Interaction of Antidepressants with the Serotonin and Norepinephrine Transporters

MUTATIONAL STUDIES OF THE S1 SUBSTRATE BINDING POCKET

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Background: SERT and NET are important targets for antidepressants.
Results: Antidepressants are differentially affected by mutations within the central S1 pocket of SERT and NET.
Conclusion: Our data indicate that many antidepressants bind within the S1 pocket, and inhibitor selectivity is determined by residues within this site.
Significance: This study provides a framework for modeling of drug binding, which may be used in future structure-based drug design.

The serotonin transporter (SERT) and the norepinephrine transporter (NET) are sodium-dependent neurotransmitter transporters responsible for reuptake of released serotonin and norepinephrine, respectively, into nerve terminals in the brain. A wide range of inhibitors of SERT and NET are used as treatment of depression and anxiety disorders or as psychostimulants. Despite their clinical importance, the molecular mechanisms by which various types of antidepressant drugs bind and inhibit SERT and NET are still elusive for the majority of the inhibitors, including the molecular basis for SERT/NET selectivity. Mutational analyses have suggested that a central substrate binding site (denoted the S1 pocket) also harbors an inhibitor binding site. In this study, we determine the effect of mutating six key S1 residues in human SERT (hSERT) and NET (hNET) on the potency of 15 prototypical SERT/NET inhibitors belonging to different drug classes. Analysis of the resulting drug sensitivity profiles provides novel information on drug binding modes in hSERT and hNET and identifies specific S1 residues as important molecular determinants for inhibitor potency and hSERT/hNET selectivity.

Serotonin (5-hydroxytryptamine; 5-HT)³ and norepinephrine (NE) are neurotransmitters in the central nervous system involved in control of human behaviors such as mood, sleep, pain, appetite, aggression, and sexual activity. The serotonin and norepinephrine transporters (SERT and NET, respectively) are integral membrane proteins that facilitate sodium- and chloride-dependent transport of released 5-HT and NE, respectively, into neurons (1, 2) and play key roles for spatiotemporal regulation of 5-HT and NE neurotransmission, maintenance of extrasynaptic monoamine levels, and pre-synaptic monoamine homeostasis (3, 4). Given these important roles of SERT and NET, pharmacological modulation of their transport activity influences a variety of neurophysiological processes and is used in the treatment of human psychiatric diseases. In particular, inhibitors of SERT and NET are widely used in the treatment of major depressive disorder with more than 30 drugs in current clinical use (see Fig. 1) (5). Also, psychostimulants such as amphetamine and 3,4-methylenedioxymethamphetamine (also known as ecstasy) have SERT and NET as primary targets (6, 7).

Despite the clinical importance of SERT and NET inhibitors, for the majority of these, key aspects of their molecular pharmacology have remained largely unknown; including location and structure of inhibitor binding sites, inhibitor binding modes, and the molecular mechanism by which inhibitor binding antagonizes transporter function. Although several residues in SERT and NET have been identified that control binding of inhibitors (for review, see Ref. 2), the lack of detailed structural information of SERT and NET has limited development of such information into structural models of inhibitor binding. However, a major step forward was provided with the crystallization of LeuT (8), a bacterial homologue to the solute carrier 6 (SLC6) family of transporters including SERT and NET. LeuT has proven an excellent model for understanding structural aspects of SLC6 transporters, including the binding sites for ligands and candidate permeation pathways. Specifically, LeuT structures have provided evidence for inhibitor binding sites located in two distinct regions; that is, the central substrate binding pocket (denoted the S1 pocket) and the S2 pocket, located in the extracellular vestibule (see Fig. 2) (9–12).

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The equivalent regions in the mammalian SLC6 transporters are natural candidates for harboring binding sites for antidepressants, and indeed several LeuT-guided studies have suggested that inhibitors bind to the S1 pocket (13–19), the S2 pocket (9–10, 20, 21), or overlapping sites in both S1 and S2 pockets (22, 23) of SERT and NET.

To better understand the role of the S1 pocket and the structural basis of drug inhibition and SERT/NET selectivity, we have examined the effect of S1 mutations in human SERT (hSERT) and NET (hNET) for a panel of compounds belonging to different antidepressant drug classes. Specifically, we studied the effect of six single point mutations of key residues surrounding the S1 pocket of hSERT and hNET on the inhibitory potency of 15 structurally and pharmacologically diverse inhibitors (see Figs. 1 and 2). Cluster analysis of the resulting drug activity profiles across each set of transporter mutants reveals distinct sets of drugs that have similar perturbation patterns, suggesting that these share similar binding modes within the S1 pocket. Hereby, we provide information aiding the general understanding of how antidepressants interact with and distinguish between hSERT and hNET at the molecular level.

**EXPERIMENTAL PROCEDURES**

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum, trypsin, and penicillin-streptomycin was purchased from Invitrogen. All DNA restriction enzymes and T4 DNA ligase enzyme were from New England Biolabs ( Ipswich, MA). Cell culture dishes were from Sarstedt AG & Co (Nümbrecht, Germany), and 96-well plates were from Nunc (Roskilde, Denmark). [3H]5-HT (27–28 Ci/mmol), [3H]dopamine (DA; 91–139 Ci/mmol), and MicroScint-20 scintillation mixture were obtained from PerkinElmer Life Sciences. Amitriptyline, atomoxetine, clonipramine, duloxetine, fluoxetine, fluvoxamine, imipramine, 2-(2-dimethylaminomethyl-phenylsulfanyl)-5-methyl-phenylamine (MADAM), maprotiline, mianserin, nisoxetine, paroxetine, (S)-citalopram, sertraline, and venlafaxine were kindly provided by H. Lundbeck A/S (Valby, Denmark).

