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Reductions in β-amyloid concentrations in vivo by the γ-secretase inhibitors BMS-289948 and BMS-299897

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Abstract

A primary pathological feature of Alzheimer's disease is β-amyloid (Aβ)-containing plaques in brain and cerebral vasculature. Reductions in the formation of Aβ peptides by γ-secretase inhibitors may be a viable therapy for reducing Aβ in Alzheimer's disease. Here we report on the effects of two orally active γ-secretase inhibitors. BMS-289948 (4-chloro-*N*-(2,5-difluorophenyl)-*N*-((1R)-{4-fluoro-2-[3-(1H-imidazol-1-yl)propyl]phenyl}ethyl)benzenesulfonamide hydrochloride) and BMS-299897 (4-[2-((1R)-1-{[(4-chlorophenyl)sulfonyl]-2,5-difluoroanilino}ethyl)-5-fluorophenyl]butanoic acid) markedly reduced both brain and plasma $A\beta_{1-40}$ in APP-YAC mice with ED_{50} values of 86 and 22 mg/kg per os (po), respectively, for BMS-289948, and 30 and 16 mg/kg po, respectively, for BMS-299897. Both compounds also dose-dependently increased brain concentrations of APP carboxy-terminal fragments, consistent with inhibition of γ-secretase. BMS-289948 and BMS-299897 (100 mg/kg po) reduced brain and plasma $A\beta_{1-40}$ rapidly (within 20 min) and maximally within 3 h. BMS-299897 also dose-dependently reduced cortical, cerebrospinal fluid (CSF), and plasma Aβ in guinea pigs with ED_{50} values of 30 mg/kg intraperitoneally, without affecting CSF levels of α-sAPP. The reductions in cortical Aβ correlated significantly with the reductions in both plasma ($r^2 = 0.77$) and CSF ($r^2 = 0.61$) Aβ. The decreases in Aβ were apparent at 3 and 6 h post-administration of BMS-299897, but not at 12 h. These results demonstrate that BMS-289948 and BMS-299897 are orally bioavailable, functional γ-secretase inhibitors with the ability to markedly reduce Aβ peptide concentrations in APP-YAC transgenic mice and in guinea pigs. These compounds may be useful pharmacologically for examining the effects of reductions in β-amyloid peptides in both animal models and in Alzheimer's disease.

Keywords: Alzheimer's disease; β-Amyloid; Secretase; Amyloid precursor protein; γ-Secretase inhibitor; Proteolytic processing

1. Introduction

The presence of extracellular neuritic plaques and intracellular neurofibrillary tangles are characteristic features of Alzheimer's disease brain. Although the precise molecular mechanisms by which these lesions develop are unclear, the principal consequences are neuronal death and cognitive impairment [1]. Mutations in the amyloid precursor

Abbreviations: Aβ, β-amyloid; APP, Amyloid precursor protein; DAPT, [*N*-(3,5-difluor-phenacetyl)-L-alanyl]-*S*-phenylglycine; BMS-289948, 4-chloro-*N*-(2,5-difluorophenyl)-*N*-((1R)-{4-fluoro-2-[3-(1H-imidazol-1-yl)propyl]phenyl}ethyl)benzenesulfonamide hydrochloride; BMS-299897, 4-[2-((1R)-1-{[(4-chlorophenyl)sulfonyl]-2,5-difluoroanilino}ethyl)-5-fluorophenyl]butanoic acid; CSF, cerebrospinal fluid; APP-YAC, amyloid precursor protein yeast artificial chromosome; ELISA, enzyme-linked immunosorbant assay

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protein (APP) or in presenilins are associated with early onset, familial forms of Alzheimer's disease [2]. Genetic defects in APP typically result in increased production of β-amyloid peptides including $A\beta_{1-40}$ and $A\beta_{1-42}$ [3], while mutations in presenilin 1 and presenilin 2 result in selective increases in $A\beta_{1-42}$ formation [4,5]. Increased production of β-amyloid peptides, in particular the highly fibrillogenic form, $A\beta_{1-42}$, may facilitate amyloidogenesis and the formation of neuritic plaques in Alzheimer's disease [6,7]. Mounting evidence also suggests that even less-aggregated protofibrils or oligomers of Aβ may produce the synaptic toxicity observed in Alzheimer's disease [8]. Decreasing the formation of Aβ peptides may limit the deposition of Aβ into insoluble plaques and may also diminish the ability of Aβ to form oligomers.

The β-amyloid peptides are formed through proteolytic cleavage of APP by several distinct secretase enzymes [9,10]. β-Secretase cleaves APP to form the amino-terminus of Aβ (contained within the 99 amino acid-residue carboxy-terminal APP fragment or β-carboxy-terminal fragment) and a soluble APP derivative (β-sAPP) [11]. α-Secretase cleaves APP within the Aβ peptide domain, thereby precluding the generation of Aβ, to produce α-sAPP and an 83-residue carboxy-terminal fragment (α-carboxy-terminal fragment) [12,13]. Both the β-secretase-cleaved and α-secretase-cleaved carboxy-terminal fragments are substrates for further proteolysis by γ-secretase, which cleaves the former fragment to produce the carboxy-terminus of Aβ and the latter fragment to form p3. Compounds that alter the proteolytic cleavage of APP, including those that inhibit β- or γ-secretase activity or facilitate α-secretase activity, can reduce the production of Aβ peptides and may have potential in treating Alzheimer;s disease [14].

