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Effect of pH and ligand charge state on BACE-1 fragment docking performance

José L. Domínguez · M. Carmen Villaverde · Fredy Sussman

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Abstract In this work we propose a protocol for estimating the effect of pH on the docking performance to BACE-1, which affords the charge state of the inhibitor as well as the protonation state of all ionisable residues in the protein at a given pH value. To the best of our knowledge, this is the first report of a protocol predicting the BACE-1 ligand docking poses not only at the neutral pH at which most crystallographic structures were obtained, but also at the optimal pH of the enzyme (in the acidic range), at which most of the BACE-1 binding affinity assays are performed. We have applied this protocol to a set of 23 fragment-like BACE-1 ligands that span four orders of magnitude in their binding affinities. The pK_a values of the BACE-1 acidic residues deviate substantially from the estimates for model compounds in solution and display a ligand dependent variability, especially in the case of the catalytic Asp dyad residues. This outcome should have a strong bearing on the design of protocols for docking based BACE-1 screening campaigns. Finally, we were able to find an explanation for the poor docking success rate of some fragments based on the availability of anchoring points, a rationale that could help to improve hit rates in BACE-1 screening campaigns.

Keywords BACE-1 inhibition · Docking protocol · Protonation state · pH dependence · Docking performance · Fragment screening

Introduction

β-Site amyloid precursor protein cleaving enzyme (BACE-1) is a prominent target in Alzheimer's disease (AD) drug discovery efforts. This enzyme (a member of the Asp protease family) cleaves the amyloid precursor protein (APP), generating hydrolytically a peptide of 42 residues in length called amyloid beta (Aβ) peptide, whose aggregation as oligomers in the inter-synapsis region is the cause of AD in accordance with the amyloid cascade hypothesis [1, 2]. Many problems have beset all the stages of the drug discovery efforts aimed at this enzyme, including failures in the early HTS campaigns for BACE-1 inhibitors [3, 4]. The use of fragment based search (FBS) methods has overcome some obstacles present in the search for hit leads for BACE. FBS seeks small molecules whose molecular weight is <300 Da, which will serve as kernels for high affinity binders after substantial modification [5–12]. Moreover, the search for low molecular weight drug leads is of special interest for CNS diseases, since it is a requirement for blood brain barrier trespassing [8, 10, 12].

One the main hurdles in the in silico FBS campaigns for BACE-1 is the prediction of the charge state of the binding site ionisable residues and the bound inhibitor, which in principle should have a strong influence on the outcome of the docking based screenings. Some of the earliest in silico HTS campaigns used a single Asp dyad protonation state in their screening campaigns, based on the hypothesis that most ligands induce a unique Asp dyad protonation state [13–15]. Our laboratory, based on a combination of surface

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J. L. Domínguez · M. C. Villaverde · F. Sussman (✉)
Departamento de Química Orgánica, Facultad de Química, Universidad de Santiago de Compostela, 15782 Santiago de Compostela, Spain
e-mail: fredy.sussman@usc.es

plasmon resonance (SPR) experiments with molecular mechanics calculations was one of the first to provide some robust evidence that the charge state of the ionisable residues of BACE-1 strongly depends on the chemical nature of the inhibitor [16, 17] and hence any ligand screening protocol would require the prediction of the active site ionisable residues protonation state (when bound to a given inhibitor) ahead of the docking calculations. In the last few years there has been a keen interest on this issue, but its focus has been on the charged state of the Asp dyad, clearly disregarding the effect of the other acidic residues buried inside this enzyme [13–15].

Very recently Kacker et al. [18] used a very sophisticated combination of quantum mechanics calculations and molecular dynamics simulations to predict the protonation state of the enzyme's Asp dyad for subsequent self-docking, cross-docking and HTS studies, while Barman and Prabhakar [19] have searched through all possible charge positions to predict which Asp dyad charge combination generates the highest enrichment for self docking simulations, but to the best of our knowledge no pH dependence calculations have been carried out until now.

The X-ray crystallographic structures of BACE-1—inhibitor complexes are predominantly obtained at a pH close to neutral. Hence, the retrospective docking calculations should display an increased success rate for the inhibitor and ionisable protein residues charge state predicted to exist at this pH value. On the other hand, the majority of the BACE-1 inhibition assays are carried out at the acidic pH at which this enzyme has its optimal activity (4.5–5.0). The actual charge state of the Asp dyad and other active site residues at the enzyme's optimal pH for activity should certainly differ from the one at which the BACE-1—inhibitor crystallographic structures were obtained. To bridge this divide and to answer some of the other open questions stated above we have used a protocol that blends the pK_a prediction for BACE-1 titratable residues with the docking simulations. The results of these calculations have enabled us to perform docking calculations on a set of 23 ligands at both acidic and neutral pH values, and hence estimate the effect of the pH on the docking performance. The results indicate that these inhibitors are mostly charged at both pH values, since the charged inhibitors have a substantial increase in the hit success rate. A large number of the inhibitors studied here have a pK_a value of 7 or higher in solution. Hence this outcome implies that the enzyme environment will retain, or may be in a few cases raise, the solution pK_a value of the inhibitors.

The fact that the highest docking performance rate is obtained at a pH close to neutral supports the charge assignment prediction for the protein ionisable residues provided by our pK_a calculations. The Asp dyad protonation state has an evident effect on the docking hit enrichment. Moreover, including the predicted charge state of the acidic residues other than those belonging to the Asp dyad has a smaller but noticeable effect on the docking enrichment, since the calculated pK_a values for all acidic residues deviate from their estimates for model compounds and present a clear ligand dependence. Hence, protocols used in the BACE-1 screening campaigns should include the pK_a value prediction for all these residues.

Finally, we were able to elaborate an explanation for the low success rate of some ligands, based on the availability of anchoring points, an idea that could be exploited in weeding out false negatives or false positives in BACE-1 screening campaigns.

Methods

The group of 23 BACE-1 inhibitor fragments studied are displayed in Fig. 1. The structures of 20 out of 23 ligands bound to BACE-1 are known, and their PDB entry names are included in the figure as ligand identifiers. Perusal of these compounds indicates that the molecular weight of most of them is <350 Da (a value slightly above than the fragment cutoff of 300 Da), making them good candidates for fragment like inhibitors, which can be enlarged to attain good affinities. The last three entries in this list (compounds 3H0B0, 3H0BM and 3H0BS) represent small modifications of the ligand in PDB entry 3H0B [12]. These variants were studied because, to the best of our knowledge, they are the only ones that have published binding affinities at two pH values (4.5 and 6.5) [12], and hence they are valuable for testing our approach. The affinities of all compounds for BACE-1 and the ligand efficiencies are shown in Table 1. As seen from this table, the binding affinities have very diverse values, spanning four orders of magnitude from millimolar to sub-micromolar range.

The BACE-1 complexes were downloaded from the protein data bank (PDB) and hydrogens were added using the Discovery Studio (DS) suite of programs [20]. Most structures have a resolution below 2.5 Å, although all the complexes present one or more residue gaps in their structure. The most frequent structural breakpoint starts around residue 157 and extends for 9–12 residues. Other gaps start at residues 310 and extend for around 6 or 7 residues. We have generated gap-less complexes using a protocol previously described, which copies the missing fragments from the complex found in pdb entry 1FKN and then optimize the structure around the insertion location by an energy minimization protocol [16].

