

Inhibitors of Cathepsin B Improve Memory and Reduce β -Amyloid in Transgenic Alzheimer Disease Mice Expressing the Wild-type, but Not the Swedish Mutant, β -Secretase Site of the Amyloid Precursor Protein*

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Elucidation of $A\beta$ -lowering agents that inhibit processing of the wild-type (WT) β -secretase amyloid precursor protein (APP) site, present in most Alzheimer disease (AD) patients, is a logical approach for improving memory deficit in AD. The cysteine protease inhibitors CA074Me and E64d were selected by inhibition of β -secretase activity in regulated secretory vesicles that produce β -amyloid ($A\beta$). The regulated secretory vesicle activity, represented by cathepsin B, selectively cleaves the WT β -secretase site but not the rare Swedish mutant β -secretase site. *In vivo* treatment of London APP mice, expressing the WT β -secretase site, with these inhibitors resulted in substantial improvement in memory deficit assessed by the Morris water maze test. After inhibitor treatment, the improved memory function was accompanied by reduced amyloid plaque load, decreased $A\beta_{40}$ and $A\beta_{42}$, and reduced C-terminal β -secretase fragment derived from APP by β -secretase. However, the inhibitors had no effects on any of these parameters in mice expressing the Swedish mutant β -secretase site of APP. The notable efficacy of these inhibitors to improve memory and reduce $A\beta$ in an AD animal model expressing the WT β -secretase APP site present in the majority of AD patients provides support for CA074Me and E64d inhibitors as potential AD therapeutic agents.

Alzheimer disease (AD)² is an age-related neurodegenerative disorder that results in loss of memory and accumulation of neurotoxic β -amyloid ($A\beta$) peptides in brain (1–4). Expression

of mutant forms of human amyloid precursor protein (APP) in mouse models of AD results in increased $A\beta$ and amyloid plaques in brain, with memory deficits that resemble AD (2, 3, 5–7). Such studies demonstrate that overproduction of $A\beta$ peptides participates as a major factor in the development of AD.

$A\beta$ peptides are produced by proteolytic processing of APP, resulting in production of $A\beta_{40}$ and $A\beta_{42}$ (1–40 and 1–42 residues, respectively). Proteases referred to as β -secretase and γ -secretase cleave at the N- and C termini of $A\beta$ within APP to generate $A\beta$ peptides. $A\beta$ peptides then undergo secretion to provide extracellular $A\beta$ that produces neurotoxic effects with aggregation and accumulation in amyloid plaques of AD brains. Compounds that inhibit β -secretase activity are particularly attractive as potential therapeutic agents for AD.

The majority of AD patients express the wild-type (WT) β -secretase site of APP. Therefore, compounds that inhibit cleavage of the WT β -secretase site are relevant to the AD population. Significantly, findings in this study showed that the endogenous β -secretase activity in $A\beta$ -containing regulated secretory vesicles (RSV) possesses high selectivity for cleaving the WT β -secretase site but does not cleave the Swedish (Swe) mutant β -secretase site. The RSV provide production and synthesis of a major portion of secreted $A\beta$ (8, 9). Although many previous studies of β -secretase have utilized substrates containing the Swe mutant β -secretase site (10–14) expressed in one extended family (15), it is most relevant to study proteolytic cleavage of the WT β -secretase site expressed in the major portion of the AD population.

The distinct preference for cleavage of the WT β -secretase site by the RSV β -secretase activity, identified as cathepsin B (9), predicts that protease inhibitors selected by the RSV assay will be effective only in animals expressing the WT β -secretase site and will have no effect in animals expressing the Swe mutant site of APP. Therefore, the goal of this study was to evaluate improvement in memory deficit by the cysteine protease inhibitors CA074Me and E64d in the London APP transgenic mouse model of AD that expresses human APP containing the WT β -secretase site (16, 17), compared with inhibitor treatment of mice that express human APP containing the Swe mutant β -secretase site (in Swedish/London mice).

Results from this study showed that administration of CA074Me or E64d to the London APP mice resulted in signif-

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² The abbreviations used are: AD, Alzheimer disease; $A\beta$, β -amyloid; APP, amyloid precursor protein; WT, wild-type; RSV, regulated secretory vesicle(s); Swe, Swedish; Z, benzyloxycarbonyl; CTF β , C-terminal β -secretase fragment; ELISA, enzyme-linked immunosorbent assay; MCA, methylcoumarinamide.

icant improvement in memory deficit, reduced amyloid plaque load in brain, reduced levels of A β 40 and A β 42 in brain, and reduced C-terminal β -secretase fragment (CTF β) derived from APP by β -secretase when compared with untreated control animals. In contrast, these cysteine protease inhibitors had no effect on any of these parameters in mice expressing the Swe mutant β -secretase site of APP (in Swedish/London APP mice). Most importantly, these results show that these cysteine protease inhibitors are efficacious for improving memory deficit in an AD mouse model representative of the majority of AD patients who express the WT β -secretase site of APP. These novel findings implicate the potential use of such cysteine protease inhibitors as candidate therapeutic agents for treatment of AD.

EXPERIMENTAL PROCEDURES

RSV and Cathepsin B Assays with Z-Val-Lys-Met \downarrow MCA Substrate That Represents the WT β -Secretase Site—RSV were purified from bovine adrenal medulla as previously described (18). The endogenous β -secretase activity in the RSV was assayed with Z-Val-Lys-Met \downarrow MCA substrate that mimics the WT β -secretase site of APP as well as with Z-Val-Asn-Leu \downarrow MCA that mimics the Swe mutant β -secretase site of APP (substrates from TheraKem). Cathepsin B (Athens Research) was also assayed using these substrates.

The peptide-MCA substrates provide β -secretase cleavage site-specific assays. The cleavage sites (indicated by arrows) within the substrates Z-Val-Lys-Met \downarrow MCA and Z-Val-Asn-Leu \downarrow MCA represent model wild-type β -secretase and Swedish mutant β -secretase cleavage sites of APP, respectively. The three amino acid residues to the left of the arrow represent the amino acid residues adjacent to the N-terminal side of the β -secretase cleavage sites of the WT and Swe APP. Such substrates are routinely used as model peptide substrates, because the residues at the positions adjacent to the N-terminal side of the cleavage site in the peptide (or protein) are important determinants of protease recognition and cleavage of peptide and protein substrates (19–22). Moreover, the RSV β -secretase activity identified using the WT β -secretase peptide-MCA substrate has also been shown to cleave the peptide bond of the β -secretase site in the larger peptide substrate SEVK \downarrow MDAEF, which includes amino acid residues on both the N- and C-terminal sides of the β -secretase site (9).

