Presenilin is implicated in the pathogenesis of Alzheimer's disease. It is thought to constitute the catalytic subunit of the γ-secretase complex that catalyzes intramembrane cleavage of β-amyloid precursor protein, the last step in the generation of amyloidogenic Aβ peptides. The latter are major constituents of amyloid plaques in the brain of Alzheimer's disease patients. Inhibitors of γ-secretase are considered potential therapeutics for the treatment of this disease because they prevent production of Aβ peptides. Recently, we discovered a family of presenilin-type aspartic proteases. The founding member, signal peptide peptidase, catalyzes intramembrane cleavage of distinct signal peptides in the endoplasmic reticulum membrane of animals. In humans, the protease plays a crucial role in the immune system. Moreover, it is exploited by the hepatitis C virus for the processing of the structural components of the virion and hence is an attractive target for anti-infective intervention. Signal peptide peptidase and presenilin share identical active site motifs and both catalyze intramembrane proteolysis. These common features let us speculate that γ-secretase inhibitors directed against presenilin may also inhibit signal peptide peptidase. Here we demonstrate that some of the most potent known γ-secretase inhibitors efficiently inhibit signal peptide peptidase. However, we found compounds that showed higher specificity for one or the other protease. Our findings highlight the possibility of developing selective inhibitors aimed at reducing Aβ generation without affecting other intramembrane-cleaving aspartic proteases.

Alzheimer's disease (AD) is characterized by the formation of senile plaques in the brain. Major constituents of these plaques are the amyloidogenic 40- and 42-residue-long Aβ peptides Aβ40 and Aβ42, respectively (1). The amyloid cascade hypothesis usually links the generation of amyloid plaques with the neuropathological changes accompanying the symptoms typical of this disease (2). Aβ peptides are generated from the type I transmembrane protein β-APP (β-amyloid precursor protein) by sequential proteolysis (3). The protein is first cleaved in the exoplasmic domain by the β-site APP-cleaving enzyme (BACE) to release the ectodomain (4, 5). The residual membrane-anchored stub of 99 residues (C99) is subsequently cleaved in the center of the transmembrane region by γ-secretase (6). The resulting cleavage products, an Aβ peptide and the amyloid intracellular domain (AICD), are liberated from the lipid bilayer toward the exoplasm and cytosol, respectively (7–9).

To date, the majority of characterized familial AD mutations are clustered along the presenilin-1 (PS1) gene (10, 11). They are thought to accelerate disease onset by increasing the Aβ42/Aβ40 ratio (12). It is not well understood how these mutations, which are essentially scattered along the entire PS1 gene, can lead to a specific increase in the production of the 42-residue-long peptide that corresponds to the most amyloidogenic form of Aβ (13). It has been shown that PS1 plays a key role in transport and maturation of β-APP (14). It is also an essential component of the γ-secretase complex (6), and several lines of evidences suggest that PS1 may constitute the catalytic subunit of this multi-subunit protease (15). For example, several aspartic protease transition state analogues have been found to inhibit γ-secretase activity and target PS1 (16–20), and conservative mutations of putative active site aspartates in PS1 result in the loss of γ-secretase activity (21, 22). Thus, in recent years, the development of small molecular weight compounds aimed at reducing γ-secretase/PS1 activity as a possible therapeutic strategy for AD has attracted major attention. Several potent inhibitors that affect γ-secretase/PS1 in cellular assays have been reported, and at least one compound has been shown to reduce plaque load in a transgenic animal model for AD-type amyloidosis (23). The major concern related to this approach is that γ-secretase/PS1 not only catalyzes the processing of C99, but it is also required for the processing of other transmembrane proteins such as CD44 (24), the tyrosine kinase receptor ErbB (25, 26), and the Notch receptor family (27, 28).

