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In silico multi-filter screening approaches for developing novel β-secretase inhibitors

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Abstract—A large database of chemical structures was screened for potential inhibitors of β-secretase was carried out using in silico multi-filter techniques. Substructure screening, computer-aided ligand docking, binding free energy calculations, and partial interaction energy analyses were performed successively to identify chemical compounds which could serve as different scaffolds from known β-secretase inhibitors for future drug design. We showed that our in silico multi-filter screening retrieved all known inhibitors from the compound database investigated, which suggests that the other compounds identified as inhibitors by this computerized screening process are potential β-secretase inhibitors.

Alzheimer's Disease (AD), the major symptom of which is dementia, is a neurodegenerative disease mainly affecting elderly people and is increasingly prevalent in a country which is rapidly progressing, especially as there is currently no effective drug therapy available. Neuritic plaque, also called senile plaque, which is the product of aggregation of β-amyloid peptides (A-β) is observed in the brains of the patients with AD and is considered to be one of the key factors in the etiology of the disease.[1–3] In the biological process generating A-β, β-secretase catalyzes the penultimate step in the plaque formation, cleaving these peptides from Amyloid Precursor Protein.[4] Accordingly, work aimed at identifying inhibitors of β-secretase has been developed rapidly because the inhibition of the generation of A-β may lead to the discovery of a potential therapeutic agent. Thus, we performed in silico multi-filter screening to discover inhibitors of novel structure which will support different structural scaffolds for future drug design than the already known inhibitors employing the procedures shown in Figure 1.

Substructure screening (First filter: 2D-query search): In this study, for the purpose of substructure screening, we first determined the major chemical substructures of ligands which are common between known inhibitors. This was performed by making a thorough investigation of the three-dimensional structures of β-secretase complexed with the known inhibitors from the Protein Data Bank (PDB ID: 1TQF, 1W51, 2B8L, 2B8V, and 2FDP). From this investigation using computer graphics, it was found that the hydroxyl groups of the inhibitors commonly formed hydrogen bonds with carboxylic group of Asp32 or Asp228 and benzene rings of all the inhibitors made hydrophobic interactions with methyl group of Thr72 and Thr231 and alkyl part of the side chain of Gln73 in β-secretase. We selected these two chemical groups as query substructures, and used UNITY-2D module of Sybyl7.3 (Tripos Inc.) to screen chemical compounds which containing these groups (hydroxyl group and benzene ring) from the database of Namiki Shoji Co. Ltd, which comprises approximately 3.5 million compounds. By applying this first filter, we found approximately 70,000 chemical compounds (first 'hits'; about 2% of the total compounds screened).

High throughput protein structure-based virtual screening (Second filter: 3D-coarse docking): The second filter was applied by screening the first 'hits' by high throughput protein structure-based virtual ligand docking. Our objective in this filter is to roughly exclude those compounds which were not expected to bind with β-secretase. In this filter, we used HTVS (High Throughput Virtual Screening) mode of GLIDE (*Schrödinger*

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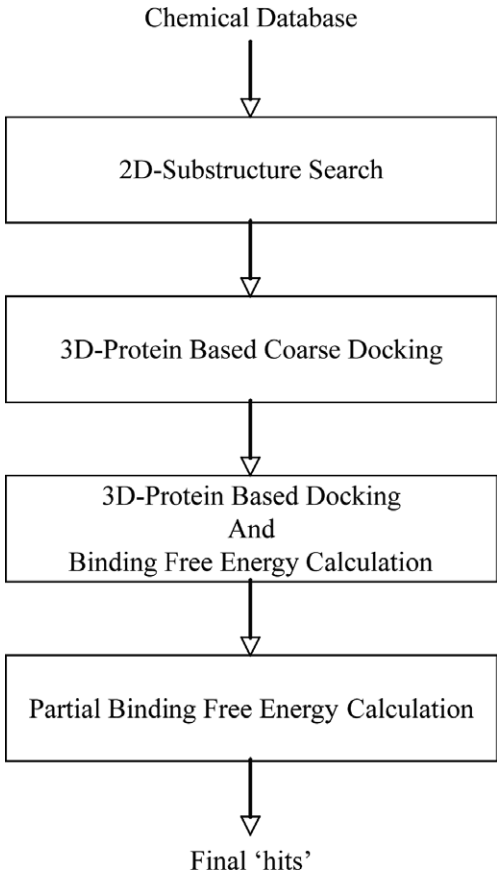