Cleavage at the model β -secretase site of the peptide-MCA substrates liberates the MCA group that is monitored by its fluorescent signal. These fluorescent protease assays were conducted as previously described (9).

Transgenic AD Mice—This study utilized two mouse models of AD, consisting of one expressing human APP containing the WT β -secretase site and mutation at the γ -secretase site (London APP mice) and the other expressing the Swe mutant β -secretase site and the London mutation (Swedish/London APP). London APP was generated using site-directed mutagenesis to insert the V717I London mutation into human APP cDNA. Swedish/London APP was generated by site-directed mutagenesis at the β -secretase site of the London APP to convert Lys-Met to the mutant Asn-Leu. Both AD mouse strains were created in a C57BL/6 mouse background using the Thy-

1.2 expression cassette driven by the Thy-1 promoter containing an SV40 polyadenylation site (23).

Treatment of Transgenic AD Mice with Cysteine Protease Inhibitors—London APP and Swedish/London APP mice (male) were treated at 12 and 6 months of age, respectively. At these ages, the London and Swedish/London APP mice show comparable levels of A β with similar pathological and behavioral effects.

An ALZET osmotic minipump (model 2004; ALZET) was implanted subcutaneously in the midscapular area of the back of each animal. A catheter was connected to the osmotic minipump and inserted into the lateral ventricle of the brain (icv) (ALZET Brain Infusion Kit 3). CA074Me or E64d (Peptide International) was administered at 1 mg/ml in saline with 1.5% Me₂SO (Sigma) at a rate of 0.25 μ l/h, corresponding to an estimated dose rate of 0.006 mg/day or 0.013 mg/day-g brain weight, which is a dose below that which causes lipofuscin formation (24). Controls received carrier solution (saline with 1.5% Me₂SO). Animals received continuous infusion of inhibitors or carrier for 28 days and were then evaluated in the Morris water maze test. The animals were sacrificed, and half of the brain was prepared for measurement of A β 40, A β 42, CTF β , and sAPP α . The other half of the brain was prepared for histological evaluation of amyloid plaque load.

Morris Water Maze Memory Test—The spatial memory capability of each animal was assessed by the Morris water maze test (25, 26) (700-0718-4W, SD Instruments). The Morris water maze test evaluates memory in a swimming test. Mice were individually trained in a 1.2-m open field water maze in a pool filled with water to a depth of 30 cm and maintained at 25 °C. An escape platform (10 cm square) was placed 1 cm below the surface of the water. All animals underwent nonspatial pretraining for 3 consecutive days, which prepared the animals for the final behavioral test to determine the retention of memory to find the platform. Two days following the nonspatial pretraining, the hidden platform was placed in the center of one quadrant of the pool, the animal was released facing the pool wall in a random fashion, the time was recorded (latency period), and the distance traveled to reach the platform was measured using video recording (Smart Video Tracking System; SD Instruments). The latency period was also determined for normal wild-type, strain-matched, and age-matched mice (nontransgenic) ($n = 3$).

Preparation of Brain Extracts—Brain extracts were homogenized (1:3 weight/volume of buffer) in buffer of 5 M guanidine HCl in 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, plus protease inhibitors (Sigma). Homogenates were diluted to 0.5 M guanidine and centrifuged (200,000 \times g for 20 min.), and supernatant and pellet fractions were collected. Protein content was determined by the Bradford method.

Analyses of A β Peptides—Enzyme-linked immunosorbent assays (ELISAs) measured A β levels. The pellet from the brain extract procedure was sonicated in 6 M guanidine and centrifuged at 200,000 \times g for 20 min at 4 °C, and the supernatant was diluted to 0.5 M guanidine. Similarly, the supernatant from the brain extract was brought to 0.5 M guanidine. The two supernatants were combined, and A β 40 and A β 42 were determined as previously described (27, 28) using ELISA kits specific for

each peptide (KHB3481/KHB3441; BIOSOURCE International). The amount of A β was calculated as ng of A β /g of protein and normalized as a percentage of A β in the control group for each experiment.

CTF β and sAPP α Analysis—CTF β was determined in the pellet fraction from the brain extract by Western blot (antibody A8717; Sigma) as described previously (29). Also, sAPP α was assessed in the supernatant fraction from the brain extract by Western blot (antibody 6E10; Signet Laboratories). Relative amounts of CTF β and sAPP α bands were assessed by densitometry.

Brain Amyloid Plaque Load Analysis—Amyloid load was assessed in brain sections by immunostaining for A β (A β antibody 10D5 (Elan Pharmaceuticals). Brain tissues were fixed in 4% paraformaldehyde and then in 4% paraformaldehyde and 30% sucrose for 24 h each at 4 °C. Tissues were washed in Tris-buffered saline and transferred to an O.C.T. medium. Cryosections were blocked with normal serum, incubated with anti-A β , and stained with diaminobenzoic acid with the Vector ABC Elite kit (Vector Laboratories). Bright field light microscopy imaged amyloid areas that were quantitated (by NIH Image software).

Statistics—Experiments in the London APP mice consisted of eight animals in each of the three treatment groups (24 animals/experiment); experiments were replicated, and data were pooled (48 animals total). The same number of animals and experiments were used for the Swedish/London APP mice experiments. Each biochemical analysis consisted of two or three replicates. Quantitative histology was conducted from eight sections per animal. Statistical analyses utilized software designed for scientific data analysis (Prism 4 GraphPad, San Diego, CA).

Animal Use—All animal studies were conducted in accordance with the guidelines and regulations set forth by the National Institutes of Health (Bethesda, MD) using procedures approved by the animal review committee, IACUC, at the Medical University of South Carolina and Veterans Affairs Medical Center (Charleston, SC).

