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Structural determinants of the alpha2 adrenoceptor subtype selectivity

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Abstract

Alpha2-adrenergic receptor (α_2 -AR) subtypes, acting mainly on the central nervous and cardiovascular systems, represent important targets for drug design, confirmed by the high number of studies published so far. Presently, only few α_2 -AR subtype selective compounds are known. Using homology modeling and ligand docking, the present study analyzes the similarities and differences between binding sites, and between extracellular loops of the three subtypes of α_2 -AR. Several α_2 -AR subtype selective ligands were docked in the active sites of the three α_2 -AR subtypes, key interactions between ligands and receptors were mapped, and the predicted results were compared to experimental results. Binding site analysis reveals a strong identity between important amino acid residues in each receptor, the very few differences being the key towards modulating selectivity of α_2 -AR ligands. The observed differences between binding site residues provide an excellent starting point for virtual screening of chemical databases in order to identify potentially selective ligands for α_2 -ARs.

Keywords

homology modeling; docking; GPCR; α_2 -adrenergic receptor

Introduction

The use of computer methods has been successfully applied in numerous studies aiming at the discovery of new potential active compounds, or at the modification of the existing ones in order to obtain a better biological effect (drug design). The two major types of drug design (ligand-based and structure-based drug design) have different approaches but an identical goal: identification of small active molecules that have complementary shape and charge distribution to the biological target they bind. Structure-based drug design (direct drug design) relies on the three dimensional structure of the biomolecular target obtained through experimental methods such as X-Ray crystallography or NMR spectroscopy. Although the number of solved protein structures is continuously growing, there are still a lot of difficulties in obtaining G-protein coupled receptor (GPCR) structures using experimental techniques, since they are membrane-bound proteins. For this reason, molecular modeling studies in general and homology modeling in particular [1] are some of the most widely used techniques for filling the gap between primary sequence and structural information. This approach has been proven extremely relevant especially for the GPCR

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area [2-5], where the need for three-dimensional information is very stringent due to the lack of high-resolution structural data of their active and inactive states. The conformational changes that switch between active and inactive states of GPCR have been intensively studied during the last years and many hypotheses have been generated. Recently published crystal structure of a photoactivated intermediate of bovine rhodopsin [6] has shown that the differences between active and inactive states of bovine rhodopsin are minor although previous facts had indicated considerable structural changes [7, 8]. On the contrary, the conformational changes triggered due to the GPCR activation by small ligands were not much known. A recent disulfide cross-linking study using the muscarinic M3 receptor revealed new insights regarding the conformational changes associated with receptor activation [9]. Therefore, helices 3, 6, 7 and 8 are involved in receptor activation and the resultant structural changes are somewhat similar, but not identical to those observed in the photoreceptor rhodopsin. Alpha2-adrenergic receptors (α_2 -ARs) have a wide distribution in the human body, being responsible for the regulation of many biological and physiological functions such as the control of central nervous system (CNS) and cardiovascular system. α_2a -AR is the most important subtype from clinical point of view, its stimulation being responsible for the most of the classical effects of α_2 -AR agonists, like hypotension, sedation, analgesia, lowering of blood pressure, etc. α_2b -AR subtype is distributed mainly in peripheral tissues and mediates important physiological responses, such as salt-induced hypertension, vasoconstrictor response to α_2 -AR agonist gastric mucosal defense, and it has an important role in developmental and reproductive processes [10,11]. The α_2c -AR subtype is present in the adrenal medulla, where it mediates the epinephrine release and in the central nervous system (CNS), where it participates with α_{2A} -AR in the presynaptic inhibition of norepinephrine release [12].

α_2 -ARs are attractive targets for the treatment of several general diseases like hypertension, pain, depression, anxiety, and obesity, thus their specific agonists and antagonists would have important therapeutic applications. However, because of the common mechanisms for the signal transduction and the very high degree of sequence homology (more than 80%) exhibited by α_2 -ARs, information regarding subtype-selective compounds is still limited. At the time of this manuscript preparation, only a few compounds have been reported to be α_2 -ARs subtype-selective: the agonists guanfacine for α_2a -AR and R-(+)-m-nitrobiphenylindole oxalate for α_2c -AR and the antagonists BRL-44408 for α_2a -AR, JP-1302 and OPC28326 for α_2c -AR [13-19]. As an accomplishment of our previous works [20, 21], this paper combines structural bioinformatics approaches like homology modeling and docking to identify the main molecular characteristics which regulate the selectivity within the α_2 -ARs subtypes. For this purpose we have built the three dimensional structure of each α_2 receptor subtype and analyzed their binding sites to find structural differences that could guide the design of new selective ligands.

Results and Discussion

Homology models of α_2 -ARs

For many years the bovine rhodopsin has been the only GPCR with experimental structural information available, and all the homology modeling efforts were focused on this structure [22, 23]. During the last few years, other GPCR protein structures have been solved: the human β_2 adrenergic receptor [24], the turkey β_1 adrenergic receptor [25], the squid rhodopsin [26] and the human adenosine A2a receptor [27]. Although the squid rhodopsin has the highest sequence similarity with α_2 -ARs, the β_2 adrenergic receptor structure (PDB code: 2rh1) was chosen as structural template because it belongs to the GPCR-class A subfamily and binds a biogenic amine like α_2 -ARs. These criteria proved to be successful in other cases, too [28].

