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Modulators of the Gamma Secretase Reciprocally Regulate Short and Long Aβ Peptides and Spare ε-Site Cleavages

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Introduction

Recent studies have shown that a subset of non-steroidal anti-inflammatory drugs (NSAIDs) preferentially reduce the production of Amyloid β1-42 (Aβ 1-42) and induce Aβ 1-38 production, while failing to inhibit Notch cleavage [20]. Potent compounds that modulate the activity of the gamma secretase in this manner may have advantages over classical gamma secretase inhibitors for Alzheimer’s disease therapeutics [5,18].

In addition, recent work has highlighted the similarities in the pattern of presenilin−dependent, intramembraneous endoproteolytic cleavage of substrates of the gamma secretase [6]. Both APP and Notch sustain so-called γ-site cleavages, resulting in the proteolysis at one of several peptide bonds that lie centrally within the transmembrane alpha helix. For APP, γ−site cleavages result in Aβ peptides of varying length; Aβ x-37, x-38, x-39, x-40, x-41, x-42, x-43 have all been demonstrated. A similar pattern of γ−site cleavage has been demonstrated for Notch proteins,
generating polypeptides termed Notch-β. A second presenilin-dependent, gamma secretase cleavage is located somewhat closer to the intracellular leaflet of the transmembrane domain and is termed the epsilon- (ε-) site. The ε-cleavage site also generates peptides of varying length due to cleavage within a stretch of amino acids. Originally demonstrated for the Notch proteins and termed the S3 site, recent work has shown that APP proteins are cleaved at ε-sites at positions 49, 50, or 51 relative to the first amino acid of Aβ. Several other substrates of APP have also recently been shown to sustain epsilon-site cleavage as a result of the presenilin-dependent gamma secretase [6].

To facilitate drug discovery efforts, assays to measure the peptides Aβ 1-38, Aβ 1-40, Aβ 1-42 generated by γ-site cleavages and reporter-transfection assays to measure the ε-site cleavages of Notch and APP were adopted for high-throughput. Using these tools and standard assays to measure the activities of the gamma secretase, NSAIDs and gamma secretase inhibitors were classified pharmacologically. The results suggest that the mechanism of action for modulators of the gamma secretase involves selective action at γ-site cleavages with relative sparing of ε-site cleavages for any substrate. The present studies are also consistent with a model in which modulators of the gamma secretase shift cleavage of the 42-43 bond to cleavage of the 38-39 bond by an unknown mechanism.

**Methods**

For analysis of the γ-site cleavage, neoepitope specific polyclonal antibodies were developed against Aβ 1-38 to allow detailed pharmacological studies in a high throughput format. Rabbits were immunized with hCys-aminohexanoyl-Ala-Ile-Ile-Gly-Leu-Met-Val-Gly-GlyOH as described previously [16]. An ELISA using mAb 6E10 (Senetek, Maryland Heights, MO) as the capture antibody and Aβ1-38 (Rb341 at 1:4000) detection was adapted for use in 96-well or 384-well format. This assay was sensitive to 30 pg/ml Aβ 1-38 but not to Aβ 1-40 and Aβ 1-42 (≤8 ng/ml tested). Neoepitope specific rabbit polyclonal antibodies to Aβ 1-40 (Rb208) and Aβ 1-42 (Rb321) were used at 1:2000 and 1:3000, respectively, with 6E10 as the capture antibody. HEK lines expressing APP-SwKK with the addition of overexpressed hPS1-M146V were used for Aβ detection experiments as described [8,11]. Table 1 summarizes high throughput results and Table 2 summarizes traditional assays run in parallel with Notch cleavage assays.

