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Cyclic *cis*-locked phospho-dipeptides reduce entry of A β PP into amyloidogenic processing pathway

Carolyn L. Fisher^a, Ross J. Resnick^a, Soumya De^b, Lucila A. Acevedo^a, Kun Ping Lu^c, Frank C. Schroeder^d, and Linda K. Nicholson^{a,*}

^aDepartment of Molecular Biology & Genetics, Cornell University, Ithaca, NY 14853, USA

^bSchool of Bio Science, Indian Institute of Technology, Kharagpur WB 721302, India

^cDivision of Translational Therapeutics, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215, USA

^dBoyce Thompson Institute, Cornell University, Ithaca, NY 14853, USA

Abstract

The *cis/trans* isomerization of X-Pro peptide bonds in proteins in some instances acts as a molecular switch in biological pathways. Our prior work suggests that the *cis* isomer of the phospho-Thr668-Pro669 motif, located in the cytoplasmic domain of the amyloid- β precursor protein (A β PP), is correlated with an increase in amyloidogenic processing of A β PP and production of amyloid beta (A β), the neurotoxic peptide fragment in Alzheimer's disease (AD). We designed a 100% *cis*-locked cyclic dipeptide composed of cyclized phospho-Thr-Pro (pCDP) as a mimic for this putative pathological conformation, and three phosphate-blocked derivatives (pCDP-diBzl, pCDP-Bzl, and pCDP-diPOM). Two H4 neuroglioma cell lines were established as AD cell models for use in testing these compounds: H4-A β PP695 for stable overexpression of wild-type A β PP695, and H4-BACE1 for stable overexpression of β -site A β PP Cleaving Enzyme-1 (BACE1). The level of the secreted A β PP fragment resulting from BACE1 activity, sA β PP β , served as a key proxy for amyloidogenic processing, since cleavage of A β PP by BACE1 is a requisite first step in A β production. Of the compounds tested, pCDP-diBzl decreased sA β PP β levels in both cell lines, while pCDP-diPOM decreased sA β PP β levels in only H4-BACE1 cells, all with similar dose-dependences and patterns of proteolytic A β PP fragments. Enzymatic assays showed that none of the pCDP derivatives directly inhibit BACE1 catalytic activity. These results suggest a model in which pCDP-diBzl and pCDP-diPOM act at a common point to inhibit entry of A β PP into the amyloidogenic A β PP processing pathway but through different targets, and provide important insights for the development of novel AD therapeutics. Keywords: Amyloid beta-Protein Precursor, Alzheimer's Disease, cyclic dipeptides, diketopiperazine, phosphorylated Thr668.

INTRODUCTION

Cyclic dipeptides (CDPs) are 2,5-diketopiperazine structures that are naturally abundant across all organisms, including mammals [1–4]. The core CDP structure, with backbone

hydrogen bond donors and acceptors, can easily bind to catalytic and regulatory sites within enzymes [4]. CDPs can be synthesized under physiological conditions using mild acid or base chemistry [4–5], which is the process by which cyclic-His-Pro is produced in mammalian nervous systems [6]. Specific CDPs act as kinase antagonists [7], chitinase inhibitors [8], cancer drugs that cause apoptosis and growth inhibition of HT-29 colon cancer tumor cells [9], and neuroprotective agents in rats with impaired motor and cognitive abilities [10]. Although the exact mechanism of action for these neuroprotective CDPs is not well understood, there is evidence suggests that increased astrocyte activity, decreased caspase-3, or reduced of microglial reactivity could explain the neuroprotective properties of certain CDPs [reviewed in 3]. CDPs are generally small (<500 Da) and can diffuse through membranes, the blood brain barrier (BBB), and can often be taken up by peptide transporters [4]. Despite their reported neuroprotective effects, the extent to which CDPs affect the production of A β peptide, the neurotoxic peptide fragment upregulated in Alzheimer's disease (AD) pathology, has not been tested.

The amyloid- β precursor protein (A β PP) is involved in a variety of cellular processes connected to the pathogenesis of AD [11]. A β PP is a type 1 transmembrane protein composed of a large N-terminal extracellular domain, a single α -helix transmembrane domain, and a cytoplasmic tail (Figure 1A) [12]. A β PP is proteolytically cleaved *in vivo* in two ways by the cell (reviewed in 13), depending on its cellular localization. At the plasma membrane, A β PP is dominantly processed via the nonamyloidogenic pathway. Here, α secretases constitutively cleave A β PP into sA β PP α and C83 fragments [14]. C83 is further cleaved by γ secretase producing p3 and A β PP intracellular domain (AICD). Alternatively, in the amyloidogenic pathway A β PP can be internalized and localized to endosomes, where β secretase (β site A β PP cleaving enzyme 1, or BACE1) cleaves A β PP to produce sA β PP β and C99 fragments [15], followed by γ secretase cleavage of C99 to produce neurotoxic A β peptide and AICD (Figure 1A).

The innate balance between nonamyloidogenic and amyloidogenic A β PP processing can be shifted by a number of factors. Cleavage of A β PP by BACE1 is enhanced by elevated BACE1 expression [16], by elevated A β PP gene dosage as occurs in trisomy 21 individuals [17–18], by familial A β PP mutations [19], and by cholesterol enrichment in membrane invaginations (reviewed in 20). Conversely, a BACE1 cleavage site mutation in A β PP identified in an Icelandic population has been found to be protective against AD [21]. In cells, this mutation reduces BACE1-mediated A β PP cleavage and shifts A β PP processing away from the amyloidogenic route [21]. Other genetic changes that protect against AD include the E2 allele of the Apolipoprotein E (APOE) [22,23], and the BACE1-knockout, which has been shown to abolish AD pathology in mice [24]. Clearly, the regulation of A β PP processing is complex, and the development of chemical probes that alter A β PP processing could serve as useful tools for the development of strategies to prevent and/or treat AD.

Within the cytoplasmic tail of A β PP (A β PPc), the level of phosphorylation of the Thr668-Pro669 (TP) motif (Figure 1B) is increased in the AD brains [25], and may be an important signaling motif that becomes dysregulated in the development of AD [25–27]. Prior to phosphorylation, the *trans*-isomer of the TP peptide bond is stabilized by the formation of a

helix-capping box structure [28] (Figure 1A, pink box) and no *cis*-TP isomer is detected [29]. Only after phosphorylation is the helix-capping box destabilized (Figure 1B) and a *cis*-phosphorylated-TP (pTP) population emerges in ~10% abundance with the *trans*-pTP isomer in ~90% abundance [29]. The exchange between *cis* and *trans* isomers of the pTP peptide bond is very slow [30], and is accelerated by ~2000 fold by the enzyme Pin1 [31,32]. Additionally, brain tissue from Pin1 knockout mice show an increase in the phosphorylation of T668 in A β PP and in amyloidogenic A β PP processing (Figure 1A) [33]. Since pT668 accumulates in AD brains [25] and is required for formation of the *cis*-pTP isomer [29], the *cis* isomer might serve as a molecular signal for putative cellular binding proteins that localize A β PP to endosomes for β -secretase cleavage [33].

To test the importance of the *cis*-pTP conformation as a signal for A β PP processing, we synthesized a small molecule, phospho-Thr-Pro cyclic dipeptide (pCDP) (Figure 1C), that is a 100% “*cis*-locked” mimic of the *cis*-pTP motif in the A β PP cytoplasmic tail. Three additional pCDP derivatives with blocking groups on the phosphate moiety were generated to test delivery and activity of these molecules in cells (Figure 2). Two distinct H4 neuroglioma cell lines that stably overexpress either A β PP695 (H4-A β PP695) or BACE1 (H4-BACE1) were generated, providing AD cell models in which the effects of pCDP compounds on A β PP processing production were investigated. The secreted product of APP cleavage by BACE1, sA β PP β , was used as a proxy for monitoring amyloidogenic processing. Testing of pCDPs in two comparative AD models revealed that, while both pCDP-diBzl and pCDP-diPOM inhibit amyloidogenic A β PP processing, they must act through different targets but at a similar point in the pathway. Together, these data suggest that pCDP-diBzl and pCDP-diPOM molecules provide intriguing tools for investigating the role of *cis*-pTP conformation in the proteolytic processing of A β PP, potentially leading to novel strategies for inhibiting the production of A β .

