

DISTINCT MOLECULAR RECOGNITION OF PSYCHOSTIMULANTS BY HUMAN
AND DROSOPHILA SEROTONIN TRANSPORTERS.

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RECOGNITION OF PSYCHOSTIMULANTS BY 5-HT TRANSPORTERS

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ABSTRACT

In this study, HEK-293 cells stably expressing human, *Drosophila*, or a chimeric serotonin (5-hydroxytryptamine, 5-HT) transporter (hSERT, dSERT, and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸, respectively) were used to explore the ability of two libraries of structurally distinct psychostimulants to inhibit 5-HT uptake. One library consisted of 3-phenyltropane analogs, whereas the second library consisted of several substituted-amphetamines. hSERT exhibited a lower K_i value for all the compounds in both libraries as compared to dSERT, whereas the chimeric SERT exhibited properties more closely resembling those of dSERT. This species-selectivity was explored using computer-generated Comparative Molecular Field Analysis (CoMFA) to model the interactions of the cocaine analogs and substituted-amphetamines at hSERT, dSERT, and the cross-species chimera. Models for the 3-phenyltropane analogs indicate that a region exists around the aromatic ring where decreased electron density is favored, particularly for hSERT. This finding may indicate pi-pi stacking with an aromatic amino acid residue in SERT. Also, electronegative substituents in the 4'-position provide favorable interactions. This structural feature was demonstrated by increased potency of analogs with electronegative substituents on the aromatic ring that withdraw electron density. For the substituted-amphetamines, key areas for interaction exist around the amine, an electrostatic component surrounding the 3-position on the aromatic ring, and a steric component surrounding the 4-position.

During the normal propagation of the neurochemical signal, the family of Na⁺- and Cl⁻-dependent neurotransmitter transporters act to modulate the spatial and temporal actions of neurotransmitter in the synapse through an uptake mechanism (Barker and Blakely, 1995). The serotonin (5-HT, 5-hydroxytryptamine) transporter (SERT) is a member of this Na⁺- and Cl⁻-dependent neurotransmitter transporter gene family. 5-HT plays an important role in the modulation of a wide variety of physiological processes, such as memory, mood, sleep, and libido (Amara and Kuhar, 1993; Barker and Blakely, 1995). SERT functions by coupling the inward movement of neurotransmitter to an electrochemical gradient. Stoichiometrically, a molecule of neurotransmitter, Na⁺, and Cl⁻ are transported into the cell, while K⁺ is counter-transported into the extracellular space. The uptake process is electrically neutral, because the neurotransmitter exists in a protonated form at physiological pH (Rudnick and Clark, 1993). In addition to regulating synaptic 5-HT concentrations, SERT is a target for structurally diverse drug families, including tricyclic antidepressants, selective 5-HT reuptake inhibitors, and psychostimulants such as cocaine and amphetamine (Owens *et al.*, 1997; Amara and Sonders, 1998; Schloss and Williams, 1998).

Cocaine and amphetamine remain two of the most abused drugs in present society. Widespread abuse of these substances carries grave physiological, psychological, and social consequences. Due to these problems, much research has focused on understanding how these two classes of abused drugs alter neurochemistry. Cocaine targets three major neurotransmitter systems in the brain: dopamine (DA), serotonin, and norepinephrine (NE) (Eshleman *et al.*, 1999; Uhl *et al.*, 2002), in each case acting to inhibit the respective neurotransmitter transporter. Amphetamine and its neurotoxic

derivatives are substrates for biogenic amine transporters, promoting non-vesicular release of neurotransmitter, with actions on dopaminergic, serotonergic, and noradrenergic systems (Amara and Sonders, 1998;Cozzi *et al.*, 1999;Jones *et al.*, 1999;Kantor *et al.*, 1999;Reneman *et al.*, 2002). Thus, these two structurally distinct drug classes both ultimately lead to increased neurotransmitter levels in the synapse.

Previous studies have shown that human and *Drosophila* SERTs exhibit different pharmacologies even though they possess approximately 51% sequence similarity (Hoffman *et al.*, 1991;Usdin *et al.*, 1991;Ramamoorthy *et al.*, 1993;Blakely *et al.*, 1994;Corey *et al.*, 1994;Demchyshyn *et al.*, 1994;Barker *et al.*, 1998). The studies presented in this work demonstrate differences in the way the human and *Drosophila* SERT species variants, as well as a chimeric SERT, recognize cocaine and substituted-amphetamines. In light of the structural diversity of these compounds, we hypothesize that distinct determinants of psychostimulant recognition by SERT exist and differences in these modes of recognition can be explored through molecular modeling and Comparative Molecular Field Analysis (CoMFA). In order to test this hypothesis, we have utilized a library of 3-phenyltropane analogs and substituted-amphetamines to examine structural components of these compounds that are critical for recognition by hSERT, dSERT, and the H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera. The structural diversity of the compound classes provides a unique collection of probes for what may be multiple binding pockets or, at minimum, unique contact sites within the same pocket, for these two prototypical psychostimulants. In our studies, both libraries of compounds were screened using a [³H]5-HT uptake inhibition assay with HEK-293 cells stably expressing hSERT, dSERT, or the H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera. The K_i values for inhibition of

[³H]5-HT transport were calculated from IC₅₀ values obtained from dose-response curves. The results of these assays were used as input for modeling using CoMFA. CoMFA has proven valuable in previous studies as a method to model protein-ligand interactions in systems that lack protein crystal structures or other definitive structural information (Carroll *et al.*, 1994; Wilcox *et al.*, 1998; Li *et al.*, 1999; Newman *et al.*, 1999). The six models presented here indicate differences in recognition on four levels. First, differences exist between the way hSERT and dSERT recognize 3-phenyltropane derivatives. Second, there are differences in the molecular recognition of the substituted-amphetamines by the two species variants. Third, and perhaps most interesting, CoMFA maps reveal differences in the way SERTs, in general, recognize these two classes of psychostimulants. Fourth, the models suggest that the H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera, which is an hSERT construct with transmembrane domains (TMDs) V-IX replaced with the dSERT sequence, exhibits distinct hybrid interactions with some components similar to hSERT and dSERT for the 3-phenyltropane analogs, whereas the determinants for recognizing the substituted-amphetamines closely parallels dSERT. These maps and accompanying data indicate fundamental differences in the recognition of psychostimulants, suggesting unique molecular interactions with SERTs that promote high-affinity interactions with these drugs of abuse.

