Pharmacological characterization of 3,4-methylenedioxyamphetamine (MDA) analogs and two amphetamine-based compounds: \( N,\alpha\)-DEPEA and DPIA

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MDA; transporter; amphetamine; dopamine; serotonin; new psychoactive

Abstract
3,4-methylenedioxyamphetamine (MDA) is a psychoactive compound chemically related to the entactogen MDMA. MDA shares some of the entactogenic effects of MDMA but also exerts stimulant effects and psychedelic properties at higher doses. Here, we examined the pharmacological properties of MDA analogs and related amphetamine-based compounds detected in street drug samples or in sport supplements. We examined the key pharmacological mechanisms including monoamine uptake inhibition and release using human embryonic kidney 293 cells stably transfected with the respective human transporters. Additionally, we assessed monoamine

Abbreviations: 1-(2H-1,3-benzodioxol-5-yl)-1-methoxypropan-2-amine, 3C-BOH; 1-(4-fluoro-1,3-benzodioxol-5-yl)propan-2-amine, 2F-MDA; 1-(7-fluoro-1,3-benzodioxol-5-yl)propan-2-amine, 5F-MDA; 1-(6-fluoro-1,3-benzodioxol-5-yl)propan-2-amine, 6F-MDA; 3,4-methylenedioxyamphetamine, MDA; 5-hydroxytryptamine, 5-HT (serotonin); di-\(\beta\)-phenylisopropylamine, DPIA; \( N,\alpha\)-diethylphenylethylamine, \( N,\alpha\)-DEPEA; norepinephrine transporter, NET; serotonin transporter, SERT; dopamine transporter, DAT; \(\alpha\)-ethyl-3,4-methylenedioxyphenethylamine, BDB.

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1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA) is a substrate at presynaptic 5-HT and norepinephrine transporters (SERT and NET, respectively). Currently, MDMA remains among the most popular drugs of abuse due to its empathogenic effects. MDMA has also regained interest as potential therapeutic agent, due to its promising effects in drug-assisted therapy for post-traumatic stress disorder and its ability to enhance fear extinction (Hake et al., 2019; Mitlohefer et al., 2019; Mitchell et al., 2021). Metabolism of MDMA includes N-demethylation to 3,4-methylenedioxymethamphetamine (MDA) and O-demethylation to the catechol metabolites 3,4-dihydroxymethamphetamine (HHMA) and 3,4-dihydroxyamphetamine (HHA) (Kreth et al., 2000; de la Torre, Farré et al., 2004; Segura et al., 2005; Meyer et al., 2008). The catechol metabolites are subsequently O-demethylated by catechol-O-methyltransferase (COMT) (de la Torre, Farré et al., 2004; de la Torre, Yubero-Lahoz et al., 2012). MDA is an active substance on its own with a similar pharmacology to MDMA (Schindler et al., 2014; Baggott et al., 2019; Luethi et al., 2019). Nevertheless, users describe subjective differences in the experienced empathogenic effects and duration of drug action. Additionally, at higher doses MDA produces weak psychodelic-like effects (Baggott et al., 2019) likely via its agonistic activity at the serotonergic 5-HT2A receptor (Rickli, Kopf et al., 2015). This combination of psychoactive effects has made MDA an appealing recreational drug, which is sold on the black market either in its own right or as a constituent of ecstasy pills (Baggott et al., 2000).

Originally, MDA was briefly examined for its potential medical use but it never reached therapeutic applications (Smith Kline and French, 1957; Friedhoff et al., 1958; Naranjo et al., 1967). Numerous other structurally related MDMA and amphetamine analogs have since been synthesized and some of them have been investigated for therapeutic use. Furthermore, such analogs have become available on the recreational drug market as new psychoactive substances (NPS) during the last few decades.

In the present study, we assessed the monoamine transporter and receptor interaction profile of several hitherto only little investigated MDA and amphetamine analogs. Namely, we assessed the monoaminergic profile of the MDA derivatives 2-fluoro-MDA (2F-MDA), 5F-MDA, and 6F-MDA, β-methoxy-3,4-methylenedioxymamphetamine (3C-BOH), the MDA side chain homolog 1-(1,3-benzodioxol-5-yl)2-aminobutane (BBB), and the amphetamine derivatives Nα-diethylphenylethylamine (Nα-DEPEA) and di(β-phenylisopropyl)amine (DPIA). The assessed profiles will contribute to a better understanding of the effects and risks associated with these compounds.

2. Experimental procedures

2.1. Drugs

MDA, 2F-MDA, 5F-MDA, 6F-MDA, 3C-BOH (stereoisomeric composition not determined), BBB, Nα-DEPEA, and DPIA (stereoisomeric composition not determined) were racemates synthesized by ReChem (Burgdorf, Switzerland) and used in hydrochloride (HCl) form, with high-performance liquid chromatography (HPLC) purities of >98.5%. MDMA and amphetamine were racemates and obtained as hydrochlorides from Lipomed (Arlesheim, Switzerland), with HPLC purities >98.5%. [3H]-norepinephrine (10.0 Ci/mmol) and [3H]-amphetamine (45.4 Ci/mmol) were purchased from PerkinElmer (Schwerzenbach, Switzerland), while [3H]-5-HT (80.0 Ci/mmol) was purchased from Anawa (Zürich, Switzerland). Mazindol and fluoxetine HCl were obtained from Lipomed (Arlesheim, Switzerland); nisoxetine HCl was purchased from Sigma-Aldrich (Buchs, Switzerland).

