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An evaluation of automated in silico ligand docking of amino acid ligands to Family C G-protein coupled receptors

Minghua Wang and David R. Hampson*

Department of Pharmaceutical Sciences, University of Toronto, 19 Russell St., Toronto, Ont., Canada M5S 2S2
Department of Pharmacology, University of Toronto, 19 Russell St., Toronto, Ont., Canada M5S 2S2

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Abstract—Family C G-protein coupled receptors (GPCRs) consist of the metabotropic glutamate receptors (mGluRs), the calcium-sensing receptor (CaSR), the T1R taste receptors, the $GABA_B$ receptor, the V2R pheromone receptors, and several chemosensory receptors. A common feature of Family C receptors is the presence of an amino acid binding pocket. The objective of this study was to evaluate the ability of the automatic docking program FlexX to predict the favored amino acid ligand at several Family C GPCRs. The docking process was optimized using the crystal structure of mGluR1 and the 20 amino acids were docked into homology models of the CaSR, the 5.24 chemosensory receptor, and the GPRC6A amino acid receptor. Under optimized docking conditions, glutamate was docked in the binding pocket of mGluR1 with a root mean square deviation of 1.56 angstroms from the co-crystallized glutamate structure and was ranked as the best ligand with a significantly better FlexX score compared to all other amino acids. Ligand docking to a homology model of the 5.24 receptor gave generally correct predictions of the favored amino acids, while the results obtained with models of GPRC6A and the CaSR showed that some of the favored amino acids at these receptors were correctly predicted, while a few other top scoring amino acids appeared to be false positives. We conclude that with certain caveats, FlexX can be successfully used to predict preferred ligands at Family C GPCRs.

1. Introduction

Family C G-protein coupled receptors (GPCRs) possess large extracellular ligand binding domains which are homologous to the bacteria periplasmic binding proteins.[1] Most Family C receptors possess a conserved binding motif recognizing L-amino acids. Family C receptors include the metabotropic glutamate receptors (mGluRs) which regulate neurotransmission and are therapeutic targets for a number of neurological and psychiatric diseases.[1] Another member of this receptor family, the calcium-sensing receptor (CaSR), plays a critical role in regulating calcium homeostasis in the body. Inactivating and activating mutations in the CaSR have been identified in inherited human hypercalcemic and hypocalcemic disorders, respectively.[2] Aromatic amino acids can enhance the response of the CaSR to calcium,[3] presumably through an amino acid binding pocket analogous to that of the glutamate binding pocket in the mGluRs.[4,5] Other Family C receptors such as the T1R1/T1R3 heteromeric taste receptor can sense most amino acids,[6] while arginine and lysine are the most potent amino acid ligands for the fish 5.24 chemosensory receptor.[7] A presumed mammalian homolog of the fish 5.24 receptor, GPRC6A, has been cloned and also shown to be activated by amino acids with preference for positively charged amino acids.[8,9]

The availability of crystal structure of the ligand binding domain of mGluR1[10] provides an excellent template to study the molecular determinants of ligand and drug binding to other Family C GPCRs. Based on extensive medicinal chemistry and pharmacophore studies, and molecular modeling and mutagenesis studies, the molecular determinants for glutamate and drug binding to mGluRs have been identified.[11–14] Although the mGluRs share several highly conserved residues in the binding pocket, subtle differences at other sites within pocket confer ligand and drug selectivity. The mode of glutamate binding in the mGluRs has shed light on

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* Corresponding author. Tel.: +1 416 978 4494; fax: +1 416 978 8511; e-mail: d.hampson@utoronto.ca

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ligand docking at other Family C GPCRs. For example, the 5.24 receptor was found to bind lysine and arginine in a manner similar to the mode of glutamate binding in the mGluRs.[15,16]

Structure-based drug design has shown promise in reducing the time and money spent in the drug discovery process.[17,18] Automatic docking using computer programs to identify small molecule receptor ligands plays an increasingly important role in the drug discovery process.[19] Although homology models are not as good as experimentally determined structures for docking applications, the use of homology models in ligand screening is gaining momentum.[20] The effective use of homology models in drug screening usually requires greater than 50% sequence identity between the target and the template.[21] Therefore, the lower sequence identities among members of the Family C GPCRs to the only available template mGluR1 (36–42% for mGluRs, except for 69% for mGluR5, and 20–30% for non-mGluR Family C receptors) have resulted in the use of primarily manual docking of ligands and drugs.[12,13,15,16] Recently, however, several studies employing automated ligand docking and screening using homology models of mGluRs have been reported.[22–24]

Here we report our experience in using FlexX[25] to dock amino acids in homology models of the CaSR, the 5.24 receptor, and GPRC6A. We evaluated the ability of this program to correctly predict the rank order of amino acids at each of these receptors. By comparing the in silico docking score with experimental data from the literature, we found that the FlexX scoring functions can be used to discriminate and predict ligand selectivity.