The γ-secretase inhibitor *N*-[*N*-(3,5-difluor-phenacetyl)-L-alanyl]-*S*-phenylglycine (DAPT) was shown to reduce brain concentrations of Aβ following systemic dosing in mice transgenic for human APP_{V717F} (PDAPP mice) [15]. More recently, DAPT was shown to diminish brain, plasma, and CSF Aβ in mice transgenic for human APP with the ''Swedish'' mutation ($APP_{K670N/M671L}$; Tg2576 mice) [16]. The purpose of the present investigation was to test the effects of two novel, γ-secretase inhibitors on both peripheral and central Aβ peptide levels in two rodent species which express normal (not mutant) human APP. This is important since sporadic forms of Alzheimer's disease, which comprise greater than 90% of all cases of the disease, are generally not associated with mutant forms of APP or over-expression of Aβ [17]. APP-YAC mice were employed since these animals express normal human APP transcripts in a tissue-specific manner and at levels comparable to endogenous mouse APP [18,19]. Guinea pigs were also utilized since their Aβ peptide sequence is identical to the human peptide [20] and expressed at comparable levels. Both APP-YAC mice and guinea pigs may therefore serve as physiological, rather than pathological, models for testing the effects of γ-secretase inhibitors on Aβ peptide formation. The present study demonstrates that the γ-secretase inhibitors BMS-289948 (4-chloro-*N*-(2,5-difluorophenyl)-*N*-((1R)-{4-fluoro-2-[3-(1H-imidazol-1-yl)propyl]phenyl}ethyl)-benzenesulfonamide hydrochloride) and BMS-299897 (4-[2-((1R)-1-{[(4-chlorophenyl)sulfonyl]-2,5-difluoroanilino}ethyl)-5-fluorophenyl]butanoic acid) [21] decrease Aβ peptide levels in plasma, CSF, and brain in these two normal APP-expressing animal models.

2. Methods

2.1. Animals

APP-YAC mice transgenic for human genomic APP were generated as described previously [19] and bred to homozygosity. Only homozygous mice (20–30 g) were utilized in this study. Both male and female mice were utilized, but only a single sex was used in individual experiments (there were no differences in Aβ levels between male and female mice). Guinea pigs (350–400 g) were purchased from Hilltop. Animals were housed on a 12 h:12 h light:dark cycle with access to food and water ad libitum. All procedures were approved by the Institutional Animal Care and Use Committee and were in accordance with *The Guide for the Care and Use of Laboratory Animals*.

2.2. Compound synthesis and administration

BMS-289948 (also known as SIB-3399) and BMS-299897 (also known as SIB-3520) (Fig. 1) were synthesized by the Medicinal Chemistry Department at SIBIA Neurosciences, Inc. Both compounds were dissolved in

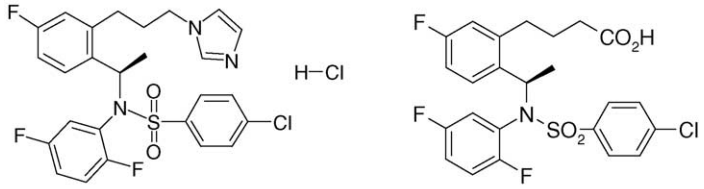

Fig. 1. Chemical structures of BMS-289948 and BMS-299897.

100% polyethylene glycol 400 and administered by either oral gavage or intraperitoneally at a dosing volume of 3 ml/kg for guinea pigs or 5 ml/kg for APP-YAC mice.

2.3. Blood, cerebrospinal fluid, and brain tissue collection

Animals were anesthetized with an intramuscular injection of a mixture containing ketamine (150 mg/kg), acepromazine (1.5 mg/kg), and xylazine (8 mg/kg). Collection of cerebrospinal fluid (guinea pigs only) was performed as described previously [22]. Briefly, animals were placed in a modified stereotaxic frame, the skin and muscles overlying the back of the neck were excised, and the atlanto-occipital membrane was exposed. A 30 gauge hypodermic needle, attached to a 1 cm^3 syringe, was inserted through the membrane and into the cisterna magna. Approximately 125–200 μl of clear CSF was obtained per animal. Blood was obtained by cardiac puncture (from all animals) and collected into syringes pre-loaded with ethylenediaminetetraacetic acid (EDTA). The blood was centrifuged at room temperature for 10 min at 2000 × *g*. Plasma was then aliquoted, frozen on dry ice, and stored at −70 °C until assayed. After collection of blood each animal was euthanized and the brain was rapidly removed and dissected over wet ice. Brain hemispheres (from APP-YAC mice) or cerebral cortices (from guinea pigs) were frozen on dry ice and stored at −70 °C until assayed.

2.4. Brain tissue processing

For determination of Aβ, full-length APP, and APP carboxy-terminal fragments, brain tissues were weighed and Dounce homogenized in 24 volumes (w/v) of 1% CHAPS/phosphate buffered saline containing 1 mM PMSF, 4.5 μg/ml leupeptin, 30 μg/ml aprotonin, and 1 μM pepstatin. Homogenates were then incubated on a tube rotator for 30 min at 4 °C followed by centrifugation (4 °C for 80 min at 80,000 × *g*) on a Beckman ultracentrifuge. Supernatants were frozen on dry ice, and stored at −70 °C until assayed by ELISA or Western blot. For determination of α-sAPP, brain tissue was homogenized in 5 volumes of ice-cold buffer (320 mM sucrose, 10 mM HEPES, 100 mM sodium chloride, and 150 mM EDTA containing protease inhibitors as described above). Homogenates were centrifuged (80,000 × *g*) for 30 min at 4 °C and supernatants were aliquoted and stored at −70 °C until assayed.

