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Significantly different effects of tetrahydroberberrubine enantiomers on dopamine D1/D2 receptors revealed by experimental study and integrated in silico simulation

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Abstract

Tetrahydroberberrubine (TU), an active tetrahydroprotoberberines (THPBs), is gaining increasing popularity as a potential candidate for treatment of anxiety and depression. One of its two enantiomers, *L*-TU, has been reported to be an antagonist of both D1 and D2 receptors, but the functional activity of the other enantiomer, *d*-TU, is still unknown. In this study, experiments were combined with in silico molecular simulations to (1) confirm and discover the functional activities of *L*-TU and *d*-TU, and (2) systematically evaluate the molecular mechanisms beyond the experimental observations. *L*-TU proved to be an antagonist of both D1 and D2 receptors (IC_{50} = 385 nM and 985 nM, respectively), while *d*-TU shows no affinity against either D1 or D2 receptor, based on the cAMP assay (D1 receptor) and calcium flux assay (D2 receptor). Results from both flexible-ligand docking studies and molecular dynamic (MD) simulations provided insights at the atomic level. The *L*-TU-bound structures predicted by MD (1) undergo an outward rotation of the extracellular helical bundles; (2) have an enlarged orthosteric binding pocket; and (3) have a central toggle switch that is prevented from rotating freely. These features are unique to the *L*-TU enantiomer and provide an explanation for its antagonistic behavior toward both D1 and D2 receptors. The present study provides new sight on the structural and functional relationships of *L*-TU and *d*-TU binding to dopamine receptors, and provides guidance to the rational design of novel molecules targeting these two dopamine receptors in the future.

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Compliance with ethical standards

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Keywords

Tetrahydroberberubine; Dopamine receptors; Antagonistic activity; Molecular dynamics simulation

Introduction

G protein-coupled receptors (GPCRs) constitute the largest family of membrane proteins that are related to the pharmaceutical science. GPCRs mediate a variety of physiological responses triggered by hormones, neurotransmitters and environmental stimulants [1], GPCR drugs account for ~27% of the global market share of therapeutic drugs and have aggregated sales of ~US\$890 billion in 2011–2015 [2]. At present, more than 200 ligand-receptor complexes covering 44 distinct GPCRs have had their structures resolved by crystallography [3], which has laid solid foundation for lead drug discovery through virtual screening and off-target rationalization [4]. Dopamine receptors, an important subgroup of GPCRs, have the neurotransmitter dopamine as their primary endogenous ligand. Dopamine exerts its physiological functions, such as eliciting emotion, cognition and movement coordination, through hve distinct dopamine receptors which are classified into D1-like (D1 and D5) and D2-like (D2, D3 and D4) families. These two families of dopamine receptors differ in their pharmacological and functional characteristics. The D1-like receptors are coupled to Gs/olf proteins that activate adenylyl cyclase and lead to increased cAMP accumulation; whereas the D2-like receptors are coupled to Gi/o proteins that inhibit adenylyl cyclase activity [5]. Due to the important roles of dopamine receptors, compounds targeting their activity have attracted a great deal of attention in drug development and clinical use.

Tetrahydropprotoberberines (THPBs) are isoquinoline alkaloids with a chiral center at C-14 and originally isolated from the Chinese herb *Corydalis yanhusuo* and various species of *Stephania*. The representative compounds of the THPB family are *L*-tetrahydropalmatine(*L*-THP), *L*-stepholidine(*L*-SPD), *L*-corydalmine(*L*-CDL), etc. *L*-THP, an natural extract, has been used in China as a pain-killer for more than 40 years and displays D1 and D2 antagonistic effects [6]. *L*-SPD is known to interact with dopamine receptors and has also been proposed as a novel antipsychotic agent [7, 8]. Another derivative of THPBs, tetrahydroberber-rubine (*d*-TU also known as nandinine, Fig. 1), which was first isolated 80 years ago, has recently attracted increasing attention because of its pharmacological activities, promising safety profiles, and low industrial production cost. Its pharmacological activities include a tissue factor inhibitory effect [9] and anti-acute lung injury [10], anti-obesity [11], antianxiety and antidepressants activities [12, 13].

It is noteworthy that the stereoisomerism has a significant impact on the binding properties of THPBs. For example, *L*-THP has high binding affinity for the D2 receptor, while the *d*-enantiomer (*d*-THP) exhibits low or absent affinity for the receptor [14]. Mi et al. [12] reported that *L*-TU had high binding affinities for the D1, D2, D3 and 5-HT1A receptors, and the compound acted as an antagonist of the D1 and D2 receptors and an agonist of the 5-HT1A receptor, utilizing cAMP and [³⁵S]GTPγS assays, respectively. However, the

molecular basis beyond the above observation is unknown and the reasons cause the different binding affinities of *l*-TU and *d*-TU to D1 and D2 receptors remain unclear. It is of great importance to study the mechanism of the molecular interactions between *d**l*-TU and D1/D2 receptors at the atomic level which can reveal (1) the impact of the conformational change of the small molecules on bioactivities, (2) the key residues that are involved in critical receptor-ligand interactions, and (3) the preferred substructures that fit the shape and physical-chemical properties of the binding pockets. These detailed information will facilitate us to rationally design novel antagonists targeting both the dopamine receptors. In the present study, we prepared *l*-TU and *d*-TU with high enantiomeric excess (*e.e.* %) using a semi-synthetic method and chemical resolution. The functional activities of *l*-TU and *d*-TU binding to D1 receptor (D1R) and D2 receptor (D2R) were determined by the cAMP assay and calcium flux assay, respectively. The molecular mechanisms of *l*-TU and *d*-TU binding to D1 and D2 receptors were then studied using a series of integrated computational methodologies, including homology modeling, molecular docking, molecular dynamics (MD) simulation and protein-ligand binding free energy calculation.

