

Pharmacological profile of novel psychoactive benzofurans

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Author contribution

A Rickli and ME Liechti designed the research study
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A Rickli, MC Hoener, and ME Liechti analysed the data
A Rickli and ME Liechti wrote the paper

Abstract

Background and purpose: Benzofurans are newly used psychoactive substances, but their pharmacology is unknown. The aim of the present study was to pharmacologically characterize benzofurans *in vitro*.

Experimental approach: We assessed the effects of the benzofurans 5-APB, 5-APDB, 6-APB, 6-APDB, 4-APB, 7-APB, 5-EAPB, and 5-MAPDB and benzodifuran 2C-B-FLY on the human noradrenaline (NA), dopamine (DA), and serotonin (5-HT) uptake transporters using HEK 293 cells that express the respective transporters. We also investigated the release of NA, DA, and 5-HT from monoamine-preloaded cells, monoamine receptor binding affinity, and 5-HT_{2A} and 5-HT_{2B} receptor activation.

Key results: All of the benzofurans inhibited NA and 5-HT uptake more than DA uptake, similar to methylenedioxymethamphetamine (MDMA) and unlike methamphetamine. All of the benzofurans also released monoamines and interacted with trace amine-associated receptor 1 (TAAR₁), similar to classic amphetamines. Most benzofurans were partial 5-HT_{2A} agonists similar to MDMA, but also 5-HT_{2B} receptor agonists, unlike MDMA and methamphetamine. The benzodifuran 2C-B-FLY very potently interacted with 5-HT₂ receptors and also bound to TAAR₁.

Conclusions and implications: Despite very similar structures, differences were found in the pharmacological profiles of the different benzofurans and compared with their amphetamine analogues. Benzofurans acted as indirect monoamine agonists that interact with transporters similarly to MDMA. The benzofurans also interacted with serotonergic receptors. This pharmacological profile likely results in MDMA-like entactogenic psychoactive properties. However, benzofurans produce 5-HT_{2B} receptor activation associated with heart valve fibrosis. The pharmacology of 2C-B-FLY indicates predominant hallucinogenic properties and a risk for vasoconstriction.

Keywords: Novel psychoactive substances, benzofuran, pharmacology, monoamine transporter, receptor

Abbreviations: 5-APB, 5-(2-aminopropyl)benzofuran; 6-APB, 6-(2-aminopropyl)benzofuran; 5-APDB, 5-(2-aminopropyl)-2,3-dihydrobenzofuran; 6-APDB, 6-(2-aminopropyl)-2,3-dihydrobenzofuran; 4-APB, 4-(2-aminopropyl)benzofuran; 7-APB, 7-(2-aminopropyl)benzofuran; bromo-dragonFLY, 1-(8-bromobenzo[1,2-b;4,5-b']difuran-4-yl)-2-aminoprop-ane; β -keto-MDA, β -keto-3,4-methylenedioxyamphetamine; 2C-B-fly, 8-bromo-2,3,6,7-benzo-dihydro-difuran-ethylamine; DA, dopamine; DAT, DA transporter; 5-EAPB, 5-(2-ethylaminopropyl)benzofuran; 5-HT, 5-hydroxytryptamine or serotonin; 5-MAPDB, 1-(2,3-dihydrobenzofuran-5-yl)-*N*-methylpropan-2-amine; MDMA, 3,4-methylenedioxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; NA, noradrenaline; NET, noradrenaline transporter; SERT, serotonin transporter; TAAR, trace amine-associated receptor.

Introduction

Novel psychoactive substances are newly used designer drugs (“internet drugs,” “research chemicals,” “legal highs”) that potentially pose similar health risks to classic illicit substances. In recent years, the number of newly detected psychoactive substances on the illicit drug market has dramatically increased. In the European Union, 41 novel psychoactive substances were identified for the first time in 2010, 49 were identified in 2011, 73 were identified in 2012, and 81 were identified in 2013 within the European Early Warning System (EMCDDA, 2014).

Benzofurans are a group of novel psychoactive substances (King, 2014) of particular interest because they are structurally very similar to the popular recreational drug 3,4-methylenedioxymethamphetamine (MDMA) and its active metabolite 3,4-methylenedioxyamphetamine (MDA; (Greene, 2013). 5-(2-Aminopropyl)benzofuran (5-APB) and 6-(2-aminopropyl)benzofuran (6-APB) are benzofuran analogues of MDA (Fig. 1). 5-(2-Aminopropyl)-2,3-dihydrobenzofuran (5-APDB) and 6-(2-aminopropyl)-2,3-dihydrobenzofuran (6-APDB) are dihydrobenzofuran analogues (Fig. 1) that were originally synthesized for research purposes (Monte *et al.*, 1993). 4-(2-Aminopropyl)benzofuran (4-APB) and 7-(2-aminopropyl)benzofuran (7-APB) are positional isomers of 5-APB and 6-APB. 1-(2,3-Dihydrobenzofuran-5-yl)-*N*-methylpropan-2-amine (5-MAPDB) is a dihydrobenzofuran analogue of MDMA, and 5-(2-ethylaminopropyl)benzofuran (5-EAPB) is a benzofuran analogue of MDMA but with an *N*-ethyl group (Fig. 1).

5-APB and 6-APB appeared on the drug market in 2010-2011 (Chan *et al.*, 2013; Jebadurai *et al.*, 2013; Stanczuk *et al.*, 2013; Archer *et al.*, 2014; Elliott *et al.*, 2014; King, 2014), with reports of intoxication (Chan *et al.*, 2013; Greene, 2013; Jebadurai *et al.*, 2013; Seetohul *et al.*, 2013). 4-APB was first reported to the EMCDDA in 2010 (King, 2014) and is typically detected in products that are sold as 6-APB as byproduct (Stanczuk *et al.*, 2013; Strano Rossi *et al.*, 2014). Users report that the effects of 5-APB and 6-APB are comparable to MDMA but more intense (Greene, 2013; Jebadurai *et al.*, 2013). Adverse effects include nausea, sympathomimetic stimulation, and agitation (Chan *et al.*, 2013; Greene, 2013). 5-

APDB and 6-APDB were first reported to the EMCDDA in 2012, and another three benzofurans, including 5-EAPB, were first reported in 2013 (King, 2014). Presently, no published studies have reported the psychotropic and toxic effects of these benzofurans, but 5-APDB, 6-APDB, and 5-EAPB are being discussed in drug user forums (Bluelight, 2013a; Bluelight, 2013b; Drugs-Forum, 2013). Little is known about the pharmacology of benzofurans. 5-APB and 6-APB have been shown to inhibit the human dopamine (DA), noradrenaline (NA), and serotonin (5-hydroxytryptamine [5-HT]) transporters (DAT, NET, and SERT, respectively; (Iversen *et al.*, 2013) and are agonists at the rat 5-HT_{2A} receptor (Dawson *et al.*, 2014) and human and rat 5-HT_{2B} receptor (Iversen *et al.*, 2013; Dawson *et al.*, 2014). Additionally, fast cyclic voltammetry experiments in rat brain slices indicated that 5-APB releases DA at high concentrations (Dawson *et al.*, 2014). 5-APDB and 6-APDB also inhibited the monoamine transporters with greater affinity for the SERT over the DAT compared with MDA in crude rat synaptosome preparations (Monte *et al.*, 1993).