2.2. Monoamine uptake transporter inhibition

Inhibition of the human norepinephrine, dopamine, and 5-HT transporters (NET, HDAT, and SERT, respectively) was assessed using stably transfected human embryonic kidney (HEK) 293 cells (In-vitrogen, Zug, Switzerland) as previously described (Luethi et al., 2018). In short, HEK 293 cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Life Technologies, Zug, Switzerland) containing 10% fetal bovine serum (FBS; Gibco) and 250 μg/mL Geneticin (Gibco). Once the cells reached a confluency of 70-90%, they were detached and then resuspended in Krebs-Ringer Bicarbonate Buffer (Sigma-Aldrich, Buchs, Switzerland) at a concentration of 3 × 10⁵ cells/mL. The uptake buffer used for the
[1H]-dopamine uptake experiments was additionally supplemented with 0.2 mg/mL ascorbic acid (Sigma-Aldrich, Buchs, Switzerland). For each monoamine uptake experiment, 100 μL of cell suspension was incubated in 25 μL buffer containing the test substances, vehicle control (dimethyl sulfoxide), or a transporter-specific inhibitor (10 μM nisoxetine [NET], mazindol [DAT], or fluoxetine [SERT]) for 10 min in round bottom 96-well plates at room temperature by shaking at 450 rpm (Thermomixer Comfort, Eppendorf, Hamburg, Germany). Monoamine uptake transport was triggered by adding 50 μL of either [1H]-norepinephrine, [1H]-dopamine, or [1H]-5-HT dissolved in uptake buffer at a final concentration of 5 nM for further 10 min. Next, 100 μL of the cell suspension mixture was transferred into 500 μL microcentrifuge tubes containing 50 μL of 3 M potassium hydroxide (Sigma-Aldrich) and 200 μL silicon oil (1:1 mixture of silicon oil type AR 20 and AR 200; Sigma-Aldrich). In order to terminate the uptake reaction, the cells were then centrifuged through the silicon oil during 3 min at 13,200 rpm. Directly after, the tubes were instantly frozen in liquid nitrogen. Next, the cell pellet was cut directly into 6 mL scintillation vials (PerkinElmer) containing 500 μL lysis buffer (5 mM EDTA, 0.05 M TRIS-HCl, 50 mM NaCl, and 1% NP-40). The vials were then shaken for 1 h at 700 rpm. Afterwards, 3 mL of scintillation fluid (Ultima Gold; PerkinElmer) was added to each vial. Monoamine uptake was measured using a Packard Tri-Carb Liquid Scintillation Counter 1900 TR. Nonspecific uptake in the presence of the selective inhibitors was subtracted from the total counts in order to determine the specific monoamine uptake.

To calculate IC50 values, the data were fitted by nonlinear regression to variable-slope sigmoidal dose-response curves using Prism software (version 8, GraphPad, San Diego, CA, USA). The DAT/SERT ratio was expressed as 1/IC50 : SERT IC50, where a ratio < 1 is indicative of a substance with stronger serotoninergic effects (more entactogenic and similar to MDMA) and a ratio of > 1 was indicative of stronger dopaminergic effects (more psychostimulant and similar to amphetamine).

2.3. Transporter-mediated monoamine efflux

Norepinephrine, dopamine, and 5-HT efflux was examined using HEK 293 cells (Invitrogen, Zug, Switzerland) stably transfected with hNET, hDAT, or hSERT, respectively. The cells were seeded at a concentration of 100,000 cells/well in poly-D-lysine coated XF24 cell culture microplates (Seahorse Biosciences, North Billerica, MA, USA) and cultured overnight. Next, the cells were exposed to 85 μL Krebs-HEPES buffer (130 mM NaCl, 1.3 mM KCl, 2.2 mM CaCl2, 1.2 mM MgSO4, 1.2 mM KH2PO4, 10 mM HEPES, 10 mM d-glucose, pH 7.5) containing 10 nM of [1H]-norepinephrine, [1H]-dopamine, or [1H]-5-HT, 1 μM unlabeled neurotransmitter for NET and DAT-mediated efflux, 10 μM pargyline, and 0.2 mg/mL ascorbic acid. The buffer was removed and the cells were washed twice before exposing them for 15 min (DAT and SERT) or 45 min (NET) to 100 μM of the test drugs dissolved in 1000 μL Krebs-HEPES buffer while on a rotatory shaker at 300 rpm and 37 °C. The substrate release was then stopped by washing the cells twice with ice-cold buffer and adding 50 μL of lysis buffer for 1 h Afterwards, 40 μL of the cell lysate was resuspended in 3 mL scintillation fluid (Ultima Gold) in 6 mL scintillation vials. The radioactivity inside the cells was measured using a Packard Tri-Carb Liquid Scintillation Counter 1900 TR. Non-specific release was subtracted from the total release to determine the specific transporter-mediated release. A single test drug concentration (100 μM) and the different release durations were chosen based on previously examined kinetic evaluations of the release-over-time curves (Hysek et al., 2012). A substance was classified as monoamine releaser if it produced monoamine efflux significantly higher than the nonspecific efflux. Statistical significance was determined by an ANOVA followed by Holm-Sidak’s test (P value < 0.05) for at least three independent experiments.

