and Matthias agreeing to co-supervise my thesis and to split my project into two parts, as a full-time position in either group was not possible at that time. Since starting my thesis, my supervisors have constantly supported me and gave me the freedom to develop and improve myself. A successful completion of my thesis would not have been possible without all the help of my supervisors and the goodwill of Prof. Anne Eckert who agreed to join the dissertation committee. At the beginning of my time in the lab, Anna introduced me to pharmacological research and Franzi introduced me to toxicological research. Soon after, I got the chance to pass on my freshly acquired knowledge to a series of students and this thesis would most likely be shorter without the contributions of Philine, Karolina, Luca, Robert, and Melanie. In addition to the support in the lab, Marius and his team at Roche played an important part by screening a high amount of our compounds. Bea, Evelyne, and Jamal were always helpful when I had questions of any kind. Urs provided valuable advice with his extensive knowledge of bioanalytics and card game tactics. Cécile was possibly the only person who delayed the completion of this thesis, but the fun we had during all the distractions were undoubtedly worth it. Riccardo and I had great discussions during our extremely short breaks in the gym. The joyful laughter of Gerda made tough times easier to swallow. David, Fabio, François, Miljenko, Noëmi, and Xun made my time in the lab a mostly pleasant experience. Friede, Patrick, and Yasmin participated in many stimulating discussions; sometimes we even talked about work. Finally, I am very happy that Deborah was and still is around to help me find my way, literally and figuratively!

TABLE OF CONTENT

RATIONALE AND MOTIVATION

In recent years, various so-called new psychoactive substances (NPSs; “legal highs”,“designer drugs”) have emerged on the recreational drug market. Such compounds are often structurally and pharmacologically related to traditional drugs of abuse but due to their novelty, they are usually not yet legally controlled when they first appear. Moreover, distributors often circumvent the law by misleading labeling such as “research chemicals”, “plant food”, “bath salts”, or “not for human consumption” (Liechti, 2015). More than 600 different NPSs were reported to the EU Early Warning System of the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) between 2005 and 2016 (European Monitoring Centre for Drugs and Drug Addiction, 2017). A variety of chemical classes with distinct pharmacological profiles have been reported, such as synthetic cathinones, synthetic cannabinoids, phenethylamines, opioids, tryptamines, arylamines, benzodiazepines, piperazines, and others (European Monitoring Centre for Drugs and Drug Addiction, 2015).

In this thesis, focus will be laid on two aspects related to NPSs use. In the first part, the interactions of various chemically distinct NPSs with monoamine transporters and receptors will be discussed. This part includes different classes of stimulants (cathinones, stimulant phenethylamines, aminoindanes, and phenidates) and psychedelic phenethylamines. Furthermore, monoaminergic transporter and receptor interactions will be discussed as off-target effects for the *N*-methyl-D-aspartate (NMDA) receptor antagonists diphenidine and methoxphenidine. Correlations between *in vitro* data and human effective doses are discussed at the end of the first part. In the second part, potential hepatotoxic mechanisms associated with synthetic cathinones will be described. At the beginning of each part, a separate introduction to the subject is included, which is followed by original research.

A detailed review of the structure and pathways of monoaminergic systems and detailed review of the liver anatomy and function would be far beyond the scope of this thesis. Therefore, the introduction of the first part only focuses on the role that monoamine transporters and receptors play in association with the mechanism of action of stimulants and psychedelics. Transporters and receptor subtypes that were not investigated are not specifically discussed. The introduction of the second part addresses potential mechanisms of stimulant-induced hepatotoxicity, with a focus on mitochondria. A short overall discussion of the research presented in this thesis is given in the concluding remarks and outlook section at the end of the thesis.

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CONTRIBUTIONS

I contributed as lead author to the publications presented in this thesis with the exception of the investigation of the receptor interaction profiles of novel *N*-2-methoxybenzyl (NBOMe) derivatives of 2,5-dimethoxy-substituted phenethylamines (2C drugs). However, this publication is included in the thesis as I contributed with a substantional number of experiments and therefore co-authored the publication. Besides myself, master students under my supervision participated in the research and several experiments have been done in collaboration with a neuroscience research laboratory at Hoffmann-La Roche. All contributors and their affiliations are listed on the respective publications.

Part I PHARMACOLOGICAL INVESTIGATIONS

1 TARGETS OF STIMULANTS AND PSYCHEDELICS

1.1 Monoaminergic systems

Monoamine neurotransmitters are widespread in the nervous system and are derived from aromatic amino acids and thyroid hormones (Bjork and Svenningsson, 2011). Monoamines have a chemical template comprised of an aromatic nucleus that is connected to an amino group by a two carbon aliphatic chain. Monoamines include norepinephrine (NE), dopamine (DA), serotonin (5-hydroxytryptamine; 5-HT), epinephrine, histamine, and trace amines. Monoaminergic signaling is modulated by various traditional and newly emerged drugs, which can be roughly divided into stimulants and psychedelics (Liechti, 2015). Such substances may either interact with transporters or with receptors, or a combination thereof.

1.2 Role of monoamine transporters in drug action

The monoamine neurotransmitters NE, DA, and 5-HT mediate a variety of functions, such as locomotion, autonomic function, hormone secretion, affect, emotion, and reward (Torres et al., 2003). Transporter-mediated reuptake of NE, DA, and 5-HT into the presynapse by neurotransmitter:sodium symporters (NSS) for NE (NET/SLC6A2), DA (DAT/solute carrier protein SLC6A3), and 5-HT (SERT/SLC6A4) is the principal mechanism of terminating signal transduction in monoaminergic neurons (Kristensen et al., 2011). These NSS are mainly expressed in the neurons containing the respective monoamines. DAT is expressed in the substantia nigra and ventral tegmental area, NET is expressed in the locus coeruleus and other brainstem nuclei, and SERT is expressed in the median and dorsal raphe nuclei (Hoffman et al., 1998). Therapeutic compounds such as antidepressants, and recreationally used psychoactive drugs like cocaine or amphetamines target NSS. These compounds may either act as transporter inhibitors that bind to the transporter or as substrate-type monoamine releasers (Rothman and Baumann, 2003), both leading to increased monoamine concentrations in the synaptic cleft and subsequently to an increased response at the respective target receptors. Cocaine, methylphenidate (MPH), and various related compounds are non-selective, competitive inhibitors of NSS (Ritz et al., 1987). Examples of selective inhibitors are desipramine, nisoxetine, and reboxetine (selective NET inhibitors), the cocaine analogues GBR 12935 and WIN 35,428 (selective DAT inhibitors), or the selective serotonin reuptake inhibitors (SSRIs) fluoxetine, citalopram, paroxetine, and sertraline (Torres et al., 2003). Compared to the transporter inhibitors, the mechanism of action of most amphetamines is different. Amphetamines act as exogenous substrates of the NSS inducing an

inwardly directed electrophysiological current, a phenomenon not observed for inhibitors (Sandtner et al., 2016; Schicker et al., 2012). The binding mode of the drug at the transporter is decisive whether a drug is an inhibitor, a substrate, or both (Sandtner et al., 2016). Exposure to SERT substrates induces a conformational change of the transporter from an outward facing to an inward facing (releasing) state (Fenollar-Ferrer et al., 2014). This conformational change of SERT results in an increase in the intramolecular distance between the N and C terminus, which can be detected using fluorescence resonance energy transfer (FRET) measurement (Schicker et al., 2012). Inside the presynaptic terminal, transporter substrates cause a release of vesicular monoamines into the cytoplasm by acting as weak bases at the vesicles (Jones et al., 1994; Seiden et al., 1993; Sitte and Freissmuth, 2015; Sulzer et al., 1995; Sulzer et al., 1993; Torres et al., 2003). According to this "weak base hypothesis", transporter substrates are transported into synaptic vesicles by the vesicular monoamine transporters (VMATs) 1 and 2, and subsequently decrease the proton gradient. This then prevents inward transport of monoamines (Sitte and Freissmuth, 2015; Sulzer et al., 1995; Sulzer et al., 1993). The elevation of cytosolic monoamine neurotransmitters is furthermore increased by inhibition of monoamine oxidase (MAO) inhibition of the drugs (Sitte and Freissmuth, 2015). In contrast to physiological efflux of neurotransmitters by exocytosis, drug-mediated elevated concentrations of cytosolic monoamines result in an occupation of the internal substrate-binding site and outward transport by NSS (Scholze et al., 2000; Sitte and Freissmuth, 2015).

The monoamine reuptake inhibition profiles can be used to predict psychotropic effects of stimulants. Dopamine plays an essential role in the reinforcing effects of drugs (Koob, 1992; Ritz et al., 1987; Wise, 1978; Woolverton and Johnson, 1992), whereas serotonergic activity is inversely linked to abuse liability (Kuhar et al., 1991; Ritz et al., 1987; Wee et al., 2005; Wee and Woolverton, 2006). Therefore, the DAT/SERT inhibition ratio (1/DAT IC_{50} : 1/SERT IC_{50}) is a marker of the reinforcing effects and abuse liability of a substance (Baumann et al., 2000).

1.3 Role of monoamine receptors in drug action

The action of monoamine neurotransmitters is almost exclusively mediated through G protein–coupled receptors (GPCRs), which make up the largest group of transmembrane proteins and have seven transmembrane domains (Kobilka, 2007). Several psychiatric and neurological diseases are caused by dysfunction of these systems (Bjork and Svenningsson,

2011) and GPCRs are targets for about a third of all Food and Drug Administration (FDA)-approved drugs (Hauser et al., 2018).

1.4 Alpha-adrenergic receptors

Adrenergic receptors that are activated by NE and epinephrine modulate stimulant-induced behavior (Schmidt and Weinshenker, 2014). Adrenergic α_{1A} receptors are located in most tissues and play an important role maintaining the function of the cardiovascular and urinary systems (Karabacak et al., 2013). Adrenergic α_{2A} receptors are expressed in pyramidal cells in the prefrontal cortex, the locus coerulus, and other regions of the brain (Giovannitti et al., 2015; MacDonald et al., 1997; Wang et al., 2007). The α_{1A} and α_{2A} receptors are thought to have opposing roles as antagonism of α_{1A} receptors decreases the acute locomotor response to stimulants, while α_{2A} receptor antagonism increases the acute locomotor response (Schmidt and Weinshenker, 2014).

1.5 Serotonergic receptors

The serotonin receptor family is the largest family of G-protein coupled neurotransmitter receptors, and their structure and function is comprehensively reviewed in (Nichols and Nichols, 2008). Serotonin 5-HT_{1A} receptors are expressed in limbic brain areas, cortical areas, and dorsal and median raphe nuclei (Valdizan et al., 2010). Azapirone-type drugs act as 5-HT_{1A} receptor agonists and partial agonists (Blier and de Montigny, 1987; Sprouse and Aghajanian, 1987), indicating that 5-HT_{1A} receptors may play a role in anxiety. Furthermore, 5-HT_{1A} receptors have been of interest as targets of antidepressant drugs (Blier and Ward, 2003). Other functions of 5-HT_{1A} receptors are related to brain development (Rojas and Fiedler, 2016), stress response (Samad et al., 2006), immune system modulation (Idova and Davydova, 2010), schizophrenia (Meltzer and Sumiyoshi, 2008), depression (Yohn et al., 2017), and drug addiction (Filip et al., 2010). The 5-HT_{2A} receptors can be found at the highest density in the neocortex, and additionally in the hippocampus, thalamic nuclei, and hypothalamus (Barnes and Sharp, 1999; Lopez-Gimenez and Gonzalez-Maeso, 2018). Serotonin 5-HT_{2A} receptor activation is associated with mind-altering effects of psychedelics (Glennon et al., 1984; Kraehenmann et al., 2017; Nichols, 2004, 2016; Titeler et al., 1988), which can be blocked by the receptor antagonist ketanserin (Preller et al., 2017). Serotonin 5-HT_{2B} receptors are expressed in the liver, kidneys, stomach, and gut, and to a lesser extent also in the lungs, cardiovascular tissue, and central nervous system (Bonhaus et al., 1995; Borman et al., 2002; Choi et al., 1994; Choi and Maroteaux, 1996; Duxon et al., 1997).

Besides coordination of proper formation of organs during development (Nebigil et al., 2000), 5-HT_{2B} receptors are thought to play a role in drug abuse (Lin et al., 2004) and function of the auditory system (Tadros et al., 2007). Serotonin 5-HT_{2C} receptors are mainly localized in the central nervous system with highest expression in epithelial cells of the choroid plexus, and additionally in limbic areas, hippocampus, substantia nigra, amygdala, and hypothalamus (Abramowski et al., 1995; Clemett et al., 2000; Lopez-Gimenez et al., 2001). Agonism at 5-HT_{2C} receptors is associated with anxiety states (Berg et al., 2008) and selective antagonists thereof may therefore potentially act as anxiolytic drugs. All known psychedelics are both 5-HT_{2A} and 5-HT_{2C} agonists; however, the role of 5-HT_{2C} receptor activation in the mechanism of action of psychedelics remains unclear (Nichols, 2004, 2016).

1.6 Dopaminergic receptors

Dopamine receptors mediate various physiological functions including voluntary movement, reward, sleep regulation, feeding, affect, attention, cognitive function, olfaction, vision, hormonal regulation, sympathetic regulation, and penile erection (Beaulieu et al., 2015). Pharmaceuticals targeting dopamine receptors are used for the management of several neuropsychiatric disorders including schizophrenia, bipolar disorder, depression, and Parkinson's disease (Beaulieu et al., 2015). Dopamine D_2 receptors are mainly localized in subcortical regions like the striatum and the nucleus accumbens (Leuner and Muller, 2006), and are targets of antipsychotics (Seeman and Kapur, 2000).

1.7 Trace amine-associated receptors

The trace amine-associated receptor 1 (TAAR1) is expressed in monoaminergic brain regions and the limbic system (Borowsky et al., 2001; Espinoza et al., 2015; Lindemann et al., 2008), and is involved in regulating the limbic network, reward circuits, cognitive processes, and mood states (Lindemann et al., 2008; Miller, 2011; Revel et al., 2013; Wolinsky et al., 2007). Agonists at TAAR1 revealed antipsychotic and antidepressant properties, and TAAR1 was proposed as potential target in the treatment of schizophrenia (Revel et al., 2013; Wolinsky et al., 2007) and dependence on stimulant drugs (Cotter et al., 2015; Di Cara et al., 2011; Jing and Li, 2015; Pei et al., 2014).

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2 PHARMACOLOGY OF STIMULANTS

2.1 Pharmacological profile of mephedrone analogs and related new psychoactive substances

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ABSTRACT

Background: Mephedrone is a synthetic cathinone and one of the most popular recreationally used new psychoactive substances. The aim of the present study was to characterize the *in vitro* pharmacology of novel analogs of mephedrone and related newly emerged designer stimulants.
Methods: We determined norepinephrine, dopamine, and serotonin transporter inhibition potencies and monoamine release in transporter-transfected human embryonic kidney 293 cells. We also assessed monoamine receptor and transporter binding affinities.
Results: Mephedrone analogs potently inhibited the norepinephrine transporter and, with the exception of 3-methylmethcathinone (3-MMC), inhibited the serotonin transporter more potently than the dopamine transporter. Similar to classic amphetamines, mephedrone analogs were substrate-type monoamine releasers. 5-(2-Aminopropyl)indole (5-IT) was a highly potent monoamine transporter inhibitor and a releaser of dopamine and serotonin. 4-Methylamphetamine (4-MA) mediated efflux of all three monoamines and inhibited the serotonin transporter more potently than the dopamine transporter, unlike amphetamine. *N*-methyl-2-aminoindane (*N*-methyl-2-AI) was a selective norepinephrine transporter inhibitor and norepinephrine releaser, whereas 5-methoxy-6-methyl-2-aminoindane (MMAI) was a selective serotonin transporter inhibitor and serotonin releaser. All of the drugs interacted with monoamine receptors.
Conclusion: The predominant actions on serotonin *vs.* dopamine transporters suggest that dimethylmethcathinones, 4-MA, and MMAI cause entactogenic effects similar to 3,4-methylenedioxymethamphetamine, whereas 3-MMC, 5-IT, and *N*-methyl-2-AI have more stimulant-type properties like amphetamine. Because of pharmacological and structural similarity to mephedrone, similar health risks can be expected for these analogs.
This article is part of the Special Issue entitled 'Designer Drugs and Legal Highs.'

1. Introduction

4-Methylmethcathinone (4-MMC, mephedrone) is a substituted synthetic cathinone (β-keto amphetamine) that has recently become popular as a party drug (Dargan et al., 2010; Green et al., 2014). Mephedrone was widely sold as a "legal high" and continued to be available on the illicit drug market after being classified as illegal (Green et al., 2014; Wood et al., 2012). Structurally and pharmacologically similar new psychoactive substances (NPS) have emerged on the drug market as legal alternatives to the newly banned mephedrone (Brandt et al., 2010). Knowledge of the effects and toxicity of NPS is often solely based on user reports and

Abbreviations: 2,3-DMMC, 2,3-dimethylmethcathinone; 2,4-DMMC, 2,4-dimethylmethcathinone; 3,4-DMMC, 3,4-dimethylmethcathinone; 3-MMC, 3-methylmethcathinone; 4-MA, 4-methylamphetamine; 4-MMC, 4-methylmethcathinone (mephedrone); 5-IT, 5-(2-aminopropyl)indole; 5-HT, 5-hydroxytryptamine (serotonin); DA, dopamine; DAT, dopamine transporter; FLIPR, fluorescence imaging plate reader; HPLC, high-performance liquid chromatography; MDMA, 3,4-methylenedioxymethamphetamine; MMAI, 5-methoxy-6-methyl-2-aminoindane; NE, norepinephrine; NET, norepinephrine transporter; *N*-methyl-2-AI, *N*-methyl-2-aminoindane; NPS, new psychoactive substances; SERT, serotonin transporter; TAAR, trace amine-associated receptor.

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clinical intoxication cases, and pharmacological and toxicological data are mostly lacking. Therefore, the assessment of *in vitro* pharmacological profiles of NPS is a first approach to better understand their clinical effects and toxicology. In the present study, we assessed monoamine transporter and receptor interaction profiles of a new series of mephedrone analogs and related designer drugs (Fig. 1) and compared them to mephedrone. Several of the tested substances were first described in the 20th century, but the widespread availability and recreational use of these substances is a rather recent phenomenon (Baumeister et al., 2015; Brandt et al., 2014; King, 2014; Liechti, 2015). The substituted cathinones 2,3-dimethylmethcathinone (2,3-DMMC), 2,4-dimethylmethcathinone (2,4-DMMC), and 3,4-dimethylmethcathinone (3,4-DMMC) have received relatively little attention to date. 3,4-DMMC has recently been sold and confiscated in various countries (Locos and Reynolds, 2012; Odoardi et al., 2016; Zancajo et al., 2014). 3-Methylmethcathinone (3-MMC) has become one of the most popular NPS in various European countries after the ban of mephedrone, and it has been associated with clinical toxicity and several fatal cases (Adamowicz et al., 2014, 2016; Backberg et al., 2015; European Monitoring Centre for Drugs and Drug Addiction, 2015). 5-(2-Aminopropyl) indole (5-IT) is an indole derivative and stimulant NPS that has been associated with numerous fatal and non-fatal intoxications in recent years (Backberg et al., 2014; Katselou et al., 2015; Kronstrand et al., 2013; Seetohul and Pounder, 2013). 5-IT has been shown to be a substrate at the transporter for norepinephrine (NET), dopamine (DAT), and serotonin (SERT) in rat brain synaptosomes with greater potency for release at NET and DAT over SERT (Marusich et al., 2016). Moreover, 5-IT produced locomotor stimulation and stimulant effects similar to 3,4-methylenedioxymethamphetamine (MDMA) in mice (Marusich et al., 2016). 4-Methylamphetamine (4-MA) is an NPS that has been detected in street amphetamine ("speed") samples across Europe and was linked to several fatalities in combination with amphetamine (Blanckaert et al., 2013). In a study comparing the monoamine releasing potencies of a series of amphetamines analogs *in vitro*, 4-MA and D-amphetamine had similar potencies as releasers of norepinephrine (NE) and dopamine (DA), but 4-MA was a more potent releaser of serotonin (5-HT) (Wee et al., 2005). 4-MA was self-administered at a lower rate by rhesus monkeys compared to D-amphetamine (Wee et al., 2005). *N*-methyl-2-aminoindane (*N*-methyl-2-AI) and 5-methoxy-6-methyl-2-aminoindane (MMAI) are two psychoactive aminoindanes that have been sold as designer drugs online. MMAI has previously been shown to have effects on the SERT similar to MDMA (Rudnick and Wall, 1993) and a high selectivity for 5-HT *vs.* NE and DA uptake inhibition (Johnson et al., 1991).

2. Material and methods

2.1. Drugs

MDMA, mephedrone, and 4-MA were purchased from Lipomed (Arlesheim, Switzerland) with high-performance liquid chromatography (HPLC) purity > 98.5%. 2,3-DMMC, 2,4-DMMC, 3,4-DMMC, 3-MMC, 5-IT, and MMAI were purchased from Cayman Chemicals (Ann Arbor, MI, USA) with purity > 98%. *N*-methyl-2-AI was provided by Dr. Christian Bissig (Forensic Institute, Zürich, Switzerland) with purity > 98%. 5-IT was obtained as racemic base; the remaining compounds were obtained as racemic hydrochlorides. Radiolabelled norepinephrine and dopamine ([^{3}H]-NE and [^{3}H]-DA, respectively) were obtained from Perkin-Elmer (Schwerzenbach, Switzerland). Radiolabeled serotonin ([^{3}H]-5-HT) was purchased from Anawa (Zürich, Switzerland).

2.2. Monoamine uptake transport inhibition

Inhibition of the human NE, DA, and 5-HT transporter (hNET, hDAT, and hSERT, respectively) was assessed in human embryonic kidney (HEK) 293 cells (Invitrogen, Zug, Switzerland) stably transfected with the respective human transporter as previously described (Hysek et al., 2012; Tatsumi et al., 1997). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Life Technologies, Zug, Switzerland) with 10% fetal bovine serum (Gibco) and 250 μg/ml Geneticin (Gibco) to 70–90% confluence, detached, and then resuspended (3×10^6 cells/ml) in Krebs-Ringer Bicarbonate Buffer (Sigma-Aldrich, Buchs, Switzerland). For [^{3}H]-DA uptake experiments, the uptake buffer was supplemented with 0.2 mg/ml ascorbic acid. The cell suspension (100 μl) was incubated with 25 μl buffer containing the test

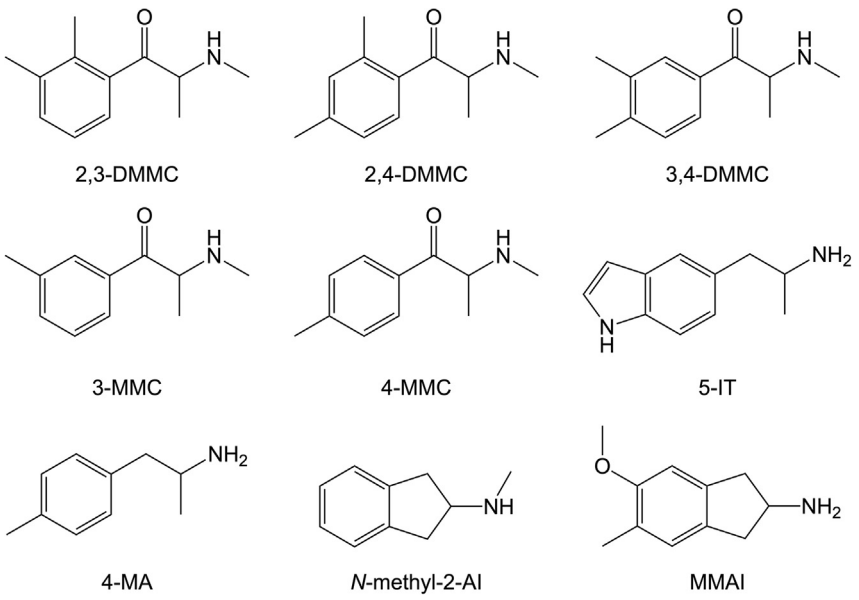

Fig. 1. Chemical structures of mephedrone analogs and related designer drugs.

drugs, vehicle control, or monoamine-specific inhibitors (10 μM nisoxetine for NET, 10 μM mazindol for DAT, and 10 μM fluoxetine for SERT) for 10 min in a round bottom 96-well plate at room temperature by shaking at 450 rotations per minute on a rotary shaker. To initiate uptake transport, 50 μl of [^{3}H]-NE, [^{3}H]-DA, or [^{3}H]-5-HT dissolved in uptake buffer were added at a final concentration of 5 nM for additional 10 min. Thereafter, 100 μl of the cell suspension was transferred to 500 μl microcentrifuge tubes that contained 50 μl of 3 M KOH and 200 μl silicon oil (1:1 mixture of silicon oil types AR 20 and AR 200; Sigma-Aldrich). The tubes were centrifuged for 3 min at 16,550 g to transport the cells through the silicone oil into the KOH. The tubes were frozen in liquid nitrogen and the cell pellet was then cut into 6 ml scintillation vials (Perkin-Elmer) that contained 0.5 ml lysis buffer (0.05 M TRIS-HCl, 50 mM NaCl, 5 mM EDTA, and 1% NP-40 in water). The samples were shaken for 1 h before 5 ml scintillation fluid (Ultimagold, Perkin Elmer, Schwerzenbach, Switzerland) was added. Monoamine uptake was then quantified by liquid scintillation counting on a Packard Tri-Carb Liquid Scintillation Counter 1900 TR. Nonspecific uptake in the presence of selective inhibitors was subtracted from the total counts.

2.3. Transporter-mediated monoamine release

Transporter-mediated monoamine efflux was assessed in HEK 293 cells stably expressing the respective transporter as previously described (Simmler et al., 2013, 2014a). Briefly, 100,000 cells per well were cultured overnight in a poly-D-lysine coated XF24 cell culture microplate (Seahorse Biosciences, North Billerica, MA, USA). Thereafter, the cells were preloaded with 10 nM [^{3}H]-NE, [^{3}H]-DA, or [^{3}H]-5-HT diluted in 85 μl Krebs-HEPES buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM HEPES, 10 mM D-glucose, pH 7.5) containing 10 μM pargyline and 0.2 mg/ml ascorbic acid for 20 min at 37 °C, washed twice, and treated with 1000 μl Krebs-HEPES buffer containing 100 μM of the test drugs for 15 min (DAT and SERT) or 45 min (NET) at 37 °C by shaking at 300 rotations per minute on a rotary shaker. The cells were then washed again with cold buffer and lysed in 50 μl lysis buffer during 1 h. Thereafter, 40 μl of the cell lysate was transferred into 4 ml scintillation vials with 3.5 ml scintillation fluid and the radioactivity inside the cells was quantified by liquid scintillation counting as described for the monoamine uptake inhibition assay. Monoamine transporter blockers (10 μM nisoxetine for NET, 10 μM mazindol for DAT, and 10 μM citalopram for SERT) were included in the experiment to determine "pseudo-efflux" caused by nonspecific monoamine release and subsequent reuptake inhibition (Scholze et al., 2000). The use of a single high concentration and the release durations were based on kinetic evaluation of the release-over-time curves for substrate-releasers in previous studies (Hysek et al., 2012; Simmler et al., 2014a).

2.4. Radioligand receptor and transporter binding assays

The radioligand binding assays were performed as previously described in detail for transporters (Hysek et al., 2012) and receptors (Revel et al., 2011). Briefly, HEK 293 cell membrane preparations (Invitrogen, Zug, Switzerland) overexpressing the respective transporters (Tatsumi et al., 1997) or receptors (human genes except rat and mouse genes for trace amine-associated receptors [TAARs]) (Revel et al., 2011) were incubated with radiolabeled selective ligands at concentrations equal to K_d and ligand displacement by the compounds was measured. The difference between the total binding and nonspecific binding that was determined in the presence of the selected competitors in excess, was defined as specific binding of the radioligand to the target. The following radioligands and competitors, respectively, were used: *N*-methyl-[^{3}H]-nisoxetine and indatraline (NET), [^{3}H]citalopram and indatraline (SERT), [^{3}H]WIN35,428 and indatraline (DAT), [^{3}H]8-hydroxy-2-(di-*n*-propylamine)tetralin and indatraline (5-HT_{1A} receptor), [^{3}H]ketanserin and spiperone (5-HT_{2A} receptor), [^{3}H]mesulgerine and mianserin (5-HT_{2C} receptor), [^{3}H]prazosin and risperidone (α_1 adrenergic receptor), [^{3}H]rauwolscine and phentolamine (α_2 adrenergic receptor), [^{3}H]spiperone and spiperone (D_2 receptor), and [^{3}H]RO5166017 and RO5166017 (TAAR1).

2.5. Activity at the serotonin 5-HT_{2A} receptor

Mouse embryonic fibroblasts (NIH-3T3 cells) expressing the human 5-HT_{2A} receptor were incubated in HEPES-Hank's Balanced Salt Solution (HBSS) buffer (Gibco; 70,000 cells/100 μl) for 1 h at 37 °C in 96-well poly-D-lysine-coated plates. To each well, 100 μl dye solution (fluorescence imaging plate reader [FLIPR] calcium 5 assay kit; Molecular Devices, Sunnyvale, CA, USA) was added and the plates were incubated for 1 h at 37 °C. The plates were placed in a FLIPR and 25 μl of the test drugs diluted in HEPES-HBSS buffer containing 250 mM probenicid were added online. The increase in fluorescence was then measured and EC_{50} values were derived from the concentration-response curves using nonlinear regression. The maximal receptor activity (efficacy) is expressed relative to 5-HT activity, which was set to 100%.

2.6. Activity at the serotonin 5-HT_{2B} receptor

HEK 293 cells expressing the human 5-HT_{2B} receptor were incubated in growth medium (DMEM high glucose [Invitrogen, Zug, Switzerland], 10 ml/l PenStrep [Gibco], 10% fetal calf serum [non-dialysed, heat-inactivated], and 250 mg/l Geneticin) at a density of 50,000 cells/well at 37 °C in poly-D-lysine-coated 96-well plates overnight. The growth medium was then removed by snap inversion, and 100 μl of the calcium indicator Fluo-4 solution (Molecular Probes, Eugene, OR, USA) was added to each well. The plates were incubated for 45 min at 31 °C before the Fluo-4 solution was removed by snap inversion, and 100 μl of Fluo-4 solution was added a second time for 45 min at 31 °C. The cells were washed with HBSS and 20 mM HEPES (assay buffer) immediately before testing using an EMBLA cell washer, and 100 μl assay buffer was added. The plates were placed in a FLIPR, and 25 μl of the test substances diluted in assay buffer was added online. The increase in fluorescence was then measured and EC_{50} values were derived from the concentration-response curves using nonlinear regression. The maximal receptor activity (efficacy) is expressed relative to 5-HT activity, which was set to 100%.

2.7. Cytotoxicity

Cytotoxicity in hSERT-, hDAT-, and hNET-transfected HEK 293 cells was assessed with the ToxiLight bioassay kit (Lonza, Basel, Switzerland) according to the manufacturer's protocol. The cells were treated for 1 h at room temperature with the drugs at the highest assay concentrations. Adenylate kinase release as a result of cell membrane integrity loss was then quantified and compared to control.

2.8. Statistical analysis

Monoamine uptake data were fit by nonlinear regression to

Table 1
Monoamine transport inhibition.

	NET IC_{50} [μM] (95% CI)	DAT IC_{50} [μM] (95% CI)	SERT IC_{50} [μM] (95% CI)	DAT/SERT ratio (95% CI)
Cathinones				
3-MMC	0.27 (0.21–0.36)	2.6 (2.0–3.3)	9.5 (6.9–13.2)	3.7 (2.1–6.6)
4-MMC	0.26 (0.19–0.35)	5.7 (4.5–7.2)	3.6 (2.8–4.6)	0.63 (0.39–1.02)
2,3-DMMC	0.53 (0.36–0.78)	7.4 (5.4–10.1)	1.2 (1.0–1.4)	0.16 (0.10–0.26)
3,4-DMMC	0.45 (0.33–0.60)	9.4 (7.6–11.7)	1.1 (0.9–1.4)	0.12 (0.08–0.18)
2,4-DMMC	1.5 (1.1–2.0)	83 (65–105)	1.5 (1.0–2.2)	0.02 (0.01–0.03)
Phenethylamines				
5-IT	0.04 (0.03–0.06)	0.68 (0.55–0.85)	1.3 (0.9–1.7)	1.9 (1.1–3.1)
4-MA	0.31 (0.24–0.42)	5.6 (4.5–6.9)	0.82 (0.64–1.05)	0.15 (0.09–0.23)
Aminoindanes				
N-methyl-2-AI	2.4 (1.9–3.1)	90 (71–113)	223 (175–284)	2.5 (1.5–4.0)
MMAI	3.6 (2.5–5.3)	193 (167–225)	0.68 (0.50–0.92)	0.004 (0.002–0.006)

Values are means and 95% confidence intervals (CI). DAT/SERT ratio = 1/DAT IC_{50}: 1/SERT IC_{50}.

variable-slope sigmoidal dose-response curves and IC_{50} values were assessed with Prism software (version 7.0a, GraphPad, San Diego, CA, USA). The DAT/SERT ratio is expressed as 1/DAT IC_{50}: 1/SERT IC_{50}. Analysis of variance followed by the Holm-Sidak test was used to analyze drug-induced release of five independent experiments. The drugs were considered monoamine releasers if they caused significantly higher ($^*p < 0.05$) efflux than the selective inhibitors. IC_{50} values of radioligand binding were determined by calculating nonlinear regression curves for a one-site model using three independent 10-point concentration-response curves for each substance. K_i (affinity) values, which correspond to the dissociation constants, were calculated using the Cheng-Prusoff equation. Nonlinear regression concentration-response curves were used to determine EC_{50} values for 5-HT_{2A} and 5-HT_{2B} receptors activation. Efficacy (maximal activity) is expressed relative to the activity of 5-HT, which was used as a control set to 100%.

3. Results

3.1. Monoamine uptake transporter inhibition

IC_{50} values for NET, DAT, and SERT inhibition are listed in Table 1, and the corresponding uptake inhibition curves are presented in Fig. 2. Mephedrone analogs potently inhibited the NET and, with the exception of 3-MMC, were more potent SERT *vs.* DAT inhibitors. 5-IT was a highly potent inhibitor of the NET and a potent inhibitor of the DAT and SERT. 4-MA and MMAI inhibited the SERT at submicromolar concentrations but were only weak inhibitors of the DAT. *N*-methyl-2-AI was a selective NET inhibitor with only very weak inhibition of the SERT and DAT.

3.2. Monoamine release

Monoamine efflux at a 100 μM concentration of the test drugs is shown in Fig. 3. All of the cathinones were releasers of all three monoamines, with the exception of 3,4-DMMC, for which 5-HT release was not significantly higher than the inhibitor control. 5-IT caused DA and 5-HT efflux. 4-MA caused NE, DA, and 5-HT efflux. *N*-methyl-2-AI was a selective NE releaser. MMAI was a selective 5-HT releaser.

3.3. Monoamine receptor and transporter binding affinities

The monoamine receptor and transporter binding affinities and receptor activation potentials of the mephedrone analogs and related designer drugs are shown in Table 2. None of the drugs interacted with the dopamine D_2 receptor, but all of the drugs had low micromolar or submicromolar affinity for α_{1A} or α_{2A} adrenergic receptors. 4-MA and *N*-methyl-2-AI interacted with the α_{2A} receptor but not the α_{1A} receptor. All other compounds interacted with the α_{1A} and the α_{2A} receptor. 3-MMC, *N*-methyl-2-AI, and MMAI had low micromolar affinities for the serotonin 5-HT_{1A} receptor, and the other drugs had only low or no affinity for this receptor. All of the drugs bound to the 5-HT_{2A} receptor, but only 2,3-DMMC, 5-IT, 4-MA, and mephedrone activated the receptor. Only 5-IT and 4-MA activated the 5-HT_{2B} receptor. *N*-methyl-2-AI did not bind to the 5-HT_{2C} receptor, whereas the other drugs bound with affinities of 1.3–8.1 μM. All of the drugs interacted with rat and mouse TAARs.

3.4. Cytotoxicity

None of the drugs were cytotoxic up to 1 h at the investigated concentrations, thus confirming cell integrity during the functional assays.

4. Discussion

4.1. Monoamine uptake transporter inhibition

Similar to mephedrone, the novel mephedrone analogs potently inhibited the NET, which likely results in similar sympathomimetic stimulation (Hysek et al., 2011). The crucial role of NE in the acute effects of psychostimulants is supported by the finding that the release of NE but not DA correlates with human doses of amphetamine-type stimulants (Rothman et al., 2001). Additionally, NET inhibition potency values strongly correlated with the psychotropic effective doses of psychostimulants including cathinones in humans (Simmler et al., 2013). Furthermore, NE has been shown to contribute to the acute subjective stimulation and cardiovascular effects of MDMA in humans (Hysek et al., 2011).

3-MMC more potently inhibited the DAT than the SERT. Mephedrone (4-MMC) had similar potency at the DAT and SERT as previously shown in some other studies (Baumann et al., 2012; Hadlock et al., 2011; Simmler et al., 2013), while others found 5–10-fold higher potency at the DAT *vs.* SERT (Eshleman et al., 2013; Mayer et al., 2016; Pifl et al., 2015). Moreover, the present high NET *vs.* DAT selectivity of mephedrone was not or less observed in some other studies (Eshleman et al., 2013; Mayer et al., 2016; Pifl et al., 2015). While the selectivity of mephedrone for the NET over the SERT in our study is similar to other *in vitro* studies (Eshleman et al., 2013; Mayer et al., 2016; Pifl et al., 2015), the NET over DAT selectivity appears to be higher compared with other labs. This has been observed for mephedrone in previous studies of our

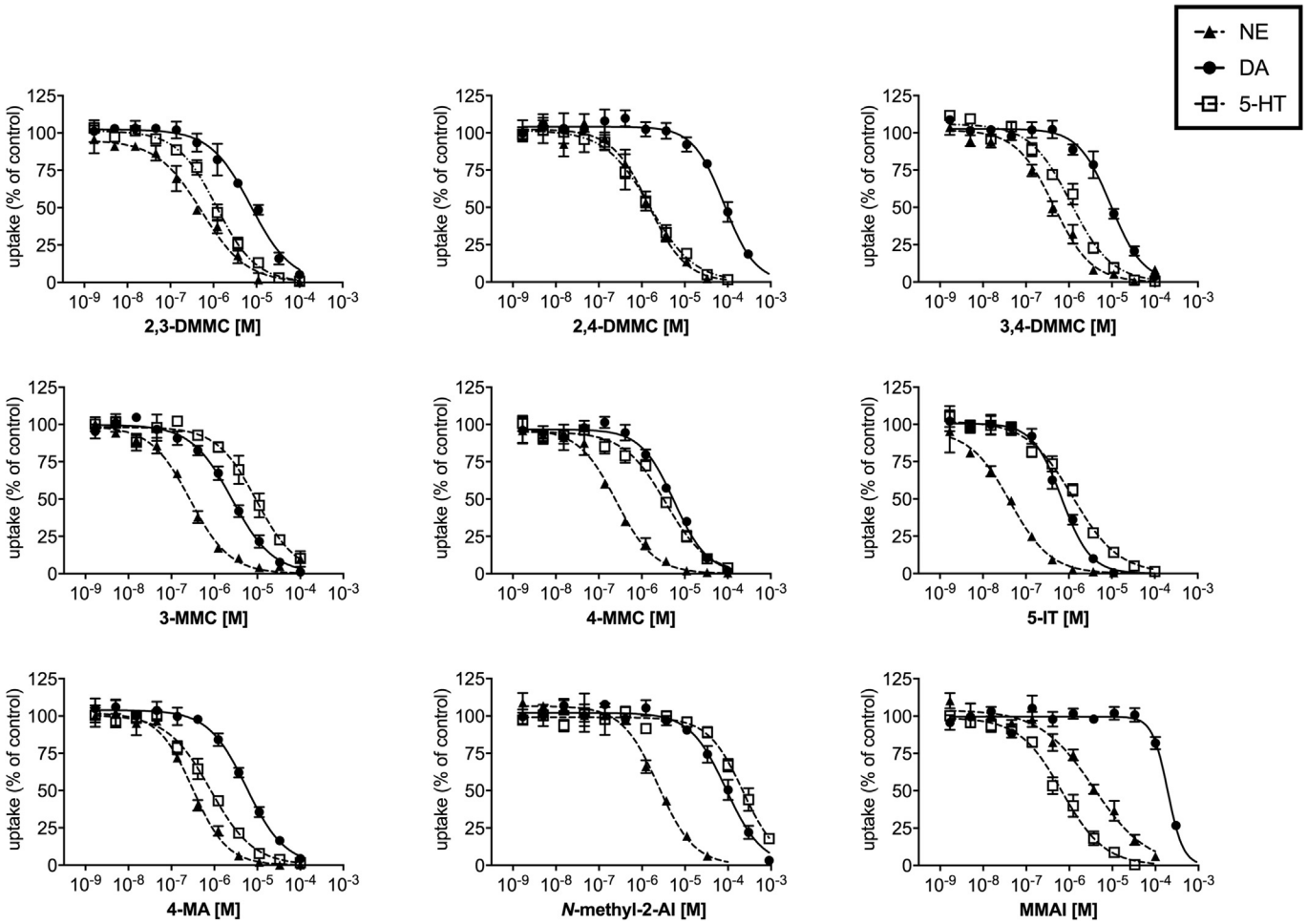

Fig. 2. Monoamine uptake inhibition in stably transfected HEK 293 cells that expressed the hNET, hDAT, or hSERT. Curves were fitted by non-linear regression, and corresponding IC_{50} values are shown in Table 1. The data are presented as the mean ± SEM. Numbers in parentheses indicate the number of individual experiments performed in triplicate (hNET/hDAT/hSERT): 2,3-DMMC (3/3/7), 2,4-DMMC (4/6/3), 3,4-DMMC (4/3/3), 3-MMC (3/3/3), 4-MMC (3/3/3), 5-IT (3/4/3), 4-MA (4/3/4), *N*-methyl-2-AI (3/6/5), MMAI (4/6/5).

lab (Rickli et al., 2015a; Simmler et al., 2013), suggesting that those differences may be explained by differences in the experimental design or the transfected cell line.

