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Potential Functional Role of Phenethylamine Derivatives in Inhibiting Dopamine Reuptake: Structure–Activity Relationship

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Abstract

Numerous psychotropic and addictive substances possess structural features similar to those of β-phenethylamine (β-PEA). In this study, we selected 29 β-PEA derivatives and determined their structure–activity relationship (SAR) to their ability to inhibit dopamine (DA) reuptake; conducted docking simulation for two selected compounds; and identified their potential functionals. The compounds were subdivided into arylethylamines, 2-(alkyl amino)-1-arylalkan-1-one derivatives and alkyl 2-phenyl-2-(piperidin-2-yl)acetate derivatives. An aromatic group, alkyl group, and alkylamine derivative were attached to the arylethylamine and 2-(alkyl amino)-1-arylalkan-1-one derivatives. The inhibitory effect of the compounds on dopamine reuptake increased in the order of the compounds substituted with phenyl, thiophenyl, and substituted phenyl groups in the aromatic position; compounds with longer alkyl groups and smaller ring-sized compounds at the alkylamine position showed stronger inhibitory activities. Docking simulation conducted for two compounds, **9** and **28**, showed that the (S)-form of compound **9** was more stable than the (R)-form, with a good fit into the binding site covered by helices 1, 3, and 6 of human dopamine transporter (hDAT). In contrast, the (R, S)-configuration of compound **28** was more stable than that of other isomers and was firmly placed in the binding pocket of DAT bound to DA. DA-induced endocytosis of dopamine D_2 receptors was inhibited when they were co-expressed with DAT, which lowered extracellular DA levels, and uninhibited when they were pretreated with compound **9** or **28**. In summary, this study revealed critical structural features responsible for the inhibition of DA reuptake and the functional role of DA reuptake inhibitors in regulating D_2 receptor function.

Key Words: Phenethylamine, Drug addiction, Dopamine transporter, Dopamine D_2 receptor, Docking simulation, Structure activity relationship

INTRODUCTION

The medial forebrain bundle contains a subset of dopaminergic neurons that project from the ventral tegmental area to the nucleus accumbens (NAc) (Coenen *et al*., 2018). Most drugs of abuse stimulate dopamine (DA) release into NAc from these dopaminergic neurons (Hernandez *et al*., 2006).

Dopamine transporter (DAT) plays a key role in terminating DA neurotransmission by reuptaking DA released into synapses (Carroll *et al*., 1999; Enyedy *et al*., 2003). DAT belongs to the solute carrier superfamily and is characterized by twelve transmembrane domains. It reuptakes synaptic DA released from presynaptic neurons by utilizing the energy of the sodium gradient generated by Na^+/K^+-ATPase (Kilty *et al*., 1991; Shimada *et al*., 1991). DAT plays a major role in clearing synaptic DA; mice lacking this protein show markedly increased locomotor activity (Giros *et al*., 1996).

DAT is the major target of various psychoactive agents used to treat depression or attention-deficit/hyperactivity disorder (Snyder, 1973; Robinson and Becker, 1986; Madras *et al*., 2005). Several drugs of abuse increase DA concentration in the synapse between medial forebrain bundle dopaminergic neurons and NAc by inhibiting DAT. For example, the ability to inhibit the reuptake of DA has been strongly implicated in the

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reinforcing properties of cocaine (Carroll *et al*., 1999). Therefore, considerable emphasis has been placed on DAT as a molecular target for the development of therapies for drug addiction and abuse.

