Limits of Ligand Selectivity from Docking to Models: In Silico Screening for A1 Adenosine Receptor Antagonists

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Abstract

G protein-coupled receptors (GPCRs) are attractive targets for pharmaceutical research. With the recent determination of several GPCR X-ray structures, the applicability of structure-based computational methods for ligand identification, such as docking, has increased. Yet, as only about 1% of GPCRs have a known structure, receptor homology modeling remains necessary. In order to investigate the utility of homology models and the inherent selectivity of a particular model in relation to close homologs, we constructed multiple homology models for the A1 adenosine receptor (A1AR) and docked (~2.2 M lead-like compounds. High-ranking molecules were tested on the A1AR as well as the close homologs A2aAR and A2bAR. While the screen yielded numerous potent and novel ligands (hit rate 21% and highest affinity of 400 nM), it delivered few selective compounds. Moreover, most compounds appeared in the top ranks of only one model. These findings have implications for future screens.


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Introduction

G protein-coupled receptors (GPCRs) are one of the pharmaceutically most important protein families, and the targets of around one third of present day drugs [1]. They mediate the transmission of signals from the exterior to the interior of a cell by binding signaling agents and, via conformational changes, eliciting intracellular responses. GPCRs consist of seven membrane-crossing helices. The binding pockets of the native small molecule ligands, i.e. orthosteric binding sites, are situated in the middle of the helical bundle in the Class A GPCR structures that have been determined so far [2]. Despite the recent advances in GPCR X-ray structure determination [3] and the substantial numbers of novel ligands identified for some GPCRs [4,5], there are still many (potential) GPCR targets for which no structure or ligands are known. In order to apply protein structure-based methods of ligand identification, in particular docking, to receptors that lack an experimentally determined structure, homology modeling is a promising avenue. Constructing homology models is facilitated by the fact that the transmembrane (TM) region of Class A GPCRs is relatively well conserved [6]. The accuracy of homology models is limited, however, by the uncertainty of modeling the extra- and intracellular loops, which greatly vary in length and amino acid composition, even between otherwise closely related GPCRs [7].

In this study, we tested the utility of homology models for docking and selecting compounds with reasonable affinity for the investigated receptor subtype. We intentionally restricted the amount of existing ligand data used to refine the binding site during model building to mimic a situation where few ligands are known (as would be the case for previously little investigated “novel” targets). In fact, except for the very first steps of model building and optimization, only the affinity data obtained in this study was used to improve the homology models. Three sequential cycles of model refinement, docking, and ligand testing were applied, using the data acquired in previous rounds to guide the receptor model optimization in the following rounds. In parallel, we also probed the tendency of the screen to identify novel ligands of other subtypes within the same receptor family, i.e. the selectivity of a homology model-based screen against a single GPCR subtype. These findings were compared with the distribution of selectivity ratios of known ligands for the same subtypes.

The adenosine receptors (ARs), which consist of the four subtypes A1, A2A, A2B, and A3, have been chosen as a suitable test case for the application of virtual screening to a closely related subtype of a known GPCR structure. There are both antagonist-bound and agonist-bound X-ray structures known for the A2aAR subtype, with various ligands co-crystallized for each case. Thus, the region for orthosteric AR ligand binding has been well characterized. The first antagonist-bound structure to be determined was co-crystallized with the high affinity ligand 4-[2-(7-amino-2-(2-furyl)-1,2,4-triazolo[1,5-a][1,3,5]triazin-5-yl-amino)ethylphenol (I, ZM241385, Fig. 4) [8,9]. An unexpected
orientation of the ligand perpendicular to the plane of the membrane bilayer was observed. Extracellular loops, as well as helical TM domains, are involved in coordinating the ligand. In silico virtual screening for A2AAR antagonists has already been demonstrated to be successful based on the inactive conformation of the A2AAR, as determined by crystallography [10,49].

Among the different subtypes, the A1AR is also an attractive pharmaceutical target. Its antagonists have been explored as kidney-protective agents, compounds for treating cardiac failure, cognitive enhancers, and antiasthmatic agents [11,12]. Structurally diverse antagonists, such as the pyrazolopyridine derivative 2 and the 7-deazaadenine derivative 3, were previously identified, and some of these compounds were under consideration for clinical use [13,14]. The prototypical AR antagonists, i.e. the 1,3-dialkylxanthines, have provided numerous high affinity antagonists with selectivity for the A1AR. One such antagonist, rolofylline 4, an alkylxanthine derivative of nanomolar affinity, was previously in clinical trials for cardiac failure [15].