The alignment of the amino acid sequences generated by the T-coffee server was manually refined using as a guide the three dimensional structure of the $\beta 2$ -AR. In order to avoid deletions or insertions in the transmembrane domains and to preserve the highly-conserved amino acid motifs specific for each transmembrane helix identified based on the conserved residues within the GPCR amino acid sequences (gray in figure 1), the three dimensional structure of $\beta 2$ -AR was used as a guidance tool. For each loop, the deletions or insertions were grouped into one single section which was placed in the most adequate point consistent with the template structure. The disulfide bridge between the second extracellular loop (EL2) and the extracellular part of the third transmembrane helix, conserved in most GPCRs, was taken in consideration during the model building process (double underlined in figure 1). In all three $\alpha 2$ -ARs, the C-terminal part and the third intracellular loop (IL3) were not modeled because these fragments do not have a correspondent in the X-ray structure.

The homology models for $\alpha 2$ -ARs have been generated based on the alignment in figure 1 using the crystal structure of the human $\beta 2$ -AR. The resulted models have been energetically minimized, including the main backbone. For easiness, in the present paper the homology models of $\alpha 2$ -ARs will be further mentioned as Ma ($\alpha 2a$ -AR), Mb ($\alpha 2b$ -AR) and Mc ($\alpha 2c$ -AR). Previous mutagenesis studies on $\alpha 2a$ -AR [29] have shown that substitution of Asp3.32 with an asparagine produces EC50 values 500-fold higher than those for the wild-type, suggesting an involvement of this amino acid in the ligand binding. Also, the biological characterization of Ser5.46Ala mutant has revealed a possible role of Ser5.46 residue in hydrogen bond formation with the *para*-hydroxyl group on the phenyl ring of catecholamines. To check if Ma, Mb and Mc models confirm and support this essential information we performed a docking test using noradrenaline (norepinephrine), the endogenous ligand of all adrenergic receptors. Using SiteMap [30], the primary binding site of Ma model was located in the extracellular part of the transmembrane domain, within a cavity defined by residues from helices 3, 5, 6 and 7. This position was identified as the active site in other GPCRs structures [22-29, 31]. For an accurate prediction of the norepinephrine geometry in the binding site we used Schrodinger's Induced Fit (IFD) method which merges the predictive power of Prime with the docking and scoring capabilities of Glide [32, 33]. The results from the Induced fit docking have shown for the $\alpha 2a$ -AR a ligand conformation which is in agreement with the mutagenesis data. The best pose of norepinephrine is located in the transmembrane domain in a crevice positioned in the extracellular half of the protein exactly where the GPCR binding pocket is located [22-29]. The ligand is oriented parallel with the membrane and the amino and hydroxyl groups point toward helices 3 and 5, respectively. The positively charged nitrogen atom is placed at about 3.13 Å from Asp3.32 side-chain, and is favorable oriented to establish an electrostatic interaction with its negatively charged COOH functional group. The benzene ring is surrounded by a hydrophobic environment defined by several aromatic amino acids placed on helices 5 and 6: Tyr5.38, Phe6.52 and Phe6.53. The hydroxyl group in the *para* position interacts through a hydrogen bond with the hydroxyl group from Ser5.46 side-chain (depicted in red in figure 2). Similar conformations of norepinephrine were found for the other two $\alpha 2$ -ARs (Mb and Mc models). The carboxylic group from the Asp3.32 side-chain forms a salt-bridge with the quaternary amino group of norepinephrine (3.13 Å in Mb and 3.34 Å in Mc), while Ser5.46 forms hydrogen bonds with the catecholic hydroxyl group.

All this information is in agreement with experimental data obtained through site-directed mutagenesis and supports the further use of the homology models in our study.

Binding site analysis

The position of the binding sites indicated by SiteMap is in agreement with what is known about GPCR binding from literature and mutagenesis data [29, 31]. Because of the high sequence similarity of $\alpha 2$ -ARs, the predicted hydrophilic and hydrophobic surfaces are

comparable between the three proteins, the major difference being the size of their binding sites. The $\alpha 2b$ -AR subtype has the biggest binding cavity ($683.3 \text{ \AA}^3$), while the $\alpha 2c$ -AR subtype contains the smallest one ($513.6 \text{ \AA}^3$).

During the inspection of the three $\alpha 2$ -ARs binding sites, a very high sequence identity was noticed for the amino acids found in the immediate vicinity of the docked norepinephrine. We found that apart from nine amino acid variations (table 1), all the residues placed at less than $6 \text{ \AA}$ around norepinephrine are identical, which suggests a reasonable explanation for the scarcity of selective ligands for this receptor family. Three of the nine amino acid variations (AAV) are located on the second extracellular loop (EL2) between helices 4 and 5, the other six being placed on the transmembrane helices. Obviously, not all the residues from this selected group of amino acids found in the close vicinity of the docked norepinephrine contribute equally to the ligand binding, but there is a high probability to find key residues involved in binding in this set. A special attention has been dedicated to these nine variations (see figure 1 of Supporting Inf) which together with the size of the binding site are the only differences we have noticed in that region.