The dimethylmethcathinones inhibited the SERT more potently than the DAT. These results suggest that 3-MMC has stronger amphetamine-like stimulant properties compared with mephedrone and especially the other more serotonergic dimethylmethcathinones. Stimulant toxicity was reported to be the main clinical feature in patients with recreational 3-MMC intoxication, although often combined with other drugs (Backberg et al., 2015). Dimethylmethcathinones presumably have entactogenic properties that are similar to MDMA because of greater activation of the 5-HT system (Hysek et al., 2012; Simmler et al., 2013). High selectivity for the SERT *vs.* DAT was also observed for the para-substituted 4-MA, whereas previous studies found high inhibition selectivity for the DAT *vs.* SERT for amphetamine (Rickli et al., 2015a; Simmler et al., 2013). The strong serotonergic activity of 4-MA has been hypothesized to decrease its reinforcing potency compared with other amphetamine analogs (Baumann et al., 2011; Wee et al., 2005). However, the strong serotonergic activity of 4-MA may have led to several fatal cases when combined with the strong dopaminergic activity of amphetamine in users of 4-MA contaminated "speed" (Blanckaert et al., 2013). Moreover, the extreme hyperthermia that is observed in such patients may be explained by the strong serotonergic potency of 4-MA, which is not shared by amphetamine (Blanckaert et al., 2013). 5-IT was a very potent inhibitor of the NET, with potent inhibition also of the DAT and SERT. 5-IT has been associated with sympathomimetic and serotonergic toxicity and was involved in numerous deaths across Europe (Backberg et al., 2014; Katselou et al., 2015; Kronstrand et al., 2013; Seetohul and Pounder, 2013). *N*-methyl-2-AI selectively inhibited the NET, with very weak inhibition potency for the DAT and SERT, suggesting mild psychoactive effects that are similar to 2-aminoindane (2-AI) (Simmler et al., 2014b). MMAI had NET inhibition potencies that were similar to *N*-methyl-2-AI. Unlike *N*-methyl-2-AI, however, MMAI potently inhibited the SERT at submicromolar concentrations.

4.2. Monoamine release

Consistent with previous studies, mephedrone caused efflux of all three monoamines (Baumann et al., 2012; Eshleman et al., 2013; Mayer et al., 2016). The cathinone analogs of mephedrone were also monoamine releasers, indicating that they are monoamine transporter substrates like most amphetamines (Sitte and Freissmuth, 2015). One exception was 3,4-DMMC, which was a potent inhibitor of the SERT but did not cause significant 5-HT efflux. The monoamine transporter inhibition profile of 3,4-DMMC is similar to MDMA (Simmler et al., 2013), but their differences in 5-HT release may partially explain their different subjective effects and potency.

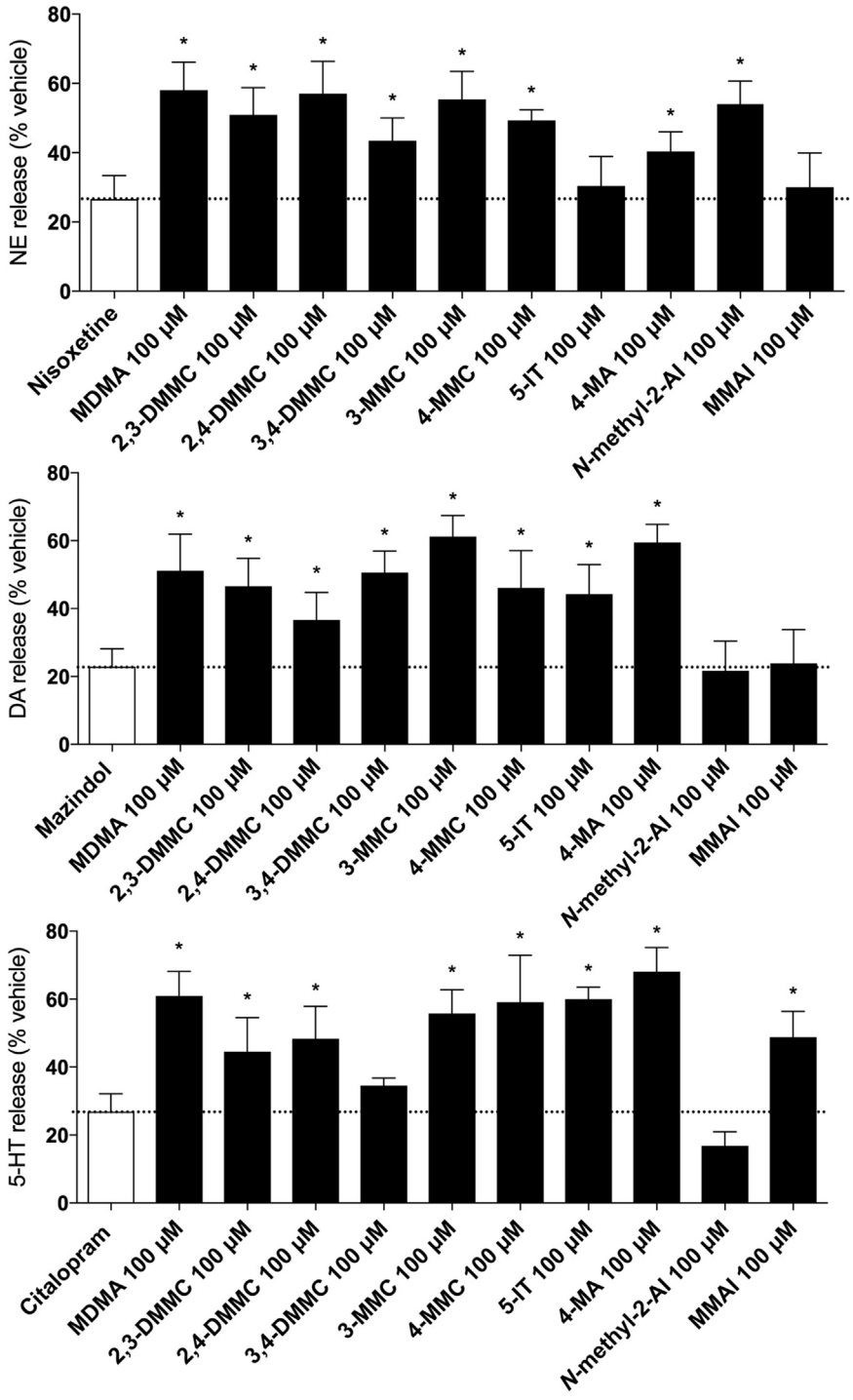

Fig. 3. Monoamine release induced by 100 µM of the drugs after preloading hNET-, hDAT-, or hSERT-expressing HEK 293 cells with radiolabeled monoamines. "Pseudo-efflux" that arose from monoamine diffusion and subsequent reuptake inhibition is marked with a dashed line. Substances that caused significantly higher monoamine efflux (*$p < 0.05$) than pure uptake inhibitors (open bars) were determined to be monoamine releasers. The data are presented as the mean ± SEM of five independent experiments.

4-MA released all three monoamines as described for amphetamine (Rickli et al., 2015a). 5-IT was a very potent inhibitor of the NET, but NE release was not observed. *N*-methyl-2-AI selectively inhibited the NET and was also a selective NE releaser. MMAI was a highly selective 5-HT releaser, consistent with previous reports (Marona-Lewicka and Nichols, 1994, 1998). The high serotonergic activation by MMAI suggests entactogenic effects. However, the lack of any effect on the DA or NE system indicates that the psychopharmacology of MMAI differs from typical entactogens like MDMA (Marona-Lewicka and Nichols, 1994).

4.3. Receptor-binding profiles

All of the drugs potently bound to adrenergic receptors, which

Table 2
Monoamine transporter and receptor binding affinities.

	NET	DAT	SERT	D_2	α_{1A}	α_{2A}	5-HT1A	5-HT_{2A}	5-HT_{2A}		5-HT_{2B}		5-HT_{2C}	$TA1_{rat}$	$TA1_{mouse}$
	Ki	*Ki*	*Ki*	*Ki*	*Ki*	*Ki*	*Ki*	*Ki*	EC_{50}	E_{max}	EC_{50}	E_{max}	*Ki*	*Ki*	*Ki*
Cathinones															
2,3-DMMC	8.4 ± 0.3	4.2 ± 0.6	6.1 ± 0.5	>11	0.78 ± 0.10	3.0 ± 0.1	>17	0.64 ± 0.19	0.13 ± 0.02	84 ± 12	>10		2.4 ± 0.9	1.2 ± 0.1	0.88 ± 0.06
2,4-DMMC	>26	>26	17 ± 1	>11	0.16 ± 0.02	3.0 ± 0.3	15 ± 3	1.3 ± 0.1	>10		>10		1.3 ± 0.3	0.59 ± 0.08	3.1 ± 0.2
3,4-DMMC	12 ± 2	7.6 ± 0.6	5.7 ± 0.3	>11	1.9 ± 0.3	3.5 ± 0.2	>17	1.9 ± 0.3	>10		>10		1.5 ± 0.2	2.6 ± 0.2	4.5 ± 0.4
3-MMC	5.6 ± 1.5	3.2 ± 0.6	>22	>12	7.9 ± 0.2	1.1 ± 0.1	4.8 ± 0.5	3.4 ± 0.8	>20		>20		3.6 ± 1.0	5.7 ± 1.4[a]	10±1[a]
4-MMC	>26	2.9 ± 0.2	>22	>11	1.1 ± 0.1	11 ± 1	>17	1.6 ± 0.2	0.36 ± 0.19	79 ± 20	>10		8.1 ± 5.4	5.0 ± 0.1	12 ± 1
Phenethylamines															
5-IT	1.3 ± 0.3	0.92 ± 0.13	10 ± 2	>25	5.4 ± 0.5	1.7 ± 0.1	11 ± 2	0.38 ± 0.11	0.49 ± 0.17	42 ± 9	1.5 ± 0.6	36 ± 5	3.0 ± 0.8	0.15 ± 0.02[a]	0.36 ± 0.15[a]
4-MA	9.4 ± 1.2	9.4 ± 0.9	13 ± 3	>25	>12	2.1 ± 0.4	18 ± 6	3.3 ± 0.5	3.3 ± 1.0	71 ± 4	0.86 ± 0.38	54 ± 8	6.3 ± 1.1	0.10 ± 0.01[a]	0.15 ± 0.07[a]
Aminoindanes															
N-methyl-2-AI	>30	>30	>30	>25	>12	0.49 ± 0.07	3.6 ± 0.1	5.4 ± 0.9	>20		>20		>15	0.53 ± 0.04[a]	2.6 ± 0.1[a]
MMAI	>26	>26	11 ± 1	>11	4.0 ± 0.2	1.0 ± 0.1	1.6 ± 0.2	8.3 ± 1.3			>10		5.4 ± 1.4	0.14 ± 0.02	4.9 ± 1.1

Ki and EC_{50} values are given as μM (mean ± SD); activation efficacy (E_{max}) is given as percentage of maximum±SD.
[a] Values are from Simmler et al. (2016).

are known to modulate stimulant-induced behavior (Schmidt and Weinshenker, 2014). Furthermore, the drugs interacted with several serotonin receptors. All of the compounds bound to the 5-HT_{2A} receptor as previously shown for mephedrone and MDMA (Eshleman et al., 2013; Simmler et al., 2013) and typically for serotonergic hallucinogens (Eshleman et al., 2014; Nichols, 2016; Rickli et al., 2015c, 2016). Additionally, 2,3-DMMC, mephedrone, and 5-IT were potent functional 5-HT_{2A} agonists in our calcium mobilization assay like MDMA (Rickli et al., 2015b) and classic serotonergic hallucinogens (Rickli et al., 2016) known to produce their psychotropic effects at least in part via 5-HT_{2A} receptor activation (Liechti et al., 2000; Preller et al., 2017; Vollenweider et al., 1998). Another study documented 5-HT_{2A} receptor antagonistic properties for mephedrone in another 5-HT-induced inositol monophosphate formation assay (Eshleman et al., 2013). However, MDMA had both agonist (Eshleman et al., 2014) and antagonist effects (Eshleman et al., 2013) in this assay indicating that the 5-HT_{2A} ligands may act as agonist and antagonists depending on assay set-up. Certain hallucinogenic properties have been described for mephedrone (Kasick et al., 2012; Schifano et al., 2011) and our results suggest that 2,3-DMMC could have hallucinogen-like properties as well. 5-IT is a positional isomer of the psychedelic tryptamine α-methyltryptamine (αMT). 5-IT has been previously suggested to also have hallucinogenic properties (Marusich et al., 2016), and its potent 5-HT_{2A} receptor activation supports this possibility. All of the substances interacted with rat and mouse TAARs. Many stimulant NPS interact with TAARs (Simmler et al., 2016), which have a modulatory role on monoaminergic activity (Revel et al., 2011, 2012). In a recent screening of a large set of NPS, cathinones were described as poor TAAR1 ligands (Simmler et al., 2016). Our results suggest that this does not apply to all cathinones as submicromolar affinity for rat and mouse TAARs was observed for 2,4-DMMC and 2,3-DMMC, respectively.

The present study has limitations. First, we did not investigate the effects of the drugs on intracellular targets such as the vesicular monoamine transporter 2 (VMAT2). Lower potency VMAT2 interactions have been reported for methcathinones compared to MDMA and methamphetamine (Eshleman et al., 2013; Fleckenstein et al., 2009; Pifl et al., 2015). It was therefore concluded that mephedrone is unlikely to cause neurotransmitter release form synaptic vesicles (Eshleman et al., 2013). Second, the static monoamine release assay used in the present study was only useful to qualitatively determine whether a drug is a substrate releaser or not, but the assay was not suitable to assess the potency of the releasers. Superfusion assays would be more suitable to also determine the potency of the substances to release monoamines (Eshleman et al., 2013). However, the potency of the substances to release monoamine is reflected by their potency to inhibit monoamine uptake in the uptake assay used in the present study (Simmler et al., 2013). Finally, we included no *in vivo* data. However, *in vivo* microdialysis studies showed that the cathinones mephedrone and methylone markedly released both 5-HT and DA at similar potencies reflecting their *in vitro* pharmacological profiles (Baumann et al., 2012; Kehr et al., 2011). Additionally, methcathinone was a more potent inhibitor of the DAT than SERT *in vitro*, more potently released monoamines via the DAT than SERT (Cozzi et al., 2013; Simmler et al., 2013), and consistently also more potently increased extracellular DA than 5-HT in rat brain nucleus accumbens dialysate (Cozzi et al., 2013). Vice versa, the more potent *in vitro* SERT than DAT inhibitor and predominant 5-HT releaser 4-trifluoromethylmethcathinone (4-TFMAP) increased 5-HT but not DA *in vivo* (Cozzi et al., 2013). Thus, for several cathinones the *in vitro* profiles accurately predicted the *in vivo* neurochemical effects.

5. Conclusion

The present study characterized a series of novel mephedrone analogs that potently interacted with monoamine transporters and receptors, suggesting their potential abuse liability, which has been previously observed for synthetic cathinones. 4-MA is a potent inhibitor of the SERT, which may explain its higher toxicity when combined with the potent DAT inhibitor amphetamine. 5-IT is a highly potent monoamine transporter inhibitor that has been associated with sympathomimetic toxicity and numerous fatalities across Europe. *N*-methyl-2-AI is a selective NET inhibitor and NE releaser, and MMAI is a selective SERT inhibitor and 5-HT releaser.

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Author contributions

D.L., S.K., and M.E.L. designed the research. D.L., K.E.K., L.D., and M.C.H. performed the research. D.L., M.C.H., and M.E.L. analyzed the data. D.L. and M.E.L. wrote the manuscript with input from all of the other authors.

Conflict of interest

M.C.H. is an employee of F. Hoffmann-La Roche. The other authors do not have any conflicts of interest to declare for this work.

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2.2 Pharmacological profile of methylphenidate-based designer drugs

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ABSTRACT

Background: Methylphenidate-based designer drugs are new psychoactive substances (NPS) that are used outside medical settings and their pharmacology is largely unexplored. The aim of the present study was to characterize the pharmacology of methylphenidate-based substances *in vitro*.
Methods: We determined the potencies of the methylphenidate-based NPS *N*-benzylethylphenidate, 3,4-dichloroethylphenidate, 3,4-dichloromethylphenidate, ethylnaphthidate, ethylphenidate, 4-fluoromethylphenidate, isopropylphenidate, 4-methylmethylphenidate, methylmorphenate, and propylphenidate and the potencies of the related compounds cocaine and modafinil with respect to norepinephrine, dopamine, and serotonin transporter inhibition in transporter-transfected human embryonic kidney 293 cells. We also investigated monoamine efflux and monoamine receptor and transporter binding affinities. Furthermore, we assessed the cell integrity under assay conditions.
Results: All methylphenidate-based substances inhibited the norepinephrine and dopamine transporters 4 to >1000-fold more potently than the serotonin transporter. Similar to methylphenidate and cocaine, methylphenidate-based NPS did not elicit transporter-mediated efflux of monoamines. Besides binding to monoamine transporters, several test drugs had affinity for adrenergic, serotonergic, and rat trace amine-associated receptors but not for dopaminergic or mouse trace amine-associated receptors. No cytotoxicity was observed after drug treatment at assay concentrations.
Conclusion: Methylphenidate-based substances had pharmacological profiles similar to methylphenidate and cocaine. The predominant actions on dopamine transporters *vs.* serotonin transporters may be relevant when considering abuse liability.
This article is part of the Special Issue entitled 'Designer Drugs and Legal Highs.'

1. Introduction

The psychostimulant methylphenidate (MPH; Ritalin®) is used for the treatment of attention-deficit/hyperactivity disorder and narcolepsy but it also has a history of being misused as a 'smart drug' and 'cognitive enhancer' (Arria et al., 2008; Liakoni et al., 2015; Maier et al., 2013). In recent years, an increasing number of MPH-based new psychoactive substances (NPS; Fig. 1) (Brandt et al., 2014) have become available as alternatives to MPH (Bailey et al., 2015; European Monitoring Centre for Drugs and Drug Addiction, 2015) and have been associated with several fatalities (Krueger et al., 2014; Maskell et al., 2016; Parks et al., 2015). Characteristic for the NPS phenomenon, many of the currently circulating MPH analogs originated from drug development efforts (Deutsch et al., 1996; Markowitz et al., 2013; Misra et al., 2010), which subsequently appeared on the streets. The pharmacological and subjective-effect profiles of MPH are very similar to cocaine (Simmler et al., 2014; Vogel et al., 2016; Volkow et al., 1999). Furthermore, some of these substances are either sold in their own right or offered in the form of branded products (Bailey et al., 2015;

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); DA, dopamine; DAT, dopamine transporter; FLIPR, fluorescence imaging plate reader; HPLC, high-performance liquid chromatography; MDMA, 3,4-methylenedioxymethamphetamine; MPH, methylphenidate; NE, norepinephrine; NET, norepinephrine transporter; NPS, new psychoactive substances; SERT, serotonin transporter; TAAR, trace amine-associated receptor.

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Parks et al., 2015). Methylphenidate predominantly inhibits the norepinephrine (NE) and dopamine (DA) transporters (NET and DAT, respectively), thus, possibly contributing to its abuse potential (Simmler et al., 2014; Vogel et al., 2016). Correspondingly, questions arise about the extent to which MPH analogs might share MPH-like characteristics. Assessing the pharmacological profile of NPS *in vitro* is an initial step to gain a better understanding of the potential clinical effects and toxicology of these substances. For this reason, the present study reports on the transporter interaction profiles of the MPH-related NPS *N*-benzylethylphenidate, 3,4-dichloroethylphenidate, 3,4-dichloromethylphenidate, and isopropylphenidate and the transporter and receptor interaction profiles of ethylnaphthidate, ethylphenidate, 4-fluoromethylphenidate, 4-methylmethylphenidate, methylmorphenate, and propylphenidate. Modafinil, a stimulant prescribed for the treatment of narcolepsy, which is frequently offered for sale as a 'neuroenhancer' (Ghahremani et al., 2011; Maier et al., 2013; Mereu et al., 2013; Müller et al., 2013), has also been included in this investigation. Stimulants may act as transporter inhibitors or as transporter substrates that cause monoamine efflux into the synaptic cleft (Rothman and Baumann, 2003; Sitte and Freissmuth, 2015). Therefore, additionally to the transporter inhibition potencies of the substances, their mechanism of action (reuptake inhibitor or transporter substrate) was determined.

2. Material and methods

2.1. Drugs

Cocaine, 3,4-methylenedioxymethamphetamine (MDMA) and MPH were purchased from Lipomed (Arlesheim, Switzerland), with high-performance liquid chromatography (HPLC) purity > 98.5%. Modafinil was purchased from Cayman Chemicals (Ann Arbor, MI, USA), with purity > 98%. Methylmorphenate and propylphenidate were obtained from reChem Labs (Ontario, Canada) and afterwards identified and tested for purity using nuclear magnetic resonance (NMR) and HPLC, which revealed purity > 95%. *N*-Benzylethylphenidate, 3,4-dichloroethylphenidate, 3,4-dichloro methylphenidate, ethylnaphthidate, 4-fluoromethylphenidate, isopropylphenidate, and 4-methylmethylphenidate were part of confiscations by German authorities and test purchases (Klare et al., 2017). The substances were fully characterized in a previous study (Klare et al., 2017) and purity values were estimated at > 95% based on spectroscopic and chromatographic methods of analysis. Ethylphenidate was provided by Dr. Christian Bissig (Forensic Institute, Zurich, Switzerland) after being confiscated by Swiss authorities and being tested for purity of >98%. Modafinil was obtained as racemic base. The other drugs were obtained as racemic hydrochloride salts. Radiolabeled [^{3}H]-NE (13.1 Ci/mmol) and [^{3}H]-DA (30.0 Ci/mmol) were obtained from Perkin-Elmer (Schwerzenbach, Switzerland). Radiolabeled [^{3}H]-5-HT (80 Ci/mmol) was purchased from Anawa (Zürich, Switzerland).

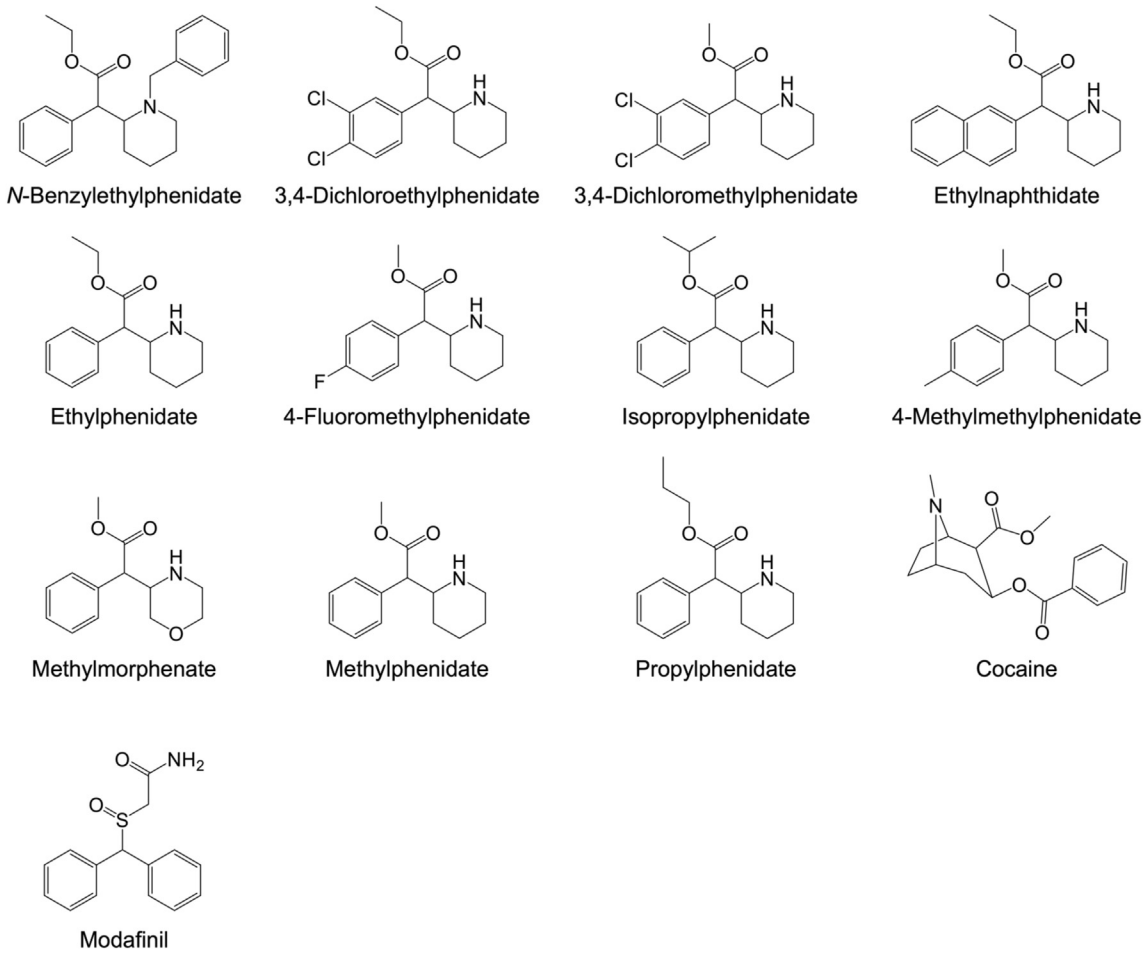

Fig. 1. Chemical structures of MPH-based NPS and related compounds.

2.2. Monoamine uptake transport inhibition

Monoamine uptake inhibition was assessed using human embryonic kidney (HEK) 293 cells that stably expressed the human SERT, DAT, or NET (Tatsumi et al., 1997) as previously described (Hysek et al., 2012). Briefly, the cells were cultured to 70–90% confluence, detached, and resuspended in Krebs-Ringer Bicarbonate Buffer (Sigma-Aldrich, Buchs, Switzerland). For [^{3}H]-DA uptake experiments, the uptake buffer was supplemented with 1.14 mM ascorbic acid. The cells were then treated with vehicle control and drug in the range of 1 nM–900 μM for 10 min at room temperature. Additionally, monoamine-specific inhibitors were added (10 μM fluoxetine for SERT, 10 μM mazindol for DAT, and 10 μM nisoxetine for NET). To initiate uptake transport, [^{3}H]-5-HT, [^{3}H]-DA, or [^{3}H]-NE were added at a final concentration of 5 nM for an additional 10 min. The cells were then separated from the uptake buffer by centrifugation through silicone oil, and the tubes were frozen in liquid nitrogen. The cell pellet was cut into scintillation vials and lysed. The samples were shaken for 1 h before scintillation fluid (Ultimagold, Perkin Elmer, Schwerzenbach, Switzerland) was added. Monoamine uptake was then quantified by liquid scintillation counting on a Packard Tri-Carb Liquid Scintillation Counter 1900 TR. Uptake in the presence of the selective inhibitors was determined to be nonspecific and subtracted from the total counts.

2.3. Transporter-mediated monoamine efflux

The potential of the drugs to initiate transporter-mediated NE, DA, or 5-HT efflux was assessed in HEK 293 cells that overexpressed the respective transporter as previously described (Simmler et al., 2013). Briefly, the cells were first preloaded with [^{3}H]-NE, [^{3}H]-DA, or [^{3}H]-5-HT dissolved in Krebs-HEPES buffer for 20 min at 37 °C. The cells were then washed and treated with 100 μM of the drugs for 15 min (DAT and SERT) or 45 min (NET). The treatment durations for [^{3}H]-NE, [^{3}H]-DA, and [^{3}H]-5-HT efflux experiments were based on kinetic evaluation of the efflux-over-time curves of MDMA (Simmler et al., 2014). The cells were washed again, and the remaining radioactivity inside the cells was quantified. The monoamine transporter blockers citalopram (SERT), mazindol (DAT), and nisoxetine (NET) were added as a negative control at a concentration of 10 μM to determine “pseudo-efflux” that was caused by nonspecific monoamine efflux and subsequent reuptake inhibition (Scholze et al., 2000).

2.4. Radioligand receptor and transporter binding assays

The radioligand binding assays were performed as previously described for transporters (Hysek et al., 2012) and receptors (Revel et al., 2011). Briefly, membrane preparations of HEK 293 cells (Invitrogen, Zug, Switzerland) that overexpressed the respective transporters (Tatsumi et al., 1997) or receptors (human genes, with the exception of rat and mouse genes for trace amine-associated receptors [TAARs]) (Revel et al., 2011) were incubated with the radiolabeled selective ligands at concentrations equal to K_d, and ligand displacement by the compounds was measured. Specific binding of the radioligand to the target receptor was defined as the difference between the total binding and nonspecific binding that was determined in the presence of the selected competitors. The following radioligands and competitors, respectively, were used: *N*-methyl-[^{3}H]-nisoxetine and 10 μM indatraline (NET), [^{3}H]citalopram and 10 μM indatraline (SERT), [^{3}H]WIN35,428 and 10 μM indatraline (DAT), [^{3}H]8-hydroxy-2-(di-*n*-propylamine)tetralin and 10 μM pindolol (5-HT$_{1A}$ receptor), [^{3}H]ketanserin and 10 μM spiperone (5-HT$_{2A}$ receptor), [^{3}H]mesulgerine and 10 μM mianserin (5-HT$_{2C}$ receptor), [^{3}H]prazosin and 10 μM chlorpromazine (α_{1A} adrenergic receptor), [^{3}H]rauwolscine and 10 μM phentolamine (α_{2A} adrenergic receptor), [^{3}H]spiperone and 10 μM spiperone (D$_2$ receptor), and [^{3}H]RO5166017 and 10 μM RO5166017 (TAAR$_1$).

2.5. Activity at the serotonin 5-HT$_{2B}$ receptor

Activity at the 5-HT$_{2B}$ receptor was assessed as previously described (Rickli et al., 2016). Briefly, human 5-HT$_{2B}$ receptor-expressing HEK 293 cells were incubated in a cell culture plate overnight. The next day, the growth medium was removed by snap inversion, and calcium indicator Fluo-4 solution (Molecular Probes, Eugene, OR, USA) was added to each well. The plates were then incubated for 45 min at 31 °C. The Fluo-4 solution was removed by snap inversion and then added a second time. The cells were then incubated for another 45 min at 31 °C. Immediately before testing, the cells were washed with HBSS and 20 mM HEPES (assay buffer; Gibco) using an EMBLA cell washer, and assay buffer was added. The plates were placed in a FLIPR. Test substances that were diluted in assay buffer were added online, and the increase in fluorescence was measured.

2.6. Cytotoxicity

Cytotoxicity was assessed with the ToxiLight bioassay kit (Lonza, Basel, Switzerland) according to the manufacturer's protocol. The kit measures adenylate kinase release as a result of cell membrane integrity loss. Human SERT-, DAT-, and NET-transfected HEK 293 cells were treated for 1 h at room temperature with the drugs at the highest assay concentrations.

2.7. Statistical analysis

Calculations were performed using Prism 7.0a software (GraphPad, San Diego, CA, USA). Monoamine transporter inhibition data were fit by nonlinear regression to variable-slope sigmoidal dose-response curves and IC$_{50}$ values were assessed. The DAT/SERT ratio is expressed as 1/DAT IC$_{50}$:1/SERT IC$_{50}$. Compound-induced monoamine efflux of five independent experiments was compared with negative controls using analysis of variance followed by the Holm-Sidak test. *P* values lower than 0.05 were considered significant and substances were considered transporter substrates if they caused significantly higher efflux than the negative controls. IC$_{50}$ values of radioligand binding were determined by calculating nonlinear regression curves for a one-site model using three independent 10-point concentration-response curves for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation. Nonlinear regression concentration-response curves were used to calculate the EC$_{50}$ values for the 5-HT$_{2B}$ receptor activation.

3. Results

3.1. Monoamine uptake transporter inhibition

Monoamine uptake inhibition curves are shown in Fig. 2, and the corresponding IC$_{50}$ values and DAT/SERT inhibition ratios are listed in Table 1. Methylphenidate was a potent inhibitor of the NET and DAT at submicromolar concentrations and a weak inhibitor of the SERT. 3,4-dichloromethylphenidate inhibited the NET more than 10-fold more potently than MPH, whereas the inhibition potency for the DAT was more than 2-fold increased. The NET and DAT inhibition potencies of 3,4-dichloroethylphenidate, ethylnaphthidate, 4-fluoromethylphenidate, and 4-methylmethylphenidate were similar to MPH in the range of

0.04–0.42 μM for the NET and 0.08–0.34 μM for the DAT. *N*-Benzylethylphenidate, ethylphenidate, isopropylphenidate, methylmorphenate, and propylphenidate inhibited the NET with 6–800-fold lower potency compared to MPH and the DAT with 4–500-fold lower potency. The SERT inhibition potency for all MPH-based NPS was lower than the NET and DAT inhibition potencies. Ethylnaphthidate inhibited the SERT at 1.7 μM with a DAT/SERT ratio of 5. The remaining compounds inhibited the SERT 40 to >1000-fold weaker than the DAT and 26 to >1000-fold weaker than the NET. Modafinil was a weak inhibitor of monoamine transporters with an IC_{50} value > 10 μM for the DAT and no relevant NET or SERT inhibition (IC_{50} values > 100 μM). Unlike the MPH-based substances and modafinil, cocaine inhibited all three transporters with similar potency in the range of 0.5–1.5 μM.

3.2. Monoamine efflux

Similar to cocaine, MPH and the MPH-based NPS and related compounds did not cause monoamine efflux (Fig. 3) and are therefore not transporter substrates.

3.3. Monoamine receptor and transporter binding affinities

The interactions between MPH-based NPS and related compounds with monoamine receptors and transporters are shown in Table 2. All MPH-based NPS bound to the NET and DAT but only *N*-benzylethylphenidate, 3,4-dichloroethylphenidate, 3,4-dichloromethylphenidate, and ethylnaphthidate bound to the SERT in the concentration range tested. 3,4-Dichloroethylphenidate, 3,4-dichloromethylphenidate, 4-methylmethylphenidate, and ethylnaphthidate bound to the α_{1A} receptor in the range of 1.7–6.5 μM and additionally to the α_{2A} receptor in the range of 7–10 μM. Ethylphenidate and propylphenidate bound to the α_{2A} receptor with 14 μM and 8.7 μM, respectively, but did not bind to the α_{1A} receptor in the investigated concentration range. 3,4-Dichloroethylphenidate, 3,4-dichloromethylphenidate, ethylnaphthidate, 4-methylmethylphenidate, and propylphenidate, had affinities of 1–17 μM for the 5-HT_{1A} receptor. Ethylnaphthidate was the only drug to bind to the 5-HT_{2A} receptor with an IC_{50} value of 4.9 μM and only 3,4-dichloromethylphenidate and ethylnaphthidate bound to the 5-HT_{2C} receptor, both with an IC_{50} of 12 μM. None of the compounds activated the 5-HT_{2B} receptor or bound to the mouse $TAAR_1$, and only 3,4-dichloroethylphenidate, 3,4-dichloromethylphenidate, and isopropylphenidate bound to the rat $TAAR_1$ with affinities in the range of 6–13 μM. None of the MPH-based NPS had relevant affinity for D_2 receptors. Modafinil and cocaine bound to the monoamine transporters but did not interact with monoamine or trace amine receptors.

3.4. Cytotoxicity

Cytotoxicity was not observed for any of the drugs in the functional assays at the concentrations tested, thus confirming cell integrity during the assays.

4. Discussion

We characterized the *in vitro* pharmacological profiles of MPH-based NPS and compared them with MPH and cocaine. All compounds inhibited the DAT substantially more potently than the SERT, suggesting predominantly stimulant-type effects similar to amphetamine and a high abuse liability (Liechti, 2015; Simmler et al., 2013).

4.1. Monoamine uptake transporter inhibition and monoamine efflux

Methylphenidate and MPH-based NPS, with the exception of *N*-benzylethylphenidate, isopropylphenidate, and methylmorphenate, inhibited the NET at submicromolar concentrations, suggesting cardiostimulant and psychostimulant properties, similar to amphetamines (Hysek et al., 2011; Simmler et al., 2013). Moreover, the NET and DAT inhibition potencies but not the SERT inhibition potency correlate with the psychotropic effective doses of psychostimulants in human (Simmler et al., 2013).

The MPH-based NPS were only monoamine transporter inhibitors and not monoamine transporter substrates, indicating a mechanism of action similar to cocaine but not amphetamines (Fleckenstein et al., 2007; Torres et al., 2003). Ethylnaphthidate inhibited the SERT at low micromolar concentrations, but the remaining MPH-based NPS displayed a clear preference for DAT over SERT, resulting in high DAT/SERT ratios frequently reported for locomotor stimulants (Simmler et al., 2013). Our results are consistent with other studies that reported potent NET and DAT inhibition for MPH (DAT/SERT ratio = 2207) and triple uptake inhibition for cocaine (DAT/SERT ratio = 3.2) (Han and Gu, 2006). Modafinil was a moderate and relatively selective DAT inhibitor, with an IC_{50} value of 11 μM. This finding is consistent with previous *in vitro* studies that reported IC_{50} values of 4–13 μM (Karabacak et al., 2015; Loland et al., 2012; Madras et al., 2006; Zolkowska et al., 2009). The interaction between modafinil and DAT is also thought to modulate the pharmacological effects of the drug (Wisor, 2013). The psychopharmacological profiles and cognitive-enhancing properties of MPH and modafinil may be different. Modafinil has been shown to improve attention and wakefulness, whereas MPH has been shown to improve memory (Repantis et al., 2010).

4.2. Transporter and receptor binding profiles

Compared with ethylphenidate, replacement of the benzene ring with naphthalene (ethylnaphthidate) increased the potency in inhibiting the SERT and increased the affinity for 5-HT receptors. Many stimulant NPS interact with TAARs (Simmler et al., 2016); however, no potent TAAR interactions were found for MPH-based NPS. 3,4-Dichloromethylphenidate and ethylnaphthidate interacted with the α_{1A} and 5-HT_{1A} receptor in the low micromolar range. The remaining MPH-based NPS did not potently interact with monoamine receptors, indicating that they exert their primary effects by inhibiting uptake transporters, similar to MPH and cocaine (Ritz et al., 1987, 1988; Volkow et al., 2002). Consistent with the monoamine uptake data, 3,4-dichloroethylphenidate, 3,4-dichloromethylphenidate, and ethylnaphthidate potently bound to the NET and DAT and had affinity for the SERT as well. 4-Fluoromethylphenidate, 4-methylmethylphenidate, and methylphenidate bound potently to the NET and DAT but had no affinity to the SERT in the tested concentration range. *N*-Benzylethylphenidate, ethylphenidate, isopropylphenidate, and propylphenidate showed high affinity for the DAT but not for the NET or SERT. Methylmorphenate did not potently bind to any transporter. Cocaine potently bound to all transporters but not to receptors. No interaction between modafinil and monoamine receptors was observed. To date, no single site of action for modafinil has been identified (Gerrard and Malcolm, 2007).

