Exploring the Inhibitory Mechanism of Approved Selective Norepinephrine Reuptake Inhibitors and Reboxetine Enantiomers by Molecular Dynamics Study

Guoxun Zheng¹, Weiwei Xue¹, Panpan Wang¹, Fengyuan Yang¹, Bo Li², Xiaofeng Li², Yinghong Li², Xiaojun Yao² & Feng Zhu¹

Selective norepinephrine reuptake inhibitors (sNRIs) provide an effective class of approved antipsychotics, whose inhibitory mechanism could facilitate the discovery of privileged scaffolds with enhanced drug efficacy. However, the crystal structure of human norepinephrine transporter (hNET) has not been determined yet and the inhibitory mechanism of sNRIs remains elusive. In this work, multiple computational methods were integrated to explore the inhibitory mechanism of approved sNRIs (atomoxetine, maprotiline, reboxetine and viloxazine), and 3 lines of evidences were provided to verify the calculation results. Consequently, a binding mode defined by interactions between three chemical moieties in sNRIs and eleven residues in hNET was identified as shared by approved sNRIs. In the meantime, binding modes of reboxetine’s enantiomers with hNET were compared. 6 key residues favoring the binding of (S, S)-reboxetine over that of (R, R)-reboxetine were discovered. This is the first study reporting that those 11 residues are the common determinants for the binding of approved sNRIs. The identified binding mode shed light on the inhibitory mechanism of approved sNRIs, which could help identify novel scaffolds with improved drug efficacy.

Norepinephrine reuptake inhibitors (NRIs) are psychostimulant which is commonly used for mood and behavioral disorders. Typical NRIs include the selective norepinephrine reuptake inhibitors (sNRIs), serotonin-norepinephrine reuptake inhibitor and others. Currently, 4 sNRIs (atomoxetine, maprotiline, reboxetine and viloxazine) have been approved and marketed by either the U. S. Food and Drug Administration (FDA) or the European Medicines Agency for treating attention deficit hyperactivity disorder and depression. Amongst these 4 sNRIs, reboxetine is a racemic mixture of (R, R)- and (S, S)- enantiomers. (S, S)-reboxetine showed 130-fold higher affinity to hNET than (R, R)-reboxetine, and was reported as the predominant influence on reboxetine’s steady state pharmacological property. Due to the existing deficiencies of currently marketed sNRIs (their delayed onset of action and non- or partial-response), new strategies were applied to enhance drug efficacy by improving their metabolic and pharmacological properties or by developing dual- and triple-acting antidepressants. The binding mode shared by all approved and marketed sNRIs could contribute to the discovery of drug-like scaffold with enhanced efficacy.

Human norepinephrine transporter (hNET), the drug target of sNRIs, was reported to be closely relevant to various mood and behavioral disorders by facilitating the reuptake of norepinephrine from the synaptic cleft. Current understanding of hNET was based on the X-ray crystal structures of bacterial and invertebrate homologs.

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including the bacterial leucine transporter LeuT\(^{18–21}\), the bacillus neurotransmitter/sodium symporter MhsT\(^{22}\) and the drosophila dopamine transporter (dDAT)\(^{23,24}\). As the most recently determined template, dDAT’s X-ray crystal structure of high resolution revealed the binding of sNRIs (reboxetine and nisoxetine)\(^{23}\) and tricyclic anti-depressant (nortriptyline)\(^{24}\). These co-crystallized structures showed a competitive binding of inhibitors to the S1 binding site by locking hNET in an outward-open conformation\(^{23,24}\). As shown in SI, Fig. S1, dDAT demonstrated the highest sequence identity among those hNET’s homologs, making it a new platform for constructing reliable models of sNRIs’ binding in hNET.

Many mutational and biomedical studies have been conducted to clarify the binding mode of sNRIs with hNET and identify key residues defining their recognition\(^{25–27}\). It was found that residue Asp75 in hNET was crucial for the interaction between sNRIs and hNET\(^{27}\). Moreover, 2 residues (Phe323 and Ser419) were identified as sensitive (with ≥5 fold-change in the loss- or gain-of-potency) to 3 sNRIs (atomoxetine, nisoxetine and maprotiline)\(^{25}\). Based on the X-ray crystal structure of hNET’s bacterial and invertebrate homologs\(^{18,23}\), 7 residues (Phe72, Asp75, Val148, Tyr152, Phe317, Phe323, Ser420) were also suggested as critical for some sNRIs (e.g. reboxetine) by visualizing the interaction distance between ligands and the target\(^{23}\). In the meantime, computational methods have been proposed and frequently used to elaborate the binding mode between sNRIs and hNET with great efficiency and accuracy\(^{28}\). These methods were applied (1) to elucidate binding mechanisms of substrates and inhibitors to monoamine transporter (MAT)\(^{29–36}\) (2) to discover novel scaffolds of MAT inhibitors by virtual screening\(^{37–39}\), and (3) to distinguish various molecular mechanisms of enantiomers binding to MAT\(^{40,41}\).

