GENERAL ARTICLE

Protective effects of BACE1 inhibitory ligand molecules against amyloid beta-induced synaptic and mitochondrial toxicities in Alzheimer's disease

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Abstract

Amyloid- β (A β) peptides are the major drivers of Alzheimer's disease (AD) pathogenesis, and are formed by successive cleavage of the amyloid precursor protein (APP) by the beta and gamma secretases. Mounting evidence suggests that A β and mitochondrial structural and functional abnormalities are critically involved in the loss of synapses and cognitive decline, in patients with AD. In AD brain, state the sequential proteolytic cleavage of APP by beta secretase 1 enzyme (BACE1) and γ -secretase leads to the production and release of A β 40 and 42. BACE1 expression and activity increased in the brains of AD patients. Structurally, β -secretase has a very large binding site (1000 Å) with fewer hydrophobic domains that makes a challenge to identify the specific targets/binding sites of BACE1. In the present study, we constructed a BACE1 pharmacophore with pepstatin and screened through molecular docking studies. We found one potential candidate (referred as ligand 1) that binds to the key catalytic residues of BACE1 and predicts to inhibit abnormal APP processing and reduce A β levels in AD neurons. Using biochemical, molecular, transmission electron microscopy, immunoblotting and immunofluorescence analyses, we studied the protective effects of ligand 1 against A β -induced synaptic and mitochondrial toxicities in mouse neuroblastoma (N2a) cells that express mutant APP. We found interaction between ligand 1 and BACE1 and this interaction decreased BACE1 activity, A β 40 and 42 levels. We also found increased mitochondrial biogenesis, mitochondrial fusion and synaptic activity and reduced mitochondrial fission in ligand 1-treated mutant APP cells. Based on these results, we cautiously conclude that ligand 1 reduces A β -induced mitochondrial and synaptic toxicities, and maintains mitochondrial dynamics and neuronal function in AD.

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Graphical Abstract

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disease, characterized clinically by memory loss, language deterioration, impaired visuospatial skills, poor judgment and difference in attitude (1). The histopathological investigation of postmortem AD brains revealed that two major pathological hallmarks—senile plaques containing amyloid beta ($A\beta$) and tau-rich neurofibrillary tangles (NFTs). The histopathological investigation of postmortem AD brains revealed that two major pathological hallmarks, including senile plaques containing amyloid- β ($A\beta$) and tau-rich neurofibrillary tangles (NFTs). The amyloid deposits consist of accumulation of both aggregated and non-aggregated forms of $A\beta$. $A\beta$ is derived from sequential proteolytic processing of $A\beta$ precursor protein ($A\beta$ PP) by β - and γ -secretases (2, 3). The NFTs in AD brain are composed of phosphorylated tau (p-tau), a microtubule associated protein that regulates polymerization and stabilization of neuronal microtubules (4). AD is a multifactorial disease, with both genetic and environmental factors implicated in its pathogenesis (5). A small proportion of AD cases show an autosomal dominant transmission mutant alleles, with mutations in $A\beta$ PP, presenilin 1 and presenilin 2 genes. These mutant alleles cause early onset of familial AD (6, 7). The best described other risk factors for AD are age, traumatic brain injury, depression, cardio-vascular factors and lifestyle factors (8).

In AD, numerous reports evidenced that the prime beta secretase 1 enzyme (BACE1) plays a significant role in the formation of $A\beta$ peptides (9, 10). APP processing occurs via two pathways. Beta secretase (or BACE1) based amyloidogenic and α -secretase based non-amyloidogenic: In non-amyloidogenic pathway, cleavage occurs by α -secretase within the $A\beta$ domain and generates the large soluble N-terminal fragment (sAPP α) and a non-amyloidogenic C-terminal fragment (CTF) of 83 amino-acid residues (C83). Further cleavage of this C-terminal fragment by

γ -secretase generates the non-amyloidogenic peptide (P3) and APP intracellular domain. In amyloidogenic pathway, cleavage occurs by β -secretase at the beginning of the $A\beta$ domain and generates a soluble N-terminus fragment (sAPP β) and amyloidogenic C-terminal fragment of 99 residues—C99). This C-terminal fragment, further cleaved by γ -secretase and generates $A\beta$. Cleavage by multiple γ -secretases can generate $A\beta$ 1–40 and $A\beta$ 1–42 fragments (11, 12). However, BACE1 is an impending target for the treatment of AD because it is responsible for cleavage of $A\beta$ PP (13). BACE2 differs from BACE1 in several aspects, including enzyme activation, binding sites of protein and functions (14–17).

$A\beta$ accumulation in cells results in a cascade of cellular changes, including oxidative damage, tau hyperphosphorylation, inflammatory responses, mitochondrial damage and synaptic failure (18–20). Changes in mitochondrial metabolism in the presence of toxic $A\beta$ and p-tau are well-documented (21). Our lab studies showed that increased oxidative damage contributes to synaptic damage before the $A\beta$ accumulation (22). Mitochondrial dysfunction is common in several neurodegenerative diseases, including Alzheimer's, Huntington's, Parkinson's, ALS, multiple sclerosis and others (23, 24). The development of mitochondrial dysfunction in AD is associated with $A\beta$ and p-tau (25, 26). Evidence shows mitochondrial abnormalities contribute to AD pathology. APP and $A\beta$ accumulate in the mitochondrial membranes and are responsible for increased reactive oxygen species (ROS) production, initiating mitochondrial dysfunction (27, 28). Other studies showed increased ROS production and decreased ATP synthesis in postmortem AD brains (29). Many studies also reported changes in the mitochondrial DNA in the brains of AD patients (30). Research confirmed that mitochondrial encoded genes were also abnormally expressed in transgenic mice, whereas other studies showed mitochondrial dysfunction is an early event in AD along with the increased demand of energy in the AD brain (31, 32). Accumulation of

A β in the outer membrane, and fragmented mitochondria were viewed via electron microscopy in AD transgenic mice (33–35). The extent of cognitive decline has also been shown to contribute to mitochondrial damage (36).

Mitochondrial dysfunction is an early cellular event in AD. A β found in the neurons of AD that disrupts synaptic signaling and neuronal growth (37). The ROS production from mitochondria increases BACE1 activity, which further increase A β secretion in AD. The secreted A β is found at the synapses, where it is translocated into synaptic mitochondria, which further induces ROS production. A β and p-tau interact with mitochondrial fission protein, Drp1, which induces excessive mitochondrial fragmentation, impaired mitochondrial dynamics that leads to synaptic damage in AD neurons (38, 39). These abnormal interactions are believed to progress disease pathology in AD. These evidences propose that the accumulation of A β in synapses and synaptic mitochondria causes synaptic mitochondrial failure and synaptic degeneration (40, 41).

The exact mechanism behind the aggregation of A β at the extracellular level and p-tau depositions in intracellular pathology are still not completely understood. The accumulation of A β protein, processed by cleavage of APP, is the vital cause for plaque formation in AD. The presently BACE1 inhibitor drugs like JNJ-54861911, CNP520, LY3202626, Elenbecestat, Lanabecestat and Verubecestat are currently being tested in human clinical trials on AD patients and preliminary results thus far show ineffectiveness in cognitive decline reduction and disease progression (42, 43). Much of the research on BACE1 inhibitor drug discovery has failed at clinical trials, and consequently many researchers and clinicians have turned on to study alternative AD therapeutics. Thus, an inhibitor drug of APP proteolytic processing by BACE1 is still a major unsolved concern in AD research.

To justify BACE1 inhibitor drug development, it was necessary to provide *in vivo* validation that BACE1 is the primary β -secretase enzyme in the brain. To do so, gene targeting in embryonic stem cells was used to produce BACE1 knockout (–/–) mice (44, 45). Initial reports showed that BACE1–/– mice were viable and fertile and did not have detectable abnormalities. Their normal morphology and behavior, tissue histology and blood cell and clinical chemistry characteristics suggested that BACE1 inhibition as a therapeutic approach for AD. Additionally, APP overexpressing transgenic mice that also lack the BACE1 gene are devoid of cerebral A β , amyloid deposition and A β -associated memory impairments (46). It is important to note that these data validate BACE1 as the major β -secretase in the CNS and indicate that BACE2 does not compensate for BACE1 loss of function, at least for the production of A β . Furthermore, they strongly suggested that BACE1 inhibition should be a safe and effective therapeutic strategy for AD.