The human A1AR subtype was investigated in this study because it shares a high level of sequence identity (40%) with the A2AAR. It should thus be possible to model the A1AR by homology with high confidence. While this homology model was the only three-dimensional structure of a protein employed in the screening, all compounds were also tested in receptor binding assays against two other AR subtypes in order to investigate the intrinsic selectivity of the model.

Methods

Homology Modeling

The 3D structure of the A1AR was generated with the software MODELLER [16,17] using the X-ray structure of the A2AAR (PDB 3EML; the only structure available at the time) [8] as a template. The overall sequence identity between the two proteins is 40%, with an additional 21% similar residues. Since the A2AAR structure was solved with the antagonist 1, water molecules, and stearic acid, these heteroatoms were included during A1AR model building to obtain a model conformation closer to the A2AAR X-ray structure.

Due to the stochastic conformational sampling used for homology modeling, an ensemble of 100 models was constructed using the same alignment. The most accurate model from this ensemble of models was selected according to the DOPE (Discrete Optimized Protein Energy) atomic distance-dependent statistical potential function [18], which is included in MODELLER. However, because DOPE had only been trained and tested on
globular proteins, its usefulness for assessing models of membrane proteins such as GPCRs was unclear. Thus, globular regions were extracted from the modeled A1AR structures by selecting residues in a 6 Å sphere around C7, C11, and C12 of \(1\). This extraction resulted in 100 approximately globular protein fragments. These fragments were scored with DOPE and DOPE_HR (DOPE high resolution) and the top five scoring models were inspected visually. Criteria in this visual inspection were the absence of obvious steric clashes with \(1\), the absence of kinks in the helices, an orientation of the sidechain of Asn254 6.55 away from the main chain, and preservation of the disulfide bonds between Cys80 3.25-Cys169 and Cys260 6.61-Cys263 7.28 (superscripts denote Ballesteros-Weinstein numbers [19]). The model that was chosen among the top five according to these criteria was denoted as model \(O\).

**Table 1.** In vitro affinity in binding to three subtypes of hARs of diverse heterocyclic derivatives identified through their high ranks in the *in silico* screen (structures are shown in Chart 2).