As one of these powerful computational methods, the molecular dynamics (MD) providing atomic description of protein dynamics and flexibility\(^{42–45}\) was employed to simulate the large scale motions of MAT\(^{27,46,47}\). However, MD simulation has not yet been carried out to explore the binding of sNRIs to hNET. Moreover, the variation on binding modes behind the affinity discrepancy of reboxetine’s enantiomers remains elusive. Thus, there is an urgent need to reveal the mechanism underlying sNRIs’ pharmacodynamics and target recognition\(^{23,25}\).

In this work, multiple computational methods were integrated to explore the inhibitory mechanism of approved sNRIs (atomoxetine, maprotiline, reboxetine and viloxazine). First, a recently reported co-crystal structure of drosophila dopamine transporter (dDAT) in complex with reboxetine\(^{47}\) was used as a template to construct the homology model of hNET. Then, 4 studied sNRIs were docked into hNET for MD simulation, and 3 lines of evidences were provided to verify the simulation results. Consequently, a binding mode shared by approved sNRIs was discovered by clustering the binding free energies of residues. Moreover, the binding modes of 2 reboxetine enantiomers with hNET were compared, and residues favoring the binding of (S, S)-reboxetine over that of (R, R)-reboxetine were discovered. The identified binding mode shed light on the inhibitory mechanism of approved sNRIs, which could help identify novel scaffolds with improved drug efficacy\(^{13}\).

**Materials and Methods**

**Homology Modeling.** The homology model of hNET was constructed by using the automated mode in SWISS-MODEL\(^{48}\), based on a recently determined dDAT’s X-ray crystal structure\(^{49}\) of 3.0 Å resolution (PDB entry: 4XNX from Arg25 to Pro596). As shown in SI, Fig. S1, dDAT\(^{50}\) demonstrated 61% sequence identity to hNET, which was much higher than that (23%) of LeuT\(^{18}\). The sequence coverage of the constructed hNET model was between Arg56 and Pro594 covering hNET’s all transmembrane (TM) regions and corresponding extracellular loops. To validate the constructed homology model, the Ramachandran plot in PROCHECK\(^{50}\) was further exploited. Finally, two functional Na\(^+\) in dDAT (PDB entry: 4XNX\(^{23}\)) were added to their interacting sites in hNET by structural superimposition via PyMOL\(^{50}\).
Molecular Docking. Initial binding conformations of sNRIs in hNET were obtained by molecular docking using Glide<sup>31</sup> of standard precision. The docking grid box was defined by centering (R, R)-reboxetine (PDB entry: 4XNX<sup>23</sup>) in the modeled hNET using the Receptor Grid Generation tool in Glide. Docking poses of sNRIs, with the most similar conformations or orientations as (R, R)-reboxetine in dDAT<sup>31</sup>, were chosen for MD simulation. Furthermore, a cross-docking approach was applied in this work to validate the docking method. Detailed supporting information was provided in SI, Methods.

System Setup of the Protein-Ligand and Membrane. The Membrane Builder in CHARMM-GUI<sup>53</sup> was used to embed the sNRIs-hNET complexes into the explicit POPC lipid bilayer. TIP3P water<sup>32</sup> was then positioned above and below the constructed bilayer (20 Å thickness). Na<sup>+</sup> and Cl<sup>−</sup> were added to keep the environmental salt concentration at 0.15 M. As a result, each periodic cell of the entire system (83 Å × 83 Å × 127 Å) contained ~96,400 atoms. Detailed supporting information was provided in SI, Methods.

MD Simulation. AMBER14<sup>43</sup> using GPU-accelerated PMEMD was applied to carry out MD simulation. The force field ff14SB<sup>54</sup> was used for protein, and Lipid14<sup>55</sup> were utilized for lipid. TIP3P water’s ions parameters were directly adopted from previous publication<sup>56</sup>. sNRIs’ parameters were generated using the General AMBER force field<sup>57</sup> and the charges of sNRIs’ atoms were derived by the Restricted Electrostatic Potential partial charges<sup>58</sup> in Antechamber<sup>49</sup>. Gaussian09<sup>60</sup> was applied to optimize the geometry and calculate the electrostatic potential at the HF/6–31G* level. In all simulations, a sequential process was executed (minimization, heating and equilibration). After this, 150 ns production MD simulation at 310 K and 1 atm was conducted in NPT ensemble with the periodic boundary conditions. Meanwhile, the long-range electrostatic interactions (cutoff = 10.0 Å) was treated using Particle-Mesh Ewald method<sup>61</sup>. The SHAKE algorithm<sup>62</sup> was applied to constrain the bond lengths involving bond to hydrogen atoms, and in simulation the integration time step was set as 2 fs. Supporting data were provided in the SI, Methods.