2.5. Western blot analysis

For measurement of full-length APP, APP carboxy-terminal fragments or α-sAPP, brain extracts were diluted in 2× Tris–glycine reducing sample buffer and boiled for 5 min. The samples were run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (120 V) using 10–20% Tris–tricine gels for analysis of full-length APP and APP carboxy-terminal fragments or 4–12% Tris–glycine gels for analysis of α-sAPP (Invitrogen). Proteins were transferred to Protran nitrocellulose membranes, the membranes were blocked in 10% milk, and probed with the C-terminal APP polyclonal antibody 369 or 7334 (both antibodies recognize C-terminal $APP_{645–694}$). Blotted membranes were developed with horseradish peroxidase (HRP)-conjugated secondary antibodies. For assessment of α-sAPP, membranes were probed with an HRP-conjugated monoclonal antibody specific for human $Aβ_{1–12}$ (26D6). For determination of Aβ peptides, brain extracts were immunoprecipitated with 26D6 monoclonal antibody, eluted, and diluted in 2× bicine sample buffer (0.72 M Bistris, 0.32 M bicine, 2% SDS, 25% glycerol, 100 mM DTT, and 0.008% bromophenol blue). The samples were boiled for 5 min and loaded onto bicine-urea gels [23]. Gels were run at 100 V for 2 h and proteins were transferred to PVDF membranes. Membranes were then boiled in PBS for 5 min, blocked in 10% milk, and probed with 26D6-HRP. The protein bands were visualized by enhanced chemiluminescence and exposed to Kodak Scientific Imaging Film X-OMAT. Band densities in developed films were quantitated with a scanning laser densitometer (Molecular Dynamics).

2.6. ELISA analysis

Standard sandwich ELISAs were used to quantify $Aβ_{1–40}$ in brain, plasma, and CSF. In brief, an $Aβ_{1–40}$ human-specific monoclonal antibody was coated onto microtiter plates. The plates were blocked with 0.1% bovine serum albumin in phosphate buffered saline and standards and samples were added and incubated overnight at 4 °C. Plasma and brain samples were typically assayed undiluted while CSF was diluted 1:4. After binding the wells were washed with phosphate buffered saline. 26D6-HRP was used for detection [24]. Optical densities of each well were determined at 450 nm using an ELISA plate reader. Concentrations in samples were determined by linear regression using the standard curve generated from each plate. For measuring plasma Aβ in APP-YAC mice, the biotinylated monoclonal antibody 4G8 ($Aβ_{17–24}$), instead of 26D6, was used for detection. The limit of sensitivity of each assay was 0.025 ng/ml. Analysis of α-sAPP in guinea pig CSF was performed by incubating samples in microtiter plates coated with an N-terminal APP polyclonal antibody. After binding, the wells were washed and 26D6-HRP was used for detection. Optical densities of each well were determined at 450 nm using an ELISA plate reader.

2.7. Statistical analysis

The values presented are mean ± standard error of the mean. In general, values are expressed as a percentage of

the mean of vehicle-treated control groups. Differences between treatment and control groups were analyzed by one-way analysis of variance, followed by Dunnett's *t*-test when a significant ($P < 0.05$) *F*-value was obtained. Correlation analysis was carried out using the Pearson Product Moment correlation analysis and ED_{50} values were estimated using non-linear regression (Prism GraphPad).

3. Results

3.1. Effects of BMS-289948 and BMS-299897 on Aβ in brain and plasma in APP-YAC mice

BMS-289948 produced dose-dependent decreases in brain and plasma $A\beta_{1–40}$ 3 h following oral administration in APP-YAC mice (Fig. 2A). The ED_{50}s for reducing brain and plasma $A\beta_{1–40}$ were approximately 86 and 22 mg/kg, respectively. BMS-299897 also produced dose-dependent reductions in brain and plasma $A\beta_{1–40}$ in APP-YAC mice (Fig. 2B), but was more efficacious than BMS-289948. The ED_{50}s for reducing brain and plasma $A\beta_{1–40}$ were 30 and 16 mg/kg, respectively. Both compounds were well-tolerated and no adverse effects were observed following acute dosing. Correlation analysis revealed a significant correlation between brain and plasma $A\beta_{1–40}$ ($r^2 = 0.60$, $P < 0.0001$) in APP-YAC mice treated with BMS-299897. The decreases in brain $A\beta_{1–40}$ as determined by ELISA were confirmed by immunoprecipitation/Western blot analysis (Fig. 2C). Treatment with BMS-299897 reduced both $A\beta_{1–40}$ and $A\beta_{1–42}$ peptide levels to an equivalent extent.

3.2. Effects of BMS-289948 and BMS-299897 on brain APP carboxy-terminal fragments in APP-YAC mice

BMS-289948 and BMS-299897 dose-dependently increased APP carboxy-terminal fragments in brain from APP-YAC mice as determined by Western blot analysis using the anti-C-terminal APP antibody 369 (Fig. 3). Significant increases were observed following dosing with 25–175 mg/kg BMS-289948 and with 50–150 mg/kg BMS-299897. The highest doses tested produced 15–20-fold increases in the APP carboxy-terminal fragments while also producing 85–95% decreases in brain Aβ peptides. BMS-289948 and BMS-299897 increased both the α- and β-cleaved fragments, although the lower molecular weight α-cleaved fragment appeared to be increased to a greater extent. Neither compound affected brain concentrations of full-length APP.

3.3. Time course of the effects of BMS-289948 and BMS-299897 on brain and plasma APP $A\beta_{1–40}$ in APP-YAC mice

Fig. 4 shows data from two separate time course experiments; a 3 h time course study following BMS-289948 (100 mg/kg po) treatment (Fig. 4A) and a 24 h time course study following BMS-299897 (100 mg/kg po) administration (Fig. 4B). BMS-289948 produced marked decreases in plasma $A\beta_{1–40}$ with a rapid onset (i.e. 20 min post-dosing), which, at the earliest time points, were larger in magnitude than the reductions in brain $A\beta_{1–40}$. By 2 h post-dosing, the reductions were similar in magnitude for both brain and plasma $A\beta_{1–40}$. The 24 h time course study with BMS-299897 revealed marked decreases in both brain and

