Identification of γ-secretase inhibitor potency determinants on Presenilin
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Running Title: Sulfonamide class of gamma-secretase inhibitors selectively target Presenilin 1
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Production of amyloid β peptides (Aβ), followed by their deposition in the brain as amyloid plaques, contribute to the hallmark pathology of Alzheimer’s disease. The enzymes responsible for production of Aβ, BACE1 and gamma-secretase, are therapeutic targets for treatment of AD. Two presenilin (PS) homologues, referred to as PS1 and PS2, comprise the catalytic core of gamma-secretase. In comparing presenilin selectivity of several classes of gamma-secretase inhibitors, we observed that sulfonamides in general tend to be more selective for inhibition of PS1-comprising gamma-secretase, as exemplified by ELN318463 and BMS299897. We employed a combination of chimeric constructs and point mutants to identify structural determinants for PS1 selective inhibition by ELN318463. Our studies identified amino acid residues L172, T281, L282 in PS1 as necessary for PS1 selective inhibition by ELN318463. These residues also contributed in part to the PS1 selective inhibition by BMS299897. Alanine scan mutagenesis of areas flanking L172, T281 & L282 identified additional amino acids that affect inhibitor potency of not only these sulfonamides, but also of the non-sulfonamide inhibitors DAPT, and L-685,458, without affecting Aβ production and presenilin endo-proteolysis. Interestingly, many of these same residues have been identified previously to be important for gamma-secretase function. These findings implicate TM3, and a second region near the carboxy-terminus of PS1 amino-terminal fragment, in mediating the activity of gamma-secretase inhibitors. Our observations demonstrate that PS selective inhibitors of gamma-secretase are feasible, and such inhibitors may allow differential inhibition of Aβ peptide production and Notch signaling.

The most common cause of dementia in the elderly is Alzheimer’s disease (AD) which bears pathological hallmarks of amyloid plaques and neurofibrillary tangles. Extensive genetic, biochemical, molecular, and cellular studies over the last two decades have implicated the production and deposition of Aβ peptides as a critical initiating event leading to the pathogenesis of AD, reviewed in (1). Aβ peptides are produced by the action of two enzymes, β-secretase, and gamma-secretase. The gamma-secretase enzyme is a large molecular complex comprised of four principal subunits, the Presenilins (PS1 or PS2), Nicastrin, Aph-1, and Pen-2 (2-6). Gamma-secretase functions as an intramembrane-cleaving aspartyl protease, reviewed in (7-9).

In addition to processing of β-amyloid precursor protein (APP) and Aβ peptide production, gamma-secretase is also involved in the processing of many additional Type I transmembrane proteins, most notably Notch, reviewed in (9,10). Gamma-secretase cleavage of Notch leads to nuclear translocation of Notch intra-cellular domain (NICD), and expression of downstream genes, a process referred to as Notch signaling. Notch signaling is critical for cellular differentiation during development, as well as tissue homeostasis in adults. Pharmacological inhibition of Notch signaling by non-selective gamma-secretase inhibitors in adult mice has been demonstrated to affect tissue differentiation and cell homeostasis in multiple systems e.g. gastrointestinal tract, pancreas, skin and lymphocytes (11-14), consistent with predictions from KO mouse models (15-22). Inhibition of Notch signaling by non-selective gamma-secretase inhibitors is a potentially limiting issue for the clinical development of this class of therapeutics. Hence, a major objective of inhibitors targeting...
gamma-secretase for treatment of AD is development of selective inhibitors that can reduce Aβ peptide production, without significantly interfering with the processing of other substrates of gamma-secretase, principally Notch.

Two presenilin homologues, referred to as PS1 and PS2, comprise the catalytic core of gamma-secretase. Observations from KO mice show that Presenilin 1 containing gamma-secretase contributes to ~80% of total Aβ production in brain, while PS2 containing gamma-secretase contributes to ~20% of total Aβ (16,17,23,24). Furthermore, the observation that PS1 KO mice exhibit perinatal lethality, whereas PS2 KO mice are viable suggests that PS2 selective inhibitors may spare Notch signaling while lowering Aβ appreciably to be viable therapeutics (16,24-27).

We employed transformed fibroblasts from presenilin double-KO mice transiently transfected with APPsw + PS1, or APPsw + PS2, to identify PS selective inhibitors. Among the different classes of inhibitors we tested, we noted that while most were equipotent for inhibition of Aβ production from PS1 or PS2 gamma-secretase, sulfonamides exemplified by ELN318463 (this report) and BMS 299897 (28-30), displayed selectivity for inhibition of PS1 gamma-secretase. We exploited this observation to map determinants for PS1 selectivity of these sulfonamides using chimeric constructs and point mutants. The results of our studies identify 3 residues as necessary and sufficient for the observed PS-1 selectivity of sulfonamide ELN318463: L172, T281, L282 in PS1. These residues also contribute in part to the observed selectivity of BMS299897.

Molecular cloning and construction of chimeras
Human PS1, PS2, and APPsw cDNA inserts were subcloned into pCF vector, which was modified from pcDNA3 (Invitrogen, CA, USA) by inserting the adenoviral tripartite leader sequence.
(31) 38 bp upstream of the starting ATG codon, between the CMV promoter and the EcoR1 site. Presenilin chimeras were constructed by blunt-end ligation of PCR amplified fragments (pfu Turbo DNA polymerase kit, Stratagene) and sequence verified prior to transfection into transformed fibroblasts derived from PS1/PS2 double knock-out mice. Alanine scan mutagenesis of the C2 and C5 sub-regions was carried out with mutagenic primers and the quick-change site directed mutagenesis kit (Stratagene).

**Cell culture & Transfection**

Mouse fibroblasts derived from the PS1–/–/PS2–/– double knockout cells, (dKO cells, gift from Dr. Bart De Strooper), (23) were grown at 37°C under 10% CO₂ in Dulbecco’s modified Eagle’s medium (DMEM) containing 2-10% fetal bovine serum (FBS) and 100 µg/ml penicillin/streptomycin (Invitrogen Corporation, Carlsbad, CA, USA), and 2 µM L-Glutamine (Gibco/Invitrogen Corporation, Carlsbad, CA, USA). Transfections were performed using Nucleofector II (Amaxa GmbH, Germany) with about 1 to 10 million of PS1–/–PS2–/– cells, using transfection reagents supplied by the manufacturer. An RPMI washed cell pellet was resuspended in 100 µl Solution R. To this cell suspension, 1-2 µg of a DNA mixture comprised of APPsw, (32), and PS1 (2) or PS2 (33) was added, and the cell-DNA mixture was electroporated immediately (program T-20). Electroporated cells were first suspended into 1 ml of warm RPMI and then transferred (within 2-5 minutes after addition of RPMI) into 5-10 ml of DMEM with 10% FBS, for plating into 96-well plates. For experiments using PS2, cells were plated at a density of ~50,000/well in 96-well plates; for experiments involving PS1, cells were plated at a density of ~15,000/well in 96-well plates. The cells were treated overnight (14-20 hours) with different concentrations of gamma-secretase inhibitors 1-3 hours following plating. During the initial phases of this work, transfection efficiencies were monitored using GFP reporter plasmid (~90%) in conjunction with Aβ production from parallel transfections. The levels of Aβ produced were normalized for APP expression levels, as well as PS expression levels, using western blots analysis (see supplemental data, Figure S1, and Figure 9). As the level of Aβ produced was highly consistent and reproducible across experiments with Nucleofector mediated transfection under the experimental conditions described above (as illustrated in figure 8), in subsequent experiments with point mutant PS constructs shown in figure 5, 6, and tables 1 & 2, Aβ production from transfected cultures was used as a surrogate for transfection efficiency.