Calculation of the Binding Free Energies. The energies of each sNRi binding to hNET (\(\Delta G_{\text{MM/GBSA}}\)) without the entropic effect were analyzed by the MM/GBSA approach with single-trajectory<sup>63,64</sup>. The last 50 ns of simulation (500 snapshots) was used for binding free energy calculation. For each snapshot, the energy of sNRi binding to hNET was computed by:

\[
\Delta G_{\text{MM/GBSA}} = \Delta F_{\text{vdW}} + \Delta F_{\text{ele}} + \Delta G_{\text{pol}} + \Delta G_{\text{nonpol}}
\]

In Eq. (1), \(\Delta F_{\text{vdW}}\) indicates the van der Waals energy, and \(\Delta F_{\text{ele}}\) denotes the electrostatic energy. \(\Delta G_{\text{pol}}\) is the polar solvent interaction energy calculated by solving the GB equation. \(\Delta G_{\text{nonpol}}\) is the non-polar solvation contribution and estimated as 0.0072 × \(\Delta \text{SASA}\) using the LCPO method<sup>65</sup>, where the \(\Delta \text{SASA}\) denotes the solvent accessible area. Supporting data were provided in the SI, Methods.

Analysis of the Per-residue MM/GBSA Free Energy Decomposition. In order to analyze the per-residue contribution to sNRIs’ binding to hNET, energy was calculated by the approach of MM/GBSA decomposition using mmpbsa.pl plugin in AMBER14<sup>43</sup>. The per-residue energy (\(\Delta G_{\text{per-residue}}\)) without entropic effect could be computed by:

\[
\Delta G_{\text{MM/GBSA}} = \Delta F_{\text{vdW}} + \Delta F_{\text{ele}} + \Delta F_{\text{pol}} + \Delta F_{\text{nonpol}}
\]

Most of the terms in Eq. (2) are defined in the same way as that in Eq. (1), but the energy of the non-polar solvent interaction (\(\Delta F_{\text{nonpol}}\)) was computed recursively approximating a sphere around an atom from an icosahedron<sup>66</sup>. Supporting data were provided in the SI, Methods.

Hierarchical Clustering of Residues Based on Their Energy Contributions. Energy contributions of certain residue to 4 approved sNRIs (atomoxetine, maprotiline, reboxetine and viloxazine) rendered a 4 dimensional vector. The clustering tree of residues contributing to at least one studied sNRI (atomoxetine, maprotiline, reboxetine and viloxazine) was depicted by the latest version of iTOL<sup>68</sup>. The residues favoring sNRI’s binding are colored in red (the highest one was colored as red and the lower contribution one was set to fade gradually to white). In the meantime, the residues hampering sNRIs’ binding are displayed in blue (the highest one was colored as blue and the lower one was set to fade gradually to white). The white color here denotes residue with no contribution to sNRIs’ binding.

Results and Discussion

Construction of the sNRIs-hNET Complexes. The homology model of the hNET was built on a 3.0 Å high-resolution crystal structure of the dDAT (PDB entry: 4XNX<sup>23</sup>). The sequence identity between the modeled fraction of hNET and dDAT was 61% (SI, Fig. S1). As shown in SI, Fig. S2, the modeled hNET contained the
### Table 1. Comparison of binding free energies between the calculated results and the experimental data of 6 sNRIs of this study binding to hNET. **ΔG** is in kcal/mol and Kᵢ value is in nM.  

| sNRIs studied | Kᵢ' | ΔΔG<sub>exp</sub> | ΔΔG<sub>Gexp</sub> | ΔΔG<sub>GMM/GBSA</sub> | ΔΔG<sub>Gcalc</sub> |
|---------------|-----|-----------------|-----------------|-----------------|-----------------|
| Atomoxetine   | 5.00 | 41.42 | -3.94 | -3.94 | -3.94 |
| Maprotiline   | 7.00 | 40.21 | -2.73 | -2.73 | -2.73 |
| Nisoxetine    | 1.60 | 46.05 | -8.57 | -8.57 | -8.57 |
| Talopram      | 2.90 | 42.47 | -4.99 | -4.99 | -4.99 |
| (S, S)-reboxetine | 0.08 | 47.67 | -10.19 | -10.19 | -10.19 |
| Viloxazine    | 73.00 | 37.48 | 0.00 | 0.00 | 0.00 |

The dynamic stabilities of the 6 systems (atomoxetine, maprotiline, nisoxetine, reboxetine, talopram and viloxazine) along MD simulation were measured by their average distance from the initial structures in terms of RMSD. The RMSD values of protein backbone atoms, ligand heavy atoms and binding site residues (around 5 Å of ligand) in the entire MD simulation trajectories were illustrated in SI, Fig. S6. All 6 simulated systems reached equilibration state after 100 ns with only little fluctuation in the monitored RMSD.

