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ARTICLE

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(2-Aminopropyl)benzo[β]thiophenes (APBTs) are novel monoamine transporter ligands that lack stimulant effects but display psychedelic-like activity in mice

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Derivatives of (2-aminopropyl)indole (API) and (2-aminopropyl)benzofuran (APB) are new psychoactive substances which produce stimulant effects in vivo. (2-Aminopropyl)benzo[β]thiophene (APBT) is a novel sulfur-based analog of API and APB that has not been pharmacologically characterized. In the current study, we assessed the pharmacological effects of six APBT positional isomers in vitro, and three of these isomers (3-APBT, 5-APBT, and 6-APBT) were subjected to further investigations in vivo. Uptake inhibition and efflux assays in human transporter-transfected HEK293 cells and in rat brain synaptosomes revealed that APBTs inhibit monoamine reuptake and induce transporter-mediated substrate release. Despite being nonselective transporter releasers like MDMA, the APBT compounds failed to produce locomotor stimulation in C57BL/6J mice. Interestingly, 3-APBT, 5-APBT, and 6-APBT were full agonists at 5-HT_2 receptor subtypes as determined by calcium mobilization assays and induced the head-twitch response in C57BL/6J mice, suggesting psychedelic-like activity. Compared to their APB counterparts, ABPT compounds demonstrated that replacing the oxygen atom with sulfur results in enhanced releasing potency at the serotonin transporter and more potent and efficacious activity at 5-HT_2 receptors, which fundamentally changed the in vitro and in vivo profile of APBT isomers in the present studies. Overall, our data suggest that APBT isomers may exhibit psychedelic and/or entactogenic effects in humans, with minimal psychomotor stimulation. Whether this unique pharmacological profile of APBT isomers translates into potential therapeutic potential, for instance as candidates for drug-assisted psychotherapy, warrants further investigation.

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INTRODUCTION

Naturally occurring tryptamines include serotonin (5-hydroxytryptamine; 5-HT), melatonin (*N*-acetyl-5-methoxytryptamine), *N*,*N*-dimethyltryptamine (DMT), and psilocybin. DMT and psilocybin are known to produce powerful psychedelic-like effects (e.g., hallucinations), and many structurally related synthetic tryptamine derivatives are used recreationally [1–4]. Most psychoactive tryptamines interact with monoaminergic receptors and transporters that can lead to untoward effects [5], which underscores the importance of thorough pharmacological profiling for this class of substances. As an historical example, the synthesis of α-methyltryptamine, also known as 3-(2-aminopropyl)indole (3-API), (Fig. 1A) was first published in 1947 [6], and the substance was marketed as an antidepressant in the former Soviet Union in the 1960s. However, it was withdrawn from clinical use after a short period of time due to adverse effects, including psychedelic-like effects such as altered perception (e.g., hallucinations) and mood [7, 8]. Due to its hallucinogenic properties, 3-API is used recreationally since the 1990s, but the drug is also associated with acute mental health disturbances and seizures [2, 7]. Studies in rat brain synaptosomes not only revealed that 3-API inhibits monoamine uptake by serotonin, dopamine, and norepinephrine transporters (SERT, DAT, and NET), but also that it potently induces transporter-mediated efflux [9], and therefore exhibits characteristics reminiscent of amphetamine-type psychostimulants [10].

In contrast to 3-API, psychedelic-like effects have rarely been reported for the positional isomers 5-(2-aminopropyl)indole (5-API) and 6-(2-aminopropyl)indole (6-API), which originated from pharmaceutical industry research [11]. The recreational use of 5-API is associated with severe sympathomimetic adverse effects and has contributed to several fatalities in Europe [12–16]. 5-API and 6-API are substrates of SERT, DAT, and NET in rat brain

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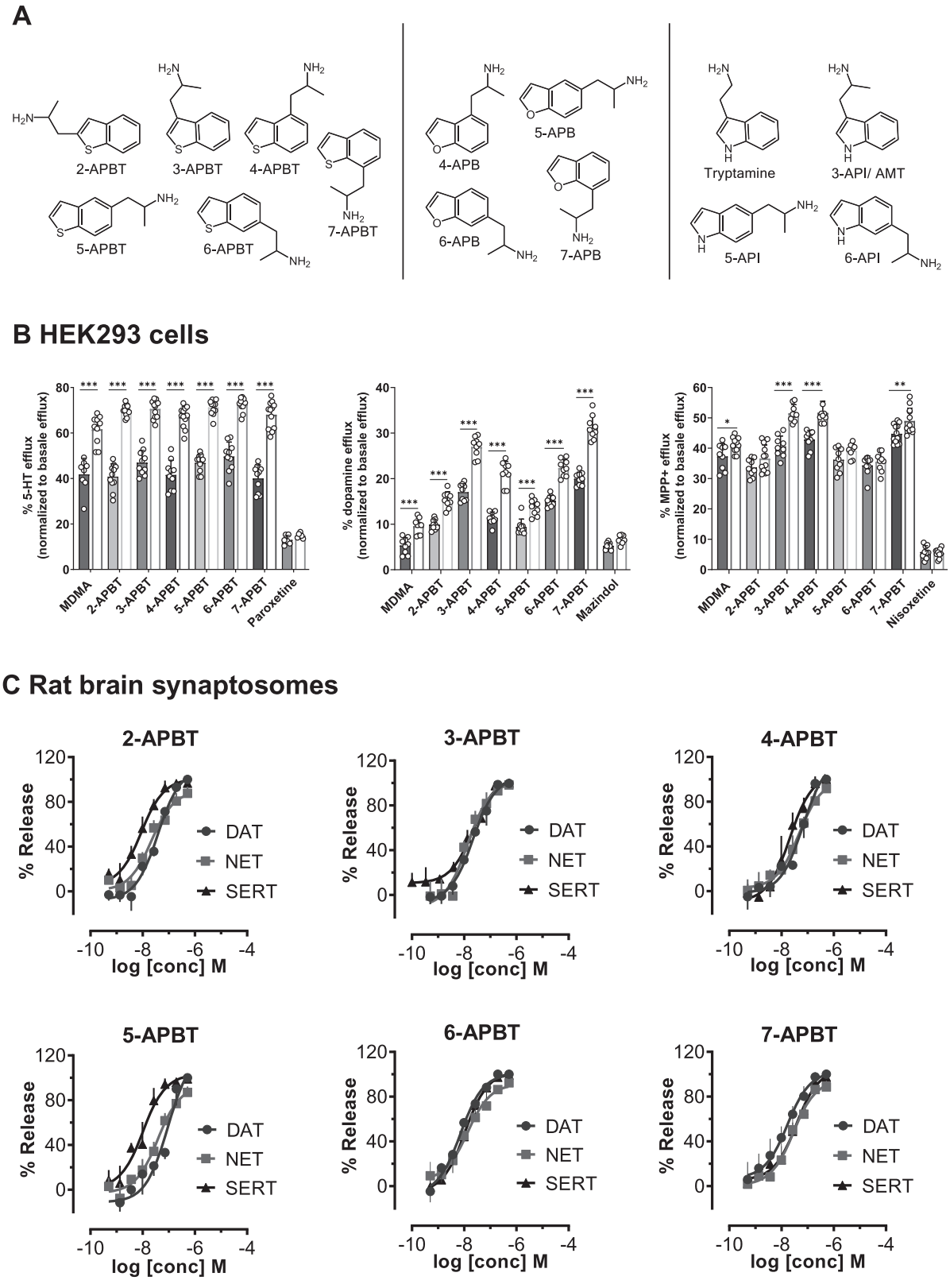

