

Transition-state analogue inhibitors of γ -secretase bind directly to presenilin-1

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The β -amyloid precursor protein (β -APP), which is involved in the pathogenesis of Alzheimer's disease, and the Notch receptor, which is responsible for critical signalling events during development, both undergo unusual proteolysis within their transmembrane domains by unknown γ -secretases. Here we show that an affinity reagent designed to interact with the active site of γ -secretase binds directly and specifically to heterodimeric forms of presenilins, polytopic proteins that are mutated in hereditary Alzheimer's and are known mediators of γ -secretase cleavage of both β -APP and Notch. These results provide evidence that heterodimeric presenilins contain the active site of γ -secretase, and validate presenilins as principal targets for the design of drugs to treat and prevent Alzheimer's disease.

The amyloid β -protein (A β) is the principal component of the neuritic plaques that characterize Alzheimer's disease, and evidence from biochemical, genetic and neuropathological studies, as well as from animal modelling, supports the hypothesis that accumulation of A β causes Alzheimer's and is not merely a disease marker^{1,2}. β -secretase and γ -secretase, the proteases that excise A β from β -APP, are therefore considered important targets for the development of therapeutics for Alzheimer's, a disease for which no

effective treatment exists³. β -secretase has recently been identified by four pharmaceutical companies⁴⁻⁷, an advance that should greatly accelerate the discovery of drugs for this target. The identification of γ -secretase would likewise be a significant advance in our understanding of the molecular mechanism of Alzheimer's disease and would expedite the discovery of agents that work by blocking this protease.

Despite extensive searching by many pharmaceutical and aca-

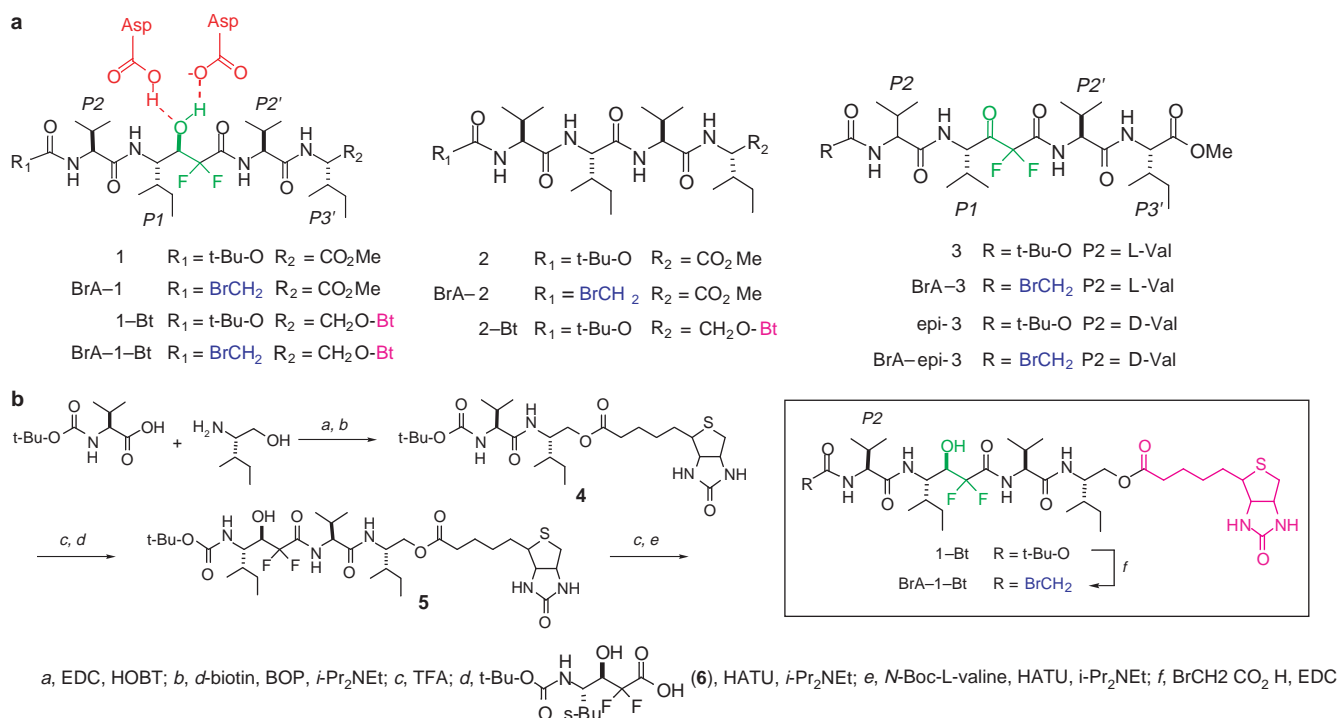


Figure 1 Design and synthesis of transition-state-analogue affinity reagents for γ -secretase. **a**, Peptidomimetics used in this study. Compounds of general structure **1** contain a non-hydrolyzable difluoro-alcohol moiety (green) that resembles the transition state of the proteolysis carried out by aspartyl proteases (aspartate residues shown in red). Compounds of general structure **2** are peptide counterparts of compound **1** that lack a transition-state-mimicking moiety. Compounds of general structure **3** contain a non-hydrolyzable difluoro-ketone moiety (green) that readily

forms a hydrate resembling the aspartyl-protease-catalysed transition state. BrA, bromoacetamide; *t*-Bu-O, tert-butoxy; Bt, biotin. **b**, Synthesis of biotinylated and bromoacetylated analogues. Synthesis of difluoro-alcohol building blocks such as that used in step d has been previously described^{9,24}. Magenta, C-terminal biotin; green, difluoro alcohol; blue, N-terminal bromoacetyl group. Key (bottom) shows the reagents used at each step (see Methods).