The ligand-based pharmacophore drug discovery approach is expected to address and to find the best potential therapeutics against BACE1 in AD. Hence, an attempt has been made with pepstatin (pharmacophore) based screening to find the most promising and effective therapeutic ligands against BACE1 inhibition in AD.

The purpose of our article is to identify the BACE1 inhibitor ligands, and study the features of ligand in AD neurons with drug discovery approach. We selected the best ligand small molecules, which exhibited the best docking scores and best interacting sites of BACE1 inhibition. Furthermore, we tested the protective effects of ligand 1 against BACE1 and A β -induced mitochondrial and synaptic properties in N2a cells and mutant APP cells (meaning N2a cells transfected with mutant APP cDNA containing both Swedish and Indiana mutations). We sought

to determine (1) cell viability, (2) the inhibition of A β 40 and 42 levels, (3) mRNA and protein levels of mitochondrial dynamics and biogenesis and synaptic genes, (4) immunohistochemistry and (5) mitochondrial number and morphology in N2a cells and mAPP cells treated and untreated with ligand 1.

Results

Active site CASTp analysis

Prediction of all possible binding sites of the protein BACE1 was analyzed through the computed atlas of surface topography of proteins (CASTp) and results explore nearly 47 binding pockets with unique area and volumes. The binding pockets 1 with 337.5 area and 424.9 vol closed to conserved catalytic residues of domain 1 of BACE1 (Fig. 1A). The active site of BACE1 contains the catalytic residues as ASP 32, THR 33 and SER 35 located at ectodomain 1 pocket (Fig. 1A) and these active site selected for pharmacophore and molecular docking studies.

Ligands screening

Beta amyloid based amyloidogenic APP processing involves two successive catalytic cleavages to release sAPP β and A β . Hence, we chose pepstatin, a well-known aspartyl protease inhibitor as the reference ligand for screening the similar structures from chemical libraries. The non-commercial chemical library PubChem contains huge ligands around 93 lakhs including, tested and non-tested, overall 15 130 clusters of structurally similar compounds of pepstatin screened with 80% cutoff among the compounds. Furthermore, *in silico* screening and validation were performed based upon the structural similarity of pepstatin ligands downloaded, and results were saved in ligand chemical format (.sdf). From the PubChem library screening, we only considered the 873 structure-related lead molecules set for the further pharmacophore validations.

Pharmacophore-validated ligands

Hexa-peptide containing the unusual amino acid statine Sta [(3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid] pepstatin used as a referenced ligand is a well-known aspartyl protease inhibitor. The molecular mechanism and catalytic cleavage with aspartic acid (D) functional group participations is visualized in (Fig. 2A). The selected group of ligand chemical structure features and associations is depicted in (Fig. 2B). In pharmacophore screening, BACE1 protein model was mechanically generated by the LS program, and includes multiple features: hydrogen bond donor (HBD) (green), hydrogen bond acceptors (red) (HBA) and hydrophobic group (yellow) (Fig. 1C). The program mechanically generated many excluded volumes (EV; gray) within the model. The HBD features pharmacophore point toward 1-proton acceptor group at ASP32 and 2-proton donors of the ligand from the THR33, respectively (Fig. 1B). Within the test database, we kept the ligand, pepstatin, present in complex structures (Fig. 1D). The pepstatin-BACE1 complex will display the binding regions of ligand part with BACE1 (Fig. 1D). First, the pepstatin was extracted and then, hydrogen atoms were additional and energy minimized by the LigandScout (LS). The minimized structure of pepstatin ligand added to the test database. When screening, the test compounds were properly mapped within the pepstatin pharmacophore models as shown in Figure 1. The result verified the validity of our pharmacophore screening library of pepstatin from PubChem 873 molecules. A

Figure 1. Pharmacophore models of BACE1 and pepstatin complexes. (A) CASTp calculations of BACE1 domain 1 interaction with selected pharmacophore residues involvement and positioned were highlighted with ball and stick models ASP 32, THR33 and SER 35. The two core binding pockets of BACE1 domain 1 region, i.e. starting from 1 to 146 residues were read and highlighted of catalytic core trait pockets domain 1 covered the catalytic residues pockets 29, 31 were highlighted with light pink and yellow (colored) ball and stick models. (B) Two-dimensional pharmacophore model of BACE1 with pepstatin was generated by LS from the complex structure of BACE1 and pepstatin. The dotted arrows indicated the HBD and HBA features with residues, THR 33A posing that two HBD (green colored dotted) and one HBA ASP 33A HBA (red colored dotted) with pharmacophore pepstatin ligand and the yellow sphere represented the hydrophobic feature (HF) in the ligand-based pharmacophore. (C) The 1D colored spheres pharmacophore models of BACE1 schematic images indicating the nature of HBD (green), 2 HBA (red) and one hydrophobic group (yellow), HF yellow spheres and gray spheres represent EV. (D) The BACE1 with pepstatin (ligand) complex model with extended wire protein ligand stick model with pharmacophore properties like HBA, HBD and HF and EV.

small number of ligand molecules fitted within the designed BACE1 pharmacophore around 16 molecules with CID numbers: 10008594, 10031313, 10034005, 10048142, 10054794, 10079786, 99647460, 98053228, 99647463, 91742649, 90468180, 90013848, 88791717, 87900731, 87794123 and 87464897, observed to be the best-fitted ligands. Based on BACE1 pharmacophore analysis, these ligands were used for further validation with molecular docking studies.

Molecular docking

Docking studies were carried out with the 16 best pharmacophore ligands by running the AutoDock 4.2v (47) PyRx virtual screening tool setup with the docking files (48). The 16 compounds considered for the molecular docking studies based on best-fitted pharmacophore fitness. After molecular docking results, only the top five leads from the molecular docking results (ranked by docking score) were visually scrutinized. The best five compounds, which showed good possession of the predicted binding pocket, having at least (minimum) two hydrogen bonds within the protein, were selected, and shown as a docking model (Fig. 3A). The top ranking molecules docking scores with respective CIDs are 1008594, CID 10048142, CID 10054794, CID 10031313 and CID 10034005. These ligands further analyzed for molecular interaction such as hydrogen bonding, residue interactions with respective ligand and protein visualized with the PyMol visualization tool as illustrated in (Fig. 3B). We are especially interested in each ligand and their molecular

interactions with BACE1 chief residues of the protein. The best ligand molecules selected through molecular docking scores. Top five ligands mostly bind with the active site of the BACE1 and inhibit its catalytic function. These ligands considered as drugs to target AD by inhibiting the BACE1 enzymatic catalytic function in APP processing. The computational validations like molecular docking; absorption, distribution, metabolism, elimination and toxicity (ADMET) and drug-likeness properties indicate that ligand 1 showed good lead properties from the top 3 qualified ligands, further, biological validations proceed to test the ligands in mouse neuronal cell (N2a) cultures in *in vitro* studies (Fig. 3C).

ELISA ligand-based inhibition of BACE1 activity, A β 40 and 42

To determine whether ligand 1 lowers BACE1 activity and reducing the A β 40 and A β 42 levels, we measured BACE1 activity, A β 40 and A β 42 levels in mutant APP cells (N2a + mutant cDNA transfected) treated with ligand 1 using Sandwich ELISA. As shown in Figure 4A and B, significantly reduced BACE1 activity found in the mAPP cells treated with ligand 1 ($P=0.001$) relative to mAPP cells. The levels of A β 40 ($P=0.01$) and A β 42 ($P=0.005$) were found to be reduced in the mAPP cells treated with ligand 1 compared with mAPP cells (Fig. 4C and D). It is interesting to note that in BACE1 activity, A β 40 and A β 42 levels were significantly reduced in the mAPP cells treated with ligand 1. Overall, these observations revealed that treatment of ligand 1 is effectively inhibits BACE1 activity and reduced both A β 40 and A β 42.