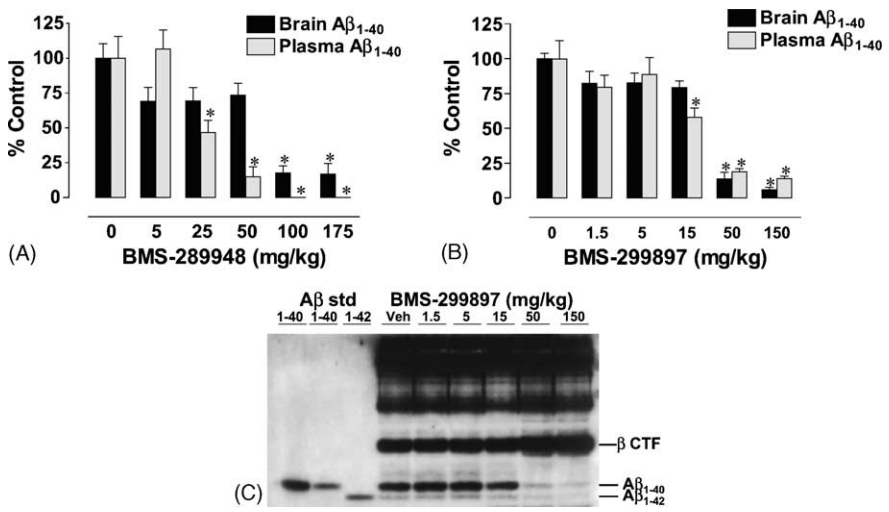

Fig. 2. Dose-dependent reductions in brain and plasma Aβ following BMS-289948 and BMS-299897 administration in APP-YAC mice. (A) Vehicle or BMS-289948 (5–175 mg/kg) was dosed orally and concentrations of brain and plasma $A\beta_{1–40}$ were determined 3 h later. Vehicle-control levels of $A\beta_{1–40}$ in brain were 8.7 ± 0.7 ng/g and in plasma were 0.5 ± 0.1 ng/ml. (B) Vehicle or BMS-299897 (1.5–150 mg/kg po) was administered and concentrations of brain and plasma $A\beta_{1–40}$ were determined 3 h later. Vehicle-control levels of $A\beta_{1–40}$ in brain were 8.1 ± 0.8 ng/g and in plasma were 0.45 ± 0.5 ng/ml. All values were expressed as a percentage of vehicle-treated controls. n = 8–9 mice per group. *Significant difference compared to control group (0 dose) by analysis of variance and Dunnett's test ($P < 0.05$). (C) Reductions in brain Aβ peptides by BMS-299897 were confirmed by immunoprecipitation/Western blot analysis. Note that this representative Western blot shows that BMS-299897 not only decreases $A\beta_{1–40}$, but also $A\beta_{1–42}$.

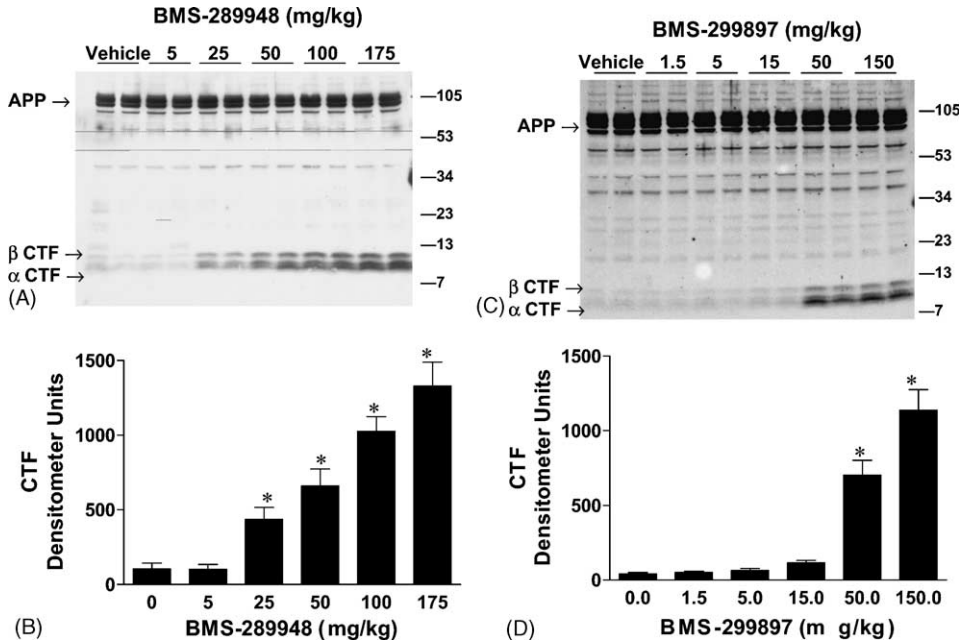

Fig. 3. Dose-dependent increases in brain APP carboxy-terminal fragments following BMS-289948 and BMS-299897 administration in APP-YAC transgenic mice. (A) BMS-289948 was dosed po (0–175 mg/kg) and brain levels of APP carboxy-terminal fragments were assayed 3 h later by Western blot analysis using the 369 carboxy-terminal APP antibody. (B) BMS-299897 was administered (0–150 mg/kg po) and brain APP carboxy-terminal fragments were assayed 3 h later. Full-length APP was not altered by either compound. Scanning laser densitometry was used to quantify the APP carboxy-terminal fragments (C, D). n = 8–9 mice/group; *Significant difference relative to vehicle control group (0 dose) by analysis of variance and Dunnett's test ($P < 0.05$).