MATERIALS AND METHODS

Materials

The human H4 neuroglioma cell line was purchased from ATCC (Manassas, VA). Dulbecco's Modified Eagle Medium (DMEM), Penicillin-Streptomycin and culture dishes were from Corning Life Sciences (Tewksbury MA); Fetal bovine serum (Premium Select) was purchased from Atlanta Biologicals (Flowery Branch, GA); DC Protein Assay Kit, Pre-stained Dual Color Protein Standards, Clarity Western ECL, Chemidoc MP System, Image Lab Software, Chemi Hi Sensitivity blot application, Immuno-Blot LF PVDF membrane, 4–20% Mini Protean TGX Stain Free precast gels, and 4–15% Mini Protean TGX Stain Free precast gels were obtained from BioRad (Hercules, CA); polyvinylidene fluoride (PVDF) transfer membrane (0.45mm) was purchased from Perkin Elmer (Waltham, MA); Whatman nitro-cellulose transfer membrane (0.2 μ m) was purchased from GE Healthcare Life Sciences; Lipofectamine 2000, Pierce ECL Western Blotting, Pierce SuperSignal West Pico substrates and G418 sulfate (Geneticin) were all from ThermoFisher Scientific (Waltham, MA); Recombinant Human BACE1 protein, CF (931-AS) and Mca-SEVNLDAEFRK(Dnp)RR-NH2 Fluorogenic Peptide Substrate (ES004) were acquired from

R&D Systems (Minneapolis, MN); the BACE1 inhibitor LY2811376 was purchased from Selleckchem (Houston, TX).

Plasmids

pCAX-A β PP⁶⁹⁵ expressing full-length human A β PP⁶⁹⁵ was a gift from Dennis Selkoe & Tracy Young-Pearse (Addgene plasmid # 30137). pCMV6-XL5-BACE1 expressing full-length human BACE1, transcript variant a, NM_012104.3 (# SC115547) was purchased from Origene, Rockville, MD. pSV2neo [34] which provides a selectable marker for resistance to the antibiotic G418 in mammalian cell lines was a gift from David Shalloway, Cornell University.

Antibodies

Rabbit anti-sA β PP β (poly8134, 813401) and the mouse monoclonal antibody 6E10 (803001) were purchased from BioLegend (San Diego, CA) and the anti- β -tubulin mouse monoclonal antibody (2G7D4, A01717-40) was acquired from GenScript (Piscataway, NJ). The rabbit monoclonal antibodies anti-A β PP (EPR5119(2), ab133588), anti-C-terminal A β PP antibody Y188 (ab32136) and anti-BACE1 (EPR3956, ab108394) were all purchased from Abcam (Cambridge, MA). Goat anti-rabbit and goat anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). The anti-pan-A β rabbit monoclonal antibody (D54D2) was purchased from Cell Signaling Technology (Danvers MA).

Cell culture

All H4 neuroglioma cell lines were routinely grown in monolayer culture in growth medium consisting of DMEM (4.5 g/L glucose, 3.7g/L sodium bicarbonate) supplemented with 10% fetal bovine serum and 100 IU /100 μ g/ml penicillin/streptomycin at 37°C in a humidified atmosphere (90%) containing 10% CO₂. Cells were isolated by trypsinization and routinely passaged to maintain stocks or plated for experiments as described below or in the figure legends.

Generation of H4 neuroglioma cell lines over-expressing A β PP⁶⁹⁵ or BACE1

Cell lines constitutively over-expressing human A β PP⁶⁹⁵ (H4-A β PP⁶⁹⁵) or human BACE1 (H4-BACE1) were created by co-transfecting either pCAX-A β PP⁶⁹⁵ or pCMV6-XL5-BACE1 (2–4 μ g) with the G418 resistance plasmid pSV2neo (0.2–0.4 μ g) into H4 neuroglioma cells using Lipofectamine 2000 according to the manufacturer's instructions. Forty-eight hours after transfection, cells were split into growth medium supplemented with 500 μ g/mL of G418 and after 2–3 weeks G418 resistant colonies were screened by immunoblotting for A β PP or BACE1 over-expression as compared to the parental H4 cell line. Positive clones were expanded and maintained in growth medium containing 200 μ g/mL G418 until frozen.

Analysis of custom-synthesized compounds

Nuclear Magnetic Resonance (NMR) Analysis—All pCDPs used in this study (pCDP, pCDP-Bzl, pCDP-diBzl, pCDP-diPOM) were custom synthesized using green

chemistry [35] and phosphorylation of Threonine completed [36–37] by Viva Biotech Ltd. (Shanghai, China) and purchased through Trilliance (Toronto, Ontario). Deuterated-methanol NMR solvent (99.8%, CD₃OD) was purchased from Cambridge Isotope Laboratories and used for each sample preparation. NMR spectra (¹H and ¹³C) were recorded at room temperature (RT) with a Bruker Avance^{III} HD 800 MHz instrument (SUNY-ESF) or a Varian Inova 600 MHz instrument (Cornell University). Chemical shifts are reported in δ (ppm) units relative to residual solvent peaks CD₃OD (3.31 ppm for ¹H, 49.0 ppm for ¹³C). Splitting patterns are assigned as s (singlet), d (doublet), t (triplet), q (quartet), p (pentet), m (multiplet), dd (doublet of doublets), ddd (doublet of doublet of doublets), dddd (doublet of doublet of doublet of doublets), dq (doublet of quartets), dt (doublet of triplets), td (triplet of doublets), qd (quartet of doublets), and pd (pentet of doublets). All NMR spectra were analyzed with MNOVA software (v.10.0). Supplemental Figures 1–4 show ¹H NMR spectra for the pCDP compounds.

Liquid Chromatography Mass Spectrometry (LCMS) Analysis—Each pCDP standard sample was diluted from 100 mM of stock pCDP in dimethyl sulfoxide (DMSO) down to 1 μ M pCDP in 50:50 methanol:water and 2 μ L was separated using reverse-phase high resolution UHPLC-MS with an Agilent Zorbax RRHD Eclipse XDB-C18 column (2.1 $\times$ 100 mm, 1.8 μ m particle diameter), 0.1% formic acid in acetonitrile (organic phase), and 0.1% formic acid in water (aqueous phase) at a rate of 0.5 mL/min for 15 minutes at 40°C (through the Chromelon Xpress software system) on a Thermo Scientific Dionex Ultimate3000 UHPLC system, equipped with a diode array detector and connected to a Thermo-Scientific Q Exactive hybrid quadrupole-Orbitrap mass spectrometer (Boyce Thompson Institute Mass Spectrometry Center, Cornell University). A solvent gradient scheme was used: 5% organic for 1.5 minutes, a linear increase to 100% organic over 11 minutes, and then a 2 minute hold at 100% organic before decreasing back to 5% organic over 0.1 minutes with a final hold at 5% organic for the last 0.4 minutes, for a total of 15 minutes. The Thermo-Scientific Xcalibur software package was used to visualize, analyze, and depict the LCMS data shown in Supplemental Figure 5.

pCDP (Supplemental Figures 1 and 5A)

¹H NMR (800 MHz, CD₃OD, 25°C)— δ 1.53 (d, J = 6.5 Hz, 3H), 1.90 – 1.96 (m, 1H) 1.98 – 2.04 (m, 2H), 2.28 – 2.34 (m, 1H), 3.45 (ddd, J = 11.9, 9.1, 3.0 Hz, 1H), 3.65 (dt, J = 11.6, 7.9, 1H), 4.12 (dt, J = 3.4, 1.6 Hz, 1H), 4.22 (ddd, J = 9.8, 6.8, 1.7 Hz, 1H). **¹³C NMR (800 MHz, CD₃OD-d₄, 25°C)**: δ 19.01, 22.85, 29.10, 46.02, 60.64, 59.89, 72.91, 165.82, 171.71. HR-LCMS (ESI+): Calculated C₉H₁₅N₂O₆P + [M + H]⁺ = 279.07471; found [M + H]⁺ = 279.07360, mass tolerance 0.1 mmu, retention time = 1.12 minutes.