RESULTS

Specificity of the RSV β -Secretase Activity for Cleaving the WT β -Secretase Site; Selection of Cysteine Protease Inhibitors—Comparison of the endogenous RSV β -secretase activity for cleaving the WT or Swe mutant β -secretase sites demonstrated the distinct selectivity of the RSV β -secretase activity for the WT β -secretase site, represented by the substrate Z-Val-Lys-Met \downarrow MCA (Table 1). The RSV assay showed essentially no activity for cleaving the Swe mutant β -secretase site represented by the substrate Z-Val-Asn-Leu \downarrow MCA (mutant residues are underlined) (Table 1). Furthermore, the β -secretase activity purified from RSV, identified as cathepsin B (9), also showed high selectivity for the WT β -secretase substrate and essentially no activity for the Swe β -secretase substrate (Table 1). The high preference of cathepsin B for the WT β -secretase site was supported by its high catalytic efficiency, indicated by its k_{cat}/K_m value of $3.17 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$, which represents a highly active enzyme. Because the majority of AD patients express the WT β -secretase site of APP, the RSV assay is rele-

TABLE 1
RSV β -secretase activity and cathepsin B selectively cleave the wild-type β -secretase substrate

The RSV contain A β and APP and were obtained as purified secretory vesicles from bovine adrenal medulla as described under "Experimental Procedures." The WT β -secretase cleavage site is represented by the substrate Z-Val-Lys-Met \downarrow MCA, and the Swe mutant β -secretase cleavage site is represented by the substrate Z-Val-Asn-Leu \downarrow MCA (mutant residues are underlined). These cleavage site-specific peptide-MCA substrates were utilized to assess β -secretase activities in the isolated RSV as well as β -secretase activity of cathepsin B.

Proteolytic activity	Relative activities ^a	
	WT β -secretase substrate Z-Val-Lys-Met \downarrow MCA	Swe mutant β -secretase substrate Z-Val- <u>Asn</u> - <u>Leu</u> \downarrow MCA
	%	%
RSV β -secretase	100	0.15
Cathepsin B	100	0.04

^a The relative β -secretase activities with the two substrates were compared relative to the WT β -secretase substrate as 100%.

TABLE 2
Cysteine protease inhibitors inhibit RSV β -secretase and cathepsin B cleavage of the wild-type β -secretase substrate

The relative potencies of the inhibitors CA074 and E64c to reduce β -secretase activity of isolated RSV and of cathepsin B were measured as the inhibitor concentrations required to reduce proteolytic activity by 50% (IC₅₀). β -Secretase activity in RSV (purified as described under "Experimental Procedures") and by cathepsin B was measured with the substrate Z-Val-Lys-Met \downarrow MCA.

Inhibitor	Potency of inhibition (IC ₅₀)	
	RSV β -secretase activity	Cathepsin B activity
	nM	
CA074	10	2.0
E64c	15	0.3

vant for selection of protease inhibitors of β -secretase for reduction of A β .

The compound CA074, a specific inhibitor of cathepsin B (30, 31), was a potent inhibitor in the RSV assay with its IC₅₀ value of 10 nM (Table 2). The cysteine protease inhibitor E64c was also highly effective, with an IC₅₀ value of 15 nM in the RSV assay. Cathepsin B was also potently inhibited by CA074 and E64c, with IC₅₀ values in the nanomolar range (Table 2). These data predicted that the cysteine protease inhibitors selected by the RSV assay would inhibit β -secretase cleavage of the WT β -secretase site, but not the mutant Swedish β -secretase site, for A β production. Therefore, cell-permeable forms of these cysteine protease inhibitors, CA074Me and E64d (29, 32), were tested in the AD mouse model that expresses human London APP containing the WT β -secretase site (with mutant γ -secretase site), which displays elevated A β and memory deficit (16, 17).

Animals Remain Healthy during Administration of Inhibitors—The CA074Me and E64d inhibitors as well as carrier solution (control) were administered directly (icv) into the brains of the mice by an implanted osmotic minipump in each mouse for 28 days. Animals remained healthy and were observed to have normal eating, body weight, grooming, and general appearance during the inhibitor treatment period. Of the 96 animals used in this study, none showed postoperative complications, such as infections; also, none died. These observations demonstrated that animals remained healthy during inhibitor treatment. Moreover, acute toxic effects were not expected, because E64d was found to be safe for patients in clinical trials for muscular dystrophy (33).

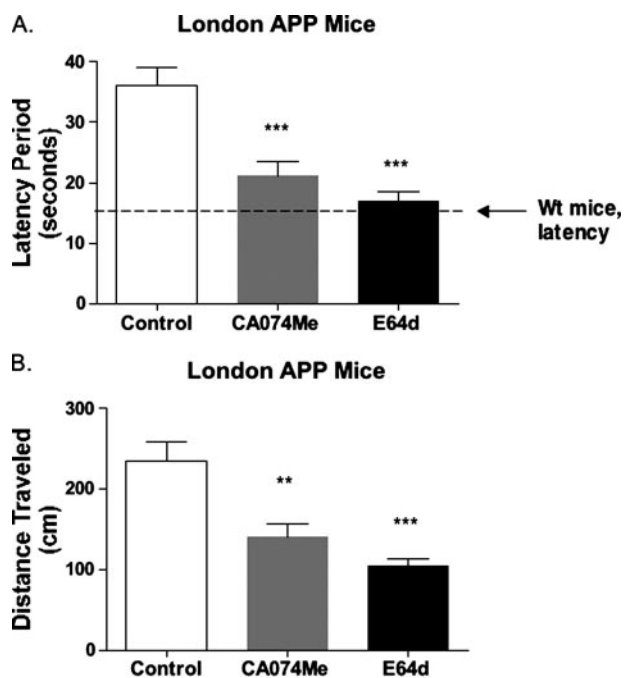


FIGURE 1. CA074Me or E64d improve memory deficit in London APP mice. A, shortened latency times for CA074Me- and E64d-treated animals. Memory was assessed by the Morris water maze test. The latency period measures the time required for the animal to swim to a submerged platform after training to learn of the platform location, with shorter times reflecting improved memory. The mean latency times and percentage S.E. for the control, CA074Me-treated, and E64d-treated London APP animals were 36 s ± 8%, 21 s ± 10%, and 17 s ± 12%, respectively. The bar graphs represent the mean latency periods (seconds), with the error bars showing the S.E. The statistical significance between the means for the London APP animals of each treated group and the control is indicated (***, $p < 0.0001$, Student's *t* test). The mean latency period for untreated wild-type, strain-matched, nontransgenic mice was 14 s ± 8%, which is shown as a dashed line. Thus, the CA074Me and E64d treatments significantly shortened the latency period by 42 and 53%, respectively, relative to the London APP controls and restored about 68 and 86%, respectively, of the memory of wild-type nontransgenic mice. B, shorter distances traveled for CA074Me- and E64d-treated animals. The distance traveled to the platform is a measure of memory, with shorter distances reflecting improved memory. The mean distance traveled and percentage S.E. for control, CA074Me-treated, and E64d-treated London APP animals was 238 cm ± 11%, 141 cm ± 8%, and 106 ± 5%, respectively. The bar graphs represent the mean distances traveled (cm) ± S.E., with significance differences for the London APP mice between each treated group and the control indicated (***, $p < 0.0001$; **, $p < 0.005$, Student's *t* test). Thus, the CA074Me and E64d treatments significantly shortened the distance traveled by 41 and 56%, respectively, relative to the London APP controls.