METHODS

[³H]5-HT uptake assays.

The H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera was generated as previously described (Rodriguez *et al.*, 2003). Briefly, it is a full-length 638 amino acid transporter, constructed from amino acids 1-281 and 477-638 with identity from hSERT and residues 282-476 with identity from dSERT.

Human embryonic kidney cells (HEK-293) stably expressing either the human or *Drosophila* SERT were generous gifts from Dr. Randy D. Blakely, Vanderbilt University. All cells were maintained in Dulbecco's modified Eagle's medium with 10% dialyzed fetal bovine serum supplemented with 2 mM glutamine, 1% penicillin/streptomycin, and 600 mg/L geneticin (G-418 sulfate) in a humidified 5% CO₂ incubator at 37°C.

Uptake assays were performed on cell suspensions in 96-well microtube racks. Confluent cells in 150 mm tissue culture dishes were washed with phosphate buffered saline (PBS), scraped, and resuspended in Krebs-Ringer-HEPES (KRH) buffer supplemented with D-glucose (120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl₂, 10 mM HEPES, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 1.8 g/L D-glucose, pH 7.4). Total protein concentration was determined using the Bradford assay (Bradford, 1976). Cell suspensions were diluted in KRH/glucose to yield a concentration of 0.25 mg/mL total protein. 400 μL (100 μg) of the cell suspension was added to each tube in a 96-well microtube rack. Amphetamine and 3-phenyltropane analogs were diluted serially in KRH buffer, and 50 μL aliquots of increasing concentrations of amphetamine or cocaine analogs were added to triplicate microtubes. When 3-phenyltropane analogs were

assayed, the cells were pre-incubated with drug at 37°C for 10 minutes. This pre-incubation was not included when the substituted-amphetamines were assayed, as these compounds have been shown to be substrates for SERT, and the intracellular accumulation of drug could affect 5-HT transport (Wall, *et al.*, 1995; Rodriguez, *et al.*, 2003). [³H]5-HT (~110 Ci/mMol) was diluted to yield a final assay concentration of 20 nM in KRH buffer supplemented with 100 μM L-ascorbic acid and 100 μM pargyline. 50 μL of the [³H]5-HT solution was added to each microtube, and uptake was allowed to proceed at 37°C for 10 minutes. Transport was terminated via vacuum filtration onto 96-well GF/B filter plates (pre-soaked with 0.3% polyethyleneimine) using a Packard Filtermate 96-well harvester (Meriden, CT). Nonspecific uptake was determined using 10 μM fluoxetine. On average, specific 5-HT uptake was approximately 95% of total uptake. The filter plates were dried at ambient temperature overnight. The next day scintillation cocktail was added prior to counting in a Packard TopCount NXT (Meriden, CT) to determine accumulated [³H]5-HT. IC₅₀ values were determined using the non-linear regression routine in GraphPad Prism v.3.0 (GraphPad Software, Inc. San Diego, CA), and K_i values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). The K_m values for 5-HT uptake at each construct were: 1.2±0.2 μM (hSERT), 0.9±0.2 μM (dSERT), and 0.8±0.1 μM (H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸). Experiments were performed in triplicate and repeated in at least three separate assays.

CoMFA for 3-phenyltropane analogs.

Computational molecular modeling studies were performed using the SYBYL v.6.8 software package (Tripos Inc, St. Louis, MO) on a Silicon Graphics O2 workstation

(Mountain View, CA) running IRIX v.6.5. The 3-phenyltropane analogs were sketched using library fragments in SYBYL. Atom types were automatically assigned by SYBYL and then manually checked. The compounds were subjected to a grid search (0-360 degrees, 10-degree increment) to find the lowest energy conformation. This conformation was then minimized for all molecules using the conjugate gradient method with no initial simplex minimization, a gradient value of 0.05, and 10,000 maximum iterations. The compound set, as well as a consensus molecule containing the common tropane ring substructure, was inserted into the database. This consensus molecule containing the core structure of all the analogs was used as the template for SYBYL's database alignment tool. All of the structures were aligned using the tropane ring carbon skeleton as anchor points. The aligned molecules were then inserted into a molecular spreadsheet and pK_i values inserted as functional data. CoMFA data were generated using the parameters listed above. For the hSERT data, three components were determined to be optimal ($q^2=0.63$), whereas the dSERT ($q^2=0.51$) and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera ($q^2=0.54$) models utilized two components that were determined to be optimal based on the partial-least squares analysis.

CoMFA for substituted-amphetamines.

The amphetamine analogs were sketched using library fragments in SYBYL. Atom types were automatically assigned by SYBYL and then manually checked. The stereochemistry was assigned as S, based on the known S>R stereoselectivity for amphetamines at monoamine transporters. The compounds were subjected to a grid search (0-360 degrees, 10-degree increment) to find the lowest energy conformation. This

conformation was then minimized for all molecules using the conjugate gradient method with no initial simplex minimization, a gradient value of 0.05, and 10,000 maximum iterations. Alignment of the database was optimized using a template molecule based on the most rigid structure of (S)-5-methoxy-6-methyl-2-aminoindan, with the aromatic ring centroid and amine nitrogen of each compound held as the anchor points. The aligned molecules were then inserted into a molecular spreadsheet with pK_i values as functional data. CoMFA data were generated using the Tripos Standard CoMFA field class, with distance dielectric, no smoothing, and a steric and electrostatic cutoff of 30.0 kcal/mol. Partial least squares (PLS) analysis was validated using the leave-one-out option. Four components were initially selected for the PLS analysis, and then the number of optimal components was determined. For the hSERT, dSERT, and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera models three components were used with q² values of 0.65, 0.73, and 0.59, respectively.