pCDP-Bzl (Supplemental Figures 2 and 5B)

¹H NMR (600 MHz, CD₃OD-d₄, 25°C)— δ 1.52 (d, J = 6.3 Hz, 3H), 1.84 – 1.96 (m, 2H), 1.97 – 2.07 (m, 1H), 2.22 – 2.30 (m, 1H), 3.39 (ddd, J = 11.9, 8.7, 3.7 Hz, 1H), 3.49 (dt, J = 11.7, 8.2 Hz, 1H), 4.02 (d, J = 5.9 Hz, 1H), 4.18 (dd, J = 8.1, 8.3 Hz, 1H), 4.73 (dt, J = 7.4, 6.1 Hz, 1H), 4.92 (d, J = 6.2 Hz, 2H), 7.24 (t, J = 7.3 Hz, 1H), 7.31 (t, J = 7.6 Hz, 2H), 7.40 (d, J = 7.4 Hz, 2H). **¹³C NMR (800MHz, CD₃OD, 25°C)**: δ 19.41, 22.98, 28.95, 45.96, 59.91, 60.73, 68.55, 71.95, 128.12 (2C), 128.49, 129.09 (2C), 139.15, 166.04, 171.84. HR-

LCMS (ESI⁺): Calculated C₁₆H₂₁N₂O₆P + [M + H]⁺ = 369.12166; found [M + H]⁺ = 369.12050, mass tolerance 0.5 mmu, retention time = 4.43 minutes.

pCDP-diBzl (Supplemental Figures 3 and 5C)

¹H NMR (800 MHz, CD₃OD, 25°C)— δ 1.51 (d, J = 6.8 Hz, 3H), 1.82 (m, 3H), 2.26 (m, 1H), 3.38 (m, 2H), 4.17 (m, 2H), 5.03 (m, 4H), 5.27 (pd, J = 1.8, 6.8 Hz, 1H), 7.35 (m, 10H). **¹³C NMR (800MHz, CD₃OD, 25°C)**: δ 18.57, 22.98, 29.58, 46.24, 60.08, 60.95, 70.60, 70.81, 75.08, 128.91 (3C), 129.11 (3C), 129.60, 129.65, 129.66, 129.68, 137.32 (2C), 164.96, 171.66. HR-LCMS (ESI⁺): Calculated C₂₃H₂₇N₂O₆P + [M + H]⁺ = 459.16861; found [M + H]⁺ = 459.16706, mass tolerance 0.1 mmu, retention time = 7.49 minutes.

pCDP-diPOM (Supplemental Figures 4 and 5D)

¹H NMR (800 MHz, CD₃OD, 25°C)— δ 1.25 (d, J = 2.5 Hz, 18H), 1.58 (d, J = 6.8 Hz, 3H), 1.93 – 1.03 (m, 2H), 2.03 – 2.08 (m, 1H), 2.34 (dddd, J = 9.7, 6.7, 4.4, 1.7 Hz, 1H), 3.50 (ddd, J = 12.0, 8.8, 3.4 Hz, 1H), 3.69 (dt, J = 12.0, 8.2 Hz, 1H), 4.25 – 4.22 (m, 2H), 5.29 (pd, J = 6.8, 1.9 Hz, 1H), 5.62 – 5.68 (m, 4H). **¹³C NMR (800MHz, CD₃OD, 25°C)**: 18.61, 27.23 (6C), 23.12, 29.58, 39.74 (2C), 46.38, 60.12, 60.80, 75.87, 84.19, 84.31, 164.86, 171.65, 177.86, 177.91. HR-LCMS (ESI⁺): Calculated C₂₁H₃₅N₂O₁₀P + [M + H]⁺ = 507.21087; found [M + H]⁺ = 507.20940, mass tolerance 0.1 mmu, retention time = 7.96 minutes.

Preparation of pCDP and inhibitor treatment solutions

All pCDPs were solubilized in DMSO to a stock concentration of 100 mM and stored at 4°C. The BACE1 inhibitor LY2811376 was solubilized in DMSO to a stock concentration of 10mM and stored at –80°C. Further dilutions of these compounds were routinely prepared in DMSO (unless indicated otherwise) and the final concentration of DMSO in media during all cell treatments or in all enzymatic assay solutions did not exceed 0.4%.

Cell treatments for analysis of A β PP processing

Cells were routinely plated and treated as described below unless otherwise indicated in the figure legends. Cells were plated at a density of 2×10^5 cells per well in six-well cluster dishes in 2 mL of complete media and grown for 16–24 hours. Cells were washed twice with DMEM only and re-fed with 1 mL of complete media containing pCDPs, inhibitors or DMSO and incubated for 24 hours. Cells were washed, re-treated for an additional 24 hours and the conditioned media (CM) was collected and cleared by centrifugation at $12,000 \times g$ for 20 min at 4°C to remove intact cells and cellular debris. An aliquot of the cleared CM was combined with 4X Laemmli SDS-sample buffer containing 25 mM dithiothreitol (DTT) and used for the analysis of secreted A β PP fragments by immunoblotting. This same 48 hour cell treatment procedure was performed to assess cell viability using a TC20 automated cell counter (BioRad), where live cells were distinguished by selective Trypan Blue staining of dead cells.

Preparation of cell lysates

Following the removal of the CM as described above, cell monolayers were washed twice with ice cold phosphate buffered saline (PBS) solution to remove residual media and lysed in 200–250 μ L of lysis buffer (LB; 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), pH 7.2, 150 mM NaCl, 2mM Ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1mM sodium orthovanadate, 50 mM sodium fluoride, 10 mM β -glycerophosphate, 500 μ M 4-(2-Aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), 10 μ g /mL Aprotinin, 10 μ g/mL Leupeptin, and 5 μ g/mL Pepstatin) for 30 minutes at 4°C with rocking. Crude lysates were collected and centrifuged at $28,000 \times g$ for 20 minutes at 4°C to remove cellular debris and insoluble material. The clarified whole cell lysates were removed, total protein was quantified using the DC Protein Assay with Bovine Serum Albumin (BSA) as a standard and then combined with 4X Laemmli SDS-sample buffer containing 25 mM DTT for analysis by immunoblotting.

Time course of pCDP-diBzl treatment

To obtain roughly the same protein levels and to harvest CM for all time points simultaneously, all cells were plated at the same time at a density of 2×10^5 cells per well in six-well cluster dishes in 2 mL of complete media and grown for 16–24 hours. All wells were then washed twice with DMEM only and two wells were re-fed with 1 mL of complete media containing 400 μ M of pCDP-diBzl, two wells were re-fed with 1mL of complete media containing DMSO only, and the rest of the wells were re-fed with complete media only (48 hour time point). After 24 hours, media was removed from four currently untreated wells, washed with DMEM only, and then two wells were re-fed with 1 mL of complete media plus 400 μ M of pCDP-diBzl while the other two wells were re-fed with 1 mL of complete media plus DMSO only (24 hour time point). At approximately the same time, the cells for the 48 hour time point were washed and re-fed with media containing DMSO or 400 μ M of pCDP-diBzl for another 24 hours (it was from this final 24 hour collection period that the 48 hour time point was collected). The same process was followed after an additional 12, 16, and 20 hours to obtain the 12, 8 and 4 hour treatment time points, respectively. At the end of the 48 hour experiment, all CM was collected and cleared by centrifugation at $12,000 \times g$ for 20 min at 4°C to remove intact cells and cellular debris. An aliquot of the cleared CM was combined with 4X Laemmli SDS-sample buffer containing 25 mM DTT and used for the analysis of secreted A β PP fragments by immunoblotting. Cells were washed and lysed as described above.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

Whole cell lysates (WCLs) and CM were prepared as described above and various amounts were analyzed by immunoblotting as indicated in the figure legends. Samples and protein standards were adjusted as needed to the desired total protein amount using LB (for WCL samples) or to equal volume using growth media (for CM samples). Housekeeping protein β -tubulin was used as a loading control for all WCL blots except those corresponding to treatments of H4-BACE1 cells, where Stain Free total protein provided a more reliable

loading control at the high total protein loading level that was needed to detect endogenous A β PP [38].

