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Revealing vilazodone’s binding mechanism underlying its partial agonism to the 5-HT\textsubscript{1A} receptor in the treatment of major depressive disorder\dag

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It has been estimated that major depressive disorder (MDD) will become the second largest global burden among all diseases by 2030. Various types of drugs, including selective serotonin reuptake inhibitors (SSRIs), serotonin-norepinephrine reuptake inhibitors (SNRIs), and serotonin receptor partial agonist/reuptake inhibitors (SPARIs), have been approved and become the primary or first-line medications prescribed for MDD. SPARI was expected to demonstrate more enhanced drug efficacy and a rapid onset of action as compared to SSRI and SNRI. As one of the most famous SPARIs, vilazodone was approved by the FDA for the treatment of MDD. Because of the great clinical importance of vilazodone, its binding mechanism underlying its partial agonism to the 5-HT\textsubscript{1A} receptor (5-HT\textsubscript{1AR}) could provide valuable information to SPARIs’ drug-like properties. However, this mechanism has not been reported to date; consequently, the rational design of new efficacious SPARI-based MDD drugs is severely hampered. To explore the molecular mechanism of vilazodone, an integrated computational strategy was adopted in this study to reveal its binding mechanism and prospective structural feature at the agonist binding site of 5-HT\textsubscript{1AR}. As a result, 22 residues of this receptor were identified as hotspots, consistently favoring the binding of vilazodone and its analogues, and a common binding mechanism underlying their partial agonism to 5-HT\textsubscript{1AR} was, therefore, discovered. Moreover, three main interaction features between vilazodone and 5-HT\textsubscript{1AR} have been revealed and schematically summarized.

In summary, this newly identified binding mechanism will provide valuable information for medicinal chemists working in the field of rational design of novel SPARIs for MDD treatment.

Introduction

It has been estimated that major depressive disorder (MDD) will become the second largest global burden among all diseases by 2030,\textsuperscript{1–3} which makes the discovery of novel and efficacious antidepressants an urgent need.\textsuperscript{4–6} Abnormality in the concentrations of monoamine, particularly 5-HT (5-hydroxytryptamine, serotonin) in MDD patients, led to the hypothesis that a dysfunction of 5-HT signaling might cause depression.\textsuperscript{7–9} Based on these theories, a variety of drugs have been approved by the food and drug administration (FDA) and become the primary or first-line medications prescribed for MDD.\textsuperscript{10–13} These drugs include the selective serotonin reuptake inhibitors (SSRIs),\textsuperscript{10,11} serotonin-norepinephrine reuptake inhibitors (SNRIs),\textsuperscript{14} and serotonin receptor partial agonist/reuptake inhibitors (SPARIs).\textsuperscript{15} The major problems of both SSRIs and SNRIs are their greatly delayed onset of action and undesired side effects,\textsuperscript{16} which substantially hamper their prescription for MDD treatment.\textsuperscript{17}

The evidence demonstrates that the pre- and post-synaptic 5-HT\textsubscript{1A} receptors play opposite roles in depression.\textsuperscript{18,19} In particular, the activation of pre-synaptic 5-HT\textsubscript{1A}Rs (autoreceptors) decreases the firing rate and serotonin secretion. In contrast, the activation of the post-synaptic receptor enhances the firing and secretion.\textsuperscript{18,20} The administration of SSRIs results in a significant increase in extraneuronal serotonin that is quickly counteracted by a negative feedback mechanism mediated by autoreceptors; this in turn leads to the desensitization of the autoreceptors, but not of...
the post-synaptic receptors. With the desensitization of the autoreceptors reduced, the autoreceptors gradually recover to release serotonin normally; thus, the greater activation of post-synaptic 5-HT1A will relieve and improve the depression symptoms. It was speculated that the delayed onset of action might result from the negative feedback mechanisms because it took time to recover the autoreceptors’ normal serotonin release. Thus, rapid desensitization of the autoreceptors is regarded as important in the fast onset of antidepressants, which is proposed as a novel strategy for treating MDD. To date, 2 SPARIs (vilazodone and vortioxetine) have been approved by the FDA for the treatment of MDD, and hundreds of active SPARIs of diverse scaffolds have been found.

