RESEARCH PAPER

Pharmacological profile of novel psychoactive benzofurans

Anna Rickli[1], Simone Kopf[1], Marius C Hoener[2] and Matthias E Liechti[1]

[1]*Psychopharmacology Research, Division of Clinical Pharmacology and Toxicology, Department of Biomedicine, University Hospital Basel and University of Basel, Basel, Switzerland, and* [2]*Neuroscience Research, pRED, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd, Basel, Switzerland*

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 and 5-HT uptake transporters using HEK 293 cells that express the respective transporters. We also investigated the release of NA, dopamine 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 dopamine uptake, similar to methylenedioxymethamphetamine (MDMA) and unlike methamphetamine. All of the benzofurans also released monoamines and interacted with trace amine-associated receptor 1 (TA$_1$ receptor), similar to classic amphetamines. Most benzofurans were partial 5-HT$_{2A}$ receptor 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-HT$_2$ receptors and also bound to TA$_1$ receptors.

CONCLUSIONS AND IMPLICATIONS

Despite very similar structures, differences were found in the pharmacological profiles of 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 5-HT receptors. This pharmacological profile probably results in MDMA-like entactogenic psychoactive properties. However, benzofurans induce 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.

Abbreviations

2C-B-FLY, 8-bromo-2,3,6,7-benzo-dihydro-difuran-ethylamine; 4-APB, 4-(2-aminopropyl)benzofuran; 5-APB, 5-(2-aminopropyl)benzofuran; 5-APDB, 5-(2-aminopropyl)-2,3-dihydrobenzofuran; 5-EAPB, 5-(2-ethylaminopropyl) benzofuran; 5-MAPDB, 1-(2,3-dihydrobenzofuran-5-yl)-N-methylpropan-2-amine; 6-APB, 6-(2-aminopropyl)benzofuran; 6-APDB, 6-(2-aminopropyl)-2,3-dihydrobenzofuran; 7-APB, 7-(2-aminopropyl)benzofuran; β-keto-MDA, β-keto-3,4-methylenedioxyamphetamine; bromo-dragonFLY, 1-(8-bromobenzo[1,2-b;4,5-b']difuran-4-yl)-2-aminopropane; DAT, dopamine transporter; MDMA, 3,4-methylenedioxymethamphetamine; MDA, 3,4-methylenedioxyamphetamine; NET, noradrenaline transporter; SERT, 5-HT transporter; TA receptor, trace amine-associated receptor

Tables of Links

TARGETS		
GPCRs[a]		**Transporters**[b]
5-HT1A receptor	D1 receptor	DAT
5-HT2A receptor	D2 receptor	NET
5-HT2B receptor	D3 receptor	SERT
5-HT2C receptor	H1 receptor	
α1A adrenoceptor	TA1 receptor	
α2A adrenoceptor		

LIGANDS		
5-HT	Noradrenaline	Pyrilamine
Butaclamol	Mazindole	Rauwolscine
Citalopram	MDMA	Risperidone
Clozapine	Mesulergine	RO5166017
Dopamine	Methamphetamine	SCH23390
Ketanserin	Mianserin	Spiperone
LSD	Phentolamine	WIN35428
Nisoxetine	Prazosin	

