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ACAT INHIBITION AND AMYLOID BETA REDUCTION

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Abstract

Alzheimer's disease (AD) is a devastating neurodegenerative disorder. Accumulation and deposition of the beta-amyloid (Aβ) peptide generated from its larger amyloid precursor protein (APP) is one of the pathophysiological hallmarks of AD. Intracellular cholesterol was shown to regulate Aβ production. Recent genetic and biochemical studies indicate that not only the amount, but also the distribution of intracellular cholesterol is critical to regulate Aβ generation. Acyl-coenzyme A: cholesterol acyl-transferase (ACAT) is a family of enzymes that regulates the cellular distribution of cholesterol by converting membrane cholesterol into hydrophobic cholesteryl esters for cholesterol storage and transport. Using pharmacological inhibitors and transgenic animal models, we and others have identified ACAT1 as a potential therapeutic target to lower Aβ generation and accumulation. Here we discuss data focusing on ACAT inhibition as an effective strategy for the prevention and treatment of AD.

Keywords

Alzheimer's disease; Aβ; ACAT; Cholesterol; Cholesteryl ester; Lipid droplet

Introduction

Progressive neurodegeneration, memory loss, cognitive impairment and personality changes are the major clinical features of Alzheimer's disease (AD) [1,2]. At the molecular level, extracellular amyloid plaque and intracellular neurofibrillary tangle formation are defining lesions of AD. Initial amyloid β (Aβ) accumulation in the brain results in plaque formation at a later time [3–6].

Several epidemiological, molecular and biochemical studies link elevated cholesterol with the onset of AD [7,8]. Total serum cholesterol and low-density lipoprotein (LDL) cholesterol are elevated in AD patients, as compared to age-matched controls [9,10]. Low cholesterol diet may lower the risk of neurodegenerative disorders including AD [11]. Genetic studies have identified more than 50 genes that influence the onset of familial as well as sporadic AD [12]. Interestingly, many of these genes are important for cholesterol metabolism and transport. Identification of Apolipoprotein E (apo ε) gene as a major risk factor for late onset AD opened a new avenue connecting cholesterol with the disease [13–

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15]. ApoE is the major apolipoprotein in the central nervous system that functions as a ligand in receptor-mediated endocytosis of lipoprotein particles. ApoE delivers cholesterol and other essential lipid particles to neurons through members of low density lipoprotein receptors (LDLR) family to support neuronal metabolism, synaptogenesis and the maintenance of synaptic connections [16–18]. In humans, the apo ε gene exists in three different polymorphic forms apo ε2, ε3 and ε4. Among the three, apo ε4 is the strongest risk factor for late onset AD [13–15]. Statins, drugs inhibiting the synthesis of cholesterol, have long been used for reducing the levels of serum cholesterol. The initial discovery of the beneficial effect of statins in AD has further strengthened the connection between cholesterol and AD [19–21]. However, currently the use of statins in AD patients is controversial. Recent randomized controlled trials using a large number of patients could not confirm a therapeutic effect of statins against AD [22–25].

High intake of dietary cholesterol resulting in hypercholesterolemia was also shown to enhance amyloid β (Aβ) deposition in animal brains. Initial studies were performed in rabbits [26,27], and these were followed by transgenic mice [28–30] and guinea pigs [31], all showing a strong correlation between plasma cholesterol and Aβ levels. One study in particular analyzed both Aβ levels and APP processing in a transgenic mouse model of AD on high fat/high cholesterol diet [32]. APP processing was elevated, therefore the authors could conclude that high cholesterol increased Aβ generation as opposed to decreasing its catabolism. This effect could be reversed by cholesterol-lowering agents [30].

Recent experiments in cellular and animal models showed dramatic reduction of Aβ generation and deposition upon inhibition of the enzyme acyl-coenzyme A: cholesterol acyltransferase (ACAT), which is critical to maintain cholesterol homeostasis by converting free cholesterol to cholesteryl ester. Here we review the current evidence that supports the involvement of ACAT activity in cholesterol homeostasis, APP processing and Aβ generation.

