Second Generation γ-Secretase Modulators Exhibit Different Modulation of Notch β and Aβ Production*

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Background: The γ-secretase complex is a drug target in the treatment of Alzheimer disease (AD).

Examples: Two novel second generation γ-secretase modulators (GSMs) modulate both Nβ and Aβ but not Notch intracellular domain (NICD) production.

Conclusion: Second generation and NSAID-based GSMs have different modes of action regarding Notch processing.

Significance: GSMs that do not affect NICD signaling are essential for the development of tolerable AD therapeutics.

The γ-secretase complex is an appealing drug target when the therapeutic strategy is to alter amyloid-β peptide (Aβ) aggregation in Alzheimer disease. γ-Secretase is directly involved in Aβ formation and determines the pathogenic potential of Aβ by generating the aggregation-prone Aβ42 peptide. Because γ-secretase mediates cleavage of many substrates involved in cell signaling, such as the Notch receptor, it is crucial to sustain these pathways while altering the Aβ secretion. A way of avoiding interference with the physiological function of γ-secretase is to use γ-secretase modulators (GSMs) instead of inhibitors of the enzyme. GSMs modify the Aβ formation from producing the amyloid-prone Aβ42 variant to shorter and less amyloidogenic Aβ species. The modes of action of GSMs are not fully understood, and even though the pharmacology of GSMs has been thoroughly studied regarding Aβ generation, knowledge is lacking about their effects on other substrates, such as Notch. Here, using immunoprecipitation followed by MALDI-TOF MS analysis, we found that two novel, second generation GSMs modulate both Notch β and Aβ production. Moreover, by correlating S3-specific Val-1744 cleavage of Notch intracellular domain (Notch intracellular domain) to total Notch intracellular domain levels using immunocytochemistry, we also demonstrated that Notch intracellular domain is not modulated by the compounds. Interestingly, two well characterized, nonsteroidal anti-inflammatory drugs (nonsteroidal anti-inflammatory drug), R-flurbiprofen and sulindac sulfide, affect only Aβ and not Notch β formation, indicating that second generation GSMs and nonsteroidal anti-inflammatory drug-based GSMs have different modes of action regarding Notch processing.

Amyloid-β precursor protein (APP) is processed into the Alzheimer-related amyloid-β peptide (Aβ) via a proteolytic event that is sequentially mediated by the membrane-integral β- and γ-secretases (1). After ectodomain shedding of APP, γ-secretase processes the membrane-bound stub, C99, generating Aβ peptides of different lengths (γ cleavage), and simultaneously, the APP intracellular domain is released into the cytosol (ε cleavage) (1). Aβ is manifested in many different forms (2), although Aβ40 and Aβ42 are among the most abundant species (3–5). The longer Aβ42 peptide is more prone to aggregate into toxic soluble oligomers (6–8), before eventually forming the insoluble plaques observed in the brains of Alzheimer disease (AD)-affected patients (9).

γ-Secretase is an unusual intramembrane-cleaving protease, composed of presenilin 1 or 2, which harbors the catalytic site, Aph-1-a or -b, nicastrin, and Pen-2 (10–13). The γ-secretase complex processes several type I membrane-bound proteins, including the Notch receptor. Notch is an important signaling molecule in cell differentiation during development as well as adulthood. Notch is processed in a manner similar to APP, generating Nβ peptides (54 cleavage) and the Notch intracellular domain (NICD) (S3 cleavage) (14, 15), which translocates to the nucleus and acts as an essential regulator of transcription factors (16).

The γ-secretase complex is an appealing drug target when the therapeutic strategy is to alter the metabolism of Aβ. It is
Second Generation GSMS Modulate Both N\(\beta\) and A\(\beta\) Formation