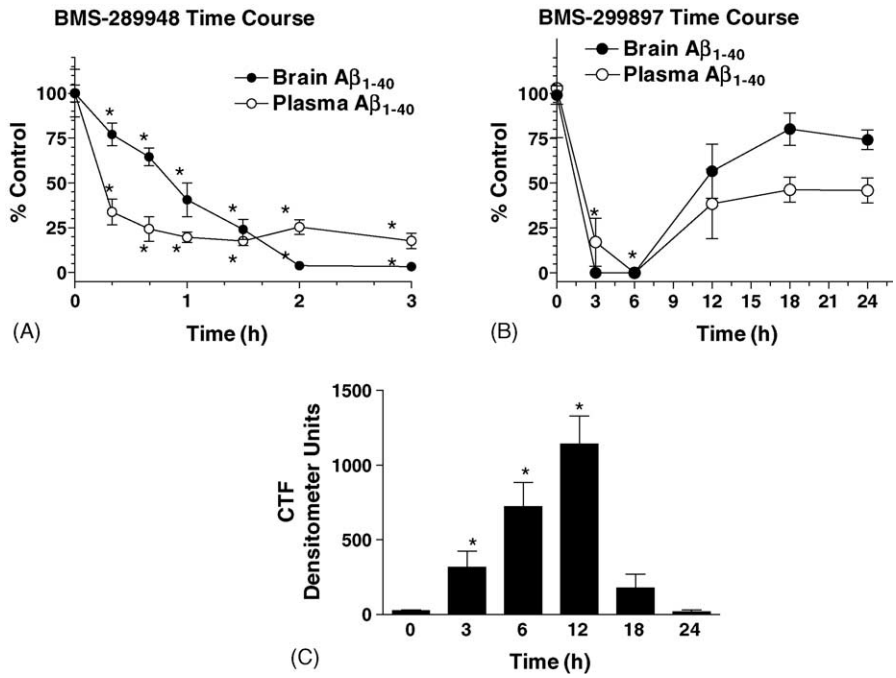

Fig. 4. Time course of the effects of BMS-289948 and BMS-299897 on brain and plasma Aβ and brain APP carboxy-terminal fragments in APP-YAC transgenic mice. (A) Brain tissue and plasma were obtained at times 20, 30, 40, 60, 120, and 180 min following oral dosing of BMS-289948 (100 mg/kg). $A\beta_{1-40}$ levels were analyzed by sandwich ELISA. Baseline (zero time point) concentrations of $A\beta_{1-40}$ in brain were 8.3 ± 0.7 ng/g and in plasma were 0.6 ± 0.1 ng/ml. Values are expressed as a percentage of baseline $A\beta_{1-40}$ concentrations. (B) Brain tissue and plasma were obtained at times 3, 6, 12, 18, and 24 h following oral dosing of BMS-299897 (100 mg/kg). $A\beta_{1-40}$ levels were analyzed by sandwich ELISA. Baseline (zero time point) concentrations of $A\beta_{1-40}$ in brain were 8.0 ± 0.7 ng/g and in plasma were 0.54 ± 0.2 ng/ml. Values are expressed as a percentage of baseline $A\beta_{1-40}$ concentrations. (C) Brain extracts were assayed for levels of full-length APP and APP carboxy-terminal fragments at 3, 6, 12, 18, and 24 h post-administration of BMS-299897 (100 mg/kg po). Extracts were assayed by Western blot using the 369 polyclonal antibody and scanning laser densitometry was used to quantify the APP carboxy-terminal fragments (both β- and α-cleaved carboxy-terminal fragments). n = 8–9 animals/group; $^{*}P < 0.05$ vs. time 0 by analysis of variance and Dunnett's t-test.

plasma $A\beta_{1-40}$ at 3 and 6 h post-administration. By 12 h, however, the effects of BMS-299897 were less pronounced with brain $A\beta_{1-40}$ returning to baseline values (although plasma levels remained diminished). The time course and reversibility of the reductions in Aβ closely paralleled the pharmacokinetics of these compounds following oral dosing in which peak brain and plasma exposure was achieved within 1–2 h of administration (data not shown).

BMS-299897 also produced time-dependent increases in APP-carboxy-terminal fragments (Fig. 4C). Significant increases were observed at 3, 6, and 12 h post-administration, although the maximal effect was observed at 12 h. Hence, there was a temporal disconnect between the maximal decreases in brain $A\beta_{1-40}$, which occurred at 6 h, and the increases in APP carboxy-terminal fragments, which occurred at 12 h.

3.4. Effect of BMS-299897 on brain levels of α-sAPP in APP-YAC mice

Concentrations of α-sAPP were determined in soluble brain extracts prepared 3 h following dosing with BMS-299897 (100 mg/kg po). There were no differences in α-sAPP levels between animals treated with vehicle or with BMS-299897 (Fig. 5).

3.5. Effects of BMS-299897 on Aβ levels in guinea pigs

Although measurement of CSF Aβ can be performed in Tg2576 [25] and PDAPP [26] transgenic mice which overexpress Aβ, the low levels of Aβ present in APP-YAC mice coupled with the small volumes of CSF that can be obtained from mice precluded our use APP-YAC mice for CSF Aβ determinations. Hence guinea pigs were utilized in subsequent studies. The baseline concentrations of $A\beta_{1-40}$ in guinea pig cerebral cortex, CSF, and plasma as determined by ELISA were 15.18 ± 0.57 ng/g ($n = 15$), 10.78 ± 1.15 ng/ml ($n = 15$), and 0.92 ± 0.09 ng/ml ($n = 15$), respectively. BMS-299897 produced dose-dependent reductions in $A\beta_{1-40}$ in cerebral cortex, plasma and CSF with ED_{50} values of approximately 30 mg/kg ip 3 h post-dosing (Fig. 6). The highest dose tested, 150 mg/kg ip, yielded 80% decreases in $A\beta_{1-40}$ in each of the three compartments examined. CSF levels of α-sAPP were not affected by BMS-299897. Correlation analysis revealed a significant correlation between cortical and CSF $A\beta_{1-40}$ ($r^2 = 0.61$, $P < 0.0001$) and a significant correlation between cortical and plasma $A\beta_{1-40}$ ($r^2 = 0.77$, $P < 0.0001$) (Fig. 7). Brain $A\beta_{1-40}$ and $A\beta_{1-42}$ were reduced to a similar extent as determined by immunoprecipitation/Western blot analysis (Fig. 8A). In addition, BMS-299897 also increased APP carboxy-terminal fragments at doses (15, 50, and 150 mg/kg) which did not affect levels of full-length APP (Fig. 8B and C). Oral administration of BMS-299897 also was efficacious in reducing Aβ peptide concentrations in guinea pigs (data not shown).