Significant Improvement in Memory Deficit in London APP Mice Treated with Cysteine Protease Inhibitors—Administration of either CA074Me or E64d caused a significant improvement in the memory deficit of London APP mice, as assessed by the Morris water maze test that measured the latency period and distance traveled to swim to a submerged platform. The mean latency periods for control, CA074Me-treated, and E64d-treated animals were 37, 21, and 17 s, respectively, showing that CA074Me and E64d resulted in a significant reduction in latency period of 43 and 54%, respectively. These results demonstrate that these inhibitors produced substantial improvements in memory deficit (Fig. 1A). The normal, wild-type mice (untreated, nontransgenic, and strain- and age-matched) had a latency period of about 14 s (Fig. 1A). Thus, the reduction in latency period after treatment of London APP mice with the inhibitors approached that of the normal wild-type mice.

The mean distances traveled for control, CA074Me-treated, and E64d-treated animals were 238, 141, and 106 cm, respectively, which indicated that CA074Me and E64d caused a significant reduction in the mean distance traveled of 41 and 56%, respectively (Fig. 1B). The mean calculated swimming speed was not significantly different for the treated and control London APP mice (data not shown), and, thus, the shorter latency period and distance traveled caused by the cysteine protease inhibitors were not due to faster swimming. Rather, the shorter latency period and distance traveled demonstrate an improved ability to remember the location of the platform. These data, thus, demonstrate that CA074Me and E64d provide significant improvement in memory deficit in the London APP mice.

Reduced Amyloid Plaque Load in London APP Mice Treated with Cysteine Protease Inhibitors—Amyloid plaque load was examined with anti-A β compared in brain sections from control, CA074Me-treated, and E64d-treated London APP mice (Figs. 2, A–C, respectively). The CA074Me- and E64d-treated mice showed substantial reduction in total brain amyloid plaque load compared with control, untreated London APP mice. This result was illustrated by quantitative image analyses conducted over the entire brain sections of inhibitor-treated and untreated controls to assess total brain amyloid plaque load (Fig. 2D). Results found that the mean percentage of tissue area of amyloid plaques for the control, CA074Me-treated, and E64d-treated animals was 0.50, 0.22, 0.21% of the tissue area, respectively. These data demonstrate a significant 55% reduction in amyloid plaque load by the CA074Me and E64d inhibitors. These results demonstrate that the significant reduction of amyloid plaque by about one-half is sufficient to result in improved “memory deficit” after treatment of London APP mice with CA074Me or E64d cysteine protease inhibitors.

It is noted that treatment with CA074Me or E64d resulted in extensive reduction of amyloid plaque load in the hippocampus region (Fig. 2, B and C) with a more modest reduction in the cortex region of the brain. With icv drug administration, diffusion from the ventricular space may provide greater access to hippocampus than to larger brain regions, such as the cortex (34). Nonetheless, results demonstrate that substantial reduction of amyloid plaque load in total brain occurs during improved “memory deficit” after treatment with CA074Me and E64d inhibitors.

Reduced Brain A β 40 and A β 42 after Treatment of London APP Mice with Cysteine Protease Inhibitors—Treatment with CA074Me or E64d resulted in significant reductions in brain levels of A β 40 and A β 42 compared with untreated controls in the London APP mice. Both inhibitors reduced brain levels of A β 40 by about 40% (Fig. 3A). Brain levels of A β 42 were reduced by 40 and 45% in CA074Me- and E64d-treated animals, respectively (Fig. 3B). Thus, CA074Me and E64d reduced A β levels in brains of London APP mice.

Reduced CTF β and Increased sAPP α Derived from APP after Treatment of London APP Mice with Cysteine Protease Inhibitors—CA074Me or E64d treatment decreased brain levels of CTF β and increased sAPP α generated by proteolysis of APP. β -Secretase processing of APP generates a C-terminal β -secretase fragment (CTF β) of 12 kDa, an intermediate required for production of A β . Western blots of CTF β qualita-