1. ACAT in intracellular cholesterol homeostasis

In addition to the *de novo* cholesterol synthesis via the HMGCoA reductase pathway and ApoE-mediated cholesterol transport via LDLRs, cellular cholesterol is found in two forms: free cholesterol (FC) and cholesteryl esters (CE). The maintenance of the dynamic equilibrium between FC and CE is critical for cholesterol homeostasis. Overproduction of free cholesterol can be toxic to cells [33]. The ER-resident enzyme ACAT uses cholesterol and long-chain fatty acyl coenzyme A as substrates to convert FC into CE. Cholesteryl ester hydrolases (CEH) are responsible for the reverse reaction converting CE into FC [34,35]. ACAT and CEH act in opposite directions to maintain the dynamic equilibrium between FC and CE. ACAT has been the focus of intense research as the enzyme responsible for the generation of CEs in atherosclerotic plaques. CEH function is likely performed by many enzymes, which have not yet been positively identified in mammalian cells. FC is stored in membrane bilayers, whereas CEs are hydrophobic in nature and require a special environment to remain stable in aqueous cytoplasm. Lipid droplets surrounded by a phospholipid monolayer serve as a microdomain storing neutral lipids [36]. In addition to their storage function, evidence indicates that lipid droplets also carry proteins commonly found on the plasma membrane.

1.1 Acyl-coenzyme A: cholesterol acyl-transferase (ACAT) and cholesteryl esters

Two ACAT (*Acat1* or *SOAT1* and *Acat2* or *SOAT2*) genes have been identified in mammals [37–39]. The gene products, ACAT1 and ACAT2, are multiple membrane-spanning ER-resident proteins and share ~50% amino acid sequence homology. ACAT1 is the enzyme responsible for foam cell formation and is linked to increased coronary artery atherosclerosis

[40–42]. Thus, ACAT1 is considered a critical target for the treatment of atherosclerosis. However, while ACAT2 knock out hyperlipidemic mice are protected from atherosclerosis, ACAT1 null mice only show a minor improvement [43–45]. This is likely due to the essential role of ACAT2 in lipoprotein formation. Conventional biochemical approaches to compare the two isoforms become exceedingly difficult due to the lack of sufficient purified proteins in most preparations [46,47].

In hepatocytes and intestinal enterocytes, ACAT activity is responsible for the generation of CE in the ER lumen. This CE constitutes the neutral lipid core of secreted lipoproteins, such as VLDL or chylomicrons [48]. However, in most other tissues, ACAT is involved in generating cytoplasmic lipid droplets. ACAT1 is ubiquitously expressed in various tissues and cell types including hepatocytes, macrophages, adrenals, skin and neurons, whereas ACAT2 is expressed mostly in the small intestine and hepatocytes [49,50]. Therefore, studying the role of ACAT in the brain is restricted to ACAT1 only. Based on ACAT1/ACAT2 mRNA and protein distribution, Rudel *et al*. hypothesize that ACAT1 is involved in the synthesis of intracellular CE and ACAT2 functions by supplying the CE to LDL for lipoprotein assembly [51,52]. To accomplish this, ACAT1 and ACAT2 would have different topologies in the ER, targeting CEs to cytoplasmic lipid droplets or to lipoprotein assembly in the lumenal side of ER, respectively. Both enzymes are ER-resident protein, spanning the ER five or seven times [53,54]. Identification of the putative active site residues of ACAT1 and ACAT2 led to the conclusion that the amino acid requirement for ACAT activity may be different for the two enzymes [55,56]. The putative active site of ACAT1 was located to the cytoplasmic side of the ER, whereas the active site of ACAT2 is located at the lumenal side of the ER [53]. The fact that ACAT1 and ACAT2 can functionally complement each other [57] indicates that their membrane topologies are not fixed. While ACAT1 is an allosteric enzyme [48], its gene does not contain the sterol regulatory element (SRE) that is widely present within the promoter regions of many cholesterol-regulatory genes. Therefore, cholesterol may not directly regulate ACAT expression [58]. More interestingly, ACAT1 does not contain a sterol-sensing domain (SSD) [59], which is the cholesterol binding motif found in almost all cholesterol regulating proteins.

1.2 Intracellular lipid droplets

In most cell types, cytoplasmic lipid droplets are prevalently occupied by CEs. In adipocytes, however, triacylglycerides represent the main component of lipid droplets [60]. Adipocytes possess a unique morphology that allows for formation of large lipid droplets. These large droplets sometimes occupy almost the entire cell volume by pushing other intracellular compartments to the cell periphery [61]. Detailed studies on lipid droplet biogenesis and function in adipocytes are being conducted to understand how lipid droplets sequester excess CEs and triacylglycerides in metabolic diseases such as obesity, diabetes and atherosclerosis caused by genetic disorders or consumption of cholesterol-rich diet.