β-phenethylamine (β-PEA), an endogenous trace amine, acts as a central nervous system stimulant in humans (Juorio *et al*., 1991). It is synthesized in neurons that contain tyrosine hydroxylase and coexists with DA in the nigrostriatal brain regions (Paterson *et al*., 1990). Substituted β-PEAs regulate monoamine neurotransmission through various pathways that include stimulation of trace amine-associated receptor 1 (Borowsky *et al*., 2001; Bunzow *et al*., 2001); inhibition of vesicular monoamine transporter 2 in the brain neurons (Erickson *et al*., 1996; Borowsky *et al*., 2001); or increasing DA release (Bailey *et al*., 1987; Nakamura *et al*., 1998). In addition, several *in vitro* and behavioral studies suggest the DAT is involved in mediating the pharmacological effects generated by β-PEA (Miller, 2011; Hossain *et al*., 2014). Accordingly, inhibition of DAT is known to mediate the abuse potential of β-PEA derivatives. However, the structural requirements of β-PEA derivatives to inhibit DA reuptake remain poorly understood.

In this study, we conducted DA reuptake inhibition assays for β-PEA derivatives and determined their structure–activity relationship (SAR). In addition, docking simulation was conducted for two compounds to predict how DAT interacts with the β-PEA derivatives and to evaluate the effect of DA reuptake inhibition on dopamine D_2 receptor (D_2R) function.

MATERIALS AND METHODS

Materials

GBR 12909 dihydrochloride was purchased from Tocris Bioscience (Bristol, UK). [^{3}H]-DA and [^{3}H]-sulpiride were purchased from PerkinElmer Life Sciences (Boston, MA, USA). Test compounds were provided by the Korean Ministry of Food and Drug Safety (Cheongju, Korea).

Cell line and culture conditions

Human embryonic kidney (HEK)-293 cells were obtained from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in minimal essential medium containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

Dopamine reuptake assay

A reuptake assay was conducted using HEK-293 cells stably expressing human (hDAT) cDNA. Cells cultured in a 24-well plate were washed with uptake buffer (5 mM Tris base, 7.5 mM HEPES, 120 mM NaCl, 5.4 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM ascorbic acid, and 5 mM glucose, pH7.1). The cells were treated with test compounds at 37°C for 20 min, then [^{3}H]-DA was added (final concentration: 20 nM) for 5 min. The cells were washed thrice with ice-cold uptake buffer, and 1% sodium dodecyl sulfate buffer was added into each well overnight. Samples were mixed into a scintillation cocktail, and radioactivity was measured using a Wallac 1450 MicroBeta® TriLux liquid scintillation counter (PerkinElmer Life Sciences). GBR 12909 was the reference compound for DA reuptake inhibition.

Dose-response assays were conducted for each compound to determine the IC_{50} values. GraphPad Prism 5 (GraphPad, San Diego, CA, USA) was used to analyze data and calculate IC_{50} values.

Docking simulation

To predict a possible binding pose of two potent DA reuptake inhibitors, **9** and **28**, a molecular docking simulation was conducted using Smina software (Koes *et al*., 2013), which is equipped with customized scoring functions such as Vina, Smina, Vinardo, and Autodock4 (AD4). Vina (Trott and Olson, 2010) is a hybrid (empirical and knowledge-based) scoring function, whereas AD4 (Huey *et al*., 2007) is based on AMBER force field. Smina and Vinardo are updated versions of Vina, for example, Smina has improved docking performance and Vinardo can perform optimized scoring function due to improved scoring, ranking, and docking performance (Quiroga and Villarreal, 2016). Because the score ranking and binding pose critically depend on the scoring function, it is important to employ a proper scoring function to enhance the computational confidence for a rigid docking simulation.

A predicted three-dimensional (3D) protein structure of hDAT (UniProt ID: Q01959, Sequence length: 620) was obtained from the AlphaFold protein structure database (AF-Q01959-F1) (Jumper *et al*., 2021). The model confidence data and the per-residue confidence score (pLDDT) showed that 12-transmembrane helices were highly structured, while the cytoplasmic N-terminus (1-58 amino acid residues) and the extracellular domain linked with the transmembrane helix 3 and 4 (192-209 amino acid residues) were in the random coiled structure with pLDDT score below 50. As with the X-ray structure of *Drosophila* DAT bound to DA (PDB ID: 4XP1, sequence length: 535) (Wang *et al*., 2015), the root-mean-square deviation (*rmsd*) of Cα atoms in range of residue 59-597 of hDAT was calculated to be about 2.8 Å, suggesting that they possess highly similar structural features. Energy minimization of the modeled hDAT was performed using AMBER99SB and AM1-BCC of Chimera software (Pettersen *et al*., 2004).