Figure 1. Flowchart of our in silico multi-filter screening approaches. A large chemical database was first screened by 2D-substructure search to obtain first 'hits'. The first 'hits' were docked into the binding site of β-secretase using High Throughput Virtual Screening (HTVS) mode of docking program GLIDE to retrieve second 'hits'. The second 'hits' were then docked using Standard Precision (SP) mode of GLIDE and their binding free energies were calculated using MM/PBSA method to select third 'hits'. Final 'hits' were determined by setting a limiting condition that partial binding free energies of the third 'hits' to Asp32 and Asp228 in the binding site of β-secretase are greater than 4 kcal/mol.

L.L.C.)[5] as a tool for virtual ligand docking. We used several conformations of β-secretase to allow for protein flexibility. To select conformations of β-secretase used for GLIDE-HTVS docking, protein structures registered in PDB were classified by root mean square deviation of active site conformations defined as amino acids within 5 Å from the ligands. The first protein structure we selected was the structure of PDB ID:2B8L[6] because the resolution of this was higher than the other crystals. The remaining protein structures were then compared with active site conformation of structure of PDB ID:2B8L. Crystal structures of which root mean square deviation was closer than 1 Å were classified into the group of PDB ID:2B8L and rejected. Subsequently, those structures with highest resolution remaining were selected. This process was reiterated until all structures were either selected or classified. We finally selected four structures (PDB ID: 1TQF,[7] 1W51,[8] 2B8L, and 2G94[9]). Missing atoms and residues of the selected four structures were added by protein structure prediction program PRIME (*Schrödinger L.L.C.*).[10] Grids used for the ligand docking were generated within 20 Å from the center of active site of these fixed structures. In order to determine the criteria score of GLIDE-HTVS-Score for this filter, we first docked ligands the binding modes of which are already known (PDB ID: 1TQF, 1W51, 2B8L, 2B8V, and 2FDP). As the differences between the lowest score and second and third from the lowest were both approximately 0.15, we set the criteria of GLIDE-HTVS-Score as −8.6, which is 0.15 lower than the lowest score we observed.

The ligand first 'hits' were then docked into the binding site of β-secretase. Figure 2 illustrates several ligands (capped sticks) docked into the binding site of β-secretase (represented by Connolly surfaces) using the HTVS mode of GLIDE. The docked ligands were ranked by GLIDE-HTVS-Score and the compounds with GLIDE-HTVS-Score lower than −8.6 were removed (second 'hits'). Concerning the evaluation of isomers here, we selected all the isomers as 'hits', in case at least one of the isomers had higher score than −8.6. In this process, we defined about 230 chemical compounds as second 'hits' (comprising 0.33% of the first 'hits').

Binding mode prediction and binding free energy calculation (Third filter: 3D standard docking): In the binding mode prediction, we used Standard Precision mode of GLIDE and MM/PBSA methods[11] to predict the binding mode of the compounds identified as second 'hits'. We used several ligands the binding modes of which were known (PDB ID: 1TQF, 1W51, 2B8L, 2B8V, and 2FDP), and the same four protein structures as GLIDE-HTVS for the purpose of establishing GLIDE-SP docking parameters. We also generated five