All CM samples (except for sA β , described below) were separated on 10% SDS-PAGE gels [39] and transferred to PVDF membrane without methanol [40] with constant cooling. Membranes were blocked with Tris-Buffered Saline with Tween (TBST; 25 mM Tris-HCl pH 7.2, 150 mM NaCl, and 0.1% Tween 20) containing 5% milk for 1–2 hours at RT and then incubated with primary antibodies (diluted in TBST containing 0.5–1% BSA) at the following dilutions: anti-sA β PP α (EPR5119(2), 1:15,000) or anti-sA β PP β (25 ng/mL). Anti-sA β PP α was incubated for 2–3 hours at RT and anti-sA β PP β antibody was incubated overnight at 4°C. Membranes were incubated for 2–3 hours at RT with the appropriate HRP-conjugated secondary antibody (diluted 1:10,000 in TBST containing 1% milk). Pierce ECL Western Blotting substrate (for sA β PP α) or Pierce SuperSignal West Pico substrate (for sA β PP β) were used to visualize the results on X-ray film. Band intensities were quantified by ImageJ and a background area of the same size was taken above each band and subtracted. These resulting values were first normalized to the averaged DMSO control for each protein and then further normalized for the total protein of the corresponding WCL for each sample (as determined by the DC Protein Assay).

All WCL samples were separated on 4–15% or 4–20% Mini Protean TGX Stain Free precast gels (Bio-Rad). Covalent coupling of the chromophore in the gel to the separated proteins was achieved using an activation time of 1 min. Proteins were transferred to Immobilon-P PVDF membrane with 10% methanol for 2 hr at constant current of 250 mA [40]. Membranes were blocked with TBST containing 5% milk for 2 hours at RT or overnight at 4°C. Each membrane was washed four times with TBST (5 min per wash). Membranes were incubated with anti-A β PP (Y188, 1:40,000) overnight at 4°C or for 2.5 hours at RT, with anti- β -tubulin (1:30,000) for 2 hrs at RT, or with anti-BACE1 (1:5,000) for 4 hr at RT. Membranes were incubated for 1.5–3 hours at RT with the appropriate HRP-conjugated secondary antibody as described above.

For WCL blots of treated H4-BACE1 cells, proteins transferred membranes were first imaged using a stain free blot application on a Chemidoc MP system (Bio-Rad), then were incubated with Pierce SuperSignal West Pico Chemiluminescent Substrate for visualization. For all other WCL blots, Pierce SuperSignal West Pico Chemiluminescent Substrate (for comparison of A β PP levels across different cell models), Clarity Western ECL (for comparison of BACE1 levels across different cell models), or Pierce ECL Western Blotting Substrate (for A β PP and β -tubulin in H4-APP695 cells) were used for visualization. All blots were visualized using Chemi Hi Sensitivity blot application in the Chemidoc MP System (Bio-Rad). Band volumes were quantified using Image Lab (Bio-Rad). For use of β -tubulin as a loading control, desired protein band volumes in a given lane were normalized to the β -tubulin signal volume in that lane. For use of stain free total protein as a loading control, desired protein band volume(s) in each lane were normalized to total protein as quantified using Image Lab (Bio-Rad). For all WCL blots of FL-A β PP where mature and immature FL-A β PP were adequately resolved to allow quantification of each, previously normalized band volumes (for β -tubulin signal or stain free total protein) were further

normalized to the average control immature A β PP band volume, as shown in the corresponding graphs.

Total secreted A β levels were determined by immunoblotting using the antigen epitope retrieval method essentially as described [41]. Briefly, 20 μ L of conditioned media was separated in a 12.5% Bis-Tris-Mes gel and transferred to 0.2 μ M nitrocellulose membranes for 90 minutes at 40V using the BioRad Trans blotting system containing 20% methanol [40]. Membranes were steamed for 15 minutes to expose latent epitopes, blocked with TBST-milk and incubated with anti-A β antibody (1:4000) for 1 hour at RT followed by overnight incubation at 4°C. Membranes were incubated with HRP-conjugated secondary antibody and results were visualized using the Pierce ECL Western Blotting substrate. Band intensities were quantified by ImageJ as described above.

Observation of pCDP uptake by LCMS

The following LCMS procedure for cell lysates was adapted from [42]. H4-A β PP695 cells were plated at a density of 1.5×10^6 cells per 10-cm dish ($\times 20$) in complete media and grown at 37°C in a humidified atmosphere (90%) containing 10% CO₂. After ~24 hours cells were re-fed with 5 mL of fresh media containing various pCDPs (400 μ M) or DMSO as indicated in the figure legend (two plates per condition). The remaining ten plates were re-fed with 5mL of fresh media and were used as a “spiked” positive control set. After 24 hours the treated cells were scraped into their conditioned media (duplicates were combined) and collected by centrifugation for 5 minutes at $2000 \times g$ at RT. The remaining 10 untreated dishes (“spiked” positive control set) were scraped, divided into five equal aliquots and cells were collected by centrifugation. Cell pellets were washed three times with cold (4°C) PBS to remove all residual conditioned media and pCDPs. Cell pellets were quick frozen in liquid nitrogen and stored at –80°C until lysis.

Frozen pellets were thawed on ice for 30 minutes, re-suspended in 500 μ L of water and 500 μ L of methanol, vortexed, and incubated on ice for 1 hour to facilitate complete lysis. The five “spiked” positive control samples were prepared by adding 1 μ L of 1 mM each pCDP or DMSO (1:100 dilution of the stocks in water) to individual pellets, vortexed and incubated on ice for one hour. Lysates were centrifuged at 15,000 RPM for 30 minutes at 4°C and the supernatants were collected and lyophilized to dryness. Each dried sample was dissolved in 300 μ L of 50:50 methanol:water and 2 μ L was separated using reverse-phase high resolution UHPLC-MS exactly as described above (see *LCMS Analysis* section of *Analysis of custom-synthesized compounds*). The Thermo-Scientific Xcalibur software package was used to visualize, analyze, and depict the LCMS data presented here.

BACE1 activity assay

BACE1 activity was determined by incubating 400 μ M of individual pCDPs, the BACE1 inhibitor LY2811376 (positive control) or DMSO (negative control) in a reaction mixture containing 25 mM sodium acetate assay buffer, pH 4.42, 1% BSA, 0.2 μ g of the catalytic domain of recombinant human BACE1 and 10 μ M of Methyl cumaryl amide (Mca) fluorogenic substrate in a final volume of 110 μ L. Fluorescence intensity was measured with a Synergy H1 hybrid reader (BioTek, Winooski, VT) (excitation 320 nm, emission 405 nm)

using a black microplate with half-area wells and an opaque bottom with continuous gentle shaking at 37°C for one hour with readings acquired every 5 minutes. Results were analyzed in Excel 2010 by averaging the DMSO control 1 hour fluorescence intensity values and then normalizing all experimental results for pCDPs and BACE1 inhibitor to this value. The normalized data was then averaged over repeated points (n=6 for all pCDP treatments and DMSO negative control, n=5 for BACE1 inhibitor data), the standard deviations were determined, and the data was graphed using Excel 2010 software.

Statistical analysis

Averages, 20% Coefficient of Variation (CV), standard deviation (S.D.), and statistical analysis for significance via the unpaired, independent Student's T test for data were determined using Excel 2010. CM and WCL data were expressed as the mean $\pm$ 20% CV. CM data were deemed significant at $p < 0.05$. 20% CV was used as an estimate for western blot variability to yield more conservative assignments of statistical significance [38]. Cell viability data were expressed as mean $\pm$ S.D. and were deemed significant at $p < 0.05$. All graphs were generated in Excel 2010. Approximate EC50 values were determined by fitting the sA β PP β level versus dose curves to the 4-parameter logistic model $Y = (d - a) / \{1 + (X/c)^b\}$, where a is the lower asymptote, d is the upper asymptote, X is the dose concentration, c is the concentration at which the sA β PP β level is midway between a and d, and b is the slope factor that describes the steepness of the central linear portion of the curve [43]. Parameters a, b, c and d were optimized by minimizing the sum of the squared differences between experimental and model-predicted values using the Solver add-in within Excel 2010 (Microsoft).