4.3. Comparison of transporter binding and transporter inhibition

No drug-mediated monoamine efflux was observed for any of the MPH-based compounds, strengthening the argument that they

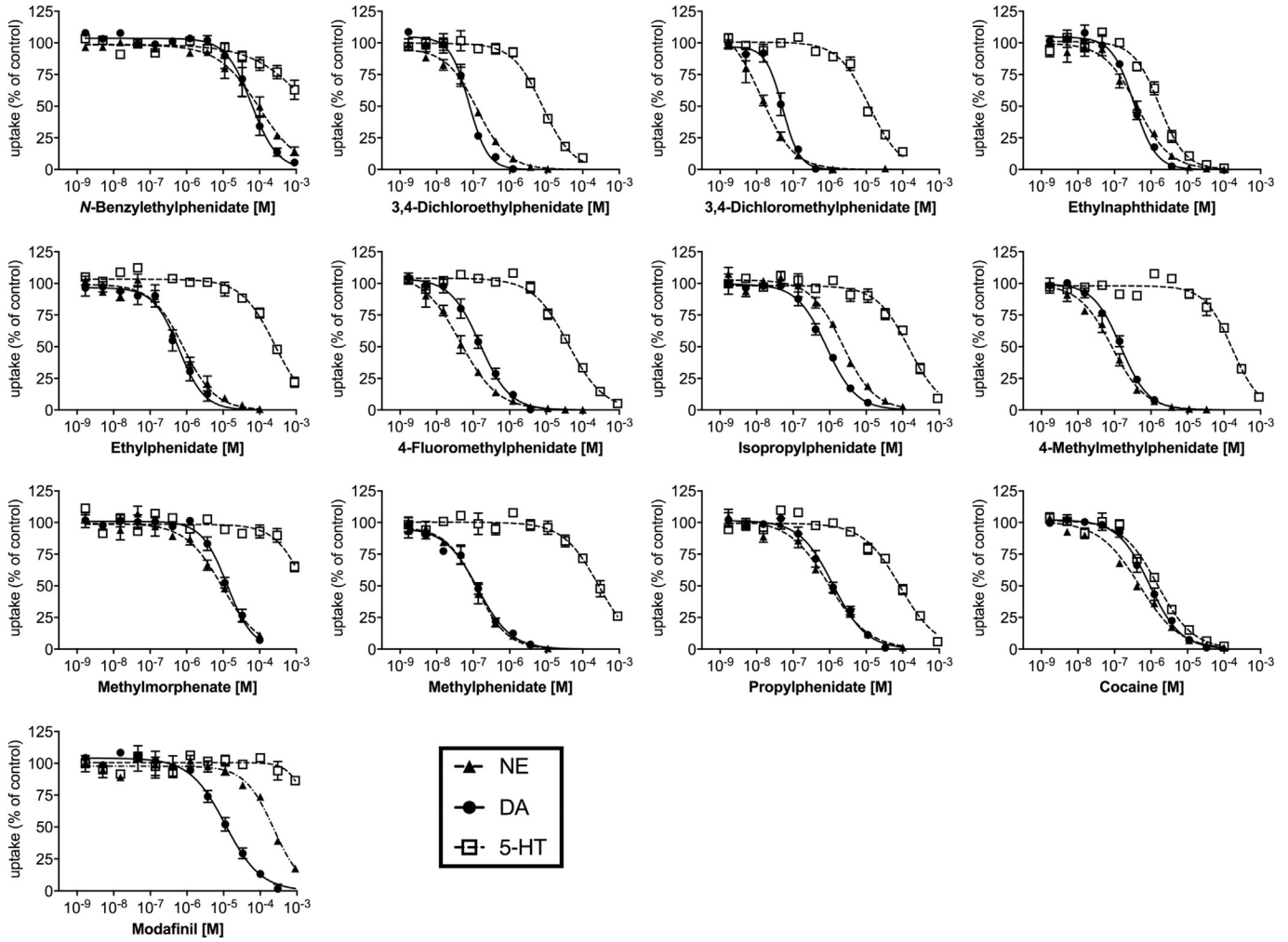

Fig. 2. Monoamine uptake inhibition in stably transfected HEK 293 cells that expressed the human NET, DAT, or SERT. Curves were fitted by non-linear regression, and corresponding IC_{50} values are shown in Table 1. The data are presented as the mean ± SEM and numbers in parentheses indicate the number of individual experiments performed in triplicate (NET/DAT/SERT): *N*-benzylethylphenidate (4/4/4), 3,4-dichloroethylphenidate (3/3/5), 3,4-dichloromethylphenidate (3/3/4), ethylnaphthidate (3/3/3), ethylphenidate (3/3/5), 4-fluoromethylphenidate (4/4/4), isopropylphenidate (3/3/4), 4-methylmethylphenidate (4/3/4), methylmorphenate (3/3/4), methylphenidate (3/3/6), propylphenidate (3/3/4), cocaine (3/4/5), modafinil (6/7/6).

Table 1
Monoamine transport inhibition.

	NET	DAT	SERT	DAT/SERT
	IC_{50} [μM] (95% CI)	IC_{50} [μM] (95% CI)	IC_{50} [μM] (95% CI)	ratio (95% CI)
Methylphenidate-based				
Methylphenidate	0.12 (0.09–0.16)	0.13 (0.10–0.18)	274 (204–366)	2108 (1133–3660)
4-Methylmethylphenidate	0.09 (0.07–0.11)	0.15 (0.12–0.18)	164 (132–204)	1093 (733–1700)
Ethylphenidate	0.81 (0.62–1.06)	0.61 (0.45–0.84)	257 (205–322)	421 (244–716)
4-Fluoromethylphenidate	0.04 (0.03–0.06)	0.15 (0.12–0.20)	40 (33–48)	267 (165–400)
3,4-Dichloromethylphenidate	0.01 (0.01–0.02)	0.05 (0.04–0.06	12 (9–15)	240 (150–375)
Isopropylphenidate	2.3 (1.8–2.9)	0.82 (0.68–1.00)	147 (112–193)	179 (112–284)
Methylmorphenate	9.3 (7.0–12.3)	13 (11–16)	1831 (932–3600)	141 (58–327)
3,4-Dichloroethylphenidate	0.13 (0.10–0.16)	0.08 (0.06–0.09)	8.0 (6.9–9.3)	100 (77–155)
Propylphenidate	0.94 (0.71–1.25)	1.2 (1.0–1.6)	84 (67–106)	70 (42–106)
N-Benzylethylphenidate	95 (59–154)	60 (41–86)	2515 (958–6605)	42 (11–161)
Ethylnaphthidate	0.42 (0.32–0.54)	0.34 (0.28–0.42)	1.7 (1.3–2.1)	5.0 (3.1–7.5)
Other				
Modafinil	231 (177–300)	11 (9–14)	2616 (250–27300)	238 (28–1950)
Cocaine	0.48 (0.36–0.64)	0.90 (0.75–1.08)	1.5 (1.2–1.9)	1.7 (1.1–2.5)

Values are means and 95% confidence intervals (CI). DAT/SERT ratio = 1/DAT IC_{50}: 1/SERT IC_{50}.

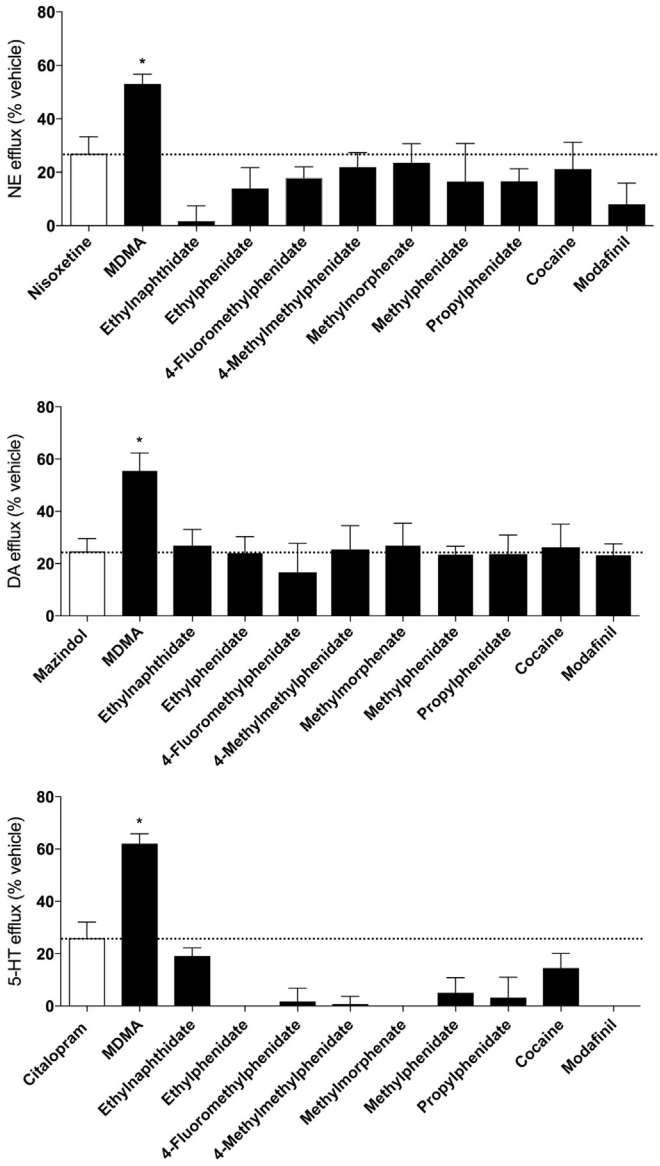

Fig. 3. Monoamine efflux induced by 100 μM of the compounds after preloading HEK 293 cells that expressed the human NET, DAT, or SERT with radiolabeled monoamine. The efflux is expressed as percentage of [^{3}H]-NE, [^{3}H]-DA, or [^{3}H]-5-HT decrease in monoamine preloaded cells compared to vehicle control. The dashed line marks nonspecific "pseudo-efflux" that arises from monoamine diffusion and subsequent reuptake inhibition. Substances that caused significantly more monoamine efflux (*$p < 0.05$) than pure uptake inhibitors (open bars) were determined to be monoamine transporter substrates. The data are presented as the mean ± SEM of five independent experiments.

are pure uptake blockers.For uptake blockers, a correlation between the monoamine uptake and radioligand binding affinities has been previously described for the NET (Cheetham et al., 1996; Lee et al., 1982), the DAT (Javitch et al., 1984; Schoemaker et al., 1985), and the SERT (D'Amato et al., 1987; Langer et al., 1980). However, discrepancies between monoamine uptake inhibition and radioligand binding have been observed for cocaine-like drugs and proposed for MPH-like drugs, when the conditions for the binding and uptake inhibition assays varied (Reith et al., 2005; Rothman et al., 1993).

Highest NET and DAT binding affinities were observed for the most potent NET and DAT inhibitor 3,4-dichloromethylphenidate. However, the increase in potency compared to MPH was much more pronounced with a 76-fold and 12-fold increase for NET and DAT binding, respectively. 3,4-Dichloroethylphenidate bound more than 10-fold more potently to the NET and DAT whereas the NE and DA uptake inhibition was similar to MPH. Ethylnaphthidate, 4-fluoromethylphenidate, and 4-methylmethylphenidate inhibited the NET and DAT with similar potency as MPH. These substances bound to the NET with affinity in the range of 0.22–0.31 μM and to the DAT with affinity in the range of 0.026–0.040 μM. MPH bound to the NET and DAT with 0.50 μM and 0.070 μM, respectively. Thus, unlike for the dichloro substituted compounds, the IC_{50} values and the K_i values for ethylnaphthidate, 4-fluoromethylphenidate, and 4-methylmethylphenidate correlate well. *N*-Benzylethylphenidate, ethylphenidate, isopropylphenidate, methylmorphenate, and propylphenidate inhibited the NET with 6–800-fold lower potency compared to MPH and the DAT with 4–500-fold lower potency. While the binding affinities for the NET were 8–48-fold decreased, the DAT binding affinities were decreased only for *N*-benzylethylphenidate, methylmorphenate, and propylphenidate (5–46-fold) whereas the DAT binding affinites of ethylphenidate and isopropylphenidate were close to MPH. Remarkably, *N*-benzylethylphenidate was by far the weakest transporter inhibitor, it did however not have the lowest NET and DAT binding affinities. In the investigated concentration range, only *N*-benzylethylphenidate, 3,4-dichloroethylphenidate, 3,4-dichloromethylphenidate, and ethylnaphthidate bound to the SERT. 3,4-dichloroethylphenidate, 3,4-dichloromethylphenidate, and ethylnaphthidate, were the most potent SERT inhibitors, *N*-benzylethylphenidate was however the weakest SERT inhibitor. Thus, as observed for the NET and DAT, the SERT binding affinity of *N*-benzylethylphenidate was much higher than might be expected from the uptake inhibition data.

To conclude, the rank order of potency of the radioligand binding and uptake inhibition was similar with the 3,4-substituted and 4-substitued compounds being among the most potent MPH-based NPS; the relative potencies of the uptake inhibition and transporter binding varied however to a certain extent.

Besides cocaine, ethylnaphthidate was the only compound to have considerable inhibition potencies and affinities for all transporters. The inhibition and binding potencies generally decreased with increasing size of the carbon side chain. Compared to MPH, the steric ring-substitution of *N*-benzylethylphenidate substantially decreased the inhibition potency for all transporters and the binding to the NET and DAT. However, higher binding affinity for the SERT was observed. Modafinil selectively inhibited and bound to the SERT.

The present study has limitations. Possible potent contaminants could theoretically have influenced the results for some drugs with lower purity. Substance-induced efflux was only tested at a high substance concentration. The absence of monoamine efflux could be the result of bell-shaped concentration-efflux curves as it has been demonstrated for amphetamine analogs with known monoamine releasing properties, including MDMA, in different *in vitro* assays (Seidel et al., 2005). However, such bell-shaped efflux curves were not observed in the assay used in the present study as previously documented (Hysek et al., 2012), strengthening the argument that the MPH-based NPS are in fact pure uptake inhibitors. Moreover, in this study the focus was laid on the NET, DAT, and SERT, as they are main targets of amphetamines and presumably many stimulant NPS (Sitte and Freissmuth, 2015). Other possible mechanisms that may contribute to the effects of NPS, such as VMAT2 inhibition (Sulzer et al., 2005), calcium-triggered exocytosis of monoamines (Mundorf et al., 1999; Sulzer et al., 2005), mRNA regulation (Douglass et al., 1995), or ion channel blockage (Bauman and DiDomenico, 2002; O'Leary and Hancox, 2010), were not investigated in this study.

Table 2
Monoamine transporter and receptor binding affinities.

	NET	DAT	SERT	D_2	α_{1A}	α_{2A}	5-HT_{1A}	5-HT_{2A}	5-HT_{2B}	5-HT_{2C}	TA_{1rat}	TA_{1mouse}
	K_i	K_i	K_i	K_i	K_i	K_i	K_i	K_i	EC_{50}	K_i	K_i	K_i
Methylphenidate-based												
Methylphenidate	0.50 ± 0.17	0.070 ± 0.020	>22	>4.4	>8.9	>15	>17	>13	>10	>15	>5.0	>4.7
4-Methylmethylphenidate	0.31 ± 0.10	0.033 ± 0.007	>22	>4.4	6.5 ± 0.3	10 ± 1	9.9 ± 0.7	>13	>10	>15	>5.0	>4.7
Ethylphenidate	4.9 ± 0.7	0.081 ± 0.007	>30	>25	>12	14 ± 1	>25	>12	>20	>15	>15[a]	>15[a]
4-Fluoromethylphenidate	0.22 ± 0.08	0.040 ± 0.007	>22	>4.4	>8.9	>15	>17	>13	>10	>15	>5.0	>4.7
3,4-Dichloromethylphenidate	0.0066 ± 0.0006	0.0060 ± 0.0005	3.0 ± 0.1	>4.4	1.7 ± 0.1	7.0 ± 0.6	1.8 ± 0.1	>13		>15	6.2 ± 0.6	>14
Isopropylphenidate	4.2 ± 0.4	0.097 ± 0.014	>23	>4.4	>11	>15	>17	>13		>15	13 ± 2	>14
Methylmorphenate	24 ± 1	3.2 ± 0.3	>22	>4.4	>8.9	>15	>17	>13	>10	>15	>5.0	>4.7
3,4-Dichloroethylphenidate	0.028 ± 0.003	0.0065 ± 0.0002	1.5 ± 0.2	>4.4	4.3 ± 0.1	7.5 ± 0.3	4.5 ± 0.4	>13		12 ± 0.3	6.6 ± 1.3	>14
Propylphenidate	3.8 ± 1.3	0.33 ± 0.07	>22	>4.4	>8.9	8.7 ± 0.5	17 ± 1	>13	>10	>15	>5.0	>4.7
N-Benzylethylphenidate	5.5 ± 0.5	0.33 ± 0.01	8.4 ± 1.0	>4.4	>11	>15	>17	>13		>15	>15	>14
Ethylnaphthidate	0.27 ± 0.06	0.026 ± 0.003	0.58 ± 0.05	>4.4	1.8 ± 0.2	8.6 ± 0.5	1.3 ± 0.2	4.9 ± 0.5	>10	12 ± 3	>5.0	>4.7
Other												
Modafinil	>26	4.0 ± 0.7	>22	>4.4	>8.9	>15	>17	>13	>10	>15	>5.0	>4.7
Cocaine	1.6 ± 0.3	0.20 ± 0.02	0.87 ± 0.04	>4.4	>8.9	>15	>17	>13	>10	>15	>5.0	>4.7

Values are given as μM (mean ± SD).
[a] From Simmler et al., 2016.

5. Conclusion

Similar to MPH and cocaine, MPH-based NPS are potent inhibitors of the NET and DAT. Furthermore, they are not monoamine transporter substrates and have only minor interactions with monoamine receptors. The high selectivity for the DAT *vs*. SERT suggests that these emerging drugs may have abuse potential. Modafinil is a weak but selective inhibitor at DAT but does not present monoamine receptor interactions.

Author contributions

D.L., S.D.B., S.K., and M.E.L. designed research. D.L., P.J.K., and M.C.H. performed research. D.L., M.C.H., and M.E.L. analysed data. D.L. and M.E.L. wrote the paper with input from all other authors.

Conflict of interest

M.C.H. is an employee of F. Hoffmann-La Roche. The other authors do not have any conflicts of interest to declare for this work.

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2.3 Effects of the new psychoactive substances diclofensine, diphenidine, and methoxphenidine on monoaminergic systems

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Neuropharmacology and analgesia

Effects of the new psychoactive substances diclofensine, diphenidine, and methoxphenidine on monoaminergic systems

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ABSTRACT

Diclofensine, diphenidine, and methoxphenidine are new psychoactive substances (NPSs) that recently appeared on the illicit drug market. Pharmacological profiling of such newly emerged drugs is crucial for a better understanding of their psychotropic effects and toxicity. We therefore investigated the potential of these NPSs to inhibit the norepinephrine, dopamine, and serotonin transporters in human embryonic kidney cells stably transfected with the respective transporters. In addition, we determined monoamine transporter and receptor affinities for the substances. Diclofensine potently bound to the monoamine transporters in the submicromolar range and had similar inhibition potential for all three transporters in the range of 2.5–4.8 μM. Moreover, diclofensine bound to adrenergic, dopamine, serotonin, and trace amine-associated receptors. Diphenidine was an equipotent inhibitor of the norepinephrine and dopamine transporters in the low micromolar range and a very weak inhibitor of the serotonin transporter. Besides binding to transporters, diphenidine bound to adrenergic α_{1A} and α_{2A} receptors and serotonin 5-hydroxytryptamine 1A (5-HT_{1A}) and 5-HT_{2A} receptors in the range of 4–11 μM. Methoxphenidine bound to all transporters, but considerable inhibition (IC_{50} < 10 μM) was observed only for the norepinephrine transporter. Moreover, methoxphenidine bound to adrenergic α_{2A} and serotonin 5-HT_{2A} and 5-HT_{2C} receptors in the range of 2.5–8.2 μM. None of the test drugs mediated substrate-type efflux of monoamines. These data demonstrate that the monoamine transporter inhibition and receptor interactions most likely mediate the psychoactive effects of diclofensine and possibly play a contributory role for diphenidine and methoxphenidine.

1. Introduction

The emergence of numerous potentially harmful new psychoactive substances (NPSs) in recent years poses a challenge to drug regulatory authorities and health personnel. Case reports are often the only source of information on the toxicity of newly emerged drugs and in vitro screenings are therefore a helpful tool to better understand the pharmacology of such substances. In the current study, we present in vitro pharmacological profiles of three NPSs (Fig. 1) that have recently reached the illicit drug market. Diclofensine was originally developed as an antidepressant and was shown to have potent monoamine transporter inhibition potencies in rat brain synaptosomes (Andersen, 1989; Funke et al., 1986; Gasić et al., 1986; Hyttel and Larsen, 1985; Keller et al., 1982) and to increase extracellular dopamine levels in rats (Nakachi et al., 1995). Diphenidine and its methoxylated derivative 2-methoxydiphenidine (methoxphenidine) are NPSs of the diarylethylamine class, which have previously been associated with adverse events including deaths (Elliott et al., 2015; Gerace et al., 2017; Helander et al., 2015; Hofer et al., 2014; Kusano et al., 2017; Lam et al., 2016; Valli et al., 2017). Diphenidine and methoxphenidine act as *N*-methyl-D-aspartate receptor antagonists (Berger et al., 2009; Wallach et al., 2015), and their effects have been described as being comparable to other dissociative anesthetic drugs, such as ketamine (Helander et al., 2015; Morris and Wallach, 2014).

2. Material and methods

2.1. Drugs

Diclofensine, diphenidine, and methoxphenidine were kindly provided by the Forensic Institute Zürich (Zürich, Switzerland) with > 98% purity. 3,4-Methylenedioxymethamphetamine (MDMA) was purchased from Lipomed (Arlesheim, Switzerland) with high-performance liquid chromatography purity of > 98.5%. Diclofensine was obtained

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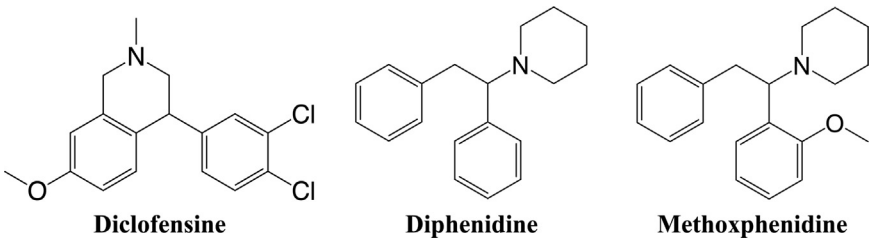

Fig. 1. Structures of NPSs included in the study.

as racemic base, the other drugs were obtained as racemic hydrochloride salts. Radiolabelled [^{3}H]norepinephrine and [^{3}H]dopamine were purchased from Perkin-Elmer (Schwerzenbach, Switzerland). Radiolabeled [^{3}H]serotonin was obtained from Anawa (Zürich, Switzerland).

2.2. Monoamine uptake transporter inhibition

Norepinephrine, dopamine, and serotonin uptake inhibition for the test drugs in the range of 1 nM to 900 µM was assessed in human embryonic kidney (HEK) 293 cells transfected with the human transporter for norepinephrine (hNET), dopamine (hDAT), or serotonin (hSERT) as previously described in detail (Hysek et al., 2012b; Tatsumi et al., 1997), with slight modifications (Luethi et al., 2017b). Briefly, cells were suspended in uptake buffer and incubated with the test drugs for 10 min before [^{3}H]norepinephrine, [^{3}H]dopamine, or [^{3}H]serotonin were added at a final concentration of 5 nM for additional 10 min to initiate uptake transport. Thereafter, the cells were separated from the uptake buffer by centrifugation through silicone oil. The centrifugation tubes were then frozen in liquid nitrogen and the cell pellet was cut into scintillation vials containing lysis buffer. Scintillation fluid was added to the vials and uptake was quantified by liquid scintillation counting. Transporter inhibitors (10 µM nisoxetine for NET, 10 µM mazindol for DAT, and 10 µM fluoxetine for SERT) were added to assess for non-specific monoamine uptake.

2.3. Drug-induced monoamine efflux

To assess whether the test drugs act as pure transporter inhibitors or as transporter substrates, drug-induced monoamine efflux was assessed at a drug concentration of 100 µM in hNET-, hDAT-, or hSERT-transfected HEK 293 cells as previously described (Simmler et al., 2013) with slight modifications. Briefly, cells were cultured in a poly-D-lysine coated microplate and preloaded with 10 nM [^{3}H]norepinephrine, [^{3}H]dopamine, or [^{3}H]serotonin for 20 min at 37 °C. The cells were then washed twice, and subsequently treated with the test drugs for 15 min (DAT and SERT) or 45 min (NET) at 37 °C on a rotary shaker. Thereafter, 300 µl of the assay buffer was transferred into scintillation vials, scintillation fluid was added, and the amount of monoamine efflux was then quantified by liquid scintillation counting. Transporter inhibitors (10 µM nisoxetine for NET, 10 µM mazindol for DAT, and 10 µM citalopram for SERT) were included to determine "pseudo-efflux" caused by nonspecific monoamine efflux and subsequent reuptake inhibition (Scholze et al., 2000). The assay set-up was based on previous kinetic evaluation of the efflux-over-time curves for monoamine transporter substrates (Hysek et al., 2012b; Simmler et al., 2014a). The transporter substrate MDMA was used as positive control.

2.4. Monoamine receptor and transporter binding affinities

Radioligand binding affinities for transporters and receptors were assessed as previously described in detail (Luethi et al., 2017c). Briefly, membrane preparations that overexpressed the respective transporters (Tatsumi et al., 1997) or receptors (human genes, with the exception of rat and mouse genes for trace amine-associated receptors (Revel et al., 2011)) were incubated with the radiolabeled selective ligands at concentrations equal to K_d, and ligand displacement by the compounds was measured. Specific binding of the radioligand to the target receptor was defined as the difference between the total binding and nonspecific binding that was determined in the presence of the selected competitors at a concentration of 10 µM. The following radioligands and competitors, respectively, were used: *N*-methyl-[^{3}H]nisoxetine and indatraline (NET), [^{3}H]WIN35,428 and indatraline (DAT), [^{3}H]citalopram and indatraline (SERT), [^{3}H]8-hydroxy-2-(di-*n*-propylamine)tetralin and pindolol (serotonin 5-HT$_{1A}$ receptor), [^{3}H]ketanserin and spiperone (serotonin 5-HT$_{2A}$ receptor), [^{3}H]mesulgerine and mianserin (serotonin 5-HT$_{2C}$ receptor), [^{3}H]prazosin and chlorpromazine (α_1 adrenergic receptor), [^{3}H]rauwolscine and phentolamine (α_2 adrenergic receptor), [^{3}H]spiperone and spiperone (dopamine D$_2$ receptors), and [^{3}H]RO5166017 and RO5166017 (trace amine-associated receptors 1).

2.5. Activity at the serotonin 5-HT$_{2A}$ receptor

Mouse embryonic fibroblasts (NIH-3T3 cells) expressing the human serotonin 5-HT$_{2A}$ receptor were incubated in HEPES-Hank's Balanced Salt Solution (HBSS) buffer (Gibco, Zug, Switzerland; 70,000 cells/100 µl) for 1 h at 37 °C in 96-well poly-D-lysine-coated plates. Thereafter, 100 µl of dye solution (fluorescence imaging plate reader [FLIPR] calcium 5 assay kit; Molecular Devices, Sunnyvale, CA, USA) was added to each well, and the plates were again incubated for 1 h at 37 °C. The plates were then placed in a FLIPR, and 25 µl of the test drugs that were diluted in HEPES-HBSS buffer containing 250 mM probenicid was added online. The increase in fluorescence was measured, and EC$_{50}$ values were derived from the concentration-response curves using nonlinear regression.

2.6. Activity at the serotonin 5-HT$_{2B}$ receptor

HEK 293 cells expressing the human serotonin 5-HT$_{2B}$ receptor were incubated in growth medium at a density of 50,000 cells per well at 37 °C in poly-D-lysine-coated 96-well plates overnight. The growth medium was then removed by snap inversion, and 100 µl of the calcium indicator Fluo-4 solution (Molecular Probes, Eugene, OR, USA) was added to each well. The plates were incubated for 45 min at 31 °C, the Fluo-4 solution was then removed by snap inversion, and 100 µl of Fluo-4 solution was added a second time for 45 min at 31 °C. The cells were washed with HBSS and 20 mM HEPES (assay buffer) using an EMBLA cell washer, and 100 µl assay buffer was added. The plates were then placed in a FLIPR, and 25 µl of the test substances that were diluted in assay buffer was added online. The increase in fluorescence was measured, and EC$_{50}$ values were derived from the concentration-response curves using nonlinear regression.

2.7. Cytotoxicity

Adenylate kinase release was measured with the ToxiLight BioAssay Kit from Lonza (Basel, Switzerland) as marker for cytotoxicity to confirm cell integrity under assay conditions. Briefly, 25,000 hSERT-, hDAT-, or hNET-transfected HEK 293 cells per well were seeded in a 96-well plate. The following day, the cells were treated with 100 µl of the

test substances dissolved in medium at the highest concentration used in the functional assays. After 1 h incubation at room temperature, 50 µl of the ToxiLight assay buffer was added to 20 µl of supernatant and after 5 min the luminescence was measured with a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland). The luminescence signal was then compared to medium control. The detergent Triton X-100 (0.5%) was used as a positive control.

2.8. Statistical analysis

Monoamine uptake data were fit by nonlinear regression to variable-slope sigmoidal dose-response curves and IC_{50} values were assessed with Prism software (version 7.0a, GraphPad, San Diego, CA, USA). The DAT/SERT ratio is expressed as 1/DAT IC_{50}: 1/SERT IC_{50}. Drug-induced monoamine efflux of five independent experiments was analyzed using analysis of variance followed by the Holm-Sidak test. The drugs were considered monoamine transporter substrates, if they caused significantly higher ($^{*}P < 0.05$) efflux than the selective inhibitors. IC_{50} values of the radioligand binding assays were assessed by calculating nonlinear regression curves for a one-site model using three independent 10-point concentration-response curves for each substance. Affinity (K_i) values, which correspond to the dissociation constants, were calculated using the Cheng-Prusoff equation. EC_{50} values for serotonin 5-HT_{2A} and 5-HT_{2B} receptor activation were determined using nonlinear regression concentration-response curves.

3. Results

3.1. Monoamine uptake transporter inhibition

Monoamine uptake inhibition curves are shown in Fig. 2 and the corresponding IC_{50} values are listed in Table 1. Diclofensine inhibited the NET, DAT, and SERT in the low micromolar range (2.5–4.8 µM). Diphenidine was an equipotent inhibitor of the NET and DAT with IC_{50} values of 3.3 and 3.4 µM, respectively, but only a very weak inhibitor of the SERT (IC_{50}: 675 µM). Methoxphenidine inhibited the NET at 7.8 µM but was a weak inhibitor of the DAT and SERT (IC_{50}: 65 and 741 µM, respectively).

3.2. Drug-induced monoamine efflux

Substances that interact with monoamine transporters may act as transporter inhibitors or as transporter substrates that cause monoamine efflux into the synaptic cleft (Rothman and Baumann, 2003; Sitte and Freissmuth, 2015). Drug-induced monoamine efflux is shown in Fig. 3. Diclofensine, diphenidine, and methoxphenidine did not mediate efflux of any monoamines. The positive control MDMA caused efflux of all monoamines.

3.3. Monoamine receptor and transporter binding affinities

Diclofensine potently bound to monoamine transporters in the range of 0.027–0.096 µM, and bound to the dopamine D_2 receptor with K_i of 2.2 µM and to adrenergic α_{1A} and α_{2A} receptors with K_i of 0.14 and 1.2 µM, respectively (Table 2). Moreover, diclofensine had affinity to the serotonin 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors in the range of 0.079–1.2 µM, but it did not activate the serotonin 5-HT_{2A} or 5-HT_{2B} receptors at the concentrations investigated (EC_{50} > 20 µM). Diclofensine also bound to the rat and mouse trace amine-associated receptors 1 with K_i of 1.3 and 6.9 µM, respectively. The dissociative drugs diphenidine and methoxphenidine had highest transporter affinity for the DAT, followed by the NET and SERT. Diphenidine bound to adrenergic α_{1A} and α_{2A} receptors with affinities of 11 and 4.4 µM, respectively. Methoxphenidine bound to the adrenergic α_{2A} receptor (2.6 µM) but not to the adrenergic α_{1A} receptor (K_i > 12 µM). Diphenidine bound to serotonin 5-HT_{1A} and 5-HT_{2A} receptors with K_i of 11 µM but

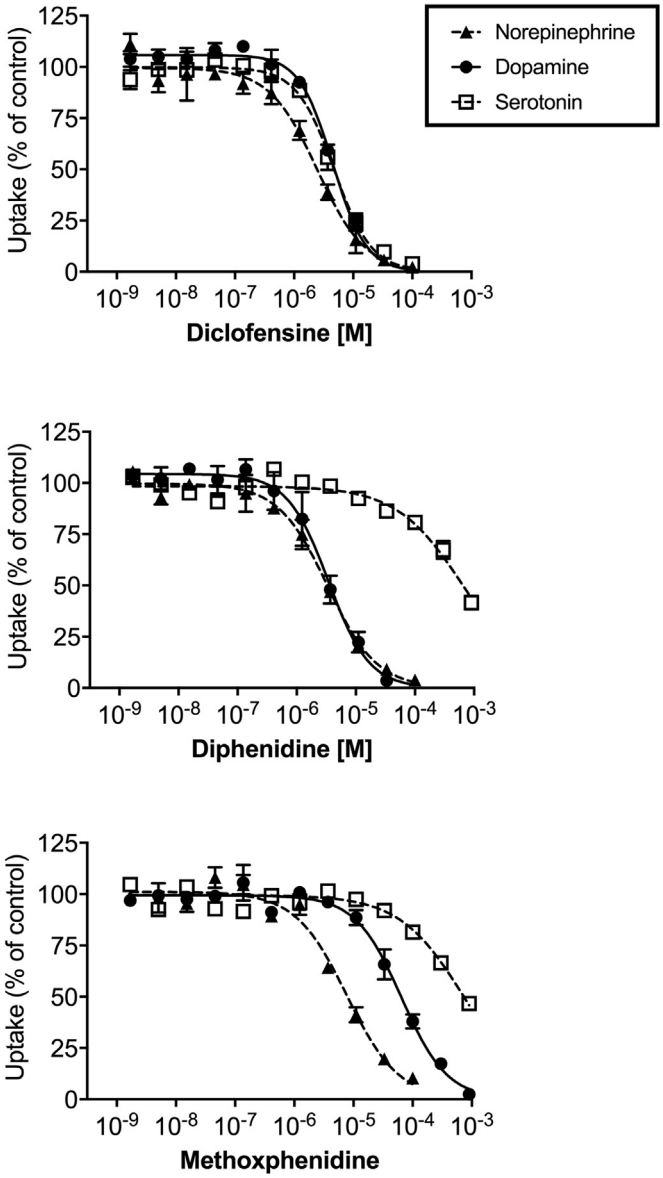

Fig. 2. Monoamine uptake inhibition in stably transfected HEK 293 cells that expressed the human NET, DAT, or SERT. Curves were fitted by non-linear regression, and corresponding IC_{50} values are shown in Table 1. The data are presented as the mean ± S.E.M. and numbers in parentheses indicate the number of individual experiments performed in triplicate (NET/DAT/SERT): diclofensine (3/3/3), diphenidine (3/3/4), methoxphenidine (3/5/4).

Table 1
Monoamine transport inhibition.

	NET IC_{50} [µM] (95% CI)	DAT IC_{50} [µM] (95% CI)	SERT IC_{50} [µM] (95% CI)	DAT/SERT ratio (95% CI)
Diclofensine	2.5 (1.8–3.3)	4.5 (3.8–5.4)	4.8 (4.0–5.6)	1.1 (0.7–1.5)
Diphenidine	3.3 (2.6–4.3)	3.4 (2.6–4.5)	675 (527–864)	199 (117–332)
Methoxphenidine	7.8 (5.9–10.1)	65 (52–79)	741 (586–938)	11 (7–18)

Values are means and 95% confidence intervals (CI). DAT/SERT ratio = 1/DAT IC_{50}:1/SERT IC_{50}.

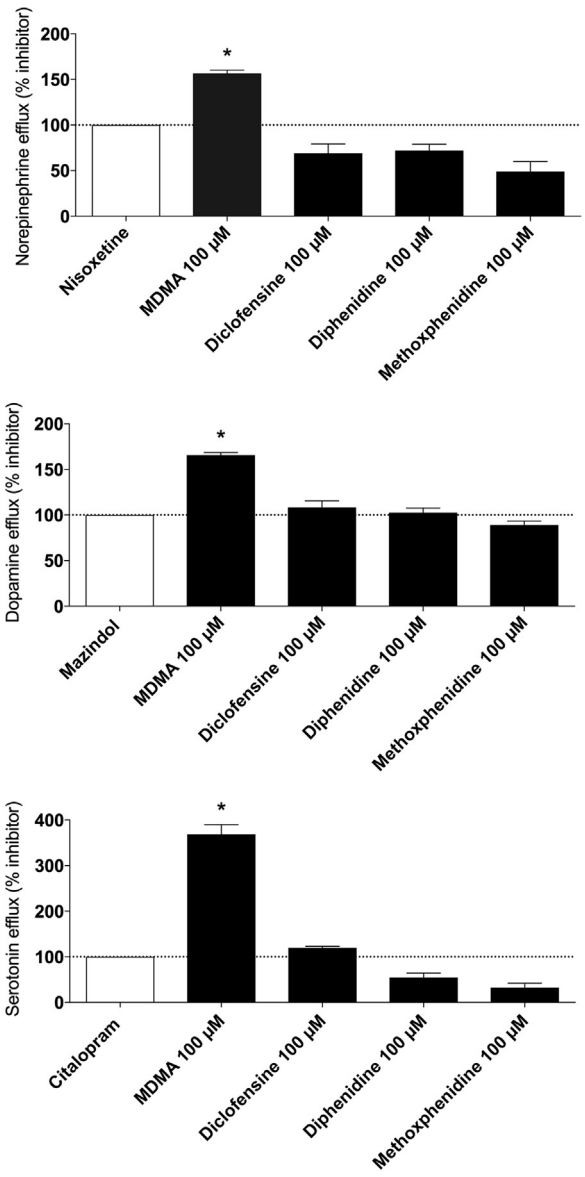

Fig. 3. Monoamine efflux induced by 100 µM of the test drugs after preloading HEK 293 cells that expressed the human NET, DAT, or SERT with radiolabeled monoamine. The dashed line marks nonspecific "pseudo-efflux" that arises from monoamine diffusion and subsequent reuptake inhibition. Substances that caused significantly more monoamine efflux ($^{*}P < 0.05$) than pure uptake inhibitors (open bars) were determined to be monoamine transporter substrates. The data are presented as the mean ± S.E.M. of five independent experiments.

did not bind to the serotonin 5-HT_{2C} receptor (K_i > 15 µM). Methoxphenidine bound to serotonin 5-HT_{2A} and 5-HT_{2C} receptors (K_i of 8.2 µM and 2.5 µM, respectively) but it did not bind to the serotonin 5-HT_{1A} receptor (K_i > 25 µM). Neither diphenidine nor methoxphenidine activated the serotonin 5-HT_{2A} or 5-HT_{2B} receptor (EC_{50} > 20 µM). Furthermore, diphenidine and methoxphenidine did not bind to the dopamine D_2 receptor (K_i > 25 µM) or to rat or mouse trace amine-associated receptors 1 (K_i > 15 µM).

3.4. Cytotoxicity

No cytotoxicity was observed for any of the drugs in the functional assays at the concentrations tested, thus confirming cell integrity during the assays.

Table 2
Monoamine transporter and receptor binding affinities.

	NET K_i	DAT K_i	SERT K_i	D_2 K_i	α_{1A} K_i	α_{2A} K_i	5-HT_{1A} K_i	5-HT_{2A} K_i	5-HT_{2A} EC_{50}	5-HT_{2B} EC_{50}	5-HT_{2C} K_i	$TAAR_1$ [a] rat K_i	$TAAR_1$ [a] mouse K_i
Diclofensine	0.027 ± 0.009	0.028 ± 0.004	0.096 ± 0.028	2.2 ± 1.7	0.14 ± 0.01	1.2 ± 0.1	1.2 ± 0.3	0.079 ± 0.010	> 20	> 20	0.50 ± 0.11	1.3 ± 0.1 [b]	6.9 ± 0.7 [b]
Diphenidine	3.4 ± 1.0	0.23 ± 0.02	27 ± 3	> 25	11 ± 1	4.4 ± 0.8	11 ± 1	11 ± 1	> 20	> 20	> 15	> 15 [b]	> 15 [b]
Methoxphenidine	6.9 ± 1.4	4.8 ± 0.8	20 ± 2	> 25	> 12	2.6 ± 0.1	> 25	8.2 ± 1.6	> 20	> 20	2.5 ± 0.6	> 15 [b]	> 15 [b]

Values are given as µM (mean ± SD).
[a] Trace amine-associated receptor 1.
[b] Values from Simmler et al. (2016).

4. Discussion

4.1. Monoamine uptake transporter inhibition and drug-induced monoamine efflux

Consistent with previous research (Keller et al., 1982), diclofensine was a potent triple monoamine transporter inhibitor and was devoid of monoamine releasing properties. The pharmacological profile of diclofensine is therefore similar to cocaine (Luethi et al., 2017a). This finding underscores the prior concern about the abuse liability of diclofensine (Lamb and Griffiths, 1990).

