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Introduction

Major depression disorder (MDD) is a serious mental health condition with the etiopathogenesis of social, psychological, biological and genetic factors.^{1–8} MDD patients demonstrate an outsized risk of suicidal behavior,^{3–6,9} seriously threatening human health. Currently, common antidepressants, such as serotonin–norepinephrine reuptake inhibitors (SNRIs), selective serotonin reuptake inhibitors (SSRIs), triple reuptake inhibitors (TRIs) and so on, could alleviate the depression mainly *via* interacting with targets hSERT, hNET or hDAT in a single, dual or triple fashion.^{10–13} However, drugs of these kinds often have serious side-effects and delayed functions, and can take weeks to manifest their full-blown antidepressant effects.^{14–17} In addition, over 30% of the patients are resistant to such available treatments,

The binding mode of vilazodone in the human serotonin transporter elucidated by ligand docking and molecular dynamics simulations[†]

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Vilazodone is a novel antidepressant used for the treatment of major depressive disorder (MDD) with a primary action mechanism of inhibiting the human serotonin reuptake transporter (hSERT) and acting as a 5-HT_{1A} receptor partial agonist. The interaction between vilazodone and the 5-HT_{1A} receptor has been reported, however, the binding mode of vilazodone in the hSERT remains elusive. In the current study, to elucidate the molecular mechanism of vilazodone binding in the hSERT, the drug and its five analogs were docked into the hSERT crystal structure as initial conformations and were sampled by 400 ns molecular dynamics (MD) simulations. Through the analysis of the profiles of protein–ligand binding free energies, interaction fingerprints, and conformational rearrangements, the binding mode of vilazodone in the hSERT, vilazodone adopted a linear pose in the binding pocket. Its arylpiperazine fragment occupies the central site (S1) and interacts with Y95, D98, I172, Y176, F335, F341, S438, and T439, while the indole fragment extends to the allosteric site (S2) *via* interacting with the ionic switch (R104/E403) between the two sites. The new insights obtained are not only helpful in understanding the binding mode of vilazodone in the hSERT, but also provide valuable guidance to the discovery of novel antidepressant drugs.

making the discovery of novel and effective antidepressants much more necessary.^{18,19} In 2013, vilazodone with a different scaffold (a bulky molecular backbone consisting of an arylpiperazine fragment and an indole fragment) than the common antidepressants^{20–23} has been approved by the FDA due to its good efficacy,^{10,24–26} high tolerance,^{14,15,25} and few side effects,^{16,27–30} enriching the diversity of drug skeletons for MDD.^{17,31–39} In addition, clinical studies further revealed the limited adverse drug effects on sexual function and bodyweight, prompting it to be further investigated in the treatment of generalized anxiety disorder (GAD).^{15,40–46}

Vilazodone exerted the pharmacological functions through a novel mechanism, not only inhibiting hSERT activity, but also partially agonizing the 5-HT_{1A} receptor.^{15,47–49} Currently, the binding mode between vilazodone and the 5-HT_{1A} receptor has provided insights into the rapid onset of the drug's action.^{49–52} However, the binding mode or conformation of vilazodone in the hSERT is still elusive and no report including computational and experimental research has explained or unveiled this problem,^{16,53} which makes it essential to reveal such a mechanism for facilitating the design of a new antidepressant.^{54,55} In addition, a recently released hSERT crystal structure clearly displayed the location of orthosteric (S1) and allosteric sites (S2), and also pointed out that ligands bound in the S2 site or interacting with key



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residues in the S2 site could enhance and improve the inhibitory activity of ligands bound in the S1 site,^{56,57} which contributed to great efforts in designing and discovering a novel allosteric drug based on the hSERT and laid the foundation for illustrating the binding mode of vilazodone in the hSERT.^{58–64}

Herein, an integrated computational approach was applied to elucidate the molecular mechanism of vilazodone with bulky fragments binding to the hSERT. First, on the basis of the resolved crystal structure of the hSERT,⁵⁶ six ligands (Fig. 1)²⁸ were docked to the binding pocket of the hSERT. Second, the obtained initial coordinate of each complex was subjected to 400 ns molecular dynamic (MD) simulations, and the proteinligand binding mode was identified by per-residue energy and interaction fingerprint calculations. Finally, it was discovered that, unlike the action mechanisms of classical SSRIs, vilazodone with special structural characteristics not only occupied the central binding site (S1) of the hSERT, but also extended to the allosteric site (S2) by disrupting or triggering the ionic switch between R104 and E403. The elucidated binding mode was further confirmed by the conformation of escitalopram in S1 and S2 sites. As a result, the putative binding mode of vilazodone was identified in this study, and as for antidepressants, this binding mode is novel and there is only one drug vilazodone with this binding mode, providing valuable guidance for the design of novel antidepressants with an innovative molecular skeleton.

Materials and methods

Generation of initial conformation

Preparation of ligands. The structures of the ligands were drawn by ChemBioDraw⁶⁵ and sorted by their inhibitory activities (Fig. 1). The structures of the ligands were then preprocessed by LigPrep (OPLS-2005 force fields)⁶⁶ to generate the 3D structures and the energies of the generated conformations were minimized. The ionized states of the ligands were assigned *via* Epik⁶⁶ at a pH value of 7.0 \pm 2.0.

Preparation of protein. The crystallographic structure of the hSERT was collected from Protein Data Bank (PDB entry: 5I73),⁵⁷ which was then processed by the Protein Preparation Wizard module in Maestro before calculating the docking grid (including adding hydrogen atoms and assigning protonation states and partial charges with the OPLS-2005 force field).

Molecular docking. The initial docking poses of the studied system were obtained using standard precision (SP) docking in Glide⁶⁷ implemented in Maestro⁶⁷ with default parameters. First, the spatial coordinates of escitalopram were used as a reference in determining the binding site and the docking grid box. Then, processed ligands were docked into the prepared hSERT receptor. 5000 poses were generated during the initial phase of the docking calculation, out of which the best 400 poses were selected for energy minimization based on 100 steps of conjugate gradient minimizations. The choice of the initial conformations of the studied systems should be based on the understanding of the hSERT binding pocket, ensuring that the docked ligands interacted with D98 with a salt bridge and some other interactions with the key residues (such as a H-bond with E493 and hydrophobic interactions with A169, I172, A173, and T439) in the hSERT. Additionally, for the similar molecular fragments to escitalopram of the docked ligands, the spatial similarity (RMSD < 0.50 Å) should be guaranteed when choosing the initial conformations, and the indole segments of vilazodone and its analogs in the S1 site with certain strain in the linker were selected as the initial conformation for further MD simulation studies. Finally, docking calculations included the searching process of dominant conformations, providing the possible binding poses of vilazodone and its analogs. Thus, the docking scores of all the studied systems should also be considered when selecting the initial conformations.

