β-secretase-1 inhibition by novel Verubecestat-derived Bacehibitol

Submitted to BIOCHEM 3381A, December 8, 2021

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Alzheimer's disease is a prevalent progressive brain disorder, characterized by loss of memory and cognitive abilities. Believed to be caused by the aggregation of β-amyloid (Aβ) plaques after amyloid precursor protein (APP) cleavage by beta secretase-1 (BACE1) in the endosome, a novel inhibitor was sought to be designed. With the scaffold of Verubecestat, a Merck BACE1 inhibitor that did not progress through clinical trials, the novel inhibitor was aimed to decrease Verubecestat's side effects by specifically directing to the BACE1 and APP pathway by adding a sterol group, which would target the novel inhibitor to the endosome. MCULE generated structurally similar compounds to Verubecestat, which were named VSMs for "Verubecestat-similar molecules," upon which a sterol group was added in regions with the least steric hindrance to improve affinity to the PLIP-determined binding site. Considering the Lipinski properties of each sterol-linked novel compound, only one violated less than two of Lipinski's rule of 5 and was named "Bacehibitol." Bacehibitol was then further analyzed for physicochemical, pharmacokinetic, and pharmacodynamic properties on SwissADME, demonstrating both poor gastrointestinal absorption and poor blood brain barrier penetration. Bacehibitol was taken to SwissTargetPrediction to search for probable macromolecular interactions with *Homo sapiens* targets and was found to have low probability of being bioactive with off-target proteins. Future studies may use Bacehibitol as a blueprint to improve interactions with key BACE1 residues in the flexible "flap" domain, as well as optimize its properties such as the molecular mass, the sterol group, and solubility. Further directions include future *in vitro* studies for the *in silico* design of a successful BACE1 inhibitor.

Introduction

Alzheimer's disease (AD) the most common type of dementia (70%), characterized by loss of memory and cognitive abilities. The disease's biomarkers include neurofibrillary tangles and amyloid plaques, which cause neuronal loss and gliosis in the cerebral cortex (1). Neurofibrillary tangles are made of a collection of abnormal cellular components, consisting mostly of an abnormally phosphorylated Tau protein. Amyloid plaques result from the abnormal cleavage of the amyloid precursor protein (APP) by gamma-secretase and beta-secretase 1 (BACE1). Normal cleavage results in small amounts of Beta-amyloid in healthy individuals (2). This sequential cleavage is important in producing APP and can be used as a possible target for drugs and inhibitors.

BACE1 cleavage is the rate-limiting step in the process of generating Aβ. As a result, BACE1 can be considered a worthy target for novel therapeutics. This protease cuts specific proteins that are usually related to neuronal function and is usually found in the plasma membrane, endoplasmic reticulum, and the Golgi apparatus. Previous research has shown that BACE1 performs different functions depending on its location in the cell, with APP cleavage happening in the early endosome (3). The enzyme mainly has two functions. First, the processing of Neuregulin-1 (NRG1), a protein responsible for a cascade of events that causes the myelination of neurons (4). And second, the processing of APP in Alzheimer's disease patients. The result of this cleavage causes the formation of secreted APP beta (sAPPβ) and a 99-amino acid long C-terminal fragment that is bound to the membrane called CTF99 (5). This is followed by the cleavage of the CTF99 fragment to an AICD fragment and an Amyloid beta fragment (6). The latter fragment binds to ApoE in order to generate amyloid plaques. This binding also causes the generation of reactive oxygen species (ROS) and interactions with different other proteins, causing blocked ion channels, distribution of Ca+ ion channels, mitochondrial oxidative stress, and neuronal loss.

Many small-molecule inhibitor candidates have been introduced in clinical trials which target BACE1; however, there have been very high failure rates (7). Verubecestat (developed by Merck) was one of the inhibitors with the highest potential. It showed a low LC50 and a high selectivity for BACE1 compared to other inhibitors (7). It also has increased blood brain barrier (BBB) permeability and bioavailability, as well as decreased efflux by P-glycoprotein in cells compared to other inhibitors. However, the inhibitor was stopped in clinical trials due to many adverse side effects and the lack of efficacy (7). It was proposed that the drug had inhibitory effects on other important proteins which affected their function and thus showed adverse side effects (8). Due to

the many functions of BACE1, blocking the cleavage of non-amyloid substrates decreases a drug's value as an AD therapeutic.

To this end, our goal is to target BACE1's endocytic pathway exclusively with improvements to Verubecestat without affecting other proteins. This can potentially be achieved by designing a novel Verubecestat similar molecule (VSM) with a sterol link bound to it. In one study, a BACE1 inhibitor, C3, was administered in two forms (sterol link vs no sterol link). In both treatments, cleavage of APP was not affected whereas the cleavage of NRG1 in cells was only inhibited when cells were subject to the C3 pan inhibitor. The C3 inhibitor, when linked to a sterol, appeared to cause a significant decrease in NRG1 cleavage (3). Making our novel VSM sterol-linked can thus potentially target it selectively to the BACE1 endocytic pathway (3). Therefore, the improvements to Verubecestat can bind to BACE1 with high affinity to stop the interaction between BACE1 and APP, without affecting other substrates in the BACE1 pathway.