2. Results

2.1. Homology modeling

Homology models of the CaSR, GPRC6A, and the 5.24 receptor were constructed and refined in parallel. The amino acids aligned in the binding pockets are shown in Table 1. The residues in the binding pocket that interact with the glycine moiety (the α-carboxyl group and α-amino group of the bound amino acid ligand) are highly conserved in Family C receptors.[1,16] In contrast, the amino acids that interact with the side chains of the bound amino acid ligands are not conserved and are responsible for conferring ligand selectivity in the mGluRs and other Family C receptors.[12–14,16,43]

Table 1. Comparison of residues in the binding pockets of receptors studied

	mGluR1	CaSR	5.24 Receptor	GPRC6A
Glycine moiety	S165	S147	S152	S149
	T188	S170	T175	T172
	Y236	Y218	Y223	Y220
	D318	E297	D309	D303
Distal sites	Y74	R66	K74	S69
	R78	W70	Q78	Q73
	S164	G146	S151	Y148
	S186	A168	A173	E170
	G319	A298	N310	N304
	R323	S302	S314	A308
	K409	I396	M389	L411

The residues in the binding pockets listed in Table 1 are divided into two categories according to their interacting partners in the ligand. One part consists of residues that interact with the glycine moiety of the ligand and the other part consists of residues that interact with the distal (side chain) part of the bound amino acid ligand. With the exception of two conservative replacements (serine 170 and glutamate 297) in CaSR , all four residues that interact with the glycine moiety of ligand are identical among the four receptors examined; in contrast, none of residues in the distal portion of the pocket are identical to the mGluR1 template. Consequently, the comparison of the binding pockets of the three homology models with the template indicates that the four residues interacting with the glycine moiety of ligand are overlaid well with little shift in the backbone. However, for the residues in the distal portion, their side chains are different from the template and the backbone atoms also drifted away from the template during the refinement. While the three positively charged residues in the distal portion of the binding pockets of mGluR1 form a positively charged environment, the equivalent residues at these positions are replaced by non-charged residues in the three homology models. Thus, the homology modeling results presented here further establish that the differences in the distal portion of the ligand binding pockets determine the ligand selectivity of each receptor.

2.2. Optimization of the docking process

Prior to docking the amino acids using into the homology models, we tested the docking procedure on the crystal structure of mGluR1. FlexX uses an incremental construction approach[25] (similar to that used in the program DOCK[26]). In addition to defining the binding pocket as described in Section 4, only a few options need to be considered in the binding process. We examined the effect of two options, 'assign formal charges' and 'place particles,' which have been shown to be important factors in previous studies.[27,28] Within FlexX, the experimenter can assign formal charges to ligands which will affect the weight of the electrostatic interaction in the binding. The 'place particles' option was developed to account for the role of solvent in the binding process.[29] By turning on and off each option, we have four combinations of docking condition to examine.

The comparison of the docked pose of glutamate with the crystallized conformation yielded a value of root mean square deviation (RMSD), a representation of the accuracy of the docking. FlexX reproduced co-crystallized glutamate conformation with RMSDs ranging from 1.55 to 1.66 Å (Fig. 1 and Table 2) under the conditions tested. As shown in Figure 1, the glycine moiety (i.e., the α-amino and α-carboxyl groups of the bound amino acid ligands) for each docked pose was overlaid unequivocally with the crystallized ligand with slight variation in the side chain. The option 'assign formal

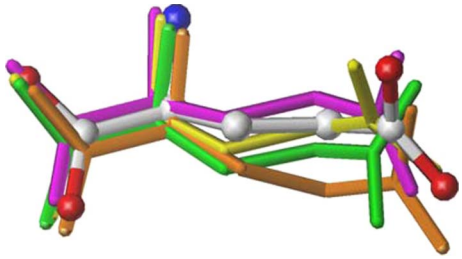

Figure 1. A comparison of glutamate poses under different docking options to the co-crystallized ligand. The co-crystallized ligand is shown as a ball and stick representation with color coding as follows: carbon, gray; oxygen, red; nitrogen blue. The reproduced poses are shown in orange (both option off), yellow ('place particles' on), green ('assign charges' on), and magenta (both options on).