The benzodifurans 8-bromo-2,3,6,7-benzo-dihydro-difuran-ethylamine (2C-B-FLY) and 1-(8-bromobenzo[1,2-b;4,5-b']difuran-4-yl)-2-aminoprop-ane) (bromo-dragonFLY) are known as “fly” drugs because of their chemical structures (Fig. 1). A series of benzodifurans were originally synthesized to study 5-HT_{2A} receptor function (Monte *et al.*, 1997; Parker *et al.*, 1998; Chambers *et al.*, 2001). The recreational use of 2C-B-FLY and bromo-dragonFLY began to be reported in 2007 (Andreasen *et al.*, 2009; Greene, 2013; King, 2014), and there are case reports of severe agitation, hallucinations, seizures, and fatalities associated with bromo-dragonFLY (Andreasen *et al.*, 2009; Wood *et al.*, 2009; Nielsen *et al.*, 2010). 2C-B-FLY and bromo-dragonFLY are potent 5-HT_{2A} agonists (Monte *et al.*, 1996; Chambers *et al.*, 2001), but interactions with other monoamine receptors and their transporters have not been tested.

Systematic evaluations of the pharmacological profiles of benzofurans are lacking. We determined the potencies of a series of benzofurans and the benzodifuran 2C-B-FLY to inhibit the DAT, NET, and SERT and tested transporter-mediated monoamine release *in vitro*. We also characterized the binding profiles at monoamine receptors and assessed 5-

HT_{2A} and 5-HT_{2B} receptor activation. The 5-HT_{2A} receptor mediates hallucinogenic effects (Nichols, 2004), and the 5-HT_{2B} receptor has been implicated in drug-associated endocardial fibrosis (Roth, 2007). MDMA, MDA, β -keto-MDA, and methamphetamine were included as comparator substances.

Methods

Drugs

MDMA, MDA, β -keto-MDA, methamphetamine, and 2C-B-Fly were obtained from Lipomed (Arlesheim, Switzerland). 6-APB, 6-APDB, 5-APB, 5-APDB, 4-APB, 7-APB, and 5-MAPDB were obtained from Cayman Chemicals (Ann Arbor, MI, USA). 5-EAPB was obtained from the Forensic Institute (Zurich, Switzerland). All of the drugs were used as racemic hydrochloride salts. Purity was at least 98% for all of the substances, with the exception of 2C-B-Fly, whose purity was approximately 95% as determined by HPLC.

Monoamine uptake transport inhibition

Inhibition of the human NET, DAT, and SERT was assessed in human embryonic kidney (HEK) 293 cells that were stably transfected with the transporters as specified previously (Hysek *et al.*, 2012c). Briefly, the cells were suspended in uptake buffer. We incubated the cells for 10 min with different concentrations of the test compounds and then added the corresponding [³H]monoamine (5 nM final concentration) at room temperature. After 10 min, we stopped uptake by separating the cells from the buffer using centrifugation through silicone oil (Hysek *et al.*, 2012c). 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 beta-counter. Nonspecific uptake was determined for each experiment in the presence of 10 μ M fluoxetine for SERT cells, 10 μ M nisoxetine for NET cells, and 10 μ M mazindol for DAT cells and subtracted from the total counts to yield specific uptake (100%). DAT:SERT inhibition ratios were calculated as 1/DAT IC₅₀:1/SERT IC₅₀. Higher relative potency at the

DAT indicates a higher abuse potential, whereas relatively increased activity of the 5-HT system is linked to a reduction of abuse potential and more MDMA-like psychotropic effects (Wee *et al.*, 2005). Stimulant amphetamines, such as methamphetamine, have a DAT:SERT inhibition ratio > 10, whereas MDMA and other substances with MDMA-like psychotropic effects have a DAT:SERT inhibition ratio close to 0.1 (Baumann *et al.*, 2012; Simmler *et al.*, 2013; Simmler *et al.*, 2014a; Simmler *et al.*, 2014b).

Transporter-mediated monoamine release

We studied the effects of a single high dose (100 μM) of the test compounds on transporter-mediated NE, 5-HT, and DA efflux in HEK 293 cells that overexpressed the respective human monoamine transporter as previously reported in detail (Simmler *et al.*, 2013). Briefly, adherent cells were incubated with the respective radiolabeled monoamine (10 nM [^3H]NA and 10 μM unlabelled NA, 10 nM [^3H]DA and 1 μM unlabelled DA, and 10 nM [^3H]5-HT) for 20 min at 37°C. We then washed the cells twice with buffer and added 1 ml of buffer that contained the test compound (100 μM final concentration). We stopped [^3H]5-HT and [^3H]DA release after 15 min and [^3H]NA release after 45 min by washing twice with ice-cold buffer. We quantified the radioactivity that remained in the cells. Nonspecific “pseudo-efflux,” which arises from nonspecific substrate release and subsequent reuptake inhibition (Scholze *et al.*, 2000), was assessed for each experiment using the transporter inhibitors nisoxetine (NET cells), citalopram (SERT cells), and mazindol (DAT cells) at 10 μM as negative control conditions.

Radioligand binding assays

The radioligand binding assays were performed as described previously (Hysek *et al.*, 2012c; Simmler *et al.*, 2013). Briefly, membrane preparations of HEK 293 cells (Invitrogen, Zug, Switzerland) that overexpress the respective transporters (Tatsumi *et al.*, 1997) or receptors (human genes, except rat and mouse genes for TAAR₁; (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 determined in the presence of selected competitors in excess. The following radioligands and competitors, respectively, were used: *N*-methyl- ^3H -nisoxetine and indatraline (NET), ^3H -citalopram and indatraline (SERT), ^3H -WIN35,428 and indatraline (DAT), ^3H -8-hydroxy-2-(di-*n*-propylamino)tetralin and indatraline (5-HT_{1A} receptor), ^3H -ketanserin and spiperone (5-HT_{2A} receptor), ^3H -mesulergine and mianserin (5-HT_{2C} receptor), ^3H -prazosin and risperidone (α_1 adrenergic receptor), ^3H -rauwolscine and phentolamine (α_2 adrenergic receptor), ^3H -SCH 23390 and butaclamol (D₁ receptor), ^3H -spiperone and spiperone (D₂ and D₃ receptors), ^3H -pyrilamine and clozapine (histaminergic H₁ receptor), and ^3H -RO5166017 and RO5166017 (TAAR₁).