CEs are generated in the cytoplasmic leaflet of the ER or between the two leaflets, before being pinched off into highly mobile cytoplasmic lipid droplets [62]. Apart from being a fundamental component of lipid homeostasis, lipid droplets also act as critical organelles during development. Novel interaction of lipid droplets with microtubules is considered a crucial feature in developmentally regulated cellular positioning of *Drosophila melanogaster* [63]. Specific proteins are found in and around the ER membrane domains adjacent to the proposed site of lipid droplets biogenesis. These proteins are part of the PAT family, consisting of perilipin, adipophilin and TIP47 (tail-interacting protein of 47 kDa) [64,65]. A number of cellular proteins involved in vesicle trafficking, membrane fusion and cytoskeletal reorganization were found associated with the lipid droplets via direct or indirect interaction with the PAT proteins. The small GTPase ARF1, which is involved in Phospholipase D (PLD)-mediated cellular trafficking, binds adipophilin (also called ADRP)

and localizes to the lipid droplets [66]. Additional small GTPases, particularly belonging to the Rab family, are known to regulate membrane trafficking in the endocytic as well as other biosynthetic pathways [67]. The possibility that several Rab proteins may link lipid droplets with other intracellular organelles is under scrutiny [68]. Rab18 has gained particular attention because of its recruitment to lipid droplets upon stimulation of lipolysis by β-adrenergic receptor activation [69]. The interesting discovery of the localization of an N-terminal truncation mutant of caveolin, $Cav3^{DGV}$ [70], to the lipid droplets provided the initial evidence that caveolin plays a role in lipid droplet regulation. Regulated localization of endogenous caveolin-1 and caveolin-2 to the lipid droplets in cultured cells and regenerating liver was later confirmed [71,72]. Caveolins are membrane bound cell surface proteins involved in protein and membrane trafficking. It is unclear how caveolins move from the cell surface to the lipid droplets. The discovery of the PAT family proteins at the plasma membrane of macrophages and adipocytes indicates that lipid droplets may be transported to the plasma membrane [73,74]. In spite of several reports suggesting the role of lipid droplets in intracellular signaling, vesicle trafficking, protein degradation and temporal protein storage [75,76], experimental evidence is scarce.

While cholesterol-rich diet has been linked with the onset of neurodegenerative diseases including AD [11,27,77–80], very little is known about the role of lipid droplets in disease progression. Inefficient detection techniques hinder the study of lipid droplets in non-adipocytes. Lipid droplets in adipocytes can be easily detected by light microscopy because of their large size reaching more than 100 μm in diameter [36]. On the other hand, lipid droplets generated in non-adipocytes are not more than a few μm in diameter [73]. We have successfully identified the presence of lipid droplets in the cytoplasm of cultured cells by labeling formalin-fixed cells with HCS LipidTOX™ red neutral lipid stain ([81] and Fig. 1). Newer techniques such as freeze-fracture cytochemistry together with light and thin-layer electron microscopy are becoming instrumental to test lipid droplet structure and function [73]. Proton magnetic resonance revealed lipid droplets in rat intracerebral glioma with diameters ranging between 1.3 to 4.3 μm [82]. Interestingly, a recent report on Aβ-positive neurons of autopsied brain tissue from AD patient not only shows the presence of lipid droplets in neurons, but also shows a positive correlation between Aβ levels and lipid droplets [83]. Detection of lipid droplets in various tissues is the first step toward understanding their function and their role in various diseases including AD.

2. ACAT and Aβ generation

The brain is the organ with the highest cholesterol content. Almost all cholesterol in the brain is synthesized *de novo* with very little cholesterol transported from the plasma because the blood brain barrier (BBB) effectively prevents cholesterol uptake from the circulation [84]. Brain cholesterol is essential for a number of biological functions such as membrane trafficking, signal transduction, myelin formation and synaptogenesis [85]. Brain cholesterol has also been implicated in pathological functions, specifically in the onset of AD [8]. Therefore, reducing brain cholesterol would be an obvious strategy to alleviate symptoms of AD. The cholesterol lowering drugs statins reduce Aβ generation *in vitro* and *in vivo* [20,31,86–88], while also exerting anti-oxidant effects on cerebral vasculature (reviewed in, [89,90]). However, recent large clinical trials using statins for AD did not confirm the initial positive data from smaller trials [91–97].