The structures of two potent inhibitors, **9** and **28**, were drawn and analyzed using MarvinSketch software (San Diego, CA, USA). Two compounds were protonated at pH 7.4 because the pKa values of compounds **9** and **28** were about 9.76 and 9.11, respectively. Compound **9**, which has a chiral center, can exist as (R)- and (S)-isomers; compound **28**, which has two chiral centers was represented as (R, R)-, (R, S)-, (S, R)-, and (S, S)-isomers.

After the preparation of 3D structures of hDAT and the ligands, the docking simulations were computed by four scoring functions. To assign the binding site, the search space box was centered around DA of the *Drosophila* DAT with each dimension extended 20 Å from the center of mass of DA. Alignment with *Drosophila* DAT showed that the binding pocket of hDAT is formed with the amino acid residues containing Phe76, Asp79, Ile148, Ser149, Leu150, Val152, Gly153, Tyr156, Asn157, Phe326, and Gly327. All docking results were analyzed and visualized using Chimera software.

Endocytosis assay

Endocytosis of D_2R was measured based on the hydrophilic properties of [^{3}H]-sulpiride (Kim *et al*., 2001). HEK-293 cells were transfected with D_2R in pCMV5 along with either a mock vector or hDAT in pcDNA3.1. One day after transfection, the cells were seeded at a density of 1.5×10^5 cells/well in 24-well plates. After 24 h, the cells were stimulated with 10 μM DA

for 60 min and washed thrice with warm serum-free medium. The cells were then incubated with 250 μL of [^{3}H]-sulpiride (final concentration 2.2 nM) at 4°C for 150 min in the absence and presence of a competitive inhibitor (10 μM haloperidol). The cells were washed thrice with ice-cold serum-free medium and then 1% sodium dodecyl sulfate was added. The samples were mixed with 2 mL scintillation fluid (Sigma-Aldrich, St. Louise, MO, USA) and counted on a liquid scintillation analyzer (1450 MicroBeta TriLux, PerkinElmer Life Sciences).

Statistical analysis

Data were expressed as means ± standard deviations. Statistical significance of the data was analyzed by one-way analysis of variance with Tukey's post hoc test using GraphPad Prism 5. A *p*-value of <0.05 was considered significant.

RESULTS

Structure–activity relationship of β-phenethylamines

We analyzed the SAR of 29 β-PEA derivatives for the inhibition of DA reuptake. Based on structural features, the compounds were divided into three groups: arylalkylamines (compounds **1-18**), 2-(alkyl amino)-1-arylalkan-1-one derivatives (compounds **19-27**), and alkyl 2-phenyl-2-(piperidin-2-yl) acetate derivatives (Table 1, 2). The compounds that inhibited DA reuptake by more than 30% in the primary screening (1 μM final) were selected, and their IC_{50} values were determined.

For the series of arylalkylamine derivatives (Table 1), the inhibition of DA reuptake was enhanced in the order of compounds having phenyl, thiophenyl, and substituted phenyl groups at the Ar position (comparing compounds **1-4** and **5-8**). A substituted phenyl group at the Ar position greatly reduced the DA reuptake inhibitory activities of the compounds compared to those of the others with identical substitutions at R1 and R2 (comparing compound **2** *vs.* **3** and **4**; compound **6** *vs.* **7** and **8**). Compounds with a methoxy group at the Ar position showed very weak or no DA reuptake inhibitory activities (compounds **7**, **8**, and **12-18**). Changes in the substituents at R2 from amino to aminomethyl groups produced the opposite effects, depending on the nature of the substituent at the Ar position (compound **1** *vs.* **5**; compound **2** *vs.* **6**).