**ELISA assays for Aβ40, Aβ42, total Aβ or Aβ1-x**

Aβ1-x ELISA employed antibody 266 (recognizing Aβ 16-23, Elan) for capture and antibody 3D6 (recognizing Aβ1-5, with specificity of position 1 of Aβ, Elan) for detection. Aβ40 ELISA employed antibodies 266 as capture and 2G3 (specific for Aβ40) as detection, respectively. Aβ42 ELISA employed antibodies 266 as capture and 21F12 (specific for carboxy-terminus of Aβ 42) as detection, respectively (34). Aβ peptide (California Peptide Research, Napa, CA) was used as concentration standard in the ELISA.

ELISA assays were performed at room temperature using 50 µl of diluted or original conditioned medium from overnight culture of transfected cells, treated with gamma-secretase inhibitors or DMSO control. Following a 1 hour incubation to capture Aβ peptide from media, plates, the plates were incubated sequentially (45 minutes per incubation) with biotinylated detecting antibody (0.5 mg/ml), followed by HRP-streapavidin, and HRP substrate (1-step slow TMB-Elisa, Pierce, Woburn, MA, USA, catalogue number: 34024). Plates were washed between incubations with Tris-buffered saline (TBS) plus 0.05% Tween-20. Substrate reactions were terminated with 2 NH₂SO₄, for absorbance readings with a SpectraMax Plus (Molecular Devices, Sunnyvale, CA, USA).

**Determination of inhibitor potencies & statistical analyses**

Dose response curves, and corresponding EC50 values for the inhibitors described in this report, were derived by curve fitting of secreted Aβ levels (determined by ELISA) from cells treated with a range of inhibitor concentration using the ELfit program (IDBS, Alameda, CA, USA). Due to experimental limitations involving transient transfection of a large number of
constructs per experiment, the inhibitor potencies against a given construct or chimera were determined from individual experiments. Conclusions regarding the effect of a chimera or a mutation on inhibitor potency were based on average EC50’s from replicate experiments. Drift in the absolute value of an inhibitor’s potency between experiments over time is evident (e.g. compare ELN318463 potency in figure 1 with figure 4). However, despite this experimental drift in EC50 values, the rank order of PS1 selectivity (ELN318463 > BMS299897) remained invariant. Furthermore, our conclusions from early studies with chimeric construct were corroborated by subsequent studies with point mutants as elaborated in the results and discussion sections. For the alanine scan mutagenesis of the C2 and C5 sub-regions, the statistical significance of compound potency against a particular alanine mutant was determined using unpaired t-test (Graphpad Prizm software package) by comparing the EC50 of the compound against the mutant versus wild type PS1 (n = 2-4 each).

RESULTS

We examined the inhibition potency of gamma-secretase inhibitors against PS1 or PS2 comprised γ-secretase using transformed primary embryonic fibroblasts derived from PS1-/-PS2-/- knockout mice (23) transfected with PS1 or PS2 expression constructs to reconstitute homogenous enzyme complexes in these cells, without the complication of endogenous presenilins. This experimental system allowed us to unambiguously compare functional properties of reconstituted wild-type or mutant PS1 or PS2 γ-secretases. In each experiment, expression constructs for either human PS1 or PS2, together with Swedish APP751 (APPsw), were transiently transfected into PS1-/-PS2-/- dKO cells. The transfected cells were then incubated with different concentrations of inhibitors overnight. Total Aβ levels in conditioned medium from transfected cells were then measured by ELISA.

Figure 1 illustrates that among the classes of gamma-secretase inhibitors we tested, sulfonamides show preferential inhibition of PS1-γ-secretase, while non-sulfonamide inhibitors only have modest selectivity for PS1- vs. PS2-γ-secretase. The dose response curves and EC50 values from a representative experiment are shown in Fig. 1. The mean values from 2 independent experiments on PS1/PS2 selectivity of the inhibitors are shown in Figure 3. ELN318463 is ~51-fold more selective for PS1, and BMS299897 is ~35-fold more selective for PS1, while L-685,458 is only ~3-fold more selective for PS1, and DAPT is actually 2-fold more selective for PS2. Additional sulfonamide inhibitors of the type represented by ELN318463 also displayed preferential PS1 selectivity (data not shown).

The observation of the differential inhibition of PS1 versus PS2, mainly by sulfonamide series of inhibitors, prompted us to examine the structural basis for this differential inhibition. We employed chimeric PS1/PS2 molecules (illustrated in Figure 2) to map the domain(s) in PS1 responsible for differences in inhibitor potencies. Evaluation of an initial set of chimeric presenilin molecules revealed that the middle third of PS1 (residues 128-298) is both necessary and sufficient for its high potency inhibition by ELN318463 and BMS299897 (Figure 3). For both ELN318463 and BMS299897, the EC50 values of PS1/2B are similar to that of PS1, while EC50 values of PS1/2A and PS1/2C are similar to those of PS2. More telling, inhibitor potencies against PS2/1C behaved just like PS1, in terms of its inhibition by ELN318463 and BMS299897, despite the fact that majority of this construct is comprised of PS2 sequence. As before (Figure 1) non-sulfonamide inhibitors, such as DAPT and L-685,458, did not display >3-fold selectivity for PS1 nor PS2, and the chimeras did not reveal any consistent basis for this low level of selectivity.

We examined the region bounded by PS1 residues 128-298 in more detail by studying inhibitor sensitivity of additional “small fragment chimeras” (PS1/2C1, PS1/2C2, PS1/2C3, PS1/2C4, and PS1/2C5, Figure 2) for the two sulfonamide inhibitors. Expression, Aβ production, and endo-proteolytic processing of the chimeric PS molecules is indistinguishable from the wild-type counterpart (supplementary data, supplemental Figure S1 part B and C). The non-sulfonamide
inhibitors were not examined further due to their lack of appreciable PS selectivity (Fig. 3). As shown in Figure 4, it is clear that for ELN318463 and BMS-299897, only the C2 (containing PS2 residues #171-200 substituted for PS1 residues #165-194) and C5 sub-region (containing PS2 residues #275-304 substituted for PS1 residues #269-298) chimeras reported inhibitor EC50 values appreciably different from that of parental PS1. Thus, the C2 and C5 sub-regions largely account for the inhibitor selectivity between PS1 and PS2. The PS2 C2 region in the context of PS1 reduces the potency of Elan G ~3X relative to wild type PS1, while the PS2 C5 region in the context of PS1 reduces the potency of Elan G ~18X relative to PS1. It is noteworthy that the product of the fold selectivity for ELN318463 reported by the C2 & C5 subregions (54X) is in close agreement to the average PS1 selectivity of ELN318463 from the experiments reported in Figures 1, 3 and 4 (39X, 51X, 65X, respectively).