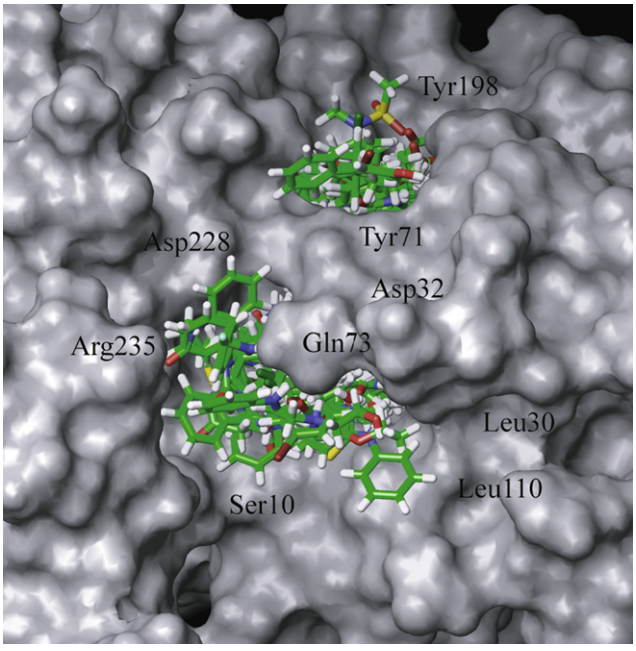

Figure 2. Illustration of several ligands (capped stick) docked into the binding site of β-secretase (Connolly surface) using HTVS mode of GLIDE. The docking poses of these ligands were evaluated and identified by GLIDE-HTVS-Score. The residues shown in this figure are some of binding site residues of β-secretase.

initial conformations of the ligands by using Conformational-Search from Macromodel (*Schrödinger L.L.C.*) because such protein structure-based ligand docking depends on the initial conformation. From the docking results obtained with each protein, conformations of the ligands were then merged, and docked poses of GLIDE-SP-Score ranking from 1 to 50 were selected as candidates of binding mode followed by minimization in vacuum to refine the ligand and protein conformation by AMBER8.[12] In the binding free energy calculation, program sender from AMBER8, DelPhi,[13] and MSMS[14] was used to calculate internal energy of the system, electric contribution to solvation and nonpolar contribution to solvation, respectively. Charges used for compounds here were determined using ab initio HF/6-31G* basis set and RESP (restrained electrostatic potential) fitting. We selected the most stable docked pose as the predicted binding pose, and the results of RMSD from the X-ray binding modes are shown in Table 1. RMSDs were calculated with all the heavy atoms. This result demonstrates that this approach successfully regenerated and selected the close binding mode of each of the ligands.

Table 1. Root mean square deviations (RMSDs) between the geometry of ligands docked by GLIDE and that in the protein–ligand complex determined by the X-ray crystallography and calculated binding free energies by MM-PBSA method

PDBID	RMSD (Å)	Binding free energy (kcal/mol)	IC_{50} (nM)
1TQF	0.948	−61.53	1400
1W51	0.692	−62.39	200
2B8L	0.278	−72.04	15
2B8V	0.583	−62.80	98
2FDP	0.508	−59.08	26 (K_i)

IC_{50} values for the ligands are also shown in the table.

Seven additional inhibitors taken from the literature[15] the binding modes of which are not known were docked into the binding site of β-secretase. Then the binding free energies were calculated by the MM/PBSA method in order to determine the criteria of binding free energy for the hit selection. As the lowest binding free energy we observed here was −45.19 kcal/mol, we determined the criteria of binding free energy to be higher than −45.00 kcal/mol. Binding modes and affinities of second 'hits' were then calculated. All the isomers were calculated individually in this filter. We found 45 chemical compounds the calculated binding energy of which was higher than the criteria determined. We classified these compounds as third 'hits', which comprised approximately 20% of second 'hits'.