Vortioxetine showed multimodal activities by broadly binding to the serotonin reuptake transporter and 5-HT1A, 5-HT1B, 5-HT1D, and 5-HT3 receptors. Compared with vortioxetine, vilazodone and its analogues were much more selective to the serotonin transporter (SERT) and 5-HT1A and thus capable of providing the most simplified and representative model of SPARIs’ mechanism. Moreover, due to the clinical importance of vilazodone, the binding mode of the privileged indolebutylpiperazine scaffold (residing in vilazodone and its analogues) in SERT and 5-HT1A offered much more information about SPARIs’ drug-like properties than that of the active SPARIs found in vitro and could facilitate the discovery of SPARI-based MDD drugs.

On the one hand, the binding mode of 5-HT in SERT has already been revealed. Due to the great structural similarity between 5-HT and the fragment indolebutylamine residing in vilazodone and its analogues, this fragment simulated 5-HT by competitively binding to SERT, and the binding mode of vilazodone and its analogues in SERT could be effectively deduced. On the other hand, to understand vilazodone’s partial agonism to 5-HT1A and the resulting faster onset of action, it is very important to elucidate the binding mechanism of vilazodone and its analogues in 5-HT1A. However, this mechanism has not been reported to date; consequently, the discovery of novel and efficacious SPARI-based MDD drugs is severely hampered.

In this study, an integrated computational strategy was adopted to identify the binding mode of vilazodone and its analogues in 5-HT1A. First, the structure of 5-HT1A was constructed by homology modelling based on the crystal structure of the β2 adrenergic receptor (β2AR). Second, molecular docking combined with the Prime/MM-GBSA approach was applied to generate the binding poses of 51 ligands (vilazodone and its analogues) in the modelled target. Third, six representative ligand-target complexes were further selected and subjected to MD simulation and binding free energy analysis. As a result, a common binding mode shared by these six complexes was characterized by clustering 5-HT1A’s residues in terms of their energy contribution to each ligand. Finally, an in silico alanine scanning mutagenesis study on these key residues contributing to drug binding was also performed to verify the reliability of the simulation results. Overall, the binding mechanism discovered herein can be regarded as a useful starting point for the design of novel chemical entities with improved antidepressant activity and pharmacological profiles.

Materials and methods

Sequence alignment and homology modelling

The sequence of human 5-HT1A was downloaded from the UniProt database (entry: P08990), and the sequence similarity search was conducted using the SWISS-MODEL server. The disclosed co-crystal structure of β2AR (PDB entry: 3SN6) was selected as a template to build 5-HT1A. The sequence of human 5-HT1A was aligned with that of human β2AR (PDB entry: 3SN6, from E30 to C341) using ClustalX 2.0 and then visualized by ESPript 3.0. The homology model of 5-HT1A was constructed using the automated mode of SWISS-MODEL based on the template. Finally, the stereochemical quality of the modelled target was evaluated by Ramachandran plot analysis using PROCHECK. The detailed protocol can be found in the ESI† for Materials and methods.

Molecular docking

The initial poses of the studied 51 ligands (Fig. 1) binding to the modelled 5-HT1A were obtained via standard precision (SP) docking in Glide with the default settings. First, these 51 ligands were preprocessed by LigPrep using OPLS-2005 force field to generate a low-energy conformation, and the ionized state was assigned by Epik at a pH value of 7.0 ± 2.0. The docking grid was calculated using the Receptor Grid Generation tool in Glide by centering P0G (a ligand in JN6302 introduced to the constructed 5-HT1A model) and simultaneously constraining an ionic bond interaction with the carboxylate of D116. The detailed protocol can be seen in the ESI† for Materials and methods.

Post-docking via prime optimization

The SP docking pose was submitted to optimization by Prime followed by calculation of the binding free energy (∆Gprime/MM-GBSA) via the following eqn (1):

$$\Delta G_{\text{Prime/MM-GBSA}} = \Delta E_{\text{MM}} + \Delta G_{\text{solv}} + \Delta G_{\text{SA}}$$

where ∆EMM denotes the difference between the minimized energies of the docking complexes and the sum of the energy of the receptor and the ligand obtained using OPLS-2005 force field, ∆Gsolv is the difference between solvation energies of the complexes and the sum of solvation energies for the receptor and ligands obtained using the GBSA continuum model, and ∆GSA indicates the difference between surface area energies of the complexes and the sum of surface area energies for the receptor and ligand.
MD simulation and binding free energy calculations

Preparing the MD simulation. Herein, 6 representative structures were selected from 51 receptor–ligand complexes for the MD simulations. Initially, the spatial orientations of these complexes were calculated by OPM and then inserted into a POPC lipid bilayer with a water layer thickness of 20 Å and a NaCl concentration of 0.15 mol L$^{-1}$ using CHARMM-GUI Membrane Builder. Finally, the LEaP module in AMBER14 was used to assign the force field parameters for the protein (ff14SB), lipid (Lipid14), and water (TIP3P). Ions ($\text{Na}^+$ and $\text{Cl}^-$) were modelled using parameters from Joung's work. For the ligands, Antechamber was adopted to assign gaff atom types and RESP partial atomic charges. The geometry optimization and the electrostatic potential calculation for the ligands were carried out using Gaussian09 at the HF/6-31G* level. The size for each system was 750 atoms, with the periodic box set as 80 Å x 80 Å x 113 Å.