While both the C2 and C5 sub-regions contribute to the inhibition potency difference between PS1 and PS2 for BMS299897, the product of the fold difference in EC50 values between parental PS1 and the chimeras, PS1/2C2 (2X) and PS1/2C5 (4X), does not fully account for the ~46X difference in potency of BMS299897 between PS1 vs. PS2. One explanation for this observation with BMS299897 may be that both the C2 and C5 sub-regions from PS1 may need to be present in cis configuration to fully account for the potency difference between PS1 & PS2. The trans arrangement of the C2 or C5 sub-regions, as it occurs in the chimeras we tested, may mask the combined contributions of the two sub-regions in native PS1 molecule. The conversion mutants in the C2 and C5 sub-regions, as well as the triple mutant constructs described below provide a test of this assumption. At this point, we can conclude that the C2 and C5 sub-regions likely make significant contributions to inhibitor selectivity of sulfonamide inhibitors.

As the C5 sub-region (encompassing the C-terminal segment of presenilin NTF) makes a greater contribution for PS1/PS2 selectivity of ELN318463 (Figure 4), we tested the five non-conserved residues within the C5 sub-region (Figure 5A) first for their contribution to the observed selectivity of the sulfonamides. We mutated and assayed five conversion mutant constructs in the context of PS1/2C2 (Figure 5B & 5C). For convenience, PS1/2C2 was used as the reference construct in place of PS1. The results obtained using PS1/2C2 as reference are validated by the triple mutation constructs described below, as well as by the alanine scan of the C2 region (see discussion).

As before, each mutant construct was assayed for inhibitor sensitivity in transiently transfected PSdKO cells treated with a range of inhibitor concentrations. As summarized in Figure 5C, of the five conversion mutants tested in the PS1/2C2 backbone, T281P and L282I mutations significantly affected the potency of ELN318463 as evidenced by >2X, and >6X increase in EC50 values, respectively, compared with parental PS1/2C2. The conversion mutations in the PS1 C5 region did not significantly affect the potency of BMS299897 as compared with the parental construct PS1/2C2. This was not surprising, because although the PS2 derived C5 sub-region lowered the potency of BMS299897 ~4X in the context of PS1 alone (Figure 4), the <2X difference between PS1/2C2 and PS1/2C5 (see Figure 4) falls within the experimental variability of our assay, and thus it would not read-out as a “significant” difference. This observation suggests that the 5 residues differing between PS1 and PS2 in the C5 sub-region do not contribute more than 2X to the loss of potency observed with BMS299897 when tested in the context of PS1/2C2. The contributions of these amino acid residues may become noticeable if tested in the context of PS1, or in combination. This latter possibility was tested experimentally by the triple-mutants described below.

We next queried the C2 sub-region for dominant residues conferring PS1 selectivity. The C2 sub-region in PS1 and PS2 encodes the majority of transmembrane domain 3 (TM3), as well as part of the loop connecting TM3 and TM4. Sequence alignment of PS1 and PS2 reveals seven non-conserved amino acid residues in the C2 sub-region (Figure 6A). Conversion mutants at each of these positions were generated in the context of PS1(L282I) (Figure 6B), and assayed...
for the impact on inhibitor potency. We selected PS1(L282I), instead of wild-type PS1, as the backbone for this analysis in order to amplify the relative contribution of any conversion mutant in the PS1 C2 sub-region, which alone contributes ~3X potency difference between PS1 & PS2. Inhibition potency of the sulfonamides against a particular conversion mutant in the C2 sub-region of PS1 was referenced against PS1/2C2(L282I), a PS1/2C2 chimera molecule with L282I mutation, as illustrated in Figure 6C.

We employed PS1/2C2(L282I) as the reference construct for assessment of PS1 C2 region conversion mutants because it established the bottom of the range of inhibitor potency for the conversion mutants. Thus, we reasoned that if any one of the seven amino acid residues in the PS1 C2 sub-region contributes to the higher inhibition potency of ELN318463 against PS1, a conversion mutant of that PS1 residue into a PS2 residue, in the context of PS1(L282I), should lead to a decrease in potency (i.e. an increase in EC50 value) comparable to that observed against PS1/2C2(L282I). Indeed, this is what we observed for PS1(L282I, L172M). Inhibitor potencies of both ELN318463 and BMS299897 were lowered approximately 3X against this particular mutant (Figure 6D), and the EC50 value of both compounds are very similar to their potencies against PS1/2C2(L282I). In contrast, potencies of ELN318463 and BMS299897 are not affected by any of the other 6 conversion mutants in the C2 sub-region.

The data strongly suggest that L172 in PS1 largely accounts for the contribution made by the C2 sub-region to the high-potency inhibition of PS1 for both ELN318463 and BMS299897. Alanine scan mutagenesis of L172 in the PS1 context lowered the potency of ELN318463 2.6X relative to wild-type PS1 (see Table 1 & alanine scan results, below), in close agreement with the 3X effect observed in the context of PS1/2C2(L281), validating use of this construct as a reference. Finally, in our experiments analyzing the C2 sub-region, the potencies of ELN318463 & BMS299897 against PS1/2C2(L282I) are within 2X the potencies determined during analysis of the C5 region (Figure 5), further illustrating the limited range of inter-experimental variability we observed during the course of this work.

Having identified L172, T281, and L282 in PS1 to be primarily responsible for the high-potency inhibition of PS1 by ELN318463, when tested individually, and partly responsible for BMS299897, we next tested whether the 3 amino acid residues, when in combination, are both necessary and sufficient in determining PS1/PS2 selectivity for the two sulfonamides. We also included the two non-sulfonamide inhibitors, DAPT and L-685,458 as controls in this experiment to confirm the effect is specific to sulfonamides. We prepared two composite mutants, triple-PS1 refers to PS1(L172M, T281P, L282I); triple-PS2 refers to PS2 (M178L, P287T, I288L). The mutated amino acid residues in triple-PS2 correspond to the three identified amino acid residues in triple-PS1.