In order to assign the protonation states of the ionisable residues in BACE-1, the pK_a values of these residues were calculated by CPIRpK [21], a protocol resident in the

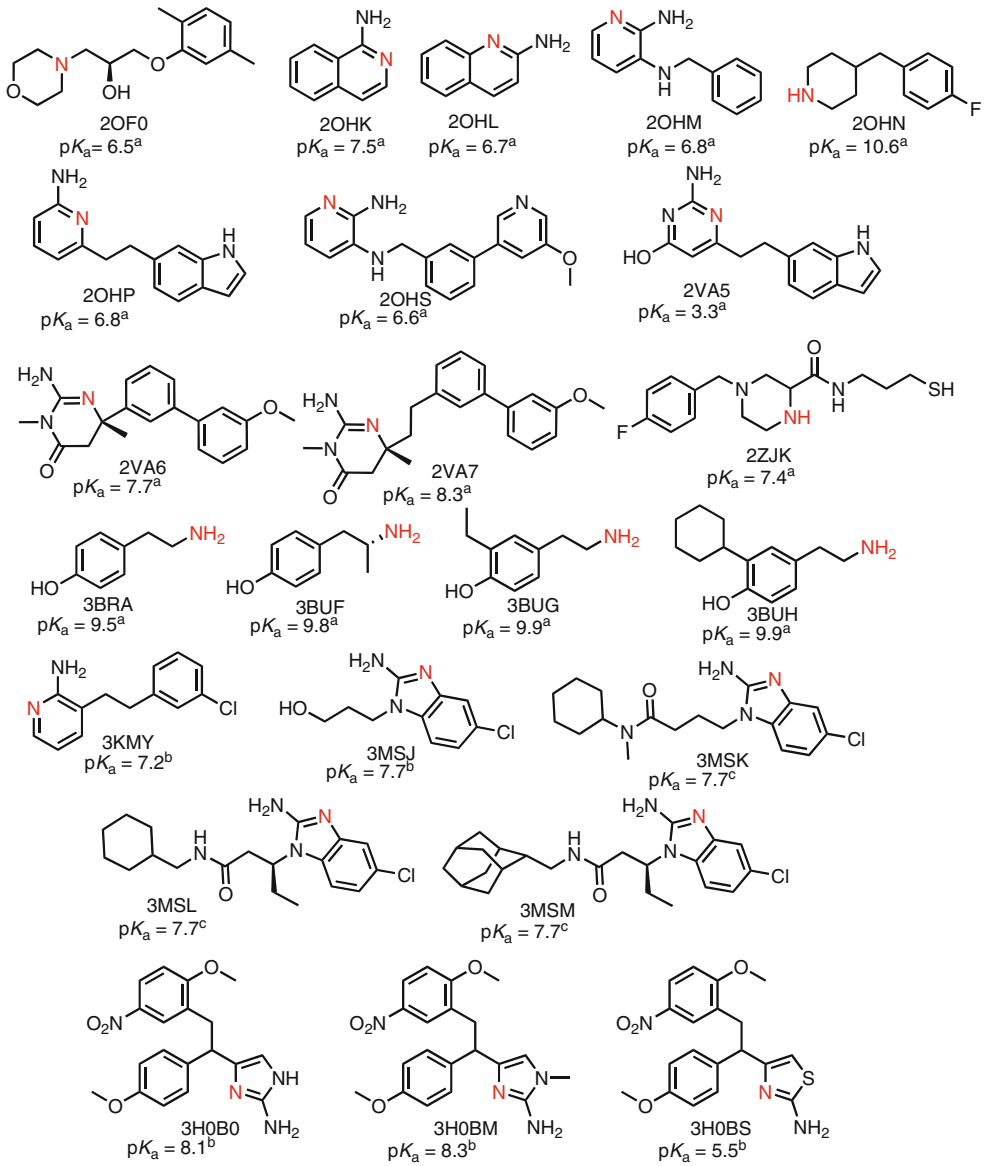

Fig. 1 Chemical structure of the inhibitor fragments used in our study named by the PDB id entries of their complexes with BACE-1. The N atom shown in *red* is the one that is most likely protonated.[a]Scifinder result [23]. [b]Literature value (see references [11] and [12]). [c]Estimation by analogy

Discovery Studio set of programs that evaluates the solvation effects by an approximate Generalized Born algorithm [22]. CPIRpK is able to afford highly accurate pK_a values with little computational cost. In the original work, this approach was tested on the pK_a prediction of all titratable residues in 24 proteins. The results indicate that the RMSD between the calculated and observed pK_a values lies for the most part below 0.5 pK_a units [21]. In our calculations we chose the charge assignments at two pH values (4.5 and 7.4). Since most of the X-ray structure studies that provided the observed ligand pose were obtained at pH values close to neutral, we proceeded to assign charge states of the ionisable residues of BACE-1 at pH 7.4 according to their pK_a values. On the other hand we thought interesting to perform docking at the acidic pH close to the one where this enzyme has its optimal activity (4.5), since most of the inhibitor binding assays are performed around this value. In the case of 3H0B ligand analogues discussed in reference 12, the authors have studied the effect of pH on inhibitor binding at pH 4.5 and 6.5. In order to test our protocol we have calculated the binding affinities for ligand 3H0BS at pH 6.5 as well.

A crucial issue in the prediction of the BACE-1 active site charge state is the inhibitor protonation state. As seen from Fig. 1 most inhibitors have one or several amino groups that could interact with the Asp dyad through electrostatic interactions. To determine the proclivity of the amino groups to be protonated we have searched either the

Table 1 BACE-1 ligand inhibition data and efficiency (L. E.) for in vitro assays

Ligand ID	IC_{50} (μM)	K_D (μM)	L. E.	References
2OF0	>2,000		<0.19	[5]
2OHK	~2,000		0.33	[5]
2OHL	~2,000		0.33	[5]
2OHM	310		0.32	[5]
2OHN	~500		0.32	[5]
2OHP	94		0.31	[6]
2OHS	40		0.26	[6]
2VA5	130		0.28	[7]
2VA6	0.38		0.36	[7]
2VA7	0.20		0.35	[7]
2ZJK		>1,000		[8]
3BRA		2,000	0.37	[9]
3BUF		800	0.38	[9]
3BUG		660	0.36	[9]
3BUH		220	0.31	[9]
3KMY		32	0.38	[10]
3MSJ	770		0.29	[11]
3MSK	26		0.27	[11]
3MSL	7		0.29	[11]
3MSM	8.9		0.26	[11]
3H0B0	5.7		0.27	[12]
3H0BM	0.47		0.31	[12]
3H0BS	1.1		0.30	[12]

original references or we performed SciFinder searches based on the ACD labs predictor [23]. In Fig. 1 the nitrogen atoms of the amino groups whose protonated form is predicted to have the highest pK_a value, are singled out in red. The BACE-1 structures with their titratable residues charge states assigned, both at pH 4.5 and 7.4, served as a template for docking calculations performed with GOLD [24]. The GOLD runs were configured to return 25 poses, with a number of iterations in the genetic algorithm ranging from a minimum of 1,000,000 to a maximum of 1,250,000. To ensure a wider range of poses, we used the 'Diverse Solutions' option in GOLD, which avoids the existence of clusters with similar solutions. The GoldScore [25, 26], ChemScore [27–29] and ChemPLP [30] scoring functions were used to generate the binding conformations. Each of the resulting poses was then rescored with each of the aforementioned scoring functions.