Figure 2. Mechanisms of pepstatin cleavage ligand studies. (A) Peptide cleavage mechanism by aspartyl proteases, (red colored) circle is ligand-interaction group and (blue colored) circle is amino acid aspartyl protease (Asp) interaction group participated in catalytic cleavage process. (B) Aspartyl protease inhibitor pepstatin (CID 5478883) used as template ligand and computational screening finalize five best ligands representing the same characteristic features of pepstatin ligand and ligand 1 finally tested in *in vitro* cell culture studies.

Cell viability

To determine the effect of ligand 1 treatment of N2a cells, we assessed cell viability. As shown in Figure 5, significantly increased levels of cell viability were found in ligand 1 treated N2a cells ($P=0.038$) relative to untreated cells. Cell Viability Section—Cell Viability was increased in mAPP cells treated with ligand 1 compared to mAPP cells alone.

mRNA levels of mitochondrial dynamics and mitochondrial biogenesis and synaptic genes

We determined the protective effects of ligand 1 on mRNA expression of mitochondrial, synaptic and AD-related genes, we measured mRNA levels of the mitochondrial dynamics genes Drp1 and Fis1 (fission); Mfn1, Mfn2, Opa1 (fusion); mitochondrial biogenesis genes PGC1 α , Nuclear respiratory factor 1 (Nrf1), Nrf2 and Mitochondrial transcription factor 1 (TFAM); synaptic and dendritic genes synaptophysin, PSD 95; APP-related genes like APP, BACE1, presenilin 1 (Table 1). We analyzed the data in two ways: (1) to determine the effect of A β , and ligand 1 (Table 2) in N2a cells and (2) to determine the preventive effects of ligand 1, if any, in the presence of mAPP (Table 3).

Comparison 1—untreated N2a cells versus N2a cells treated with ligand 1

Mitochondrial fission genes Drp1 (by 1.1-fold) and Fis1 (by 1.1-fold) were reduced, but no statistical significance (Table 2) in ligand 1 treated N2a cells relative to untreated cells. By contrast, fusion genes Mfn1 (by 1.1-fold), Mfn 2 (by 1.2-fold) and Opa1 (by 1.4-fold) were increased in ligand 1 treated cells relative to ligand

1 untreated cells (Table 2). Both mitochondrial biogenesis and synaptic genes were increased in ligand 1 treated cells relative to untreated cells; however, mRNA levels of PGC1 α significantly increased (by 1.6 fold, $P<0.05$). mRNA levels of BACE1 (by 1.2-fold), APP (by 1.8-fold) and PS1 (by 1.3-fold) were decreased in ligand 1 treated N2a cells relative to untreated cells.

As shown in Table 2, mitochondrial fission genes Drp1 (by 2.3-fold, $P<0.005$) and Fis1 (1.3-fold) were increased and fusion genes Mfn1 (by 1.7, $P<0.05$), Mfn2 (1.3-fold) and Opa1 (1.3-fold) were reduced in mAPP cells relative to N2a cells. Mitochondrial biogenesis genes PGC1 α (by 1.7-fold, $P<0.05$), Nrf1 (by 1.7-fold, $P<0.05$), Nrf2 (by 1.5-fold $P<0.05$) and TFAM (by 1.5-fold, $P<0.05$) were significantly reduced in mAPP cells relative to N2a cells. These observations indicate that mutant APP reduces mitochondrial biogenesis activity. mRNA levels of synaptic genes synaptophysin (by 1.5-fold, $P<0.05$), and PSD95 (by 1.3-fold) were reduced in mAPP cells relative to N2a cells. As expected, mRNA levels of BACE1 (by 1.5-fold, $P<0.05$), APP (by 2.1-fold, $P<0.005$) and PS1 (by 1.3-fold) were increased in mAPP cells relative to N2a cells (Table 2).

Comparison 2—mAPP versus mAPP + ligand 1

The levels of mRNA expression of mitochondrial fission genes Drp1 (by 2.3-fold, $P<0.005$), Fis1 (by 1.5-fold, $P<0.05$) were significantly reduced in mAPP cells treated with ligand 1 relative to mAPP cells (Table 3). On the other hand, fusion genes Mfn1 (by 1.3-fold), Mfn 2 (by 1.5-fold, $P<0.05$) and Opa1 (by 1.2-fold) were reduced in mAPP cells with treated ligand 1 relative to mAPP cells (Table 3). These observations indicate that ligand 1 reduces mitochondrial fission activity and enhances fusion activity in mAPP cells.

Figure 3. A BACE1 molecular docking studies with ligands. (A) The predicted docking structures of lead molecules with the target protein based on lamarkin geometric algorithm and PyRx analyses: the five best leads, which include (ligand 1) CID 10008594, (ligand 2) CID 10048142, (ligand 3) CID 10054794, (ligand 4) CID 100031313 and (ligand 5) CID 10034005. Crystal structure of BACE1 superposed on the docking predictions of top ranking ligands models (1–5). (B) These five ligands lead H-bond interactions displayed in yellow dashed lines and ligand molecules in cyan color stick models purple color residues with labels. (C) Based on the molecular docking studies, drug-likeness and ADMET predicted only three ligands models, i.e. 1, 4 and 5 considered and one ligand 1 highlighted in red colored box selected for in vitro cell culture studies.

As shown in Table 3, mitochondrial biogenesis, genes PGC1 α (by 2.0-fold, $P < 0.005$), Nrf1 (by 2.1-fold, $P < 0.005$), Nrf2 (by 2.1-fold, $P < 0.005$) and TFAM (by 1.8-fold, $P < 0.05$) were reduced in mAPP cells treated with ligand 1 treated cells relative mAPP cells.

These observations indicate that ligand 1 increases mitochondrial biogenesis activity in the presence of mutant APP.

In mRNA, expression levels of synaptic genes synaptophysin (by 1.9-fold, $P < 0.05$), and PSD95 (by 1.3-fold, $P < 0.05$) were

Figure 4. BACE1 activity and Aβ40, 42 levels. (A and B) Using Sandwich ELISA, we measured BACE1 activity levels in mAPP cells, mAPP treated with ligand 1, significantly lower levels of BACE1 activity with ($P=0.001$). (C) Represents the levels of soluble Aβ40 levels. Ligand 1 treated mAPP cells showed significantly lower in mAPP cells ($P=0.01$) compared with untreated mAPP cells. (D) Represents the levels of soluble Aβ42 levels. Ligand 1 treated mAPP N2a cells showed significantly lower levels of Aβ42 ($P=0.005$) compared with untreated mAPP cells.

Figure 5. Details of cell viability (MTT) assay. Cell was assessed between mouse neuroblastoma (N2a) cells and N2a cells treated with ligand 1, mAPP cells and mAPP cells treated with ligand 1. Significantly increased cell viability levels were found in N2a cells treated with ligand 1 relative to N2a cells.

increased in mAPP cells treated with ligand 1 relative to mAPP cells. These observations indicate that ligand 1 improves the synaptic activity in mAPP cells.

mRNA levels were significantly decreased for APP (by 1.5-fold, $P < 0.05$), BACE1 (1.4-fold, $P < 0.05$) and PS1 (1.5-fold, $P < 0.05$) in ligand 1 treated mAPP cells relative to mAPP cells. These observations indicate that ligand 1 reduces abnormal APP processing activity.

Immunoblotting analysis

To determine the effects of ligand 1 on protein levels of BACE1, full length (FL) APP and Aβ, we quantified proteins levels of BACE1, FL APP and CTFs (C99 and C83) in four independent treatments of cells with N2a cells, N2a+ligand 1, mAPP and mAPP+ligand 1.