Methods

Preparation and resolution of *l*-TU and *d*-TU

The compounds of *d**l*-TU were synthesized by pyrolysis monodemethylation of berberine hydrochloride and reduction reactions as the previously reported [9]. Then *d*-TU and *l*-TU were obtained through the classical chemical resolution using (+)-di-para-toluoyl-D-tartaric acid ((+)-DTTA, CAS: 32634–68-7) in 95% ethanol and repeated recrystallization for several rounds [15].

The purities were determined by OJ-RH chiral column HPLC (Chiralcel®), Eluent: A, 0.1 M NH₄OAc (0.05% TFA); B, Acetonitrile (0.05% TFA); A/B initial 80:20, 10 min 60:40, 20 min 60:40, 30 min 0:100.

Functional activity assay at D1R and D2R

The functional activity assays of *d*-TU and *l*-TU against D1 and D2 receptors were performed by GenScript Corp (Piscataway, NJ, USA). Agonistic and antagonistic activities on D1 and D2 receptors were tested by the cAMP assay with a CISBIO Kit and calcium flux assay with FLIPR® calcium-4 Kit of Molecular Device, respectively. Test articles and control articles were dissolved in DMSO and stored at –20 °C. The stock solutions were diluted in HBSS buffer (with 20 mM HEPES buffer, pH 7.4) to make 5 × working solution before use. The final concentrations of the test articles in the assay were 16 nM, 80 nM, 400 nM, 2 μM and 10 μM. CHO-K1 cells expressing D1 and D2 receptors were cultured in the 15-cm dishes and maintained at 37 °C/5% CO₂. When the cell confluency reached 90%, the cells were collected and inoculated into 384 microplates for experiments. CHO-K1/D1 was cultured with Ham's F12 containing 10% fetal bovine serum and 200 μg/ml Zeocin. CHO-K1/Gα15/D2 was cultured with Ham's F12 containing 10% fetal bovine serum, 200 μg/ml Zeocin and 100 μg/ml Hygromycin B.

cAMP assays on D1 receptor

The protocols were detailed in the manual of the CISBIO Kit. In brief, we seeded 3000 cells/5 μ l (CHO-K1 cells expressing D1 receptor) in 384-well plates and 5 μ l 2 $\times$ compound solution before 0.5-h incubation at RT (For agonistic assays, only test articles were included; in the case of inhibitory assays, a mixture of 4 $\times$ test articles and 4 $\times$ EC₈₀ positive agonist volume was added). Then 10 μ l detection reagents were added and incubated for another hour before the signal of the plate was read by PheraStar and the data of the 665 nM and 620 nM in each well were recorded by Excel. The ratio of the 2 readouts was then plotted as a function of the log of the cumulative doses of compounds. Finally, data analysis wizard written by GenScript was used to analyze the EC₅₀ and IC₅₀. Dose response curves of agonist were fitted with four-parameter-logistic-equation by the software GraphPad Prism 6. The percentage of compound effect was calculated as:

$$\% \text{Stimulation} = 100\% - \frac{(R_{\text{Compound}} - R_{\text{Agonist } 100})}{(R_{\text{Background}} - R_{\text{Agonist } 100})} \times 100\% \quad (1)$$

$$\% \text{Inhibition} = \frac{(R_{\text{Compound}} - R_{\text{Agonist } 80})}{(R_{\text{Background}} - R_{\text{Agonist } 80})} \times 100\% \quad (2)$$

Calcium flux assay on D2 receptor

CHO-K1 cells expressing D2 receptor were seeded in a 384-well black-wall, clear-bottom plate at a density of 15,000 cell per well in 20 μ l of growth medium for 18 h prior to the day of experiment and maintained at 37 °C/5% CO₂.

Agonist activity test

20 μ l of dye-loading solution was added into the well. Then the plate was placed into a 37 °C incubator for 60 min, followed by a 15-min incubation at room temperature. At last, 10 μ l compounds or control agonist were added into respective wells of the assay plate during reading in FLIPR. The plate containing 5 $\times$ compound or control agonist solution was placed in FLIPR. Solutions were added into the cell plate automatically at the 20 s and the fluorescence signal was monitored for an additional period of 100 s (21 s to 120 s).

Antagonist activity test

20 μ l of dye-loading solution and 10 μ l 5 $\times$ compound solution were added into the well. Then the plate was placed into a 37 °C incubator for 60 min, followed by a 15-min incubation at room temperature. At last, 12.5 μ l 5 $\times$ EC₈₀ control agonist were added into respective wells of the assay plate during reading in FLIPR. The plate containing 5 $\times$ EC₈₀ control agonist solution was placed in FLIPR. Solutions were added into the cell plate automatically at the 20 s and the fluorescence signal was monitored for an additional period of 100 s (21 s to 120 s.)