For the first variation we have examined, the presence of a valine residue in $\alpha 2a$ -AR and $\alpha 2c$ -AR and an isoleucine residue in $\alpha 2b$ -AR at the position 2.57 (Val86/Ile65/Val104, AAV1), does not make a big difference in the properties of the binding site. Valine and isoleucine display similar topological and physicochemical characteristics like a C-beta branch, non-reactive side chains, and similar hydrophobicity, but also some differences like volumes, sizes and surfaces. Although a valine residue allows for the ligand to accommodate a larger substituent than an isoleucine residue does, it is expected that a ligand which can easily discriminate between these two residues would be difficult to find. An identical circumstance was observed at position 5.39 occupied by a valine residue in $\alpha 2a$ -AR and an isoleucine residue in $\alpha 2b$ -AR and $\alpha 2c$ -AR (Val197/Ile173/Ile211, AAV5). Similarly, the presence at the same position on EL2 of an isoleucine residue in $\alpha 2a$ -AR and a leucine residue in $\alpha 2b$ -AR and $\alpha 2c$ -AR (Ile190/Leu166/Leu204, AAV3) does not dramatically affect the properties of the binding site, because in both situations the pocket contains an aliphatic, hydrophobic residue with identical size (the same molecular weight), volume, and a comparable surface. However, a first significant difference in the $\alpha 2$ -ARs binding sites was noticed at position 5.43, filled by a Cys residue in $\alpha 2a$ -AR and $\alpha 2c$ -AR and a Ser residue in $\alpha 2b$ -AR (AAV6). Compared to cysteine, serine is a smaller amino acid, less hydrophobic and slightly polar, even if it is rather neutral. Cysteine has a bulky side-chain, very hydrophobic, which can also function as a nucleophile group. The hydroxyl group in the serine side-chain could be involved in the formation of hydrogen bonds with the protein backbone or with diverse polar functional groups from ligands. Due to its location and vicinity in $\alpha 2a$ -AR and $\alpha 2c$ -AR, Cys5.43 cannot be involved in disulfide bond formation with other cysteine residues. This "mutation" can be explored in the design of new $\alpha 2$ -ARs subtype selective compounds by selecting the right substituent for each type of receptor: a polar substituent for $\alpha 2b$ -AR which interacts with Ser5.43 side-chain through a hydrogen bond or a sulfur-containing substituent or a related bioisoster for $\alpha 2a$ -AR and $\alpha 2c$ -AR. Also, the contribution of this residue to the agonist binding mode at AR receptor has been recently reported [34].

The pair aspartate-glutamine on EL2 (AAV4) also suggests a possible direction in the process of designing new selective compounds for $\alpha 2b$ -AR. Both aspartic acid and glutamine residues are definitely polar, but their size and side-chain charging are totally different. Asp192 and Asp206 in $\alpha 2a$ -AR and $\alpha 2c$ -AR subtypes contain a side-chain carboxyl group which is negatively charged unlike Gln168 in $\alpha 2b$ -AR that is a polar but uncharged amino acid. A positively charged substituent might be the perfect pair to create a stabilizing salt-bridge with the ligand in the case of $\alpha 2a$ -AR and $\alpha 2c$ -AR, and a hydroxyl or

ether substituent might be a suitable partner for a hydrogen bond formation in $\alpha 2b$ -AR. A similar association of amino acids is noticed at the beginning of helix 7, in the 7.36 position: Lys7.36 in $\alpha 2a$ -AR and $\alpha 2c$ -AR and Gln7.36 in $\alpha 2b$ -AR (AAV9). Compared with lysine, glutamine is a smaller, uncharged, and slightly polar amino acid, and may be involved in hydrogen bond interactions with functional groups from ligands. Lysine is a positively charged amino acid which is prone to form salt-bridge interactions. We believe that all these differences revealed through binding site analysis represent an excellent starting point for further studies aiming at discovery of selective $\alpha 2$ -ARs ligands. One might argue that all these differences might occur because of an inaccurate alignment and/or modeling of the loops or transmembrane helices. But an incorrect alignment of the transmembrane domains is very unlikely to happen because we did not allow insertions or deletions within the transmembrane domain and the highly conserved residues of each transmembrane helix were aligned according to Baldwin's model for the alpha-carbon positions in the seven transmembrane helices of the GPCR family [35]. Six out of the nine mentioned variations are located in the transmembrane region, only three being situated on the second extracellular loop. Except for the cysteine residue from EL2, there is no other known conserved residue on this extracellular loop across GPCR family. Thus, we have decided to check if the location of AAV2, AAV3 and AAV4 variations on EL2 is a real fact or it is the consequence of a wrong alignment. For this purpose, the entire EL2 of each $\alpha 2$ -AR model (Glu173-Gln193 in Ma, Gly152-Glu169 in Mb and Tyr191-Glu207 in Mc) was rebuilt *de novo* without any alignment with template structure. The new obtained models will be further mentioned as M1a, M1b and M1c, respectively (depicted in gray in figure 3). Given that the spatial arrangement of the EL2-s differs significantly in M1 receptors as a consequence of different lengths of EL2 in each subtype, no similarity between the positions of these three variations was noticed (figure 3).