MD simulations

System setup. Before MD simulations, the 6 complexes were optimized by Prime and then nested in a transmembrane system in the Amber specified format. Namely, OPM^{68} was applied to determine the spatial orientations of the studied systems, which were then inserted into the POPC lipid bilayer (192 lipids in the membrane systems) and immersed into water (TIP3P) with a 20 Å thickness. Moreover, sodium chloride solution (NaCl with a concentration of 0.15 mol L⁻¹) was applied to simulate the physiological environment by CHARMM-GUI Membrane Builder.⁶⁵

MD production. MD production was performed using GPUaccelerated PMEMD in AMBER16.⁶⁹ All the components of the studied complexes were processed by force field parameters (ff14SB⁷⁰ for the protein and Lipid14 for the lipid) to form the corresponding coordinate files (.inpcrd) and topology file (.prmtop) using the LEaP module in AMBER16, and parameters from Joung's work⁷¹ were applied to model Na⁺ and Cl⁻. As for the selected ligand, the geometry optimization and electrostatic potential calculation of the ligands were carried out at the HF/6-31G* level via Gaussian 09,72 and the generated files (*.frcmod, and *.mol2) were used to assign gaff atom types and the RESP partial charges through the antechamber embedded in AMBERTOOLS16. Prior to the MD production, two procedures were applied to minimize the initial energies of the prepared systems: (1) a harmonic restraint processed all the solute atoms (force constant = 10 kcal mol⁻¹ Å⁻²), (2) then the atoms were released to move freely in the second step. During these 2 procedures, the energy minimization was conducted using the steepest descent approach for the first 5000 steps and the subsequent 5000 steps were processed by a conjugated gradient method. Afterwards, the studied systems were heated gradually from 0 K to 300 K, and the periodic boundary condition of the studied system was equilibrated via 5 ns unrestrained MD production. Finally, all the systems created by LEaP were subjected to 400 ns simulations in NPT ensembles with a pressure of 1 atm and at a temperature of 1 atm, controlled using a Monte Carlo barostat and Langevin dynamics, respectively.

Binding free energy estimation and per-residue binding energy decomposition

The molecular mechanics/generalized born surface area (MM/GBSA) method using a single molecular dynamic trajectory was applied to calculate the binding free energy ($\Delta G_{\text{MM/GBSA}}$) regardless of the entropic influence between the docked ligands and the hSERT.^{73,74} Herein, 1000 snapshots were extracted from the equilibrium trajectories (100–200 ns) for calculations using mm_mpsa.pl as follows.

$$\Delta G_{\rm MM/GBSA} = \Delta E_{\rm vdW} + \Delta E_{\rm ele} + \Delta G_{\rm pol} + \Delta G_{\rm nonpol}$$

where $\Delta E_{\rm vdW}$ represented the van der Waals interactions contribution, $\Delta E_{\rm ele}$ stood for the electrostatic energy contribution, $\Delta G_{\rm pol}$ was the polar solvent interaction energy calculated by the GB model (igb = 2), and $\Delta G_{\rm nonpol}$ was nonpolar solvation free energy, which was evaluated using the LCPO method (0.0072 × Δ SASA, SASA is the solvent accessible area with a probe radius of 1.4 Å).^{75,76} The per-residue energy contribution between a hSERT residue and a ligand was decomposed by:

$$\begin{split} \Delta G_{\rm MM/GBSA}^{\rm per-residue} &= \Delta E_{\rm vdW}^{\rm per-residue} + \Delta E_{\rm ele}^{\rm per-residue} + \Delta G_{\rm pol}^{\rm per-residue} \\ &+ \Delta G_{\rm nonpol}^{\rm per-residue} \end{split}$$

where the first three terms were defined in the same way as the corresponding terms in the previous formula, and the last term was calculated based on the ICOSA method.

Interaction fingerprint analysis

Interaction fingerprints between the ligands and the hSERT were calculated based on IChem.^{77–79} 500 snapshots of the ligand and residues within 6 Å of the ligand's mass center were extracted from the final 50 ns trajectory and saved by the mol2

format. During calculations, seven interactions (hydrophobic, aromatic, H-bond donor, H-bond acceptor, positively ionizable, negatively ionizable, and metal coordination) were applied to evaluate interaction fingerprints between the ligand and the hSERT *via* parsing atoms and bond connectivity fields in the form of one-dimensional (1D) descriptors made up of 1 and 0, and detailed information about the rules of detecting the interactions between proteins and ligands is shown in Table S2 (ESI[†]).⁸⁰ In addition, the results were shown by radar plots.

Results and discussion

Ligand docking pose analysis

Initial conformation selection from docking is key for the successful prediction of protein-ligand binding using MD simulations.49,81,82 In this study, the initial conformations of vilazodone and its five analogs (Fig. 1) in the hSERT binding pocket are obtained using Glide docking and followed by Prime optimizations. To guarantee the reliability of the selected binding conformations of the six ligands in the hSERT, three standards were comprehensively considered. They were (i) the spatial similarity of ligands' docking poses with escitalopram in the crystal (PDB: 5I73⁵⁷), which was defined by root mean square deviation (RMSD < 0.5 Å),⁸¹ (ii) the salt bridge between ligands and D98 in the hSERT,^{82,83} and (iii) the low docking scores of ligands.⁷⁴ The selected binding conformations of 6 ligands in the hSERT are demonstrated in Fig. 2. The protonated nitrogen atom of each ligand forms a salt bridge interaction with the carboxyl in D98 (Fig. 2), which was verified by the previous study that this salt bridge played a key role in maintaining the binding of vilazodone in the active pocket.¹² Moreover, the residues in the hSERT binding pocket (Y95, R104, A169, I172, Y175, Y176, F335, F341 and T439) had hydrophobic interaction and π - π stacking with the six docked ligands. However, it is noted that there is a certain fold of ligand docking poses in the binding pocket. This is because the line linker consisting of four carbon atoms increased the flexibility of ligands. Moreover, the central site (S1) of the hSERT binding pocket was triangular, which made it difficult to accommodate such long-chain molecules. Thus, the inconsistent folding directions of the ligands' linkers in the hSERT showed some degrees of variations of their binding conformations (Fig. 2).

