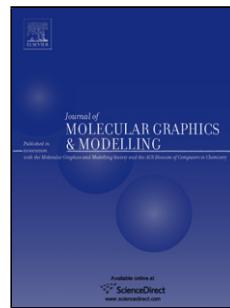


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Author: Xinli Duan Min Zhang Xin Zhang Fang Wang Ming Lei



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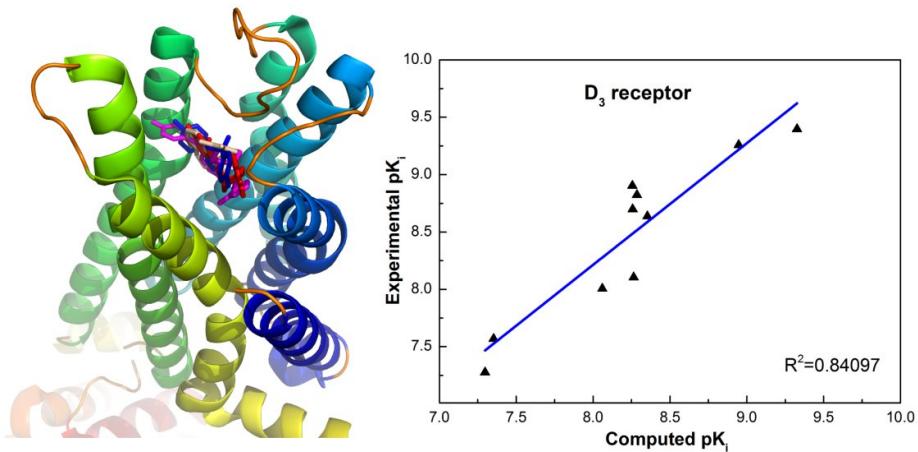
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Graphical Abstract

D₂, D₃, D₄ and 5-HT_{2A} receptors were modeled and refined by molecular dynamics simulations. Docking studies show the correlations between computed and experimental pKi is matched well. The homology models can be used as 3D templates for antipsychotic drug design and virtual screening in the future.



Highlights

- D₂-like and 5-HT_{2A} receptors were modeled using β2-adrenergic receptor as template.
- Homology model refined by molecular dynamics simulations.
- Docking studies show the correlation between computed and experimental pKi is good.
- Evidenced D₃R model is reliable by comparing modeled D₃R with crystal structure.
- These models will be useful in virtual screening and design of antipsychotic drugs.

Molecular Modeling and Docking Study on Dopamine D₂-like and Serotonin 5-HT_{2A} Receptors

Xinli Duan,^{¶a} Min Zhang,^{¶a} Xin Zhang,^a Fang Wang,^{*b} Ming Lei^{*a}

a. State Key Laboratory of Chemical Resource Engineering, Institute of Materia Medica, College of Science, Beijing University of Chemical Technology, Beijing, 100029, PR China

b. College of Life Science and Technology, Beijing University of Chemical Technology, Beijing, 100029, PR China

¶ Co-first author

* Corresponding authors:

Ming Lei

State Key Laboratory of Chemical Resource Engineering

Institute of Materia Medica, College of Science

Beijing University of Chemical Technology

Beijing, 100029

PR China

Phone: 86-10-6444-6598

Fax: 86-10-6444-6598

Email: leim@mail.buct.edu.cn

Fang Wang

School of Life Science and Technology

Beijing University of Chemical Technology

Beijing 100029

PR China

Phone: 86-10-6443-4815

Fax: 86-10-6443-4815

Email: wangfang@mail.buct.edu.cn

Abstract

Psychiatric disorders, such as schizophrenia, bipolar disorder and major depression, are paid more and more attention by human due to their upward tendency in modern society. D₂-like and 5-HT_{2A} receptors have been proposed as targets of antipsychotic drugs. Atypical antipsychotic drugs have been deemed to improve the treatment of positive, negative and extrapyramidal symptoms. Unfortunately, no experimental structures for these receptors are available except D₃ receptor (D₃R). Therefore, it is necessary to construct structures of D₂-like and 5-HT_{2A} receptors to investigate the interaction between these receptors and their antagonists. Accordingly, homology models of dopamine D₂, D₃, D₄ and serotonin 5-HT_{2A} receptors have been built on the high-resolution crystal structure of the β₂-adrenergic receptor, and refined by molecular dynamics simulations. The backbone root-mean-square deviation (RMSD) of D₃R model relative to crystal structure is 1.3 Å, which proves the reliability of homology modeling. Docking studies reveal that the binding modes of four homology models and their antagonists are consistent with experimental site-directed mutagenesis data. The calculated pKi values agree well with the experimental pKi ones. Antagonists with linear structures such as butyrophenones and benzisoxazolyl piperidines are easily docked into D₂-like and 5-HT_{2A} receptors. Polycyclic aromatic compounds have weaker affinity with four receptors. Homology models of D₂-like and 5-HT_{2A} receptors will be helpful for predicting the affinity of novel ligands, and could be used as three-dimensional (3D) templates for antipsychotic virtual screening and further drug discovery.

keywords: Dopamine receptor; 5-Hydroxytryptamine receptor; Homology modeling; Molecular dynamics; Docking.

1. Introduction

Psychiatric disorder is a serious system disease mainly characterized by behavior and mental activity disorders, which affects almost 1% of the world's population [1,2]. Schizophrenia is one of the most mysterious mental disorders, patients of schizophrenia die at 12-15 years old before the average age [3]. Every country is taking measures to prevent and treat schizophrenia in order to avoid social contradictions and relieve people's suffering [4]. The symptoms of schizophrenia consist of positive, negative and cognitive symptoms [5]. Positive symptoms include delusions, hallucinations, thought disorder and disorganized behavior, while negative symptoms include social withdrawal, apathy, anhedonia, alogia and behavioral perseveration. Finally, cognitive symptoms are characterized by disturbances in executive functions, working memory impairment, and inability to sustain attention.

Pharmacotherapy is the main treatment for the psychotic symptoms of mental conditions such as schizophrenia, bipolar disorder and major depression [6]. Antipsychotic drugs are usually classified into two major groups: typical antipsychotics, all of which are dopamine D₂ receptor blockers [7,8], are effective for treating the positive symptoms but relating with extrapyramidal symptoms (EPS), tardive dyskinesia and other movement disorder side effects [9]. Another group, atypical antipsychotics, are considered as multitarget profiles [6,10,11]. They are used to treat positive and negative symptoms with a lower induction of EPS and other new side effects (weight gain, metabolic adverse). As the origin of schizophrenia remains unclear, current researches in this field are mainly focused on its neurogenesis pathology and genetics [12]. Many evidences including pharmacology studies indicate that schizophrenia is associated with an imbalance of the central nervous system (CNS) [13]. Chlorpromazine, the first antipsychotic drug developed in 1952, boomed the research & development (R&D) of typical antipsychotics. In the 1970s, researchers developed clozapine owing to typical antipsychotics failing in treating negative symptoms and cognitive disorders of [14,15]. From the beginning of clozapine as early atypical antipsychotics, drugs such as loxapine and clothiapine were developed based on skeleton benzodiazepine ring [16,17].

Interestingly, one of the most important features of atypical antipsychotics is that they have a high blocking K_i(5-HT_{2A})/K_i(D₂) ratio [9,18,19]. It is related to the strong 5-HT_{2A} receptor antagonism and weak D₂ receptor antagonism that atypical antipsychotics might increase the release

of dopamine (DA) in prefrontal cortex (PFC) instead of nucleus accumbens [20]. DA, which could be able to directly activate 5-HT_{1A}, 5-HT_{2C}, and 5-HT₃ receptors, is a partial agonist of 5-HT_{2A} receptor and could be expressed in DA neuron. Serotonin (5-HT) and DA work together in the pathogenesis of various mental disorders [21]. Thus, drugs with antagonistic effects of D₂ and 5-HT_{2A} receptors can significantly improve the treatment of negative symptoms of psychosis and reduce EPS side effect [22]. Targeting 5-HT_{2A} and D₂ receptors, some antipsychotics were successfully discovered such as risperidone, sertindole and aripiprazole.

D₃ receptor antagonists, which could inhibit EPS without producing metabolic adverse effects [23], affect electrical activity of DA neurons in the ventral tegmental area and enhance cortical DA and acetylcholine release. This mechanism was proposed to explain the fact that D₃ receptor antagonists could improve the treatment of negative symptoms [24,25]. D₃ receptor has been considered as one of the key targets of antipsychotic drug. ABT-925, the only selective D₃ receptor antagonist, was in clinical phase II in patients with acute exacerbation schizophrenia [26]. The experimental basis for efficacy in social and cognitive behaviors of selective D₃ receptor antagonists is meaningful and still rapidly growing [27,28,29]. It would be useful to screen new selective D₃ receptor antagonists especially in treating negative and cognitive symptoms.