Recently, we identified the intramembrane-cleaving protease SPP (for signal peptide peptidase) that contains motifs YD and LGLGD characteristic for GXGD aspartic proteases (29). These identical motifs are present in the predicted transmembrane regions of PS1, supporting its function as an intramembrane-cleaving aspartic protease and hence a catalytic subunit of the γ-secretase/PS1 complex (6, 30). SPP promotes intramembrane proteolysis of distinct signal peptides after they have been cleaved off from newly synthesized secretory or membrane proteins in the endoplasmic reticulum (ER) membrane of higher eukaryotes (29, 31). In humans, SPP is essential for the generation of signal sequence-derived human lymph...
Inhibition of SPP with γ-Secretase/PS1 Inhibitors

EXPERIMENTAL PROCEDURES

Synthesis of Inhibitors—L-685,485 (34), L-852,646 (19), DAPT (WO 9822494), LY411575 (WO 9828268), (Z-LL)2-ketone (35), and TBLK (29) were synthesized as described previously. NVP-AHW700-NX was synthesized according to methods reported for a related compound (36). The purity of each compound was checked by 1H nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, high-pressure liquid chromatography, and thin-layer chromatography, and the results were consistent with the expected structures. JLK2 (37) was kindly provided by F. Checher and peptatin A was purchased from Sigma.

γ-Secretase Assays—Inhibition of γ-secretase activity in live cells was assayed by quantification of the generation of secreted Aβ. In brief, human embryonic kidney cells (HEK-293) cells stably transfected with β-APP carrying the Scw2 mutation (38, 39) were plated in microtiter plates. After 1 day, the inhibitors were added in fresh medium, and the cells were incubated for another 24 h. 10 μl of conditioned medium were removed for determination of Aβ levels by sandwich enzyme-linked immunosorbent assay using the Aβ40-specific monoclonal antibody 25H10 raised against the free C-terminal peptide, MVGGVV, of Aβ40. The monoclonal β1 antibody (39) was biotinylated and used as a detection antibody with alkaline phosphatase coupled to streptavidin. For chemiluminescence, substrate CSPD (disodium 3-(5-chloro)tricyclo[3.3.1.1^3,7]decan}-4-yl)phenyl phosphate) and the enhancer EmeraldII (Tropix) were applied. Standard curves with synthetic Aβ40 peptide (Bachem) were run in parallel. For testing γ-secretase in vitro, detergent-solubilized γ-secretase activity was prepared from HEK-293 cells (40) and incubated with substrate Met-C99, which was synthesized by in vitro translation (see below), and either MeSO (2%) or inhibitor at the indicated concentration. After incubation, samples were subjected to immunoprecipitation with antibody 25H10 and analyzed by SDS-PAGE and phosphorimaging using 15% polyacrylamide Tris-Bicine-urea acrylamide gels (41) and a STORM PhosphorImager (Amersham Biosciences). Reaction peptide Met-Aβ40 was synthesized by in vitro translation.

SPP Assay and Affinity Labeling—γ-Secretase inhibitors were tested on SPP in a previously established in vitro assay (35). In brief, 2 μl of cell-free translation mixture containing [35S]methionine-labeled peptide Met-C99 (41) were diluted with 35 μl of SPP buffer (25 mM HEPES-KOH, pH 7.6, 100 mM KOAc, 2 mM Mg(OAc)2, 1 mM dithiothreitol) and supplemented with 1 μl of 100× concentrated inhibitor in MeSO. Reactions were initiated by the addition of 2 μl of CHAPS-solubilized ER membrane proteins, and samples were incubated for 1 h at 30 °C. Samples were analyzed next by SDS-PAGE and phosphorimaging using 15% polyacrylamide Tris-Bicine-urea acrylamide gels (41) and a STORM PhosphorImager (Amersham Biosciences). Quantification was performed with IQMac version 1.2 software (Amersham Biosciences). For affinity labeling, CHAPS-solubilized ER membrane proteins were incubated in SPP buffer in the presence of 50 nM TBLK or 25 nM L-852,646 and the indicated concentrations of competitor (29). Samples were spotted at 30 °C for 30 min and subsequently irradiated with UV light (30 s for TBLK, 5 min for L-852,646; 350-watt high pressure mercury lamp, 10-cm distance to lamp) (29). Samples were analyzed by SDS-PAGE on 12% polyacrylamide Tris-glycine gels (42), and biotinylated proteins were visualized by enhanced chemiluminescence (Amersham Biosciences) after Western blotting with a polyclonal anti-biotin antibody (Bethyl).
Inhibition of SPP with γ-secretase/PS1 Inhibitors