RESULTS

H4 neuroglioma cells stably overexpressing A β PP695wt or BACE1 enable reliable detection of sA β PP β

BACE1 cleavage of A β PP produces sA β PP β and C99 fragments (Figure 1A), and is essential for the production of A β . The C-terminal fragment, C99, is a transient intermediate in the amyloidogenic pathway that reflects the relative balance of multiple pathways. C99 is processed by γ -secretase [44], is also a substrate for α -secretase [45], and is turned over by both ERAD and ubiquitin-independent lysosomal degradation pathways [46]. Moreover, C99 can be additionally cleaved by C89 to by BACE1 and BACE2 [47], and the proportion of C89 relative to C99 increases with increasing BACE1 expression [48]. In contrast, sA β PP β (~100 kDa) is secreted to the medium and can be detected (and distinguished from sA β PP α) by antibodies specific for the C-terminus of sA β PP β [49]. The sA β PP β fragment therefore serves as an effective proxy for the maximum possible amyloidogenic processing, since for every A β peptide molecule produced, a corresponding sA β PP β fragment must be generated. Importantly, sA β PP β reflects the amount of A β PP that undergoes the entry step (ie. β -secretase cleavage) into the amyloidogenic pathway (Figure 1). Our goal was to generate two distinct cell lines, via overexpression of A β PP695 or BACE1 that recapitulate two distinct causative mechanisms of AD [16–17, 50], to enable reliable detection of sA β PP β for evaluating the effects of pCDP treatment on A β PP amyloidogenic processing.

The human H4 neuroglioma cell line, derived from a neuroglioma tumor and adapted for growth in culture [51–52], has been broadly used in AD research [53–56]. In H4 cells, the endogenous A β PP751 isoform (Figure 3A) predominantly undergoes nonamyloidogenic processing, as shown by the absence of sA β PP β in 15 μ L of conditioned media (CM) (Figure 3B) and the abundance of sA β PP α detected in just 2 μ L of the same CM (Figure 3C). To generate detectable levels of sA β PP β , we chose to take two separate approaches: (1) increase the level of the A β PP695 isoform, since this is the predominant form found in neurons [57] and overexpression of A β PP695 is known to increase amyloidogenic processing [58], and (2) increase the level of BACE1 to shift the balance toward amyloidogenic processing [50].

To this end, we generated two distinct H4 cell lines that serve as AD models: the H4-A β PP695 cell line that overexpresses A β PP695 (wild-type) and the H4-BACE1 cell line that overexpresses BACE1 (wild-type) (Figure 3A). The quantity of A β PP695 is increased approximately 15-fold in H4-A β PP695 lysates compared to endogenous A β PP751 in H4 and H4-BACE1 cell lysates, using β -tubulin for normalization across lanes (Figure 3A). BACE1 is robustly detected in 6.8 μ g of H4-BACE1 cell lysate, whereas endogenous BACE1 was not detected in 6.8 μ g of H4 cell lysate (Figure 3A). Additionally, the level of mature A β PP751 is reduced in the H4-BACE1 cells (Figure 3A), which is consistent with previous results that have shown that most cleavage of A β PP via BACE1 happens after O-glycosylation [59].

As anticipated, both H4-A β PP695 and H4-BACE1 cell lines enable sensitive detection of sA β PP β (Figure 3B), and produce different relative levels of sA β PP α (Figure 3C). With only 2 μ L of CM, sA β PP α is detected in H4-A β PP695 cells after a 3 second exposure time (Figure 3C, top blot). After a 2 minute exposure of the same blot, the less abundant sA β PP α from both H4 and H4-BACE1 cells is detectable (Figure 3C, bottom panel), at the expected (different) molecular weights. Due to robust overexpression of A β PP695 in the H4-A β PP695 cell line, the highly abundant sA β PP α secreted into CM (Figure 3C) is not conducive to observing small changes in nonamyloidogenic processing. However, since A β PP expression remains at an endogenous level in the H4-BACE1 cell line, changes in both sA β PP β (Figure 3B) and sA β PP α (Figure 3C) can be detected in CM, thereby allowing amyloidogenic and non-amyloidogenic processing of A β PP to be simultaneously monitored in this cell line. Collectively, these data demonstrate the utility of H4-A β PP695 and H4-BACE1 cells for monitoring sA β PP β as a proxy for the amyloidogenic processing of A β PP.

The *cis*-locked pCDP mimic was derivatized to aid in cellular uptake

Since the *cis*-locked pCDP (Figure 1C) is charged and is not expected to be taken up by cells, we prepared three derivatives that block the phosphate group and are more hydrophobic (Figure 2). The phosphate group in pCDP (Figure 2A) was protected by a single benzyl group (Figure 2B), two benzyl groups (Figure 2C), or two pivaloyloxymethyl (POM) groups (Figure 2D). Notably, since the POM groups are easily removed by cellular esterases [60], the pCDP-diPOM molecule is a “pro-drug” version of the pCDP (Figure 2A).

All pCDPs were analyzed by ^1H NMR and LCMS to verify their structure and MW (Supplemental Figures 1–5).

Only pCDP-diBzl reduces sA β PP β in the conditioned media of H4-A β PP695 cells

Using sA β PP β as a proxy for monitoring amyloidogenic processing of A β PP, the effects of the pCDP compounds (Figure 2) were investigated in H4-A β PP695 cells. H4-A β PP695 cells were treated with 400 μM of pCDP, pCDP-Bzl, pCDP-diBzl, and pCDP-diPOM for a total of 48 hours and 15 μL of CM from the final 24 hours of treatment was analyzed by Western blot for changes in sA β PP β (Figure 4A). Only pCDP-diBzl had a potent effect on the quantity of sA β PP β present in the media, severely reducing the amount of detectable sA β PP β to less than 1% of the control without change in the levels of mature or immature FL-A β PP (Figure 4A). The well-characterized BACE1 inhibitor (LY2811376) was also used to treat cells at 2.5 μM (Figure 4A) and exhibited a similar effect on H4-A β PP695 cell line as the pCDP-diBzl. The other CDP variants (pCDP, pCDP-Bzl, pCDP-diPOM) had little to no effect on the amount of sA β PP β , despite their related structure. To assess the impact of pCDP-diBzl treatment on cell viability, H4-A β PP695 cells were treated with 200 μM and 400 μM of pCDP-diBzl for 48 hours, trypsinized, stained with Trypan Blue, and counted. Although growth seemed to be slowed in the presence of 200 μM and 400 μM pCDP-diBzl, no significant toxicity was observed (>96% of all cells counted were alive) (Supplemental Figure 6).