MATERIALS

Dulbecco's modified Eagle's medium, pargyline, and L-ascorbic acid were purchased from Sigma (St. Louis, MO). Dialyzed fetal bovine serum was purchased from Hyclone (Logan, UT). Trypsin, L-glutamine, penicillin/streptomycin and geneticin were from Life Technologies (Grand Island, NY). Bradford Protein Assay Dye Concentrate was from Bio-Rad Laboratories (Hercules, CA). Cell culture flasks and 150 mm dishes were from Falcon/B-D Labware (Mountain View, CA). 96-well microtube racks and tubes were purchased from Marsh Bio Products (Rochester, NY). [³H]5-HT Trifluoroacetate (~110 Ci/mMol) was purchased from Amersham Biosciences (Piscataway, NJ). Cocaine and CPT-D-tartrate were purchased from Research

Biochemical International (Natick, MA). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) or Fischer Scientific (Pittsburg, PA) and were of the highest grade available. 3-phenyltropane analogs RTI-142 and RTI-121 (Neumeyer *et al.*, 1994) , RTI-55 and RTI-112 (Carroll *et al.*, 1994), β -CFT (Neumeyer *et al.*, 1996), RTI-32 (Holmquist *et al.*, 1996), RTI-31 (Carroll *et al.*,1995), RTI-83 (Blough *et al.*, 1996), and RTI-311 (Scheffel *et al.*, 1997), were synthesized as previously described.

Substituted-amphetamines were synthesized using conventional methodologies and their structures confirmed with NMR, MS, and elemental analysis, all of which matched expected criteria.

RESULTS

Comparison of 3-phenyltropane analogs and substituted-amphetamines at hSERT, dSERT, and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera.

Initial studies determined the sensitivities of hSERT, dSERT, and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ to cocaine and amphetamine derivatives. The potency of the 3-phenyltropane analogs and substituted-amphetamines for inhibition of [³H]5-HT uptake was determined (Figure 1). The K_i value for inhibition of 5-HT uptake at HEK-293 cells expressing hSERT (hSERT-HEK) was 10- to 100-fold lower, depending on the compound, as compared to dSERT and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ expressing cells (dSERT-HEK and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸-HEK, respectively) (Tables 1 and 2). Interestingly, potencies at H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ generally paralleled dSERT for all compounds assayed. For the 3-phenyltropane analogs, the rank order of K_i values at hSERT was RTI-55 < [RTI-] 112 < -311 < -31 < -142 < -83 < -32 < -121 < cocaine < β-CFT and < CPT-D-tartrate. At dSERT, the rank order of K_i values was: RTI-55 < RTI-142 < -121 < -311 < -32 < -112 < Cocaine, < -31, < -83 < β-CFT and < CPT-D-tartrate. At the H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera, the rank order of K_i values was: RTI-55 = RTI-142 < cocaine < -121 < -112 < -311 < -83 < -31 < β-CFT < -32 and < CPT-D-tartrate.

For the substituted-amphetamines, the order of increasing K_i values at hSERT was: DCA < 4-MTA < 2-Me-MDA < MMAI < MDA < 3-MTA < AMMI < DFA < 6-Me-MDA < 4-TFMA < AMMT. The order of increasing K_i values for the substituted-amphetamines at dSERT was: DCA < 4-MTA < 3-MTA < AMMT < MMAI < MDA < 6-Me-MDA < 2-Me-MDA < DFA < 4-TFMA. The order of increasing K_i values for H¹⁻

$^{281}\text{D}^{282-476}\text{H}^{477-638}$ chimera was: DCA < 4-MTA < 3-MTA = MDA = 6-Me-MDA < AMMT = AMMI < MMAI = 2-Me-MDA < 4-TFMA.

CoMFA of 3-phenyltropane analogs at hSERT, dSERT, and $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$ chimera.

A Silicon Graphics O2 workstation and the SYBYL software package were used to generate CoMFA maps for distinct models of 3-phenyltropane analog interaction with human (Fig. 2A), *Drosophila* (Fig. 2B), and the $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$ chimeric (Fig. 2C) SERTs, using RTI-55 as a model compound for reference. Overall, upon inspection, a high degree of similarity exists between the dSERT (Fig. 2B) and $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$ (Fig. 2C) models. Also, the compounds assayed share very similar potencies for dSERT and the $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$ chimera. Four prominent features stand out in all three models. First, the region of disfavored steric bulk, near the bridgehead amine, depicted in yellow is reduced in size in the dSERT and $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$ models (Fig. 2B and 2C), as compared to hSERT (Fig. 2A). The pharmacological data indicate that addition of bulky groups to the amine nitrogen or to the C2 carboxylic acid carbon results in a loss of potency. For example, RTI-121 and RTI-311 are congeners of RTI-55 with bulky substitutions at position C2 or the amine nitrogen, respectively. Both of these compounds showed profound loss of activity at all three constructs as compared to RTI-55 (Table 1). A second prominent feature is the blue field, denoting positive charge and hydrogen bond donors being favored. In hSERT (Fig. 2A), the blue field surrounds the aromatic ring, whereas in dSERT and $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$ (Fig. 2B and 2C) the blue region is reduced in size and does not envelop the aromatic ring as completely as in the hSERT model. The

third region of interest is the area denoting favored steric bulk, in green, near the 4'-substituted position of the aromatic ring. All three models share this green field. The effect of this region of steric bulk is evident when comparing β -CFT, RTI-31, and RTI-55. The potency of the compound increases in going from F-, to Cl-, to I-, following increasing hydrophobicity of the substituent. The favorability of steric bulk is evident in that the 4'-ethyl substitution (RTI-83) was more potent than the 4'-methyl congener (RTI-32) of the 3-phenyltropane series (60 nM vs. 110 nM at hSERT). The $H^{1-281}D^{282-476}H^{477-638}$ model shows this region of favored steric bulk that is larger than either parental hSERT or dSERT. In both hSERT and $H^{1-281}D^{282-476}H^{477-638}$ lengthening the 4'-substituent from methyl to ethyl affords a modest increase in potency. By contrast, at dSERT, this changes lead to decreased affinity. The small yellow regions in this location in dSERT may indicate a reduced tolerance to steric bulk at this site, relative to hSERT and $H^{1-281}D^{282-476}H^{477-638}$. The fourth region of interest is the red field region of favored negative charge on the $H^{1-281}D^{282-476}H^{477-638}$ model (Fig. 2C) also located near the 4'-position that is shared by the hSERT model (Fig. 2A) but not the dSERT model (Fig. 2B). The somewhat higher potency of RTI-31 (4'-Cl) over RTI-32 (4'-CH₃) at the hSERT and $H^{1-281}D^{282-476}H^{477-638}$ chimera may reflect this favorability for negative substituents (such as chlorine or iodine) at the 4'-position.

