Molecular docking study of A3 adenosine receptor antagonists and pharmacophore-based drug design

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1. Introduction

Adenosine is known to act as a neuromodulator by suppressing synaptic transmission in the central and peripheral nervous system. A3 adenosine receptor (A3AR) antagonists were recently considered as potential drugs for the treatment of cardiac ischemia and inflammation diseases. To better understand the chemical features responsible for the recognition mechanism and the receptor–ligand interaction, we have performed the molecular simulation study combined with a virtual library screening process to develop novel A3AR antagonists. A series of A3AR selective antagonists, including triazolopurines, imidazopurines, pyrrolopurines, and quinazolines were employed to dock into the A3AR binding site via AUTODOCK software. The putative binding mode for each compound was proposed. Three main hydrophobic pockets, one hydrogen bonding with Asn250, and one π–π interaction with Phe168 for all antagonists were identified. The most favorable binding conformations served as the templates for pharmacophore modeling with Catalyst 4.11 and a virtually generated library have been screened for novel antagonist development.

2. Materials and methods

Like most other transmembrane GPCRs, the high-resolution A3AR structure has not been solved to date, thus a model of the human A3AR was obtained from the Protein Data Bank (code 1OEA). It was originally constructed by homology modeling to the X-ray structure of bovine rhodopsin with 2.8 Å resolutions (Gao et al., 2002a,b). Based on the published literature, a series of compounds were selected according to their affinity and selectivity to dock into the receptor binding site. These A3AR antagonists. Understanding the molecular recognition mechanism involved in the antagonist binding is crucial. Previous docking studies of some A3AR antagonists have been reported in the literatures (Biagi et al., 2005; Moro et al., 2005). However, the molecular models for the highly selective A3AR antagonists, including triazolopurines, imidazopurines, pyrrolopurines, and quinazolines have never been studied. In order to obtain more detailed binding modes and figure out the origin of the pharmacophore, we were interested in studying these molecules to extract the critical interactions using structure-based docking analysis. Putative bioactive conformations obtained from this study are used as the templates to build the database searchable pharmacophore models, and a new series of a focused compound library has been generated based on synthetic chemistry. Successful virtual screening process with the obtained pharmacophore models resulted in a small numbers of potential hits which having different scaffold to the known A3AR antagonists.
antagonists, including triazolopurines, imidazopurines, pyrrolopurines, and quinazolines that covered structural diversity and chemical aspects that might be important for the accuracy of interaction analysis and determining essential pharmacophores. Their chemical structures and biological activities (Kᵢ values) were listed in Fig. 1 (Baraldi et al., 2005a; Okamura et al., 2002; Saki et al., 2002; Van Muijlwijk-Koezen et al., 2000).

The structures were prepared for docking study as follows: for the protein, waters were removed from the PDB file and hydrogen atoms were added; Gasteiger charges, atomic solvation parameters and fragmental volumes were merged to the receptor. For all antagonists, structures were built by using a 2D/3D editor sketcher available in Catalyst software and were minimized to a local energy minimum using the CHARMm-like force field. Gasteiger charges were assigned and non-polar hydrogen atoms were merged. All torsions were allowed to rotate during docking. The docking energy grid was produced with the auxiliary program AutoGrid. The grid dimensions were 50 × 40 × 50 points along the x-, y- and z-axes, with points separated by 0.375 Å. The grids were chosen to be sufficiently large to cover significant portions of the active pocket of A3AR. The center of the antagonists was positioned at the grid center. All graphic manipulations and visualizations were performed by means of the Chimera program, while ligand docking was performed using the empirical energy function implemented in AUTODOCK 4.0.1. The Lamarckian genetic algorithm was utilized and the energy evaluations were set at 2.5 × 10⁶. Other parameters were set to default values. Generation of database searchable pharmacophores was executed using Catalyst 4.11 which installed on a IBM6223I2C work station equipped with a Intel Xeon processor (3.0 GHz) and 1 GB of RAM running the RedHat WS3.0 operating system.