Linz et al., who found that elevated levels of D₄ receptor in the post-mortem brains of schizophrenic patients relative to controls, suggested that D₄ receptor and schizophrenia had a connection [30]. Antipsychotic compounds targeting D₄ subtype was observed that clozapine prefers to bind with D₄ receptor. As a consequence, the development of selective D₄ receptor antagonists is also becoming a challenging field in atypical antipsychotics discovery [31].

The serotonergic system plays an important role in the regulation of the PFC and is strongly associated with emotional control, cognitive behavior, and working memory [32,33]. It has been reported that 5-HT_{2A} receptor antagonists could increase the firing rate of midbrain dopaminergic neurons in a state-dependent manner [34]. Moreover, 5-HT_{2A} receptor antagonists increase the activity of nigrostriatal DA-containing neurons following moderate D₂ receptor blockade associated with antipsychotic drugs [35]. Therefore, 5-HT_{2A} receptor antagonism could enrich the atypical antipsychotic profile although the latest research observed that mice genetically depleted of brain serotonin did not display a depression-like behavioral phenotype [36].

DA and 5-HT receptors belong to the family of G-protein coupled receptors (GPCRs) which are

one of the most important protein families. They are similar in structures with seven transmembrane helices domain (TM helix I-VII), extracellular loops (ECL) and intracellular loops (ICL). There are five different receptor subtypes of DA receptors which could be divided into two families, D₁-like (D₁ and D₅ subtypes) and D₂-like (D₂, D₃ and D₄ subtypes) [37,38,39,40,41,42]. The mental disorder diseases, such as schizophrenia, major depression and bipolar disorder, are associated with D₂-like receptors [43]. Among three D₂-like receptors, only D₃ subtypes crystal structure was resolved due to complicated structures of GPCRs [44]. In 2013, the crystal structures of 5-HT_{1B} and 5-HT_{2B} receptor were also released [45,46]. For the structures of other D₂-like receptors and serotonin receptors, Javitch and Shi modeled the D₂ structure based on the high resolution of bovine rhodopsin. Molecular modeling and mutated to cysteine in ECL2 of D₂R (D₂R denotes D₂ receptor) revealed ECL2 likely contributes to the binding site and probably in other aminergic GPCRs as well [47]. Yuriev et al. constructed homology models of dopamines (D₂R, D₃R and D₄R) and serotonin (5-HT_{1B}, 5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) based on β₂-adrenergic receptor and addressed the required modeling of ECL2 implicating in ligand binding [48]. Chen and Wang et al. employed molecular dynamics (MD) simulations to identify the predicted D₂ receptor structure. Homology models of the protein were developed on the basis of four available receptor crystal structures. Docking studies revealed the possible binding mode and five other residues (Asp72, Val73, Cys76, Leu183 and Phe187) which were responsible for the selectivity of the tetralindiol derivatives [49]. Reichert et al. reported homology models for both human D₂ and D₃ receptors in complex with haloperidol. The receptor models show that although D₂ and D₃ share highly similar folds and three-dimensional (3D) conformations, the slight sequence differences in extracellular loop regions result in the binding cavity in D₂R being shallower than that in D₃R, which might explain that some large ligands could bind to D₃R with greater affinity than D₂R [50]. Bucolo et al. emphasize Reichert's point using homology modeling of D₂R and D₃R integrated with MD simulation and docking evaluation [51].

In general, previous theoretical researches on dopamine and serotonin receptors provide insights for antipsychotic drug discovery, but the exact structures of D₂, D₄ and 5-HT_{2A} are still unknown. The latest crystallized β₂-adrenergic receptor has been already investigated as an alternative template to model other Class-A GPCRs for potential drug discovery applications , which may be better as a basis for homology model generation [52]. In this paper, 3D structures of D₂, D₃, D₄ and 5-HT_{2A} receptors were generated by homology modeling based on the latest β₂-adrenergic receptor, and were

optimized using MD simulations. The antagonists of the DA and 5-HT_{2A} receptors were docked into their respective refined structures to investigate the interactions between the ligands and receptors. The homology modeling, molecular dynamics and docking studies of D₂-like and 5-HT_{2A} receptors could provide lights for the structure-based antipsychotic drug virtual screening and discovery in the future.

2. Methods

2.1. Homology modeling

Homology modeling was performed using SYBYL-X 1.3 software package [53]. The human sequences of receptors were retrieved from the National Center for Biotechnology Information (NCBI) protein database (<http://www.ncbi.nlm.nih.gov/gene>). The retrieved protein sequences of D₂, D₃, D₄ and 5-HT_{2A} receptors are NP_000786.1 , NP_000787.2, NP_000788.2 and NP_000612.1, respectively. All receptors were modeled using crystal structure of β₂-adrenergic receptor as the template by FUGUE searching, which was obtained at 2.4 Å resolution (PDB code: 2RH1). The N terminus (residues 1 to 28) and the majority of the C terminus (residues 343 to 365) were so disordered that they were not resolved in the crystal structure of 2RH1 [54]. The sequence identities between 2RH1 with D₂, D₃, D₄ and 5-HT_{2A} receptors are more than 30% in the structurally conserved regions. The sequence alignment between the template receptor and the target receptors was performed through RUGUE module using PSI-BLAST method [55]. A multiple sequence format file was generated. The sequences and structures are structurally aligned using ORCHESTRAR program using BATON method [56]. All receptors were built by recognizing structure of conserved regions (SCR) though ORCHESTRAR module, searching the gaps and adding side chains. The disulfide bridge was built by Biopolymer in SYBYL-X 1.3.

2.2. Molecular Dynamics

MD simulations on homology models of D₂, D₃, D₄ and 5-HT_{2A} receptors were carried out using AMBER 12.0 package [57] and AMBER FF10 force field. The periodic boundary conditions, and cuboid box of TIP3P water [58] at constant temperature (300 K) and constant pressure (1 bar) were employed in the systems. The minimum distance of a protein atom to the edge of the

rectangular water box was 10 Å. The protonation state of ionizable groups was used at the neutral pH. Cl⁻ counterions were added to keep the four systems in neutral condition. The temperature was controlled by Langevin dynamics with the collision frequency $\gamma=1.0 \text{ ps}^{-1}$ [59]. The system pressure was maintained by Berendsen's coupling algorithm [60]. The long-range electrostatic interactions were treated by using Periodic Boundary Conditions (PBC) and Particle Mesh Ewald method [61]. The short-range nonbonded interactions were cut off at 12 Å. Bond lengths involving H atoms were constrained using the SHAKE algorithm [62]. First stage minimization was performed using 1000 cycles of the steepest descent algorithm and 1000 cycles of the conjugate gradient following. After energy minimization, the system was gradually heated from 0 K to 300 K. After 100 ps equilibration, 2 ns MD simulation was continued using P traj module for further analysis.

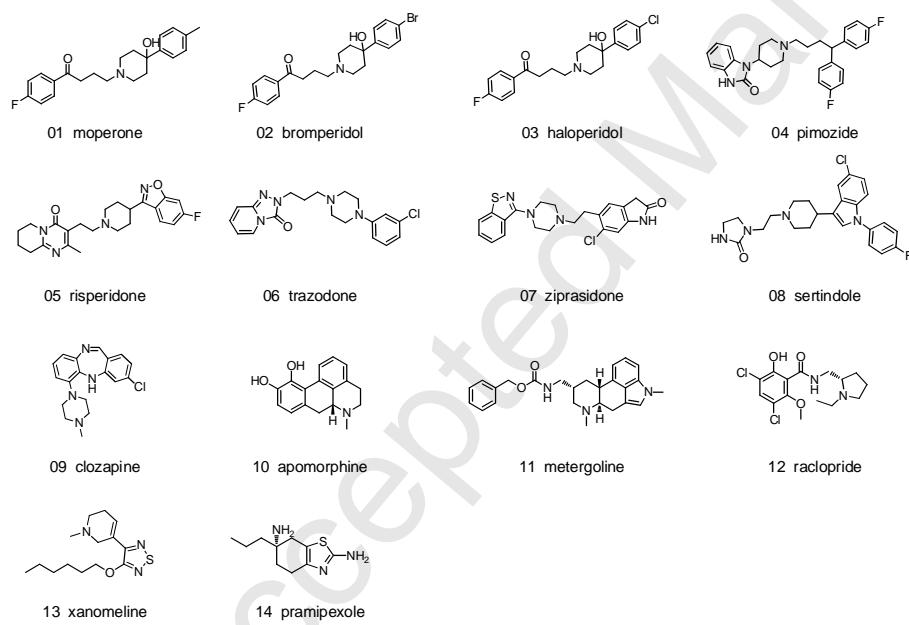


Fig. 1. Antagonists of D₂-

like and 5-HT_{2A} receptors.