For ε-site analysis, Notch- and APP-Gal4-VP16 (GV) promoter transfection assays [1,9] were adapted for an automated 384-well plate format. Co-transfection of either Notch- or APP-GV expression vectors and UAS-firefly luciferase into HEK293 APPSwKK cells along with TK-renilla luciferase as a control for transfection efficiency were performed as described [11]. ε-Site cleavage in Notch was measured by NICD production using western blot of HEK293 APP-SwKK cells transfected with Notch1ΔE (M-V substitution) after incubating in the presence of inhibitor compounds for 18 hr [1]. AICD production was measured in microsomal membrane preparations of HEK293 APPSwKK cells incubated at 37°C in the presence of inhibitors for 30 min [4]. T47D cells, a breast carcinoma line, and the anti-ErbB4 antibody Ab2 (Neomarkers) were used to measure ErbB4 CTF accumulation, an indicator of ε-cleavage [10,15].
Results

Pharmacology of gamma secretase inhibitors and NSAIDs in modulating subspecies production

High throughput ELISA assays for the three Aβ subspecies Aβ 1-38, 1-40, and 1-42 were used to classify NSAIDs and gamma secretase inhibitors with regard to their behavior at three subsites within the γ-site of APP. Detailed dose-response curves are presented in Fig.1. A highly potent inhibitor γ-secretase inhibitor, LY411575 [19], showed coordinate inhibition of all three Aβ subspecies with very similar potencies for each subspecies in the subnanomolar range. Similarly a peptidomimetic inhibitor L685,458 [17] elicited coordinate blockade of the three Aβ subspecies. However, a small degree of induction of Aβ 1-42 above its baseline levels (maximum 1.5-fold) was also observed. Other published gamma secretase inhibitors [19] showed similar behavior with coordinate inhibition of Aβ 1-38, 1-40, and 1-42 production with or without some degree of induction of Aβ 1-42 (Table 1).

Table 1. Inhibition or induction of Aβ subspecies by gamma secretase inhibitors

<table>
<thead>
<tr>
<th>Compound</th>
<th>Cell based IC50 or ED50* (nM)</th>
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<tbody>
<tr>
<td></td>
<td>Aβ 1-40</td>
</tr>
<tr>
<td>LY411575</td>
<td>0.36</td>
</tr>
<tr>
<td>L685,458</td>
<td>271</td>
</tr>
<tr>
<td>DAPT</td>
<td>287</td>
</tr>
<tr>
<td>Fenchylamine</td>
<td>9,900</td>
</tr>
<tr>
<td>MW167</td>
<td>17,600</td>
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</table>

A markedly different profile was found for certain NSAIDs, including sulindac sulfide, indomethacin, ibuprofen and flurbiprofen which have been termed gamma secretase modulators. The pharmacologic profile of modulatory NSAIDs is characterized by a marked (1.5-2-fold) induction of Aβ 1-38, an inhibition of Aβ 1-42 and little effect on Aβ 1-40 production. In all cases, the range of induction for Aβ 1-38 was very similar to the range of inhibition for Aβ 1-42. Although Aβ 1-40 was not affected at the concentrations tested, at higher concentrations production of all three Aβ subspecies was reduced. However, cell viability also declined at these concentrations making it difficult to distinguish between cellular damage and selective inhibition. In many cases the ED50 and IC50s for the modulatory NSAIDs could not be calculated because a complete curve could not be generated. Cell lines expressing a presenilin mutant PS1-M146V were used to enhance the effect of modulators [21,11]

Sulindac sulfide showed the most potent modulatory behaviour with inhibition of Aβ 1-42 (IC50 31µM) and induction of Aβ 1-38 in the same concentration range. Indomethacin was also potent with an IC50 for Aβ 1-42 of 49 µM, and an ED50 for Aβ 1-38 induction of 49 µM. Both stereoisomers of ibuprofen showed similar potencies as the racemic ibuprofen which showed an IC50 near the limit of detection (429 µM) [13]. Fenoprofen was also a weak modulator. Other NSAIDs showed a second profile in these assays: weak or no effects upon Aβ subspecies production. In contrast to sulindac sulfide, its prodrug, sulindac sulfoxide, was virtually inactive at concentrations tested. Naproxen, dapsone, and piroxicam were also inactive at modulating Aβ production despite their potency at inhibition of COX-1 and ~2.
A third pharmacological class of NSAIDs included meclofenamic acid and diclofenac. These compounds showed coordinate inhibition of all three \( \text{A}\beta \) subspecies in a similar concentration range, and are termed inhibitory NSAIDs because of their similarity to the classical gamma secretase inhibitors. Diclofenac showed an IC50 for \( \text{A}\beta \) 1-42 of 86 \( \mu \)M, for \( \text{A}\beta \) 1-40 of 120 \( \mu \)M and >300 \( \mu \)M for \( \text{A}\beta \) 1-38. Meclofenamic acid showed an IC50 for \( \text{A}\beta \) 1-42 of 69 \( \mu \)M, for \( \text{A}\beta \) 1-40 of 114 \( \mu \)M and for \( \text{A}\beta \) 1-38 of >300 \( \mu \)M.