**Analysis of the Binding Free Energy.** The MM/GBSA calculations were performed to quantitatively analyze the binding free energies of hNET in complex with sNRIs (Table 1). The predicted binding free energy (ΔGD<sub>GMM/GBSA</sub>) for hNET with atomoxetine, maprotiline, nisoxetine, talopram, viloxazine and (S, S)-reboxetine were -41.42, -40.21, -46.05, -42.47, -37.48 and -47.67 kcal/mol, respectively. Meanwhile, ΔGD<sub>exp</sub> was used to convert the experimental Kᵢ values<sup>27,69–71</sup> to the binding free energies (ΔG<sub>exp</sub>). As shown in Table 1, binding free energies for each system were overestimated compared to the experimental results. If one is only interested in the relative order of binding affinities of structurally similar ligands of resemble binding modes, the entropy effects could be omitted<sup>22</sup>. Therefore, the energy differences (Table 1) calculated (ΔΔGD<sub>GMM/GBSA</sub>) and estimated by experimental data (ΔΔG<sub>exp</sub>) among 4 sNRIs could tell whether these overestimations were originated from the omission of entropy effects. ΔGD<sub>GMM/GBSA</sub> was shown in Fig. 2 to correlate well with ΔGD<sub>exp</sub> (R² = 0.9008). In spite of an overestimation of ΔGD<sub>GMM/GBSA</sub> comparing to the ΔGD<sub>exp</sub><sup>7,27,69–71</sup>, the ascending trend of ΔGD<sub>exp</sub> was reproduced well by ΔGD<sub>GMM/GBSA</sub>. The overestimated energy in our study was in agreement with the reported over-estimation using the approach of MM/GBSA<sup>7,27,69–71</sup>. Each component of energy in Eq. (1) was illustrated in SI, Table S1. In particular, ΔE<sub>pol</sub> and ΔE<sub>disp</sub> mainly contributed to the binding of sNRIs with hNET, while the polar solvent energy (ΔG<sub>pol</sub>) impeded the binding.

**Verifying the Resulting Models of MD Simulation.** Besides the good correlation between the results of simulation and experiments in previous section, 3 lines of evidence further verified our resulting model of MD simulation. The first line of evidence was from the recently identified key residues that control sNRIs’ selectivity in hNET<sup>76</sup>. As reported, a mutational analysis of 6 diverging residues (Ala145, Tyr151, Ile315, Phe316, Ser420 and Ala426) in the central binding site (S1 site) of hNET to the complementary residues in the human dopamine transporter (hDAT) transferred a hDAT-like pharmacology to hNET, showing that those 6 residues were collectively key residues for sNRIs’ selectivity<sup>96</sup>. To investigate the influence on the binding of 6 studied sNRIs to the hDAT-like hNET<sup>76</sup>, in silico mutations on these 6 residues in hNET to the identity of the corresponding residues in hDAT (A145S-Y151F-I315V-F316C-S420A-A426S) were conducted in this work. As illustrated in SI, Fig. S7, 6 studied sNRIs (atomoxetine, maprotiline, nisoxetine, talopram, viloxazine and (S, S)-reboxetine) in complex with the hDAT-like hNET were analyzed by adding 20 ns simulation to the models of the wild type hNET constructed in this work. The resulting binding free energy of 6 sNRIs was calculated, and the fold-changes in their binding affinity induced by hDAT-like hNET mutations from Andersen’s experimental study<sup>76</sup> were listed in Table 2 (detail information of each energy term can also be found in SI, Table S3). As shown, the fold-changes in binding affinities of 4 sNRIs (atomoxetine, nisoxetine, reboxetine and talopram) reported in Andersen’s study<sup>76</sup> were reproduced well by our calculated ΔΔGD<sub>GMM/GBSA</sub> (ΔΔG<sub>calc</sub>). In particular, the ΔΔG<sub>calc</sub> were 0.47, 1.15, 2.89 and 3.70 kcal/mol.
for reboxetine, atomoxetine, nisoxetine and talopram, respectively, which were comparable and followed the same trends as the reported increases in binding free energies (ΔΔGexp) induced by hDAT-like hNET mutations (1.38, 1.97, 3.04 and 3.16 kcal/mol for reboxetine, atomoxetine, nisoxetine and talopram)76. Since those additional 20 ns simulations were all based on the resulting hNET models constructed in this work, this reproduction of experiments by simulation could act as one line of evidence for verifying our resulting simulation models. In the meantime, the binding free energies of 2 sNRIs (maprotiline and viloxazine) not included in Andersen’s study76 were also calculated and listed in Table 2 and SI, Table S3. The bindings of both sNRIs were affected significantly by hDAT-like hNET mutations. These results supported Andersen’s findings that 6 residues in the S1 site of hNET controlled sNRIs’ selectivity76. SI, Fig. S8 illustrated changes in the conformation and orientation of both hNET’s binding site and those 6 studied sNRIs within it.

