Supporting Information

A Prochelator Activated by β-Secretase Inhibits Aβ Aggregation and Suppresses Copper-Induced Reactive Oxygen Species Formation

Drew S. Folk and Katherine J. Franz*

Department of Chemistry, Duke University, PO Box 90346, Durham, North Carolina 27708
Email: katherine.franz@duke.edu

Experimental

Materials and Instrumentation

All chemicals and solvents were obtained from Sigma-Aldrich and used without further purification unless otherwise noted. All water was nanopure. Peptides were synthesized on a Protein Technologies PS3 automated peptide synthesizer. Liquid chromatography-electrospray mass spectrometry (LC-MS) data were collected on an Agilent 1100 Series HPLC in line with an LC/MSD trap and a Daly conversion dynode detector. UV-Vis spectra were recorded on a Cary 50 UV-Vis spectrophotometer. Turbidity, deoxyribose, and Amplex Red assays were conducted on a Perkin Elmer Victor 1420 plate reader. Fluorescence data were recorded on a FluoroLog-3 fluorimeter from HORIBA Jobin Yvon.

Preparation of Peptides

Peptides were synthesized in 0.1 mmol scale on PAL-PEG-PS resin (Applied Biosystems). Standard Fmoc (9-fluorenylmethoxy-carbonyl)-protected amino acids (Chem-Impex and Novabiochem) were coupled in 20 min cycles with HBTU (*O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate) (Novabiochem) and *N*-methylmorpholine (NMM) (Acros) in *N*,*N*′-dimethylformamide (DMF) (Caledon). Fmoc protecting groups were removed by using 20% piperidine in DMF. The N-termini of peptides designated with an 'Ac' were acetylated using acetic anhydride and NMM. Cleavage from the resin and removal of side chain protecting groups was accomplished by treating resin with a 10-mL mixture of 95% trifluoroacetic acid (TFA) and 2.5% triisopropylsilane (TIS) under nitrogen while shaking for 4 h. Peptide was precipitated from solution by evaporating off TFA with a nitrogen stream, followed by three washes with diethyl ether (Caledon). Purification was accomplished by semi-preparative reversed-phase HPLC on a YMC C18 column with a linear 40 min gradient from 7 to 70% acetonitrile in water with 0.1% TFA. Purity was validated to be greater than 95% by analytical HPLC. Mass of each peptide was determined by ESI-MS.

Preparation of Aβ

The $A\beta_{1\text{-}42}$ peptide was purchased from either Genscript or EZBioLab and validated by the supplier to be > 95% pure (DAEFRHDSGYEVHHQKLVFFAEDVGSNKGAII-GLMVGGVVIA). A portion (1 mg) of Aβ was dissolved in 600 µL of 1% ammonium hydroxide in de-ionized water by sonicating 1 min on, 30 s off, and 1 min on. The solution was filtered through a syringe filter (GE Water, Nylon 0.22 µM, 3 mm). A second aliquot of 400 µL 1% ammonium hydroxide was used to rinse the vial and filter of any residual peptide. The concentration of this stock solution was determined by the Pierce MicroBCA assay and validated by measuring the tyrosinate absorption (295 nm, ε = 2480 $M^{-1}cm^{-1}$) at pH 12. The stock solution was portioned into aliquots and stored at -20 °C and used within 5 days of preparation.

LC-MS Kinetic Assay of BACE Activity

SWH prochelator and Swedish mutant APP control peptide (SW) were dissolved in 10% glacial acetic acid and the pH was adjusted to 4.5 with 100 mM NaOH. Water was added to achieve final concentrations of 155 µM. Aliquots of 350 µL were pipetted into

