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Interaction between a hydroxypiperidine analogue of 4-(2-benzhydryloxy-ethyl)-1-(4-fluorobenzyl)piperidine and Aspartate 68 in the human dopamine transporter

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Abstract

Compound (+)-*R*,*R*-D-84 is an optically active *trans*-hydroxy-substituted derivative of 4-(2-benzhydryloxy-ethyl)-1-(4-fluorobenzyl)piperidine (D-164). As a hydroxypiperidine analog of GBR 12935, (+)-*R*,*R*-D-84 is a candidate dopamine transporter compound for the treatment of cocaine dependence. The present work addresses the functional activity of (+)-*R*,*R*-D-84 at monoamine transporters and its potential molecular mechanism involving acidic amino acids (D and E). The selectivity for the dopamine vs. serotonin transporter of (+)-*R*,*R*-D-84 was greater than that of (−)-*S*,*S*-D-83, its enantiomer, and the selectivity of both compounds was greater than that of GBR 12909 (diphenyl-fluorinated GBR 12935). Only (+)-*R*,*R*-D-84 displayed improved selectivity vs. the norepinephrine transporter. D313N or E215Q mutation did not alter the pattern of affinities (measured by membrane binding of the cocaine analog [^{3}H]CFT) for the dopamine transporter of (+)-*R*,*R*-D-84, (−)-*S*,*S*-D-83, D-164 (non-hydroxylated analog), or GBR 12909. In contrast, D68N mutation specifically lowered the affinity of (+)-*R*,*R*-D-84, pointing to a role for D68 in the interaction with (+)-*R*,*R*-D-84, possibly through hydrogen bonding between the hydroxyl and the carboxyl group of D68 which is lacking in N68. The present results, combined with behavioral data, implicate D68 in the dopamine transporter in cocaine antagonist activity of (+)-*R*,*R*-D-84.
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Keywords: GBR 12935 derivative; Dopamine transporter; Monoamine uptake; Hydrogen bonding; Cocaine antagonist; Striatum; (Rat)

1. Introduction

Psychostimulant drugs such as cocaine block the dopamine transporter thereby increasing the level of extracellular dopamine (Chen and Reith, 1994; Hurd et al., 1988; Wu et al., 2001; Zahniser et al., 1999). In developing pharmacotherapies for the treatment of cocaine dependence, the dopamine transporter is one of the targets (see recent review of Dutta et al., 2003). It appears that dopamine transporter compounds with a slow onset and long duration of action could be useful as treatment medications (Gorelick, 1998; Howell and Wilcox, 2001). In this respect, 1-(2-(di(4-fluorophenyl)-methoxy)-ethyl)-4-(3-phenylpropyl)piperazine (GBR 12909) (Fig. 1) has generated interest as a compound targeting the dopamine transporter with high affinity and moderate selectivity (Van der Zee et al., 1980; Lewis et al., 1999), developing peak locomotor activation later and exerting activity longer than cocaine (Elmer et al., 1996), attenuating the effect of cocaine in enhancing accumbal extracellular dopamine (Baumann et al., 1994), and reducing cocaine self-administration in rhesus monkeys (Glowa et al., 1996). GBR 12909 binds not only to the dopamine transporter, but also to a piperazine acceptor site likely associated with the cytochrome P450IID1 system (Andersen et al., 1987;

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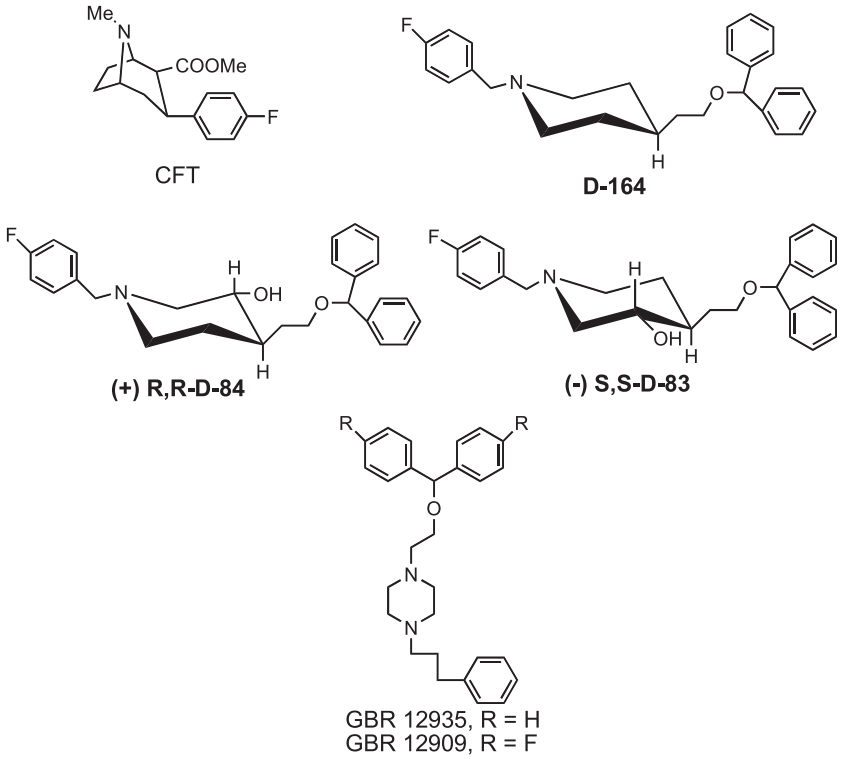

Fig. 1. Structures of CFT, GBR 12935, GBR 12909, D-164, (+)-*R*,*R*-D-84, and (−)-*S*,*S*-D-83.

