Signal Peptide Peptidase and γ-Secretase Share Equivalent Inhibitor Binding Pharmacology*

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The enzyme γ-secretase has long been considered a potential pharmaceutical target for Alzheimer disease. Presenilin (the catalytic subunit of γ-secretase) and signal peptide peptidase (SPP) are related transmembrane aspartyl proteases that cleave transmembrane substrates. SPP and γ-secretase are pharmacologically similar in that they are targeted by many of the same small molecules, including transition state analogs, non-transition state inhibitors, and amylid β-peptide modulators. One difference between presenilin and SPP is that the proteolytic activity of presenilin functions only within a multisubunit complex, whereas SPP requires no additional protein cofactors for activity. In this study, γ-secretase inhibitor radioligands were used to evaluate SPP and γ-secretase inhibitor binding pharmacology. We found that the SPP enzyme exhibited distinct binding sites for transition state analogs, non-transition state inhibitors, and the nonsteroidal anti-inflammatory drug sulindac sulfide, analogous to those reported previously for γ-secretase. In the course of this study, cultured cells were found to contain an abundance of SPP binding activity, most likely contributed by several of the SPP family proteins. The number of SPP binding sites was in excess of γ-secretase binding sites, making it essential to use selective radioligands for evaluation of γ-secretase binding under these conditions. This study provides further support for the idea that SPP is a useful model of inhibitory mechanisms and structure in the SPP/presenilin protein family.

Signal peptide peptidase (SPP) 2 and presenilin, the catalytic subunit of γ-secretase, are structurally conserved transmembrane aspartyl proteases and belong to a protein family encoded by seven genes in humans (1–3). γ-Secretase is essential for the production of amyloid β-peptide (Aβ), which plays a key role in Alzheimer disease (4, 5). Active γ-secretase enzyme is a membrane-bound protein complex requiring four different subunits: presenilin-1 or presenilin-2, nicastrin, Aph-1, and Pen-2 (6–9). The presenilin subunit appears to carry the active site of the enzyme because it contains aspartyl protease active-site motifs essential for γ-secretase activity (10, 11) and can be photolabeled using transition state and substrate analogs (12–14). In addition to the transition state analog inhibitors, there are also non-transition state γ-secretase inhibitors that target presenilin (15, 16). These compounds are proposed to mediate inhibition through a distinct allosteric site on the basis of inhibitor cross-competition kinetics and radioligand displacement studies (17, 18). Differences in binding pharmacology between the transition state analogs and non-transition state inhibitors were also inferred from photolabeling studies (16, 19). As well as the apparent differences in binding pharmacology, the two classes of inhibitors have different effects on the γ-secretase enzyme, such that only the non-transition state inhibitors cause accumulation of enzyme-bound processing intermediates, which are longer forms of Aβ (20–24). Thus, the small molecule γ-secretase inhibitors fall into two groups that have different effects on Aβ generation as well as distinct binding characteristics. An additional class of compounds, the “Aβ modulators,” shift the position of the γ-secretase cleavage sites, resulting in Aβ with different C termini. For example, the nonsteroidal anti-inflammatory drug (NSAID) sulindac sulfide increases the production of Aβ-(1–38) while lowering Aβ-(1–42) (25). Aβ modulators appear to target yet another distinct binding site on γ-secretase (18).

SPP is proposed to cleave membrane-embedded signal peptides left over from the translocation of secretory proteins (26, 27). Its activity is required for histocompatibility antigen E (HLA E) epitope formation (28); maturation of hepatitis C virus (29); and, in zebrafish, neuronal cell survival (30). Like presenilin, SPP can be photolabeled by a γ-secretase cleavage site analog inhibitor, indicating conservation of active-site structure within the two enzymes (31, 32). Furthermore, some of the non-transition state inhibitors also inhibit SPP (31, 33), and even Aβ modulators affect SPP (34). In contrast to presenilin, which requires additional subunits for enzyme activity, the active SPP enzyme does not require additional subunits for activity (32). This implies that the additional subunits of γ-secretase, which are essential for enzyme maturation, contribute only to non-catalytic functions, such as the recognition and binding of substrates by nicastrin (35, 36). SPP therefore represents a simplified model for γ-secretase.