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\text{Met-A} \quad \text{pressed in HEK cells in the presence of indicated inhibitors. IC50 values}
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\text{labels used in this study. IC50 values were determined by measuring the levels of A}
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\text{/H9253} \quad \text{/H9252} \quad \text{were determined by measuring the levels of A}
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\text{derivative of (Z-LL)2-ketone, is thought to be converted}
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\text{in situ}
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\text{Active Site Labeling of SPP and Competition with}
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\text{To test whether the effective γ-secretase/PS1 inhibitors target SPP, we used the photoactive compound L-852,646, a derivative of L-852,458, that was applied previously to label PS1 in detergent-solubilized HeLa total cell membranes (19). When incubated with detergent-solubilized ER membrane proteins and activated with UV light, L-852,646 selectively labeled an ~40-kDa protein such as TBL1K (Fig. 3B). The addition of increasing amounts of the SPP inhibitor (Z-LL)2-ketone progressively reduced labeling. Consistently, compounds that inhibited SPP in the cell-free in vitro SPP assay competed with TBL1K and L-852,646 for binding to the SPP active site. This finding is further evidence that PS1 and SPP are of the same type of aspartic protease (30, 44, 45).}

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\text{Potency of γ-Secretase/PS1 Inhibitors on SPP in Live Cells—}
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\text{We next tested the inhibitory potency of γ-secretase/PS1 inhibitors on SPP in a cell culture system. Besides cleaving signal peptides, SPP also catalyzes the processing of HCV core protein and promotes its release from the ER membrane and trafficking to lipid droplets in the cytosol (33). When SPP is inhibited, the core protein is not processed and remains anchored in the ER membrane by the C-terminal hydrophobic transmembrane region. We therefore could investigate SPP activity in tissue culture cells expressing HCV proteins and monitor the processing of core protein either by detecting core protein by Western blot analysis (Fig. 4A), or by visualizing}
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its intracellular localization using indirect immunofluorescence (Fig. 4, B and C).

As depicted in Fig. 4A, (Z-LL)₂-ketone, L-685,458, NVP-AHW700-NX, and LY411575 inhibited the processing of HCV core protein. Apparent IC₅₀ values varied from ∼10 nM (for LY411575) to ∼5 μM (for L-685,458). The IC₅₀ values observed with the less membrane-permeable compounds, (Z-LL)₂-ketone and L-685,458, were much higher than in the in vitro assays. These compounds most likely penetrate the plasma membrane to a lower extent compared with the less peptidic and therefore more permeable compounds LY411575 and NVP-AHW700-NX, which showed comparable IC₅₀ values in both assays. DAPT and pepstatin A did not inhibit the processing of HCV core protein and hence did not affect SPP, as already observed in the cell-free in vitro assay. Also JKL2 did not affect the processing of HCV core protein at concentrations up to ∼10 μM, at which level the compound started to become cytotoxic (not shown).

The consequences of SPP inhibition on the processing of HCV core protein were next visualized by indirect immunofluorescence. When processed and released from the ER membrane, core protein was found associated at the surface of lipid droplets in the cytosol and appeared in characteristic ring-like structures (Fig. 4B). When expressed in the presence of (Z-LL)₂-ketone, L-685,458, NVP-AHW700-NX, and LY411575, all of which inhibit SPP, HCV core protein did not localize to lipid droplets and appeared in a reticular staining pattern, indicating retention in the ER membrane. DAPT and pepstatin A did not affect the processing of HCV core protein, had also no effect on its intracellular distribution. Taken together, (Z-LL)₂-ketone and the γ-secretase/PS1 inhibitors L-685,458, LY411575, and NVP-AHW700-NX efficiently inhibit SPP in the detergent-solubilized state as well as in living cells. These compounds prevent intramembrane proteolysis of SPP substrates, which, in turn, cannot be released from the ER membrane, and fulfill associated functions in the cell (46).

**DISCUSSION**

In the present study we have demonstrated that aspartic protease inhibitors directed against γ-secretase/PS1 are not necessarily specific and can affect the related intramembrane-cleaving aspartic protease SPP. This finding has implications for the therapeutic strategy in the treatment of AD. To date, the therapeutic potential of small compound inhibitors of γ-secretase/PS1 was scored mainly against the possible side effects that could be expected by the concomitant inhibition on the Notch-1 signaling pathway (28). This was evaluated by measuring the inhibition of fetal T cell maturation in the presence of γ-secretase/PS1 inhibitors (47–49). However, the results presented in this study suggest that some of the most potent γ-secretase/PS1 inhibitors can also block SPP. At first glance, our data are discouraging in respect to developing γ-secretase/PS1 inhibitors as therapeutics, because SPP plays
a key role in the processing of distinct signal peptides (30), which can have post-targeting functions such as that of reporting proper biosynthesis of antigen-presenting major histocompatibility class I molecules to the immune system (32, 50). Our study, however, also identified compounds that are more selective against either γ-secretase/PS1 or SPP, indicating that specific inhibitors may be designed but need to be tested against the individual intramembrane-cleaving aspartic proteases.

