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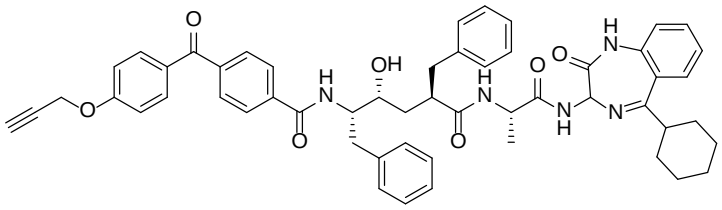

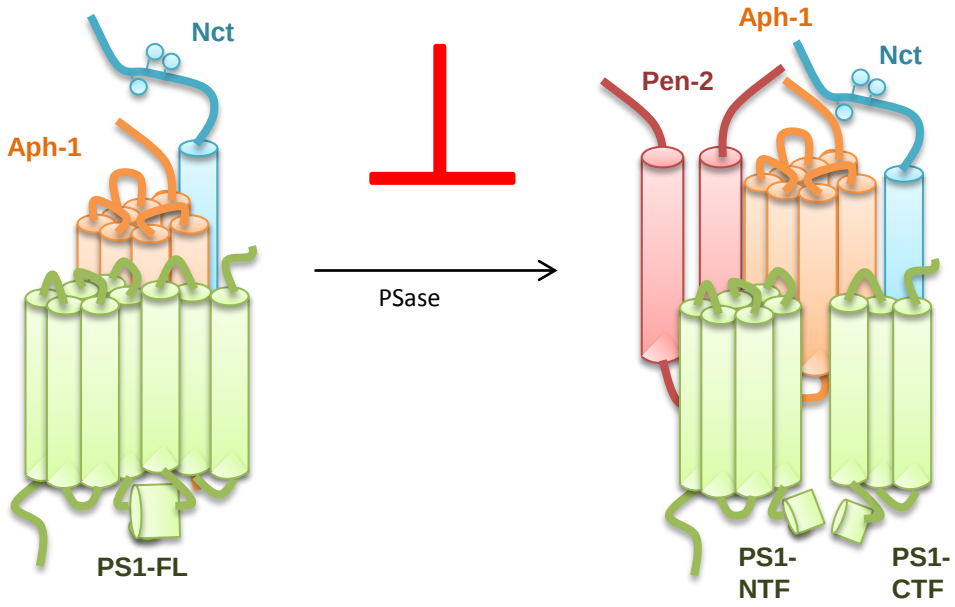

CBAP-BPyne, a dual presenilinase and γ-secretase clickable probe, provides a novel means to investigate the mechanism of endoproteolysis.

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ARTICLE TYPE

Development of CBAP-BPyne, a probe for γ-secretase and presenilinase

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γ-Secretase undergoes endoproteolysis of its catalytic subunit, presenilin (PS), to form PS N-terminal and C-terminal fragments (PS1-NTF/CTF), which generate the active site. PS endoproteolysis, catalyzed by presenilinase (PSase), remains poorly understood and requires novel chemical approaches for its mechanistic study. CBAP is a dual inhibitor that suppresses both γ-secretase and PSase activities. To probe γ-secretase and PSase activity in cells, we have synthesized the clickable photoaffinity probe CBAP-BPyne. We found that CBAP-BPyne specifically labels PS1-NTF and signal peptide peptidase (SPP). CBAP-BPyne is a valuable tool to directly study the mechanism of endoproteolysis.