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2 The abbreviations used are: SPP, signal peptide peptidase; Aβ, amyloid β-peptide; NSAID, nonsteroidal anti-inflammatory drug; ZLL, ketone, 1,3-di-[N-carboxybenzoyl-L-leucyl-L-leucylamino]acetone; DAPT, N-[N-(3,5-di-fluorophenacetyl)-L-alanyl]-(S)-phenylglycine t-butyl ester; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonic acid; MES, 4-morpholinoethanesulfonic acid; PrP-PP, prolactin signal peptide diproline mutant.
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Thus, a variety of small molecules target both presenilin and SPP, but it has not been determined to what extent the inhibitor binding pharmacology is similar for the two enzymes. To address this question, we utilized an active site-directed γ-secretase radioligand (17, 18, 37) and took advantage of our finding that it binds with high affinity to both SPP and γ-secretase. In the course of this study, we found that SPP family protein binding is abundant in cell homogenates, presenting a challenge to the evaluation of γ-secretase and SPP binding under these conditions. Using this approach, we show that γ-secretase inhibitors exhibit equivalent small molecule binding pharmacology for SPP, implying conserved structure and mechanism in the SPP/presenilin protein family.

EXPERIMENTAL PROCEDURES

Compounds and Radioligands—L-685,458 (13), (Z-LL₂)-ketone (38), and DAPT (39) were purchased from Calbiochem (catalog nos. 565771, 421050, and 565770, respectively). BMS-299897 (40), IN973 (15, 41), BMS-433796 (42), and the radioligands [³H]L-685,458 (17) and [³H]IN973 (41) were prepared as described previously. Other chemicals were purchased from Sigma.

Cell Culture—THP-1 cells were grown in roller bottles in RPMI 1640 medium containing D-glutamine (Invitrogen) and 10 μM β-mercaptoethanol to a density of 1 × 10⁶/ml. Cells were harvested by centrifugation, and cell pellets were quick-frozen in dry ice/ethanol and stored at −80 °C prior to use. For expression of human SPP, HEK293 cells were seeded into T-175 flasks at a density of 13 × 10⁵ cells/flask (Falcon). At 24 h post-seeding, cells were transiently transfected using Lipofectamine Plus (Invitrogen) following the manufacturer’s instructions. Briefly, 12 μg of SPP cDNA expression construct was diluted in Opti-MEM I (Invitrogen) and then combined with 60 μl of Plus reagent/flask and 90 μl of Lipofectamine reagent/flask. The medium was aspirated from the flasks and replaced with 15 ml of Opti-MEM I. DNA complexes were added to the cells and incubated for 6 h, and then cells were fed growth medium. Cells were harvested 48 h post-transfection by rinsing with 10 ml of Dulbecco’s phosphate-buffered saline (Invitrogen catalog no. 14190), followed by scraping into 10 ml of Dulbecco’s phosphate-buffered saline. Cells were collected by centrifugation, and pellets were frozen and stored at −80 °C.

cDNA Expression Construct for SPP—An HM13 Gateway entry clone was purchased from the Invitrogen UltimateORF collection (catalog no. IOH6087). The sequence of this clone was then verified before transfer into the Gateway expression vector pcDNA3.1/Myc-His by site-specific recombination using LR Clonase I (Invitrogen). The vector pcDNA3.1/Myc-His allows for the in-frame addition of a Myc and His₆ tag at the C terminus of a protein, but in this case, a stop codon was added to prevent translation of the tag. The resulting clone was completely sequenced to confirm identity to the SPP sequence (GenBank™ accession number BC008959.2).