Methods

Identification of Novel Verubecestat Similar Molecules (VSMs)

Merck's drug, Verubecestat, was loaded in to the "*1-Click Scaffold Hop*" virtual screen software on MCULE to identify novel small molecule compounds with structural similarity to Verubecestat. Verubecestat was loaded into MCULE under the IUPAC name, *N*-[3-[(5*R*)-3-amino-2,5-dimethyl-1,1-dioxo-6*H*-1,2,4-thiadiazin-5-yl]-4-fluorophenyl]-5-fluoropyridine-2-carboxamide, and was analyzed and compared against MCULE's diverse subset of 10,000 purchasable compounds using the default advanced options. Compound similarity was determined by an FTree similarity score that analyzed Verubecestat based on pharmacophore properties and molecular topology. Compounds with an FTree similarity score of 0.85 were kept and labelled Verubecestat Similar Molecules (VSM).

Identification of BACE1 Binding Pockets

Identification of reliable binding surfaces for VSMs to bind to BACE1 (PDB 1TQF) was determined by CavityPlus. A geometrical and chemical property method was employed by CavityPlus to identify ligand binding sites within BACE1 based on its three-dimensional structure. Binding associations, drug scores, and druggability were estimated for each identified binding site using CavityPlus. Associated PDB files of each potential binding site were collected and were referenced when docking potential inhibitors to BACE1.

MCULE Docking of Identified VSMs to BACE1

The identified VSMs from the MCULE "*1-Click Scaffold Hop"* were then docked to BACE1 (PDB 1TQF) using the "*1-Click Docking*" software on MCULE. No advanced options were changed. A predicted docking score (kcal/mol) was obtained for each VSM. The poses with the best score (lowest ΔG) for each VSM were collected and viewed in PyMOL. The binding site of each VSM docked with BACE1 was then compared to the binding surface PDB file generated by CavityPlus. The comparison of each VSM docked with BACE1 was done visually on PyMOL and was used to verify that each VSM docked in the binding pocket with highest potential for druggability (highest drug score, predicted average and max pK_d).

Addition of Sterol Groups to Identified VSMs

The pose PDB files collected from docking each VSM with BACE1 were viewed in PyMOL with the surface of the protein shown. Sterics of each binding pocket were observed with each VSM docked in BACE1. The visualization of each binding pocket allowed for the identification of locations where sterol groups could be linked. Multiple iterations of adding a sterol group (2,3,4,5,6,7,8,9,10,11,12,13,14,15,16,17-hexadecahydro-1H-cyclopenta[a]phenanthren-3-ol) to each identified location on each VSM were conducted to produce potential sterol-linked VSM BACE1 inhibitors. MCULE molecule drawer tool was used to add sterol groups to the VSM scaffolds.

Chemical Properties of Sterol-Linked VSMs

The VSMs with sterol linkages were analyzed using MCULE to determine their physicochemical property profiles. The potential sterol-linked VSM BACE1 inhibitors were analyzed to determine their molecular mass, number of hydrogen bond acceptors, hydrogen bond donors, and octanol-water partition coefficient.

MCULE Docking of Potential VSMs with Sterol Groups to BACE1

The sterol-linked VSM BACE1 inhibitors that had no more than one violation of Lipinski's rule of 5 were docked with BACE1 (PDB 1TQF) using MCULE under the same default conditions as described in previous MCULE protocols. The docking poses of the lowest docking scores were collected and visualized using PyMOL.

Docking Interactions with BACE1 Determined by PLIP

Specific interactions between sterol-linked VSM BACE1 inhibitors and BACE1 were identified using Protein-Ligand Interaction Profiler (PLIP). Docking pose of the lowest binding score was uploaded to PLIP and analysis was run under default conditions. Resulting identified interactions were collected and represented using PyMOL.

Pharmacokinetic, and Pharmacodynamic Properties of VSMs

Sterol-linked VSMs that successfully docked with BACE1 (PDB 1TQF) were analyzed with SwissADME to determine physicochemical, pharmacokinetic, and pharmacodynamic properties, as well as drug-like nature and medicinal chemistry friendliness. Each sterol-linked VSM was drawn into the molecular drawer and analysis was run under default conditions.

Prediction of Off-Target Interactions

Sterol-linked VSMs analyzed by SwssADME were sent to SwissTargetPrediction. SwissTargetPrediction ran a high frequency virtual screen to identify the most probable

macromolecular targets within the *Homo sapiens* species. Sterol-linked VSMs analyzed by SwissTargetPrediction were compared with other molecules with two- and three-dimensional similarity in a library of 370,000 active sites of proteins found within *Homo sapiens*.

Results

Identification of Novel Verubecestat Similar Molecules (VSMs)

Novel molecules that were similar to Verubecestat are shown in Figure 1. There were 5 VSMs that had pharmacophore properties and molecular topology similar to Verubecestat, as determined by MCULE "*1-Click Scaffold Hop"* software (Table 1). Each of these novel VSMs had an FTree similarity score greater than 0.85, suggesting a meaningful similarity to Verubecestat. The development of the novel BACE1 inhibitor used these 5 VSMs as scaffolds.