As revealed by the data shown in Figure 7, the EC50 for inhibition of triple-PS1 by ELN318463 is indistinguishable from wild type PS2. Thus, in terms of inhibitor sensitivity, Triple-PS1 behaved like PS2, despite the fact that it is largely a PS1 molecule, with only 3 amino acid residues converted into PS2 sequence (L172M, T281P, L282I). Similarly, the EC50 for inhibition of triple-PS2 by ELN318463 is indistinguishable from wild type PS1, despite the fact that it is largely a PS2 molecule, with only 3 amino acid residues converted into PS1 sequence (M178L, P287T, I288L). Furthermore, the gamma-secretase activity of each Triple-PS construct is indistinguishable from that of its wild-type counterpart, as measured by Aβ production (supplementary data, supplemental Figure S1 part D). The data strongly suggest that the 3 identified amino acid residues are both necessary and sufficient in determining PS1>PS2 selectivity for ELN318463. For BMS299897, the triple mutations had slightly less dramatic effect than that seen for ELN318463. Still, the effect was stronger than what one would have predicted based on the additive effects of single mutations in both the C2 and C5 regions (compare EC50 values for BMS299897 in Fig. 6D and Fig. 5C to those in Fig. 7B for mutations L172M, T281P, & L282). As expected, the triple mutations did not have detectable effect on inhibition potencies of the
non-sulfonamide inhibitors, DAPT and L-685,458, confirming that the effects of the three identified amino acid residues were specific to sulfonamide inhibitors.

In order to test if additional residues flanking L172, T281, and L282 of PS1 contribute to inhibitor potencies of ELN318463 and BMS299897, we conducted alanine scan mutagenesis in the C2 & C5 sub-regions. We also tested the alanine scan mutants to identify any amino acid residues in these sub-regions for their effect on potencies of non-sulfonamide inhibitors, as this knowledge may shed light on the structural determinants for both types of inhibitors. We first performed alanine scan around L172 in the C2 sub-region of PS1. Briefly, each of the 15 amino acid residues other than an alanine residue at positions 161 through 176 in human PS1 was individually mutated into an alanine residue. The mutants were then individually co-transfected with APPsw into the PS1^-/-PS2^-/- dKO cells, and dose response profiles were established with each mutant to determine EC50 values for four γ-secretase inhibitors: ELN318463, BMS299897, DAPT, and L-685,458. To normalize for inter-experiment variation in EC50 values, we included PS1 as a control in each experiment, and expressed the inhibitor potency against a given mutant as a ratio of EC50 value against the mutant PS1 over wild-type PS1 within each experiment. The effect of mutations, revealed by inspecting this ratio, identifies residues that lower potency of all 4 inhibitors tested (black arrows in Table 1 and Table 2), as well as residues that increased potency of the certain inhibitor(s) (red arrow in Table 1 and Table 2). Arrowheads in table 1 and table 2 indicate residues identified as sites of FAD mutations.

Three residues identified by alanine scan to affect inhibitor potency in the C2 sub-region have previously described as sites of FAD mutations in PS1 (arrowheads in Table 1). L166A had the most dramatic effect on all four compounds tested, including sulfonamide and non-sulfonamide inhibitors. Relative to wt PS1, PS1(L166A) lowered the potency of the sulfonamides by ~10X, while the potency of the non sulfonamides was lowered ~ 4X. This position is associated with a highly aggressive FAD mutation, L166P, which has been shown to lead to a 6-fold increase in Aβ42, and decrease both AICD generation and Notch cleavage (35). Consistent with earlier results (35), production of total Aβ relative to wt PS1 was not affected by this mutation (Fig 8A), and L166A led to elevation of Aβ42/Aβ total ratio (Fig 8C). Interestingly, alanine mutation of the other two FAD associated residues in the C2 sub-region (S169 and L173) significantly affected potency of sulfonamides and DAPT, but not L-685,458. S169A increased inhibition potency of sulfonamides and DAPT slightly more than 2X, with no effect on the potency of L-685,458, whereas L173 lowered potency of sulfonamides and DAPT, but not L-685,458. Consistent with FAD mutations S169P and S169L, the S169A mutant exhibited a modest 1.5-2X elevation in Aβ42/total ratio (Figure 8C), while presenilins endoproteolysis was not affected (Figure 9B). The S169A and L173A mutants did not affect production of total Aβ relative to wild-type PS1 (Figure 8A).

In addition to L166A discussed above, I162A and W165A had a modest effect (3-4X reduction of potency) on both sulfonamide and non-sulfonamide inhibitors, while S170A primarily affected sulfonamide inhibitors (lowered potency 2.6 – 4.5X). These mutants were indistinguishable from PS1 with respect to activity, as assessed by Aβ production (Figure 8A). Although L172A mutation lowered potency of ELN318463 2.6X, consistent with the 3X effect of L172M conversion mutant (Figure 6), the result did not reach statistical significance in this experiment (p = 0.14).

Results from a similar alanine scan mutagenesis of L282 flanking residues in the C5 sub-region of PS1 are summarized in Table 2. Inhibitor potencies against 21 alanine mutants were determined as described above. Five residues in the C5 sub-region affected potency of all four inhibitors tested: L271, R278, L282, L286, & I287 (denoted by black arrows adjacent to Table 2). Of particular note, R278A had the most dramatic effect on all the compounds tested. This mutation reduced the inhibition potency for L-685,458 by ~120-fold and about 10-fold for ELN318463, BMS 299897, and DAPT. R278 is situated near the amino-terminus of cytosol-localized
hydrophobic region 7 (36-38). One study reported that R278T was associated with early-onset familial Alzheimer’s disease (FAD) (39), but no further molecular or biochemical studies were reported for this PS1 FAD mutant. Two other FAD mutations R278K & R278I have been described at this site. Aβ production from this mutant was 50% of wild-type PS1 (Fig 8B). The mutant exhibited a 5X elevation in Aβ42/total ratio over wild-type PS1 (Fig 8C), and a concomitant decrease in PS1 endoproteolysis (Figure 9B). The FAD associated L271A also affected both sulfonamide and non-sulfonamide inhibitor potencies.

The Y288A mutation is also noteworthy as it led to complete inactivation of PS1, despite normal endoproteolysis (Figure 9B). Our findings corroborate the functional significance of Y288 described previously (40). Interestingly, our mutational scan of two upstream residues, L286A and I287A revealed an effect on the potency of all classes of inhibitors, without significantly affecting Aβ production (figure 8B). PS endoproteolysis was not affected by the L286A mutation (figure 9B). Consistent with FAD mutations at codon 286, L286A displayed elevation in Aβ42/total ratio (Fig 8C).

In contrast with the T281P conversion mutation identified above (Figs 5 & 7), T281A mutation did not affect potency of ELN318463 in the context of PS1. However, and consistent with the 6.5X loss of potency effected by the conversion mutation L282I (Figure 5), L282A lowered the potency of ELN318463 10X, with lesser effects on the other 3 inhibitors. Interestingly, V272A also reveals a strong effect on potency of ELN318463 as L282A, with no effect on BMS299897 inhibition, suggesting that V272 may also be involved in ELN318463 activity. Aβ production was not affected by V272A, T281A, and L282A mutants relative to wild-type PS1 (Figure 8B). Taken together, these observations support our studies with chimeric constructs, which mapped residues in the C5 sub-region as contributing to the PS1 selectivity of ELN318463.