Radioligand Binding Assays—Cell pellets were homogenized in 10 ml of 50 mM HEPES (pH 7.0) with 0.1% mammalian protease inhibitor mixture (Sigma catalog no. P8340) using a Dounce homogenizer at 4 °C. The homogenate was centrifuged at 48,000 × g for 20 min. The pellet was resuspended in buffer to yield a protein concentration of 5 mg/ml. To prepare P2 membranes, cell homogenate was centrifuged at 1000 × g for 10 min; the supernatant was centrifuged at 48,000 × g for 30 min; and the pellet was then resuspended in buffer at 5 mg/ml. Protein determinations were carried out using the Bio-Rad protein assay (catalog no. 500-006). [³H]IN973 binding was performed in 50 mM HEPES and 0.1% CHAPS (pH 7.0) at a concentration of 240 μM/ml total homogenate protein. [³H]L-685,458 binding was carried out in 50 mM MES, 150 mM NaCl, 5 mM MgCl₂, and 0.5% CHAPS (pH 6.5) at protein concentrations of 40 μg/ml for THP-1 cells and 5 μg/ml for human SPP-expressing HEK293 cells. The use of P2 membranes or cell homogenate and different buffer systems were found not to affect the binding parameters (Bₘₐₓ and Kᵢ) of the two radioligands. The P2 membrane and 0.5% CHAPS combination was used because it decreased assay variability with the [³H]L-685,458 radioligand. Binding assays were performed in polypropylene 96-deep well plates (Beckman Instruments) in a final volume of 0.25 ml containing 5% (v/v) dimethyl sulfoxide. Assays were initiated by the addition of 25 μl of assay buffer containing radioligand to 12.5 μl of dimethyl sulfoxide containing various concentrations of unlabeled compounds, followed by 212 μl of cell homogenate. Unless noted otherwise, nonspecific binding was defined in the presence of 1000 nM BMS-433796 for [³H]IN973 or 600 nM unlabeled L-685,458 for [³H]L-685,458. After incubation at 25 °C for 1.5 h, the separation of bound from free radioligand was accomplished by filtration over GF/B glass fiber filters (Brandel, Gaithersburg, MD) presoaked in 0.5% polyethyleneimine and 0.3% Triton X-100 solution (for [³H]L-685,458) or cold wash buffer (for [³H]IN973) using a cell harvester (Brandel). Filters were washed four times with 1.0 ml of ice-cold phosphate-buffered saline (pH 7.0) and then assessed for radioactivity by liquid scintillation counting using a Wallac MicroBeta Trilux (PerkinElmer Life Sciences). IC₅₀ values of competing compounds were calculated using the XLfit program in Microsoft Excel. Equilibrium saturation data were analyzed using the KELF software package (Biosoft, Cambridge, UK). The resulting IC₅₀, Kᵢ, and Bₘₐₓ values are expressed as the means ± S.E.

SPP Enzyme Assay—SPP enzyme activity was measured using the synthetic substrate Prl-PP and Western blotting based on the procedure described by Sato et al. (34). Briefly, n-dodecyl β-D-maltoside-solubilized cell extracts were incubated in the assay at a final total extract protein concentration of 0.08 mg/ml with 2 μM Prl-PP peptide substrate for 30 min at 37 °C. Under these conditions, the extent of conversion of substrate to product was confirmed to be time-dependent and dependent on the amount of cell extract used. Cleaved peptide product was separated from Prl-PP substrate by gel electrophoresis in the presence of 8 M urea, followed by chemiluminescence imaging of the Western blot.

RESULTS

The structures of the radioligands [³H]L-685,458 (17) and [³H]IN973, previously referred to as “compound D” (15, 41), represent a transition state analog and a non-transition state analog inhibitor, respectively (Fig. 1A). To evaluate binding of the radioligands to SPP, the SPP protein was overexpressed in
HEK293 cells by transient transfection using a cDNA expression vector. Radioligands were added at a concentration of 1.5 nM, and nonspecific binding was determined by the addition of 1 μM unlabeled compound. Homogenates made from SPP-transfected cells showed a 12-fold increase in binding activity for the radioligand [3H]L-685,458 relative to parental vector controls (Fig. 1B), indicating that binding of radioligand to SPP accounted for >90% of the specific signal observed for [3H]L-685,458. In contrast, binding of the selective γ-secretase inhibitor [3H]IN973 was not increased by overexpression of SPP, indicating that this compound did not bind significantly to SPP.