Niznik et al., 1991). Our work has demonstrated that the piperazine ring of GBR 12909 can be converted into a piperidine ring without loosing affinity for the dopamine transporter while reducing its affinity for the piperazine binding site (Dutta et al., 1993; Madras et al., 1994). Compound D-164 (Fig. 1) is a piperidine derivative of GBR 12909 (GBR 12935), defluorinated at the biphenyl ring system but fluorinated at the single phenyl ring, along with an additional shortening of the carbon chain between the piperidine and single phenyl ring. Its affinity for the dopamine transporter is close to that of GBR 12909 but it is more selective for the dopamine vs. serotonin transporter than GBR 12909 in in vitro binding assays (Ghorai et al., 2003). Compound (+)-*R*,*R*-D-84 is an optically active *trans*-hydroxy-substituted analog of D-164 (Fig. 1) with a 37-fold higher binding affinity for the dopamine transporter compared with compound D-164 and a 71-fold higher binding selectivity for the dopamine vs. serotonin transporter (Ghorai et al., 2003). Compound (+)-*R*,*R*-D-84 produces partially cocaine-like responding in cocaine drug discrimination testing, and it stimulates locomotor activity for hours longer than cocaine (Ghorai et al., 2003). Thus, (+)-*R*,*R*-D-84 is a candidate dopamine transporter compound for further study.

The present study addresses two questions regarding the interaction of (+)-*R*,*R*-D-84 with monoaminergic transmission. The first question is how selective the compound is in blocking the dopamine vs. serotonin or norepinephrine transporter in functional assays measuring transmitter uptake rather than radioligand binding. Although uptake and binding potencies are correlated well for the dopamine and serotonin transporter (Eshleman et al., 1999; Madras et al., 1989; Javitch et al., 1984), the correlation is substantially lower for the norepinephrine transporter (Eshleman et al., 1999). In particular, cocaine- and GBR 12909-like compounds are appreciably more potent in inhibiting norepinephrine uptake than [^{3}H]nisoxetine binding to the norepinephrine transporters (Eshleman et al., 1999; Kuhar et al., 1999; Reith et al., 2004). Much of the difference can be accounted for by the difference in temperature commonly used for the two assays, with smaller contributions from the assay buffer and type of preparation (intact vs. broken membranes) (Reith et al., 2004). The present study addresses the uptake activity of (+)-*R*,*R*-D-84 at the three monoamine transporters for a pharmacologically more relevant selectivity profile than that derived from binding assays.

The second question addressed by the present study is what molecular mechanism underlies the enhanced binding interaction between hydroxylated (+)-*R*,*R*-D-84 and the dopamine transporter compared with non-hydroxylated compound D-164 or GBR 12909. It could be thought that the hydroxyl group of (+)-*R*,*R*-D-84 forms hydrogen bonding with the carboxyl group of acidic amino acids D and E in the dopamine transporter, especially those in transmembrane domains 1–2 found to be critical for interaction with azido-labeled iodinated GBR 12935 or transmembrane domains 4–6 additionally involved in

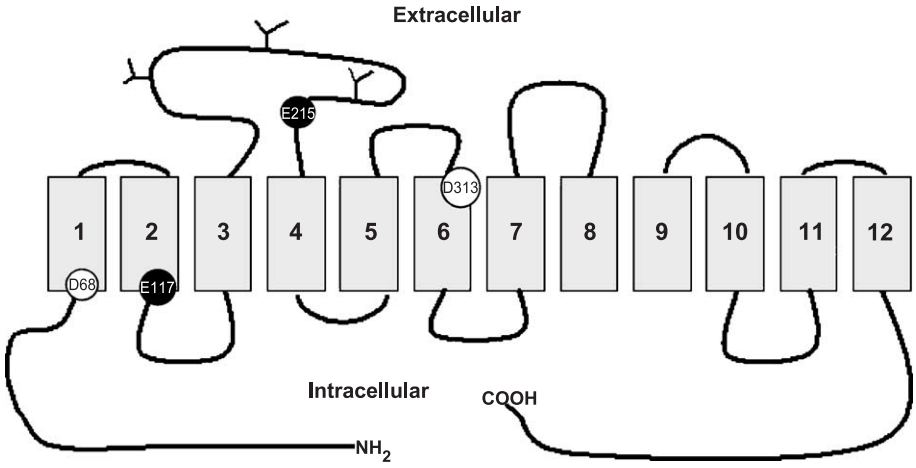

Fig. 2. Conserved acidic amino acid residues in N-terminal half of the membrane topology model of the human dopamine transporter. The N-terminal half up to transmembrane domain 6 was assessed for D or E residues that are absolutely conserved within the family of Na^+,Cl^- dependent neurotransmitter transporters (dark circles) or conservatively replaced (white circles). E117Q was not functionally active (Chen et al., 2001).