Data were recorded by ScreenWorks (version 3.1) as FMD files with FLIPR and stored on the GenScript computer network for off-line analysis. Data acquisition and analyses was performed using the ScreenWorks (version 3.1) program and exported to Excel. The average value of the first 20 s (1 s to 20 s) readings was calculated as the baseline and the relative fluorescent units (RFU) intensity values were calculated by subtracting the average value of baseline from the maximal fluorescent units (21 s to 120 s) .The percentage of compound effects was calculated with the following equation:

$$\%Stimulation = \frac{(\Delta RFU_{Compound} - \Delta RFU_{Background})}{(\Delta RFU_{Agonist\ control} - \Delta RFU_{Background})} \times 100\% \quad (3)$$

$$\%Inhibition = 100\% - \frac{(\Delta RFU_{Compound} - \Delta RFU_{Background})}{(\Delta RFU_{Agonist\ control} - \Delta RFU_{Background})} \times 100\% \quad (4)$$

D2 receptor and homology modeling of D1 receptor

The crystal structure of the D2 receptor [16] (PDB entry: 6CM4) was downloaded from the Protein Data Bank (PDB) [17]. This crystal structure was released recently [16] and it is currently the only available crystal structure of D2 receptor. The Schrödinger software package [18] was applied for protein model preparation, including energy minimization and residue repair.

The full amino acid sequence of D1 receptor (UniProt Code: P21728) was obtained from the web site of Uni-ProtKB (<http://www.uniprot.org/uniprot/>) [19] and sequence similarity searches for D1 receptor were performed using the NCBI BLAST server (<https://blast.ncbi.nlm.nih.gov/>) [20]. The inactive-state structure of D3 receptor (PDB code: 3PBL) was selected as the template to construct the antagonistic conformations of D1 receptor.

The homology modeling and loop refinement were optimized with Modeler 9.17 [21, 22]. As the D1 orthosteric binding pocket was defined by the transmembrane helices, some sequence segments in the loop regions that apparently did not affect ligand binding was removed. Truncations in the sequence of D1 receptor were made at the N-terminus before ASP19 and at the C-terminus after CYS347. Moreover, since the third extracellular loop (ECL3, between TM4 and TM5) and the third intracellular loop (ICL3, between TM5 and TM6) had long-flexible sequences, 19 residues from PR0168 (ECL3) to CYS186 (ECL3) and 35 residues from GLN222 (ICL3) to PR0256 (ICL3) were also truncated. Therefore, the sequence of D1 receptor was from ASP19 to LYS167 (ECL3), from ASP187 (ECL3) to ALA 221 (ICL3), and from GLU257 (ICL3) to CYS347. The disulfide bridge/bond between CYS298 and CYS307 in TM6 and TM7 of D1 receptor was considered and constructed. The sequence alignments and homology modeling were based on this truncated sequence of D1 receptor.

The D1 receptor model was evaluated with Discrete Optimized Protein Energy (DOPE) [23] measurement and Ramachandran plot [24, 25] following our standard process [26]. DOPE, which reflects the energy profiles of individual residues, is a key parameter to determine the quality of a homology model compared to the templates. Ramachandran plot, on the other hand, demonstrates if the dihedral angles ψ against ϕ of amino acid residues on the backbone structure of the protein fall within the energetically allowed regions. The Ramachandran plot was measured through the web server RAMPAGE (<http://mordred.bioc.cam.ac.uk/~rapper/rampage.php>) [27].

Enrichment test for D1 receptor model

A docking study could be considered as a success once the process can differentiate active compounds from a large number of random or inactive compounds and rank the active compounds as top hits. Drug-like Zinc database (<http://zinc.docking.org>) [28] was used to randomly select five hundred compounds as decoys. ChEMBL database (<https://www.ebi.ac.uk/chembl/>) [29] was used to acquire ten active compounds for D1 receptor. The virtual screening using homology protein model of D1 receptor for the mixed sample of random and active compounds was conducted to determine whether this D1 homology model can have docking process enrich active compounds as top hits. This protocol was successfully applied in previous studies [26, 30]. Surflex-Dock Screen, the suite implemented in SYBYL-X 1.3, was employed for the in silico screening. A conserved orthosteric binding pocket among the dopamine receptors was defined by selecting corresponding surrounding residues. The Kollman all-atom approach was adopted to calculate atomic charges for the protein, and the Gasteiger-Hückel approach for the ligand following our usual operation routine. The hydrogen atoms of the protein were not allowed to move. No additional starting conformation was applied. The distance to expand search grid was set to 6 Å. The maximum number of poses per ligand was set to 3 and the minimum RMSD between final poses was set to 0.05 Å.