Partial interaction energy analysis (Fourth filter: binding hot spot analysis): To determine the most important areas on the ligand binding to β-secretase (hot spots), we performed computational alanine scanning[16] to the crystal structures of PDB ID: 1TQF, 1W51, 2B8L, 2B8V, and 2FDP. The amino acids in the active site we defined in the second process were replaced by alanine, respectively, and only hydrogens of the substituted alanine were refined before the analysis. The MM/PBSA method was used to estimate the binding free energies of each alanine-substituted protein and the differences of binding free energies between alanine-substituted proteins and the wild-type protein were considered as partial interaction energies of each scanned residue. As shown in Figure 3, the results of this calculation indicated that partial interaction energies between ligands and Asp32 and Asp228, the catalytic cores of β-secretase, were especially strong. This suggests that high partial interaction energies with these two catalytic cores were more important for ligand binding than the other residues in active site. Both partial interaction energies were stronger than 4 kcal/mol therefore we defined

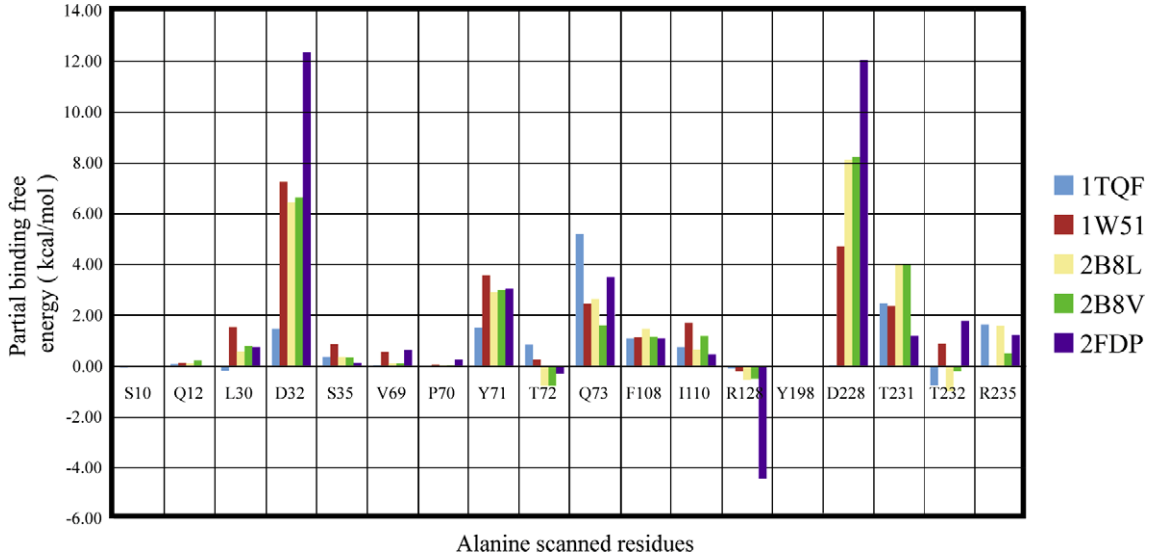

Figure 3. The interaction energies of amino acids in the active site with inhibitors the binding modes of which are known by the X-ray crystallography. The contribution of each amino acid to the binding free energy was calculated using the MM/PBSA method computing the difference of binding free energy between the wild-type protein and the alanine-mutated protein.

fourth threshold of screening by interaction energy contribution with Asp32 and Asp228 to be stronger than 4 kcal/mol, respectively. The interaction contributions with Asp32 and Asp228 of third 'hits' were then calculated and the results are shown in Table 2.

Table 2. The calculated binding free energies of third 'hit' compounds to β-secretase and contributions of two aspartic acids to the binding free energies