Performing the MD simulations. MD simulations were performed with GPU-accelerated PMEMD in AMBER14. Prior to the simulations, operational procedures (minimization, heating, and equilibration) were conducted, and the detailed protocol used herein is provided in the ESI. Methods. Subsequently, 150 ns MD simulation was carried out in the NPT ensemble (310 K and 1 atm) under the periodic boundary condition. The Particle-mesh Ewald algorithm was adopted to deal with the long-range electrostatic interaction (cut-off = 10 Å). The bond distance involving the bond to the hydrogen atom was constrained using the SHAKE algorithm and the integration time step was set as 2 fs in the simulations.

Calculating the binding free energy. The binding free energy ($\Delta G_{\text{MM/GBSA}}$) of the ligands to the receptor, excluding the entropic effect, was calculated by the MM/GBSA approach using a single trajectory. In this study, 500 images of the last 50 ns equilibrated trajectory were used. For each image, $\Delta G_{\text{MM/GBSA}}$ was computed as follows:

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

where, $\Delta E_{\text{vdW}}$ denotes the van der Waals interaction energy, $\Delta E_{\text{ele}}$ stands for the electrostatic energy, $\Delta G_{\text{pol}}$ is the polar solvent interaction energy calculated via the GB model ($\sigma_{gb}$ = 2), and $\Delta G_{\text{nonpol}}$ is the nonpolar solvation free energy, which has been evaluated as 0.0072 $\times$ SASA using the LCPO method, where SASA indicates the solvent accessible area determined with a probe radius of 1.4 Å.

Calculating the per-residue energy contribution. The per-residue energy contribution ($\Delta G_{\text{MM/GBSA per-residue}}$) of the receptor to ligand binding was decomposed by the following equation:

$$\Delta G_{\text{MM/GBSA per-residue}} = \Delta E_{\text{vdW per-residue}} + \Delta E_{\text{ele per-residue}} + \Delta G_{\text{pol per-residue}} + \Delta G_{\text{nonpol per-residue}}$$

where the way to define $\Delta E_{\text{vdW per-residue}}$, $\Delta E_{\text{ele per-residue}}$, and $\Delta G_{\text{pol per-residue}}$ was the same as that in eqn (2), but the nonpolar solvent interaction energy ($\Delta G_{\text{nonpol per-residue}}$) was calculated by a
Hierarchical clustering

Hierarchical clustering was performed by the R statistical analysis software. At first, a 6-dimensional vector was generated by the energy contributions of a certain residue to at least one ligand binding (≠ 0 kcal mol⁻¹), and the similarity among the vectors was then calculated in terms of the Manhattan distance:

\[ \text{Distance}(a, b) = \sum_i |a_i - b_i| \]  

where \( i \) indicates the dimension of the energy contribution of the residues \( a \) and \( b \). The Ward’s minimum variance algorithm was used to minimize the total within-cluster variance. Finally, the cluster outcomes were displayed using the web-server iTOl.

Computational alanine scanning mutagenesis

Computational alanine scanning (CAS) is acknowledged as an effective approach for identifying hot spots at the binding sites of protein–protein, protein–DNA, and protein–ligand interactions. Herein, CAS was conducted to verify the identified key residues contributing to the binding. For alanine mutation, the variation of the binding free energies between the mutant and wild-type was defined as follows:

\[ \Delta G_{\text{mutant/wild-type}} = \Delta G_{\text{mutant}} - \Delta G_{\text{wild-type}} \]  

where \( \Delta G_{\text{mutant}} \) and \( \Delta G_{\text{wild-type}} \) were calculated from 500 images extracted from the MD simulations. The alanine mutation was generated by truncating the side chain of mutated residues at \( C_r \) and replacing this with a hydrogen atom (C=H). The alanine scanning conducted herein was applied only to the selected residues with a high absolute binding energy contribution (>0.50 kcal mol⁻¹).