Table 1. Novel compounds with scaffold similarities to Merk's Verubecestat MCULE "1-Click Scaffold Hop." All novel compounds were discover based on pharmacophore property and molecular topology similarity with Verubecestat

Rank	Novel Compound	Given Name[1]	Similarity Score [2]
1	MCULE-9782190592-0	VSM1	0.9136
2	MCULE-5237437595-0	VSM2	0.8929
3	MCULE-6817545231-0	VSM3	0.8920
4	MCULE-9683553185-0	VSM4	0.8865
5	MCULE-1014957285-0	VSM5	0.8857

1 VSM = Verubecestat Similar Molecule

2 FTrees similarity scores are calculated by pharmacophore properties and molecular topology.

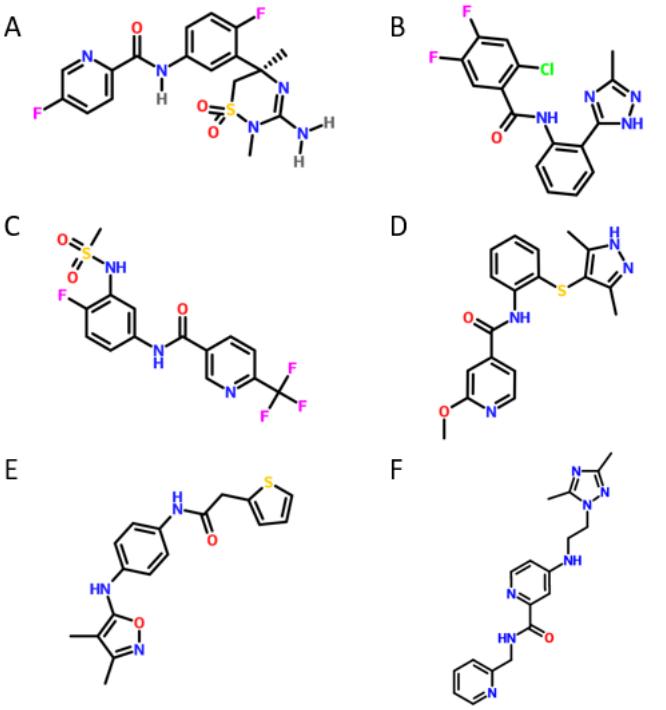

Figure 1. Stick Diagrams of Verubecestat and novel Verubecestat similar molecules (VSM) with FTree scores greater than 0.85. MCULE *"1-Click Scaffold Hop"* identified five novel compounds with scaffold similarities to Verubecestat (CAS accession code: 1286770-55-5). Each VSM had an FTree similarity score greater than 0.85 when referenced to Verubecestat. (A) Verubecestat (B) VSM1 (C) VSM2 (D) VSM3 (E) VSM4 (F) VSM5. MCULE ID numbers can be found in Table 1.

Identification of BACE1 Binding Pockets

CavityPlus identified 10 potential binding sites on BACE1 (Table 2). This score was based on cavity surface area, cavity volume, and availability of both hydrophilic and hydrophobic interactions. Each of these binding sites had varying druggability scores with most being identified as poor sites for inhibition (weak druggability and pKd scores less than 6 suggest the site is not suiTable for binding). These sites with poor potential for binding are shown in Figure 2 in purple. Only one site, labelled red in Figure 2, on BACE1 was indicated as having high potential for binding. This site is BACE1 active site where APP and the other associated substrates are cleaved.

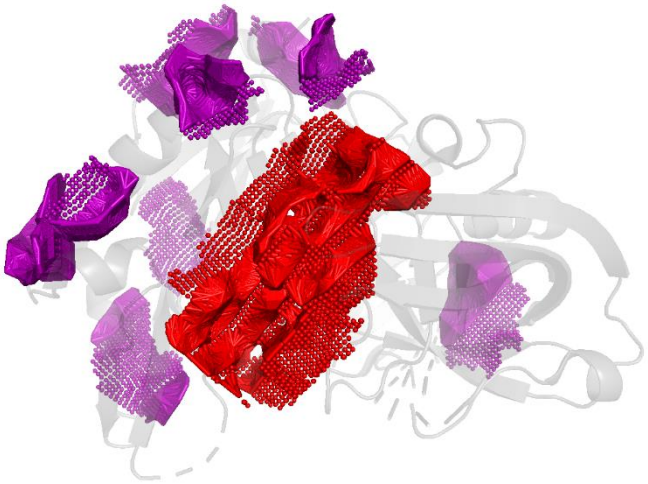

Figure 2. Identification of various BACE1 binding sites. CavityPlus identified 10 binding sites on BACE1 (PDB 1TQF) outlined in Table 1. Purple surfaces indicate 9 binding sites of low "druggability." The red surface indicates the binding site 1 which had the highest "druggability." Image was generated in PyMOL.

Table 2. BACE1 binding surface identification and associated cavity results determined by CavityPlus. Cavity identification based on cavity surface area, cavity volume, and availability of both hydrophilic and hydrophobic interactions.