Next, we determined the inhibitory activities of 2-(alkyl amino)-1-arylalkan-1-one derivatives (compounds **19-27**) and alkyl 2-phenyl-2-(piperidin-2-yl)acetate derivatives (compounds **28-29**) on DA reuptake, which are shown in Table 2. As the size of the heterocyclic ring at R2 increased, the DA reuptake inhibitory activities of the compounds became weaker. For example, a compound with a five-membered ring (compound **19**, pyrrolidine ring, IC_{50}=398.6 nM) showed much stronger inhibitory activity than that with a seven-membered ring (compound **20**, azepane, IC_{50}=4,594.0 nM). Likewise, a derivative with pyrrolidine (compound **21**, five-membered, IC_{50}=413.4 nM) was more potent than that having piperazine (compound **22**, six-membered, IC_{50}=6,011.0 nM). In the series of compounds that have carbon chains at the R2 position and amino methyl groups at R3 (compounds **23-26**), the number of carbons at the R2 position played important roles in determining the inhibitory activities, that is, the larger the stronger (hexyl>pentyl>propyl>ethyl). Replacement of the substituent at R1 from a phenyl ring to naphthyl ring influenced the inhibitory activity in a favorable way (compound **23** *vs.* **27**).

Compounds **28** and **29**, which have distinct R1 and R2 groups compared with the other members in Table 2, showed strong inhibitory activities for DA reuptake. However, the number of compounds in this category was too small to derive any meaningful SAR. Detailed structural features of these compounds and the substituents that determine the inhibition of DA reuptake are summarized in Fig. 1.

Docking study

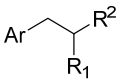
Because the compounds used in the reuptake assay were likely to be racemic mixtures, docking simulation was conducted for the most stable isomer in the mixture. To predict a more reasonable binding pose of a ligand, docking simulation was performed using various customized scoring functions, such as Vina, Smina, Vinardo, and AD4. Table 3 shows the scores obtained using those score functions for compounds **9** and **28**.

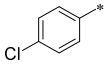
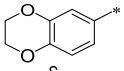
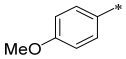
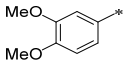
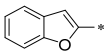
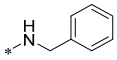
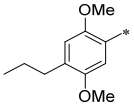
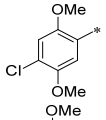
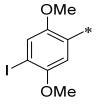
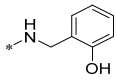
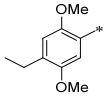
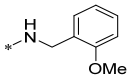
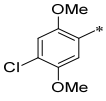
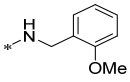
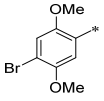
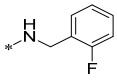
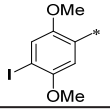
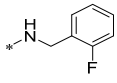
All the scoring functions showed that the (S)-form of compound 9 was more stable than the (R)-form and the difference in docking score between them was about 0.2 kcal/mol. In case of compound **28**, (R, S)-configuration was the most stable among possible isomers and the difference in docking score from (R, R) isomer, which is the second most stable isomer, was about 0.9 kcal/mol. Thus, we presume that R and S configurations of compound **9** bind hDAT similarly and that the (R, S)-form of compound **28** bind DAT more dominantly than other isomers.