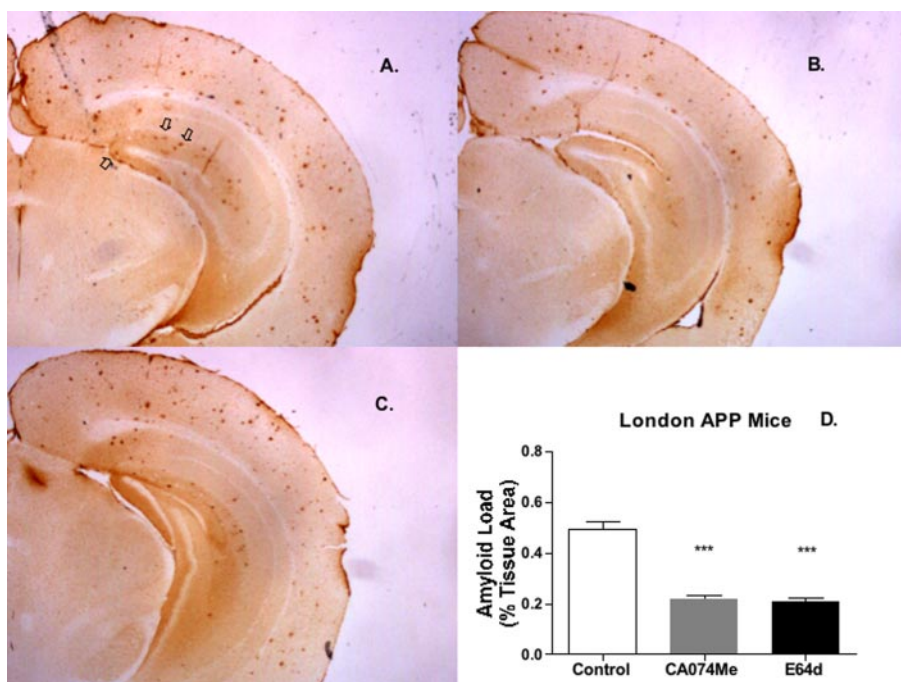


FIGURE 2. CA074Me or E64d reduce brain amyloid plaque load in London APP mice. A–C, reduced amyloid plaques observed immunohistochemically in brains of CA074Me- and E64d-treated animals. *Micrograph A* presents a typical brain section (hippocampus and cortex) from untreated control APP London mice showing amyloid plaques revealed by anti- $A\beta$ (see *arrows* pointing to plaques in hippocampus). *Micrographs B* and *C* are typical sections from CA074Me-treated and E64d-treated APP London mice showing substantial reduction of amyloid plaques in the hippocampus, with the cortex showing a modest reduction. *D*, reduction of total amyloid plaque load in the brain after treatment with inhibitors. Relative amyloid plaque load in the entire brain section was quantitated by computer image analyses, expressed as a percentage of the tissue area. The relative amyloid loads are displayed as the mean (percentage of tissue area) and S.E. The percentage of tissue area and the percentage S.E. for the control, CA074Me-treated, and E64d-treated animals were 0.50 ± 6 , 0.22 ± 10 , and $0.21 \pm 5\%$, respectively, with the statistical significance in the difference between each treated group and control indicated (***, $p < 0.0001$, Student's *t* test). Thus, inhibitor treatments reduce the plaque load by about 45%.

tively showed that both CA074Me and E64d resulted in lower levels of CTF β (Fig. 4A). Densitometric quantitation of the blots showed that both inhibitors significantly reduced CTF β by 40% compared with untreated controls (Fig. 4B). The observed decreases in CTF β are consistent with inhibition of β -secretase activity by the cysteine protease inhibitors.

Cleavage at the α -secretase site within the $A\beta$ domain within APP results in production of sAPP α and precludes formation of $A\beta$. Western blots showed that treatment with either inhibitor resulted in increased production of sAPP α (Fig. 5A). Densitometric quantitation of the sAPP α bands showed that CA074Me and E64d increased sAPP α by 45 and 55%, respectively, relative to control (Fig. 5B). These results demonstrate that increased α -secretase processing of APP occurs with decreased β -secretase production of CTF β , resulting in the overall reduction of brain $A\beta$ levels during treatment with the cysteine protease inhibitors in the London APP mouse model.

No Effect in Swedish Mutant APP Mice Treated with Cysteine Protease Inhibitors—The CA074Me or E64d inhibitors produced no effects in the Swedish/London APP mice, which express human APP containing the Swe mutant β -secretase site. No significant change in memory, amyloid plaque load, $A\beta_{40}$, $A\beta_{42}$, CTF β , or sAPP α resulted from treating Swedish/London APP mice with these cysteine protease inhibitors (Table 3).

Importantly, the CA074Me and E64d inhibitors were efficacious in London APP mice expressing the WT β -secretase site, present in the majority of AD patients, for improvement in memory deficit accompanied by reductions in amyloid plaque load and $A\beta$ peptides.

DISCUSSION

Significant results show the effectiveness of CA074Me and E64d cysteine protease inhibitors to improve memory and reduce $A\beta$ in the London APP mouse model of AD that represents the majority of AD patients who express the WT β -secretase site of APP. These inhibitors reduced the latency period and distance traveled in the Morris water maze test by about 50%, indicating improvement in spatial memory capability. Inhibitor treatment reduced amyloid plaque load in brain by about 50%, showing that the compounds reduced a neuropathological hallmark of AD. Furthermore, the inhibitors substantially reduced both $A\beta_{40}$ and $A\beta_{42}$ in brain. These novel results demonstrate the *in vivo* effectiveness of these cysteine protease inhibitors to improve memory deficit, with

reduction in brain $A\beta$ and amyloid plaque load, in the London APP mouse model of AD.

CA074Me and E64d treatment of the London APP mice also caused a significant reduction in brain levels of CTF β that is derived from APP by β -secretase processing. The reduction in CTF β and $A\beta$ suggests that CA074Me and E64d reduced brain $A\beta$ by inhibiting β -secretase activity in the London APP mice. Inhibition of β -secretase processing of APP also resulted in increased brain levels of sAPP α derived from APP by α -secretase, indicating that the inhibitors influenced α -secretase activity possibly due to enhanced availability of APP during decreased CTF β production. Thus, inhibition of β -secretase decreases the amyloidogenic β -secretase pathway for $A\beta$ production, with simultaneous increase in the nonamyloidogenic α -secretase pathway.

In addition to the London APP mice, the effectiveness of CA074Me and E64d to reduce $A\beta$ production has been demonstrated in other model neuronal systems of guinea pig brain *in vivo* and in bovine neuronal-like chromaffin cells in primary culture. Notably, guinea pig and bovine neurons produce the human $A\beta$ sequence derived from processing of the WT β -secretase site of APP (35, 36). *In vivo* icv administration of CA074Me and E64d to normal guinea pigs resulted in substantial reduction of brain $A\beta$ levels and inhibited β -secretase activity (29). In addition, CA074Me and E64d inhibited $A\beta$ produc-