**cDNA Constructs and Site-directed Mutagenesis**—The mammalian expression plasmids pCDNA3.1 and pCI-IRE-neo containing hSERT (pCDNA3.1-hSERT) and hNET (pCI-IRE-neo-hNET) cDNA, respectively, have been described previously (24). Generation of point mutations in pCDNA3.1-hSERT and pCI-IRE-neo-hNET was performed by site-directed mutagenesis using the QuikChange mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s recommendations followed by sequencing of the entire hSERT or hNET coding sequence of each mutant construct (MWG Biotech, Martinsried, Germany). To compare cell-surface expression patterns of wild-type (WT) and mutant hSERT and hNET using fluorescence microscopy, transporter constructs with green fluorescent protein (GFP) fused to the intracellular N-terminal region of the transporters were created. For hSERT constructs, the hSERT coding domain sequence (CDS) was PCR-amplified from pCDNA3.1-hSERT with restriction enzyme recognition sites XhoI and HindIII contained upstream and downstream, respectively, to the CDS. The PCR fragment was XhoI/HindIII-digested and ligated into a modified version of the GFP fusion protein cloning vector pGFP2 (PerkinElmer Life Sciences) using XhoI and HindIII sites to generate pGFP2- hSERT, in which the GFP and hSERT CDS are fused. Subsequently, the GFP-hSERT CDS was excised from pGFP2-GFP-hSERT by Nhel and ligated into Nhel-digested pCDNA3.1-hSERT generating pCDNA3.1-GFP-hSERT. Subsequently, fragments containing the individual point mutations in the hSERT CDS were excised from their parent mutant pCDNA3.1-hSERT constructs using BlpI and XbaI and ligated into BlpI/XbaI-digested pCDNA3.1-GFP-hSERT, generating pCDNA3.1-GFP-hSERT constructs containing point mutations in the hSERT CDS.

For generation of GFP-tagged hNET constructs, the CDS of GFP was excised from pGFP2-C1 (PerkinElmer Life Sciences) with Nhel and EcoRI and ligated into Nhel/EcoRI-digested pCI-IRE-neo-hNET generating pCI-IRE-neo-GFP-hNET. Subsequently, the CDS containing the individual point mutations in hNET was excised from the mutant pCI-IRE-neo-hNET constructs using EcoRI and NotI and ligated into EcoRI/NotI digested pCI-IRE-neo-GFP-hNET providing pCI-IRE-neo-GFP-hNET constructs containing point mutations in the hNET CDS. The sequence integrity of all GFP-fusion constructs was verified by DNA sequencing (MWG Biotech).

**Cell Culturing and Expression**—COS7 cells (American Type Culture Collection, Manassas, VA) were cultured in growth medium (DMEM supplemented with 10% v/v fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin) at 37 °C in a humidified 5% CO₂ environment. COS7 cells were transfected using TransIT DNA transfection reagent (Mirus, Madison, WI). Before transfection, confluent cells growing in 150-mm tissue culture dishes were washed twice with 10 ml of phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.3), and the cells were then detached with 2 ml of trypsin followed by resuspension of cells in growth medium at a concentration of 6 × 10⁵ cells/ml. Cells were then immediately mixed with transfection reagent/DNA complex, which was prepared immediately before use according to the instructions supplied by the manufacturer. Briefly, per 96-well plate, 5 μg of DNA and 15 μl of transfection reagent were mixed in 0.5 ml of DMEM and incubated at 20 °C for 20 min. The combined cell suspension and transfection reagent/DNA mixture was subsequently plated into white 96-well tissue culture plates (50 μl of cell suspension per well corresponding to 30,000 cells) and transferred into the 37 °C humidified 5% CO₂ environment until use in functional assays. Under these conditions, the transfection efficiency was between 40 to 90% as assessed by visual inspection of cells transfected with GFP-hSERT and GFP-hNET (data not shown).

**[3H]5-HT and [3H]DA Transport Measurements**—Uptake assays were performed 40–48 h after transfection, and all washing steps were performed using an automated plate washer (ELx50, Bio-Tek Instruments, Inc., Winooski, VT). Before uptake experiments, cells were washed twice with 300 μl of PBS containing 0.1 mM CaCl₂ and 0.5 mM MgCl₂ (PBSCM) and allowed to pre-equilibrate at 20 °C for 20 to 30 min in 50 μl of PBSCM per well. In all NET assays, the PBSCM buffer was supplemented with 1 mM l-ascorbic acid. The time course of uptake into transfected COS7 cells was initially assessed for
each construct by measuring accumulation of radiolabeled substrate over increasing time periods (supplemental Figs. S1 and S2). 40 μl PBSCM was added to each well, and uptake was initiated by the addition of 10 μl of PBSCM containing [3H]5-HT (for SERT assays) or [3H]DA (for NET assays), respectively, giving a final substrate concentration of 50 nM. For time kinetics experiments, eight sets of triplicate wells were incubated at 20 °C for increasing time periods (0.5–20 min), and uptake was terminated by aspiration and rapid washing of wells three times with 300 μl of PBSCM. For transport inhibition experiments to determine IC_{50} (for calculation of K_{i}), eight sets of triplicate wells were preincubated in 40 μl of PBSCM per well with 0 or increasing concentration of inhibitors at 20 °C for 30 min before the addition of 20 μl of PBSCM containing [3H]5-HT (for SERT assays) or [3H]DA (for NET assays) giving a final substrate concentration of 50 nM. Uptake was allowed to proceed at 20 °C for 5 min before uptake were terminated as described above. For determination of K_{m} values, eight sets of triplicate wells were incubated with increasing concentrations of unlabeled 5-HT (for SERT assays) or DA (NET assays) in PBSCM and a fixed concentration of 50 nM [3H]5-HT (for SERT assays) or [3H]DA (for NET assays) at 20 °C for 5 min. For all transport assays, the amount of accumulated radioligand per well was determined by solubilizing cells in 50 μl of scintillant solution (MicroScint20) and subjected to at least 1 h of vigorous shaking. Radioactivity was determined by counting of plates in a Packard TopCounter (Packard Instrument Co.). For cells transfected with WT transporters, total specific uptake of [3H]substrate typically ranged between 4000 and 6000 counts per min (cpm) per well, corresponding to 125–180 and 40–55 fmol of substrate per well for WT hSERT and WT hNET, respectively. For each experiment, nonspecific uptake (“background”) was determined by assaying triplicate wells with non-transfected cells in parallel. Typically, accumulated radioactivity in non-transfected cells was between 100 and 500 cpm per well. Specific uptake was calculated by subtracting nonspecific uptake from total uptake. The uptake level was in all experiments no more than 10% that of total added [3H] substrate, and experiments yielding a ratio of specific to nonspecific uptake less than 4:1 were discarded.

Confocal Laser Scanning Microscopy—A Leica SP2 confocal microscope equipped with an argon laser, a helium/neon laser, and a 63×/1.17 oil immersion objective was used. 24 – 48 h after transfection, COS7 cells were re-plated in 12-well glass-bottom plates (MatTek Corp., Ashland, MA) and incubated for 2 h to adhere. 30 min before imaging, cells were stained with CellMask™ Deep Red plasma membrane stain (Molecular Probes, Inc., Eugene, OR) following the instructions provided by the manufacturer. GFP-tagged WT and mutant transporters were visualized using the 488-nm argon laser line at 25–35% input power as the excitation source and measurement of emission in the 500–560-nm spectrum range. Cell membrane stain was visualized using the 633-nm helium/neon laser line at 25–35% input power and collection of emission in the 640–700-nm spectrum range. Overlay images were produced with Leica LAS AF lite software (Leica Microsystems GmbH, Wetzlar Germany).