eppendorf tubes and 10 µL β-secretase (C-terminal FLAG-tagged, extracellular domain; Sigma-Aldrich) (0.6 mg/mL) was added to initiate the reaction. Samples were allowed to vortex slowly at 25 °C. Aliquots of 16 µL were removed from the reaction mixture every 5–30 min and diluted into 25 µL of 8 M urea in HPLC vials to quench the reaction. Samples were analyzed on a Varian Polaris C_{18} reverse phase column (150 mm x 1 mm) with a linear 20-min gradient from 3–55% acetonitrile in water with 0.3% formic acid. Signal was detected at 280 nm on a UV detector. The only amino acids that significantly absorb light at this wavelength are tryptophan and tyrosine. Both the intact prochelator (SWH = Ac-EVNLDAHFWADR) and the C-terminal cleavage product (i.e. chelator, CP = DAHFWADR) each contain a single tryptophan and thus have similar extinction coefficients at 280 nm, allowing for direct comparison of the peak areas of prochelator and CP product. Likewise, the SW control peptide (Ac-EVNLDAEFWHDR) and its C-terminal cleavage product (DAEFWHDR) also contain a single tryptophan. The masses for the starting peptides and cleavage products were validated by in-line mass spectroscopy. Although the N-terminal cleavage products were not visible on the 280 nm chromatograms, these peaks were identified in the mass spectra, verifying that BACE cleavage occurred at the expected sites. **Figure S1** shows the % product as a function of time with the slope of the linear fit being the initial rate**.** As expected, the replacement of E in SW to H in SWH does not diminish the reactivity of SWH as a substrate for BACE. The percent product formed was calculated from the following equation:

$$\%\text{Product} = \frac{\text{Peak Area of CP}}{\text{Peak Area of CP + Peak Area of SWH}}$$

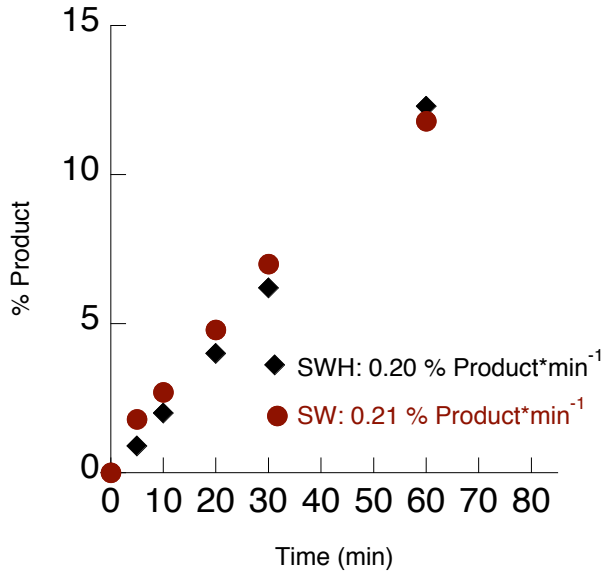

Figure S1. Cleavage of 155 µM SWH and SW in sodium acetate buffer pH 4.5 by BACE display similar initial rates by LC-MS.

Copper Binding

CP: Cu(II) sulfate solution (100 mM) was prepared from solid $Cu(SO_4)\cdot 5H_2O$ and standardized with 0.0500 M EDTA to a murexide endpoint in ammonia buffer. Lyophilized CP was dissolved in H_2O and the concentration was determined

spectrophotometrically by pipetting small aliquots (3-5 μL) into 1 mL 8 M urea and recording the absorbance at 280 nm. The extinction coefficient of tryptophan under these conditions is 5635 $M^{-1}cm^{-1}$.[1] The extinction coefficient for the d-d transition band of CP(Cu) was determined by titration of Cu^{2+} into 1 mM CP in 50 mM HEPES (N-(2-hydroxyethyl)-piperazine-N'-2-ethansulfonic acid) buffer pH 7.4. The complex shows an absorption maximum at 526 nm with a molar absorptivity of 110 M^{-1} cm^{-1}. Competition studies were performed by preparing 1 mL of 1 mM CP(Cu) in 50 mM HEPES buffer. The reaction vessel was a 3 mL cuvette and all titrations were carried out at 25 °C. Aliquots (1-2 μL) of the competitive chelator NTA (1 M) were pipetted into CP(Cu) solutions and monitored spectrophotometrically. After each addition, solutions were manually mixed and equilibrated for 5 min before data were collected. A typical titration is shown in **Figure S2**. Conditional stability constants were calculated on Microsoft Excel using the equations listed below. The conditional stability constant (K') for NTA was calculated from the overall log β of 12.7 for the 1:1 Cu:NTA complex[2] to be 10.68 at pH 7.4.