The second line of evidence, coming from the reported mutagenesis experiments on the sensitivity profiles of hNET’s residues, indicated that our resulting models of MD simulation were capable of distinguishing sensitive residues from non-sensitive ones. As reported, the sensitivity profiles could shed light on the binding mode of sNRIs25. The residues’ sensitivity to the binding of sNRIs could be estimated by the variation in the binding free energy before and after the in silico mutation. In this work, 2 sensitive mutations (S419T and F323Y with ≥5-fold changes in binding affinity) and 2 non-sensitive mutations (F72Y and N153S with markedly change in potency) of 2 sNRIs (atomoxetine and maprotiline) identified by previous experiments25 were selected, and their sensitivities were explored by in silico mutation study. Particularly, hNET of single-point mutations (S419T, F323Y, F72Y and N153S) in complex with those 2 sNRIs were analyzed by additional 20 ns simulation based on the models of the wild type hNET constructed in this work (SI, Fig. S9). The calculated binding free energies of 2 sNRIs and the fold-changes in their binding affinity from Sorensen’s experiments25 induced by those single-point mutations were shown in Table 3, and information of each energy term was listed in SI, Table S4. As shown, the sensitivity profiles of 4 mutations reported in Sorensen’s work25 were successfully discovered by the calculated ΔΔGcalc in this work. Particularly, our simulation discovered F72Y and N153S as non-sensitive mutations to both sNRIs.

![Figure 2. Correlation between energy difference of 6 studied sNRIs calculated in this work (ΔΔGcalc) and that estimated based on experiments (ΔΔGexp).](image_url)
≥13, 10, 11, 10 and 12 residues were recognized as high contribution ones (energy decomposition could help identify key residues in the binding of sNRIs to hNET. As shown in Fig. 3, 12, 8, 5 and 2 residues were identified as good binding residues comparing to their corresponding docking poses (SI, Table S4). This could be the third line of evidence verifying our resulting simulation models.

Analysis of the sNRIs’ Binding Mode in hNET. The representative structures of 6 sNRIs extracted from the equilibrated simulation trajectory slightly shifted in conformation comparing to their corresponding docking poses (SI, Fig. S11), and key interactions between the ligands and Asp75 of hNET were retained. Moreover, per-residue free energy decomposition could help identify key residues in the binding of sNRIs to hNET. As shown in Fig. 3, 12, 13, 10, 11, 10 and 12 residues were recognized as high contribution ones (≥0.5 kcal/mol) for the binding of atomoxetine, maprotiline, nisoxetine, talopram, viloxazine and (S, S)-reboxetine. The first number in the bracket indicated the minimum fold-changes, while the second one indicated the maximum fold-changes. Numbers out of the bracket indicated the fold-changes derived from the experimental values of both K<sub>i</sub> wild type and K<sub>i</sub> mutation. The first number indicated the minimum fold-changes, while the second one indicated the maximum fold-changes.

Table 3. The calculated and experimental changes in binding free energies of 8 sNRIs-hNET complexes (2 sNRIs against 4 single-point mutations) before and after those mutations in hNET<sup>25</sup> (ΔG is in kcal/mol).