The poses of (S)-form, compound **9** overlapped with and tightly occupied the binding site of hDAT (Fig. 2A). The poses based on Vina, Smina, and Vinardo (Vina family) but not that derived from AD4 perfectly superimposed probably because they are fundamentally different score functions. The docking poses based on Vina family and AD4 differed at the position of the branched methyl (R1) and at the terminal ethylamine (R2) groups. The binding pose predicted by Vina family showed that the methyl group was located close to Phe76 and the ethylamine was attached to Tyr156; whereas, the pose predicted by AD4 showed opposite orientation. Considering the space size of the binding site of the binding site, it is possible that the branched methyl group is replaced with an ethyl group or a propyl group in β-PEA. The binding pose predicted by Vina family not AD4 showed that the amine of compound **9** was connected to the polar sidechain of Asp79 and the backbone of Phe320 via two hydrogen bonds. Also, the binding pattern of Vina family was similar to X-ray structure of *Drosophila* DAT bound to DA, suggesting that Vina family scoring function is suitable for the docking simulation of hDAT. More specifically, the benzofuran (Ar) of compound **9** was surrounded by hydrophobic residues on the helix 1 (Phe76), helix 3 (Val152 and Tyr156), and helix 8 (Ser422 and Ala423). In addition, Phe326 of the helix 6 held up the chiral center of the ligand. Overall, the (S)-form of compound **9** showed a good fit into the binding site covered by helices 1, 3, 6 and 8.

As shown in Fig. 2B, the (R, S)-form of compound **28** was stably placed in the binding pocket of hDAT bound to DA. The docking based on Vina family commonly showed that the 4-methylphenyl ring (R2) of compound **28** was attached to Phe76 of helix 1, Val152 and Tyr156 of helix 3, and Ser422 of helix 8. The R2 of compound **9**, benzofuran, is consistent with the catechol ring of DA. In addition, piperidine (R3), a six-membered ring with the amine, was adjacent to Phe320 and Phe326 of helix 6; and the methyl ester (R1) group was squeezed between Phe65 and Ala81 of helix 1. A hydrogen

Table 1. Inhibitory activities of arylethylamines against dopamine reuptake

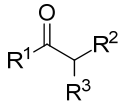


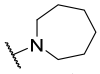
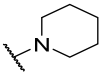
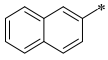
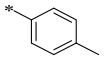
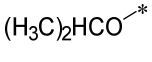
Compound	Ar	R1	R2	IC_{50} (nM)	% of inhibition at 1 μM
1		CH_3	NH_2	1,230.0	
2		CH_3	NH_2	1,090.0	
3		CH_3	NH_2	3,838.0	
4		CH_3	NH_2		–6.0
5		CH_3	$NHCH_3$	1,650.0	
6		CH_3	$NHCH_3$	878.5	
7		CH_3	$NHCH_3$		–2.7
8		CH_3	$NHCH_3$		28.5
9		CH_3	$NHCH_2CH_3$	360.5	
10		CH_3		947.9	
11		CH_3		7,553.0	
12		-	NH_2		–11.1
13		-	NH_2		–2.4
14		-			–15.7
15		-			–7.8
16		-			–3.5
17		-			–3.4
18		-			–9.0

*Compounds which inhibited 30% of dopamine reuptake inhibition were selected for IC_{50} determination.

bond was formed between the charged amine of piperidine and the polar side chain of Asp79. Considering the inhibitory activities of the β-PEA derivatives on DA reuptake (Table 2), the formation of the hydrogen bond with the amine of compound **28** could play an important role for the protein-ligand binding. According to the pose obtained from the Vina family, the hydrophobic pocket bound to the methyl ester group is limited by size, so there would be no space to bind to the iso-

Table 2. Dopamine reuptake inhibitory effects of 2-(alkyl amino)-1-arylalkan-1-one derivatives and alkyl 2-phenyl-2-(piperidin-2-yl)acetate derivatives



Compound	R^1	R^2	R^3	IC_{50} (nM)
19		$CH_2CH_3CH_3$		398.6
20		$CH_2CH_3CH_3$		4,594.0
21		CH_3		413.4
22		CH_3		6,011.0
23		CH_2CH_3	$NHCH_3$	1,872.0
24		$CH_2CH_3CH_3$	$NHCH_3$	1,008.0
25		$CH_2(CH_2)_3CH_3$	$NHCH_3$	493.2
26		$CH_2(CH_2)_4CH_3$	$NHCH_3$	421.0
27		CH_3	$NHCH_3$	742.4
28				68.1
29				479.1

*Compounds which inhibited 20% of dopamine reuptake inhibition were selected for IC_{50} determination.