In contrast to statins, ACAT inhibitors act by reducing CE formation without affecting total cholesterol levels [98]. ACAT inhibitors have been developed against lipid-disorder diseases, and tested for the treatment of atherosclerosis and hypercholesterolemia [99]. Clinical trials are still ongoing. Regarding AD, accumulating evidence is showing that ACAT activity regulates amyloid pathology in the brain [98,100]. Our data, followed by

others, clearly support ACAT inhibition as a strategy for the treatment of AD. Here we summarize data linking ACAT inhibition with reduced Aβ generation.

2.1 ACAT inhibition in animal models of AD

The first animal study was performed with transgenic mice expressing human APP_{751} containing the London (V717I) and Swedish (K670M/N671L) mutations ($hAPP_{FAD}$ mice). Two months treatment with the ACAT inhibitor CP-113,818 not only reduced amyloid plaques in 6 months old $hAPP_{FAD}$ mice, but also improved their spatial learning and memory in a Morris Water Maze test [100]. The antiamyloid effect of CP-113,818 was gender independent. Reduction of plasma cholesterol was previously linked with reduction in Aβ generation [9], but CP-113,818-treatment reduced serum cholesterol levels by only 29% compared to the 90 and 96% reduction of insoluble $Aβ_{40}$ and $Aβ_{42}$ levels in the animal brain, respectively. However, brain CE levels were reduced by 86% in CP-113,818-treated animals, which is comparable to the reduction of Aβ in the same brains. These data indicate that reduced brain ACAT1 activity decreased Aβ levels, instead of an indirect effect of reduced plasma cholesterol. The dramatic reduction in the brain Aβ load was possibly due to the effective delivery strategy of the inhibitor, using implantable slow-release biopolymer pellets, and/or to the ability of the drug to penetrate the BBB. Levels of CP-113,818 in the brain have not been measured. Similarly to statins, ACAT inhibitors appear to indirectly modulate Aβ generation without directly affecting the proteolytic activities of the APP processing enzymes β- and γ-secretases. APP C-terminal fragments (APP-CTFs) were reduced in the brains of treated animals, indicating that CP-113,818 inhibited APP processing with a consequent decrease in Aβ generation. These data were in agreement with our earlier cell-based studies [98]. However, the possibility that ACAT inhibition may also affect Aβ catabolism by reducing its degradation and/or clearance via yet unidentified mechanism(s) has not been excluded.

Recently, *Bryleva et al.* reported greatly improved AD-like pathology when triple transgenic AD mice (3XTg-AD) were crossed with an ACAT null mouse model [101]. In addition, 12 months old $ACAT^{-/-}$/3XTg-AD mice performed at the level of non-transgenic animals in a memory test [101]. $Aβ_{42}$ levels were decreased by 78% in $ACAT^{-/-}$/3XTg-AD mice. These data are consistent with our studies using the ACAT inhibitor CP-113,818 in $hAPP_{FAD}$ mice [100]. Complete lack of ACAT activity in $ACAT^{-/-}$/3XTg-AD also resulted in a decrease in full length APP levels, with a similar decrease in APP-CTF generation [101]. This observation contradicts our data that show reduction of APP-CTF and Aβ levels upon CP-113,818-treatment without largely affecting the levels of full length APP [100]. The discrepancy may be due to the two different approaches, one using a general pharmacological inhibitor of ACAT1, and the other using complete ablation of the ACAT1 gene. Interestingly, $ACAT^{-/-}$/3XTg-AD mice exhibit an approximately 27% increase of 24-hydroxycholesterol (24SOH) levels with unchanged levels of the enzyme catalyzing its synthesis, 24-hydroxylase [101]. 24SOH and 27-hydroxycholesterol are endogenous activators of liver X receptors (LXRs) that upon stimulation cause degradation of $Aβ_{42}$ [102,103]. 24SOH appears to induce α-secretase-mediated processing of APP, whereas 27-hydroxycholesterol upregulates APP and BACE [104]. Treatment of hippocampal neurons with 24SOH reduces APP holoprotein levels [101]. Since 24-hydroxylase is a brain-specific enzyme, changes in 24SOH may contribute to reduced amyloid pathology *in vivo*. However, cell-based data using non-neuronal cells are not explained by this mechanism.