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

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 (Figure 1). 5-(2-Aminopropyl)-2,3-dihydrobenzofuran (5-APDB) and 6-(2-aminopropyl)-2,3-dihydrobenzofuran (6-APDB) are dihydrobenzofuran analogues (Figure 1) that were originally synthesized for research purposes (Monte *et al.*, 1993). 4-(2-
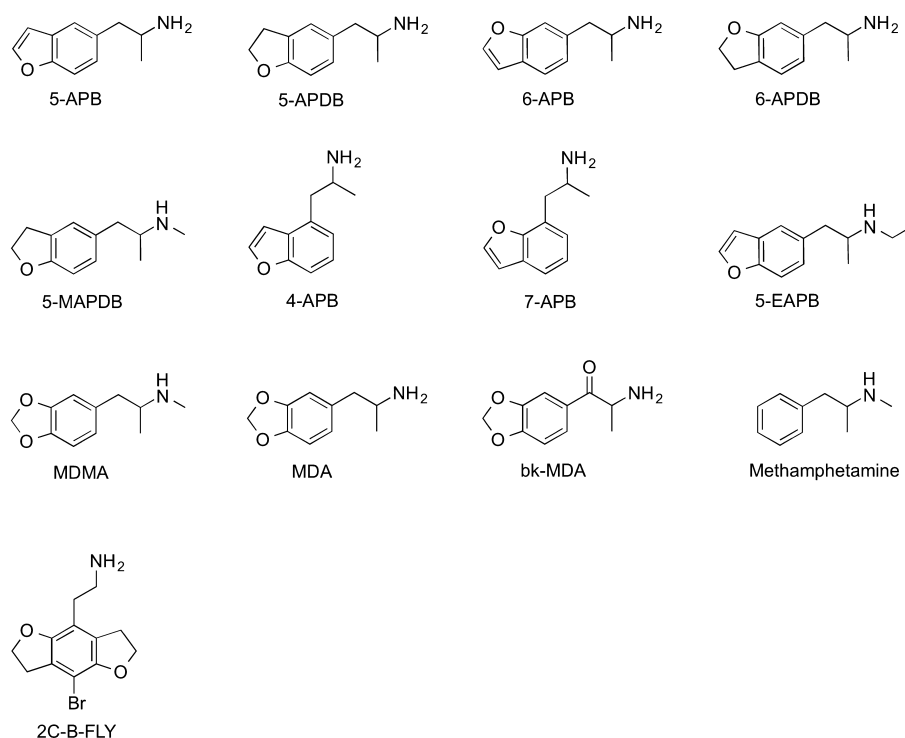

Figure 1
Chemical structures of benzofurans and related amphetamines.

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 (Figure 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 and Evans, 2014; King, 2014), with reports of intoxication (Chan *et al.*, 2013; Greene, 2013; Jebadurai *et al.*, 2013; Seetohul and Pounder, 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 a by-product (Stanczuk *et al.*, 2013; Strano Rossi *et al.*, 2014). Users report that the effects of 5-APB and 6-APB are comparable with 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,b; Drugs-Forum, 2013). Little is known about the pharmacology of benzofurans. 5-APB and 6-APB have been shown to inhibit the human dopamine, noradrenaline and 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 dopamine 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-aminopropane) (bromo-dragonFLY) are known as 'fly' drugs because of their chemical structures (Figure 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$_{2A}$ receptor 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

Monoamine uptake transport inhibition

Inhibition of the human NET, DAT and SERT was assessed in 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 β-counter. Non-specific 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, and IC$_{50}$ values were calculated using Prism software (GraphPad, San Diego, CA, USA). DAT : SERT inhibition ratios were calculated as 1/DAT IC$_{50}$:1/SERT IC$_{50}$. 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 in 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; 2014a,b).

Transporter-mediated monoamine release

We studied the effects of a single high dose (100 μM) of the test compounds on transporter-mediated NA, 5-HT and dopamine 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]-dopamine and 1 μM unlabelled dopamine, 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]-dopamine 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. Non-specific 'pseudo-efflux', which arises from non-specific 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.

ANOVA 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.

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 TA$_1$ receptor; 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 non-specific binding 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*-propylamino)tetralin and indatraline (5-HT$_{1A}$ receptor), [^{3}H]-ketanserin and spiperone (5-HT$_{2A}$ receptor), [^{3}H]-mesulergine and mianserin (5-HT$_{2C}$ receptor), [^{3}H]-prazosin and risperidone (α_1 adrenoceptor), [^{3}H]-rauwolscine and phentolamine (α_2 adrenoceptor), [^{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 (histamine H$_1$ receptor), and [^{3}H]-RO5166017 and RO5166017 (TA$_1$ receptor). 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.

Functional 5-HT$_{2A}$ and 5-HT$_{2B}$ receptor 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 HBSS (Gibco) and 20 mM HEPES (assay buffer; Gibco, Life Technologies, Zug, Switzerland) 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. EC$_{50}$ values were derived from the concentration–response curves using non-linear regression. 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 DMSO 0.1% (v:v) which is non-toxic and was also used as negative control. Triton™ X-100 (0.1%, Sigma-Aldrich, Buchs, Switzerland) 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 IC$_{50}$ values were calculated using Prism software (GraphPad, San Diego, CA, USA). ANOVA 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. IC$_{50}$ values for radioligand binding were determined by calculating nonlinear 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. EC$_{50}$ values for 5-HT$_2$ receptor activation were derived from the concentration-response curves using nonlinear regression.

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, with the exception of d-methamphetamine. 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.