3. Results and discussion

During the docking analysis, we identified that the effective binding site of A3AR antagonists occur in the upper region of transmembrane (TM) helical bundle surrounded by TMs 3, 5, 6 and 7 (Fig. 2). All training set compounds were docked into the binding site, and the docking data were analyzed using the lowest-energy docking modes. A detailed comparison of interaction pattern from the docking results for each compound as well as their predicted total binding energies (Eₘ) were summarized in Fig. 1. The outcome of the docking analysis suggested that all ligands adopted a similar binding poses in the A₃AR (Fig. 2), and some common interaction specificities were observed in all models, namely, the hydrogen bonding with Asn250 and the π–π stacking interaction with residues Phe168 or Phe182.

Additionally, a Y-shaped active site that composed of three hydrophobic pockets (P1, P2 and P3) was predicted (Fig. 3). P1 is mainly comprised of three non-polar side chains, namely Leu90, Trp243 and Leu246. This has shown an agreement with the previous mutagenesis studies in which Trp243 was found to be...
crucial for receptor activation (Gao et al., 2002a,b), P2 included different types of hydrophobic amino acids: Met172, Ile253 and Tyr254. And another conserved hydrophobic interaction surface area P3 mainly involving residues Phe182, Ile186 and Val178, and the interaction varies depending upon different antagonistic ligands.

For compound 1, the imidazole ring and the methoxy substituent of the phenyl ring were oriented toward the hydrophobic pocket P1 and P2 respectively. The n-butyl group was located in P3 and formed a hydrophobic interaction with apolar amino acids Phe182 and Ile186 (Fig. 2). In addition, the 9-N of the triazole ring was within the H-bonding distance from the amino group of Asn250, and the triazole ring underwent an edge-to-face stacking interaction with Phe168 (Fig. 4). These two interactions appeared to be the key stabilization driving force for the triazole ring. It was in accordance with the previous site-directed mutagenesis studies which had shown the inability of the N250A mutant of A3AR to bind radio-labeled antagonists (Gao et al., 2002a,b), and thus proposing a direct interaction of Asn250 with the ligand.

Compound 2 and 3 (KF26777, 2-(4-bromophenyl)-7,8-dihydro-4-propyl-1H-imidazo[2,1-$i$]purin-5(4$H$)-one dihydrochloride) shared similar binding modes compared to compound 1. The partial negative charge on the fluorine atoms of compound 2 did not seem to be stabilized by any residues in pocket P2. This could explain both of its lower affinity and poorer docking energy compared to compound 1.

For compound KF26777, in addition to the H-bonding interaction of Asn250 with the imidazole N atom, an extra H-bond relationship was observed between the hydroxyl group of the residue Ser247 and the nitrogen on position 9 at 2.8 Å (Fig. 5). These interactions might play a crucial role in stabilizing the imidazopurine scaffold, and may be responsible for the favorable docking energy ($E_b = -7.50$ kcal/mol) and binding affinity ($K_i = 0.20$ nM).

The imidazopurine and pyrrolopurine derivatives (4, 5 and 6) were docked into the A3AR binding site in the similar orientation. For all three compounds, a hydrogen bonding interaction was predicted between a nitrogen atom of the imidazole ring and the amino group of Asn250. Edge-to-face stacking interactions were observed between Phe168 and the pyrimidone ring of compound 4 and 5, while compound 6 instead undergo a parallel displaced interaction by the phenyl group with Phe182 (Fig. 6). Similar hydrophobic interactions were obtained for all three molecules and the docking energy was well correlated to their antagonist binding affinities.

The quinazoline derivative VUF5574 (N-(2-methoxyphenyl)-N-(2-[3-pyridyl]quinoxalin-4-yl)urea, $K_i = 0.50$ nM) (Fossetta et al., 2003), showed the best docked result ($E_b = -8.15$ kcal/mol) due to it was engaged in several attractive interactions (Fig. 7). Hydrogen bonding was predicted between the 2-N of the quinazoline scaffold and the amino group of Asn250 at 2.8 Å. The o-methoxyphenyl group of the ligand was placed in hydrophobic pocket P2, which constituted by Phe168, Met172 and Val178. The oxygen atom of the o-methoxyphenyl group was within the H-bonding distance from the amino group of Gln167 at 2.9 Å, and an edge-to-face CH/$\pi$ interaction between o-methoxyphenyl aromatic ring and Phe168 methyl group was observed. The pyridine ring was buried in hydrophobic pocket P1, and in addition, the quinazolinophenyl group was predicted to undergo hydrophobic interactions with Thr94 and Ile186 side chains.