Although our alanine scan analysis of the C2 and C5 sub-regions was driven by our observation of PS1 selective inhibition by sulfonamides, we identified certain residues that affected potencies of DAPT, as well as L-685,4858. As noted, many of these residues coincided with sites of FAD mutations in the C2 and C5 subregions (see discussion). Interestingly, many of the non-FAD residues that affected inhibitor potency also elevated Aβ42/Aβ1-x ratio (supplementary data, Figure S2, and discussion). Furthermore, our study also identified certain residues that affect potencies of the non-sulfonamides exclusively. In this last category, P284A is noteworthy for affecting potency of DAPT and L-685,458, while alanine mutation of R278 flanking residues E277A and N279A, affected potency of L-685,458 exclusively. Curiously, alanine mutation of the F283, the residue upstream of P284, increased potency of BMS299897. Mutation of the FAD residue E280 lowered potency of ELN318463, DAPT, and L-685,458, with a concomitant reduction in Aβ production (relative to wt PS1, Fig 8B).

In summary, alanine scan mutagenesis of the C2 and C5 sub-regions in PS1 largely corroborates our studies mapping selectivity determinants for sulfonamides to these regions. In addition, the experiments reveal determinants in common with sulfonamides that affect potency of additional classes of gamma-secretase inhibitors, namely DAPT and L-685,458.

DISCUSSION

We report the first observation of PS1 selective inhibition of gamma-secretase by sulfonamides. Using a series of chimeric constructs and point mutations, we identify the structural determinants for PS1 selective inhibition by ELN318463, and to a lesser extent, BMS299897. Chimeric constructs offer a powerful & convenient solution for identifying domains in a protein that contribute to a particular phenotype when there is significant divergence between homologues. Our chimera studies identified two sub-regions in PS1 (designated C2 and C5, encompassing TM3, and hydrophobic region 7 following TM6, respectively), as comprising the principal determinants for PS1 selectivity of sulfonamide inhibitors. The divergence between PS1 & PS2 in the C2 and C5 sub-regions is among the lowest between the two presenilins. This implies that if
other regions share similar degree of divergence with the C2 or C5 sub-regions between PS1 and PS2, and make similar contributions to inhibition potency of sulfonamides, they would have been revealed in our chimera studies.

We identified 3 amino acid residues in PS1: L172, T281, & L282, that are largely responsible for the observed PS1 selectivity of sulfonamide inhibitor ELN318463, and to a lesser extent BMS299897. Since we did not exhaustively test all double mutant combinations of these residues, we cannot state with certainty that all 3 residues are necessary for completely effecting the potency of the sulfonamides we tested. However, the double mutant we did test, PS1(L282I, L172M) in Figure 6D, had an intermediate effect on the potency of both sulfonamides tested when compared with the triple PS1 mutant (Figure 7). This observation suggests that the other combinations of double mutant PS constructs would likewise have had an intermediate effect on inhibitor potency.

The difference in inhibition potency between PS1 and PS2 by sulfonamides could reflect either a difference in compound binding affinity, or a difference in allosteric inhibition subsequent to compound binding. Our experimental approach does not to distinguish between the two possibilities. However, the highly conservative L282I mutation in PS1 led to a 6.4-fold reduction in inhibition potency for ELN318463, while a non-conservative T281P mutation of the adjacent residue, a potentially much more disruptive change, only led to a ~2-fold decrease in potency for ELN318463 (Figure 7C). This observation suggests that L282 in PS1 may make physical contact with ELN318463, while other amino acid residues (L172 and T281 in PS1) influence potency of ELN318463 indirectly. Verification of this assumption must await direct binding studies with cross-linkable compounds. Alternatively, the impact of the triple-mutants on inhibitor potency could reflect a difference in the underlying biology of the presenilins as described by Mastrangelo et al. (41), e.g. a difference in sub-cellular localization, or in affinity for interacting proteins which indirectly affect sensitivity to inhibitors.

Our conclusions regarding selectivity determinants from chimeric constructs & conversion point mutants in the C2 & C5 sub-regions were confirmed by the subsequent results with the triple mutants (triple-PS1 & triple-PS2). Likewise, the data from conversion mutants in the C2 & C5 sub-regions was corroborated by the results of the alanine scan experiments in these regions. Specifically, alanine scan of every residue throughout the C2 and C5 regions did not reveal additional non-conserved residues that affected inhibitor potency. The additional residues revealed by alanine scan to affect inhibitor potencies in the C2 and C5 sub-regions were all conserved between PS1 and PS2. Overall, the alanine mutations did not grossly affect function, as measured by total Aβ levels normalized relative to that of wild-type PS1 (Figure 8A and 8B). Two noteworthy exceptions in the C5 sub-region that affected Aβ production, R278 and Y288, are discussed below.

Alanine scan mutagenesis of conserved residues in the C2 and C5 sub-regions revealed a preponderance of FAD associated amino acids among the residues which affected potencies of both sulfonamide and non-sulfonamide inhibitors. A concordance between FAD residues and inhibitor sensitivity has been noted previously using peptidomimetic (42), active site (43), and most recently NSAID (44) class of gamma-secretase inhibitors. We therefore examined whether the FAD as well as non-FAD residues in the C2 and C5 sub-regions that affected inhibitor potencies in our alanine-scan studies impacted Aβ42/Aβtotal ratio. Interestingly, many (but not all) alanine mutants of FAD as well as non-FAD residues elevated Aβ42/Aβtotal ratio (Figure 8C, and supplementary data, supplemental figure S2). The results from this analysis revealed that in addition to the known FAD residues noted in Table 1 and Table 2, alanine mutation of non-FAD residues W165, F283 and I287 also elevated Aβ42/Aβtotal ratios. In contrast, I162A and F283A did not result in elevation of Aβ42/ Aβ total ratio relative to wt PS1. Thus, there is a strong (but not strict), concordance between inhibitor sensitivity, and elevation of Aβ42/Aβtotal. We also observe that under our experimental conditions, alanine mutation of FAD
residue T274 caused neither an elevated Aβ42/Aβ total ratio, nor a difference in inhibitor sensitivity relative to wt-PS1 (Table 2, and supplementary data figures S2 and S3).

Taken together, the observed concordance between inhibitor sensitivity and elevation of Aβ42/Aβ1-x ratio suggests that the different classes of inhibitors not only share common structural determinants affecting potency, but also that these common determinants coincide with residues in PS1 previously known to be functionally significant. The mutants L166A, R278A and L286A had very large (>5X) effects on the inhibition potency of all classes of inhibitors we tested. The FAD residue L166 has been demonstrated to differentially influence ε-(S3-like) and γ (S4-like)-cleavage of substrates (35). The effects we observe upon alanine mutation of residue R278 (reduced presenilin endoproteolysis, and Aβ production) are similar to the observations of Nakaya et al. from the FAD R278I mutation of PS1 (45), whereas mutagenesis of L286 has been documented to differentially affect Aβ42 production and Notch cleavage (46). Alanine mutation of L271 also affected sulfonamide and active site inhibitor potencies, and to a lesser extent DAPT, and the FAD L271V has been associated with increased expression of exon 8 deleted transcripts, producing non-functional protein with regard to GSK and τ- binding activities of PS1 (47).