Binding Site	Predicted Max pKd[1]	Predicted Average pKd	Drug Score	Druggability[2]
1	11.81	6.67	594.00	Medium
2	10.49	6.22	-901.00	Weak
3	8.55	5.55	-493.00	Weak
4	7.24	5.10	-1276.00	Weak
5	7.10	5.05	-980.00	Weak
6	6.91	4.99	-1075.00	Weak
7	6.76	4.94	-963.00	Weak
8	6.72	4.92	-1152.00	Weak
9	6.67	4.91	-1398.00	Weak
10	5.51	4.51	-1297.00	Weak

1 pKd = -log10(Kd)
2 Indicated the possibility of a cavity binding site to be druggable or not

MCULE Docking of Identified VSMs to BACE1

The VSMs identified in Figure 1 were docked in binding site 1 (refer to Figure 2) of BACE1 using MCULE's *"1-Click Docking"* software. Table 3 shows the estimated free energy of binding Vina score of each VSM with BACE1. Binding affinity scores ranged from -7.5kcal/mol to -8.3 kcal/mol (Table 3). Without any modifications to VSMs, VSM1 docked with the highest affinity. All 5 VSMs were verified to bind preferentially to binding site 1 of BACE1.

Table 3. Free energy of binding of novel Verubecestat similar compounds *"1-Click Docking"* via MCULE with BACE1. VSMs were docked into BACE1 binding site 1.

Novel Compound[1]	Docking Vina Score[2]
VSM1	-8.3
VSM2	-8.0
VSM3	-7.8
VSM4	-8.2
VSM5	-7.5

1 VSM = Verubecestat Similar Molecule. Refer to Table 1 for molecule ID.
2 Docking Vina Score is a rough estimate of the free energy of binding (kcal/mol)

Addition of Sterol Groups to Identified VSMs

The docking of each novel VSM without modification into binding site 1 was observed in PyMOL. Visual scanning of the fit of each molecule into binding site 1 was used to identify locations where a sterol group (PubChem CID - 1107) could be added. These locations were identified based on steric hindrance and depth within the binding pocket. Carbon atoms closest to the exterior of the binding pocket were labelled as ideal locations to add sterol groups. Figure 3 shows an example of this process where VSM1 is docked into binding site 1. The orange-coloured carbons of VSM1 were identified as prime locations to add the sterol group.

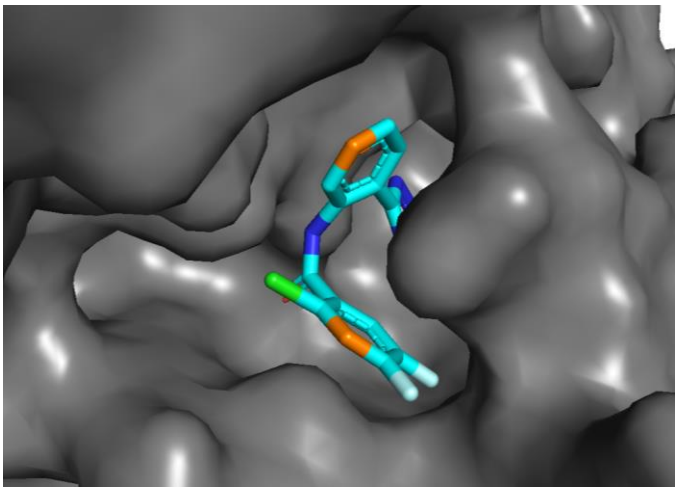

Figure 3. Docking of VSM1 into binding site 1 of BACE1 for location of sterol linkage identification. VSM1 is docked into binding site 1 (highest "druggability") using MCULE *"1-Click Docking"* and imaged using PyMOL. Surface of BACE1 (PDB 1TQF) binding site 1 is coloured grey. VSM1 is shown in stick representation. For the atoms of VSM1, cyan, blue, red, light blue, and green correspond with carbon, nitrogen, oxygen, fluorine, and chlorine respectively. The orange carbons represent where sterol groups can potentially be added.

For each VSM, multiple locations to add a sterol group were identified and shown in Figure 3. MCULE was used to add a sterol group to the corresponding locations VSM. Table 1A shows how many VSM derivatives/iterations were developed. All the VSM derivatives/iterations were docked into binding site 1 of BACE1 to determine their estimated free energy of binding Vina score. Figure 4 shows a derivative/iteration of each VSM with added sterol groups that resulted with the lowest docking score.