Functional serotonin 5-HT_{2A} and 5-HT_{2B} activity

The 5-HT_{2B} receptor functional assay was performed as described previously (Jensen *et al.*, 2008). Briefly, human 5-HT_{2B} receptor-expressing HEK 293 cells were incubated at 37°C in 96-well plates coated with poly-D-lysine. 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 Hank's Buffered Saline Solution (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 buffer was added online. The increase in fluorescence was then measured. Efficacy (maximal activity) is expressed relative to the activity of 5-HT, which was used as a control set to 100%.

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 for measuring cytolysis (Crouch *et al.*, 1993; Hysek *et al.*, 2012c; Felser *et al.*, 2014). Cells grown in 96-well plates were exposed to the compounds at a high concentration of 100 µM. All test conditions contained dimethyl sulfoxide (DMSO) 0.1% (v:v) which is non-toxic and was also used as negative control. Triton™ X-100 (0.1%) lyses cells and was used as positive control. After 4 h of incubation at 37°C 10 µl of supernatant per well was removed and combined with 50 µl of ToxiLight™ reagent and luminescence recorded using a Tecan Infinite™ 200 Pro (Tecan, Männedorf, Switzerland) plate reader.

Statistical analyses

The uptake transporter inhibition data were fit by non-linear regression to variable-slope sigmoidal dose-response curves, and IC₅₀ values were calculated using Prism software (GraphPad, San Diego, CA, USA). Analysis of variance followed by the Holm-Sidak test was used to compare compound-induced release with the negative controls. Substances that induced significantly higher monoamine efflux compared with the negative control were considered monoamine releasers. IC₅₀ values for radioligand binding were determined by calculating nonlinear regression curves for an 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. EC₅₀ values for the 5-HT₂ receptor activation were derived from the concentration-response curves using nonlinear regression.

Results

Monoamine uptake transporter inhibition

Uptake inhibition curves are depicted in Fig. 2, and the corresponding IC_{50} values and DAT:SERT inhibition ratios are listed in Table 1. All of the benzofurans inhibited the NET at submicromolar concentrations, similar to MDMA, MDA, and methamphetamine. All of the benzofurans were weak DAT inhibitors compared with methamphetamine and more similar to MDMA, which was also a weak DAT inhibitor. Only 5-APB, 6-APB, and 5-EAPB were more potent at the DAT compared with MDMA and MDA. In contrast, the dihydrobenzofurans 5-APDB, 6-APDB, and 5-MAPDB were inactive at the DAT ($IC_{50} > 30 \mu\text{M}$). 5-APB, 5-APDB, 6-APB, and 5-EAPB inhibited the SERT at submicromolar concentrations and more potently than MDMA. 6-APDB and 5-MAPDB inhibited the SERT in the 1-3 micromolar concentration range, similar to MDMA. 4-APB and 7-APB exhibited low potency at the SERT, more similar to methamphetamine. The DAT:SERT inhibition ratio for all of the benzofurans was low, consistent with greater serotonergic vs. dopaminergic activity that is overall similar to MDMA. The dihydrobenzofurans (5-APDB, 6-APDB, and 5-MAPDB) and 5-APB exhibited the lowest DAT:SERT inhibition ratios (lower than MDMA). In contrast, 4-APB and 7-APB exhibited the highest DAT:SERT inhibition ratios, consistent with their low potency at the SERT and showing a profile that is between MDMA and methamphetamine with regard to serotonergic vs. dopaminergic activity. In terms of structure-activity relationships, the dihydro-compounds 5-APDB and 6-APDB had similar noradrenergic and serotonergic activities compared with their analogues 5-APB and 6-APB but were markedly less potent at the DAT. The monoamine transporter inhibition potencies of the positional isomers 4-APB and 7-APB were reduced, particularly for the SERT, compared with their analogues 5-APB and 6-APB. Additionally, the oxygen in the para-position for 5-APB and 5-APDB resulted in higher absolute and relative potency at the SERT compared with 6-APB and 6-APDB, respectively. Beta-keto-substitution in the β -keto-MDA vs. MDA structures increased dopaminergic vs. serotonergic activity. The benzodifuran 2C-B-FLY was inactive at all of the monoamine transporters ($IC_{50} > 50 \mu\text{M}$).

Monoamine release

At high concentrations, all of the benzofurans released at least one of the monoamines through the respective monoamine transporter, similar to the amphetamines (Fig. 3). In contrast, 2C-B-FLY was not a monoamine releaser.

Binding affinities

The benzofurans interacted with the monoamine transporters but also with several monoamine receptors (Table 2 and 3). All of the benzofurans exhibited submicromolar affinity for TAAR₁, except for 5-EAPB, which was inactive at mouse TAAR₁. Benzofurans showed mostly higher potency at TAAR₁ than the classic amphetamines. All of the benzomonofurans exhibited binding affinities for the 5-HT_{2A} receptor in the micromolar range (0.8-3.4 μM). Functionally, most of them acted as low-potency partial agonists similar to MDMA and MDA but unlike methamphetamine. Most of the benzofurans were also partial agonists at the 5-HT_{2B} receptor. In contrast MDMA and methamphetamine did not stimulate 5-HT_{2B} receptors. With the exception of 7-APB and 5-EAPB, the benzofurans exhibited submicromolar binding affinities at the 5-HT_{2C} receptor. Binding potencies at the 5-HT₁ receptor varied among the different benzofurans. Only 7-APB showed submicromolar binding affinity. Potent binding to most of the assessed 5-HT receptor subtypes distinctly discriminated the benzofurans from the pharmacological profiles of their related amphetamines, which exhibited no or low 5-HT_{1A} affinity and did not bind to 5-HT_{2B} or 5-HT_{2C} receptors except for MDA with a K_i value of 3 μM at 5-HT_{2C}. Most of the benzofurans bound to α₁- and α₂-adrenergic receptors in the 3-12 μM and 0.1-6 μM ranges, respectively. There was no binding to DA receptors and only low-affinity binding to histamine H₁ receptors (> 10 μM for most of the drugs). The benzodifuran 2C-B-FLY did not bind to the monoamine transporters but interacted with all of the receptors tested in the present study and particularly exhibited high affinity for TAAR₁ and all of the 5-HT₂ receptors. Importantly, 2C-B-FLY was a very potent agonist at the 5-HT_{2A} receptor. 2C-B-FLY thus exhibited a pharmacological profile that was distinct from the mono-benzofurans and related amphetamines.

Cytotoxicity

None of the investigated compounds produced cytotoxicity, thus confirming cell integrity during the functional assays in this study.

Discussion

We determined the *in vitro* pharmacological profiles of new benzofurans that are recreationally abused compared with their well-known amphetamine analogues. The benzofurans blocked monoamine transporters and induced transporter-mediated monoamine release similarly to MDMA. More than MDMA and methamphetamine, the benzofurans also directly stimulated adrenergic and 5-HT receptors. The benzodifuran 2C-B-FLY was a potent agonist at 5-HT_{1A}, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} receptors, consistent with the reported hallucinogenic properties of 2C-B-FLY.