Molecular docking between *l*-TU, *d*-TU and D1R, D2R

Molecular docking studies were performed using the Glide/XP (extra precision) module of the Schrodinger software package [31–33]. Small molecules were prepared with Lig-Prep [34]. Protein targets were processed with Protein Preparation Wizard [18] and force field was set to OPLS3 [35] for both the small molecule and protein target. The specified chiralities were retained for the small molecules. Bond orders were assigned, and the hydrogen atoms were added to fill in open valences for the protein target. For D1 receptor, the energy grid center was specified as the centroid of selected residues, including D103, T108, S197, I201, and W285. The grid box was set to a cube, with the cubic length being set to 16 Å. For D2 receptor, on the other hand, the energy grid center is specified as the centroid of the co-crystallized ligand (PDB entry: 6CM4). The scaling factor was set to 0.80 (default value) and the partial charge cutoff was set to 0.15 Å (default value) to soften the potential for nonpolar parts of the ligand. Maximum number of poses for each ligand was limited to 20. Risperidone was used as the positive control to validate the docking protocol and was first extracted from the D2 receptor-risperidone crystal structure and then docked back to the binding pocket. Encouragingly, the RMSD of the ligand is only 0.3 Å, indicating that our flexible docking protocol is reasonable. As shown in Fig. SI 1, the risperidone

occupied the binding site with a similar binding mode as that of the crystal structure: the salt bridge with ASP114 was able to form, and the fluorobenzene ring occupies the hydrophobic pocket.

Molecular dynamics simulation and protein-ligand binding free energy calculations

The web-based tool CHARMM-GUI [36, 37] was used to construct the initial configurations for MD simulations. The D1 or D2 receptor protein was embedded in a bilayer of approximately 256 POPC lipids. The POPC membrane was placed on the X-Y plane and the protein was aligned along the Z axis. The water thickness was at least 17.5 Å on the top and bottom sides of the system. Ions of Na⁺ and Cl⁻ were added to minimize the interaction energies in an iterative fashion until the ion concentration of 0.15 M was reached and the system was neutralized. The sizes of all simulation boxes were approximately 100 × 100 × 100 Å.

The ANTECHAMBER module in AMBERTOOLS16 [38] was utilized to generate topologies for use in MD. The atom types and parameters for ligands were assigned according to the General Amber Force Field (GAFF) [39]. The partial charges for ligands were derived using the AM1-BCC method [40] and the Restrained Electrostatic Potential (RESP) model [41] to fit the HF/6-31G* electrostatic potential generated using the GAUSSIAN 16 software package [42]. The ff14SB force-field [43] was adopted for proteins and AMBER Lipid17 force field [44] was applied to lipids. Water molecules were treated with TIP3P water model [45].

The MD simulations were carried out using the PMEMD.mpi and PMEMD.cuda modules in the AMBER16 package [46–48]. After a set of minimization steps to remove possible steric clashes, each system was gradually heated from 0 to 300 K and then kept at 300 K in the following equilibrium and production stages. The periodic boundary condition was employed to produce a constant temperature and pressure (NPT) ensemble. The pressure was controlled at 1 atm by the anisotropic (X-, Y-, Z-) pressure scaling protocol implemented in AMBER16 [48] with a pressure relaxation time of 1 ps. The Particle Mesh Ewald (PME) method [49, 50] was adopted to handle the long-range electrostatics and a 10 Å cutoff was set to treat real-space interactions. All the covalent bonds involving hydrogen atoms were constrained with the SHAKE algorithm [51]. The simulation time for each system was 200 ns with a time step of 2 fs.

Saved snapshots of MD simulations were subject to the Molecular Mechanics Poisson-Boltzmann Surface Area (MM/PBSA) free energy calculations [52, 53] with lipids, ions and water molecules removed first. The Poisson-Boltzmann (PB) calculations were performed with the Delphi program [54]. The PB calculations employed the Parse radii for all atoms with a grid spacing of 0.5 Å and a probe radius of 1.4 Å. The value of the exterior dielectric was set to 80 and that of the solute interior dielectric was set to 1. The nonpolar solvation energy was calculated with the solvent-accessible surface area (SASA) approach and the rescaling equation $G_{nonpol} = \gamma \times SASA + \beta$, where $\gamma = 0.00542 \text{ kcal/mol/Å}^2$ and $\beta = 0.92 \text{ kcal/mol}$. The NMode module in AMBER16 was applied to derive the entropy contribution during protein-ligand binding.

Results and discussion

Preparation of *l*-TU and *d*-TU

d-TU was obtained with 65% yield and the structure was determined by MS, ¹HNMR and ¹³CNMR [9]. Their absolute configuration was confirmed through chiral column HPLC and CD spectrum [55, 56]. As shown in Fig. 2, the retention times of HPLC chromatograms for *d*-TU and *l*-TU are 17.685 min and 20.669 min. The *e.e.*% value for each configuration was greater than 99%.

The functional activities of *l*-TU and *d*-TU at D1 and D2 receptors

The functional activities of *l*-TU and *d*-TU at D1 receptor were evaluated by determining the change in cAMP levels in the cells with a CISBIO Kit which includes 2 elements of the HTRF technology. As shown in Fig. 3a, the D1 receptor agonist SKF38393 induced a dose-dependent increase in cAMP production in CHO-K1/D1 cells, with an EC₅₀ of 49.1 nM. But *l*-TU and *d*-TU did not exhibit significant agonistic activity and the EC₅₀ values of them were both greater than 10,000 nM, indicating that they are not D1 receptor agonists. In contrast, *l*-TU, as well as the known D1 antagonist SCH23390, inhibited the cAMP production in a dose-dependent manner with an IC₅₀ of 385 nM and 1.42 nM respectively, while the IC₅₀ value of *d*-TU is greater than 10,000 nM (Fig. 3b). The results suggest that *l*-TU has an antagonistic effect on D1 receptor while *d*-TU does not.