2.3. Molecular Docking

Molecular docking method is widely used to predict the binding mode of ligand with protein. In this study, the homology models of D₂, D₃, D₄ and 5-HT_{2A} receptors were used to dock with molecules in order to validate the reliability of optimized homology models. The antagonists were obtained from the GLIDA database (<http://pharminfo.pharm.kyoto-u.ac.jp/services/glida/>). The structures of antagonists (see Fig. 1) were retrieved from PubChem [63] and minimized in SYBYL-X

1.3 program [53] using Tripos force field with Gasteiger-Hückel charges.

Before performing ligand docking, it is critical to search for the binding pockets of prepared proteins. In this paper, docking studies were performed using Surflex-Dock implemented in SYBYL-X 1.3. Ligands were docked into corresponding receptor's binding site automatically using a protomol-based method and an empirically derived scoring function [64,65]. The protomol is constructed by merging sticky spots into a pocket based on a set of protein-free spheres [66]. The protomol can be generated automatically or defined based on a cognate ligand or known active site. Residues mode was adopted to generate the protomol in Surflex-Dock program. This mode defines the active site by considering a reasonable distance around selected residues. It was reported that Asp3.32 is one of the most conserved amino acids among aminergic GPCRs and is supposed to form a reinforced hydrogen bond with the basic amine of the ligand [43]. So we chose the pocket including residue Asp3.32 as the binding site. Then we generate the protomol automatically based on the residues belong to the pocket we chose above. In addition, parameters of threshold and bloat values could significantly affect the size and extent of the generated protomol.

The Surflex-Dock scoring function, which includes several terms such as hydrophobic, polar, repulsive, entropic, solvation and crash, is based on the binding affinities of protein-ligand complexes. Total score could be expressed as $-\log(K_d)$, herein Surflex-Dock scores (total scores) were expressed in $-\log(K_d)$ to represent binding affinities and evaluate the docking results. K_d represents the dissociation constant of ligand with receptor.

Another docking method (AutoDock 4.2 software package) was also employed to evaluate the accuracy of the homology models and molecular docking methods above [67,68]. The AutoDockTools 1.5.4 package was employed to generate the docking input files. Possible favorable interaction regions of residues in possible binding sites were determined by Lamarckian Genetic

Algorithm. Prepared ligands were docked within the grid region (grid size was set to 40*40*40

points with grid spacing of 0.375 Å), which was set to the center of active gorge of receptors. Ten independent docking runs were carried out for each ligand.

3. Results and discussion

3.1. Homology modeling

D_2 , D_3 , D_4 and 5-HT_{2A} receptors have a wide distribution in the nervous system. Antipsychotic drugs have binding potency with dopamine receptors and hydroxytryptamine receptors to treat mental diseases. Till now the structures of D_2 , D_4 and 5-HT_{2A} receptors are not resolved, a homology model could be useful to study the pharmacology of antipsychotic drugs interacting with D_2 , D_3 , D_4 and 5-HT_{2A} receptors of the human brain. In this study, four receptors were built to predict the interaction modes between target proteins and the tested compounds. All homology models were built based on the multiple structure-sequence alignment method utilizing all target receptors.

In D_2 receptor, only the conserved disulfide bridge was modeled, and this disulfide bond between Cys107 in the third helix and the conserved Cys182 in ECL2 connecting with transmembrane (TM) dopamine. Another disulfide bridge was not modeled because two conserved cysteine residues (Cys399 and Cys401) are too close to form a stable disulfide bond. As ECL2 is supposed to contribute to the binding site in D_2 and probably in other aminergic GPCRs, this modeled disulfide bond is crucial to the structural integrity and function of many GPCRs. One disulfide bridge was modeled in D_3 receptor involving residues Cys103 and Cys181 of ECL2 and TM3. In D_4 receptor, the disulfide bond connecting TM3 (Cys108) and ECL2 (Cys185) was modeled. Another disulfide bond between Cys148 in TM3 and Cys227 in ECL2 was built in 5-HT_{2A} receptor.

Two repeating torsion angles along the backbone chain, Φ and Ψ , were used to describe the conformations of the built models. By comparing the Φ and Ψ dihedral angles of the homology models, the statistical Ramachandran maps were obtained using ProTable module to evaluate the backbone conformation of the constructed models and detect dissatisfactory residues [53]. Conformationally unreasonable residues fall in the disallowed regions of the statistical Ramachandran maps. Glycine residues often locate in disallowed regions. As glycine contains two hydrogen atoms in α -positions, one hydrogen atom in the side chain has an extremely small van der Waals radius and is more flexible than other residues. As shown in Fig. 2, percentages of allowed region residues are 99.77%, 99.50%, 98.57% and 99.22% for $D_2\text{R}$, $D_3\text{R}$, $D_4\text{R}$ and $5\text{-HT}_{2A}\text{R}$, respectively. Most of residues are located around Φ value of -60° and Ψ value of -45° . This phenomenon agrees well with the fact that four models are mainly made up by α helices.

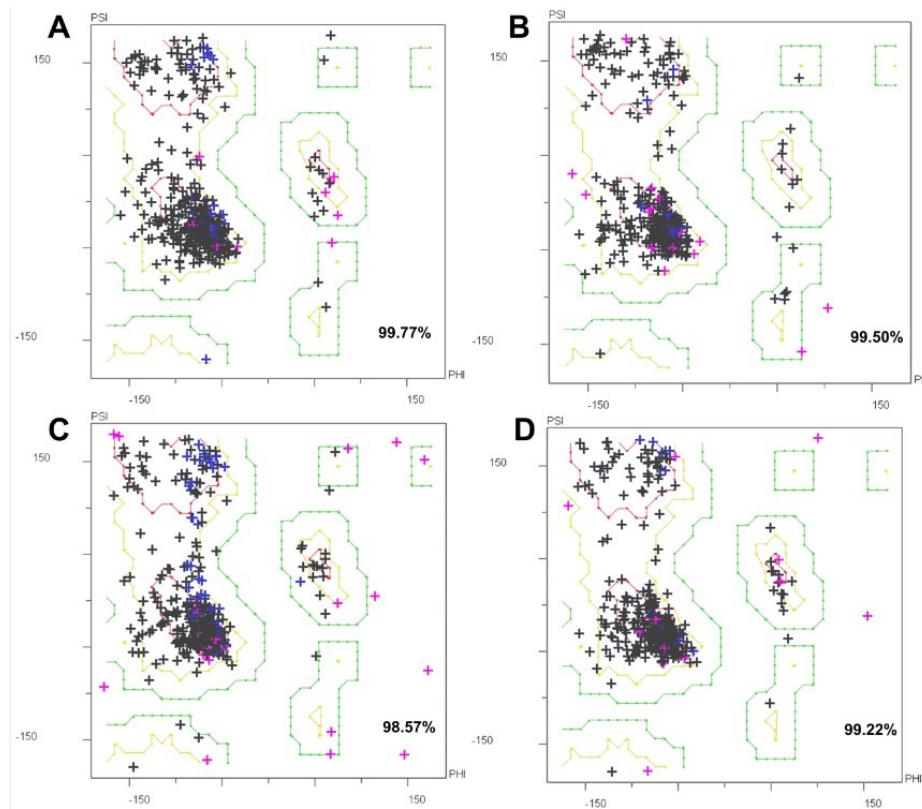


Fig. 2. Ramachandran plots of D₂R (A), D₃R (B), D₄R (C) and 5-HT_{2A}R (D). Different colored areas indicate “most favored” (red), “additional allowed” (yellow), “generally allowed” (green) and “disallowed” (white) regions. Residue color: proline (blue), glycine (magenta), other residues (black).

Table 1

The residues of disallowed region in the D₂, D₃, D₄ and 5-HT_{2A} receptors.

Receptors	The disallowed residue
D ₂ R	His313
D ₃ R	Glu280, Gly313
D ₄ R	Gly17, Gly99, Gln220, Gly259, Gly275, Gly291
5-HT _{2A} R	Gly141, Gly189, Ser221

All residues in disallowed regions are listed in Table 1. His313 of D₂R, Glu280 of D₃R, Gln220 of D₄R and Ser221 of 5-HT_{2A}R are situated in ICL3, which was replaced by a lysozyme (T4L) in

order to stabilize the protein structure [54]. ICL3 parts of proteins are far away from the binding sites of D₂, D₃, D₄ and 5-HT_{2A} receptors. These residues in disallowed regions should have little impact on proteins' function. Accordingly, these constructed four models should be conformationally reasonable and could be used for further MD simulations or virtual screening.

