Development of Peptidic Dopamine Transporter Inhibitors via Aromatic Modification-Mediated Conformational Restriction

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Abstract: The dopamine transporter plays an important role in the molecular mechanism of cocaine dependence. It is suggested that inhibitors of the dopamine transporter would have strong therapeutic potential. Here we report that aromatic modification can constrain a linear peptide into the β-turn conformation, which is preferred by the dopamine transporter. On the basis of this finding, a novel selective and competitive peptidic inhibitor of the dopamine transporter was developed. The peptide binds to the dopamine- and cocaine-binding site of the dopamine transporter and has behavioral effects different from those of cocaine in mice.

Cocaine abuse and addiction is a major public health problem and leads to many severe social problems in the world. The key target of cocaine is the dopamine transporter (DAT),[1,2] which is located in the plasma membrane of dopaminergic neuron cells. Together with the norepinephrine transporter (NET) and the serotonin transporter (SERT), DAT forms a subfamily of Na^+/Cl^- dependent monoamine transporters containing 12 putative transmembrane domains. Rapid uptake of released dopamine in the synaptic cleft by DAT terminates the activity of the neurotransmitter.[3,4] The blockade of the presynaptic DAT by cocaine results in an increased dopamine activity in the mesolimbic dopamine reward system of the brain. A variety of structural classes of nonpeptide DAT inhibitors such as benztropine, GBR 12909, and even cocaine itself have been used as templates for the design and synthesis of potential cocaine abuse therapeutic agents, but a clinically useful agent has yet to be identified.[5−8]

Previous works have demonstrated that short peptides could inhibit both DAT and SERT with IC_{50} values in the micromolar range.[9,10] The structural requirements for peptides binding to DAT and the behavioral effects of these peptides are still not clear. In this report, the design and synthesis of aromatically modified peptidic DAT inhibitors using H-Phe-Phe-Tyr-Thr-Pro-Lys-Thr-OH (**1**) as template are described. We also explored the interaction of these peptides with DAT and other monoamine transporters to gain an insight into their structure−activity relationship, binding site, and selectivity. In addition, the behavioral effects of one of these DAT inhibitor peptides were studied in mice.

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Our early studies showed that peptide **1** could inhibit DAT at high concentrations.[11] Recently, we found that the minute amount of benzylated byproduct in the peptide product is the bioactive component while **1** has no bioactivity by itself. On the basis of this serendipitous discovery and the fact that aromatic modifications of peptidic ligands have long been implicated in improving binding efficacy,[12−17] we speculated that addition of aromatic groups in discrete positions of the peptide might produce its DAT binding affinity. To test the hypothesis directly, systematic aromatic modification based on peptide **1** was performed. Table 1 shows the primary structure of peptides **1−15** we prepared and their IC_{50} values in the ^{3}H-dopamine uptake assay using CHO cells stably transfected with rat DAT (D8 cells).[11] Figure 1 shows a concentration−response curve for the inhibition of DAT uptake by peptide **13** as an example.

Peptide **1** has no bioactivity, while peptide **2** with the deletion of Phe[1] and the replacement of Phe[2] by 4,4′-biphenylalanine (Bip) in **1** apparently inhibits DAT function (Table 1). Far-ultraviolet circular dichroism (CD) revealed that peptide **1** gives an ellipticity minimum at approximately 205 nm characteristic of a minor conformation. Notably, the CD spectrum of peptide **2** closely resembles that observed for a well-characterized β-turn conformation, with a strong negative CD band at approximately 218 nm (Figure 2).[18] β-Turns constitute four consecutive residues that cause a reversal of direction of the peptide chain. The β-turn conformation content of each peptide was expressed as the mean residue ellipticity at 218 nm in the CD spectrum (Table 1). Additional benzylation of Tyr[2] (**3**) and Thr[3] (**4**) in peptide **2** has weak effects on their conformations and bioactivities. The Gly[7] in peptide **4−14** was added to the C-terminus to facilitate the chemical peptide synthesis. Thr[6] benzylation (**5**) produced a 10-fold increase in DAT inhibition relative to peptide **2**, indicating that this residue could interact with DAT. The β-turn conformation was destroyed when Bip[1] in peptide **5** was replaced by other aromatically modified amino acids, such as 1-naphthylalanine (Nal[1]) (**6**), 2-naphthyl-alanine (Nal[2]) (**7**), or 4-benzoylphenylalanine (Bpa) (**8**). These results excluded the possible contributions of aromatic groups per se or peptide self-association to the CD spectra. Peptides **6−8** have weak DAT inhibitory activity more likely due to the loss of β-turn conformation.