The dissociative drug diphenidine was an equipotent inhibitor of the NET and DAT, with IC_{50} values in the low micromolar range. Methoxphenidine was slightly less potent in inhibiting the NET and significantly less potent in inhibiting reuptake via DAT. Both drugs are thought to exert their dissociative mind-altering effects mainly via *N*-methyl-D-aspartate receptor antagonism (Morris and Wallach, 2014). Diphenidine and methoxphenidine did not mediate efflux of any monoamines, as observed for cocaine and methylphenidate-based NPSs (Luethi et al., 2017a).

In a recent study, IC_{50} values in the low micromolar range were reported for diphenidine but not for methoxphenidine, using a fluorescence-based neurotransmitter uptake kit (Wallach et al., 2016). Compared to the IC_{50} values measured with the fluorescence-based kit (Wallach et al., 2016), DAT inhibition by diphenidine and methoxphenidine in the present study was 1.7-fold and 2.2-fold, respectively, decreased; NET inhibition potency for diphenidine and methoxphenidine in the present study was however 2.8-fold and 4.5-fold, respectively, increased.

4.2. Monoamine receptor and transporter binding affinities

Diclofensine had high affinity for all monoamine transporters and additionally bound to the dopamine D_2 receptor, as observed for other tetrahydroisoquinoline derivatives (Mach et al., 2004; Silvano et al., 2010). Furthermore, diclofensine had affinity for adrenergic α_{1A} and α_{2A} receptors at 0.14 and 1.2 μM, respectively. These receptors modulate norepinephrine efflux and sympathomimetic activity (Hysek et al., 2012a, 2013). However, a clinical trial found that diclofensine did not significantly influence heart rate or blood pressure after an oral dose of 50 mg (Culig et al., 1983). The observed affinity values of diclofensine for serotonin 5-HT_{1A} and 5-HT_{2A} receptors were 1.2 and 0.079 μM, respectively. Both receptors are involved in body temperature regulation (Blessing et al., 2003; Liechti et al., 2000), and serotonin 5-HT_{2A} receptors are also targets of psychedelic drugs (Nelson et al., 1999; Nichols, 2016; Vollenweider et al., 1998). However, with an EC_{50} value > 20 μM, diclofensine is not a serotonin 5-HT_{2A} receptor agonist and no psychedelic effects are to be expected from activation of this receptor. The submicromolar affinity for the serotonin 5-HT_{2C} receptor may contribute to the antidepressant properties of diclofensine (Kim et al., 2010; Opal et al., 2014). With rat and mouse trace amine-associated receptor 1 affinities of 1.3 and 6.9 μM, respectively, the trace amine-associated receptor 1 binding potencies of diclofensine were weaker than for most amphetamines but stronger than for most cathinones (Simmler et al., 2016).

Diphenidine and methoxphenidine had low micromolar affinity for the adrenergic α_{2A} receptor (4.4 and 2.6 μM, respectively) and methoxphenidine also had appreciable affinity for the serotonin 5-HT_{2C} receptor (2.5 μM). In the receptor binding affinity screening by Wallach and colleagues (Wallach et al., 2016), similar binding affinity to the adrenergic α_{2A} receptor was reported for methoxphenidine (2 μM) but not for diphenidine (K_i > 10 μM). Furthermore, no potent interactions with serotonin receptors were found for either diphenidine or methoxphenidine in that study. For these two substances the highest affinity values were reported for the *N*-methyl-D-aspartate receptor (Wallach et al., 2016).

The strength of the present study is that the determined transporter inhibition potencies and receptor affinities for diclofensine, diphenidine, and methoxphenidine can be directly compared to a large set of data for NPSs, such as psychedelics (Luethi et al., 2017c; Rickli et al., 2015c, 2016) synthetic cathinones (Luethi et al., 2017b; Simmler et al., 2013, 2014a), amphetamines (Rickli et al., 2015a), and other designer stimulants (Luethi et al., 2017a; Rickli et al., 2015b; Simmler et al., 2014b), all measured with the same methods.

5. Conclusion

Diclofensine inhibited the NET, DAT, and SERT with similar potencies, similar to cocaine. Unlike cocaine, however, it had high affinity for several monoamine receptors. The pharmacological profile indicates stimulant properties and a potential for abuse for diclofensine. Diphenidine was an inhibitor of the NET and DAT, whereas its methoxylated derivative methoxphenidine was mainly an inhibitor of the NET. These drugs mediate their dissociative psychoactive effects via potent *N*-methyl-D-aspartate receptor antagonism (Wallach et al., 2016), monoamine transporter inhibition could however contribute to their psychoactive properties.

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Conflict of interest

M.C.H. is an employee of F. Hoffmann-La Roche. The other authors do not have any conflicts of interest to declare for this work.

Author contributions

D.L. and M.E.L. designed the research. D.L. and M.C.H. performed the research. All authors analyzed the data and contributed substantially to the writing of the manuscript.

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3 PHARMACOLOGY OF PSYCHEDELICS

3.1 Receptor interaction profiles of novel *N*-2-methoxybenzyl (NBOMe) derivatives of 2,5-dimethoxy-substituted phenethylamines (2C drugs)

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ABSTRACT

Background: *N*-2-methoxybenzyl-phenethylamines (NBOMe drugs) are newly used psychoactive substances with poorly defined pharmacological properties. The aim of the present study was to characterize the receptor binding profiles of a series of NBOMe drugs compared with their 2,5-dimethoxy-phenethylamine analogs (2C drugs) and lysergic acid diethylamide (LSD) *in vitro*.
Methods: We investigated the binding affinities of 2C drugs (2C-B, 2C-C, 2C-D, 2C-E, 2C-H, 2C-I, 2C-N, 2C-P, 2C-T-2, 2C-T-4, 2C-T-7, and mescaline), their NBOMe analogs, and LSD at monoamine receptors and determined functional 5-hydroxytryptamine-2A (5-HT_{2A}) and 5-HT_{2B} receptor activation. Binding at and the inhibition of monoamine uptake transporters were also determined. Human cells that were transfected with the respective human receptors or transporters were used (with the exception of trace amine-associated receptor-1 [$TAAR_1$], in which rat/mouse receptors were used).
Results: All of the compounds potently interacted with serotonergic 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors and rat $TAAR_1$ (most K_i and EC_{50}: <1 μM). The *N*-2-methoxybenzyl substitution of 2C drugs increased the binding affinity at serotonergic 5-HT_{2A}, 5-HT_{2C}, adrenergic α_1, dopaminergic $D_{1\text{-}3}$, and histaminergic H_1 receptors and monoamine transporters but reduced binding to 5-HT_{1A} receptors and $TAAR_1$. As a result, NBOMe drugs were very potent 5-HT_{2A} receptor agonists (EC_{50}: 0.04–0.5 μM) with high $5\text{-HT}_{2A}/5\text{-HT}_{1A}$ selectivity and affinity for adrenergic α_1 receptors (K_i: 0.3–0.9 μM) and $TAAR_1$ (K_i: 0.06–2.2 μM), similar to LSD, but not dopaminergic D_{1-3} receptors (most K_i: > 1 μM), unlike LSD.
Conclusion: The binding profile of NBOMe drugs predicts strong hallucinogenic effects, similar to LSD, but possibly more stimulant properties because of α_1 receptor interactions.

Abbreviations: 25B-NBOMe, 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25C-NBOMe, 2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25D-NBOMe, 2-(4-methyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25E-NBOMe, 2-(4-ethyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25H-NBOMe, 2-(2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25I-NBOMe, 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25N-NBOMe, 2-(4-nitro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25P-NBOMe, 2-(4-propyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25T2-NBOMe, 2-(2,5-dimethoxy-4-ethylthiophenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25T4-NBOMe, 2-(2,5-dimethoxy-4-isopropylthiophenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 25T7-NBOMe, 2-(2,5-dimethoxy-4-*n*-propylthiophenyl)-*N*-[(2-methoxyphenyl)methyl] ethanamine; 2C-B, 4-bromo-2,5-dimethoxyphenethylamine; 2C-C, 2-(4-chloro-2,5-dimethoxy)ethanamine; 2C-D, 2-(2,5-dimethoxy-4-methyl) ethanamine; 2C-E, 1-(2,5-dimethoxy-4-ethylphenyl)-2-aminoethane; 2C-H, 2,5-dimethoxyphenethylamine; 2C-I, 4-iodo-2,5-dimethoxyphenethylamine; 2C-N, 2-(2,5-dimethoxy-4-nitro)ethanamine; 2C-P, 2-(2,5-dimethoxy-4-propylphenyl)ethanamine; 25CN-NBOH, 2-([2-(4-cyano-2,5-dimethoxyphenyl)ethylamino]-methyl)phenol; 2C-T-2, 2-[2,5-dimethoxy-4-(ethylthio)phenyl]ethanamine; 2C-T-4, 2,5-dimethoxy-4-isopropylthiophenethylamine; 2C-T-7, 2-[2,5-dimethoxy-4-(propylthio)phenyl]ethanamine; 5-HT, 5-hydroxytryptamine (serotonin); DA, dopamine; DAT, dopamine transporter; mescaline, 2-(3,4,5-trimethoxyphenyl)ethanamine; DOI, 2,5-dimethoxy-4-iodoamphetamine; NBOMe, *N*-(2-methoxy)benzyl; NE, norepinephrine; NET, norepinephrine transporter; SERT, serotonin transporter; TAAR, trace amine-associated receptor; LSD, lysergic acid diethylamide.

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1. Introduction

New psychoactive substances are constantly emerging on the illicit drug market and typically sold via the Internet. Of particular interest are *N*-2-methoxybenzyl-phenethylamines (NBOMe drugs), which are novel and reportedly very potent hallucinogens that have been increasingly used recreationally (Forrester, 2014; Hill et al., 2013; Ninnemann and Stuart, 2013; Rose et al., 2013; Walterscheid et al., 2014; Wood et al., 2015; Zuba, 2012), with additional potential use as radiotracers (Ettrup et al., 2011, 2010). Recreationally used NBOMe drugs include 25I-NBOMe, 25C-NBOMe, 25B-NBOMe, and 25D-NBOMe (Armenian and Gerona, 2014; Poklis et al., 2014; Rose et al., 2013), which are derivatives of 2,5-dimethoxy-4-substituted phenethylamines (2C drugs; Dean et al., 2013; Hill and Thomas, 2011; Shulgin and Shulgin, 1991) (see Fig. 1). *N*-2-methoxybenzyl substitution enhances the potency of 2C drugs at serotonergic 5-hydroxytryptamine-2A (5-HT_{2A}) receptors, resulting in exceptionally potent 5-HT_{2A} receptor agonists (Braden et al., 2006; Heim, 2004; Nichols et al., 2015) with strong hallucinogenic properties in animals and humans (Halberstadt and Geyer, 2014; Srisuma et al., 2015). Pharmacological interactions between NBOMe drugs and 5-HT_2 receptors have been well characterized for some compounds of this novel drug family (Blaazer et al., 2008; Braden et al., 2006; Ettrup et al., 2011, 2010; Hansen et al., 2014; Nichols et al., 2008). However, systematic characterizations of the effects of a larger series of NBOMe drugs at a wider range of relevant human receptors and comparisons with their 2C parent drugs are lacking. Importantly, NBOMe drugs have been reported to produce psycho- and cardiovascular stimulant effects, in addition to hallucinations. Specifically, sympathomimetic toxicity, including tachycardia, hypertension, mydriasis, agitation, and hyperthermia, is commonly reported in cases of acute NBOMe drug intoxication (Hill et al., 2013; Rose et al., 2013; Srisuma et al., 2015; Stellpflug et al., 2014; Wood et al., 2015). Pharmacologically, compounds of the 2C series, including 2C-C, 2C-E, and 2C-I, inhibit the norepinephrine (NE) and serotonin transporters (NET and SERT, respectively), similar to amphetamines, although with only very low potency (Eshleman et al., 2014; Nagai et al., 2007). These findings raise the question of whether NBOMe drugs may have similar but more potent stimulant-type pharmacological properties, including inhibition of the NET, dopamine (DA) transporter (DAT), and SERT, or interactions with adrenergic α_1 receptors that lead to vasoconstriction.

We assessed the *in vitro* pharmacology of a series of NBOMe drugs compared with their 2C parent drugs. We characterized the binding affinity profiles at monoamine receptors and DAT, NET, and SERT inhibition potencies. We also determined the functional 5-HT_{2A} receptor activation potencies because 5-HT_{2A} receptors mediate hallucinogenic effects (Nichols, 2004). The prototypical serotonergic hallucinogen lysergic acid diethylamide (LSD) was included as a comparator drug (Nichols, 2004; Passie et al., 2008).

2. Methods

2.1. Drugs

2C-B, 2C-C, 2C-D, 2C-E, 2C-H, 2C-I, 2C-N, 2C-P, 2C-T-2, 2C-T-4, 2C-T-7, mescaline, 25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, 25I-NBOMe, 25N-NBOMe, 25P-NBOMe, 25T2-NBOMe, 25T4-NBOMe, 25T7-NBOMe, and mescaline-NBOMe were synthesized by Lipomed (Arlesheim, Switzerland) for this study at no cost. All of the compounds were used as hydrochloride salts. Purity was >98% for all of the substances. [^{3}H]NE and [^{3}H]DA were obtained from Perkin–Elmer (Schwerzenbach, Switzerland), and [^{3}H]5-HT was obtained from Anawa (Zürich, Switzerland).

2.2. Radioligand receptor and transporter binding assays

The radioligand binding assays were performed as described previously (Hysek et al., 2012; Simmler et al., 2013). Briefly, membrane preparations of human embryonic kidney (HEK) 293 cells (Invitrogen, Zug, Switzerland) that overexpress the respective transporters (Tatsumi et al., 1997) or receptors (human genes, with the exception of rat and mouse genes for trace amine-association receptor 1 [$TAAR_1$]; (Revel et al., 2011)) were incubated with the radiolabeled selective ligands at concentrations equal to K_d, and ligand displacement by the compounds was measured. Specific binding of the radioligand to the target receptor was defined as the difference between the total binding and nonspecific binding that was determined in the presence of selected competitors in excess. The following radioligands and competitors, respectively, were used: *N*-methyl-[^{3}H]-nisoxetine and indatraline (NET), [^{3}H]citalopram and indatraline (SERT), [^{3}H]WIN35,428 and indatraline (DAT), [^{3}H]8-hydroxy-2-(di-*n*-propylamine)tetralin and indatraline (5-HT_{1A} receptor), [^{3}H]ketanserin and spiperone (5-HT_{2A} receptor), [^{3}H]mesulgerine and mianserin (5-HT_{2C} receptor), [^{3}H]prazosin and risperidone (adrenergic α_1 receptor), [^{3}H]rauwolscine and phentolamine (adrenergic α_2 receptor), [^{3}H]SCH 23390 and butaclamol (D_1 receptor), [^{3}H]spiperone and spiperone (D_2 and D_3 receptors), [^{3}H]pyrilamine and clozapine, (histaminergic H_1 receptor), and [^{3}H]RO5166017 and RO5166017 ($TAAR_1$). IC_{50} values were determined by calculating non-linear regression curves for a one-site model using three to five independent 10-point concentration–response curves for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation.

2.3. Activity at serotonin 5-HT_{2A} receptor

Human 5-HT_{2A} receptor-expressing NIH-3T3 cells were incubated in HEPES- Hank's Balanced Salt Solution (HBSS) buffer (70′000 cells/100 μl) for 1 h at 37 °C in 96-well poly-D-lysine-coated plates. To each well 100 μl of Dye solution (FLIPR calcium 5 assay kit; Molecular Devices, Sunnyvale, CA, USA) was added and plates were incubated for 1 h at 37 °C. The plates were then placed in a fluorescence imaging plate reader (FLIPR), and 25 μl of the test substances diluted in HEPES-HBSS buffer containing 250 mM probenicid were added online. The increase in fluorescence was then measured. EC_{50} values were derived from the concentration–response curves using nonlinear regression. Efficacy (maximal activity) is expressed relative to the activity of 5-HT, which was used as a control set to 100%.

2.4. Activity at serotonin 5-HT_{2B} receptor

Human 5-HT_{2B} receptor-expressing HEK293 cells were incubated in growth medium (DMEM high glucose [Invitrogen, Zug, Switzerland], 10 ml/l PenStrep [Gibco, Life Technologies, Zug, Switzerland]), 10% FCS non dialyzed heat inactivated and 250 mg/l geneticin) at a density of 50′000 cells/well at 37 °C in 96-well poly-D-lysine-coated plates over-night. On the next day the growth medium was removed by snap inversion, and 100 μl of Fluo-4 solution (calcium indicator; Molecular Probes, Eugene, OR, USA) was added to each well. The plates were incubated for 45 min at 31 °C. The Fluo-4 solution was removed by snap inversion, and 100 μl of Fluo-4 solution was added a second time. The cells were then incubated for another 45 min at 31 °C. Immediately before testing, the cells were washed with HBSS (Gibco) and 20 mM HEPES (assay buffer; Gibco) using an EMBLA cell washer, and 100 μl assay buffer was added. The plate was placed in a fluorescence imaging plate reader (FLIPR), and 25 μl of the test substances diluted in assay

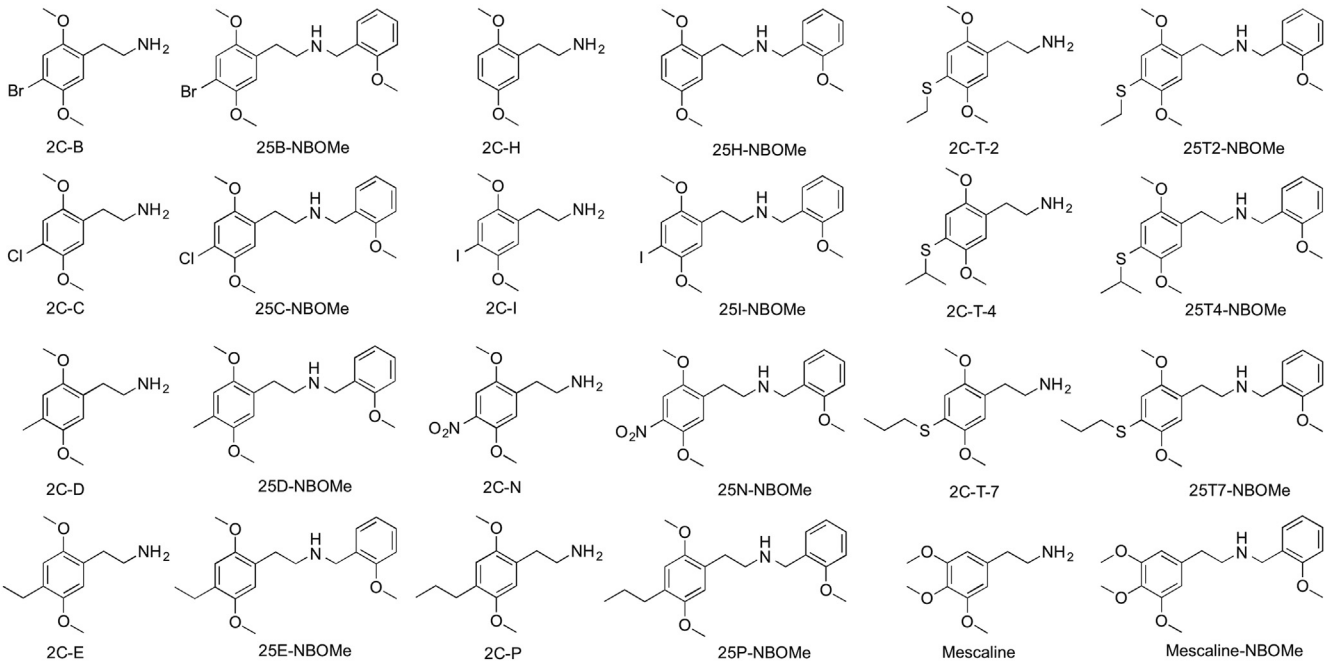

Fig. 1. Chemical structures of 2,5-dimethoxyphenethylamines (2C drugs) and their *N*-2-methoxybenzyl-substituted analogs (NBOMe drugs).

buffer was added online. The increase in fluorescence was then measured. EC_{50} values were derived from the concentration–response curves using nonlinear regression. Efficacy (maximal activity) is expressed relative to the activity of 5-HT, which was used as a control set to 100%.

2.5. Monoamine uptake transporter inhibition

Inhibition of the human NET, DAT, and SERT was assessed in HEK 293 cells that were stably transfected with transporters as specified previously (Hysek et al., 2012). Briefly, the cells were suspended in uptake buffer and incubated for 10 min with different concentrations of the test substances. The corresponding radiolabeled [^{3}H] monoamine (5 nM final concentration) was then added at room temperature. After 10 min, uptake was stopped by separating the cells from the buffer using centrifugation through silicone oil (Hysek et al., 2012). The centrifugation tubes were frozen in liquid nitrogen and cut to separate the cell pellet from the silicone oil and assay buffer layers. The cell pellet was then lysed. Scintillation fluid was added, and radioactivity was counted on a β-counter. Nonspecific uptake was determined for each experiment in the presence of 10 μM fluoxetine for SERT cells, 10 μM nisoxetine for NET cells, and 10 μM mazindol for DAT cells and subtracted from the total counts to yield specific uptake (100%). The data were fitted by non-linear regression to variable slope sigmoidal dose–response curves (bottom = 0%), and IC_{50} values were calculated using Prism software (GraphPad, San Diego, CA, USA).

2.6. Cytotoxicity

To confirm cell integrity during the pharmacological assays, cytotoxicity was assessed using the ToxiLight bioassay (Lonza, Basel, Switzerland) according to the manufacturer's instructions. The assay quantitatively measures the release of adenylate kinase from damaged cells, providing a highly sensitive method of measuring cytolysis (Crouch et al., 1993). Cells that were grown in 96-well plates were exposed to the compounds at a high concentration of 100 μM. All of the test conditions contained 0.1% (v:v) dimethylsulfoxide, which is non-toxic at this concentration and was also used as a negative control. Triton X-100 (0.1%, Sigma–Aldrich, Buchs, Switzerland) lyses cells and was used as a positive control. After 4 h incubation at 37 °C, 10 μl of the supernatant per well was removed and combined with 50 μl of ToxiLight reagent, and luminescence was recorded using a Tecan Infinite 200 Pro plate reader (Tecan, Männedorf, Switzerland).

3. Results

3.1. Interactions with serotonin receptors

Table 1 shows binding to serotonin 5-HT_{1A}, 5-HT_{2A}, and 5-HT_{2C} receptors, activation potency and efficacy at 5-HT_{2A} and 5-HT_{2B} receptors, and 5-HT receptor binding ratios. All of the compounds exhibited high binding affinity for 5-HT_{2A} and 5-HT_{2C} receptors (K_i < 1 μM, with the exception of 2C-H and mescaline). *N*-2-methoxybenzyl substitution further increased the average binding affinity for both 5-HT_{2A} and 5-HT_{2C} receptors 26- and 14-fold (range: 6–100 and 8–32, respectively), leading to compounds with up to 8.4-fold higher affinity for these receptors compared with LSD. Moderate 5-HT_{2A} over 5-HT_{2C} receptor binding preference was observed, with 5-HT_{2A}/5-HT_{2C} receptor binding ratios of 3–16 for the 2C drugs and slightly more selective ratios of 5–26 for the NBOMe drugs. All of the compounds also potently activated 5-HT_{2A} receptors and typically more potently than LSD (EC_{50} < 1 μM, with the exception of 2C-H, mescaline, and mescaline-NBOMe). However, in contrast to the robust effect on binding to 5-HT_{2A} receptors, *N*-2-methoxybenzyl substitution did not consistently change the activation potency at 5-HT_{2A} receptors and even reduced the activation efficacy, with the exception of 2C-H. All of the compounds potently activated the 5-HT_{2B} receptor (EC_{50} < 1 μM, with the exception of 2C-H, mescaline, mescaline-NBOMe, and LSD). *N*-2-methoxybenzyl substitution increased 5-HT_{2B} receptor activation 5-fold (range: 0.8–18) but reduced activation efficacy. All of the 2C drugs potently bound to 5-HT_{1A}

Table 1
Serotonin receptor interactions.

	5-HT$_{1A}$	5-HT$_{2A}$			5-HT$_{2B}$		5-HT$_{2C}$	Selectivity (binding ratios)	
	Receptor binding $K_i \pm SD$ [μM]	Receptor binding $K_i \pm SD$ [μM]	Activation potency $EC_{50} \pm SD$ [μM]	Activation efficacy % maximum ± SD	Activation potency $EC_{50} \pm SD$ [μM]	Activation efficacy % maximum ± SD	Receptor binding $K_i \pm SD$ [μM]	5-HT$_{2A}$/ 5-HT$_{1A}$	5-HT$_{2A}$/ 5-HT$_{2C}$
2Cs									
2C-B	0.24 ± 0.04	0.0086 ± 0.003	0.08 ± 0.02	45 ± 7	0.13 ± 0.06	89 ± 13	0.047 ± 0.009	28	4.7
2C-C	0.19 ± 0.01	0.0130 ± 0.005	0.20 ± 0.06	49 ± 10	0.28 ± 0.11	81 ± 14	0.090 ± 0.026	15	6.9
2C-D	0.44 ± 0.01	0.0324 ± 0.005	0.35 ± 0.18	41 ± 3	0.23 ± 0.07	77 ± 17	0.15 ± 0.03	14	4.6
2C-E	0.36 ± 0.04	0.0105 ± 0.001	0.11 ± 0.03	40 ± 2	0.19 ± 0.04	66 ± 7	0.10 ± 0.02	34	10
2C-H	0.07 ± 0.02	1.6 ± 0.3	9.4 ± 0.5	28 ± 5	6.2 ± 2.8	46 ± 18	4.1 ± 0.9	0.04	2.6
2C-I	0.18 ± 0.01	0.0035 ± 0.001	0.06 ± 0.03	45 ± 8	0.15 ± 0.10	70 ± 18	0.040 ± 0.009	51	11
2C-N	2.2 ± 0.1	0.0235 ± 0.011	0.17 ± 0.04	48 ± 10	0.73 ± 0.09	74 ± 20	0.37 ± 0.02	94	16
2C-P	0.11 ± 0.04	0.0081 ± 0.001	0.09 ± 0.06	63 ± 5	0.13 ± 0.01	72 ± 18	0.040 ± 0.005	14	4.9
2C-T-2	0.37 ± 0.04	0.0090 ± 0.002	0.08 ± 0.03	67 ± 16	0.13 ± 0.09	75 ± 14	0.069 ± 0.018	41	7.7
2C-T-4	0.47 ± 0.13	0.0279 ± 0.012	0.22 ± 0.13	87 ± 7	0.16 ± 0.06	68 ± 10	0.18 ± 0.07	17	6.5
2C-T-7	0.52 ± 0.05	0.0065 ± 0.002	0.13 ± 0.05	76 ± 10	0.35 ± 0.25	45 ± 10	0.039 ± 0.013	80	6.0
Mescaline	4.6 ± 0.4	6.3 ± 1.8	10 ± 1.8	56 ± 15	>20	NA	17 ± 2.0	0.73	2.7
N-benzylphenylethylamines (NBOMes)									
25B-NBOMe	3.6 ± 0.3	0.0005 ± 0.0000	0.04 ± 0.01	28 ± 7	0.01 ± 0.01	19 ± 5	0.0062 ± 0.0022	7200	12
25C-NBOMe	5.0 ± 0.1	0.0007 ± 0.0002	0.15 ± 0.06	32 ± 2	0.10 ± 0.13	16 ± 5	0.0052 ± 0.0026	7143	7.4
25D-NBOMe	7.1 ± 0.5	0.0010 ± 0.0004	0.09 ± 0.03	27 ± 7	0.10 ± 0.07	22 ± 6	0.013 ± 0.004	7100	13
25E-NBOMe	3.5 ± 0.2	0.0006 ± 0.0001	0.16 ± 0.11	28 ± 15	0.06 ± 0.03	26 ± 10	0.0072 ± 0.0029	5833	12
25H-NBOMe	6.0 ± 0.7	0.0164 ± 0.0014	0.49 ± 0.07	38 ± 10	0.34 ± 0.14	11 ± 5	0.13 ± 0.02	366	7.9
25I-NBOMe	1.8 ± 0.3	0.0006 ± 0.0002	0.24 ± 0.12	27 ± 7	0.13 ± 0.08	32 ± 12	0.0046 ± 0.0020	3000	7.7
25N-NBOMe	4.2 ± 0.6	0.0008 ± 0.0002	0.07 ± 0.03	34 ± 3	0.07 ± 0.03	26 ± 14	0.021 ± 0.003	5250	26
25P-NBOMe	1.8 ± 0.1	0.0011 ± 0.0002	0.22 ± 0.11	42 ± 7	0.17 ± 0.13	23 ± 8	0.0060 ± 0.0015	1636	5.5
25T2-NBOMe	2.2 ± 0.2	0.0006 ± 0.0002	0.10 ± 0.03	38 ± 6	0.04 ± 0.04	31 ± 12	0.0065 ± 0.0006	3667	11
25T4-NBOMe	2.5 ± 0.3	0.0016 ± 0.0004	0.13 ± 0.05	46 ± 8	0.20 ± 0.10	27 ± 11	0.016 ± 0.005	1563	10
25T7-NBOMe	1.8 ± 0.2	0.0011 ± 0.0002	0.26 ± 0.16	41 ± 6	0.31 ± 0.23	14 ± 5	0.0064 ± 0.0013	1636	5.8
Mescaline-NBOMe	21 ± 5.7	0.14 ± 0.03	3.0 ± 0.6	33 ± 11	>20	NA	0.64 ± 0.04	147	4.5
LSD	0.0030 ± 0.0005	0.0042 ± 0.0013	0.26 ± 0.15	28 ± 10	12 ± 0.35	71 ± 31	0.015 ± 0.003	0.71	3.6

Values are K_i given as μM (mean ± SD); NA, not assessed.

receptors ($K_i < 0.52$ μM, with the exception of 2C-N and mescaline), although none exhibited the very high affinity of LSD. *N*-2-methoxybenzyl substitution decreased binding to 5-HT$_{1A}$ on average 17-fold (range: 2–86). The 2C drugs preferentially bound to 5-HT$_{2A}$ over 5-HT$_{1A}$ receptors with binding ratios of 14–94, with the exception of 2C-H and mescaline (Table 1). Receptor selectivity was markedly increased for 5-HT$_{2A}$ over 5-HT$_{1A}$ receptors for all of the compounds with *N*-2-methoxybenzyl substitution, with 5-HT$_{2A}$/5-HT$_{1A}$ ratios >100 for 25H-NBOMe and mescaline-NBOMe and >1000 for all of the other NBOMe drugs.

3.2. Binding to monoamine receptors and transporters

Table 2 shows the binding affinities for monoamine receptors and transporters. Compared with the 2C drugs, the NBOMe analogs exhibited higher binding affinities for all receptors and transporters, with the exception of TAAR$_1$. Specifically, all of the NBOMe drugs and LSD showed high-affinity binding to adrenergic α_{1A} receptors ($K_i < 1$ μM, with the exception of mescaline-NBOMe) and 19-fold (range: 11–38) higher binding affinity compared with the 2C drugs (not including mescaline). Most of the compounds also potently bound to α_{2A} receptors ($K_i < 1$ μM, with the exception of 2C-H, 2C-N, and mescaline). *N*-2-methoxybenzyl substitution did not appreciably alter α_{2A} receptor binding. LSD was the only substance that exhibited high-affinity binding to dopamine D_1-D_3 receptors. Most of the 2C and NBOMe drugs showed low-affinity binding to D_2 receptors, and NBOMe drugs also showed low-affinity binding to D_2 and D_3 receptors. *N*-2-methoxybenzyl substitution also increased histamine H_1 receptor binding 65-fold (range: 2–267) compared with the 2C analogs, resulting in high-affinity binding for several NBOMe drugs (Table 2). All of the 2C and NBOMe drugs showed high-affinity binding to TAAR$_{1rat}$ ($K_i < 1$ μM, with the exception of mescaline, 25-H-NBOMe, 25-N-NBOMe, and mescaline-NBOMe). *N*-2-methoxybenzyl substitution decreased binding to TAAR$_{1rat}$ 4-fold (range: 2–9). Binding affinity to monoamine transporters was low for 2C drugs ($K_i > 10$ μM). *N*-2-methoxybenzyl substitution increased binding to all monoamine transporters, resulting in low-affinity interactions for most of the NBOMe drugs ($K_i < 1$–10 μM, with the exception of mescaline-NBOMe). LSD did not interact with any of the monoamine transporters.

3.3. Monoamine uptake transporter inhibition

IC_{50} values for monoamine uptake inhibition are listed in Table 3. The 2C drugs did not inhibit or only very weakly inhibited ($IC_{50} > 10$ μM) monoamine uptake. *N*-2-methoxybenzyl substitution consistently enhanced monoamine uptake inhibition potency approximately two-to 15-fold for the NET, two-to five-fold for the DAT, and two-to 26-fold for the SERT. As a result, 25B-NBOMe, 25C-NBOMe, 25D-NBOMe, 25E-NBOMe, 25H-NBOMe, and 25I-NBOMe blocked the NET and/or SERT at 5–10 μM concentrations. LSD did not inhibit any of the monoamine transporters.

3.4. Cytotoxicity

None of the compounds produced cytotoxicity after 4 h incubation at 37 °C, with the exception of 25T7-NBOMe. 25T7-NBOMe became toxic after 4 h incubation at 100 μM (but not 10 μM). Because the assays lasted less than 4 h, this toxicity did not affect the data.

Table 2
Monoamine transporter and receptor-binding affinities.

	α_{1A}	α_{2A}	D_1	D_2	D_3	H_1	$TAAR_{1rat}$	$TAAR_{1mouse}$	NET[a]	DAT[b]	SERT[c]
2C-series											
2C-B	8.2 ± 2.2	0.32 ± 0.01	12 ± 1.2	2.2 ± 0.3	10 ± 2.0	14 ± 0.5	0.09 ± 0.01	3.0 ± 0.3	31 ± 6.6	>30	9.7 ± 0.3
2C-C	13 ± 1.9	0.53 ± 0.06	13 ± 1.0	2.1 ± 0.4	17 ± 0.3	24 ± 0.9	0.11 ± 0.02	4.1 ± 0.3	>30	>30	24 ± 4.1
2C-D	12 ± 3.2	0.29 ± 0.03	24 ± 5.2	7.1 ± 1.7	>17	>25	0.15 ± 0.03	3.5 ± 0.1	>30	>30	31 ± 2.2
2C-E	7.4 ± 2.8	0.10 ± 0.02	15 ± 0.6	3.2 ± 1.0	19 ± 4.4	>25	0.07 ± 0.01	1.2 ± 0.1	33 ± 2.7	>30	29 ± 4.4
2C-H	7.9 ± 1.8	1.0 ± 0.05	>14	9.0 ± 1.5	>17	>25	0.90 ± 0.16	11 ± 2.2	>30	>30	>30
2C-I	5.1 ± 1.1	0.07 ± 0.01	13 ± 4.1	2.7 ± 0.58	5.0 ± 0.1	6.1 ± 0.5	0.12 ± 0.02	3.3 ± 0.1	15 ± 3.5	>30	4.9 ± 0.3
2C-N	>15	1.3 ± 0.2	19 ± 5.2	6.1 ± 2.7	20 ± 3.1	>25	0.34 ± 0.02	>20	>30	>30	32 ± 3.1
2C-P	3.5 ± 0.5	0.09 ± 0.01	8.4 ± 0.9	2.3 ± 0.7	5.2 ± 0.5	21 ± 3.2	0.02 ± 0.01	0.28 ± 0.03	18 ± 2.4	40 ± 4.0	19 ± 0.2
2C-T-2	17 ± 6.4	0.23 ± 0.01	15 ± 1.7	5.1 ± 1.0	11 ± 0.6	>25	0.04 ± 0.01	2.2 ± 0.6	>30	>30	13 ± 0.6
2C-T-4	11 ± 4.4	0.13 ± 0.04	20 ± 6.3	16 ± 2.1	19 ± 1.4	>25	0.05 ± 0.01	4.5 ± 0.9	17 ± 1.1	>30	>30
2C-T-7	13 ± 5.0	0.18 ± 0.001	15 ± 3.1	5.0 ± 0.8	7.5 ± 0.3	>25	0.03 ± 0.01	0.56 ± 0.1 2	27 ± 9.8	34 ± 6.2	12 ± 0.7
Mescaline	>15	1.4 ± 0.2	>14	>10	>17	>25	3.3 ± 0.5	11 ± 3.6	>30	>30	>30
N-benzylphenylethylamines (NBOMes)											
25B-NBOMe	0.43 ± 0.10	0.43 ± 0.03	9.3 ± 2.0	0.84 ± 0.27	2.7 ± 0.3	0.08 ± 0.02	0.28 ± 0.002	4.5 ± 1.7	1.1 ± 0.3	7.2 ± 0.5	0.84 ± 0.06
25C-NBOMe	0.81 ± 0.26	0.56 ± 0.08	12 ± 1.6	1.6 ± 0.4	3.5 ± 0.3	0.09 ± 0.01	0.52 ± 0.10	15 ± 1.9	1.6 ± 0.6	14 ± 3	1.5 ± 0.1
25D-NBOMe	0.70 ± 0.26	0.37 ± 0.05	8.7 ± 1.4	2.6 ± 0.4	6.4 ± 0.9	0.63 ± 0.06	0.81 ± 0.10	13 ± 4.4	2.2 ± 0.3	14 ± 2.4	1.4 ± 0.2
25E-NBOMe	0.53 ± 0.20	0.26 ± 0.07	4.9 ± 0.9	1.5 ± 0.2	3.2 ± 0.2	1.4 ± 0.2	0.26 ± 0.03	1.1 ± 0.3	3.0 ± 0.2	8.1 ± 0.6	1.7 ± 0.1
25H-NBOMe	0.55 ± 0.05	0.53 ± 0.04	14 ± 2.4	7.7 ± 1.7	20 ± 4.5	4.1 ± 0.4	1.4 ± 0.2	>20	5.5 ± 0.9	35 ± 1.7	2.3 ± 0.1
25I-NBOMe	0.37 ± 0.02	0.32 ± 0.01	6.7 ± 1.1	0.90 ± 0.13	2.1 ± 0.2	0.09 ± 0.01	0.44 ± 0.07	4.0 ± 0.8	1.3 ± 0.5	5.4 ± 0.5	1.0 ± 0.2
25N-NBOMe	0.85 ± 0.11	0.59 ± 0.07	18 ± 6.7	2.4 ± 0.1	4.5 ± 0.8	0.21 ± 0.04	2.2 ± 0.1	>20	7.2 ± 0.5	13 ± 1.2	5.1 ± 0.3
25P-NBOMe	0.31 ± 0.08	0.41 ± 0.07	3.1 ± 0.1	0.87 ± 0.08	2.3 ± 0.3	1.7 ± 0.2	0.06 ± 0.01	0.24 ± 0.03	2.8 ± 0.3	4.7 ± 0.4	5.2 ± 0.4
25T2-NBOMe	0.55 ± 0.17	0.45 ± 0.04	7.7 ± 0.4	1.6 ± 0.3	3.0 ± 0.4	0.49 ± 0.04	0.35 ± 0.02	4.2 ± 0.6	5.9 ± 0.4	8.6 ± 1.8	5.0 ± 0.2
25T4-NBOMe	0.58 ± 0.25	0.26 ± 0.03	4.9 ± 0.5	1.7 ± 0.5	1.9 ± 0.3	5.4 ± 0.3	0.12 ± 0.02	1.6 ± 0.4	4.3 ± 0.8	6.2 ± 1.5	8.1 ± 0.3
25T7-NBOMe	0.34 ± 0.06	0.36 ± 0.02	4.1 ± 0.2	1.0 ± 0.2	1.4 ± 0.2	1.2 ± 0.1	0.09 ± 0.03	1.0 ± 0.2	3.7 ± 1.1	4.8 ± 1.4	3.2 ± 0.2
Mescaline-NBOMe	3.0 ± 1.2	0.81 ± 0.05	>14	9.6 ± 2.6	>17	14 ± 1.2	13 ± 5.6	>20	46 ± 7.5	>30	24 ± 1.3
LSD	0.67 ± 0.18	0.012 ± 0.002	0.31 ± 0.1	0.025 ± 0.0004	0.096 ± 0.005	1.1 ± 0.2	0.45 ± 0.05	10 ± 2.9	>30	>30	>30

[a] Values are K_i given as μM (mean ± SD). Comparative Ki values for known monoamine transporter inhibitors were: 0.015 ± 0.01 μM for reboxetine at the NET.
[b] 0.06 ± 0.01 μM for methylphenidate at the DAT.
[c] 0.005 ± 0.001 μM for citalopram at the SERT.