Mutant FL-APP and CTFs (CTF99, CTF83) levels were significantly reduced in ligand 1 treated cells, FL-APP ($P=0.03$); CTF-99

Table 1. Summary of qRT-PCR oligonucleotide primers used in measuring mRNA expression of mitochondrial dynamics, mitochondrial biogenesis, synaptic and APP-related genes in untreated-N2a, cDNA mAPP-N2a, ligand1-N2a and cDNA mAPP + ligand 1 cells

Gene	DNA sequence (5'-3')	PCR product size
Mitochondrial dynamics genes		
Drp1	Forward primer ATGCCAGCAAGTCCACAGAA Reverse primer TGTCTCGGGCAGACAGTTT	86
Fis1	Forward primer CAAAGAGGAACAGCGGGACT Reverse primer ACAGCCCTCGCACATACTTT	95
Mfn1	Forward primer GCAGACAGCACATGGAGAGA Reverse primer GATCCGATTCCGAGCTTCCG	83
Mfn2	Forward primer TGCACCGCCATATAGAGGAAG Reverse primer TCTGCAGTGAAGTGGCAATG	78
Opa1	Forward primer ACCTTGCCAGTTTAGCTCCC Reverse primer TTGGGACCTGCAGTGAAGAA	82
Mitochondrial biogenesis genes		
PGC1 α	Forward primer GCAGTCGCAACATGCTCAAG Reverse primer GGAACCCCTTGGGGTCATTT	83
Nrf1	Forward primer AGAAACGGAAACGGCCTCAT Reverse primer CATCCAACGTGGCTCTGAGT	96
Nrf2	Forward primer ATGGAGCAAGTTTGGCAGGA Reverse primer GCTGGGAACAGCGGTAGTAT	96
TFAM	Forward primer TCCACAGAACAGCTACCCAA Reverse primer CCACAGGGCTGCAATTTTCC	84
Synaptic genes		
Synaptophysin	Forward primer CTGCGTTAAAGGGGGCACTA Reverse primer ACAGCCACGGTGACAAAGAA	81
PSD95	Forward primer CTTTCATCCTTGCTGGGGGTC Reverse primer TTGCGGAGGTCAACACCATT	90
APP-related genes		
APP-human	Forward primer TGGAGGTACCCACTGATGGT Reverse primer TGTGCATGTTCACTCTGCCA	81
BACE1	Forward primer GCGAATTGGCTTTGCTGTCA Reverse primer TGTCTGCCGTAACAAACGGA	86
Presenilin1	Forward primer AGACCTACAATGTCGCCGTG Reverse primer AGTGGATGGCAATCATCCCG	84
Housekeeping genes		
B-actin	Forward primer AGAAGCTGTGCTATGTTGCTCTA Reverse primer TCAGGCAGCTCATAGCTCTTC	91
GAPDH	Forward primer TTCCCGTTTCAGCTCTGGG Reverse primer CCCTGCATCCACTGGTGC	59

Table 2. Fold changes of mRNA expression in mitochondrial dynamic, biogenesis, synaptic and APP-related genes in N2a + ligand 1, N2a + mAPP and N2a + mAPP + ligand 1 cells relative to the untreated-N2a cells

Genes		mRNA fold changes		
		Ligand 1	mAPP	mAPP + ligand 1
Mitochondrial structural genes	Drp1	-1.1	-2.3**	1.1
	Fis1	-1.1	1.3	1.0
	Mfn1	1.1	-1.7*	-1.2
	Mfn2	1.2	-1.3	1.1
	OPA1	1.4	-1.3	1.1
Biogenesis genes	PGC1 α	1.6*	-1.7*	1.2
	Nrf1	1.2	-1.7*	1.2
	Nrf2	1.3	-1.5*	1.4
	TFAM	1.2	-1.5*	1.5
	Synaptophysin	1.4	-1.5*	1.3
Synaptic genes	PSD95	1.2	-1.3	1.3
	APP	-1.2	2.1**	1.3
APP-related genes	BACE1	-1.8**	1.5*	1.1
	Presenilin1	-1.3	1.3	-1.2

*P < 0.05; **P < 0.005.

Table 3. Fold changes of mRNA expression in mitochondrial dynamic, biogenesis, synaptic and APP-related genes in N2a + mAPP + ligand 1 cells relative to the mAPP N2a cells

Name of the gene	Genes	mRNA fold changes mAPP + ligand 1
Mitochondrial structural genes	Drp1	−2.3**
	Fis1	−1.5*
	Mfn1	1.3
	Mfn2	1.5*
	OPA1	1.2
Biogenesis genes	PGC1 α	2.0**
	Nrf1	2.1**
	Nrf2	2.1**
	TFAM	1.8*
Synaptic genes	Synaptophysin	1.9*
	PSD95	1.8*
APP-related genes	APP	−1.5*
	BACE1	−1.4*
	Presenilin1	−1.5*

* $P < 0.05$; ** $P < 0.005$.

($P = 0.001$); CTF-83 ($P = 0.002$) relative to ligand 1 untreated mAPP cells (Fig. 6).

Relative control N2a cells, ligand 1 treated 1 N2a cells showed significantly reduced BACE1 levels ($P = 0.01$). On the other hand, mAPP cells showed significantly increased BACE1 levels ($P = 0.001$) relative to control N2a cells. BACE1 levels were also significantly reduced in ligand 1 treated mAPP cells ($P = 0.002$) relative to ligand 1 untreated mAPP cells (Fig. 6).

In mAPP cells compared with ligand 1 treated mAPP cells, Drp1 protein levels were significantly decreased ($P = 0.01$) and Fis1 ($P = 0.001$) (Fig. 7A). In contrast, increased levels of mitochondrial fusion proteins, Mfn1 ($P = 0.002$), Mfn2 ($P = 0.002$) and Opa1 ($P = 0.01$), were found in mAPP cells treated with ligand 1 compared with mAPP cells (Fig. 7A).

As shown in Figure 7B, mitochondrial biogenesis proteins PGC1 α ($P = 0.01$); Nrf1 ($P = 0.004$); Nrf2 ($P = 0.004$) and TFAM ($P = 0.01$) were increased in mAPP cells with ligand 1 relative to mAPP cells, indicating that ligand 1 reverse the mitochondrial biogenesis.

The levels of synaptic and dendritic protein levels, synaptophysin ($P = 0.02$), PSD95 ($P = 0.02$) and MAP 2 ($P = 0.04$), were significantly increased in mAPP cells treated with ligand 1 relative to mAPP cells (Fig. 7C).

Immunofluorescence analysis of BACE1, 6E10 and CTF

To determine the effect of ligand 1 on BACE1, 6E10 and C-terminal fragments, immunofluorescence analysis was performed on four different groups of cells: (1) untreated N2a cells versus; (2) N2a + ligand 1; (3) cells transfected with mAPP and (4) mAPP cells treated with ligand 1.

Comparison with untreated cells

mAPP and CTFs, BACE1 levels were significantly reduced in ligand 1 treated cells relative to mAPP cells, BACE1 ($P = 0.01$); 6E10 ($P = 0.01$); CTF ($P = 0.01$) (Fig. 8A), indicating that ligand 1 reduces mutant APP/A β levels, BACE1 and CTF.

Transmission electron microscopy

To determine the effects of ligand 1 on the number and length of mitochondria, we used transmission electron microscopy (TEM) on cells with untreated N2a cells, N2a + mAPP, mAPP and mAPP cells treated with ligand 1.

Mitochondrial number in ligand 1 treated N2a cells

Significantly increased number of mitochondria were found in mAPP cells ($P = 0.002$) relative to N2a cells (Fig. 8B). Relative to mAPP cells, ligand 1 treated mAPP cells showed significantly reduced number of mitochondria ($P = 0.01$), indicating that ligand 1 reduces mitochondrial fission activity.

Mitochondrial length in ligand 1-treated N2a cells

Mitochondrial length was also measured, in order to understand whether ligand 1 treatment alters mitochondrial length (represented in μm) in both N2a cells and mAPP cells. As shown in (Fig. 8B), mitochondrial length was significantly increased in control N2a cells treated with ligand 1 ($P = 0.02$) relative to untreated cells. Mitochondrial length was significantly reduced in mAPP cells ($P = 0.01$) relative to N2a cells. Mitochondrial length was increased in ligand 1 treated mAPP cells relative to mAPP cells, but not significant.