To evaluate the functional role of the nonmodified residues in peptide **5** precisely, we individually substituted the residues by an alanine. The replacements at Tyr[2] (**9**) and Thr[3] (**10**) greatly decreased the potency while the global conformation was retained, which could indicate interactions between these two residues and DAT. The potency reduction could also be due to the loss of intramolecular interactions in the peptide. The replacement at Pro[4] (**11**) lost the conformational stability and uptake inhibiting, indicating a rigidification of the peptide structure is important for its DAT binding action. Pro serves as a β-turn inducer in peptides because of the pyrrolidine ring. This provided another important evidence to deduce the peptide conformation as β-turn in addition to the CD data. The substitution at Lys[5] (**12**) had no noticeable effects on conformation and potency.

The synthesis of peptide **13** with the deletion of Lys[5] resulted in a 5-fold enhancement in bioactivity compared to **5**. Basic side chain of Lys[5] may be a steric hindrance to binding with DAT in addition to weakening the molecular hydrophobic

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Table 1. DAT Inhibition and CD Data of Peptide Analogues[a]

no.	compound	IC_{50} (μM)[b]	$[\Phi]_{218}$obsd[c]
	cocaine	0.3	
1	Phe-Phe-Tyr-Thr-Pro-Lys-Thr	>1000	−4
2	Bip-Tyr-Thr-Pro-Lys-Thr	19	−35
3	Bip-Tyr(3′Bzl)-Thr-Pro-Lys-Thr	10	−40
4	Bip-Tyr-Thr(OBzl)-Pro-Lys-Thr-Gly	20	−35
5	Bip-Tyr-Thr-Pro-Lys-Thr(OBzl)-Gly	1.8	−50
6	Nal^1-Tyr-Thr-Pro-Lys-Thr(OBzl)-Gly	199	−6
7	Nal^2-Tyr-Thr-Pro-Lys-Thr(OBzl)-Gly	184	−4
8	Bpa-Tyr-Thr-Pro-Lys-Thr(OBzl)-Gly	239	−3
9	Bip-Ala-Thr-Pro-Lys-Thr(OBzl)-Gly	173	−50
10	Bip-Tyr-Ala-Pro-Lys-Thr(OBzl)-Gly	11	−44
11	Bip-Tyr-Thr-Ala-Lys-Thr(OBzl)-Gly	>1000	−2
12	Bip-Tyr-Thr-Pro-Ala-Thr(OBzl)-Gly	1.7	−34
13	Bip-Tyr-Thr-Pro-Thr(OBzl)-Gly	0.3	−43
14	Bip-Tyr-Thr-Ala-Thr(OBzl)-Gly	>1000	−2
15	Bip-Tyr-Thr-Pro-Phe	0.9	−34

[a] N-terminus is amine group, C-terminus is carboxy group. Bip: 4,4′-biphenylalanine. Nal^1: 1-naphthylalanine. Nal^2: 2-naphthylalanine. Bpa: 4-benzoylphenylalanine. [b] IC_{50}, half-maximal (50%) inhibitory concentration obtained in ^{3}H-dopamine uptake assay. Standard deviations were within 10% of the mean of 2−6 experiments performed in triplicate. [c] The β-turn conformation content of each peptide was expressed as $[\Phi]_{218}$obsd, the mean residue ellipticity at 218 nm.

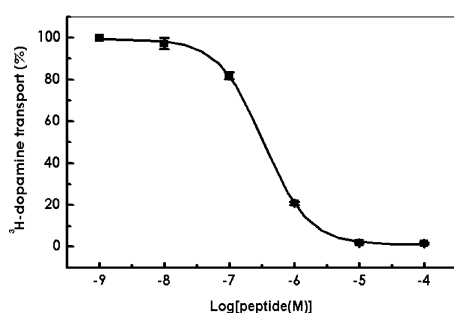

Figure 1. Inhibition by peptide **13** for ^{3}H-dopamine uptake by rat DAT expressed in CHO cells. A concentration−response curve for the inhibition by peptide **13** is represented as the mean of three determinations each done in triplicate. ^{3}H-dopamine uptake determined in reactions without peptide was used to define 100% uptake.