A dose-dependence experiment using 25, 50, 100, 200, and 400 μM of pCDP-diBzl was performed to monitor changes in sA β PP β in H4-A β PP695 cells (Figure 4B). As shown, 48 hours of treatment with 400 μM of pCDP-diBzl has the most potent impact on sA β PP β levels (Figure 4B) with an approximate EC₅₀ value of 126 μM (Figure 4B). As described above, the robust production of sA β PP α in these cells (Figure 3C) prevents reliable evaluation of small changes in the non-amyloidogenic pathway. The mature and immature A β PP signal for the DMSO and 400 μM pCDP-diBzl samples from Figures 4A and 4B were combined ($n=4$) to this highest level of treatment with pCDP-diBzl had no significant impact on FL-A β PP levels (Figure 4C). A same dose-dependent response was also observed on sA β levels in H4-A β PP695 cells (Figure 4D) with no significant impact on FL-A β PP signal. Additionally, a time-dependence study was performed to see if 48 hours of treatment was crucial for the observed effect on sA β PP β reduction (Figure 4E). A short exposure time (30 seconds) shows clear reduction of sA β PP β in CM of pCDP-diBzl treated cells for 12, 24 and 48 hr total time periods (Figure 4E, left). A much longer exposure (10 minutes) reveals additional observable reduction of sA β PP β at 4 hours (Figure 4E, right), whereas sA β PP β was not yet detectable even in untreated cells at 2 hours (data not shown). These data demonstrate that pCDP-diBzl treatment of H4-A β PP695 cells reduces secreted sA β PP β levels without significantly changing FL-A β PP levels, and that this reduction is evident at the earliest time point at which sA β PP β can be detected.

pCDP-diBzl and pCDP-diPOM reduce sA β PP β in the conditioned media of H4-BACE1 cells

As was observed in the H4-A β PP695 cells, treatment of H4-BACE1 cells with 400 μM of pCDP-diBzl yielded a similar decrease in sA β PP β without a significant impact on FL-A β PP levels (Figure 5A). Additionally, sA β PP α (derived from endogenous A β PP751 in these

cells) does not significantly change upon treatment with any of the pCDP compounds, and shows no significant dose-dependence with pCDP-diBzl treatment (Figure 5). The dose-dependent reduction of sA β PP β induced by pCDP-diBzl treatment of H4-BACE1 cells (Figure 5B) is similar to what was observed in H4-A β PP695 cells, with an EC50 value of approximately 67 μ M (Figure 5B). Again, mature and immature FL-A β PP signal for the DMSO and 400 μ M pCDP-diBzl samples from Figures 5A and 5B were combined (n=4) to show no significant impact on FL-A β PP levels (Figure 5C). The similar dose dependence and EC50 values in these distinct cell lines, where either A β PP or BACE1 are significantly overexpressed, indicate that pCDP-diBzl does not act through direct competition for a binding partner of either A β PP or BACE1. These data further suggest that pCDP-diBzl might act in both distinct cell lines via a common mechanism to reduce sA β PP β . In contrast, while 400 μ M pCDP-diPOM treatment of H4-A β PP695 cells showed no significant effect, treatment of H4-BACE1 cells with 400 μ M pCDP-diPOM significantly reduced the amount of sA β PP β without changing FL-APP signal (Figure 5A and B). Although this 5-fold reduction is approximately the same as the effect of pCDP-diBzl treatment in these cells, the observation that pCDP-diPOM is ineffective in cells overexpressing A β PP is consistent with a mechanism of action in which pCDP-diPOM blocks A β PP interaction with an amyloidogenic binding partner.

As expected, treatment with 2.5 μ M BACE1 inhibitor LY2811376 again inhibits sA β PP β production in H4-BACE1 cells. Interestingly, a clear, significant increase in both sA β PP α and mature (glycosylated) full length A β PP is observed in this cell model, where only endogenous A β PP751 is expressed (Figure 5A). This suggests that direct inhibition of BACE1 catalytic cleavage of A β PP might increase recycling of A β PP back to the plasma membrane via trafficking of endocytosed A β PP back to the trans-Golgi network and subsequent secretory pathway, where glycosylation and β -secretase activities are active. This effect is not observed in treatments with 400 μ M pCDP-diBzl or 400 μ M pCDP-diPOM, suggesting a different mechanism for these molecules.

Association of pCDPs with and their conversion by H4-A β PP695 cells

To gain insight regarding the fate of pCDPs inside cells, LCMS was used to detect the presence of each compound in lysates from H4-A β PP695 cells treated with the various pCDPs. Importantly, these lysates were extracted using 1:1 methanol/water by volume, which is expected to at least partially solubilize most polar lipids and to denature some proteins. Although the pCDP variants are chemically very similar, LCMS was highly effective for detecting not only the pCDP variant, but also its conversion products. The neutral-charge MW of each pCDP molecule is shown in Figure 2 and the electrospray ionization in positive mode (ESI+) MW for each pCDP is the following: 279 for pCDP, 369 for pCDP-Bzl, 459 for pCDP-diBzl, and 507 for pCDP-diPOM.

For comparison, lysates from cells treated with 400 μ M of each pCDP variant (Figure 6A) and lysates from untreated cells that were spiked with 100 μ M of respective pCDPs (Figure 6B) were analyzed by LCMS. Lysate from cells treated with 400 μ M of pCDP-diBzl displays not only the expected MW 459 but also a substantial amount of MW 369, corresponding to pCDP-Bzl. In contrast, lysate from untreated cells spiked with pCDP-diBzl

shows predominantly MW 459 and significantly lower MW 369. This suggests that pCDP-diBzl is converted to pCDP-Bzl inside the cell. Similarly, treatment with 400 μ M of pCDP-diPOM shows an elevated amount of MW 279 and no detectable MW 507, while the untreated cell lysate spiked with pCDP-diPOM shows a substantial peak only for MW 507 (Figure 6). This supports successful entry of pCDP-diPOM into cells where active esterases efficiently cleave the POM groups *in vivo* [60], whereas esterase activity in the spiked case could be inhibited in 1/1 methanol/water. Lysate from pCDP-Bzl treated cells does show MW 369, demonstrating that the pCDP-Bzl molecule does associate with cells, although as shown above it does not significantly reduce sA β PP β (Figure 4A). Cells treated with pCDP do not show MW 279 significantly above the DMSO control, but spiked cells with pCDP do, indicating that although pCDP itself is detectable it does not associate with treated cells sufficiently enough to be detected. Together, these results show that pCDP-diBzl, pCDP-Bzl, and pCDP-diPOM associate with H4-A β PP695 cells, and that pCDP-diBzl and pCDP-diPOM are modified by the cell.

pCDPs do not specifically inhibit the catalytic activity of BACE1

Since a reproducible effect on reduction of sA β PP β by pCDP-diBzl and pCDP-diPOM was observed, we sought to determine whether these compounds directly inhibit the activity of the BACE1 catalytic domain. Using recombinant human BACE1 catalytic domain and an intramolecularly quenched Mca fluorogenic substrate as part of a FRET-based assay [61], we tested 400 μ M of each pCDP variant for inhibition of BACE1 catalytic activity. Additionally, we tested the known BACE1 inhibitor LY2811376 at 2.5 μ M as a positive control for reduced BACE1 catalytic activity. As shown in Figure 7, while LY2811376 showed a potent decrease as expected, we saw no significant decrease in the catalytic activity of recombinant, purified BACE1 catalytic domain (as measured by fluorescence intensity) with any of pCDP variants at 400 μ M. These results demonstrate that pCDP-diBzl and pCDP-diPOM do not act as a direct inhibitor of BACE1 catalytic activity, and point to an alternate mechanism by which they inhibit amyloidogenic A β PP processing in H4 cell models of AD.

DISCUSSION

The effective treatment of AD will require an in-depth understanding of key interactions that mediate the amyloidogenic processing of A β PP. Although BACE1 cleavage of A β PP initiates this process, therapeutic strategies to inhibit this catalytic reaction are complicated by the essential functions of BACE1 that include myelination, axon guidance, muscle spindle formation, and neuronal network functions (reviewed in 62). Hence, an agent that could specifically reduce BACE1 cleavage of A β PP without reducing its cleavage of other normal cellular targets is highly desirable. In this study, we have investigated the possibility that the *cis* conformation of a phospho-Thr-Pro peptide bond in the cytoplasmic tail of A β PP might function as a signal for increased amyloidogenic A β PP processing. We designed and synthesized four “*cis*-locked” pCDP compounds and tested their effect on sA β PP β production in two distinct AD cell models. Using this approach, we have demonstrated that one specific pCDP derivative, pCDP-diBzl, is active in suppressing amyloidogenic processing of A β PP in both H4-A β PP695 and H4-BACE1 cell models. Additionally, a

second derivative, pCDP-diPOM, has a similar effect but only in H4-BACE1 cells, where solely endogenous A β PP is expressed. Moreover, we have demonstrated that neither the pCDP-diBzl nor the pCDP-diPOM directly inhibit the BACE1 catalytic site *in vitro*. These findings support a pathogenic role of the *cis* conformation in promoting amyloidogenic A β PP processing, and open new avenues toward the development of A β PP-specific therapeutic agents to inhibit this role.