directly involved in the A\(\beta\) formation, and it also determines the pathogenic potential of A\(\beta\). Because \(\gamma\)-secretase mediates cleavage of many substrates involved in cell signaling, such as the Notch receptor, it is crucial to sustain these pathways while inhibiting toxic A\(\beta\)-secretion. However, investigations attempting to find \(\gamma\)-secretase inhibitors (GSIs) with a sufficient therapeutic window between APP and Notch processing has been extremely challenging (17). Moreover, severe side effects, probably caused by abrogated Notch signaling, were present, causing a recent large clinical phase 3 trial to be interrupted (reviewed in Ref. 18). Alternative strategies to combat A\(\beta\) production is therefore clearly needed, and \(\gamma\)-secretase modulators (GSMs) represent a growing and promising class of anti-amyloidogenic drugs. Importantly, Notch inhibition is avoided using GSMS instead of GSIs. Typically, GSMS do not affect the overall rate of Notch and APP processing (\(\varepsilon\) and S3 cleavage). Instead, by shifting the cleavage preference of the enzyme from producing the amyloid-prone A\(\beta\)42 variant to shorter and less toxic A\(\beta\) species, GSMS change the proportions of various A\(\beta\) peptides that are formed (19, 20). The first GSMS, subsets of nonsteroidal anti-inflammatory drugs (NSAIDs), such as sulindac sulfide and ibuprofen, were identified in 2001 (19). Since then, many second generation GSMS have brought improvement. These compounds, which are generally structurally distinct from the NSAID family by the absence of an acidic carboxyl group, are more potent and efficient in the central nervous system than were the early GSMS (21–24). Some studies reported NSAID-based GSMS interacting with APP-derived C99 peptides (25–27), but other groups have challenged this implied substrate targeting hypothesis (28–31). During the last year, we and others identified \(\gamma\)-secretase instead of APP as the principal target of second generation GSMS (21, 32–36). However, even though the pharmacology of A\(\beta\) generation with respect to first and second generation GSMS has been thoroughly studied, little is known about their overall effect on \(\gamma\)-secretase-mediated cleavage of other substrates, such as the Notch receptor. In this study, we have developed an assay studying N\(\beta\) production and investigated, head to head, Notch and APP processing in presence of both first and second generation GSMS. These studies provide compelling evidence that it is possible to obtain selectivity between N\(\beta\) and A\(\beta\) production, a feature that may be important in development of GSMS for chronic treatment in AD.

**EXPERIMENTAL PROCEDURES**

**Compounds**—The GSIs dibenzazepine (DBZ) and L-685,458, as well as GSMS R-flurbiprofen and sulindac sulfide, were obtained from Sigma-Aldrich. AZ1136 was prepared as previously described (32), as was AZ4126, according to patent number WO2010132015.

**Ethical Permission**—All animal experiments were performed in accordance with relevant guidelines and regulations provided by the Swedish Board of Agriculture. The ethical permission was provided by the Stockholm Södra Animal Research Ethical Board.

**Cell Culture**—HEK293 cells stably expressing human FLAG-Notch1-\(\Delta E\) (FLAG-N\(\Delta\)E) (32) or APPswe were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, nonessential amino acids, 10 \(\mu\)M Heps, and 300 \(\mu\)g/ml hygromycin or 100 \(\mu\)g/ml Zeocin, respectively. For each experiment, the cells were counted and plated in T75 flasks, 6- or 384-well plates (for N\(\beta\), A\(\beta\), and NICD experiments, respectively) the day before treatment. On the following day, the GSM, R-flurbiprofen (200 \(\mu\)M), sulindac sulfide (125 \(\mu\)M), AZ1136 (25 \(\mu\)M), AZ4126 (400 nM), or vehicle control (Me2SO) was separately added to fresh cell media and incubated for 24, 16, or 5 h (for N\(\beta\), A\(\beta\), and NICD experiments, respectively) before conditioned media or cells were analyzed.

**Immunoblotting**—The cells were treated as described above and lysed in cell lysis buffer (10 mM Tris, pH 8.1, 1 \(\mu\)M EDTA, 150 mM NaCl, 0.65% Igepal CA-630) supplemented with protease inhibitor mixture (Roche Applied Science). The protein levels were determined by the BCA protein assay kit (Pierce), separated on NuPAGE 4–12% gradient Bis-Tris gels with MES buffer (both Invitrogen), and transferred to nitrocellulose membranes (Bio-Rad), which were probed with \(\alpha\)-FLAG M2 (Sigma), Val-1744 (Cell Signaling), or \(\alpha\)-GAPDH (Acris Gmbh) primary antibodies. The blots were developed using horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) followed by Immobilon Western chemiluminescent HRP substrate (Millipore) using Amersham Biosciences Hyperfilm™ ECL (GE Healthcare).