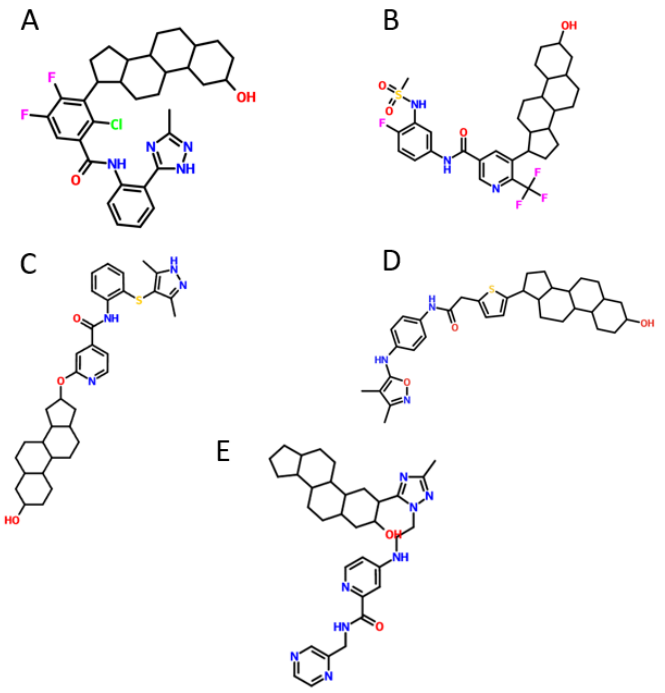

Figure 4. Stick Diagrams of sterol-lined VSMs with the most successful dockings scores determined by MCULE. Stick diagrams of the novel VSMs with sterol links MCULE "1-Click Scaffold Hop" (A) VSM1+sterol link (iteration 1.7, ΔG= -10.9 kcal/mol) (B) VSM2+sterol link (iteration 2.1, ΔG= -8.9 kcal/mol) (C) VSM3+sterol link (iteration 3.2, ΔG= -10.5 kcal/mol) (D) VSM4+sterol link (iteration 4.4, ΔG= -9.7 kcal/mol) (E) VSM5+sterol link (iteration 5.4, ΔG= -9.7 kcal/mol).

Chemical Properties of Sterol-Linked VSMs

To determine the viability of each sterol-linked VSM as a drug, MCULE was used to determine each VSM derivative's physicochemical property profile. Table A1 shows the toxicity results, molecular weight, number of hydrogen bond acceptors and donors, and octanol-water partition coefficient (log P). For an oral drug to have certain biological or pharmacological activity, the number of Lipinski rule of 5 violations were counted for each sterol-linked VSM derivative. A drug must not violate more than 1 rule to have some degree of bioavailability: molecular weight less than 500 Daltons, no more than 5 hydrogen bond donors and 10 acceptors, and an octanol-water partition coefficient that is less than or equal to 5. Of all the sterol-linked VSM derivatives, VSM 5.4 was the only molecule to only meet the requirement of the rule.

VSM 5.4, shown in Figure 4, was the focal small molecule inhibitor of BACE1 for the rest of development. VSM 5.4 was given the industry name Bacehibitol.

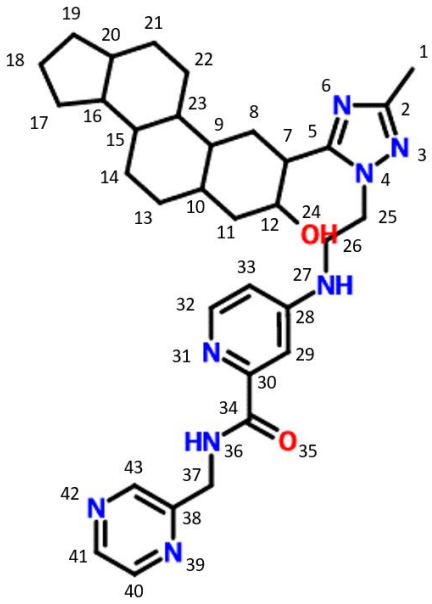

Figure 5. Stick diagram of VSM 5, iteration 4 (Bacehibitol) with one violation of Lipinski's rule of 5. VSM 5.4 analyzed by MCULE property checker showed one violation of Lipinski's rule of 5. Molecular weight 584.7543 Da, *log*P 4.9845, hydrogen bond acceptors 10, hydrogen bond donors 3. Stick diagram was generated via MCULE and heavy atom numbers were added via Microsoft PowerPoint.

MCULE Docking of Potential VSMs with Sterol Groups to BACE1

Bacehibitol was docked in binding site 1 of BACE1 to give a docking score of -9.7 kcal/mol. Interactions between Bacehibitol and BACE1 were determining by Protein-Ligand Interaction Profiler (PLIP) (Table A2). Figure 6 showed that a variety of interactions occur between Bacehibitol and BACE1. It is also shown that the sterol link apart of Bacehibitol interacts with BACE1 via hydrophobic interactions. 5 of the interactions where hydrophobic and the other 5 was hydrophilic.