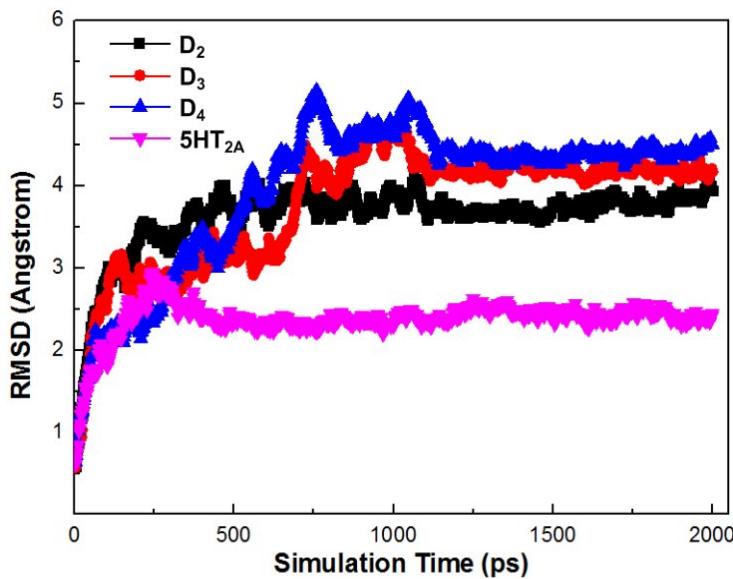


Fig. 3. Backbone root-mean-square deviations (RMSDs) of D₂, D₃, D₄ and 5-HT_{2A} receptors in molecular dynamics simulations. RMSDs are relative to corresponding starting structures of homology models (D₂ (black squares), D₃ (red circles), D₄ (blue triangle) and 5-HT_{2A} (magenta triangle)).

3.2 Molecular dynamic simulations

A 2.0 nano-second (ns) molecular dynamic (MD) simulations were performed for D₂, D₃, D₄ and 5-HT_{2A} homology models in a water environment that could reproduce the biological milieu in which these four GPCRs are located, in order to further refine and discriminate their structural difference. As shown in Fig. 3, the root-mean-square deviations (RMSDs) of proteins relative to the corresponding starting homology models, reach a relative stable conformation after 1.1 ns, 1.1 ns, 1.1 ns and 500 ps at a value of approximately 3.8 Å, 4.0 Å, 4.3 Å and 2.4 Å for D₂, D₃, D₄ and 5-HT_{2A} receptors, respectively. Total energies (denoted as E_{tot}) and potential energies (denoted as E_p) of four systems gradually become stable during MD simulations (see Fig. S1 and S2 in Supporting

Information SI). Energies of D₄R are slightly lower compared to those of other D₂-like subtypes and 5-HT_{2A} receptors. Herein conformations at 2.0 ns were extracted from the trajectories and were used for docking studies.

3.2.1. Comparison of D₃ receptor model with crystal structure

The crystal structure of D₃R (PDB code: 3PBL, resolution: 2.89 Å) was released in the 2010s. The structure indicates that the binding pocket is controlled by side chains of helices I, II, III, VII, and ECL2 [44]. In order to evaluate the reliability of homology modeling method used in this study before and after dynamics simulation with the D₃R crystal structure 3PBL, the calculated backbone RMSDs of D₃R homology model and the final structure after 2.0 MD run relative to crystal structure are 1.3 Å and 2.2 Å, respectively (see Fig. 4). The RMSD value is increased after MD run because some helical structures near extracellular region of TM4 skewed outwards after MD simulation as shown in Fig. 4B. But TM3, TM5 and TM6 parts close to the binding site align well with the crystal structure. In Fig. 4A, seven TM helices of D₃R homology model before MD simulation matched well with the crystal structure. The small RMSD demonstrates a high structural similarity of D₃R homology model and crystal structure, which proves the reliability of the homology modeling used in this study and implies reasonable structures of built D₂-like and 5-HT_{2A} receptors.

3.2.2. Docking eticlopride into optimized D₃R model

We docked eticlopride into the D₃R model after MD simulation by Surflex-Dock module of SYBYL-X 1.3. The docking result indicates that eticlopride's binding mode with D₃R model is similar to that in crystal structure. That is to say, the eticlopride binding conformation could be reproduced perfectly. The low RMSD (0.7 Å) and internal H-bonds were calculated as shown in Fig. 5. Andrei et al also docked agonists into both a new homology modeled receptor and the A_{2A} AR crystallographic structure and reproduced good results [69]. This demonstrates that the binding pocket of D₃R was built successfully.

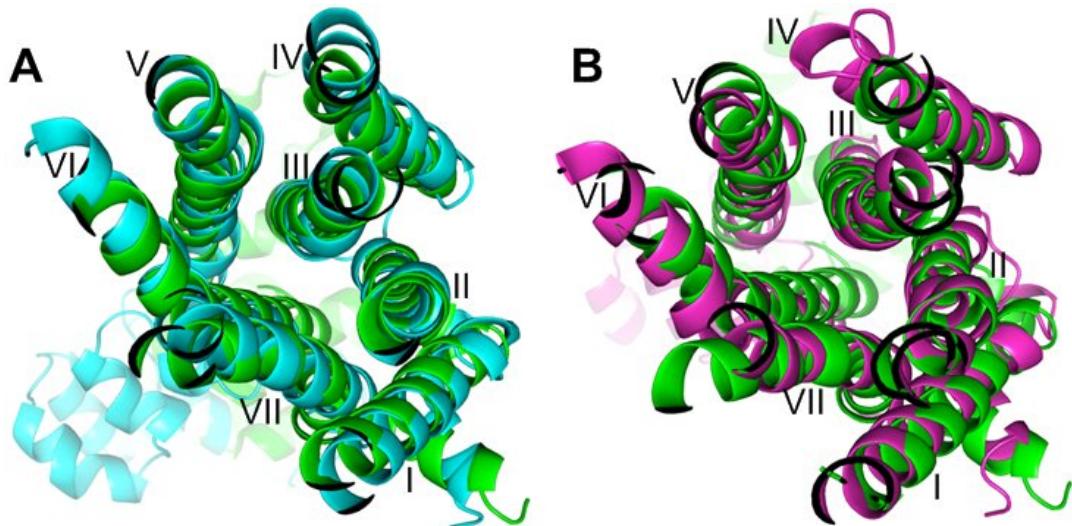


Fig. 4. Structural alignment of crystal structure (3PBL in green cartoon) with the homology model of D₃R homology model colored by cyan cartoon (A) and that after MD simulation colored by magenta cartoon (B).

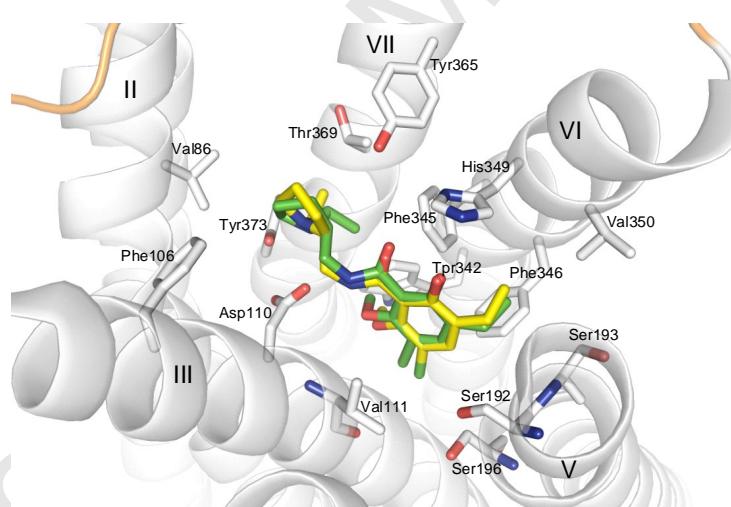


Fig. 5. Superimposition of eticlopride (C atoms are colored by yellow stick) docked into D₃R model by Surflex-Dock and that (C atoms are colored by green stick) of the crystal structure (3PBL).