synaptosomes, potently inducing substrate release [17]. The position of the alkylamine side chain strongly impacts the selectivity of API isomers at DAT vs. SERT, resulting in eightfold higher DAT selectivity for 5-API and eightfold higher SERT selectivity for 6-API [17]. In human transporter-transfected cells, 5-API also potently inhibits monoamine transport at SERT and DAT [18]; in addition, 5-API acts as a releaser of dopamine and serotonin. However, in contrast to studies in rat brain synaptosomes, 5-API was reported to strongly inhibit norepinephrine uptake without inducing significant norepinephrine efflux in

Fig. 1 Chemical structures of APBT isomers and chemically related compounds and effects of APBT isomers on transporter-mediated efflux in HEK293 cells and in rat brain synaptosomes. A Chemical structures of (2-aminopropyl)benzo[β]thiophens and examples of the 5-(2-aminopropyl)indoles 3-API, 5-API, and 6-API and the (2-aminopropyl)benzofuran isomers 4-APB, 5-APB, 6-APB, and 7-APB. **B** Transporter-mediated efflux in HEK293 cells expressing MATs. Monoamine release was induced by 10 μM compound in the absence (filled bars) or presence (empty bars) of 10 μM monensin after preloading the transporter-transfected cells with the respective radiolabeled substrate. Transporter blockers (paroxetine, mazindol, and nisoxetine for hSERT, hDAT, and hNET, respectively) were used to assess nonspecific efflux. Data are expressed as mean ± SD from at least three experiments performed in triplicate. Data were analyzed by two-way ANOVA followed by Sidak's multiple comparison test (***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ when compared to the corresponding condition without monensin). **C** Transporter-mediated efflux in rat brain synaptosomes. Data are mean ± SD expressed as a % of maximal release response for three experiments performed in triplicate.

human NET-transfected cells [18]. In addition to its interactions with monoamine transporters (MATs), 5-API inhibits human monoamine oxidase A (MAO-A) [19]. Furthermore, 5-API binds to adrenergic and serotonergic receptors, and partially activates serotonergic 5-HT_{2A} and 5-HT_{2B} receptors [18]. The extant literature illustrates the complex pharmacology of API positional isomers.

The structurally related (2-aminopropyl)benzofurans (APBs) share the heterocyclic core ring structure found in API analogs but with bioisosteric replacement of the nitrogen by an oxygen atom. Pharmacological studies with 4-APB, 5-APB, 6-APB, and 7-APB revealed potent norepinephrine uptake inhibition combined with a more pronounced 5-HT vs. dopamine uptake inhibition in human transporter-transfected cells [20]. In rat brain synaptosomes, 5-APB and 6-APB were found to be nonselective monoamine releasers, serving as efficacious substrates for SERT, DAT, and NET [21]. Furthermore, 4-APB, 5-APB, 6-APB, and 7-APB are strong monoamine releasers that also interact with various monoaminergic receptors and the trace amine-associated receptor 1 [20].

Derivatives of (2-aminopropyl)benzo[β]thiophene (APBT) are sulfur-based analogs of APIs and APBs that have recently been synthesized and analytically characterized [22]. However, the pharmacological profile of these APBTs has not yet been investigated. APBTs share the heterocyclic core ring structure also found in API and APB analogs but with bioisosteric replacement of the nitrogen (APIs) or oxygen (APBs) by a sulfur atom (Fig. 1A). The pharmacological profile of APBTs may vary depending on the location and composition of side chains or the presence of ring substituents. The impact of side chain substitution has previously been described for the amphetamine scaffold, which essentially alters the potency and affinity of the resulting derivatives at different monoaminergic targets [23].

Previous studies with APBTs include a patent issued to Smith Kline & French Laboratories in 1960, which reported various central nervous effects [24]. In studies using mitochondrial suspensions from rat brain, 3-APBT inhibited MAO-A but had no effect on MAO-B [25]. The current study aimed to assess the pharmacological profile of six APBT positional isomers 2-, 3-, 4-, 5-, 6-, and 7-APBT (Fig. 1A). Based on the structural similarities to their indole and benzofuran counterparts, the APBT isomers featured in the present study are hypothesized to modulate monoaminergic neurotransmission similar to other stimulants such as 5-API/6-API or 5-APB/6-APB by interacting with MATs and possibly monoaminergic receptors.