γ-Secretase is an aspartyl protease that belongs to the I-CLiPs family (intramembrane-cleaving proteases), a class of membrane-embedded enzymes that performs transmembrane (TM) hydrolysis on its substrates.[1] γ-Secretase cleaves a wide array of type-1 TM substrates that have undergone ectodomain shedding. Some important γ-secretase substrates include amyloid precursor protein (APP), Notch, and E-cadherin. γ-Secretase plays a pivotal role in Alzheimer's disease (AD) and cancer and is an important target for prospective drug development.[2,3]

γ-Secretase is composed of at least four subunits: PS, nicastrin, Aph-1, and Pen-2.[4] PS is the catalytic subunit of γ-secretase.[5-8] The assembly, stabilization, trafficking, and maturation of the γ-secretase complex are tightly controlled and well regulated. The final step of γ-secretase activation occurs via Pen-2-mediated endoproteolysis of PS.[9-11] Specifically, PS is translated as a single polypeptide chain and then, upon Pen-2 insertion into the complex, processed into two fragments, PS1-NTF and PS1-CTF. The two fragments of PS form a stable heterodimer, with each fragment contributing an aspartate residue to generate the active site of γ-secretase (Figure 1).

The enzyme responsible for the endoproteolytic cleavage of PS is termed PSase. Current evidence suggests that PSase is actually PS itself, and endoproteolysis is an autocatalytic cleavage event. This is illustrated by the following observations: First, mutation of PS's catalytic aspartate residues not only blocks γ-secretase activity, but also PSase activity.[5] Second, pepstatin A, an aspartyl protease inhibitor, suppresses PSase activity, further suggesting that PSase is an aspartyl protease.[12] However, the coexpression of WT PS1 with PS1 D257A (a γ-secretase and PSase deficient mutant) does not restore endoproteolysis of the mutant, indicating that endoproteolysis occurs *in cis* and is an autocatalytic event.[13] Finally, an *in vitro* reconstitution study showed that bimolecular interaction of PS1 and Pen-2 is necessary and sufficient for PS1 endoproteolysis.[8] Collectively, these studies strongly indicate that PS has PSase activity. Notwithstanding findings that PS possesses γ-secretase and PSase activities, it has been a formidable challenge to characterize both activities and understand their differences due to their complex interdependence. While many γ-secretase active site-based inhibitors exist to directly probe γ-secretase, no successful PSase-directed probes exist to date. CBAP (Figure 2A) is a γ-secretase inhibitor that also causes a "pharmacological knock-down" of PS1 NTF/CTF with a concomitant accumulation of full-length PS1 (PS1-FL) in the cell.[14] However, the mechanism of action of CBAP in γ-secretase and PSase remains to be investigated. We have synthesized CBAP-BPyne, a clickable, photoreactive form of CBAP, as a tool to understand the mechanism of PSase (Figure 2A).

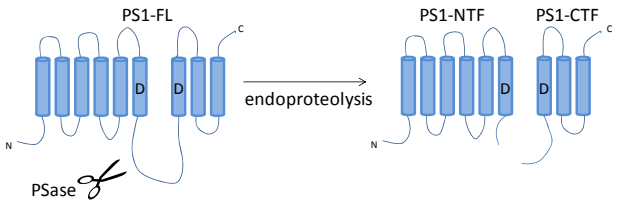

Figure 1. Endoproteolysis of PS1. PS1-FL (full-length) is endoproteolysed by PSase in a hydrophobic stretch of the cytoplasmic loop, to form an ~27 kDa NTF and ~16 kDa CTF. Endoproteolysis and subsequent PS1-NTF/CTF heterodimer formation are required for γ-secretase activity, as PS1-FL is a zymogen, activated by autocleavage. (D: catalytic Asp residues)

The CBAP intermediate TBS-protected alcohol (**4**) was synthesized by coupling amino benzodiazepinone **3** to carboxylic acid **1** as previously reported.[14] To synthesize CBAP-BPyne, we initially investigated the selective removal of the NHBoc group

from **4**, but all conditions examined resulted in poor product formation where removal of the silyl and Boc protecting groups occurred at competitive rates. It was determined that selective Boc group removal or one-pot global deprotection strategies were not viable to produce the CBAP-BPyne in sufficient yields and purity. CBAP-BPyne was ultimately synthesized by removing the silyl protecting group in **4** with TBAF to yield CBAP followed by a rapid deprotection of the NHBoc group at 0 °C in dilute TFA to produce the fully deprotected scaffold. The crude amino alcohol was then immediately coupled with the NHS ester of propargyl benzophenone **2** to afford CBAP-BPyne.[15‡]