Sampling of the initial conformations by MD simulations

Stabilities of the simulation complex. The selected initial conformations of the six studied complexes were sampled by 400 ns MD simulations. The RMSD fluctuations of the protein backbone-atoms, heavy atoms of the ligand and backbone-atoms atoms of the binding pocket (within 6.0 Å of the ligands), loop regions, TM1–12 domains, and TM1, 3, 6, 8, and 10 surrounding the S1 binding pocket over the simulation time were calculated to monitor the dynamic stability. As demonstrated in Fig. 3, the RMSD values of the ligands 15, 20 and 22 (red) exhibited relatively large fluctuations (1.0–2.0 Å),



Fig. 2 The docking poses of the ligands binding to the hSERT.

indicating that their docking conformations were well adjusted by MD samplings during the initial process of simulations. In all the studied systems, it could be found that the fluctuation trends of the protein (green line) and the loop domain (yellow line) were basically consistent, indicating that the larger fluctuation of the protein backbone-atoms was mainly caused by the loop region with larger flexibility. In addition, although the loop region had relatively large fluctuations, such fluctuations had little impact on the binding sites (gray line) and TM1-12 domains (blue line), especially TM1, 3, 6, 8, and 10 (dark line) surrounding the S1 binding pocket, indicating the few influences on the interactions between ligands and receptors brought by the fluctuant loop region in the constructed systems. Moreover, it was worth mentioning that all the systems have been subjected to independent simulations at least 3 times, and taking the ligand 29 (vilazodone) system as an example, it could be learnt that the system could also reach the equilibrium state around 100 ns, indicating the convergence of the trajectory after 100 ns (Fig. S1, ESI[†]).

Comparison of the docking and MD simulated conformations. To investigate the conformational rearrangements of the proteinligand complex before and after MD sampling, structural alignment between the selected initial conformations and the corresponding representative snapshots from the equilibrated trajectories was conducted and shown in Fig. 4. In consistence with the RMSD value (Fig. 3), the conformations of the docked ligands were all shifted to a different degree after the MD sampling, among which the larger displacements occurred in the segment of the indole rings of the ligands. Except for the ligand 47 bound complex, the protonated nitrogen atoms of the other 5 ligands maintained a stable salt bridge interaction with the carboxyl group of D98 (Fig. 4). As expected, the linear structure of the four carbon atoms led to the larger flexibility of the ligands, which resulted in the spatial shift compared with the docking conformations. For example, the indole fragment of ligand 29 (vilazodone) stretched into the S2 site, while the indole segments of other systems (ligand 15 and 22) fold to some extent in the S1 site to adjust the relative position of the piperazine ring to D98 (Fig. 4). In general, among the six simulation complexes, ligand 29 (vilazodone) not only adjusted the relative position of the arylpiperazine ring to D98 located at the S1 site, but also extended the indole fragment into the S2 site to maintain the linear state of the molecule, which may be essential to ensure the stability of the binding conformation.

Accurate ranking of the six complexes by calculated binding free energies

For the 6 studied complexes, the binding free energies (BFEs) were calculated using the MM/GBSA approach⁸⁴ on the basis of snapshots extracted from the 100-200 ns dynamic trajectories, and the $\Delta G_{MM/GBSA}$ values were -76.45, -71.48, -68.53, -68.18, -65.30, and -64.54 kcal mol⁻¹ for ligand 29, 39, 47, 15, 22 and 20 bound hSERT, respectively. In addition, the binding energies could be further deduced from the experimental IC₅₀ values using the formula: $\Delta G_{exp} = RT \ln (IC_{50})$ (Table 1). Compared with ΔG_{exp} , the values of $\Delta G_{MM/GBSA}$ were relatively larger, which was mainly related to the ignorance of the calculation of entropy.⁸⁵ To further verify the stability and reliabilities of the predicted model, linear fitting between $\Delta\Delta G_{MM/GBSA}$ and $\Delta\Delta G_{exp}$ (calculated using ligand 20 as the reference) was carried out using the least square method, and a correlation coefficient (R^2) of 0.96 was obtained (Fig. 5), suggesting the estimated BFEs from the simulated models could reproduce the ranking trends of the experimental values. The sub-energy



Fig. 3 The RMSDs of protein (green line), ligand (red line), binding sites (gray line), loop regions (yellow line), TM1-12 domains (blue line), and TM1, 3, 6, 8, 10 (dark line) surrounding the S1 binding pocket as a function of the simulation time.

terms of the calculation results are shown in Table 1, where electrostatic (ΔE_{ele}) and van der Waals (ΔE_{vdW}) interactions were the main components contributing to the binding of the ligands, but polar-solvent interactions (ΔE_{pol}) were unfavorable to the binding of the ligands.

In addition, the binding free energies during the different stages (200–300 ns and 300–400 ns) of trajectories for 6 studied systems were also calculated and the results are displayed in Fig. S2 (ESI \dagger). From the perspective of energy fluctuations, all the 6 studied systems could reach the convergence after the trajectory of 100 ns, which was consistent with the values of RMSD. Moreover, for the Ligand 29 (Vilazodone) system,

the binding free energies of different simulation periods on the basis of the three independent trajectories were calculated, and the very small energy changes also indicated that the interactions between the receptor and the ligand changed little during the dynamic simulation process, guaranteeing the reliability of the selected dynamic trajectories (Fig. S3, ESI[†]).

Analysis of the key residue energy contribution to ligand binding

To identify the hSERT residues playing a key role in vilazodone and its five analogs binding, the total BFEs of the six complexes were decomposed at the per-residue level. As shown in Fig. 6,

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Fig. 4 The superimposition of the representative snapshots (deep green) and the initial poses (dim gray) of six modeled complexes: (A)–(F) the ligand 29 (vilazodone), 39, 47, 15, 22 and 20 systems, respectively. Red dashed line indicates the salt bridge interaction between ligands and D98.

