Discovery of Cell-Permeable Non-Peptide Inhibitors of β-Secretase by High-Throughput Docking and Continuum Electrostatics Calculations[#]

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Abstract: A fragment-based docking procedure followed by substructure search were used to identify active-site β-secretase inhibitors from a composite set of about 300 000 and a library of nearly 180 000 small molecules, respectively. EC_{50} values less than 10 μM were measured in at least one of two different mammalian cell-based assays for 12 of the 72 purchased compounds. In particular, the phenylureathiadiazole **2** and the diphenylurea derivative **3** are promising lead compounds for β-secretase inhibition.

Alzheimer's disease is the most common neurodegenerative disease and accounts for the majority of the dementia diagnosed after the age of 60.[1] Amyloid plaques, which are found in the post-mortem brain of Alzheimer's disease patients,[2] consist mainly of fibrillar aggregates of the Aβ peptide, a proteolytic cleavage product of the β-amyloid precursor protein (APP). Two enzymes, γ- and β-secretase (β-site APP cleaving enzyme, or BACE-1), are responsible for the sequential processing of APP.[3] Although it is not clear whether the plaques or oligomeric prefibrillar species are responsible for neuronal loss and dementia,[4] the pepsin-like aspartic protease BACE-1 has become one of the major Alzheimer's disease targets.[1,5] BACE-1 is a very difficult target as is witnessed by the very small number of known nonpeptidic inhibitors.[1,5−7] Moreover, not a single BACE-1 inhibitor was found in a library containing more than 1800 renin inhibitors,[8] despite the fact that both BACE-1 and renin are pepsin-like enzymes. In addition, a single molecule (1,3,5-trisubstituted benzene) emerged as a BACE-1 inhibitor from a multimillion compound library submitted to a high-throughput screening campaign.[9]

Here, we report the identification of a dozen BACE-1 inhibitors with a common phenylurea scaffold by our in silico screening approach that consists of four steps (details of the methods are in Supporting Information). First, each molecule is automatically decomposed into rigid fragments by the program DAIM (decomposition and identification of molecules; P. Kolb and A. Caflisch, manuscript in preparation). In a second step the fragments are docked into the rigid binding site by the program SEED,[10,11] which approximates solvation effects by continuum electrostatics.[12] As an improvement with respect to previous versions of SEED,[10,11,13] the screened electrostatic interaction and fragment desolvation energy were evaluated using an empirical correction of the Coulomb field approximation, i.e., eq 8 of ref 14. In the third step the optimal SEED binding modes of the fragments are then used as binding site descriptors to guide the placement of the flexible molecules by the docking program FFLD (fragment-based flexible ligand docking), which is based on a genetic algorithm.[13,15] The most favorable FFLD binding modes are further minimized in the rigid protein using the CHARMM program.[16]

The final step of our approach is the evaluation of the binding free energy with solvation effects,[17] which is an essential element of the in silico screening procedure. Computer-aided approaches for docking libraries of small molecules into proteins of known structure require fast and accurate methods for the evaluation of binding free energies.[18−22] Rigorous approaches to evaluate relative binding affinities such as free energy perturbation and thermodynamic integration have sampling and convergence problems that prevent them from being used routinely.[23] Moreover, it is very difficult to handle large 2D structural diversity between ligands, e.g., in the case of completely different core structures.[18] Several semiempirical methods based on linear approximations to the free energy have been introduced and used with success.[22] A decade ago Åqvist and co-workers proposed the LIE (linear interaction energy) method to calculate free energies of binding by averaging interaction energies from molecular dynamics (MD) simulations of the ligand and the ligand/protein complex.[24,25] To improve efficiency, which is essential for evaluating large libraries of compounds, we have replaced the MD sampling with a simple energy minimization and combined the LIE method with a rigorous treatment of continuum electrostatics, i.e., numerical solution of the Poisson equation by the finite-difference technique.[26] The modified LIE approach, termed LIECE where the last two letters stand for continuum electrostatics, was shown to have an accuracy in the binding energy prediction of about 1 kcal/mol for a set of 13 and 29 peptidic inhibitors of BACE-1 and HIV-1 aspartic protease, respectively.[17] It was also shown that a LIECE model parametrized on HIV-1 aspartic protease is not transferable to BACE-1 and vice versa.[17] Hence, in general the LIECE approach cannot be used in virtual screening against a target for which no inhibitor is known. On the other hand, a recent application to three different kinases indicates transferability of the LIECE parameters (Huang, Kolb, and Caflisch, unpublished results).