interaction with an azido-analog of compound D-164 (Vaughan et al., 2001). It would therefore be of interest to study the interaction between (+)-*R*,*R*-D-84 and dopamine transporters with D and E mutated in these regions. For other purposes, our laboratory had generated D and E mutations (Chen et al., 2001), and those situated in the transmembrane regions targeted by GBR-like compounds (see Fig. 2) were therefore selected for the present study, i.e. D68N and D313N (E117 was not functionally active). An additional mutation was assessed in the current study: E215Q, located in the large second extracellular loop. Because extracellular or intracellular loops are not commonly thought to carry binding sites for transporter blockers or substrates (Smicun et al., 1999; see also Chen and Reith, 2002), this mutation was included as a control. GBR 12909 was included as a structure related to (+)-*R*,*R*-D-84 but without the hydroxyl group on the piperazine ring, and so was compound D-164, which is equal to (+)-*R*,*R*-D-84 except for the hydroxyl group. Also included were (−)-*S*,*S*-D-83, the enantiomer of (+)-*R*,*R*-D-84 which has the hydroxyl pointing in a different direction, and 2β-carbomethoxy-3β-(4-fluorophenyl)tropane (CFT), a cocaine-like compound from a different class of dopamine transporter compounds incorporating the phenyltropane structure (see Dutta et al., 2003).

2. Materials and methods

2.1. Materials and animals

Male Sprague–Dawley rats (250–350 g) were purchased from Taconic Germantown, NY, USA. The animal protocols were approved by the Institutional Animal Care and Use Committee of the New York University Medical Center. Wild-type and mutant dopamine transporters were prepared as described in our previous work (Chen et al., 2001). D-164, (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 were synthesized as described previously (Ghorai et al., 2003). Unlabeled CFT naphthalene sulfonate was from the Research Triangle Institute (Research Triangle Park, NC). Citalopram was a research sample from H. Lundbeck. The trypsin–EDTA solution was from BioWhittaker (Walkersville, MD, USA). Fetal bovine serum and bovine calf serum was obtained from Hyclone (Logan, UT, USA). [^{3}H]CFT (WIN 35,428, 85 Ci/mmol), [^{3}H]dopamine (54.8 Ci/mmol), [^{3}H]serotonin (23.7 Ci/mmol), and [^{3}H]norepinephrine (49.7 Ci/mmol) were from PerkinElmer Life Sciences (Boston, MA, USA). Glass fiber filtermats A and B and Betaplate Scint scintillation cocktail were purchased from PerkinElmer Life Sciences. Whatman GF/C filters were from Whatman (Gaithersburg, MD, USA) and CytoScint fluid was from ICN Biomedicals (CostaMeca, CA, USA). All other chemicals were from Sigma (St. Louis, MO, USA) or Fisher (Fairlawn, NJ, USA).

2.2. Monoamine uptake into rat brain synaptosomes

Synaptosome-containing P_2 fractions were prepared as we described previously (Zimányi et al., 1989; Xu et al., 1995). Briefly, rats were decapitated, striatum was dissected, and the tissue was homogenized in 15 volume of an ice-cold 0.32-M sucrose solution in a glass homogenizer with motor-driven Teflon pestle. The homogenizer and Teflon pestle were rinsed with 30 volumes of ice-cold sucrose solution. This fluid and homogenate were combined and centrifuged at 1000×*g* for 10 min at 4 °C. The supernatant was transferred into a clean centrifuge tube and centrifuged at 17,000×*g* for 20 min at 4 °C. The resultant pellet (P_2) was re-suspended in ice-cold 0.32 M sucrose. Aliquots were incubated at 21 °C with test drug for 5 min in a total volume of 180 μl of "uptake buffer A" containing 122 mM NaCl, 5 mM KCl, 1.2 mM $MgSO_4$, 10 mM dextrose, 1 mM $CaCl_2$, 0.01 mM nialamide, 1 μM ascorbic acid, and 30 mM Na^+

from a mixture of primary and secondary phosphate buffer, pH 7.4. Tritiated monoamine (20 μl) was added and the incubation was continued for 4 min. The uptake assays were terminated by filtration followed by five washes with ice-cold uptake buffer A without ascorbic acid and nialamide ("buffer B") on 0.1% polythyleneimine pre-soaked Wallac B filtermats (PerkinElmer Life Sciences) with a 96-well Brandel cell harvester (Brandel, Gaithersburg, MD, USA) under reduced pressure (0.29 lb/in.^{2} or 15 mm Hg). The radioactivity was counted at 40% efficiency in a 6 Detector Microbeta Trilux liquid scintillation counter (PerkinElmer) after adding 10 ml of Betaplate Scint to each filtermat. The definition of nonspecific uptake of [^{3}H]dopamine, [^{3}H]serotonin, and [^{3}H]norepinephrine, respectively, was 100 μM cocaine, 10 μM citalopram, and 10 μM desipramine. The tissue protein concentration was 30–40 μg/0.2 ml of assay mixture.

2.3. Binding of [^{3}H]CFT to membranes prepared from HEK-293 cells expressing wild-type and mutant human dopamine transporter