2.4. Radioligand receptor and transporter binding assays

The receptor and transporter binding was assessed by radioligand displacement as previously described (Luethi et al., 2018). In short, cell membrane preparations transfected with the respective transporters (human genes) or receptors (human genes, except for rat and mouse trace amine-associated receptor 1, [TAAR1]) were incubated with respective radiolabeled ligands at a concentration equal to the dissociation constant (KD). The following cell lines were used for the membrane preparations: HEK 293 cells (5-HT1A, 5-HT2A, 5-HT2C, TAAR1, D2, NET, DAT, SERT), Chinese hamster lung cells (α2A adrenergic receptor), and Chinese hamster ovary cells (α1A adrenergic receptor). Ligand displacement was measured for each test drug; to calculate the specific binding of the radioligand to the targets site, nonspecific binding (in the presence of a competitor in excess) was subtracted from total binding. The following radioligand and competitor pairs were used: 0.90 nM [3H]–8-hydroxy-2-(dipropylamino)tetratin (8-OH-DPAT) and 10 μM pindolol (5-HT1A receptor), 0.40 nM [3H]-ketanserin and 10 μM spiperone (5-HT2A receptor), 1.4 nM [3H]-mesulergine and 10 μM mianserin (5-HT2C receptor), 3.5 nM or 2.4 nM [3H]-RO5166017 and 10 μM RO5166017 (rat and mouse TAAR1, respectively), 0.11 nM [3H]-prazosin and 10 μM chlorpromazine (α1A adrenergic receptor), 2 nM [3H]-rauwolscine and 10 μM phenotamine (α2 adrenergic receptor), 1.2 nM [3H]-spiperone and 10 μM spiperone (dopaminergic D2 receptor), 2.9 nM N-methyl-[3H]-nisoxetine and 10 μM indatraline (NET), 1.5 nM [3H]-citalopram and 10 μM citalopram (SERT), 3.3 nM [3H]-WIN35,428 and 10 μM mirtazapine (DAT). The IC50 values of the radioligand binding experiments were extrapolated by calculating nonlinear regression curves for a one-site model using at least three independent 10-point dose-response curves for each substance. The binding affinity (Ki) or dissociation constant values were determined using the Cheng-Prusoff equation. Ki < 50 nM was defined as high affinity binding, Ki = 50–1000 nM as moderate affinity binding, and Ki > 1000 nM as low affinity binding.

2.5. Activity at the serotonin 5-HT3 and 5-HT2B receptors

The activity at 5-HT3A and 5-HT2B receptors was assessed as previously described by (Luethi et al., 2018). In short, the human 5-HT2A receptor was expressed in mouse embryonic fibroblasts (NIH-3T3 cells) and seeded into poly-D-lysine coated 96-well plates at a concentration of 70,000 cells per 100 μL. The cells were then incubated in HEPES-Hank’s Balanced Salt Solution (HBSS) buffer (Gibco) for 1 h at 37 °C, before 100 μL dye solution (Calcium 5 Assay Kit; Molecular Devices, Sunnyvale, CA, USA) was added for 1 h at 37 °C inside the fluorescence imaging plate reader [FLIPR]. Next, the plates were exposed to 25 μL test drugs diluted in HEPES-HBSS buffer and 250 mM probenecid online. The dose-response curves were fitted using nonlinear regression and EC50 values were determined.

The human 5-HT2B receptor was expressed in HEK 293 cells and seeded in poly-D-lysine coated 96-well plates at a concentration of 50,000 cells per well. The cells were incubated in high glucose DMEM containing 10% FBS (non-dialized, heat-inactivated), 250 mg/L Geneticin, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco) overnight at 37 °C. The growth medium was replaced by snap inversion and the cells were incubated with 100 μL calcium
indicator Fluo-4-solution (Molecular Probes, Eugene, OR, USA) for 45 min at 31 °C. Thereafter, the Fluo-4 solution was removed by snap inversion and 100 μL of fresh Fluo-4 solution was added and incubated for 45 min at 31 °C. The cells were washed with HBSS containing 20 mM HEPES using an EMBLA cell washer before being exposed to 100 μL of assay buffer. The plate was then inserted into the FLIPR and 25 μL of the test compounds diluted in assay buffer were added online. The dose-response curves were fitted using nonlinear regression and EC₅₀ values were determined. The efficacy (maximal activity) was calculated relative to 5-HT, which was defined as 100%. Activation efficacy (85% was defined as partial agonism while) 85% was defined as full agonism.

2.6. Activity at the human TAAR1

The activity at the human TAAR1 was examined as previously described (Luehli et al., 2018). In short, recombinant HEK 293 cells expressing the human TAAR1 were grown in culture flasks in high glucose DMEM composed of 10% heat inactivated FBS, 500 μg/mL Genetecin (Gibco, Zug, Switzerland), and 500 μg/mL hygromycin B at 37 °C and 5% CO₂. At 80-90% confluency, the cells were washed with PBS and detached with trypsin/EDTA solution (5 min at 37 °C). Afterwards, fresh medium was added (45 mL) and the cells were transferred into a 50 mL tube and centrifuged at 900 rpm for 3 min at room temperature. The supernatant was aspirated and the cell pellet was resuspended in medium to a concentration of 500,000 cells/mL. Next, 100 μL of cell suspension was transferred into a 96-well plate (BIOCOAT 6640, Becton Dickinson, Allschwil, Switzerland) and incubated for 20 h at 37 °C. Thereafter, the medium was aspirated and replaced with 50 μL PBS without Ca₂⁺ and Mg²⁺ ions. The PBS was removed by snap inversion and the plate was gently tapped against tissue. Next, 90 μL of Krebs-Ringer Bicarbonate buffer (Sigma-Aldrich) with 1 mM IBMX was added and the plate was incubated for 60 min at 37 °C and 5% CO₂. Test compounds were investigated in duplicate at a concentration range between 300 pM and 30 μM. Each 96-well plate contained a standard curve of cAMP concentrations ranging from 0.13 nM to 10 μM. An individual experiment was supplemented with a plate that contained the reference substances ROS526390, β-phenylethylamine, and ptyramine. Cells were introduced to either 30 μL of compound solution, basal control in PBS (containing 1 mM IBMX), or 30 μL of β-phenylethylamine (as maximal response) for 40 min at 37 °C. The cells were then lysed for 120 min at room temperature by exposing them to 50 μL of detection mix solution (composed of Ru-cAMP Alexa700 anti-cAMP antibody and lysis buffer) while under forceful shaking. The fluorescence and FRET signal were determined with a NanoScan reader (Innovative Optische Messtechnik, Berlin, Germany; 456 nm excitation wavelength; 630 and 700 nm emission wavelengths) using the following equation: FRET (700 nm) = P × FRET (630 nm), where P = Ru (700 nm) / Ru (630 nm).