Earlier work has acknowledged that the use of molecular mechanics (MM) force fields enhanced the number of poses closer to the observed ones, and specifically in screening campaigns reduces the number of false positives and false negatives [31, 32]. Following this idea we have rescored our set of poses obtained from GOLD-based docking simulations for every compound by two molecular mechanics CHARMM rescoring functions based on the protein–ligand interaction energy. In the first MM rescoring function (referred to as Charmm) the poses for each ligand were subjected to a proton only energy minimization run, performed in two steps: 2,000 steps Steepest Descent minimization protocol, which was followed by a 50,000 step ABNR minimization, both with a 0.002 gradient tolerance. The second re-scoring function (referred to as h.Charmm) involved a MM protocol in which the atoms were restrained to move under a harmonic potential with a force constant value of twice the value of the atomic mass of each atom. The minimization schedule was the same as in the first MM protocol. The structures were ranked by the MM binding energy in the rigid body approximation that includes an implicit generalized Born model with a simple smoothing function (GBSW) desolvation term [22]. The force field chosen in all our MM calculations was CHARMM, due to the large availability of parameters for non-peptidic fragments in this force field [33], and the suite of programs used to run these calculations was CHARMM version 34b2 [34].

Docking performance assessment

The metrics for the native docking calculations carried out here starts with the evaluation of the RMSD between one or more of the top-ranked pose solutions and the X-ray binding mode. Docking performance was defined as the percentage of complexes in a set for which the RMSD between the three top-ranked solutions and the X-ray binding mode is below a certain cut-off. In the literature, an RMSD cutoff of 2.0 Å is used widely to assess docking performance. Nevertheless, in the case of fragments it was found recently that a lower cut-off (1.5 Å) is more appropriate, since the poses that fall in the range of RMSD values spanning from 1.5 to 2.0 Å may contain meaningful mistakes and the overall orientation of the ligand may be substantially different from those found in the observed structures [35].

In our calculations the success rate for a given simulation will depend on many parameters, which include the docking scoring function, the environmental pH, the inhibitor charged state, and the re-scoring function. For any of the possible combination of factors we have defined the success rate for compound i [SR(i)] by the following expression:

$$SR(i) = N_C(i)/N_T \quad (1)$$

where $N_C(i)$ is the number of poses found to have a RMSD <1.5 Å amongst top N_T ranking positions.

The total success rate, given by the hit percentage rate (hit %), for all ligands studied here at a given pH value by each of the rescoring functions can be calculated by the following expression:

$$\text{hit}\,\% = \sum_i \text{SR(i)}/\text{N}_\text{L} \quad (2)$$

where N_L is total number of ligands.

An optional statistic used by other groups is to calculate the success rate by finding for every compound one pose amongst the top N_T ranking positions (within a RMSD value) and then apply Eq. 2. Increasing the number of poses that may be screened for hits improves substantially the enrichment rate. Recently, Kacker et al. [18] obtained at 90 % hit rate when using the top ten ranking docking exits as possible hits.

Results

Protonation state of the inhibitor amino groups and BACE-1 acidic residues

Perusal of the X-ray structures of the BACE-1 bound fragments listed in Fig. 1 indicate that these compounds anchor themselves to the Asp dyad through their amino groups by hydrogen bonds or by direct ion pair interactions. As it can be seen from Fig. 1, many compounds contain more than one amino group. Hence, identifying the amino group that is most likely to be protonated is essential for predicting the binding pose.

As seen from Fig. 1, many of the fragments have the protonated amino groups with a pK_a value in solution close or above 7.0. Taking the solution pK_a values as a guide, 15 of the compounds studied will be charged both at pH 4.5 and 7.4, while 8 will be charged only at an acidic pH (e.g., 3H0BS). Nevertheless, the actual pK_a value of the fragment, when bound to BACE-1, may vary due to their proximity to charged residues like those of the Asp dyad. For instance, our literature survey of 2-amino pyrimidine fragment found in inhibitor 2VA5, indicates that the nitrogen group at position 3 (closest to the hydroxyl group) is the one with largest pK_a value. However, the nitrogen that makes a hydrogen bond contact with one of the Asp dyad oxygen carboxylates is at position 1 in the pyrimdine ring, and hence it should be the amino group that is most likely protonated (see Fig. 1). As we shall see below this protonation state is the one that provides a better docking performance.

Once we had a preliminary identification of the protonated amine group in these fragments, we calculated the pK_a values of all titratable residues in BACE-1 either for a neutral or a charged inhibitor. Table 2 displays the pK_a values only for the Asp dyad residues in BACE-1 (Asp 32/Asp 228) when the fragments are either neutral or protonated. As seen from this table, the charged states of the inhibitor have a marked effect upon the Asp dyad pK_a values. For instance, assigning a charge to the inhibitor lowers substantially the pK_a values of the Asp dyad residues. The resulting pK_a values have allowed us to predict the protonation state of these residues at both pH values (4.5 and 7.4) needed for our studies. Previous studies performed in our laboratory on the effect of the pH on the binding affinity of some peptidomimetic BACE-1 inhibitors predicted that Asp 32 always had a higher proclivity to stay protonated than did Asp 228, over a larger pH value span [16]. The results shown in this Table indicate that for a large number of ligands, the aforementioned pK_a order still holds. Nevertheless, in some cases, especially when the ligand is protonated, the pK_a ranking amongst the Asp dyad residues is inverted. For instance, that is the case of BACE-1 bound to charged inhibitors 2OHL, 3BRA and 3BUG. Hence, the Asp dyad adopts a wide variety of protonation states depending on the charge state of the inhibitor and the environmental pH value. When a charge of +1 is assigned to the inhibitors at the enzyme's optimal pH (4.5), most of complexes are predicted to have a monoprotonated Asp dyad, although a certain number of alternative states (i.e., diprotonated and doubly charged) are possible. If the pH is raised to a value close to neutral (7.4), we predict that the inhibitor binding elicits mostly a doubly charged Asp dyad state.

The presence of the ligands not only affects the pK_a values of the Asp dyad but has a strong influence on the protonation state of some of the other acidic residues (see Online Resource 1). Many of these residues have pK_a values that show strong upwards and downwards shifts from the estimates for model Asp and Glu residues in solution. There also seems to be a pK_a variability for a given BACE-1 residue when bound to different inhibitors. In some cases these pK_a differences could reach more than one pK_a unit.

Effect of the protonation state of the inhibitor and the charge of the protein residues on the docking performance

To gauge the effect of the inhibitor charge state and the protein residues protonation states at two pH values onto the docking performance, we have calculated the RMSD from the poses resulting from the self docking simulations with respect to the observed crystallographic poses. The results are shown in Fig. 2, which displays the effect of the pH through a histogram distribution at increasing RMSD value, for both neutral and charged ligands. The RMSD distribution was calculated from Eqs. 1 and 2 with the N_T value set to 25 (the number of pose exits). As seen from this figure, there is a marked effect of the inhibitor's protonation state in their self-docking success. The charged inhibitors increase substantially the percentage of hits at the lowest RMSD bin (<1.5 Å) and decrease the number of poses with hits with the highest RMSD values (i.e. >6.0 Å).