Table 1 Comparison between the calculated ($\Delta G_{MM/GBSA}$) and experimental (ΔG_{exp}) binding free energies of studied systems (ΔG : kcal mol⁻¹, IC₅₀: nM)

Ligands	Calculated values						Experimental values		
	$\Delta E_{ m ele}$	$\Delta E_{ m vdW}$	$\Delta G_{ m pol}$	$\Delta G_{ m nonpol}$	$\Delta G_{ m MM/GBSA}$	$\Delta\Delta G_{ m calc}{}^{a}$	$\Delta G_{\exp}{}^{b}$	$\Delta\Delta G_{\exp}^{a}$	IC ₅₀
29	-21.92 ± 0.22	-72.15 ± 0.14	25.70 ± 0.20	-8.04 ± 0.01	-76.45 ± 0.13	-11.91	-12.69	-2.83	0.5
39	-22.23 ± 0.11	-66.67 ± 0.10	25.55 ± 0.09	-8.12 ± 0.01	-71.48 ± 0.10	-6	-11.56	-1.70	3.4
47	-26.58 ± 0.12	-66.65 ± 0.09	32.46 ± 0.10	-7.77 ± 0.01	-68.53 ± 0.10	-3.99	-10.64	-0.78	16.0
15	-26.88 ± 0.16	-66.97 ± 0.11	23.05 ± 0.14	-7.38 ± 0.01	-68.18 ± 0.12	-3.64	-10.51	-0.65	20.0
22	-25.54 ± 0.12	-61.02 ± 0.11	28.34 ± 0.12	-7.07 ± 0.01	-65.30 ± 0.11	-0.76	-10.10	-0.24	40.0
20	-17.77 ± 0.17	-62.76 ± 0.14	23.10 ± 0.15	-7.11 ± 0.01	-64.54 ± 0.13	0.00	-9.86	0.00	60.0
Esc.	-22.10 ± 0.15	-55.95 ± 0.10	26.06 ± 0.12	-6.16 ± 0.01	-58.14 ± 0.10	d	-10.07	d	42.0

 a $\Delta\Delta G$ represents the relative binding free energy with the ligand 20 bound complex as the reference. b ΔG_{exp} was calculated based on the equation $\Delta G_{exp} = RT \ln IC_{50}$, where $R = 8.314 \text{ J} (\text{mol K})^{-1}$ and T = 310 K. $^{c} IC_{50}$: half maximal inhibitory concentration, obtained from a previous study.^{28 d} Not calculated due to the large difference between the molecular skeletons of Esc and other compounds.

a total of 24 residues with high energy contributions were mainly distributed in the transmembrane (TM) regions (such as TM1, TM3, TM6, TM8 and TM10), and the global distribution of the key residues contributing to vilazodone's binding is shown in Fig. S4 (ESI[†]). Moreover, the energy contributions of the residues in TM1, TM3, TM6, TM8 and TM10 together with their proportion of the contribution of the 24 residues were analyzed (Tables S2–S6, ESI[†]).

As illustrated in Table S2 (ESI[†]), except for the ligand 47 bound complex, the proportion of the energy contributions from four residues (Y95, A96, D98 and R104) located in TM1 in other complexes ranged from 21.6% to 26.3%. The value of the proportion for the ligand 47 bound complex was only 13.3%. This was because the salt bridge between the protonated nitrogen atom of the ligand and the D98 carbonyl group was attenuated during MD simulations (Fig. 7D), failing to maintain the key interaction with the recognizing site. For comparison, based on the data reported in our previous study,⁸² the proportion of the energy contribution from the four residues in the TM1 of the hSERT for escitalopram (a representative SSRI) binding was calculated. It is noted that the value of the escitalopram bound complex (38.8%) was much higher than that of vilazodone analogues.

As illustrated in Table S3 (ESI[†]), the energy contributions of the six residues (A169, I172, A173, Y175, Y176 and N177) in TM3 in all the studied complexes accounted for more than 26%



Fig. 5 The linear fitting diagram of the predicted ($\Delta G_{MM/GBSA}$) and the experimental (ΔG_{exp}) relative binding free energies of the six simulated complexes.

of the total energy contributions, and the proportion of the ligand 15 bound complex equaled to 34.43%. Among the six residues, I172 and Y176 contributed more energy (Fig. 6), and this was consistent with the reported findings that both I172 and Y176 played an important role in the binding of small molecules to the hSERT.^{11,57,86} It is clear (Fig. 4) that I172 stabilized the binding conformation of the indole group of the ligands mainly through hydrophobic interactions, and Y176 was located above the piperazine ring of the ligands forming π -ionic interactions.

As illustrated in Table S4 (ESI[†]), it is found that eight residues (A331, Q332, F334, F335, S336, G338, F341 and V343) in TM6 contributed more energy to the binding of vilazodone and its analogues accounting from 19.1% to 29.7%. However, due to the lack of specific contacts such as the π - π stacking interaction between F335 and the indole fragment of vilazodone and its analogs, the value of the proportion of the energy contributions from TM6 in the escitalopram bound complex (15.95%) was relatively low. More importantly, F335 and Y176 acted as the hydrophobic switch⁸⁷ between the binding site and the extra-membrane regions.

Table S5 (ESI[†]) showed that the proportion of energy contributions of four residues (S438, T439, G442 and L443) in TM8 to vilazodone's binding was the highest (22.3%), while that of escitalopram's binding was the lowest (14.7%). Among these amino acids, the backbone carbonyl group (–CO–) and the side chain hydroxyl group (–OH) of S438 could interact with the aromatic ring and piperazine moiety on vilazodone *via* π - π interactions and polar interactions, respectively, which resulted in the relatively large energy contribution (–3.03 kcal mol⁻¹) to the binding of vilazodone (Table S5, ESI[†]). However, due to the scaffold difference of two molecules, S438 did not make strong interactions with escitalopram, leading to the minimum energy to the ligand binding (–0.85 kcal mol⁻¹).