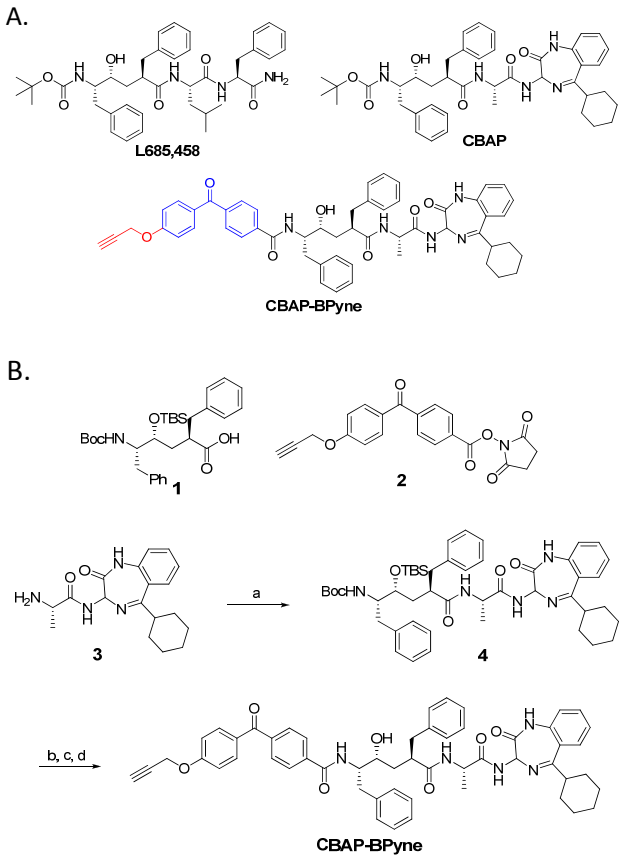

Figure 2. A. Structures of L685,458, CBAP, and CBAP-BPyne. Red: clickable alkyne; blue: crosslinkable benzophenone B. Reagents and conditions for synthesis of CBAP-BPyne. a) **1**, HATU, DIPEA, DMF, 24 h, RT, 83%; b) TBAF, THF, 6 h, RT, 84%; c) TFA, CH_2Cl_2, 5 min, 0 °C; d) **2**, DIPEA, DMF, 18 h, 79%.

CBAP-BPyne contains a photophore for photoaffinity labeling and an alkyne for click chemistry (copper catalyzed azide-alkyne cycloaddition). This clickable probe approach facilitates the design of functional probes that can selectively label and detect proteins in complex cellular systems with minimal modification to the original compound.[16-19] First, using our *in vitro* γ-secretase activity assay with recombinant APP and Notch1 substrates,[20-24] we determined that both CBAP and CBAP-BPyne are equipotent γ-secretase inhibitors. Specifically, both compounds potently inhibit γ-secretase activity for both the production of Aβ40 and Notch1-NICD (Figure 3A). Next, we examined CBAP and CBAP-BPyne's cellular activity in inhibiting PS1 processing. HeLa cells were treated for four days in a 12-well plate with CBAP, CBAP-BPyne, L685,458 (an active site-directed γ-secretase inhibitor), or vehicle control at concentrations of 1, 3, or 10 μM. Cells were lysed with RIPA buffer (50 mM Tris base pH 8.0, 150 mM NaCl, 0.1% SDS, 1% NP-40, 0.5% deoxycholate) and 15 μg of cell lysate was separated on a 12% Bis-Tris gel in MES buffer. Proteins were transferred to Immobilon-FL PVDF membrane, probed with anti PS1-NTF antibody, and imaged on Odyssey (LI-COR Biosciences). CBAP and CBAP-BPyne caused a much greater accumulation of PS1-FL at the expense of PS1-NTF/CTF than did L685,458 (Figure 3B for PS1-NTF and data not shown for PS1-CTF). This indicates that CBAP and CBAP-BPyne are capable of inhibiting PSase activity, while L685,458 has nearly no effect on PSase activity under these conditions. CBAP is a more potent PSase inhibitor than CBAP-BPyne as seen by the nearly complete depletion of PS1-NTF in CBAP treated cells compared to the incomplete PS1-NTF depletion in CBAP-BPyne treated cells (Figure 3B). This effect is not due to a difference in the ability of the compounds to permeate the cell membrane because CBAP and CBAP-BPyne were equipotent in a cell-based γ-secretase activity assay (Aβ42 IC_{50} = 28 nM and 20 nM, for CBAP and CBAP-BPyne, respectively, in CHO-APP cells).