Table 3
Monoamine transporter inhibition.

	NET	DAT	SERT
	IC_{50} [μM] (95% CI)	IC_{50} [μM] (95% CI)	IC_{50} [μM] (95% CI)
2C-series			
2C-B	44 (33–58)	231 (196–271)	18 (12–27)
2C-C	93 (64–137)	305 (243–383)	74 (58–95)
2C-D	45 (28–72)	626 (536–730)	77 (60–98)
2C-E	26 (18–37)	275 (221–343)	62 (52–74)
2C-H	125 (97–161)	857 (752–976)	311 (238–408)
2C-I	22 (16–31)	126 (103–155)	13 (10–16)
2C-N	287 (223–369)	>900	154 (112–213)
2C-P	94 (73–120)	198 (136–287)	30 (22–41)
2C-T-2	153 (152–154)	332 (332–332)	62 (62–62)
2C-T-4	134 (92–195)	294 (242–357)	113 (92–138)
2C-T-7	135 (115–163)	261 (210–324)	44 (36–52)
Mescaline	>900	841 (590–1200)	367 (291–462)
N-benzylphenylethylamines (NBOMes)			
25B-NBOMe	6.7 (5.6–8.1)	117 (89–154)	7.1 (5.7–8.8)
25C-NBOMe	5.9 (4.4–7.8)	70 (56–87)	7.3 (5.6–9.6)
25D-NBOMe	4.0 (3.0–5.3)	106 (81–140)	3.9 (2.6–5.7)
25E-NBOMe	11 (8.3–14)	100 (88–112)	8.3 (6.2–11)
25H-NBOMe	10 (7.8–13)	120 (101–144)	12 (9.7–14)
25I-NBOMe	10 (7.4–14)	65 (46–89)	6.8 (4.8–9.5)
25N-NBOMe	33 (25–44)	245 (194–310)	20 (15–26)
25P-NBOMe	14 (11–16)	82 (61–110)	12 (9.3–16)
25T2-NBOMe	25 (15–42)	67 (54–84)	20 (14–29)
25T4-NBOMe	28 (22–35)	58 (43–80)	14 (11–18)
25T7-NBOMe	34 (29–40)	55 (45–68)	17 (13–23)
Mescaline-NBOMe	89 (61–130)	449 (303–665)	85 (63–116)
LSD	>900	>900	>900
Monoamine transporter inhibitors			
Reboxetine	0.036 (0.030–0.044)	ns	ns
Methylphenidate	ns	0.12 (0.09–0.16)	ns
Citalopram	ns	ns	0.045 (0.037–0.057)

Values are means of three to four independent experiments and 95% confidence intervals (CI). ns, not shown.

4. Discussion

We pharmacologically characterized the *in vitro* receptor interaction profiles of novel recreationally abused hallucinogenic *N*-2-methoxybenzyl-substituted phenethylamines compared with their 2C phenethylamine analogs. Both the NBOMe and 2C drugs potently interacted with serotonin 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors and $TAAR_{1rat}$. We also found several consistent and potentially important structure-affinity relationships for the NBOMe drugs, their 2C analogs, and several targets. Specifically, *N*-2-methoxybenzyl substitution increased the binding affinity for and/or activation potency at serotonergic 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C} receptors, adrenergic α_1 receptors, dopaminergic $D_{1\text{-}3}$ receptors, histaminergic H_1 receptors, and monoamine transporters but reduced binding to 5-HT_{1A} receptors and $TAAR_1$.

The 5-HT_{2A} receptor mediates hallucinogenic drug properties (Halberstadt and Geyer, 2011; Nelson et al., 1999; Nichols, 2004; Vollenweider et al., 1998) and is therefore considered the key target of hallucinogenic phenethylamines, including 2C and NBOMe drugs (Braden et al., 2006; Halberstadt, 2015; Halberstadt and Geyer, 2014). *N*-2-methoxybenzyl substitution consistently increased the already high *in vitro* affinity of 2C drugs for 5-HT_{2A} receptors, in agreement with data on 25H-NBOMe and 25I-NBOMe *vs*. 2C-H and 2C-I, respectively (Braden et al., 2006; Heim, 2004). All of the NBOMe drugs exhibited low nanomolar or even sub-nanomolar affinity for 5-HT_{2A} receptors, confirming studies on 25B-NBOMe, 25C-NBOMe, 25H-NBOMe, 25I-NBOMe, and 25B-NBOMe that used rat receptors (Braden et al., 2006; Ettrup et al., 2011, 2010; Nichols et al., 2015) or human receptors (Braden et al., 2006; Hansen et al., 2014; Nichols et al., 2015). Generally, 5-HT_{2A} receptor affinity correlates with hallucinogenic drug potency in humans (Halberstadt, 2015; Titeler et al., 1988), and NBOMe drugs can be expected to be extremely potent hallucinogens *in vivo*. Indeed, higher incidences of hallucinations and delusions have been reported in patients with NBOMe compared with 2C drug intoxication (Forrester, 2013, 2014; Srisuma et al., 2015).

Surprisingly, the consistent six-to 100-fold increase in 5-HT_{2A} receptor affinity that was produced by *N*-2-methoxybenzyl substitution did not translate into a similar increase in 5-HT_{2A} receptor activation potency, and the activation efficacy was even reduced compared with the 2C drugs in our functional assay. In contrast, others found that *N*-2-methoxybenzyl substitution in 2C-H or 2C-I increased the potency for rat or human 5-HT_{2A} receptor activation in the inositol phosphate hydrolysis assay *in vitro* (Braden et al., 2006). However, high-affinity agonist binding does not correlate well with inositol phosphate turnover (Acuna-Castillo et al., 2002; Roth et al., 1997), suggesting that additional ligand–receptor interactions contribute to receptor activation (Halberstadt, 2015; Nichols, 2004). Additionally, marked discrepancies between inositol phosphate hydrolysis activation and other *in vitro* assays and the *in vivo* effects of hallucinogens in laboratory animals or humans are well recognized (Nichols, 2004; Saez et al., 1994; Villalobos et al., 2004). Thus, although most of the effects of hallucinogens are clearly mediated by 5-HT_{2A} receptor activation (Halberstadt, 2015; Nichols, 2004), the signaling pathways that mediate these effects have not yet been conclusively identified (Halberstadt, 2015).

Currently unknown pharmacokinetic characteristics of NBOMe drugs may also influence drug potency *in vivo*. For example, differences in the *in vivo* brain binding properties of *N*-2-methoxybenzyl-substituted positron emission tomography tracers were reported for substances with similar *in vitro* 5-HT_{2A} receptor binding properties (Ettrup et al., 2011). Most importantly, NBOMe drugs are used recreationally at higher doses than LSD (Bersani et al., 2014; Halberstadt and Geyer, 2014), despite their higher 5-HT_{2A} receptor binding affinities. The lower *in vivo* potency of orally administered NBOMe drugs could be explained by their lower hepatic stability that reduced oral bioavailability compared with 2C drugs (Leth-Petersen et al., 2014). Thus, high 5-HT_{2A} receptor binding or activation *in vitro* is only one factor that potentially predicts hallucinogen potency *in vivo*. In the first *in vivo* studies that evaluated NBOMe drugs in mice, 25I-NBOMe was 14-times more potent than its analog 2C-I in inducing 5-HT_{2A} receptor-mediated head-twitch responses (Halberstadt and Geyer, 2014), consistent with the higher 5-HT_{2A} receptor binding in the present study. In contrast, 25I-NBOMe was slightly less potent in inducing head twitches than expected, based on its high 5-HT_2 binding potency (Nichols et al., 2015) and compared with LSD (Halberstadt and Geyer, 2013, 2014), consistent with the similar 5-HT_{2A} receptor activation potency of the two compounds in the present study but not reflecting the higher receptor binding potency of 25I-NBOMe compared with LSD. Additionally, 2-([2-(4-cyano-2,5-dimethoxyphenyl)ethylamino]-methyl)phenol (25CN-NBOH), which is structurally similar to the NBOMe drugs that were tested in the present study, was a more potent 5-HT_{2A} receptor agonist than 2,5-dimethoxy-4-iodoamphetamine (DOI) *in vitro* (Hansen et al., 2014) but less effective in inducing head-twitch responses in mice (Fantegrossi et al., 2015). Thus, more *in vivo* studies are needed to determine the *in vivo* potency of novel NBOMe drugs.

Within the 2C or NBOMe drug series, para-phenyl substitutions compared with 2C-H or 25H-NBOMe, respectively, enhanced 5-HT_2 receptor binding and activation potency, which was expected based on previous studies (Blaazer et al., 2008; Eshleman et al., 2014; Hansen et al., 2014; Shulgin and Shulgin, 1991). Interestingly, 5-HT_{2A} receptor activation potency increased with the size of the 4-substituent (2C-D < 2C-E < 2C-P) within the 2C series (Blaazer et al., 2008; Eshleman et al., 2014), whereas it decreased within the NBOMe series (25D-NBOMe > 25-E-NBOMe > 25P-NBOMe). Similarly, activation potency increased with halogen size for the 4-halogen-substituted 2C drugs (2C-C < 2C-B < 2C-I) but not consistently for the NBOMe analogs. Thus, *N*-2-methoxybenzyl substitution interacted with 4-phenyl substitution to affect 5-HT_{2A} receptor activation potency.

In the present study, all of the compounds were partial agonists at 5-HT_{2A} receptors, but receptor activation efficacy was consistently decreased for the *N*-2-methoxybenzyl-substituted compounds in the assay used in the present study. The high 5-HT_{2A} receptor affinity and reduction of partial activation efficacy of the NBOMe drugs suggest 5-HT_{2A} antagonistic properties of these compounds, as similarly described for LSD (Nichols, 2004). In fact, 2C drugs have been shown to act as 5-HT_{2A} receptor antagonists that inhibit 5-HT-induced currents in *Xenopus laevis* oocytes (Villalobos et al., 2004). Therefore, 5-HT_{2A} receptor antagonism has been suggested to also play a role in the mechanism of action of hallucinogens (Villalobos et al., 2004). Alternatively, other receptors, such as 5-HT_{2C} and 5-HT_1 receptors, may contribute to the mechanism of action of hallucinogens, or signaling pathways other than inositol phosphate hydrolysis may be involved (Nichols, 2004). Consistently, *N*-2-methoxybenzyl substitution increased binding affinity for 5-HT_{2C} receptors. All of the NBOMe drugs very potently bound to 5-HT_{2C} receptors, with only low (five-to 26-fold) selectivity for 5-HT_{2A} receptors over 5-HT_{2C} receptors in the binding assay, as previously shown for some NBOMe drugs (Ettrup et al., 2010; Hansen et al., 2014) and generally observed with hallucinogenic phenethylamines (Eshleman et al., 2014; Glennon et al., 1992). *N*-2-methoxybenzyl substitution only slightly increased 5-HT_{2A} over 5-HT_{2C} receptor binding selectivity. In contrast, *N*-2-methoxybenzyl substitution consistently decreased 5-HT_{1A} receptor binding, thus markedly altering 5-HT_{1A} over 5-HT_{2A} receptor binding ratios for the NBOMe drugs compared with the 2C drugs.

Thus, NBOMe drugs are unlike LSD, which is a potent 5-HT_{1A} receptor ligand and full agonist at 5-HT_{1A} receptors (Nichols, 2004). Importantly, 5-HT_{1A} receptors have been shown to contribute to the discriminative stimulus effects of some hallucinogens (Halberstadt, 2015; Nichols, 2004). Additionally, 5-HT_{1A} antagonism markedly enhanced the hallucinogenic effects of DMT in humans (Strassman, 1996). Accordingly, 5-HT_{1A} receptor stimulation has been hypothesized to counteract hallucinogenic activity (Halberstadt and Geyer, 2011; Nichols, 2004), and lower 5-HT_{1A} receptor stimulation for the NBOMe drugs may further enhance their hallucinogenic drug properties. *N*-2-methoxybenzyl substitution increased 5-HT_{2B} activation, but this is likely not relevant for the psychotropic properties of the NBOMe drugs (Blaazer et al., 2008). However, 5-HT_{2B} receptors have been implicated in substance-induced heart valve fibrosis (Bhattacharyya et al., 2009; Setola et al., 2003), and the 2C and NBOMe drugs may therefore have cardiac toxicity if used chronically.

Because NBOMe drugs produce marked sympathomimetic cardiovascular effects in humans (Wood et al., 2015), we tested whether these drugs interact with monoamine transporters similarly to cocaine or amphetamines (Simmler et al., 2013, 2014a) and other novel psychoactive substances (Rickli et al., 2015a, 2015b; Simmler et al., 2014a; Simmler et al., 2014b). *N*-2-methoxybenzyl substitution enhanced monoamine transporter inhibition compared with the 2C drugs. However, the potency of even the most potent NBOMe drugs at the NET and SERT was low and only in the 5–10 μM range, indicating that amphetamine-type monoamine transporter interactions contribute only little to the cardiostimulant effects of NBOMe drugs.

In addition to their very high 5-HT_{2A} binding affinity, we found that the NBOMe drugs and LSD had high binding affinity for adrenergic α_{1A} receptors. 2C drugs have been shown to contract blood vessels (Saez et al., 1994) through direct interactions with serotonergic 5-HT_2 and adrenergic α_1 receptors (Lobos et al., 1992). The vasoconstrictive potency of 2C drugs does not appear to correlate well with hallucinogenic potency in humans (Saez et al., 1994) or 5-HT_{2A} receptor activation. For example, 2C-D had higher affinity for 5-HT_{2A} receptors compared with 2C-H in the present study but lower potency in contracting the rat aorta (Saez et al., 1994). Additionally, 2C-N, which exhibited high affinity for 5-HT_{2A} receptors but not α_1 receptors in the present study, did not present vasoconstrictive activity (Saez et al., 1994). These findings and the relatively high affinity of the NBOMe drugs for adrenergic α_1 receptors indicate that these receptors might contribute to the stimulant-type cardiovascular effects that are typically seen in cases of NBOMe drug intoxication (Srisuma et al., 2015; Wood et al., 2015). Additionally, the behavioral effects of 25I-NBOMe in mice showed a rapid peak (within minutes), whereas the response to 2C-I was relatively flat (Halberstadt and Geyer, 2014). Thus, such substance characteristics as the higher lipophilicity of NBOMe drugs may further accentuate the clinical drug response. As a result, there is likely a high risk of overdose with NBOMe drugs, and several fatalities have been reported (Hill et al., 2013; Srisuma et al., 2015; Walterscheid et al., 2014; Wood et al., 2015).

Both the 2C and NBOMe drugs bound to $TAAR_1$, with few exceptions. *N*-2-methoxybenzyl substitution slightly decreased $TAAR_1$ binding affinity as previously shown for other N-substitutions in phenethylamines (Lewin et al., 2008). $TAAR_1$ modulates psychotropic drug actions. Importantly, methylenedioxymethamphetamine inhibits its own stimulant effects via $TAAR_1$ activation (Di Cara et al., 2011). Whether similar $TAAR_1$-mediated "auto-inhibition" exists for hallucinogens remains to be determined. One hypothesis is that the lower $TAAR_1$ activity that is associated with *N*-2-methoxybenzyl substitution may also enhance psychostimulant drug properties *in vivo*.

LSD exhibited high affinity for D_1, D_2 and D_3 receptors, as previously shown (Watts et al., 1995) and in contrast to phenethylamines. D_2 receptors have been shown to contribute to the interoceptive effects of LSD in rats (Halberstadt and Geyer, 2013, 2014). Although *N*-2-methoxybenzyl substitution increased $D_{1\text{-}3}$ receptor binding affinity compared with 2C drugs, NBOMe drugs were less potent at $D_{1\text{-}3}$ receptors compared with LSD, indicating that LSD has a unique mixed dopaminergic-serotonergic binding profile.

In summary, NBOMe drugs are highly potent 5-HT_{2A} receptor ligands and partial 5-HT_{2A} receptor agonists, similar to the classic hallucinogen LSD, but with 5-HT_2 over 5-HT_1 receptor selectivity, unlike LSD. NBOMe drugs bind to adrenergic α_1 receptors and $TAAR_1$, similar to LSD, but do not bind to dopaminergic $D_{1\text{-}3}$ receptors, unlike LSD. The *in vitro* binding profiles of NBOMe drugs suggest that they have higher hallucinogenic effects and potency compared with their parent 2C drugs and are similar to the very potent hallucinogen LSD because of their similar or even higher potency at 5-HT_{2A} receptors. At higher doses, NBOMe drugs may also exhibit additional stimulant properties through α_1 receptor interactions.

Conflicts of interest

M.C.H. is an employee of F. Hoffmann-La Roche.

Authorship contributions

Participated in research design: Rickli, Liechti.
Conducted experiments: Rickli, Luethi, Reinisch, Buchy.
Performed data analysis: Rickli, Hoener, Liechti.
Wrote or contributed to the writing of the manuscript: Rickli, Liechti.

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3.2 Monoamine receptor interaction profiles of 4-thio-substituted phenethylamines (2C-T drugs)

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ABSTRACT

Background: 4-Thio-substituted phenethylamines (2C-T drugs) are potent psychedelics with poorly defined pharmacological properties. Because of their psychedelic effects, 2C-T drugs are sometimes sold as new psychoactive substances (NPSs). The aim of the present study was to characterize the monoamine receptor and transporter interaction profiles of a series of 2C-T drugs.
Methods: We determined the binding affinities of 2C-T drugs at monoamine receptors and transporters in human cells that were transfected with the respective receptors or transporters. We also investigated the functional activation of serotonergic 5-hydroxytryptamine 2A (5-HT_{2A}) and 5-HT_{2B} receptors, activation of human trace amine-associated receptor 1 ($TAAR_1$), and inhibition of monoamine uptake transporters.
Results: 2C-T drugs had high affinity for 5-HT_{2A} and 5-HT_{2C} receptors (1–54 nM and 40–350 nM, respectively). With activation potencies of 1–53 nM and 44–370 nM, the drugs were potent 5-HT_{2A} receptor and 5-HT_{2B} receptor, respectively, partial agonists. An exception to this were the benzylthiophenethylamines, which did not potently activate the 5-HT_{2B} receptor (EC_{50} > 3000 nM). Furthermore, the compounds bound to serotonergic 5-HT_{1A} and adrenergic receptors. The compounds had high affinity for the rat $TAAR_1$ (5–68 nM) and interacted with the mouse but not human $TAAR_1$. The 2C-T drugs did not potently interact with monoamine transporters (K_i > 4000 nM).
Conclusion: The receptor binding profile of 2C-T drugs predicts psychedelic effects that are mediated by potent 5-HT_2 receptor interactions.
This article is part of the Special Issue entitled 'Designer Drugs and Legal Highs.'

1. Introduction

Substituted phenethylamines are a class of drugs that includes several potent psychedelics that exert their effects through interactions with the serotonergic 5-hydroxytryptamine 2 (5-HT_2) receptor site (Glennon et al., 1982, 1984; Nelson et al., 1999; Nichols, 2004; Titeler et al., 1988). Many psychedelic phenethylamines were first synthesized by Alexander Shulgin during the 1970s and 1980s and were described in the book *PiHKAL: A Chemical Love Story* (Shulgin and Shulgin, 1995). 2C drugs are a subfamily of substituted phenethylamines, consisting of 2,5-dimethoxy-4-substituted phenethylamines. The term 2C refers to the two carbon atoms between the benzene ring and amino group (Shulgin and Shulgin, 1995). Originally proposed as psychotropic agents for psychotherapy (Shulgin and Shulgin, 1995; Shulgin and Carter, 1975), 2C drugs are now popular among recreational drug users because of their psychedelic and entactogenic properties (de Boer and Bosman, 2004;

Abbreviations: 4-bromo-2,5-dimethoxyphenethylamine, 2C-B; 2,5-dimethoxy-4-methylthiophenethylamine, 2C-T-1; 2,5-dimethoxy-4-(β-methallyl)thiophenethylamine, 2C-T-3; 2,5-dimethoxy-4-isopropylthiophenethylamine, 2C-T-4; 2,5-dimethoxy-4-propylthiophenethylamine, 2C-T-7; 2,5-dimethoxy-4-allylthiophenethylamine, 2C-T-16; 2,5-dimethoxy-4-*n*-butylthiophenethylamine, 2C-T-19; 2,5-dimethoxy-4-(2,2-difluoroethylthio)phenethylamine, 2C-T-21.5; 2,5-dimethoxy-4-(2,2,2-trifluoroethylthio)phenethylamine, 2C-T-22; 2,5-dimethoxy-4-isobutylthiophenethylamine, 2C-T-25; 2,5-dimethoxy-4-benzylthiophenethylamine, 2C-T-27; 2,5-dimethoxy-4-(3-fluoropropylthio)phenethylamine, 2C-T-28; 2,5-dimethoxy-4-(4-fluorobutylthio)phenethylamine, 2C-T-30; 2,5-dimethoxy-4-(4-trifluoromethylbenzylthio)phenethylamine, 2C-T-31; 2,5-dimethoxy-4-(3-methoxybenzylthio)phenethylamine, 2C-T-33; 5-hydroxytryptamine (serotonin), 5-HT; dopamine, DA; dopamine transporter, DAT; fluorescence imaging plate reader, FLIPR; high-performance liquid chromatography, HPLC; lysergic acid diethylamide, LSD; norepinephrine, NE; norepinephrine transporter, NET; new psychoactive substance, NPS; serotonin transporter, SERT; trace amine-associated receptor 1, $TAAR_1$.

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Gonzalez et al., 2015). Today, the Internet appears to be the main source for both acquiring information on and purchasing 2C drugs and other NPSs (Brandt et al., 2014; Orsolini et al., 2017; Schifano et al., 2005). Although classic 2C drugs are considered physiologically safe, several incidences, including sympathomimetic toxicity, psychosis, and death, have been documented (Bosak et al., 2013; Curtis et al., 2003; Huang and Bai, 2011; Miyajima et al., 2008; Stoller et al., 2017). Additionally, several 2C fatalities have been reported in the media (Dean et al., 2013). Moreover, newly emerged highly potent phenethylamine hallucinogens, including *N*-(2-methoxybenzyl)-2,5-dimethoxy-4-substituted ("NBOMe") phenethylamines, were found to be unexpectedly toxic and recently associated with several fatalities (Nichols, 2016; Nikolaou et al., 2015; Poklis et al., 2014; Rose et al., 2013; Suzuki et al., 2015). We previously reported the receptor and transporter interaction profiles of 2C drugs compared with their NBOMe analogs (Rickli et al., 2015). The sulfur-containing 2C drugs (2C-T-2, 2C-T-4, and 2C-T-7) that were included in the study proved to be potent agonists at 5-HT_2 receptors (Rickli et al., 2015). Several other compounds of the 2C-T series have been described (Shulgin and Shulgin, 1995; Trachsel, 2003), but little information is available regarding their interactions with monoamine receptors and transporters. On Internet drug discussion websites such as bluelight.org, the most commonly discussed 2C-T drugs are 2C-T-2, 2C-T-4, 2C-T-7, and 2C-T-21. Other compounds of the series are only sporadically mentioned and their use does currently not seem to be widespread. However, NPSs constantly emerge and it is possible that several other 2C-T compounds will appear on the drug market in the future.

In the present study, we determined and compared the monoamine receptor and transporter affinities of 14 compounds of the 2C-T series (Fig. 1). The numbering of the compounds of the 2C-T series describes the sequence of construction and has no structural relationship (Shulgin and Shulgin, 1995). 2C-T-3 was first named 2C-T-20; however, because its amphetamine analog 2,5-dimethoxy-4-(beta-methallylthio)amphetamine was originally named Aleph-3, 2C-T-20 was later renamed 2C-T-3 to maintain consistency between the 2C-T and Aleph series (Shulgin and Shulgin, 1995). The unusual number of 2C-T-21.5 is based on the fact that with its difluoroethylthio substitution, 2C-T-21.5 lies between the mono-fluorinated 2C-T-21 and tri-fluorinated 2C-T-22 (Shulgin and Shulgin, 1995).

2. Material and methods

2.1. Drugs

The 2C-T drugs were synthesized as hydrochlorides as described previously (Shulgin and Shulgin, 1995; Trachsel, 2003) and provided by ReseaChem GmbH. High-performance liquid chromatography (HPLC) purity was >98.5%. 4-Bromo-2,5-dimethoxyphenethylamine (2C-B) hydrochloride, D-methamphetamine hydrochloride, and lysergic acid diethylamide (LSD) were purchased from Lipomed (Arlesheim, Switzerland), with high-performance liquid chromatography (HPLC) purity > 98.5%.

2.2. 5-HT_{1A} and 5-HT_{2A} receptor radioligand binding assays

For membrane preparations, HEK 293 cells that were transiently transfected with the 5-HT_{1A} or 5-HT_{2A} receptor were released from the culture flasks using trypsin/ethylenediaminetetraacetic acid (EDTA), harvested, washed twice with ice-cold phosphate-buffered saline (PBS; without Ca^{2+} and Mg^{2+}), pelleted at 1000 rotations per minute (rpm) for 5 min at 4 °C, frozen, and stored at −80 °C. Frozen pellets were suspended in 20 ml HEPES-NaOH (20 mM, pH 7.4) that contained 10 mM EDTA and homogenized with a Polytron (PT 6000, Kinematica, Luzern, Switzerland) at 14,000 rpm for 20 s. The homogenates were centrifuged at 48,000 × g for 30 min at 4 °C. Subsequently, the supernatants were removed and discarded, and the pellets were resuspended in 20 ml HEPES-NaOH (20 mM, pH 7.4) that contained 0.1 mM EDTA using the Polytron (20 s at 14,000 rpm). This procedure was repeated, and the final pellets were resuspended in HEPES-NaOH that contained 0.1 mM EDTA and homogenized using the Polytron. Typically, aliquots of 2 ml membrane portions were stored at −80 °C. With each new membrane batch, the dissociation constant (K_d) was determined by a saturation curve.

For the competitive binding assays, [^{3}H]-8-OH-DPAT and [^{3}H]-ketanserin were used as 5-HT_{1A} and 5-HT_{2A} receptor radioligands, respectively, at concentrations equal or close to the K_d values. Specific binding of the radioligands to the target receptors was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 μM pindolol (for the 5-HT_{1A} receptor radioligand) or 10 μM spiperone (for the 5-HT_{2A} receptor radioligand). The compounds were tested at a broad range of concentrations (30 pM–30 μM) in duplicate. The test compounds were diluted in binding assay buffer at pH 7.4 (50 mM Tris/HCl, 10 mM $MgCl_2$, and 1 mM EGTA), and dilution curves were constructed in assay microplates (Greiner, 96-well, U-bottom, PS). Radioligand (50 μl) and the membrane suspension (100 μl) were added to the assay plates to a final volume of 200 μl in each well and incubated and shaken for 30 min at room temperature. Incubations were terminated by rapid filtration through Unifilter-96 plates (Packard Instrument Company, PerkinElmer, Schwerzenbach, Switzerland) and GF/C glass filters (PerkinElmer) that were presoaked for a minimum of 1 h in 0.3% polyethylenimine and washed three times with ice-cold washing buffer (50 mM Tris/HCl, pH 7.4). After the addition of Microscint 40 (45 μl/well, PerkinElmer), the Unifilter-96 plates were sealed. After 1 h, radioactivity was counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC_{50} values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. K_i values are presented as means ± SD (in μM).

2.3. 5-HT_{2C} receptor radioligand binding assay

For membrane preparations, HEK 293 cells that were transiently transfected with the 5-HT_{2C} receptor were released from the culture flasks using trypsin/EDTA, harvested, washed twice with ice-cold PBS (without Ca^{2+} and Mg^{2+}), pelleted at 1000 rpm for 5 min at 4 °C, frozen, and stored at −80 °C. Frozen pellets were suspended in 20 ml HEPES/NaOH (20 mM, pH 7.4) that contained 10 mM EDTA and homogenized with a Polytron (PT 6000, Kinematica) at 14,000 rpm for 20 s. The homogenates were centrifuged at 48,000 × g for 30 min at 4 °C. Subsequently, the supernatants were removed and discarded, and the pellets were resuspended in 20 ml HEPES-NaOH (20 mM, pH 7.4) that contained 0.1 mM EDTA using the Polytron (20 s at 14,000 rpm). This procedure was repeated, and the final pellets were resuspended in HEPES/NaOH that contained 0.1 mM EDTA and homogenized using the Polytron. Typically, 2 ml aliquots of membrane portions were stored at −80 °C. With each new membrane batch, the dissociation constant (K_d) was determined by a saturation curve.

For the competitive binding assay, [^{3}H]-mesulergine was used as the 5-HT_{2C} receptor radioligand at a concentration equal or close to the K_d value. Specific binding of the radioligand to the target

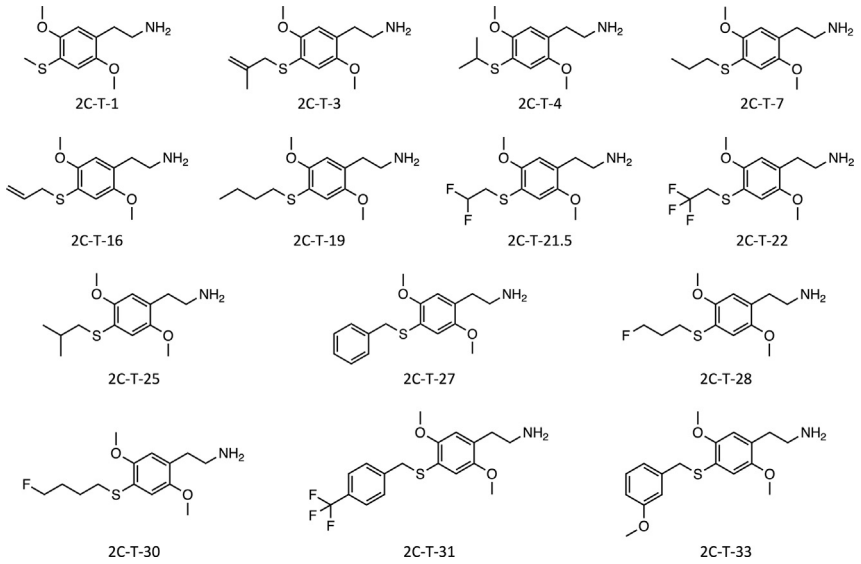

Fig. 1. Structures of 4-thio-substituted phenethylamines (2C-T drugs).

receptor was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 μM mianserin. The compounds were tested at a broad range of concentrations (30 pM–30 μM) in duplicate. The test compounds were diluted in binding assay buffer at pH 7.4 (50 mM Tris/HCl, 10 mM $MgCl_2$, 1 mM EGTA, and 10 μM pargyline), and dilution curves were constructed in 96-well white polystyrene assay plates (Sigma-Aldrich, Buchs, Switzerland). Membrane stocks were thawed and resuspended to a concentration of approximately 0.04 mg protein/ml binding assay buffer using a Polytron tissue homogenizer. The membrane homogenate (40 μg/ml) was then lightly mixed for 5–30 min with YSi-poly-L-lysine (PerkinElmer) at 0.5 mg beads/well. The membrane/bead mixture (50 μl) was added to each well of the assay plate that contained the radioligand (50 μl) and the test compounds (final volume in each well, 200 μl) to start the assay. The assay plates were sealed, incubated for 2 h at room temperature with agitation, and then counted in the PVT SPA counting mode of a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC_{50} values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. K_i values are presented as means ± SD (in μM).

2.4. Rat and mouse $TAAR_1$ receptor radioligand binding assays

HEK 293 cells that stably expressed rat or mouse $TAAR_1$ were used as described previously (Revel et al., 2011). All of the cell lines were maintained at 37 °C and 5% CO_2 in high-glucose Dulbecco's modified Eagle's medium (DMEM) that contained 10% fetal calf serum (heat-inactivated for 30 min at 56 °C), 1% penicillin/streptomycin, and 375 μg/ml Geneticin (Gibco, Zug, Switzerland). For membrane preparation, the cells were released from the culture flasks using trypsin/EDTA, harvested, washed twice with ice-cold PBS (without Ca^{2+} and Mg^{2+}), pelleted at 1000 × *g* for 5 min at 4 °C, frozen, and stored at −80 °C. Frozen pellets were suspended in buffer A (20 ml HEPES-NaOH [20 mM, pH 7.4] that contained 10 mM EDTA) and homogenized with a Polytron (PT 6000; Kinematica) at 14,000 rpm for 20 s. The homogenate was centrifuged for 30 min at 48,000 × g at 4 °C. The supernatant was removed and discarded, and the pellet was resuspended in buffer A using the Polytron (20 s at 14,000 rpm). The centrifugation and removal of the supernatant was repeated, and the final pellet was resuspended in buffer A and homogenized using the Polytron. Typically, 2-ml aliquots of membrane portions were stored at −80 °C. With each new membrane batch, the dissociation constant (K_d) was determined by a saturation curve.

For the competitive binding assays, the $TAAR_1$ agonist [^{3}H]-RO5166017 was used as a $TAAR_1$ radioligand at a concentration equal or close to the K_d values, which were usually around 0.7 nM (mouse $TAAR_1$) and 2.3 nM (rat $TAAR_1$). Nonspecific binding was defined as the amount of radioligand that bound in the presence of 10 μM RO5166017. Compounds were tested at a broad range of concentrations (30 pM–30 μM) in duplicate. Compounds (20 μl/well) were transferred to a 96-deep-well plate (TreffLab, Degersheim, Switzerland), and 180 μl of binding buffer (20 mM HEPES-NaOH, 10 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 7.4), 300 μl of radioligand, and 500 μl of membranes (resuspended at 60 μg protein/ml) were added. The plates were incubated at 4 °C for 90 min. Incubations were terminated by rapid filtration through Unifilter-96 plates (Packard Instrument Company) and GF/C glass filters (PerkinElmer) that were presoaked for 1 h in 0.3% polyethylenimine and washed three times with 1 ml of cold binding buffer. After the addition of Microscint 40 (45 μl/well, PerkinElmer), the Unifilter-96 plate was sealed. After 1 h, radioactivity was counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC_{50} values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation. K_i values are presented as means ± SD (in μM).

2.5. Adrenergic α_{1A} and α_{2A} receptor radioligand binding assays

CHO cells were stably transfected with the adrenergic α_{1A} receptor and maintained at 37 °C and 5% CO_2 in HAM's F12 medium (Invitrogen) that contained 10% fetal calf serum (heat inactivated for 30 min at 56 °C), 150 μg/ml Geneticin (Gibco, Zug, Switzerland),

and 1% penicillin/streptomycin. CHL cells that stably expressed the adrenergic α_{2A} receptor were maintained at 37 °C and 5% CO_2 in high-glucose DMEM that contained 5% fetal calf serum (heat inactivated for 30 min at 56 °C) and 250 μg/ml Geneticin (Gibco, Zug, Switzerland).

For membrane preparations, the cells were released from the culture flasks using trypsin/EDTA, harvested, washed twice with ice-cold PBS (without Ca^{2+} and Mg^{2+}), pelleted at 1000 rpm for 5 min at 4 °C, frozen, and stored at −80 °C. Frozen pellets were suspended in 20 ml HEPES-NaOH (20 mM, pH 7.4) that contained 10 mM EDTA and homogenized with a Polytron (PT 6000, Kinematica) at 14,000 rpm for 20 s. The homogenates were centrifuged at 48,000 × *g* for 30 min at 4 °C. Subsequently, the supernatants were removed and discarded, and the pellets were resuspended in 20 ml of HEPES-NaOH (20 mM, pH 7.4) that contained 0.1 mM EDTA using the Polytron (20 s at 14,000 rpm). This procedure was repeated, and the final pellets were resuspended in HEPES-NaOH that contained 0.1 mM EDTA and homogenized using the Polytron. Typically, 2 ml aliquots of membrane portions were stored at −80 °C. With each new membrane batch, the dissociation constant (K_d) was determined by a saturation curve.

For the competitive binding assays, [^{3}H]-prazosin and [^{3}H]-rauwolscine were used as adrenergic α_{1A} and adrenergic α_{2A} receptor radioligands, respectively, at concentrations equal or close to the K_d values. Specific binding of the radioligands to the target receptors was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 μM chlorpromazine (for the α_{1A} receptor radioligand) and 10 μM phentolamine (for the α_{2A} receptor radioligand). The compounds were tested at a broad range of concentrations (30 pM–30 μM) in duplicate. The test compounds were diluted in binding assay buffer (50 mM Tris/HCl, pH 7.4), and dilution curves were constructed in assay microplates (Greiner, 96 well, U-bottom, PS). Radioligand 50 μl) and the membrane suspension (100 μl) were added to the assay plates and incubated and shaken for 1 h at room temperature. Incubations were terminated by rapid filtration through Unifilter-96 plates (Packard Instrument Company) and GF/C glass filters (PerkinElmer) that were presoaked for 1 h in 0.3% polyethylenimine and washed three times with 1 ml cold binding assay buffer. After the addition of Microscint 40 (45 μl/well, PerkinElmer), the Unifilter-96 plates were sealed. After 1 h, radioactivity was counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC_{50} values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. K_i values are presented as means ± SD (in μM).

2.6. D_2 receptor radioligand binding assay

For membrane preparations, HEK 293 cells that were transiently transfected with the dopamine D_2 receptor were released from the culture flasks using trypsin/EDTA, harvested, washed twice with ice-cold PBS (without Ca^{2+} and Mg^{2+}), pelleted at 1000 rpm for 5 min at 4 °C, frozen, and stored at −80 °C. Frozen pellets were suspended in 20 ml of HEPES-NaOH (20 mM, pH 7.4) that contained 10 mM EDTA and homogenized with a Polytron (PT 6000, Kinematica) at 14,000 rpm for 20 s. The homogenates were centrifuged at 48,000 × *g* for 30 min at 4 °C. Subsequently, the supernatants were removed and discarded, and the pellets were resuspended in 20 ml of HEPES-NaOH (20 mM, pH 7.4) that contained 0.1 mM EDTA using the Polytron (20 s at 14,000 rpm). This procedure was repeated, and the final pellets were resuspended in HEPES-NaOH that contained 0.1 mM EDTA and homogenized using the Polytron. Typically, 2 ml aliquots of membrane portions were stored at −80 °C. With each new membrane batch, the dissociation constant (K_d) was determined by a saturation curve.

For the competitive binding assays, [^{3}H]-spiperone was used as the dopamine D_2 receptor radioligand at a concentration equal or close to the K_d value. Specific binding of the radioligand to the target receptor was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 μM spiperone. The compounds were tested at a broad range of concentrations (30 pM–30 μM) in duplicate. The test compounds were diluted in binding assay buffer at pH 7.4 (50 mM Tris/HCl, 5 mM $MgCl_2$, 1 mM EDTA, 5 mM KCl, 1.5 mM $CaCl_2$, and 120 mM NaCl), and dilution curves were constructed in assay microplates (Greiner, 96 well, U-bottom, PS). Radioligand (50 μl) and the membrane suspension (100 μl) were added to the assay plates (final volume in each well, 200 μl) and incubated and shaken for 1 h at room temperature. Incubations were terminated by rapid filtration through Unifilter-96 plates (Packard Instrument Company) and GF/C glass filters (PerkinElmer) that were presoaked for a minimum of 1 h in 0.3% polyethylenimine and washed three times with ice-cold washing buffer (50 mM Tris/HCl, pH 7.4). After the addition of Microscint 40 (45 μl/well, PerkinElmer), the Unifilter-96 plates were sealed. After 1 h, radioactivity was counted using a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC_{50} values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. K_i values are presented as means ± SD (in μM).

2.7. Monoamine transporter radioligand binding assays

HEK 293 cells that stably expressed the human norepinephrine transporter (hNET), human serotonin transporter (hSERT), or human dopamine transporter (hDAT) were cultured, and cells were collected and washed three times with ice-cold PBS, pelleted at 1000 rpm for 5 min at 4 °C, frozen, and stored at −80 °C. Frozen pellets were then resuspended in 400 ml of HEPES-NaOH (20 mM, pH 7.4) that contained EDTA (10 mM) at 4 °C. After homogenization with a Polytron (PT 6000, Kinematics) at 10,000 rpm for 15 s, the homogenates were centrifuged at 48,000 × *g* for 30 min at 4 °C. Aliquots of the membrane stocks were frozen at −80 °C.