Inhibitory Potency of Antidepressants at SERT and NET

Data and Statistical Analysis—Data and statistical analyses were performed using Prism 4.0 (GraphPad Inc., San Diego, CA) and R (25) software. For determination of IC_{50} values, dose-response data obtained from [3H]5-HT or [3H]DA uptake inhibition experiments were fitted by the equation,

\[
\% \text{ Specific uptake} = 100/(1 + 10^{\log IC_{50} - \log (\text{inhibitor}) \times \text{Hill slope}})
\]

where IC_{50} is the concentration of inhibitor that produces a half-maximal inhibition of uptake. For determination of the Michaelis-Menten constant, data from K_{m} assays were fitted by the equation,

\[
\% \text{ Specific uptake} = 100/(1 + 10^{\log K_{m} - \log (\text{inhibitor}) \times \text{Hill slope}})
\]

where [L] is the concentration of substrate. WT transporters were always assayed in parallel with mutants, and K_{i}, fold changes were calculated from paired determinations.

Cluster Analysis—For both SERT and NET, the -fold changes in K_{i} of the 15 inhibitors between mutant and WT transporter were hierarchically clustered using mapping of Euclidean distance between each pair of mutant vectors, consisting of six positional mutants per vector. The implementation was carried out using the statistical software R (25) with the stats and pvclust packages (27) with standard settings. To assess the confidence of the generated clusters, we used cluster bootstrapping, resampling the data 1000 times using the pvclust package with p > 0.95 as a threshold to select stable clusters, meaning that the cluster is present in >95% of the bootstrap replicates (27).

RESULTS

Selection of Antidepressant Compounds and S1 Residues—To investigate the role of the S1 binding pocket in hSERT and hNET for the inhibitory function of antidepressant drugs, we characterized a panel of 15 SERT and NET inhibitors (Fig. 1) at hSERT and hNET single point mutations of key residues within the S1 pocket (Figs. 2 and 3; see Tables 2 and 3). Our selection of compounds aimed to cover structurally diverse SERT/NET inhibitors with distinct selectivity profiles and included two highly selective pharmacological tool compounds (MADAM and nisoxetine) and 13 antidepressant drugs, classified according to their pharmacological profile as selective 5-HT reuptake inhibitors (SSRIs), selective NE reuptake inhibitors (NRIs), or dual acting 5-HT and NE reuptake inhibitors (SNRIs) (Fig. 1). The selection of S1 residues for mutation as well as the amino acid substitutions (Fig. 4) were guided by results from previous mutational studies of rodent and human SERT and NET (17, 18, 28–31) and LeuT-based homology models of SERT and NET with various ligands docked into the S1 binding pocket (15, 17, 18, 31). In homology models of hSERT and hNET, the S1 pockets are formed by residues located in TM1, TM3, TM6, and
TM8, which in both transporters form a predominantly non-polar, hydrophobic cavity except for the sub region (here denoted subsite A; Fig. 2) that is believed to accommodate the polar aminoalkyl chain, which is present in SERT/NET substrates and almost all SERT/NET inhibitors (13–19, 22, 28, 32, 33). The polar surroundings in subsite A are formed by side chains from TM1 and TM6 residues and a break in the helical structure of both TM1 and TM6, which expose backbone carboxyl oxygen and nitrogen atoms as potential hydrogen bonding partners (2). Previous mutational analysis of residues in subsite A in hSERT have shown that Y95A, D98E, and S438T greatly perturb inhibitor binding and that these mutations confer differential effects on inhibitors (17, 18, 28). Thus, these mutations are highly promising candidates for probing potential inhibitor interactions within the subsite A region. Furthermore, these mutations do not change transporter maturation and cell membrane expression (supplemental Figs. S6 and S7) or change the basal 5-HT transport function when heterologously expressed in cells (Table 1), and therefore, allow determination of inhibitory potency (Ki) in [3H]-5-HT uptake assays (“Experimental Procedures”). In contrast to SERT, much less mutational data is available for subsite A residues in NET. Our initial mutational analysis of the hNET residues Phe-72, Asp-75, and Ser-419 (corresponding to the selected subsite A residues in hSERT; Fig. 2) showed that transport function of hNET expressed in COS7 cells was highly sensitive to mutation at all three residues. In agreement with previous findings (28), we found that expression of D75E and D75A mutants did not produce measureable [3H]-DA uptake in COS7 cells (Fig. 3). Confocal imaging of GFP-tagged variants of these hNET mutants showed loss of cell-surface expression for D75A compared with GFP-tagged WT hNET (Fig. 3), suggesting that this mutation disrupt the ability of the transporter protein to express and traffic to the cell surface membrane. In agreement with previous findings (28), we found that D75E displayed surface expression patterns comparable to WT hNET, indicating that D75E disrupts transport function. Similarly, the F72A mutation disrupted transport function as COS7 cells did not display [3H]-DA uptake but retained cell membrane expression of GFP-tagged transporter (Fig. 3). In contrast, the F72Y mutant robustly transported [3H]-DA, although the transport activity was reduced ~50% as compared with WT hNET (Table 1). Thus, the transport active mutants F72Y and S419T were included as probes for C subsite A in hNET.

The regions located opposite to subsite A within the central S1 pocket (here denoted subsites B and C; Fig. 2) are generally non-polar and formed by hydrophobic side chains from resi-
dues in TM3 and TM8. To modify subsite B in hSERT, we selected the N177S mutant that previously has been shown to perturb inhibitor binding (15, 17) and the corresponding hNET mutant N153S. For subsite B in hNET, we also included the G149A mutant, which we recently found to affect inhibitor binding in hNET (31). For subsite C, we selected the hSERT mutants I172M and F341Y, which have been found to greatly affect inhibitor potency (15, 17, 30, 34). The corresponding mutants in hNET (V148M and F323Y) were generated and tested for transport function (Table 1). The F323Y mutant retained 10% transport activity, but as found previously (30), the V148M mutation did not produce measureable specific uptake in COS7 cells but retained cell membrane expression (Fig. 3). Two additional mutants in this position (V148F and V148I) were subsequently generated. Whereas the V148F mutant showed no measureable uptake activity and lacked cell-surface expression (Fig. 3), V148I retained 30% transport activity as compared with WT hNET (Table 1) and was included together with F323Y in the mutant library as probe for subsite C in hNET.