MATERIAL AND METHODS

Experimental procedures for cell culture, uptake inhibition, batch release, and FRET experiments in transporter-transfected human embryonic kidney 293 (HEK293) cells were performed as described previously [26–28]. Uptake inhibition experiments in rat brain synaptosomes have been reported earlier [17] and were conducted as described. All experiments using animal tissue were performed according to the ARRIVE guidelines. Receptor binding affinity and activation potency assays were performed as described in previous literature [29–31]. In vivo behavioral experiments using male C57/BL6J mice, including assessment of locomotor activity and temperature changes as well as head-twitch response (HTR) post ABPT administration, were performed as described previously [17, 32–34] or as described in the supplemental methods. All in vivo behavioral procedures were approved by the Animal Care and Use Committee of the NIDA, IRP. More detailed information on all experimental procedures, including in silico docking simulations, release experiments in rat brain synaptosomes, details of statistical analyses, and all reagents used, can be found in the supplemental file.

RESULTS

Benzothiophene isomers inhibit transporter-mediated uptake in HEK293 cells

To assess whether the six APBTs interact with hSERT, hDAT, or hNET, we performed uptake inhibition experiments in transporter-transfected HEK293 cells at the respective MAT. All six isomers were fully efficacious inhibitors of 5-HT uptake with IC_{50} values ranging from 0.79 to 3.87 μM. IC_{50} values obtained for dopamine uptake inhibition ranged from 0.90 to 7.62 μM. Similar to the values obtained for hSERT and hDAT, IC_{50} values for MPP^+ uptake inhibition at hNET were between 0.53 and 1.75 μM (Fig. S1A, Table 1). The APBTs were more potent inhibitors at hSERT vs. hDAT except for 7-APBT. In contrast, only 2-APBT and 5-APBT inhibited hSERT more potently than hNET. Compared to MDMA, all six isomers were more potent inhibitors at hSERT, hDAT, and hNET. Importantly, different side chain positions attached to the benzothiophene heterocycle did not dramatically affect inhibition potency at the different MATs. IC_{50} values for inhibition of GABA uptake at hGAT1 for 5-APBT, 6-APBT, and 7-APBT were weak (>1 mM) (Fig. S2, Table S1).

Benzothiophene isomers inhibit transporter-mediated uptake in rat brain synaptosomes

The uptake inhibition activity of the APBTs was tested in rat brain synaptosomes in order to examine the effects in a native tissue preparation containing plasma membrane transporters in situ. As shown in Fig. S1B and Table 1, all six isomers were potent inhibitors of [^{3}H]5-HT uptake in rat brain synaptosomes with IC_{50} values ranging from 32.9 to 184.2 nM. Likewise, all tested APBTs were fully efficacious inhibitors of [^{3}H]dopamine (IC_{50} values between 161 and 917 nM) and [^{3}H]norepinephrine (IC_{50} values between 198 and 486 nM) uptake in rat brain synaptosomes (Table 1). In general, the APBTs were nonselective in their effects on uptake inhibition, although 2-APBT and 5-APBT displayed some preference for rSERT. Compared to MDMA, all six isomers were more potent inhibitors of any tested neurotransmitter uptake in rat brain synaptosomes.

Benzothiophenes evoke transporter-mediated release in HEK293 cells

To distinguish between monoamine uptake blockers and substrate-type releasers, the effect of the APBTs on monoamine reverse transport was investigated. Release assays in cells were performed at a single concentration of 10 μM for each isomer with the same MAT-expressing cell lines used for uptake inhibition experiments. MDMA (10 μM) was used as a reference substance for comparison. All tested isomers were releasers at SERT, DAT,

and NET (Fig. 1B). APBT-mediated release at SERT was similar for all tested isomers and slightly stronger compared to the effect of MDMA at the same concentration. Likewise, all tested isomers induced release at DAT comparable to MDMA, whereas 3-APBT and 7-APBT stimulated release at DAT the most. At NET, 3-APBT, 4-APBT, and 7-APBT were stronger releasers than MDMA, whereas 2-APBT, 5-APBT, and 6-APBT-induced efflux in a similar or slightly lower manner. At SERT and DAT, the Na^+/H^+-ionophore monensin significantly enhanced efflux for all six isomers (Fig. 1B). At NET, monensin significantly increased the efflux of the stronger norepinephrine releasers 3-APBT, 4-APBT, and 7-APBT as well as of MDMA, but not of the remaining isomers (Fig. 1B).

Benzothiophenes evoke transporter-mediated release in rat brain synaptosomes

The dose-effect efflux curves for APBTs in rat brain synaptosomes are shown in Fig. 1C and the corresponding EC_{50} values are displayed in Table 2. All tested isomers were potent releasers of preloaded [^{3}H]5-HT with EC_{50} values ranging from 8.9 to 36.9 nM, compared to 75.9 nM for MDMA. Similar EC_{50} values were found for [^{3}H]MPP^+ release at rDAT, which ranged from 7.2 to 92.8 nM, compared to 118.4 nM for MDMA. APBT-induced efflux of preloaded [^{3}H]MPP^+ at rNET occurred with EC_{50} values ranging from 13.4 to 46.2 nM, which is similar to the EC_{50} values at rSERT and rDAT. APBTs displayed more potent substrate-releasing properties at rNET in comparison to the effects of MDMA (EC_{50} 113.7 nM). In agreement with the uptake inhibition findings from synaptosomes, the APBTs were generally nonselective in their releasing effects, with 2-APBT and 5-APBT showing some preference for rSERT.

Benzothiophenes induce the inward-facing conformation of SERT

To assess potential conformational changes induced by APBTs, intramolecular FRET was recorded in HEK293 cells stably expressing an hSERT construct with a fluorescence donor (CFP) and

Table 1. Inhibition of transporter-mediated uptake by APBT isomers compared to MDMA in HEK293 cells and rat synaptosomes.