A.

IC_{50} (nM)		
	Aβ40	Notch1
CBAP	0.70	0.56
CBAP-BPyne	0.91	1.0

B.

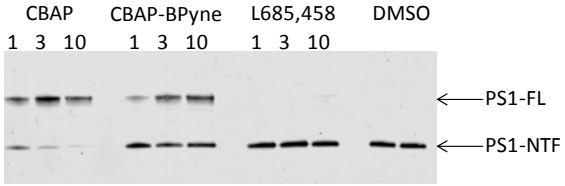

Figure 3. A. *In vitro* inhibitory potency (IC_{50}) of CBAP and CBAP-BPyne. B. HeLa cells were treated with 1, 3, and 10 μM of CBAP, CBAP-BPyne, and L685,458, or vehicle control (DMSO), for 4 days. Protein concentration was determined and same amount of cell lysate (15 μg) was run on SDS-PAGE. Western blot analysis was performed with an anti PS1-NTF antibody.

Finally, we determined that CBAP-BPyne is a functional probe, as it specifically labels PS1-NTF (Figure 4A). Briefly, 600 μg of HeLa cell membrane, diluted with PBS to a volume of 500 μL in a 12-well plate, was incubated with either 2 μM CBAP or vehicle control for 15 min at 37 °C. 20 nM CBAP-BPyne was added for 1 hour at 37 °C followed by UV irradiation (350 nm) for 45 min to promote benzophenone-protein crosslinking. Membrane was pelleted by centrifugation at 100,000 ×*g* for 30 min and resuspended in PBS using Qiagen TissueLyser. Click chemistry reagents [1 mM tris(2-carboxyethyl)phosphine, 1 mM $CuSO_4$, 0.1 mM tris-(benzyltriazolylmethyl)amine, and 0.1 mM biotin azide in 5% *t*-butyl alcohol with 1% DMSO] were added and the mixture was rotated for 1 hour at room temperature. Membranes were pelleted by centrifugation at 100,000 ×*g* for 30 min, resuspended in 500 μL PBS and solubilized with the addition of RIPA buffer. Samples were centrifuged at 13,400 ×*g* and supernatant was added to Pierce Streptavidin Plus UltraLink

Resin and rotated overnight at 4 °C. Proteins were eluted with 2 mM biotin in SDS sample buffer at 70 °C for 10 min, separated on a 12% Bis-Tris gel or a 4-20% TGX gel, transferred to Immobilon-FL PVDF, probed with the relevant antibody, and visualized on Odyssey (LI-COR Biosciences). CBAP-BPyne was also found to label SPP, a protein structurally similar to PS (Figure 4A). CBAP-BPyne does not label PS1-CTF or any of the other three subunits of γ-secretase (data not shown). Photoaffinity labeling studies followed by click chemistry with TAMRA-azide confirmed the specific labeling of PS1-NTF and SPP, and showed that CBAP-BPyne binds additional proteins, although PS1-NTF is the primary target (Figure 4B). The additional unidentified proteins that are specifically labeled (denoted with a star) may play a role in endoproteolysis and will be studied further for their identity and function. Whether bands that migrated in the range of high molecular weight represent aggregated PS1-NTF, SPP or novel proteins also remains to be investigated.