Table 2 Predicted Asp dyad pK_a values

Ligand ID	Residue	pK_a value	
		Neutral ligand	Charged ligand
2OF0	Asp 32	8.6	6.4
	Asp 228	6.4	4.2
2OHK	Asp 32	8.8	4.0
	Asp 228	5.1	4.3
2OHL	Asp 32	8.3	3.5
	Asp 228	5.7	6.0
2OHM	Asp 32	6.6	5.2
	Asp 228	8.1	4.4
2OHN	Asp 32	6.4	5.9
	Asp 228	4.5	2.9
2OHP	Asp 32	9.5	5.7
	Asp 228	5.9	5.3
2OHS	Asp 32	7.3	7.2
	Asp 228	7.8	4.3
2VA5	Asp 32	8.7	5.7
	Asp 228	5.3	4.1
2VA6	Asp 32	8.4	5.9
	Asp 228	5.8	3.8
2VA7	Asp 32	8.8	6.1
	Asp 228	5.8	3.9
2ZJK	Asp 32	6.2	6.1
	Asp 228	4.8	4.6
3BRA	Asp 32	6.5	4.2
	Asp 228	4.7	5.2
3BUF	Asp 32	7.0	4.5
	Asp 228	4.7	5.4
3BUG	Asp 32	6.5	4.4
	Asp 228	5.1	5.7
3BUH	Asp 32	7.0	4.5
	Asp 228	5.0	5.7
3H0B0	Asp 32	6.8	5.2
	Asp 228	7.0	5.6
3H0BM	Asp 32	7.6	5.0
	Asp 228	6.9	5.7
3H0BS	Asp 32	7.6	4.3
	Asp 228	6.7	5.3
3KMY	Asp 32	7.2	7.2
	Asp 228	8.4	3.7
3MSJ	Asp 32	9.0	6.2
	Asp 228	6.7	5.0
3MSK	Asp 32	8.4	5.3
	Asp 228	6.0	4.3
3MSL	Asp 32	8.1	5.6
	Asp 228	6.0	3.9
3MSM	Asp 32	8.6	5.6
	Asp 228	6.0	3.9

To study the effect of the scoring function on hit enrichment we have proceeded to rescore the original GOLD hits not only with some of the GOLD resident fitness functions (GoldScore, ChemScore and ChemPLP), but with Charmm scoring functions as well (see "Methods" section). One of the main differences between both types of scoring functions resides in the representation of hydrogen bonds and direct ion pair interactions. While the MM based scoring functions represent these non-bonded interactions by a Coulombic interaction screened by an implicit generalized Born desolvation term, the fitness scoring functions (resident in GOLD) represent them by typical short range hydrogen bond functions. Hence, when the GOLD resident scoring functions are used, the ionisable protein residues that are not at a hydrogen bond distances with the functional groups of the inhibitor do not participate in ranking the ligand's pose when the distances between donors and acceptors are beyond a certain cutoff.

One of the major problems when comparing the results of docking predictions of different labs is that each group uses diverse metrics for pose acceptance. Perusal of the literature indicate that one of the most glaring differences in the docking studies (even to the same protein) is the number of top ranking poses allowed in the search for a low RMSD candidate. Recent studies related to the effect of the protonation state of the Asp dyad in docking calculations to BACE-1 [18, 19] have allowed a set of ten top ranking poses for a RMSD hit, while other authors use less permissive metrics that includes search hits only at the top ranking pose in three parallel docking runs [35]. To determine the effect of the ranking set size on our results, we have calculated the effect of the inhibitor protonation state and pH for increasing the pose set size. The results are displayed in Figs. 3, 4 and 5. While Fig. 3 displays the percentage of hits at RMSD <1.5 Å that are found amongst the top three poses, Fig. 4 depicts the success rate resulting of finding at least one hit at this RMSD cutoff amongst the top three poses. Finally, Fig. 5 displays the single hit success amongst the top ten poses. The results of Figs. 3, 4 and 5 include the hit rate for the various re-scoring functions for both protonated and neutral ligand at two pH values used in our studies. For reference we have performed as well docking simulations with the protein having all ionogenic residues charged, regardless of their degree of burial (referred to as no CPI in Figs. 3, 4, 5).

One of the most outstanding results observed in Fig. 3 relates to the marked effect of the inhibitor protonation state. As seen from this figure, charged ligands exhibit a marked success rate enhancement over neutral ligands, for all re-scoring functions and pH values used. One of the highest hit percentage increases is observed for those that resulted from the docking carried out with the Charmm and

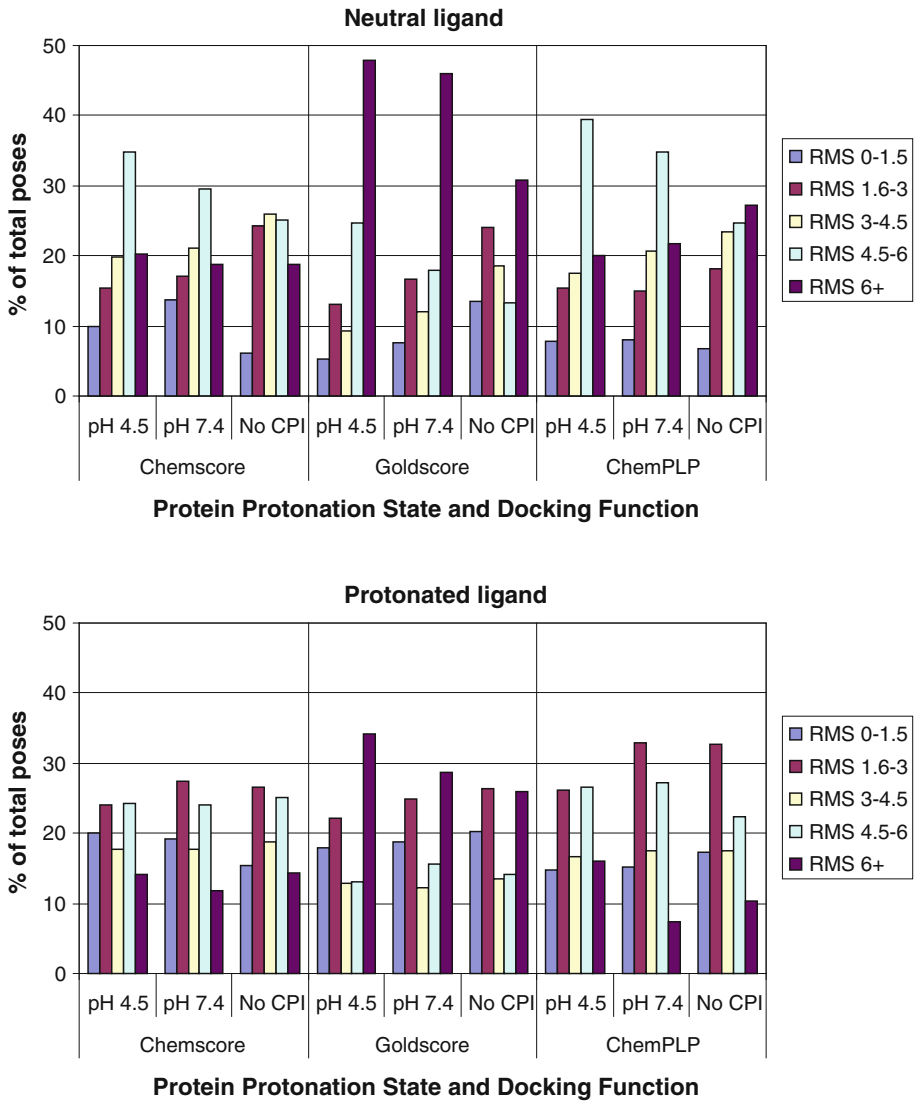

Fig. 2 Percentage of poses found at a given RMS value (see *colour code*), resulting from the docking calculation with a given scoring function (Chemscore, Goldscore and ChemPLP) performed at the indicated pH value.Calculations referred to as No CPI were performed with all protein ionisable residues charged

h.Charmm re-scoring of the ChemPLP poses at pH 7.4 and for the no CPI charge state. As seen from Fig. 3, the percentage of hits doubles from ~30 to 60 % when the ligand adquires a charge. For protonated ligands, the highest success rates are provided by MM based rescoring functions. For instance, rescoring by the harmonic Charmm (h.Charmm) protocol, leads to a 75 % success rate for docking calculations performed with Chemscore at pH 7.4.