<table>
<thead>
<tr>
<th>Compound ID</th>
<th>(A_1) (K_i) (nM)</th>
<th>(A_{2A}) (K_i) (nM)</th>
<th>(A_3) (K_i) (nM)</th>
<th>Model</th>
<th>Closest ChEMBL (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>17±9%</td>
<td>3310±270</td>
<td>43±3%</td>
<td>(A)</td>
<td>0.53</td>
</tr>
<tr>
<td>8</td>
<td>3460±420</td>
<td>11±6%</td>
<td>35±4%</td>
<td>(A)</td>
<td>0.64</td>
</tr>
<tr>
<td>9</td>
<td>2±2%</td>
<td>2360±260</td>
<td>4860±330</td>
<td>(A)</td>
<td>0.47</td>
</tr>
<tr>
<td>10</td>
<td>9±9%</td>
<td>37±1%</td>
<td>9060±1100</td>
<td>(A)</td>
<td>0.57</td>
</tr>
<tr>
<td>11</td>
<td>13±9%</td>
<td>35±3%</td>
<td>13,700±2200</td>
<td>(A)</td>
<td>0.56</td>
</tr>
<tr>
<td>12</td>
<td>28±9%</td>
<td>3655±870</td>
<td>2780±920</td>
<td>(A)</td>
<td>0.72</td>
</tr>
<tr>
<td>13</td>
<td>10±10%</td>
<td>10,900±2200</td>
<td>3480±1100</td>
<td>(A)</td>
<td>0.60</td>
</tr>
<tr>
<td>14</td>
<td>19±10%</td>
<td>6540±1090</td>
<td>49±1%</td>
<td>(A)</td>
<td>0.25</td>
</tr>
<tr>
<td>15</td>
<td>20±4%</td>
<td>5±3%</td>
<td>9330±1800</td>
<td>(A)</td>
<td>0.30</td>
</tr>
<tr>
<td>16</td>
<td>13±2%</td>
<td>36±0.2%</td>
<td>13,400±1900</td>
<td>(A)</td>
<td>0.46</td>
</tr>
<tr>
<td>17</td>
<td>400±60</td>
<td>740±390</td>
<td>48±7%</td>
<td>(B)</td>
<td>0.49</td>
</tr>
<tr>
<td>18</td>
<td>3430±1030</td>
<td>2130±720</td>
<td>1760±210</td>
<td>(B)</td>
<td>0.41</td>
</tr>
<tr>
<td>19</td>
<td>3340±560</td>
<td>6660±860</td>
<td>23±3%</td>
<td>(B)</td>
<td>0.41</td>
</tr>
<tr>
<td>20</td>
<td>45%**</td>
<td>3560±510</td>
<td>1520±360</td>
<td>(B)</td>
<td>0.71</td>
</tr>
<tr>
<td>21</td>
<td>980±90</td>
<td>1340±310</td>
<td>205±30</td>
<td>(B)</td>
<td>0.39</td>
</tr>
<tr>
<td>22</td>
<td>36%**</td>
<td>9300±700</td>
<td>42±6%</td>
<td>(B)</td>
<td>0.32</td>
</tr>
<tr>
<td>23</td>
<td>1220±340</td>
<td>3780±830</td>
<td>70±20</td>
<td>(B)</td>
<td>0.50</td>
</tr>
<tr>
<td>24</td>
<td>33±9%</td>
<td>6140±1690</td>
<td>40±6</td>
<td>(D)</td>
<td>0.42</td>
</tr>
<tr>
<td>25</td>
<td>2930±480</td>
<td>1450±170</td>
<td>550±70</td>
<td>(D)</td>
<td>0.30</td>
</tr>
<tr>
<td>26</td>
<td>3940±390</td>
<td>1370±470</td>
<td>3850±590</td>
<td>(D)</td>
<td>0.27</td>
</tr>
</tbody>
</table>

*a* Binding in membranes of CHO (\(A_1\) and \(A_3\)ARs) or HEK293 (\(A_{2A}\)AR) cells stably expressing a hAR subtype. Total and nonspecific binding at the \(A_1\)AR determined using \(^{[3}H\)DPCPX in the absence and presence of 10 \(\mu\)M CGS15943 (N-[9-chloro-2-(2-furanyl)[1,2,4]triazolo[1,5-c]quinazolin-5-amine), respectively.

*b* ECFP4 Tanimoto similarity for the most structurally similar known AR ligand (Table S3).

*p* percent inhibition at 10 \(\mu\)M compound concentration.

**n = 1.

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**Figure 2.** Calculated binding mode of compound 8, the ligand hit with the highest selectivity towards \(A_1\)AR. The protein is model \(A\). Orange dotted lines denote hydrogen bonds formed with Asn254 6.55. Helices are labeled with roman numerals. doi:10.1371/journal.pone.0049910.g002

**Table 2.** Performance of the four homology models against the three AR subtypes.

<table>
<thead>
<tr>
<th>MODEL</th>
<th>(A_1) (A/T^a)</th>
<th>(A_{2A}) (A/T^b)</th>
<th>(A_3) (A/T^c)</th>
<th>(Round)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1/15 7% 33% 7/15</td>
<td>5/15 33% 5/15</td>
<td>1/15 7% 33% 7/15</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>5/12 42% 58% 4/12</td>
<td>7/12 58% 7/12</td>
<td>5/12 42% 58% 4/12</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>0/6 0% 0% 0/6</td>
<td>0/6 0% 0/6</td>
<td>0/6 0% 0% 0/6</td>
<td>3</td>
</tr>
<tr>
<td>D</td>
<td>2/6 33% 50% 3/6 33% 50% 3/6 33%</td>
<td>3/6 50% 3/6 50% 3/6 50% 3/6 50%</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>8/39 21% 38% 14/39</td>
<td>15/39 38% 15/39</td>
<td>8/39 21% 38% 14/39</td>
<td>36%</td>
</tr>
</tbody>
</table>

*a* number of actives (A) over number of molecules tested (T).