Results and discussion

Homology model of 5-HT₁₆R in an agonistic conformation

The construction of the correct binding pocket conformation has been reported to be critical for the rational design of drugs targeting GPCR. The ligands studied herein act as partial agonists of 5-HT₁₆R, and the active state crystal structure of an agonist-stabilized β₂AR (in an agonistic conformation, PDB entry: 3SN6) has already been adopted as a template for constructing the homology models of 5-HT₁₆R in several virtual screening studies. Therefore, the agonist-stabilized β₂AR 3SN6 was selected herein as the template for the homology modelling. The sequence alignment between the target and template (ESI† Fig. S1) revealed an overall residue identity of 34% and a binding pocket residue conservation of 41%. The binding pocket in the modelled receptor was identified by the interaction between P0G and β₂AR and surrounded by the residues in the vicinity of the ligand (<6 Å, ESI† Fig. S2). As shown in ESI† Fig. S3, the homology model constructed in this study was superimposed well with its template. As expected, this model adopted a conformation similar to that of the active state β₂AR that covered the transmembrane helices (TM1-7) associated with the intracellular (LCL) and extracellular (ECL) loop regions (ESI† Fig. S3). Moreover, the overall quality of the constructed model was evaluated by the Ramachandran plot analysis. As shown in ESI† Fig. S4, the majority (98.5%) of the residues were located in the allowed regions. Therefore, the modelled 5-HT₁₆R in the agonistic conformation was qualified enough to study its interactions with vilazodone and its analogues.

Docking of the ligands into 5-HT₁₆R

A total of 51 ligands (Fig. 1) were studied herein. According to their diverse structural features, these ligands (11 to 61, with their original ligand ID retained) were grouped into four classes: (I) 11–21, (II) 22–31, (III) 32–48, and (IV) 49–61, in a similar way as that reported in Heinrich’s work (ESI† Tables S1–S4), and vilazodone was numbered as ligand 29. In first, these 51 ligands were docked into the binding pocket to obtain the initial receptor–ligand complexes and then subjected to post-processing optimization. The post-processed docking poses of these 51 ligands are depicted by classes in Fig. 2. As illustrated, the positively charged ammonium moiety of all 51 ligands directly interacted with the carboxylate of D1163.32 via a salt bridge, and the ligands’ indole formed a hydrogen bond with S199. The salt bridge represented conserved protein–ligand interaction (anchor site) throughout a wide range of mammalian biogenic amine GPCRs, and the hydrogen bond was found to be essential for the agonism of these partial agonists studied herein.

The post-docking scores (ΔG_{Prime/MM-GBSA}) of all 51 complexes are listed in Table S5 (ESI†). The correlation coefficient \( R^2 \) between \( \Delta G_{\text{exp}} \) (binding energy deduced from the experimental data) and \( \Delta G_{\text{bind}} \) was 0.84 (Fig. 3). This high correlation was in accordance with the previous studies stating that molecular docking combined with the Prime/MM-GBSA approach enabled a rapid estimation of the binding affinity of a series of ligands to their corresponding receptor. Moreover, two outliers (ligands 17 and 18 shown in Fig. 3) have also been observed, which may have originated from the unfavorable contribution of their substituents on indole at position 5 to their binding to 5-HT₁₆R. In particular, as shown in ESI† Fig. S3, a strong polar group (-C==NOH and -COOH circled by red dash line) was orientated into the hydrophobic cavity between TM6 and TM7 constituted by 13 hydrophobic residues (F361, F362, I363, V364, A365, L366, L367, V367, L380, L381, G382, A383, I384, I385, and I385). The hydrophobic region may repel these polar moieties and hence may not stabilize the ligands in the binding pocket. Thus, the local region did not favor the binding of these two ligands with 5-HT₁₆R.

MD simulations and binding free energy calculations of the receptor–ligand complexes

MD simulation can be used as a post-processing tool for validating or refining docking solutions. To explore more detailed and reliable information on the binding mode, 6 representative complexes were selected for the MD simulations. The docking poses of these 6 complexes are shown in Fig. 4. As specified, complexes 1, 4, and 6 were chosen for single-molecule MD simulations, whereas 3, 5, and 7 were chosen for the ensemble approach. A total of 1000 images from 500 images were extracted from the MD simulations of the receptor–ligand complexes with vilazodone, and the average values of the MD simulations were calculated for each of the 51 complexes. The correlation coefficient \( R^2 \) between \( \Delta G_{\text{exp}} \) and \( \Delta G_{\text{docking}} \) is 0.84 (Fig. 3), which is similar to the value obtained from the docking scores (ΔG_{Prime/MM-GBSA}), which is 0.84 (Fig. 3). Therefore, the MD simulations can be used as a post-processing tool for validating or refining docking solutions.
complexes from the docking study were selected and subjected to MD simulations followed by binding energy calculations. The selected ligands included 14 from class I, 29 (vilazodone) from class II, 36, 39, and 48 from class III, and 51 from class IV, whose binding affinities to 5-HT₁₆R ranged from 0.3 nM to 60.0 nM (Table 1).