The correct prediction of the ionization states of the acidic residues should help to increase the number of poses at lower RMSD values. Since the original crystallographic structures were obtained at pH close to neutral, it is expected that the assignment of the charge state of the protein residues at this pH value should produce the largest hit enrichment. Results shown at the lower panel of Fig. 3 support (as expected) the higher success rate at pH 7.4, especially for docking calculations performed with ChemScore. In this case the additional enrichment could be as much as 20 % when the rescoring is performed with h.Charmm over those calculated at pH 4.5 and ca. 14 % over the fully charged protein (i.e., no CPI). Hit rates obtained when the docking of protonated fragments is performed by either GoldScore or ChemPLP scoring functions still indicate that the highest enrichment rates are obtained at pH 7.4 but the differences are smaller, especially when compared to the no CPI results. These reduced differences with respect to the no CPI calculations can be partially explained by the pK_a values of the active site residues. As seen from Table 2, in the case that the ligand is protonated at neutral pH, the Asp dyad (in many cases)

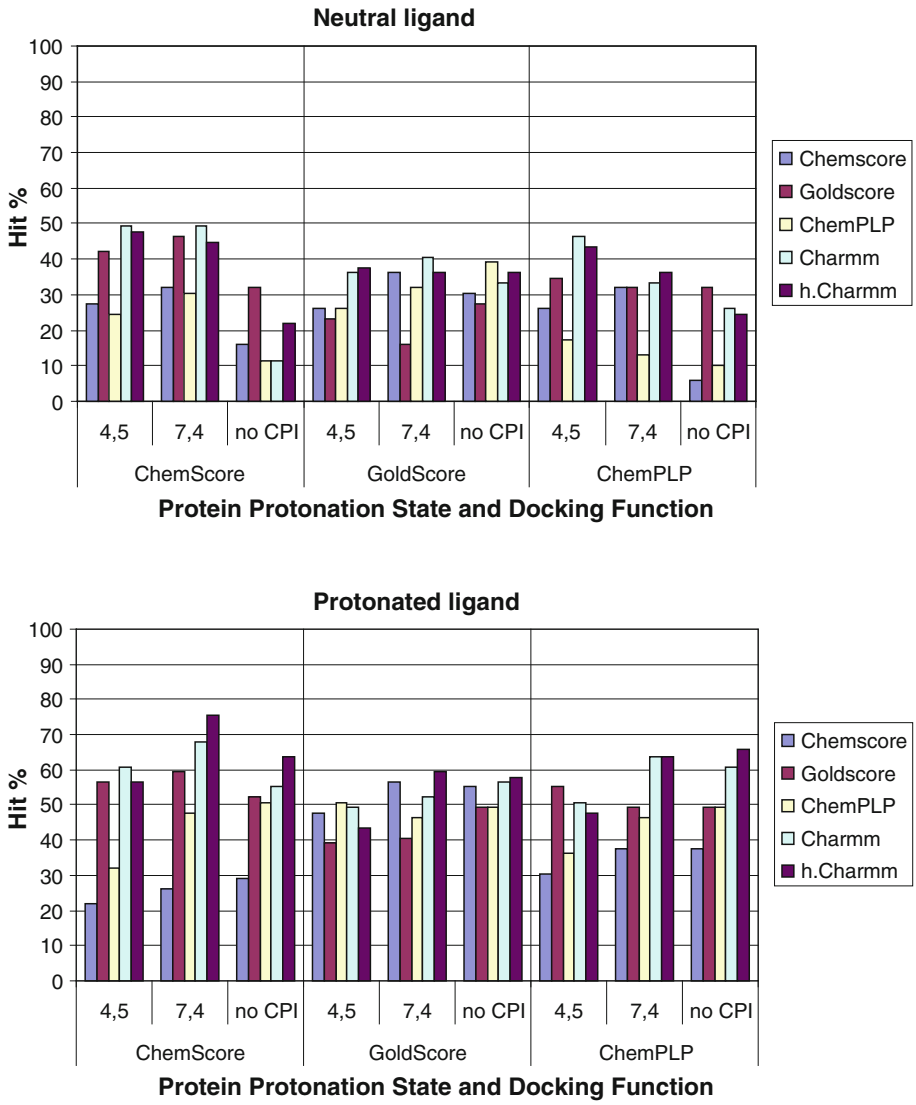

Fig. 3 The success rate (percentage of compounds with RMSD < 1.5 Å) amongst the top three ranking poses after rescoring with the functions shown at the right of every panel.Results for neutral ligands are shown in the *upper panel* and results for protonated ligands are shown in the *lower panel*

has a charge of -2, the same value that it would have when there is no charged assignment for this pair of residues. Still, for some docking protocols there is a noticeable difference between the docking success rate at pH 7.4 and the one where no charge assignment has been made. As mentioned above, that is the case of the Charmm and h.Charmm rescoring of docking pose generation with ChemScore (see Fig. 3). As observed from Fig. 3, another factor that has a strong effect on the success rate is the quality of the scoring functions. As pointed out before, many GOLD's off-the-shelf scoring functions, reduce the range of the electrostatic interactions that are crucial for fully translating the effect of the acidic residues charge state into pose location, since they replace the Coulombic interactions present in MM approaches by hydrogen bond type functions. The results shown in Fig. 3 indicate that the MM fare in many cases better than the original fitness functions and hence their use in the original pose generation may improve even more the hit enrichment, indicating that the Coulombic type of representation for electrostatic interactions are important for translating the effect of the charge state of the ionisable residues into finding a correct binding pose.

Allowing a search for the pose hit amongst a larger number of ranking poses improves the hit rate to levels (as seen from the comparison of Figs. 3 and 5) observed in other studies with similar pose number cutoff values [18]. Nevertheless, an increase in the number of acceptable poses seems to reduce the performance differences between the docking calculations carried out at dissimilar