Human embryonic kidney-293 cells (HEK-293) were stably transfected with wild-type or mutant human dopamine transporter, and grown in Dulbecco's modified Eagle's medium/Hank's F12 medium supplemented with 5% (v/v) fetal bovine serum and 5% (v/v) bovine calf serum and 2 mM L-glutamine as described previously (Chen et al., 2001, 2004a). Once cells had reached confluence in T150 flasks, growth medium was removed by aspiration. Cells were washed with cold phosphate-buffered saline once, 3 ml of cold lysis buffer (2 mM HEPES and 1 mM EDTA, adjusted to pH 7.6 with a 1-N NaOH stock) was added to the flask. Lysate was collected and combined with another 3 ml of lysis buffer used to rinse the flask. The solution was transferred to a Beckman polycarbonate thick-wall centrifuge tube and centrifuged at 21,000×g for 30 min at 4 °C. Pellets were homogenized with a Brinkmann Polytron at setting 6 for 15 s in "binding buffer" [above buffer B with 0.1 mM tropolone for inhibition of catechol-*O*-methyltransferase (Eshleman et al., 1999)]. The general protocol for the [^{3}H]CFT binding assay with wild-type and D313N dopamine transporter was as described by us previously (Li et al., 2004; Chen et al., 2004b). Briefly, aliquots of membrane suspension (20 μl) were assayed in triplicate with drugs and [^{3}H]CFT (4 nM) for 15 min at room temperature in a total volume of 200 μl binding buffer. The membrane suspension aliquot delivered ~15 μg of protein when compound D-164 or (+)-*R*,*R*-D-84 was tested, and 40–70 μg when CFT, GBR 12909, or (−)-*S*,*S*-D-83 was tested. The assays were terminated on 0.05% (v/v) polyethyleneimine-presoaked Wallac filtermats A with the MACH3-96 TomTec Cell Harvester (Wallac, MD, USA); filters were washed with ice-cold buffer B. Filtermats were assayed for radioactivity as described above in the 6 Detector Microbeta Trilux liquid scintillation counter. Nonspecific binding was defined with 100 μM cocaine. For the mutants E215Q and D68N the same protocol was followed for measuring the affinity of CFT, GBR 12909, or (−)-*S*,*S*-D-83. Compounds (+)-*R*,*R*-D-84 and D-164 were tested under upscaled assay conditions with all components present in the assay at four times higher amounts (see Results). Thus, the final assay volume was 0.8 ml, and the protein level was ~60 μg, which was reduced in half of the experiments to ~30 μg with the same results. Incubations were performed in borosilicate glass culture tubes (12×75 mm), and worked up in sets of 24 for harvesting in a 24-pin Brandel cell harvester with Whatman GF/C filters pretreated with 0.1% (v/v) polyethyleneimine. After addition of ice-cold buffer B, samples were filtered, and filters were washed with buffer B. The filters then were suspended in 5 ml of CytoScint fluid for 6 h and assayed for radioactivity by liquid scintillation counting (Beckman model LS 6500 Multipurpose Scintillation Counter).

2.4. Molecular modeling

Molecules were built with the SYBYL molecular modeling program (version 6.9, 2002, Tripos Associates, St. Louis, MO). The BUILT/EDIT mode with SKETCH option was used. Molecular mechanics minimizations were performed in the MAXMIN minimization mode with Tripos force field and the Powell minimizer options, and with 2000 iterations or until convergence, defined as energy gradient of 0.005 kcal/mol or less. Electrostatic interactions were taken into consideration by the use of a distant dependent dielectric function. Following initial minimization, each molecule was subjected to further rigorous conformational search to investigate lower energy conformations. For this purpose, molecules were subjected to the RANDOM conformational search routine to identify lowest energy conformational structures. In this search method, all torsional angles of rotatable bonds are perturbed randomly and a resulting conformation is optimized at each step of the search. The optimized conformation generated is routinely compared with the conformation already stored in the database and stored in the database if it is found to be unique. It is important to point out that the lowest energy conformation may or may not represent the bioactive conformational structure.

2.5. Data analysis

IC_{50} values for the inhibition of monoamine uptake or [^{3}H]CFT binding by test drugs were estimated with the ALLFIT equation (DeLean et al., 1978) which equals the logistic model in the ORIGIN software (OriginLab, Northampton, MA). IC_{50} values were converted to K_i values by the Cheng–Prusoff equation (Cheng and Prusoff, 1973), which takes into account the level of radioligand and K_m of uptake or K_d of [^{3}H]CFT binding under the particular condition examined. The latter was

obtained with the nonlinear computer-fitting program KELL, a newer version of LIGAND (Biosoft, Ferguson, MO), as described previously (Chen et al., 1997). All results are expressed as mean±S.E.M. Statistical analysis consisted of one-way analysis of variance (ANOVA) followed by the Dunnett multiple comparison test for comparing multiple groups with one control group. Data were log-transformed where appropriate for comparing groups with the same variation. The accepted level of significance was 0.05.

3. Results

3.1. Activity at monoamine transporters in functional assays

GBR 12909 was approximately 10-fold more active at the dopamine than serotonin or norepinephrine transporter, thus displaying a greater selectivity for the dopamine transporter than CFT (Table 1). The dopamine transport inhibitory activity of nonhydroxylated D-164 was retained in the (+)-enantiomer of its *trans*-hydroxylated version, (+)-*R*,*R*-D-84, with the (−)-enantiomer being considerably less active at the dopamine transporter. The selectivity of (+)-*R*,*R*-D-84 and (−)-*S*,*S*-D-83 for the dopamine vs. serotonin transporter was greater than that of GBR 12909, with (+)-*R*,*R*-D-84 being an order of magnitude more selective than (−)-*S*,*S*-D-83. Furthermore, (+)-*R*,*R*-D-84, but not (−)-*S*,*S*-D-83, displayed an improved selectivity for the dopamine vs. norepinephrine transporter (Table 1).