Compared with TM1, 3, 6 and 8, the three residues (E493, T497 and V501) in TM10 contributed less energy to ligands' binding. As can be seen from Table S6 (ESI[†]), the values of the proportion ranged from 5.2% to 13.7%. E493 in TM10

contributed a large amount of energy to vilazodone's binding $(-1.54 \text{ kcal mol}^{-1})$. It is known that E493 can form a salt bridge interaction with R104 in TM1 and act as an ionic switch for the S1 and S2 sites of the hSERT.⁸⁷ Interestingly, the interaction energy between R104 and vilazodone was also higher $(-2.01 \text{ kcal mol}^{-1})$. Besides, from the binding conformational analysis, the indole fragment in vilazodone had a strong interaction with the ionic switch consisting of R104 and E493.

Conformational changes of vilazodone and its analogs in the hSERT binding pocket

Insight from interaction fingerprint analysis. To quantitatively characterize the conformational changes of vilazodone and its analogs in the hSERT binding pocket, the interaction fingerprints of the six studied complexes before and after simulation were calculated. As shown in Fig. 7, it is observed that six ligands had stable interactions with two residues (Y176 and F335) acting as hydrophobic switches of the hSERT binding site and extra-membrane region. Except for the ligand 20 bound complex, the other five ligands could maintain or form relatively stable interactions with Y95 and I172. Moreover, the interactions between D98 and ligands 47 and 15 were slightly attenuated after MD simulations. However, compared to the initial docking conformation, the aryl piperazine fragment of vilazodone formed stable hydrophobic and salt bridge interactions with Y95 and D98 through positional adjustment after MD simulations. Moreover, the ionic switch (R104-E493) played an important role in connecting the S1 and S2 binding sites,⁸⁷ and only ligands 29 (vilazodone) and 15 had stable interactions with two residues (Fig. 7), which was consistent with the energy contribution analysis results of the residues (Fig. 6). Thus, interaction fingerprint analysis suggested that vilazodone not only occupies the S1 site of the hSERT, but also interacts with residues located at the S2 site. So, it was necessary to further analyze the binding conformation of ligand 29 (vilazodone) and the analogs in the hSERT.

Insight from the structural alignment analysis. To analyze the binding conformation of vilazodone and its analogs in the hSERT, the representative structures of six complexes obtained from the equilibrated MD trajectories were aligned with the X-ray structure of the hSERT complexed with escitalopram at the S1 and S2 sites (PDB: 5I73⁵⁷) and are illustrated in Fig. 8. Apparently, only ligands 29 (vilazodone) and 15 adopted a linear pose in the hSERT binding pocket (Fig. 8A and D). The arylpiperazine fragment in vilazodone had high spatial overlap with escitalopram at the S1 site of the hSERT, and the indole fragment of vilazodone extended to the hSERT S2 site, interacting with the carbonyl group of E493 by hydrogen bonding interactions and having π - π interactions with R104. Moreover, by comparing the vilazodone bound complex with the co-crystal structure (Fig. 9), it could be found that the R104 of the former had a lateral shift due to ligand binding, leading to the distance between R104 and E493 being increased from \sim 3.0 to 6.0 Å (the opening of the ionic switch). Thus, it is proposed that vilazodone not only occupied the S1 site of the hSERT, but also extended to the S2 site through interaction with the ionic



Fig. 6 The per-residue binding free energy decomposition of the 6 simulation systems and the values of Escitalopram obtained from the previous studies.

switch residues (R104 and E493), which was confirmed by the structural alignments of vilazodone and escitalopram binding modes shown in Fig. 10.

Elucidating the binding mode of vilazodone in the hSERT

The common antidepressant SSRIs (selective serotonin transporter inhibitors) exhibited inhibitory activities against the hSERT mainly *via* being accommodated in 3 sub-sites (sub-site A, sub-site B and sub-site C) of the S1 site with their three pharmacophores including R1, R2 and R3,^{12,57} which made the conformation of the

hSERT keep the occluded state and prevented the substrate (serotonin) from being reuptaken from the synaptic cleft. However, occupying the S1 site alone couldn't have exerted such strong inhibitory activities as vilazodone. In addition, the resolved hSERT crystal structure clearly displayed the location of orthosteric (S1) and allosteric sites (S2) in the hSERT, and the ligand bound in the S2 site or interacting with key residues lining in the S2 site could enhance and improve the inhibitory activity of ligand binding in the S1 site. Thus, the binding patterns of vilazodone in the hSERT presented by the representative conformation were reliable, not

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Fig. 7 The comparison of interaction fingerprints of the hSERT and six ligands in the final 50 ns simulations with the docking poses (green color represents the interaction fingerprints between the six ligands and the hSERT in the final 50 ns, and the light grey represents the interaction fingerprints between the six ligands and the hSERT in the final 50 ns, and the light grey represents the interaction fingerprints between the six ligands and the hSERT in the final 50 ns, and the light grey represents the interaction fingerprints between the six ligands and the hSERT from the docking conformations with minimization). (A) hSERT-ligand 29 (vilazodone); (B) hSERT-ligand 39; (C) hSERT-ligand 47; (D) hSERT-ligand 15; (E) hSERT-ligand 22 and (F) hSERT-ligand 20. The ionic switch (R104-E493) and the hydrophobic switch (Y176-F335) were shown in red and blue, respectively.



Fig. 8 Structural alignment of representative snapshots (green) of the six modeled complexes to the co-crystal structure of hSERT complexed escitalopram (grey). (A–F) the ligand 29 (vilazodone), 39, 47, 15, 22 and 20 bound complexes, respectively.





Fig. 9 The monitored distance between R104 and E493 of the hSERT in vilazodone (green) and escitalopram (gray) bound complexes



Fig. 10 The comparison of pose of vilazodone (green stick) and escitalopram (black stick) in the hSERT binding pocket consisting of S1 and S2 sites. (A) The binding poses of escitalopram (black stick) in the S1 and S2 sites of the hSERT; (B) the superimposition of the binding poses of vilazodone and escitalopram in the hSERT.

S1 Binding Site R104TM1 F335TM6 Y175TM3 Y175

Salt Bridge ----- Hydrogen Bond ----- Hydrophobic Interaction ----Fig. 11 The schematic diagram of the action mode of vilazodone in the hSERT binding pocket.

only binding to the S1 site of hSERT, but also extending to the S2 site.