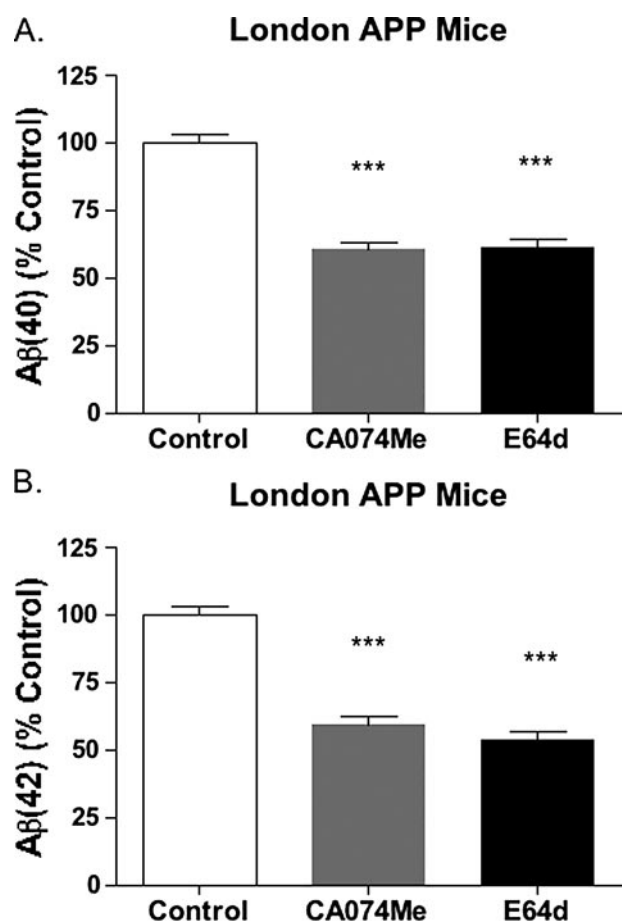


FIGURE 3. CA074Me or E64d reduces brain Aβ₄₀ and Aβ₄₂ in London APP mice. *A*, quantitative reduction of brain Aβ₄₀ after inhibitor treatment. London APP mice were treated with CA074Me or E64d, and Aβ₄₀ levels in brain were measured by ELISAs and expressed as a percentage of the control Aβ₄₀ levels. The mean percentage of control and percentage S.E. for the control, CA074Me-treated, and E64d-treated animals were 100 ± 3, 61 ± 5, and 61 ± 5%, respectively. Displayed is the mean (percentage of control) and the S.E., with statistical significance between the mean of either treated group and the control indicated (***, *p* < 0.0001, Student's *t* test). Thus, both inhibitor treatments reduced brain Aβ₄₀ by about 40%. *B*, quantitative reduction of brain Aβ₄₂ after inhibitor treatment. After treatment of London APP mice with CA074Me or E64d, Aβ₄₂ levels in brain were measured by ELISAs and expressed as a percentage of the control Aβ₄₂. The mean percentage of control and percentage S.E. for the control, CA074Me-treated, and E64d-treated animals were 100 ± 3, 54 ± 5, and 49 ± 5%, respectively. Displayed is the mean (% control) and the S.E., with statistical significance between the mean of either treated group and the control indicated (***, *p* < 0.0001, Student's *t* test). Thus, the CA074Me and E64d treatments reduced brain Aβ₄₂ by about 46 and 51%, respectively.

tion in RSV isolated from bovine chromaffin cells (9); inhibitor treatment of these chromaffin cells in primary culture reduced Aβ production with reduced β-secretase activity for WT APP in the regulated secretory pathway (9). These data demonstrate the effectiveness of CA074Me and E64d to reduce endogenous Aβ and β-secretase activity in guinea pig and bovine species, as well as in transgenic London APP mice, that express human APP with the WT β-secretase site.

The ability of cysteine protease inhibitors to reduce Aβ and inhibit processing of the WT β-secretase site of APP was predicted based on their ability to inhibit endogenous RSV β-secretase activity that produces the majority of Aβ for regulated secretion. Notably, the RSV β-secretase activity, identified as cathepsin B (9), possesses the unique biochemical prop-

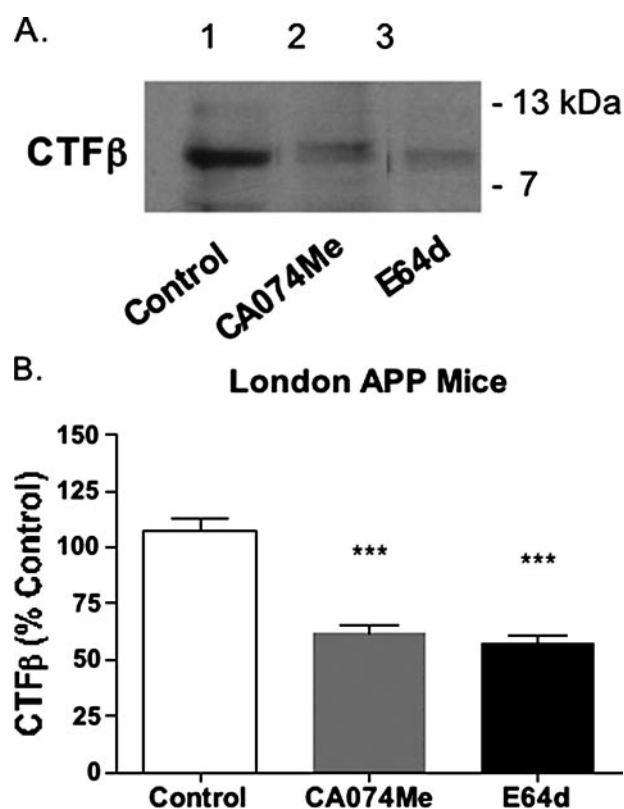


FIGURE 4. CA074Me or E64d reduce brain CTFβ in London APP mice. *A*, reduction in CTFβ after inhibitor treatment illustrated by Western blots. Western blots for analyses of brain CTFβ in London APP mice were performed with brain extracts (30 μg of protein/gel lane). Representative Western blots are shown for control, CA074Me-treated, and E64d-treated mice. *B*, quantitative reduction in brain CTFβ caused by inhibitors. Relative levels of brain CTFβ (12 kDa) were analyzed by quantitation of Western blots. The mean percentage of control and percentage S.E. for the control, CA074Me-treated, and E64d-treated animals were 108 ± 5, 62 ± 5, and 58 ± 7%, respectively. Graphically displayed is the mean (percentage of control) and the S.E., with statistical significance between the mean of either treated group and the control indicated (***, *p* < 0.0001, Student's *t* test). Thus, the CA074Me and E64d treatments reduced brain CTFβ by about 45%.

erty of specificity for cleaving the WT β-secretase site but not the Swe mutant β-secretase site. This specificity predicts that inhibitors of the RSV β-secretase activity, represented by cathepsin B, would be effective in animals expressing the WT β-secretase site as demonstrated in the London APP AD mice but not in animals expressing the Swe mutant β-secretase site of APP.