Monoamine uptake transporter inhibition and monoamine release

All of the benzofurans inhibited the NET at submicromolar concentrations, similar to MDMA, MDA, and methamphetamine. Noradrenaline mediates sympathomimetic stimulation (Hysek *et al.*, 2011), and this finding predicts the cardiostimulant and psychostimulant properties of these benzofurans, similar to MDMA and methamphetamine. Unlike the relatively constant NET inhibition, the potencies of the benzofurans to inhibit the DAT and SERT notably varied, resulting in DAT:SERT inhibition ratios that ranged from 0.01 to 0.65. Specifically, the dihydrobenzofurans 5-APDB and 5-MAPDB exhibited the highest preference for the SERT vs. DAT (more selective than MDMA), followed by 5-APB, 6-APDB, and 5-EAPB, which exhibited a DAT:SERT inhibition ratio similar to MDMA. With DAT:SERT ratios of 0.46 and 0.65, 4-APB and 7-APB were the benzofurans with the most dopaminergic profiles and were relatively more dopaminergic than MDMA. Stimulants like methamphetamine exhibit a DAT:SERT ratio > 10, whereas MDMA and other entactogens exhibit a DAT:SERT ratio of 0.01-1 (Simmler *et al.*, 2013; Liechti, 2014b; Simmler *et al.*, 2014a; Simmler *et al.*, 2014b).

Accordingly, based on their DAT:SERT inhibition ratios, all of the benzofurans can be expected to produce MDMA-like entactogenic subjective effects in humans. In contrast to the benzofurans, the benzodifuran 2C-B-FLY blocked monoamine transporters only at very high concentrations but had high affinity for 5-HT receptors. Thus, monoamine transporter inhibition is unlikely to contribute to the mechanism of action of 2C-B-FLY as is also the case for the structurally similar substance 2C-B and related compounds of the 2C phenethylamine series containing methoxy-groups at positions 2 and 5 of the benzene ring (Acuna-Castillo *et al.*, 2002; Hill *et al.*, 2011; Eshleman *et al.*, 2014).

Only a few other studies determined the monoamine transporter inhibition profiles of some of the benzofurans. Consistent with our findings, 5-APDB and 6-APDB inhibited the SERT more potently than the DAT in rat synaptosomes (Monte *et al.*, 1993). The oxygen in the para position in the 5-APDB and 5-APB structures enhanced the serotonergic vs. dopaminergic properties compared with 6-APDB and 6-APB, respectively, as shown in the present study and previously for 5-APDB vs. 6-APDB in rat synaptosomes (Monte *et al.*, 1993). Drug discrimination studies in rats showed that 5-APDB and 6-APDB substituted for MDMA-like serotonergic drugs but not the more dopaminergic stimulant amphetamine (Monte *et al.*, 1993). These behavioural findings support our hypothesis that 5-APDB and 6-APDB produce subjective effects that are similar to MDMA, and entactogenic effects have been reported by users (Bluelight, 2013a; Bluelight, 2013b; Drugs-Forum, 2013). The monoamine transporter inhibition profiles for 5-APB and 6-APB were determined in one previous study (Iversen *et al.*, 2013). In contrast to our results, this study showed that 5-APB and 6-APB inhibited the DAT more potently than the SERT (Iversen *et al.*, 2013). However, MDMA did not show the serotonergic preference that is typically reported by others (Rothman *et al.*, 2001; Han *et al.*, 2006; Hysek *et al.*, 2012c; Simmler *et al.*, 2013). Consistent with the present results, the inhibition profiles for 5-APB and 6-APB were similar to MDMA and unlike methamphetamine (Iversen *et al.*, 2013). The reinforcing effects of benzofurans have not yet been studied in drug self-administration studies. There is a decrease in reinforcing potency and efficacy among monoamine releasing agents when 5-

HT releasing potency is increased relative to DA (Wee *et al.*, 2005). The relatively serotonergic *in vitro* properties of the benzofurans would indicate lower addictive properties (Wee *et al.*, 2005; Liechti, 2014b), more similar to MDMA, which is not a strong reinforcer in self-administration studies (Lamb *et al.*, 1987; Cole *et al.*, 2003), than to methamphetamine.

All of the benzofurans also released 5-HT, NA, and DA through their respective transporters, similar to their amphetamine analogues and other amphetamine derivatives (Simmler *et al.*, 2013; Simmler *et al.*, 2014a; Simmler *et al.*, 2014b). Dopamine release has also been previously documented for 5-APB in voltammetric studies of rat brain slices (Dawson *et al.*, 2014). In contrast to the benzofurans, 2C-B-FLY did not release monoamines. Our release assay was designed to qualitatively assess monoamine release because we used only one high concentration of the substances to induce transporter-mediated monoamine efflux. Additional studies that include the assessment of transporter-mediated ionic currents and *in vivo* microdialysis could be useful to further characterize and quantify monoamine release and its contribution to the mechanism of action of the benzofurans.

Receptor binding profiles

The present study found several relevant high-potency interactions between the benzofurans and various monoamine receptors. 6-APB, 6-APDB, 4-APB, 7-APB, and 2C-B-FLY all bound to α_{2A} -adrenergic receptors, which are known to modulate NA release and sympathomimetic activity (Hysek *et al.*, 2012a). As expected (Monte *et al.*, 1996), 2C-B-FLY potently interacted with 5-HT₂ receptors. Specifically, 2C-B-FLY potently bound to the human 5-HT_{2A} receptor ($K_i = 0.01 \mu\text{M}$), consistent with the previously documented nanomolar affinity for rat cortical 5-HT_{2A} receptors (Monte *et al.*, 1996). Even higher-potency binding to 5-HT_{2A} receptors has been shown for the benzodifuran bromo-dragonFLY in rat (Monte *et al.*, 1996; Chambers *et al.*, 2001) and human (Monte *et al.*, 1996) 5-HT_{2A} receptors. In the present study, 2C-B-FLY was also a very potent functional 5-HT_{2A} receptor agonist. 2C-B-FLY resembles the structures of the 2C series phenethylamines, which are

also potent 5-HT_{2A} receptor agonist (Nelson *et al.*, 1999; Acuna-Castillo *et al.*, 2002; Hansen *et al.*, 2014).