$$[CP]_{Total} = [CP]_{Free} + [CP(Cu)]$$

$$[NTA]_{Total} = [NTA]_{Free} + [NTA(Cu)]$$

$$[Cu]_{Total} = [Cu]_{Free} + [CP(Cu)] + [NTA(Cu)]$$

$$CP_{Free} + Cu^{2+} \rightarrow CP(Cu); \qquad K^{app}_{CP(Cu)} = \frac{[CP(Cu)]}{[Cu^{2+}][CP_{Free}]}$$

$$NTA_{Free} + Cu^{2+} \rightarrow NTA(Cu); \qquad K'_{NTA} = \frac{[NTA(Cu)]}{[NTA_{Free}][Cu^{2+}]} = 10^{10.68}$$

$$CP(Cu) + NTA_{Free} \leftrightarrow NTA(Cu) + CP_{Free}; \qquad K_{ex} = \frac{[CP_{Free}][NTA(Cu)]}{[CP(Cu)][NTA_{Free}]} = \frac{K'_{NTA}}{K_{CP(Cu)}}$$

$$K^{app}_{CP(Cu)} = \frac{K'_{NTA}}{K_{ex}}$$

The competition experiment revealed $K^{app}_{CP(Cu)}$ to be 10^{12}. This apparent value can be converted to a conditional constant (K′) by accounting for the ternary complex that forms between NTA, copper, and HEPES. The conditional stability constant for CP with copper(II) at pH 7.4 was therefore obtained as K′ = $10^{12.6}$, according to the following equation, where log K_T for the ternary complex was take to be 0.6.[3]

$$\log(K'_{CP(Cu)}) = \log K^{app}_{CP(Cu)} + \log K_T$$

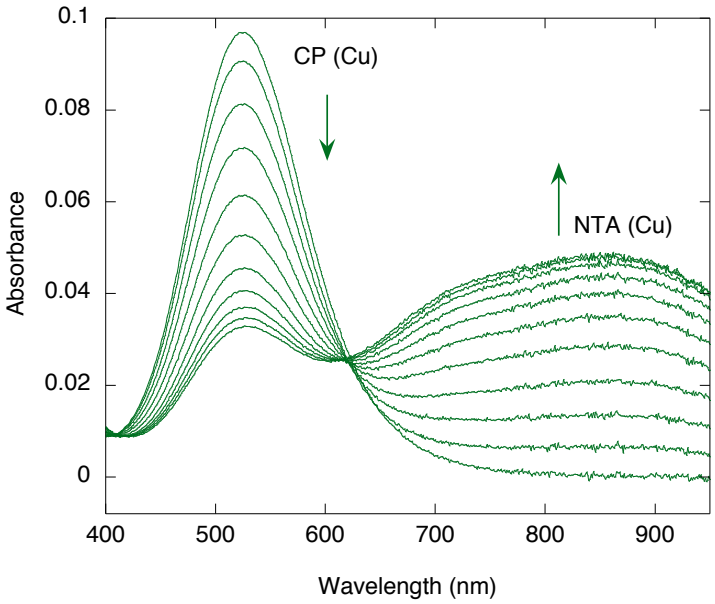

Figure S2: Titration of CP(Cu) with competitive chelator NTA in HEPES buffer at pH 7.4. Conditions: 50 mM HEPES, 1 mM CP, 1 mM $CuSO_4$, NTA from 1 mM to 20 mM.

SWH: The conditional copper binding of the prochelator (SWH) at pH 7.4 was evaluated using a fluorescence quenching assay. 3 mL of 10 μM SWH in 50 mM HEPES buffer was prepared in a fluorescence cuvette. 1 equiv of $CuSO_4$ was added to quench the fluorescence signal through copper binding. The excitation and emission wavelengths were 280 nm and 361 nm respectively. 1 equivalent of competitive chelators NTA and Glycylglycine (GlyGly) were added to individual samples of SWH(Cu). Restoration of fluorescence indicated removal of copper from SWH. The results shown in **Figure S3** indicate SWH does not bind copper as strongly as GlyGly thus making its affinity (log K') towards metal ions at pH 7.4 less than 4.7 (calculated from overall logβ of 5.55.[2]