The peptidomimetic gamma secretase inhibitor MW167 [22] displayed a unique pharmacological pattern with regard to the three \( \text{A}\beta \) subspecies. At lowest concentrations, MW167 elicited an inhibition of \( \text{A}\beta \) 1-38 and a significant induction (2-fold) of \( \text{A}\beta \) 1-42. The IC50 for \( \text{A}\beta \) 1-38 inhibition was 2.0 \( \mu \)M and the ED50 for \( \text{A}\beta \) 1-42 induction was 3.9 \( \mu \)M. However, at higher concentrations, \( \text{A}\beta \) 1-40 was inhibited (IC50 17.6 \( \mu \)M). At higher concentrations still, all \( \text{A}\beta \) production was blocked [22]. The pharmacological profile of this compound below 20 \( \mu \)M is opposite to that of the NSAID modulators and is therefore termed an inverse modulator of \( \text{A}\beta \) subspecies production.

**Classification of gamma secretase inhibitors and NSAIDS at the epsilon site**

Chimeric constructs encoding APP and Notch fused to Gal4-VP16 were used to measure the presenilin-dependent gamma secretase cleavage of the \( \epsilon \)-site of APP and Notch [1,9]. These assays have been previously used to measure total gamma secretase cleavage of Notch and APP. However, since \( \epsilon \)-site cleavage alone is sufficient to release the intracellular domain of a chimeric APP or Notch protein, a reduction in cleavage and release of the chimeric protein would require an inhibition of the \( \epsilon \)-site cleavage. Detailed dose-response curves for gamma secretase inhibitors and NSAIDs are presented in Fig. 2. The potent inhibitors LY411575 and L685458 showed a single phase of inhibition with virtually identical potency for both Notch- and APP-GV constructs. The potency of these compounds in the Notch and APP-GV assays closely matched their potencies in inhibiting production of the three \( \text{A}\beta \) subspecies (see Fig 1).

The NSAID modulators, sulindac sulfide, indomethacin, flurbiprofen and ibuprofen showed inhibition of Notch and APP-GV assays only at very high concentrations (0.3-1 mM) at which cell viability was uncertain (Fig 2). At concentrations that induced \( \text{A}\beta \) 1-38 and blocked \( \text{A}\beta \) 1-42, the modulators had no effect upon the Notch or APP-GV assays, consistent with a selective sparing of the \( \epsilon \)-site cleavages. Naproxen and meclofenamic did not show any significant potency in Notch- or APP-GV assays, consistent with their lack of effect upon the gamma secretase.

The inverse modulator MW167 showed inhibitory potency in the range of 10-100 \( \mu \)M in both Notch- and APP-GV assays. This range of potency is 10-100 fold weaker than that observed for its induction of \( \text{A}\beta \) 1-42 and inhibition of \( \text{A}\beta \) 1-38, and was more similar to its potency for its \( \text{A}\beta \) 1-40 inhibition (See Fig 1). Fenchylamine showed an inhibition of APP and Notch-GV assays at about 3 \( \mu \)M, approximately equivalent to its inhibitory potency for the three \( \text{A}\beta \) subspecies, but somewhat higher than its partial induction of \( \text{A}\beta \) 1-42.

For confirmation, traditional assays for \( \epsilon \)-site cleavages were performed in the presence of NSAIDs and inhibitors. Notch cleavage and the production of NICD are shown in Table 2.
For the gamma secretase inhibitors LY411575, DAPT and fenchylamine, the ratio of concentrations for Aβ 1-42 inhibition and NICD inhibition is approximately one. A somewhat increased ratio was seen for BMS-494, a potent sulfonamide inhibitor [19]. For the NSAID modulators sulindac sulfide, flurbiprofen, indomethacin and ibuprofen, no inhibition was observed for NICD production at the concentrations tested, resulting in ratios for which only the lower limit was determined. For the most potent modulator tested sulindac sulfide, the ratio was at least 10, while for weaker compounds solubility or cell toxicity limited an accurate determination of the ratio.