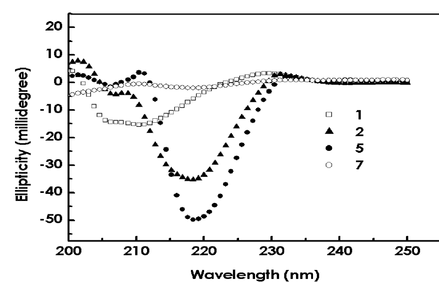

Figure 2. Circular dichroism spectra of peptide **1**, **2**, **5** and **7** at the concentration of 0.4 mg/mL. Each peptide was dissolved in aqueous potassium phosphate pH 7 solution containing 10% trifluoroethanol.

property. A Pro^4 to Ala^4 change (**14**) in peptide **13** reduced the activity almost completely and **14** served as a useful control in following experiments. It is interesting that the replacement of benzylated Thr^5 by Phe (**15**) in peptide **13** had no obvious effects on its activity or conformation.

To identify the specificity of these peptides, we determined the IC_{50} values of the peptides for both ^{3}H-monoamine and ^{3}H-γ-aminobutyric acid uptake in parallel using gene-engineered transporter assay systems (Table 2). Peptide **2** had no action on NET, SERT, or γ-aminobutyric acid transporter 1 (GAT1), a member of Na^+/Cl^- dependent transporters. Peptide **13** retained more than 300-fold selectivity over NET, SERT, or GAT1, while peptide **15** lost the selectivity significantly.

Table 2. IC_{50} (μM) of Peptides Acting on Monoamine Transporters[a]

compound	DAT[b]	NET[b]	SERT[b]	GAT1[b]
cocaine	0.3	0.9	0.4	nd
2	19	>1000	>1000	>1000
13	0.3	90	110	>1000
15	0.9	25	32	>1000

[a] Standard deviations were within 10% of the mean of 2−6 experiments performed in triplicate. [b] DAT: dopamine transporter. NET: norepinephrine transporter. SERT: serotonin transporter. GAT1: γ-aminobutyric acid transporter1. nd means not detected.

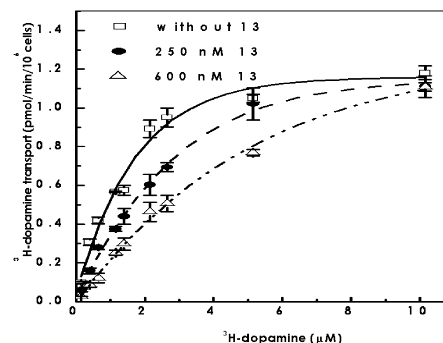

Figure 3. Effect of peptide **13** on the saturability of ^{3}H-dopamine transport by rat DAT expressed in CHO cells. Symbols and bars represent the mean ± SEM of three independent experiments performed in triplicate. K_m (μM) and V_{max} (pmol per min per 10^6 cells) are, respectively, 1.38 ± 0.27, 1.27 ± 0.13 without the peptide; 2.42 ± 0.36, 1.29 ± 0.11 with 250 nM peptide; 3.60 ± 0.20, 1.25 ± 0.08 with 600 nM peptide.

Our data showed that aromatic modification of peptide **1** might possibly constrain the peptide into a β-turn, which was thought to be very important in the recognition process between the peptide and DAT. In nature, the β-turn regions generally lie on the surface of proteins and peptides and frequently mediate molecular recognition. It is interesting to note that NET has exhibited the same ligand preference in a study of conopeptides.[19] Although Tyr^2, Thr^3, and benzylated Thr^5 in peptide **13** do not determine the global conformation, these residues on the β-turn form the pharmacophore of peptide **13**, and the benzylated Thr^5 is crucial for its selectivity.

β-Turns are frequently investigated as potential leads for drug discovery, and there are many elegant strategies to constrain β-turn peptides.[20−22] Regardless of the exact chemical mechanism, one can conclude in our case that the β-turn conformation is promoted by the Bip. The CD data obtained with peptides containing Nal^1, Nal^2, or Bpa led to the proposal that geometric topography of the N-terminal residue is also important to introduce the β-turn conformation. The finding that simple aromatic modification could stabilize a linear peptide into a global rigid structure offers a new example for β-turn peptide design.