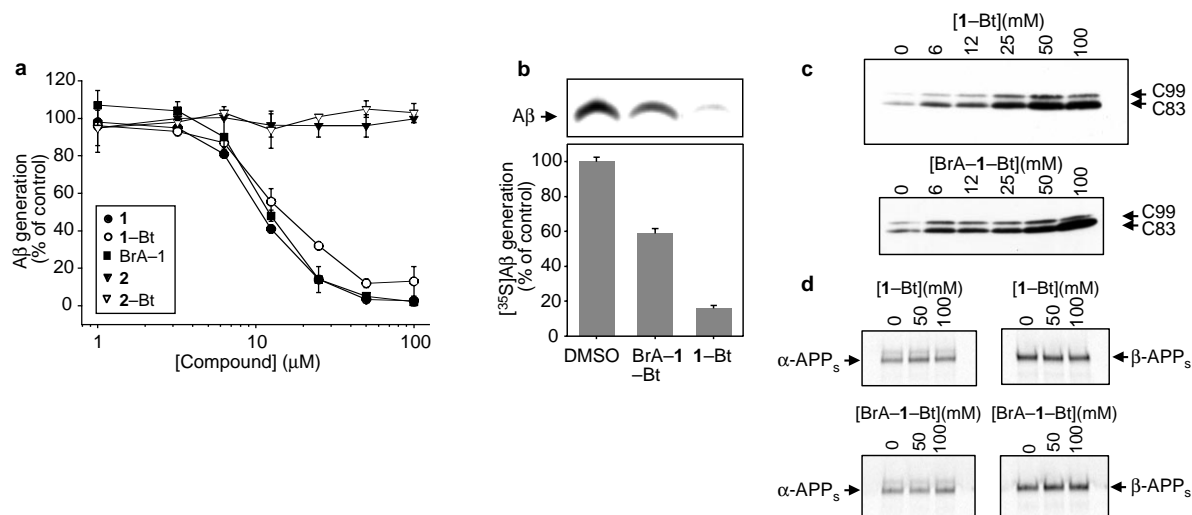


Figure 2 Effects of designed peptidomimetic inhibitors on β -APP processing.

Effect of biotinylation of inhibitors on A β production in living cells. CHO 7W cells stably transfected with human β -APP were treated with various concentrations of the indicated compound for 4 h, whereupon media were removed for analysis by A β sandwich ELISA. Values are means \pm s.d. from three separate experiments and are expressed relative to control values. **b**, Effects of 1-Bt and BrA-1-Bt on A β production in metabolically labelled cells. CHO 7W cells were labelled with [35 S]Met for 2 h in the presence or absence (dimethylsulphoxide only; DMSO) of 50 μ M of the indicated compound, and A β was immunoprecipitated from the media with antibody R1282. The resulting A β bands (corresponding to M_r ~4K) were quantified by densitometry. Error bars represent the range of values from two experiments; values

are expressed relative to control. **c**, Dose-dependent effects of 1-Bt and BrA-1-Bt on levels of γ -secretase substrates C83 and C99. After treatment of CHO 7W cells with the indicated concentrations of 1-Bt or BrA-1-Bt for 4 h, cell lysates were analysed by immunoprecipitation with antibody C7 and western blotting with antibody 13G8, both directed against the β -APP C terminus. **d**, Effects of 1-Bt and BrA-1-Bt on levels of α -APP $_s$ and β -APP $_s$. Human 293 cells expressing the K595N/M596L 'Swedish' double mutation of β -APP were metabolically labelled overnight in the presence of the indicated concentrations of 1-Bt or BrA-1-Bt. Proteins were immunoprecipitated from the media using specific antibodies against α -APP $_s$ (1736) or Swedish-mutant β -APP $_s$ (192sw) and visualized by fluorography.

demographic laboratories, γ -secretase has not been definitively identified, largely because of its unusual characteristics as an enzyme that apparently catalyses an intramembranous hydrolysis^{8,9}. The identification of γ -secretase is also critical for understanding the biochemical events involved in signalling from the Notch receptor. The ectodomains of both β -APP and Notch are shed by tumour necrosis factor- α converting enzyme (TACE)^{10,11}, and the membrane-anchored fragments left behind undergo further processing within their transmembrane domains by γ -secretases that are similar, if not identical, to each other¹². In the case of Notch, this intramembranous proteolysis releases an intracellular domain that can activate transcription of specific genes involved in cell-fate determination¹³. Both intramembranous cleavage events require presenilin-1 (PS1; refs 12, 14), a protein with eight transmembrane domains^{15,16} that was first identified because it is the site of missense mutations causing autosomal dominant, early-onset Alzheimer's¹⁷. Moreover, mutation of either of two conserved aspartate residues in transmembrane domains 6 and 7 of PS1 or its homologue PS2 blocks both γ -secretase processing of β -APP¹⁸⁻²¹ and related cleavage of Notch^{20,22,23}. γ -secretase inhibitors designed to mimic the cleavage site in β -APP also inhibit the analogous proteolysis of Notch^{12,23}.