The second noteworthy exception of alanine mutation which affected Aβ production in our studies is Y288A, which resulted in undetectable levels of Aβ (despite normal PS1 expression and processing Figure 9B). Our findings on gamma-secretase activity and presenilinase processing of PS1 from this mutant are consistent with those of Laundon et al. (40), who reported that this mutation significantly lowered Aβ production from a C99 substrate. Residues 286-288 lie proximal to the presenilinase cleavage site (48,49) and in addition to the effects of L286 mutation on inhibitor potency noted above, alanine mutation of I287 also significantly affected inhibitor potencies. These residues fall within the PALIY motif identified by Laudon et al as important for assembly of PS1 NTF and CTF into functional gamma-secretase high molecular weight complex (40). The preponderance of residues in the conserved cytosolic loop/hydrophobic region 7 of PS1 which affect the potency of all classes of inhibitors tested reveal a new role for this domain, and suggest this region is an important site for effecting inhibition of gamma-secretase by multiple classes of inhibitors.

The relationship among different types of inhibitors (sulfonamide: BMS299897; peptidic: DAPT; and transition state isostere: L-685,458) revealed in our studies was not anticipated by earlier investigations into compound binding sites nor mechanism of action. For example, it was reported that BMS299897 did not compete with L-685,458, suggesting that sulfonamide inhibitors are allosteric inhibitors, in contrast to transition state isosteres, which directly bind to the enzyme’s active site (50). Separately, it was suggested that DAPT mainly inhibits γ-site cleavage, while L-685,458 mainly inhibits ζ- and ε-site cleavage (51).

On the other hand, our alanine scan experiments revealed several amino acid residues in TM3, as well as in the C-terminus of PS NTF, that affected the inhibition potency of all the inhibitors tested (discussed above). This finding suggests that TM3 (the C2 sub-region) and the C-terminus of PS NTF (the C5 sub-region) may be in close proximity, analogous to recent reports based on competition studies (52) and cysteine scan mutagenesis (53), which revealed: a) the binding sites for DAPT and transition state analogues partially overlap; and b) the proximity of inhibitor interacting residues in TMD6 and TMD7. Hence, our data are not inconsistent with the recent finding on the binding site for DAPT (53,54). In fact, the two results may complement each other. Morohashi et al. employed biochemical labeling to identify protein fragments that are in the vicinity of DAPT-binding site, and found that (1) DAPT mainly labels TMD7 of PS. (2) sulfonamide inhibitor can compete effectively with DAPT; and (3) L-685,458 attenuated DAPT labeling only at higher concentration. In a follow-up study, Sato et al identified L250 in TMD6 & L383 in TMD7 as the primary residues for DAPT binding.
Hence, based on the information generated by this and the aforementioned studies, we can speculate that the binding pocket for gamma-secretase inhibitors may be composed of residues from several regions of presenilin. It is conceivable that the TMD6 and TMD7 binding sites (identified by Morohashi et al., and Sato et al.), and the TMD3 and the C-terminus of PS-NTF found by us, are close to each other in 3-dimensional space, and form an extended binding site for different classes of inhibitors. This extended binding site may be spatially close to the active site, or even overlap with the active site (comprised of the catalytic aspartates in TMD6 and TMD7), because mutations in the C2 and C5 sub-regions led to changes in potencies for transition state analogue inhibitor. Biochemical labeling is a more direct approach for identifying binding site residues than the inhibition potency studies we performed. Since inhibition potency difference can be due to either a difference in compound binding or a difference in subsequent allosteric changes, we can not definitively conclude that those identified residues are directly involved in compound binding. Hence, our findings provide an initial mechanistic understanding of how inhibitors interact with the gamma-secretase. More extensive structure-function studies, e.g. direct chemical labeling studies with different compounds, are needed to advance our understanding of inhibitor/gamma-secretase interaction.

In summary, the novel observation of PS1 selective inhibition of gamma-secretase by sulfonamides reported here illustrates the possibility of discovering isoform selective gamma-secretase inhibitors. Constitutive as well as conditional knockout mouse studies have demonstrated that mice with selective ablation of different subunits exhibit different severity of Notch deficient phenotypes depending upon the age and subunit targeted (10,41,55-62). Hence, these studies suggest that isoform selective inhibitors offer another avenue for circumventing Notch related toxicity observed with first generation gamma-secretase inhibitors (11-14).
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The abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β peptide; APP, amyloid precursor protein; APPsw, Swedish mutant version of APP751 isoform; CTF, C-terminal fragment; FAD: familial Alzheimer’s disease; dKO cells, double knockout cells; NTF, N-terminal fragment; PS, presenilin; PS1, presenilin 1; PS2, presenilin 2; SEM, standard error of the mean; TMD, trans-membrane domain; wt, wild-type.

FIGURE LEGENDS

Figure 1. Sulfonamide inhibitors of gamma-secretase show differential inhibition of PS1- and PS2-comprised gamma-secretase. Inhibitor potencies were assessed in PS1−/−PS2−/− cells transfected with human PS1+APPsw or PS2+APPsw. Cells were treated with a range of inhibitor concentrations, and dose response of inhibition was determined by ELISA of Aβ in conditioned media.

Figure 2. Schematic illustration of PS1/PS2 chimeras and sequence junctions of constructs used in these studies. PS1 contains 467 amino acid residues, and PS2 contains...
Numbers in the table indicate beginning and end positions of each chimeric fragment in source human PS1 or PS2 sequences.

Figure 3. Mapping selectivity determinants for differential inhibition potency on presenilin using chimeric constructs. EC50 values (nM, \( \pm \) SEM, n=2 per compound) for inhibition potencies of different compounds are shown in table. The results map selectivity determinants to the middle third of the PS1 molecule between residues 128-298.

Figure 4. PS1 C2 and C5 sub-regions in the middle third of presenilin-1 are dominant contributors of selective inhibition of gamma-secretase by sulfonamides. Potencies of compounds are from n=2 independent experiments, all constructs were assayed in triplicate in each experiment, and values are reported as mean EC50 (\( \pm \) SEM).

Figure 5. Fine mapping selectivity determinants in the C5 sub-region. (A) Amino acid sequence alignment in the C5 sub-region between PS1 and PS2. The C5 sub-region contains half of the loop connecting TM6, and the conserved segment following TM6, in PS1 or PS2. The amino acid residues differing between PS1 and PS2 are boxed and highlighted in red boldface. (B) Shows the diagram of parental construct: PS1/2C2, the 5 arrows above the C5 sub-region illustrate the 5 amino acid residues different between PS1 and PS2. Each of the 5 positions was mutated in the chimera PS1/2C2 backbone to convert a PS1 amino acid residue into a PS2 amino acid residue. (C) Shows the EC50 values determined from cells individually transfected with the control construct PS1/2C2, or one of the 5 point mutants, and treated with ELN318463 or BMS299897. The EC50 values (nM) are shown as mean \( \pm \) SEM (n=2).