A 24 h time course study with BMS-299897 (100 mg/kg ip) revealed substantial reductions in cortical, CSF, and plasma $A\beta_{1-40}$ at 3 and 6 h post-administration (Fig. 9). However, the effects were reversible since by 12 h the concentrations of $A\beta_{1-40}$ in cerebral cortex, CSF, and plasma were similar to baseline levels.

4. Discussion

A number of approaches are currently being investigated for reducing the production or deposition of Aβ peptides as a possible treatment for Alzheimer's disease. These potential pharmacotherapies include inhibitors of γ- or β-secretase activity [9], copper/zinc metal chelators [27], statins [28], and certain non-steroidal anti-inflammatory agents [29]. In addition, immunization therapies are being explored as a means to facilitate clearance of Aβ deposits from brain [30]. Small molecule inhibitors of γ-secretase have been sought since the discovery that Aβ is derived from the larger precursor protein APP [31] and the elucidation that γ-secretase cleavage of APP is an important proteolytic event in the formation of Aβ peptides [32]. Here we describe the Aβ lowering effects of two small molecule, γ-secretase inhibitors, BMS-289948 and BMS-299897. These structurally related, orally bioavailable compounds were effective in reducing brain and plasma

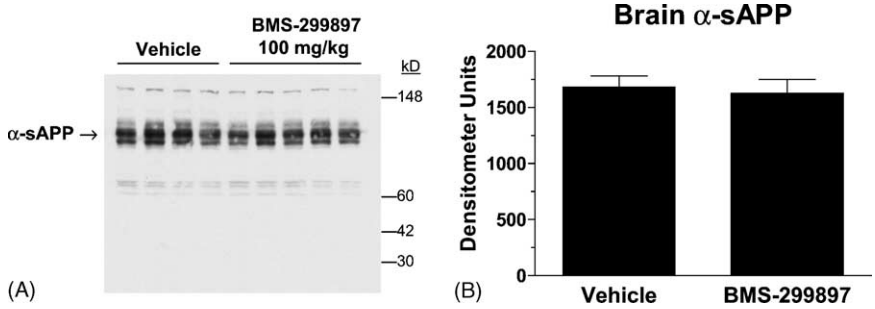

Fig. 5. BMS-299897 did not alter levels of α-sAPP in soluble brain extracts in APP-YAC transgenic mice. Brain tissue was obtained 3 h following oral administration of BMS-299897. Soluble brain extracts were prepared by extracting the tissue in non-detergent buffer followed by ultracentrifugation. (A) Western blot of α-sAPP levels as assessed by immunoblot analysis using the 26D6 monoclonal antibody conjugated to HRP. (B) Scanning laser densitometry was used to quantify the α-sAPP bands. Values are mean ± S.E.M.; $n = 4$–5 mice/group; $^*P < 0.05$ vs. vehicle by analysis of variance and Dunnett's t-test.

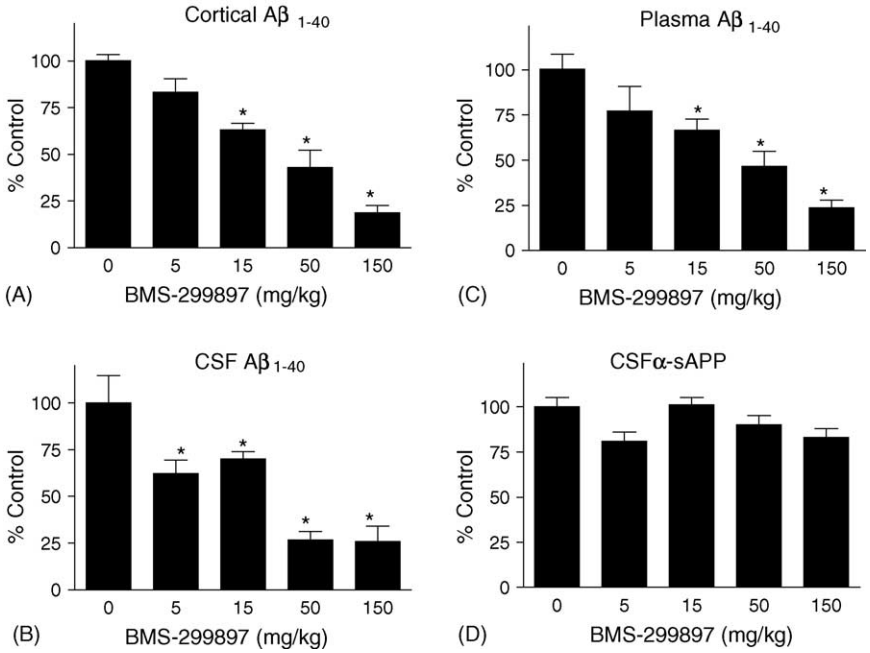

Fig. 6. Dose-dependent reductions in brain, plasma, and CSF Aβ following BMS-299897 administration in guinea pigs. Animals were dosed ip with vehicle or BMS-299897 (5, 15, 50, or 150 mg/kg). Brain tissue, plasma, and CSF were obtained 3 h post-administration. $A\beta_{1–40}$ in cerebral cortex (A), CSF (B), and plasma (C) and α-sAPP in CSF (D) was measured by ELISA. Values are expressed as a percentage of vehicle-treated control $A\beta_{1–40}$ or α-sAPP concentrations. Vehicle-treated control concentrations of $A\beta_{1–40}$ in brain were 18.1 ± 0.7 ng/g, in CSF were 15.2 ± 1.4 ng/ml, and in plasma were 0.69 ± 0.8 ng/ml. Vehicle-treated control levels of α-sAPP in CSF were 320 ± 15 ng/ml. Values are mean ± S.E.M.; $n = 6–8$ animals/group; $^{*}P < 0.05$ vs. vehicle group by analysis of variance and Dunnett's test.

Aβ in APP-YAC transgenic mice and in decreasing brain, CSF, and plasma Aβ in guinea pigs.