In the $\beta 2$ -AR crystal structure and also in Ma, Mb and Mc models, EL2 is folded above the binding cavity, covering the top of the active site like a lid. In M1s models obtained by *de novo* modeling of EL2 from the M models, EL2 is placed outside of the transmembrane domain and the top of the binding site is wide-open. In these circumstances, it is not important if the previously mentioned amino acid differences are really aligned or not, because they would have little or no influence on the ligand binding mode.

Furthermore, in all of the new models (gray in figure 3) the conserved cysteine in EL2 is unable to form the disulfide bridge with the cysteine residue located on the extracellular side of the helix 3. This disulfide bridge is highly conserved in the GPCR family, being considered critical for stabilization of the ligand in the binding site and/or proper receptor folding. To preserve this characteristic, another *de novo* modeling of EL2 was performed and this time a constraint was added - disulfide bridge formation. For this purpose, the sulfur-sulfur bond was used as an anchor point by keeping rigid the coordinates of Cys188 ($\alpha 2a$ -AR), Cys164 ($\alpha 2b$ -AR) and Cys202 ($\alpha 2c$ -AR), similarly with the initial Ma, Mb and Mc models (black in figure 3). The two resulted fragments for EL2 were modeled concurrently. Fragment 1 contains the residues located between the last amino acid on helix 4 and cysteine residue on EL2 (Cys188 in Ma, Cys164 in Mb and Cys202 in Mc), and also includes the AAV2 variation (Lys174/Asp153/Arg192). Fragment 2 contains the amino acids positioned after the disulphide bridge up to the first residue on helix 5 and includes the other two differences enclosed in EL2: Ile190/Leu166/Leu204 (AAV3) and Asp192/Gln168/Asp206 (AAV4). The resulted models will be further called M2a, M2b and M2c receptors.

In each M2 model, the second half of EL2 (fragment 2) was predicted *de novo* in a similar manner with Ma, Mb and Mc models (see table 1 of Supporting Inf). On the contrary, when fragment 1 was built without any alignment between $\alpha 2$ -ARs and $\beta 2$ -AR sequences (gray in figure 4), the spatial arrangements of fragment 1 are pretty different than those resulted via

homology modeling (black in figure 4). This remarkable discrepancy can be explained by EL2 similarity between α 2-ARs and β 2-AR: fragment 1 exhibits no sequence similarity while fragment 2 shows around 30% similarity.

Most likely, the AAV3 and AAV4 variations enclosed in fragment 2 are preserved very well also in M2 models (figure 4). The involved amino acids, Ile190/Leu166/Leu204 (AAV3) and Asp192/Gln168/Asp206 (AAV4), occupy analogous positions on EL2 and have the same orientation of the side-chains, toward the interior of the transmembrane helical bundle. On the opposite, the critical variation AAV2 enclosed in fragment 1, presents a different alignment of the involved amino acids. The positively charged residues Lys174 in α 2a-AR and Arg192 in α 2c-AR have similar positions and side-chain orientations in M2 models, but we cannot say the same thing about Asp153 from α 2b-AR. This residue is found at a different position when compared with its initial position from M2b models, thanks to a wider curvature at the beginning of EL2, caused by the presence of Gly152. Probably, this arrangement is more realistic than the initial model obtained using homology, because in the *de novo* modeling the conformational flexibility of glycine is taken in consideration. For the same reason, we have chosen to use these models (M2) in further docking experiments.

Virtual screening experiment

To test the ability of M2a, M2b and M2c models to discriminate between active and inactive compounds a virtual screening experiment was carried out using a library which contains α 2-ARs active compounds seeded between a set of randomly selected decoys. The actives represent approximately 8% of the entire set of compounds and they were selected based on the biological activity expressed against alpha2 subtypes.

The docking simulations were performed on each model using the library of 1730 compounds seeded with 129 actives selected from Wombat database [36, 37] and 1601 compounds selected from the Database of Useful Decoys (DUD) [38, 39]. The experimental values of biological activities range from high micromolar to high nanomolar. The decoys selection process was conducted in part randomly and in part toward the drug-like molecules using cutoffs for molecular weight, logP and number of rotatable bonds. Also, it has to be emphasized that we have tried to select the set of decoys having a molecular weight distribution similar to that of the actives set.

The models ability to recover the actives from the library was assessed by enrichment validation. The enrichment factor quantifies the number of active compounds from a selected subset of the ranking list relative to the entire database. In this study the enrichment factors (EF) were calculated using the following equation:

$$EF = \text{Hits}_{\text{selected}} / \text{Hits}_{\text{total}} * N_{\text{total}} / N_{\text{selected}}$$

where Hits_{selected} represents the number of actives found at a specific threshold level (percentage) of the database screened, Hits_{total} represents the total number of actives in database, N_{total} represents the total number of compounds in the entire database and N_{selected} represents the number of compounds in the selected subset of the ranked database.