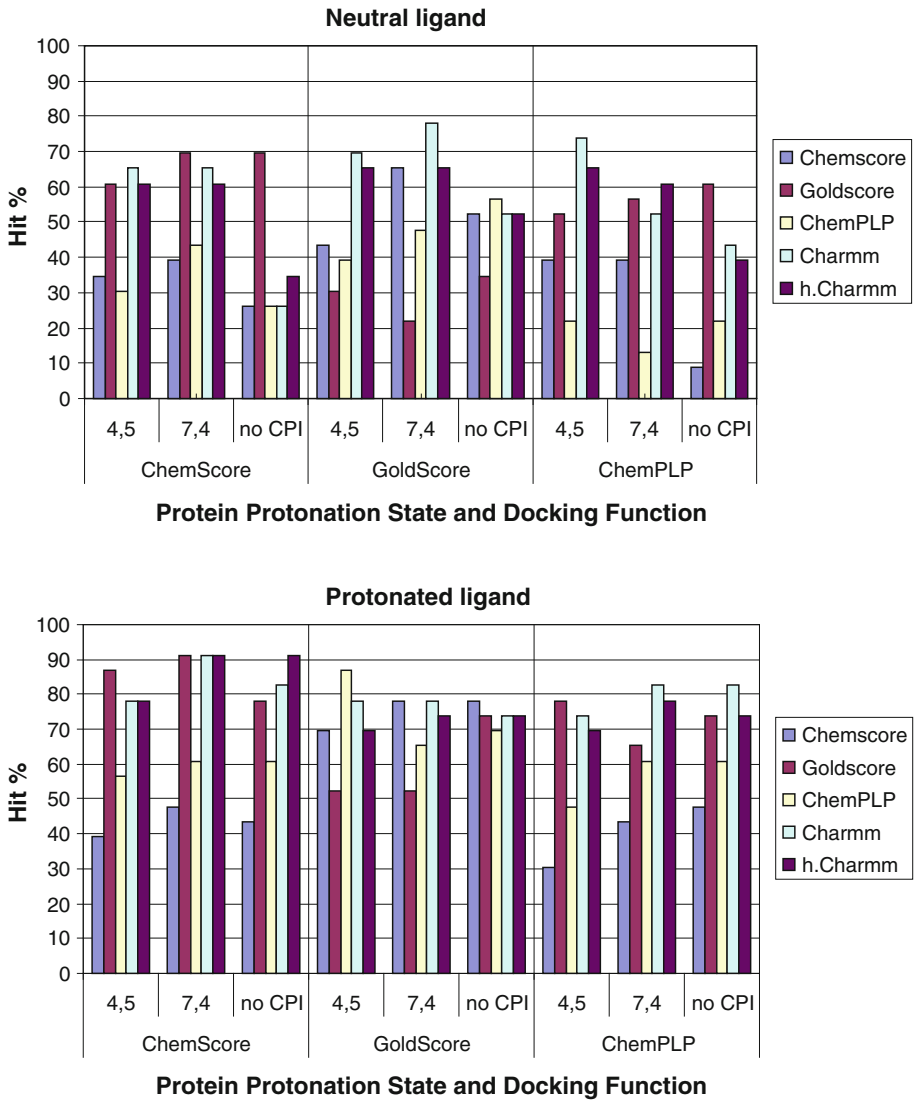

Fig. 4 Percentage of fragments that have at least one pose hit (within less that 1.5 Å from the crystal structure) amongst the three top poses as ranked by rescoring value.Results for neutral ligands are shown in the *upper panel* and results for protonated ligands are shown in the *lower panel*

pH values or rescored with different functions (see Fig. 5), a result that lends support for docking simulations carried out with more stringent searches.

Rationale for pH dependence

Very rarely the pH dependence on ligand binding to a protein is studied. For the set of ligands studied here the only case where this effect has been analyzed is in the case of 3H0B derivatives. In that work, the binding of amino-heterocyclic based compounds was studied at both pH 4.5 and 6.5. For the derivative containing a thiazole moiety (3H0BS), the experimental results indicate that the binding affinity observed at pH 4.5 ($IC_{50} = 1.1$ μM) is reduced drastically upon a pH increase (0 % inhibition at pH 6.5) [12]. To test our protocol we have re-scored the 25 top GOLD exit docking poses with our Charmm and h.Charmm scoring function values for this ligand at both pH values, when the inhibitor is charged and neutral. Figure 6 displays the binding ranking prediction at both pH values for the top ranked re-scored pose. As seen from this figure, the molecular mechanics based scoring results indicate that the drop in affinity observed experimentally can only be clearly seen in our calculations when we assume that at the higher pH value the ligand becomes neutral (see Fig. 6). This outcome supports the possibility that the pK_a in solution (5.5) is kept when the ligand is bound to BACE-1, and indicates that our protocol should be useful in predicting approximate figures for the pK_a value of the inhibitor when bound to the active site of BACE-1.

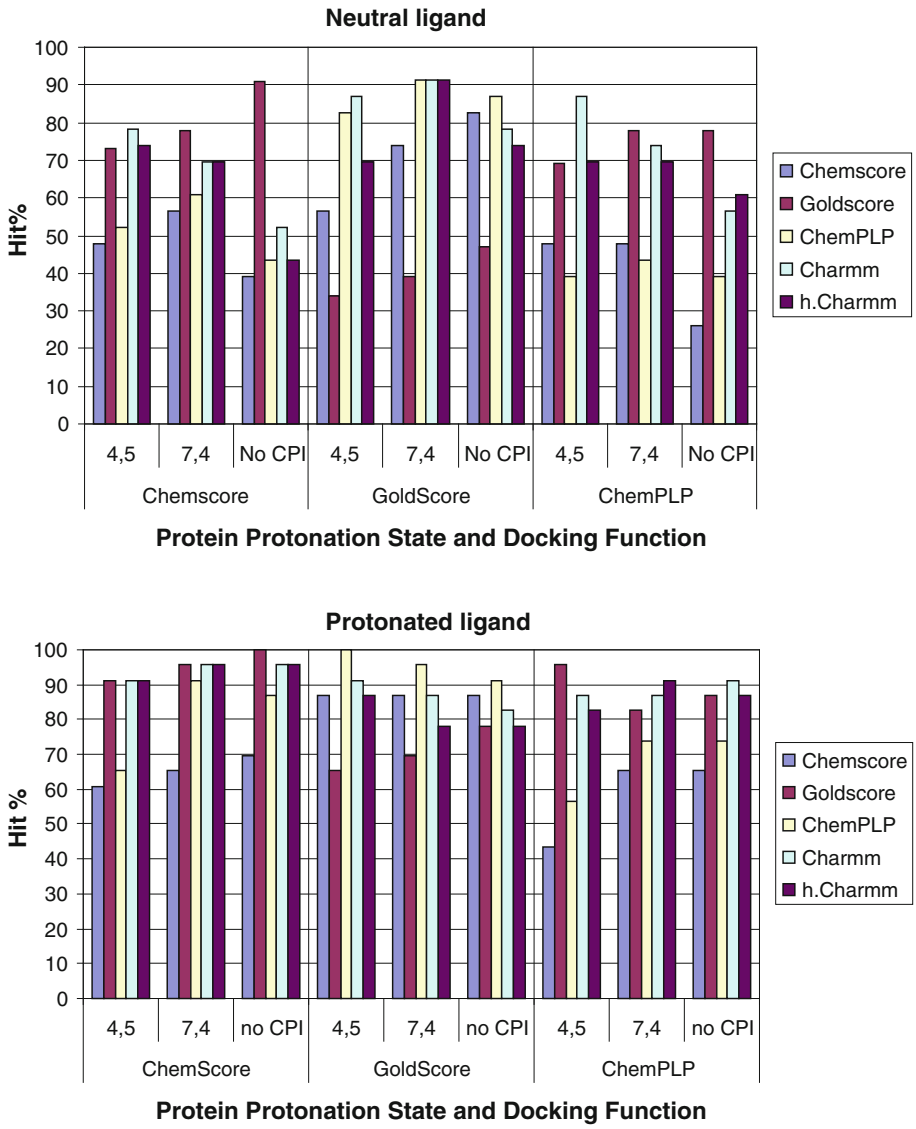

Fig. 5 Percentage of fragments that have at least one pose hit (within less that 1.5 Å from the crystal structure) amongst the ten top poses as ranked by rescoring value.Results for neutral ligands are shown in the *upper panel* and results for protonated ligands are shown in the *lower panel*

Rationale behind self-docking success

The high throughput search for non-peptidic ligands for BACE has been beset by many setbacks, due to the special features of the enzyme's binding site, which relate in part to its plasticity and its high exposure to solvent. Table 3 lists the overall docking performance for neutral and protonated ligands at both pH values. As seen from this table many compounds have good success rates across the board, regardless of the protonation state of the ligand and/or the environmental pH. (e.g., compounds 2OHN, 2VA7, 2VA6). We have found that the success rate is clearly linked to a proper assignment of the protonated amino group in the inhibitor. For instance, the final assignment in ligand 2VA5 (protonated at position 1) improves the overall hit rate by $\sim$30 % over the one that places the charge at position 3 (results not shown).

A small set of compounds seem to have overall poor self-docking success rates. That is the case for compounds 2ZJK, 3BRA, 3BUG, 3BUF, whose success rates are sometimes well below 30 %. Finding an explanation for the low success rate of these compounds on the basis of their chemical structure and/or the type of interactions they generate with the enzyme could be very valuable in informing future BACE-1 targeted screening campaigns. Below we shall discuss this issue in some detail.