For the competitive binding assays, *N*-methyl-[^{3}H]-nisoxetine, [^{3}H]-citalopram, and [^{3}H]-WIN35,428 were used as hNET, hSERT, and hDAT radioligands, respectively, at concentrations equal or close to the K_d values. Specific binding of the radioligand to the target transporters was defined as the difference between total binding (binding buffer alone) and nonspecific binding that was determined in the presence of 10 μM indatraline. The compounds were tested at a broad range of concentrations (30 pM–30 μM) in duplicate. The test compounds were diluted in binding assay buffer at pH 7.4 (126 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4), and dilution curves were constructed in 96-well OptiPlates (PerkinElmer). Membrane stocks were thawed and resuspended to a concentration of approximately 60 μg protein/ml binding assay buffer using a Polytron tissue homogenizer. The membrane homogenates were then lightly mixed for 5–30 min with polyvinyl toluene (PCT) wheatgerm agglutinin-coated scintillation proximity assay beads (WGA-SPA, Amersham Biosciences) at 11.5 mg beads/well. The membrane/bead mixture (50 μl) was

added to each well of the assay plate that contained the radioligand (50 μl) and the test compounds (final volume in each well, 200 μl) to start the assay. The assay plates were sealed, incubated for 2 h at room temperature with agitation, and counted in the PVT SPA counting mode of a TopCount Microplate Scintillation Counter (Packard Instrument Company). IC_{50} values were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves, run in duplicate, for each compound. K_i (affinity) values, which correspond to the dissociation constants, were determined using the Cheng-Prusoff equation: $K_i = IC_{50} / (1 + \text{radioligand concentration} / K_d)$. K_i values are presented as means ± SD (in μM).

2.8. Functional activity at the serotonin 5-HT_{2A} receptor

Mouse embryonic fibroblasts (NIH-3T3 cells) that expressed the human 5-HT_{2A} receptor were incubated in HEPES-Hank's Balanced Salt Solution (HBSS) buffer (Gibco, Zug, Switzerland; 70,000 cells/100 μl) for 1 h at 37 °C in 96-well poly-D-lysine-coated plates. To each well, 100 μl of dye solution (fluorescence imaging plate reader [FLIPR] calcium 5 assay kit; Molecular Devices, Sunnyvale, CA, USA) was added, and the plates were incubated for 1 h at 37 °C. The plates were placed in a FLIPR, and 25 μl of the test drugs that were diluted in HEPES-HBSS buffer that contained 250 mM probenicid was added online. The increase in fluorescence was then measured, and EC_{50} values were derived from the concentration-response curves using nonlinear regression. The maximal receptor activity (efficacy) is expressed relative to 5-HT activity, which was set to 100%.

2.9. Functional activity at the serotonin 5-HT_{2B} receptor

HEK 293 cells that expressed the human 5-HT_{2B} receptor were incubated in growth medium (high-glucose DMEM; Invitrogen, Zug, Switzerland), 10 ml/L penicillin/streptomycin (Gibco, Zug, Switzerland), 10% fetal calf serum (non-dialyzed, heat-inactivated), and 250 mg/L Geneticin at a density of 50,000 cells/well at 37 °C in poly-D-lysine-coated 96-well plates overnight. The growth medium was then removed by snap inversion, and 100 μl of the calcium indicator Fluo-4 solution (Molecular Probes, Eugene, OR, USA) was added to each well. The plates were incubated for 45 min at 31 °C before the Fluo-4 solution was removed by snap inversion, and 100 μl of Fluo-4 solution was added a second time for 45 min at 31 °C. The cells were washed with HBSS and 20 mM HEPES (assay buffer) immediately before testing using an EMBLA cell washer, and 100 μl assay buffer was added. The plates were placed in a FLIPR, and 25 μl of the test substances that were diluted in assay buffer was added online. The increase in fluorescence was then measured, and EC_{50} values were derived from the concentration-response curves using nonlinear regression. The maximal receptor activity (efficacy) is expressed relative to 5-HT activity, which was set to 100%.

2.10. Functional activity at the human $TAAR_1$ receptor

Recombinant HEK 293 cells that expressed human $TAAR_1$ were grown at 37 °C and 5% CO_2/95% air in 250 ml Falcon culture flasks in 30 ml of culture medium. The cell culture medium contained high-glucose DMEM, 10% fetal calf serum (heat inactivated for 30 min at 56 °C), 500 μg/ml Geneticin (Gibco), and 500 μg/ml hygromycin B. Cells were harvested when 80–90% confluence was reached. The culture medium was removed from the culture flasks, and the cells were washed once with 5 ml of PBS. After removing the wash solution, 5 ml of trypsin/EDTA solution was added for 5 min at 37 °C. Afterward, 45 ml of culture medium was added to the 5 ml detached cell solution, and 50 ml was transferred to a Falcon tube. The tube was centrifuged at 900 rpm for 3 min at room temperature, and the supernatant was removed. The cell pellet was resuspended in fresh culture medium and brought to 5×10^5 cells per ml. The cells were then plated into 96-well plates (BIOCOAT 6640, Becton Dickinson, Allschwil, Switzerland) with a multipipette (100 μl/well, 80,000 cells/well) and incubated for 20 h at 37 °C. For the cAMP assay, the cell culture medium was removed, and 50 μl of PBS (without Ca^{2+} and Mg^{2+}) was added. Afterward, PBS was removed by snap inversion followed by gently tapping of the plate on a tissue. Krebs-Ringer bicarbonate buffer (90 μl; Sigma-Aldrich) that contained 1 mM IBMX was added, and the plates were incubated for 60 min at 37 °C and 5% CO_2/95% air. All of the compounds were tested at a broad range of concentrations (300 pM–30 μM) in duplicate, and a standard curve (0.13 nM–10 μM cAMP) was constructed on each plate. A reference plate that included RO5256390, β-phenylethylamine, and p-tyramine also accompanied each experiment. Typically, 30 μl of a compound solution, 30 μl of β-phenylethylamine (as maximal response), or a basal control in PBS that contained 1 mM IBMX was then added, and the cells were incubated for 40 min at 37 °C. Afterward, the cells were lysed with 50 μl of 3× detection mix solution that contained Ru-cAMP Alexa700 anti-cAMP antibody and lysis buffer for 120 min at room temperature under strong shaking using black lids. Fluorescence was measured using a NanoScan (IOM reader; 456 nm excitation wavelength; 630 and 700 nm emission wavelengths). The FRET signal was calculated as the following: FRET (700 nM) − P × FRET (630 nM), where P = Ru (700 nM) / Ru (630 nM).

2.11. Monoamine uptake transporter inhibition

The monoamine transporter inhibition potential of the 2C-T drugs was assessed for a single high concentration of 10 μM to exclude activity. Monoamine uptake was determined in HEK 293 cells that stably expressed the hNET, hDAT, and hSERT (Tatsumi et al., 1997) as previously described in detail (Hysek et al., 2012). Briefly, cells were cultured to 70–90% confluence in DMEM (10% fetal calf serum and 250 μg/ml Geneticin, both from Gibco), detached, and resuspended in Krebs-Ringer bicarbonate buffer (Sigma-Aldrich) at a density of 3×10^6 cells/ml. For [^{3}H]-DA uptake experiments, the uptake buffer was supplemented with 0.2 mg/ml ascorbic acid. The cell suspension (100 μl) was incubated with 25 μl of the test drugs, vehicle control, and transporter-specific inhibitors (10 μM nisoxetine for NET, 10 μM mazindol for DAT, and 10 μM fluoxetine for SERT) dissolved in buffer for 10 min in a round-bottom 96-well plate at room temperature at 450 rpm on a rotary shaker. Monoamine uptake transport was then initiated by adding 50 μl of [^{3}H]-NE (13.1 Ci/mmol; PerkinElmer), [^{3}H]-DA (30.0 Ci/mmol, PerkinElmer), or [^{3}H]-5-HT (80.0 Ci/mmol; Anawa, Zürich, Switzerland) dissolved in buffer at a final concentration of 5 nM for an additional 10 min. The cell suspension (100 μl) was then transferred to 500 μl microcentrifuge tubes that contained 50 μl of 3 M KOH and 200 μl silicon oil (1:1 mixture of silicon oil types AR 20 and AR 200; Sigma-Aldrich). To separate the cells from the uptake buffer, they were centrifuged through silicone oil for 3 min at 16,550 × *g*, and the tubes were frozen in liquid nitrogen immediately afterward. The cell pellet was then cut into 6 ml scintillation vials (PerkinElmer) that contained 0.5 ml lysis buffer (0.05 M Tris-HCl, 50 mM NaCl, 5 mM EDTA, and 1% NP-40 in water), and the samples were shaken for 1 h before 5 ml of scintillation fluid (Ultimagold, PerkinElmer) was added. Monoamine uptake was then quantified by liquid scintillation counting on a Packard 1900 TR Tri-Carb Liquid Scintillation Counter (Packard Instrument Company). Nonspecific uptake that was determined in the presence of selective inhibitors was subtracted from the total counts, and

monoamine uptake was compared with the vehicle control.

2.12. Statistical analysis

IC_{50} values of radioligand binding were determined by calculating nonlinear regression curves for a one-site model using at least three independent 10-point concentration-response curves for each substance. K_i (affinity) values, which correspond to the dissociation constants, were calculated using the Cheng-Prusoff equation. Nonlinear regression concentration-response curves were used to determine EC_{50} values for 5-HT_{2A} and 5-HT_{2B} receptor activation. Efficacy (maximal activity) is expressed relative to the activity of 5-HT, which was used as a control set to 100%. Monoamine uptake of three independent experiments was compared with vehicle controls using analysis of variance followed by Dunnett's multiple-comparison test.

3. Results

3.1. Interactions with serotonin receptors

The binding affinities and activation potency of 2C-T drugs to serotonin receptors are listed in Table 1. All of the drugs bound to the 5-HT_{1A} receptor in the range of 660-2370 nM and bound to the 5-HT_{2A} receptor in the range of 1–54 nM, with an activation potency of 1–53 nM and activation efficacy of 3–75%. All of the drugs except 2C-T-27 and 2C-T-33 activated the 5-HT_{2B} receptor, with an activation potency of 44-3310 nM and activation efficacy of 28–75%. All of the drugs bound to the 5-HT_{2C} receptor with affinities of 40–350 nM. 2C-B and LSD were included in Table 1 as reference psychedelics for comparison.

3.2. Binding to monoamine receptors and transporters

Binding affinities of 2C-T drugs to monoamine transporters and receptors are listed in Table 2. None of the drugs bound to the tested binding region of the NET at the investigated concentration range. Only 2C-T-27, 2C-T-31, and 2C-T-33 bound to the DAT, with affinities of 4.8–7.7 μM. Only 2C-T-3 bound to the SERT, with a K_i of 7.2 μM. No affinity for adrenergic α_{1A} or dopamine D_2 receptors was detected *in vitro*, with the exception of 2C-T-28, 2C-T-30, 2C-T-31, and 2C-T-33, which bound to the α_{1A} receptor with a K_i of 2.3–3.6 μM. However, the drugs bound to the α_{2A} receptor in the range of 97–804 nM. Furthermore, the drugs bound to rat and mouse $TAAR_1$, with affinities of 5–68 nM and 55-2340 nM, respectively, but did not activate the human $TAAR_1$ at the investigated concentration range. As reference, the $TAAR_1$ interactions of the partial agonist (Simmler et al., 2016) D-methamphetamine are listed in Table 2.

3.3. Monoamine uptake transporter inhibition

No significant NE, DA, or 5-HT uptake inhibition was observed for any of the 2C-T drugs at 10 μM (data not shown).

4. Discussion

The compounds had 17- to 830-fold higher affinity for the 5-HT_{2A} *vs.* 5-HT_{1A} receptor and 4- to 44-fold higher affinity for the 5-HT_{2A} *vs.* 5-HT_{2C} receptor. Similarly, selectivity for the 5-HT_2 receptor site has been shown for 2C drugs, NBOMe derivatives, and phenylisopropylamine hallucinogens (substituted amphetamines) but not for other psychedelics of the tryptamine class or LSD (Fantegrossi et al., 2005; Halberstadt and Geyer, 2011; Pierce and Peroutka, 1989; Rickli et al., 2015, 2016; Titeler et al., 1988). In accordance with those findings, in our study 2C-B had selectivity ratios in the range of the 2C-T drugs and LSD was less selective with 5-HT_{2A} *vs.* 5-HT_{1A} and 5-HT_{2A} *vs.* 5-HT_{2C} ratios of 0.28 and 2.6, respectively.

The three benzylthiophenethylamines (2C-T-27, 2C-T-31, and 2C-T-33) most potently bound to the 5-HT_{2A} receptor, with affinities of 1.6–3.8 nM. However, they had the lowest receptor activation potential for the 5-HT_{2A} receptor (26–53 nM) and negligible or no activation potential for the 5-HT_{2B} receptor. These three benzylthiophenethylamines also most potently bound to the 5-HT_{2A} receptor in a previous test series of 11 2C-T drugs using [^{3}H]-LSD as the radioligand (Trachsel et al., 2013). High affinity and antagonistic properties could be expected because of the bulky and lipophilic 4-substituent (Hansen et al., 2014; Nichols et al., 1977; Seggel et al., 1990). The remaining compounds all activated the 5-HT_{2A} and 5-HT_{2B} receptors in the range of 1–15 nM and 44–370 nM, respectively, with an activation efficacy of 36–75% and 28–75%, respectively. Drugs of the 2C-T series can therefore be classified as partial agonists as previously shown for other 2C drugs (Moya et al., 2007; Rickli et al., 2015) and similar psychedelic effects may be expected. Several fluorine-containing compounds were investigated. The 5-HT_{2A} and 5-HT_{2C} receptor affinity of 2C-T-7 was higher (3.2- and 2.5-fold, respectively) compared with its monofluorinated analog 2C-T-28. Compared with its monofluorinated analog 2C-T-30, 5-HT_2 binding affinity also increased for 2C-T-19 (1.4- and 1.6-fold increase in affinity for 5-HT_{2A} and 5-HT_{2C} receptors, respectively). A decrease in affinity by fluorine has previously been described for 2C-T-30 in radioligand binding assays using [^{3}H]-LSD-labeled cloned 5-HT_{2A} receptors (Trachsel, 2012). However, although 2C-T-7 had a higher activation potential for 5-HT_{2A} and 5-HT_{2B} receptors compared with 2C-T-28, the activation potential of 2C-T-19 for both receptors was lower compared with 2C-T-30. The difluoroethyl derivative 2C-T-21.5 and trifluoroethyl derivative 2C-T-22 had comparable affinities for 5-HT_{2A} and 5-HT_{2C} receptors. However, 2C-T-21.5 had a higher activation potential for the 5-HT_{2A} receptor, whereas 2C-T-22 had a higher activation potential for the 5-HT_{2B} receptor. In a previous study (Rickli et al., 2015), 2C-T-2, the non-fluorinated analog of 2C-T-21.5 and 2C-T-22, had slightly higher affinity for 5-HT_{2A} and 5-HT_{2C} receptors (9 and 67 μM, respectively) compared with 2C-T-21.5 and 2C-T-22. In the present study, however, the 5-HT_{2A} and 5-HT_{2B} receptor activation potency of 2C-T-21.5 and 2C-T-22, respectively, was higher than the previously determined receptor activation potency for 2C-T-2 (Rickli et al., 2015). Therefore, although terminal fluorinating appears to decrease 5-HT receptor affinity, it may increase activation potential in some cases.

In the radioligand binding assay, none of the substances bound to the investigated NET binding site, and none of the compounds significantly inhibited NE uptake at 10 μM. Only 2C-T-27, 2C-T-31, and 2C-T-33 bound to the DAT, with affinities of 4.8–7.7 μM. Only 2C-T-3 bound to the SERT, with an affinity of 7.2 μM. However, no DA or 5-HT uptake inhibition was observed for any of the compounds at 10 μM. Transporter binding and inhibition may occur at higher concentrations, which has been shown previously for 2C-T-2, 2C-T-4, and 2C-T-7 (Rickli et al., 2015). However, given the potent interactions with serotonergic receptors, the monoamine transporter interactions of 2C-T drugs are likely not clinically relevant and were not investigated at higher concentrations in the present study. No interactions with dopaminergic D_2 or adrenergic α_{1A} receptors were observed in the radioligand binding assays, with the exception of low-affinity binding of 2C-T-28, 2C-T-30, 2C-T-31, and 2C-T-33 to the α_{1A} receptor. However, all of the drugs bound to the α_{2A} receptor, with an affinity of 97–804 nM. The selectivity for α_{2A} over α_{1A} receptors has previously been reported for 2C drugs but not NBOMe compounds, which bound to both receptors with

Table 1
Serotonin receptor binding affinities and activation potencies.

	5-HT_{1A}	5-HT_{2A}			5-HT_{2B}		5-HT_{2C}	Selectivity (binding ratios)	
	Receptor binding	Receptor binding	Activation potency	Activation efficacy	Activation potency	Activation efficacy	Receptor binding	5-HT_{2A}/5-HT_{1A}	5-HT_{2A}/5-HT_{2C}
	K_i ± SD [nM]	K_i ± SD [nM]	EC_{50} ± SD [nM]	max ± SD [%]	EC_{50} ± SD [nM]	max ± SD [%]	K_i ± SD [nM]		
2C-T-1	1035 ± 125	49 ± 21	2.0 ± 0.1	75 ± 3	57 ± 38	58 ± 11	347 ± 129	21	7.1
2C-T-3	812 ± 161	11 ± 5	7.7 ± 1.6	44 ± 6	44 ± 8	28 ± 7	40 ± 6	74	3.6
2C-T-4	916 ± 86	54 ± 21	5.5 ± 0.7	56 ± 5	63 ± 11	75 ± 10	295 ± 28	17	5.5
2C-T-7	878 ± 97	5.3 ± 0.6	1.2 ± 0.4	49 ± 12	52 ± 10	46 ± 12	54 ± 25	166	10
2C-T-16	660 ± 74	9.2 ± 3.6	1.3 ± 0.6	57 ± 9	47 ± 32	36 ± 1	67 ± 15	72	7.3
2C-T-19	1019 ± 129	6.9 ± 3.3	12 ± 2	55 ± 6	369 ± 188	40 ± 3	101 ± 25	148	15
2C-T-21.5	1321 ± 193	14 ± 3	4.6 ± 1.4	66 ± 7	182 ± 12	40 ± 4	159 ± 77	94	11
2C-T-22	1915 ± 53	16 ± 1	15 ± 5	36 ± 2	110 ± 55	35 ± 15	151 ± 45	120	9.4
2C-T-25	1036 ± 134	21 ± 7	12 ± 2	49 ± 7	108 ± 35	32 ± 11	80 ± 32	49	3.8
2C-T-27	1166 ± 147	1.6 ± 0.5	26 ± 2	27 ± 4	>10,000		52 ± 12	729	33
2C-T-28	1904 ± 42	17 ± 6	5.7 ± 0.3	45 ± 7	81 ± 23	34 ± 16	135 ± 38	112	7.9
2C-T-30	2368 ± 22	9.5 ± 2.0	5.7 ± 2.4	40 ± 1	51 ± 34	61 ± 10	158 ± 45	249	17
2C-T-31	1063 ± 51	3.8 ± 1.1	53 ± 12	2.8 ± 0.7	3309 ± 1084	44 ± 13	157 ± 8	280	41
2C-T-33	1411 ± 38	1.7 ± 1.3	26 ± 8	40 ± 1	>10,000		75 ± 6	830	44
2C-B	311 ± 46	6.9 ± 1.8	2.1 ± 0.8	92 ± 8	75 ± 14	52 ± 26	43 ± 4	45	6.2
LSD	1.5 ± 0.4	5.3 ± 3.4	44 ± 14	73 ± 2	>10,000		14 ± 3	0.28	2.6

*K*i and EC_{50} values are given as nM (mean ± SD); activation efficacy (E_{max}) is given as percentage of maximum ± SD.

Table 2
Monoamine receptor and transporter binding affinities.

	human $TAAR_1$	rat $TAAR_1$	mouse $TAAR_1$	α_{1A}	α_{2A}	D_2	NET	DAT	SERT
	EC_{50} [nM]	K_i [nM]	K_i [nM]	K_i [nM]	K_i [nM]	K_i [nM]	K_i [nM]	K_i [nM]	K_i [nM]
2C-T-1	>30,000	52 ± 8	1877 ± 661	>6510	334 ± 43	>4400	>9710	>8710	>8580
2C-T-3	>30,000	8.0 ± 1.3	465 ± 236	>6510	97 ± 12	>4400	>9710	>8710	7221 ± 470
2C-T-4	>30,000	19 ± 5	2337 ± 911	>6510	217 ± 36	>4400	>9710	>8710	>8580
2C-T-7	>30,000	10 ± 0	311 ± 61	>6510	335 ± 49	>4400	>9710	>8710	>8580
2C-T-16	>30,000	17 ± 7	453 ± 133	>6510	229 ± 17	>4400	>9710	>8710	>8580
2C-T-19	>30,000	4.8 ± 1.5	96 ± 33	>6510	458 ± 45	>4400	>9710	>8710	>8580
2C-T-21.5	>30,000	68 ± 11	1674 ± 185	>6510	383 ± 26	>4400	>9720	>8710	>7510
2C-T-22	>30,000	38 ± 15	974 ± 17	>6510	592 ± 39	>4400	>9720	>8710	>7510
2C-T-25	>30,000	11 ± 1	359 ± 95	>6510	279 ± 6	>6270	>9720	>8710	>7510
2C-T-27	>30,000	10 ± 2	596 ± 197	>6510	351 ± 43	>6270	>9720	4760 ± 569	>7510
2C-T-28	>30,000	62 ± 19	426 ± 116	2730 ± 653	331 ± 31	>6270	>9720	>8710	>7510
2C-T-30	>30,000	29 ± 3	182 ± 52	2297 ± 134	408 ± 42	>6270	>9720	>8710	>7510
2C-T-31	>30,000	5.2 ± 0.6	55 ± 9	2534 ± 88	804 ± 126	>6270	>9720	5474 ± 54	>7510
2C-T-33	>30,000	38 ± 0	761 ± 117	3628 ± 308	343 ± 72	>6270	>9720	7706 ± 249	>7510
d-Methamphetamine	3707 ± 587	257 ± 15	889 ± 49						

K_i and EC_{50} values are given as nM (mean ± SD).

submicromolar affinity (Rickli et al., 2015). All of the drugs potently bound to the rat $TAAR_1$ in the range of 5–68 nM and less potently to the mouse $TAAR_1$ in the range of 55-2340 nM. However, the compounds were inactive at the human $TAAR_1$ in the functional assays (EC_{50} > 30 μM). Consistent with our results, a rank order affinity for rat > mouse > human $TAAR_1$ has previously been described for substituted phenethylamines with bulky residues (Lewin et al., 2008; Simmler et al., 2016; Wainscott et al., 2007).

The psychoactive dose of phenethylamines cannot be explained solely by data from *in vitro* assays. For example, Shulgin proposed a dose of 60–100 mg and duration of 3–5 h for 2C-T-1; for 2C-T-4, he proposed a human dose of 8–20 mg and duration of 12–18 h (Shulgin and Shulgin, 1995). However, receptor binding affinities in the radioligand assays for these two compounds were comparable, and the activation potential for the 5-HT_{2A} receptor was even higher for 2C-T-1 than for 2C-T-4. Therefore, other factors, such as lipophilicity, functional selectivity, and monoamine oxidase (MAO) and cytochrome P450 (CYP) metabolism, may influence the dose and effect of the compounds. Shulgin mentioned an unusual subjective variability for 2C-T drugs in the book *PiHKAL* (Shulgin and Shulgin, 1995). MAO-A, MAO-B, and to a lesser extent CYP2D6 were identified as the main enzymes that are involved in the deamination of 2C-T-2 and 2C-T-7 (Theobald and Maurer, 2007). A study with methoxylated and alkylthio amphetamine derivatives, however, did not find a correlation between the MAO inhibitory potential of the drugs and hallucinogenic potency reported in humans (Scorza et al., 1997). Other human doses were reported for most of the 2C-T drugs, and dose estimates started at 8 mg for the most potent compounds (Shulgin and Shulgin, 1995; Trachsel et al., 2013). A higher human dose (80–130 mg) was described for 2C-T-27 (Trachsel, 2012), consistent with the lower 5-HT_{2A} receptor activation potency that was found in the present study.

5. Conclusion

We characterized sparsely studied potent psychoactive drugs, providing insights into the relationship between the structure and 5-HT_2 receptor binding and activation of psychedelic phenethylamines. 2C-T drugs potently bound to 5-HT_{2A} and 5-HT_{2C} receptors and had affinity for 5-HT_{1A} and α_{2A} receptors. Furthermore, 2C-T

drugs were potent 5-HT_{2A} and 5-HT_{2B} partial agonists with the exception of benzylthiophenethylamines, which had no or negligible activation potential for the 5-HT_{2B} receptor.

Author contributions

D.L., D.T., and M.E.L. designed the research. D.L. and M.C.H. performed the research. D.L., M.C.H., and M.E.L. analyzed the data. D.L., M.C.H., and M.E.L. wrote the paper.

Conflict of interest

D.T. is an employee of ReseaChem GmbH. M.C.H. is an employee of F. Hoffmann-La Roche. The other authors do not have any conflicts of interest to declare for this work.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuropharm.2017.07.012.

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4 CORRELATION BETWEEN IN VITRO DATA AND HUMAN DOSES

4.1 Monoamine transporter and receptor interaction profiles *in vitro* predict reported human doses of novel psychoactive stimulants and psychedelics

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Brief Report

BRIEF REPORT

Monoamine Transporter and Receptor Interaction Profiles in Vitro Predict Reported Human Doses of Novel Psychoactive Stimulants and Psychedelics

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Abstract

Background: Pharmacological profiles of new psychoactive substances can be established rapidly in vitro and provide information on potential psychoactive effects in humans. The present study investigated whether specific in vitro monoamine transporter and receptor interactions can predict effective psychoactive doses in humans.
Methods: We correlated previously assessed in vitro data of stimulants and psychedelics with human doses that are reported on the Internet and in books.
Results: For stimulants, dopamine and norepinephrine transporter inhibition potency was positively correlated with human doses, whereas serotonin transporter inhibition potency was inversely correlated with human doses. Serotonin 5-hydroxytryptamine-2A (5-HT_{2A}) and 5-HT_{2C} receptor affinity was significantly correlated with psychedelic doses, but 5-HT_{1A} receptor affinity and 5-HT_{2A} and 5-HT_{2B} receptor activation potency were not.
Conclusions: The rapid assessment of in vitro pharmacological profiles of new psychoactive substances can help to predict psychoactive doses and effects in humans and facilitate the appropriate scheduling of new psychoactive substances.

Keywords: new psychoactive substance, stimulants, psychedelics, receptor, transporter

Introduction

The unprecedented proliferation of new psychoactive substances (NPSs) over the last decade has introduced a variety of substance classes to recreational drug users worldwide. The Internet plays a major role in the distribution of such compounds and in acquiring information about their effects and reported subjective effective doses in substance users. From 2011 to 2017, we assessed the monoamine transporter and receptor interaction profiles of more than 100 NPSs and related classic amphetamine-type and psychedelic drugs of abuse using the same in vitro assays and procedures in our laboratory (Simmler et al., 2013; Simmler et al., 2014a, 2014b; Rickli et al., 2015a, 2015b, 2015c, 2016; Luethi et al., 2018a, 2018b, 2018c, 2018d). The compounds that we investigated can predominantly be classified as stimulants or psychedelics based on their pharmacological and reported psychoactive effect profiles. Stimulants exert their pharmacological effects mainly by interacting with transmembrane monoamine transporters (i.e., norepinephrine [NE], dopamine [DA], and serotonin [5-hydroxytryptamine (5-HT)] transporters [NET, DAT, and SERT, respectively]), either as inhibitors or as transporter substrates that mediate the non-exocytotic release of neurotransmitters (Rothman and Baumann, 2003). Psychedelics mediate their mind-altering effects by interacting with 5-HT receptors, mainly 5-HT_{2A} receptor agonism (Nichols, 2016; Liechti, 2017). The present study

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investigated whether (1) in vitro monoamine transporter inhibition potencies and (2) in vitro serotonin receptor binding and activation can be used to predict human doses of stimulants and psychedelics, respectively, that are reported on online drug information websites and in books.

Methods

Drugs

The present study included drugs for which we previously investigated and published in vitro pharmacological profiles using identical assays and procedures in our laboratory (Simmler et al., 2013; Simmler et al., 2014a, 2014b; Rickli et al., 2015a, 2015b, 2015c, 2016; Luethi et al., 2018a, 2018b, 2018c, 2018d). These drugs could be categorized as either psychostimulants or psychedelics based on their chemical structure and reported pharmacological effects. Substances that predominantly inhibited monoamine transporters were classified as stimulants. Substances that most potently bound to 5-HT_2 receptors were pharmacologically classified as psychedelics. Five aminoindanes, 8 benzofurans, 28 cathinones, 3 piperazines, 10 piperidines, and 6 other NPSs were categorized as psychostimulants. One benzodifuran, 1 ergoline, and 7 tryptamines were categorized as psychedelics. The class of phenethylamines was further divided into 15 stimulant phenethylamines (amphetamine-type substances) and 36 psychedelic phenethylamines (ring-substituted phenethylamines, including 2C drugs and their methoxybenzyl [NBOMe] analogs). The stimulants are listed in supplementary Table 1. The psychedelics are listed in supplementary Table 2.

Dose Estimates

Dose estimates for human psychoactive doses were based on information that is found on the websites erowid.org, psychonautwiki.org, and tripsit.me (accessed December 17, 2017) and in published books and other publications (Shulgin and Shulgin, 1995, 1997; Simmler et al., 2013; Trachsel et al., 2013). The average midrange of the common dose range that is reported on the websites or in the books was taken as the dose estimate. Unless stated otherwise, oral doses of the racemic mixtures were used for this study.

Monoamine Transporter Inhibition

Norepinephrine, DA, and 5-HT uptake inhibition was assessed in human embryonic kidney 293 cells that were transfected with the human NET, DAT, or SERT as previously described in detail (Luethi et al., 2018c). Briefly, the cells were suspended in buffer and incubated with the drugs for 10 minutes before [^{3}H]-NE, [^{3}H]-DA, or [^{3}H]-5-HT at a final concentration of 5 nM was added for an additional 10 minutes to initiate uptake transport. The cells were then separated from the uptake buffer by centrifugation through silicone oil. The centrifugation tubes were frozen in liquid nitrogen, and the cell pellet was cut into scintillation vials that contained lysis buffer. Scintillation fluid was added, and uptake was quantified by liquid scintillation counting. Transporter inhibitors (10 μM nisoxetine for the NET, 10 μM mazindol for the DAT, and 10 μM fluoxetine for the SERT) were added to assess nonspecific monoamine uptake. Monoamine uptake data were fit by nonlinear regression to variable-slope sigmoidal dose-response curves, and IC_{50} values were determined using Prism 7.0a software (GraphPad).

5-HT Receptor Binding Affinities

Radioligand binding affinities for 5-HT receptors were assessed as previously described in detail (Luethi et al., 2018d). Briefly, membrane preparations overexpressing the respective human receptors were incubated for 30 minutes (5-HT_{1A} and 5-HT_{2A} receptors) or 2 hours (5-HT_{2C} receptor) with radiolabeled selective ligands at concentrations equal to K_d, and ligand displacement by the compounds was measured. Specific binding of the radioligand to the target receptor was defined as the difference between total binding and nonspecific binding that was determined in the presence of competitors. The following radioligands and competitors, respectively, were used: 1.39 nM [^{3}H]8-hydroxy-2-(di-*n*-propylamine)tetralin and 10 μM pindolol (5-HT_{1A} receptor), 0.45 nM [^{3}H]ketanserin and 10 μM spiperone (5-HT_{2A} receptor), and 1.6 nM [^{3}H]mesulgerine and 10 μM mianserin (5-HT_{2C} receptor).

Activity at the 5-HT_{2A} Receptor

Activity at the 5-HT_{2A} receptor was assessed as previously described in detail (Luethi et al., 2018a). Briefly, NIH-3T3 cells expressing the human 5-HT_{2A} receptor were incubated in buffer for 1 hour at 37°C before 100 μL of dye solution (fluorescence imaging plate reader [FLIPR] calcium 5 assay kit; Molecular Devices) was added to each well, and the plates were again incubated for 1 hour at 37°C. The plates were then placed in a FLIPR, and 25 μL of the test drugs that were diluted in buffer was added online. The increase in fluorescence was measured for 51 s. EC_{50} values were derived from the concentration-response curves using nonlinear regression.

Activity at the 5-HT_{2B} Receptor

Activity at the 5-HT_{2B} receptor was assessed as previously described in detail (Luethi et al., 2018a). Briefly, human embryonic kidney 293 cells that expressed the human 5-HT_{2B} receptor were incubated in growth medium overnight. The growth medium was then removed by snap inversion, and 100 μL of the calcium indicator Fluo-4 solution (Molecular Probes) was added to each well. The plates were incubated for 45 minutes at 31°C. The Fluo-4 solution was then removed by snap inversion, and 100 μL of Fluo-4 solution was added a second time for 45 minutes at 31°C. The cells were washed using an EMBLA cell washer, and 100 μL of assay buffer was added. The plates were then placed in a FLIPR, and 25 μL of the test substances that were diluted in buffer was added online. The increase in fluorescence was measured for 51 seconds. EC_{50} values were derived from the concentration-response curves using nonlinear regression.

Statistical Correlation

Mean estimated dose values were correlated with previously published mean IC_{50} values for the monoamine transporter inhibition of stimulants and the mean serotonin receptor affinity (K_i) and receptor activation (EC_{50}) values of psychedelics. The Spearman rank-order correlation coefficient (r_s) was computed using Prism 7.0a software (GraphPad). Values of $P < .05$ (2-tailed) were considered statistically significant. Multiple regression analysis was conducted to assess the relative contribution of different predictors to the dose estimate using Statistica 12 software (StatSoft) after logarithmic transformation of the data.

Results

Based on the reported information, dose estimates could be made for 54 of 75 stimulants and 35 of 45 psychedelics. The doses apply to the oral route of administration if not indicated otherwise (supplementary Tables 1 and 2). References for the information sources of the pharmacological data and for the dose estimates for each substance are listed in supplementary Tables 1 (stimulants) and 2 (psychedelics).

Stimulants

Correlations between transporter inhibition potencies (mean IC_{50} values) of stimulants and their mean dose estimates are shown in Figure 1. Inhibition potency values of the NET and DAT were significantly correlated with the human dose estimates ($r_s = 0.48$, $P < .001$, n = 54, and $r_s = 0.60$, $P < .001$, n = 54, respectively). Furthermore, the NET and DAT inhibition potencies were significantly intercorrelated ($r_s = 0.61$, $P < .001$, n = 74). In contrast, the inhibition potency values of the SERT were inversely correlated with the dose estimates ($r_s = -0.41$, $P < .01$, n = 54) and inversely intercorrelated with DAT inhibition ($r_s = 0.26$, $P < .05$, n = 73) but not NET inhibition. When DAT and NET inhibition was used as the predictor within a multiple regression analysis to predict the dose, DAT inhibition and NET inhibition alone were significant predictors ($R = 0.55$, $P < .001$, and $R = 0.51$, $P < .001$, respectively) when entered alone, but adding NET to DAT inhibition only minimally and nonsignificantly increased the overall prediction (multiple $R = 0.59$, $P < .001$). However, SERT inhibition was inversely correlated with dose when analyzed alone ($R = 0.36$, $P < .01$) and relevantly and significantly increased the overall prediction when it was added to NET and DAT inhibition (multiple $R = 0.63$, $P < .001$, n = 54).

Psychedelics

Correlations between 5-HT receptor affinities (mean K_i values) and their dose estimates are shown in Figure 2. Reported human doses for psychedelics were significantly correlated with 5-HT_{2A} and 5-HT_{2C} receptor binding ($r_s = 0.62$, $P < .001$, n = 35, and $r_s = 0.69$, $P < .001$, n = 35, respectively) but not with 5-HT_{1A} receptor binding ($r_s = -0.18$, $P = .3$, n = 35). The 5-HT_{2A} and 5-HT_{2C} affinity values were significantly intercorrelated ($r_s = 0.90$, $P < .001$, n = 45), and the 5-HT_{1A} and 5-HT_{2A} affinity values were inversely intercorrelated (-0.32, $P < .05$, n = 45). No correlation was found between 5-HT_{1A} receptor binding and 5-HT_{2c} receptor binding.

5-HT_{2A} receptor activation potencies (mean EC_{50} values) did not correlate with reported human doses ($r_s = -0.08$, $P = .6$, n = 35). Four substances did not activate the 5-HT_{2B} receptor in the investigated concentration range, and these substances thus could not be included in the statistical analysis. The 5-HT_{2B} receptor activation of the remaining psychedelics did not correlate with the dose estimates ($r_s = 0.25$, $P = .2$, n = 31).

Discussion

Stimulants

In the present study, we found that both NET and DAT inhibition potencies were correlated highly significantly with human doses that are reportedly used across a larger set of psychoactive, mostly amphetamine-type stimulants. In contrast, SERT inhibition potency was inversely correlated with human doses, a finding that is consistent with the notion that serotonergic

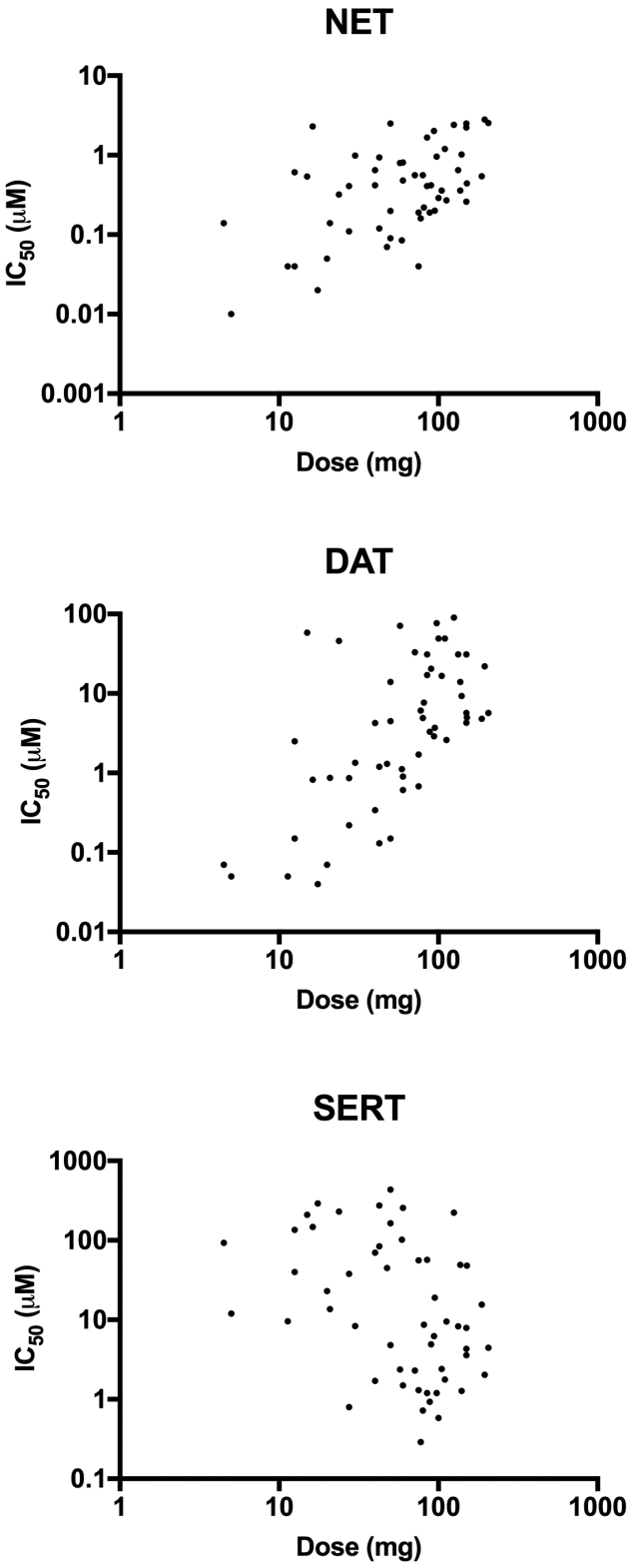

Figure 1. Correlations between dose estimates of stimulants and their transporter inhibition potencies (mean IC_{50} values).

activity is inversely linked to the drug abuse liability of amphetamine-type substances (Ritz et al., 1987; Kuhar et al., 1991; Wee et al., 2005; Wee and Woolverton, 2006). We also found a significant intercorrelation between NET and DAT inhibition potencies

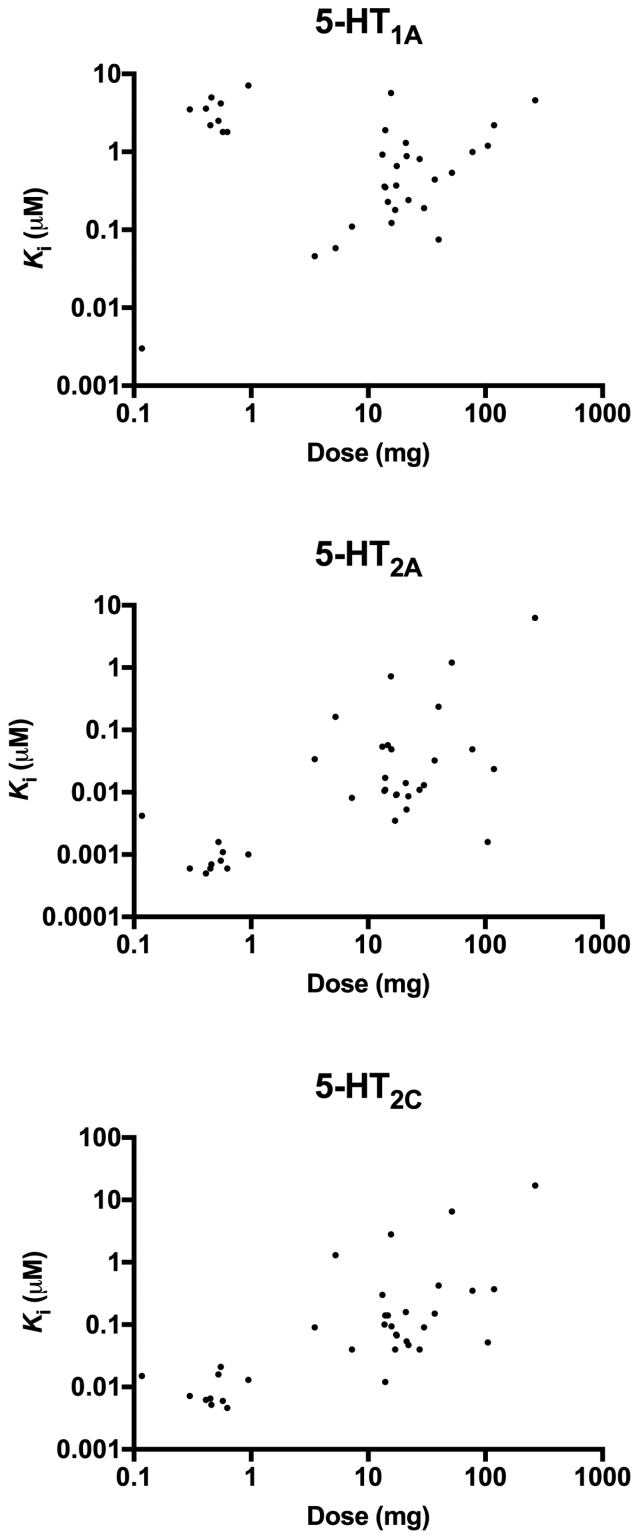

Figure 2. Correlations between dose estimates of psychedelics and their serotonin 5-HT receptor affinities (mean K_i values).

across substances, which is unsurprising given their similarity (i.e., high amino acid sequence similarity [Andersen et al., 2015]) and the ability of both transporters to transport NE and DA across the cell membrane (Gu et al., 1994). The present data are consistent with a small previous study that reported that oral doses of 5 classic amphetamine-type stimulants used in clinical studies correlated with their NE-releasing potencies, although no significant correlation was shown for DA release (Rothman et al., 2001). In another study, Iversen and colleagues found no correlation between uptake inhibition potency and doses of stimulant drugs producing subjective effects (Iversen et al., 2013). The lack of correlation may relate to the small number of compounds tested.