Because in the current study a dataset enriched by approximately 8% (129 actives and 1601 inactives) was screened, the best reachable performance for each model will be 100% (129 of 129) at top 8%, corresponding to an EF=13.4. At this threshold the maximum enrichment factor has not been achieved for any of the models. Therefore, enrichment factors were calculated at top 2%, 5% and 10% of the scored and ranked dataset.

As can be seen from Table 2, the virtual screening experiments performed on the three models resulted in a significant prioritization of ligands versus decoys. When top 2% of the library was considered the maximum enrichment factor for each model was obtained (Table 2).

In the case of M2a model a similar performance was observed at the 5% threshold level, but the enrichment factor has decreased to 8.3 at 10% threshold. The M2b and M2c models perform almost similar in retrieving actives at 5% and 10% levels, with comparable values of the EFs, but poorer when compared with M2a.

The virtual screening experiments conducted for the α 2-AR homology models have shown that these models perform well, identifying known actives hidden in a database of decoys. This exercise proves that the models are robust and reliable and can be unwaveringly used in further experiments.

Docking

A docking experiment was set up to check if AAV1-AAV9 variations are involved in the binding mode of several α 2-AR subtype-selective antagonists (figure 5). Data analysis was performed on the top ranked poses for each ligand in each α 2-AR subtype, according to their docking scores.

A freely interference of AAV2 and AAV7 variations with ligands has not been anticipated, mainly because of their positions compared to the active site. AAV2 is positioned on the EL2 very close to the extracellular end of helix 4 and it is somehow separated from the active site due to the helix 3 leaning present in the GPCR family [40] and AAV7 is located at the border between helix 6 and EL3, high above the binding cavity. Surprisingly, they proved their interference with some ligands as it will be shown next.

Induced fit docking of BRL-44408 has revealed that AAV2, AAV3 and AAV4 variations work together to differentiate the binding mode of this compound that acts as an α 2a-AR selective antagonist [41,42]. Thus, in the α 2a-AR binding site (figure 6a), the imidazoline ring of BRL-44408 is positioned between helices 3 and 6 and the rest of the molecule, containing an isoindole moiety, is placed in a hydrophobic pocket defined by Phe6.52, Phe6.53 and Val3.33 residues. Furthermore, the protonated nitrogen atoms of the imidazoline ring interact with the receptor as follows: one atom is hydrogen bonded to the Tyr 6.55 residue, whereas the other one is engaged in an electrostatic interaction with Asp3.32 side-chain (figure 6a). The presence of a leucine residue on EL2 in α 2b-AR instead of an isoleucine like in the α 2a-AR subtype (AAV3) pushes the imidazoline ring away from helix 6 and the hydrogen bond with Tyr6.55 cannot be established. Still, a new hydrogen bond is formed with Gln168 corresponding to AAV4 (figure 6b). The α 2c-AR subtype contains also a leucine residue at AAV3 position but BRL-44408 adopts a totally different orientation in the α 2c-AR binding site compared to the other two AR subtypes. Accordingly, the best pose of BRL-44408 in the α 2c-AR binding site is aligned parallel to the membrane and not perpendicular as for α 2a-AR and α 2b-AR subtypes (figure 6c). This particular arrangement is caused by the swing of Asp206 side-chain (AAV4) toward Arg192 (AAV2) to whom it forms a salt bridge, leaving more space in the α 2c-AR binding site. Even if position AAV4 from α 2a-AR subtype is also occupied by an aspartic acid, position AAV2 is not filled with an arginine residue, and the salt bridge contact cannot be established. Moreover, the aspartic acid side-chain points toward the interior of the transmembrane domain, and a parallel orientation of the ligand to the membrane, is not possible. Besides all these aspects, BRL-44408 has the best complementary orientation against the α 2a-AR binding site (see figure 2 of Supplemental Inf).

The molecular docking experiments of JP-1302, a selective antagonist of the $\alpha 2c$ -AR subtype [18, 19], have yielded different binding modes for each $\alpha 2$ -ARs binding sites (figure 7), mostly due to AAV8 variation, but also AAV7 and AAV9 variations have played an important role. The glycine residue found at position AAV8 in the $\alpha 2c$ -AR subtype, allows the accommodation of the ligand's acridine ring into a hydrophobic pocket located in the extracellular part of the receptor, between the upper parts of helices 6 and 7. In the case of $\alpha 2a$ -AR and $\alpha 2b$ -AR subtypes the same position is occupied by larger residues, like histidine and arginine respectively, which obstruct the acridine ring, and implicitly the entire ligand, to adopt a similar orientation as in $\alpha 2c$ -AR binding site. The JP-1302 binding mode at the $\alpha 2c$ -AR binding site is characterized by the formation of a salt bridge between Asp3.32 and the protonated nitrogen atom of piperidine ring and a hydrogen bond between Tyr6.58 (AAV7) and the nitrogen atom of acridine ring. Additionally, the hydrophobic parts of the ligand are favorably oriented in the hydrophobic cavities of the binding sites. For example the acridine ring is surrounded by aromatic residues like Phe7.35, Tyr6.55, Tyr6.58 and the hydrophobic part of Lys7.36 side-chain (AAV9), the phenyl linker is lined by the Phe7.39 and Phe6.51 residues, while the methyl substituent of piperidine ring is flanked by the Trp6.48 and Cys3.37 residues (see figure 3 of Supplemental Inf).