D2 functional activities of *l*-TU and *d*-TU were investigated by the detection of calcium flux with FLIPR[®] calcium-4 Kit (Molecular Device) and the results are shown in Fig. 4a. Compared with the D2 agonist dopamine (EC₅₀ of 4.07 nM), *l*-TU and *d*-TU had significantly higher EC₅₀ values (greater than 10,000 nM). No significant agonistic activities could be observed for either *l*-TU or *d*-TU, indicating that they are not conventional D2 receptor agonists. However, *l*-TU exhibited stronger antagonistic activity than the positive control SCH23390, with IC₅₀ values of 985 nM and 4058 nM respectively. Only very weak antagonistic activity was observed for *d*-TU, with an IC₅₀ value greater than 10,000 nM (Fig. 4b).

In summary, *l*-TU, but not *d*-TU, demonstrated antagonistic effects on both D1 receptor and D2 receptor. Our experimental results are consistent with previous results from Mi et al. [12] who used cAMP and [³⁵S]GTPγS assays to determine the functional activities of *l*-TU at D1 and D2 receptors, respectively. Moreover, we demonstrated that neither *l*-TU nor *d*-TU is an agonist for D1 and D2 receptors.

Homology modeling of the D1 receptor

There is no available crystal structure for the D1 receptor. Modeller [57] was used for building up the homology models for this protein target. Sequence extraction was from the UniProt. The inactive-state structure of D3 receptor (PDB entry: 3PBL) was selected as the template to construct the antagonist-bound conformation of the D1 receptor.

The alignment between the template and the target sequence was performed with the structural information of the template being taken into account by the dynamic programming

algorithm, Align2d. Then one or more target model(s) were created using the Automodel function of the Modeller homology modeling software. The sequence alignment between the D1 receptor and the D3 receptor is shown in Fig. SI 2a. The seven α -helices for the transmembrane region are marked with the number “9”. A high correlation was observed between the discrete optimized protein energy (DOPE) profiles [58] of the template and the target protein models (Fig. SI 2b). No positive energy was observed for each individual residue. And the positions of sequence truncations gave observable gaps on the DOPE plot. Ramachandran plot [59] was used to evaluate the quality of the protein models by mapping the calculated ψ and ϕ torsional angles into different regions of the plot. (Fig. SI 2c). 258 residues (97.0%) are in the favored region, 7 residues (2.6%) are in the allowed region, and only one (0.4%) residue is in the outlier region. The outlier, SER162, is located on ECL2, which is not involved in formation of the binding pocket.

Model validation with enrichment test

In order to determine the ability of the newly developed D1 receptor model to distinguish active compounds from random compounds, the enrichment test was conducted. Surflex-Dock Screen [60] was used to predict the binding affinities between small molecules and protein models through virtual screenings.

As specified in the “Methods” part, 10 active compounds were downloaded through the ChEMBL database, and 500 compounds were randomly selected and downloaded from the Zinc database for D1 receptor model (Tables SI 1 and SI 2). The docking scores were applied to rank the 510 compounds and compounds with higher scores ranked higher. All 10 active compounds are ranked within Top 89, i.e. Top 17%. The result suggests that active D1 receptor compounds can form favorable interactions with the D1 receptor protein model (Fig. SI 3a). Docking scores of the random and active compounds were compared (Fig. SI 3b) and the asymptotic significance (two-tailed) by Mann-Whitney test was less than 0.01, indicating that active compounds tend to have better docking scores than randomly selected ones. The calculated enrichment factor, 5.7, is a typical value for a GPCR receptor that has crystal structure [61].

L-TU shows promising binding affinity on D1 and D2 receptors

We attempted to interpret the experimental results by using molecular docking studies. As the enantiomers of TU have distinct conformations as shown in Fig. 5, it is not a surprise that their binding affinities toward specific targets varied dramatically. For D1 receptor, as shown in Fig. 5a, b, both *L*-TU and *d*-TU can have the A ring (marked in Fig. 1) face up toward the extracellular space and the D ring (marked in Fig. 1) faced down toward the intracellular space, but the chiral center resulted in two distinct conformations leading to different binding modes. *L*-TU can have hydrogen bonding with SER191 on ECL2, and a salt bridge interaction with ASP103 on TM3 (Fig. 5a). Aromatic ring systems on the residues TRP285, PHE288, and PHE289 on TM6, and TRP321 on TM7 created the hydrophobic environment in the bottom of the binding pocket. *L*-TU had the D ring flipped toward the TM6 and TM7 to fit in this hydrophobic region. The hydrophobic interactions including π - π stacking were observed between the *L*-TU and these surrounding residues. Unlike *L*-TU, the unfavorable angle caused by the chiral center prevented *d*-TU from digging into the bottom

of the binding pocket (Fig. 5b). Instead, only the hydrophobic interactions with PHE288 and TRP321 observed and hydrophilic interactions with SER191 and ASP103 could no longer be maintained, replaced by a hydrogen bond formed between the hydroxyl group of *d*-TU and the amide functional group of ASN292 on TM6. The pose of the *l*-TU was generally favored according to the Glide calculation with the Glide docking score of -7.7 , while -5.8 for *d*-TU.