Table 2. RMSD and FlexX scores of glutamate pose under different docking conditions

'Assign formal charges' option	'Place particles' option	RMSD (in Å)	FlexX Score
Off	Off	1.66	−29.69
Off	On	1.55	−34.59
On	Off	1.62	−37.48
On	On	1.56	−45.43

The best scored pose (FlexX score) was selected under each condition. RMSD values were calculated by FlexX using the co-crystallized glutamate-mGluR1 structure as the reference.

charges' had little effect on the RMSD value, while the 'place particles' option improved the fitness of the docked pose to the co-crystallized glutamate. When the FlexX scoring function was used to evaluate these poses, it appears that turning on either option decreased the score (lower score means higher affinity) approximately by 5 U and an additive effect was obtained when both options are turned on (Table 2), suggesting that either option has beneficial effect on the evaluation of scoring and a better effect was obtained with both options on.

In addition to reproducing the co-crystallized conformation of the ligand accurately, a useful docking algorithm should be capable of excluding false positives. To test the effect of the two docking options in predicting preferred ligands, the 20 amino acids were docked into the binding pocket of mGluR1. The FlexX scores for the best ranked pose of each amino acid were compared in Figure 2. When both options were off (Fig. 2A) or when only the 'place particles' option was turned on (Fig. 2B), glutamate was not ranked as the best ligand. With the 'assign charges' option on (Fig. 2C), glutamate was ranked as the best ligand, albeit only slightly better than several other amino acids. The best results in terms of ranking glutamate as the best ligand at mGluR1 were obtained when both options were turned on (Fig. 2D).

In addition to the built-in FlexX scoring function, the CScore module within Sybyl includes four more scoring functions, that is, G-Score, D-Score, ChemScore, and PMF score. To see how these scoring functions perform in ranking the ligands, the best ranked poses of ligands by FlexX score were re-scored using these functions. It was observed that only D-score could discriminate glutamate from other amino acids in mGluR1 (Fig. 3). Since the FlexX scoring function performed the best for mGluR1, we used it to evaluate the docking results for the three homology models.