| sNRIs studied | Single point mutations in hNET | Calculated value | Experimental value<sup>25</sup> |
|---------------|-------------------------------|------------------|---------------------------------|
|               | ΔΔG<sub>cal</sub><sup>a</sup> | Fold-change of potency<sup>b</sup> | Fold-change of potency<sup>c</sup> | ΔΔG<sub>exp</sub><sup>d</sup> |
| Atomoxetine   | F72Y                          | −0.03            | 0.95                            | 0.78 (0.55–1.14) | −0.15 (−0.35–0.08) |
|               | N153S                         | −0.31            | 0.59                            | 2.33 (1.73–3.29) | 0.50 (0.33–0.71) |
|               | F323Y                         | 1.93             | 25.95                           | 3.89 (2.45–6.14) | 0.81 (0.53–1.08) |
|               | S419T                         | 1.27             | 8.52                            | 12.67 (8.73–18.86) | 1.51 (1.28–1.74) |
| Maprotiline    | F72Y                          | −0.31            | 0.59                            | 0.59 (0.34–0.99) | −0.31 (−0.64–−0.01) |
|               | N153S                         | 0.05             | 1.09                            | 2.34 (1.44–3.75) | 0.50 (0.22–0.78) |
|               | F323Y                         | 0.96             | 5.05                            | 4.97 (3.21–7.75) | 0.95 (0.69–1.21) |
|               | S419T                         | 1.36             | 9.92                            | 5.88 (3.61–9.43) | 1.05 (0.76–1.33) |

sNRIs. In Table 3, the ΔΔG<sub>cal</sub> were between −0.31 and 0.05 kcal/mol. The corresponding range of fold-changes in potency (FC potency) could thus be estimated as from 0.59 to 1.09 by the equation ΔΔG<sub>cal</sub> = RT ln (FC potency), which were comparable to the experimentally estimated non-sensitive FC potency (from 0.59 to 2.34)<sup>25</sup>. Meanwhile, S419T and F323Y were identified as sensitive to both sNRIs. Their ΔΔG<sub>cal</sub> were between 0.96 and 1.93 kcal/mol. The corresponding range of FC potency were estimated as from 5.05 to 25.95, which were also comparable to those experimentally estimated sensitive FC potency (from 3.89 to 12.67)<sup>25</sup>. The distinct difference in FC potency between sensitive and non-sensitive mutations indicated that our resulting models were capable of distinguishing the sensitive mutations (S419T and F323Y) from the non-sensitive ones (F72Y and N153S). As those <i>in silico</i> mutational studies were based on models constructed in this work, their ability to identify the sensitivity profiles of hNET’s residues could be considered as another line of evidence to verify our resulting models. SI, Fig. S10 illustrated the changes in the conformation and orientation of both hNET’s binding site and 2 sNRIs within it.

The above evidence was supported further by the third line of evidence from the crystallography study, which reported co-crystallized structures of ligands (nisoxetine and reboxetine) with dDAT<sup>23</sup>. Based on their structures, these 2 sNRIs appeared to have very similar modes of binding and action. Of these 2 complexes, the amino groups of both ligands interact with the residues Phe43 and Asp46 (the corresponding residues Phe72 and Asp75 in hNET)<sup>23</sup>. Moreover, the cavity formed by residues Phe43, Ala44, Phe319 and Ser320 (the corresponding residues Phe72, Ala73, Phe317 and Ser318 in hNET) was occupied by the amine group of nisoxetine and the morpholine nitrogen of reboxetine<sup>31</sup>. In addition, two aromatic rings of both sNRIs were involved in the hydrophobic clef bordered by residues Val120, Tyr123, Tyr124, Phe319, Phe325 and Ser422 (the corresponding residues Val148, Tyr151, Tyr152, Phe317, Phe323 and Ser420 in hNET), which further stabilized both sNRIs in the S1 binding site<sup>33</sup>. In this study, all of those residues contributed significantly (≥0.5 kcal/mol) to the binding of sNRIs (illustrated in Fig. 3), which could be the third line of evidence verifying our resulting simulation models.

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Identification of the Shared Binding Mode of Approved sNRIs. Among those 538 residues in hNET, 238 were with energy contribution to at least 1 approved sNRI. To characterize the most favorable binding mode shared by approved sNRIs, hierarchical clustering with ward algorithm<sup>6</sup> was exploited to identify hot spots from those 238 residues based on their energies. In Fig. 4, 4 groups of residues (A, B, C and D) were discovered. The residues favoring sNRI’s binding were colored in red. The residue with the highest contribution (~3.91 kcal/mol) was colored as standard red. The color of the lower contribution one was set to fade gradually towards white (no contribution). In the meantime, the residues hampering sNRI’s binding were displayed in blue. The highest one was colored as standard blue (0.20 kcal/mol) and the color of the lower one was set to fade gradually towards
white. Importantly, it should be noticed that the highest energy contribution of residue favoring sNRIs' binding is much larger (about 19 times) than that hampering the binding.

As shown in Fig. 4, energy contribution of group A (Phe72, Asp75, Ala145, Val148, Gly149, Tyr152, Phe317, Phe323, Ser419, Ser420, Gly423) were consistently higher across all approved sNRIs than that of group B, C and D, indicating a crucial role played by group A in sNRIs' binding. In particular, the sum of group A's energy contributions accounted for the major part of the total energy (77.80% for atomoxetine, 78.06% for maprotiline, 78.95% for (S, S)-reboxetine and 75.27% for viloxazine). Those 11 residues revealed a similar pattern in drug binding in spite of their distant chemical scaffolds, and therefore were identified as hot spots for sNRIs' binding.