Additional support for the role of ACAT in amyloid pathology derives from a genetic study showing that a single nucleotide polymorphism rs 1044925 in the ACAT1-encoding gene SOAT1 (sterol O-acyltransferase 1) is associated with reduced risk for AD [105]. This study shows decreased amyloid load in patients harboring the rs 1044925 polymorphism. Other genetic studies contradict the association of SOAT1 polymorphism with AD risk [106].

However, this may be due to the diverse genetic backgrounds of the populations analyzed in different studies.

Finally, in a recent unpublished work we have confirmed that ACAT inhibitors reduce amyloid pathology in a mouse model of AD. Here, we tested a less potent ACAT1 inhibitor, CI-1011, to study its effect on Aβ generation. We have compelling data substantiating that CI-1011 also reduces APP processing and Aβ generation in both cell- and animal-based AD models (unpublished data).

2.2. Mechanism of ACAT-inhibition in Aβ generation

The cellular mechanism underlying ACAT inhibitor-mediated reduction of Aβ generation remains largely unknown. CP-113,818 and CI-1011 reduce generation of APP-CTFs and Aβ in cell- and animal-based AD models ([98,100], and unpublished data). In addition, a 50% reduction of ACAT1 expression by RNAi results in 48%, 27% and 28% decrease in amyloidogenic APP-βCTF, non-amyloidogenic APP-αCTF and secreted $A\beta_{42}$ levels in human H4 neuroglioma cells, respectively [107]. Data obtained so far indicate that reduction of ACAT activity affects processing of APP at the level of all three secretases (α-, β- and γ-secretases). However, CP-113,818 does not alter BACE1 or γ-secretase activities in *in vitro* assays [100]. Instead, our studies strongly indicate that the ACAT inhibitors reduce APP processing by an indirect modulation of the secretase activities on APP, possibly by disrupting APP trafficking. Like most type I transmembrane proteins, nascent APP is generated in the ER (immature APP) and undergoes maturation in the ER-Golgi *en route* to the plasma membrane via the secretory pathway. α-secretase-mediated ectodomain shedding occurs primarily at the plasma membrane. Plasma membrane-bound APP is rapidly endocytosed to enter the endosomal compartments, where it is sequentially cleaved by β- and γ-secretases to generate Aβ [108]. We have demonstrated that CP-113,818 treatment delays APP maturation and increases ER-retention of immature APP, suggesting that the inhibition of ACAT activity targets newly synthesized APP in the early secretory pathway and limits its availability for the secretases [109]. This mechanism supports our previous observation that ACAT inhibition does not directly inhibit secretase activities. However, this may not be the major mechanism by which ACAT activity regulates APP processing. Although ER-retention, and ER-associated degradation has gained significant attention towards understanding APP metabolism in the early secretory pathway [110,111], amyloidogenic processing of APP mostly occurs in the acidic compartments of the endocytic pathway or in the lipid rafts [108]. Therefore, targeting APP in the early secretory pathway may not be the primary mechanism by which ACAT inhibitors reduce Aβ generation. Further studies are needed to unravel the full picture of ACAT's impact on Aβ generation.

Inactivation of ACAT1 only leads to a slight accumulation of membrane cholesterol, as excess cholesterol is rapidly exported from the cell [112,113]. The excess cholesterol may be found in the ER, Golgi, or plasma membrane. This cholesterol pool has not yet been identified. It is possible that the free cholesterol pool may be present in lipid raft-like structures at the Golgi-plasma membrane. If so, they may act as docking sites for APP or APP secretases causing their mislocalization and improper processing.

In addition to the accumulation of cholesterol, disruption of CE synthesis by ACAT inhibition may disturb ER-associated cytoplasmic or lumenal lipid droplet biogenesis and transport. A competitive inhibitor of ACAT1, Sandoz 58-035, was shown to cause the formation of lamellar bodies descending from lipid droplets [114,115]. The possibility that altered lipid droplet formation may affect APP trafficking and/or processing needs to be explored.