*b* sum: overall hit rate for all tested ligands.

doi:10.1371/journal.pone.0049910.t002

In Silico Screening for \(A_1\)AR Antagonists
Model Refinement

As shown previously, adapting the orthosteric sites of GPCR homology models to known ligands improves pose fidelity and hit rates [20]. Thus, for optimization of model O, binding site residues within a 6 Å radius around the equivalent position of I (the ligand in 3EML) were iteratively refined with CHARMM [21] and MODELLER. The residues selected for optimization were also compared to mutagenesis studies of the A1AR in recognition of agonists and antagonists [22,23]. Residues that caused major changes in binding affinity (up to 100-fold decrease) after alanine substitution were checked against the selection of residues within 6 Å of the ligand. In all cases, the residues that contributed to a loss of binding affinity after alanine substitution were included in the selection.

For the part of the refinement using CHARMM, the CHARMM22 force field (Accelrys, Inc.) was used, and harmonic restraints with a force constant of 50 kcal/mol-Å² and a minimum at 2.4 Å were assigned to the hydrogen bonds formed between the respective ligand and Asn2546.55, the key recognition residue in the A1AR binding pocket. A known ligand of the A1AR (4-[(3-benzyl-3-phenyl-triazolo[4,5-c]pyrimidin-7-yl)amino)cyclohexane-1-ol; 5, [24]) was placed manually in the binding site (to ensure correct orientation, i.e. maintenance of the two hydrogen bonds with Asn2546.55 and force-field minimized while keeping the adjacent residues fixed. The optimized ligand pose was then included in the following re-modeling step with MODELLER. This procedure of force-field minimizing the ligand and remodeling with MODELLER was repeated until the atomic positions of the active site residues and the ligand converged. To check for bias introduced by the optimization with the reference triazolo-pyrimidine derivative 5, a second AR antagonist (1-(8-butyl-2-furan-2-yl)-8H-pyrazolo[4,3-e] [1,2,4]triazolo[1,5-c]pyrimidin-5-yl)-3-(4-nitro-phenyl)-urea, 6 [25]) was manually placed in the binding site, again making making sure that the hydrogen bonds with Asn2546.55 are formed, and minimized with PLOP [26,27].

Residues whose interaction with the ligand had unfavorable force field energy values ( Ala612.64, Ile692.24, Phe171, Leu2506.31, and Ile2747.39) were sidechain-optimized followed by minimization together with the ligand. Both ligands had been part of a set of 3276 A1AR binders extracted from the WOMBAT database [28]. They were selected for the refinement process because they docked in poses interacting with Asn2546.55 and ranked highly when docked to model O. The final refined structure, termed model A, was used in the first docking round (see below and Fig. 1A).

Using the ligand data acquired in round one, the orthosteric binding site of the A1AR was optimized a second time. In this round of refinement (resulting in model B; Fig. 1B), residues were chosen based on their deviation from the corresponding residues in the A1AR structure. In particular, extracellular loop 3 (ECL3; residues Phe2596.66 to Cys2637.28) and adjacent residues in helix 7 (up to Ser2677.32) were rebuilt to maintain the salt bridge between His2647.29 and Glu172. Moreover, the “toggle switch” Trp2479.48 and the adjacent His2516.52, which showed large deviations of up to 140° in their χ₁ angles, were manually flipped and then minimized. No restraints were applied during loop rebuilding, but all loop conformations that did not place the CS and Cγ atoms of His2647.29 within 0.8 Å of the equivalent positions of these atoms in the A2AAR structure 3EML were discarded. The sidechain orientations for all other residues were sampled and minimized together with 8 (Figure 5), the ligand that was used in this refinement. All optimizations in this and the third round were done with PLOP and the pose of 8 was the one obtained from docking.

Refinements in the third round again used the most selective ligand identified in the previous rounds (8) and optimized the sidechains of the same residues as before. Multiple structures were generated, clustered by sidechain conformations and assessed by calculating their ability to rank the ligands over the decoys of rounds one and two (assessed via the value of the area under the curve [logAUC] of receiver-operator characteristic [logROC] plots). For each sidechain conformation cluster, the best structure according to the logAUC criterion was kept and used in docking (models C and D; Fig. 1C and 1D, respectively).