Simulation stability evaluation. The root-mean-square deviation (RMSD) of protein backbone atoms, ligand heavy atoms, and binding site residues atoms (extending 6 Å around the ligand) for 6 simulation systems against the simulation time was calculated, and the results are illustrated in ESI,† Fig. S6. These RMSD values (fluctuation range within 4 Å) manifested that all systems achieved their equilibrium state after 100 ns simulation. Moreover, the salt bridge between the positively charged nitrogen of all the ligands and the carboxyl of D116 \(^{36} \) in the receptor \(^{48} \) was analyzed by superimposing the initial docking poses with the MD simulation results [ESI,† Fig. S7]. It was noted that although there was a certain level of structural shifts on both the target and ligands, the conserved salt bridges were maintained very well during the whole MD simulation.

Binding free energy calculations. The free energy of binding \( (\Delta G_{\text{MM/GBSA}}) \) of vilazodone and its analogues to 5-HT₁₆R was quantitatively calculated by the MM/GBSA method. As shown in Table 1, the calculated \( \Delta G_{\text{MM/GBSA}} \) corresponding to 29, 36, 51, 14, 39, and 48 bound complexes was \(-62.73, -59.63, -58.10, -57.29, -56.17, \) and \(-51.65 \) kcal mol \(^{-1} \), respectively. Moreover, the binding free energies of these 6 complexes could be deduced by experiment. In particular, the experimental IC₅₀ values \(^{29} \) could be converted into binding energies \((\Delta G_{\text{exp}})\) using the equation \( \Delta G_{\text{exp}} = RT\ln IC_{50} \) based on IC₅₀ values. The experimental IC₅₀ values from the previous study \(^{29} \) are in nM (Table 1).

Table 1 The calculated and experimentally determined binding free energies throughout 6 complexes (\( \Delta G \) in kcal mol \(^{-1} \) and IC₅₀ is in nM)

<table>
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<th>Ligands</th>
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<th>( \Delta G_{\text{exp}} )</th>
<th>( \Delta G_{\text{exp}} )</th>
<th>IC₅₀</th>
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Fig. 2 The post-docking poses of 4 classes of vilazodone and its analogues in the binding pocket of the human 5-HT₁₆ receptor. Receptors are displayed in pink cartoons and the residues in the orthostatic binding pocket are shown as pink sticks. Ligands are shown by various colors. Residues labeled in red background represent the ionic bond interactions existing between the ligands and receptor.

Fig. 3 Correlation between the experimentally determined binding free energy \( (\Delta G_{\text{calc}}) \) and post-docking binding energy \( (\Delta G_{\text{calc}}) \) obtained by Prime/MM-GBSA.

Table 1 The calculated and experimentally determined binding free energies throughout 6 complexes (\( \Delta G \) in kcal mol \(^{-1} \) and IC₅₀ is in nM)

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\( \Delta G \) denotes the difference in binding energies \((\Delta G)\) as a reference. Estimated free energy values are computed by \( \Delta G_{\text{exp}} = RT\ln IC_{50} \) based on IC₅₀ values. Experimental IC₅₀ values from the previous study \(^{29} \).
resembled binding conformation was taken into consideration, the entropy contribution could be ignored, and the above-mentioned overestimation was inevitable, as shown in the literature using the MM/GBSA method. In Table S6 (ESI†), values for the detailed energy terms shown in eqn (1) are provided. As shown, the van der Waals ($\Delta E_{\text{vdW}}$), the electrostatic interaction ($\Delta E_{\text{ele}}$), and the nonpolar solvation ($\Delta G_{\text{nonp}}$) terms were favorable for the ligands' binding, whereas the polar solvation component $\Delta G_{\text{pol}}$ was unfavorable. Among these favorable terms, $\Delta E_{\text{vdW}}$ accounted for the major part of the energy contributions for all 6 studied complexes, with the ligand 29-bound complex being at the top rank ($\Delta E_{\text{vdW}} = -58.41 \text{ kcal mol}^{-1}$) and the ligand 48-bound complex being at the lowest rank ($\Delta E_{\text{vdW}} = -50.10 \text{ kcal mol}^{-1}$). The lowest $\Delta E_{\text{vdW}}$ of ligand 48 might originate from the two electron-donating groups (−OCH₃) on its aromatic ring of arylpiperazine, which enhanced the electron density of the ring and in turn hampered the binding of ligand 48 to the binding pocket of 5-HT₁AR.