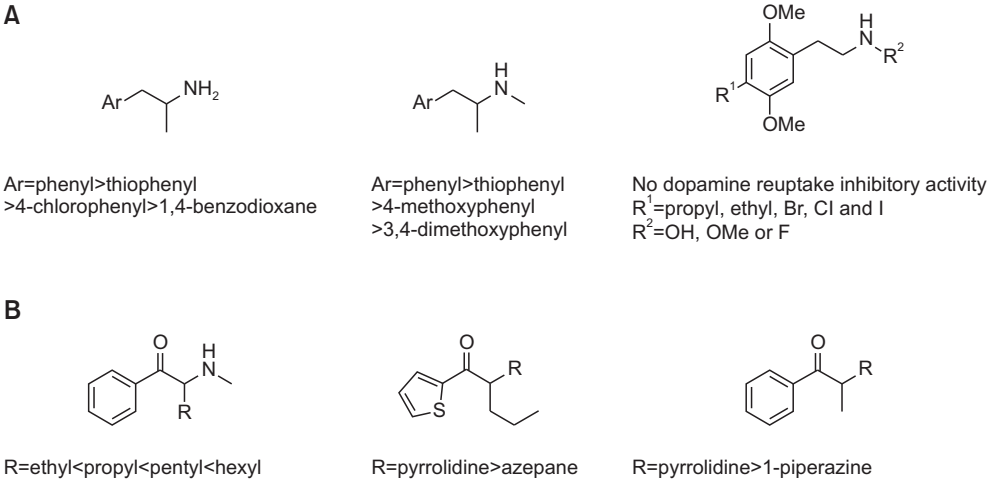


Fig. 1. Structure–activity relationship of β-phenethylamine derivatives (**1-29**) with respect to the inhibition of dopamine reuptake.

Table 3. Docking scores (kcal/mol) of the chiral isomers of compounds **9** and **28** calculated using four scoring functions: Vina, Smina, Vinardo, and Autodock4 (AD4)

Scoring function	Compound **9**		Compound **28**				DA (substrate)
	R	S	RR	RS	SR	SS	
Vina	–7.5	**–7.7**	–8.4	**–9.3**	–7.6	–8.3	–6.8
Smina	–7.6	**–7.8**	–8.4	**–9.3**	–7.6	–8.3	–7.3
Vinardo	–8.0	**–8.3**	–7.7	**–8.7**	–6.9	–7.9	–7.0
AD4	–42.6	**–43.3**	–44.1	**–44.6**	–43.4	–41.4	–30.6