As a potent specific DAT inhibitor, peptide **13** was chosen to study its bioactivity on D8 cells in detail. The peptide acts as a full and reversible inhibitor of dopamine uptake. The rate of cellular accumulation of ^{3}H-dopamine via the rat DAT was reduced 80% by 1 μM peptide **13**, and the inhibition was completely reversed upon washing twice with peptide-free assay solution. Saturation analysis suggested that peptide **13** is a competitive inhibitor to DAT, as it (the peptide concentration was 250 nM) reduced the affinity of the substrate by 43% without affecting the maximum rate of ^{3}H-dopamine uptake (Figure 3). Accordingly, the kinetic mode of peptide **13** on ^{3}H-dopamine uptake is similar to that of cocaine. These two inhibitors also showed a competitive synergism effect when

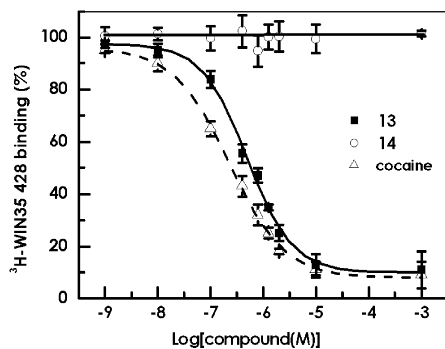

Figure 4. Inhibition of the binding of cocaine analogue ^{3}H-WIN35,-428 to the rat DAT expressed in CHO cells by peptide **13**. The binding of ^{3}H-WIN35,428 to DAT was examined in the presence of the indicated concentrations of peptide **13**, **14**, or cocaine. Radioligand binding determined in reactions without compounds was used to define 100% binding. Symbols and bars represent the mean ± SEM of three independent experiments performed in triplicate.

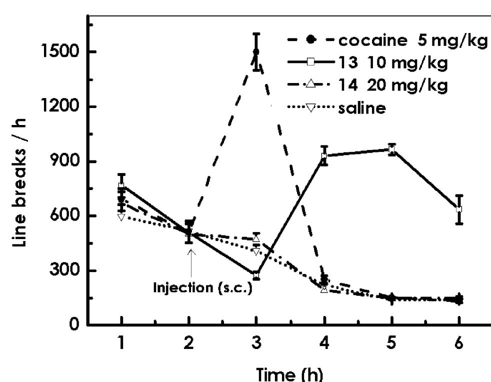

Figure 5. Peptide **13** elevated locomotor behavior of mice. Locomotor activities of mice during a habituation period (1−2 h) and after subcutaneous (s.c.) injection of saline, peptide **13**, **14**, and cocaine (2−6 h). Line breaks scores shown represent the horizontal distance traveled. $n = 8$ mice per group, *, $P < 0.01$ vs saline, Student's test and analyses of variance.

acting on DAT at the same time**.** Peptide **13** did not change the IC_{50} value of cocaine, but increased the inhibiting activity of cocaine when cocaine was at low concentration. In addition, the peptide inhibited the binding of radiolabeled cocaine analogue ^{3}H-WIN35,428 to the D8 cells with an IC_{50} of 494 ± 49 nM and acted as a full inhibitor, which was analogous to that achieved by cocaine (the IC_{50} was 226± 18 nM) (Figure 4). These results indicate that peptide **13** binds to the dopamine- and cocaine-binding site of DAT.

To evaluate the psychostimulant actions of potent peptide **13** as a DAT inhibitor, the locomotor activities of mice injected subcutaneously with 10 mg/kg peptide **13** after 2 h of habitation was examined. As shown in Figure 5, an initial 1 h suppression of locomotor activity was followed by a long-lasting stimulant effect. Peptide **13** elevated locomotor activity with a slower onset and a longer duration of action than cocaine after systemic administration. These results are probably due to the aromatic modification of the peptide. The aromatic group may confer slow absorption and elimination property on peptide **13**, and help the peptide to penetrate blood-brain barrier. In addition, the unnatural amino acids at both ends and the deletion of Lys were thought to resist amino-, carboxypeptidases and trypsin. The measurement of brain levels of peptide **13** will be addressed by subsequent pharmacokinetic studies.