4. Pharmacophore-based drug design

To develop new A3AR antagonist, we performed a new approach called "pharmacophore-based virtual library screening". The
elucidated binding modes allowed us to gain a comprehensive insight into the interactions between antagonists and A3AR. According to the docked results, we could extract the pharmacophore models for each compound based on the essential ligand-receptor interactions. The purpose of pharmacophore models is to perform in silico screening searches in a 3D database of a virtual compound library to find diverse structures with desired A3AR activity and selectivity.

The pharmacophore-based virtual screening process includes the following steps: (1) Generate and validate pharmacophore models. (2) Generate virtual compound library and build 3D database that can be used for pharmacophore-based screening. (3) Database search to find and define primary hits. (4) Biological evaluation to identify true lead compounds from the primary hits.

We consider the four to five featured hypothesis models will be reasonable to characterize the important binding points between the antagonists and the receptor. Therefore, three hydrophobic features were selected corresponding to the binding pockets P1, P2, and P3, and two hydrogen bond (HB) acceptors were extracted to represent the dipole–dipole force between electronegative atoms and the hydrogen from the residues of A3AR. Using the hypothesis generation method implemented within the Catalyst software package, multiple queries can be built with combinations of the features we have selected. This report we discuss the use and the in silico screening result for the four-featured pharmacophore models.

From total six of four and five featured pharmacophore combinations (combination from three hydrophobic features and two HB acceptors but keep at least one HB feature in the model), we selected three four-featured pharmacophore models, which represent different binding patterns in 3D space and are able to cover such important interaction points that mostly contributed to the binding energies. In Fig. 8, we illustrated the selected pharmacophore models that mapped with the docking study compounds. Each pharmacophore has generated using Catalyst 4.11 based on the docked low energy conformation of the antagonists. Hypo-1 is generated from the compounds 1, 2, and 3, and it maps the low energy conformations very well for these antagonists. Hypo-2 represents the recognition modes for another three compounds: 4, 5 and 6. And for Hypo-3, however it comes from the conformation of compound 7 that showed the best docking study result. These three pharmacophore models cover different binding modes for docking study molecules (the known A3AR antagonists) and we decided to use them as queries for the virtual screening applications.

Our strategy to generate a pharmacophore searchable database is to design a focused compound library from chemistry reaction expertise. This approach is effective to find lead molecules with novel scaffolds compared to commercially available compound database and is practical for medicinal chemists. Start from a commercially available starting material 4-nitro-3-pyrazolecarboxylic acid, we can easily construct a pyrazole derivative library with great variation in diversified R1, R2 and R3 function groups. The total number of this focused library is about 1500 analogs depends on the chemical reagents for R1, R2 and R3 substituents (Fig. 9).

Chemical structures of the enumerated library compounds were generated using 2D/3D editor sketcher in Catalyst 4.11soft-

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**Fig. 6.** Docking complexes of compound 6. The antagonist is represented by ball and stick model. The antagonist and receptor residues are colored according to atom type. Phe182 are colored in purple. H-bond interactions are colored in cyan. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

**Fig. 7.** Docking complexes of VUF5574. The antagonist is represented by ball and stick model. The antagonist and receptor residues are colored according to atom type. Phe168 is colored in magenta and hydrogen bond interactions in cyan. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

**Fig. 8.** Extracted pharmacophore models from docked A3 antagonists. (a) Hypo-1 mapped with compound 1 (pink), 2 (yellow), and 3 (brown). (b) Hypo-2 mapped with compound 4 (pink), 5 (yellow), and 6 (brown). (c) Hypo-3 mapped with compound 7. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
ware package and are minimized to the closest local minimum using the CHARMM-like force field implemented in the program. A stochastic research coupled to a poling method (Smellie et al., 1995) was applied to generate conformers for each compound by using 'Best conformer generation' option with 20 kcal/mol energy cutoff (20 kcal/mol maximum compared to the most stable conformer).