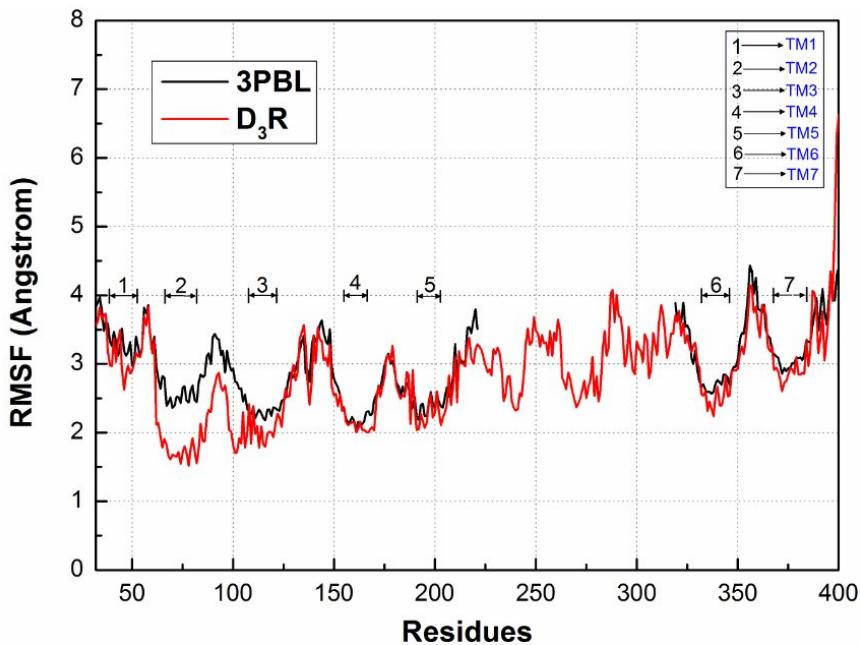


Fig. 6. Plots of the root-mean-square fluctuations (RMSFs) of D₃R homology model compared with experimental RMSF (PDB code: 3PBL) converted from thermal B-factors per residue.

3.2.3. RMSF and B-factors of D₃R

Both root-mean-square fluctuations (RMSFs) based on MD simulation and thermal B-factors based on experiment could provide a profile of the D₃R structural flexibility. The relationship of RMSFs and B-factors is that B-factors equal to $8\pi/3$ (RMSFs)². RMSFs reflect the flexibility of protein structure. As shown in Fig. 6, the RMSF values of Arg222-Arg318 in black curve are missed because a part of ICL3 (Val213-Gln329) regions is not resolved by experiment. Most of ICL3 (Arg222-Arg318) in 3PBL are replaced with T4-lysozyme to crystallize the 3PBL structure [44]. We predicted the RMSFs of ICL3 in D₃R, and the extents of flexibilities are higher than other helix and loop regions. The predicted results exactly show that the residues in ICL3 are less stable than other regions. RMSFs of 3PBL in Thr62-Cys102 are higher than those of D₃R but the trend of red curve (this work) is similar to black one (experimental work). The RMSFs of Arg58-Thr64 (from 3.8 Å to 1.5 Å) change largely as this region belongs to ICL1. The loops in protein own higher flexibility than helices etc.. This also explains the peak position of Leu89-Cys102 (ECL1) residues region. Strands in Tyr66-Tyr88 region fluctuate little as these residues are composed of TM1. The RMSFs of other regions except Thr62-Cys102 in D₃R have similar curves and main peaks in the same positions with

experimental data. Meanwhile, the flexibilities of residues in the loop of D₃R are higher than those in helix regions as anticipated. Strands at stationary regions constitute the base of RMSF curve. The residues in peak regions are located on ICLs or ECLs. Combining with calculated RMSDs above, RMSF analyses imply the reasonability of refined D₃R homology model.

Table 2

RMSDs (unit: Å) of C α atoms of transmembrane (TM) helices of D₂, D₃, D₄ and 5-HT_{2A} receptors after MD simulations from corresponding homology models.

TM helix	D ₂ R	D ₃ R	D ₄ R	5-HT _{2A} R
I	0.636	0.941	0.699	1.320
II	1.531	1.296	1.245	1.742
III	1.676	1.471	1.180	0.720
IV	2.290	1.278	1.703	0.771
V	2.846	2.372	2.209	1.233
VI	1.593	1.349	0.927	1.305
VII	0.408	0.950	1.209	1.239

3.2.4. Comparison in structures of four receptors before and after MD simulations

Seven TM helices of receptors before and after MD simulations have been compared by alignment of C α (see Fig. 7A). The calculated RMSDs of C α atoms of TM helices of D₂, D₃, D₄ and 5-HT_{2A} receptors after MD simulations from corresponding homology models are listed in Table 2. The RMSDs of TM helix V of D₂R, D₃R and D₄R are larger than other TM helices (see Fig. 7B). This is in that TM helix V of D₂R, D₃R and D₄R are located at the cytoplasmic end which is far away from the center of the receptors. The RMSDs of TM helix IV of D₂R and D₄R are 2.290 Å and 1.703 Å, respectively. The other TM helices of four receptors align well with each other. It indicates the reasonability of built homology models of four receptors and those after MD simulations.

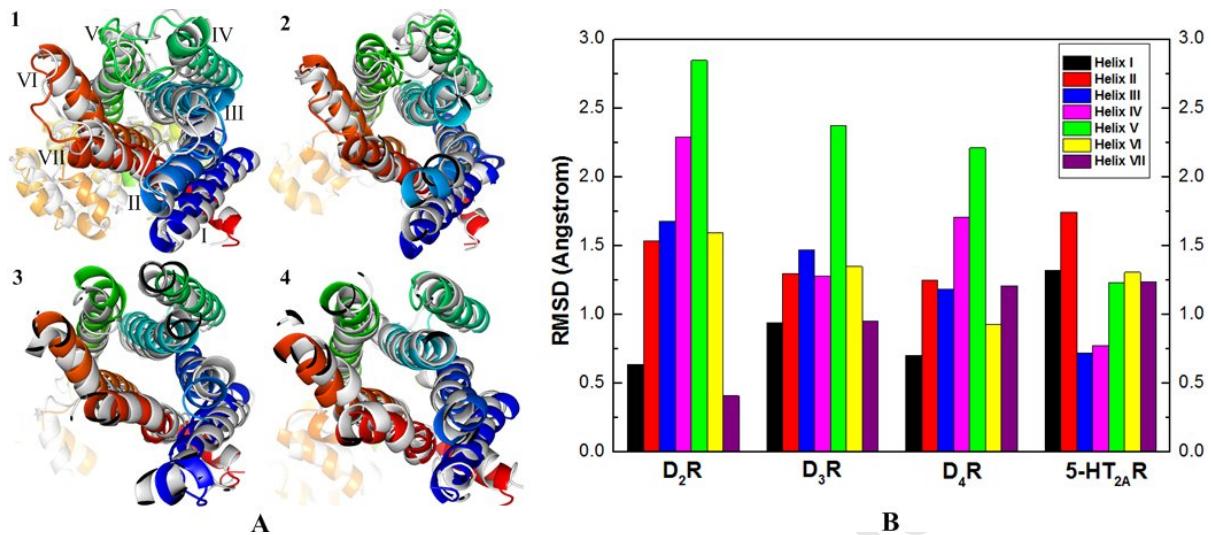


Fig. 7. A) Superimposition of D₂R (A1), D₃R (A2), D₄R (A3) and 5-HT_{2A}R (A4) before (colored by rainbow cartoon) and after (colored by white cartoon) MD simulation. B) RMSDs of TM helices of D₂, D₃, D₄ and 5-HT_{2A} receptors after MD simulation from corresponding homology models (helix I (black color), II (red color), III (blue color), IV (black color), V (green color), VI (yellow color) and VII (purple color)).

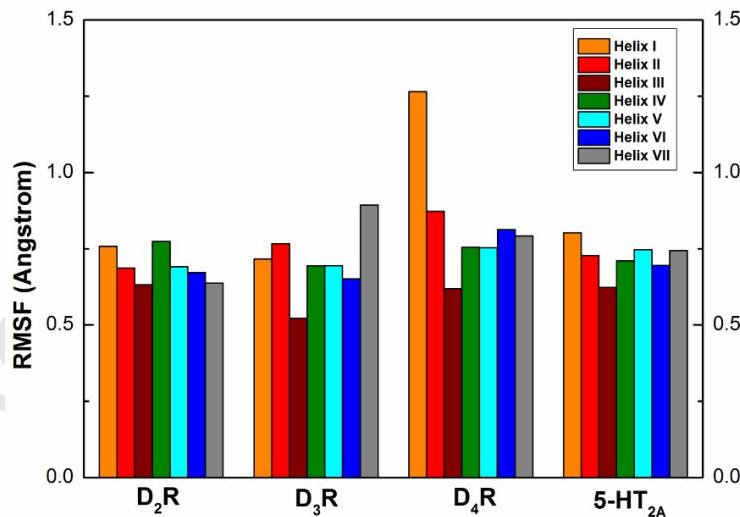


Fig. 8. RMSFs of TM helices of D₂, D₃, D₄ and 5-HT_{2A} receptors in the last 500ps MD simulations from corresponding average structures (helix I (orange color), II (red color), III (wine color), IV (olive color), V (cyan color), VI (blue color) and VII (grey color)).