Indeed, CA074Me and E64d had no effect in mice expressing the Swe mutant β-secretase site of the Swedish/London APP mouse AD model. After treatment with CA074Me or E64d, Swedish/London APP mice showed no change in memory deficit, amyloid plaque load, brain levels of Aβ₄₀ and Aβ₄₂, or CTFβ and sAPPα. These novel results demonstrate the unique property of the specificity of the CA074Me and E64d inhibitors for improvement of "memory deficit" and reduction of Aβ, concomitant with reduced amyloid load and CTFβ, in animals expressing the WT β-secretase site but not in animals expressing the Swe mutant β-secretase site of APP. Since the majority of the AD population expresses the WT β-secretase site of APP, the CA074Me and E64d are relevant to the human AD disease condition.

The selectivity of cathepsin B for the WT but not the Swe mutant β -secretase site predicts that knocking out cathepsin B in mice expressing Swe mutant APP would have no effect on CTF β . Indeed, in cathepsin B knock-out mice that express Swe mutant APP, there was no change in CTF β , which indicates that cathepsin B does not cleave the Swe β -secretase site of APP *in vivo* (37). These results are consistent with findings of this study showing that inhibitors of cathepsin B have no effect in mice expressing Swe mutant APP.

In contrast to cathepsin B, the BACE 1 β -secretase shows preference for cleaving the Swe mutant β -secretase site rather than the WT site (10–12, 38). Comparison of BACE 1 with cathepsin B with the cleavage site-specific substrates for the WT β -secretase site (represented by Z-Val-Lys-Met \downarrow MCA) and for the Swe mutant β -secretase site (represented by Z-Val-Asn-Leu \downarrow MCA) demonstrated the distinct differences in cleavage site specificities of these two different proteases (Table 4). Examination of the enzyme-specific activities showed that BACE 1 prefers cleavage of the Swe mutant site, with no cleavage of the WT β -secretase site (Table 4). The higher specific activity of cathepsin B demonstrated its clear preference for the WT but not the Swe mutant β -secretase site (Table 4). Cathepsin B cleavage of the WT β -secretase substrate shows high catalytic efficiency with k_{cat}/K_m value of $3.71 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ that is similar to other known active proteases for endogenous substrates, such as angiotensin-converting enzyme (39). However, BACE 1 shows low catalytic efficiency, represented by its k_{cat}/K_m value of only $\sim 50 \text{ M}^{-1} \text{ s}^{-1}$ (Table 5), which is similar to protease activity for a nonsubstrate (40). The contrasting cleavage specificities and catalytic efficiencies of cathepsin B and BACE 1 lead to the hypothesis of distinct proteases for cleaving the WT compared with the Swe mutant β -secretase sites of APP, respectively.

In the Swedish/London mice, it is, therefore, hypothesized that BACE 1 provides β -secretase activity for processing Swe mutant APP, since cathepsin B shows essentially no cleavage of the Swe mutant site. The finding that the cysteine protease inhibitors of cathepsin B, CA074Me and E64d, had no effects in the Swedish/London mice is consistent with the prediction that these inhibitors do not inhibit BACE 1. Indeed, it is known that E64c and CA074 do not inhibit BACE 1 activity *in vitro* (29). Thus, it is not likely that CA074Me and E64d inhibit BACE 1 β -secretase activity *in vivo*.

However, in the London APP mice expressing the WT β -secretase site, CA074Me and E64d are probably reducing A β by inhibiting the β -secretase activity of cathepsin B. CA074Me enters cells and is converted intracellularly by esterases to CA074, a specific inhibitor of cathepsin B (31). Therefore, the effects of CA074Me to improve “memory deficit” and reduce A β in the London APP mice is probably due to inhibition of cathepsin B in RSV of neurons that produce A β (9). In addition, the selective and potent cathepsin B inhibitor, Ac-LVK-alde-

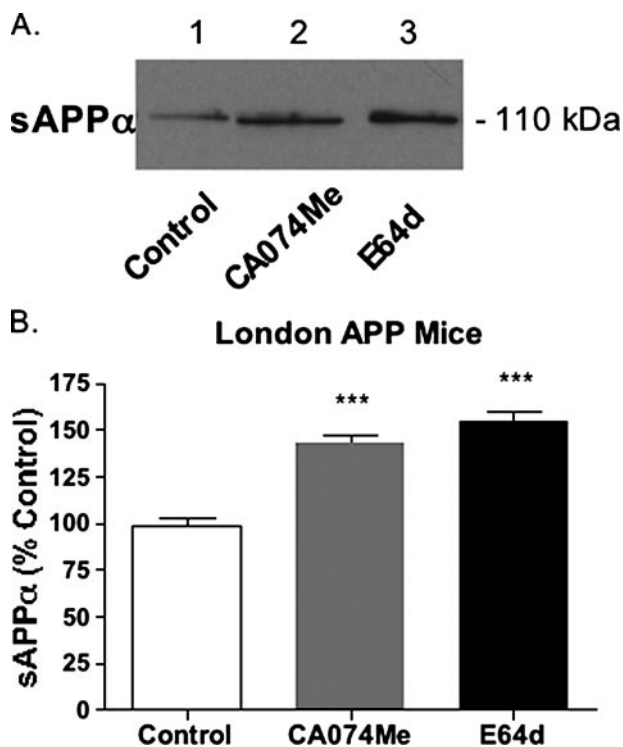


FIGURE 5. CA074Me or E64d increase brain sAPP α in London APP mice. A, increase in sAPP α after inhibitor treatment illustrated by Western blots. The sAPP α fragment derived from APP in brain extracts was detected by Western blots (30 μ g of protein/gel lane). Representative Western blots are shown for control, CA074Me-treated, or E64d-treated mice. B, quantitative increase in sAPP α caused by inhibitors. Relative levels of the sAPP α derived from APP were assessed by quantitation of Western blots by densitometry. The mean percentage of control and percentage S.E. for the control, CA074Me-treated, and E64d-treated animals were $99 \pm 5\%$, $144 \pm 3\%$, and $155 \pm 3\%$, respectively. Graphically displayed is the mean (percentage of control) and the S.E., with statistical significance between the mean of either treated group and the control indicated (***, $p < 0.0001$, Student's t test). Thus, the CA074Me and E64d treatments increased brain CTF β by about 50%.