We previously showed that difluoro ketone and difluoro alcohol transition-state analogues, rationally designed on the basis of the γ -secretase cleavage site in β -APP, are selective inhibitors of this proteolytic activity in whole cells^{9,24}. Inhibition by difluoro alcohol peptidomimetics in particular have provided pharmacological evidence of an aspartyl-protease mechanism⁹. Modification of these analogues showed that γ -secretase has low sequence specificity for inhibitors⁹, as it does for its substrates^{8,25-27}. More recently, we found that installing large, hydrophobic P1 substituents (such as *sec*-butyl or cyclohexylmethyl) into these transition-state mimics enhances their potency, indicating that there may be a relatively large complementary S1 pocket in γ -secretase capable of accommodating these

bulky substituents²⁸. Because these transition-state analogues are designed to interact specifically with the diasparyl active site of γ -secretase, we aimed to convert them into tagged affinity reagents to identify their molecular target.

Results

Design of a γ -secretase affinity reagent and its effects on β -APP processing. We designed and synthesized a version of one of our transition-state analogue inhibitors, difluoro alcohol compound 1 (Fig. 1a), with biotin at the carboxy terminus (1-Bt; Fig. 1a, b). Previous structure-activity studies showed that C-terminal modification of these analogues is tolerated by γ -secretase⁹. Indeed, we found that 1-Bt retained inhibitory potency towards A β production in Chinese hamster ovary (CHO) cell line 7W transfected with human β -APP (IC_{50} ~15 μ M) compared with the parent compound 1 (IC_{50} ~10 μ M; Fig. 2a). In contrast, compounds 2 and 2-Bt (Fig. 1a), analogues of 1 and 1-Bt that lack the transition-state-mimicking difluoro alcohol moiety, did not inhibit A β production at doses up to 100 μ M (Fig. 2a). Compound 1-Bt increased in a dose-dependent manner the 83- and 99-amino-acid β -APP C-terminal fragments (C83 and C99) that are substrates of γ -secretase, which is consistent with its IC_{50} value with respect to A β production (Fig. 2c). Thus, this compound blocks A β production at the level of γ -secretase. In contrast, 1-Bt did not affect levels of the β -APP metabolites α -APP $_s$ and β -APP $_s$, generated respectively by α - and β -secretase proteolysis (Fig. 2d), indicating that the activities of these other β -APP-cleaving proteases were not altered.

We then converted compound 1-Bt to an amino-terminally modified bromoacetamide, BrA-1-Bt (Fig. 1a, b). Bromoacetamides are relatively stable compounds that are susceptible to nucleophilic attack, and similar derivatization of other bioactive compounds has provided affinity-labelling reagents for target

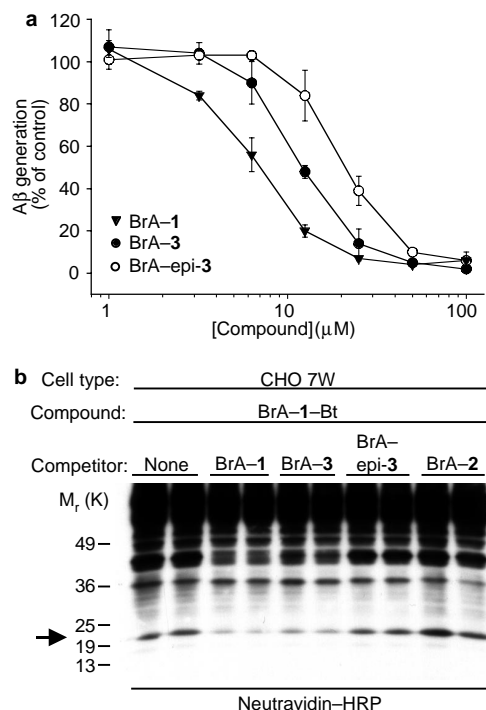


Figure 3 Specific covalent binding of γ -secretase inhibitor BrA-1-Bt to an M_r ~21K protein in cell lysates. a, Effects of unbiotinylated bromoacetamide peptidomimetics on A β production in CHO 7W cells. Values are means \pm s.d. from three experiments and are expressed relative to control values. These compounds were used in subsequent experiments to compete with BrA-1-Bt for binding. **b**, Western blot showing biotinylation of proteins in CHO 7W cell lysates by BrA-1-Bt and competition by four distinct unbiotinylated analogues. Arrow denotes biotinylated M_r ~21K protein. The apparent competition by analogues in the M_r 36–49K region observed in cell lysates was not seen in isolated microsomes, a semi-purified γ -secretase preparation (Fig. 5b).

receptors and enzymes^{29–31}. We therefore reasoned that whereas the difluoro alcohol moiety of BrA-1-Bt should interact with the residues in γ -secretase that are directly involved in catalysis (the two critical aspartates; Fig. 1a), the bromoacetamide group would be available for attack by any nearby nucleophilic residues (such as serine, threonine and cysteine), allowing covalent attachment to the enzyme. Replacement of the N-terminal *t*-butoxycarbonyl group of γ -secretase inhibitors 1, 3, and epi-3 with a bromoacetyl group (to form BrA-1, BrA-3 and BrA-epi-3; Fig. 1a) did not diminish γ -secretase inhibitory activity (Fig. 3a); potencies were similar to those of their respective parent compounds (Fig. 2a and ref. 9). BrA-1-Bt was incompatible with the A β enzyme-linked immunosorbent assay (ELISA; in which a biotinylated antibody was used as a receptor); however, metabolic labelling revealed that this bromoacetylated derivative still inhibited A β production, but with somewhat lower potency than that of 1-Bt (Fig. 2b). Moreover, this compound increased the levels of γ -secretase substrates C83 and C99 in a dose-dependent manner, as was observed with 1-Bt (Fig. 2c), and it likewise did not affect formation of α -APP_s or β -APP_s (Fig. 2d).