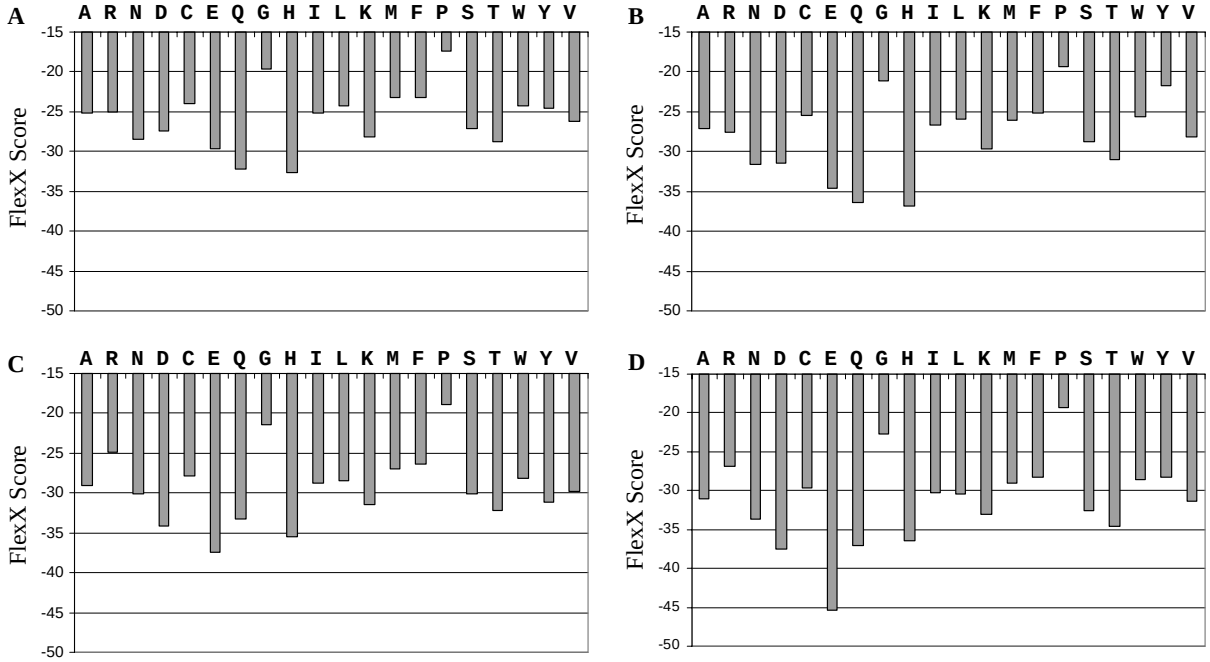

Figure 2. FlexX scores of the 20 amino acids docked into the binding pocket of the mGluR1 crystal structure under different conditions. (A) Both options off; (B) the option 'place particles' on; (C) the option 'assign charges' on; (D) both options on. The 20 amino acid ligands were docked and the scores of the top-scoring poses for each ligand are shown.

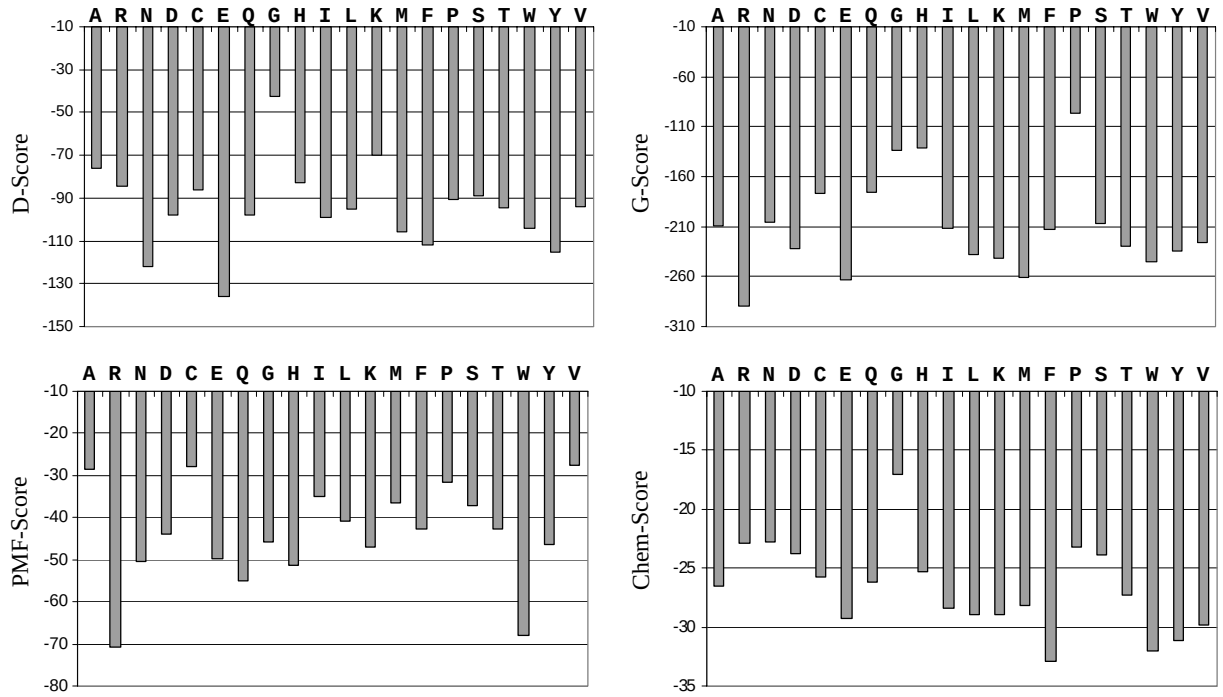

Figure 3. Comparison of different scoring functions in ranking the 20 amino acids docked in mGluR1. The docking was performed with both options 'assign charges' and 'place particles' on, and the top-scored poses using the FlexX scoring function were selected for re-scoring by the other scoring functions (D-Score, G-Score, PMF-Score, and Chem-Score) in the CScore module of Sybyl.