Docking

All calculations were carried out using DOCK3.5.54 [29–32] and the approximately 2.2 M molecules of ZINC’s lead-like subset [33]. The molecules in this subset are between 250 and 350 g/mol in molecular weight, have less than 7 rotatable bonds, and have an xlogP between 2.5 and 3.5.

The docking spheres used as anchor points in the binding site to position the database molecules in the orthosteric pocket were calculated based on the heavy-atom positions of carazolol and I when superimposing the backbone atoms of the β2-adrenergic receptor (PDB code 2RH1) and A2AAR, respectively, with the A1AR model. Where necessary, spheres were moved manually to obtain a more homogenous distribution. During docking, every molecule was fitted onto spheres chosen by the algorithm based on the similarity of the distances between the spheres and corresponding heavy atoms in the molecules. Each molecule pose was minimized for 25 steps with the simplex method. Finally, the binding affinity was estimated by adding the electrostatic and van der Waals interaction energies and correcting for solvation penalty. These energy terms were obtained from precalculated values stored on cubic grids. To emphasize the highly conserved interaction of adenosine with Asn2546.55, partial charges on the polar side chain atoms were amplified by 0.4 units in such a way that the overall charge of the residue remained neutral. After docking, the top 500 poses were inspected visually to filter out
molecules with unsatisfied hydrogen bond donors or acceptors, incorrect protonation states, unlikely binding modes due to incorrect parametrization, or highly strained conformations. The selected molecules were acquired from their respective vendors as listed in the ZINC database.

Selectivity Ratios of known AR Ligands

All ligands annotated with an activity value against at least one of the investigated AR subtypes were downloaded from version 12 of the ChEMBL database [34]. The data was made uniform by keeping only affinities measured as Ki. Ki-values described as “greater than” a threshold (ranging from 10^{-8} M to 10^{-2} M, depending on the study the data originated from) were treated as “inactive”. For molecules with more than one independently measured Ki value, the average was calculated. Cases with at least one “active” and one “inactive”, i.e. inconsistent, classification with respect to a particular receptor were discarded. The selectivity ratio was calculated by dividing the respective Ki values of one ligand against two different receptor subtypes, and binned according to their ratio. The Ki-value of an inactive molecule was arbitrarily set to 1 M, except for cases where a molecule was inactive against both investigated subtypes, and was thus not considered further in the analysis. The choice of the numerical value for inactive compounds had no influence on the conclusions drawn, as we only compared data that had been obtained with the same settings.

**Figure 4. Chart 1.** Reference compounds (known selective A_{1}AR antagonists) mentioned in the text. Ki values are as follows, with targets other than human A_{1}AR in parentheses: 1: K_i 0.8 nM [8]; 2: K_i 18 nM; 3: K_i 1 nM; 4: K_i 1 nM [11]; 5: K_i 3 nM (bovine A_{1}AR [20]); 6: K_i 584 nM [21].

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Figure 5. Chart 2. Molecules identified in this study. Binding affinity data at three AR subtypes are presented in Table 1.
doi:10.1371/journal.pone.0049910.g005
Experimental Assays

Binding affinity for three human AR (hAR) subtypes was measured using standard radioligand assays and membrane preparations from Chinese hamster ovary (CHO) cells [35] (A1 and A3) or human embryonic kidney (HEK) 293 cells (A2A) stably expressing a hAR subtype (Table 1).

Receptor binding assays: [3H]8-Cyclopentyl-1,3-dipropylxanthine ([3H]DPCPX, 120 Ci/mmol) and [125I]-N6-(4-aminomethylbenzyl)adenosine-5'-N6-methyluronamide ([125I]I-AB-MECA, 2200 Ci/mmol) were purchased from Perkin-Elmer Life and Analytical Science (Boston, MA). [3H][2-[p-[2-Carboxethyl]phenyl-ethylamino]-5'-N6-ethylcarboxamido-adenosine] ([3H]CGS21680, 39 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO). Other pharmacological reagents were purchased from Tocris-R&D Systems, Inc. (Minneapolis, MN). Test compounds were prepared as 5 mM stock solutions in DMSO and stored frozen.