The virtual screening process was then carried out by using the best flexible database search option in Catalyst, and use of a 'Best fit' cutoff value as 3.0, resulted in total 150 compounds as primary hits (about 13% hit rate according to this specific best fit value cutoff). According to our previous study on A2A adenosine receptor antagonists (Ye et al., 2008; Wei et al., 2007, and unpublished biological data), we found that the pharmacophore models derived from the docking analysis were more predictive compared with models simply generated from ligand-based common feature analysis.

We picked up some representative library compounds from the primary hits and performed a mapping validation test (Fig. 10), and their mapping results with the pharmacophore models have summarized in Table 1. All molecules except compound 4 with Hypo-2 exhibited a significant high numbers of fit values toward all three of the pharmacophore models (a complete geometric fit to a four-featured pharmacophore model will give a number of 4.0 as fit value, see Catalyst version 4.11 software; ACCELERYS Inc: 2005). As an example, the mapping results for compound L3, L5 and L6 with the pharmacophore models are illustrated in Fig. 11. The

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Hypo-1</th>
<th>Hypo-2</th>
<th>Hypo-3</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1</td>
<td>3.735</td>
<td>3.420</td>
<td>3.792</td>
</tr>
<tr>
<td>L2</td>
<td>3.843</td>
<td>3.604</td>
<td>3.820</td>
</tr>
<tr>
<td>L3</td>
<td>3.852</td>
<td>3.680</td>
<td>3.896</td>
</tr>
<tr>
<td>L4</td>
<td>3.682</td>
<td>2.868</td>
<td>3.744</td>
</tr>
<tr>
<td>L5</td>
<td>3.821</td>
<td>3.680</td>
<td>3.896</td>
</tr>
<tr>
<td>L6</td>
<td>3.793</td>
<td>3.556</td>
<td>3.916</td>
</tr>
</tbody>
</table>

* a Compound numbers and chemical structures are depicted in Fig. 10. The number represents the best fit of each molecule to the corresponding pharmacophore model. The higher the best fit value, the better a molecule maps the features of the pharmacophore. The best fit value 4.00 means a perfect mapping of the molecule to the pharmacophore model.

* Hypo-1 was generated from the docked conformation of A2AR antagonists 1, 2 and 3.

* Hypo-2 was generated from the docked conformation of A2AR antagonists 4, 5 and 6.

* Hypo-3 was generated from the docked conformation of A2AR antagonist 7.
molecular docking to study the binding modes of a series of different structural scaffolds. We performed an automated approach for the purpose of developing novel A3 antagonists with different structural scaffolds. We found that the binding site consists of three main hydrophobic pockets, which leverage the steric interference between the residues and substituted groups on the A3 antagonists. The residues involved in hydrophobic interactions with the ligands were Leu90, Met172, Trp243, Leu246, Ile253 and Tyr254. In addition to the lipophilic effect, our docking results also indicated that the π–π stacking interaction with Phe168 and the intermolecular hydrogen bonding with Asn250 appeared to be the key stabilization driving force for the A3AR antagonists.

Based on the docking analysis results, we extracted a series of database searchable pharmacophore models that represent the important binding modes for A3 antagonists. These pharmacophore models were used for in silico screening process to develop potential new A3 antagonists. A new series of pyrazole core structure derivatives as a focused compound library has been designed and generated based on synthetic chemistry. Successful virtual screening process with the obtained pharmacophore models resulted in a small numbers of primary hits, which showed high potential for the human A3 adenosine receptor antagonists.

References


Fig. 11. Results from mapping validation analysis for representative library compounds as primary hits. (a) Selected library compound L3 mapped to Hypo-1 (fit value = 3.852). (b) Selected library compound L5 mapped to Hypo-2 (fit value = 3.680). (c) Selected library compound L6 mapped to Hypo-3 (fit value = 3.916).