**Comprehensive analysis of the receptor–ligand interactions**

Fig. 5 shows the 6 representative images obtained from the MD simulations of the receptor–ligand complexes. The detailed binding mode of each ligand is described in the ESI† for Results and discussion. In this section, the receptor–ligand interactions are systematically analyzed from four different perspectives: first, the residues in the vicinity of the studied

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Fig. 4 Correlation between the differences in the calculated ($\Delta G_{\text{calc}}$) and experimentally determined ($\Delta G_{\text{exp}}$) binding free energies using ligand 48 as the reference.

Fig. 5 The representative images of 6 ligands (dark sticks) with residues (green sticks) in the binding pocket of the 5-HT₁A receptor. Residues labeled in red, yellow, and green backgrounds represent the ionic bonds, hydrophobic interactions, and polar interactions existing between the residues and ligands, and the ionic bridges and hydrogen bonds are shown by the red dashed lines and green dashed lines, respectively.
ligands are discussed based on the MD simulations; second, the key residues making a high contribution to the ligands’ binding are identified by the per-residue energy decomposition; third, the hotspots consistently contributing to the binding of all the studied ligands are discovered by hierarchical clustering analysis; and finally, these identified hotspots are further validated by computational alanine scanning.

**Polar and nonpolar residues in the vicinity of the studied ligands.** Fig. 5 shows the representative snapshots of the 6 receptor–ligand complexes. The detailed binding modes of all the ligands are provided in the ESI† for Results and discussion. As shown, the studied ligands interacted with 9 residues in their close vicinity (<6 Å), including 5 polar (D1163.32, C1203.36, T1213.37, S1995.42, and Y3907.43) and 4 nonpolar (I1133.29, V1173.33, F3616.51, and F3626.52) residues.

Ionic (salt bridge) interaction was preserved between the positively charged nitrogen of the studied ligand and the carboxyl oxygen on D1163.32 of the receptor (Fig. 5), which was validated by the previous site-directed mutagenesis experiment.95 Moreover, –OH of Y3907.43 formed a hydrogen bond with the charged center of each ligand. As known, these abovementioned polar interactions could result in an enthalpy gain, which in turn drove the ligands’ binding in 5-HT1AR.45,89,96–98 Moreover, the hydrogen atom attached to nitrogen in the indole ring of each ligand formed a hydrogen bond with either T1213.37 or C1203.36 (both with a polar side chain). In addition, polar-substituted groups with an electron-withdrawing profile (–F or –CN) at the 5-position of indole rings formed a hydrogen bond with the –OH of S1995.42, which were, therefore, speculated to facilitate the ligands’ binding.99

As illustrated in Fig. 5, I1133.29, V1173.33, F3616.51, and F3626.52 made hydrophobic interactions with all the studied ligands via their nonpolar side chain. The side chains of F3616.51 and F3626.52 were below the distal indole ring of the ligand and almost perpendicular (83–89°) to the plane of the ligand’s heterocyclic ring, thereby fostering edge-to-face π–π interactions between the residues and ligands. Different from F3616.51 and F3626.52, V1173.33 resided above the plane of the ligand’s indole ring. I1133.29 in the vicinity of the arylpiperazine moiety stabilized the aromatic ring via the −CH–π interaction. As shown in Fig. 5, all these residues were located on TM3, TM5, TM6, and TM7 and played essential roles in stabilizing ligand binding via the hydrophobic interactions to 5-HT1aR.