Initially, about 300 000 molecules with at least one hydroxyl group were selected from a collection of chemical libraries containing about six million compounds. The in silico screening of these 300 000 molecules, i.e., docking and LIECE energy evaluation, took about 10 days on a Beowulf cluster of 100 1.8-GHz Opteron

[#] This paper is dedicated to Martin Karplus on the occasion of his 75th birthday.

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Table 1

Compounds	Structures	MW (g mol^{-1})	BACE-1[a] IC_{50} (μM)	Abeta(sw)[b] EC_{50} (μM)	SEAP[c] EC_{50} (μM)	Cytotoxic[d] CC_{50} (μM)	LIECE[e] K_i (μM)
1		561.5	57.8 ± 10.3	3.0 ± 0.6	3.5 ± 0.7	12.5	8.1
2		322.4	>25 [f]	2.6 ± 0.9	11.4 ± 0.9	22.3	28.8
3		418.9	97.0 ± 21.4	2.6 ± 1.1	23.3 ± 8.1 [g]	11.1	9.8
4		506.5	283.9 ± 36.8	3.2 ± 0.2	27.0 ± 9.5 [g]	24.6	9.4

[a] The BACE-1 fluorescence resonance energy transfer assay kit was purchased from PanVera (Madison, WI; no. P2985). BACE-1 activity assays were carried out according to the manufacturer's instructions. Average value and standard deviation are from three independent experiments. [b] Cell-based assay.[28] Average value and standard deviation are from three independent experiments. [c] Cell-based assay.[29] Average value and standard deviation are from three independent experiments. [d] Cytotoxic concentration.[33] [e] See ref 17. [f] Interference at concentrations higher than 25 μM. [g] Percentage inhibition at 3 μM.

CPUs. The rigid conformation of BACE-1 from its complex with the OM00-3 inhibitor (PDB code 1m4h [27]) was used for the docking. Interestingly, only 10 compounds had a LIECE-predicted affinity in the high-nanomolar range and most of them were phenylurea derivatives with the two NH groups involved in hydrogen bonds with one of the two catalytic aspartates. Unfortunately, these 10 compounds were no longer available from the original vendor. Therefore, we decided to select from the six million molecule collection all of the nearly 32 000 compounds with a phenylurea moiety, i.e., those with (only 1233 molecules) and without a hydroxyl group. These nearly 32 000 compounds were docked; the poses with the most favorable FFLD energy were further minimized by CHARMM,[16] and the energetically most favorable 50 000 poses (8558 different molecules) were evaluated by the LIECE approach.[17] The LIECE binding energy evaluation was performed in two steps using first a grid spacing of 1.0 Å in the finite-difference Poisson calculation followed by a more accurate calculation with a grid spacing of 0.3 Å for the best 2000 poses. The two-step LIECE procedure required about 20 h on the Beowulf cluster of 100 CPUs. Upon visual inspection of the top 200 poses (131 different molecules), 10 compounds were purchased and tested in an enzymatic assay with purified BACE-1 and in two cell-based assays. We first tested the cellular activity of the selected compounds by measuring Aβ peptide secretion.[28] To confirm BACE-1 inhibition in an additional mammalian cellular assay we established the so-called SEAP (secreted alkaline phosphatase) system. For this system, HEK 293 cells were transfected with a SEAP-APP fusion protein bearing the SEAP enzyme moiety localized in the topologically extracellular space, such as ER/Golgi lumen and endosomes, or also at the cell surface.[29] This protein is anchored to cellular membranes via a portion of APP harboring the Swedish mutation at the β-site and the K612V mutation at the α-site. Endogenous β-secretase activity causes liberation and subsequent secretion of the SEAP enzyme, whose activity in the supernatant is measured via a chemiluminescent read-out. In this way, the diphenylurea derivative **1** (Table 1) was identified as a low-micromolar inhibitor of BACE-1. Two of the remaining nine compounds showed low-micromolar activity in at least one of the two cell-based assays and the enzymatic assay (data not shown).