Discussion and conclusions

Many authors have investigated the effect of the charge state of the inhibitor and acidic residues on the docking success rate for BACE-1 [14, 15, 18, 19] and other proteins

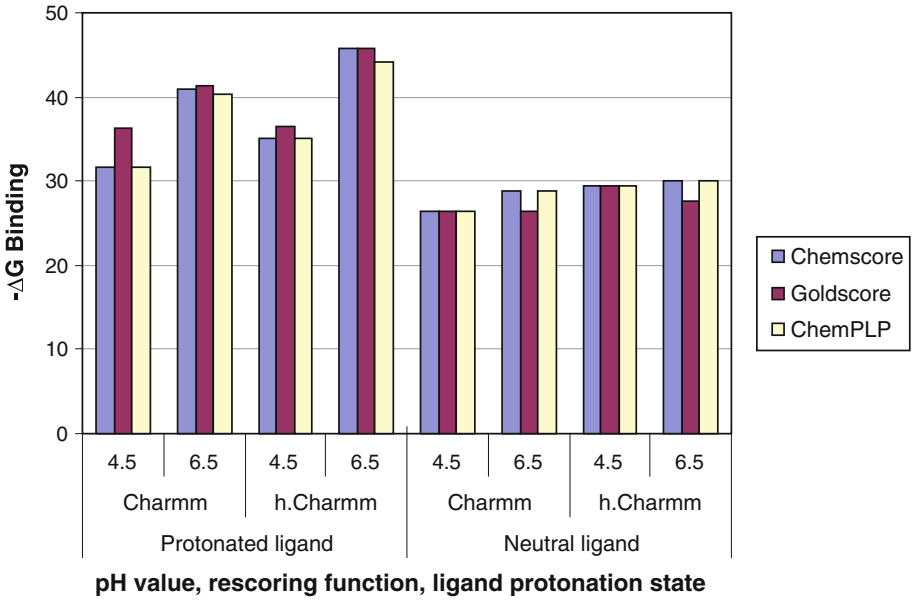

Fig. 6 Rescoring results (referred to as ΔG binding) for the top ranked 3H0BS pose, docked originally by the resident GOLD scoring functions (GoldScore, ChemScore and ChemPLP).The revaluation was performed for both neutral and protonated ligands at both pH 4.5 and 6.5, using the Charmm and h.Charmm scoring functions. ΔG rescoring values are in kcal/mol

Table 3 Percentage hit rates at RMSD <1.5 Å across all protocols

Ligand	Neutral	Protonated	Average
2OF0	17.8	55.6	36.7
2OHK	4.4	80	42.2
2OHL	6.7	93.3	50.0
2OHM	60	91.1	75.6
2OHN	80	97.8	88.9
2OHP	51.1	82.2	66.7
2OHS	64.4	91.1	77.8
2VA5	28.9	64.4	47.0
2VA6	84.4	82.2	83.3
2VA7	95.6	95.6	95.6
2ZJK	17.8	24.4	21.1
3BRA	17.8	37.8	27.8
3BUF	24.4	13.3	18.9
3BUG	15.6	11.1	13.3
3BUH	66.7	51.1	58.9
3KMY	66.7	91.1	78.9
3MSJ	22.2	73.3	47.8
3MSK	28.9	51.1	40.0
3MSL	55.6	91.1	73.3
3MSM	77.8	97.8	87.8
3HOB0	73.3	73.3	73.3
3HOBM	66.7	68.9	67.8
3HOBS	64.4	77.8	71.1

[36]. Nevertheless, in BACE-1 the effect of acidic residues other than the ones belonging to the Asp dyad has been disregarded. One of the first studies by Polgar et al. [15] investigated the effect of the inhibitor protonation state on the screening of BACE-1 ligands. The screening was performed by docking the putative ligands into a BACE-1 template with a charge assignment of −1 for the Asp dyad, regardless of the chemical nature of the inhibitor. As pointed in the introduction we have shown recently that the charge assignment for the Asp dyad strongly depends on the chemical structure of the ligand [16]. A corollary for this result is that BACE-1 screening campaigns should require the prediction of the charge state of BACE-1 acidic residues ahead of the docking stage. This idea has been recently incorporated in part to self-docking, cross-docking and screening studies to BACE-1 [18, 19], however to the best of our knowledge, the present study is the first time that the effect of pH on docking performance has been studied and the protonation state of *all* titratable residues in BACE-1 has been predicted ahead of the docking simulations by using a very fast predictive algorithm [21]. Our results indicate that although there are very few acidic residues (other than those belonging to the Asp dyad) that establish direct contacts with the inhibitor, many residues that are negatively charged in solution undergo upward or downward shifts in their pK_a values with respect to those used for model compounds of Asp and Glu residues in solution [16]. This outcome may be the result of the microenvironment in which these residues find themselves. Those residues that undergo downward shift would find themselves in a polar microenvironment, while those that experience an upward shift could find themselves buried in a hydrophobic pocket. Moreover, the pK_a value of an acidic

residue (other than those of the Asp dyad) may differ sometimes by more than one pK_a unit when one ligand is replaced by other in a BACE-1-inhibitor complex (see Online Resource 1). Hence, given the long-range nature of the electrostatic interactions, the charge state of these acidic residues may influence the actual binding pose of different ligands in very diverse ways. In order to take into account these long-range interactions, new scoring functions that include electrostatic interactions like the MM based one discussed above should be used, even for pose generation. As mentioned in the results section, our calculations show in some cases an increment in the success rate when the whole enzyme is charge typed according to the pK_a predictions, especially when the MM re-scoring functions are used (see Fig. 3), indicating that the long range electrostatic interactions may play a role in finding the correct binding pose as well.

Underlying rationale for docking performance

Many authors have elaborated proposals that could explain the failures at the pose search and re-scoring stages in docking simulations. Docking performance has been related to the ligand affinity for the enzyme, since high-affinity inhibitors may be 'easier' to dock than weaker binding compounds. Other authors suggested an alternative hypothesis that related improved docking performance with enhanced observed ligand binding efficiency (LE) [35]. These authors set a cutoff value for LE of 0.4, below which the probability for poor docking performance increases. Examination of Tables 1 and 3 clearly indicates that although the compounds with the lowest docking performance are amongst the ones that have lowest binding affinities, there is not a direct correlation between docking performance and binding strength. The experimental values for LE lie in the range between 0.19 and 0.38 (see Table 1), below the cutoff value of 0.4 proposed earlier for good docking performance [35]. Actually, some of the compounds with a LE close to 0.4 (3BRA, 3BUF and 3BUG) have resulted amongst the worst 'dockers' in our calculations.