HEK293 cells						
	SERT IC_{50} [nM] (95% CI)	**DAT IC_{50} [nM] (95% CI)**	**NET IC_{50} [nM] (95% CI)**	**DAT/SERT ratio**	**DAT/NET ratio**	**NET/SERT ratio**
2-APBT	786.8 (663.8–932.5)	1344 (1010–1788)	924.0 (766.4–1114)	0.59	0.69	0.85
3-APBT	1871 (1571–2229)	3258 (2398–4427)	536.4 (413.5–695.8)	0.57	0.16	3.49
4-APBT	2824 (2069–3854)	7617 (5753–10,086)	1295 (1048–1602)	0.37	0.17	2.18
5-APBT	1263 (1043–1530)	2101 (1553–2843)	1748 (1245–2453)	0.60	0.83	0.72
6-APBT	796.7 (678.2–935.8)	903.9 (688.2–1187)	655.2 (534.2–803.5)	0.88	0.72	1.22
7-APBT	3872 (3068–4886)	992.4 (803.0–1226)	527.7 (417.3–667.3)	3.90	0.53	7.34
MDMA	4531 (3314–6195)	19030 (14,090–25,700)	4570 (2930–7128)	0.24	0.24	0.99
Rat brain synaptosomes						
	SERT IC_{50} [nM] (95% CI)	**DAT IC_{50} [nM] (95% CI)**	**NET IC_{50} [nM] (95% CI)**	**DAT/SERT ratio**	**DAT/NET ratio**	**NET/SERT ratio**
2-APBT	32.9 (19.9–54.5)	322.3 (186.7–556.5)	267.0 (201.0–354.7)	0.10	0.83	0.12
3-APBT	91.2 (61.6–134.8)	371.8 (264.3–523.0)	247.0 (182.1–335.1)	0.24	0.67	0.37
4-APBT	152.3 (95.3–243.3)	917.0 (776.2–1083.3)	486.3 (351.9–672.1)	0.17	0.53	0.31
5-APBT	46.5 (32.2–67.2)	430.9 (334.0–555.9)	295.2 (216.7–402.3)	0.11	0.69	0.16
6-APBT	66.3 (43.9–100.1)	160.6 (136.6–188.7)	198.1 (138.6–283.2)	0.41	1.23	0.33
7-APBT	184.2 (126.1–269.1)	212.8 (145.3–311.6)	291.5 (230.4–368.8)	0.86	1.37	0.63
MDMA	471.9 (251.9–884.1)	1826 (1270–2626)	1107 (852.8–1437)	0.26	0.61	0.43

IC_{50} values are given as mean and 95% confidence intervals obtained from nonlinear curve fits obtained from at least three independent experiments, performed in triplicate (data shown in Fig. S1A, B). DAT/SERT ratio is expressed as 1/(DAT IC_{50}) divided by 1/(SERT IC_{50}). DAT/NET ratio is expressed as 1/(DAT IC_{50}) divided by 1/(NET IC_{50}). NET/SERT ratio is expressed as 1/(NET IC_{50}) divided by 1/(SERT IC_{50}).

Table 2. Transporter-mediated efflux by APBT isomers in rat synaptosomes.

Rat brain synaptosomes						
	SERT EC_{50} [nM] (95% CI)	**DAT EC_{50} [nM] (95% CI)**	**NET EC_{50} [nM] (95% CI)**	**DAT/SERT ratio**	**DAT/NET ratio**	**NET/SERT ratio**
2-APBT	8.9 (5.6–14.2)	38.6 (27.3–54.6)	21.6 (13.6 –34.2)	0.23	0.56	0.41
3-APBT	21.9 (14.6–32.8)	21.7 (16.0–29.6)	13.4 (7.9 –22.8)	1.00	0.62	1.62
4-APBT	21.2 (12.7–35.2)	66.6 (42.0–105.5)	46.2 (28.9 –73.7)	0.32	0.69	0.46
5-APBT	10.3 (5.6–19.0)	92.8 (48.4–178.1)	38.4 (23.8 –62.2	0.11	0.41	0.27
6-APBT	10.7 (7.5–15.4)	7.2 (4.5–11.7)	13.6 (9.1 –20.6)	1.50	1.90	0.78
7-APBT	36.9 (23.4–58.3)	16.8 (9.7–28.9)	28.5 (17.0 –47.8)	2.20	1.70	1.29
MDMA	75.9 (41.3–139.2)	118.4 (80.2–174.9)	113.7 (69.3 –186.3)	0.64	0.96	0.67

EC_{50} values are given as mean and 95% confidence intervals obtained from nonlinear curve fits obtained from at least three independent experiments, performed in triplicate (data shown in Fig. 1C). DAT/SERT ratio is expressed as 1/(DAT EC_{50}) divided by 1/(SERT EC_{50}). DAT/NET ratio is expressed as 1/(DAT EC_{50}) divided by 1/(NET EC_{50}). NET/SERT ratio is expressed as 1/(NET EC_{50}) divided by 1/(SERT EC_{50}).

acceptor (YFP) attached to the N terminus and C terminus, respectively. This construct can be used to detect conformational changes of SERT exposed to its substrates. Hence, an accumulation of inward-facing SERT conformations, which results in decreased FRET, is an indicator for substrate-type substances [28]. All six isomers reduced the NFRET between 6 and 10%, greater than the effect produced by MDMA (3%), but less than the effect of PCA (19%) (Fig. S3A). The observed change in FRET indicates a conformational change to an inward-facing conformation [28], supporting the substrate-like behavior and stimulatory effects in in vitro efflux experiments, indicating that all tested isomers are substrates at SERT (Fig. S3A).

Benzothiophenes fit into the orthosteric binding site of MATs in silico

APBTs and the respective native substrates were docked in silico into the primary substrate binding pocket S1 of SERT, DAT, and NET. The MATs were in the ligand-binding competent outward-open conformation [35] in the presence of bound Na^+ and Cl^- ions. For each transporter and compound, 1000 poses with estimated docking energies were obtained to allow for achieving extensive sampling. Representative poses of highly populated clusters with low binding energies containing at least 100 docking poses are shown in Fig. S3B (right panel). The distribution of estimated binding energies of all docked poses is shown in Fig. S3B (left panel). Each APBT showed overlapping docking poses in hSERT, hDAT, and hNET with seemingly indistinguishable binding energies. Moreover, the poses overlapped with those of the respective native substrate, supporting the consistency of the docking results. Of note, the docking pose of dopamine at hDAT was identical to the binding conformation observed in the drosophila DAT crystal structure [36]. Docking energies of the APBTs ranged from −35 to −20 kJ/mol, which correspond to estimated K_i values ranging from 0.7 to 313 µM. The native substrates, which were used as controls, showed weaker affinity for the transporters. The indistinguishable binding energies of the tested isomers was consistent with our experimental and FRET microscopy data despite the differences in chemical structure.