3.2.5. RMSFs of TM helices in D₂, D₃, D₄ and 5-HT_{2A} receptors

RMSFs of TM helices were calculated for D₂, D₃, D₄ and 5-HT_{2A} receptors in the last 500 ps MD simulations from corresponding average structures as shown in Fig. 8. The RMSF of TM helix I of D₄R is 1.25 Å due to no N-terminal connecting with helix I in D₄R. The RMSFs of other helices in four receptors are around or below 0.75 Å. That implies stability of conformations of D₂R, D₃R, D₄R and 5-HT_{2A}R in the last 500 ps MD runs. The analysis on RMSFs of TM helices in D₂, D₃, D₄ and 5-HT_{2A} receptors illustrate that the refined homology models could be used for the following docking studies.

3.3. Docking studies

Based on refined models after MD simulations above, we docked antagonists into the binding pockets of D₂-like and 5-HT_{2A} receptors. The binding pocket should be formed mainly by helix II, III, V, VI and VII. Surflex-Dock module was used as well as that used in the docking test of eticlopride into D₃ homology model. Total Score are expressed in $-\log(K_d)$ to evaluate the docking results. K_d, which represents a dissociation constant of a ligand from protein, equals approximately to the value of K_i. Total Score of antagonists docked in D₂-like and 5-HT_{2A} receptors correlates with their corresponding experimental pK_i values (Table 3). The experimental K_i values (retrieved from PDSP K_i database, <http://pdsp.med.unc.edu/pdsp.phe>) of antagonists were compared with the predicted values (see Table S1 in SI). Fig. 9 shows a good correlation by linear regression coefficients. The R² of D₂, D₃, D₄ and 5-HT_{2A} receptors are 0.913, 0.841, 0.820 and 0.940, respectively. Meanwhile, AutoDock software was also used to cross-validate the docking results. The correlation of predicted pK_i and experimental pK_i values targeting with D₂ receptor is using AutoDock shown in Fig. 10 (see Fig. S3 of SI for D₃, D₄ and 5-HT_{2A} receptors). The calculated pK_i of D₂ receptor agreed with experimental pK_i ($R^2=0.79$) using AutoDock (the R² values of D₂ receptor using Surflex-Dock is 0.91). It is obvious that the calculated results using AutoDock and those using Surflex-Dock method in this manuscript are not different. This also indicates that the docking method is reliable in this work. Structure-based docking calculations are strictly related to the reliability of the receptor structures. Hence, the good correlations of experimental and computed K_i values for four receptors suggests that the binding pockets of homology models are successfully modeled and further reflects the reliability of the refined models.

Table 3

The experimental pK_i values (nM) and total scores of antagonists with four receptors using Surflex-Dock.

antagonists	Ki (nM)/Total score of D ₂	Ki (nM)/Total score of D ₃	Ki (nM)/Total score of D ₄	Ki (nM)/Total score of 5-HT _{2A}
01 moperone	-	9.3/8.9	7.6/7.9	-
02 bromperidol	8.8/8.8	8.9/8.3	7.3/8.0	-
03 haloperidol	8.7/8.7	9.4/9.3	7.7/8.2	7.3/6.7
04 pimozide	8.8/9.1	8.6/8.4	8.7/8.8	7.7/7.6
05 risperidone	8.6/8.1	8.7/8.3	7.7/8.0	9.2/9.0
06 trazodone	-	-	-	7.4/6.9
07 ziprasidone	8.3/7.8	8.1/8.3	-	9.6/9.7
08 sertindole	8.1/7.6	8.0/8.1	7.6/7.5	9.4/9.0
09 clozapine	7.5/7.3	7.3/7.3	7.4/7.1	7.8/7.7
10 apomorphine	7.1/6.2	7.6/7.4	8.4/8.4	6.9/6.9
11 metergoline	-	-	-	8.6/8.4
12 raclopride	8.2/8.4	8.8/8.3	5.8/6.0	-
13 xanomeline	-	-	-	6.9/7.1
14 pramipexole	5.8/4.6	-	7.5/7.2	-

Antagonists used in this docking study could be classified into four categories according to their structures: Butyrophenones including 01, 02 and 03 compounds, benzisoxazolyl piperidines including 04, 05, 06, 07 and 08, polycyclic aromatic compounds such as 09, 10 and 11, and other compounds containing 12, 13 and 14 (see Fig. 1). The binding modes of haloperidol (03), risperidone (05), clozapine (09) with four receptors were investigated in detail.

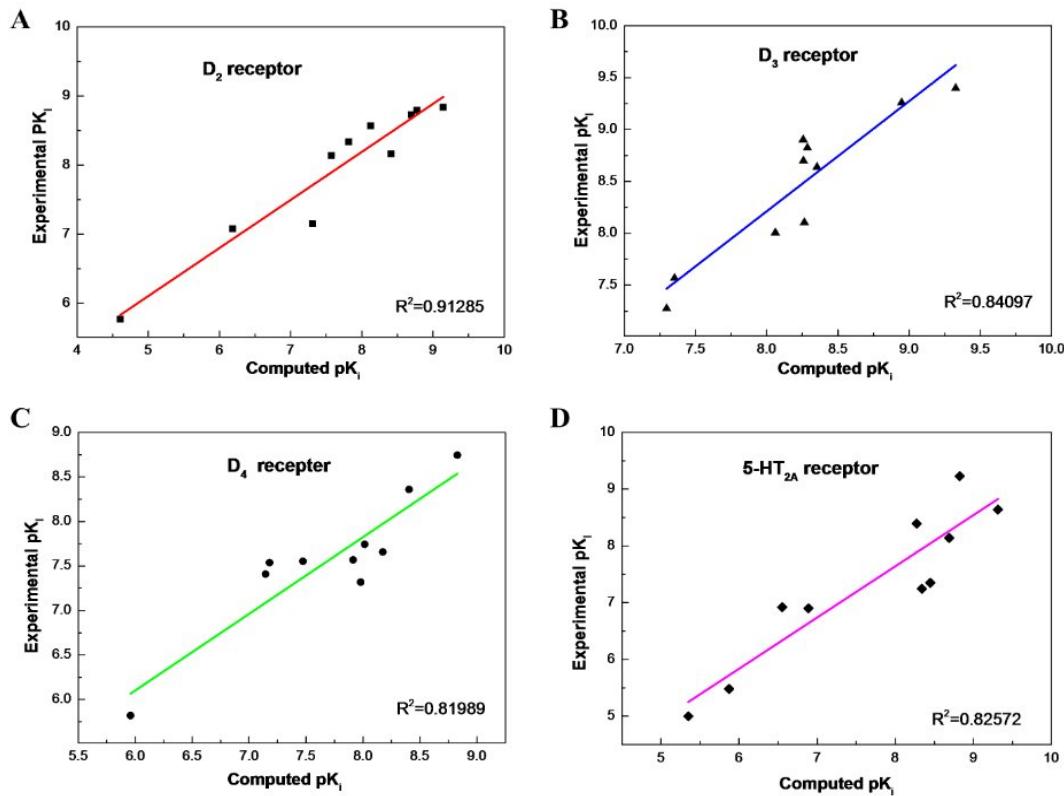


Fig. 9. Correlation of predicted pKi values using Surfflex-Dock and experimental pKi values of antagonists binding with D₂R (A), D₃R (B), D₄R (C) and 5-HT_{2AR} (D).

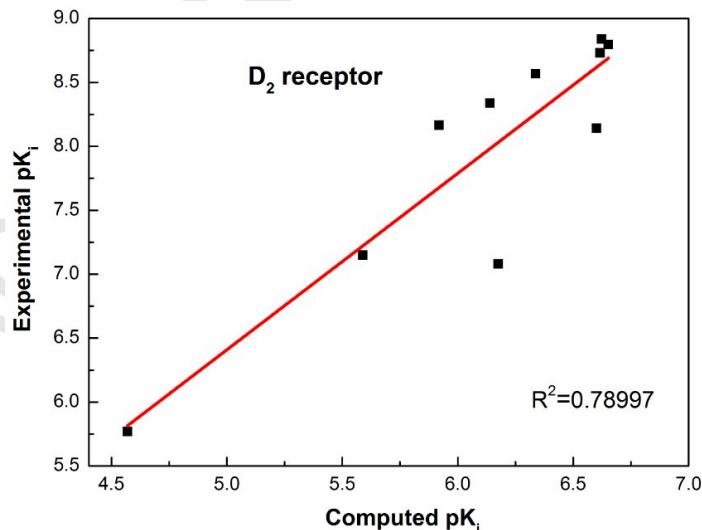


Fig. 10. Correlation of predicted pKi values using AutoDock 4.2 and experimental pKi values of antagonists binding with D₂R.