3. Results

3.1. Monoamine uptake transporter inhibition

Functional monoamine uptake inhibition at the hNET, hDAT, and hSERT is shown in Figure 2. The corresponding IC₅₀ values are listed in Table 1. Similar to MDA, all fluorinated MDA analogs potently inhibited NET (IC₅₀ < 1 μM) and had a preference to inhibit 5-HT vs. dopamine uptake (DAT/NET ratio = 0.12–0.66). The β-methoxy and α-ethyl MDA analogs (3C-BOH and BDB) inhibited NET one order of magnitude less potently (IC₅₀ = 1.8 μM) when compared to MDA. Like MDMA and MDA, BDB preferentially inhibited 5-HT vs. dopamine uptake (DAT/SERT ratio = 0.15). In contrast, 3C-BOH showed a slight preference to inhibit dopamine vs. 5-HT uptake (DAT/SERT ratio = 2.3). The amphetamine analog N₃,N₁-dePEA potently inhibited NET (IC₅₀ = 0.25 μM) and like amphetamine, showed preference to inhibit dopamine vs. 5-HT uptake (DAT/SERT ratio = 6.2). In contrast, DPA was a weak NET inhibitor (IC₅₀ = 3.1 μM) and showed slight preference to inhibit 5-HT vs. dopamine uptake (DAT/SERT ratio = 0.55), thereby differing from amphetamine (DAT/SERT ratio = 49).

3.2. Monoamine efflux

The monoamine efflux observed at 100 μM drug concentration is shown in Figure 3. Like MDMA and MDA, all fluorinated MDA analogs were triple monoamine releasers at high concentrations with the exception of 6F-MDA, for which norepinephrine efflux was not significant (P > 0.05). In contrast, DPA did not significantly release any of the three monoamines, while 3C-BOH only induced 5-HT efflux. BDB induced 5-HT and dopamine efflux but not norepinephrine efflux. N₃,N₁-dePEA was a selective 5-HT releaser.

3.3. Monoamine receptor and transporter binding affinities and activation potencies

Serotonin receptor binding affinities and activation potencies of the investigated substances are shown in Table 2. The binding affinities (Kᵢ values) at other monoaminergic targets are shown in Table 3. Positive controls used in the receptor and transporter binding assays are listed in Supplementary Table S1. MDA, 6F-MDA, N₃,N₁-dePEA, and DPA showed low micromolar affinities for the 5-HT₁₆ receptor (Kᵢ = 3.5–9.0 μM), while 2F-MDA, 5F-MDA, 3C-BOH, and BDB showed no affinity at this receptor at investigated concentrations (Kᵢ > 5 μM). The majority of the test substances bound to the 5-HT₂₅ receptor in the range of 0.94–10 μM with 6F-MDA being the most potent substance. For N₃,N₁-dePEA and amphetamine no affinity could be determined at investigated concentrations (Kᵢ > 12 μM). However, only MDA, 2F-MDA, 5F-MDA, and 3C-BOH activated the receptor with EC₅₀ values in the range of 1.7–17 μM, either as partial agonists (Aᵢ = 53–80%) or in the case of 3C-BOH as full agonist (Aᵢ = 103%). MDA, 2F-MDA, 6F-MDA, and 3C-BOH additionally activated the 5-HT₂₅ receptor in the submicromolar range (EC₅₀ = 0.2–0.32 μM) as partial agonists (Aᵢ = 40–58%), while the remaining drugs did not activate the receptor. MDA, the fluorinated MDA analogs, and 3C-BOH bound with low micromolar affinities to the 5-HT₂₃ receptor (Kᵢ = 2.2–6.3 μM). N₃,N₁-dePEA, DPA, and BDB did not bind to the 5-HT₂₃ receptor (Kᵢ > 5.1 μM).

None of the MDA analogs bound to the monoamine uptake transporters at investigated concentrations except for 3C-BOH, which showed low micromolar binding to NET and DAT (Kᵢ = 7.6 and 2.0 μM, respectively). Similarly, N₃,N₁-dePEA showed low micromolar binding to NET (Kᵢ = 1.5 μM) and submicromolar binding affinity at DAT (Kᵢ = 0.5 μM). DPA bound to all three monoamine transporters in the low micromolar range (Kᵢ = 1.8–2.6 μM). None of the drugs bound
to the D₂ receptor ($K_i > 13 \mu M$). MDA bound to the α₁A receptor with affinity of 8.7 μM, whereas none of its analogs interacted with the receptor at investigated concentrations ($K_i > 8.8 \mu M$). On the other hand, Nₐα-DEPEA showed low micromolar binding affinity ($K_i = 4.1 \mu M$) and DPA even submicromolar affinity ($K_i = 0.12 \mu M$) at the α₁A receptor. The test compounds bound in the low micromolar range to the α₁A receptor ($K_i = 0.95-4.6 \mu M$) with the exception of BDB, which did not bind at investigated concentrations ($K_i > 4.7 \mu M$). All of the MDA analogs interacted with the rat and mouse TAAR1 in the submicromolar or low micromolar range ($K_i = 0.07-3.8 \mu M$). Nₐα-DEPEA and DPA bound to the rat TAAR1 only ($K_i = 1.5-3.5 \mu M$). The only substances to activate the human TAAR1 were the fluorinated MDA analogs and 3C-BOH ($EC_{50} = 6.5-11 \mu M$).