An important consideration to address is what the active form of each compound is in the cell. Our mass spectrometry data of WCL from cells treated with pCDP-diPOM shows full deprotection of pCDP-diPOM to pCDP, which allows us to conclude that the effective form of pCDP-diPOM in treated H4-BACE1 cells is the deprotected pCDP. Of the molecules tested, pCDP is the closest mimic of phosphoThr-Pro, thus it is expected to be less effective in a background of excess A β PP (as in H4-A β PP695 cells), providing a plausible explanation for why it does not work in H4-A β PP695 cells. For the pCDP-diBzl compound, our mass spectrometry data of WCL from cells treated with this compound shows that pCDP-diBzl is partially converted to pCDP-Bzl, but not to pCDP. Importantly, direct treatment with pCDP-Bzl did not display significant activity in either cell type, even though it is observed in WCL from H4-A β PP695 cells. Thus, the active form in pCDP-diBzl treated cells is most likely pCDP-diBzl, although the prominent level of pCDP-Bzl in these cells cannot be ruled out as possibly contributing to activity.

We have employed two distinct cell models to investigate what are potentially multi-target mechanisms of these compounds. Our H4-A β PP695 and H4-BACE1 cell lines provide comparative disease models in which two distinctly different perturbations, the over expression of A β PP or BACE1, both recapitulate the disease state as measured by elevated amyloidogenic A β PP processing. Since AD is a complex disease with many potential targets that can influence A β PP processing [63], and given the known pleiotropic effects of diketopiperazine molecules [3], it is plausible that the observed effects of these compounds involve multiple targets. Indeed, the use of both AD cell models reveals that pCDP-diBzl and pCDP-diPOM have at least partially different mechanisms of activity. The observation that pCDP-diBzl has a similar dose-dependence in both models suggests that its primary target is not a direct binding partner of either A β PP or BACE1, which are each robustly over expressed in their respective cell lines. This would potentially rule out numerous cellular factors as pCDP-diBzl targets known to directly bind to A β PP [64]. However, the inability of pCDP-diPOM to alter amyloidogenic processing of A β PP in H4-A β PP695 cells suggests that the target of pCDP-diPOM may indeed be an amyloidogenic-promoting binding partner of A β PP, whereby the robust over expression of A β PP in H4-A β PP695 cells prevents effective inhibition of this pathogenic interaction. Moreover, the effectiveness of pCDP-diPOM in H4-BACE1 cells, where BACE1 is robustly over expressed, supports the possibility that pCDP-diPOM blocks an A β PP-specific binding partner.

The pattern of A β PP proteolytic products induced by compound treatments offers clues regarding potential mechanisms of pCDP-diBzl and pCDP-diPOM activities. Specifically, both compounds reduced sA β PP β and total secreted A β levels without significantly changing sA β PP α or matA β PP levels. These observations suggest that these compounds do not significantly increase levels of A β PP at the plasma membrane (which should increase

sA β PP α), and do not increase A β PP levels in the ER/Golgi secretory pathway (which should increase matA β PP). The observation of this same pattern of A β PP proteolytic products with both compounds suggests that pCDP-diPOM and pCDP-diBzl might act at a common point in the complex system of networks that govern A β PP processing. BACE1 cleavage of A β PP, which produces sA β PP β and is the first requisite step in the production of A β , involves additional regulatory factors that could serve as targets of pCDP-diBzl and pCDP-diPOM. While BACE1 endocytosis is ARF6-dependent [65], A β PP is internalized via clathrin-dependent endocytosis (reviewed in 64). The sorting of A β PP and BACE1 into common intracellular acidic vesicles via different pathways [66], which is central to BACE1 cleavage of A β PP [67–69], offers many potential targets [64]. Together, these results suggest a model in which both pCDP-diBzl and pCDP-diPOM inhibit the association of A β PP and BACE1 and subsequent generation of sA β PP β , but that their activities are mediated through different targets (Figure 8).

While additional studies are underway to elucidate the specific mechanisms by which pCDP-diBzl and pCDP-diPOM inhibit amyloidogenic processing, it is informative to consider mechanisms deduced for other small molecules that alter A β PP proteolytic processing. For example, treatment of rat primary cortical neurons with statins similarly decreases secreted A β 40 and A β 42, but significantly reduces matA β PP via a cholesterol-independent mechanism that involves selective reduction of A β PP phosphorylation at Thr668 [70], pointing to a central role of T668 phosphorylation in A β PP processing. In another example, treatment of HEK293 cells with the natural product 2,2',4'-trihydroxychalcone (TDC) from *Glycyrrhiza glabra* (licorice) root, reduced sA β PP β without significant change in matA β PP (as we observe here), by serving as a non-competitive inhibitor of BACE1 catalytic activity [71]. Finally, the metal chelator clioquinol and various derivatives potently inhibit A β accumulation in cell models and in mice [72–77]. Treatment of A β PP-CHO cells with clioquinol alone reduced total sA β PP (sA β PP α and sA β PP β were not distinguished), but the robust reduction of secreted A β 40 by clioquinol required co-treatment with copper (Cu²⁺) [77]. The deduced mechanism was via activation of PI3K-Akt and JNK signaling pathways, culminating in the upregulation of secreted matrix metalloproteases (MMPs) that degrade extracellular A β [77]. However, the mechanism by which total sA β PP was reduced was not elucidated.

Interestingly, there is an additional mechanism by which clioquinol upregulates MMPs and A β clearance, with a possible link to A β PP proteolytic processing. Quinol family compounds, including clioquinol, inhibit the hydroxylation activity of Factor Inhibiting HIF-1 (FIH-1), one of the two distinct enzymes that initiate ubiquitin-mediated proteasomal degradation of hypoxia-inducible factor-1 α (HIF-1 α) [78–81]. Under low oxygen levels where FIH-1 activity is low, the stabilized HIF-1 α induces expression of numerous genes, including MMPs [82,83]. Hence, the inhibition of FIH-1 by clioquinol stabilizes HIF-1 α , which leads to the upregulation of MMPs and subsequent rapid degradation of extracellular A β . HIF-1 α is stabilized by direct interaction with COPS5 (also known as Jab1), a protein implicated in amyloidogenic processing of A β PP [77,84]. Thus, an additional impact of clioquinol might be to induce elevated HIF-1 α levels that compete for COPS5 binding to and stabilization of an amyloidogenic factor, thereby indirectly reducing amyloidogenic processing of A β PP. The multiple signaling pathways that respond

to clioquinol, and the COPS5-mediated link between HIF-1 α and amyloidogenic A β PP processing, exemplify the many interconnected systems of networks that govern the proteolytic fate of A β PP.

In conclusion, AD is a multifactorial disease that involves factors at the genetic, epigenetic, interactome, and environment levels [63]. To understand such a multi-scale complex system, eukaryotic cell models that simulate the disease state are of great value. Here, we have used two distinct H4 neuroglioma cell lines, each with elevated expression of a single component involved in A β PP processing, to test compounds that mimic the *cis* conformation of a phospho-Thr-Pro peptide bond in the cytoplasmic tail of A β PP. The activity of pCDP, when delivered to the cell as its precursor pCDP-diPOM, suggests that the *cis* conformation of the phospho-Thr668-Pro669 motif in A β PP serves as a signal that increases association of A β PP and BACE1, since pCDP is the closest mimic of phosphoThr-Pro and is only active when endogenous levels of APP are present. Conversely, the similar activity of pCDP-diBzl in both comparative AD cell models points to a different target for this compound and suggests that the *cis* conformation is a broadly used signal, consistent with the wide array of diketopiperazine bioactivities [3]. Based on these results we propose a model in which our two identified active compounds act through different targets, but at comparable points in A β PP processing system. This model provides a framework for investigation of pCDPs binding to specific components of putative association complexes, and for further development of compounds that effectively block interactions that promote A β production. Overall, our studies support the idea that the *cis* isomer is a pathogenic conformation in the determination of A β PP proteolytic fate, and show that small molecules that mimic this conformation reduce amyloidogenic processing of A β PP. These findings provide important insights for guiding the future development of novel AD therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Putative role of the phospho-Thr668-P669 *cis* isomer as a signal in the proteolytic processing of A β PP