Figure 6. Fine mapping selectivity determinants in the C2 sub-region. (A) Amino acid sequence alignment in the C2 sub-region between PS1 and PS2. The C2 region contains majority of the trans-membrane 3 (TM3) segment, indicated by the line above the sequences, and the loop between TM3 and TM4. The amino acid residues different between PS1 and PS2 are boxed and highlighted in red boldface. (B) Shows the diagram of parental construct: PS1(L282I), in which L282 is replaced by Ile from PS2. The arrows above the C2 region indicate the 7 amino acid residues different between PS1 and PS2. Each of the 7 positions was mutated in the PS1(L282I) backbone to convert a PS1 amino acid residue into a PS2 amino acid residue. (C) PS1/2C2(L282I) and PS1(L282I) are the same, except that, in the C2 region, PS1/2C2(L282I) harbors PS2 sequence, while PS1(L282I) harbors PS1 sequence. (D) Shows the EC50 values determined from cells individually transfected with the control construct PS1/2C2(L282I), or one of the 7 point mutants, and treated with either ELN318463 or BMS299897. The EC50 values (nM) are shown as mean \( \pm \) SEM (n=2).
Figure 7. Triple-PS1 refers to PS1(L172M, T281P, L282I), which is PS1-based; and Triple-PS2 refers to PS2(M178L, P287T, I288L), which is PS2-based. The table shows EC50 values (± SEM) determined for PS1 and PS2, as controls, and for the two triple mutants, for 2 sulfonamide inhibitors (ELN318463, BMS299897), as well as 2 non-sulfonamide inhibitors (DAPT and L-685,458).

Figure 8. Activity of alanine scan mutants as assessed by Aβ production. Graphs above show the relative Aβ levels of the mutants, expressed as a ratio of Aβ produced from the mutant relative to wild type PS1. The activity of Ala-scan mutants in the C2 and C5 regions of PS was assessed by measuring total Aβ produced (1-x) from the mutant compared with total Aβ from wild type PS1, within the same experiment. The arrows denote the select mutations identified in Table 1 & 2, which affected the potency of multiple compounds. Red arrows indicate mutants that increased potency >2X, black arrows indicate mutants that decreased potency >2X. Y288A mutation is inactive. A. A-beta production from alanine-scan mutants in the C2 sub-region relative to wild-type PS1. B. A-beta production from alanine-scan mutants in the C5 sub-region relative to wild-type PS1. In A and B, the values for A-beta production are derived from n=3 independent experiments, and the error bars denotes standard error of the mean. C. Assessment of Aβ42/Aβ1-x ratio from select alanine mutants of FAD residues (n=2).

Figure 9. (A) APP expression levels in cells transfected with different chimeric PS constructs were determined by semi-quantitative western blots using an APP C-term antibody (Sigma). The relative APP level from different PS chimeras is expressed relative to the signal intensity of APP from wt PS1. (B) Western blot of cell lysates (10 µg/lane) from cells transfected with PS1 alanine scan constructs, indicated above each lane, probed with PS1 NTF, PS1-CTF, and APP C-term antibodies, respectively.

Table 1. Effect of alanine scan mutagenesis of residues in the C2 sub-region on inhibitor potencies. After deriving EC50 values for each compound from every mutant construct, the EC50 value for a given Ala mutation was normalized to the EC50 value of the wild-type PS1 (derived from the same experiment) to obtain a ratio. The effect of mutations can be analyzed by examining this ratio. Statistically significant differences in EC50 ratios (see methods) are indicated in solid black font color, ratios >5X are denoted in boldface, and non-significant ratios (p>0.05) are denoted in light-grey font color. FAD associated residues are indicated by arrowheads. Black arrows identify residues that significantly lowered potencies of all 4 inhibitors tested, red arrows denote residues which significantly increased potency of the inhibitor(s) tested. The data in this figure are derived from n = 3 independent transfections and EC50 determinations. We included two PS1 transfections as a control within each experimental set (9-12 constructs total/set) to enable comparing mutant PS constructs among different experiments.
Table 2. Effect of alanine scan mutagenesis of residues conserved between PS1 & PS2 in the C5 sub-region on inhibitor potencies. Designation of residues, and inhibitor potencies are same as in Table 1.
Figure 1

A. ELN318463

- PS1, EC50: 12 nM
- PS2, EC50: 656 nM

B. BMS299897

- PS1, EC50: 8.0 nM
- PS2, EC50: 308 nM

C. DAPT

- PS1, EC50: 56 nM
- PS2, EC50: 25 nM

D. L685,458

- PS1, EC50: 17 nM
- PS2, EC50: 44 nM
| Chimeras   | Fragment origins                        |
|-----------|----------------------------------------|
| PS1/2A    | 1-127 (PS1), 134-448 (PS2)            |
| PS1/2B    | 1-298 (PS1), 305-448 (PS2)            |
| PS1/2C    | 1-127 (PS1), 134-304 (PS2), 299-467 (PS1) |
| PS2/1C    | 1-133 (PS2), 128-298 (PS1), 305-448 (PS2) |
| PS1/2C1   | 1-127 (PS1), 134-170 (PS2), 165-467 (PS1) |
| PS1/2C2   | 1-164 (PS1), 171-200 (PS2), 195-467 (PS1) |
| PS1/2C3   | 1-194 (PS1), 201-221 (PS2), 216-467 (PS1) |
| PS1/2C4   | 1-215 (PS1), 222-274 (PS2), 269-467 (PS1) |
| PS1/2C5   | 1-268 (PS1), 275-304 (PS2), 299-467 (PS1) |

**Figure 2**
### Figure 3

| Constructs | ELN318463 | BMS299897 | DAPT  | L685,458 |
|------------|-----------|-----------|-------|----------|
| PS1        | 16 ± 4    | 8 ± 0     | 58 ± 2| 17 ± 0   |
| PS2        | 813 ± 157 | 278 ± 31  | 27 ± 2| 48 ± 5   |
| PS1/2A     | 1125 ± 248| 234 ± 1   | 34 ± 1| 30 ± 4   |
| PS1/2B     | 19 ± 4    | 10 ± 1    | 47 ± 9| 31 ± 2   |
| PS1/2C     | 1797 ± 15 | 217 ± 5   | 38 ± 2| 20 ± 2   |
| PC2/1C     | 14 ± 5    | 9 ± 0.5   | 77 ± 10| 54 ± 4  |
| Construct | ELN318463 (EC50, nM) | BMS299897 (EC50, nM) |
|-----------|----------------------|----------------------|
| PS1       | 24±2                 | 8±1                  |
| PS2       | 1578±262             | 369±28               |
| PS1/2C1   | 18±0.5               | 5±1                  |
| PS1/2C2   | 78±8                 | 20±0.5               |
| PS1/2C3   | 31±4                 | 8±1                  |
| PS1/2C4   | 21±8                 | 7±3                  |
| PS1/2C5   | 430±24               | 34±2                 |