Inhibitory Potency of Antidepressants at SERT and NET

Inhibitory Potency of Antidepressants at SERT and NET—To determine the impact on inhibitory potency of selective perturbations in subsite A, B, and C of the S1 pocket in hSERT and hNET (Figs. 2 and 3), we determined the $K_i$ for each of the 15 selected compounds at hSERT and hNET mutants expressed in COS7 cells in an uptake inhibition assay (Fig. 5;
TABLE 1

Uptake kinetics for hSERT and hNET mutants

WT hSERT and hNET were expressed in COS7 cells grown in 96-well plates. \( K_i \) values for transport were determined as described under "Experimental Procedures." Under these conditions, transporter-specific uptake of radiolabeled substrate was linear for WT hSERT, WT hNET, and all mutants (supplemental Figs. S1 and S2). Relative transport activity was determined from paired uptake experiments and is expressed as the percentage of mutant-to-WT uptake for 5 min with 50 nM substrate concentration. Data represent the mean ± S.E.; \( N \) is the number of independent experiments, each performed in triplicate.

### hSERT mutants

| Mutant | \( K_i \) \( \mu M \) | \( N \) | Transport activity | \( \% \) of WT |
|--------|-----------------|------|-------------------|------------|
| hSERT WT | 0.98 ± 0.09 \( b \) | 30 | 66 ± 3 | 102 |
| Y95A | 0.22 ± 0.04 \( b \) | 3 | 47 ± 2 | 108 |
| D98E | 0.22 ± 0.06 \( b \) | 4 | 86 ± 3 | 102 |
| S438T | 4.24 ± 0.12 \( b \) | 3 | 66 ± 3 | 106 |
| F341Y | 0.08 ± 0.02 \( b \) | 3 | 48 ± 3 | 102 |
| S438T | 0.14 ± 0.02 | 3 | 32 ± 2 | 102 |

### hNET mutants

| Mutant | \( K_i \) \( \mu M \) | \( N \) | Transport activity | \( \% \) of WT |
|--------|-----------------|------|-------------------|------------|
| hNET WT | 0.57 ± 0.09 \( b \) | 18 | 50 ± 2 | 48 |
| F72Y | 0.26 ± 0.16 \( b \) | 4 | 29 ± 3 | 24 |
| V148I | 0.35 ± 0.05 \( b \) | 3 | 48 ± 3 | 24 |
| N153S | 1.67 ± 0.20 | 4 | 19 ± 2 | 16 |
| F323Y | 1.35 ± 0.18 | 4 | 10 ± 1 | 13 |
| S419T | 2.52 ± 0.40 | 4 | 11 ± 1 | 14 |

\( a \) Values are from Andersen et al. (17).
\( b \) Values are from Andersen et al. (31).
\( c \) Values are from Andersen et al. (18).

TABLE 1

The impact of the D98E and S438T mutants in subsite A of hSERT and hNET on inhibitor potency was assessed by measuring IC\(_{50}\) values for transport. The IC\(_{50}\) values were determined from concentration-inhibition curves constructed from measurements of \([^3]H\)5-HT (hSERT) or \([^3]H\)DA (hNET) uptake with increasing concentrations of inhibitor (Fig. 5). When larger than symbols, \( K_i \) values were calculated using the Cheng-Prusoff approximation (26) (“Experimental Procedures”). We used the resulting data set to construct a sensitivity profile for each compound toward the tested mutants in hSERT and hNET. Specifically, for each compound and mutant combination, we calculated the fold change in \( K_i \) from paired experiments as \( K_i(\text{mutant})/K_i(\text{WT}) \) or \(-K_i(\text{WT})/K_i(\text{mutant}) \) for mutants decreasing or increasing inhibitor potency, respectively. These values are displayed in Fig. 6 as a heat map.

### Impact of Subsite A Mutations on Inhibitor Potency

We probed the role of subsite A for inhibitor potency by the Y95A, D98E, and S438T mutants in hSERT and F72Y and S419T mutants in hNET (Figs. 2 and 3). In hSERT, the Y95A mutation removes an aromatic ring and a potential hydrogen bonding hydroxyl group from the S1 pocket and likely alters the size and shape of the pocket within region A (22) (Fig. 4). Notably, Y95A promotes 10–100-fold loss of potency for the majority of the tested SSRI and SNRI compounds with exception of sertraline (6-fold loss), fluvoxamine (8-fold loss), and the structurally similar tricyclic antidepressants imipramine (8-fold loss), clomipramine (4-fold loss), and amitriptyline (no significant change) (Fig. 6 and Table 2). Interestingly, the NRIs are the least affected inhibitor class, displaying no significant change in potency except for a 6-fold loss of potency for nisoxetine. Thus, these results verify that Tyr-95 has a key role for high affinity inhibitor binding of SSRIs and SNRIs in hSERT (17, 22, 29, 30).

The lack of transport activity for the equivalent mutant in hNET (F72A) prevents similar analysis in hNET. Alternative perturbation of this residue in the form of the F72Y mutation did not markedly decrease potency of any compound. However, the F72Y mutation has previously been shown to induce a 6-fold gain of potency for \((R,S)\)-citalopram (29), and accordingly we find that F72Y displays a 6- and 18-fold gain of potency for \((S)\)-citalopram and the SNRI milnacipran, respectively (Fig. 6 and Table 3). As F72Y introduces only a hydrogen bond donating hydroxyl group into the S1 pocket, it is unlikely to interact with the inhibitors in hNET. However, the high selectivity gain of potency may suggest that the hydroxyl group enables hydrogen bond interaction to these two inhibitors. This idea is supported by the observation that removal of the hydroxyl group in WT hSERT by the Y95A mutation promotes 114- and 16-fold loss of potency for milnacipran and \((S)\)-citalopram, respectively, indicating that milnacipran and \((S)\)-citalopram bind in close proximity to this residue in both transporters.