Consistent with the predicted lysergic acid diethylamide (LSD)-like properties of substances with high 5-HT_{2A} receptor affinity, both 2C-B-FLY and bromo-dragonFLY completely substituted for LSD in drug discrimination studies (Monte *et al.*, 1996). The affinity of 2C-B-FLY for the 5-HT_{1A} receptor was relatively low, which has also been shown for rat 5-HT_{1A} receptors (Monte *et al.*, 1996). The 5-HT_{2A} receptor is thought to mediate the alterations in perception induced by hallucinogens (Vollenweider *et al.*, 1998; Nelson *et al.*, 1999; Nichols, 2004) and therefore is likely the key target in the mechanism of action of benzodifuran hallucinogens. Interestingly, some of the benzofurans also exhibited micromolar affinity for the 5-HT_{2A} receptor and were low-potency 5-HT_{2A} receptor partial agonists similar to MDMA and MDA, but in contrast to methamphetamine. Binding to 5-HT_{2A} receptors at micromolar concentrations has also been previously shown for 5-APB and 6-APB (Iversen *et al.*, 2013). 5-APB also constricted rat aorta via 5-HT_{2A} agonist action (Dawson *et al.*, 2014). Thus, some benzofurans could have hallucinogenic properties because of 5-HT_{2A} receptor stimulation, in addition to their MDMA-like entactogenic subjective effects. Psychosis and hallucinations have been reported after the use of 6-APB (Chan *et al.*, 2013; Greene, 2013). However, in drug discrimination studies, 5-APDB and 6-APDB did not substitute for LSD in rats (Monte *et al.*, 1993), consistent with their lower binding affinity compared with 5-APB and 6-APB. In terms of clinical toxicity, the 5-HT_{2A} agonist and possible α_1 -adrenergic agonist action could enhance the risk for vasoconstriction, hyperthermia, and hypertension. Both α_1 and 5-HT_{2A} receptors are implicated in substance-induced vasoconstriction (Blessing *et al.*, 2003; Docherty *et al.*, 2010; Dawson *et al.*, 2014) and associated hypertension (Hysek *et al.*, 2013) and hyperthermia (Liechti *et al.*, 2000; Hysek *et al.*, 2012b; Liechti, 2014a) in humans. In fact, hypertension, hyperpyrexia, and cases with severe limb ischemia have been reported after use of bromo-dragonFLY (Thorlacius *et al.*, 2008; Wood *et al.*, 2009; Nielsen *et al.*, 2010), a benzodifuran structurally similar to 2C-B-FLY. Direct agonist actions at the 5-HT_{2A} receptor compared with an indirect action via 5-HT release can also be expected to result in

longer-lasting effects as described for serotonergic hallucinogens (Schmid *et al.*, 2014) and compared with MDMA (Hysek *et al.*, 2012c).

In humans, MDMA is mainly inactivated by *O*-demethylation but also *N*-demethylated to the minor but active metabolite MDA (Hysek *et al.*, 2013). Similarly, 5-MAPDB and 5-EAPB are *N*-dealkylated (Welter *et al.*, 2014) to 5-APDB and 5-APB, respectively. As shown in the present study, the *N*-dealkylated substances MDA, 5-APDB, and 5-APB activate 5-HT_{2A} and 5-HT_{2B} receptors more potently and also more potently bind to 5-HT_{2C} receptors than their parent compounds MDMA, 5-MAPDB, and 5-EAPB, respectively. Thus, the formation of active metabolites likely adds enhanced 5-HT_{2A} and 5-HT_{2B}-receptor-associated toxicity in these cases.

2C-B-FLY and several of the benzofurans acted as partial agonists at the 5-HT_{2B} receptor as previously shown for 5-APB (Iversen *et al.*, 2013; Dawson *et al.*, 2014) and 6-APB (Iversen *et al.*, 2013). In contrast, no such 5-HT_{2B} agonist properties were observed for the classic amphetamines MDMA and methamphetamine in the present study. 5-HT_{2B} receptors have been implicated in substance-induced heart valve fibrosis (Setola *et al.*, 2003; Bhattacharyya *et al.*, 2009). 5-HT_{2B} receptor activation by 2C-B-FLY, 5-APB, 6-APB, 6-APDB, and 7-APB occurred at submicromolar concentrations that are likely present when these drugs are used by drug users to induce subjective effects.

All of the benzofurans bound to TAAR₁, many at even higher potency than MDMA or methamphetamine. MDMA and methamphetamine inhibit their own neurochemical and locomotor stimulant effects via TAAR₁ activation (Di Cara *et al.*, 2011). Similar TAAR₁-mediated “auto-inhibition” may therefore modulate the effects of benzofurans. In contrast, for cathinones (e.g., β -keto-amphetamines), more stimulant-like and addictive properties would be expected based on their lower affinity for TAAR₁ compared with their amphetamine analogues (Simmler *et al.*, 2013; Simmler *et al.*, 2014a).

In terms of structure-activity relationships, 3,4-substitution on the benzene ring (methylenedioxy-group in MDMA and MDA, furans, or dihydrofurans) strongly reduced the DAT/SERT inhibition ratio confirming previous studies (Nichols, 1994; Han *et al.*, 2006;

Iversen *et al.*, 2013; Simmler *et al.*, 2013). Additionally, the DAT/SERT inhibition ratio depended on the position of the oxygen on the benzene ring and was lowest for compounds with the oxygen in the para-(4)-position and highest for those with the oxygen in the ortho-(2)-position. This finding was consistent with the high serotonergic activity of other para-substituted amphetamines (Nichols, 1994; Rickli *et al.*, 2015). The dihydrobenzofurans (5-APDB, 6-APDB, and 5-MAPDB) exhibited reduced monoamine transporter inhibition potency in particular at the DAT resulting in relatively more serotonergic properties compared to their furan analogues. *N*-alkylation (MDMA, 5-MAPDB, 5-EAPB, methamphetamine) moderately reduced activity at 5-HT_{2A/B} receptors and binding at 5-HT_{2C} receptors. This has previously been shown for other phenethylamines for simple *N*-alkylation (e.g., methyl, ethyl) (Nelson *et al.*, 1999). *N*-alkylation had no relevant effect on the interactions with the monoamine transporter as previously noted for related amphetamines (Nichols, 1994). 2,5-substitution on the benzene ring strongly increased activity at the 5-HT₂ receptors and reduced interactions with the monoamine transporters as seen in 2C-B-FLY in the present study and many other 2,5-substituted phenethylamines (2C series) (Hill *et al.*, 2011; Eshleman *et al.*, 2014). Transporter inhibition potency was also moderately reduced when the oxygen was in the ortho-(2)-position (similar to the 2 series) at the benzene ring as in 7-APB compared with 5-APB, 6-APB, or 4-APB. Beta-keto-substitution increased dopaminergic vs. serotonergic activity extending previous similar findings (Simmler *et al.*, 2013; Simmler *et al.*, 2014a).

Conclusions

Benzofurans are monoamine transporter blockers and monoamine releasers, similar to MDMA, but they also interact with serotonergic receptors. This mechanism of action predicts psychotropic and clinical toxicological effects that are similar to the entactogen MDMA but with additional hallucinogenic properties. The benzodifuran 2C-B-FLY is a potent hallucinogen, likely also associated with a risk for clinical complications related to vasoconstriction (e.g., ischemia and hypertension). Although structure-activity relationships

exist, the present study showed that structurally very similar compounds may exhibit distinct pharmacological profiles, illustrating the need for pharmacotoxicological profiling of each novel psychoactive substance.