For D2 receptor, conformational differences among *l*-TU and *d*-TU can be visualized (Fig. 5c, d). Given that the protein model is based on the crystal structure and the binding pocket is directly defined from the co-crystallized ligand, favorable interactions between *d*-TU and D2 receptor are supposed to be discovered. Indeed, better docking scores were obtained for the D2 receptor than the D1 receptor. The Glide docking scores for *l*-TU and *d*-TU are -8.7 and -7.1 kcal/mol, respectively. Again, *l*-TU is a more potent binder than *d*-TU. Similar with the D1 receptor, the favorable conformation for *l*-TU (Fig. 5c) here indicated that the compound can have the D ring face down toward the intracellular space and A ring face up toward the extracellular space. Hydrophilic interactions can be observed between (1) ASP114 on TM3 and the nitrogen atom and the hydroxyl group on *l*-TU, and (2) HIS396 on TM6 and the dioxymethylene group. Meanwhile π - π stacking is observed between the A ring and D ring of *l*-TU and TYR408 on TM7, TRP386, PHE390 on TM6 in the binding pocket. For *d*-TU, a conformation flip was observed for the ligand bound to D1 and D2 receptors as shown in Fig. 5d. The interpretations for the different binding modes are as the follows: (1) D1-like (D1 and D5) and D2-like (D2, D3 and D4) receptors possess distinctive structural features, thus surrounding residues of the binding pocket can create diverse physical-chemical environments respectively; (2) both orientations of *d*-TU may be reasonable, yet, the docking studies were performed using a static binding pocket without taking the flexibility of the receptor into account. Similarly, the hydrophilic interactions with ASP114 and HIS396 can be observed for *d*-TU. While π - π stacking no longer existed due to the twisted angle and the enlarged distance.

The direct comparison between the two enantiomers indicates stronger receptor-ligand interactions for *l*-TU and the protein models, which can help explain the differences among the protein binding affinities. The preferable chirality was further evaluated in parallel with agonists of D1 and D2 receptors through MD simulations in an effort to explore the mechanism of *l*-TU binding to D1 and D2 receptors as an antagonist.

***l*-TU has the antagonistic effects on D1 and D2 receptors**

Two hundred nanoseconds (ns) molecular dynamics simulations were performed for four systems, *l*-TU-D1 receptor complex, *l*-TU-D2 receptor complex, SKF38393-D1 receptor complex, and Dopamine-D2 receptor complex (Fig. 6). SKF38393 and dopamine are known agonists for D1 receptor and D2 receptor. The conformational changes among the protein structures caused by the agonist binding and the *l*-TU binding were focused and compared.

SKF38393-D1 receptor, *l*-TU-D1 receptor, and *l*-TU-D2 receptor systems achieved equilibrium stages quickly within the first 25 ns of simulation. The average coordinates of the last 50 ns for SKF38393-D1 receptor and *l*-TU-D2 receptor systems, and the average coordinates of 100 ns to 150 ns for *l*-TU-D1 receptor system were calculated and extracted

for the detailed comparisons. For Dopamine-D2 receptor system, two equilibrium stages can be observed for the Dopamine (Fig. 6d), which reflects two stable conformations (Fig. SI 4). The average coordinates of 100 ns to 150 ns for Dopamine-D2 receptor system were calculated and extracted.

Comparisons between the *L*-TU-bound and SKF38393-bound D1 receptor reveal significant rearrangement in the protein-ligand complex structure (Fig. 7). The extracellular helical bundles from the *L*-TU-bound structure undergoes outward rotation compared with SKF38393-bound structure (Fig. 7a). And the conformational changes in the intracellular part causes the helical bundles from the *L*-TU-bound structure rotate inward (Fig. 7b). Because of the dramatic conformational changes especially in the extracellular part, there is an expansion on the orthosteric binding pocket for the *L*-TU-bound structure (Fig. 8a, b). The volume of the binding pocket is expanded from 406.8 Å³ for the SKF38393-bound D1 receptor to 671.3 Å³ for the *L*-TU-bound D1 receptor. Also, in the *L*-TU-bound structure, the central toggle switch, TRP285, points toward the *L*-TU to form potential anion- π interactions with the oxygen from the methoxy group of the *L*-TU (Fig. 7c). The interaction can further limit the free rotation of the TRP285 which is critical for the class A GPCR activation [62]. On the other hand, in the SKF38393-bound structure, the D1 receptor agonist SKF38393 possesses a position to have limited hydrophobic interactions with the TRP285 (Fig. 7d). There would be fewer obstacles for the TRP285 to rotate freely. The direct comparison of the TRP285 from the two binding modes (Fig. 7f) reveals that although the orientation keeps the same, but the outward rotation of the helix swung out the residues for the *L*-TU-bound structure.