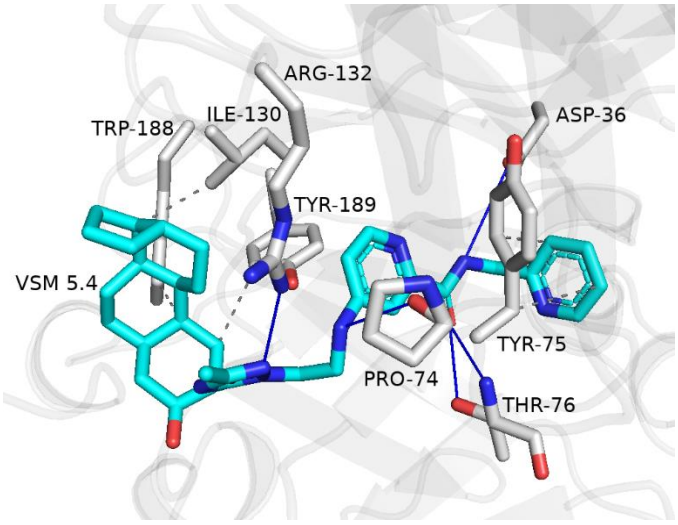

Figure 6. Docking of Bacehibitol in binding site 1 of BACE1 with interacting residues shown. Bacehibitol was docked into BACE1 (PDB 1TQF) using MCULE "1-Click Docking." PLIP identified all interactions between Bacehibitol and BACE1 (Table A2). Image was generated in PyMOL. The structure of BACE1 is shown as a cartoon with 90% transparency; however, the various residues that participate in polar interactions (side-chain or main-chain atoms) with Bacehibitol are shown in stick representation. The structure of Bacehibitol is also in stick representation. Dashed grey lines indicate the hydrophobic interaction. Solid blue lines indicate the hydrogen bonds. For the atoms on the interreacting residues of BACE1, light-grey, blue, red, and orange correspond with carbon, nitrogen, oxygen, and sulphur respectively. For the atoms on Bacehibitol, cyan, blue, red, and orange correspond with carbon, nitrogen, oxygen, and phosphate respectively.

Bacehibitol docked with BACE1 had shown that residues from various domains interact to coordinate the ligand in binding site 1 (Figure 7). Half of the interactions appear to be within the "flap" region of BACE1, whereas the other half of the interactions appear to be originating from residues in the catalytic domain and non-functionally relevant domains.

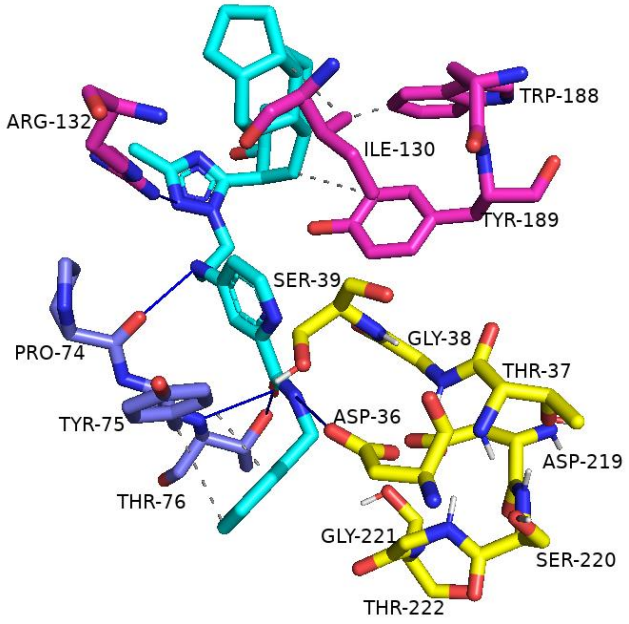

Figure 7. BACE1 residues Interacting with Bacehibitol coloured by domain function. All residues of BACE1 (PDB 1TQF) are shown in stick representation. Yellow residues are involved in catalytic function, dark blue residues are involved with the dynamic flap domain in BACE1, and pink residues are residues not involved in any functionally relevant domain. The structure of Bacehibitol is also in stick representation. Dashed grey lines indicate the hydrophobic interaction. Solid blue lines indicate the hydrogen bonds. For the atoms on Bacehibitol, cyan, blue, red, and orange correspond with carbon, nitrogen, oxygen, and phosphate respectively. For the atoms on the interreacting residues of BACE1, blue, red, and orange correspond with, nitrogen, oxygen, and sulfur respectively. Carbon atoms of BACE1 residues are coloured according to the domain to which they belong.

Pharmacokinetic, and Pharmacodynamic Properties of VSMs

SwissADME analyzed the physicochemical, pharmacokinetic, and pharmacodynamic properties, as well as druglike nature and medicinal chemistry friendliness of Bacehibitol. Bacehibitol had a calculated topological polar surface area (TPSA) of 130.75Å² and a Wildman-Crippen Log P (WLOGP) score of 4.18. These values provide an understanding of Bacehibitol's polar and lipophilic nature in regards to its bioavailability. Using these calculated values, Figure 8 shows the predicted absorption of Bacehibitol via the intestine and the permeability through the blood brain barrier (BBB). The coloured zones demonstrate zone of optimal absorbance and BBB permeability, as provided by SwissADME and based on the properties of clinically available drugs.