Results

Monoamine uptake transporter inhibition

Uptake inhibition curves are depicted in Figure 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
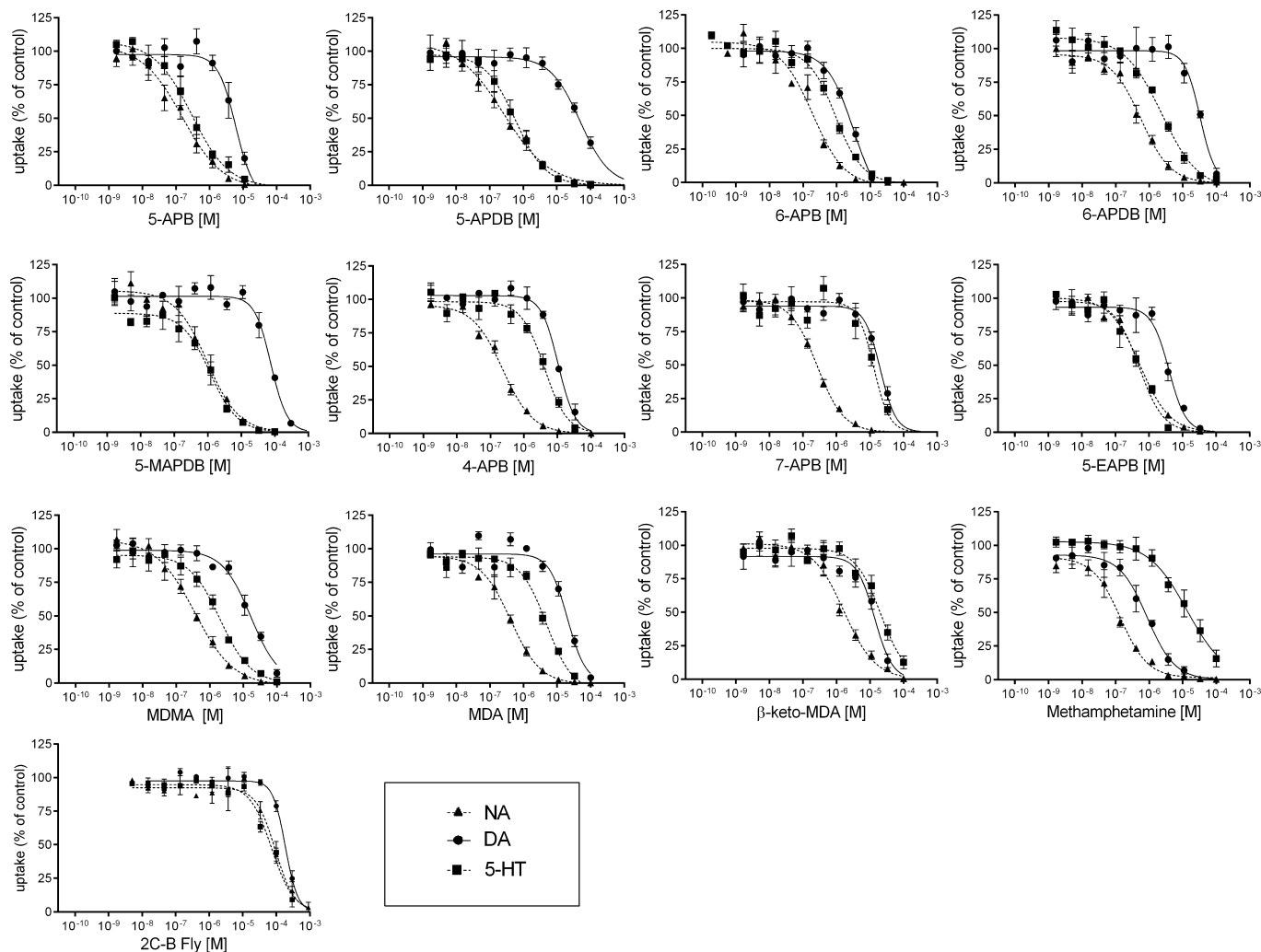
Figure 2

Monoamine uptake transporter inhibition. Concentration–response curves show the uptake inhibition of [³H]-NA, [³H]-dopamine and [³H]-5-HT in HEK 293 cells transfected with the respective monoamine transporter. The data are expressed as the mean ± SEM of three to four independents experiments. Curves were fitted to the data with non-linear regression. The corresponding IC_{50} values are shown in Table 1.