TABLE 3
CA074Me and E64d cysteine protease inhibitors have no effect in Swedish/London APP mice

CA074Me is the methylated, cell-permeable form of CA074 that is a specific inhibitor of cathepsin B. E64d is the ester form of E64c that inhibits cysteine proteases. Swedish/London APP transgenic mice were treated with CA074Me or E64d or without inhibitors (control) as described under “Experimental Procedures.” After inhibitor treatments, animals were evaluated for improvement in “memory deficit” by the Morris water maze test that measured latency period (s) and distance traveled (cm). Brains were assessed for levels of amyloid plaque load by quantitative immunohistochemistry, A β 40, and A β 42 by ELISAs; also, CTF β and sAPP α were assessed by quantitation of Western blots.

Parameter	Mean \pm percentage S.E.			Significance ($p < 0.05$)
	Control	CA074Me	E64d	
Latency period (s)	38 \pm 8%	37 \pm 12%	40 \pm 10%	NS ^a
Distance traveled (cm)	253 \pm 10%	246 \pm 13%	270 \pm 12%	NS
Amyloid plaque load in brain (% area)	1.14 \pm 4%	1.07 \pm 4%	1.06 \pm 4%	NS
A β 40 levels in brain (% control)	103 \pm 5%	91 \pm 4%	92 \pm 7%	NS
A β 42 levels in brain (% control)	100 \pm 4%	97 \pm 3%	101 \pm 3%	NS
CTF β levels in brain (% control)	96 \pm 4%	97 \pm 3%	100 \pm 3%	NS
sAPP α levels in brain (% control)	105 \pm 5%	101 \pm 4%	98 \pm 3%	NS

^a NS, means not significantly different by analysis of variance.

TABLE 4
Specific activities of cathepsin B and BACE 1 for cleaving the wild-type β -secretase site of APP

Peptide-MCA substrates that mimic the wild-type and Swedish mutant β -secretase cleavage sites are represented by Z-Val-Lys-Met \downarrow MCA and Z-Val-Asn-Leu \downarrow MCA (mutant residues are underlined), respectively. Purified cathepsin B and BACE 1 were assessed for specific activities for cleaving the representative substrates in cleavage site-specific assays for the wild-type and Swedish mutant β -secretase sites.

Protease	Specific activity	
	WT site Z-Val-Lys-Met \downarrow MCA	Swedish mutant site Z-Val-Asn-Leu \downarrow MCA
	<i>pmol AMC/min/μg enzyme</i>	
Cathepsin B	547	0.2
BACE 1	0	0.01

TABLE 5
Kinetic efficiencies of cathepsin B and BACE 1 for cleaving the wild-type β -secretase site of APP

The kinetic catalytic efficiency, k_{cat}/K_m ($M^{-1} s^{-1}$), of cathepsin B was determined for cleaving the representative wild-type β -secretase substrate Z-Val-Lys-Met \downarrow MCA.

Protease	Catalytic efficiency, k_{cat}/K_m^a
	$M^{-1} s^{-1}$
Cathepsin B	3.17×10^5
BACE 1	~ 50

^a The k_{cat}/K_m value for BACE 1 cleavage of the wild-type β -secretase site is shown as the average from two reports (10, 52).

hyde, reduces A β and CTF β *in vivo* in guinea pig brains (41). Significantly, the *in vivo* effectiveness of the cysteine protease inhibitors mirrors their *in vitro* effectiveness for inhibition of the β -secretase activity of cathepsin B in regulated secretory vesicles. These results suggest that the cysteine protease inhibitors may be reducing β -secretase activity by inhibiting cathepsin B.

BACE 1 has been proposed to be a major β -secretase based on BACE 1 knock-out experiments (42–45). However, the knock-out results (43–45) do not preclude cathepsin B as a candidate β -secretase that cleaves the WT β -secretase site of APP in the regulated secretory pathway. Roberds *et al.* (45) describes BACE 1 knock-out mice that lack β -secretase activity, but the β -secretase assays contained E64, a potent inhibitor of cysteine proteases, which precluded detection of cysteine protease β -secretase activity in the assay. Therefore, Roberds *et al.* (45) do not rule out cathepsin B as a β -secretase. Luo *et al.* (43) found that knocking out BACE 1 in transgenic mice expressing Swe mutant human APP resulted in no production of A β or C99 (CTF β). However, this study did not evaluate WT β -secretase site APP cleavage. Thus, Luo *et al.* (43) do not rule out cathepsin B β -secretase cleavage of APP containing the WT β -secretase site. Cai *et al.* (44) found that “conditioned media” lacked A β from embryonic brain neurons of BACE 1 knock-out animals expressing APP containing the WT β -secretase site. However, the conditioned media reflected only material secreted by constitutive secretion, because the neurons were not stimulated with a triggering agent and, therefore, no regulated secretion occurred. Thus, Cai *et al.* (44) do not rule out cathepsin B β -secretase activity in the regulated secretory pathway. Taken together, the knock-out BACE 1 experiments did not address cysteine protease β -secretase cleavage of APP containing the WT β -secretase in the regulated secretory pathway. Therefore, they do not rule out cathepsin B as a possible β -secretase.

Studies in human AD brains support a role for cathepsin B in the “disease situation.” Cathepsin B is colocalized with A β in amyloid plaques of AD brains (46–48) and is elevated in CSF of AD patients (49). Also, age-related changes in cathepsin B expression are consistent with the late age of onset of AD (50, 51). These findings indicate a role for cathepsin B in AD.

In conclusion, this study shows that the cysteine protease inhibitors CA074Me and E64d are efficacious in the London APP mouse model by reducing memory deficit, amyloid plaque load, brain A β , and β -secretase activity. However, these cysteine protease inhibitors have no effect in Swedish/London APP mice. The reason for this difference is attributed to the expression of APP containing the WT β -secretase site in the London APP mouse, rather than the Swe mutant β -secretase site expressed in the London/Swedish APP mouse. The difference was expected, because the RSV β -secretase and cathepsin B assays used to select the compounds identify those that inhibit β -secretase cleavage of the WT β -secretase substrate and not the Swe β -secretase substrate. Inhibition of cathepsin B as a β -secretase is a likely mechanism by which the cysteine protease inhibitors improve memory deficit and reduce A β in the London APP mice. Since most AD patients express APP containing the WT β -secretase, the efficacy in the London APP mouse demonstrates the potential of these cysteine protease inhibitors as therapeutic agents for AD.

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