Benzothiophenes bind to and activate 5-HT_2 receptor subtypes

For receptor binding affinity and activation potency assays as well as for subsequent in vivo behavioral investigations, only 3-APBT, 5-APBT, and 6-APBT were included; these isomers correspond to the most frequently encountered substitution profile of benzofurans and aminopropyl indoles. The binding affinities and agonist potencies of 3-APBT, 5-APBT, and 6-APBT at 5-HT_2 subtypes are listed in Table 3 and corresponding curves are shown in Fig. S4. All tested APBTs bound to the 5-HT_{2A} and 5-HT_{2C} receptors with affinities in the range of 196–461 nM. 3-APBT and 5-APBT bound to the 5-HT_{2B} receptor with high nanomolar affinity (3-APBT: K_i = 5.88 nM; 5-APBT: K_i = 3.98 nM). The three APBT isomers were highly efficacious agonists at all three 5-HT_2 subtypes, but they had at least an order of magnitude higher potency at the 5-HT_{2B} receptor compared to the 5-HT_{2A} and 5-HT_{2C} subtypes.

In vivo behavioral studies in mice

Benzothiophene isomers fail to stimulate locomotor activity. Treatment with 3-APBT or 6-APBT across a broad range of doses surprisingly did not increase locomotor activity compared to saline-treated control mice (Fig. 2A, C; $F_{8,36} = 0.86$ n.s., $F_{8,36} = 0.93$ n.s.). Similarly, 5-APBT did not increase locomotor activity at any dose tested and reduced the total distance traveled significantly ($F_{8,36} = 4.62$ $P = 0.0006$) at 10 mg/kg s.c. vs. vehicle controls ($P < 0.05$, Dunnett's multiple comparisons test; Fig. 2B), but not at the highest dose. In contrast, 10 mg/kg s.c. 5-APB and 6-APB as well as 30 mg/kg s.c. MDMA all produced significant locomotor stimulation vs. saline controls ($F_{3,16} = 18.69$ $P = 0.0006$; $^*P < 0.05$, Dunnett's multiple

Table 3. Serotonin receptor binding affinities and activation potencies.

	5-HT_{2A}				5-HT_{2B}				5-HT_{2C}			
	K_i ± SEM [nM]	EC_{50} [nM]	pEC_{50} ± SEM	E_{max} ± SEM [%]	K_i ± SEM [nM]	EC_{50} [nM]	pEC_{50} ± SEM	E_{max} ± SEM [%]	K_i ± SEM [nM]	EC_{50} [nM]	pEC_{50} ± SEM	E_{max} ± SEM [%]
5-HT	N.D.	0.34	9.47 ± 0.03	100	N.D.	0.20	9.69 ± 0.03	100	N.D.	0.40	9.40 ± 0.03	100
3-APBT	461 ± 50	44.4	7.35 ± 0.03	94.3 ± 1.1	5.88 ± 0.65	3.40	8.47 ± 0.02	89.2 ± 0.5	231 ± 52	25.4	7.60 ± 0.02	96.4 ± 0.7
5-APBT	400 ± 76	14.4	7.84 ± 0.03	93.3 ± 0.8	3.98 ± 0.59	0.79	9.10 ± 0.02	94.2 ± 0.5	321 ± 74	21.6	7.67 ± 0.02	104.4 ± 0.8
6-APBT	196 ± 29	6.69	8.18 ± 0.03	93.1 ± 0.4	N.D.	0.45	9.35 ± 0.02	94.2 ± 0.4	288 ± 104	10.0	8.00 ± 0.03	105.8 ± 0.9

Binding data were generated according to PDSP protocols. K_i values represent means ± SEM from three independent experiments performed in triplicate. Calcium flux data were collected in HEK T-Rex-293 inducible cell lines stably expressing either human 5-HT_{2A}, human 5-HT_{2B}, or human 5-HT_{2C} receptors. Data represent EC_{50}, pEC_{50}, and E_{max} means ± SEM from three independent experiments performed in triplicate. E_{max} is defined as percent of 5-HT maximum response.