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 μM). 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 5-hydroxytryptaminergic versus 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 5-hydroxytryptaminergic versus dopaminergic activity. In terms of structure–activity

relationships, the dihydro-compounds 5-APDB and 6-APDB had similar noradrenergic and 5-hydroxytryptaminergic 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. β-Keto-substitution in the β-keto-MDA versus MDA structures increased dopaminergic versus 5-hydroxytryptaminergic activity. The benzodifuran 2C-B-FLY was inactive at all of the monoamine transporters (IC_{50} >50 μM).

Monoamine release

At high concentrations, all of the benzofurans released at least one of the monoamines through the respective

Table 1

Monoamine transporter inhibition

	NET	DAT	SERT	DAT/SERT inhibition ratio
	IC_{50} (µM) (95% CI)	IC_{50} (µM) (95% CI)	IC_{50} (µM) (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)
β-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/DAT IC_{50}: 1/SERT IC_{50}.

monoamine transporter, similar to the amphetamines (Figure 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 (Tables 2 and 3). All of the benzofurans exhibited submicromolar affinity for the TA_1 receptor, except for 5-EAPB, which was inactive at mouse TA_1 receptos. Benzofurans showed mostly higher potency at TA_1 receptors 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 $5-HT_1$ receptor varied among 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 α_{1A}- and α_{2A}-adrenoceptors in the 3–12 and 0.1–6 µM ranges respectively. There was no binding to dopamine receptors and only low-

affinity binding to histamine H_1 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 TA_1 receptors and all of the $5-HT_2$ 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 compounds investigated 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 adrenoceptors 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.
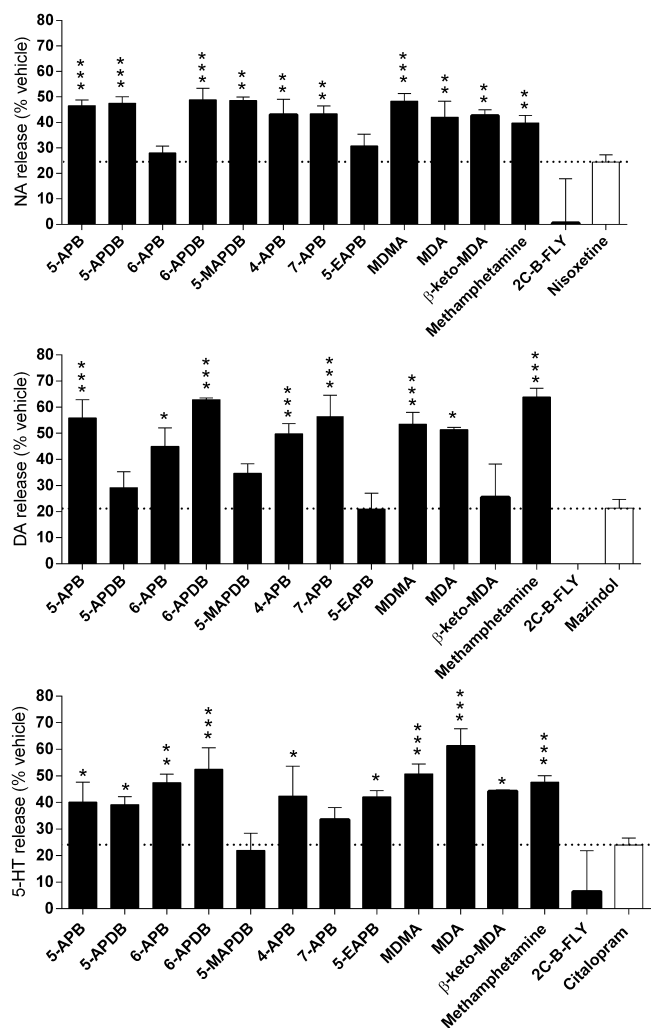
Figure 3

Monoamine release. Monoamine release was induced by a high concentration of the compound (100 μM) after preloading the transporter-transfected cells with the respective radiolabelled monoamine. All of the benzofurans released NA, dopamine and 5-HT similarly to methamphetamine and MDMA. In contrast, the benzodifuran 2CB-FLY was not a monoamine releaser. Transporter blockers induced non-specific '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 ± SEM of three to four independent experiments.