Conflict of interest

None.

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Figure Legends

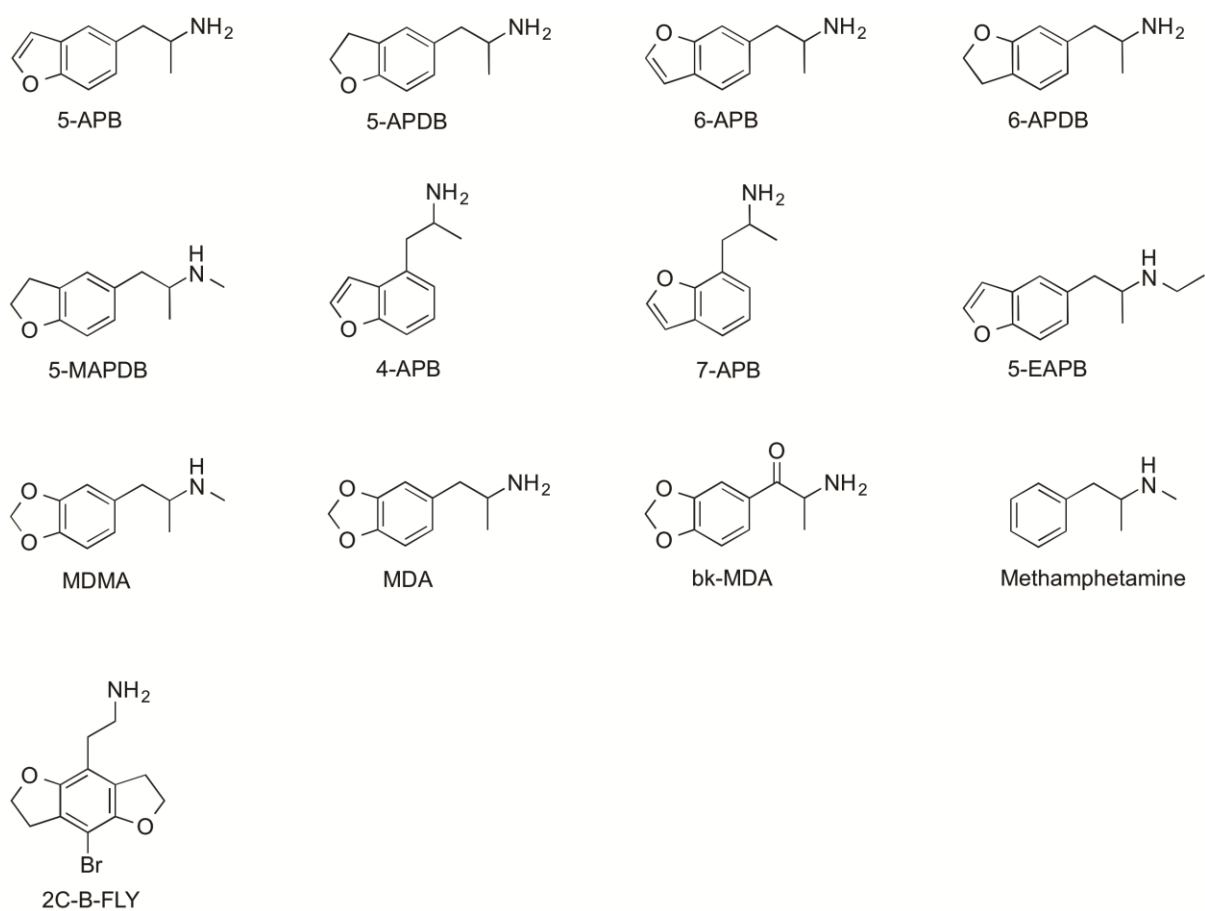


Figure 1. Chemical structures of benzofurans and related amphetamines.

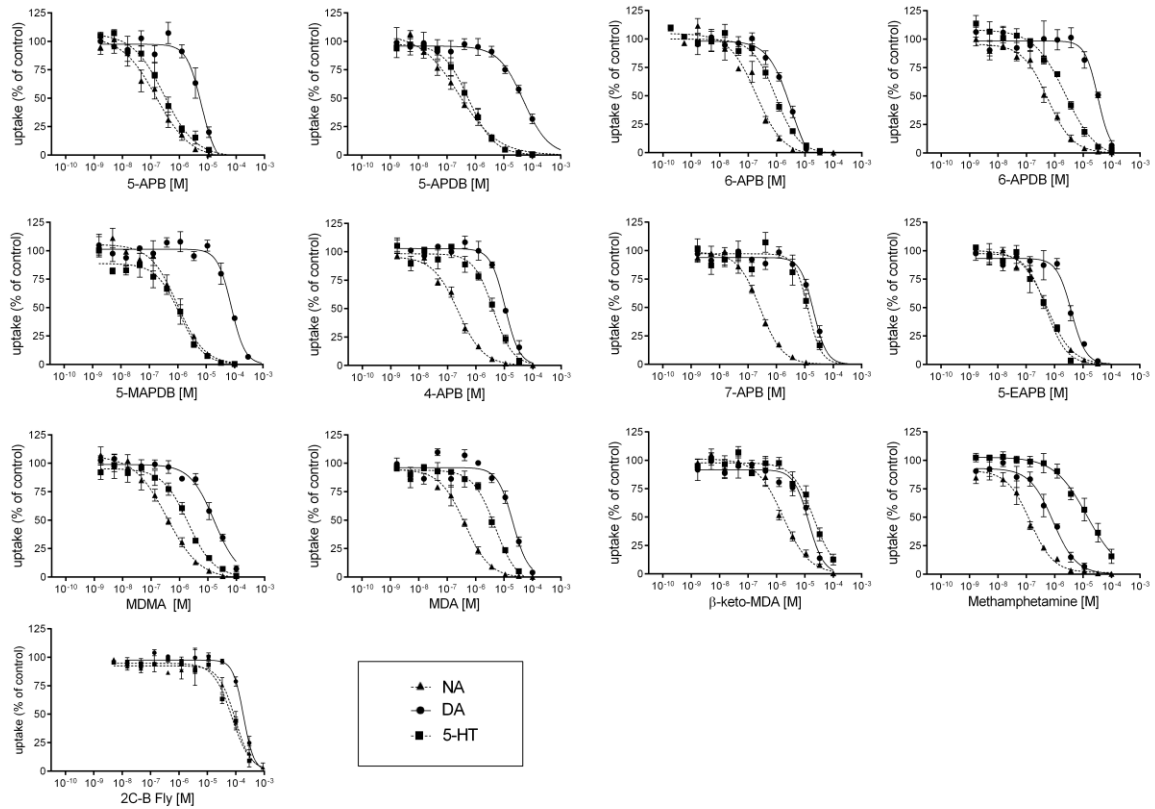


Figure 2. Monoamine uptake transporter inhibition. Concentration-response curves show the uptake inhibition of [³H]NA, [³H]DA, and [³H]5-HT in HEK 293 cells transfected with the respective monoamine transporter. The data are expressed as the mean ± SEM of 3-4 independent experiments. Curves were fitted to the data with non-linear regression. The corresponding IC₅₀ values are shown in Table 1.