3.2. Interaction with dopamine transporter mutants

In experiments with a protein concentration of wild-type dopamine transporter in HEK-293 cell membranes below 20 μg per 0.2-ml assay, (+)-*R*,*R*-D-84 consistently displayed a subnanomolar affinity (K_i~0.5 nM, see Discussion). Therefore, protein concentrations for wild-type and mutant transporters were held below 20 μg/0.2 ml for (+)-*R*,*R*-D-84, and for compound D-164 as well for comparison.

Table 2
Effect of mutation of D68, D313, or E215 in the human dopamine transporter on affinity of CFT, GBR 12909, D-164, (−)-*S*,*S*-D83, and (+)-*R*,*R*-D-84

Compound	Dopamine transporter	K_i (nM)	K_i ratio (mutant/wild type)
CFT	Wild type	24.7±1.7 (6)	
	D68N	87.5±19.3 (4)[a]	3.54±0.82
	D313N	5.92±0.41 (4)[a]	0.24±0.02
	E215Q	20.0±2.3 (4)	0.81±0.11
GBR 12909	Wild type	44.6±6.3 (6)	
	D68N	63.7±10.0 (4)	1.42±0.30
	D313N	28.6±3.1 (4)	0.64±0.11
	E215Q	27.7±5.7 (4)	0.62±0.16
D-164	Wild type	5.61±0.47 (3)	
	D68N	8.25±1.34 (4)	1.47±0.27
	D313N	3.07±0.26 (3)	0.55±0.06
	E215Q	5.58±0.66 (5)	0.99±0.14
(−)-*S*,*S*-D-83	Wild type	33.6±3.6 (3)	
	D68N	67.3±7.1 (5)[a]	2.0±0.3
	D313N	26.4±2.8 (3)	0.79±0.12
	E215Q	38.9±5.3 (5)	1.16±0.20
(+)-*R*,*R*-D-84	Wild type	0.473±0.085 (3)	
	D68N	7.89±1.39 (5)[a]	16.7±4.2
	D313N	0.200±0.017 (3)	0.42±0.08
	E215Q	0.455±0.064 (4)	0.96±0.22

Cell membrane preparations were assayed for [^{3}H]CFT binding as described in Materials and methods. Results are mean±S.E. for the number of experiments with independent cell membrane preparations indicated between parentheses.

[a] $P<0.01$ compared with wild type (one-way analysis of variance followed by Dunnett multiple comparisons test).

Mutants D68N and E215Q showed low binding values (as determined by combination of [^{3}H]CFT concentration, K_d and B_{max}) and were therefore studied under upscaled conditions in four-times larger assay volumes providing higher *absolute amounts* of membrane protein but the same (or lower) *concentrations* of protein. Our previous work has shown that the different harvesting and filter counting procedures for the 0.2- and 0.8-ml binding assays, by themselves, do not impact the experimental outcome (Wang et al., 2003). Under conditions of low protein levels as detailed above, the binding affinity of (+)-*R*,*R*-D-84 for the

Table 1
Potency of CFT, GBR 12909, D-164, (−)-*S*,*S*-D83, and (+)-*R*,*R*-D-84 in inhibiting synaptosomal uptake of dopamine in rat striatum, and serotonin or norepinephrine in rat cerebral cortex

Compound	Dopamine uptake (K_i (nM))	Serotonin uptake (K_i (nM))	Norepinephrine uptake (K_i (nM))	K_i ratio, serotonin/dopamine	K_i ratio, norepinephrine/dopamine
CFT	18.4±3.3 (4)	72.4±8.1 (3)	12.4±1.4 (4)	3.9	0.7
GBR 12909	10.6±2.2 (3)	91.1±12.8 (4)	102±32 (4)	8.6	9.6
D-164	1.84±0.44 (3)[a]				
(−)-*S*,*S*-D-83	28.3±4.5 (6)[a]	1,263±180 (3)	184±10 (3)	45	6.5
(+)-*R*,*R*-D-84	3.01±0.54 (7)[a]	1,083±196 (3)	199±18 (3)	360	66

Uptake assays were conducted with P_2 synaptosomal fractions of rat striatum as described in Materials and methods. Results are mean±S.E. for the number of experiments with independent striatal P_2 preparations indicated between parentheses. K_i values, calculated from IC_{50} values reported by us previously for the inhibition of [^{3}H]CFT binding to rat striatal membranes were for CFT, 18.9±2.6 nM; GBR 12909, 8.56±1.54 nM; D-164, 13.9±3.8 nM; (−)-*S*,*S*-D-83, 47.4±5.2 nM; (+)-*R*,*R*-D-84, 0.372±0.040 nM (Ghorai et al., 2003). The protein levels in the binding assays were ~60 μg/0.2 ml and in the uptake assays 30–40 μg/0.2 ml. The dopamine uptake K_i for (+)-*R*,*R*-D-84 was unchanged with a lower protein level of 6–7 μg/0.2 ml.

[a] K_i values computed from Ghorai et al. (2003).