**Table 2.** Comparison of Inhibition of Aβ 1-42 and Notch cleavage

<table>
<thead>
<tr>
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<th>Cell Based IC50 (nM)</th>
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<tr>
<td></td>
<td>Aβ 1-42</td>
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<tr>
<td>LY411575</td>
<td>0.08</td>
</tr>
<tr>
<td>BMS-494</td>
<td>80</td>
</tr>
<tr>
<td>DAPT</td>
<td>100</td>
</tr>
<tr>
<td>Fenchylamine</td>
<td>2,500</td>
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<tr>
<td>Sulindac sulfide</td>
<td>31,000</td>
</tr>
<tr>
<td>Indomethacin</td>
<td>49,000</td>
</tr>
<tr>
<td>Flurbiprofen</td>
<td>253,000</td>
</tr>
<tr>
<td>Ibuprofen (rac)</td>
<td>429,000</td>
</tr>
</tbody>
</table>

AICD production assays (Fig. 3a) demonstrated a marked sensitivity to the potent gamma secretase inhibitors LY411575, DAPT, and L685458. The concentration range for inhibition of AICD production was similar to those found for inhibition of the Aβ subspecies as well as APP- and Notch-GV. As seen for the Notch and APP-GV assays, NSAID modulators, as well as inactive NSAIDs, failed to block AICD production at the concentrations tested (<500 µM). Also consistent with the observations from Notch and APP-GV assays, MW167 was weak at blocking AICD, showing only a partial response at 100 µM, considerably higher than its potency in blocking Aβ 1-38.

ErbB4 is also a substrate of the presenilin-dependent gamma secretase, and its cleavages at the ε-site may be monitored by the accumulation of a polypeptide lacking its extracellular domain, the ErbB4-CTF. In the presence of inhibitors, ErbB4-CTFs accumulate because its cleavage to smaller soluble products is blocked [15]. The dose-response of cells exposed to NSAIDs and gamma secretase inhibitors (Fig 3b) shows that the potent gamma secretase inhibitors caused an accumulation of ErbB4-CTFs, while all NSAIDs tested failed to cause accumulation at the concentrations tested, confirming their inactivity at the ε-site of ErbB4.

**Discussion**

Use of traditional and high throughput assays for γ-site and ε-site cleavages of the presenilin-dependent gamma secretase has led to a pharmacologic classification of NSAIDs and γ-secretase inhibitors into modulators, reverse modulators, inhibitors, and inactive compounds. At the γ-site, modulators showed complex behavior with induction of Aβ 1-38 in the same concentration range as inhibition of Aβ 1-42 production. Cleavage at the ε-site of Notch, APP
and Erb-B4 was preferentially spared by modulators compared to the efficacy of modulators in blocking Aβ1-42 production. Inhibitors showed little or no selectivity toward the Aβ subspecies, and also blocked ε-site cleavage in the same concentration range, consistent with a coordinate reduction of gamma-secretase-mediated activities at all the ε- and γ-sites. The peptidomimetic inhibitor of the gamma secretase MW167 had inverse modulatory properties at low concentrations at which it induced Aβ 1-42 [23] and, in the same concentration range, inhibited Aβ 1-38 production. It is worth noting that other inhibitors showed some modulatory behavior, mainly consisting of an induction of Aβ 1-42.

The mechanism by which modulators elicit these characteristic changes in the Aβ subspecies is an open question that will likely be answered when the multiple transmembrane components of the gamma secretase complex [7] are purified in an active form, and studied structurally and pharmacologically. At present, major questions remain as to what determines substrate specificity of the gamma secretase, whether or not proteins are cleaved at one or more sites sequentially, and whether or not additional enzymatic activities outside of the presenilin complex serve important roles in the different functions that have been attributed to the gamma secretase.