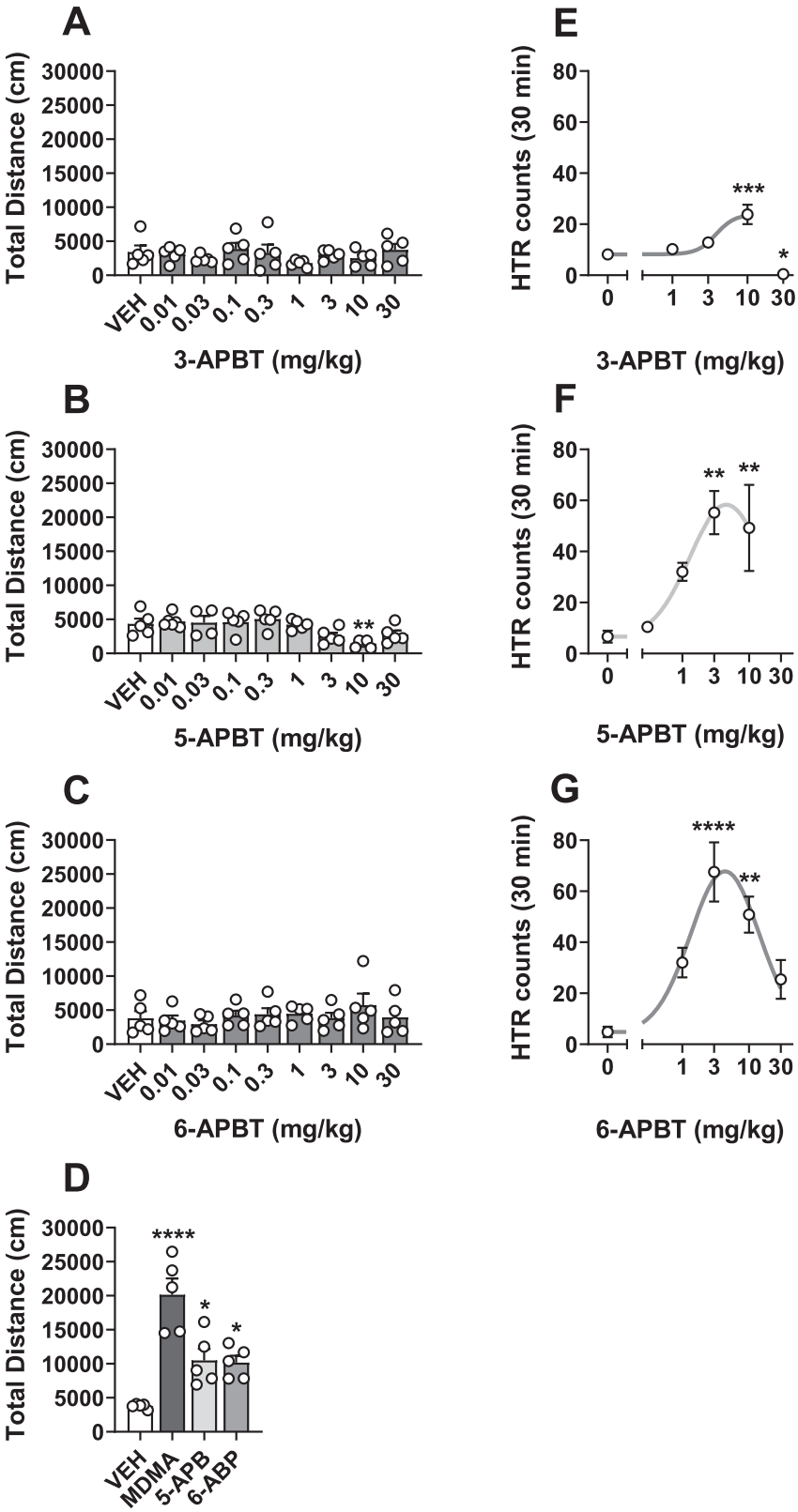

Fig. 2 Effects of 3-APBT, 5-APBT, and 6-APBT on locomotor activity and HTR in mice. A–D APBT-treated mice received s.c. injections of 0.01–30 mg/kg s.c. 3-APBT, 5-APBT, 6-APBT, or vehicle (saline 0.9%). Locomotor activity was assessed over 60 min following habituation and drug injections. MDMA (30 mg/kg), 5-APB (10 mg/kg), and 6-APB (10 mg/kg) were administered s.c. as positive controls that are known to produce locomotor stimulation in mice. Data are mean ± SEM for $n = 5$ mice per group and were compared via one-way ANOVA with Dunnett's post hoc test comparing all groups to 0 mg/kg vehicle controls (****$P < 0.0001$, **$P < 0.01$, *$P < 0.05$). **E–G** Effect of 3-APBT, 5-APBT, and 6-APBT on the HTR in mice. Data are presented as group means ± SEM. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$ indicate significant difference from the vehicle control group (Dunnett's test).

comparisons test; Fig. 2D). Outside of increased rest time induced by 10 mg/kg 5-APBT, none of the APBT compounds at any doses tested differed from vehicle controls in number of movements ($F_{8,36} = 0.29$ n.s., $F_{8,36} = 0.07$ n.s., $F_{8,36} = 0.70$ n.s.), rest time ($F_{8,36} = 0.56$ n.s., $F_{8,36} = 0.01$ n.s., $F_{8,36} = 0.75$ n.s.), or stereotypy episodes ($F_{8,36} = 0.70$ n.s., $F_{8,36} = 0.03$ n.s., $F_{8,36} = 0.48$ n.s.) in locomotor experiments (Fig S5A–I).

These data support that lack of locomotor activity by APBT compounds is not due to masking by stereotypies or differences in activity. Despite having no effect on locomotor activity, 3-APBT ($F_{8,35} = 15.97$ $P < 0.0001$), 5-APBT ($F_{8,34} = 20.42$ $P < 0.0001$), and 6-APBT ($F_{8,35} = 28.92$ $P < 0.0001$) significantly decreased core body temperature at the two highest doses vs. vehicle controls ($P < 0.05$, Dunnett's multiple comparisons test; Fig. S6A–C), attesting to the bioavailability of the drugs at the doses tested. Comparatively, 10 mg/kg 5-APB or 6-APB also reduced core body temperature ($F_{3,17} = 13.09$ $P = 0.0001$; $P < 0.05$, Dunnett's multiple comparisons test), but this effect was not observed in mice given 30 mg/kg s.c. MDMA (Fig. S6D).

Benzothiophene isomers induce the head-twitch response (HTR). The HTR serves as a measure of 5-HT_{2A} activation in mice [37]. Given the potent agonist activity of 3-APBT, 5-APBT, and 6-APBT at the 5-HT_{2A} receptor, HTR studies were conducted in C57BL/6J mice to determine whether those isomers can activate the receptor in vivo. As shown in Fig. 2E–G, 3-APBT ($F(4,20) = 17.2$, $P < 0.0001$), 5-APBT ($F(4,20) = 6.40$, $P = 0.0017$), and 6-APBT ($F(4,20) = 10.23$, $P = 0.0001$) induced the HTR with inverted U-shaped dose-response functions. The ED_{50} of 3-APBT was calculated by nonlinear regression as 3.70 (95% CI 1.97–6.96) mg/kg, which is equivalent to 16.2 µmol/kg. 5-APBT was more potent, inducing the HTR with $ED_{50} = 1.05$ (95% CI 0.51–2.13) mg/kg, which is equivalent to 4.60 µmol/kg. The ED_{50} for 6-APBT was 1.08 (95% CI 0.63–1.82) mg/kg, which equals 4.72 µmol/kg. In summary, 5-APBT and 6-APBT are equipotent, and both compounds have about four times higher potency than 3-APBT.

DISCUSSION

The aim of the current study was to determine the pharmacological effects of six APBT isomers, as compared to structurally similar APIs and APBs reported in the literature. All six APBTs exhibited a similar ability to inhibit uptake at MATs and induced transporter-mediated substrate release. The in vitro results from HEK293 cells and rat brain synaptosomes showed that APBTs inhibit substrate uptake in a concentration-dependent manner at human and rat SERT, DAT, and NET, whereas they had no effect at human GAT1, a transporter from the same family that was included as a negative control. The different potencies of APBTs in HEK293 cells compared to rat brain synaptosomes may be based on the different species and tissue origin, as it has been observed for other stimulants [38]. Uptake inhibition potencies in HEK293 cells were in the low micromolar range, with potencies typically being higher at SERT than at DAT, which is comparable to the activity of the benzofurans [20]. In HEK293 cells, 5-APBT was nearly equipotent at SERT and DAT, similar to the nitrogen analog 5-API; however, 5-APBT was around 40-fold less potent at NET than its indole analog [18]. Overall, replacing the oxygen (APBs) or nitrogen (APIs) atoms with sulfur (APBTs) had no overt effect on the in vitro SERT uptake inhibition potency. However, the potency of NET inhibition decreased after replacing oxygen and nitrogen with sulfur, whereas the potency of DAT inhibition decreased when replacing nitrogen with sulfur [18, 20].