Compound	Chirality	Binding free energy (kcal/mol)	Contribution of Asp32 (kcal/mol)	Contribution of Asp228 (kcal/mol)
A	(*R*)	−56.45	1.22	1.23
	(*S*)	−59.36	0.87	0.05
B	(*R*)	−55.39	8.48	11.59
	(*S*)	−52.90	2.79	4.14
C	(*R*, *R*)	−55.43	2.43	2.84
	(*R*, *S*)	−55.93	8.31	5.73
	(*S*, *R*)	−55.27	3.30	4.05
	(*S*, *S*)	−56.16	0.90	2.17
D	No chiral	−51.24	−3.82	−2.37
E	(*R*, *R*)	−49.11	−0.33	−0.13
	(*R*, *S*), (*S*, *R*)	−49.83	−0.68	−0.78
	(*S*, *S*)	−48.30	−0.09	0.08
F†	(*R*, *R*)	−47.43	12.57	9.43
	(*R*, *S*)	−48.02	9.30	6.39
	(*S*, *R*)	−47.78	11.13	5.07
	(*S*, *S*)	−47.44	10.49	6.16
G	(*R*, *R*)	−49.34	0.83	−0.60
	(*R*, *S*), (*S*, *R*)	−44.72	−0.33	−4.13
	(*S*, *S*)	−35.35	−3.88	−0.61
H†	(*R*, *R*)	−46.25	12.01	9.81
	(*R*, *S*), (*S*, *R*)	−48.19	6.83	8.56
	(*S*, *S*)	−47.80	8.45	5.64
I	(*R*, *R*)	−47.25	8.28	5.29
	(*R*, *S*)	−46.48	8.53	6.60
	(*S*, *R*)	−46.06	9.66	6.19
	(*S*, *S*)	−45.61	1.81	1.76
J	(*R*, *R*)	−44.73	0.41	0.43
	(*R*, *S*)	−43.89	0.63	0.60
	(*S*, *R*)	−46.18	−0.28	−1.12
	(*S*, *S*)	−45.45	−1.09	−1.19
K	(*R*)	−43.79	−0.16	0.50
	(*S*)	−45.78	0.98	0.86
L	(*R*, *R*)	−46.81	1.84	1.73
	(*R*, *S*)	−45.32	1.23	1.80
	(*S*, *R*)	−39.39	0.93	4.49
	(*S*, *S*)	−45.97	−0.10	2.95
M	(*R*)	−45.08	0.71	0.78
	(*S*)	−44.69	−0.82	−0.58
N	(*R*, *R*)	−45.27	0.37	−0.08
	(*R*, *S*)	−41.23	−2.15	−2.04
	(*S*, *R*)	−42.78	−4.20	−0.99
	(*S*, *S*)	−39.06	−4.92	0.12

Compounds with dagger, *F* and *H*, were already approved as β-secretase inhibitors in the international patents. Energies greater than 4.0 kcal/mol are shaded which are threshold to select the final hits.

We determined five chemical compounds as final 'hits' the binding affinities of which with Asp32 and Asp228 were stronger than threshold (shading columns in Table 2); compounds *B*, *C*, *F*, *H*, and *I* (approximately 11% of the previous screen). Two compounds of the 5 final 'hits' we selected, compounds *F* and *H*, were already approved in international patent as inhibitors of β-secretase, the IC_{50}s of which were 5 and 7 μM, respectively (the isomeric states of two chiral carbons were not shown in patent).[17] This suggests that our in silico multi-filter screening approaches are effective for developing β-secretase inhibitors. The chemical formulas of these two compounds are shown in Figure 4a and b. The binding mode of compound *H*-(*S*, *R*) which had the strongest binding free energy is shown in Figure 5. It is suggested that the hydroxyl group and protonated nitrogen of compound *H*-(*S*, *R*) can make hydrogen bonds with Asp228 (right half of orange region in Fig. 5) and an ionic bond with Asp32 (left half of orange region in Fig. 5). These hydrogen bonds are common in the known inhibitors. The compound *H*-(*S*, *R*) also makes hydrogen bonds with main-chain of Lys107 and Gly230, and forms an ionic bond with side-chain of Asp32 and makes hydrophobic interactions with Tyr71, Ile110, Ile118, Tyr198, and Ile226. As compounds *B*, *C*, and *I* have similar energy profiles, these three compounds are expected to inhibit the activity of β-secretase. The compound *B* interacts with S1, S2, S3, and S1′ subsites of β-secretase (the hydrophobic interaction with Phe108, Ile110, Trp115, Thr231, and the hydrophilic interaction with Gln73 and Asp228), the compound *C* interacts with