CoMFA of substituted-amphetamines at hSERT, dSERT, and $H^{1-281}D^{282-476}H^{477-638}$ chimera.

A Silicon Graphics O2 workstation and the SYBYL software package were used to generate CoMFA maps for distinct models of amphetamine derivative interaction with

the 5-HT transporters. Figure 3 is the pharmacological data as a CoMFA model, using 3,4-dichloroamphetamine for orientation, for interactions with the human (Fig. 3A), *Drosophila* (Fig. 3B), and the H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimeric (Fig. 3C) SERTs. The compounds in this training set all possess the aromatic core and ethylamine of the parent compound *d*-amphetamine, but vary by either single substitutions on the aromatic ring or the creation of a fused, sterically constrained bicyclic system with the aromatic ring and another saturated five- or six-membered ring. As with the 3-phenyltropane derivatives, inspection of Table 2 shows that the majority of compounds have similar potencies at dSERT and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ that are, in general, lower than their potencies at hSERT. Inspection of the CoMFA fields shows a general similarity between those of dSERT and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ consistent with the experimental data. The CoMFA fields for all three substituted-amphetamine models show a region of favored steric bulk (green) in the vicinity of the 4-position of the aryl ring. This field changes size very slightly among the three models. In addition, a red region exists near the 3-position of the aryl ring, indicating a region where electronegative substituents would increase potency. This red region is considerably larger on the hSERT model (Fig. 3A) as compared to the models in Figures 3B and 3C. There is also a sterically disfavored yellow region in the vicinity of the 5,6-positions of the aryl ring on all three models. The hSERT CoMFA (Fig. 3A) differs principally in that it has a much larger negative field at the 3-aryl position. In addition, the disfavored yellow region is displaced from its hSERT location in both the dSERT and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ models (Fig. 3B and 3C). Each of the amphetamines tested in this training set contain the ethylamine moiety. The region around the amine differs in each of the three models. For hSERT (Fig. 3A), the region

around the amine shows a small red field (negative electrostatics favored) as well as a slightly distal small blue (positive electrostatics favored) field. The dSERT and $H^{1-281}D^{282-476}H^{477-638}$ models show much larger red fields in this region. These results suggest a contact site with the amphetamine amine moiety exists on hSERT, but is different in dSERT and the $H^{1-281}D^{282-476}H^{477-638}$ chimera.

DISCUSSION

The studies described in this paper seek to examine the structural features of two different psychostimulants in the context of their common ability to interact with SERTs. Due to the striking differences in their molecular structure, yet common target, one might predict some overlapping features responsible for molecular recognition if shared contact sites exist.

Structure-activity relationships of 3-phenyltropane analogs at hSERT, dSERT, and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera.

The most potent 3-phenyltropane analog tested in this study at both hSERT and dSERT was RTI-55 (Carroll *et al.*, 1994), whereas RTI-55, RTI-142, and cocaine are equipotent at the H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera. The 3-phenyltropane analog RTI-55 differs from cocaine in two ways. First, it lacks the ester linkage between the tropane substructure and the aromatic ring. Second, RTI-55 contains an iodine atom at the 4'-(para) position on the aromatic ring. These two changes to cocaine's structure combine to promote an increase in potency approximately 40-fold at hSERT (250 nM vs. 7 nM) and greater than 3-fold at dSERT (190 nM vs. 750 nM), but little difference at H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸, possibly indicating a unique mode of recognition by the chimera. Using RTI-55 and our CoMFA models, several possible conclusions were drawn about the high-potency interaction between 3-phenyltropane analogs and SERTs. Substitutions at the 4' position on the aromatic ring appear to be important for potent interactions with SERT (as reflected by the red fields in Figs 2A and 2C). The 4'-fluoro is the most electron withdrawing substituent, followed by the 4'-chloro and 4'-iodo. In rank order of potency, the iodine-containing compound RTI-55 is most potent, followed by the chlorine-

containing (RTI-31) and then the fluorine-containing analog (β -CFT). These results are similar to those reported in previous studies focused on the cocaine binding site in purified rat brain striatum, a rich source of dopaminergic neurons (Carroll *et al.*, 1994). This study, while performed on tissue containing DAT, supports the hypothesis that the importance of the substitution at the 4'- position may be general for high-affinity 3-phenyltropane analog interactions with biogenic amine neurotransmitter transporters. (Carroll *et al.*, 1994).

Our CoMFA models revealed similarities among hSERT, dSERT, and the H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera interaction with the aromatic ring of the 3-phenyltropane analogs. In the models, the electron withdrawing substituents seem to exert their effect through a delocalization of electrons in the aromatic ring. Interestingly, the dSERT and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera models (Fig. 2B and 2C) show a much smaller area of favorable interactions around the aromatic ring faces. It has been proposed that pi-pi stacking interactions with residues in DAT may contribute to high-potency interactions with cocaine analogs (Blough *et al.*, 2002). Aromatic amino acids present in hSERT, but absent in dSERT and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸, could be important points of interaction or provide unique conformations. The H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera may have contact points for cocaine in both its central dSERT (TMDs V-IX) and its hSERT (TMDs I-IV and X-XII) regions. The effect of alkyl substitutions on the aromatic ring have been shown previously to effect binding to both SERT and NET (Blough *et al.*, 1996). The green region of favorable steric bulk in the vicinity of the 4-position is common to all three models (Fig. 2A-C). The effect of this steric region is evident when comparing β -CFT, RTI-32, and RTI-55. The potency of the compounds increases when going from F-, to Cl-

, to I-, with increasing hydrophobicity of substituents. Steric bulk effects have also been observed for DAT binding sites in rat striatum, where a 4'-bromo substitution had similar potency as a 4'-methyl substitution (1.8 nM vs. 1.7 nM) (Carroll *et al.*, 1991). Similar Van der Waals radii of the bromine and methyl substituent could explain the similarity in potency. This steric constraint has been observed with other cocaine derivatives at biogenic amine transporters (Neumeyer *et al.*, 1996). Interestingly, our CoMFA models do not suggest a significant role for the charge associated with the amine nitrogen in contributing to potency. This observation is supported by the recent synthesis of non-nitrogen containing phenyltropanes by Goulet and coworkers that effectively and potently inhibit 5-HT transport (Goulet *et al.*, 2001).