The DAT:SERT inhibition ratio (defined as [1/DAT IC_{50}]/[1/SERT IC_{50}]) can be used to predict the reinforcing potential and abuse liability of psychostimulant drugs, with higher DAT selectivity indicating greater propensity for addictive properties [39–44]. Entactogenic substances, such as MDMA, typically exhibit a DAT:SERT ratio of <1 and display lower abuse liability when compared to drugs like amphetamine and methamphetamine [43, 45, 46]. The observed DAT:SERT inhibition ratios of APBTs tested in rat brain synaptosomes and in HEK293 cells were generally <1, which suggests that their pharmacological effects are more MDMA-like rather than methamphetamine-like. The predominantly serotonergic properties of the tested APBTs observed in vitro may also indicate lower abuse liability, similar to what has been observed for MDMA [42, 47], which is a weak reinforcer in self-administration studies [48]. Furthermore, MDMA has been proven effective as an adjunct to psychotherapy in patients suffering from posttraumatic stress disorder and other neuropsychiatric diseases [49–59].

The APBTs were not only efficacious uptake inhibitors, but they also acted as potent transporter substrates by way of releasing preloaded substrate at human and rat SERT, DAT, and NET. Similar results have been reported for their benzofuran counterparts in transporter-transfected HEK293 cells [20, 60]. The sodium-proton ionophore monensin augmented the APBT-triggered outward transport of preloaded MAT substrates, a clear indication of the substrate activity of APBTs [10]. To further explore the substrate activity of the compounds, we performed FRET microscopy to examine the conformational equilibrium induced by the six isomers using a SERT-construct tagged at the N- and C-termini with CFP and YFP, respectively [61]. Indeed, similar to previous findings with known transporter substrates [62], the APBT isomers induced an inward-facing conformation of SERT, as seen with PCA and MDMA.

FRET and efflux measurements showed that APBTs are MAT substrates, which suggests a binding mode similar to the native substrate(s). Accordingly, the best poses for all APBTs were very similar to each other and fully consistent with the known binding mode of dopamine and various amphetamines as observed in complexes with drosophila DAT [36]. The best binding poses of the APBTs show that their positively charged amino group interacts with the conserved and functionally essential aspartate (D79 in hDAT, D98 in hSERT, D75 in hNET) in TM1, while the hydrophobic benzothiophene ring structure binds into the dominantly hydrophobic pocket between TM3 and TM8. The cumulative interactions of the hydrophobic benzothiophene ring with the hydrophobic pocket in the S1 are similar as all APBT poses occupy the same volume in the substrate binding site. They can tolerate small difference in their binding mode without a big change in affinity, because of the absence of orientation-defining hydrogen bonds. Importantly, these small adjustments allow for optimally positioning the amine nitrogen for all APBT isomers, which is important for substrate transport.

In rat brain synaptosomes, 5-API and 6-API have previously been shown to act as substrates at SERT, DAT, and NET [17] though 5-API was a more potent releaser at DAT compared to SERT with eightfold higher selectivity for DAT. In contrast, 5-APBT was more potent at releasing 5-HT in rat brain synaptosomes when compared to DAT, with a tenfold higher selectivity for SERT (Table 2). Overall, replacement of the oxygen or nitrogen with sulfur in the drug scaffold led to enhanced 5-HT releasing properties at SERT.

Since 3-APBT, 5-APBT, and 6-APBT potently induced efflux at DAT and NET in HEK293 cells and rat brain synaptosomes, we expected a corresponding increase in locomotor activity consistent with a typical psychostimulant profile. Surprisingly, in contrast to locomotor stimulant effects of 5-APB, 6-APB, and MDMA in the present and previous studies [21, 63–65], none of the tested APBTs produced locomotor stimulation, and 5-APBT was even observed to reduce the distance traveled at the higher dose of 10 mg/kg. Importantly, the compounds at various doses did not differ from vehicle controls in number of movements, rest time, or stereotypy episodes throughout the testing session supporting that the lack of locomotor activity is not due to competing behaviors (stereotypies) or differences in activity. Thus, APBTs are potent transporter releasers much like indoles, benzofurans, and MDMA,

yet the APBTs do not induce locomotor stimulation. The lack of locomotor stimulation may be related to the potent activation of 5-HT_{2C} receptors, which has been shown to decrease locomotor activity [66, 67]. Additionally, although interactions with the 5-HT_{1A} receptor were not assessed, activation of the 5-HT_{1A} receptor by the APBT isomers could potentially reduce locomotor stimulation, as has been demonstrated for selective and nonselective 5-HT_{1A} agonists [68, 69]. Furthermore, potential antagonism at the histamine receptor H_1 might depress locomotor activity [70, 71]. Interestingly, the benzofuran analogs 5-APB and 6-APB produce time- and dose-dependent stimulation of locomotor activity [63] and have been shown to bind to 5-HT_{1A}, 5-HT_{2C}, and H_1 receptors, but with unknown activation potential [20]. Furthermore, the indole analog 5-API strongly induced locomotor activation, while 6-API did not, which correlated well with the corresponding dopamine releasing potencies in rat brain synaptosomes (5-API $EC_{50} = 12.9$ nM; 6-API $EC_{50} = 164$ nM) [17]. The DAT EC_{50} value of 5-API was around sevenfold lower than the corresponding value of 5-APBT, hence, pointed to a higher efficacy of 5-API to induce dopamine release. Importantly, 5-API displayed a higher DAT:SERT ratio than any APBT assessed in the current study [17]. Hence, potentially none of the APBTs features a DAT:SERT ratio that was sufficient to elicit substantial motor stimulation. 6-APBT, which had the highest efflux potency at DAT in rat brain synaptosomes, was also a very potent 5-HT releaser. Hence, locomotor stimulation by 6-APBT may have been depressed by its strong serotonergic effects [72–74], potential activation of H_1 receptors, or other yet unidentified or unexamined downstream effects, which impact on monoamine release. Since compounds that increase locomotor activity are likely to be reinforcing [75] and the assessed APBTs lacked motor stimulatory effects and displayed predominantly low DAT:SERT ratios, these substances can be expected to have a low abuse potential [41, 42, 45, 46]. However, further behavioral assays are needed to confirm this anticipation.