Discussion

Using bioinformatics and molecular docking tools, we investigated pharmacophore ligands for BACE1. BACE1 protein contains many catalytic residues, which serve as targets for new drugs. In order to develop drugs for these targets, we identified catalytic core domain active site of BACE1 through CASTp analysis. The resultant CASTp calculations revealed that major core catalytic residues in domain 1 are located at binding pockets, i.e. 29, 31 and these pocket motifs are ASP 32, GLY 34, SER 35, SER 36, ASN 37, VAL 69 and ARG 128. The powerful protease ligand pepstatin (aspartyl protease inhibitor) selected as the pharmacophore ligand for BACE1 screening. Ligand Scout pharmacophore analysis of BACE1 in presence of pepstatin gave the three best ligand associated pharmacophore residues: ASP 32, THR33 and SER 35. These three key residues set to filter from the LigandScout screening, resulting the outcome with 16 best-fitted ligands. Furthermore, molecular docking studies performed for the 16 ligands to find best BACE1 small molecule inhibitor precisely, through molecular docking studies. The molecular docking tool PyRx optimized with 16 pharmacophore ligands, with minimal binding energies and interacted residues. Molecular docking studies of BACE1 results gives the best lead molecules based on docking energies. We selected the top five ligands as best lead

Figure 6. Immunoblotting analysis of FL-APP, CTFs C99 and C83 and BACE1. Mouse neuroblastoma (N2a) cells were transfected with mutant APP cDNA and immunoblotting analysis was conducted using protein lysates from transfected with mAPP (1) N2a cells, (2) N2a + ligand 1, (3) mAPP and (4) mAPP + ligand 1 cells. A protein band of 110 kDa of full-length APP was found in N2a cells transfected with mutant APP cDNA, but not N2a cells treated and untreated with ligand 1. Significantly decreased full-length APP was found in mAPP cells treated with ligand 1 ($P=0.03$). BACE1 ($P=0.002$) and CTFs C99 ($P=0.001$) and C83 ($P=0.002$) were significantly decreased in ligand 1 treatment in mAPP cells relative to ligand 1 untreated mAPP cells.

molecules with docking scores and hydrogen bonding with the BACE1 protein. These top five ligand lead molecules expected to inhibit the of BACE1 activity by inhibiting abnormal APP processing and A β toxicity in AD neurons.

The chemical library screening of PubChem relative structures of pepstatin around 873 molecules was set for pharmacophore screening. Only 16 pharmacophore ligands selected for the molecular docking studies. Further, only five lead molecules considered as best ligands based on the best binding energy and molecular interactions. Ligand molecular interactions with BACE1 were as follows: CID 10008594 with ASP32, THR 33, ARG 56; CID 10048142 with ASP 32, THR 33, LYS 107; CID 10054794 with THR 33, TYR 51, THR 33; CID 100031313 with THR 33, SER 35, THR 95 and CID 100034005 with ASP 32, TYR 123, GLU 134. The complete ligand bond length and docking energies are listed in (Table 4). The complete pharmacophore core site, where BACE1 inhibition takes place is most likely within the domain1 region, involving catalytic residues ASP 32, GLY 34, SER 35, SER 36, ASN 37 and ARG 128. The five selected ligand-protein interactions are visualization illustrated in (Fig. 3A–C).

Drug likeness is a basic property for prospective drugs and includes Lipinski's rule of five (molecular weight, log P-value, no. of rotatory bonds and no. of H-donor 5 and acceptors 10). Ligand properties were tested Lipinski rule of five by submitting to Molsoft server to predict drug-likeness properties for top ranking five ligands. Only CID 1008594, CID 100031313, CID 10054794 and CID 10034005 showed good drug-likeness properties with likeness of 1.21, 0.65, 0.31 and 0.30, respectively. The remaining ligand, CID 10048142, showed non-drug like property with -0.69 (Fig. 9).

All five top molecules were tested again for ADMET, using admetSAR prediction for the toxicity to confirm their toxic properties. The admetSAR web-based analysis gave selected ligands based on the canonical SMILE notation of molecules. Among the best five molecules, only three molecules qualified, i.e. CID 1008594, CID 100031313 and CID 10034005 showing the non-toxic and good lead properties (Table 5A). Based on all the above aspects of proposed small molecules, only three presented useful and important properties. These included sufficient blood-brain barrier transported capabilities (Table 5b), and computational validations like molecular docking, ADMET/TOX, admetSAR and drug like properties. The most promising, ligand 1 suggested for *in vitro* validations.

Using cell cultures, the protective effects of ligand 1 were studied in the presence and absence of A β . We measured mRNA and protein levels of mitochondrial dynamics, mitochondrial biogenesis, synaptic genes using real time RT-PCR and immunoblotting and immunofluorescence analysis. A β found to impair the mitochondrial dynamics (increased fission and decreased fusion). Mitochondrial biogenesis, synaptic activities and mitochondrial function were abnormal in mAPP cells. On the other hand, ligand 1 treated cells showed enhanced fusion activity and reduced fission machinery and increased biogenesis and synaptic proteins. Mitochondrial function and cell viability were elevated in ligand 1 treated cells. Interestingly, ligand 1 treated mAPP cells showed reduced mitochondrial dysfunction, maintained cell viability and mitochondrial dynamics, mitochondrial biogenesis and synaptic activity.

A β 40 and 42 levels were significantly reduced in ligand 1 treated mAPP cells (Fig. 4C and D) relative to ligand 1 untreated

Figure 7. Representative immunoblotting analysis of ligand 1 protection of mitochondrial structural proteins. (A) Representative immunoblots of mouse neuroblastoma (N2a) cells were transfected with mutant APP cDNA and ligand 1 + mAPP treated cells immunoblotting analysis was conducted using protein lysates from transfected with mAPP (1) N2a cells, (2) N2a + ligand, (3) mAPP and (4) ligand 1 + N2a cells with mutant A β PP cDNA. (B) Quantitative densitometry analysis of mitochondrial dynamics proteins Drp1 ($P = 0.01$) and Fis1 ($P = 0.001$) were significantly decreased; and the fusion proteins Mfn1 ($P = 0.002$), Mfn2 ($P = 0.02$) and Opa1 ($P = 0.01$) were significantly increased in ligand 1 treated mAPP cells relative to mAPP cells, indicating the presence of ligand 1 protective mitochondrial dynamics in mAPP cells. (C) Quantitative densitometry analysis of mitochondrial biogenesis proteins biogenesis proteins PGC1 α ($P = 0.01$), Nrf1 ($P = 0.004$), Nrf2 ($P = 0.004$) and TFAM ($P = 0.001$) were significantly increased in ligand 1 treated mAPP cells relative to mAPP cells, indicating that ligand 1 enhances mitochondrial biogenesis in mAPP cells. (D) Quantitative densitometry analysis of synaptic proteins Synaptophysin ($P = 0.02$), PSD95 ($P = 0.02$) and MAP 2 ($P = 0.04$) were significantly increased in ligand 1 treated mAPP cells relative to mAPP cells, indicating the presence of ligand 1 protective neuronal protection in mAPP cells.

mAPP cells. These observations are interesting and may have therapeutic value for AD. It is possible that ligand 1 blocks/reduces the activity of the BACE1 cleavage site (that is responsible for A β 40 and 42 production) at C-terminal region of A β in mutant APP cells. Additional research is still needed in order to determine how ligand 1 reduces the levels of A β 40 and 42 in APP mouse models.

It is well established that A β causes lower cell survival in AD neurons (49). In the current study, we found mAPP cells showed reduced cell survival. On the other hand, ligand 1 molecule enhanced cell survival in mAPP cells. This effect presents strong evidence that ligand 1 is protective against AD neurons. These observations warrant further *in vivo* studies using mouse models of AD.

In cells treated with ligand 1, mitochondrial biogenesis, mitochondrial fusion and synaptic activities were enhanced, mitochondrial fission activity was reduced, suggesting that ligand 1 prevents mitochondrial structural, biogenesis and synaptic genes from expressing abnormally. Overall, ligand 1 appears to inhibit BACE1 activity and protect the mitochondrial structure by regulating mitochondrial fission and fusion activities. Furthermore, the protective effects of ligand 1 need to

be tested in APP transgenic mouse models to validate the *in vivo* efficacy of ligand 1 for therapeutic treatments of AD.