2.3. Docking amino acid ligands in the homology models

With the optimized docking procedure for mGluR1 in place, we proceeded to test the ability of FlexX to dock 20 amino acids into the homology models of the CaSR, 5.24 receptor, and GPRC6A. FlexX docked the amino acid ligands in the binding pockets of the homology models with the glycine moiety in the ligand interacting with the conserved residues in the receptor as observed in the crystal structure of mGluR1. The ranking of the ligands is shown in Figure 4, while Figure 5 graphically depicts the best five amino acid ligands for each receptor. The top ranked ligands reflect the property of the ligand binding pocket of each model in terms of size and molecular interactions. For example, hydrophobic aromatic amino acid ligands are preferred in the CaSR pocket. The location of the amino acid binding site in the CaSR is equivalent to the glutamate binding site in the mGluRs,[4,5] while the calcium binding site has recently been localized to an adjacent site in close proximity to the amino acid binding pocket.[30] However, to our knowledge, the orientation of amino acid ligand docking in the CaSR has not been reported. In our homology model of the CaSR, the binding pocket contains a hydrophobic distal portion which is composed in part of the aromatic side chain of tryptophan 70 and the aliphatic carbon chain of arginine 66 (Fig. 5B). This hydrophobic environment accommodates the side chain of hydrophobic amino acid ligands such as phenylalanine and tryptophan, and thus likely explains preference for aromatic amino acids in potentiating the CaSR.[3,5]

The 5.24 receptor and GPRC6A are both most potently activated by the positively charged amino acids arginine and lysine. We and others have shown that a negatively charged residue (aspartate 388) in the distal portion of the binding pocket determines, in part, the selectivity of fish 5.24 receptor for basic amino acids such as arginine and lysine.[15,16] However, the binding pocket of GPRC6A differs from that of the 5.24 receptor in that there is no negatively charged residue in GPRC6A at the equivalent position of aspartate 388 in the 5.24 receptor. The model of GPRC6A shows that glutamate 170 is the determinant for binding positively charged ligands (see Fig. 5D), indicating the importance of electrostatic interactions in determining the ligand selectivity.

In contrast to the results of amino acid docking at mGluR1 which showed that glutamate was by far the optimal ligand (Fig. 2D), the scores of the docked amino acids in the models of the CaSR, 5.24, and GPRC6A yielded several good hits with similar scores (Figs. 4A–C). This is consistent with the experimental data demonstrating that these receptors can each be activated or potentiated by more than one amino acid.[3,8,9,15,16] Tryptophan is ranked as the best ligand and other hydrophobic amino acids are among the top ranked ligands for the CaSR (Fig. 4A). Arginine and lysine are among the best ligands for the 5.24 receptor and GPRC6A (Figs. 4B and C). As noted above, for these two receptors, not only does the charge environment differ between the two receptors, but also the shapes of the binding pockets differ from each other; GPRC6A has a more restricted shape because of the presence of tyrosine 148 in the binding pocket, which may explain why the GPRC6A pocket excludes branched amino acids with low ranking scores (Fig. 4C). This is