Imiloxan has been reported as a $\alpha 2b$ -AR subtype-selective compound [43] but it has been tested only against rat $\alpha 2a$ -AR and $\alpha 2b$ -AR subtypes, thus its selectivity refers mainly to $\alpha 1$ -ARs subtypes. pKi values against human $\alpha 2$ -ARs show imiloxan as a potent antagonist of $\alpha 2b$ -AR, but the selectivity is not convincing [44]. Other compounds with a pharmacological profile similar to imiloxan have been recently reported [45, 46] but again they have not been evaluated against all three $\alpha 2$ subtypes. ARC-239 shows a similar biological profile with imiloxan and it exhibits good activity against $\alpha 2b$ -AR and $\alpha 2c$ -AR subtypes and weak activity on $\alpha 2a$ -AR subtype [42]. The molecular docking has revealed that AAV2 and AAV4 variations are determinant factors for the ARC-239 binding to $\alpha 2$ -ARs subtypes, the most significant differences consisting in the spatial arrangement of the best poses. In $\alpha 2b$ -AR subtype, the ligand fits the cleft between the second extracellular loop and helices 3, 4 and 5 being perpendicular on the membrane. The anisole moiety is positioned right under EL2 and it points towards the upper parts of the helices 3 and 4. In addition to the hydrogen bonds between isoquinoline-1,3-diketone ring and Tyr 5.38 and Tyr6.55, a hydrogen bond is formed between one of the two nitrogen atoms of piperidine ring and Gln168 (AAV4). In the $\alpha 2a$ -AR and $\alpha 2c$ -AR binding sites, ARC-239 adopts an orientation parallel to the membrane and the factors responsible for these spatial arrangement differences are AAV2 and AAV4 variations. In $\alpha 2a$ -AR and $\alpha 2c$ -AR these positions are occupied by an aspartic acid residue (AAV2) and a lysine or arginine residue (AAV4). The side chains of Asp192 and Lys174 in $\alpha 2a$ -AR and Asp206 and Arg192 in $\alpha 2c$ -AR respectively are pointing to each other due to their charges and they are filling the cavity where the anisole ring lays when ARC-239 is binding to $\alpha 2b$ -AR.

Methods

Sequence Alignment

The sequences of the human $\alpha 2a$ -AR, $\alpha 2b$ -AR and $\alpha 2c$ -AR were extracted from the SWISS-Prot database [47, 48] (accession codes: P108913, P18089 and P18825) in Fasta format and were automatically aligned using the T-coffee server [49, 50] with the sequence of the human $\beta 2$ -adrenergic receptor ($\beta 2$ -AR) taken from RSCB Protein Data Bank (accession code: 2RH1). The lysozyme T4 fragment was removed from the sequence of the $\beta 2$ -AR crystal structure, and the final alignment was further manually refined. Specific amino acids from the transmembrane helices were named using the numbering system proposed by Ballesteros and Weinstein in 1995 [51]. Shortly, for the most highly conserved residue in each transmembrane a value of 50 was assigned, preceded by the number of the

transmembrane helix: Asn1.50, Asp2.50, Arg3.50, Trp4.50, Pro5.50, Pro6.50, and Pro7.50 (see figure 1). The other residues on the same helix will have a value corresponding to their position relative to the most conserved residue in transmembrane. The amino acids belonging to the intra- or extracellular loops are identified by the corresponding number in the sequence (Val36, Asp135, etc).

Model building and loop modeling

The homology modeling package Modeller [52, 53] (version 9v6) was used to generate the three-dimensional models for $\alpha 2$ -ARs based on the X-ray structure of $\beta 2$ -AR using the sequence alignment presented in figure 1. The resulted models were first geometrically refined in order to reduce the side chains sterical clashes and then the whole receptors, including the main backbone were energetically minimized. The final models have over 90% of residues in the favorable regions of the Ramachandran map and all the main-chain parameters, like peptide bond planarity, bad non-bonded interactions, C α distortion, overall G-factor, bond length distribution and side-chain parameters, are in the normal range. When necessary, the second extracellular loop (EL2) of $\alpha 2$ -AR subtypes was built without any alignment with $\beta 2$ -AR, using the Modeller's routine MODLOOP, explicitly designed for loop modeling [54, 55]. Also, in these cases the final models were energetically minimized.

Binding site identification

The location of the primary binding site in the $\alpha 2$ -ARs was determined using results from mutagenesis studies and computational prediction with the SiteMap 2.1 application from the Schrödinger software package [29]. In the first step of the SiteMap procedure, a grid is set up and one or more regions on the protein surface that are suitable for ligand binding are identified. Next, contour maps are drawn generating hydrophobic and hydrophilic regions, and in the final step for each site various properties are evaluated.