We have found retrospectively a possible structural rationale for the poor performance of the worst 'dockers' based on the crystallographic ligand poses. In some instances (e.g., 3BRA, 3BUG and 3BUF) the primary amine group makes hydrogen bonds with only one of the residues belonging to the Asp dyad (Asp 32). The other hydrogen bond is found between the hydroxyl group of the phenol moiety and Phe 108. Figure 7 displays the overlap of one of highest scoring function poses obtained with the h.Charmm rescoring function and the X-ray crystal structure for the 3BUG ligand. As seen from this figure, the lack of an additional hydrogen bond interaction with Asp 228 seems to allow for the rotation of the aliphatic chain and the phenol moiety. Although the RMSD value for the pose displayed here is $\sim$3.3 Å, the hydrogen bond anchoring pattern is well reproduced. The lack of hydrogen bond anchoring motifs is most evident for ligand 2ZJK, which does not generate any sort of interactions with the Asp dyad and makes only one hydrogen bond with Tyr 71, leaving the ligand very unconstrained. This fragment was not generated

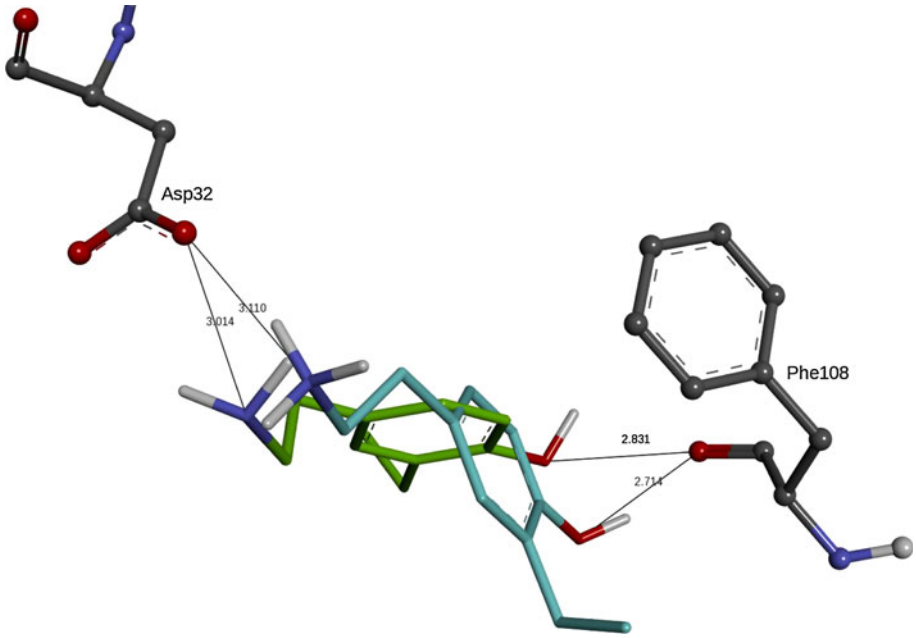

Fig. 7 Overlap of the 3BUG highest-ranking pose (*light blue*) with the crystallographic (*green*) structure.*Notice* that the HB's with anchoring residues are kept for both poses

by HTS in silico or in vitro, but was one of the kernels of a di-sulfide bond based tethering technology, used to obtain larger inhibitors. This method entails the introduction of individual Cys mutations in a binding site of interest, followed by screening against libraries of thiol-linker containing fragments that could produce disulfide bonds with the Cys residues introduced in the target protein [8]. The resulting crystallographic pose contains an enzyme-ligand di-sulfide bond, which should be important in generating the observed ligand conformation. Performing (as we did) docking simulations in the absence of this bond seems to reduce substantially the docking performance of this ligand.

It has been found that including into the docking simulations the crystallographic active site water molecules that make hydrogen bond interactions with the ligand could help improve the docking pose close to the observed crystallographic pose [37]. Perusal of the crystal structure the BACE-1—inhibitor complexes, indicate that some of them (e.g., 3BRA, 3BUF, 3BUG and 3BUH) have indeed one or sometimes two water molecules in the active site. Since these crystallographic waters make hydrogen bond interactions with the only amine group of the inhibitor (which already has been anchored by one of the Asp dyad members), we do not expect that including them into the docking simulations will help restrict the conformational space of the ligand enough to come close to the crystallographic pose. We tested this hypothesis by performing again docking calculations for one case (3BRA), in which the two water molecules present in the active site of the crystallographic structure were included. The results indicate that in this new round of calculations the number of RMSD hits remains almost unchanged (results not shown), an outcome that validated our informed supposition.

Our results predict that the best way of assuring a pose close to the observed one relies on having a number of hydrogen bond or other electrostatic interactions with the Asp dyad and polar functional groups of other residues. Nevertheless, other alternatives that favour a 'correct' pose are possible. For instance, 3BUH has a better performance than ligands 3BRA, 3BUG and 3BUF. It would seem that the cyclohexane moiety absent in the above mentioned compounds, but present in 3BUH (see Fig. 1) reduces the conformational flexibility by generating steric clashes that orient the molecule into a pose close to the observed one.

Our results indicate that the best docking enrichment is obtained at pH 7.4, in line with the fact that the original structures were obtained at a neutral pH. Nevertheless, all the binding affinities listed in Table 1 (obtained by FRET or SPR methods) were obtained at the optimal pH value which lies in the acidic range. We have investigated whether our docking protocol is able to predict at least in part the binding ranking of our ligands. The results indicate that our algorithm is only able to place the inhibitors with the highest binding affinities at the top of the ranking list and the worst binders at the bottom of the ranking. Nevertheless the overall ranking of the ligands is not in line with the experimental order (results not shown). There may be various reasons for this outcome, some of which may be related to deficiencies in the pose generation. In this regard, we believe that these results could be improved by the use of a MM based function in GOLD as a pose generator, a line of work that will be undertaken in our lab in the near future.

In summary, we have presented in this work a protocol that for the first time (in the case of BACE-1), takes into account directly the effect of pH on the native docking performance of a set of fragment-like inhibitors.

The pK_a values of the BACE-1 acidic residues deviate substantially from the estimates for model compounds in solution and display a ligand dependent variability, especially in the case of the Asp dyad residues, a result that should be taken into account in docking based ligand screening campaigns.

The biggest influence on the hit rate success is produced by the assignment of a charge state to the inhibitor and the choice of pH value. The enrichment provided by assigning a positive charge to the inhibitor probably is the result of a polar micro-environment created in part by the Asp dyad, which enables the ligand ionisable fragment to keep or increase its pK_a value predicted in solution. In one case (ligand 3H0BS) we have shown that our calculations have enabled us to predict an approximate pK_a figure for the ionisable fragment of the inhibitor, and in some other cases our protocol has allowed us to identify the most likely protonated N atom in charged inhibitors.

As seen from our results, the highest success rate is reproduced at a pH close to neutral. This outcome stands to reason and supports our pK_a assignment since the observed poses (obtained by X-ray crystallography) were obtained at this pH value. Nevertheless, most of the inhibitor binding assays for BACE-1 are performed at acidic pH values, where the enzyme has its optimal activity. Preliminary results indicate that at the enzyme's optimal pH our protocol is able to cluster the experimentally observed best binders at the top of the ranking, and the worst binders at the bottom. It would be desirable to perform the screening campaigns at this range of pH, since the candidate ranking position probably changes with this medium acidity. Presently, we are looking for modifications of the protocol that will allow for screening campaigns at the enzyme's optimal pH value. Our calculations on BACE-1 bound to fragments (presented here) indicate that at pH 4.5, the Asp dyad monoprotonated charge state (protonated at Asp 32) is the most prevalent. Nevertheless, as seen from Table 2 and our previous calculations on peptidomimetic inhibitors bound to BACE-1 [16], other options (e.g., diprotonated Asp dyad) are possible. Hence, an option aimed at enriching the docking results could be based

on a ensemble docking with a set of protein structures that contain our most commonly predicted Asp dyad protonation states and include the protonation state for the other acidic residues at pH 4.5. Ideally, the pose screening should be carried out with MM protocols that allow for the flexibility of the active site residues, since we believe it will enhance the success rate.

Finally, we have found a possible rationale for the poor docking performance of some ligands across the board irrespective of the docking protocol used. The results indicate that the worst 'behaved' compounds have few anchoring interactions with the active site residues than those that have high success rates, missing HB contacts with one or both residues of the Asp dyad. This result could be used in the screening campaigns to weed out fragments that do not attain a certain number of anchoring interactions and hence could produce false negatives poses.

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