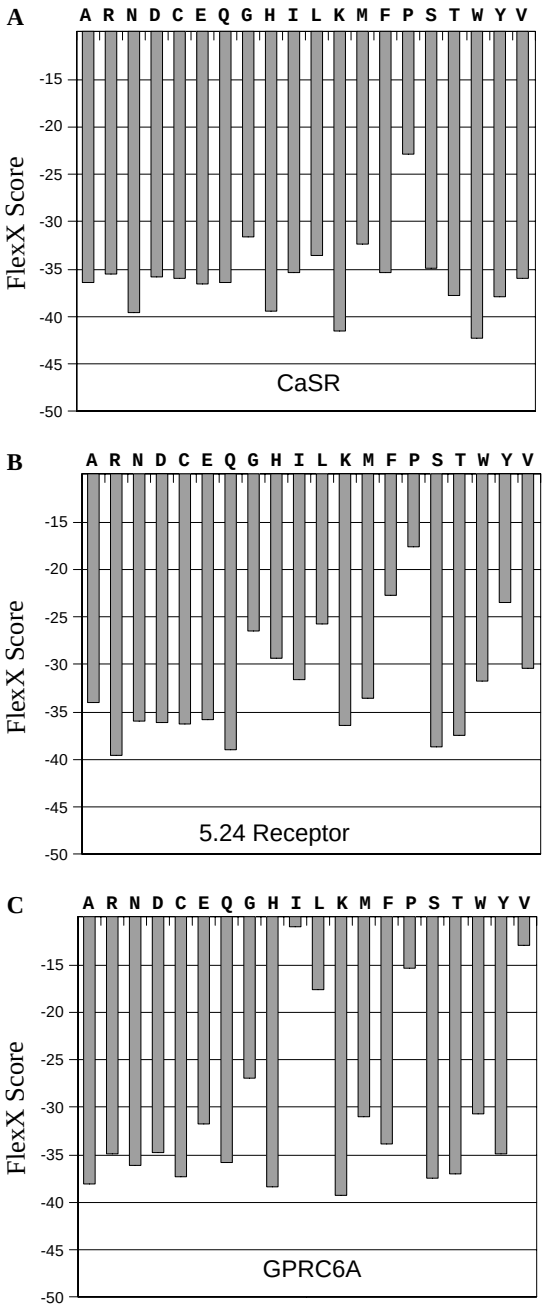

Figure 4. Score profiles of the amino acids docked into the homology models of the CaSR (A), 5.24 (B), and GPRC6A (C). The score for each amino acid is given for the best pose scored by FlexX Score.

consistent with the fact that GPRC6A, in contrast to the 5.24 receptor, cannot be activated by branched amino acids.[8,9]

3. Discussion

3.1. Optimization of the docking parameters

Among the ligand–receptor docking programs available, FlexX,[25] Dock,[26] Gold,[31] and Glide[32] are the most widely used. The performances of these programs have varied depending on the system investigated, and the intrinsic physicochemical properties of the ligand–protein interactions involved.[32–34] Thus, optimization of the individual docking system and its parameters is important in drug and ligand docking studies. In the present study, the optimization of FlexX in Sybyl was performed by taking into the account two options, 'assign charges' on the ligand and 'place particles'. The two options represent the electrostatic interactions (assign charges) and water mediated interaction in the ligand–receptor interactions (place particles). At physiological pH, amino acids exist in zwitterionic forms and therefore salt bridges make important contributions to the energy of amino acid binding to the receptors. However, although assigning charges on the ligand had little effect on the RMSD values of the docked glutamate poses for mGluR1 (see Table 2), this maneuver did improve the ranking of glutamate such that it became the best-ranked ligand out of the 20 amino acids (Figs. 2A and C).

Although water molecules are present in the X-ray structure of mGluR1, they were removed prior to ligand docking. Water molecules observed in the ligand binding site in crystal structures can provide bridging interactions between a ligand and a receptor, and they are involved in the solvation and desolvation processes in ligand binding.[35] Although the effect of adding water molecules is difficult to assess in the docking process, it has been shown that both X-ray water molecules and predicted water molecules improved the quality of prediction accuracy significantly of two enzyme–substrate systems, cytochrome P450 and thymidine kinase.[28] FlexX includes an option for placing water molecules during docking process; turning this option on alone did not change the rank order but it did lower the scores for all the ligands (Figs. 2A and B). When combined with the option 'assign charges' on the ligands, glutamate acquired a substantially lower score and was ranked as the best ligand (Figs. 2C and D). These results highlight the importance of electrostatic interactions and water bridged interactions in ligand binding of mGluR1.

Our results demonstrated that the ligand rankings varied substantially depending upon the scoring function used. The CScore module in Sybyl includes several scoring functions such as D-score[26] and G-score[31] which are also components of the docking programs Dock and Gold, respectively. These two scoring functions utilize force field-based methods in which the coefficients for terms such as hydrogen bonding and van der Waals interactions between ligand and protein are derived from the Tripos force field. In contrast, Chem-Score[36] estimates binding energy with empirical terms in which the coefficient is obtained by regression on protein–ligand complexes with known affinities, in a similar way to that for the built-in scoring function in FlexX. PMF-Score[37] was developed using knowledge-based potentials of mean force to measure the Helmholtz free energies of interactions for protein–ligand atom pairs. Our data showed that only D-score and the FlexX scoring functions ranked glutamate as the best ligand at mGluR. In a recent study, these scoring functions were