The rewarding effects of peptide **13** were studied using conditioned place preference paradigm.[23] The peptide or cocaine

Table 3. Effect of Peptide **13** on Cocaine-Induced Place Preferences in Mice

drug before daily place-conditioning sessions, altering with saline	time difference between pre- and proconditioning (s)
saline	15 ± 2
20 mg/kg **13**	−16 ± 2
20 mg/kg **14**	−14 ± 3
10 mg/kg cocaine	194 ± 20*
10 mg/kg cocaine and 10 mg/kg **13**[a]	78 ± 8 **
10 mg/kg cocaine and 10 mg/kg **14**[a]	200 ± 15
10 mg/kg **13** after cocaine induction[b]	61 ± 4***
10 mg/kg **14** after cocaine induction[b]	209 ± 21

[a] Mice were injected subcutaneously with peptides 2 h before each injection of cocaine. [b] Mice with place preference induced by cocaine were injected subcutaneously with peptides daily for 2 days. *The mice displayed significant place preference associated with cocaine, $P < 0.05$ vs saline. **Effect of peptide **13** on the acquisition of cocaine-induced place preference, $P < 0.05$ vs **14**. ***Effect of peptide **13** on the expression of cocaine-induced place preference, $P < 0.05$ vs **14**. Student's test and analyses of variance, $n = 15$ mice per group.

administration and conditioning, and saline administration and conditioning, were carried out on in a day. The process was repeated for 4 days. On day 5 a single conditional place preference assessment session followed. As shown in Table 3, the mice displayed a significant place preference associated with 10 mg/kg cocaine, whereas 20 mg/kg peptide **13** induced no place preference. Considering that peptide **13** has a long duration of action in vivo, we evaluated the peptide using a "nonstandard" conditioning procedure, in which peptide and saline pairing was carried out on alternating days, leaving 24 h for washout of the drug before the next saline session.[24] Five cycles later, peptide **13** still showed no place preference effect. As the rapid rise and decline of extracellular dopamine levels may contribute to the reinforcing properties and abuse liability of cocaine, the slower onset and offset effects of peptide **13** make it be less reinforcing than cocaine. This phenomenon had been observed in the study of benztropine analogues and was further explained that those compounds had slower apparent rates of occupancy with DAT.[25, 26]

Some selective DAT inhibitors with a slow onset and a long duration of action can suppress the reinforcing and euphorigenic effects of cocaine.[26−28] To investigate the interaction of peptide **13** and cocaine in conditioned place preference, mice were injected subcutaneously with 10 mg/kg peptide **13** 2 h before each injection of cocaine during the conditioning sessions. The result showed that peptide **13** produced a blockade of the acquisition of cocaine-induced place preference (Table 3). In another experiment, mice with place preference induced by 10 mg/kg cocaine were injected subcutaneously with 10 mg/kg peptide **13** daily for 2 days, and the animals were tested for place preference. As shown in Table 3, the systemic administration of peptide **13** produced a robust blockade of the expression of cocaine-induced place preference. The blockades of the acquisition and expression of cocaine-induced place preference cannot be attributed to peptide **13** induced place aversion, because the peptide by itself produced neither a significant place preference nor aversion at a dose as high as 20 mg/kg. Although drugs of various mechanisms can affect cocaine-induced conditioned place preference,[29,30] this result could be explained in two ways. First, peptide **13** could bind to DAT with the potency similar to that of cocaine, thus creating a blockade of the conditioned place preference induction effects of cocaine mediated via elevation of extracellular dopamine acutely. Second, as a DAT inhibitor, peptide **13** mimics the effect of cocaine, and the substitution treatment might suppress cocaine seeking.

Daily observations also showed that animals given peptide **13** subcutaneously (daily 50 mg/kg for 7 days) maintained normal body weights, showed no altered reactivity to novel stimuli and situations, and survived in an ordinary way.

In summary, peptide **13** containing Bip and benzyl group were synthesized to explore a small peptide inhibitor for DAT. Through this study, we found that the aromatic modification could restrict global peptide conformation, and the β-turn conformation is critical for the peptide−DAT interaction. In addition, the particular surface of the β-turn, which comprised the residue Tyr^2, Thr^3, and benzylated Thr^5, deeply participated in the binding to DAT. This information would be helpful to the improvement of the peptide potency. Furthermore, it was found that peptide **13** acted as a selective and competitive DAT inhibitor in vitro and had different behavioral effects compared to cocaine in vivo. Like cocaine, the peptide does increase locomotor activity, although with slower onset. The peptide does not induce place preference and blockades cocaine-induced place preference. Peptide **13** is, to our knowledge, the first DAT peptidic inhibitor without cocaine-like behavioral effects. We expect peptide **13** to be a new tool in future studies of DAT and a new lead peptide for cocaine abuse therapy.

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Supporting Information Available: LC/MS data for peptides **1**−**15** and the experimental details for CD, uptake, binding, and behavioral tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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