| Table 1 | Monoamine uptake inhibition. |
|-----------------|-----------------|-----------------|-----------------|-----------------|
|                | NET   | DAT   | SERT  | Dat/SERT ratio |
| **MDA analogs** |       |       |       |                |
| MDA             | 0.15  | 12    | 4.2   | 0.36           |
| 2F-MDA          | 0.35  | 33    | 6.4   | 0.19           |
| 5F-MDA          | 0.34  | 21    | 2.5   | 0.12           |
| 6F-MDA          | 0.42  | 23    | 15    | 0.66           |
| 3C-BOH          | 1.8   | 8.9   | 21    | 2.3            |
| BDB             | 1.8   | 36    | 5.6   | 0.15           |
| **Amphetamine analogs** |       |       |       |                |
| Nₐα-DEPEA       | 0.25  | 2.5   | 15    | 6.2            |
| DPA             | 3.1   | 18    | 10    | 0.55           |
| **Comparator substances** |       |       |       |                |
| d-Amphetamine   | 0.092 | 1.4   | 68    | 49             |
| MDA             | 0.40  | 16    | 1.9   | 0.11           |

4. Discussion

4.1. Inhibition and release properties of the MDA analogs at monoamine uptake transporters

MDA inhibited all three monoamine transporters with the highest inhibition potency observed at NET and slight selectivity for SERT vs. DAT (DAT vs. SERT ratio = 0.36). MDA induced the efflux of all three monoamines, thus confirming its previously reported classification as a non-selective monoamine substrate-type releaser in vitro and in vivo (Baumann et al., 2007; Rickli, Kopf et al., 2015; Sandtner et al., 2016). In contrast to transporter blockers (e.g., cocaine), which mediate their effects by binding to the transporter, substrate-type releasers like MDA are transported into the cells and cause the reversal in the direction of the transporter (Rothman and Baumann 2003; Sitte and Freimuth 2015). This profile suggests psychoactive properties related to both MDMA and d-amphetamine and mixed entactogenic-stimulant like subjective effects in humans. Whether MDA is indeed more stimulant-like than MDMA in humans remains to be determined in a head-to-head comparison (Baggott et al., 2019).

Introduction of fluorine atoms onto the structure of psychoactive substances is used as a medicinal chemistry concept on different phenethylamine templates, including MDA analogs (Trachsel et al., 2006; Trachsel 2012; Trachsel et al., 2013). This was investigated in order to attenuate or strengthen their psychoactive effects, to change duration of action, modify their metabolic stability, lipophilicity, acidity/basicity, and their interaction with molecular targets (Shulgin and Shulgin 1991; Böhm et al., 2004; Trachsel 2012; Luethi and Liechti 2020). For the compounds investigated herein, fluorination slightly decreased the activity at all three monoamine transporters (2- to 4-fold decrease). One exception to this was a 2-fold increased potency observed for 5F-MDA at SERT. Moreover, fluorination did overall not affect the monoamine-releasing properties of the derivatives. As for MDMA and MDA, the higher SERT vs. DAT selectivity suggests subjective MDMA-like entactogenic effects and reduced abuse liability for the fluorinated MDA analogs (Rothman and Baumann 2006; Baumann et al., 2011). Fluorine-containing analogs of MDMA and MDA (i.e., difluorinated methylenedioxy moiety, DF-MDMA and DF-MDA) have previously been prepared and discussed in order to examine the potential of reducing the formation of neurotoxic metabolites (Trachsel et al., 2006). The primary investigations of DF-MDA revealed a weaker binding affinity at SERT (Walline et al., 2008) and inactivity in humans beyond the range observed for the fluoro-free MDA (Shulgin and Shulgin 1991). The MDA analogs 2F-MDA, 5F-MDA, and 6F-MDA have also been examined at the monoamine uptake transporters in vitro where they bound to the transporters in the high nanomolar range (Trachsel, 2012). In the present study, none of the fluorinated MDA analogs bound to any monoamine transporter at investigated concentrations ($K_i > 7.4 \mu M$), which is in support of their substrate-type releaser profiles. The reason for the differences between the individual studies may be different experimental approaches (Illic et al., 2020). Furthermore, for transporter substrates the functional transporter inhibition is a better predictor of their clinical potency compared to transporter binding (Luethi and Liechti 2018).

Compared to MDA, 3C-BOH displayed a decreased activity at monoamine transporters; it induced 5-HT efflux and bound to NET and DAT in the micromolar range. 3C-BOH has been described to have amphetamine-like ef-
Table 2  Serotonin receptor binding affinities and activation potencies.

| MDA analogs | h5-HT1A | h5-HT2A | h5-HT2B | h5-HT2C | Selectivity |
|--------------|---------|---------|---------|---------|-------------|
| MDA          | 9.0 ± 0.8 | 3.2 ± 0.8 | 1.7 ± 0.4 | 57 ± 5 | 4.8 ± 0.4 | 2.8 | 1.5 |
| 2F-MDA       | > 5.5   | 4.5 ± 1.2 | 7.4 ± 0.4 | 80 ± 3 | 3.2 ± 0.5 | >1 | 0.7 |
| 6F-MDA       | > 5.5   | 7.9 ± 1.2 | 17 ± 3 | 53 ± 5 | 6.3 ± 1.6 | NA | 0.8 |
| 3C-BOH       | > 5.5   | 10 ± 2 | 11 ± 2 | 103 ± 8 | 4.4 ± 1.0 | NA | 0.4 |
| BDB          | > 17    | 8.2 ± 1.6 | >20    | >10   | >14 | >1 | 1 |