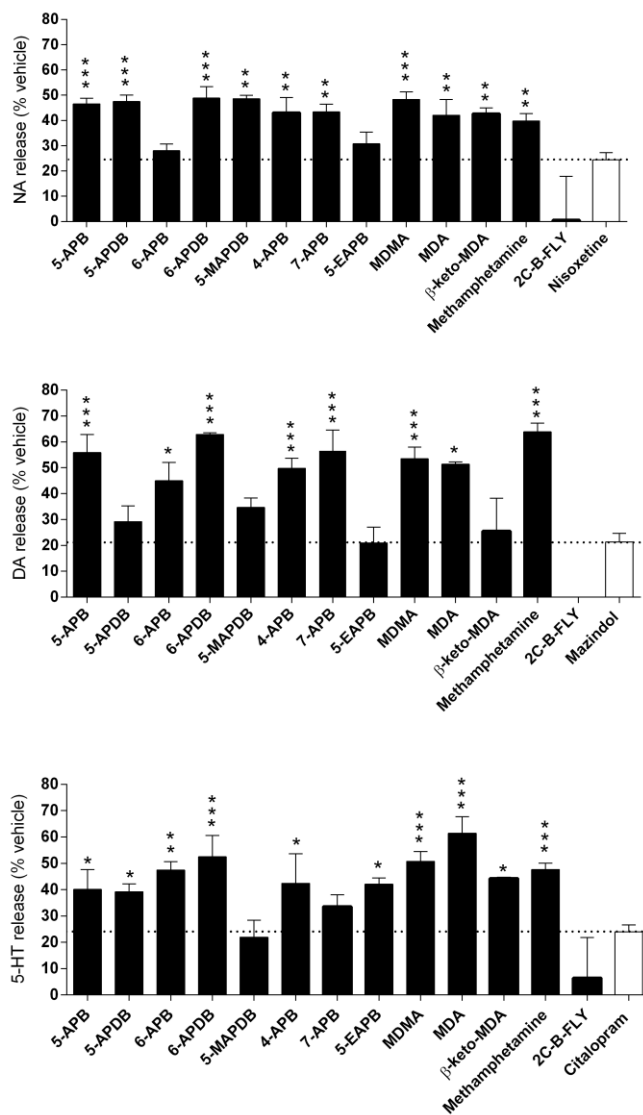


Figure 3. Monoamine release. Monoamine release was induced by a high concentration of the compound (100 μ M) after preloading the transporter-transfected cells with the respective radiolabeled monoamine. All of the benzofurans released NA, DA, and 5-HT similarly to methamphetamine and MDMA. In contrast, the benzodifuran 2CB-FLY was not a monoamine releaser. Transporter blockers induced nonspecific “pseudo-efflux” (horizontal dashed line, open bars), which arises from substrate that diffuses out of the cells and from subsequent reuptake inhibition. Compounds that produced significantly more monoamine efflux ($*P < 0.05$, $*P < 0.01$, $***P < 0.001$) compared with the respective non-releasing uptake inhibitors (negative controls, open bars) were considered monoamine releasers. The data are expressed as the mean \pm SEM of 3-4 independent experiments.

Table 1. Monoamine transporter inhibition.

	NET	DAT	SERT	DAT/SERT inhibition ratio
	IC50 [μ M] (95% CI)	IC50 [μ M] (95% CI)	IC50 [μ M] (95% CI)	Ratio (95% CI)
Benzofurans				
5-APB	0.16 (0.08-0.3)	6.1 (4-9)	0.29 (0.17-0.5)	0.05 (0.02-1.2)
5-APDB	0.29 (0.2-0.5)	49 (33-73)	0.58 (0.4-0.9)	0.01 (0.005-0.03)
6-APB	0.19 (0.1-0.3)	3.3 (2.4-4.5)	0.93 (0.7-1.3)	0.29 (0.16-0.54)
6-APDB	0.56 (0.4-0.8)	33 (25-43)	2.3 (1.4-3.9)	0.07 (0.03-0.16)
5-MAPDB	0.96 (0.5-1.7)	77 (62-96)	1.2 (0.7-2)	0.02 (0.01-0.03)
4-APB	0.24 (0.2-0.3)	12 (9-16)	5.5 (3.4-8.7)	0.46 (0.21-1.0)
7-APB	0.27 (0.2-0.3)	20 (16-26)	13 (9-18)	0.65 (0.35-1.1)
5-EAPB	0.56 (0.4-0.7)	4.9 (3-8)	0.72 (0.5-1.1)	0.15 (0.07-0.35)
Benzodifuran				
2C-B-FLY	94 (72-124)	187 (161-217)	73 (58-92)	0.39 (0.27-0.57)
Related amphetamines				
MDMA	0.36 (0.2-0.6)	16.7 (16.3-17)	2.4 (1.4-3.0)	0.14 (0.08-0.18)
MDA	0.42 (0.3-0.6)	20.5 (20.3-20.6)	4.9 (3.5-6.8)	0.24 (0.17-0.33)
beta-keto-MDA	1.6 (1.1-2.3)	14 (10-18)	21 (15-28)	1.5 (0.8-2.8)
Methamphetamine	0.14 (0.09-0.2)	0.87 (0.84-0.91)	13.6 (13.5-13.8)	15.6 (14.8-16.4)

Values are means of three to four independent experiments and 95% confidence intervals (CI).

DAT/SERT inhibition ratio = $1/\text{DAT IC}_{50} : 1/\text{SERT IC}_{50}$.

Table 2. Monoamine transporter and receptor binding affinities.