(A) A β PP is a type 1 transmembrane protein with a large N-terminal ectodomain and a short C-terminal cytoplasmic tail. At the plasma membrane, α and γ secretases are abundant to cleave A β PP into sA β PP α , C83, p3, AICD fragments in the dominant, nonamyloidogenic processing pathway. A β PP can alternatively be internalized and localized to acidic compartments where BACE1 (β secretase) and γ secretase cleave A β PP into sA β PP β , C99, A β , and AICD fragments via amyloidogenic processing. Within the cytoplasmic tail of A β PP, the amino acids T668-P669-E670-E671 form a transient helix capping box (pink box, A). Upon phosphorylation of T668, the helix-capping box is destabilized and two different structures emerge (B). These two structures are distinguished by their *cis* vs. *trans* isomer state of the pT668-P669 peptide bond. Pin1 has been identified as a peptidyl prolyl isomerase that rapidly establishes the equilibrium concentrations of 90% *trans*-pTP and 10% *cis*-pTP. By creating a covalent bond between the α -carbon of the T668 and the amine of the

E670, a “100% *cis*-locked” phosphorylated cyclic-dipeptide (pCDP) was generated to mimic the *cis*-pTP motif (C).

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Figure 2. The *cis*-locked pCDP mimic was derivatized to aid in more favorable cellular uptake. Chemical structure variations of the *cis*-locked pCDP mimic, shown in (A), include the attachment of one benzyl (Bzl) group to produce pCDP-Bzl (B), two benzyl groups (diBzl) to produce pCDP-diBzl (C), or two pivaloyloxymethyl (POM) groups to produce pCDP-diPOM (D). Molecules generated using ChemDraw 15.0.

Figure 3. H4 neuroglioma cell lines transfected with AβPP695wt (H4-AβPP695) or BACE1 (H4-BACE1) show increased production of AβPP proteolytic products
 Western blot analysis of full-length AβPP in cell lysates (A), and of sAβPPβ and sAβPPα in conditioned media (B,C). MW differences reflect exogenous AβPP695 in H4-AβPP695 versus endogenous AβPP751 in H4 and H4-BACE1. A) Full-length AβPP (top panel), BACE1 (middle panel) and β-tubulin (bottom panel) in WCL from H4, H4-BACE1 and H4-AβPP695 cells (total protein loaded: 6.8 μg, 6.8 μg and 0.6 μg, respectively). B) sAβPPβ in 15 μL CM from H4, H4-AβPP695, and H4-BACE1 cells. C) sAβPPα in 2 μL CM from H4, H4-BACE1, and H4-AβPP695 cells (top: 3 sec exposure time; bottom: 2 min exposure time).

Figure 4. pCDP-diBzl reduces sAβPPβ levels in dose- and time-dependent manners in H4-AβPP695 cells

Western blot analysis in H4-AβPP695 cells of sAβPPβ levels (A, B and E) resulting from treatment with different pCDP compounds (A), treatment with different doses of pCDP-diBzl (B), and time-dependence of pCDP-diBzl treatment (E), and of sAβ levels resulting from treatment with different doses of pCDP-diBzl (D). A) sAβPPβ in 15 μL CM (top panel) and full-length AβPP in 0.6 μg total WCL protein (bottom panel) resulting from duplicate treatments with 400 μM of pCDP-diBzl (diBzl), pCDP-Bzl (Bzl), pCDP, and pCDP-diPOM (diPOM), with 2.5 μM of BACE1 inhibitor LY2811376 (Inhib) and DMSO (control) for 48 hours total. Quantified levels of sAβPPβ (Mean ± 20% CV, n=4, each) were statistically analyzed by the Student's T test (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001). Quantified levels of full length AβPP (mature and immature) were normalized to β-tubulin (Mean ± 20% CV, n=2, each). B) As in A, resulting from dose-dependence of pCDP-diBzl treatment. Duplicate treatments were performed with 25, 50, 100, 200, and 400 μM of pCDP-diBzl and DMSO (control) for 48 hours total. Data were quantified and statistically analyzed as in A. C) DMSO and 400 μM diBzl data points for mature-AβPP and immature-AβPP (from A and B) were combined (n=4, each) and statistically analyzed as in A. D) sAβ in 20 μL CM (top panel) and full-length AβPP in 1 μg total WCL protein (bottom

panel) resulting from treatment with 100, 200 and 400 μM of pCDP-diBzl for 48 hours total. Data were quantified and statistically analyzed as in A. E) sA β PP β in 15 μL CM resulting from treatment with pCDP-diBzl (400 μM) for 4, 12, 24, and 48 consecutive hours (left panels: 30 sec exposure time; right panels: 10 min exposure time).

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Figure 5. pCDP-diBzl reduces sAβPPβ levels in a dose-dependent manner in H4-BACE1 cells
 Western blot analysis of sAβPPβ and sAβPPα levels in H4-BACE1 cells resulting from treatment with different pCDP compounds (A) and with different doses of pCDP-diBzl (B). A) sAβPPβ and sAβPPα in 15 μL CM (top panels), and full-length AβPP in 6.8 μg total WCL protein with corresponding stain free total protein image (bottom panels) resulting from duplicate treatments with 400 μM of pCDP-diBzl (Bzl), pCDP-Bzl (Bzl), pCDP, and pCDP-diPOM (diPOM), with 2.5 μM of BACE1 inhibitor LY2811376 (Inhib), and DMSO (control) for 48 hours total. Quantified sAβPPα and sAβPPβ (Mean ± 20% CV, n=4, each)

were statistically analyzed by the Student's T test (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$). Quantified levels of full length A β PP (mature and immature) were normalized to stain free total protein (Mean $\pm$ 20% CV, $n=2$, each). B) As in A, resulting from dose-dependence of pCDP-diBzl treatment. Duplicate treatments were performed with 25, 50, 100, 200, and 400 μ M of pCDP-diBzl and DMSO (control) for 48 hours total. Data were quantified and statistically analyzed as in A. C). DMSO and 400 μ M diBzl data points for mature-A β PP and immature-A β PP (from A and B) were combined ($n=4$, each) and statistically analyzed as in A.

Figure 6. ESI+ LCMS results show selective uptake and conversion of pCDPs

H4-A β PP695 cells were treated with 400 μ M of DMSO (control), pCDP- diBzl (diBzl), pCDP-Bzl (Bzl), pCDP, and pCDP-diPOM (diPOM) for 24 hours and lysates are analyzed by ESI+ LCMS (A). Untreated H4-A β PP695 cells were also lysed and spiked with 100 μ M DMSO, diBzl, Bzl, pCDP, and diPOM to act as positive controls (B). Total Ion Chromatograms (TICs) for treated and positive controls were analyzed to find MWs 279 (279.07360, mass tolerance 0.1 mmu), 369 (369.12050, mass tolerance 0.5 mmu), 459 (459.16706, mass tolerance 0.1 mmu), and 507 (507.20940, mass tolerance 0.1 mmu) (A,

B). All of the graphs (A, B) show relative abundance of these specific MWs and peaks are labeled with retention time.

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Figure 7. pCDPs do not specifically inhibit the catalytic activity of BACE1

The activity of the BACE1 catalytic domain is observed through the cleavage of the Mca fluorogenic substrate. For each time point, the average is plotted (n=6, and n=5 for BACE1 inhibitor data) and error (+/- standard deviation) was determined. Intensity is normalized to the 1 hour fluorescence intensity for the DMSO control sample.

Figure 8. Proposed model for pCDP-diBzl and pCDP-diPOM mechanisms of action

A) The disease state, induced by elevated expression of either AβPP695 or BACE1, increases the association of AβPP and BACE1 via a cytoplasmic association complex that promotes sAβPPβ production. B) pCDP-diBzl is proposed to inhibit formation of the association complex, independent of AβPP and BACE1 binding surfaces. C) pCDP-diPOM is proposed to inhibit formation of the association complex by directly blocking the AβPP binding partner in the association complex.