The impact of the D98E and S438T mutants in subsite A of the S1 pocket in hSERT for recognition of 5-HT (32), the tricyclic antidepressants imipramine (8-fold loss), clomipramine (4-fold loss), and amitriptyline (no significant change) (Fig. 6 and Table 2). Interestingly, the NRIs are the least affected inhibitor class, displaying no significant change in potency except for a 6-fold loss of potency for nisoxetine. Thus, these results verify that Tyr-95 has a key role for high affinity inhibitor binding of SSRIs and SNRIs in hSERT (17, 22, 29, 30).
Inhibitory Potency of Antidepressants at SERT and NET

**TABLE 2**
Impact of hSERT mutations on the inhibitory potency of the 15 compounds

The compounds are grouped according to their SERT/NET selectivity. **Kₗ** values were determined in a [³H]-5-HT uptake inhibition assay as described under “Experimental Procedures.” Results are presented as the mean ± S.E. from 5–12 independent experiments.

| Inhibitor | WT | Y95A | D98E | S438T | Subsite B | Subsite C |
|-----------|----|------|------|-------|----------|----------|
| SSRIs     |     |      |      |       | **Kₗ**  | **Kₗ**  |
| Fluvoxamine | 167 ± 22 | 1397 ± 165ᵃ | 59 ± 15ᵃ | 36 ± 9ᵃ | 659 ± 131ᵃ | 1418 ± 41ᵃ |
| Fluoxetine | 255 ± 61 | 2830 ± 177ᵃ | 857 ± 113ᵃ | 438 ± 102 | 2238 ± 244ᵃ | 4304 ± 92ᵃ |
| MADAM    | 9 ± 3 | 905 ± 188ᵃ | 12 ± 2 | 67 ± 27ᵃ | 54 ± 16ᵃ | 299 ± 39ᵃ |
| Paroxetine | 24 ± 6 | 1759 ± 306ᵃ | 195 ± 37ᵃ | 2885 ± 559ᵃ | 143 ± 31ᵃ | 61 ± 12ᵃ |
| Sertraline | 242 ± 33 | 1455 ± 331ᵃ | 472 ± 110 | 651 ± 1435ᵃ | 712 ± 146ᵃ | 1918 ± 295ᵃ |
| Venlafaxine | 147 ± 48 | 10862 ± 1668ᵃ | 775 ± 104ᵃ | 88 ± 19 | 1454 ± 208ᵃ | 14740 ± 3182ᵃ |
| (S)-Citalopram | 32 ± 1³ | 408 ± 54ᵃᵇ | 856 ± 117ᵃᵇ | 7693 ± 874ᵃ | 332 ± 25ᵃᵇ | 13946 ± 3167ᵃ |
| SNRIs     |     |      |      |       | **Kₗ**  | **Kₗ**  |
| Amtriptyline | 188 ± 36 | 227 ± 36 | 712 ± 100ᵃ | 2655 ± 425ᵃ | 929 ± 190ᵃ | 1705 ± 215ᵃ |
| Clomipramine | 91 ± 9 | 324 ± 40ᵃ | 158 ± 28 | 641 ± 127ᵃ | 394 ± 27ᵃ | 1519 ± 182ᵃ |
| Duloxetine | 62 ± 4 | 1488 ± 90ᵃ | 161 ± 36 | 28 ± 5ᵃ | 314 ± 39ᵃ | 1541 ± 155ᵃ |
| Imipramine | 163 ± 24 | 1310 ± 151ᵃ | 530 ± 129ᵃ | 1241 ± 106ᵃ | 850 ± 140ᵃ | 3227 ± 617ᵃ |
| Milnacipran | 68 ± 8 | 6989 ± 1452ᵃ | 87 ± 6 | 570 ± 48ᵃ | 917 ± 90ᵃ | 2071 ± 342ᵃ |
| NRIs      |     |      |      |       | **Kₗ**  | **Kₗ**  |
| Atomoxetine | 329 ± 47 | 498 ± 122 | 1335 ± 431ᵃ | 1535 ± 292ᵃ | 1242 ± 185ᵃ | 7694 ± 1269ᵃ |
| Nisoxetine | 977 ± 210 | 4790 ± 645ᵃ | 2915 ± 567ᵃ | 1959 ± 222 | 2662 ± 273ᵃ | 16815 ± 3362ᵃ |
| Maprotiline | 10682 ± 1363 | 8044 ± 1901 | 7258 ± 1334 | 4477 ± 1098ᵃ | 11837 ± 2809 | 10878 ± 1605 |

ᵃ **Kₗ** is significantly different from WT (false discovery rate < 0.05 from a two-sided Wilcoxon test versus WT; see supplemental Fig. S3 for summary of significance levels).

ᵇ Values are from Andersen et al. (17).

**TABLE 3**
Impact of hNET mutations on the inhibitory potency of the 15 compounds

The compounds are grouped according to their SERT/NET selectivity. **Kₗ** values were determined in a [³H]-DA uptake inhibition assay as described under “Experimental Procedures.” Results are presented as the mean ± S.E. from 6–12 independent experiments each performed in triplicate.

| Inhibitor | WT | F72Y | S419T | Subsite B | Subsite C |
|-----------|----|------|-------|----------|----------|
| SSRIs     | **Kₗ** | **Kₗ** |       |          |          |
| Fluvoxamine | 3332 ± 522 | 6212 ± 1217 | 286 ± 43ᵃ | 579 ± 74ᵃ | 4512 ± 632 |
| Fluoxetine | 5813 ± 520 | 8975 ± 831ᵃ | 10769 ± 610ᵃ | 1974 ± 243ᵃ | 8655 ± 1104 |
| MADAM    | 364 ± 65 | 287 ± 44 | 101 ± 13 | 60 ± 13ᵃ | 1541 ± 369ᵃ |
| Paroxetine | 385 ± 89 | 587 ± 100 | 1866 ± 262ᵃ | 378 ± 86 | 641 ± 164 |
| Sertraline | 8556 ± 1384 | 4972 ± 825 | 15200 ± 3192 | 5694 ± 1466 | 4334 ± 866 |
| Venlafaxine | 1830 ± 64 | 1770 ± 141 | 4747 ± 750ᵃ | 1163 ± 78ᵃ | 2044 ± 534 |
| (S)-Citalopram | 23421 ± 2102 | 5649 ± 1356ᵃ | 53842 ± 7845ᵃ | 19357 ± 2737 | 21717 ± 3528 |
| SNRIs     |     |      |      |          |          |
| Amtriptyline | 477 ± 95 | 480 ± 80 | 1593 ± 312ᵃ | 515 ± 58 | 1269 ± 145ᵃ |
| Clomipramine | 420 ± 27 | 462 ± 55 | 747 ± 67ᵃ | 326 ± 58ᵃ | 939 ± 123 |
| Duloxetine | 81 ± 19 | 100 ± 26 | 65 ± 7 | 211 ± 27ᵃ | 256 ± 51ᵃ |
| Imipramine | 130 ± 31 | 230 ± 54 | 558 ± 324 | 316 ± 60ᵃ | 348 ± 60ائه |
| Milnacipran | 52 ± 9 | 5 ± 1ᵃ | 2072 ± 298ᵃ | 118 ± 22 | 76 ± 18 |
| NRIs      |     |      |      |          |          |
| Atomoxetine | 9 ± 2 | 7 ± 1 | 114 ± 18ᵃ | 31 ± 5ᵃ | 21 ± 2³ | 2 ± 1ᵃ |
| Nisoxetine | 5 ± 1 | 2 ± 1ᵃ | 139 ± 58 | 30 ± 5ᵃ | 11 ± 1³ | 2 ± 1³ |
| Maprotiline | 153 ± 34 | 91 ± 27 | 899 ± 223 | 275 ± 77ᵃ | 358 ± 88 | 756 ± 237ᵃ |