APP-YAC mice express human genomic APP that contains the transcriptional regulatory elements required for the proper spatial and temporal expression of all human APP transcripts (e.g. 695, 751, 770) [19]. Other Alzheimer's disease-related transgenic mouse models rely on insertions of 'Indiana' or 'Swedish' mutant APP cDNA with associated promoters which drive the expression levels of human APP to 5–10-fold over endogenous APP [33,34], resulting in very high levels of human Aβ peptides in brain. Tg2576 mice, for example, have 10–60-fold higher levels of brain $A\beta_{1–40}$ than APP-YAC mice [25]. We have also shown that BMS-299897 reduces brain, plasma, and CSF Aβ in Tg2576 mice [35]. Transgenic mice over-expressing mutant forms of APP may most closely model genetically inherited forms of Alzheimer's disease, which account for less than 10% of all cases of the disease. APP-YAC mice, because of their expression of non-mutant forms of human APP at levels comparable to endogenous proteins, and guinea pigs, which express only endogenous APP and Aβ which is identical in sequence to human Aβ [20], may provide models which most closely mimic sporadic, non-inherited forms of the disease which account for the vast majority of all cases of Alzheimer's disease. The present findings demonstrate that small molecule compounds like BMS-289948 and BMS-299897 can reduce the formation of Aβ peptides in these species, and compounds such as these could be used to directly test the amyloid cascade hypothesis in Alzheimer's disease.

The reductions in brain $A\beta_{1–40}$ produced by these compounds were accompanied by increases in both the α-cleaved- and the β-cleaved-carboxy-terminal fragments in brain tissue. At the same time, there was no effect on

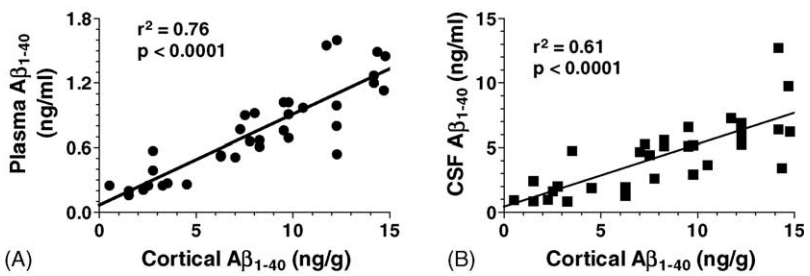

Fig. 7. Correlation analysis of $A\beta_{1–40}$ between cerebral cortex and plasma (A) and between cerebral cortex and CSF (B) in guinea pigs. Data were plotted from Fig. 6 and analyzed by Pearson Product Moment correlation analysis.

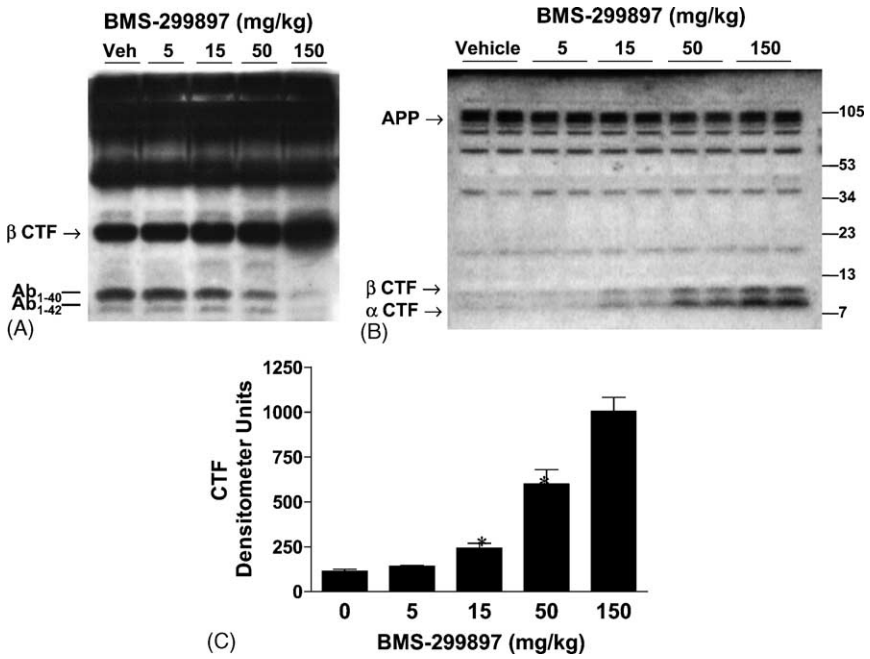

Fig. 8. Effects of BMS-299897 on cortical Aβ and APP carboxy-terminal fragments in guinea pigs as determined by Western blot analysis. (A) Representative Western blot showing reductions in $A\beta_{1–40}$ and $A\beta_{1–42}$ in cerebral cortex following administration of BMS-299897. (B) Representative Western blot showing increases in cortical APP carboxy-terminal fragments 3 h following administration of BMS-299897. (C) Scanning laser densitometry was used to quantify the APP carboxy-terminal fragments (both β- and α-cleaved carboxy-terminal fragments). Values are mean ± S.E.M., $n = 6–8$ animals/group; $^{*}P < 0.05$ vs. dose 0 by analysis of variance and Dunnett's t-test.

full-length APP or on α-sAPP levels in brain in APP-YAC mice or on α-sAPP levels in CSF in guinea pigs. Therefore, these results are consistent with γ-secretase inhibition as the mechanism of action of BMS-289948 and BMS-299897. These sulfonamide-containing compounds have been shown to directly modulate γ-secretase activity in the nM range in vitro [35] and were identified as non-competitive inhibitors of γ-secretase [36]. Recently, the peptidyl compound DAPT was shown to reduce brain Aβ levels in PDAPP mice in a manner consistent with γ-secretase inhibition [15]. This compound has also been shown to reduce Aβ peptides in brain, plasma, and CSF in Tg2576 mice [16]. Both BMS-289948 and BMS-299897 appear to have better in vivo efficacy than DAPT (85–95% decreases in brain $A\beta_{1–40}$ versus 50% reductions for DAPT 3 h following oral administration of 100 mg/kg) and are also useful tools for studying γ-secretase inhibition in vivo.