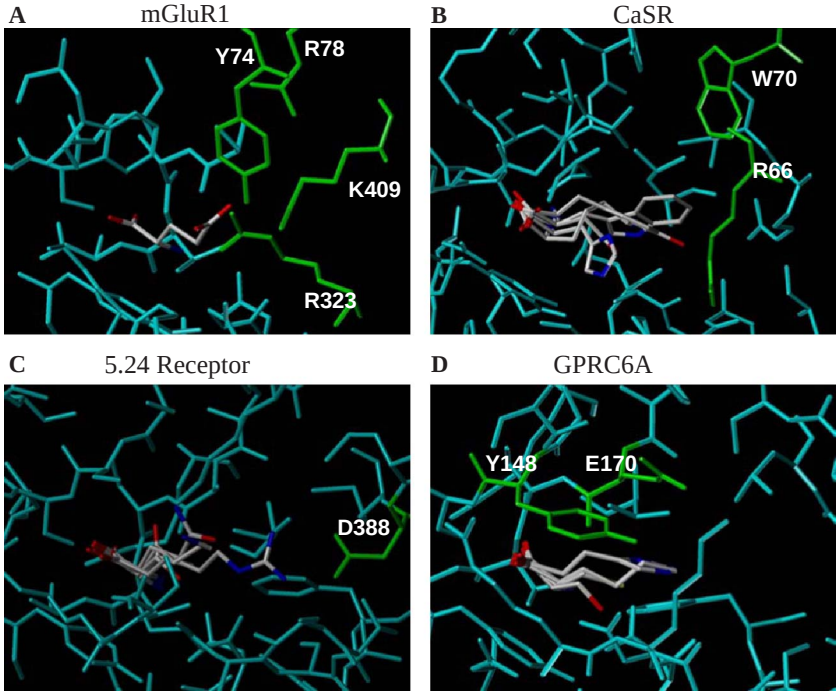

Figure 5. The best pose for glutamate in mGluR1 (A) and the best poses of the top five ranked amino acid ligands docked into the binding pockets of the three homology models (B–D). Selected residues in the pockets mediating key interactions with the ligands are labeled and shown in green. Color coding for the ligands: carbon, gray; oxygen, red; nitrogen, blue; sulfur, yellow.

evaluated in a virtual screening experiment of inhibitors for Factor Xa.[27] It was found that only PMF among the five scoring functions failed to reproduce the crystal complex of ligand and Factor Xa, and that the combination of the scoring functions (consensus score) did not improve the results. These results and the data presented in the present study suggest that scoring functions should be used with caution and systematically examined before drawing conclusions on drug or ligand rankings or relative affinities.

3.2. Ligand selectivity predicted by FlexX

The scoring profiles of the 20 amino acids docked in mGluR1 ranked glutamate as the best ligand with a significantly lower (better) score, suggesting a highly selective pocket. This is in agreement with the observation that mGluRs are activated only by glutamate but not by other amino acids (D. Kuang and D. R. Hampson, unpublished observation). In contrast, the scoring profile using homology models of CaSR, GPRC6A, and the 5.24 receptor (Fig. 4) each gave similar high scores with multiple amino acids. Thus, the predictions made by FlexX agree well with the experimental data reported in the literature indicating that multiple amino acids activate or potentiate these three receptors. Inspection of the top five ranked ligands for each receptor indicated that most of them are favored ligands for the corresponding receptor. For example, the top ranked ligands for the 5.24 receptor are arginine, glutamine, serine, threonine, and lysine, which are also favored ligands of this receptor.[7] Lysine, histidine, alanine, serine, and cysteine were predicted by FlexX for the GPRC6A, and they can all activate the receptor.[8,9] The affinities of all the amino acids for CaSR are not available, but based on the potentiation effects of amino acids on its response to calcium, this receptor has preference for aromatic amino acids.[3] This was confirmed in our docking experiment with only one false positive prediction, lysine.

Although FlexX successfully predicted the active ligands, accurately predicting the precise rank order of the active ligands presented a challenge. The most preferred amino acid ligand for each receptor was included during the refinement of the models; this is crucial for maintaining the residues in the binding pockets in the conformation that interacts favorably with the ligand during the refinement using dynamics simulations. However, the preferred ligand only came up as the best hit in the case of the 5.24 receptor. For the CaSR and GPRC6A receptors, neither phenylalanine nor arginine was scored as the best ligand, respectively.