| Amphetamine analogs | h5-HT1A | h5-HT2A | h5-HT2B | h5-HT2C | Selectivity |
|---------------------|---------|---------|---------|---------|-------------|
| Nₐ,α-DEPEA         | 5.9 ± 1.9 | >12     | >10     | >10     | >5.1 | NA | NA |
| DPIA                | 3.5 ± 0.3 | 4.2 ± 1.1 | >10    | >10     | >5.1 | NA | 0.8 |

| Comparator substances | h5-HT1A | h5-HT2A | h5-HT2B | h5-HT2C | Selectivity |
|-----------------------|---------|---------|---------|---------|-------------|
| d-Amphetamine        | 6.7 ± 1.4<sup>c</sup> | >13<sup>c</sup> | 9.4 ± 1.6<sup>b</sup> | >13<sup>c</sup> | NA | >1 |
| MDMA                  | 11 ± 2<sup>a</sup> | 6.3 ± 2.4<sup>b</sup> | 6.1 ± 0.3<sup>b</sup> | 4.4 ± 0.8<sup>a</sup> | 1.7 | 0.7 |

*Ki* and *EC₅₀* values are given as mean ± SD.

Data taken from the following publications: <sup>a</sup>Luethi et al. (2019), <sup>b</sup>Rickli, Kopf et al. (2015), <sup>c</sup>Simmler et al. (2013).

NA, not assessed.
Table 3  Monoamine transporter and receptor binding affinities.

|                | hNET | hDAT | hSERT | hD₂ | hα₁A | hα₂A | hTAAR1 | rTAAR1 | mTAAR1 |
|----------------|------|------|-------|-----|------|------|--------|--------|--------|
|                | Kᵢ ± SD [µM] | Kᵢ ± SD [µM] | Kᵢ ± SD [µM] | Kᵢ ± SD [µM] | Kᵢ ± SD [µM] | Kᵢ ± SD [µM] | EC₅₀ ± SD | E_max ± SD | Kᵢ ± SD [µM] | Kᵢ ± SD [µM] |
| **MDA analogs** |      |      |       |     |      |      |        |        |        |        |
| MDA            | >8.8 | >8.5 | >7.4  | >13 | 8.7 ± 0.6 | 2.6 ± 0.1 | >30     | 0.22 ± 0.03 | 0.18 ± 0.03 |
| 2F-MDA         | >8.7 | >8.5 | >7.4  | >14 | >8.8 | 1.6 ± 0.1 | 6.5 ± 1.9 | 32 ± 4 | 0.09 ± 0.01 | 0.07 ± 0.03 |
| 5F-MDA         | >8.7 | >8.5 | >7.4  | >14 | >8.8 | 1.7 ± 0.1 | 9.3 ± 2.0 | 17 ± 3 | 0.27 ± 0.04 | 0.21 ± 0.13 |
| 6F-MDA         | >8.7 | >8.5 | >7.4  | >14 | >8.8 | 3.6 ± 0.4 | 11 ± 2   | 21 ± 2 | 0.20 ± 0.03 | 0.22 ± 0.01 |
| 3C-BOH         | 7.6 ± 1.0 | 2.0 ± 0.2 | >7.4 | >14 | >8.8 | 4.6 ± 0.4 | 9.9 ± 3.7 | 8.1 ± 2.5 | 0.35 ± 0.07 | 1.7 ± 0.6 |
| BDB            | >8.7 | >8.5 | >7.4  | >13 | >8.8 | >4.7   | >30     | 0.64 ± 0.12 | 3.8 ± 0.5  |

**Amphetamine analogs**

|                |      |      |       |     |      |      |        |        |        |
|----------------|------|------|-------|-----|------|------|--------|--------|--------|
| Nₐα-DEPEA      | 1.5 ± 0.5 | 0.5 ± 0.1 | >7.4 | >13 | 4.1 ± 0.4 | 4.3 ± 0.5 | >30     | 3.5 ± 1.2 | >4.4     |
| DPIA           | 1.8 ± 0.5 | 2.3 ± 0.2 | 2.6 ± 0.1 | >13 | 0.12 ± 0.01 | 0.95 ± 0.08 | >30     | 1.5 ± 0.6 | >4.4     |

**Comparator substances**

|                |      |      |       |     |      |      |        |        |        |
|----------------|------|------|-------|-----|------|------|--------|--------|--------|
| d-Amphetamine  | 1.0 ± 0.6c | 5.7 ± 3.8c | >25c | >30c | >6.0c | 2.8 ± 0.8c | 2.8 ± 0.8b | 91 ± 15b | 0.23 ± 0.18c | 0.09 ± 0.06c |
| MDMA           | >8.7a | >8.5a | >7.4a | >13a | 6.9 ± 1.2a | 4.6 ± 1.1a | 35 ± 21b | 26 ± 8b  | 0.25 ± 0.01a | 3.1 ± 0.7a   |