The decreases in brain and plasma $A\beta_{1–40}$ produced by the compounds tested here correlated significantly in APP-YAC mice. There was also a significant correlation between the reductions in cortical Aβ and decreases in both plasma and CSF Aβ in guinea pigs produced by BMS-299897. These results support the notion that plasma and CSF Aβ may serve as surrogate biochemical markers of brain tissue Aβ, at least in terms of monitoring in vivo efficacy of test compounds. Having a readily accessible and predictive measure of biochemical efficacy of a test drug in brain could be quite useful in clinical studies. This

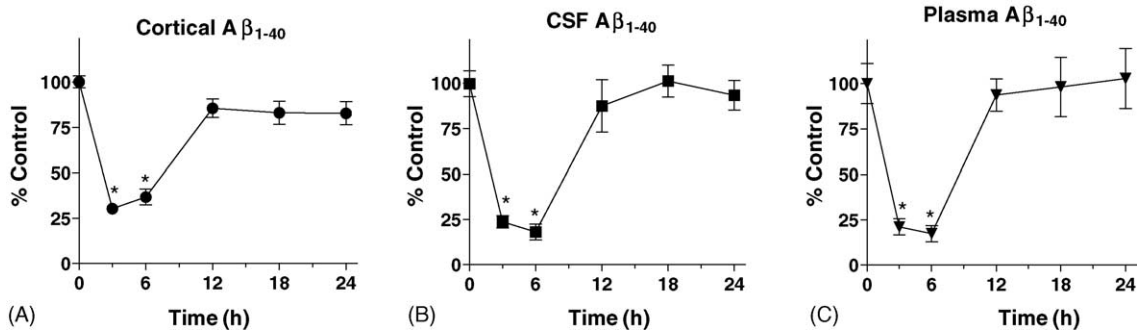

Fig. 9. Time course of the effects of BMS-299897 on brain, CSF, and plasma Aβ in guinea pigs. Animals were dosed ip with BMS-299897 (100 mg/kg) or vehicle. Cortical brain tissue (A), CSF (B), and plasma (C) were collected at the times indicated and $A\beta_{1–40}$ levels were assessed by ELISA. Values are expressed as a percentage of vehicle-treated control $A\beta_{1–40}$ concentrations. Vehicle-treated control concentrations of $A\beta_{1–40}$ in brain were 18.0 ± 0.7 ng/g, in CSF were 15.4 ± 0.9 ng/ml, and in plasma were 0.68 ± 0.8 ng/ml. Values are the mean ± S.E.M.; $n = 6–8$ animals/group; $^{*}P < 0.05$ vs. vehicle control group by analysis of variance and Dunnett's test.

is distinct from the notion that plasma or CSF Aβ may serve as a diagnostic marker for Alzheimer's disease [6,37]. We have shown that platelets contain human APP in APP-YAC mice (data not shown), and platelet APP may be a source of $A\beta_{1-40}$ detected in plasma [38,39]. Other sources of circulating $A\beta_{1-40}$ include peripheral organs where APP is found (i.e. kidney) or brain where $A\beta_{1-40}$ may enter the bloodstream from the cerebrospinal fluid or by directly crossing the brain endothelium [40].

Our findings also suggest that plasma $A\beta_{1-40}$ may be more sensitive to decreases than brain $A\beta_{1-40}$, at least at initial time points following dosing. This may result from differences in turnover of the protein between brain and blood compartments, or conceivably, to a higher degree of exposure of peripheral APP to the compounds compared to brain. By later time points the attenuation of Aβ between brain and plasma correlate more closely. Evidence suggests that CNS and plasma Aβ exist in a dynamic equilibrium which can be disrupted by peripheral administration of an anti-Aβ antibody [41] and by deposition of cerebral Aβ into plaques [26]. Hence decreases in plasma Aβ could conceivably help drive the efflux of Aβ from the brain and CSF to the plasma. A peripheral ''sink'' phenomenon was not apparent in this study since parallel reductions in Aβ occurred in plasma, CSF, and brain likely due to the suitably brain penetrant nature of these compounds. The decreases in brain and plasma $A\beta_{1-40}$ occurred as early as 20 min following oral administration of BMS-289948 suggesting rapid turnover of human $A\beta_{1-40}$ since, presumably, the ability to detect decreases hinges on the clearance of already-formed peptide. Previous estimates of $A\beta_{1-40}$ synthesis and turnover suggested that the $t_{1/2}$ for brain Aβ was 1–2.5 h [42]. Microdialysis studies revealed that turnover of extracellular human Aβ in PDAPP transgenic mice, determined following systemic administration of a γ-secretase inhibitor, was approximated 2 h in young mice [43]. We observed a 50% reduction in brain tissue Aβ by 40 min following oral dosing in APP-YAC mice, suggesting that the rate of turnover is even more rapid. In addition, there was an apparent temporal disconnect between the decreases observed in brain Aβ and the increases in APP carboxy-terminal fragments. Maximal reductions in brain Aβ were observed at 3 and 6 h post-administration, while maximal increases in APP carboxy-terminal fragments were observed at 12 h. This may result from a longer turnover of the accumulated carboxy-terminal fragments compared to Aβ. Some evidence suggests that carboxy-terminal fragments of APP can disrupt calcium homeostasis and render neurons more vulnerable to excitotoxic insults [44]. Hence, the potential for acute and chronic toxicity of γ-secretase inhibitors via accumulation of these fragments requires full characterization.

In summary, the present results demonstrate that BMS-289948 and BMS-299897 markedly reduced the formation of Aβ in brain and in plasma in APP-YAC mice and in brain, plasma, and CSF in guinea pigs. These γ-secretase inhibitors may be useful in testing whether reducing concentrations of Aβ peptides can impair the deposition of Aβ into amyloid plaques in both transgenic mice and in the clinic.

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