To determine the binding parameters, [3H]L-685,458 and [3H]IN973 were added to SPP-overexpressing cell homogenates at a range of concentrations (Fig. 1, C and D). Binding was concentration-dependent and saturable. For [3H]L-685,458, the binding isotherm revealed a single binding site with $K_d = 5.1 \pm 0.7$ nM and $B_{\text{max}} = 96 \pm 3.5$ pmol/mg of total protein. For [3H]IN973, the binding isotherm exhibited $K_d = 0.91 \pm 0.02$ nM and $B_{\text{max}} = 339 \pm 17$ fmol/mg of total protein (Fig. 1D), indicating that SPP binding sites were ~280-fold more abundant than γ-secretase binding sites in this homogenate. To determine whether the SPP-transfected cells expressed increased amounts of active SPP enzyme, enzyme activity was assayed using the synthetic peptide substrate Prl-PP (34). SPP activity was detected only in SPP-transfected cells, whereas the parental vector control cells revealed no detectable activity (Fig. 1E). The threshold for detection of product in this assay was 10% relative to the amount detected in SPP-transfected cells. This indicated an at least 10-fold increase in activity in SPP-transfected cells, consistent with the observed increase in radioligand binding. The SPP inhibitor (Z-LL)2-ketone blocked the activity, albeit at high concentrations (Fig. 1E, lane 7). Additional experiments indicated IC50 values of ~2 and 10 μM for the inhibitors (Z-LL)2-ketone and L-685,458, respectively, whereas no inhibition was observed in the presence of 10 μM DAPT, BMS-299897, BMS-433796, or IN973 (data not shown). These potencies are lower than those reported previously (31, 34). Although we do not understand the exact cause, it seems likely that differences in the assay conditions would provide the explanation.

To evaluate the potencies of other compounds in SPP binding, [3H]L-685,458 radioligand was added to SPP-overexpressing cell homogenate at a fixed concentration of 2.5 nM, and inhibition of binding was quantified in the presence of unlabeled compounds. In principle, this approach can be used to

**FIGURE 1. Radioligand binding in cell homogenates from cells overexpressing SPP.** A, shown are the chemical structures of [3H]L-685,458 and [3H]IN973. B, HEK293 cell cultures were transfected with the SPP overexpression construct (black bars) or the parental vector control (white bars), and cell homogenates were prepared. The radioligands [3H]L-685,458 and [3H]IN973 were added at 1.5 nM to 2.5 μg and 25 μg of total homogenate protein, respectively, and specific binding was determined. C, a saturation binding isotherm for [3H]L-685,458 in homogenate from cells overexpressing SPP was determined from the specific binding over a range of radioligand concentrations. The binding parameters ($K_d = 5.1 \pm 0.7$ nM and $B_{\text{max}} = 96 \pm 3.5$ pmol/mg) were averaged from three independent experiments. protein, D, shown is a saturation binding isotherm for [3H]IN973 in the same cell homogenate. The binding parameters ($K_d = 0.91 \pm 0.02$ nM and $B_{\text{max}} = 339 \pm 17$ fmol/mg) were averaged from three independent experiments. Note the ~280-fold difference in $B_{\text{max}}$ values between the two radioligands. E, shown are the results from the SPP enzyme activity assay. Extracts from HEK293 cell cultures transfected with the parental vector control (lanes 1 and 2) or the SPP overexpression construct (lanes 3–7) were incubated in the presence of the Prl-PP peptide substrate at 4°C (lanes 1 and 3) or 37°C (lanes 2 and 4–7). The inhibitor (Z-LL)2-ketone was added at concentrations of 0.1 μM (lane 5), 1 μM (lane 6), and 10 μM (lane 7).
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FIGURE 2. Radioligand displacement from SPP. The radioligand [3H]L-685,458 (2.5 nM) was added to homogenates of cells overexpressing SPP in the presence of the unlabeled compounds (Z-LL)2-ketone (O), L-685,458 (■), IN973 (▲), and sulindac sulfide (▼). Error bars represent the means ± S.D. from three or more independent experiments.

TABLE 1
Summary of IC50 values for inhibition of radioligand binding

| Compound                  | [3H]L-685,458 IC50 (nm) | [3H]IN973 IC50 (nm) |
|---------------------------|-------------------------|---------------------|
| (Z-LL)2-ketone            | not eval                | not eval            |
| L-685,458                 | 10 ± 1.1                | 72 ± 23             |
| IN973                     | 560 ± 200               | 6.4 ± 0.5           |
| BMS-299897                | 28,700 ± 9500           | 12 ± 4.1            |
| BMS-433796                | 510 ± 196               | 1.2 ± 0.26          |
| DAPT                      | 34,200 ± 5000           | 6.3 ± 1.8           |
| Sulindac sulfide          | 45,200 ± 9300           | 332,000 ± 12,100    |