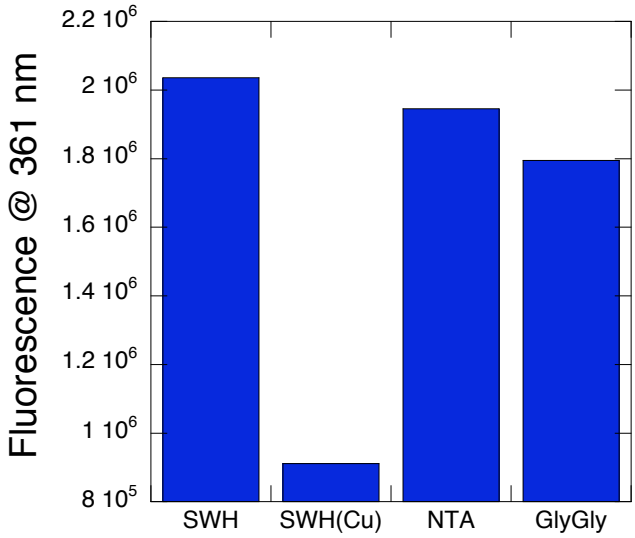

Figure S3: Quenching of tryptophan fluorescence signal in SWH by Cu^{2+} and subsequent restoration upon the addition of competitive chelators NTA and Glycylglycine (GlyGly). Conditions: 50 mM HEPES, 10 μM SWH, 10 μM $CuSO_4$, 10 μM NTA, 10 μM GlyGly. λ_{ex} = 280 nm.

Assessment of Cu Transfer from Aβ to CP by Fluorescence Quenching

Stock solutions of Aβ were added to a 3 mL fluorescence cuvette and diluted to 1 mL with HEPES buffer (50 mM HEPES, pH 7.4) to achieve a final concentration of 10 μM. $CuSO_4$ (10 μM) was added and a quenching of the fluorescence signal of tyrosine was observed indicating copper binding. Aliquots (0.2 equiv) of CP were titrated into the solution, mixed, and allowed to equilibrate for 5 min. A fluorescence scan was then taken with excitation at 280 nm. Fluorescence at 361 nm was monitored to observe tryptophan signal quenching upon displacement of copper from Aβ to CP **(Figure S4)**. This response is consistent with CP extracting Cu^{2+} from Aβ, which prevents Trp emission until the concentration of CP exceeds that of Cu^{2+}. The corresponding increase in Aβ's tyrosine fluorescence upon removal of Cu^{2+} is not observed due to the much broader and more intense tryptophan signal. The experiment was repeated with the prochelator being titrated into solution (0.2 equiv). Copper-negative controls for all trials were also performed (not shown).