Ligand Docking

Ligand setup—LigPrep 2.1 application [56] was used to prepare the ligands for docking. The hydrogen atoms were added to the ligand compounds and the possible tautomers at physiological conditions were generated. Finally, the ionization states were set and the final geometries were minimized using OPLS_2005 force field [57].

Protein setup—Protein Preparation Workflow [58] was used to add all the hydrogen atoms to the homology models of $\alpha 2$ -ARs, to optimize the charge state of Asp, Glu, Arg Lys and His residues and the orientation of hydroxyl and amide groups. At the end a restrained minimization was performed by Impref utility, which is based on the Impact molecular mechanics engine [59] and the OPLS2005 force field, setting a max RMSD of 0.20.

Docking protocol—The binding properties of the endogenous ligand and subtype-selective compounds were explored with the Induced Fit protocol based on Glide 4.5 [32] and Prime 1.6 [33] applications. The procedure starts with a preliminary docking of the ligands at the receptor binding site, when 20 poses per ligand are retained, followed by the refinement of the side chains of residues found within 8Å of an atom in any of the 20 poses. The complexes within 30 kcal/mol of the minimum energy structure are further used in the final docking step when ligands are redocked into each of the refined receptor structures. The final poses are ranked by a combined score composed of Glide score (receptor-ligand interaction energy) and Prime energy (receptor strain and solvation energy).

The grid-box was chosen to include the entire protein, when the endogenous ligand was docked (preliminary run). In the following runs, the docking grid was centered on the 3.33 position of each $\alpha 2$ -AR subtype and the size of the box was set at 30Å on each side.

Virtual screening experiment

Ligands set preparation—The set of ligands active against α_2 -ARs was compiled from Wombat 2007.1 database [36, 37] which is a collection of chemical and biological information carefully curated from the medicinal chemistry literature. The compounds were selected to satisfy the following criteria:

- tested against at least one of α_2 -AR subtype;
- biological activity expressed as pIC_{50} , pK_i to be greater than 8

A set of decoys was prepared by randomly selecting compounds from DUD decoys database [38, 39]. Shortly, the all decoy sets for all 40 targets were gathered and a superset containing 124,431 molecules was obtained. After removal of duplicates, tautomers and structures that we could not process a set of 91311 was compiled with the help of InstantJchem tool [60] from ChemAxon. The compounds were also chosen by applying drug-like rules in the selection process:

- molecular weight between 200 and 600 Da;
- number of rotatable bonds between 1 and 10 Da;
- at least one polar atom (N, O, S);
- logP value between -6 and +6.

Finally, a set of almost 1600 compounds was compiled. By combining these compounds with the set of 129 receptor-active ligands a database of 1730 compounds was gathered and subjected to ligand preparation with LigPrep utility[56], as previously described. Thus, a set of actives and decoys ready for docking studies was obtained.

Protein preparation—The 3D-models of the receptors were prepared with Protein Preparation workflow as previously described.

Docking setup—Glide receptor grids were generated from the prepared receptor models, with the docking grids centered on position 3.33 of each α_2 -AR subtype: Val114 (α_2a), Val93(α_2b), and Val132 (α_2c). The binding site was defined by a cubing docking box with a dimension sufficient to accommodate ligands with a length 20 Å, while the ligand-midpoint box a side of 14 Å was given. The docking runs were performed with Glide 4.5 [32] in SP mode using default settings for parameters. No scaling factors were applied to the van der Waals radii of the receptor atoms, while the default value of the scaling factor of 0.8 was applied to the non-polar atoms of the ligands database. The free rotation of the hydroxyl groups of residues from the binding cavity was allowed.

Conclusions

Molecular and homology modeling of the α_2 -ARs subtypes still represents a very motivating topic as confirmed by the high number of studies published so far. The present study shows the successful application of homology modeling for the building of three dimensional models for α_2 -ARs subtypes based on the X-ray structure of the β_2 -adrenergic receptor. The models have reproduced the experimental data regarding the binding of the endogenous ligand and also performed excellent in a virtual screening experiment using a library of 1700 compounds. These provide evidence that *in silico* models developed in this work are reliable and robust, being powerful tools which can be used in further virtual screenings. Docking runs performed with different selective ligands against α_2 -ARs subtypes allowed a thoroughly analysis of the common and more important, the specific elements of these receptors' binding cavities. The analysis revealed a strong identity

between the important amino acids in each receptor binding site, still very few differences have been noticed, and these are the key elements toward the discovery of selective α_2 -AR ligands. The observed differences between binding site residues provide an excellent starting point for virtual screenings of chemical databases in order to identify potentially selective ligands for α_2 -AR receptors. Our results suggest that the residue differences in the binding site presented in this study should impose a certain weight in the process of searching for α_2 -AR subtype-selective compounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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2RH1 : DEVVVVGMGIVMSLIVLAIVFGNVLVITAIKFERLQTVNYFITSLACDILVMGLAVVP
alpha2a: SLQVTLTLVCLAGLIMLLTVFGNVLVIAVFTSRALKAPQNLFLVSLASDILVATLIVIP
alpha2b: SVQATAAIAAAITFLILFTIFGNALVILAVLTSRSLRAPQNLFLVSLAANDILVATLIIP
alpha2c: SAGAVAGLAADVGFLLVFTVVGQNLVVIIVLTSRALRAPQNLFLVSLASDILVATLVMP