3. Concluding remarks

Cholesterol is a *bona fide* risk factor for AD pathogenesis. While numerous studies indicate close relationship between dietary cholesterol and cognitive impairment, a consensus still needs to be reached as to how increased plasma cholesterol affects amyloid pathogenesis in the brain. Identifying molecular targets in cholesterol biosynthesis and/or cholesterol homeostasis has become an important focus for AD research. Statins, relatively safe and available, have so far produced mixed results in clinical trials. In contrast, ACAT1 has an excellent potential to become a drug target for AD, as ACAT inhibitors only reduce CE levels and therefore affect APP processing by a mechanism different from statins. Experimental evidence pointing to reduced ACAT activity as an effective method to reduce Aβ levels and/or plaque formation is accumulating (summarized in Table 1). ACAT inhibitors are currently not marketed, but their testing in clinical trials against lipid-disorder diseases is ongoing. The underlying mechanism of how ACAT inhibitors reduce Aβ generation is currently under intense investigation. Combination of statins and ACAT inhibitors may become an effective strategy for the prevention and/or treatment of the devastating effects of AD.

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Abbreviations

AD	Alzheimer's Disease
Aβ	beta-Amyloid
APP	Amyloid precursor protein
A	Acyl-coenzyme
ACAT	cholesterol acyl-transferase
FC	Free cholesterol
CE	Cholesteryl ester
ER	Endoplasmic reticulum
BBB	Blood Brain Barrier
Tg	Transgenic

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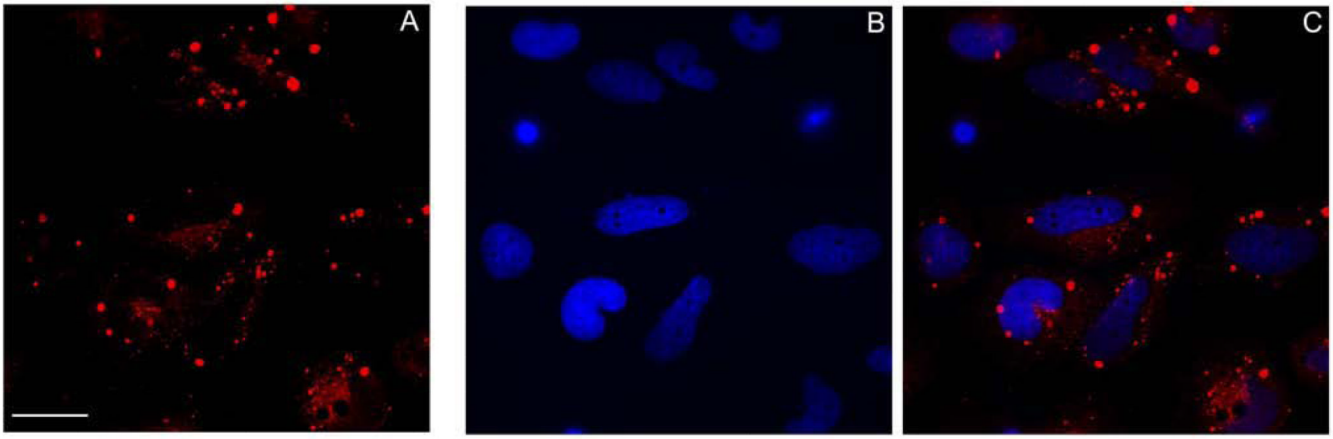

Figure 1. Staining of lipid droplets in cultured cells

B104 cells were fixed with 3% paraformaldehyde and labeled with HCS LipidTOX™ red neutral lipid stain (panel A). Hoechst 33342 stain labeled the nucleus (panel B). Panel C represents the merged image. Bar = 10 μM.

Table 1

Summary of the effect of reduced ACAT activity on Aβ generation and plaque formation in cellular and animal models of AD.

Cell lines/ Animal models	Treatment/ Method	CE (%reduction)	Aβ (%reduction)		Plaque density (%reduction)
			$Aβ_{Tot}$ or $Aβ_{40}$	$Aβ_{42}$	
CHO-APP_{751} [98]	CP	45%	$Aβ_{Tot}$: 30%	50%	
Primary neurons (Tg2576 mice)[98]	CP	~50%	$Aβ_{Tot}$ 40%	40%	
H4-APP_{751}[81]	ACAT1-RNAi (3μg)	22%	$Aβ_{Tot}$ 39%	28%	
Tg-hAPP mice[100]	CP	86%	$Aβ_{40}$ 92%	83%	88%
$ACAT1^{-/-}$/3XTg-AD (A1-/AD) mice[116]	ACAT1-KO	~100%	$Aβ_{40}$ ~75%*	78%	77%

Abbreviations: CP, CP-113,818; $Aβ_{Tot}$, Total Aβ; KO, Knock out; N.A., Data not available.

*Statistically not significant.