The tested APBTs displayed high affinity for all tested 5-HT_2 receptor subtypes and activated each receptor in the low nanomolar range. The 5-HT_{2A} receptor is the primary target for LSD, psilocybin, and other hallucinogenic drugs in the brain [76, 77]. Other hallucinogenic drugs acting strongly at 5-HT_{2A} include 3-API [78, 79], while, by contrast, 5-API displays a much lower potency and efficacy [18]. Hence, the interaction of APIs with the 5-HT_{2A} receptor is dependent on the position of the alkylamine side chain on the indole ring. Although relevant data have not been reported for 3-APB, the 5-methoxy-substituted derivative 5-methoxy-3-(2-aminopropyl)benzofuran has high affinity for 5-HT_{2A} sites [80]. In line with the activity of 5-API, both 5-APB and 6-APB have been shown to be active at 5-HT_{2A} with relatively low potency and efficacy [20]. Given those previous findings, it is notable that 3-APBT, 5-APBT, and 6-APBT are highly efficacious 5-HT_{2A} agonists. Hence, compared to APIs and APBs, the ability of APBTs to activate the 5-HT_{2A} receptor does not depend on side chain position. Furthermore, the switch to the benzothiophene heterocyclic ring system led to increased binding affinity, activation potency, and efficacy at 5-HT_2 receptors. HTR studies confirmed that 3-APBT, 5-APBT, and 6-APBT activate 5-HT_{2A} in vivo, indicating those compounds may have psychedelic-like psychopharmacology (the HTR is used as a behavioral proxy in rodents for human psychedelic effects [33]). The investigated APBT isomers did not show a clear selectivity for either 5-HT_{2A} or 5-HT_{2C}. Previous studies showed that 5-HT_{2C} agonists may act to mask behavioral effects induced by 5-HT_{2A} receptor activation in rats [66, 81]. However, these agonists displayed much higher selectivity for 5-HT_{2C} receptors vs. 5-HT_{2A} receptors. For example, the 5-HT_2 agonist Ro 60–0175, which does not induce the HTR, shows a 30-fold selectivity for the 5-HT_{2C} receptor vs. the 5-HT_{2A} receptor [82], unless administered in combination with a 5-HT_{2C}-selective antagonist [83]. Additionally, the well characterized psychedelic 2,5-dimethoxy-4-methylamphetamine (DOM), an equipotent 5-HT_{2A} and 5-HT_{2C} receptor agonists, has been shown to induce the HTR [37, 84]. Hence, the investigated APBT isomers did not display enough 5-HT_{2C} selectivity to mask the behavioral effects induced by 5-HT_{2A} receptor activation. Like most psychedelics and various stimulants, including benzofuran and indole derivatives, the tested APBT isomers displayed relatively potent agonist activity at 5-HT_{2B} receptors [85]. The activation of 5-HT_{2B} receptors has, among others, been linked to cardiac valvulopathy [86, 87]. However, several studies concluded that long-term regular substance exposure is needed to induced this adverse effect. Hence, potential future medical application of APBTs is not excluded as cardiac valvulopathy is an unlikely adverse effect if these compounds are only sporadically used [88].

Further studies are needed to assess affinities at adrenergic, dopaminergic, and histaminergic receptors to evaluate possible systemic effects. Additionally, assessment of inhibitory effects on MAO, which has already been shown for 3-APBT and MAO-A [25], is needed to estimate the risk for drug–drug interactions. The ability of APBTs to simultaneously release 5-HT and potentially inhibit MAO-A and/or MAO-B could exacerbate the serotonergic effects of co-administered drugs or could potentially result in serotonin toxicity [89].

The potential psychedelic-like properties of these novel APBT isomers combined with their lack of locomotor stimulation and therefore anticipated low abuse potential raises interesting questions for further research regarding their potential development as medications for use in drug-assisted psychotherapy [90].

CONCLUSION

In summary, the APBT isomers investigated here inhibit monoamine uptake and stimulate substrate release, but without inducing locomotor activity typically seen with structurally related APIs, ABPs, and MDMA. Interestingly, the position of the aminopropyl side chain of APBTs had little influence on transporter selectivity. When compared to APBs and APIs, replacement of the ring oxygen or nitrogen with a sulfur atom as in the APBT isomers led to enhanced SERT releasing effects and 5-HT receptor activities that fundamentally changed the in vivo profile of the compounds in mice. The investigated APBTs may be expected to exhibit psychedelic and entactogenic effects combined with low abuse potential. Whether this pharmacological profile translates into potential therapeutic activity, for instance as candidates for drug-assisted psychotherapy, warrants further investigation.

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AUTHOR CONTRIBUTIONS

SDB, GD, and PVK synthesized the APBT compounds. DR, TL, MH, and KJ performed uptake inhibition and efflux experiments in HEK293 cells and analyzed the data. DW conducted uptake inhibition and efflux experiments in rat brain synaptosomes and analyzed the data with assistance of MHB. DL performed FRET microscopy and analyzed the data. DS implemented and analyzed docking simulations at human SERT, DAT and NET with the supervision of TS. JDM conducted 5-HT_2 functional experiments and analyzed the data. GCG complemented in vivo locomotor activity and temperature assessment experiments in mice and analyzed the data. ALH conducted HTR experiments in mice and analyzed the data. DR, GCG, DL, DS, TS, SDB, MHB, ALH, and HHS wrote the manuscript with significant inputs from JDM, GD, and PVK.

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COMPETING INTERESTS

The authors declare no competing interests.

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