Similarly, the comparisons between the *L*-TU-bound and dopamine-bound D2 receptor illustrate drastic conformational changes (Fig. 9). Like the observation in D1 receptor, the extracellular part of the helices in the *L*-TU-bound structure, especially for TM5, TM6, and TM7, undergoes an outward rotation (Fig. 9a), and the intracellular part overall moves inward (Fig. 9b) compared to the dopamine-bound structure. The significant structural rearrangement increases the size of the binding pocket for *L*-TU-bound structure (Fig. 8c, d). The volume of the binding pocket for dopamine-bound D2 receptor is 554.3 Å³, while for *L*-TU-bound D2 receptor it is 795.1 Å³. The central toggle switch TRP386 on helix 6 forms favorable hydrophobic interactions with the ring system of the *L*-TU (Fig. 9c), which in turn prevents the rotation of this residue to some extent. Unlike the *L*-TU-bound structure, the dopamine-bound structure has the free rotation of the TRP386 (Fig. 9d). Dopamine itself cannot form strong interaction with the central toggle switch to prevent the rotation. The direct comparison of the TRP386 clearly shows the prevention of rotation in *L*-TU-bound structure (Fig. 9f).

The ASP^{3.32} (ASP103 for D1 receptor, and ASP114 for D2 receptor) was also investigated (Figs. 7e, 9e). The crystallization of D2 receptor reveals a salt bridge interaction between this ASP^{3.32} in the receptor and the co-crystallized ligand, risperidone [16]. In our MD simulation trajectories, the ASP^{3.32} undergoes upward movement on both D1 and D2 receptors with *L*-TU binding. No hydrophilic interaction with the ASP^{3.32} is formed from agonists either because of the distance or the undesirable angle, at least according to the average coordinates. The salt bridge with *L*-TU on both receptors, which were revealed

through docking studies, is maintained at the equilibrium stages of the MD simulation (Figs. 7e, 9e). This hydrophilic interaction combined with the hydrophobic interaction from TRP^{6.48} function as the hinge, which greatly restricts the movement of *L*-TU. In the other word, the *L*-TU occupies the binding pocket, holds the ASP^{3.32} through a salt bridge, and prevents the movement of TRP^{6.48} by hydrophobic interactions. This illustrates a distinctive protein conformation from the agonist binding.

In summary, there are three major differences revealed through the comparison. The *L*-TU binding to both the D1 and D2 receptors (1) causes the outward rotation of the extracellular helical bundles; (2) enlarges the orthosteric binding pocket; and (3) prevents the free rotation of the central toggle switch. These unique features of the protein structure caused by the *L*-TU binding are distinct from those induced by agonist binding, which may be applied to explain why *L*-TU is an antagonist of both D1 and D2 receptors and to guide us to rational design novel antagonists targeting the two dopamine receptors. The outward rotation of the extracellular helixes which expands the binding pocket is the largest conformational change observed for class A GPCRs. Hua et al. [63] made the summary on direct comparison for binding pocket volumes of agonist- and antagonist-bound pairs among seven class A GPCRs. The antagonist-bound complexes generally have significantly larger pocket volumes as the result of outward rotation of extracellular helices. In the *L*-TU-bound structures of D1 receptor and D2 receptor, the TRP^{6.48} can be stabilized by forming hydrophobic interactions with the bound ligand. In contrast, the agonist-bound structures had limited influence on the TRP^{6.48} and as a matter of fact, the TRP^{6.48} was observed to swing away from the bound ligand during the MD simulation for D2 receptor after 200 ns.

Binding free energy calculations further support that *L*-TU is an active inhibitor for both D1 and D2 receptors

The binding free energies of *L*-TU or *d*-TU ligands in D1 or D2 receptors were calculated by the MM/PBSA method from snapshots of MD simulations. As shown in Table 1, the D1R-*d*-TU and D2R-*d*-TU complexes both have positive values of binding free energies, indicating *d*-TU is not an active inhibitor for either D1 or D2 receptor, which agrees well with the experimental results. On the contrary, the D1R-*L*-TU and D2R-*L*-TU complexes both have negative binding free energies indicating *L*-TU is an active inhibitor, which again agrees well with the experimental results. Due to the limitation of MM/PBSA methods, the absolute values of calculated free energies may not be very accurate, but the relative differences of binding affinities between *L*-TU and *d*-TU in both D1 receptor and D2 receptor reveal the relative binding potencies of the two stereoisomers which are well aligned with the experimental results.

Conclusions

In this study, chemical synthesis, in vitro validation, and in silico modeling and simulation were combined to study the functional activities of *L*-TU and *d*-TU on D1 and D2 receptors. *L*-TU and *d*-TU with high *e.e.*% value and high purity were first prepared by semi-synthetic method and chemical resolution. The functional assays indicated that *L*-TU but not *d*-TU shows promising antagonistic effects on the two dopamine receptors. To understand the