Nonetheless, the studies presented here give evidence that a reciprocal relationship exists between cleavage of the 38-39 and the 42-43 peptide bonds of APP (numbered as in Aβ) by the gamma secretase. This relationship results in reciprocal rates of production of Aβ 1-38 and Aβ 1-42 regulated by modulators and inverse modulators. Although the mechanism is not understood, some structural insights are provided by a helical wheel diagram of the membrane-spanning domain of APP that places the 38-39 and 42-42 peptide bonds on the same side of the α-helix, and the 40-41 bond on the opposite side (Fig 4). If an active site residue such as an aspartyl residue of the gamma secretase complex were positioned on the same side of the substrate α-helix as the 38-39 and the 42-43 bonds, its relative ability to hydrolyze one bond or the other may be determined by its relative proximity to the one or the other. In such a model, a shift orthogonal to the membrane of the relative position of the transmembrane α-helix of APP with respect to the substrate binding cleft of the gamma secretase complex would reduce the proximity to one bond and increase it to the other, possibly resulting in reduced cleavage of one of the bonds and enhanced cleavage of the other. Such a shift could be caused by a conformational change of the gamma secretase, or a shift orthogonal to the membrane of either the substrate or enzyme complex. The data presented here are consistent with a model where modulators and reverse modulators elicit such a shift, and thereby reciprocally regulate the production of Aβ 1-38 and Aβ 1-42. In addition, the positioning of the shifting active site residue opposite to the 40-41 bond could also explain why Aβ 1-40 production is not altered by modulators. It should also be noted that an enhancement of Aβ 1-42 production is a key mechanistic consequence of missense mutations that are associated with familial Alzheimer’s disease mutations in the presenilin proteins. Under the model proposed above, it is also conceivable that missense mutations cause a shift of substrate relative to active site that resembles the inverse modulators resulting in an enhancement of Aβ 1-42 production with little effect on Aβ1-40, and in some cases a reduction in Aβ 1-38 [14]. This model fits with recent observations showing a conformational shift in PS1 and in the the distance between PS1 and APP after NSAID treatment [12].
Finally, the development of drugs that modulate the gamma secretase is likely to have advantages over gamma secretase inhibitors for Alzheimer’s disease therapeutics [20,5]. Such compounds would selectively reduce the production of the toxic Aβ (x-42) peptides, opposite to the effects of the familial PS mutations. Modulators would operate without affecting the epsilon site cleavages that are known to be essential for Notch signal transduction, the release of the transcriptional co-activator function of AICD [3] and possibly other proteins that are activated by regulated intracellular proteolysis [2]. Although the modulator mechanism has several attractive advantages, it is presently unclear whether beneficial or toxic effects will result from the induction of Aβ 1-38 or the sparing of Aβ 1-40.

**Figure Legends**

**Figure 1. Effect of Inhibitors and NSAIDs on Aβ 1-38, Aβ 1-40, Aβ 1-42 production**

Zero percent inhibition is defined as the value in the absence of compound. A negative % inhibition reflects an induction. IC50 values were calculated in cases where complete curves were generated and cell viability was unimpaired. Symbols: Aβ 1-38 (square), Aβ 1-42 (triangle point down), Aβ 1-40 (triangle point up).
Figure 2. Effect of Inhibitors and NSAIDs on APP- and Notch-Gal4-VP16 transcription-signaling assays.
Zero percent inhibition is defined as the normalized luciferase activity in the absence of compound. Normalized luciferase activity was determined by dividing UAS-firefly luciferase by TK-renilla luciferase. Symbols: APP-CT (square), Notch-IC (triangle).
Figure 3. Effect of Inhibitors and NSAIDs on ε-site cleavage assays:

Figure 3A. AICD production,

Figure 3B. Accumulation of ErbB4-CTFs
Figure 4. Helical wheel representation of the transmembrane domain from APP and a model for the shifting orientation of scissile peptide bonds with respect to active site residues
(A) Helical wheel diagram shows 38-39 and 42-43 peptide bonds on the same side of the APP transmembrane α-helix and opposite to the 40-41 bond (numbering with respect to Aβ). (B) Gamma secretase modulators shift the position of an active site aspartyl residue away from the 42-43 peptide bond and closer to the 38-39 bond (from left to right in the diagrams) producing greater endoproteolytic production of Aβ fragments terminating in residue 38 (e.g., Aβ 1-38) than 42 (Aβ 1-42). The aspartyl residue is shifted by an inverse modulator and certain PS mutations to a position closer to the 42-43 bond and away from the 38-39 bond (right to left), increasing cleavage of the 42-43 bond and increasing Aβ1-42 production at the expense of Aβ 1-38.

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References