γ -secretase affinity reagent binds to the C-terminal subunit of PS1. To identify proteins covalently bound to the γ -secretase inhibitor BrA-1-Bt using an unbiased method, we treated lysates from CHO 7W cells (expressing only endogenous presenilins) with this compound, electrophoresed them on SDS-polyacrylamide gel and blotted them onto polyvinylidene difluoride (PVDF). Biotinylated proteins were detected using neutravidin/horseradish peroxidase

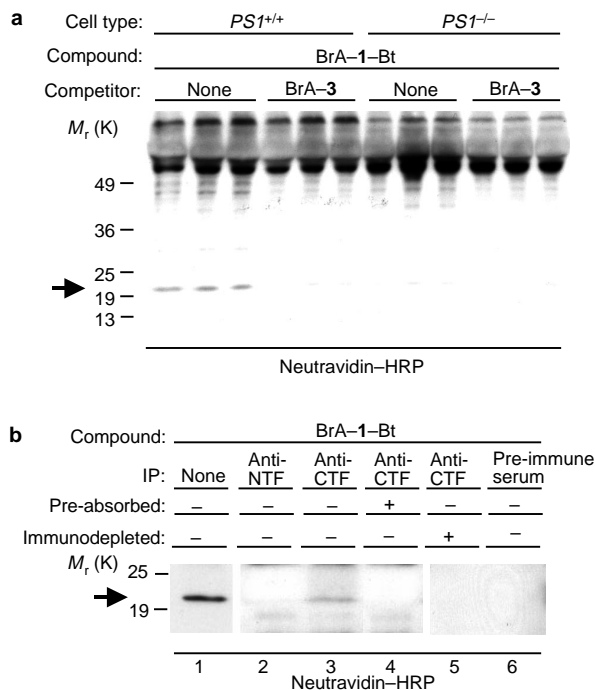


Figure 4 Identification of the affinity-labelled M_r ~21K protein as the C-terminal subunit of PS1. a, Western blot showing biotinylation of proteins in lysates of PS1^{+/+} and PS1^{-/-} mouse fibroblasts upon binding of BrA-1-Bt and competition by the unbiotinylated analogue BrA-3. **b**, Western blot showing biotinylated proteins immunoprecipitated (IP) with antibodies against the PS1 NTF (lane 2), CTF (lane 3) or CTF pre-absorbed with peptide immunogen (lane 4), compared with those in whole-cell 7W lysates (lane 1). The ~21K band was not observed when the PS1 CTF was immunodepleted before incubation with BrA-1-Bt (lane 5) or when pre-immune serum was used for precipitation (lane 6). Arrows denote biotinylated M_r ~21K protein.

conjugate (neutravidin-HRP). A biotinylated band corresponding to relative molecular mass ~21,000 (M_r ~21K) was detected after BrA-1-Bt treatment; the intensity of this band was markedly reduced by pretreatment of lysates with the unbiotinylated active inhibitors BrA-1 and BrA-3 (Fig. 3b). Pretreatment with BrA-2, a bromoacetylated version of the inactive compound 2, which lacks the transition-state mimicking difluoro alcohol moiety (Fig. 1a), did not affect the intensity of this band (Fig. 3b). In contrast, pretreatment with BrA-epi-3, which has D-valine in the P2 position (Fig. 1a) and inhibits A β production less potently than does compound 3 (Fig. 3a), led to a partial reduction in the intensity of the ~21K band (Fig. 3b). In fact, the ability of these closely related peptide analogues to compete with BrA-1-Bt for binding to this protein of M_r ~21K correlated directly with their ability to block γ -secretase activity (BrA-1 > BrA-3 > BrA-epi-3 > BrA-2; Fig. 3a, b). Because the difluoro alcohol and difluoro ketone moieties of these compounds mimic the transition state of the proteolysis catalysed by aspartyl proteases, the observed protein labelled by BrA-1-Bt is a candidate for γ -secretase.

In the mouse, deletion of the gene that encodes PS1 results in marked reduction in A β generation and substantial elevation in levels of β -APP derivatives C83 and C99, demonstrating that PS1 somehow mediates γ -secretase activity¹⁴. We therefore tested the ability of BrA-1-Bt to label proteins in lysates from PS1 knockout (-/-) murine fibroblasts compared with those from wild-type (+/+) littermates. Again, a band corresponding to M_r ~21K was observed upon treatment of wild-type cell lysates with BrA-1-Bt, and the