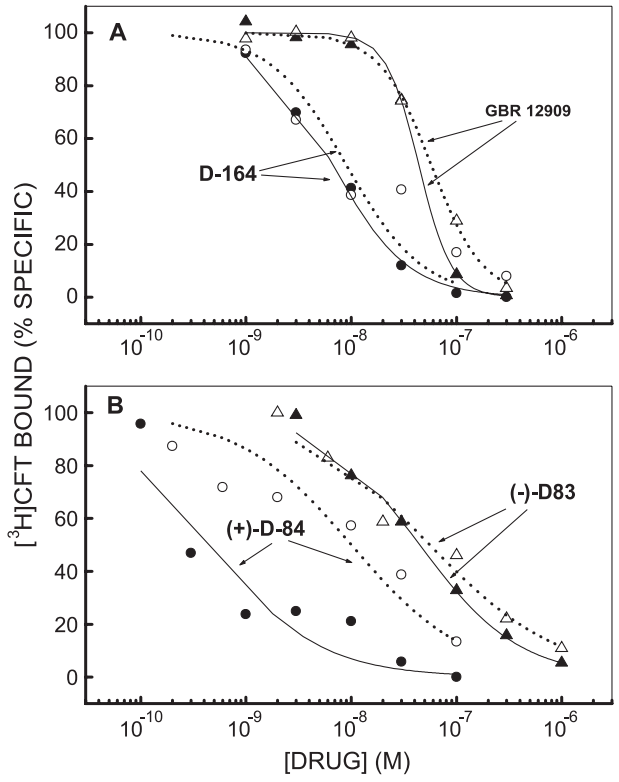

Fig. 3. Inhibition by GBR-like compounds of [^{3}H]CFT binding to membranes containing wild-type and D68N dopamine transporter. Shown are typical curves of single experiments that are part of the averages presented in Table 2. Sigmoidal curves represent the results of fitting the data to the ALLFIT equation (see Materials and methods). Closed symbols with straight lines are for wild-type, open symbols with dotted lines for D68N. (A) ●–●, compound D-164, wild-type; ○–○, compound D-164, D68N; ▲–▲, GBR 12909, wild-type; △–△, GBR 12909, D68N. (B) ●–●, compound (+)-*R*,*R*-D-84, wild-type; ○–○, compound (+)-*R*,*R*-D-84, D68N; ▲–▲, compound (−)-*S*,*S*-D-83, wild-type; △–△, compound (−)-*S*,*S*-D-83, D68N.

dopamine transporter in the present experiments with membranes of HEK-293 cells was similar (~0.5 nM) as that observed for rat striatal membrane preparations (Ghorai et al., 2003).

The pattern of K_i values for the compounds in binding to the wild-type human dopamine transporter, as measured by competition for [^{3}H]CFT binding to membrane preparations of cells expressing the transporter (Table 2), was similar to that observed with rat striatal membrane preparations (see legend to Table 1): (−)-*S*,*S*-D-83>D-164>(+)-*R*,*R*-D-84. The non-hydroxylated compound D-164 and GBR 12909 displayed a similar pattern of changes in K_i values by mutation of D68 (1.4-fold increase) (Fig. 3A), D313 (0.6-fold), or E215 (0.6- to 1-fold) (Table 2, last column). Similar changes (see also Fig. 3B) were observed for (−)-*S*,*S*-D-83 with an hydroxyl group pointing in a different direction compared with (+)-*R*,*R*-D-84) (Fig. 4). In contrast, a totally different pattern was noted for (+)-*R*,*R*-D-84, which became 17-fold weaker at the dopamine transporter upon mutation of D68 (Table 2, Fig. 3B). The response of (+)-*R*,*R*-D-84 to mutation of D313 or E215 was similar to that of compounds D-164, (−)-*S*,*S*-D-83, or GBR 12909. The pattern for CFT was unlike that of the GBR-like compounds with D68N mutation causing a somewhat greater increase in K_i but mutation of D313 a somewhat greater decrease.

4. Discussion

4.1. High-affinity binding and membrane protein concentration

In a preliminary set of experiments with high concentrations (>60 μg of protein/0.2 ml assay volume) of membranes of HEK-293 cells expressing wild-type dopamine transporter, we noted IC_{50} values for (+)-*R*,*R*-D-84 in inhibiting [^{3}H]CFT binding that were increased one order of magnitude or more over the value previously observed for rat striatal membranes (at ~60 μg of protein/0.2 ml) IC_{50} of 0.46 nM=K_i of 0.37 nM, (Ghorai et al., 2003). In contrast, at ~15 μg of HEK cell protein per 0.2 ml, the K_i of (+)-*R*,*R*-D-84 was 0.47 nM. The latter value was not affected by assay temperature (21 or 4 °C) or buffer (uptake buffer A or sodium phosphate), and the results pointed towards an impact of protein concentration on the K_i of (+)-*R*,*R*-D-84. The phenomenon of high protein concentrations increasing the apparent equilibrium binding constant, has been described years ago for homologous competition experiments (Jacobs et al., 1975; see also Bennett and Yamamura (1985). However, this phenomenon for a heterologous inhibitor, with a K_d different from that of the radioligand, has been largely ignored despite its coverage by Munson and Rodbard (1988). According to the formula offered by Munson and Rodbard (1988) (note Errata for proper formula), an approximate 4- to 5-fold increase in the K_i of (+)-*R*,*R*-D-84 can occur in our experiments with high protein. In some experiments, larger shifts occurred; possibly, at high protein levels the lipophilic nature of GBR-like compounds can cause nonspecifical adsorption to cell membranes. In the present experiments, therefore, protein concentrations were kept low (<20 μg of protein/0.2 ml assay volume) for (+)-*R*,*R*-D-84, and for compound D-164 as well, for comparison. GBR 12909 itself did not display differences in K_i between experiments with high or low protein. We interpret the somewhat higher value observed in the present experiments with cell membranes compared with earlier observations (Ghorai et al., 2003) as caused by different degrees of solubility between commercially available batches (see also Chen et al., 2004b; Little et al., 1995). With striatal membranes, but not HEK-293 cell membranes, a high protein concentration of 60 μg per 0.2 ml of binding assay mixture can be present while still allowing the detection of the high-affinity subnanomolar binding potency of (+)-*R*,*R*-D-84; the likely explanation is