*Inhibition of [3H]L-685,458 binding in SPP-overexpressing HEK293 cell homogenate.
*b Inhibition of [3H]IN973 binding in non-transfected THP-1 cell homogenate.
*c Inhibition of [3H]L-685,458 binding in non-transfected THP-1 cell homogenate.

calculate dissociation constants; however, to avoid assumptions about the multiple different potential mechanisms represented by this group of compounds, the results of this experiment are presented as IC50 curves (Fig. 2). Potent inhibition of radioligand binding was observed in the presence of the SPP inhibitor (Z-LL)2-ketone and the unlabeled ligand L-685,458. In contrast, low potency inhibition of radioligand binding was observed for the non-transition state analog γ-secretase inhibitor IN973. The radioligand displacement assay results for a range of γ-secretase inhibitors using either the SPP/[3H]L-685,458 or γ-secretase/[3H]IN973 displacement binding assay are summarized in Table 1. Additional non-transition state analogs, including BMS-299897, BMS-433796, and DAPT, showed relatively weak inhibition of SPP radioligand binding, the most potent being BMS-433796 with IC50 = 510 ± 196 nM, indicating a high degree of binding selectivity between SPP and γ-secretase. This is consistent with the relatively weak inhibition of the SPP enzyme reported for this class of inhibitors (32, 33). At high concentrations, the NSAID sulindac sulfide also inhibited radioligand binding in both SPP/[3H]L-685,458 and γ-secretase/[3H]IN973 binding assays.

As shown above, when SPP was overexpressed, SPP binding overwhelmed any other high affinity binding of the radioligand [3H]L-685,458 that might have been present. However, it was found that SPP-like binding also predominated in non-transfected cell homogenates. Saturation binding isotherms were determined for the radioligands [3H]L-685,458 and [3H]IN973 in THP-1 cell homogenates (Fig. 3). The binding affinities (Kd) were 1.2 ± 0.26 nM and 2.2 ± 0.36 nM, respectively, consistent with the affinities of these compounds for inhibition of γ-secretase and/or SPP (13, 15, 31). However, the Bmax values were significantly different: 5300 ± 1200 fmol/mg of protein for [3H]L-685,458 and 752 ± 46 fmol/mg of protein for [3H]IN973, which amounts to 7-fold more binding sites for the former compound. One possible explanation for these Bmax differences would be that most of the [3H]L-685,458 binding sites than [3H]IN973 binding sites on presenilin. Another possibility would be that most of the [3H]L-685,458 binding sites were contributed by SPP family proteins. The apparently higher affinity of [3H]L-685,458 in THP-1 cell homogene compared with SPP-expressing HEK293 cell homogenate is consistent with both hypotheses.

To evaluate the contribution of the presenilins to binding of each radioligand, homogenates were prepared from mouse embryonic fibroblast cell lines lacking expression of presenilin-1, presenilin-2, or both (43, 44). High affinity binding was evaluated for [3H]IN973 and [3H]L-685,458 using a radioligand...
concentration of 3 nM, and nonspecific binding was evaluated in the presence of 1 μM BMS-433796 and L-685,458, respectively. For [3H]IN973, presenilin-1 deficiency decreased specific binding, and deficiency in both presenilins decreased specific binding by 90%. In contrast, presenilin deficiency had no detectable effect on [3H]L-685,458 binding (Fig. 4). Thus, presenilins do not contribute significantly to [3H]L-685,458 binding under these conditions. Any presenilin-dependent binding of [3H]L-685,458 was apparently overwhelmed by a much larger amount of presenilin-independent binding.

To determine whether [3H]L-685,458 binding in THP-1 cell homogenates was predominantly to SPP or to proteins with SPP-like characteristics, displacement assays for both radioligands were carried out using selective SPP and γ-secretase inhibitors. Potent displacement of the selective γ-secretase ligand [3H]IN973 was observed for the γ-secretase inhibitors DAPT, BMS-299897, and BMS-433796 (Fig. 5 and Table 1). As expected, the SPP inhibitor (Z-LL)₂-ketone showed only weak displacement of [3H]IN973 with IC₅₀ = 2 μM. In contrast, the radioligand [3H]L-685,458 was potently displaced by (Z-LL)₂-ketone with IC₅₀ = 5 nM, consistent with SPP-like binding, but not consistent with presenilin binding. Furthermore, the γ-secretase inhibitors caused only weak, if any, displacement of [3H]L-685,458. Thus, although [3H]L-685,458 is known to bind to presenilin, its binding characteristics in cell homogenates are dominated by the more abundant SPP family proteins.