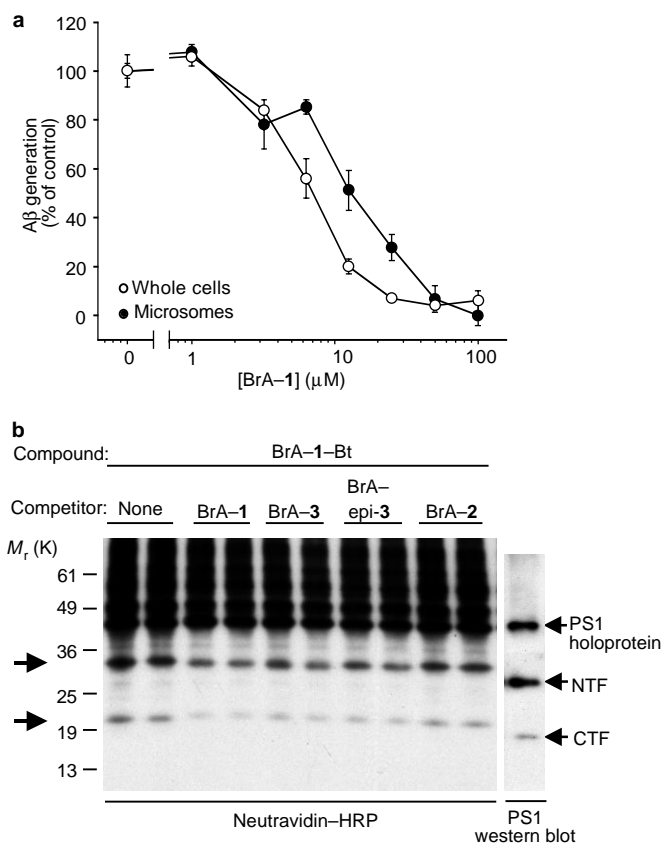


Figure 5 Specific covalent binding of γ -secretase inhibitor BrA-1-Bt to M_r ~21K and ~31K proteins in isolated microsomes. a, Effect of BrA-1 on *de novo* A β generation in isolated microsomes and in living cells. Isolated microsomes were taken up in Tris buffer, pH7.5, and incubated at 37°C for 4 h in the presence or absence (control) of BrA-1. Values are means \pm s.d. from three experiments and are expressed relative to control values. **b**, Western blot showing biotinylation of proteins in microsomes with BrA-1-Bt and competition by unbiotinylated analogues. Rightmost lane shows the migration of PS1 NTF, CTF and holoprotein (no compound treatment) in these microsomes by immunoprecipitation and western blotting. Left-hand arrows denote biotinylated NTF and CTF.

intensity of this band was markedly decreased in lysates from *PS1*^{-/-} fibroblasts (Fig. 4a). Pretreatment of wild-type lysates with BrA-3 reduced the intensity of this biotinylated band to levels observed in lysates of knockout fibroblasts (Fig. 4a). These results show that specific labelling of the M_r ~21K protein by a transition-state analogue inhibitor of γ -secretase is dependent on the presence of PS1, which is consistent with the requirement of presenilins for γ -secretase activity^{14,18-21}.

PS1 undergoes endoproteolysis, giving rise to an N-terminal fragment (NTF) and a C-terminal fragment (CTF), which form a stable, heterodimeric complex that is widely thought to be the bioactive form of the protein³²⁻³⁶. The size of the M_r ~21K biotinylated protein is similar to that reported for the PS1 CTF (~18K), with covalent modification by BrA-1-Bt possibly accounting for the difference in their electrophoretic mobilities. We therefore tested the ability of PS1-specific antibodies to immunoprecipitate this biotinylated protein from the CHO 7W lysates. Immunoprecipitation with an antibody against the PS1 C terminus (4627), but not with one directed against the N terminus (Ab14), brought down a biotinylated protein that co-migrated with the band detected in BrA-1-Bt-treated lysates (Fig. 4b, lanes 1-3). The ability of antibody 4627 to bring down the M_r ~21K biotinylated protein was blocked by pre-absorption with its corresponding peptide immunogen (Fig.

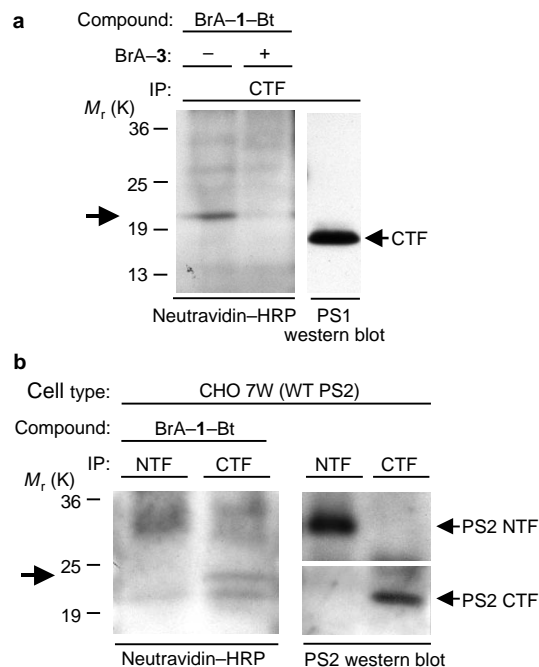


Figure 6 γ -secretase affinity reagent binds to the C-terminal subunits of PS1 and PS2. a, Immunoprecipitation of a biotinylated ~21K band by a specific antibody against the PS1 CTF. Left panel, microsomes were treated with BrA-1-Bt with or without competition by BrA-3, immunoprecipitated (IP) with antibody 4627, and detected with neutravidin-HRP. Left-hand arrow denotes biotinylated PS1 CTF. Right panel, western blot showing unmodified PS1 CTF. **b**, As in **a**, using CHO 7W cells stably expressing wild-type (WT) human PS2. Immunoprecipitations were carried out using antibody 2972 against the PS2 NTF and PS2L against the PS2 CTF; western blotting was carried out with antibody 2972 (upper panel) and antibody 2C20 against the PS2 CTF (lower panel). The ~23K band was immunoprecipitated by antibody PS2L but not by antibody 2972, whereas the band immediately below this was precipitated by both antibodies. The ~23K band was also specifically eliminated upon immunodepletion with antibody PS2L (data not shown). The difference in mobility between this biotinylated protein (left-hand arrow) and the unmodified PS2 CTF is similar to that between biotinylated and unmodified PS1 CTF (**a**).

4b, lane 4). Moreover, prior immunodepletion of PS1 CTF using antibody 4627 prevented BrA-1-Bt from labelling the M_r ~21K protein (Fig. 4b, lane 5). Together, these experiments identify this biotinylated protein as the PS1 CTF, indicating that our biotinylated γ -secretase affinity reagent bound directly and specifically to this PS1 subunit.