An essentially identical screening approach based on FFLD docking and LIECE postprocessing was applied to the 2476 compounds in a protease-focused chemical library. Intriguingly, seven among the 20 compounds with the most favorable LIECE-predicted affinity had a phenylurea scaffold. These 20 compounds were purchased and tested. The phenylurea derivative **2** showed low-micromolar activity in two different mammalian cell-based assays (Table 1). One of the remaining 19 compounds showed low-micromolar activity in both cell-based assays and an IC_{50} of 490 μM in the enzymatic assay (data not shown).

In a third in silico screening, 391 compounds from a libary of about 180 000 small molecules were first selected by similarity search using the phenylurea scaffold. After the FFLD docking and LIECE postprocessing, 42 compounds were purchased and tested. At 10 μM, 38 of the 42 compounds showed more than 20% inhibition in at least one of the two cell-based assays. Moreover, 10 of them have EC_{50} < 10 μM in the Abeta(sw) assay. The two most potent BACE-1 inhibitors obtained by the similarity search and docking approach (**3** and **4**) are shown in Table 1. Despite its smaller size, **3** is as active as **4** in the two cell-based assays and a factor of about 3 more active in the enzymatic test.

It is interesting to compare **2** with the known non-peptidic inhibitors of BACE-1 which, as mentioned above, are rare.[1,5] A series of hydroxyethylamine deriva-

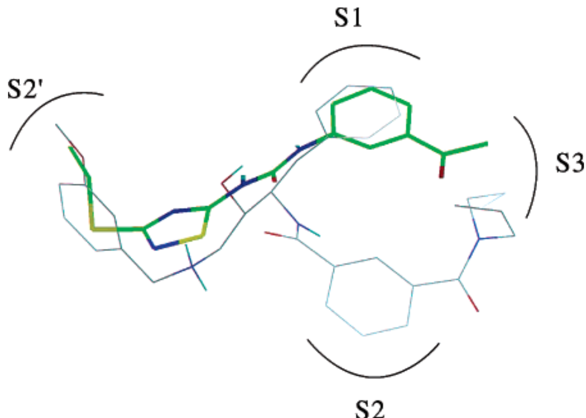
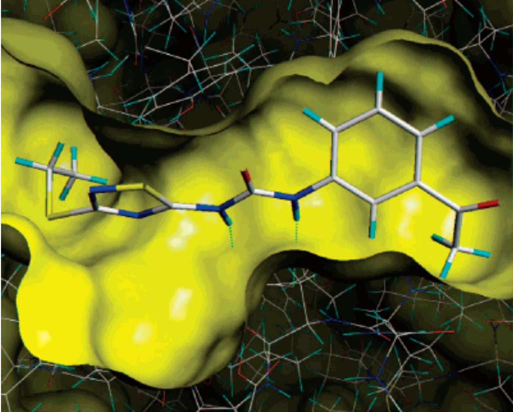


Figure 1. Superposition of a known nanomolar inhibitor of BACE-1[31] (thin lines and carbon atoms in gray) and **2** (thick lines with carbon atoms in green) in one of its two possible orientations obtained by docking in the flexible binding site. The C_{α} atoms of BACE-1 were used for the structural alignment.