Thus, the binding modes of vilazodone in the hSERT could be elucidated and a schematic diagram is illustrated in Fig. 11 through the comprehensive analysis of per-residue binding free energy and the protein–ligand interaction fingerprints as well as the conformation behaviors of the studied complex. Compared with the pharmacophores of the classical SSRIs or SNRIs (R_1 , red; R_2 , yellow and R_3 , dashed gray),^{12,88} there was some difference in the distribution of the vilazodone's pharmacophore (R_4 , orange-yellow), which extended to the hSERT S2 site by interacting with the ionic switch residues (R104 and E403), making vilazodone adopt a linear pose (Fig. 11). In addition, the R_1 and R_2

pharmacophore binding in the S1 site was basically consistent with that of classical SSRIs or SNRIs. For example, D98 (recognition site for the S1 site of the hSERT) interacted with the protonated nitrogen atom of vilazodone *via* the salt bridge and hydrogen bonding, and the previously identified key residues located in the S1 site (I172, F335, F341, Y95, Y175, Y176, S438 and T439) could interact with vilazodone through hydrophobic interactions, stabilizing vilazodone in the hSERT binding pocket.

Conclusion

As identified in this study, unlike the classical SSRIs or SNRIs, vilazodone adopted a linear pose in the hSERT's binding pocket,

not only occupying the S1 binding site, but also extending to the S2 site by interacting with the ionic switch residues (R104 and E403). The proposed binding mode of vilazodone in the hSERT provided a deep understanding of the inhibitory mechanism of the drug at the atomic level, and the obtained structural and energetic information has significance for guiding the design of novel antidepressants targeting the hSERT.

Abbreviations

BFEs	Binding free energies	
hSERT	Human serotonin transporter	
MD	Molecular dynamics	
MDD	Major depression disorder	
MM/GBSA	Molecular mechanics/generalized born surface	
	area	
SNRIs	Serotonin and norepinephrine reuptake	
	inhibitors	
SSRIs	Selective serotonin reuptake inhibitors	
ТМ	Transmembrane region	

Conflicts of interest

The authors declare no competing financial interest.

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References

- 1 E. F. Coccaro, N. Engl. J. Med., 2019, 381, 980-981.
- 2 K. Recourt, P. de Boer, R. Zuiker, R. Luthringer, J. Kent,
 P. van der Ark, I. Van Hove, J. van Gerven, G. Jacobs,
 L. van Nueten and W. Drevets, *Transl. Psychiatry*, 2019,
 9, 216.
- 3 P. F. Saltiel and D. I. Silvershein, *Neuropsychiatr. Dis. Treat.*, 2015, **11**, 875–888.
- 4 V. Krishnan and E. J. Nestler, Nature, 2008, 455, 894-902.
- 5 D. J. Kupfer, E. Frank and M. L. Phillips, *Lancet*, 2012, **379**, 1045–1055.
- 6 O. F. O'Leary, T. G. Dinan and J. F. Cryan, *Eur. J. Pharmacol.*, 2015, **753**, 32–50.
- 7 A. Caspi, K. Sugden, T. E. Moffitt, A. Taylor, I. W. Craig, H. Harrington, J. McClay, J. Mill, J. Martin, A. Braithwaite and R. Poulton, *Science*, 2003, **301**, 386–389.

- 8 J. Yin, W. Sun, F. Li, J. Hong, X. Li, Y. Zhou, Y. Lu, M. Liu, X. Zhang, N. Chen, X. Jin, J. Xue, S. Zeng, L. Yu and F. Zhu, *Nucleic Acids Res.*, 2020, 48, D1042–D1050.
- 9 Q. Yang, B. Li, J. Tang, X. Cui, Y. Wang, X. Li, J. Hu, Y. Chen, W. Xue, Y. Lou, Y. Qiu and F. Zhu, *Briefings Bioinf.*, 2019, DOI: 10.1093/bib/bbz049.
- 10 G. Wagner, M. T. Schultes, V. Titscher, B. Teufer, I. Klerings and G. Gartlehner, J. Affective Disord., 2018, 228, 1–12.
- 11 H. Wang, A. Goehring, K. H. Wang, A. Penmatsa, R. Ressler and E. Gouaux, *Nature*, 2013, **503**, 141–145.
- 12 W. Xue, P. Wang, B. Li, Y. Li, X. Xu, F. Yang, X. Yao, Y. Z. Chen, F. Xu and F. Zhu, *Phys. Chem. Chem. Phys.*, 2016, **18**, 3260–3271.
- 13 E. A. Hoge, A. Ivkovic and G. L. Fricchione, *BMJ*, 2012, 345, e7500.
- 14 M. J. Millan, Neurotherapeutics, 2009, 6, 53-77.
- 15 M. Stuivenga, E. J. Giltay, O. Cools, L. Roosens, H. Neels and B. Sabbe, *Expert Opin. Pharmacother.*, 2019, **20**, 251–260.
- 16 P. L. McCormack, Drugs, 2015, 75, 1915-1923.
- 17 B. Li, J. Tang, Q. Yang, S. Li, X. Cui, Y. Li, Y. Chen, W. Xue, X. Li and F. Zhu, *Nucleic Acids Res.*, 2017, 45, W162–W170.
- 18 S. Chaki, Trends Pharmacol. Sci., 2017, 38, 569-580.
- A. J. Rush, M. H. Trivedi, S. R. Wisniewski, A. A. Nierenberg, J. W. Stewart, D. Warden, G. Niederehe, M. E. Thase, P. W. Lavori, B. D. Lebowitz, P. J. McGrath, J. F. Rosenbaum, H. A. Sackeim, D. J. Kupfer, J. Luther and M. Fava, *Am. J. Psychiatry*, 2006, **163**, 1905–1917.
- 20 Z. A. Hughes, K. R. Starr, C. J. Langmead, M. Hill, G. D. Bartoszyk, J. J. Hagan, D. N. Middlemiss and L. A. Dawson, *Eur. J. Pharmacol.*, 2005, **510**, 49–57.
- 21 S. M. Wang, C. Han, S. J. Lee, A. A. Patkar, P. S. Masand and C. U. Pae, *Chonnam Med. J.*, 2016, **52**, 91–100.
- 22 C. Moret, M. Isaac and M. Briley, *J. Psychopharmacol.*, 2009, 23, 967–974.
- 23 Y. H. Li, X. X. Li, J. J. Hong, Y. X. Wang, J. B. Fu, H. Yang, C. Y. Yu, F. C. Li, J. Hu, W. W. Xue, Y. Y. Jiang, Y. Z. Chen and F. Zhu, *Briefings Bioinf.*, 2019, DOI: 10.1093/bib/ bby130.
- 24 L. Shi, J. Wang, S. Xu and Y. Lu, *Drug Des., Dev. Ther.*, 2016, 10, 3899–3907.
- M. E. Thase, J. Edwards, S. Durgam, C. Chen, C. T. Chang, M. Mathews and C. P. Gommoll, *Int. Clin. Psychopharmacol.*, 2017, 32, 281–288.
- 26 Y. Wang, S. Zhang, F. Li, Y. Zhou, Y. Zhang, Z. Wang, R. Zhang, J. Zhu, Y. Ren, Y. Tan, C. Qin, Y. Li, X. Li, Y. Chen and F. Zhu, *Nucleic Acids Res.*, 2020, 48, D1031–D1041.
- 27 G. D. Bartoszyk, R. Hegenbart and H. Ziegler, *Eur. J. Pharmacol.*, 1997, **322**, 147–153.
- 28 T. Heinrich, H. Bottcher, R. Gericke, G. D. Bartoszyk, S. Anzali, C. A. Seyfried, H. E. Greiner and C. Van Amsterdam, *J. Med. Chem.*, 2004, 47, 4684–4692.
- 29 T. Heinrich, H. Bottcher, K. Schiemann, G. Holzemann, M. Schwarz, G. D. Bartoszyk, C. van Amsterdam, H. E. Greiner and C. A. Seyfried, *Bioorg. Med. Chem.*, 2004, **12**, 4843–4852.