2RH1 : FGAAHILMKMTFGNFWCEFWTSIDVLCVTASIELCVIAVDYFAITSPFKYQSLTKN
alpha2a: FSLANEVMGYWYFGKAWCEIYLALDVLCTSSIVHLCAISLDEYWSITQAEYNLKRTPR
alpha2b: FSLANELLGWYFRRTWCDEVYALDVLCTSSIVHLCAISLDEYWAVSRALEYNSKRTPR
alpha2c: FSLANELMAYWYFGQVWCDEVYALDVLCTSSIVHLCAISLDEYWSVTQAVEYNLKRTPR

2RH1 : KARVILMVNLVSGLTSLFLIQMHWRATHQEA INCYAEETCDFFTNQAYAIASSIVSF
alpha2a: RIKAIITVNLVIAVISFPPLISIEKKGG---GGGPQPAEPCEINDQKWVITSSCIGSF
alpha2b: RIKCIILTVMILIAVISLPPLIYKGDQG-----PQPRGRPQCKLNQEAWYILASSIGSF
alpha2c: RVKATIVAVNLISAVISFPPLVSLYRQP-----DGAAYPQCGLNDETWYILSSCIGSF

2RH1 : YVELVIMVFVYSRVFQEAQRQK-FCLKEHKALKTLGIIMGFTLCLWLEFFIVNIVHV--
alpha2a: FAPCLIMILVYVRTYQIAKRRT-RVQREKRFTFVLAVVIGVFVVCWFFFFFTYTLTA--
alpha2b: FAPCLIMILVYLRITLYIAKRSNRRQLTREKRFTFVLAVVIGVFVLCWFFFFFSYSLGAIC
alpha2c: FAPCLIMGLVYARTYRVAKLRTR--TAREKRFTFVLAVVMGVFVLCWFFFFFSYSLYGC

2RH1 : IQDNLRKEVYILLNWIGYVNSGFNELIYCRS-PDFRIAFQELLCL
alpha2a: -VGCSVPRTLKFFFWFGYCNSSLNEVIYTI FNHDFRRAFKKILCR
alpha2b: PKHCKVPHGLFQFFFWIGYCNSSLNEVIYTI FNQDFRRAFRRILCR
alpha2c: REACQVPGLFKFFFWIGYCNSSLNEVIYTVFNQDFRRSFKHILFR

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Figure 1.

Sequence alignment between human $\beta 2$ -AR and $\alpha 2$ -ARs. The transmembrane domains in the template structure are shown in italics and the highly conserved residues in gray. The sulfur bridge between the two cysteine residues from helix 3 and EL2 is double underlined. Positions x.50 are enclosed into a black square.

Figure 2.
Docked nor-epinephrine in the $\alpha 2a$ -AR (A), $\alpha 2b$ -AR (B) and $\alpha 2c$ -AR (C).

Figure 3.

Superposition of the homology models of the α 2-ARs built based on the alignment in figure 1 (black) and the new models with EL2 modeled *de novo* (gray). For clarity only extracellular part of the transmembranes is shown and the other loops were hidden. A- α 2a-AR, B- α 2b-AR, C- α 2c-AR.

Figure 4.

Superposition of the homology models of the α_2 -ARs built based on the alignment in figure 1 (black) and the homology models with EL2 modeled de novo (gray). For clarity only the extracellular part of helices 4 and 5 are shown. A- α_2a -AR, B- α_2b -AR, C- α_2c -AR

Figure 5.
Chemical structures of α_2 -AR-selective antagonists used in the docking tests

Figure 6.
BRL-44408 (green) into the α_2a -AR (A), α_2b -AR (B) and α_2c -AR (C) binding sites.

Figure 7.
JP-1302 into the $\alpha2a$ -AR (blue), $\alpha2b$ -AR (orange) and $\alpha2c$ -AR (green) binding sites.

Table 1

Sets of different amino acids occupying the same position around the endogenous ligand (6Å)

α2a-AR	α2b-AR	α2c-AR	Location	Position on TM	Amino acid variation
V86	I65	V104	TM2	2.57	AAV1
K174	D153	R192	EL2		AAV2
I190	L166	L204	EL2		AAV3
D192	Q168	D206	EL2		AAV4
V197	I173	I211	TM5	5.39	AAV5
C201	S177	C215	TM5	5.43	AAV6
T397	G394	Y405	TM6	6.58	AAV7
R405	H405	G416	TM7	7.32	AAV8
K409	Q409	K420	TM7	7.36	AAV9

Table 2

The enrichment factors calculated for each model at different levels of sampling of the dataset.

Model	Enrichment factors (EF)		
	2%	5%	10%
M2a	13.0	13.0	8.3
M2b	13.0	11.86	6.12
M2c	13.0	12.02	6.05