N-terminal and C-terminal PS subunits are affinity-labelled in microsomes and whole cells. We analysed the ability of BrA-1-Bt to bind directly to proteins under *in vitro* conditions known to preserve γ -secretase activity. We have documented *de novo* generation of A β in microsomes isolated from β -APP-transfected CHO cell lines stably expressing endogenous hamster or exogenous human presenilins^{18,37}. This A β production is substantially reduced in microsomes of cell lines stably expressing presenilins lacking the two critical transmembrane aspartates^{18,37}. Moreover, microsomal generation of A β was blocked by our γ -secretase inhibitors in a dose-dependent manner closely similar to that seen in whole cells (Fig. 5a, shown for BrA-1). We therefore treated isolated microsomes from CHO 7W cells with BrA-1-Bt under these same conditions in the presence or absence of competitors and then lysed them with 2% CHAPS detergent. Electrophoresis on SDS-polyacrylamide gel, transfer to PVDF and probing with neutravidin-HRP revealed the biotinylated ~21K band seen in cell lysates, as well as a

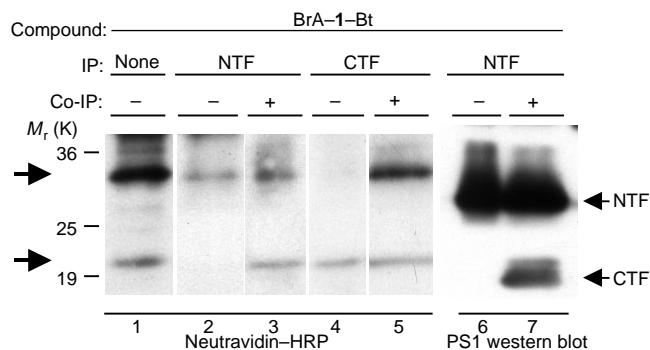


Figure 7 γ -secretase affinity reagent binds to PS1 NTF-CTF heterodimers. BrA-1-Bt-tagged proteins in isolated microsomes were immunoprecipitated (IP) with antibody 4627 against the PS1 CTF or antibody Ab14 against the PS1 NTF, under normal immunoprecipitation conditions or co-immunoprecipitation conditions (see Methods). Note the comparable shifts in mobility of PS1 NTF and CTF (lanes 6, 7) upon covalent binding by the biotinylated γ -secretase affinity reagent (lanes 1-5). Left-hand arrows denote biotinylated NTF and CTF.

further biotinylated band at \sim 31K (Fig. 5b). The intensities of both labelled bands were reduced by competitor analogues in correlation with their abilities to block γ -secretase activity (Figs 3a, 5b), demonstrating the specificity of covalent binding of BrA-1-Bt in microsomes, as was observed in lysates (Figs 3b, 5b). Whereas several biotinylated proteins of greater molecular masses were also observed, the binding of these proteins to BrA-1-Bt did not seem to be specific, that is, it was not competed for by unbiotinylated analogues. Although the endogenous PS1 holoprotein ($M_r \sim$ 44K) is present in this cell line (Fig. 5b, PS1 western blot), we were unable to detect a specific association between the inhibitors and this form of PS1, which is consistent with findings implicating presenilin heterodimers as the bioactive forms^{32,34,35}. However, the presence of other nonspecifically labelled proteins in this region of the gels, which was expected given the micromolar potency of our inhibitor, does not allow confirmation that the affinity reagent does not bind to PS1 holoprotein.

The size of the biotinylated $M_r \sim$ 31K protein is similar to that of the PS1 NTF (\sim 28K), and, like the biotinylated PS1 CTF identified above (Fig. 4b), exhibited mobility corresponding to an M_r that is \sim 3K greater than that of the corresponding unbiotinylated PS1 protein (Fig. 5b). We therefore tested again the ability of PS1-specific antibodies to immunoprecipitate these two biotinylated proteins. We treated isolated microsomes with BrA-1-Bt as before, and then with antibody 4627 against the PS1 C terminus. We subjected the resulting immunoprecipitate to SDS-polyacrylamide gel electrophoresis and blotting, and probed it with neutravidin-HRP. The biotinylated \sim 21K band obtained from cell lysates (Fig. 4b) was also immunoprecipitated by antibody 4627; this band was effectively absent when microsomes were pretreated with BrA-3 (Fig. 6a). Thus, the biotinylated $M_r \sim$ 21K protein was again clearly identified as the PS1 CTF, with a slightly lesser electrophoretic mobility as a result of covalent modification by BrA-1-Bt. These results indicate that our γ -secretase inhibitors bind directly and specifically to the PS1 CTF. A similar experiment using 7W cells stably overexpressing wild-type human PS2 revealed that our affinity reagent also labelled the human PS2 CTF (Fig. 6b). The band corresponding to affinity-labelled PS2 CTF was shifted by \sim 3K relative to that of the unlabelled PS2 CTF, a similar result to that obtained upon labelling of the PS1 CTF (Fig. 6a, b).