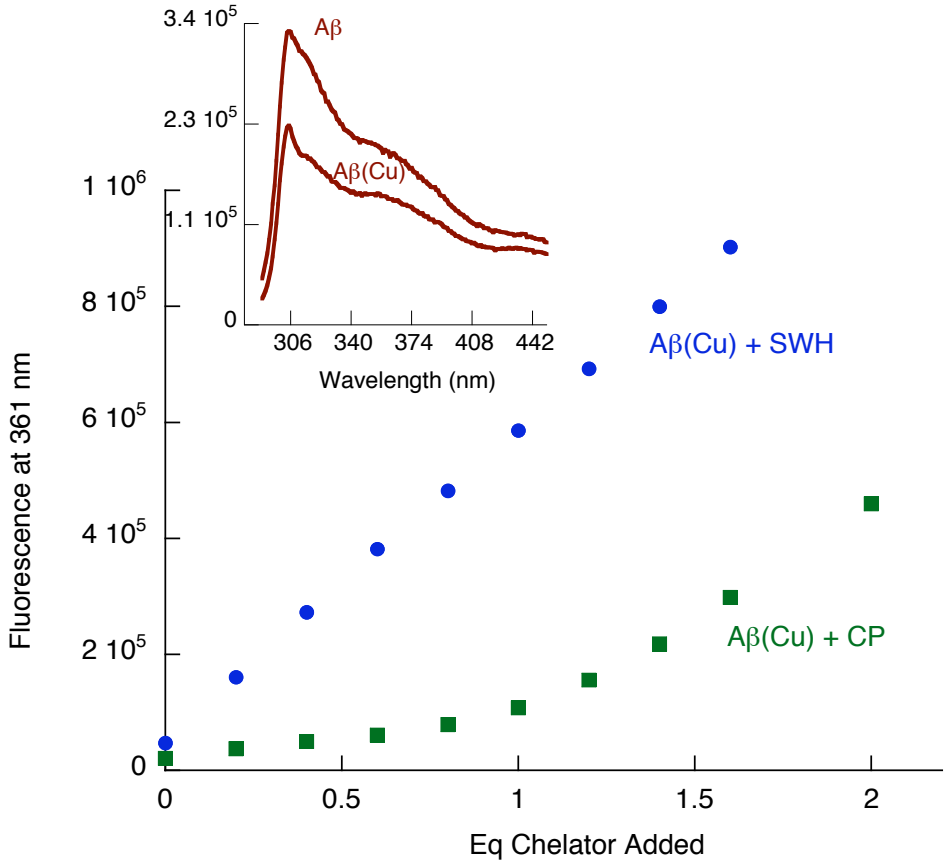

Figure S4. Fluorescence quenching of tryptophan signal of CP due to transfer of copper from Aβ to CP. Inset: Fluorescence quenching of the tyrosine in Aβ upon addition of copper. Conditions: 50 mM HEPES pH 7.4, 10 μM Aβ, 10 μM $CuSO_4$, 0–20 μM CP, 0–16 μM SWH, λ_{ex} = 280 nm, λ_{em} = 361 nm.

Assessment of Aβ Aggregation: Turbidity Assay

All solutions were prepared using Chelex-treated HEPES buffer (50 mM HEPES, 150 mM NaCl, pH 7.4) Metal solutions were prepared fresh daily. Enzymatic cleavage of SWH and SW were performed by incubating 155 μM SWH or SW in 0.1M sodium acetate buffer pH 4.5 with BACE at rt. After 19 h incubation, an aliquot was removed, quenched in 8M urea and analyzed by LC-MS to determine concentration of cleavage product formation.

Aggregation prevention assays (blue solid bars of Figure 2 in the main text) were performed by adding reagents to individual wells of a 96-well plate in the following order, with the final concentrations given in parentheses: HEPES buffer to give a final total volume of 200 μL, $Cu(Gly)_2$ (prepared from $CuCl_2$ with 2 eq. glycine) or $ZnCl_2$ (10 μM), SWH prochelator (10 μM), CP chelator (10 μM), BACE proteolysis products (10 μM), and lastly Aβ (10 μM). Samples were incubated at 37 °C for 2 h with the plate lid on to minimize evaporation. After incubation, the turbidity was measured by light scattering at 405 nm on a plate reader set to collect one reading every min for 4 min with 30 s of shaking in between each reading. Solution turbidity was determined by

subtracting the Aβ-negative control from each well and averaging these values over each 4-min period. Error bars represent the standard deviation from a minimum of 3 independent samples.

Dissaggregation assays (green dotted bars of Figure 2 in the main text) were performed by adding the enzyme proteolysis products (10 μM), to wells of Aβ(Cu) and the corresponding control wells which had been allowed to aggregate for 2 h. The plate was incubated for an additional 2 h with the lid on to limit evaporation and the degree of light scattering was measured at 405 nm as described above.

Assessment of H_2O_2 Formation: Amplex Red Assay

Hydrogen peroxide quantitation was done by following the general assay directions for the Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit available from Invitrogen. Reagents were added to individual wells of a 96-well plate in the following order, with final concentrations given in parentheses and a final volume of 50 μL: sodium phosphate buffer (50 mM), CP (50–400 nM), SWH (200 nM), H_2O_2 (50 nM – 5 μM), $A\beta_{1-42}$ (200 nM), $CuGly_2$ (200 nM), and ascorbic acid (10 μM). Amplex Red (50 μM; 0.1 U/mL HRP) was added immediately and the plate was incubated at 37 °C for 1 h. The amount of H_2O_2 produced was quantified by comparison to a standard curve measured immediately before sample analysis. Error bars represent standard deviation from runs done in at least triplicate. The results are shown in **Figure S5**.