Monoamine uptake transporter inhibition and monoamine release

All of the benzofurans inhibited the NET at submicromolar concentrations, similar to MDMA, MDA and methamphetamine. NA 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 con-

stant 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 versus 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; 2014a,b; Liechti, 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 and Thomas, 2011; Eshleman *et al.*, 2014).

Only a few other studies have 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 5-hydroxytryptaminergic versus dopaminergic properties compared with 6-APDB and 6-APB, respectively, as shown in the present study and previously for 5-APDB versus 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 5-hydroxytryptaminergic 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,b; 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 5-hydroxytryptaminergic preference that is typically reported by others (Rothman *et al.*, 2001; Han and Gu, 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 dopamine (Wee *et al.*, 2005). The relatively high 5-hydroxytryptaminergic properties of the benzofurans *in vitro* would indicate lower addictive properties (Wee *et al.*, 2005; Liechti, 2014b), more similar to MDMA, which is

Table 2

Monoamine transporter and receptor-binding affinities

	NET	DAT	SERT	α_{1A}	α_{2A}	D_1	D_2	D_3	H_1	TA_{1rat}	TA_{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
β-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 μM (mean ± SD).

[a]Values are from Simmler et al., 2014a.

Table 3

5-HT receptor interactions

	5-HT$_{1A}$	5-HT$_{2A}$			5-HT$_{2B}$		5-HT$_{2C}$
	Receptor-binding K_i (µM)	Receptor-binding K_i (µM)	Activation potency EC$_{50}$ (µM)	Activation efficacy % maximum	Activation potency EC$_{50}$ (µM)	Activation efficacy % maximum	Receptor-binding K_i (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
β-Keto-MDA	>17	>13	>20	0	>20	0	>13
Methamphetamine	8.1 ± 0.7[a]	>13	>20	0	>20	0	>13[a]

Values are K_i given as µM (mean ± SD).

[a]Values are from Simmler et al., 2014a and were included for comparison.

not a strong reinforcer in self-administration studies (Lamb and Griffiths, 1987; Cole and Sumnall, 2003) than to methamphetamine.

All of the benzofurans also released 5-HT, NA and/or dopamine through their respective transporters, similar to their amphetamine analogues and other amphetamine derivatives (Simmler et al., 2013; 2014a,b). 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 important 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}-adrenoceptors, 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-HT$_2$ receptors. Specifically, 2C-B-FLY potently bound to the human 5-HT$_{2A}$ receptor ($K_i = 0.01$ μM), 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 agonists (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 to be 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 constricts the rat aorta via an agonist action on 5-HT$_{2A}$ receptors (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$_{2A}$ receptor agonist and possible α_1-adrenoceptor 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 and Green, 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 of severe limb ischaemia have been reported after the 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 5-hydroxytryptaminergic 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., 2015) 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 probably 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$_{2B}$ receptor 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 to be present when these drugs are used by drug users to induce subjective effects.

All of the benzofurans bound to TA$_1$ receptors, many at even higher potency than MDMA or methamphetamine. MDMA and methamphetamine inhibit their own neurochemical and locomotor stimulant effects via TA$_1$ receptor activation (Di Cara et al., 2011). Similar TA$_1$ receptor-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 TA$_1$ receptors compared with their amphetamine analogues (Simmler et al., 2013; 2014a).

In terms of structure–activity relationships (Table 4), 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 and Gu, 2006; Iversen et al., 2013;

Table 4

Structure–activity relationships

Structure	Present in	Not present in	Pharmacolocial (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 dopamine (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 5-hydroxytryptaminergic)
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)

[a]Simmler *et al.*, 2013.
[b]Simmler *et al.*, 2014a.

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 5-hydroxytryptaminergic 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 5-hydroxytryptaminergic properties compared with 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-HT$_2$ 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 and Thomas, 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. β-Keto-substitution increased dopaminergic versus 5-hydroxytryptaminergic activity extending previous similar findings (Simmler *et al.*, 2013; 2014a).

Conclusions

Benzofurans are monoamine transporter blockers and monoamine releasers, similar to MDMA, but they also interact with 5-HT 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, probably also associated with a risk for clinical complications related to vasoconstriction (e.g. ischaemia 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.

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Author contributions

A. R. and M. E. L. designed the research study. A. R., S. K. and M. C. H. performed the research. A. R., M. C. H. and M. E. L. analysed the data. A. R. and M. E. L. wrote the paper.

Conflict of interest

None.

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