- 30 D. S. Robinson, D. K. Kajdasz, S. Gallipoli, H. Whalen,
 A. Wamil and C. R. Reed, *J. Clin. Psychopharmacol.*, 2011,
 31, 643–646.
- 31 C. Gommoll, G. Forero, M. Mathews, R. Nunez, X. Tang,
 S. Durgam and A. Sambunaris, *Int. Clin. Psychopharmacol.*, 2015, 30, 297–306.
- 32 C. Gommoll, S. Durgam, M. Mathews, G. Forero, R. Nunez, X. Tang and M. E. Thase, *Depress. Anxiety*, 2015, 32, 451–459.
- 33 A. Khan, A. J. Cutler, D. K. Kajdasz, S. Gallipoli, M. Athanasiou, D. S. Robinson, H. Whalen and C. R. Reed, *J. Clin. Psychiatry*, 2011, 72, 441–447.
- 34 K. Rickels, M. Athanasiou, D. S. Robinson, M. Gibertini, H. Whalen and C. R. Reed, *J. Clin. Psychiatry*, 2009, 70, 326–333.
- 35 H. A. Croft, N. Pomara, C. Gommoll, D. Chen, R. Nunez and M. Mathews, J. Clin. Psychiatry, 2014, 75, e1291–e1298.
- 36 M. Mathews, C. Gommoll, D. Chen, R. Nunez and A. Khan, *Int. Clin. Psychopharmacol.*, 2015, **30**, 67–74.
- 37 Y. H. Li, C. Y. Yu, X. X. Li, P. Zhang, J. Tang, Q. Yang, T. Fu, X. Zhang, X. Cui, G. Tu, Y. Zhang, S. Li, F. Yang, Q. Sun, C. Qin, X. Zeng, Z. Chen, Y. Z. Chen and F. Zhu, *Nucleic Acids Res.*, 2018, 46, D1121–D1127.
- 38 J. Tang, J. Fu, Y. Wang, B. Li, Y. Li, Q. Yang, X. Cui, J. Hong, X. Li, Y. Chen, W. Xue and F. Zhu, *Briefings Bioinf.*, 2019, DOI: 10.1093/bib/bby127.
- 39 J. Tang, J. Fu, Y. Wang, Y. Luo, Q. Yang, B. Li, G. Tu, J. Hong, X. Cui, Y. Chen, L. Yao, W. Xue and F. Zhu, *Mol. Cell. Proteomics*, 2019, **18**, 1683–1699.
- 40 S. Durgam, C. Gommoll, G. Forero, R. Nunez, X. Tang, M. Mathews and D. V. Sheehan, *J. Clin. Psychiatry*, 2016, 77, 1687–1694.
- 41 Z. T. Sahli, P. Banerjee and F. I. Tarazi, *Expert Opin. Drug Discovery*, 2016, **11**, 515–523.
- 42 A. F. Carvalho, M. S. Sharma, A. R. Brunoni, E. Vieta and G. A. Fava, *Psychother. Psychosom.*, 2016, 85, 270–288.
- 43 L. Citrome, Int. J. Clin. Pract., 2012, 66, 356-368.
- 44 A. L. Montejo, L. Montejo and F. Navarro-Cremades, *Curr. Opin. Psychiatry*, 2015, **28**, 418–423.
- 45 L. Citrome, J. Affect. Disord., 2016, 196, 225-233.
- 46 T. Chang and M. Fava, J. Clin. Psychiatry, 2010, 71, 971–975.
- 47 C. R. Hopkins, ACS Chem. Neurosci., 2011, 2, 554.
- 48 P. Celada, A. Bortolozzi and F. Artigas, *CNS Drugs*, 2013, 27, 703–716.
- 49 G. Zheng, W. Xue, F. Yang, Y. Zhang, Y. Chen, X. Yao and
 F. Zhu, *Phys. Chem. Chem. Phys.*, 2017, 19, 28885–28896.
- 50 A. Khan, Expert Opin. Investig. Drugs, 2009, 18, 1753-1764.
- 51 M. Liebowitz, H. A. Croft, D. K. Kajdasz, H. Whalen, S. Gallipoli, M. Athanasiou and C. R. Reed, *Psychopharmacol. Bull.*, 2011, 44, 15–33.
- 52 J. E. Frampton, CNS Drugs, 2011, 25, 615-627.
- 53 A. Heinz, K. Mann, D. R. Weinberger and D. Goldman, *Alcohol.: Clin. Exp. Res.*, 2001, 25, 487–495.
- 54 X. Li, H. Wang, Y. Xu, W. Liu, Q. Gong, W. Wang, X. Qiu, J. Zhu, F. Mao, H. Zhang and J. Li, ACS Chem. Neurosci., 2017, 8, 2708–2721.