Moreover, residues in subgroup A1 (Phe72, Asp75, Val148, Tyr152, Phe317 and Phe323) contributed much higher to sNRIs' binding than those in subgroup A2 (Ala145, Gly149, Ser419, Ser420 and Gly423). The sum of subgroup A1's energy contributions constituted 60.15%, 58.87%, 53.54% and 50.27% of the total energies for atomoxetine, maprotiline (S, S)-reboxetine and viloxazine, respectively.

As shown in Fig. 5, the amino group of all 4 sNRIs pointed to the residue Asp75, and the rest of hot spot residues formed the hydrophobic part of the binding pockets. To measure the conformational shift among the binding pockets of 4 sNRIs, the RMSDs of the 11 hot spot residues were calculated. The resulting RMSDs of different sNRIs' binding pockets were all <3.0 Å with the highest one of 2.8 Å and the lowest one of 1.7 Å. In addition, in

Figure 3. Per-residue binding free energy decomposition of 6 studied sNRIs-hNET complexes. Residues with high energy contribution (the absolute energy contribution ≥0.5 kcal/mol) were labeled.
order to compare the orientations of these sNRIs, the superimposition of all sNRIs were shown in SI, Fig. S12, which gave a resembled orientation among them. Thus, the generalized binding mode of sNRIs with hNET was schematically displayed in Fig. 6. As illustrated, the binding mode was represented by the interactions of salt bridge, hydrogen bond and hydrophobic contact between three chemical moieties and eleven hot spot residues (Phe72, Asp75, Ala145, Val148, Gly149, Tyr152, Phe317, Phe323, Ser419, Ser420, Gly423). In Fig. 6, these three chemical moieties were illustrated by the color of red (R1), light blue (R2) and blue (R3). Residues with strong (subgroup A1) energies were marked in black, and residues with relatively strong (subgroup A2) energies were shown in gray (Figs 5 and 6). Particularly, the moiety R1 mainly engaged in the formation of salt bridge interaction and the hydrogen bond with Asp75 and Phe72 or Phe317; R2 formed hydrophobic interactions with Val148, Gly149, Tyr152 and Phe323 and also contacted with Phe317; R3 contacted hydrophobically with Ala145, Ser419, Ser420 and Gly423.

Among those 11 identified hot spot residues, 5 (Phe72, Val148, Gly149, Phe323 and Ser419) were studied in Sorensen’s work. By measuring the changes on the inhibitory potencies before and after the mutation, Sorensen et al. found 2 sensitive mutations (S419T and F323Y, ≥5-fold changes for atomoxetine and maprotiline) and 2 mutations (F72Y and N153S) without markedly decrease in potency of both sNRIs. Moreover, recently determined co-crystallized structures of sNRIs in dDAT (a homologous structure of hNET) could shed light on their binding mechanism. However, besides (R, R)-reboxetine and nisoxetine, no structure of any sNRIs complexed with hNET or its homologous structure was reported. According to (R, R)-reboxetine’s co-crystallized structure, 7 residues (Phe72, Asp75, Val148, Tyr152, Phe317, Phe323, Ser420) out of those 11 hot spots were suggested as

Figure 4. Tree of 238 residues with contribution to at least one studied sNRI in binding hNET by hierarchically clustering their energies. The residues favoring sNRI’s binding are colored in red (the one with the highest contribution was colored as standard red and the lower contribution one was set to fade gradually to white). In the meantime, the residues hampering sNRI’s binding are displayed in blue (the highest one was colored as standard blue and the lower one was set to fade gradually to white). The white color here denotes residue with no contribution to sNRIs’ binding.
critical residues by visualizing interaction distances between ligand and dDAT. In addition, crystal structures of LeuBAT (engineered LeuT) in complex with 4 selective serotonin reuptake inhibitors (SSRIs), 2 serotonin–noradrenaline reuptake inhibitors (SNRIs) and 1 tricyclic antidepressant (TCA) were determined and reported to harbor a human monoamine transporter-like pharmacology. Analysis of these SSRIs, SNRIs and TCA ligands complexed with LeuBAT helped to identify the binding pocket defined by Tyr21, Asp24, Val104, Ala105, Tyr108, Phe253, Gly256, Phe259, Ser355, Gly359, Asp404 and Thr408 (corresponding residues in hNET were Phe72, Asp75, Val148, Gly149, Tyr151, Phe317, Gly320, Phe323, Ser419, Gly423, Asp473 and Ala477). Among these corresponding residues, 8 (Phe72, Asp75, Val148, Gly149, Phe317, Phe323, Ser419, Gly423) were overlapped with those 11 hot spots identified by this study. Overall, these 11 residues were reported for the first time as the common determinants for the binding of all approved sNRIs.