molecular mechanisms leading to the dramatic differences on the functional activities of the two TU enantiomers, we performed both the static (docking) and dynamic (molecular dynamics) simulations for the two dopamine receptors. Because there is no crystal structure of D1 receptor is available, a homology model of D1 receptor was constructed and verified with an enrichment test; the only available crystal structure of D2 receptor (PDB Code 6CM4) was adopted. Flexible docking simulations of *L*-TU and *d*-TU binding to D1 and D2 receptors were performed and predicted that *L*-TU is a stronger binder for both protein targets. The conformation difference caused by the chirality prevent *d*-TU from forming favorable interactions with the surrounding residues. Binding free energy calculations further demonstrated that *L*-TU has strong interactions with D1 and D2 receptors, but *d*-TU does not, which agrees well with experimental data. Trajectories from molecular dynamics simulations were used to compare the agonist-bound structures and the *L*-TU-bound structures with the full flexibility of the system being taken into account. There are three major differences revealed by the direct comparisons. The *L*-TU binding to D1 and D2 receptors (1) causes the outward rotation of the extracellular helical bundles; (2) enlarges the orthosteric binding pocket; and (3) prevents the free rotation of the central toggle switch. These unique features of the protein structure caused by the *L*-TU binding distinct from those of agonist binding, and the differences may explain why *L*-TU is an antagonist but not an agonist for both D1 and D2 receptors. In summary, the antagonistic effects of *L*-TU on both D1 and D2 receptors were observed through the wet-lab experiments, and molecular mechanisms were studied through *in silico* modeling and simulations. Our findings provide new sight on the understanding of the structures and functional relationship for *L*-TU and *d*-TU binding to the D1 and D2 receptors and could guide the rational design of novel antagonists targeting these two dopamine receptors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
Structures of *l*-TU and *d*-TU

Fig. 2.
a HPLC chromatograms of *d*-TU with chiral column, **b** HPLC chromatograms of *l*-TU with chiral column

Fig. 3.

a Agonist effect of positive control SKF 38,393, *l*-TU and *d*-TU on D1/CHO-K1 cells. **b** Antagonist effect of positive control SCH 23,390, *l*-TU and *d*-TU on D1/CHO-K1 cells

Fig. 4.

a Agonist effect of positive control dopamine, *l*-TU and *d*-TU on D2/CHO-K1 cells. **b.** Antagonist effect of positive control SCH 23,390, *l*-TU and *d*-TU on D2/CHO-K1 cells

Fig. 5.

Molecular docking and binding analysis for *l*-TU and *d*-TU on both D1 and D2 receptors. **a** Binding pose of *l*-TU (green) in D1 receptor (extracellular view with residues shown in gray surface of the binding pocket, and membrane view with helices shown in blue cartoon). **b** Binding poses of *d*-TU (magenta) in D1 receptor. **c** Binding pose of *l*-TU (green) in D2 receptor (extracellular view with residues shown in gray surface of the binding pocket, and membrane view with helices shown in cyan cartoon). **d** Binding poses of *d*-TU (magenta) in D2 receptor. Critical residues are marked in sticks. Hydrogen bonds and hydrophobic interactions are indicated through red and blue dashed lines

Fig. 6.

The 200-ns MD simulation for the protein-ligand complexes. **a** Simulation system using *L*-TU-D2 receptor complex as one example. Water molecules, sodium ions, chlorine ions, membrane lipids, protein, and *L*-TU are shown as red spots, yellow balls, green balls, cyan sticks, purple cartoon, and cyan spheres. **b** RMSD change for SKF38393 and D1 protein model. **c** RMSD change for *L*-TU and D1 protein model. **d** RMSD change for Dopamine and D2 protein model. **e** RMSD change for *L*-TU and D2 protein model

Fig. 7.

Structural comparison of l-TU- and SKF38393-bound D1 receptor. **a** The extracellular view of l-TU-bound structure (blue) and SKF38393-bound structure (pink). **b** The intracellular view of l-TU-bound structure (blue) and SKF38393-bound structure (pink). **c** The TRP285 for l-TU-bound structure is shown in blue sticks. **d** The TRP285 for SKF38393-bound structure is shown in pink sticks. **e** Membrane view of TRP285 and ASP103 with l-TU (green) and SKF38393 (yellow) binding on D1 receptor (blue cartoon and sticks for l-TU binding, and pink cartoon and sticks for SKF38393 binding). **f** Direct comparison on TRP285 between two structures

Fig. 8.
The shape and the size of the binding pockets. **a** D1R and SKF38393. **b** D1R and l-TU. **c** D2R and dopamine. **d** D2R and l-TU

Fig. 9.

Structural comparison of l-TU- and dopamine-bound D2 receptor. **a** The extracellular view of l-TU-bound structure (cyan) and dopamine-bound structure (orange). **b** The intracellular view of l-TU-bound structure (cyan) and dopamine-bound structure (orange). **c** The TRP386 for l-TU-bound structure is shown in cyan sticks. **d** The TRP386 for dopamine-bound structure is shown in orange sticks. **e** Membrane view of TRP386 and ASP114 with l-TU (green) and SKF38393 (yellow) binding on D2 receptor (cyan cartoon and sticks for l-TU binding, and orange cartoon and sticks for Dopamine binding). **f** Direct comparison on TRP386 between two structures

Table 1Calculated bind free energies (kcal/mol) for *l*-TU/*d*-TU in D1/D2 receptors

System	<i>l</i> -TU	<i>d</i> -TU	<i>l</i> -TU- <i>d</i> -TU
D1R	- 1.91±0.38	5.36±0.70	- 7.27±0.80
D2R	- 3.23±0.47	2.46±0.49	- 5.69±0.68

The uncertainties were calculated by dividing saved snapshots into three sub-blocks