In summary, mutant APP cells treated with ligand 1 were found to have reduced levels of BACE1 activity, FL mutant APP and decreased A β 40 and 42 levels. Ligand 1 treated cells showed reduced mRNA and proteins levels of mitochondrial fission genes; and increased levels of mitochondrial fusion, biogenesis and synaptic genes relative to neurons transfected with mAPP alone. This indicates that ligand 1 is a BACE1 inhibitor and protective against A β -mitochondria- and synaptic-induced toxicities in AD neurons. To our knowledge, this is the first small molecule report of a (pepstatin-based ligand mechanism) capable of recapitulating catalytic APP inhibition in AD neurons. These observations strongly suggest further verification using APP transgenic mouse models before clinical studies using AD patients.

Why BACE ligand 1 is better than BACE inhibitors: Currently, BACE1 inhibitors failed at clinical trials; however, our approach is different and we used pharmacophore based ligand(s) with supramolecular properties that directly binds to specific biological targets of BACE1. In the current study, we selected a template ligand (pepstatin) as well-known aspartic protease inhibitor that

Figure 7. Continued

selectively targets BACE1 activity. Our current study ligand tested in control and mutant APP cells. Our proof concept of ligand 1 study showed reduced BACE1 activity, reduced A β 40 and 42 levels and enhanced mitochondrial biogenesis and synaptic activities. This approach is better and worth testing in different stages of disease progression in APP transgenic mice and ongoing study findings will provide new insights for BACE1 in AD pathogenesis.

Materials and Methods

Computational screening and validations

Active site CASTp calculations. Prediction of active site of the proteins provides valuable information regarding the ligand binding sites and active sites of functional residues. Amyloidogenic pathway protein BACE1 active site information achieved through the CASTp calculations (50). CASTp calculations of BACE1 provide a valuable information and

core tunnels with functional residues. CASTp measures the BACE1 associated with structural pockets and cavities based on weighted delaunay triangulation and the alpha complex geometry algorithm. BACE1 illustrations and the listing of wall atoms and mouth atoms for each pockets were visualized in CASTp calculations (50).

Ligands selection screening. The aspartyl protease inhibitors like pepstatin (3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid are very well-known inhibitors of aspartic proteinase with the most literature available (Fig. 1A). However, very few experimental validations of BACE1 inhibitors for pharmacological drug intervention are available. Molecular neuronal toxicity with pathological associations of mitochondrial interactions of A β proteins was poorly understood in AD brains (51). Hence, we attempted to design a pharmacophore model of pepstatin structurally similar to the ligand BACE1 inhibitors with proximal

Figure 7. Continued

validations (Fig. 1B). The pepstatin-based pharmacophore was set to find the best-fitted and molecular interactions with prospective BACE1 inhibitors to find better molecules to treat AD. Structurally the BACE1 protein contains two domains: one external domain, starting from 14 A to 144 A—the residues of domain1, and the second, from 149 to 389—the residues of domain 2. Here, AD pathology presents clear evidence that catalytic cleavage of external APP caused by the β -secretase leads to A β peptide. At domain 2 starting from 147, there is successive internal cleavage within the membrane processed by the second catalytic cleavage enzyme γ -secretase leading to the formation of A β peptides with different sizes. Pharmacophore design of BACE1 inhibitors is a novel method for diminishing the activity of APP formation, and is gaining special attention in AD drug discovery.

The structure of pepstatin (CID 5478883) an aspartic protease inhibitor used as reference ligand BACE1 inhibitor along with its related analogs were screened and from PubChem database (<http://pubchem.ncbi.nlm.nih.gov/>), which is a non-commercial storehouse of small compounds (52). In the structure, data format (.sdf) relative that small structure related of compounds of pepstatin was downloaded and prepared a chemical library data for pharmacophore-based screening.

Pharmacophore design. LS pharmacophore tool helps to automated construction of BACE1 3-D pharmacophore models of structural knowledge of macromolecules/inhibitor complexes (53). A pharmacophore derived model of BACE1 was developed

on the basis of X-ray crystal structures of protein in complex with pepstatin ligand (CID 5478883). Based on LS rule, compound options embody HBD and acceptors as focused vectors along with negative and positive ionizable spheres, furthermore as lipophilic regions are taken an account to create a pepstatin-BACE1 pharmacophore. Thus, the common pharmacophore features properties of all the generated list of ligands prepared a separate sheet, and used as the qualified ligands, in further molecular docking studies of BACE1 protein.

Molecular docking. Molecular docking studies were carried out by the Pyrx AutoDock Vina docking tool using 16 minimized pharmacophore leads molecules against the predicted binding site of the optimized conformation of the ligands through AutoDock in PyRx software (47). Active site dimensions were set as grid (XYZ-axis) size center X: 61.9702, Y: 29.2373, Z: -0.4286 and dimensions (angstrom) X: 58.6169, Y: 30.7325, Z: 48.9286 to dock with ligands. The molecular docking simulations were set with 10 maximum exhaustiveness for each ligand molecule in PyRx docking setup. The ligands binding energies, conformations and hydrogen-bond interactions, bond distance of amino acid residues were calculated through molecular 3D visualization tool PyMol.

In silico drug-likeness properties. Drug-likeness explorer Molsoft tool (<http://www.molsoft.com/mprop/>) works to investigate the best docking top five lead molecules were subjected for drug discovery process to test their drug-likeness, before going to

Figure 7. Continued

further *in vitro* evaluations of BACE1 inhibitors. Keeping in all aspects to lead molecules (ligands) must obey the Lipinski Rule of five (RO5), as well as drug like properties during the drug discovery process. The best leads compounds must qualify the other possible designate characters, as their molecular mass is a smaller amount than 500 Da. Lipophilicity of ligands expressed in an amount referred to as Log P is a smaller amount than five is referred. The number of groups within the compound that may give hydrogen atoms to hydrogen bonds must be a smaller amount than five. The number of groups that may settle for hydrogen atoms to make hydrogen bonds is a smaller amount than 10 were considered as best possible therapeutic ligand features.

AdmetSAR properties. The computational predictions of pharmacokinetic properties of ligands like those that drug-like properties and ADMET/TOX were investigated through online ADMET property explorer server AdmetSAR (54), must curate small compound libraries and perform computational prediction, with support computed atom contributions.

***In vitro* biological validation of ligands.** Based on the computational validations like molecular docking scores and better binding interactions, ADMET/TOX, drug-likeness properties ligand 1, showed good properties for further biological validations. Ligand 1 exhibited a good molecular docking score and better binding interactions compared with the other molecules. Therefore, we decided to quantify the biological effects of ligand 1 in AD pathogenesis. Therefore, we treated mutant

APP cells (N2a cells transfected mutant APP cDNA) with ligand 1 and quantified the effect of ligand 1 on Sandwich ELISA studies of A β 40 and 42, and confirm that ligand 1 is more promising in BACE1 inhibition in AD process. Furthermore, we tested ligand 1 properties by checking like cell viability, mRNA and protein levels of mitochondrial dynamics, biogenesis and synaptic genes, immunoblotting assays and immunohistochemistry and electron microscopy of mitochondrial morphology structure.

Cell culture work

Chemicals and reagents. The ligand 1 was purchased from TimTech, LCC Chemical Company (Newark, DE, USA); Mutant APPSwe/Ind cells: We purchased mutant APPSwe cDNA clone (pCAX-APP Swe/Ind) from Addgene. <https://www.addgene.org> and verified expression of mutant APP APPSwe/Ind cDNA and further sub-cloned into a mammalian expression vector; and N2a cells were purchased from American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM) and Minimum Essential Medium, penicillin/streptomycin, Trypsin-EDTA and fetal bovine serum were purchased from GIBCO (Gaithersburg, MD, USA).