Structure-activity relationships of substituted-amphetamines at hSERT, dSERT, and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera.

Amphetamine and its neurotoxic derivatives are known to interact with the biogenic amine transporters to cause an increase in synaptic neurotransmitters. Structure-activity relationships for substituted-amphetamines have been studied at SERT, DAT, and NET. For instance, amphetamines with dual substitutions in the 3- and 4-positions become more selective for serotonergic effects (Nichols, 1994). Also, primary amines are the most potent for the biogenic amine neurotransmitter transporter family, but N-ethyl substitutions can differentially improve interactions with SERTs (Nichols, 1994). Our studies have focused on these two molecular areas to contribute to the understanding of substituted-amphetamine structure-activity relationships.

In this study, we have generated CoMFA maps from pharmacological data for hSERT, dSERT, and the $H^{1-281}D^{282-476}H^{477-638}$ chimera using a set of substituted-amphetamines. Similarities and differences in their characteristics can be identified from these analyses. At all three SERT constructs the most potent substituted-amphetamine assayed was 3,4,-dichloroamphetamine, although the compound was 10-fold more potent at hSERT than dSERT and the $H^{1-281}D^{282-476}H^{477-638}$ chimera (0.20 μ M for hSERT vs. 1.5 μ M for dSERT and $H^{1-281}D^{282-476}H^{477-638}$). Structurally, the closest derivative in this study to 3,4-dichloroamphetamine was 3,4-difluoroamphetamine, which differs only by the identity of the halide substitution. This fluorine-substituted compound, however, had markedly decreased potency at species variants as compared to the chlorine-substituted variant (4.3 μ M at hSERT, 56 μ M at dSERT, and 12 μ M at $H^{1-281}D^{282-476}H^{477-638}$). The CoMFA models indicate a region of favored negative charge (in red, Fig. 3) in the area around the 3-position of the aromatic ring on each of the three models. However, this field is slightly displaced from the 3-position, indicating that this electrostatic effect may not be solely determined by the substitution at the 3-position. The green field near the 4-position indicates that the bulk of the substitution plays a major role in potency. The role of this position was examined in a previous study that revealed that bulk at the 4-position is critical in determining potency (Marona-Lewicka, *et al.*, 1995). The second most potent compound at hSERT, dSERT, and the $H^{1-281}D^{282-476}H^{477-638}$ chimera was 4-methylthioamphetamine (0.45 μ M, 4.6 μ M, and 3.8 μ M, respectively). The position of the methylthiol group affects potency, with the 4-methylthioamphetamine exhibiting higher potency as compared to 3-methylthioamphetamine, due to favorable steric interactions as indicated by the green region in Figure 3.

In addition to distinctions surrounding the aromatic ring, differences in molecular fields are present near the amine. These species-specific interactions revealed similarities between dSERT and the H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera. The *Drosophila* SERT sequence in the H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera is localized to the TMD V-IX region, implicating these domains as possessing residues involved with interacting with the amine functionality. Previously published reports have identified TMD I as an important region of SERT involved with recognition of the amine of both 5-HT (Barker *et al.*, 1999) and tryptamine derivatives (Adkins *et al.*, 2001). Together, our data and these previous studies indicate that substrates likely interact with multiple TMDs, including TMD I and the TMD V-IX region, and, consequently, any model of SERT structure should consider multiple points of contact within the transporter to form a substrate translocation pathway. The models show two additional sites of similarity among the three SERTs. The green field (steric bulk favorability) could suggest that these bulky, hydrophobic substituents interact with a corresponding hydrophobic region on SERT. In addition, a sterically-disfavored yellow region in the vicinity of the 5,6-positions of the aryl ring is common to all three models, however its location is displaced in the dSERT and H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ model as compared to hSERT.

In conclusion, comparing and contrasting the two models of psychostimulant recognition by SERT provides a unique opportunity to reveal evidence of unique interaction sites for cocaine and substituted-amphetamines at SERT. For the 3-phenyltropane analog models, differences exist in the way SERT species variants recognize these compounds on several levels: recognition of the phenyl ring, different substituents in the 4'-position of the aromatic ring, and the electrostatics of the phenyl

ring. Similarity among these three models exists in the steric bulk components around the bridgehead amine and the 4'-position substitutions. For the substituted-amphetamines favored negative charge around the 3- and 4-position is shared by all three SERTs. Also, differences in the way the three SERTs recognize the amine functionality exist. Since all known substrates for SERTs contain amine groups, the site involved with coordinating the amine may be critical for substrate recognition and translocation. The overall dissimilarity of the CoMFA fields for 3-phenyltropane and substituted-amphetamine interactions is suggestive that the sites for interaction on SERT are very different. It is possible that the regions that bind to the 4'-aryl of the 3-phenyltropane analogs and the 4-aryl position of the substituted-amphetamines could be the same, but there are no other structural features to suggest other similarities. We would argue, therefore, that the sites of interactions for these two families of psychostimulants are distinct, but have major determinants within the TMD V-IX region. Also, the uniqueness of the molecular interactions can likely be explained by differences in mechanism of action for these psychostimulants; the substituted-amphetamines can act as substrates for the transporter, whereas the 3-phenyltropanes act as transport blockers (Wall, *et al.*, 1995). Future molecular studies should identify specific amino acids involved with recognition of psychostimulants and provide important new information about SERT function.