- 55 D. E. Nichols and C. D. Nichols, *Chem. Rev.*, 2008, **108**, 1614–1641.
- 56 H. Rannversson, J. Andersen, B. Bang-Andersen and K. Stromgaard, ACS Chem. Biol., 2017, 12, 2558–2562.
- 57 J. A. Coleman, E. M. Green and E. Gouaux, *Nature*, 2016, 532, 334–339.
- 58 W. Xue, T. Fu, G. Zheng, G. Tu, Y. Zhang, F. Yang, L. Tao, L. Yao and F. Zhu, *Curr. Med. Chem.*, 2018, DOI: 10.2174/ 0929867325666181009123218.
- 59 I. Erol, B. Aksoydan, I. Kantarcioglu, R. E. Salmas and S. Durdagi, J. Mol. Graphics Modell., 2017, 74, 193–202.
- 60 S. Kortagere, A. C. Fontana, D. R. Rose and O. V. Mortensen, *Neuropharmacology*, 2013, 72, 282–290.
- 61 S. Topiol, B. Bang-Andersen, C. Sanchez, P. Plenge, C. J. Loland, K. Juhl, K. Larsen, P. Bregnedal and K. P. Bogeso, *Bioorg. Med. Chem. Lett.*, 2017, 27, 470–478.
- M. A. Larsen, P. Plenge, J. Andersen, J. N. Eildal, A. S. Kristensen, K. P. Bogeso, U. Gether, K. Stromgaard, B. Bang-Andersen and C. J. Loland, *Br. J. Pharmacol.*, 2016, 173, 925–936.
- 63 A. Brinko, M. T. Larsen, H. Koldso, L. Besenbacher, A. Kolind, B. Schiott, S. Sinning and H. H. Jensen, *Bioorg. Med. Chem.*, 2016, 24, 2725–2738.
- 64 S. Topiol, B. Bang-Andersen, C. Sanchez and K. P. Bogeso, *Bioorg. Med. Chem. Lett.*, 2016, 26, 5058–5064.
- 65 E. L. Wu, X. Cheng, S. Jo, H. Rui, K. C. Song, E. M. Davila-Contreras, Y. Qi, J. Lee, V. Monje-Galvan, R. M. Venable, J. B. Klauda and W. Im, *J. Comput. Chem.*, 2014, 35, 1997–2004.
- 66 Epik, 2.0, Schrödinger, LLC, New York, NY, 2009.
- 67 Glide, 5.5, Schrödinger, LLC, New York, 2009.
- 68 M. A. Lomize, I. D. Pogozheva, H. Joo, H. I. Mosberg and A. L. Lomize, *Nucleic Acids Res.*, 2012, **40**, D370–D376.
- 69 AMBER 16, University of California, San Francisco, 2016.
- 70 C. J. Dickson, B. D. Madej, A. A. Skjevik, R. M. Betz, K. Teigen, I. R. Gould and R. C. Walker, *J. Chem. Theory Comput.*, 2014, **10**, 865–879.
- 71 I. S. Joung and T. E. Cheatham, 3rd, J. Phys. Chem. B, 2008, 112, 9020–9041.
- 72 Gaussian 09 D.01, Gaussian, Inc., Wallingford, CT, 2009.
- 73 E. Wang, H. Sun, J. Wang, Z. Wang, H. Liu, J. Z. H. Zhang and T. Hou, *Chem. Rev.*, 2019, **119**, 9478–9508.
- 74 P. Wang, T. Fu, X. Zhang, F. Yang, G. Zheng, W. Xue,
 Y. Chen, X. Yao and F. Zhu, *Biochim. Biophys. Acta, Gen. Subj.*, 2017, 1861, 2766–2777.
- 75 J. Weiser, P. S. Shenkin and W. C. Still, J. Comput. Chem., 1999, 20, 217–230.
- 76 G. Zheng, W. Xue, P. Wang, F. Yang, B. Li, X. Li, Y. Li, X. Yao and F. Zhu, *Sci. Rep.*, 2016, 6, 26883.
- 77 G. Marcou and D. Rognan, J. Chem. Inf. Model., 2007, 47, 195–207.
- 78 F. Da Silva, J. Desaphy and D. Rognan, *ChemMedChem*, 2018, 13, 507–510.
- 79 C. Southan, ChemMedChem, 2018, 13, 470-481.

- 80 J. Desaphy, E. Raimbaud, P. Ducrot and D. Rognan, J. Chem. Inf. Model., 2013, 53, 623–637.
- 81 Y. Zhang, J. B. Ying, J. J. Hong, F. C. Li, T. T. Fu, F. Y. Yang, G. X. Zheng, X. J. Yao, Y. Lou, Y. Qiu, W. W. Xue and F. Zhu, *ACS Chem. Neurosci.*, 2019, **10**, 2467–2480.
- 82 G. Zheng, F. Yang, T. Fu, G. Tu, Y. Chen, X. Yao, W. Xue and F. Zhu, *Phys. Chem. Chem. Phys.*, 2018, 20, 29513–29527.
- 83 L. Celik, S. Sinning, K. Severinsen, C. G. Hansen, M. S. Moller, M. Bols, O. Wiborg and B. Schiott, *J. Am. Chem. Soc.*, 2008, 130, 3853–3865.
- 84 T. Hou, J. Wang, Y. Li and W. Wang, J. Chem. Inf. Model., 2011, 51, 69–82.
- 85 S. Genheden and U. Ryde, *Expert Opin. Drug Discovery*, 2015, 10, 449–461.
- 86 J. A. Coleman and E. Gouaux, Nat. Struct. Mol. Biol., 2018, 25, 170–175.
- 87 S. Manepalli, C. K. Surratt, J. D. Madura and T. L. Nolan, *AAPS J.*, 2012, 14, 820–831.
- 88 W. Xue, F. Yang, P. Wang, G. Zheng, Y. Chen, X. Yao and F. Zhu, ACS Chem. Neurosci., 2018, 9, 1128–1140.