ᵃ **Kₗ** is significantly different from WT (false discovery rate < 0.05 from a two-sided Wilcoxon test versus WT; see supplemental Fig. S4 for summary of significance levels).

clic antidepressants imipramine, clomipramine, and amitriptyline (13, 18, 22), and the SSRIs paroxetine and (S)-citalopram (15–18, 28) has been characterized previously. In general agreement with previous results, we find that the D98E mutation has a substantial effect (≥10-fold) on the potency of (S)-citalopram and paroxetine, indicating that Asp-98 is a key contact point for these two inhibitors (Fig. 6 and Table 2). In contrast, D98E does not significantly change the inhibitory potency for the SSRIs MADAM and sertraline, the SNRIs clomipramine, duloxetine, and milnacipran, or the NRI atomoxetine (Table 2 and supplemental Fig. S3). We have previously described the effect of S438T on the potency of the majority of the inhibitors included in the present study (17, 18). Further characterization of fluvoxamine, milnacipran, atomoxetine, and maprotiline at the S438T mutation confirms our previous findings and shows that S438T has a highly selective effect on inhibitor potency (Fig. 6 and Table 2).

In hNET, the S419T mutation (equivalent to S438T in hSERT) also showed highly selective effects across the tested inhibitors by inducing large decreases in potency for paroxetine (11-fold), milnacipran (43-fold), atomoxetine (10-fold), and nisoxetine (22-fold), whereas a 14-fold gain of potency was observed for the SSRI fluvoxamine (Fig. 6 and Table 3). Interestingly, atomoxetine and nisoxetine are markedly more affected by S419T in hNET than the equivalent mutation in hSERT (S438T), suggesting that these NRIs are oriented differently within subsite A of the S1 pocket in hSERT and hNET. In contrast, milnacipran suffered a huge loss of potency at the
Ser-to-Thr mutants in both hSERT and hNET (10- and 43-fold, respectively). Combined with the 114-fold loss of potency for the Y95A hSERT mutant and 18-fold gain of potency for the F72Y mutant in hNET, this suggests that milnacipran occupies and adopts similar binding modes within subsite A of the S1 pocket in hSERT and hNET (Fig. 6).

Impact of Subsite B Mutations on Inhibitor Potency—In subsite B, Asn-177 in hSERT has been suggested to contribute to accommodation of polar ligand moieties (15, 17, 35), and the corresponding residue in dopamine transporter (DAT) (Asn-157) has further been proposed to have an important role for inhibitor recognition in the S1 pocket of DAT (36). To probe for inhibitor interactions in subsite B of the S1 pocket, we therefore employed the N177S mutation in hSERT and the equivalent N153S mutation in hNET, hereby changing the potential hydrogen bonding properties of the side chain (Fig. 4). In hSERT, N177S decreased the inhibitory potency of the majority of the tested SSRIs and SNRIs (5–15-fold) (Fig. 6 and Table 2). These results suggest subsite B in hSERT as a generally important region for accommodation of inhibitors.

In hNET, the N153S mutant did not markedly affect any of the inhibitors tested (Fig. 6 and Table 3). We, therefore, included the G149A mutation, which we recently found to be involved in high affinity binding of the NRI talopram in hNET (37), indicating that Gly-149 likely is contributing directly to shaping the properties of the subsite B region in hNET. The G149A mutation promotes a subtle increase in hydrophobicity and a decrease in free space in subsite B (Fig. 4), thus potentially decreasing the ability of ligands to occupy this region of the S1 pocket in hNET. We found that G149A induced no or less than a 3-fold change in the inhibitory potency for the majority of the compounds except for the NRIs atomoxetine and nisoxetine, which displayed a 4–6-fold decrease in potency, respectively, and the SSRIs fluvoxamine and MADAM, which displayed a 6–8-fold increase in potency, respectively (Fig. 6 and Table 3). Thus, these data suggest that subsite B is important for accommodation of these inhibitors in the hNET S1 pocket. Furthermore, Gly-149 appears to be an important S1 residue for determining SERT/NET selectivity of inhibitors, as we observe that changing the residue to the equivalent hSERT residue (Ala-173) increases potency of SERT-selective compounds and decreases potency for NET-selective compounds.

Impact of Subsite C Mutations on Inhibitor Potency—Previous mutational analyses of SERT and NET have identified the residues Ile-172 and Phe-341 in hSERT and the hNET residue Val-148 (equivalent to Ile-172 in hSERT) as key residues for inhibitor binding within subsite C of the S1 binding pockets (17, 30, 38). In models of hSERT, the side chain of Ile-172 together with the side chain of Phe-341 forms a hydrophobic ridge (Fig. 2) that has been suggested to be a key determinant for the shape of the S1 pocket (13, 17, 30, 34). In models of hSERT, the side chain of Ile-172 together with the side chain of Phe-341 forms a hydrophobic ridge (Fig. 2) that has been suggested to be a key determinant for the shape of the S1 pocket (13, 17, 30, 34). The I172M mutation was identified by Henry et al. (30) as having a marked selectivity in terms of its impact on the affinity of SERT substrates and inhibitors; I172M produces more than a 700-fold loss of affinity for (R,S)-citalopram, whereas high affinity binding of other SSRIs and SNRIs such as paroxetine and amitriptyline is not affected.
Characterization of the 15 inhibitors included in the present study across I172M showed that all compounds except paroxetine and maprotiline suffered a marked decrease in potency (9–127-fold). Eleven of the tested compounds have previously been tested at I172M (30, 34) and the observed -fold changes in potency correspond with the previously reported changes. Although the effects from the I172M mutation may result from a change in the tertiary structure of the S1 pocket rather than disruption of direct inhibitor-protein interactions (34), our results expand the previous findings of Ile-172 and strongly support the role of the side chain of Ile-172 as key for accommodation of structurally diverse inhibitors within the S1 pocket in hSERT.