Kᵢ and EC₅₀ values are given as mean ± SD.
Data taken from the following publications: aLuethi et al. (2019), bSimmler et al. (2016), cSimmler et al. (2013).
NA, not assessed.
fects in humans at doses of 40–70 mg, with a duration of 3–6 h (Trachsel et al., 2013). 3C-BOH can exist as four stereoisomers; the pharmacological assessment of each individual isomer is therefore necessary to gain insight into their effects. BDB inhibited SERT more potently than DAT (DAT/SERT ratio = 0.15) and induced efflux of dopamine and 5-HT, indicating a profile similar to MDA and MDMA, although with overall weaker potency. BDB (also known as MDB or J) was initially synthesized by Alexander Shulgin and is reported to induce entactogenic effects (Shulgin and Shulgin, 1991). This α-ethyl analog of MDA is also formed by N-demethylation of N-methyl-1,3-benzodioxolylbutamine (MBDB), a controlled designer drug that causes similar but milder MDMA-like psychoactive effects (Nichols 1986; Kronstrand 1996). Previously, BDB was detected in ecstasy tablets and urine of drug users; however, it is unclear whether the BDB detected in urine samples was a metabolic product or whether it was originally present in the ingested tablets (Kronstrand 1996). In vivo, BDB induces psychoactive effects similar to stimulants and psychedelics (Bronson, Jiang et al., 1995a; Bronson, Jiang et al., 1995b). BDB and MBDB have a similar pharmacological profile (Simmler et al., 2013), indicating that the effect of the N-demethylation does not substantially alter pharmacological profile similar as observed for MDMA and its N-demethylated analog MDA (Luethi et al., 2019). The results of this study are in line with previous reports of Nagai et al. (2007) who showed that BDB induces norepinephrine and 5-HT efflux more potently than dopamine efflux. Montgomery et al. (2007), showed that BDB inhibited NET and SERT equipotently (IC₅₀ = 39.5 µM and 37 µM, respectively) in two different cell lines expressing rat transporters. However, when compared to MBDB, BDB was a significantly more potent inhibitor of SERT which is in contrast to our results (Montgomery et al., 2007). The differences can in part be explained by the use of rat vs. human monoamine transporters and different assay procedures (Barker and Blakely 1996; Ilic et al., 2020). User reports support the described pharmacological profile of BDB, which reportedly produces mild entactogenic MDMA-like psychoactive effects (Shulgin and Shulgin 1991).

4.2. Inhibition and release properties of the amphetamine analogs at monoamine uptake transporters

N₁,α-DEPEA is an amphetamine analog that was patented in 1988 and was later identified in material seized for drug trafficking (Lee et al., 2014). Furthermore, it has been detected as an ingredient in various sport supplements like “Craze” or “Detonate” (Cohen et al., 2014; Lee et al., 2014; Uralets et al., 2014; Wojtowicz et al., 2015). This has led the FDA to remove N₁,α-DEPEA-containing supplements from the market. Furthermore, N₁,α-DEPEA was detected in urine samples of professional competitors (Uralets et al., 2014) and was therefore prohibited by the World Anti-Doping Agency (WADA). In vitro, N₁,α-DEPEA differs in various aspects from the structurally related amphetamine. While both substances potently inhibited norepinephrine uptake, N₁,α-DEPEA was less dopaminergic than amphetamine (DAT/SERT ratio > 10) (Simmler et al., 2013). Moreover, as a transporter blocker, N₁,α-DEPEA also bound with the highest affinity to NET and DAT. This finding is in line with previous studies that showed a correlation between functional inhibition and binding affinity for transporter blockers (Langer et al., 1980; Lee et al., 1982; Javitch et al., 1984; Schoemaker et al., 1985; D’Amato et al., 1987; Cheetham et al., 1996). At SERT, N₁,α-DEPEA behaved as a substrate-type releaser. Furthermore, unlike amphetamine (Rickli, Hoener et al., 2015), N₁,α-DEPEA did not mediate significant norepinephrine and dopamine efflux. The reduced substrate activity at cat-echolaminergic transporters might be due to steric hindrance by the two ethyl groups. Significantly decreased potency of α-ethyl vs. α-methyl-substituted phenethylamines was previously reported (van der Schout, Ariens et al. 1962; Shulgin and Shulgin 1991). For instance, van der Schout, Ariens et al. (1962) reported that α-ethylamphetamine does not affect locomotion in mice, indicating a decreased potency compared to amphetamine. However, N₁,α-DEPEA may have similar adverse effects as methamphetamine in vivo, such as a high addiction potential, withdrawal symptoms after drug cessation, or cardiovascular and dental toxicity (Kaye et al., 2007; Shetty et al., 2010; Simmler et al., 2013). Furthermore, N₁,α-DEPEA is a weak inhibitor of monoamine oxidase A (MAO-A).

DPIA is a known byproduct and impurity produced in the synthesis of amphetamine via the Leuckart reaction (Blachut et al., 2001). Furthermore, research indicates that DPIA is possibly transformed to amphetamine in vivo (Ottaviano et al., 2002). Reported to the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) in the early 2000s (King 2014), DPIA has been found in seized methamphetamine or amphetamine tablets (Lomonte et al., 1976; Huizer et al., 1985; Ottaviano et al., 2002). However, DPIA’s pharmacological and toxicological properties in humans remain unknown. In rodents, DPIA has been reported to induce similar but less potent central effects and toxicity as amphetamine (Ketema et al., 1990). Opposite to the observed amphetamine-induced cardiovascular effects, DPIA caused a decrease in heart rate and blood pressure. Furthermore, Ketema et al. (1990) showed that DPIA toxicity was comparable to amphetamine and that a combination of both displayed subadditive effects. The authors therefore hypothesized that the presence of DPIA in amphetamine pills would not lead to an enhanced toxicity compared to pure amphetamine (Ketema et al., 1990). The decreased potency compared to amphetamine is reflected by decreased activity at monoamine transporters. Specifically, DPIA acts as transporter blocker, interacting 34-fold less potently with NET and 13-fold less potently with DAT when compared to amphetamine. At SERT, however, DPIA is 7-fold more potent than amphetamine but still not as potent as serotonergic amphetamines such as MDMA. It is important to note that interpretation of the data is complicated and to be related to the fact that DPIA can exist in a total of three stereoisomers (an enamioneric pair [RR/SS] and one meso compound [RS]); each isomer is most likely to show a specific interaction with the targets investigated. Our sample was prepared by a non-stereoselective reductive amination using racemic amphetamine and phenylacetone, potentially leading to a distribution of stereoisomers. Since no determination of stereoisomeric composition was performed, our
data has to be viewed with a certain amount of caution. Nevertheless, the DPIA used in this study is likely representative of DPIA found in black market samples since it is probable that non-stereoselective conditions also apply for the illegal preparation of amphetamine.