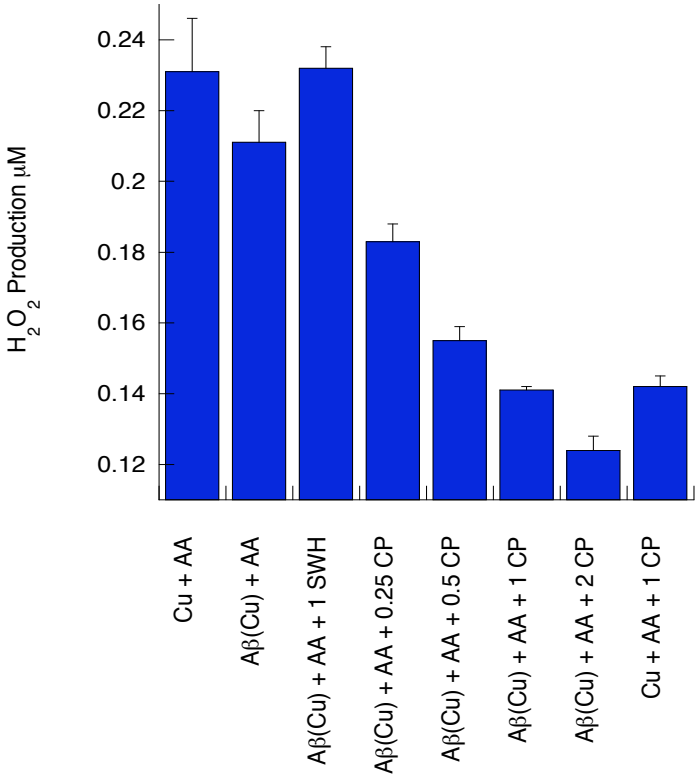

Figure S5. H_2O_2 production from Aβ(Cu) (200 nM) in the presence of ascorbic acid (AA) (10 μM), SWH (200 nM) and CP (50-400 nM). Aβ-negative control is also shown.

Assessment of $OH^{\bullet}$ Production: Deoxyribose Assay

The 2-deoxyribose assay was used to measure hydroxyl radical formation. A mixture of copper, ascorbic acid, and H_2O_2 generates hydroxyl radicals by Fenton-like chemistry. Hydroxyl radicals attack 2-deoxyribose to form malondialdehyde (MDA), which upon heating with thiobarbituric acid (TBA) produces a pink chromophore (λ_{max} = 532 nm). Chelators that prevent copper from reacting with ascorbic acid and hydrogen peroxide result in less chromophore formation. All assays were performed using 50 mM NaH_2PO_4 buffered to pH 7.4. The following reagents were added sequentially to obtain a 100 μL buffered solution with these final concentrations: SWH and CP chelators (3–100 μM), $Cu(SO_4)$ (10 μM), 2-deoxyribose (15 mM), H_2O_2 (100 μM), and ascorbic acid (2 mM). Stock solutions of ascorbic acid and H_2O_2 were prepared fresh daily and reactions were carried out in a polystyrene 96-well cell culture plate. The reaction mixtures were agitated at 37 °C for 1 h, then 100 μL of TBA (1% w/v in 50 mM NaOH) and 100 μL of trichloroacetic acid (2.8% w/v in water) were added to quench the reaction. The temperature was increased to 100 °C for 20 min, then cooled to room temperature and the absorbance at 490 nm was recorded using a PerkinElmer 1420 plate reader. Values are reported as normalized absorbance (A/A_0) where A_0 is the absorbance without chelator present and A is the absorbance with chelator added. The value for $Cu(SO_4)$ alone is $A/A_0 = 1$. Error bars represent standard deviations from measurements done in at least quadruplicate.

References

(1) Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T. *Protein Sci.* **1995**, *4*, 2411-2423.

(2) Martell, A. E.; M., S. R. In *NIST Standard Reference Database 46*; 6.0 ed.; Motekaitis, R. J., Ed.; NIST: Gaithersburg, MD, 2001.

(3) Rózga, M.; Sokolowska, M.; Protas, A. M.; Bal, W. *J. Biol. Inorg. Chem.* **2007**, *12*, 913-918.