In hNET, the equivalent mutation (V148M) renders the transporter nonfunctional (30). Instead, we introduced the V148I mutation to increase side chain bulk size in this position of subsite C in hNET (Fig. 4). In contrast to the loss of potency observed for all compounds except paroxetine and maprotiline by the I172M mutation in hSERT, we found no significant loss of potency for any compound by the V148I mutation in hNET except for a 5-fold decrease for the NRI maprotiline (Fig. 6 and Table 3). However, V148I produced marked gain of potency for several compounds (Fig. 6 and Table 3). Specifically, V148I induced a 4–18-fold gain of potency for all tested SSRIs with the exception of paroxetine, hereby identifying Val-148 as a major determinant for SERT/NET selectivity for these compounds as the equivalent residue in hSERT is Ile-172. Gain of potency was also observed for the SNRIs duloxetine (6-fold) and milnacipran (25-fold) and the NRI atomoxetine (6-fold). Taken together, these results suggest a key role of Val-148 in hNET, potentially similar to Ile-172 in hSERT as a major determinant for inhibitor accommodation in the S1 pocket in hNET.

Introduction of a hydroxyl group at Phe-341 (F341Y) in hSERT has been proposed to perturb the shape of the hydrophobic ridge formed by Ile-172 and Phe-341 as well as introducing polarity to the region (Fig. 4), thereby potentially altering the accessibility to the hydrophobic crevice in subsite C of the S1 pocket (14, 15, 17). Notably, we observe very selective effects on compound potency by the F341Y mutation in hSERT and the equivalent mutation in hNET (F323Y). The majority of the tested compounds were not affected by these mutations in hSERT and hNET (Fig. 6; Tables 2 and 3). However, large decreases in potency were found for (S)-citalopram (56-fold) and paroxetine (42-fold) at hSERT and milnacipran (13-fold) at hNET (Fig. 6; Tables 2 and 3), whereas 6 – 8-fold decreases were observed for the SSRI venlafaxine and the SNRIs amitriptyline and milnacipran at hSERT and the NRIs nisoxetine and maprotiline as well as the SNRI amitriptyline at hNET. Interestingly, all of the SNRIs experienced similar decreases in potency at hSERT F341Y and hNET F323Y. This may indicate that these compounds have common orientations in hSERT and hNET relative to subsite C in the S1 pockets. For hSERT, it is only (S)-citalopram among the evaluated SSRIs for which the loss of potency produced by F341Y correlates with the loss of potency observed for the second subsite C mutant, I172M. Generally, this might indicate that subsite C contributes differentially to accommodation of the tested SSRIs in the S1 pocket in hSERT.

In accordance with this idea, we also observe striking differences for hNET between the effects of the V148I and F323Y mutations on the potency of the NRIs atomoxetine and nisoxetine and in particular the SNRI milnacipran, for which V148I produced a 25-fold gain of potency and F323Y produced a 13-fold loss of potency, respectively (Fig. 6).

**Cluster Analysis Reveals Distinct Groups of Compounds with Similar Sensitivity Patterns**—To identify groups of compounds with similar sensitivity patterns toward the mutant panels, we hierarchically clustered the -fold change data from the heat map (Fig. 6). This analysis places compounds that behave similarly close to one another in a dendrogram, and larger clusters of compounds with similar effects can be inferred from this. We did this for all 15 compounds for hSERT and for hNET and also assessed the stability of the inferred clusters using bootstrapping (“Experimental Procedures” and supplemental Fig. S5).

For hSERT, this analysis reveals two distinct clusters with high stability. Cluster I (Fig. 7 and supplemental Fig. S5) contains five compounds, which are the SNRI milnacipran and the SSRIs venlafaxine, MADAM, paroxetine, and (S)-citalopram. This cluster is characterized by high sensitivity (>5-fold loss of potency) to Y95A and N177S and at least two of the S438T, I172M, and F341Y mutations (Fig. 6). Furthermore, this cluster also contains all compounds that are highly sensitive to the D98E mutation (paroxetine, (S)-citalopram, and venlafaxine). Cluster II (Fig. 7 and supplemental Fig. S5) contains the remaining 10 compounds, which have in common that they are markedly affected (=5-fold change in potency) by a maximum four mutations within the S1 pocket in hSERT (Fig. 6). Compounds in cluster II are generally not highly affected by the N177S (except fluoxetine) and F341Y (except amitriptyline) mutations. On the other hand, the majority of cluster II compounds are highly affected by the Y95A and I172M mutations, although to a lesser degree than the cluster I compounds.

Similar analysis of compound sensitivity profiles at the hNET mutant panel reveals only a single cluster with high stability (cluster II; Fig. 7 and supplemental Fig. S5), which contain paroxetine, amitriptyline, imipramine, atomoxetine, and maprotiline. The remaining compounds do not assemble into any cluster with high stability. Compounds in cluster III are not markedly affected by mutations in subsite B of hNET but only by subsite A and C mutations; mainly S419T and F323Y, as all five compounds experience a ≥5 loss of potency for one or both of these mutations (Fig. 6).

Thus, the cluster analysis suggests for hSERT that cluster I inhibitors have interaction points with residues within all three subsites of the S1 pocket of hSERT, whereas inhibitors in cluster II have common contact points within subsites A and C of the S1 pocket in hSERT (Fig. 7). For hNET, the cluster analysis provides little information regarding potential common inhibitor contact points within the S1 pocket of hNET for the five compounds contained in this cluster.

**Discussion**

The LeuT x-ray crystal structures and their validation as templates for comparative modeling of mammalian SLC6 transporters have provided a tool to translate mutational and structure-activity relationship studies into atomic-level transporter models. These can provide insight into the molecular
basis for recognition of inhibitors by the transporters and the mechanism of transport antagonism. Recently, such LeuT-based models of human monoamine transporters have been instrumental for studies of the binding modes and molecular mechanisms of SLC6 transporter inhibitors such as cocaine at DAT (36) and the prototypical antidepressants \((S)-\text{citalopram}\) (15, 17) and imipramine (13, 22) at hSERT and has furthermore helped delineate the molecular details underlying the SERT/NET selectivity of structurally closely related inhibitors (31). Although these studies utilize extensive experimentation to guide ligand docking approaches and to validate the resulting molecular models, they also benefit from a large body of existing data for these inhibitors. A similar level of information is currently not available for the majority of clinically relevant SERT and NET inhibitors. Thus, we here report the characterization of 15 prototypical SERT and NET inhibitors, most of which are in clinical use as antidepressants, at a panel of S1 pocket mutants in hSERT and hNET. This data set, which describes specific molecular determinants within hSERT and hNET for recognition of a range of antidepressants, represents the first systematic mutational analysis of the interactions with the transporters for many of the included inhibitors.