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Footnotes

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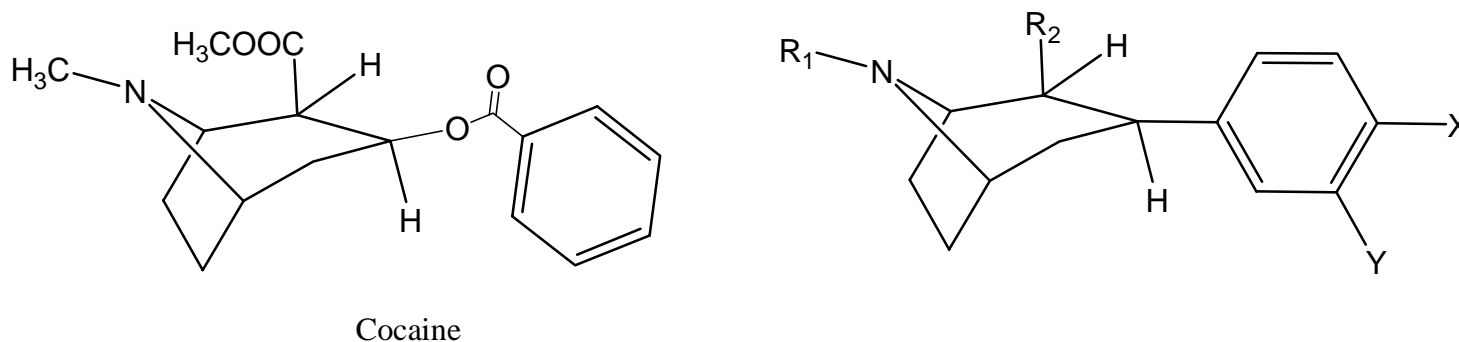
Figure Legends

Figure 1. (A) Diagram of wild-type hSERT, dSERT, and the $H^{1-281}D^{282-476}H^{477-638}$ chimera. The transporters were constructed as described in Materials and Methods, with hSERT sequence indicated in black and dSERT sequence indicated in gray. (B) Inhibition of [3H]5-HT uptake at (■) hSERT, (▲) dSERT, and (◆) $H^{1-281}D^{282-476}H^{477-638}$ using RTI-55 and 3,4-dichloroamphetamine. [3H]5-HT uptake assays were performed as described in Materials and Methods. Nonspecific uptake was determined with 10 μ M fluoxetine. Data were plotted as a percentage of specific [3H]5-HT uptake. Dose-response curves depicted are representative of at least three experiments performed in triplicate.

Figure 2. Comparative molecular field analysis (CoMFA) maps of cocaine analog interactions with (A) hSERT, (B) dSERT, and (C) $H^{1-281}D^{282-476}H^{477-638}$ chimera, arranged for cross-eyed viewing. RTI-55 is modeled within the field for directional orientation. Colored fields represent characteristics of the ligand that favorably or unfavorably affect high-affinity interactions. Steric contours are shown in green and yellow, whereas electrostatic contours are shown in blue and red. Green fields indicate molecular regions where steric bulk is favored for high-affinity interactions, whereas regions indicated by yellow are areas in which steric bulk is disfavored for high-affinity interactions. Blue fields indicate regions of the molecule where more positive charge and hydrogen bond donors are favored, and negative charge and hydrogen bond acceptors are disfavored for high-affinity interactions. Red fields indicate regions where negatively charged substituents and hydrogen bond acceptors are favored, and positive charge and hydrogen bond donors are disfavored.

Figure 3. Comparative molecular field analysis (CoMFA) maps of substituted-amphetamine interactions with (A) hSERT, (B) dSERT, and (C) H¹⁻²⁸¹D²⁸²⁻⁴⁷⁶H⁴⁷⁷⁻⁶³⁸ chimera, arranged for cross-eyed viewing. 3,4-dichloroamphetamine is modeled within the field for directional orientation. Colored fields represent characteristics of the ligand that favorably or unfavorably affect high-affinity interactions. Steric contours are shown in green and yellow, whereas electrostatic contours are shown in blue and red. Green fields indicate molecular regions where steric bulk is favored for high-affinity interactions, whereas regions indicated by yellow are areas in which steric bulk is disfavored for high-affinity interactions. Blue fields indicate regions of the molecule where more positive charge and hydrogen bond donors are favored, and negative charge and hydrogen bond acceptors are disfavored for high-affinity interactions. Red fields indicate regions where negatively charged substituents and hydrogen bond acceptors are favored, and positive charge and hydrogen bond donors are disfavored.

Table 1. 3-phenyltropane derivative K_i values for [^3H]5-HT uptake inhibition at hSERT, dSERT, and $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$ chimera.