Additional experiments with other cell lines, including non-transfected HEK293, HeLa, and A431 cells, also led to the conclusion that [3H]L-685,458 binds predominantly to SPP family proteins in cell homogenates. A431 cells have been reported not to express SPP (45). However, in A431 cells, [3H]L-685,458 still had a greater Bₘₐₓ compared with [3H]IN973 and was not significantly displaced by γ-secretase inhibitors, such as DAPT (data not shown), further suggesting that L-685,458 can bind to multiple members of the SPP family, such as SPPL2b, which it has been reported to inhibit (46).

To evaluate the interactions between different classes of inhibitors, isotherms for [3H]L-685,458 binding to SPP were determined in the presence of the non-transition state inhibitor BMS-433796, the transition state analog (Z-LL)₂-ketone, and the NSAID sulindac sulfide. The SPP inhibitor (Z-LL)₂-ketone exhibited a competitive mode of radioligand displacement, consistent with binding at the active site (Fig. 6A). In contrast, BMS-433796 and sulindac sulfide were found to affect the apparent number of sites (Bₘₐₓ) with little effect on the apparent binding affinity (Kₐ) (Fig. 6B and C). Similar experiments were carried out for the γ-secretase radioligand [3H]IN973 in THP-1 cell homogenates (Fig. 7). The binding isotherms for [3H]IN973 showed that displacement by L-685,458 was noncompetitive. In contrast, the non-transition state analog BMS-433796 showed competitive displacement. Thus, the different classes of inhibitors, the transition state analogs and non-transition state analogs, display the same noncompetitive binding interactions for both SPP and γ-secretase.
In addition, the NSAID sulindac sulfide shows the same noncompetitive mode of radioligand displacement from SPP as reported previously for γ-secretase (18).

**DISCUSSION**

The SPP enzyme is related in structure to presenilin, the catalytic subunit of γ-secretase, and the conservation of mechanism is implied by the range of small molecules that interact with both SPP and presenilin. In this study, a radioligand binding approach was used to characterize the binding of different classes of inhibitors to further explore the similarities and differences with respect to inhibitory mechanisms between these two enzymes. The results revealed a predominance of SPP family over γ-secretase binding sites in cell homogenates, which likely included a mixture of multiple members of the SPP family (1–3). Even A431 cells, which do not express SPP (45), displayed predominantly SPP family binding pharmacology (data not shown), indicating the likely abundance of other members of the SPP protein family. This presented challenges for the radioligand binding approach that were solved either by the use of compounds that were selective for a specific enzyme or by the use of cell cultures in which a specific enzyme was highly abundant so that a nonselective radioligand could be used.

The SPP radioligand binding assay described here utilized the γ-secretase inhibitor [3H]-L-685,458 and cell homogenates in which SPP was overexpressed by means of transient transfection with a cDNA clone. Overexpression of SPP increased the abundance of both radioligand binding sites and enzyme activity by ~10-fold relative to control transfection and by 280-fold relative to the γ-secretase binding sites that could be
detected using the selective γ-secretase radioligand [3H]IN973. L-685,458 is a transition state analog directed at the active site on the presenilin subunit of γ-secretase (13), and it therefore seemed likely that [3H]L-685,458 was binding to the active site of SPP. First, SPP and presenilin share a conserved polytopic transmembrane structure containing aspartyl protease motifs (1–3); second, L-685,458 is an effective inhibitor of SPP (31); and third, we showed that the radioligand [3H]L-685,458 was competitively displaced by (Z-LL)2-ketone, an active site-directed inhibitor of SPP (38). Thus, [3H]L-685,458 appears to bind to the active site of SPP. Some non-transition state γ-secretase inhibitors were also able to displace [3H]L-685,458 from SPP, consistent with the observation that some compounds in this class can inhibit SPP enzyme activity (31, 33). Furthermore, we showed that one of these non-transition state inhibitors, BMS-433796, exhibited noncompetitive displacement of [3H]L-685,458 from SPP, suggesting a distinct binding site, which is potentially equivalent to the allosteric inhibitor binding site proposed for γ-secretase (17,18). In addition, we found that the NSAID sulindac sulfide could displace the radioligand [3H]L-685,458 from SPP at high concentrations, consistent with its reported modulation of SPP enzyme activity (34). NSAIDs modulate the cleavage site positions of SPP and γ-secretase on their substrates without inhibiting enzyme activity, and it therefore seems likely that they affect the active site indirectly, if at all. Consistent with this, it was reported that displacement of [3H]L-685,458 by NSAIDs from γ-secretase is noncompetitive (18, 37). We showed that noncompetitive displacement of [3H]L-685,458 by sulindac sulfide also occurred for SPP (Fig. 6C). Thus, SPP appears to mimic the binding pharmacology of γ-secretase for modulator compounds as well as for the different types of inhibitors. SPP exhibits these characteristics with a far simpler subunit structure and without the need for complex assembly and maturation, which should be a considerable advantage in studying the three-dimensional structures of and relationships between the binding sites for different compounds.