We investigated further the presence of biotinylated, inhibitor-bound presenilins, under conditions in which presenilin NTFs and CTFs are known to co-immunoprecipitate as complexes³⁴. After incubation with BrA-1-Bt, we treated microsomes from CHO 7W

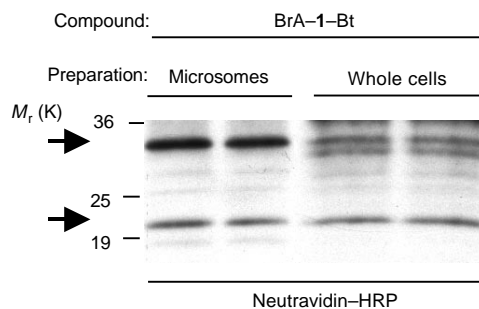


Figure 8 PS1 subunits are labelled by γ -secretase affinity reagent in living, intact cells. Western blot showing biotinylation of proteins by BrA-1-Bt in microsomes and in living CHO 7W cells. Microsomes were treated with BrA-1-Bt at 4°C in Tris buffer, pH 7.5, for 1.5 h; living cells were treated at 37°C in DMEM with 10% FBS for 2 h. Arrows denote biotinylated NTF (upper) and CTF (lower).

cells with 2% CHAPS detergent in Tris buffer. Subsequent immunoprecipitation with antibody 4627 brought down the biotinylated protein at $M_r \sim$ 21K, as expected, as well as the biotinylated $M_r \sim$ 31K protein (Fig. 7, lane 5). Conversely, immunoprecipitation under these conditions with antibody Ab14 against the PS1 N terminus resulted in detection of the biotinylated $M_r \sim$ 31K as well as the coprecipitated $M_r \sim$ 21K (Fig. 7, lane 3). When we carried out the immunoprecipitations under normal (not co-immunoprecipitation) conditions (with NP40 rather than CHAPS as the detergent), only the $M_r \sim$ 31K protein immunoprecipitated with antibody Ab14, and only the $M_r \sim$ 21K protein came down with antibody 4627 (Fig. 7, lanes 2 and 4). These results provide strong evidence that these biotinylated proteins are the presenilin NTF and CTF, respectively, and that these proteins are able to bind to transition-state analogues of γ -secretase when they are physiologically associated with each other. The PS1 NTF is apparently labelled more effectively by the inhibitor in microsomes than it is in cell lysates, as we were unable to consistently immunoprecipitate PS1 NTFs from lysates treated with BrA-1-Bt. Finally, we tested the ability of this γ -secretase affinity reagent to bind to PS1 fragments in living cells. We treated intact CHO 7W cells with BrA-1-Bt for 2 h and then lysed them; we then subjected the lysates to SDS-PAGE, blotting, and probing with neutravidin-HRP. Biotinylated proteins of $M_r \sim$ 21K and \sim 31K comigrated with the same biotinylated proteins observed in isolated microsomes (Fig. 8). Thus, the NTF and CTF of PS1 are both covalently modified by this transition-state analogue inhibitor of γ -secretase in isolated microsomes and in living cells.

Discussion

Over 60 different missense mutations in the presenilins cause autosomal dominant, early-onset, familial Alzheimer's disease (FAD)^{1,3}, which is otherwise indistinguishable from the sporadic form of Alzheimer's disease that affects millions worldwide. Presenilin FAD mutations alter γ -secretase activity to increase production of the more fibrillogenic, 42-amino-acid form of A β , the primary protein component of the early, diffuse plaques that form before the dense, senile plaques that characterize Alzheimer's disease^{1,3}. Moreover, fibroblasts from PS1-deficient mice exhibit markedly reduced γ -secretase processing of β -APP substrates¹⁴, demonstrating that presenilins somehow mediate this proteolytic activity. We recently found that two transmembrane aspartate residues in presenilins, residues that are fully conserved from worms to man, are each essential for two different proteolytic events — γ -secretase processing of β -APP and normal endoproteolysis of presenilins^{18,21}. Together, these findings support the idea that presenilins serve either as essential diasparyl cofactors for γ -secretase or as the cata-

lytic component of γ -secretase, a polytopic, intramembranous, aspartyl protease that is activated through autoproteolysis. The observations reported here, that transition-state analogue inhibitors of γ -secretase, targeted to the diaspartyl active site of the protease, bind specifically and directly to the heterodimeric form of presenilins, validate these heterodimers as targets for therapeutic design and provide evidence that they contain the active site of γ -secretase. Our compounds that lack the transition-state mimicking moiety do not inhibit γ -secretase and do not compete for binding to presenilins, whereas our active-site-directed compounds bind with specificity to presenilin forms that are associated with bioactivity (NTF-CTF heterodimers)^{32–35}.

Unequivocal certainty that presenilins contain the active site of γ -secretase must await the identification of other essential cofactors, subsequent reconstitution of proteolytic activity, and detailed structural studies with enzyme-inhibitor complexes. However, the biochemical and pharmacological evidence presented here, together with our earlier studies on the function of the two transmembrane aspartate residues, makes it all but certain that presenilins contain the active site of γ -secretase^{18,21}. As a necessary corollary, presenilins would also represent the catalytic portion of proteases responsible for cleavage of the transmembrane region of Notch, an essential signalling event during embryogenesis³⁹. Double knockout of PS1 and PS2 is lethal at the embryonic stage in mice, and causes a phenotype almost identical to that exhibited by Notch1-deficient mutants⁴⁰. Moreover, deletion of the *PS1* gene^{12,41,42}, mutation of either of the conserved aspartate residues in presenilins^{20,22} and treatment with our γ -secretase inhibitors^{12,23} all result in marked reduction of the intramembranous proteolysis of Notch. Finally, the discovery that presenilins are the proteins that catalyse the final step in the generation of A β provides a lynchpin of the 'amyloid hypothesis' of Alzheimer's disease — all known forms of autosomal dominant FAD are caused either by mutations close to the cleavage sites of the substrate precursor of A β (β -APP) or by mutations in a protease that generates A β (presenilin, alias γ -secretase). With the identification of both β - and γ -secretases, the development of effective therapeutic agents targeted to these proteases can proceed in a rational, systematic and efficient manner. □

Methods

Synthesis of affinity reagent Br-1-Bt.