tives of an isophthalamide scaffold have been shown to have nanomolar affinity by enzymatic[30−32] and cell-based assays.[32] The crystal structures of two of these inhibitors in complex with BACE-1 show that they have a very similar binding mode despite the different stereochemistry at the hydroxyl group.[31,32] In the catalytic site, the hydroxyl functionality and the protonated secondary amino group are involved in hydrogen bonds with the side chain of the catalytic Asp32 and Asp228, respectively. Moreover, the benzyl functionality close to the hydroxyl group of the two inhibitors occupies the S1 pocket in both complexes. The molecular weight of the hydroxyethylamine compounds (MW = 531 g mol^{-1} (ref 30) and MW = 579 g mol^{-1} for **3** (ref 32)) is larger than the one of compound **2** reported here (MW = 322 g mol^{-1}). Furthermore, the binding mode is different except for the phenyl group of the inhibitor **2** which occupies the S1 pocket (Figure 1) and overlaps with the corresponding ring of the benzyl functionality of the hydroxyethylamine inhibitors. Because of the small size and rather symmetric overall shape of **2**, we decided to perform minimization in the flexible binding site (library docking had been performed in the rigid protein) starting from the two end-to-end flipped orientations obtained by the FFLD docking. An alternative binding mode of inhibitor **2** is observed upon minimization in the flexible binding site with protonated Asp32 (instead of Asp228, which was protonated in all other calculations). In the alternative binding mode, the two NH groups of the urea scaffold are involved in hydrogen bonds with Asp228 (instead of Asp32), but the overall orientation is flipped end-to-end such that the ethylthioether functionality and the phenyl group occupy the S1 and S1′ pockets, respectively (Figure 2). It is not possible to apply LIECE to evaluate the two different binding modes because the LIECE approach requires a single protein conformation as reference state. Hence, the CHARMM in vacuo interaction energy supplemented by the finite-difference Poisson solvation was calculated for both binding modes, but the preferred orientation cannot be determined because the energy difference of 2.4 kcal/mol is within the limited accuracy of the estimation due to the flexible protein treatment. It is important to note that **2** has only two rotatable



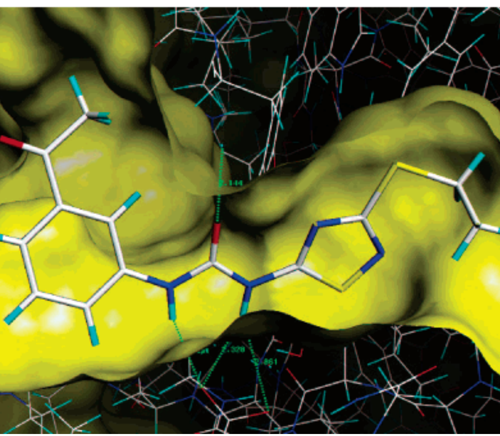


Figure 2. Two possible binding modes of **2** in the BACE-1 active site. Hydrogen bonds are shown by green dotted lines. The binding mode in the top picture corresponds to the one of Figure 1.

bonds. Its limited flexibility and the marginal loss of entropy upon binding are consistent with its rather high binding affinity given the small size.

In conclusion, high-throughput docking into the BACE-1 active site and continuum electrostatics calculations were used to select for experimental testing 72 compounds from an initial set of about 500 000. Fifty-nine of these 72 compounds are phenylurea derivatives. Twelve of the 72 compounds inhibit BACE-1 in at least one of two different mammalian cell-based assays at concentration values less than 10 μM. It is important to note that for almost all of the 12 compounds, for which an EC_{50} value could be measured, the discrepancies between LIECE-predicted affinity and the experimental value is within the LIECE accuracy of about 1 kcal/mol.[17] Given their very small size, the phenylureathiadiazole **2** (MW = 322 g mol^{-1}) and diphenylurea derivative **3** (MW = 419 g mol^{-1}) may serve as starting points for further optimization to evaluate their therapeutic potential for Alzheimer's disease.

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Supporting Information Available: Details on computation approach and experimental tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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