The nature of the catalytic site of the γ-secretase complex has been probed intensively, but it still remains somewhat controversial. Biotinylated photoaffinity labels, based on aspartic protease transition-state analogues that mimic the γ-secretase cleavage site in β-APP/C99, can be covalently cross-linked to PS1 (19, 20). Furthermore, γ-secretase activity is abolished by mutations of two critical aspartate residues (ASP-257 and ASP-385) located in the predicted transmembrane domains of PS1 (21, 22). Although such findings support the hypothesis that PS1 is the catalytic component of the complex, this notion was hampered by the fact that PS1 did not share any sequence homology with other known aspartic proteases. A limited relationship to the bacterial type IV prepilin peptidase, as revealed by Haass and co-workers (44), and the discovery of SPP, an intramembrane-cleaving aspartic protease with active site motifs identical to the putative ones in PS1 (29), overruled this objection and provided further evidence that PS1 is a protease.

Additional indirect evidence that PS1 is a protease was provided by the present study reporting on overlapping inhibitor activities. Compounds, including transition state analogues, were found to efficiently inhibit both γ-secretase/PS1 and SPP. Furthermore, the active site-directed affinity probe L-852,646, previously applied to label PS1 in solubilized total cell membranes (19), selectively labeled SPP when applied on detergent-solubilized ER membrane proteins. The latter also contained PS (not shown) but only in the unprocessed form, which cannot be labeled by L-852,646 (19). In fact, all of the effective inhibitors competed with labeling of SPP by the transition state analogue L-852,646 and the photoaffinity label TBL4K, which mimics the gem-diol intermediate upon hydration in the active site. These results suggest that the compounds investigated in this study target the active site of SPP, and it is likely that they similarly interact with PS1.

Although three compounds, pepstatin A, DAPT, and (Z-L-L)2-ketone, could discriminate between γ-secretase/PS1 and SPP, the other tested inhibitors affected both proteases to a variable degree. Thus despite overlapping inhibitor activities, the two proteases clearly differ in the way they interact with the inhibitors. The small number of compounds investigated, however, does not allow us make predictions about the specificity of a particular compound. Modifications on a lead compound may not only significantly increase its inhibitory potency but also can influence compound selectivity, as shown for DAPT and its second-generation derivative, LY411575. The new derivative is indeed ~400 times more potent against γ-secretase/PS1, but it also became an efficient inhibitor of SPP. The potency of LY411575 against SPP, however, was less than against γ-secretase/PS1. Similarly, the transition state analogue L-685,458 was less potent against SPP, whereas the related compound NVP-AHW700-NX was equally effective against SPP and γ-secretase/PS1. Thus, SPP and γ-secretase/PS1 interact differently with various compounds, but to determine what makes an inhibitor selective against one or the other protease will be a major challenge for future drug design.

SPP and γ-secretase/PS1 are both of pharmaceutical interest. SPP is essential for the processing of the HCV core protein (33), and γ-secretase/PS1 is implicated in the cause of AD (51). Drugs against either protease may be useful for the treatment of HCV infection or AD, but they should discriminate between the two proteases in order to minimize side effects. An added complication, however, is that the human genome encodes four additional homologues of SPP (29, 52, 53). It is likely that these candidate aspartic proteases catalyze intramembrane proteolysis of a far unidentified substrate proteins. In analogy to known intramembrane-cleaving proteases, they may promote the release of bioactive peptides and proteins such as signaling molecules and transcription factors (30). Because all of these proteins contain motifs identical to the active site motifs of SPP and γ-secretase/PS1, compounds like the ones tested in the present study may well target the SPP-like candidate proteases too. Therefore, compound specificity will be even more important. In the future, the development of effective therapeutic agents targeting γ-secretase/PS1 or SPP will challenge the chemists and may require systematic probing of all human intramembrane-cleaving aspartic proteases.

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