Figure 4
Figure 5
Figure 6
|                | ELN318463 EC50 (nM) | BMS299897 EC50 (nM) | DAPT EC50 (nM) | L-685,458 EC50 (nM) |
|----------------|---------------------|---------------------|----------------|---------------------|
| PS1            | 21 ± 2              | 8 ± 0.6             | 58 ± 3         | 13 ± 1              |
| PS2            | 850 ± 58            | 286 ± 22            | 23 ± 2         | 32 ± 5              |
| Triple PS1     | 824 ± 102           | 108 ± 9             | 85 ± 3         | 19 ± 2              |
| Triple PS2     | 28 ± 3              | 29 ± 6              | 24 ± 4         | 20 ± 3              |

**Figure 7**
Figure 8

A. Relative A-beta from C2 sub-region

B. Relative A-beta from C5 sub-region

C. Alanine mutation of FAD residues

- A-beta ratio (mutant/PS1)

Constructs

- PS1
- L166A
- S169A
- R278A
- L286A
- V234A

Normalized A\(\beta\)42/A\(\beta\)1-x

0.0 0.5 1.0 1.5

0.0 0.5 1.0 1.5

0 1 2 3 4 5

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Figure 9

A

Relative APP level

DNA constructs

B

µg cell lysate

Anti-PS1N

Anti-PS1C

Anti-APP

PS1, L166A, S169A, R278A, R278A+L286A, L286A, Y288A

5µg, 10µg, 20µg, 30µg
| Compound | ELN318463 | BMS299897 | DAPT | L685,458 |
|----------|-----------|-----------|------|---------|
| Mutant/PS1 | Mutant/PS1 | Mutant/PS1 | Mutant/PS1 | Mutant/PS1 |
| V161A | 1.0 | ns | 1.3 | ns | 1.5 | ns | 1.6 | ns |
| I162A | 3.1 | 0.0003 | 3.3 | 0.002 | 2.4 | 0.005 | 4.4 | 0.013 |
| H163A | 1.3 | ns | 1.5 | ns | 1.3 | ns | 1.7 | ns |
| W165A | 2.3 | 0.003 | 2.5 | 0.002 | 3.7 | 0.002 | 2.0 | 0.030 |
| L166A | 11.2 | 0.0003 | 9.1 | 0.003 | 5.1 | 0.009 | 3.5 | 0.006 |
| I167A | 0.9 | ns | 1.0 | ns | 1.4 | ns | 2.3 | ns |
| I168A | 1.4 | ns | 1.8 | 0.001 | 1.7 | 0.036 | 1.1 | ns |
| S169A | 0.4 | 0.008 | 0.4 | 0.003 | 0.4 | 0.040 | 0.9 | ns |
| S170A | 4.5 | 0.005 | 2.6 | 0.003 | 0.8 | ns | 1.4 | ns |
| L171A | 1.0 | ns | 1.1 | ns | 0.6 | ns | 0.8 | ns |
| L172A | 2.6 | ns | 2.0 | ns | 0.7 | ns | 1.5 | ns |
| L173A | 5.8 | 0.002 | 3.3 | 0.001 | 2.2 | 0.0002 | 0.9 | ns |
| L174A | 1.7 | 0.009 | 1.3 | ns | 1.6 | 0.021 | 2.0 | 0.021 |
| F175A | 1.3 | ns | 2.4 | 0.025 | 0.9 | ns | 1.6 | ns |
| F176A | 0.7 | ns | 0.9 | ns | 0.7 | ns | 1.5 | ns |
| PS1 | 1.0 | ns | 1.0 | ns | 1.0 | ns | 1.0 | ns |

ns: p > 0.05
Table 2. C5 sub-region Alanine scan

| Compound | ELN318463 | BMS299897 | DAPT | L685,458 |
|----------|-----------|-----------|------|----------|
| Mutant   | Mutant/PS1| Mutant/PS1| Mutant/PS1| Mutant/PS1|
|          | EC50 ratio| p        | EC50 ratio| p        | EC50 ratio| p        | EC50 ratio| p        |
| L271A    | 6.1       | 0.001    | 13.7   | <0.0001  | 2.2       | 0.019    | 13.2     | <0.0001  |
| V272A    | 23.2      | <0.0001  | 0.9     | ns       | 0.9       | ns       | 2.5       | 0.005    |
| E273A    | 2.0       | 0.045    | 1.3     | ns       | 1.8       | ns       | 3.2       | 0.001    |
| T274A    | 11.1      | ns       | 1.1     | ns       | 1.5       | ns       | 1.3       | ns       |
| Q276A    | 1.8       | ns       | 0.8     | ns       | 0.9       | ns       | 1.6       | ns       |
| E277A    | 1.2       | ns       | 1.4     | ns       | 0.8       | ns       | 1.8       | 0.010    |
| R278A    | 17.5      | <0.0001  | 12.6    | 0.010    | 8.6       | <0.0001  | 119.2     | <0.0001  |
| N279A    | 1.6       | ns       | 1.2     | ns       | 1.1       | ns       | 2.7       | 0.0001   |
| E280A    | 5.4       | <0.0001  | 2.1     | ns       | 2.7       | 0.0002   | 2.8       | 0.002    |
| T281A    | 0.7       | ns       | 1.0     | ns       | 0.6       | 0.012    | 1.0       | ns       |
| L282A    | 10.1      | <0.0001  | 2.3     | 0.006    | 2.8       | 0.006    | 1.5       | 0.006    |
| F283A    | 1.6       | ns       | 0.3     | 0.011    | 1.2       | ns       | 1.8       | 0.032    |
| P284A    | 2.5       | ns       | 1.8     | ns       | 1.7       | 0.012    | 3.2       | 0.0004   |
| L286A    | 22.1      | <0.0001  | 17.1    | <0.0001  | 6.2       | <0.0001  | 10.2      | <0.0001  |
| I287A    | 4.2       | <0.0001  | 7.1     | <0.0001  | 6.9       | <0.0001  | 2.5       | 0.001    |
| Y288A    | x         | x        | x       | x        |          |          |          |          |
| S289A    | 1.6       | ns       | 1.1     | ns       | 0.6       | ns       | 1.5       | ns       |
| S290A    | 0.8       | ns       | 0.7     | ns       | 0.5       | ns       | 0.9       | ns       |
| M292A    | 1.4       | ns       | 1.0     | ns       | 0.7       | ns       | 1.5       | ns       |
| V293A    | 1.2       | ns       | 0.9     | ns       | 0.9       | ns       | 1.3       | ns       |
| W294A    | 1.6       | ns       | 1.8     | ns       | 1.4       | ns       | 2.2       | 0.002    |
| PS1      | 1.0       | 1.0      | 1.0     | 1.0      |

ns: p > 0.05
Identification of γ-secretase inhibitor potency determinants on Presenilin
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