The N2a cells grown for 6 days in a serum-free medium (1:1 mixture of DMEM and OptiMEM, plus penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) until the cells developed neuronal processes. As shown in (Fig. 10), these cells were used for four groups—one control group and three treatment groups. (1) untreated N2a cells (the control group), (2) N2a cells treated with ligand 1 (N2a + ligand 1), (3) N2a cells transfected

Figure 8. Immunofluorescence analysis of BACE1, 6E10 and CTF from transfected with mAPP cells. (A) Representative immunofluorescence images of BACE1 and Aβ in four cell types. (1) N2a cells, (2) N2a + ligand 1, (3) mAPP and (4) mAPP + ligand 1 N2a cells. Immunoreactivity levels of BACE1, 6E10 and CTF were significantly lower in the ligand 1-treated mAPP N2a cells relative to the untreated mAPP N2a cells, BACE1 ($P=0.01$), 6E10 ($P=0.01$) and CTF ($P=0.01$) indicating that ligand 1 reduces mutant APP and soluble/insoluble Aβ in mAPP N2a cell cultures. (B) Representative electron microscopy images of four groups, i.e. (1) N2a cells, (2) N2a + ligand 1, (3) mAPP and (4) mAPP + ligand 1 N2a cells. With mutant AβPP cDNA, mitochondrial number significantly increased in mAPP cells ($P=0.002$) relative to N2a cells; mitochondrial number is significantly reduced mAPP cells treated with ligand 1 ($P=0.01$). Mitochondrial length was significantly increased in ligand 1 treated cells N2a cells relative to untreated cells. On the other hand, mitochondrial length was significantly reduced in mAPP cells ($P=0.01$) relative to N2a cells.

Table 4. Docking table with bonding characterization and binding energies in kcal/mol with top five therapeutic ligands involved in BACE1 inhibition

S.No	Ligand ID	Structures	Molecular Formula	Residues interaction	Bond length (Å)	Bonding energy (Kcal/Mol)
1.	CID 10008594		C ₃₃ H ₄₈ Cl ₂ N ₂ O ₃	ASP32, ARG 56, THR 33	(3.97), (3.13), (4.23)	-8.2
2.	CID 10048142		C ₂₀ H ₂₄ Cl ₂ N ₂ O ₄	ASP 32, THR 33, LYS 107	(3.39), (3.98), (3.28)	-7.8
3.	CID 10054794		C ₃₅ H ₃₁ Cl ₂ NO ₆	THR 33, TYR 51, THR 33	(4.37), (3.44, 2.90), (3.04, 3.15, 3.18)	-7.4
4.	CID 100031313		C ₁₄ H ₁₆ ClFN ₄ O	THR 33, SER 35, THR 95	(2.22), (3.03), (3.02, 3.52)	-6.9
5.	CID 100034005		C ₂₅ H ₂₇ NO	ASP 32, TYR 123, GLU134	(3.42), 3.47,(3.11)	-6.8

Figure 9. The Molsoft drug likeness prediction for top five ranking ligand molecules (ligand 1) CID 10008594, (ligand 2) CID 10048142, (ligand 3) CID 10054794, (ligand 4) CID 100031313 and (ligand 5) CID 10034005. Blue curve represents the non-drugs, and purple color curve represents the drugs and vertical red line indicates the ligand property. From all the five ligand molecules drug likeness property plots, only one (ligand 2) CID 10048142 shows in negative value highlighted with (red color) circle, remaining all ligands showed good drug likeness properties in Molsoft property explorer.

Table 5. (a) Molsoft results tabulated for top five BACE1 ligands molecular properties including Lipinski rule of FIVE (RO5) and drug-likeness. (b) admetSAR toxicity results for top five BACE1 ligands properties including ADMET/TOX data

S. No.	Property	CID 1008594	CID 10048142	CID 10054794	CID 100031313	CID 10034005
(a) Molsoft						
1.	Molecular formula	C ₃₃ H ₄₈ Cl ₂ N ₂ O ₃	C ₂₀ H ₂₄ Cl ₂ N ₂ O ₄	C ₃₅ H ₃₁ Cl ₂ NO ₆	C ₁₄ H ₁₆ ClFN ₄ O	C ₂₅ H ₂₇ NO
2.	Molecular weight	442.19	426.11	631.15	310.10	357.21
3.	Number of HBA	5	4	6	3	1
4.	Number of HBD	1	0	2	1	1
5.	Mol LogP	5.44	3.94	7.61	2.40	5.79
6.	Molecular polar surface area A ²	59.28	53.99	80.72	49.29	24.01
7.	Drug-likeness model score	1.21	-0.69	0.65	0.31	0.34
(b) admetSAR						
1.	Blood-brain barrier	BBB+	BBB+	BBB+	BBB+	BBB+
2.	Human ether-a-go-go-related gene inhibition	Weak inhibitor	Weak inhibitor	Strong inhibitor	Weak inhibitor	Weak inhibitor
3.	AMES toxicity	Non-AMES toxic	Non-AMES toxic	AMES toxic	Non-AMES toxic	Non-AMES toxic
4.	Carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens	Non-carcinogens
5.	Fish toxicity	High FHMT	High FHMT	High FHMT	High FHMT	High FHMT
6.	Acute oral toxicity	III	III	III	III	III
7.	Aqueous solubility	-3.9083	-3.1683	-4.2638	-2.6624	-3.1052

Figure 10. Experimental design of BACE1 inhibition and protective effects of mitochondria structural integrity and biogenesis of ligand 1 in AD.

mAPP with the (lipofectamine) (20 μ M final concentration for 6 h (mAPP) and (4) N2a cells transfected with mAPP cDNA for 6 h, treated with ligand 1 (mAPP + ligand 1 treatment group), N2a cells treated with ligand 1 for 24 h and then treated with the mAPP cDNA for 6 h (ligand 1 + mAPP treatment group). As shown in Figure 10, we performed four independent cell cultures and treatments for all experiments ($n=4$).

Sandwich ELISA for A β levels. Soluble A β 40 and 42 levels were measured using sandwich ELISA as described in Manczak *et al.* (55). Briefly, protein lysates were from cell pellets in a Tris-buffered saline (pH 8.0) containing protease inhibitors [20 mg/ml

pepstatin A, aprotinin, phosphoramidon and leupeptin; 0.5 mM phenylmethanesulfonyl fluoride and 1 mM ethyleneglycol-bis (flaminoethyl ether)-NN tetra acetic acid]. Samples were sonicated briefly and centrifuged at 10 000g for 20 min at 4°C. The soluble fraction was used to determine the soluble A β by ELISA. For each sample, A β 1–40 and A β 1–42 were measured with commercial colorimetric ELISA kits (Biosource International, Camarillo, CA, USA) specific for human. A 96-well plate was used, following the manufacturer's instructions. Each sample was run in duplicate. Protein concentrations of the homogenates were determined following the BSA method, and A β was expressed as pg A β /mg protein.

Cell viability (MTT assay). In ligand 1 treated N2a cells, MTT assay was performed using Cellometer Vision CBA Image Cytometry System (Nexcelom Bioscience LLC, Lawrence, MA) with two fluorophore Annexin V-FITC and propidium iodide (PI) staining solution, according to manufacturer's instructions. Briefly, cells were harvested using trypsin, then spin down at 300g for 3 min and pellets were washed with 1XPBS, cells were counted using hemacytometer. Collected 100 000–150 000 cells and cells/pellet was resuspended in 40 µl of Annexin V binding buffer. About 5 µl each of Annexin V—FITC reagent (green) and PI (red) were added to binding buffer containing cells; gently mix solution by pipetting up and down 10 times, then incubate for 15 min at RT in the dark; after incubation, add 250 µl of 1XPBS and spin down at 300g for 3 min, then re-suspended the cell pellets in 50 µl of (Annexin V) binding buffer, then assess the cells apoptosis. Gate purple represents live cells, gate green represents the positive apoptotic cells, gate blue represents for the detection of positive necrotic cells and gate red represents debris.