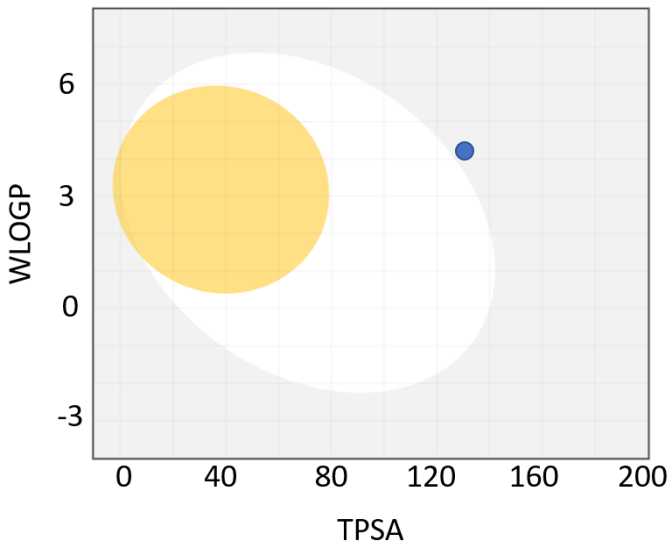

Figure 8. BOILED-Egg prediction of gastrointestinal absorption and blood brain barrier penetration of Bacehibitol. The molecular structure of Bacehibitol was analyzed using SwissADME, providing a topological polar surface area score (TPSA) of 130.75Å² and a Wildman Crippen Log P score (WLOGP) of 4.18. The white oval, like "egg-whites," represents the area where compounds have sufficient gastrointestinal absorption in humans. The yellow oval, like the "yolk," represents the area where compounds have sufficient blood brain barrier permeability in humans. The blue dot represents the topological polar surface area score and Wildman Crippen *Log* P value of Bacehibitol.

Prediction of Off-Target Interactions

Bacehibitol was analyzed by SwissTargetPrediction to determine the probability of inhibitory effects on proteins other than BACE1. Figure 9 shows the top three classes of proteins within *Homo sapiens* to which Bacehibitol may bind and inhibit. 66.7 percent of the identified potential non-BACE1 protein targets of Bacehibitol were kinases, 26.7 percent were proteases, and 6.7 percent were G-protein coupled receptors. Of the 370,000 active sites of proteins found within *Homo sapiens* analyzed with Bacehibitol, inhibition of β-secretase 2 (BACE2) was found to occur with the highest probability at 6.61 percent.

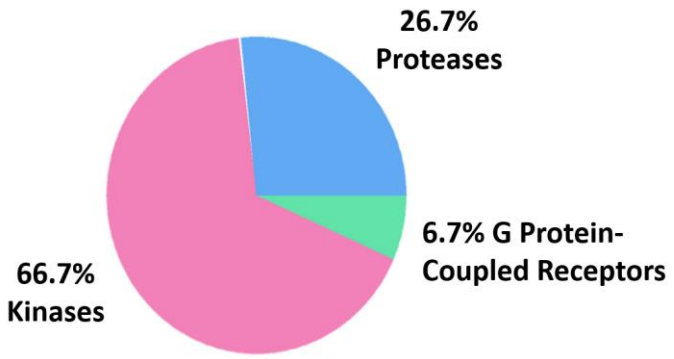

Figure 9. Classes of *Homo sapiens* proteins that have potential to be bound by Bacehibitol. The SMILES ID of Bacehibitol (Cc1nc(n(n1)CCNc1ccnc(c1)C(=O)NCc1nccnc1)C1CC2C(CC1O)CCC1C2CCC2C1CCC2) was analysed by SwissTargetPrediction. Pie chart shows the classes of proteins that may be inhibited by Bacehibitol. The highest probability of off-target binding was 6.61% with beta-secretase 2.

Discussion

At first glance, Bacehibitol is a successful small molecule inhibitor of BACE1. It had a docking score of -9.7 Kcal/mol with the binding interface of interest where APP cleavage is known to occur, additionally, the score is relatively high compared to the other Verubecestat similar molecules (Table 3) (9). A more detailed analysis of the interactions of the molecule with BACE1 identified some limitations of the molecule's binding. Figures 6 and 7 depict the binding interactions of the molecule where half of the interactions appear to be with the "flap" region of BACE1. The flap region is composed of the amino acid's residues V71- E83. Bacehibitol made most of its hydrogen bonds with residues P74 (1 bond) and T76 (2 bonds) in this region (10). Furthermore, the molecule also made 2 hydrophobic interactions at residue Y75. Association with this region of the enzyme would be a concern for inhibitor binding due to the flexibility of the region and supposed function of shielding the active site (10). *In vivo* or *in vitro* analysis of the molecule would likely report a lower ability for the molecule to inhibit the active site of BACE1 accredited to these strong interactions with the flexible domain. The remaining interactions of Bacehibitol were made with residues within the interface of interest. However, only one interaction was made with the protease active site: the hydrogen bond formed with the catalytic aspartic acid residue D36. The protease site, which consists of amino acids D36-S39 and D228-T231, would be rendered non-accessible and therefore non-functional due to steric hindrance of Bacehibitol at the site (7, 9). Future BACE1 inhibitors should optimize interactions with these residues due to their function in APP proteolysis or with residues surrounding this site while ignoring the flap region. The need for optimization is supported by SwissTargetPrediction, as shown in Figure 9. The prediction identified 78 other proteins found in *Homo sapiens* that

have a similar likelihood of binding to the inhibitor as BACE1. *In vitro* analysis may show a smaller number of off-target interactions, as the sterol-link would target the molecule to the endosomal pathway (3). Further optimization of the molecule with the protease site would result in an improved docking score and avoid implications of associating with the flexible flap region.