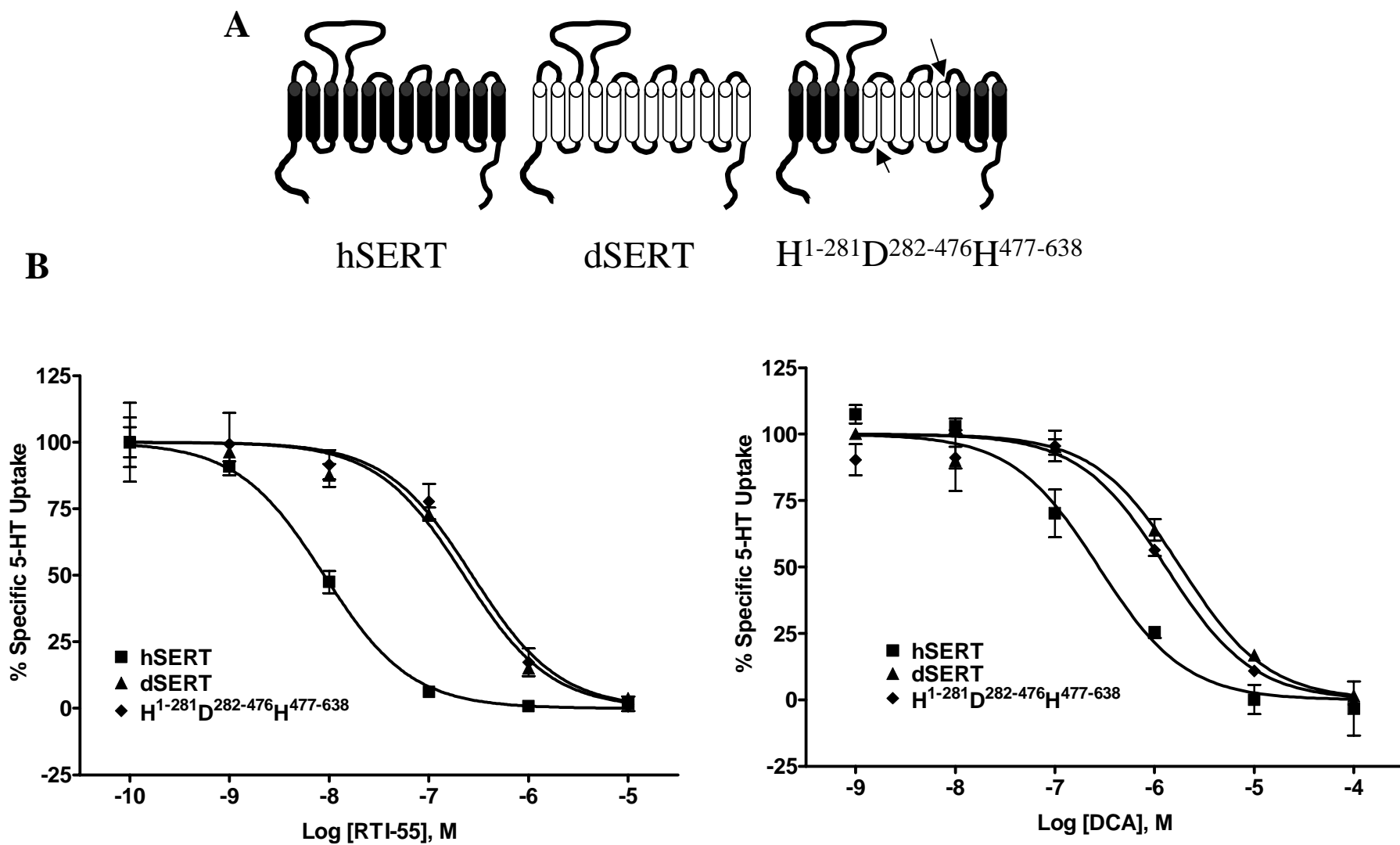
Name	R_1	R_2	X	Y	K_i , nM \pm SEM		
					hSERT	dSERT	$\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$
Cocaine	-	-	-	-	250 \pm 90	750 \pm 140	220 \pm 60
CPT-D-tartrate	CH_3	COOCH_3	H	H	1500 \pm 100	38000 \pm 10000	14000 \pm 10000
RTI-142	H	COOCH_3	F	H	45 \pm 10	320 \pm 90	200 \pm 40
β -CFT	CH_3	COOCH_3	F	H	990 \pm 110	4300 \pm 1300	2600 \pm 450
RTI-31	CH_3	COOCH_3	Cl	H	40 \pm 7	800 \pm 140	890 \pm 270
RTI-112	CH_3	COOCH_3	Cl	CH_3	12 \pm 2	580 \pm 90	550 \pm 210
RTI-32	CH_3	COOCH_3	CH_3	H	110 \pm 30	580 \pm 240	5800 \pm 1600
RTI-83	CH_3	COOCH_3	CH_2CH_3	H	60 \pm 10	2600 \pm 1300	660 \pm 320
RTI-55	CH_3	COOCH_3	I	H	7 \pm 2	190 \pm 10	200 \pm 30
RTI-121	CH_3	$\text{CO}_2\text{CH}(\text{CH}_3)_2$	I	H	180 \pm 40	340 \pm 150	360 \pm 170
RTI-311	$\text{CH}_2\text{CH}=\text{CH}_2$	COOCH_3	I	H	30 \pm 4	470 \pm 160	600 \pm 160

Table 2. Substituted-amphetamine K_i values for [^3H]5-HT uptake inhibition at hSERT, dSERT, and $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$ chimera.

Name	Abbrev.	Structure	$K_i, \mu\text{M} \pm \text{SEM}$		
			hSERT	dSERT	HDH ¹
3,4-Dichloro-amphetamine	DCA		0.20 ± 0.1	1.5 ± 0.5	1.5 ± 0.2
3,4-Difluoro-amphetamine	DFA		4.3 ± 2.2	56 ± 30	12 ± 6
4-Methylthio-amphetamine	4-MTA		0.45 ± 0.1	4.6 ± 0.2	3.8 ± 0.6
3-Methylthio-amphetamine	3-MTA		1.8 ± 0.9	7.3 ± 2.1	14 ± 0.9
1-Aminomethyl-5-methoxyindan	AMMI		2.1 ± 0.3	13 ± 3.1	16 ± 4.7
1-Aminomethyl-6-methoxytetralin	AMMT		7.0 ± 1.3	11 ± 1.9	15 ± 2.8
5-Methoxy-6-methyl-2-aminoindan	MMAI		0.83 ± 0.1	19 ± 1.0	20 ± 3.2
3,4-Methylenedioxy-amphetamine	MDA		1.1 ± 0.3	19 ± 2.4	13 ± 2.0
2-Methyl-3,4-methylenedioxy amphetamine	2-Me-MDA		0.70 ± 0.1	27 ± 2.0	19 ± 3.0
6-Methyl-3,4-methylenedioxy amphetamine	6-Me-MDA		4.4 ± 0.2	26 ± 1.9	12 ± 2.0
4-Trifluoromethyl-amphetamine	4-TFMA		5.2 ± 1.0	86 ± 19	55 ± 22

¹ $\text{H}^{1-281}\text{D}^{282-476}\text{H}^{477-638}$

Figure 1.