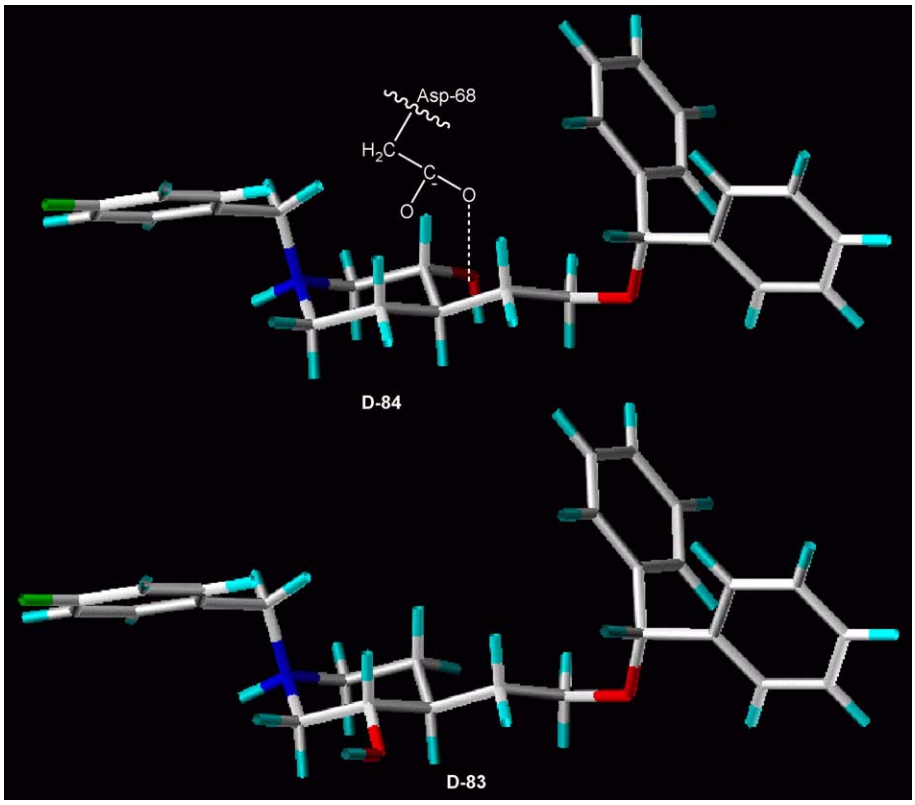

Fig. 4. Lowest energy conformational structure of (−) D-83 and (+)-*R*,*R*-D-84 upon molecular minimization by RANDOM search routine in the SYBYL molecular modeling program. The oxygen atoms are displayed in red and the nitrogen atom is displayed in blue color. The hydroxyl functional group in D-83 and D-84 are oriented in opposite directions, allowing the hydroxyl group in D-84 to interact with the Aspartate-68 residue in transmembrane domain 1 of the dopamine transporter.

that the density of the wild-type dopamine transporter in membranes of the current cell line is approximately twice as much as that in striatal membranes.

4.2. Functional activity of GBR 12935 derivatives

In functional assays, both (−)-*S*,*S*-D-83 and (+)-*R*,*R*-D-84 displayed a higher selectivity for the dopamine transporter compared with the serotonin transporter than GBR 12909 (Table 1): the K_i ratios were 45 for (−)-*S*,*S*-D-83, and considerably higher, 360, for (+)-*R*,*R*-D-84, compared with 9 for GBR 12909. In contrast, only (+)-*R*,*R*-D-84 showed higher selectivity for the dopamine vs. norepinephrine transporter (ratio of 66 compared with GBR 12909 (ratio of 9.6); the ratio was 6.5 for (−)-*S*,*S*-D-83. This is potentially important, as inhibitory potency at the norepinephrine transporter could precipitate sympathicomimetic effects. For example, reboxetine is a selective norepinephrine transporter blocker, reported to induce in human subjects hyperadrenergic symptoms resembling the syndrome of orthostatic intolerance produced by a genetic defect in the norepinephrine transporter (Schroeder et al., 2002). For many of the newer compounds reported on in the literature, functional activity at the norepinephrine transporter has been ignored by reliance on the [^{3}H]nisoxetine binding assay which underestimates the potency at this transporter for compounds in the cocaine, GBR 12909, and likely also the methylphenidate category (Reith et al., 2004). In fact, many compounds, when monitored for functional activity, are quite potent at the norepinephrine transporter, a phenomenon reported many years ago for a wide variety of compounds in the classical paper of Koe (1976). Thus, in functional assays (+)-*R*,*R*-D-84 is more selective for the dopamine vs. norepinephrine transporter than other compounds advanced in recent years as cocaine treatment candidates with slow onset and long duration of action, 3β-(4-chlorophenyl)tropane-2β-carboxylic acid phenyl ester (RTI-113) (IC_{50} ratio of 10.3, Kuhar et al., 1999), 3β-(4-iodophenyl)tropane-2β-carboxylic acid isopropyl ester (RTI-121) (ratio of 2.1, Kuhar et al., 1999), or 2β-propanoyl-3β-(4-tolyl)-tropane (PTT) (ratio of 1.0, Bennett et al., 1995). Thus, we consider (+)-*R*,*R*-D-84 a reasonable starting point for cocaine medication development.