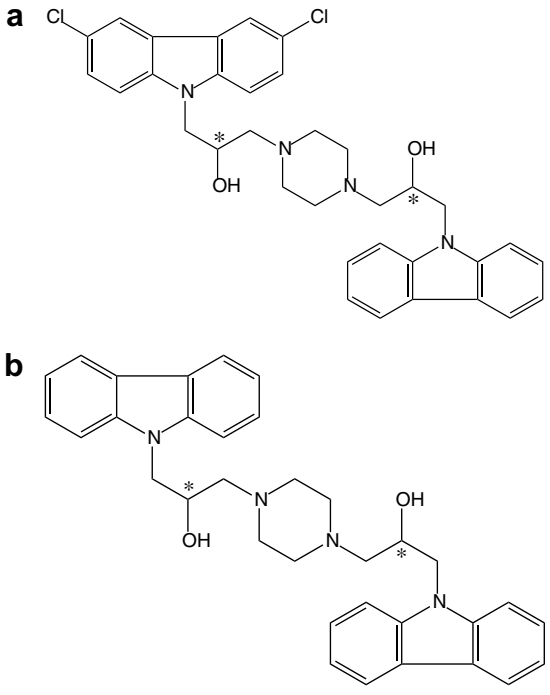

Figure 4. (a) The chemical formula of final 'hit' compound *F* the inhibitory activity of which is known to be IC_{50} = 5 μM. Although the inhibition activities of isomers were not indicated in the reference, we predicted isomer (*R*, *S*) to be the strongest. (b) The chemical formula of final 'hit' compound *H* the inhibitory activity of which is known to be IC_{50} = 7 μM. We predicted isomer (*S*, *R*) to be the strongest.

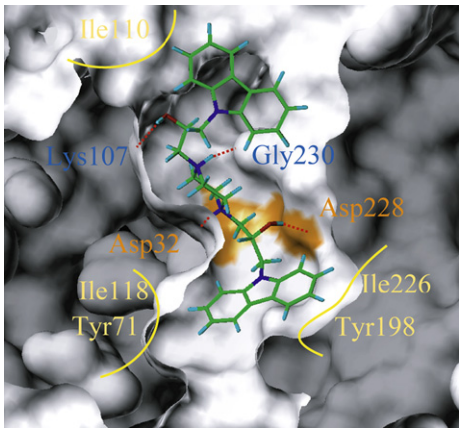

Figure 5. Predicted binding mode of known inhibitor *H*-(*S*, *R*) which is calculated to have the largest binding free energy to β-secretase among the isomers. Active site residues of β-secretase are shown in orange letter, hydrogen-bonded residues are shown in blue, residues involving in hydrophobic contacts are shown in yellow letters. Hydrogen bonds are shown in red dotted lines, and hydrophobic contacts are shown in yellow lines.

S1, S2, S3, S1′, S2′, and S3′ subsites of β-secretase (the hydrophobic interaction with Leu30, Tyr71, Gln73, Ile110, Tyr198, and the hydrophilic interaction with Asp228) and the compound *I* interacts with S1, S2, S3, S1′, S2′, and S3′ subsites of β-secretase (the hydrophobic interaction with Leu30, Try71, Thr72, Gln73, Phe108, Ile118, Tyr198, and the hydrophilic interaction with Asp228 and Thr231). As compounds *B*, *C*, and *I* are now being pharmacologically characterized by a pharmaceutical company, the chemical formulas will be shown after those are approved in international patent.

Our in silico multi-filter screening approach illustrated in Figure 1 finally defined five compounds as 'hits': of which two were the known inhibitors of β-secretase which were already approved in international patent. Finally, we also searched for inhibitors the chemical formulas of which are known in the chemical database of Namiki Shoji, and any known inhibitors but compounds *F* and *H* were not found there. This implies that we successfully retrieved all the known inhibitors from the database (Namiki Shoji) and furthermore, that we also identified three compounds that are expected to allow the identification of novel β-secretase inhibitor templates for future drug discovery.

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