To determine the druggability of Bacehibitol, the properties of the molecule were analyzed. Initial properties of the molecule were promising. Bacehibitol fulfilled the majority of the Lipinski properties including logP, hydrogen bond donors, and hydrogen bond acceptors. There was one violation, which was the mass of the molecule being greater than 500 Daltons. This violation is significant in regards to the druggability of Bacehibitol. The target of Bacehibitol, BACE1, is found in neurons in the brain, therefore the molecule must cross the blood-brain-barrier (BBB) to successfully fulfill its purpose. The BBB is a semi-permeable barrier that limits access of molecules found in the blood from reaching the brain. Figure 8 confirms this concern with Bacehibitol. Results of the SwissADME analysis determined that the molecule has very low BBB permeability, therefore the molecule would be unsuccessful in reaching its target location. Furthermore, SwissADME identified additional limitations of Bacehibitol. It was found to have low gastrointestinal absorption, limiting its use as an oral medication. The molecule's low absorption may be circumvented by administering the drug via intravenous injection. However, this would limit the application of the medicine in the population as healthcare professionals would be required in its administration. The large hydrophobic sterol group also impacted the molecule's properties by reducing its solubility.

Conclusions

BACE1 inhibitors have numerous factors that must be overcome to achieve a molecule successful in inhibiting APP cleavage in Alzheimer's disease. Firstly, the addition of a sterol group is promising in selectively targeting BACE1 cleavage of APP while minimizing off-target effects found in traditional inhibitors that target this enzyme. The application of the sterol group in small molecule inhibitors requires more research to determine scaffolds that allow molecules to overcome gastrointestinal absorption, solubility, and BBB permeability problems found in Bacehibitol. Applications of the sterol group extend beyond BACE1 inhibition. The study's purpose was to target the molecule to the endosomal pathway, which could be applied to other small molecule inhibitors as well. High-throughput screening is recommended to determine a BACE1 inhibitor that targets the binding interface more directly, avoids the flap region that shields the active site, and improves binding affinity with the catalytic site. Screening also improves the likelihood of determining a molecule with smaller molecular weight which would improve the BBB permeability and GI absorption. The flexibility of the flap domain and static nature of structures determined by X-ray crystallography is not ideal. *In vitro* analysis of inhibitors or use of structures determined by NMR spectroscopy are recommended to determine how the inhibitor will interact with BACE1 while accounting for the dynamic nature of the flap.

Acknowledgments

Large thanks to teaching assistant Michael Kovacevic for providing guidance and feedback for improvement throughout the project. Another thanks to Dr. Murray Junop and Dr. James Choy for proving the skills to complete this small molecule BACE1 inhibitor.

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Appendix

Table A1. Chemical and molecular properties of all Verubecestat similar molecules. MCULE property checker analysis of various VSM derivatives.

VSM Derivative[1]	Toxic	Molecular Weight	No. of Hydrogen Bond Donors	No. of Hydrogen Bond Acceptors	Octanol-Water Partition Coefficient	No. of Lipinski Rule Violations
1.1	No	595.1194	3	6	7.7440	2
1.2	No	610.1342	3	7	7.9074	2
1.3	No	611.1225	3	6	7.3024	2
1.4	No	598.0842	3	9	5.9290	2
2.1	No	623.7030	3	7	7.7971	2
2.2	No	638.7178	4	8	7.9605	2
2.3	No	639.7061	4	9	7.3555	2
2.4	No	653.7326	5	9	8.1239	2
3.1	No	600.8150	3	7	7.6136	2
3.2	No	586.7885	3	7	7.2688	2
3.3	No	600.8150	3	7	7.6136	2
3.4	No	586.7885	3	7	7.3052	2
3.5	No	600.8150	3	7	7.3803	2
3.6	No	600.8150	3	7	7.6136	2
4.1	No	573.7896	3	6	8.1307	2
4.2	No	573.7896	3	6	8.2044	2
4.3	No	573.7896	3	6	7.8974	2
4.4	No	573.7896	3	6	8.1307	2
4.5	No	573.7896	3	6	8.1307	2
5.1	No	597.7926	3	9	5.8979	2
5.2	No	583.7661	3	9	5.5895	2
5.3	No	584.7543	3	10	5.1302	2

5.4 (Bacehibitol)	No	584.7543	3	10	4.9845	1

Table A2. Binding interaction of Bacehibitol with BACE1 determined by Protein-Ligand Interaction Profiler. Bacehibitol was docked into BACE1 at binding site 1 using MCULE "*1-One Click Docking*" and then related PDB pose file was analyzed by PLIP.

BACE1 Residue & Atom	Bacehibitol Atom[1]	Distance (Å)	Bond Type
TYR-75/CD1	43	3.37	Hydrophobic Interaction
TYR-75/CB	41	3.94	Hydrophobic Interaction
ILE-130/CG2	16	3.53	Hydrophobic Interaction
TRP-188/CZ3	9	3.35	Hydrophobic Interaction
TYR-189/CE2	8	3.85	Hydrophobic Interaction
ASP-36/OD2	36	3.99	Hydrogen Bond
PRO-74/O	27	3.21	Hydrogen Bond
THR-76/N	35	3.07	Hydrogen Bond
THR-76/OG1	35	2.94	Hydrogen Bond
ARG-132/NH2	3	3.08	Hydrogen Bond

1 Refer to Figure 4 for corresponding atoms of Bacehibitol