We previously showed that DAT and NET inhibition potency but not SERT inhibition potency (IC_{50} values) were correlated with psychotropic effective doses within a subset of substances that were also included in the present analysis (Simmler et al., 2013). Altogether, the present study showed that DAT and NET inhibition potency values that are defined in vitro can be used to estimate whether a novel substance is psychoactive in humans, and the dose can be predicted when other known substances are co-analyzed as references. This finding has important implications because it indicates that relatively fast and simple in vitro measures are useful for legally scheduling novel substances as psychoactive and thus as illegal NPSs. Both the DAT and NET may serve as predictors of the human dose, whereas SERT inhibition potency can be used as an additional indicator, predicting lower clinical potency of the substance. Furthermore, the DAT/SERT inhibition ratio, which is defined as 1/DAT IC_{50}: 1/SERT IC_{50} (Baumann et al., 2012), is a marker of the reinforcing effects and abuse liability of a substance (Baumann et al., 2000). Compounds with higher SERT vs DAT inhibition potency are typically associated with 3,4-methylenedioxymethamphetamine-like entactogenic effects, whereas drugs with high DAT vs SERT inhibition potency exert amphetamine-type psychostimulant effects and pose a higher risk for addiction (Simmler et al., 2013, 2014a; Liechti, 2014; Suyama et al., 2016).

Psychedelics

We showed that the doses of psychedelics were correlated with 5-HT_{2A} receptor affinity (K_i values) but not with receptor activation potency in the calcium release assay used to determine EC_{50} values. 5-HT_{2A} receptor activation is assumed to mediate the mind-altering effects of psychedelics (Glennon et al., 1984; Titeler et al., 1988) and such effects can be blocked by 5-HT_{2A} receptor antagonists, such as ketanserin (Preller et al., 2017). All of the psychedelics that were included in our study were receptor agonists, and the correlation with receptor binding but not activation might be explained by higher sensitivity of the ligand-binding assays compared with the receptor activation assay. There are different 5-HT_{2A} receptor activation assays, and the potencies for inducing calcium release in the assay that was used in the present study may not reflect the same pathway or mechanism that mediates the subjective effects of hallucinogens in humans. In fact, others have also reported that high-affinity agonist binding did not correlate well with the receptor activation of 5-HT_2 receptors (Roth et al., 1997; Acuña-Castillo et al., 2002). Despite the lack of utility for predicting doses, the determination of 5-HT_{2A} receptor activity remains crucial for determining whether a NPS has receptor agonist properties and may thus be classified as a psychedelic or whether it is an antagonist that only binds to the receptor. The present study showed that 5-HT_{2A} receptor binding allows an estimate of the dose at which the substance is psychoactive in humans. Besides the correlation of the dose estimates for psychedelics with 5-HT_{2A} receptor affinities, we also found a correlation with 5-HT_{2C} receptor affinities. Today, it is widely accepted that 5-HT_{2A} receptor activation is crucial for the action of psychedelics (Preller et al., 2017); the

role of 5-HT_{2C} receptor activation, however, remains enigmatic. As all known psychedelics are both 5-HT_{2A} and 5-HT_{2C} agonists, a contribution of 5-HT_{2C} activation to psychedelic effects cannot be excluded (reviewed in Nichols, 2004, 2016).

Limitations

The outcomes of the present analysis highly depended on the types of substances that were included and may be different for other sets of psychoactive compounds. Although valid pharmacological data were used, the dose estimates were mainly derived from user reports. No controlled studies are currently available for most NPSs, but doses for some of the substances included in the present analysis are available from clinical studies. These doses were comparable to the reported recreational doses. Doses derived from clinical studies are available for mephedrone (200 mg; Papaseit et al., 2016), 3,4-methylenedioxymethamphetamine (100–125 mg; Tancer and Johanson, 2003; Papaseit et al., 2016; Vizeli and Liechti, 2017); MDAI (3 mg/kg; V. Auwärter et al., personal communication); cathinone (0.5 base mg/kg; Brenneisen et al., 1990); 4-fluoroamphetamine (100 mg; K. Kuypers et al., personal communication); D-amphetamine (15–40 mg; Martin et al., 1971; Brauer and de Wit, 1996; Dolder et al., 2017b); methamphetamine (15–30 mg; Martin et al., 1971; Gouzoulis-Mayfrank et al., 1999); MDEA (2 mg/kg; Gouzoulis-Mayfrank et al., 1999); BZP (100 mg; Lin et al., 2011); mCPP (0.5–0.75 mg/kg; Tancer and Johanson, 2003); methylphenidate (40–60 mg; Schmid et al., 2014); cocaine (48–96 mg; Volkow et al., 2000); diclofensine (50 mg; Funke et al., 1986); LSD (0.1 mg; Dolder et al., 2017a); 2C-B (20 mg; Gonzalez et al., 2015); mescaline sulfate (500 mg; Hermle et al., 1992); and psilocin/psilocybin (5–20 mg; Studerus et al., 2012). Therefore, even though the dose estimates of the current study were not derived from clinical studies, they are in accordance with the available clinical data.

Not accounted for in the in vitro assays were in vivo factors (e.g., bioavailability, route of administration, distribution, and brain penetration), which may influence clinical potency.

Conclusion

The present study found that in vitro pharmacological profiles of substances that interact with monoaminergic systems allow the characterization of substances as stimulants or psychedelics and may be used to predict human psychoactive doses. For stimulants, potent DAT and NET inhibition was associated with lower pharmacological doses in humans. In contrast, higher SERT inhibition potency was an additional indicator of lower stimulant potency and higher human doses. The potency of psychedelics was best predicted by 5-HT_{2A} and 5-HT_{2C} binding affinity. In contrast, the calcium mobilization assay used to determine 5-HT_{2A} receptor activation potency did not predict the clinical potency of psychedelics. However, it is a necessity to determine whether a drug is a 5-HT_{2A} agonist and therefore likely a psychedelic in humans.

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Statement of Interest

None.

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Part II TOXICOLOGICAL INVESTIGATIONS

5 STIMULANT-INDUCED HEPATOTOXICITY

The potential of amphetamine to induce liver injury has been known since the beginning of its use as therapeutic drug and today there are many studies showing an association between acute and chronic amphetamine use and hepatotoxicity (Carvalho et al., 2012). Reports of liver injury for other amphetamine-type stimulants followed with their appearance on the recreational drug market and MDMA has so far been the most frequently associated drug (Andreu et al., 1998; De Carlis et al., 2001; Ellis et al., 1996; Fröhlich et al., 2011; Garbino et al., 2001; Jones et al., 1994; Kamijo et al., 2002). However, stimulant-induced liver damage is not yet completely understood and different mechanisms may contribute (Carvalho et al., 2012).

5.1 Hyperthermia

Hyperthermia is a frequently observed complication with stimulant use and may lead to potentially fatal complications such as rhabdomyolysis, acute renal failure, acidosis, or multiple organ failure (Henry, 1992; Kalant, 2001; Kendrick et al., 1977). The cause of stimulant-induced hyperthermia is multifaceted as stimulants act on monoaminergic systems (discussed in Part I), which regulate body temperature in a variety of ways (Docherty and Green, 2010). Norepinephrine release generates heat through activation of uncoupling protein 3 (UCP3) along with a loss in heat dissipation through vasoconstriction (Mills et al., 2004; Parrott, 2012). An increase in the metabolic rate has additionally been reported to cause elevated core body temperature after stimulant use (Freedman et al., 2005; Parrott, 2012). Furthermore, prolonged excessive dancing, dehydration, and high ambient temperatures contribute to hyperthermia. It is likely that a combination of mechanisms and susceptibility factors are the cause of stimulant-induced severe hyperthermia in most cases (Patel et al., 2005).

5.2 Respiratory chain dysfunction

Oxidative phosphorylation is part of cellular respiration and therefore a process of ATP production in cells. Oxidative phosphorylation depends on different complexes that transfer electrons from donors to acceptors in a series of redox reactions while transferring protons across the membrane; thereby, a proton gradient across the inner mitochondrial membrane is maintained. This proton gradient drives ATP synthesis by the enzyme ATP synthase (Complex V). Of the remaining complexes, three act as proton pumps: complex I (NADH:ubiquinone oxidoreductase), complex III (ubiquinol:cytochrome c oxidoreductase),

and complex IV (cytochrome c oxidase) (Nicholls and Ferguson, 2013). The succinate dehydrogenase forms complex II (Nicholls and Ferguson, 2013).

An impermeable inner mitochondrial membrane and integrity of the electron transport chain complexes are crucial for cellular respiration. Drugs may potentially disrupt cellular respiration by inhibition of the electron transport chain complexes, by uncoupling of oxidative phosphorylation from ATP synthesis, or both (Felser et al., 2013; Fromenty et al., 1990; Krähenbühl, 2001; Serviddio et al., 2011). Consequences of such events can be a decrease in ATP production, disruption of the mitochondrial membrane potential ($\Delta\Psi_m$), or apoptosis.

5.3 Oxidative stress

Reactive oxygen species are byproducts of mitochondrial respiration, and complex I and III have been discussed as potential sites for ROS generation (Antico Arciuch et al., 2012; Drose and Brandt, 2012; Votyakova and Reynolds, 2001). Stimulants that disrupt these complexes could induce an increased production of ROS and therefore potentially mitochondrial dysfunction (Brown and Yamamoto, 2003). Furthermore, drugs that interfere with the natural antioxidant response, for example by depleting or decreasing the activity of GSH, Cu–Zn superoxide dismutase, catalase, peroxidase, glutathione reductase, glutathione S-transferase, or peroxiredoxins, may further increase toxicity caused by oxidative stress (Cadet and Krasnova, 2009; Carvalho et al., 1996; Carvalho et al., 2002; Carvalho et al., 2004).

5.4 Metabolism

Metabolism is thought to play an important role in the hepatotoxicity of stimulants but due to structural differences, different mechanisms may contribute to the toxicity for different drugs (Carvalho et al., 2012). Currently, most research regarding metabolism in stimulant-induced hepatotoxicity has focused on MDMA. Catechol metabolites formed via demethylenation mainly by CYP2D6 are assumed to be main contributors to MDMA-induced liver injury (Antolino-Lobo et al., 2011; Carmo et al., 2006). Such catechol metabolites are formed for other methylenedioxy-substituted stimulants as well (Meyer et al., 2010; Negreira et al., 2015; Pedersen et al., 2013) and it is therefore likely that these metabolites induce hepatic toxicity similarly as observed for MDMA. Enzyme polymorphism could therefore render some users more susceptible to hepatotoxicity, due to increased formation of toxic metabolites.

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6 HEPATOTOXICITY OF SYNTHETIC CATHINONES

6.1 Mechanisms of hepatocellular toxicity associated with new psychoactive synthetic cathinones

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Mechanisms of hepatocellular toxicity associated with new psychoactive synthetic cathinones

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ABSTRACT

Synthetic cathinones are a new class of psychostimulant substances. Rarely, they can cause liver injury but associated mechanisms are not completely elucidated. In order to increase our knowledge about mechanisms of hepatotoxicity, we investigated the effect of five frequently used cathinones on two human cell lines. Bupropion was included as structurally related drug used therapeutically. In HepG2 cells, bupropion, MDPV, mephedrone and naphyrone depleted the cellular ATP content at lower concentrations (0.2–1 mM) than cytotoxicity occurred (0.5–2 mM), suggesting mitochondrial toxicity. In comparison, methedrone and methylone depleted the cellular ATP pool and induced cytotoxicity at similar concentrations (≥2 mM). In HepaRG cells, cytotoxicity and ATP depletion could also be demonstrated, but cytochrome P450 induction did not increase the toxicity of the compounds investigated. The mitochondrial membrane potential was decreased in HepG2 cells by bupropion, MDPV and naphyrone, confirming mitochondrial toxicity. Bupropion, but not the other compounds, uncoupled oxidative phosphorylation. Bupropion, MDPV, mephedrone and naphyrone inhibited complex I and II of the electron transport chain, naphyrone also complex III. All four mitochondrial toxicants were associated with increased mitochondrial ROS and increased lactate production, which was accompanied by a decrease in the cellular total GSH pool for naphyrone and MDPV. In conclusion, bupropion, MDPV, mephedrone and naphyrone are mitochondrial toxicants impairing the function of the electron transport chain and depleting cellular ATP stores. Since liver injury is rare in users of these drugs, affected persons must have susceptibility factors rendering them more sensitive for these drugs.

1. Introduction

In recent years, various synthetic cathinones ("bath salts", research chemicals) with amphetamine-like properties have emerged on the illicit drug market and have become popular alternatives to classic stimulants among drug users (Baumann et al., 2013; Prosser and Nelson, 2012). Cathinone designer drugs are derivatives of cathinone (Fig. 1), a naturally occurring β-keto-amphetamine found in the leaves of the Catha edulis plant, and are chemically and pharmacologically similar to classic illicit stimulants (Baumann et al., 2012; Rickli et al., 2015; Simmler et al., 2013, 2014). Therefore, the health risks posed by synthetic cathinones may be similar to the classic stimulants (Liechti, 2015). A rare, but potentially severe adverse reaction of stimulant use is hepatotoxicity (Andreu et al., 1998; De Carlis et al., 2001; Ellis et al., 1996; Garbino et al., 2001; Jones et al., 1994; Kamijo et al., 2002). Most research concerning stimulant hepatotoxicity has so far focused on 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy"). However, the hepatotoxic mechanism of MDMA is currently not entirely understood and multiple factors including polydrug abuse, hyperthermia, and metabolism appear to be associated with liver injury in MDMA users (Antolino-Lobo et al., 2011b; Carvalho et al., 2012; Dias da Silva et al., 2013a,b). In comparison, data on hepatotoxicity of the newly used synthetic cathinones is currently scarce. A case of acute liver failure after synthetic cathinones use has been described (Fröhlich et al., 2011) and *in vitro* studies showed that mitochondrial dysfunction and oxidative stress contribute to hepatic injury associated with these compounds

Abbreviations: ADP, adenosine-diphosphate; AK, adenylate kinase; BSO, buthionine sulfoximine; CYP, cytochrome P450; DMEM, Dulbecco's Modified Eagle Medium; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FCCP, trifluoromethoxy carbonylcyanide phenylhydrazone; FCS, fetal calf serum; GR, glutathione reductase; GSH, glutathione (reduced); GSSG, glutathione (oxidized); KPE, potassium phosphate buffer; MDMA, 3,4-methylenedioxymethamphetamine; MDPV, 3,4-methylenedioxypyrovalerone; O_2^-, superoxide; OCR, oxygen consumption rate; PBS, phosphate-buffered saline; PCA, perchloric acid; SRB, sulforhodamine B; TMRM, tetramethylrhodamine methyl ester; tGSH, glutathione (total); β-NADPH, β-nicotinamide adenine dinucleotide 2′-phosphate; $\Delta\psi_m$, mitochondrial membrane potential

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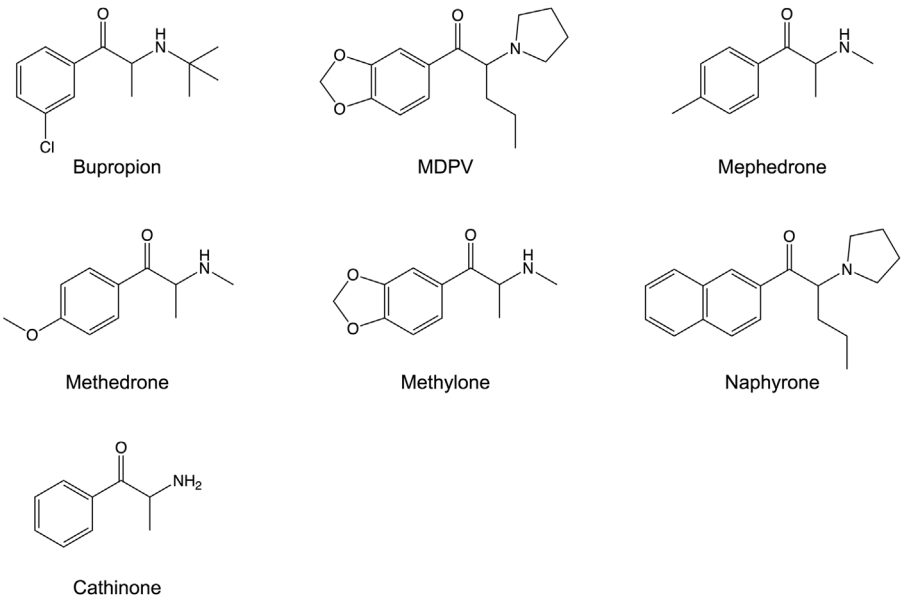

Fig. 1. Structures of the synthetic cathinones used in the study.

(Valente et al., 2016a,b).

In the current study, we aimed to investigate in more detail the mechanisms of hepatocellular toxicity of several synthetic cathinones (Fig. 1) with a focus on mitochondrial toxicity. We therefore investigated the toxicity of the cathinone designer drugs 3,4-methylenedioxypyrovalerone (MDPV), 4-methylmethcathinone (4-MMC; mephedrone), 4-methoxymethcathinone (4-MeOMC; methedrone), 3,4-methylenedioxymethcathinone (βk-MDMA; methylone), and naphthylpyrovalerone (naphyrone) in two human hepatocyte cell lines. In addition, we included bupropion, a synthetic cathinone used as an antidepressant and as a smoking cessation aid.

2. Methods

2.1. Test substances

Naphyrone was synthesized as previously described by Meltzer et al. (2006). Methylone, mephedrone, methedrone, and MDPV were purchased from Lipomed (Arlesheim, Switzerland) with HPLC purity of > 98.5%. Bupropion was purchased from Cayman Chemicals (Ann Arbor, MI, USA), with a purity of > 98%. All drugs were obtained as racemic hydrochloride salts. Drug stocks were made in autoclaved Milli-Q water and were freshly prepared for each assay.

2.2. Cell line and culture

The HepG2 cell line was obtained from the American Type Culture Collection (ATCC, Manassas, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM, 1 g/l glucose) supplemented with 10% heat inactivated fetal calf serum (FCS), 10 mM HEPES buffer, 2 mM GlutaMAX™, 1% MEM non-essential amino acids, and penicillin-streptomycin (10,000 U/ml corresponding to 10 mg/ml). Cell culture medium and supplements were purchased from Invitrogen (Basel, Switzerland).

The HepaRG cell line was obtained from Biopredic International (Saint Grégoire, France) and cultured in William's E medium (no glutamine) supplemented with 10% FCS, 2 mM L-glutamine, 50 µM hydrocortisone hemisuccinate (Sigma Aldrich, Buchs, Switzerland), 0.05% human insulin (9.5–11.5 mg/ml insulin, Sigma Aldrich), and penicillin-streptomycin (10,000 U/ml corresponding to 10 mg/ml). The cells were cultured at 37 °C in a 5% CO_2 humidified atmosphere and passaged using trypLE™ Express reagent (Invitrogen) when they reached 70–80% confluency.

2.3. Cytotoxicity of HepG2 cells

Cytotoxicity was assessed with the ToxiLight BioAssay Kit from Lonza (Basel, Switzerland) and conducted according to the manufacturer's manual. Briefly, 25,000 HepG2 cells per well were seeded in a 96-well plate. The following day, the cells were treated with 100 µl of the test substances dissolved in medium (0.01 mM, 0.1 mM, 1 mM, and 2 mM for each drug and additionally 0.2 mM and 0.5 mM for bupropion and naphyrone). Treatment with 0.5% Triton X-100 was used as a positive control. After 24 h, 50 µl of the ToxiLight assay buffer was added to 20 µl of supernatant and luminescence was measured with a Tecan M200 Pro Infinity plate reader (Männedorf, Switzerland) after 5 min incubation. The luminescence signal was then compared to medium control.

2.4. ATP content in HepG2 cells

The ATP content was assessed with the CellTiter-Glo Luminescent Cell Viability Assay from Promega (Dübendorf, Switzerland) according to the manufacturer's manual. Briefly, 25,000 HepG2 cells per well were seeded in a 96-well plate and cultured overnight. The cells were then treated for 24 h with 100 µl of the test substances dissolved in medium (concentrations as for cytotoxicity). Treatment with 0.5% Triton X-100 was used as a positive control. Thereafter, 50 µl of the supernatant was discarded and 50 µl of CellTiter-Glo reagent was added to each well. The plate was then shaken for 15 min at room temperature to induce cell lysis. Thereafter, the luminescent signal was measured with a Tecan M200 Pro Infinity plate reader and compared with medium control.

2.5. Cytotoxicity and ATP content in HepaRG cells

HepaRG cells were cultured and differentiated as follows. 10,000 cells per well were seeded in a 96-well plate and the growth medium was replaced with fresh medium every 3–4 days for 2 weeks. Thereafter, the medium was replaced with medium containing 2% of DMSO in order to differentiate the cells into cholangiocyte- and hepatocyte-like cells. The medium was again replaced with fresh medium every 3–4 days for 2 weeks and finally replaced with medium containing no DMSO and only 2% FCS. After three days, the medium of some cell preparations was replaced with the same medium containing 20 µM rifampicin (Sigma Aldrich) every 24 h for cytochrome P450 (CYP) induction. The uninduced cell preparations were treated

identically with medium not containing rifampicin. After 72 h, the cells were treated with the test drugs (0.01 mM, 0.1 mM 1 mM, and 2 mM), medium control or 0.5% Triton X-100. The treatment and experimental procedures were the same as described for HepG2 cells.

2.6. Mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\psi_m$) can be regarded as a global indicator of mitochondrial function (Felser et al., 2013). Tetramethylrhodamine methyl ester (TMRM; Thermo Scientific, Wohlen, Switzerland) staining was used to measure $\Delta\psi_m$. TMRM is a non-cytotoxic cationic red-orange fluorescent dye that permeates into the mitochondria. 150,000 HepG2 cells were cultured in a 24-well plate overnight and then treated for 24 h with 500 µl of medium containing the test drugs (0.01 mM, 0.1 mM 0.2 mM, 0.5 mM, and 1 mM). The uncoupling agent trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP, Sigma Aldrich) at a concentration of 9 µM served as a positive control. After treatment, the cells were washed with phosphate-buffered saline (PBS, Invitrogen) and detached with 0.05% trypsin-EDTA (Invitrogen) at 37 °C. 500 µl medium were added to the wells and the content was then transferred into polypropylene tubes. The cells were centrifuged at 1500*g* for 2.5 min, washed with PBS and centrifuged again. The supernatant was removed and the cells were re-suspended in PBS containing 100 nM TMRM. After 20 min of incubation at 37 °C in the dark, the cells were centrifuged at 300*g* for 5 min, re-suspended in 300 µl PBS, and transferred into polypropylene microtubes. Fluorescence (FL-2) of 10,000 live cells was measured with a FACSCalibur flow cytometer (Becton Dickinson, Allschwil, Switzerland).

2.7. Cellular total glutathione content

Glutathione is an important antioxidant agent; the cellular total glutathione (tGSH) pool consists of reduced (GSH) and oxidized (GSSG) glutathione. Oxidation of GSH is a defense mechanism against cellular stress and a decrease in the GSH content makes cells more vulnerable to toxic agents. The tGSH content was determined with the enzymatic recycling method described by Rahman et al. (2006). The assay is based on the oxidation of GSH by 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), which is reduced to the yellow 5′-thio-2-nitrobenzoic acid. Briefly, 750,000 HepG2 cells per well were seeded in a 6-well plate and left to attach overnight. The following day, the cells were treated with 1 ml of test drugs dissolved in medium. After 24 h, the cells were washed twice with 1 ml of cold PBS and then harvested with 0.05% trypsin-EDTA. Culture medium was added, the cell suspension was transferred into pre-chilled polypropylene tubes, and centrifuged at 1000*g* and 4 °C for 5 min. The supernatant was discarded and the cells re-suspended in cold PBS. The cells were centrifuged again and re-suspended in 1 ml of cold extraction buffer (0.1% Triton X-100 and 0.6% sulfosalicylic acid in 0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5 [KPE]). After two freeze-thaw cycles, the cells were centrifuged at 3000*g* and 4 °C for 4 min and the supernatant was then immediately transferred into a fresh polypropylene tube. At this point, the cell extracts could be frozen at −80 °C until further use for up to 10 days. To measure tGSH, 20 µl of standards (0.103–26.4 µM GSH in KPE and blank) and 20 µl of the cell extracts were added to a 96-well plate. 100 µl of a DTNB-glutathione reductase (GR) solution (2 mg DTNB and 10 µl of GR [9.5 mg protein/ml; 189 units/mg protein] in 6 ml KPE) were added to each well. After 30 s, 50 µl of a β-nicotinamide adenine dinucleotide 2′-phosphate (β-NADPH) solution (2 mg of β-NADPH tetrasodium salt hydrate in 3 ml of KPE) was added to each well. Immediately thereafter, the absorbance at 412 nm was measured with a Tecan M200 Pro Infinity plate reader every 0.5 min for 2 min. The protein content was determined with the Pierce Protein Assay Kit (Thermo Fisher) and the tGSH content was then adjusted to protein.

2.8. Oxygen consumption

The oxygen consumption rate (OCR) was measured with a Seahorse XF24 extracellular flux assay kit (Seahorse Biosciences, North Billerica, MA, USA). 100,000 HepG2 cells per well were seeded in a Seahorse XF24 cell culture microplate coated with poly-D-lysine. The following day, the cells were treated with the test drugs. After 24 h, the medium was removed and the cells were washed with unbuffered DMEM (4 mM L-glutamate, 1 mM pyruvate, 1 g/l glucose, 63.3 mM sodium chloride, pH 7.4), pre-warmed to 37 °C. Thereafter, unbuffered DMEM was added to the cells and the plate was incubated for 40 min at 37 °C in a CO_2 free incubator. The ports of the XF24 assay cartridge were then loaded with oligomycin, FCCP, or rotenone in order to reach a final concentration of 1 µM for each of these compounds. The XF24 Assay cartridge was loaded into a Seahorse XF24 Analyzer (Seahorse Biosciences) and the program was run according to the instructions. The oxidative leak was determined after inhibition of the mitochondrial phosphorylation by oligomycin and the maximal respiration was determined after stimulation of the electron transport chain by FCCP. Extramitochondrial respiration was determined after complex I inhibition by rotenone and subtracted from basal, leak, and maximal respiration. The protein content was determined using sulforhodamine B (SRB) staining. The cells were fixed with 100 µl of 50% (w/v) trichloroacetic acid (TCA) added directly to the assay medium of each well. After incubation for 1 h at 4 °C, the cells were washed with deionized water and then stained with 0.4% (w/v, in 1% [v/v] acetic acid) SRB. After 20 min, the cells were rapidly washed with 1% (v/v) acetic acid and the incorporated dye was solubilized with 100 µl of 10 mM TRIS base. The absorbance was then measured at 490 nm.

2.9. Activity of specific enzyme complexes of the mitochondrial electron transport chain

The activity of specific enzyme complexes of the mitochondrial respiratory chain was analyzed using an Oxygraph-2k high-resolution respirometer equipped with DataLab software (Oroboros instruments, Innsbruck, Austria). HepG2 cells were treated with test drugs for 24 h and afterwards re-suspended in MiR05 (mitochondrial respiration medium containing 0.5 mM EGTA, 3 mM magnesium chloride, 20 mM taurine, 10 mM potassium dihydrogen phosphate, 20 mM HEPES, 110 mM sucrose, 1 g/l fatty-acid free bovine serum albumin, and 60 mM lactobionic acid, pH 7.1). The cells were then transferred into the pre-calibrated Oxygraph chamber (Pesta and Gnaiger, 2012). Digitonin (10 µg/1 million cells) was used to permeabilize the cells. Complexes I and III were analyzed using L-glutamate and malate (10 and 2 mM, respectively) as substrates followed by the addition of adenosine-diphosphate (ADP; 2.5 mM) and rotenone (0.5 µM) as an inhibitor of complex I. Duroquinol (0.5 mM) was then added as a substrate of complex III. Complexes II and IV were analyzed using succinate (10 mM) as substrate and rotenone (0.5 µM) as a complex I inhibitor, followed by the addition of ADP (2.5 mM). After the addition of the complex III inhibitor antimycin A (2.5 µM), *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylendiamine and ascorbate (0.5 and 2 mM, respectively) were added to investigate complex IV activity. The absence of a stimulatory effect of exogenous cytochrome *c* (10 µM) on respiration confirmed integrity of the outer mitochondrial membrane. Protein content was determined with the Pierce Protein Assay Kit. Respiration was expressed as oxygen consumption per mg protein.

2.10. ROS production

ROS production was determined with the red mitochondrial superoxide (O_2^-) indicator MitoSOX (Invitrogen) according to the manufacturer's manual. MitoSOX red is a live-cell permeant fluorogenic dye that targets the mitochondria and exhibits red fluorescence upon oxidation by superoxide. The seeding and treatment conditions were the

same as described for cytotoxicity and ATP assays with HepG2 cells; however, 50 µM amidarone was used as a positive control. Briefly, 100 µl of MitoSOX reagent (2.5 µM) were added to each well of the 96-well plate and incubated for 10 min at 37 °C in the dark. After that, the fluorescence was measured at 510/580 nm with a Tecan M200 Pro Infinity plate reader. The protein content was assessed with the Pierce Protein Assay Kit.

2.11. Lactate determination

Lactate concentrations in the cell culture assay were determined with an enzymatic assay after protein precipitation (Olsen, 1971). Briefly, 50 µl of supernatant from the MitoSOX assay were diluted with 50 µl of 6% (v/v) perchloric acid (PCA), vortexed, and centrifuged at 3000*g* for 15 min. Reagent buffer consisting of 100 µl hydrazine buffer (6.8 mM EDTA, 100 mM hydrazine sulphate 1 M hydrazine hydrate, pH 9), 0.2 mg NAD^+ (free acid; Roche diagnostics, Rotkreuz, Switzerland) and 1 µl LDH (5 mg/ml; Roche diagnostics) were added to 10 µl of sample in a 96-well plate. After 30 min incubation at room temperature, the absorbance was measured and compared to a lactic acid standard curve.

2.12. Statistics

Data are presented as mean ± SEM and statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA). The activity of the enzyme complexes of the mitochondrial electron transport chain was compared to vehicle control with an unpaired two-tailed Student's *t*-test. For the remaining assays, means were compared to control and differences between control and test drugs were calculated with ANOVA followed by Dunett's test. P-values below 0.05 were considered statistically significant.

3. Results

3.1. Cytotoxicity and ATP content in HepG2 cells

AK release and cellular ATP content were determined as markers for cytotoxicity and mitochondrial function, respectively. In HepG2 cells after 24 h of exposure, all cathinones were cytotoxic and decreased the cellular ATP content in a concentration-dependent manner (Figs. 2 a–d; 3 a, b; Table 1). Bupropion was cytotoxic starting at 2 mM while a decrease in ATP was observed already at 0.5 mM (Fig. 2a). For MDPV, we observed cytotoxicity starting at 2 mM and a decrease in ATP already at 1 mM (Fig. 2b). Mephedrone was cytotoxic starting at 2 mM and decreased ATP at 1 mM (Fig. 2c). Naphyrone was cytotoxic at 0.5 mM and decreased ATP levels at 0.2 mM (Fig. 2d). For methedrone, we observed cytotoxicity and a decrease in ATP content starting at 2 mM (Fig. 3a). Methylone was cytotoxic at 2 mM without significant alteration in ATP content (Fig. 3b). In summary, a decrease in the cellular ATP content at non-cytotoxic drug concentrations, which is compatible with mitochondrial toxicity (Felser et al., 2013; Kamalian et al., 2015), was observed for bupropion, MDPV, mephedrone, and naphyrone.

3.2. Cytotoxicity and ATP content in HepaRG cells

HepaRG were less susceptible than HepG2 cells to the toxicants investigated. After 24 h of drug exposure, we observed cytotoxicity only for bupropion and naphyrone and a decrease in ATP content additionally for MDPV (Fig. 2a, b, d; Table 1). Bupropion was cytotoxic starting at 2 mM with a decrease in the ATP content at 1 mM (Fig. 2a). MDPV decreased ATP at starting 2 mM without being cytotoxic (Fig. 2b). For naphyrone, we observed cytotoxicity and a decrease in ATP content starting at 1 mM (Fig. 2d). As in HepG2 cells, bupropion and MDPV decreased ATP content at non-cytotoxic drug concentrations, underlining possible mitochondrial toxicity for these toxicants (Felser et al., 2013; Kamalian et al., 2015).

Induction of drug-metabolizing enzymes with 20 µM rifampicin (Berger et al., 2016) prior to treatment with the toxicants did not cause a difference in cytotoxicity or decrease in the ATP content compared to non-induced cells (data not shown). Since CYP induction in HepaRG cells did not seem to play a major role in toxicity and HepG2 cells were more sensitive to the toxicants, we decided to continue our investigations in HepG2 cells only.

3.3. Effect on mitochondrial membrane potential

Next, we determined the effect of the synthetic cathinones on the mitochondrial membrane potential ($\Delta\psi_m$) to further investigate a possible role of mitochondria in cellular ATP reduction. HepG2 cells treated for 24 h showed a decreased $\Delta\psi_m$ for bupropion, MDPV, and naphyrone (Fig. 4a, b, f). Bupropion reduced the $\Delta\psi_m$ starting at 0.5 mM (Fig. 4a), MDPV at 1 mM (Fig. 4b), and naphyrone at 0.2 mM (Fig. 4f). Mephedrone, methedrone, and methylone did not cause a decrease of $\Delta\psi_m$ at concentrations up to 1 mM (Fig. 4c–e). The uncoupler FCCP (9 µM) that we included as positive control reduced the $\Delta\psi_m$ by 42% (data not shown).

3.4. Effect on cellular oxygen consumption

A decrease in cellular ATP and $\Delta\psi_m$ can be caused by impairment and/or uncoupling of the mitochondrial respiratory chain (Felser et al., 2013; Krähenbühl, 2001). We therefore assessed the effect of

Table 1
Summary of the toxicity associated with the cathinones investigated. The concentrations (mM) indicated in Table correspond to the lowest concentration where a significant toxic effect was recorded. The data are obtained from Figs. 2–7. Where no concentration is listed, no significant toxicity was observed in the concentration range investigated.

	Bupropion (mM)	MDPV (mM)	Mephedrone (mM)	Methedrone (mM)	Methylone (mM)	Naphyrone (mM)
HepaRG cells						
Cytotoxicity	2					1
ATP depletion	1	2				1
HepG2 cells						
Cytotoxicity	2	2	2	2	2	0.5
ATP depletion	0.5	1	1	2		0.2
Decrease of $\Delta\psi_m$	0.5	1				0.2
Superoxide production	0.5	2	1			0.2
Lactate production	0.5	1	1			0.2
GSH depletion		2				0.2
Complex I disruption	0.5	1	1			0.2
Complex II disruption	0.5	1	1			0.2
Complex III disruption						0.2
Complex IV disruption						

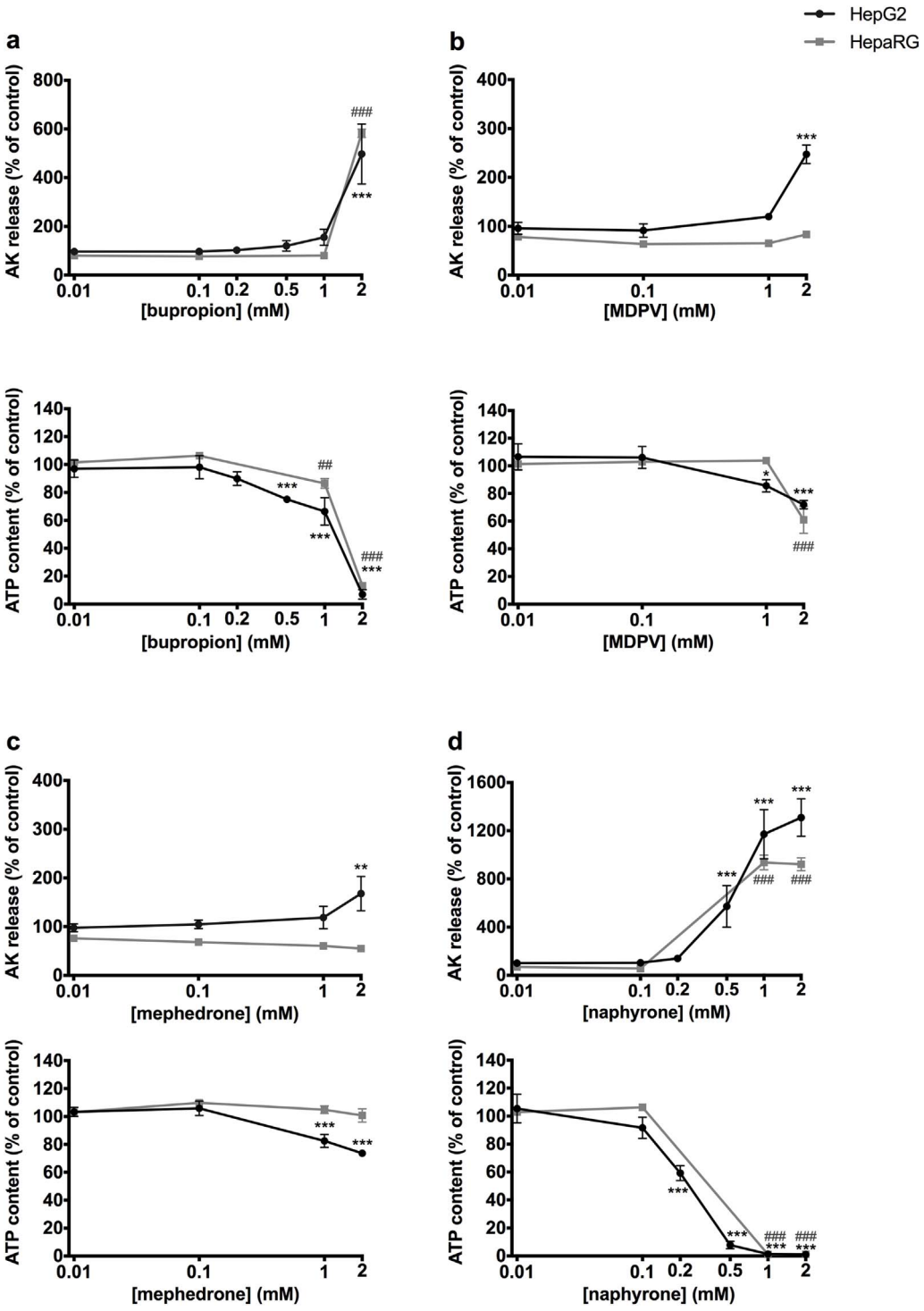

Fig. 2. Compounds depleting the cellular ATP pool at lower concentrations than induction of cytotoxicity.
Intracellular ATP content and cytotoxicity expressed as release of adenylate kinase after drug exposure for 24 h in HepG2 and non-induced HepaRG cells. Data are expressed as mean ± SEM of at least three independent experiments. Differences between control and test drugs were calculated with ANOVA followed by Dunett's test. Significance levels for HepG2 cells are given as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. Significance levels for HepaRG cells are given as $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$.

cathinones on the mitochondrial respiratory chain using a XF24 analyzer. Considering the observed decrease in the cellular ATP content at non-cytotoxic concentrations and/or decreased $\Delta\psi_m$, we tested bupropion, MDPV, mephedrone, and naphyrone at various concentrations (Fig. 5a–d). Bupropion caused an increase in leak respiration at 0.8 mM (Fig. 5a), suggesting uncoupling of oxidative phosphorylation. Mephedrone decreased the basal respiration at 1 mM and the maximal respiration at 0.5 and 1 mM (Fig. 5c). Naphyrone decreased the maximal respiration at 0.05 mM, 0.1 mM, and 0.2 mM (Fig. 5d). MDPV decreased the maximal respiration as well, but without reaching statistical significance (Fig. 5b).