Further analysis on energy contributions of sNRIs’ different chemical moieties reveals a vital role of chemical moiety R1 in sNRIs-hNET recognition. R1 forms salt bridge with Asp75 and hydrogen bonds with Asp75, Phe72 and Phe317, which in total consists of 34.29%, 23.04%, 28.04% and 22.70% of binding free energies for atomoxetine, maprotiline, (S, S)-reboxetine and viloxazine, respectively. To further understand these interactions anchoring different sNRIs into the binding site, salt bridge and hydrogen bond were monitored along the entire MD simulation. Detail information can be found in SI, Results and Discussion.

Two Reboxetine Enantiomers Distinguished by Their Binding Modes. (S, S)-reboxetine showed 130-fold higher affinity to hNET than its (R, R) enantiomer, and was reported as the predominant influence on reboxetine’s steady state pharmacological properties. However, the variation on binding modes of 2 enantiomers with hNET remains elusive. In this work, a collective computational method was applied to identify binding modes of 2 enantiomers and distinguish their conformational variations in hNET. As shown in SI, Fig. S13, interactions of salt bridge, hydrogen bond and hydrophobic contact between sNRIs and 11 hot spot residues were essential for both enantiomers in hNET’s recognition, which could be further analyzed by the energy decomposition of those 2 enantiomers (SI, Fig. S14).

However, the calculated binding free energies of (R, R)-reboxetine and (S, S)-reboxetine were −34.69 and −47.67 kcal/mol, respectively, which were consistent with the experimental results. To understand the difference
in binding affinities of 2 enantiomers, per-residue energy contribution analysis was performed to find out residues with significant variation in energy contribution (absolute variation ≥0.5 kcal/mol) between 2 enantiomers. In this work, 6 residues (Asp75, Val148, Tyr152, Ser419, Ser420 and Gly423) favoring the binding of (S, S)-reboxetine over (R, R)-reboxetine were identified. For the residue Asp75, the average interaction distance between the polar nitrogen of (S, S)-reboxetine and the carboxyloxygen of Asp75 was 2.8 Å, while the distance
was 4.2 Å for (R, R)-reboxetine (Fig. 7). A shorter interaction distance for (S, S)-reboxetine guaranteed a much stronger salt bridge and hydrogen bond interaction than (R, R)-reboxetine. Meanwhile, (S, S)-reboxetine formed strong hydrophobic interactions with other 5 residues (Val148, Tyr152, Ser420, Gly423 and Met424). Comparing to (S, S)-reboxetine’s binding, (R, R)-reboxetine and those 5 residues suffered from obvious conformation shifts (Fig. 8), which significantly increased the hydrophobic interaction distance between the ligand and residues.

**Conclusion**

In this study, multiple computational methods were integrated to explore the inhibitory mechanism of approved sNRIs (atomoxetine, maprotiline, reboxetine and viloxazine). First, a recently reported co-crystal structure of *drosophila* dopamine transporter (dDAT) in complex with reboxetine was utilized to construct the homology model of hNET. Then, those studied sNRIs were docked into hNET for MD simulation. 3 lines of evidences were further provided to verify the simulation results. Consequently, a binding mode shared by approved sNRIs was discovered by clustering the binding free energies of residues. Eleven residues (Phe72, Asp75, Ala145, Val148, Gly149, Tyr152, Phe317, Phe323, Ser419, Ser420, Gly423) in hNET were identified as crucial for revealing the binding mode of sNRIs-hNET complex. Moreover, the binding modes of reboxetine’s enantiomers with hNET were compared, and 6 residues (Asp75, Val148, Tyr152, Ser420, Gly423 and Met424) favoring the binding of (S, S)-reboxetine over that of (R, R)-reboxetine were identified. This is the first study reporting that those 11 residues are the common determinants for the binding of approved sNRIs. The identified binding mode shed light on the binding mechanism of approved sNRIs, and might therefore help identify novel scaffolds with improved drug efficacy.

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Author Contributions

W.X. and F.Z. designed research. G.Z., W.X. and F.Z. performed research. G.Z., W.X., P.W., F.Y., B.L., X.L., Y.L., X.Y. and F.Z. analyzed the data. G.Z., W.X. and F.Z. wrote the manuscript. All authors reviewed the manuscript.

Additional Information

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