Cell Culture and Membrane Preparation: CHO cells stably expressing the recombinant hA1 and hA2ARs, and HEK-293 cells stably expressing the hA2AR were cultured in Dulbecco’s modified Eagle medium (DMEM) and F12 (1:1) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 μmol/mL glutamine. In addition, 800 μg/mL gentamicin was added to the A2A media, while 500 μg/mL hygromycin was added to the A1 and A2 media. After harvesting, cells were homogenized and suspended in PBS. Cells were then centrifuged at 240 g for 5 min, and the pellet was resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM MgCl2. The suspension was homogenized and was then ultra-centrifuged at 14,330 g for 30 min at 4°C. The resultant pellets were resuspended in Tris buffer and incubated with adenosine deaminase (3 units/mL) for 30 min at 37°C. The suspension was homogenized with an electric homogenizer for 10 sec, pipetted into 1 mL vials and then stored at -80°C until the binding experiments. The protein concentration was measured using the BCA Protein Assay Kit from Pierce Biotechnology, Inc. (Rockford, IL) [36].

Binding assays: Standard radioligand binding assays for A1, A2A, and A3ARs were used [37–39]. Into each tube in the binding assay was added 50 μL of increasing concentrations of the test ligand in Tris-HCl buffer (50 mM, pH 7.5) containing 10 mM MgCl2, 50 μL of the appropriate agonist radioligand, and finally 100 μL of membrane suspension. For the A1AR (22 μg of protein/tube) the radioligand used was [3H]DPCPX (final concentration of 0.5 nM). For the A2AR (20 μg/tube) the radioligand used was [3H]CGS21680 (final concentration 10 nM). For the A3AR (21 μg/tube) the radioligand used was [125I]I-AB-MECA (final concentration 0.2 nM). Nonspecific binding was determined using a final concentration of 10 μM NECA diluted with the buffer. The mixtures were incubated at 25°C for 60 min in a shaking water bath. Binding reactions were terminated by filtration through Brandel GF/B filters under a reduced pressure using a M-24 cell harvester (Brandel, Gaithersburg, MD). Filters were washed three times with 3 mL of 50 mM ice-cold Tris-HCl buffer (pH 7.5). Filters for A1 and A2AR binding were placed in scintillation vials containing 5 mL of Hydrofluor scintillation buffer and counted using a Perkin Elmer Liquid Scintillation Analyzer (Tri-Carb 2810TR). Filters for A3AR binding were counted using a Packard Cobra II γ-counter.

Data analysis: Binding and functional parameters were calculated using Prism 5.0 software (GraphPAD, San Diego, CA, USA). IC50 values obtained from binding inhibition curves were converted to Ki values using the Cheng-Prusoff equation [40]. Data were expressed as mean ± standard error or percentage inhibition at 10 μM.
corresponding selectivity ratios (inner donuts in Fig. 3). Although the numbers have to be viewed with caution because of the limitations of statistics of small numbers, these observations contrast those for the ChEMBL ligands, which tended to be more selective for A1.

**Discussion**

Three main results emerge from this study. First, as has been shown previously [45,46], different models (or X-ray structures) of the same receptor yield different ligand sets, even when screening the same diverse library. Interestingly, the performance of the various models, both in absolute number of actual ligands as well as in terms of selectivity, differed widely. This fact is both en- and discouraging. It is encouraging, because it means that even using models with large structural deviations from a closely related template (i.e. the conformation of ECL3, the lack of the conserved salt bridge between His264-H2 and Glu172, and the orientation of Trp247-H4) such as model A, docking is likely to find pharmacologically validated ligands. Conversely, it is discouraging, as the presumably refined model C did not yield any ligands. This is particularly striking considering the small differences between models C and D. We did not exclude the molecules tested in earlier rounds of screening during the subsequent ones, yet the vast majority of ligands identified in one model did not appear in the top ranks of a screen against another one (data not shown). Such behavior is a testament to the conformational flexibility of GPCRs, but also to the sensitivity of docking to small changes in the protein structure. In combination, it can be exploited to identify larger numbers of ligands by docking to more than one protein conformation. Any model of a protein structure (including the X-ray solution) represents only one possibility from the continuum of conformations. Thus, using differently optimized models (e.g. obtained by slightly different ligand placements or different force field parameters), the set of identified ligands would have changed. Yet, the overall performance, with some models being able to recognize ligands and some not, would be similar. This fact might also be considered disheartening for approaches that aim to include receptor flexibility via docking to multiple conformations of a receptor and calculating the average rank of a molecule across all structures.