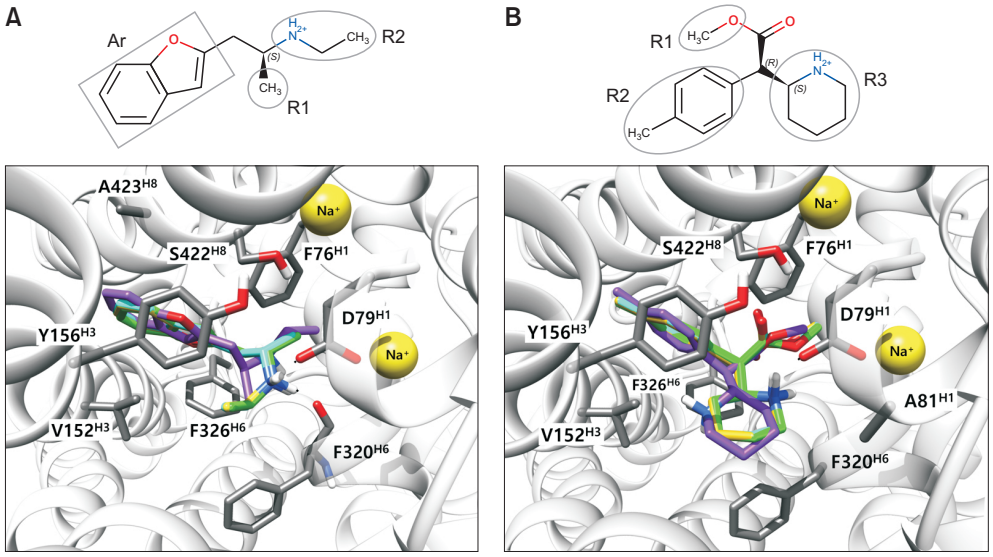


Fig. 2. Best binding poses of compounds **9** (A) and **28** (B) for human dopamine transporter (hDAT) based on Vinardo scoring function. The ball of the ligand is a chiral center and the configurations of compounds **9** and **28** indicate (S)- and (R, S)-forms, respectively. The colored ligands represent the best pose based on a scoring function: Vina (cyan), Smina (yellow), Vinardo (green), and AD4 (purple). Overall, the predicted binding pose was suitable to the binding site of hDAT. The dashed line indicates a hydrogen bond.

propyl group of the compound **29**.

When the score values of (S) form, compound **9** and (R, S) form, compound **28** were compared, compound **28** seems to form more stable interactions with hDAT since the piperidine of compound **28** is attached to the edge of Phe320 through an extra hydrophobic interaction. If piperidine is replaced with a smaller ring or short alkyl chain, it will be difficult to reach Phe320 and the binding affinity will be lowered.

Functional studies of dopamine reuptake inhibition

Reuptake of neurotransmitters released into presynaptic nerve terminals is one of the most important mechanisms responsible for the rapid termination of neurotransmission in synapses (Masson *et al*., 1999; Torres *et al*., 2001). It has long been established that the DA released into the synaptic cleft following an action potential positively feeds back onto the DAT function to increase the DA clearance rate. In accordance with this hypothesis, studies from mouse brain showed that DAT exerts inhibitory activities on D_2R signaling activities (Ghisi *et al*., 2009) but the presynaptic D_2R exerts stimulatory influences on DAT activity (Meiergerd *et al*., 1993; Dickinson *et al*., 1999). To determine whether the β-PEAs that inhibited the DAT activity altered the functional activity of D_2R, we selected two β-PEAs and determined their effects on the endocytosis of D_2R.

Most G protein-coupled receptors, including D_2R, are phosphorylated after agonist stimulation, and undergo endocytosis (Zhang and Kim, 2017). The functional role of D_2R endocytosis was proposed to be the re-sensitization of tolerant receptors (Cho *et al*., 2010). As shown in Fig. 3, treatment of cells expressing D_2Rs evoked approximately 40% of receptor endocytosis when they were co-expressed with GRK2. Additional expression of DAT significantly inhibited this endocytosis of D_2R, and pretreatment with 4-methylmethylphenidate (**28**) and isopropylphenidate (**29**), which showed potent inhibition of DA reuptake, significantly restored the DAT-mediated inhibition of D_2R endocytosis, suggesting that β-PEAs can profoundly affect the signaling of monoamine neurotransmitters, for example, DA signaling via D_2R.

DISCUSSION

β-PEA is a primary amine with the amino group attached to a benzene ring through a two-carbon or ethyl group. Some derivatives of β-PEA, such as 3C-E, 3C-P, allylescaline, escaline, isoproscaline, and methallylescaline, are designer drugs (Dean *et al*., 2013). A designer drug is a structural or functional

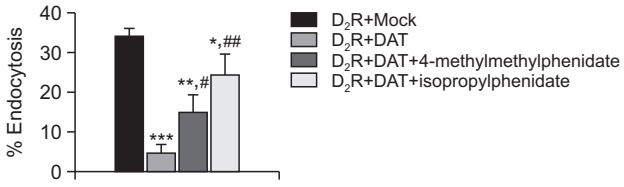


Fig. 3. Disinhibitory effects of compounds **28** and **29** on DAT-mediated inhibition of dopamine D_2 receptor (D_2R) endocytosis. Cells expressing D_2R (about 2.1 pmol/mg protein) and GRK2 were transfected with either mock vector or hDAT. Cells were treated either with vehicle, 1 μM 4-methylmethylphenidate, or 1 μM isopropylphenidate for 20 min, followed by 10 μM dopamine for 1 h. $^{*}p<0.1$, $^{**}p<0.01$, $^{***}p<0.001$ compared to Mock group. $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared to DAT group (n=3).