An inherent caveat of the use of site-directed mutagenesis to probe potential ligand-protein interactions in allosteric proteins is that an observed effect on apparent ligand affinity can be caused indirectly by long range perturbations of the ligand binding site or an induced shift in equilibrium between the conformational states that the transporter can assume. However, with the possible exception of Y95A in hSERT, all mutations employed in this study introduce conservative changes that are entirely confined within the S1 pocket and separated from the S2 pocket (Figs. 2 and 3). Therefore, compounds that experience consistent changes in potency produced by two or more of these substitutions seem highly likely to represent localized direct effects on compound accommodation in the S1 pocket and not by long range allosteric effects on a distinct binding pocket.

Understanding variations in the molecular mechanism between the wide range of SERT and NET inhibitors that are currently in use as antidepressants may offer key insight into
the neurophysiological basis underlying differences in their in vivo properties. Recent studies of the closely related DAT suggest that differences in the binding mode and molecular mechanism of action among DAT inhibitors correlate with differences in important aspects of their in vivo properties (36, 39). An important first step toward understanding similar aspects of the molecular pharmacology of antidepressants includes identification of compound binding sites and binding modes of well known inhibitors such as those included in the current study. With this in mind, analysis of the present data set within the framework of previously generated homology models of SERT and NET allows us to derive several notable features regarding SERT and NET inhibition by the tested compounds. First, our data set is useful for assessing probabilities of the S1 pocket as the primary site for high affinity binding of the various compounds. As the S1 pocket harbors the central substrate binding site, classical interpretation of the pharmacologically well established competitive mode of inhibition of most antidepressants (40, 41) is that their binding sites overlap with the substrate binding site within the S1 pocket. However, recent structural and biophysical studies of LeuT have suggested that the S2 pocket contains a functionally important substrate binding site (42–46) that can also be occupied by inhibitors (9–11, 47). Furthermore, studies of the role of the equivalent S2 pocket in mammalian SLC6 transporters, including SERT and NET (9, 10), have identified S2 mutations that can perturb high affinity inhibitor binding, possibly by removing direct ligand interactions with S2 residues or introducing steric hindrance. It is, therefore, important to establish antidepressant binding site locations in hSERT and hNET. Generally, we observe that most of the compounds are significantly affected by mutations in the S1 pocket, suggesting that the primary binding site is located there or partly overlaps into the S1 pocket. Cluster analysis of the obtained patterns of compound sensitivities toward our panel of S1 mutations in hSERT and hNET revealed that inhibitors cluster into multiple separate groups according to their sensitivity profile. In particular, one group (cluster I) contains compounds found to display profound and consistent sensitivity toward mutational perturbations in all three subsites of the S1 pocket of hSERT (Figs. 5 and 6). For compounds with this level of sensitivity, it seems clear that the S1 pocket must harbor their high affinity binding site. (S)-Citalopram has previously been extensively studied by us and others (15, 17), and it was established that (S)-citalopram most likely assumes a binding mode where it is deeply buried within the S1 pocket and occupies all three subsites of the pocket. The other four highly sensitive compounds (paroxetine, venlafaxine, MADAM, and milnacipran) have not yet been subject to similar mutational analysis and modeling studies. However, on the basis of their similar sensitivity profile to (S)-citalopram, we propose that these compounds bind within the S1 pocket. In contrast, we find four compounds (maprotiline, nisoxetine, atomoxetine, and fluvoxamine) that display no or limited sensitivity toward the majority of the S1 mutations in hSERT (Fig. 6), suggesting that these inhibitors bind only partly or not at all in the S1 pocket in hSERT. Alternatively, these compounds can adopt distinctive binding modes compared with the inhibitors displaying high sensitivity toward the six evaluated mutations that allow them to better tolerate modifications at the three evaluated subsites.

We find that fluoxetine, sertraline, and imipramine display medium sensitivity to S1 mutations in hSERT (Fig. 6). Binding modes of these inhibitors have recently been proposed from ligand docking studies supported by biostuctural, mutational, and kinetic analyses (9, 10, 13, 14, 22). On the basis of mutational analysis of S2 residues in hSERT, Zhou et al. (9, 10) have proposed that the binding sites for sertraline, fluoxetine, and imipramine in hSERT are located in the lower part of the S2, similarly to what has been found for LeuT. Recently, Sarket et al. (22) proposed a model for imipramine binding in hSERT, where the binding site overlaps both the S2 and S1 pocket, whereas Sinning et al. (13) suggested a binding mode for imipramine in which the inhibitor molecule binds exclusively within the S1 pocket. It is noteworthy that we find sertraline, fluoxetine, and imipramine to display medium sensitivity toward S1 perturbations in hSERT, suggesting that these compounds do not occupy the entire S1 pocket. This highlights the complexity of SLC6 transporter inhibition that is currently emerging, which is further exemplified by recent findings suggesting that structurally closely related inhibitors might have distinct binding sites on the same transporter protein (31).

Another notable implication of our dataset concerns previous efforts in development of pharmacophore models on the basis of structure-activity relationships for various classes of antidepressants. Although certain mutations seem to be generally important, it is notable that compounds from the same inhibitory class (SSRI, SNRI, and NRI, respectively) are differentially affected by some of the S1 mutations. This suggests that these compounds are differentially oriented and/or have different contact points in the S1 pocket, and efforts to generate a unifying pharmacophore model for these compounds are, therefore, in vain (48, 49).

A negatively charged Asp residue located in subsite A of SERT (Asp-98) has been suggested to be an important contact point in the S1 pocket by forming a direct ionic interaction with the positively charged amino group of 5-HT and SERT inhibitors (13, 15, 18, 28, 32, 33). Here, we find that the D98E mutation only has substantial (≥10-fold) impact on the potency of (S)-citalopram and paroxetine (Fig. 6 and Table 2). This result is notable as all 15 tested inhibitors contain an amino group that in principle can coordinate the acidic side chain of Asp-98. The equivalent mutation in hNET (D75E) renders the transporter nonfunctional (28), but the corresponding D79E mutation in the closely related DAT has previously been found to have only a weak effect on a range of structurally diverse DAT inhibitors (50). Because the negative charge on the carboxylic acid side chain is retained in these Asp-to-Glu mutants (Fig. 4), the ligands might still form an ionic interaction with the mutated residue, which could explain the relatively weak effect from the Glu mutants. This is supported by radioligand binding experiments showing that removal of the negative charge from Asp-79 in DAT (by mutating to Ala) completely abolished high affinity binding of several prototypical DAT inhibitors (51). Mutational studies have further indicated that the negative charge on the conserved Asp is essential for maintaining a functional transporter, as the activity is severely impaired in SERT,
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