**Key residues making a high energy contribution to the ligands’ binding.** To identify the key residues contributing to the binding of vilazodone and its analogues, the total energy was projected to each residue of 5-HT1aR. As illustrated in Fig. 6, the 21, 19, 21, 19, and 19 residues with a high absolute energy contribution (≥0.5 kcal mol⁻¹) were identified to favor the binding of the ligands 29, 36, 51, 14, 39, and 48, respectively. On the one hand, the energy contribution of different residues to the same ligand varied significantly. For example, Y962.64 made one of the highest contribution (−3.03 kcal mol⁻¹) to the binding of 29, whereas A932.61 made one of the lowest contribution (−0.55 kcal mol⁻¹). On the other hand, the contributions of the same residue to various ligands also differed greatly. For instance, the energy contributions of D1163.32 to the binding of 14 and 48 were −1.33 and −0.42 kcal mol⁻¹, respectively. Moreover, the energy contributions of the 9 residues (I1133.29, D1163.32, V1173.33, C1203.36, T1213.37, S1995.42, F3616.51, F3626.52, and Y3907.43), as discussed in the previous section, in the vicinity of the studied ligands were larger than −0.50 kcal mol⁻¹, and 10 residues (M932.60, A922.61, Y962.64, W1029E1, I1899E2, T1965.39, T2005.41, A303, W3586.48, and N3867.49) were discovered to favor the binding of at least 4 ligands (|energy contribution| ≥ 0.5 kcal mol⁻¹).

![Fig. 6](image-url) Per-residue binding energy decomposition of 6 ligands complexed with the 5-HT1A receptor. A union set of residues with an energy contribution (the absolute value) larger than 0.50 kcal mol⁻¹ to at least one studied ligand are displayed. Black and grey bars indicate the energy contributions larger and lower than 0.50 kcal mol⁻¹, respectively.
Identification of hotspots consistently favoring the ligands’ binding. Per-residue energy contribution decomposition analysis indicated that there were common features for the binding of all the ligands to human 5-HT_1A R. To characterize the key residues consistently favorable for the 6 ligands’ binding, hierarchical clustering analysis was employed to categorize the residues in terms of their energy contributions. As shown in Fig. 7, the residues in cluster A contributed much higher than other residues to the binding of all the ligands, which could be further grouped into cluster A_1 and cluster A_2 based on the clustering results. In total, the residues in cluster A provided 61.18%, 69.01%, 63.32%, 67.87%, 62.88%, and 63.73% contributions to the binding of 29, 36, 51, 14, 39, and 48, respectively. Among these, the energy contributions offered by the residues in cluster A_1 to the binding of ligands 29, 36, 51, 14, 39, and 48 amounted to 39.48%, 29.23%, 40.85%, 33.13%, 41.30%, and 28.89%, respectively.

Moreover, as shown in Fig. 6, T188_ECL2 contributed −2.70 kcal mol\(^{-1}\) energy to the binding of 36, but much less energy to the others. W102_ECL1, M92, N386, C109, and W358 contributed to the binding of 36 by −0.56 kcal mol\(^{-1}\) in total. From the tree cluster, it was apparent that the energy of cluster A might compensate the lower energy originating from W102_ECL1, M92, N386, C109, and W358 (as shown in Fig. 6D and 7). Y96 contributed much less to the binding of 48 (−0.07 kcal mol\(^{-1}\)), whereas the contribution of T188_ECL2 (−2.86 kcal mol\(^{-1}\)) might compensate the lower energy offered by Y96 (as shown in Fig. 6E and 7).

Verification of the identified hotspots by computational alanine scanning. As shown in Fig. 7, 22 hotspots were identified and could be further verified by computational alanine scanning. As shown in Fig. S8 (ESI†), the energy differences (\(\Delta G_{\text{mut-wild}}\)) of all the mutated residues were positive; this indicated that these alanine mutations disfavored the ligands’ binding. The \(\Delta G_{\text{mut-wild}}\) values of the residues in cluster A_1 and cluster A_2 are displayed in Fig. S8A and S8B-E, respectively. Generally, the more positive \(\Delta G_{\text{mut-wild}}\) indicated the more importance in terms of the corresponding residue for ligands’ binding. The binding free energies for all 6 ligands dropped dramatically when the residues in cluster A_1 were mutated to
alanine. For ligand 36, the mutation of Y96^{2.64} and I113^{3.29} to alanine had a subtle effect ($\Delta G_{mut-wild} = 0.97 \text{ kcal mol}^{-1}$) on the binding free energy (Fig. S8A, ESI†), but the alanine mutation of F112^{3.28} (Fig. S8D, ESI†) exerted obvious impacts on the binding of 36. Compared to the residues in cluster A3, D116^{3.32} in cluster A2 was also essential for the ligands’ binding due to the large variations ($\Delta G_{mut-wild} > 3.0 \text{ kcal mol}^{-1}$) resulted from the alanine mutation. In particular, the mutation D116^{3.32} to alanine for the ligand 51-bound complex resulted in the largest change ($\Delta G_{mut-wild} = 5.98 \text{ kcal mol}^{-1}$); this demonstrated that D116^{3.32} as a recognition site played a paramount role in the ligands’ binding (Fig. S8B, ESI†). Y390^{7.43} and F362^{6.52} also produced great effects on the binding of the 6 ligands. Mutations of their aromatic rings to a small side chain ($-\text{CH}_3$) decreased the hydrophobic interactions between the ligands and 5-HT_{1A}R, thereby reflecting the significant role of this interaction in the ligands’ binding. F362^{6.52} mutation resulted in a little change in the binding energy for ligand 39 ($\Delta G_{mut-wild} = 0.17 \text{ kcal mol}^{-1}$). There might be no interactions between indole and phenyl of F362^{6.52} in the 39 complex due to the long interaction distance (>4.5 Å) (Fig. 5E). The mutation of N386^{7.39} caused <2.0 kcal mol\(^{-1}\) energy change (Fig. 7B), but had modest effects on the binding free energy of all the ligands. These results showed the importance of N386^{7.39} in the ligand–receptor interaction, and alanine scanning mutation converted the polar side chain to a nonpolar side chain with the polar interactions between ligands and receptor decreased.