Second, docking to GPCRs, even using “only” homology models, works well. The screen against the A1AR was successful by all criteria, with a hit rate of 21% and potent compounds with Ki values as low as 400 nM for the 2H-chromen-2-imine derivative 17. Some of the ligands also represent novel chemotypes for the A1AR, such as 17 and related, albeit only weakly active, derivatives quinoxalin-4(3H)-ones [14, 22, 25] and a pyrido[2,3-d]pyrimidin-4(3H)-one [26]. In particular, the ligands identified with model D tend to have ECFP4 Tanimoto similarity values of less than 0.40 when compared to the 7173 AR ligands in the ChEMBL database. The reason for the relatively few genuinely novel ligands presumably lies in the bias of the library, as shown before [47]. However, the overall performance of this screen is in line with previous docking studies that identified numerous and potent GPCR ligands [9,45–49]. As was the case here, most of these campaigns targeted a Class A GPCR that binds small organic molecules. Such receptors tend to have rather narrow, well-defined binding sites – in contrast to the CXCR4 receptor, the only peptide-bound GPCR structure elucidated so far [50]. Smaller binding pockets make for narrower physical search spaces which is likely one of the reasons behind the tractability of these GPCRs by docking and similar approaches.

Third, for receptors with high degrees of similarity, such as the ARs, selective compounds cannot be predicted solely by docking to one receptor subtype. Most of the ligands identified as A1AR hits also bound to one of the other AR subtypes, and vice versa. In fact, the screen directed toward the A1AR worked even better against the A2AR, with a hit rate of 36% and the most potent compound inhibiting with a Ki of 36 nM. This is an advantage if it is desired to discover ligands for other related GPCR subtypes within a single screening process.

However, there is one compound, 8, which appears selective for the A1AR by the criteria used in this screen. In addition, some of the ligands were also moderately selective in binding to the A3AR, which may be due to the fact that the binding pocket of the A3AR is the most divergent one when comparing the three AR subtypes (Table S3), suggesting the relative ease of achieving A3AR selectivity. This tendency to cross over to other subtypes in the screening process can be expected from the similarity of the binding sites. It is difficult to estimate, however, to what degree the use of homology models affected the selectivity of the compounds. Bias stemming from the template used (the A2AR) cannot be ruled out, but cannot be the only factor as evidenced by the many compounds binding to A3AR. Very likely, even computational screens employing X-ray structures result in similarly nonsubtype-selective hit compounds. However, because biochemical testing is limited to the targeted subtype in most studies, this does not become apparent. As a further example of this observation, in the A3AR screen by Carlsson et al. [10], which is based on a crystal structure, several ligands were found that had mixed selectivity for the A2A and A3ARs.

Docking will undoubtedly continue to play a significant role in the quest for novel GPCR ligands, as it has been able to consistently identify potent and chemically novel ligands for a variety of receptors. The targeted identification of selective compounds by combining multiple approaches to model the same receptor and closely related members of the same protein family will be the topic of future investigations. Furthermore, the most promising hits from this study, such as a mixed A1/A2AAR ligand, i.e. the 2H-chromen-2-imine derivative 17, or a moderately potent and slightly selective A3AR ligand, i.e. 1,3,5-triazine derivative 24, could now be optimized structurally for AR affinity and selectivity.

**Supporting Information**

Table S1 Ligands that were tested and replaced less than 50% of radioligand at 10 μM in all targets. **n=2.** (PDF)

Table S2 Compounds in ChEMBL most similar to the ligands identified in this study. (PDF)

Table S3 Comparison of binding site residues between A1AR, A2AAR and A3AR. * superscripts give the Balleseros-Weinstein numbers. (PDF)

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**Author Contributions**

Conceived and designed the experiments: PK AS KAJ. Performed the experiments: PK KP ZG ACM. Analyzed the data: PK ACM KAJ. Wrote the paper: PK ACM AS KAJ.
References


39. Cher GY, Prussoff HR (1975) Relationship between the inhibition constant (K) and the concentration of inhibitor which causes 50 per cent inhibition (I₅₀) of an enzymatic reaction. Biochim Biophys Acta 11680.