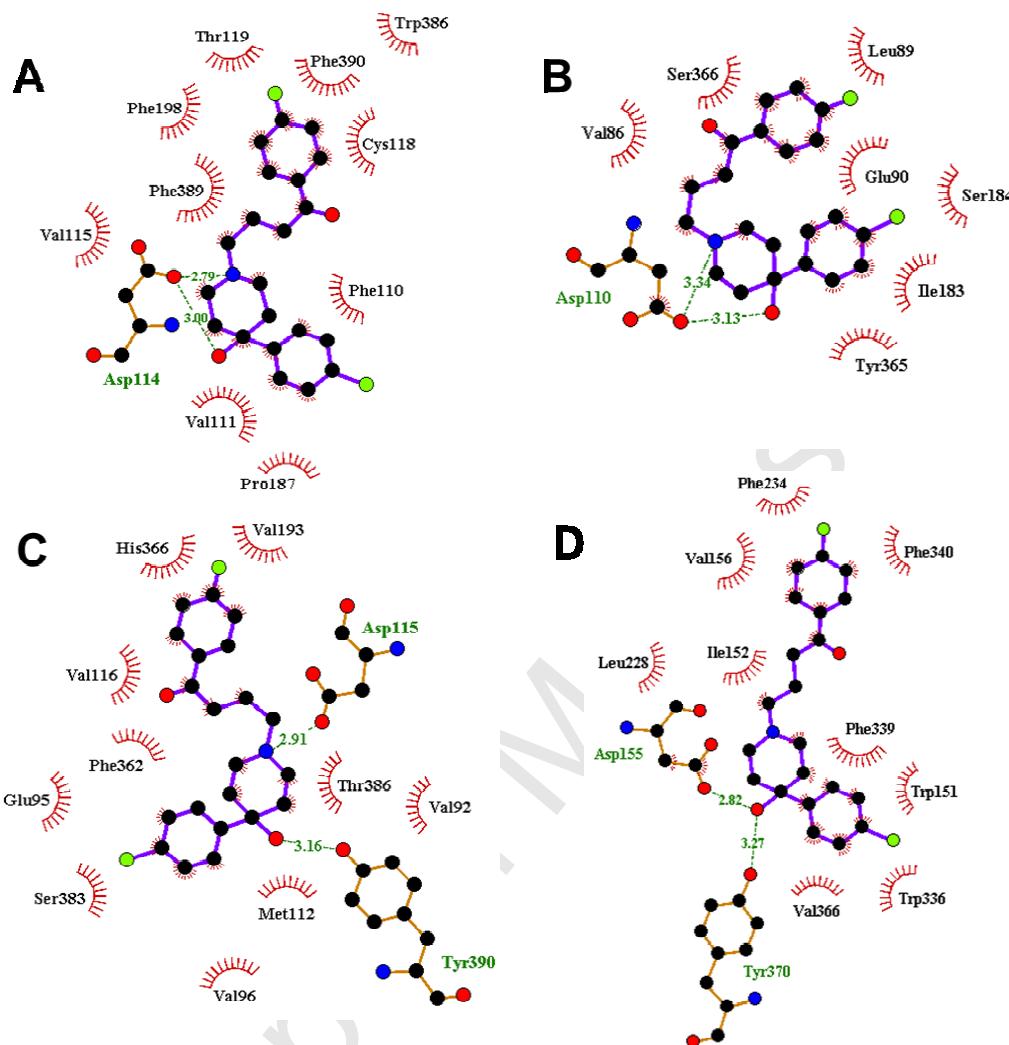


Fig. 11. Binding modes between compound 03 and D₂R (A), D₃R (B), D₄R (C) and 5-HT_{2A}R (D). Hydrogen atoms omitted for clarity. The compound (purple), residues involved in hydrogen bonding with the ligand (brown) along with their hydrogen bonds (dashed green lines), and nonbonded interactions (red spokes).

Compound 03 was docked into D₂ refined model. As shown in Fig. 11, key interactions were consistent with site-directed mutagenesis data [70,71,72]. The hydrogen bond between the tertiary hydroxyl group of 03 and Asp114 was observed (see Fig. 11A). Compound 03 has a vdW interaction with D₂R, particularly with the aromatic network involving residues Trp386, Phe389 and Phe390 located in TM6. The importance of this aromatic network has been identified by site-directed mutagenesis [73]. Meanwhile, the vdW interaction between 03 and Cys118 is present because

Cys118 is exposed to the binding site crevice. This result also agrees well with experimental report [74]. Many site-directed mutagenesis experiments have indicated that the significance of Asp3.32 residue (Asp114 in D₂R, Asp110 in D₃R, Asp115 in D₄R and Asp155 in 5-HT_{2A}R in this study) in aminergic GPCRs due to its crucial interaction with the protonated nitrogen of the ligand [75,76,77]. The same interaction was achieved in this docking study of 03 and four receptors. Compound 03 forms two hydrogen bonds with Asp110 of D₃R (see Fig. 11B). It forms one hydrogen bond with Asp115 of D₄R and Asp155 of 5-HT_{2A}R (see Fig. 11C and Fig. 11D). In addition, Tyr390 of D₄R form a hydrogen bond with 03 and Tyr370 of 5-HT_{2A}R forms one with 03. But the N-H-O between 03 and Asp114 of D₂R is stronger than O-H-O between 03 and Tyr370 of 5-HT_{2A}R. The distance of hydrogen bond in the latter becomes longer. That may explain the fact that haloperidol has a higher affinity with D₂R (total score is 9.0) than 5-HT_{2A}R (total score is 8.1). This observation is in agreement with experimental reports on this atypical antipsychotic haloperidol as a D₂R blocker.

Similar to the vdW interaction of 03 with D₂R, the nonbonded interactions between 03 and Val86, Thr365 of D₃R, those between 03 and His366, Val192, Val193, Thr386, Tyr390, Val196 and Phe362 of D₄R, and those between 03 and Val156, Val366, Trp336, Trp151, Phe339, Phe340, Phe234, Ile156 and Leu228 of 5-HT_{2A}R are observed. Higher total scores are obtained in 01-03 with four receptor models. This is in that linker alkyl group of linear structure makes these compounds be flexible and easy to form hydrogen bond and strong nonbonded interaction with surrounding residues.

In the binding mode of compound 05 and four receptors, a hydrogen bond between 05 and Asp3.32 of four receptors were found (see Fig. 12). The nonbonded interactions of D₂, D₃, D₄ and 5-HT_{2A} receptors with 05 could be observed. The binding affinity of 05 with 5-HT_{2A}R (total score is 9.0) increases about 8-fold relative to that with D₂R (total score is 8.1). This demonstrates that atypical antipsychotics risperidone owning a high blocking K_i(5-HT_{2A})/K_i(D₂) ratio could benefit the treatment of psychiatric disorders.