mRNA levels of mitochondrial dynamics, mitochondrial biogenesis and synaptic genes. Using the reagent TriZol (Invitrogen), we isolated total RNA from four independent treatments of N2a cells ($n = 4$) from control (untreated N2a cells) and experimental treatments (N2a cells treated with ligand 1; N2a cells transfected with mAPP; N2a cells transfected with mAPP and then treated with ligand 1). Using primer express Software (Applied Biosystems), we designed the oligonucleotide primers for the housekeeping genes β -actin, GAPDH; APP-related genes (APP, BACE1 and PSN1); mitochondrial structural genes; fission (Drp1 and Fis1); fusion genes (MFN1, MFN2 and Opa1) [54], mitochondrial biogenesis genes (PGC1a, Nrf1, Nrf2 and Tfam) [56] and synaptic proteins (SYN, PSD95 and MAP 2). The primer sequences and amplicon sizes are listed in (Table 1). With SYBR-Green chemistry-based quantitative real-time RT-PCR, we measured mRNA expression of the genes mentioned above as described by Manczak et al. [56]. Briefly, 2 µg of DNase-treated total RNA was used as starting material, to which we added 1 µl of oligo (dT), 1 µl of 10 mM dNTPs, 4 µl of 5 $\times$ first strand buffer, 2 µl of 0.1 M DTT and 1 µl RNase out. The reagents RNA, dT and dNTPs were mixed first, then heated at 65°C for 5 min, and finally chilled on ice until the remaining components were added. The samples were incubated at 42°C for 2 min, and then 1 µl of Superscript II (40 U/µl) was added. The samples were then incubated at 42°C for 50 min, at which time the reaction was inactivated by heating at 70°C for 15 min.

Quantitative real-time PCR amplification reactions were performed in N2a cells in an ABI Prism 7900 sequence-detection system (SDS; Applied Biosystems, Foster City, CA) in a 25-µl volume of total reaction mixture. The reaction mixture consisted of 1 $\times$ PCR buffer containing SYBR-Green; 3 mM MgCl₂; 100 nM of each primer; 200 nM of dATP, dGTP and dCTP each; 400 nM of dUTP; 0.01 U/µl of AmpErase UNG and 0.05 U/µl of AmpliTaq Gold. A 20-ng cDNA template was added to each reaction mixture.

The C_T -values of β -actin was tested to determine the unregulated endogenous reference gene in N2a cells. The C_T -value is an important quantitative parameter in real-time PCR analysis as described in Gutala and Reddy [57]. All RT-PCR reactions were carried out in triplicate, with no template control. The PCR conditions were: 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescent spectra were recorded during the elongation phase of each PCR cycle.

To distinguish specific amplicons from non-specific amplifications, a dissociation curve was generated. The C_T -values were calculated with SDS software V1.7 (Applied Biosystems) and an automatic setting of base line, which was the average value of PCR, cycles 3–15, plus C_T generated 10 times its standard deviation. The amplification plots and C_T -values were exported from the exponential phase of PCR directly into a Microsoft Excel worksheet for further analysis.

The mRNA transcript level was normalized against β -actin at each dilution. The standard curve was the normalized mRNA transcript level, plotted against the log-value of the input cDNA concentration at each dilution. To compare β -actin, and ligand 1, relative quantification was performed according to the C_T method [Applied Biosystems [58, 59]]. Briefly, the comparative C_T method involved averaging triplicate samples, which were taken as the C_T values for β -actin, and ligand 1. β -actin normalization was used in the present study. The ΔC_T -value was obtained by subtracting the average β -actin C_T value from the average C_T -value for the ligand 1 treated cells for mitochondrial dynamics, biogenesis and synaptic genes. The ΔC_T of N2a cells was used as the calibrator. The fold change was calculated according to the formula $2^{-(\Delta \Delta C_T)}$, where $\Delta \Delta C_T$ is the difference between ΔC_T and the ΔC_T calibrator value.

To determine the statistical significance of mRNA expression in untreated N2a cells and N2a cells treated with ligand 1, and N2a cells transfected with mAPP cDNA, a combination of N2a + ligand 1, and mAPP + ligand 1, the C_T value difference between untreated N2a cells and treated N2a cells was used in relation to β -actin normalization, and statistical significance was calculated using one-way ANOVA.

Immunoblotting analysis. Immunoblotting analysis was performed using protein lysates prepared from N2a transfected and transfected mutant A β PP cDNA using 6E10 antibody that recognizes mutant full-length A β PP and A β , as described in Kumar et al., 2019 [59]. About 20 µg protein lysates were resolved on a 4–12% Nu-PAGE gel (Invitrogen). The resolved proteins were transferred to nylon membranes (Novax Inc., San Diego, CA, USA) and were then incubated for 1 h at room temperature with a blocking buffer (5% dry milk dissolved in a TBST buffer). The nylon membranes were incubated overnight with the primary antibody for mutant APP and CTFs (6E10-1500 monoclonal, BioLegend, San Diego, CA, USA). We used dilutions and conditions for mitochondrial biogenesis, dynamics and synaptic proteins as described in Manczak et al., 2019 [56]. The membranes were washed with a TBST buffer three times at 10-min intervals and were then incubated for 2 h with appropriate secondary antibody Sheep anti-mouse HRP 1:10 000, followed by three additional washes at 10-min intervals. Proteins were detected with chemiluminescence reagents (Pierce Biotechnology, Rockford, IL, USA), and the bands from immunoblots were visualized.

Immunofluorescence analysis of BACE1, 6E10 and CTF. To determine BACE1, and Abeta levels, immunofluorescence analysis was performed using mutant A β PP cells and untransfected N2a as described in [35]. The fixed cells were washed with warm PBS, fixed in freshly prepared 4% paraformaldehyde in PBS for 10 min, then washed with PBS and permeabilized with 0.1% Triton-X100 in PBS. They were blocked with a 1% blocking solution (Invitrogen) for 1 h at room temperature. All sections were incubated overnight with BACE1 (1:250 dilution, Abcam, Cambridge, MA, USA) and 6E10 (1:250 dilution, BioLegend, San Diego, CA, USA)

antibodies. After incubation, the cells were washed three times with PBS, for 10 min each. The cells were incubated with a secondary antibody conjugated with Fluor 488 (Invitrogen) for 1 h at room temperature. The cells were washed three times with PBS and mounted on slides. Photographs were taken with a multiphoton laser scanning for each treatment, 10–15 photographs were taken at $\times 40$ magnifications, and statistical significance was assessed, using one-way ANOVA.

Transmission electron microscopy of mitochondria. To determine the protective effects of ligand 1, the number and morphology of mitochondria and the rescue effects of ligand 1 on mutant APP/A β induced mitochondrial toxicity. We conducted TEM of N2a cells from control and experimental treatments ($n=4$). Treated and untreated cells were fixed in 100 mM sodium cacodylate (pH 7.2), 2.5% glutaraldehyde, 1.6% paraformaldehyde, 0.064% picric acid and 0.1% ruthenium red. They were gently washed and post-fixed for 1 h in 1% osmium tetroxide plus 0.8% potassium ferricyanide, in 100 mM sodium cacodylate, pH 7.2. After a thorough rinsing in water, the N2a cells were dehydrated, infiltrated overnight in 1:1 acetone: Epon 812, infiltrated for 1 h with 100% Epon 812 resin, and embedded in the resin. After polymerization, 60–80-nm thin sections were cut on a Reichert ultramicrotome and stained for 5 min in lead citrate. They were then rinsed and post-stained for 30 min in uranyl acetate, and then rinsed again and dried. EM was performed at 60 kV on a Philips Morgagne TEM, equipped with a CCD, and images were collected at original magnifications of $\times 1000$ – $\times 37\,000$. The numbers of mitochondria were counted in the N2a cells (number of cells = 50) and experimental groups (number of cells per group = 40), and statistical significance was determined, using one-way ANOVA.

Quantification and statistical analyses. Statistical analyses were conducted in two ways: (1) untreated cells versus cells transfected with mAPP, N2a + ligand 1, mAPP + ligand 1 and (2) cells treated with mAPP versus mAPP + ligand 1 for mRNA and protein levels, cell viability and mitochondrial structural and biogenesis using appropriate statistical analysis.

Data and software availability. The PDB files that support the findings of this study have been available in Protein Data Bank under accession codes 2ZHT.

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Conflict of Interest statement

None declared.

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