As shown, methedrone and methylone neither decreased ATP at non-cytotoxic concentrations (Fig. 3a, b) nor did they disrupt $\Delta\psi_m$ (Fig. 4d, e). We therefore investigated the oxygen consumption of these substances only at the highest non-cytotoxic concentration (1 mM) and we did not find any difference compared to control incubations (data not shown).

We then determined the activity of the complexes of the mitochondrial electron transport chain for bupropion, MDPV, mephedrone, and naphyrone using a high-resolution respiratory system (Fig. 6a–h). Bupropion (0.5 mM), MDPV (1 mM), and mephedrone (1 mM) inhibited complex I and complex II activity (Fig. 6a–f). At 0.2 mM, naphyrone inhibited complex I, complex II, and complex III activity (Fig. 6g, h).

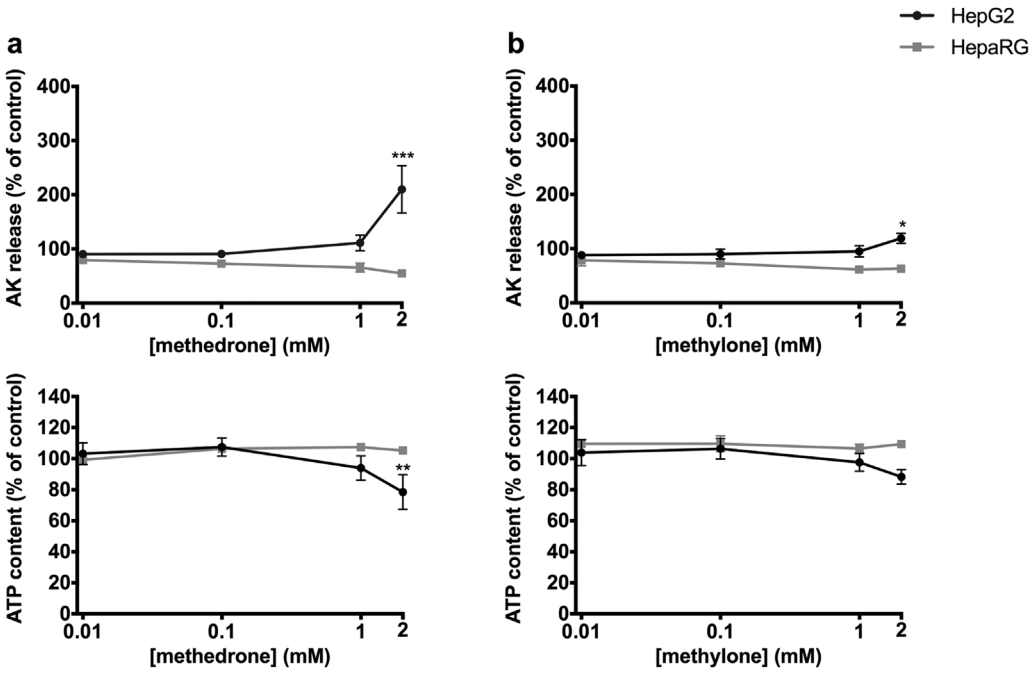

Fig. 3. Compounds inducing cytotoxicity at lower or at the same concentration than cellular ATP depletion.
Intracellular ATP content and cytotoxicity expressed as release of adenylate kinase after drug exposure for 24 h in HepG2 and non-induced HepaRG cells. Data are expressed as mean ± SEM of at least three independent experiments. Differences between control and test drugs were calculated with ANOVA followed by Dunett's test. Significance levels for HepG2 cells are given as *p < 0.05, **p < 0.01, ***p < 0.001.

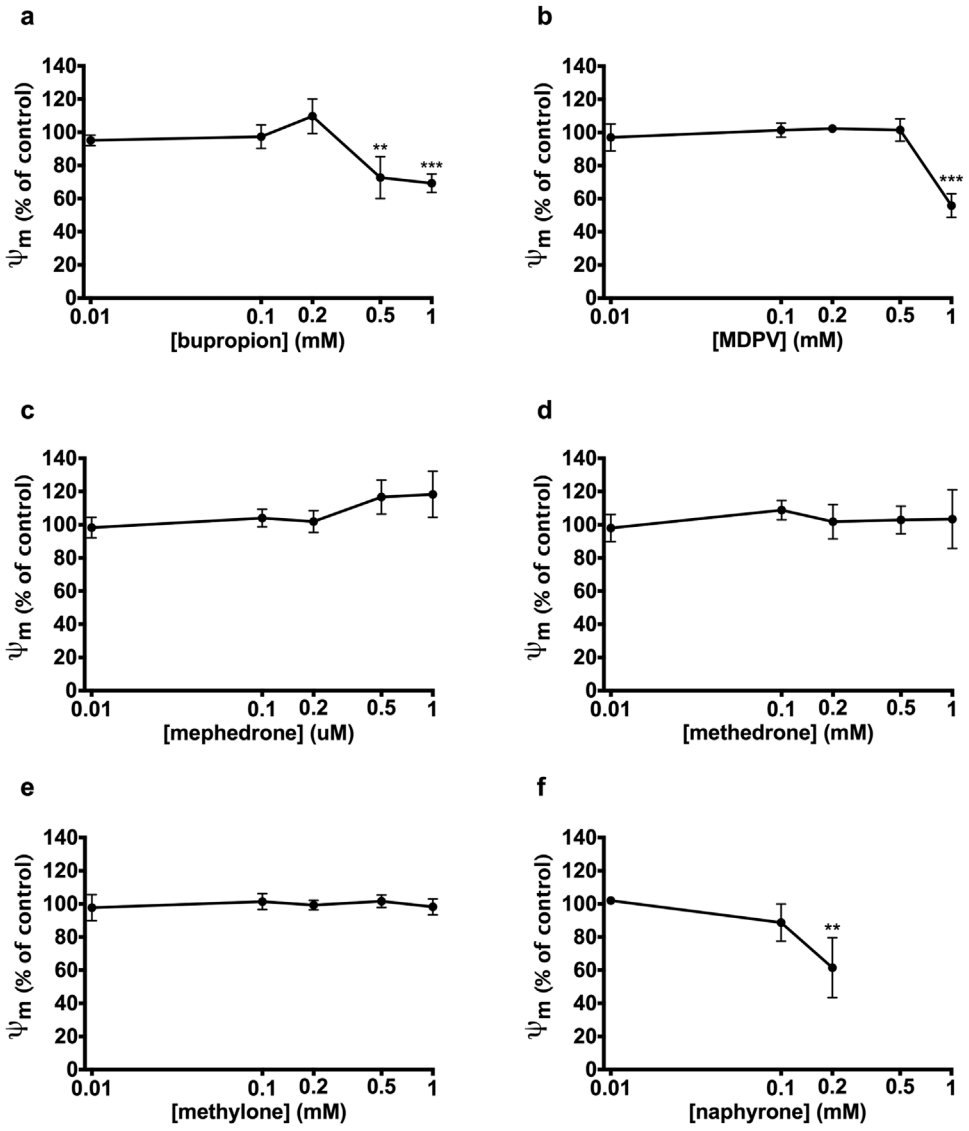

Fig. 4. Effect on mitochondrial membrane potential of HepG2 cells.
Mitochondrial membrane potential after 24 h drug exposure expressed as mean ± SEM of at least three independent experiments compared to vehicle control. Differences between control and test drugs were calculated with ANOVA followed by Dunett's test. Significance levels are given as **p < 0.01, ***p < 0.001.

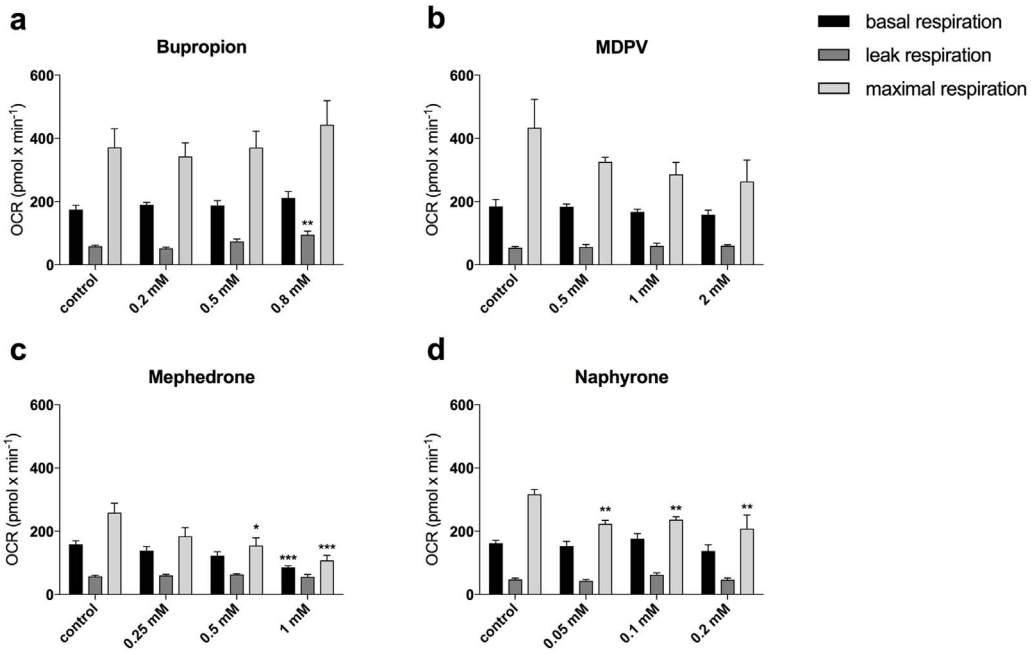

Fig. 5. Function of the respiratory chain in HepG2 cells.
Oxygen consumption rate (OCR) in HepG2 cells after 24 h drug exposure. Data are expressed as mean ± SEM of at least four independent experiments. Basal respiration, leak respiration, and maximal respiration were determined after drug treatment as specified in Methods and were compared to the respective vehicle control values. Statistical significance was calculated with ANOVA followed by Dunett's test and significance levels are given as *p < 0.05, **p < 0.01, ***p < 0.001.

3.5. Effect on lactate and ROS production and cellular GSH

Depending on its extent, the inhibition of the electron transport chain can have several metabolic consequences. Cells try to compensate the loss of mitochondrial ATP production by increasing glycolysis, resulting in increased lactate concentrations in the supernatant, the end product of glycolysis (Felser et al., 2014). In addition, inhibition of complex I and complex III of the electron transport chain can stimulate intra-mitochondrial ROS production (Dröse and Brandt, 2012). Accumulating superoxide anion is then metabolized by SOD2 to hydrogen peroxide, which can be reduced by gluthione peroxidase, leading to a decrease in reduced (GSH) and an increase in oxidized glutathione (GSSG). The cellular content of GSH is therefore a measure of oxidative stress (Rahman et al., 2006). Bupropion caused an accumulation of superoxide and lactate at 0.5 mM but without affecting cellular GSH (Fig. 7a). For MDPV, we detected increased lactate concentrations at 1 mM as well as increased superoxide levels which were accompanied by a decrease in GSH at 2 mM (Fig. 7b). Mephedrone stimulated lactate and superoxide production at 1 mM without a significant decrease in GSH up to 2 mM (Fig. 7c). Naphyrone stimulated lactate and superoxide production already at 0.2 mM with a simultaneous decrease in GSH (Fig. 7d). Buthionine sulfoximine (BSO), which we used as a positive control, decreased GSH to 14.1 nmol per mg protein, which corresponds to an 83% decrease compared to control incubations (data not shown).

4. Discussion

Our investigations demonstrate that in HepG2 cells all cathinones investigated were cytotoxic and, except methylone, decreased the cellular ATP content in a concentration-dependent fashion. Bupropion, MDPV, mephedrone, and naphyrone decreased the cellular ATP content at lower concentrations than cytotoxicity occurred, compatible with mitochondrial toxicity. For these substances, impairment of the mitochondrial respiratory chain could be demonstrated directly and/or indirectly (mitochondrial ROS production and lactate production) at similar concentrations as ATP depletion. In HepaRG cells, the compounds investigated were less toxic than in HepG2 cells, but with a similar pattern concerning cytotoxicity and ATP depletion. CYP induction by rifampicin in HepaRG cells did not incease cytotoxcitiy or depletion of the ATP pool. Thus, more active metabolite formation by CYPs did not enhance toxicity.

Regarding CYP induction, it is however important to be aware that CYP2D6 cannot be induced with rifampicin (Berger et al., 2016). CYP2D6 has been shown to be an important enzyme regarding the metabolism of synthetic cathinones (Helfer et al., 2015; Meyer et al., 2010; Negreira et al., 2015; Pedersen et al., 2013a,b) and it is therefore possible that the production of toxic metabolites may have been missed. For instance, the catechol metabolite of MDMA which is formed by CYP2D6, appears to be more hepatotoxic than the parent compound (Antolino-Lobo et al., 2011a; Carmo et al., 2006).

In accordance with our results, a recently published study has also shown that HepaRG cells are quite resistant to cytotoxicity elicited by synthetic cathinones, with EC_{50} concentrations > 2 mM and > 5 mM for MDPV and methylone, respectively, determined through the MTT reduction assay (Valente et al., 2016a). We therefore also used HepG2 cells for our investigations, as they represent a good *in vitro* model for studying the effect of toxicants on mitochondrial function (Felser et al., 2013; Kamalian et al., 2015). In the current study, cytotoxicity was observed in HepG2 cells for all test drugs in a concentration-dependent manner, which, with the exception of methylone, was accompanied by ATP depletion. For bupropion, MDPV, mephedrone, and naphyrone, ATP depletion was observed at lower concentrations than cytotoxicity, suggesting mitochondrial dysfunction (Felser et al., 2013; Kamalian et al., 2015). For bupropion, MDPV, and naphyrone, ATP depletion was accompanied by a decrease in $\Delta\psi_m$, further supporting the hypothesis that these compounds cause mitochondrial dysfunction. Since a decrease in $\Delta\psi_m$ can be due to impaired activity of the mitochondrial electron transport chain, we investigated the effects of the toxicants on oxidative metabolism of HepG2 cells in more detail. For bupropion, the cellular oxygen consumption in the presence of the complex I substrates L-glutamate and pyruvate revealed an increase in leak respiration, suggesting uncoupling of oxidative phosphorylation. For mephedrone, we observed a decrease in basal and maximal respiration and for naphyrone a decrease in maximal respiration, compatible with inhibition of the electron transport chain. For MDPV, there was a numeric decrease in maximal respiration, which did not achieve statistical significance due to a high variation in the control samples. MDPV was associated with an increase in mitochondrial ROS production and cellular generation of lactate, both indirect markers of a decreased function of the mitochondrial electron transport chain and mitochondrial ATP production (Felser et al., 2013, 2014). Using high-resolution

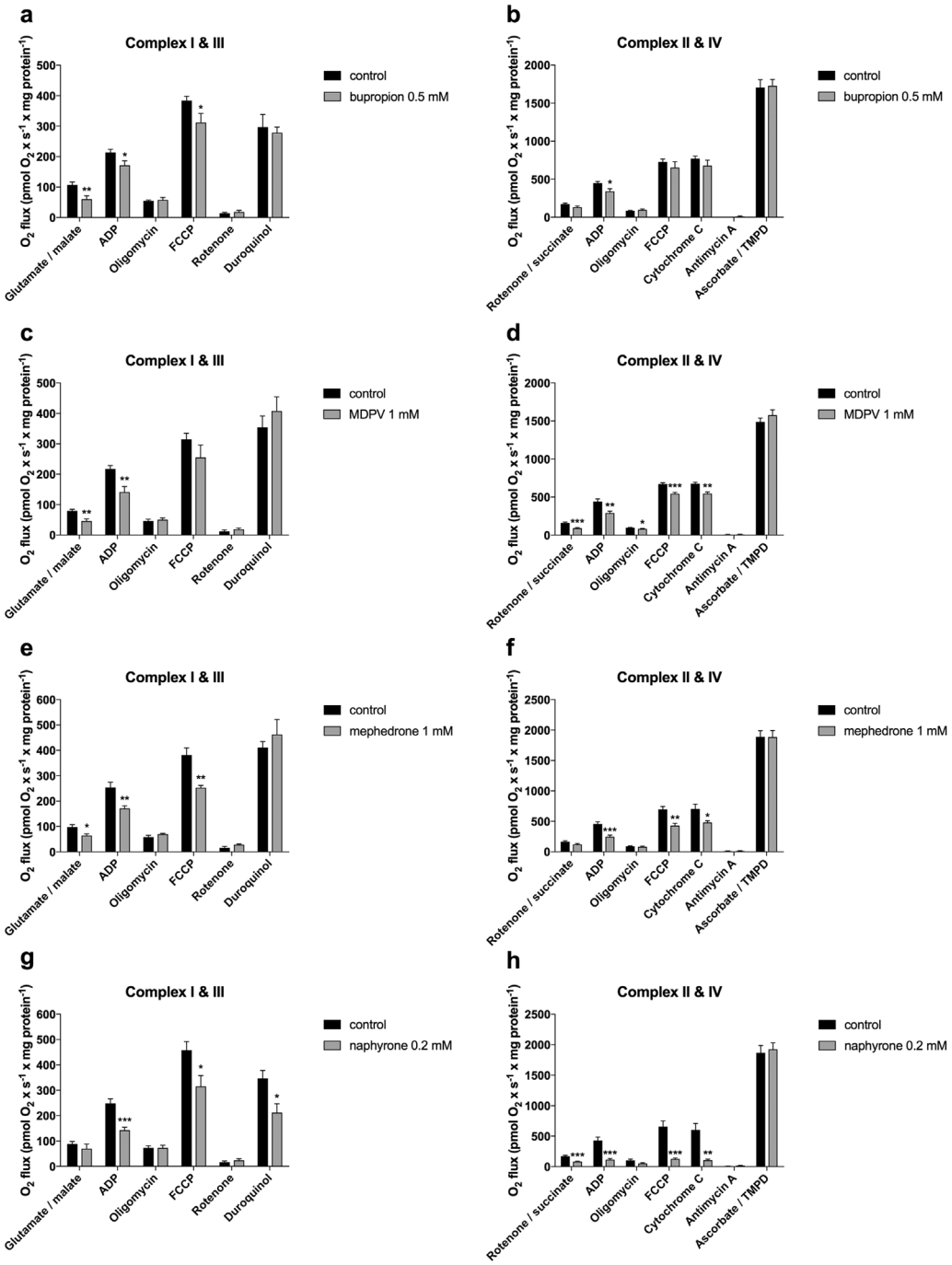

Fig. 6. Effect on the activity of the enzyme complexes of the mitochondrial electron transport chain measured using the Oxygraph-2k-high-resolution respirometer in HepG2 cells.
Respiratory capacities of HepG2 cells after 24 h treatment of bupropion (a, b), MDPV (c, d), mephedrone (e, f), and naphyrone (g, h). Data are expressed as mean ± SEM of at least six independent experiments. Treatment was compared to vehicle control with an unpaired two-tailed Student's *t*-test. Significance levels are given as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

respirometry, inhibition of complex I and II of the electron transport chain by bupropion, MDPV, mephedrone, and naphyrone and inhibition of complex III by naphyrone could be shown directly. In comparison, methedrone and methylone did not affect the enzyme complexes of the respiratory chain. Methedrone and methylone did not cause ATP depletion at non-cytotoxic concentrations either, nor did they alter $\Delta\psi_m$ or the cellular oxygen consumption at 1 mM. For methedrone and methylone, effects on mitochondria can therefore most likely not explain cytotoxicity.

The inhibition of complex I and III of the mitochondrial respiratory chain can be associated with increased mitochondrial ROS production (Dröse and Brandt, 2012), which can lead to a reduction of the mitochondrial and cellular GSH stores and opening of the mitochondrial membrane permeability transition pore (Green and Reed, 1998). In the current study, increased ROS production was observed for all mitochondrial toxicants (bupropion, MDPV, mephedrone, and naphyrone), but a significant decrease in the cellular GSH pool was only detected for MDPV and naphyrone. In support of our results, MDPV has previously been shown to stimulate ROS and RNS production and to deplete the cellular GSH stores in primary rat hepatocytes (Valente et al., 2016b).

A second consequence of the inhibition of the mitochondrial function is a shift in the ATP production from mitochondria to glycolysis with a concomitant increase in lactate production (Felser et al., 2014). As expected, the lactate concentration in the supernatant of the incubations containing one of the four compounds affecting mitochondrial function increased significantly, paralleling the increase in ROS production (Fig. 7).

Among the four compounds affecting mitochondrial function, bupropion has most often been associated with liver injury (Alonso

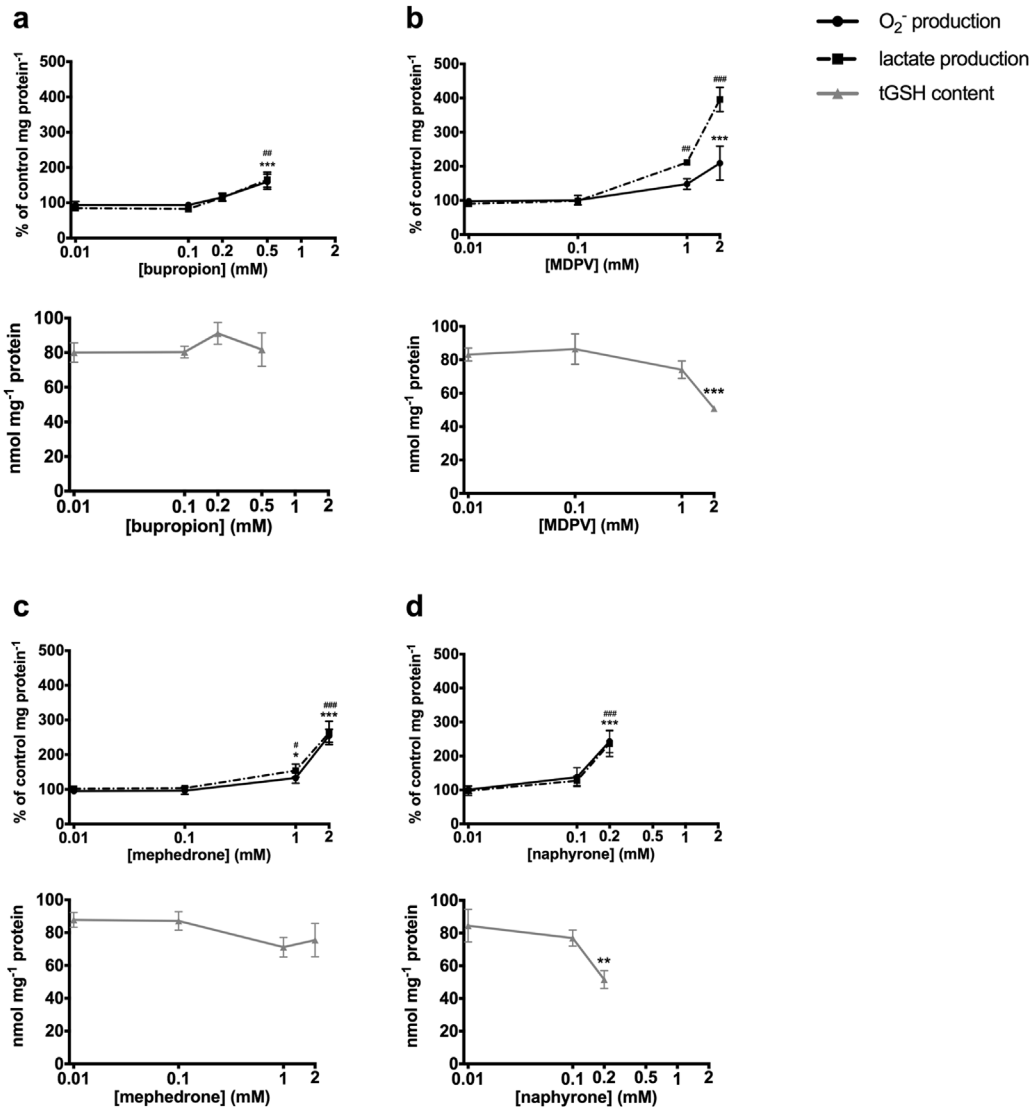

Fig. 7. Cellular stress and tGSH content in HepG2 cells.
Superoxide production, lactate concentrations and total GSH levels after 24 h drug treatment. Data are expressed as mean ± SEM of at least three independent experiments compared to vehicle control. Statistical significance was calculated with ANOVA followed by Dunett's test and significance levels are given as $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ for superoxide production and GSH and $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ for lactate production.

Rodriguez et al., 2010; Alvaro et al., 2001; Carlos Titos-Arcos et al., 2008; Hu et al., 2000; Humayun et al., 2007; Khoo et al., 2003). The results of the current study suggest that this may more likely be due to the broad use of this compound as a drug than due to a more pronounced mitochondrial toxicity compared to the other cathinones investigated. Bupropion was the only compound, however, that not only inhibited complex I and II of the electron transport chain, but also uncoupled oxidative phosphorylation, which may increase its toxicity.

For all compounds investigated, hepatic toxicity is a rare, but potentially life-threatening event. Certain factors may therefore increase the probability to develop liver injury when ingesting these compounds. One of these factors is the dose, as suggested by the concentration-dependent toxicity observed in the current study. Other factors reported in the literature suggested to increase the risk of liver toxicity include polydrug abuse and hyperthermia (Armenian et al., 2013; Borek and Holstege, 2012; Dias da Silva et al., 2013a,b; Green et al., 2004; Prosser and Nelson, 2012). CYP2D6 activity could also play a role, since, as discussed previously, the catechol metabolites of methylenedioxy cathinones (MDPV and methylone in the current study) may be more toxic than the respective parent compounds (Antolino-Lobo et al., 2011a; Carmo et al., 2006). CYP2D6 ultrarapid metabolizers may therefore be at risk for hepatotoxicity associated with these compounds. If mitochondrial dysfunction is a main toxicological mechanism, as suggested by the current study, also preexisting mitochondrial dysfunction may render patients more suceptible. This has been shown for severe hepatotoxicity induced by valproic acid, where preexisting mitochondrial dysfunction is an established suceptibility factor (Krahenbuhl et al., 2000; Stewart et al., 2010).

Blood concentrations determined in drug users or post mortem cases were typically lower than the toxic concentrations in our cellular assays (Cawrse et al., 2012; Cosbey et al., 2013; Torrance and Cooper, 2010; Wikström et al., 2010). However, a case with post-mortem mephedrone blood concentrations > 100 µM has been described (Torrance and Cooper, 2010) and in a series of methylone associated fatalities, drug concentrations in the liver were higher than in the blood, with an average liver-to-blood ratio of 2.68 (Cawrse et al., 2012). Moreover, HepG2 cells appear to be more robust to toxicants than human hepatocytes (Gerets et al., 2012) and the presence of suceptibility factors may shift the blood or liver concentration-toxicity curve to lower concentrations.

In conclusion, our investigations give a closer insight into the mechanism of cathinone-induced hepatotoxicity and demonstrate that bupropion, MDPV, mephedrone and naphyrone are associated with mitochondrial dysfunction due to interactions with enzyme complexes of the electron transport chain. Since liver injury is rare in persons ingesting these compounds, users with liver injury have to have risk factors rendering them more suceptible.

Conflict of interest

None.

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DISCUSSION

7 CONCLUDING REMARKS AND OUTLOOK

The pharmacological profiles of a variety of traditional and newly emerged drugs have been described within the scope of this thesis. The choice of substances to characterize was based on their popularity and frequency of use on the one hand, and the lack of available information on the other hand. The profiles of the investigated NPSs revealed how diverse the current drug market is.

Mephedrone was and still is one of the most popular designer stimulants; when it was put on the list of controlled substances in most countries, structurally and pharmacologically slightly different analogs emerged on the drug market, which have been described in chapter 2.1. The fatalities caused by 4-MA contaminated street amphetamine (Blanckaert et al., 2013) show the consequences that slight structural changes potentially can have. Amphetamine displays a distinct dopaminergic *vs.* serotonergic activity (Simmler et al., 2013), whereas the methylated analog 4-MA is a more potent 5-HT *vs.* DA reuptake inhibitor. As shown in chapter 4, DAT and SERT inhibition are inversely correlated. A combination of two drugs with distinct pharmacological profiles may therefore potentially dampen the reinforcing effects while increasing the adverse effects such as the hyperthermia observed in patients intoxicated with a combination of amphetamine and 4-MA (Blanckaert et al., 2013).

The MPH-based NPSs characterized in chapter 2.2 represent a group of compounds derived from MPH, a frequently prescribed stimulant for the treatment of attention-deficit/hyperactivity disorder (ADHD). As observed for MPH, designer drug analogs may potentially not only be used in a recreational setting (Bruggisser et al., 2011; Garland, 1998; Jaffe, 1991; Massello and Carpenter, 1999; Parran and Jasinski, 1991) but also as so-called “smart drugs” for cognitive enhancement (Arria et al., 2008; Beharry and Gibbons, 2016; Liakoni et al., 2015; Maier et al., 2013; Repantis et al., 2010). Unlike the prescription drug MPH, MPH-based NPSs reach the drug market often without being controlled substances, which makes the distribution and purchase via the Internet and other sources easier compared to MPH. The MPH-based designer drugs displayed pharmacological profiles similar to MPH and cocaine, but with a high range of transporter inhibition potencies. This shows one of the dangers in today's fast changing drug market: as new compounds constantly emerge, there is often only limited information on their effects and potency available, which puts users at risk of adverse effects or overdosing.

Phenethylamines with a 2,5-dimethoxy substitution pattern paired with a substituent at the 4-position (2C drugs) make up a large group of psychedelics. The profiles of 2C drugs

discussed in chapter 3.1 and 3.2 suggest that highest affinity and activation potency for the 5-HT_{2A} receptors are observed for 2C drugs with small lipophilic substituents at the 4-position. Compounds with bulky and lipophilic 4-substituents are potentially potent 5-HT_{2A} ligands as well but with decreased or no activation potency at this receptor, suggesting antagonistic behavior. The newly emerged NBOMe analogs discussed in chapter 3.1 turned out to be highly potent and compared to the relatively safe 2C drugs, NBOMes have been associated with increased clinical toxicity and numerous fatalities (Nichols, 2016; Nikolaou et al., 2015; Poklis et al., 2014; Rose et al., 2013; Suzuki et al., 2015).

In comparison to traditional drugs, increased potency and related adverse effects associated with various newly emerged designer drugs may put users at an increased health risk. This exemplifies the dilemma associated with the current designer drug crisis: many popular "legal highs" are eventually scheduled to prohibit the uncontrolled legal trade and use; however, controlled drugs will often just be replaced by new drugs, which are potentially more hazardous and for which there is usually even less information about risks and harms available. In recent years, in various countries efforts have been made to schedule NPSs not on an individual basis but based on structure class. This approach can help to decrease the use of novel compounds, as without the advantage of being legal, newly emerged compounds are often inferior to traditional illicit drugs in many aspects. In fact, since 2016 the number of compounds reported for the first time in the European Union seems to decrease (European Monitoring Centre for Drugs and Drug Addiction, 2018).

A limitation of the pharmacological investigations was the focus on monoaminergic transporters and receptors only, as possible interactions of the drugs with other targets could have been missed. Moreover, other factors such as bioavailability, route of administration, volume of distribution, or brain penetration can substantially influence the effects and clinical potency of a drug but these aspects were not accounted for in the *in vitro* assays described in this thesis. However, as shown in chapter 4, the *in vitro* monoamine transporter and receptor interaction data correlate well with reported human doses for stimulants and psychedelics and are therefore a valuable predictor for the clinical potency of the substances and may help in the scheduling process of authorities.

The toxicological investigations were focused on the liver, as it has been shown to be a target of stimulant toxicity (Andreu et al., 1998; De Carlis et al., 2001; Ellis et al., 1996; Garbino et al., 2001; Jones et al., 1994; Kamijo et al., 2002). However, the liver is just one of many organs that can be affected by stimulants, and the investigation of neurotoxicity, myotoxicity, nephrotoxicity, cardiotoxicity, and other kinds of toxicity would be needed for a

better assessment of the risks associated with new drugs. But since detailed toxicological investigations are time-consuming, comprehensive toxicological investigations for all novel compounds are currently not feasible due to the high rate of emergence of NPSs on the drug market. In chapter 6, the potential of several cathinones to induce mitochondrial damage in human hepatic cell lines have been discussed. Four of the six investigated cathinones displayed signs of mitochondrial toxicity and it is likely that other cathinones may elicit mitochondrial damage and liver injury as well. High-throughput *in vitro* methods and *in silico* models could be approaches to profile organ toxicity of NPSs in the future. Until then, knowledge on the toxicity of newly emerged drugs will continue to be derived mainly from case reports of clinical toxicity.

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APPENDIX

A LIST OF DRUGS

A.1 Stimulants

Aminoindanes

MMAI; 5-methoxy-6-methyl-2-aminoindane

***N*-methyl-2-AI;** *N*-methyl-2-aminoindane

Cathinones

2,3-DMMC; 2,3-dimethylmethcathinone

2,4-DMMC; 2,4-dimethylmethcathinone

3-MMC; 3-methylmethcathinone

3,4-DMMC; 3,4-dimethylmethcathinone

Mephedrone; 4-MMC; 4-methylmethcathinone

Phenethylamines

4-MA; 4-methylamphetamine

***d*-Methamphetamine**

MDMA; 3,4-methylenedioxymethamphetamine

Piperidines

3,4-Dichloroethylphenidate

3,4-Dichloromethylphenidate

4-Fluoromethylphenidate

4-Methylmethylphenidate

Ethylnaphthidate

Ethylphenidate

Isopropylphenidate

MPH; Methylphenidate

***N*-benzylethylphenidate**

Propylphenidate

Other

5-IT; 5-(2-aminopropyl)indole

Diclofensine

Methylmorphenate

Modafinil

A.2 Dissociatives

Diarylethylamines

Diphenidine

Methoxphenidine; 2-methoxydiphenidine

A.3 Psychedelics

Ergoline

LSD; lysergic acid diethylamide

Phenethylamines

25B-NBOMe; 2-(4-bromo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25C-NBOMe; 2-(4-chloro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25D-NBOMe; 2-(4-methyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25E-NBOMe; 2-(4-ethyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25H-NBOMe; 2-(2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25I-NBOMe; 2-(4-iodo-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25N-NBOMe; 2-(4-nitro-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25P-NBOMe; 2-(4-propyl-2,5-dimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25T2-NBOMe; 2-(2,5-dimethoxy-4-ethylthiophenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25T4-NBOMe; 2-(2,5-dimethoxy-4-isopropylthiophenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
25T7-NBOMe; 2-(2,5-dimethoxy-4-n-propylthiophenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine
2C-B; 4-bromo-2,5-dimethoxyphenethylamine
2C-C; 4-chloro-2,5-dimethoxyphenethylamine
2C-D; 2,5-dimethoxy-4-methylphenethylamine
2C-E; 2,5-dimethoxy-4-ethylphenethylamine
2C-H; 2,5-dimethoxyphenethylamine
2C-I; 4-iodo-2,5-dimethoxyphenethylamine
2C-N; 2,5-dimethoxy-4-nitrophenethylamine
2C-P; 2,5-Dimethoxy-4-propylphenethylamine
2C-T-1; 2,5-dimethoxy-4-methylthiophenethylamine
2C-T-2; 2,5-Dimethoxy-4-(ethylthio)phenethylamine
2C-T-3; 2,5-dimethoxy-4-(β-methallyl)thiophenethylamine
2C-T-4; 2,5-dimethoxy-4-isopropylthiophenethylamine
2C-T-7; 2,5-dimethoxy-4-propylthiophenethylamine
2C-T-16; 2,5-dimethoxy-4-allylthiophenethylamine
2C-T-19; dimethoxy-4-n-butylthiophenethylamine
2C-T-21.5; 2,5-dimethoxy-4-(2,2-difluoroethylthio)phenethylamine
2C-T-22: 2,5-dimethoxy-4-(2,2,2-trifluoroethylthio)phenethylamine
2C-T-25; 2,5-dimethoxy-4-isobutylthiophenethylamine,
2C-T-27; 2,5-dimethoxy-4-benzylthiophenethylamine
2C-T-28; 2,5-dimethoxy-4-(3-fluoropropylthio)phenethylamine
2C-T-30; 2,5-dimethoxy-4-(4-fluorobutylthio)phenethylamine
2C-T-31; 2,5-dimethoxy-4-(4-trifluoromethylbenzylthio)phenethylamine
2C-T-33; 2,5-dimethoxy-4-(3-methoxybenzylthio)phenethylamine
Mescaline; 3,4,5-Trimethoxyphenethylamine
Mescaline-NBOMe; 2-(3,4,5-Trimethoxyphenyl)-*N*-[(2-methoxyphenyl)methyl]ethanamine

B CURRICULUM VITAE

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Research history

10/2014 – 08/2018	**PhD at University of Basel, Psychopharmacology research group** *Summa cum laude*. PhD thesis project: Pharmacological and toxicological investigations of new psychoactive substances, supervised by Prof. Matthias Liechti and Prof. Stephan Krähenbühl.
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Institutional responsibilities

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Supervision of students/junior researchers

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Contributions to books

Luethi, D. and Liechti, M.E. *Clinical aspects related to methylphenidate-based new psychoactive substances*. Chapter in book *Novel Psychoactive Substances: What clinicians should know*. Routledge, New York, NY. ISBN: 9781315158082.

Oral contributions to conferences

Luethi, D., Hoener, M.C., Liechti, M.E. The dissociative new psychoactive substances diphenidine and methoxphenidine interact with monoaminergic transporters and receptors 38th International Congress of the European Association of Poison Centres and Clinical Toxicologists (EAPCCT), 22 – 25 May, Bucharest.

Zhou, X., Paech, F., **Luethi, D.**, Liechti, M.E., Krähenbühl, S. Psychoactive synthetic cathinones elicit mitochondrial myotoxicity by different mechanisms. 38th International Congress of the European Association of Poison Centres and Clinical Toxicologists (EAPCCT), 22 – 25 May, Bucharest.

Luethi, D., Brandt, S.D., Hoener, M.C., Liechti, M.E., 2017. Pharmacological profile of methylphenidate-based novel psychoactive substances. Fifth International Conference on Novel Psychoactive Substances, 23 – 24 October, Vienna.

Luethi, D., Krähenbühl, S., Liechti, M.E., 2016. Hepatotoxicity of novel psychoactive substances structurally related to 3,4-methylenedioxymethamphetamine (MDMA) and methamphetamine. 36th International Congress of the European Association of Poison Centres and Clinical Toxicologists (EAPCCT), 24 – 27 May, Madrid.

Poster contributions to conferences

Luethi D., Kolaczynska K.E., Rice, K.C., Blough, B.E., Baumann, M.H., Liechti, M.E., 2018. Phase I and II metabolites of methylenedioxy-substituted stimulants interact with human monoamine transporters. Inaugural Conference of the International Transmembrane Transporter Society (ITTS), 17 – 21 September, Vienna.

Luethi, D., Hoener, M.C., Liechti, M.E., 2017. Monoamine transporter and receptor interaction profiles of novel mephedrone analogs. Fifth International Conference on Novel Psychoactive Substances, 23 – 24 October, Vienna.

Luethi, D., Kaeser, P.J., Rickli, A., Hoener, M.C., Liechti, M.E., 2016. Monoamine transporter and receptor interaction profiles of novel phenethylamine derivatives. 29th international congress of the European College of Neuropsychopharmacology (ECNP), 17 – 20 September, Vienna.

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