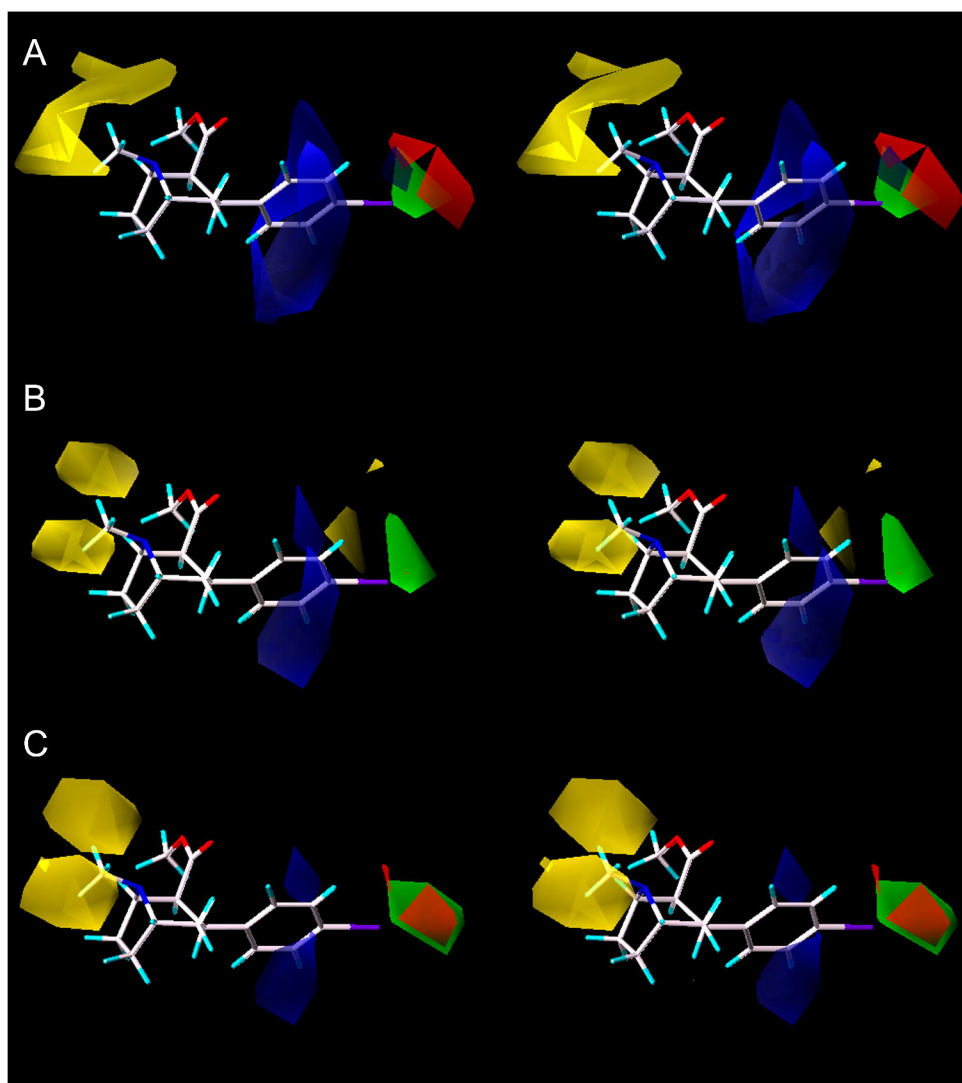


Figure 2.

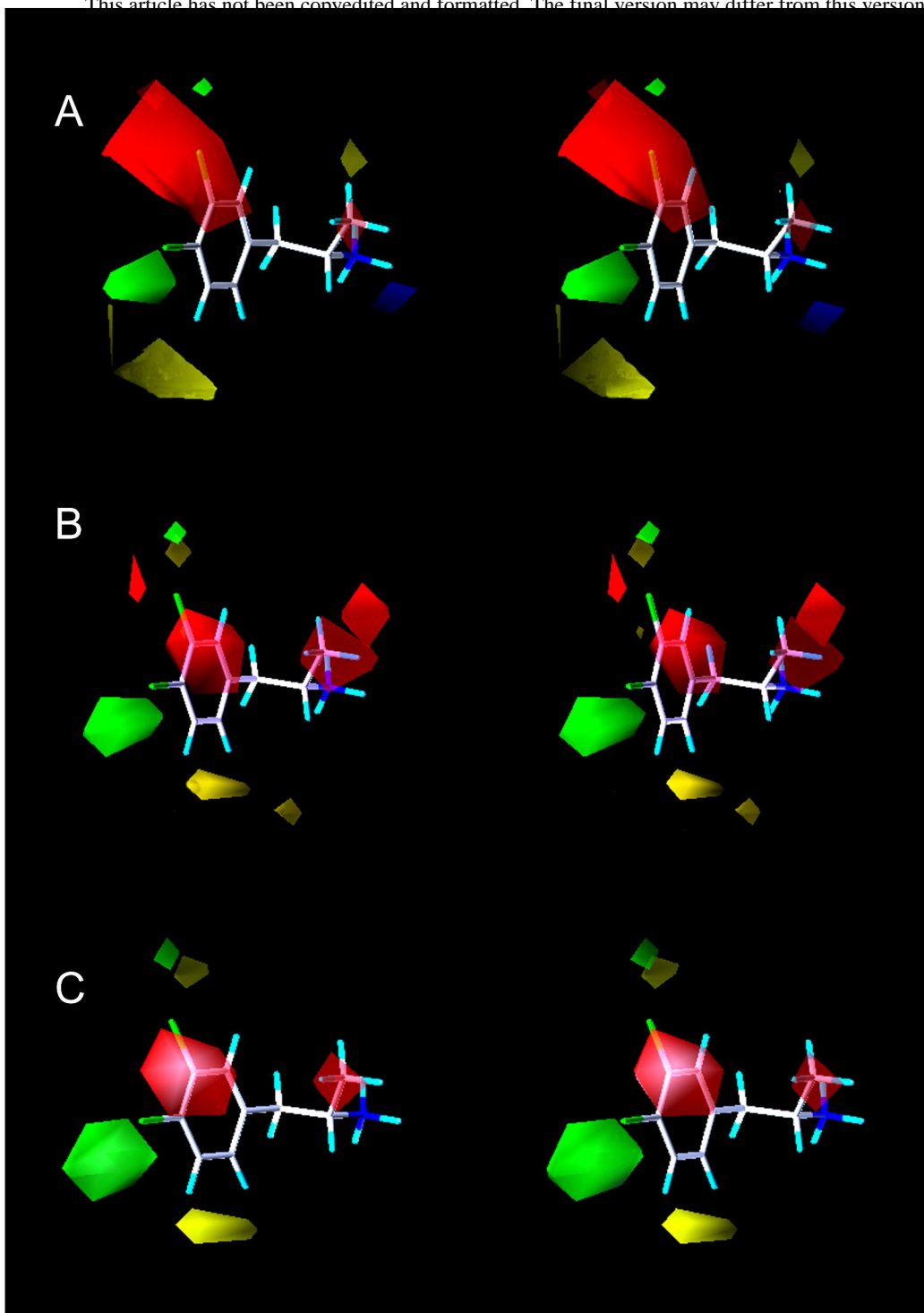


Figure 3.