Evaluation of [3H]L-685,458 binding to γ-secretase was complicated by two factors, the first being that this compound binds with high affinity to both γ-secretase and SPP and the second being that cell homogenates contained an overwhelmingly large amount of SPP family protein binding activity. A recent approach to this problem was to simultaneously overexpress all four subunits of γ-secretase, thereby increasing the number of γ-secretase binding sites well in excess of other binding activities present in the parental cell line (18). However, the multiple rounds of selection necessary to obtain stable overexpression of all four γ-secretase subunits may have resulted in additional changes in gene expression, including increases in SPP or other SPP family members. If so, the presence of SPP might account for the incomplete displacement of [3H]L-685,458 observed for selective γ-secretase inhibitors (18), thus calling into question the conclusion that the catalytic active site and the allosteric binding site are present in a 2:1 molar ratio (18). Furthermore, it seems likely that earlier studies of this radioligand in whole cell extracts may have detected predominantly SPP binding rather than γ-secretase binding (17, 37). To avoid this issue in our study, we used the radioligand [3H]IN973 (15, 41), a selective γ-secretase inhibitor representative of the non-transition state analog class of compounds. This compound has a high affinity binding site in cell homogenates (Kd ~ 1 nM), which was dependent on the presence of presenilin. In contrast to its high affinity binding to γ-secretase, the [3H]IN973 radioligand showed no directly detectable binding to SPP, indicating a low affinity, if any, for SPP. Consistent with this, unlabeled IN973 displaced [3H]L-685,458 from SPP only at higher concentrations, with IC50 ~ 500 nM, considerably less potent than its inhibition of γ-secretase (15). A low concentration of [3H]IN973 could therefore be used as a selective probe for γ-secretase binding in cell homogenates. Displacement of [3H]IN973 by the γ-secretase inhibitor BMS-433796 was found to be competitive, indicating that these compounds bind at the same site, whereas displacement by L-685,458 was noncompetitive, suggesting a separate binding site. Thus, presenilin and SPP display mechanistically equivalent binding characteristics for transition state analogs and non-transition state inhibitors.

From the drug discovery perspective, similar pharmacology between γ-secretase and SPP suggests the potential for off-target liabilities. Additional studies would be needed to understand to what extent inhibitor binding affects enzyme function in vivo and to determine what consequences, if any, would arise from dosing a nonselective drug. Nevertheless, despite compounds like L-685,458, which binds with high affinity to both γ-secretase and SPP, many of the inhibitors appear to be selective. This is expected given the amino acid sequence divergence between the two enzymes. For example, (Z-LL)2-ketone binds with >100-fold selectively to SPP, whereas the non-transition state inhibitors used in this study bind with ≈100-fold selectivity to γ-secretase. For the NSAIDs and other Aβ modulators, it will require compounds with increased potency to address the selectivity issue accurately. The in vivo consequences of inhibition of each protein in the SPP family in mammals have not been reported, but in zebrafish, deficiency in SPP or SPPL3 results in cell death in the central nervous system (30), suggesting that for drugs targeting γ-secretase, selectivity against SPP family proteins would be desirable. For γ-secretase, toxicity due to inhibition of Notch signaling is known (47), and therefore, an experimental therapy targeting an SPP family protein could benefit from a selective compound that does not target γ-secretase. The radioligand binding assays described here potentially provide a convenient approach for the identification of selective compounds.

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