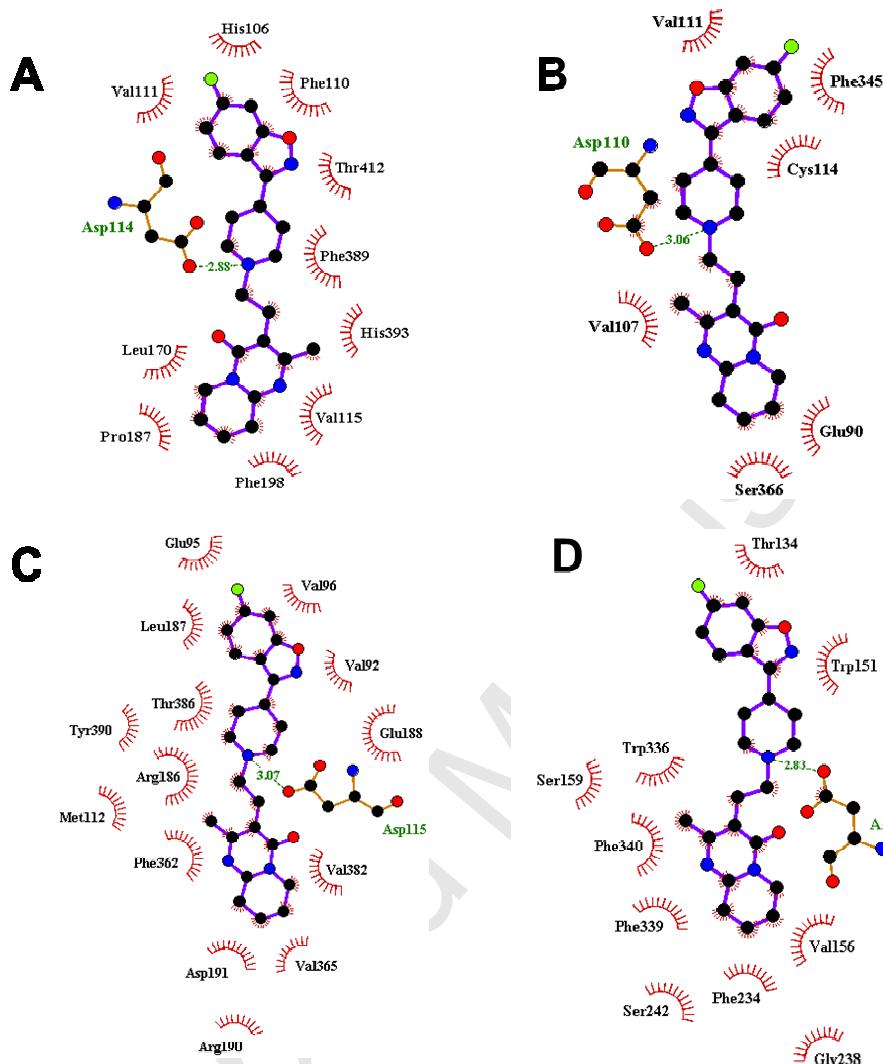


Fig. 12. Binding modes between compound 05 and D₂R (A), D₃R (B), D₄R (C) and 5-HT_{2A}R (D). Hydrogen atoms omitted for clarity. The compound (purple), residues involved in hydrogen bonding with the ligand (brown) along with their hydrogen bonds (dashed green lines), and nonbonded interactions (red spokes).

It should be noted that the binding conformations of 05 are not completely the same in D₂R, D₃R, D₄R and 5-HT_{2A}R. It is similar for the binding modes of 05 with D₄R and 5-HT_{2A}R but not for those with D₂R and D₃R. This indicates that benzisoxazolyl piperidine antagonists such as risperidone own a more flexible structure. It is possible for this kind of compounds to rotate their conformations more flexible to fulfill a favorable binding mode.

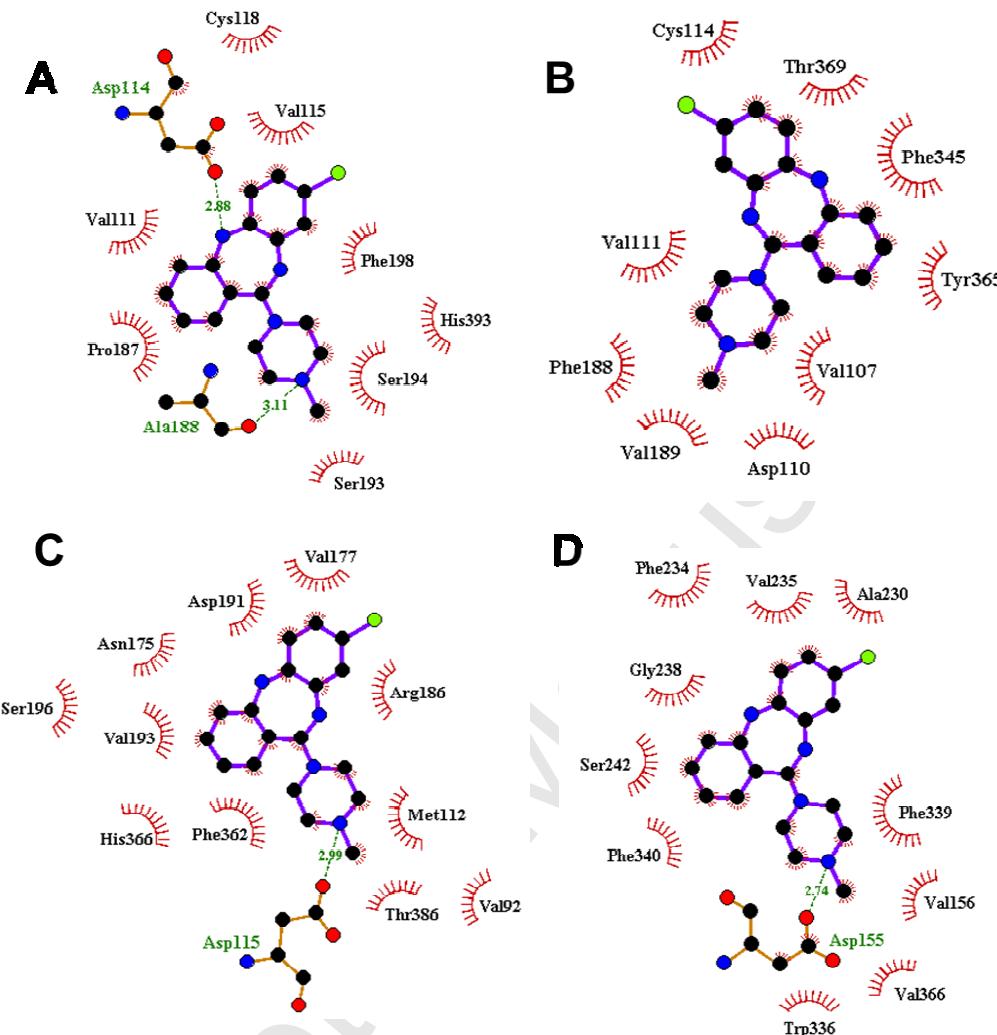


Fig. 13. Binding modes between compound 09 and D₂R (A), D₃R (B), D₄R (C) and 5-HT_{2A}R (D). Hydrogen atoms omitted for clarity. The compound (purple), residues involved in hydrogen bonding with the ligand (brown) along with their hydrogen bonds (dashed green lines), and nonbonded interactions (red spokes).

In the binding modes of compound 09 with four receptors, the hydrogen bonds between 09 and Asp3.32 of receptors except D₃R exist as shown in Fig. 13. This might be due to the structure feature of clozapine, which is a kind of polycyclicaromatic compound with poor flexibility. Phe389, which is a key residue in TM6 forming a hydrophobic interaction, was not found in 09-D₂R binding model (see Fig. 13A). It is because of the large steric hindrance of 09 and it is difficult for 09 to reach the binding site completely. The lower binding affinity for clozapine, apomorphine, and metergoline with receptors are also partly due to the poor flexibility in structures, which couldn't enter the binding site

completely.

Integrating with investigation on the binding modes of haloperidol (03), risperidone (05) and clozapine (09), we could summarize that the key residues contributed to the receptor-ligand interactions include Asp114, Phe198, Phe389, His393, Val111, Val115 and Pro187 in the binding site of D₂R, Asp110, Tyr369, Phe345 and Tyr365 in that of D₃R, Asp115, Val192, Thr389, Phe362, Val196 and Tyr390 in that of D₄R, and Asp155, Val156, Phe340, Val368, Phe339 and Trp336 in that 5-HT_{2A}R.

4. Conclusions

In summary, 3D homology models of D₂, D₃, D₄ and 5-HT_{2A} receptors were generated based on crystal structure of β₂-adrenergic receptor. The homology models were proved to be reliable using different methods such as conformational analysis, MD simulation and docking studies. The calculated and experimental pKi values have a good correlation coefficient in docking studies. R² values of D₂, D₃, D₄ and 5-HT_{2A} receptors are 0.913, 0.841, 0.820 and 0.940, respectively. The backbone RMSD of homology model of D₃R from its crystal structure is 1.3 Å which prove the reliability of homology models. Meanwhile, butyrophenones and benzisoxazolyl piperidines antagonists could be docked into D₂-like and 5-HT_{2A} receptors easily because of their linear structures. The linker alkyl group makes those drug compounds flexible and easily to form hydrogen bond and strong nonbonded interaction with surrounding residues. Polycyclic aromatic compounds have a weak affinity with D₂-like and 5-HT_{2A} receptors. The reason of this phenomenon is multivariate heterocyclic structures have large steric hindrance, the ligands such as clozapine, apomorphine and metergoline, which are difficult to approach Asp3.32 of receptors completely. The binding mode analyses reveal that Asp3.32 is the key residue in four homology receptors. We hope the homology models of D₂-like and 5-HT_{2A} receptors could be useful in virtual screening and design of atypical antipsychotic drugs.

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