4.3. Serotonin receptor binding affinities and activation potencies

MDA, 6F-MDA, \(N,\alpha\text{-DEPEA}\), and DPIA exhibited moderate affinity \(K_i = 3.5-9.0 \mu M\) at the 5-HT\(_{2A}\) receptor, which may contribute to the facilitation and/or dampening of addiction behaviors associated with these substances (Muller et al., 2007). The 5-HT\(_{2A}\) receptors are the primary sites responsible for psychedelic effects induced by potent 5-HT\(_{2A}\) receptor agonists like LSD or psilocybin (Nichols 2004; Preller et al., 2017; Madsen et al., 2019; Luethi and Liechti 2020; Holze et al., 2021; Rudin et al., 2021). A recent study confirmed that MDA indeed produces a mix of psychedelic and MDMA-like effects in healthy volunteers and with a greater duration of action than MDMA (Baggott et al., 2019). It is suggested that MDA could be a potential therapeutic agent used in drug-substance-assisted psychotherapy similar
Fig. 3 Monoamine efflux induced by 100 μM of drugs in preloaded HEK 293 cells expressing hNET, hDAT, or hSERT. The nonspecific “pseudo-efflux” that arises from monoamine diffusion and subsequent reuptake inhibition was defined in presence of transporter-specific inhibitors. Compounds which exhibited significantly more monoamine efflux (*P < 0.05) than pure uptake inhibitors were classified as monoamine transporter substrates. The data are presented as mean ± SEM from at least three independent experiments.

to MDMA and psychedelics (Baggott et al., 2019). The clinical potential of the partial 5-HT₂A receptor agonists 2F-MDA and 5F-MDA has hitherto not been studied and therefore remains unknown. It should be noted that the in vitro activation potency at the 5-HT₂A receptor measured by calcium mobilization poorly predicts the hallucinogenic potency in humans; binding affinity for agonists appears to be a better predictor of human doses that mediate psychedelic effects (Luethi and Liechti 2018).

At the 5-HT₂B receptor, 2F-MDA, 6F-MDA, and 3C-BOH exhibited submicromolar activation potency as partial agonists. Chronic 5-HT₂B receptor activation by amphetamine-type stimulants has previously been linked to an increased risk of developing valvulopathies or cardiopulmonary ad-
verse effects (Fitzgerald et al., 2000; Rothman et al., 2000). The observed potent receptor activation by 2F-MDA, 6F-MDA, and 3C-BOH suggests that these compounds may have the potential to induce such 5-HT₂₅ receptor-associated adverse effects if used chronically (Luethi et al., 2021).

4.4. Interactions with the non-serotonergic monoamine transporters and receptors

No relevant D₂ receptor binding was observed for any of the herein investigated substances. DPIA bound with moderate affinity ($K_i = 0.12 \mu M$) to the $\alpha_{1A}$ adrenoceptor, whereas MDA and N₂α-DEPEA bound with weak affinity ($K_i > 1 \mu M$); these interactions may modulate stimulant-induced vasoconstriction and hyperthermia (Hysek et al., 2012). Overall, most analogs bound with low micromolar affinity to the $\alpha_{1A}$ adrenoceptor, which may modulate norepinephrine release and adrenergic toxicity (Hysek et al., 2012), similar to amphetamine.

In addition to the derivatives investigated in the present study, many other stimulants interact with human or rodent TAAR1 in a similar way (Simmler et al., 2016). These receptors may be involved in the drug-induced psychoactive effects as they negatively modulate dopaminergic and serotonergic neurotransmission (Lindemann et al., 2008; Revel et al., 2011; Revel et al., 2012). In rodents, drug-induced TAAR1 activation leads to auto-inhibition of the stimulant effects of amphetamine (Di Cara, Maggio et al., 2011).

4.5. Limitations

The present study has some limitations. Monoamine efflux was assessed as a qualitative measure to determine whether a substance is a substrate-type releaser at a single high concentration. The potency of releasers was not determined. Nevertheless, previous studies of our group demonstrated that IC₅₀ values of our transporter inhibition assay correlate with EC₅₀ values of substrate-type releasers (Hysek et al., 2012). For transporter blockers, it might be expected that IC₅₀ and $K_i$ values correlate. Still, we observed some discrepancies, which may be explained by different assay setup conditions (Rothman et al., 1993; Reith et al., 2005; Luethi et al., 2018). Moreover, it needs to be considered that in vivo effects may not always be completely predicted by in vitro pharmacological profiles of stimulants (Rudin et al., 2021).

5. Conclusions

MDA and some of its fluorinated and nonfluorinated analogs exhibited monoamine transporter inhibition profiles similar to the prototypical entactogen MDMA. However, a better understanding of the pharmacokinetics and pharmacodynamics is crucial to make predictions of whether these substances might potentially be further explored as prospective therapeutics. In contrast, the $\beta$-methoxy MDA analog 3C-BOH and the amphetamine analog N₂α-DEPEA displayed dopaminergic monoamine interaction profiles similar to the psychostimulant amphetamine. The pharmacological similarity to amphetamine underscores the concerns of N₂α-DEPEA being used as ingredient of workout supplements. In contrast, the weak monoaminergic activity of DPIA suggest no major pharmacological contribution when present in street amphetamine.

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Author contributions

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

Conflict of interest

D.T. is an employee of ReseaChem GmbH and M.C.H. is an employee of F. Hoffmann-La Roche. M.E.L. is a consultant for Mind Medicine, Inc. The other authors declare no competing interests.

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Supplementary materials

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