To a solution of equimolar amounts of *N*-t-Boc-L-valine and L-isoleucine in methylene chloride (CH₂Cl₂; 1.7 M) was added coupling agents EDC and HOBt (1.1 eq each). Overnight stirring and aqueous work-up⁹ afforded the dipeptide alcohol (84% yield), which was coupled to *d*-biotin without further purification. *d*-biotin was dissolved in dimethylformamide (DMF, 0.18 M) at 60 °C, whereupon 1 eq of the dipeptide alcohol in DMF (1.8 M) was added, followed by BOP (1.1 eq) and diisopropylethylamine (*i*-Pr₃NEt, 2 eq). Overnight stirring at 55 °C, aqueous work-up⁹ and silica-gel chromatography (CH₂Cl₂:MeOH, 15:1) gave biotinylated dipeptide analogue 4 (35% yield; poor yield was due to emulsions and insolubility during extraction). After deprotection of compound 4 with 50% trifluoroacetic acid (TFA) in CH₂Cl₂ for 1 h, the free amine was isolated by removal of solvent and extraction between CH₂Cl₂ and saturated aqueous NaHCO₃. This amine was then coupled to carboxylic acid 6 (ref. 9; 1.1 eq) in DMF (0.3 M) with *N*-[(dimethylamino)-1H-1,2,3-triazolo[4,5-b]-pyridin-1-ylmethyl]-*N*-methyl-methanaminium hexafluorophosphate *N*-oxide (HATU, 1.2 eq) and *i*-Pr₃NEt (2 eq). Overnight reaction, aqueous work-up and silica-gel chromatography (CH₂Cl₂:MeOH, 9:1) afforded intermediate 5 (63% yield). Deprotection of compound 5 with TFA in CH₂Cl₂ as above was followed by coupling to *N*-t-Boc-L-valine (1.1 eq) in DMF (0.15 M) with HATU (1.1 eq) and *i*-Pr₃NEt (3 eq). Overnight reaction was followed by dilution with CH₂Cl₂ and washing with aqueous citric acid, NaHCO₃ and brine. Concentration and silica-gel chromatography (CH₂Cl₂:MeOH, 15:1) afforded 1-Bt (45% yield). This compound was deprotected (TFA) and coupled to bromoacetic acid (3 eq) with EDC (3 eq) in CH₂Cl₂ (40 mM) overnight, diluted in CH₂Cl₂ and washed twice with cold 1 M HCl and once with brine. Concentration and silica-gel chromatography (CH₂Cl₂:MeOH, 15:1) gave BrA-1-Bt (74% yield). Final compounds were characterized by ¹H nuclear magnetic resonance (NMR) and mass spectroscopy.

Preparation of cell lysates and microsomes, and treatment with compounds.

CHO cell line 7W, stably transfected with human β -APP751 and a neomycin-resistance gene⁶, were lysed in buffer containing 50 mM Tris pH 7.6, 1% NP-40, 150 mM NaCl, 2 mM EDTA and protease inhibitors. For microsome isolation, cell homogenates prepared as described⁴⁶ were centrifuged at 3,000g, and the supernatant was spun at 100,000g. This and all subsequent operations were carried out at 4 °C. The microsomal pellet was taken up in 50 mM Tris and 150 mM NaCl, pH 7.5. A stock solution of BrA-1-Bt in dimethylsulphoxide (DMSO) was added to cell lysates or microsomes, to a final concentration of 30 μ M. Treated lysates or microsomes were incubated with mixing for 1.5 h. Competition was effected by pre-incubation with 75 μ M of unbiotinylated compounds in DMSO or DMSO alone for 30 min before

addition of BrA-1-Bt. Final DMSO concentrations were \leq 3% (constant within each experiment). After incubation, microsomes were lysed with 2% NP-40 on ice for 20 min, or with 2% CHAPS as described⁴⁴. Washing out of free bromoacetamides before lysis did not affect results (data not shown).

Inhibition of A β production, immunoprecipitation, blotting and detection.

Antibodies against PS1, PS2, β -APP, APP, and A β were as described^{21,34,46}. Treatment of 7W cells with compounds and sandwich ELISA for A β were as described⁴. Metabolic labelling of A β in cells using [³⁵S]Met was carried out for 2 h in the presence of compound or vehicle; A β was immunoprecipitated with antibody R1282. Immunoprecipitations were carried out as described^{34,47}. Co-immunoprecipitations in 2% CHAPS were carried out by preclearing with protein A/sepharose for 30 min and then precipitating with primary antibody and protein A/sepharose for 3 h. Immunoprecipitates were washed 3 times for 20 min with 0.5% CHAPS wash buffer as described⁴⁴. Precipitated proteins were resolved by SDS-PAGE on 8–16% Tris-glycine or 10–20% Tris-tricine gels. Polyvinylidene-difluoride immunoblots were developed with peroxidase-conjugated secondary antibody or peroxidase-conjugated neutravidin (neutravidin-HRP, Pierce) and enhanced chemiluminescence (ECL+, Amersham).

RECEIVED 27 APRIL 2000; REVISED 17 MAY 2000; ACCEPTED 22 MAY 2000; PUBLISHED 9 JUNE 2000.

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ACKNOWLEDGEMENTS

We thank D. Miller and D. Walsh for helpful discussions, W. Ye for help with cell cultures, J. Shen for providing *PS1^{-/-}* mouse fibroblasts, S. Gandy for antibody Ab14, C. Haass for antibody 2972 and T. Iwatsubo for antibody PS2L. This work was supported by NIH grants NS37537 (to M.S.W.) and AG12749 (to D.J.S.) and by a Pioneer Award from the Alzheimer's Association (to D.J.S.). Correspondence and requests for materials should be addressed to M.S.W.