Although the cause(s) for the inaccuracies in ranking the best ligands could be due to a problem intrinsic to the docking program, it may have also been caused by uncertainties in the homology models. Success in using homology models in automatic docking studies is dependent on the quality of the model, which is in turn determined in large part by the sequence identity between the template and target protein. Although the receptors we studied here have relatively low sequence identities to the mGluR1 template (the average is around 24%), they are members of the same receptor family and the ligand binding domains adopt the same protein fold (Venus Flytrap configuration). A close examination of the binding pockets indicates that CaSR and GPRC6A are slightly more divergent from that of mGluR1 than the

5.24 receptor (see Table 1). In the subregion of the binding pockets that accommodate the glycine moiety of the ligand, only two residues are different in the CaSR (S170 and E297); while in the distal portion of the pocket that dictates amino acid selectivity, the only one residue that is identical to the template is serine 151 of the 5.24 receptor. All of these differences may introduce more uncertainties and inaccuracies in ligand ranking in the models of CaSR and GPRC6A compared to that of the 5.24 receptor.

In conclusion, the program FlexX was used to dock the 20 amino acid ligands into the crystal structure of the ligand binding domain of mGluR1, and in homology models of the related CaSR, 5.24, and GPRC6A receptors. We found that glutamate was ranked as the best ligand for mGluR1 using the scoring function under conditions that included charges on the ligands and water molecules in the binding process. The results obtained with the three homology models revealed that most, but not all, of the known favored amino acid ligands were among the top scoring amino acids, and that some false positives were observed with GPRC6A and the CaSR. We conclude that (1) in silico ligand predictions using FlexX with Family C receptors generally gave good results that were consistent with the experimental data reported in the literature, and (2) that caution is required in ligand identification studies and that ideally, several different docking and scoring functions should be used and evaluated in conjunction with positive controls.

4. Methods

4.1. Molecular modeling

Homology models of the fish 5.24 receptor, the mouse CaSR, and mouse GPRC6A were generated using the X-ray crystal structure of the extracellular domain of rat mGluR1 as the template (PDB coordinates, 1EWK[10]) and version 6.0 of the MODELER program[38] to generate the models. The sequences of the receptors were aligned using ClustalX and Sybyl 6.9 (Tripos Inc., St. Louis, MO) was used to view, analyze, and manipulate the structure. The preferred highest affinity ligands (arginine for fish 5.24 receptor and GPRC6A, and phenylalanine for the CaSR) were manually docked into each model and the structure of the complex was subjected to refinement using AMBER 7.0[39] with Cornell force field as described below. The force field parameters including atom charges were derived with the aid of Antechamber[40] in AMBER. The quality of the homology models was examined using PROCHECK.[41]

Molecular dynamics simulations were applied to refine the model in the presence of explicit solvent water molecules. To prevent deviation from the initial structure, the molecular dynamics was performed in two stages as described in a recent work.[42] The complex was prepared and solvated as described previously for work conducted on the mGluRs.[43] In the first stage, the solvent was equilibrated with an energy minimization and a 500 ps molecular dynamics run at 300 K and 1 atmosphere constant pressure. The system was further energy-minimized by restraining the backbone of the protein and gradually reducing the restraints from 200 to 2 kcal/mol. In the second stage, the whole system was relaxed by 500 ps molecular dynamics at 1 atmosphere constant pressure with 2 kcal/mol restraints on the backbone atoms of the receptor in the first 50 ps and 1 kcal/mol in the remainder of the simulation. In all dynamics simulations, bond lengths involving hydrogen atoms were constrained with the SHAKE algorithm.[44] A 12-Å cutoff was applied for non-bonded interactions and the particle mesh Ewald method[45] was used for the calculation of the long-range electrostatic interactions. The entire system was energy-minimized for 20,000 steps by gradually reducing the restraints on the backbone to zero.

For ligand docking, the 20 amino acids were constructed and energy-minimized using Sybyl. All of the amino acids in the text refer to the natural L-type amino acids. The binding pockets were defined as all residues within 6.5 Å of the ligand in the model, using the docking program FlexX implemented in Sybyl 6.9. Water molecules and ligands in the crystal structure of mGluR1 and in the homology models of the other three receptors were removed prior to the docking experiment. The 30 best docked conformers ranked by the built-in FlexX scoring function were then analyzed further.

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