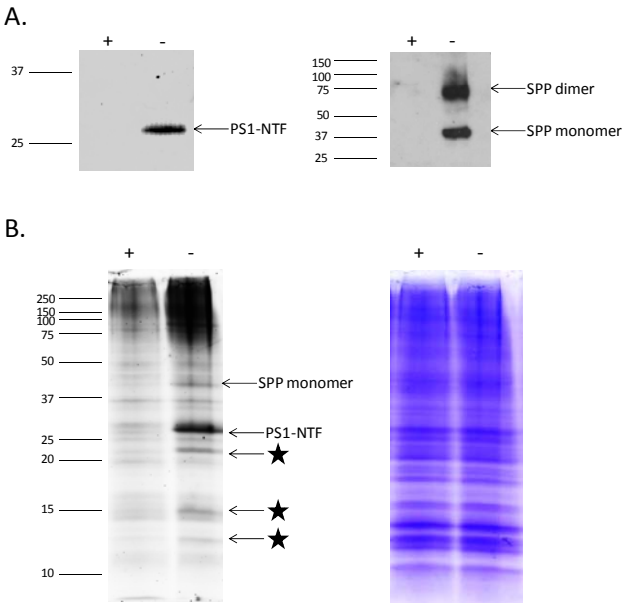

Figure 4. HeLa membranes were photolabeled with CBAP-BPyne (20 nM) in the presence (+) or absence (-) of CBAP (2 μM), followed by click chemistry with: A. biotin-azide, streptavidin pull down, and Western blot analysis with either anti PS1-NTF (left), or SPP (right) antibody or B. TAMRA-azide, in-gel fluorescence (left) and Coomassie blue gel staining (right). ★ - represents unidentified proteins that are specifically labeled by CBAP-BPyne.

CBAP-BPyne is the first clickable, photoreactive probe that inhibits both γ-secretase and PSase activities. Of note, based on current clinical investigation of non-selective γ-secretase inhibition (i.e. the case of semagacestat), PSase may not be a viable drug target for the treatment of AD since PSase inhibition also blocks γ-secretase activity, leading to toxicity.[25] However, PSase could serve as a target for cancer therapy. Furthermore, this probe can be used to investigate PSase and γ-secretase activation, which appear to play a role in disease states, as evidenced by reports that some familial AD PS1 mutations affect PSase activity.[26-28] CBAP-BPyne provides a novel means to investigate the mechanism of PSase as it has the capacity not only to bind and inhibit γ-secretase, but also to inhibit the endoproteolysis of PS1-FL, a novel function not observed in other γ-secretase probes. CBAP-BPyne may aid in the identification and characterization of PSase, revealing the mechanism of γ-secretase activation and uncovering PSase as a potential target in cancer therapy.

Notes and references

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‡ Characterization of CBAP-BPyne: ^{1}H NMR (400 MHz, CD_3OD) δ 8.61 - 8.43 (m, 1H), 8.26 (d, *J* = 8.8 Hz, 1H), 8.18 (t, *J* = 7.9 Hz, 1H), 7.82 - 7.75 (m, 2H), 7.73 - 7.67 (m, 5H), 7.55 - 7.48 (m, 1H), 7.29 (t, *J* = 7.7 Hz, 1H), 7.24 - 7.08 (m, 13H), 7.08 - 7.00 (m, 1H), 5.15 - 5.08 (m, 1H), 4.85 (2H, buried in solvent), 4.49 (dt, *J* = 13.5, 6.9 Hz, 1H), 4.34 - 4.22 (m, 1H), 3.76 (td, *J* = 8.5, 4.3 Hz, 1H), 3.12 - 3.03 (m, 1H), 3.01 (t, *J* = 2.3 Hz, 1H), 3.00 - 2.93 (m, 1H), 2.88 (d, *J* = 9.2 Hz, 1H), 2.82 - 2.69 (m, 2H), 2.04 - 1.74 (m, 4H), 1.69 - 1.54 (m, 2H), 1.49 - 1.21 (m, 8H), 1.15 (d, *J* = 10.4 Hz, 1H), 1.08 - 0.94 (m, 1H); LRMS calcd for $C_{54}H_{55}N_5O_7$ (M+H) 886.4, found 886.5; HPLC Purity: 99.9%.

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