**Discovering the inhibitory mechanism of vilazodone and its analogues**

As illustrated in Fig. 8, 22 hotspots identified in this study were primarily mapped to TM2, TM3, TM5, TM6, TM7, ECL1, and ECL2. Among these hotspots, some (such as D116^{3.32} and N386^{7.39}) had also been discovered in site-directed mutagenesis studies\(^{39,79,100-103}\) and all were located in the general orthostatic binding pocket in GPCR\(^{80,104,105}\). In this study, three main interactions between vilazodone’s analogues and 5-HT_{1A}R were identified and are schematically represented in Fig. 8. These have been described as follows: (1) salt bridges and polar interactions with the piperazine group of vilazodone and its analogues. In particular, carbonyl of D116^{3.32} recognized vilazodone’s analogues via a salt bridge, and two residues (N386^{7.39} and Y390^{7.43}) stabilized the positively charged center of these analogues via polar interactions; (2) hydrogen bonds and hydrophobic interactions with the indole ring moiety of vilazodone and its analogues. In particular, the indole ring moiety engaged in hydrogen bonds with T121^{1.37}, C120^{1.36} and S199^{5.42}, and the residues (V117^{3.33}, T188^{5.22}, I189^{5.22}, T196^{5.39}, S199^{5.42}, T206^{5.41}, A303, W358^{6.48}, F361^{5.31}, and F362^{6.52}) around it provided energies to the binding of vilazodone’s analogues via hydrophobic interactions; (3) hydrophobic interactions and hydrogen bonds with the polar group on the arylpiperazine ring of vilazodone and its analogues. Y96^{2.64} formed a hydrogen bond with the ligands’ polar group on their arylpiperazine ring, and 5 residues (M92^{2.60}, A93^{2.61}, W102^{4.11}, F112^{3.28}, and I113^{3.29}) stabilized the ligands via hydrophobic interactions. The agonism of vilazodone and its analogues to 5-HT_{1A}R, as shown in Fig. 8, will provide substantial information to medicinal chemists working in the field of rational design of novel SPARIs for MDD treatment. First, polar or hydrophilic groups substituted in the aryl moiety need to point into 5-HT_{1A}R’s polar extracellular region. Second, a heterocyclic moiety structurally similar to 5-HT_{2A} with the electron-withdrawing group could increase SPARIs’ binding affinity to 5-HT_{1A}R. Third, the polar group on the butyl linker could enhance SPARIs’ interaction with some residues (N386^{7.39} and Y390^{7.43}) in 5-HT_{1A}R. Fourth, the strategy of balancing the arylpiperazine ring and heterocyclic moiety in target recognition could provide insights into the design of highly active SPARIs when binding to 5-HT_{1A}R.

**Conclusion**

The binding mechanism underlying the partial agonism of vilazodone and its analogues to 5-HT_{1A}R was revealed in this study using an integrated computational strategy. Herein, 22 residues of 5-HT_{1A}R were identified as hotspots, consistently favoring the binding of vilazodone and its analogues, and a common binding mode underlying their partial agonism to 5-HT_{1A}R was, therefore, discovered. Moreover, three main interaction features between vilazodone and 5-HT_{1A}R were revealed and have been schematically summarized. This identified binding mechanism underlying the partial agonism of vilazodone and its analogues to 5-HT_{1A}R will provide substantial insights into the design of highly efficacious SPARIs while binding to 5-HT_{1A}R.

**Conflicts of interest**

There are no conflicts to declare.
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