4.3. Mutations and affinity of GBR 12935 derivatives for the dopamine transporter

The D and E mutations in the dopamine transporter we considered for the present study are in the transmembrane domains implicated previously in the interaction with

GBR-like compounds (Vaughan et al., 2001): domains 1–2 and 4–6. The mutants generated in our laboratory were based on a search for conserved (strictly D or E) or conservatively replaced (D for E or E for D) acidic amino acids in the Na^+,Cl^--dependent neurotransmitter transporter family (Chen et al., 2001). Of course, for the interaction of dopamine transporter selective GBR-like compounds with the dopamine transporter one would like to consider D or E residues specific for the dopamine transporter. However, such residues do not exist; the only acidic amino acid in transmembranes 1–2 or 4–6 that is not conserved in the family is D79, but D79 also occurs in the serotonin and norepinephrine transporter (see Chen and Reith, 2002). D79 has been reported to be crucial for the interaction of cocaine-like compounds (10-fold drop in CFT K_d upon mutation) and dopamine (37-fold drop in dopamine K_i) with the dopamine transporter (Kitayama et al., 1992), but no information is available on the impact of D79 mutation on the binding of GBR-like compounds. There are D and E residues in the dopamine transporter that are not conserved in the family in intracellular loop 1 and extracellular loops 2 and 3, but loops are not generally implicated as carrying direct contact points for the interaction with transporter blockers (Smicun et al., 1999; see also Chen and Reith, 2002).

It is noteworthy that the hydroxyl group did not make (+)-*R*,*R*-D-84 more potent than compound D-164 in dopamine uptake assays (Table 1), as it did in dopamine transporter binding assays (see legend to Table 1 and Ghorai et al., 2003). Clearly, the potency measured with the [^{3}H]CFT binding assay can deviate from that measured in the dopamine uptake assay, even when both assays are performed under the same conditions (Xu et al., 1995). This could happen if the CFT and dopamine binding domains partially overlap, with the test compound acting on a portion of the CFT domain not shared by the dopamine domain. It is also entirely possible that the CFT and dopamine domains are separate and distal (Chen and Reith, 2004), with CFT binding impacting dopamine recognition allosterically. Be that as it may, a higher potency in inhibiting CFT binding than dopamine uptake could signal cocaine antagonist activity at the level of the dopamine transporter, and the present results suggest that D68 plays an important role in (+)-*R*,*R*-D-84's ability to bind to the dopamine transporter, with a 17-fold drop in binding potency when D68 is replaced by N as compared with 1.4 - to 2-fold decreases observed for compounds (−)-*S*,*S*-D-83, D-164, or GBR 12909 (Table 2). It is important to recall that GBR 12909 and compound D-164 do not have the hydroxyl group. Most importantly, (−)-*S*,*S*-D-83 is identical in structure to (+)-*R*,*R*-D-84 with the exception that the hydroxyl group is pointing in opposite directions in the two compounds (Fig. 4). The lack of effect of D68N mutation on the binding potency of two compounds, D-164 and (−)-*S*,*S*-D-83, that are structurally identical to (+)-*R*,*R*-D-84 except for the hydroxyl group, strongly argues in favor of an interaction between the hydroxyl moiety of (+)-*R*,*R*-D-84 and D68, most likely through a hydrogen bonding between the hydroxyl group of (+)-*R*,*R*-D-84 and the carboxyl group of D68 which is lacking in N68 in the mutant D68N. This was further supported by our earlier results which indicated substantial loss of affinity in interaction of D-84 with the wild-type dopamine transporter when the hydroxyl functionality was converted into an ester group making it no longer available for H-bonding.

The interaction with (+)-*R*,*R*-D-84 appears to be specific for D68, as mutation of D313 did not differentiate between compounds D-164, (−)-*S*,*S*-D-83, (+)-*R*,*R*-D-84, or GBR 12909 (with K_i values of 0.4 to 0.8 times those in wild type, Table 2), and nor did mutation of E215 (0.6 to 1.2 times). CFT binding responded to D68N and E215Q mutation similarly as the binding of the GBR-like compounds, but D313N mutation appeared to differentially lower the CFT K_d (Table 2). This suggests that D313 impedes the binding of CFT by impacting a portion of the CFT binding pocket that is separate from the binding pocket for GBR-like compounds, although substantial overlap between these two binding pockets is still very likely. We interpret the effect of (+)-*R*,*R*-D-84 on CFT binding as a competitive effect (with possible kinetic peculiarities as pointed out for GBR 12783 by Do-Rego et al. (1999) involving a portion beyond the dopamine recognition domain bestowing potential cocaine antagonist activity upon (+)-*R*,*R*-D-84. Indeed, in preliminary tests in rhesus monkeys, (+)-*R*,*R*-D-84 inhibited moderate- to high-dose cocaine self-administration (Beardsley et al., 2004). However, a more complex mode of action of (+)-*R*,*R*-D-84 is suggested by its ability to enhance low-dose cocaine self-administration on the ascending portion of the U-shaped self-administration cocaine curve; this agrees with a potential capability of (+)-*R*,*R*-D-84 to be used as a substitution approach for treating cocaine dependence. The present results implicate D68 in the dopamine transporter in the cocaine antagonist activity of (+)-*R*,*R*-D-84.

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