analog of a controlled substance that has been designed to mimic the pharmacological effects of the original drug while avoiding classification as illegal and/or detection in standard drug tests (Wohlfarth and Weinmann, 2010; Weaver *et al*., 2015).

DAT plays a role in the functional effect of β-PEA (Sotnikova *et al*., 2004). Hence, in this study, we analyzed 29 derivatives of β-PEA prepared by replacing, or substituting, one or more hydrogen atoms in the β-PEA core structure with substituents. With respect to DA reuptake, we determined their SAR (Table 1, 2).

Compounds in the first group (Table 1, compounds **1-18**) that have no substituents in the benzene ring (compounds **2**, **6**, and **10**) or have a methyl benzene ring attached to R2 (compound **11** *vs.* **14-18**) showed relatively potent inhibitory effects on DA reuptake. Methamphetamine, a highly addictive psychostimulant drug that principally affects the monoamine neurotransmitter systems of the brain (Panenka *et al*., 2013), has a structure similar to that of the first group of compounds.

The presence of an amino or aminomethyl group at R2 caused mixed effects on DA reuptake inhibition depending on the nature of the Ar group (compound **1** *vs.* **5**; compound **2** *vs.* **6**). Compounds with similar structures were shown to have similar inhibitory activities for DA reuptake (Iversen *et al*., 2013).

Overall, 19 of 29 β-PEA derivatives showed inhibitory activities against DAT reuptake and provided structural activity information, which can be utilized in the design of DA reuptake inhibitors.

According to docking score values (binding affinity) for compounds **9** and **28**, the (S)-form of compound **9** was more stable than the (R)-form and the (R, S)-configuration of compound **28** was more stable than other isomers. As shown by the binding poses of two potent inhibitors, they form hydrogen bond with the targeted hDAT and fit into the binding site covered by helices 1, 3, 6 and 8. Although docking simulation assumes that direct physical interactions between small molecules and transporters are the major determinants for the selectivity difference between transporters and compounds, other indirect mechanisms may also be practically involved.

DAT lowers the concentration of DA in the synaptic cleft, and thereby influences the signaling or regulatory processes of dopaminergic neurons. As shown in Fig. 3, DAT inhibited the endocytosis of D_2R, which functions as an autoreceptor of dopaminergic neurons, by lowering the DA concentration in the synaptic cleft. As the endocytosis of D_2R mediates the re-sensitization of desensitized receptors (Yu *et al*., 1993; Cho *et al*., 2010), the roles of DAT inhibitors in dopaminergic function may be more complex. Unexpectedly, compound **28**, which exhibited higher inhibitory activity on DA reuptake than compound **29**, exerted weaker disinhibitory effects on the DAT-mediated inhibition of D_2R endocytosis. Because the regulation of this receptor endocytosis involves multiple steps, these compounds may affect certain cellular components that directly regulate D_2R endocytosis.

Overall, this study provides a theoretical background to identify candidate compounds with selective inhibitory effects on DAT. Additionally, the compounds characterized in this study can be utilized for developing therapeutic agents against various neuropsychiatric disorders. However, it should be mentioned that many of phenethylamine derivatives including those used in this study belong to new psychoactive substances which could possess yet unidentified pharmacological activities (Xie and Miller, 2008; Cocchi *et al*., 2020). Thus, the possibility that these compounds act not only on DAT but also on other targets should not be excluded.

CONFLICT OF INTEREST

The authors state no conflict of interest exists.

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