	NET	DAT	SERT	a _{1A}	a _{2A}	D ₁	D ₂	D ₃	H ₁	TAAR _{1rat}	TAAR _{1mouse}
Benzofurans											
5-APB	3.1 ± 0.2	2.6 ± 0.3	3.2 ± 0.4	3.5 ± 0.5	2.9 ± 0.1	> 14	> 10	> 17	8.4 ± 0.8	0.04 ± 0.01	0.11 ± 0.01
5-APDB	28 ± 5	> 30	4.0 ± 0.3	11 ± 3	4.2 ± 0.5	> 14	> 10	> 17	21 ± 2	0.49 ± 0.05	0.77 ± 0.06
6-APB	1.8 ± 0.4	0.60 ± 0.05	12 ± 1	7.3 ± 3.4	0.38 ± 0.02	> 14	> 10	> 17	15 ± 2	0.05 ± 0.02	0.06 ± 0.02
6-APDB	18 ± 1	>30	23 ± 1	> 15	0.65 ± 0.07	> 14	> 10	> 17	> 25	1.0 ± 0.04	0.21 ± 0.04
5-MAPDB	26 ± 5	>30	6.3 ± 0.6	4.9 ± 1.5	6.4 ± 1.8	23 ± 3	> 10	> 17	4.9 ± 0.1	0.67 ± 0.09	3.5 ± 0.1
4-APB	3.9 ± 0.5	7.4 ± 0.6	7.7 ± 0.5	12 ± 3	0.87 ± 0.22	> 14	> 10	> 17	16 ± 1	0.11 ± 0.02	2.08 ± 0.14
7-APB	5.3 ± 0.1	14 ± 2	14 ± 1	9.6 ± 2.4	0.14 ± 0.02	> 14	8.2 ± 3.2	> 17	25 ± 5	0.07 ± 0.01	0.13 ± 0.02
5-EAPB	1.0 ± 0.3	0.34 ± 0.02	0.52 ± 0.03	3.3 ± 0.5	2.7 ± 0.7	16 ± 3	> 10	> 17	2.4 ± 0.4	0.81 ± 0.08	> 15
Benzodifuran											
2C-B-FLY	17 ± 4	> 26	10 ± 3	11 ± 1	0.78 ± 0.3	1.4 ± 0.2	1.9 ± 0.3	6.8 ± 1.2	3.4 ± 0.5	0.03 ± 0.01	0.71 ± 0.23
Related amphetamines											
MDMA ^a	27 ± 9	8.4 ± 3.3	13 ± 2	> 5	15 ± 10	> 12	> 20	> 17	> 13	0.37 ± 0.12	2.4 ± 1.1
MDA	13 ± 3.7	> 26	5.6 ± 1.5	> 5	1.1 ± 0.1	> 12	> 20	> 17	> 13	0.25 ± 0.04	0.16 ± 0.01
beta-keto-MDA	> 30	11 ± 2	>30	> 5	15 ± 2	> 12	> 20	> 17	> 13	4.8 ± 0.9	6.5 ± 2.8
Methamphetamine ^a	3.0 ± 2.2	1.8 ± 0.7	25 ± 10	> 5	6.1 ± 1.6	> 12	> 20	> 17	> 13	0.35 ± 0.12	0.55 ± 0.24

Values are K_i given as microM (mean ±SD); ^avalues are from Simmler et al. 2014a.

Table 3. Serotonin receptor interactions.

	5-HT _{1A}	5-HT _{2A}		5-HT _{2B}		5-HT _{2C}	
	receptor binding Ki (μM)	receptor binding Ki (μM)	activation potency EC ₅₀ (μM)	activation efficacy % maximum	activation potency EC ₅₀ (μM)	activation efficacy % maximum	receptor binding Ki (μM)
Benzofurans							
5-APB	3.3 ± 0.2	0.84 ± 0.27	6.3 ± 2.1	54 ± 35	0.28 ± 0.12	61 ± 17	0.88 ± 0.33
5-APDB	20 ± 4	3.4 ± 1.0	11 ± 2	24 ± 17	1.2 ± 0.6	50 ± 21	0.06 ± 0.02
6-APB	1.5 ± 0.2	0.97 ± 0.23	5.9 ± 1.8	43 ± 23	0.14 ± 0.06	70 ± 9	0.27 ± 0.05
6-APDB	9.2 ± 1.5	2.0 ± 1.0	5.9 ± 1.1	62 ± 36	0.12 ± 0.03	66 ± 17	0.06 ± 0.02
5-MAPDB	26 ± 6	4.8 ± 2.1	> 20	0	> 20	0	0.10 ± 0.02
4-APB	1.2 ± 0.1	0.96 ± 0.17	13 ± 2	30 ± 9	1.0 ± 0.5	38 ± 16	0.06 ± 0.02
7-APB	0.28 ± 0.05	0.91 ± 0.20	5.7 ± 2.0	43 ± 21	0.28 ± 0.52	52 ± 17	3.3 ± 0.3
5-EAPB	3.1 ± 0.6	2.7 ± 1.5	7.6 ± 3.2	29 ± 7	> 20	0	4.6 ± 1.3
Benzodifuran							
2C-B-FLY	0.35 ± 0.04	0.011 ± 0.002	0.0015 ± 0.0002	82 ± 12	0.040 ± 0.036	56 ± 3	0.012 ± 0.004
related amphetamines							
MDMA	12 ± 0.8 ^a	6.3 ± 2.4	6.1 ± 0.3	55 ± 9	> 20	0	> 13 ^a
MDA	4.9 ± 0.9	3.3 ± 0.8	0.63 ± 0.24	77 ± 16	0.85 ± 0.11	52 ± 12	3.0 ± 0.9
beta-keto-MDA	> 17	> 13	> 20	0	> 20	0	> 13
Methamphetamine	8.1 ± 0.7 ^a	> 13	> 20	0	> 20	0	> 13 ^a

Values are Ki given as microM (mean±SD). ^avalues are from Simmler et al. 2014a and were included for comparison.

Table 4. Structure-activity relationships

structure	present in	not present in	pharmacological (clinical) activity
3,4-substitution on benzene ring (methylenedioxy or furan)	MDMA, 5-MAPDB, 5-EAPB MDA, 5-APB, 6-APB, 5-APDB, 6-APDB, 7-APB, β -keto-MDA	methamphetamine ^a amphetamine	reduced DAT/SERT inhibition ratio, ^a increased potency to release 5-HT, ^a reduced potency to release DA (more entactogenic, less stimulant)
oxygen in para-(4)-position	5-APB, 5-APDB, 5-MAPDB	7-APB, 4-APB, 6-APB, 6-APDB	reduced DAT/SERT inhibition ratio (more serotonergic)
dihydrobenzofuran	5-APDB, 6-APDB, 5-MAPDB	5-APB, 6-APB, 4-APB, 7-APB	reduced DAT/SERT inhibition ratio (more serotonergic)
N-alkyl group	MDMA, 5-EAPB, 5-MAPDB	MDA, 5-APB, 5-APDB	reduced 5-HT _{2A/B} receptor activation and 5-HT _{2C} receptor binding potency (less hallucinogenic)
2,5-oxy-substitution on benzene ring	2C-B-FLY	all other compounds	strongly increased 5-HT _{2A/B} receptor activation, strongly increased 5-HT _{2C} receptor binding potency (more hallucinogenic)
β -keto group	β -keto MDA	MDA	^{a,b} increased DAT/SERT ratio (more dopaminergic)

^aSimmler et al 2013, ^bSimmler et al. 2014a