**Immunoprecipitation (IP) and MS Analysis of N\(\beta\) and A\(\beta\) After a clarifying spin and addition of complete protease inhibitor (Roche Applied Science), collected media were immunoprecipitated with 40 \(\mu\)l of \(\alpha\)-FLAG M2-agarose (Sigma) or 5 \(\mu\)g of 4G8 (Covance) that were preincubated with a mixture of protein A- and G-Sepharose beads (GE Healthcare) for FLAG-tagged N\(\beta\) (F-N\(\beta\)) and A\(\beta\), respectively. The samples containing N\(\beta\) were incubated at 4 °C, washed, and eluted using a FLAG-tagged protein immunoprecipitation kit (Sigma) according to the manufacturer’s instructions. The A\(\beta\)-containing samples were incubated at room temperature for 2 h and washed three times for 10 min at 4 °C in PBS (pH 7.4). The immunoprecipitated peptides were then eluted in 100 \(\mu\)l of 0.1% TFA in 20% acetonitril. Eluted N\(\beta\) and A\(\beta\) peptides were concentrated using C18 ZipTips (Millipore) and re-eluted with 0.1% TFA in 50% acetonitril saturated with \(\alpha\)-cyano-4-hydroxy-cinnamic acid matrix and analyzed using MALDI-TOF MS. The experiments were performed in triplicate for N\(\beta\) and duplicate for A\(\beta\) and repeated five or six times. Control experiments included IP of media from cells without FLAG-N\(\Delta\)E (HEK293 cells) or beads without 4G8 antibodies attached, as well as cell media treated with two different \(\gamma\)-secretase inhibitors, L-685,458, and DBZ. MALDI-TOF MS measurements were performed using a Micromass M@LDI instrument (Waters). Each spectrum represents an average of 1000 shots acquired 10 at a time. In the spectrum a total peak height of +5 m/z, relative to the monoisotopic peak, was calculated for each isoform. Prior to analysis the peak heights were normalized to the sum of the peak height of all isoforms in the spectra, and an average was obtained from duplicated and triplicated samples, respectively. The data show relative changes among all the different isoforms in response to GSM treatment. It should be noted that a relative quantification cannot be interpreted as a direct reflection of an absolute or relative abundance of a species because the ionization efficiency
might be different for different isoforms and because different isoforms are more hydrophobic than others.

For tandem mass spectrometry (MS/MS) analysis, F-\(\text{N}\beta\) peptides were immunoprecipitated using the \(\alpha\)-\FLAG M2 antibody (Sigma) coupled to magnetic beads (2). Briefly, 10 \(\mu\)g of the antibody was added to 50 \(\mu\)l of magnetic Dynabeads M-280 sheep anti-mouse IgG (Invitrogen). Then antibody-coated beads were added to cell media and incubated at 4 °C for 2 h. After washing using the KingFisher magnetic particle processor, the F-\(\text{N}\beta\) peptides were eluted using 100 \(\mu\)l of 0.5% formic acid. Mass spectrometry measurements were performed using a Bruker Daltonics UltraFleXtreme MALDI-TOF/TOF instrument, and all of the samples were analyzed in duplicate. By result of MS analysis, several compounds were selected for tandem MS/MS, and 2000 single shot spectra were recorded of the precursor ions and 10000 of the fragment ions.

**Quantification of Secreted A\(\beta\) from Cells—HEK/APPswe cells were plated in 384-well plates and on the following day exposed to GSMs or vehicle control for 5 h, before conditioned media were analyzed as described previously (32). Briefly, A\(\beta\) levels were determined using Meso Scale Discovery (MSD) technology with C-terminally specific antibodies measuring A\(\beta\)1–X (where X indicates 37–42). For total A\(\beta\) levels, the 4G8 antibody was used.

**Animals and Animal Handling—**Female C57BL/6 mice (Harlan Laboratories), 10 weeks old at the time of drug administration, were kept in conventional housing and fed standard chow (Harlan Laboratories), 10 weeks old at the time of drug administration, were kept in conventional housing and fed standard (Harlan Laboratories), 10 weeks old at the time of drug administration, were kept in conventional housing and fed standard (Harlan Laboratories), 10 weeks old at the time of drug administration, were kept in conventional housing and fed standard (Harlan Laboratories), 10 weeks old at the time of drug administration, were kept in conventional housing and fed standard (Harlan Laboratories), 10 weeks old at the time of drug administration, were kept in conventional housing and fed standard (Harlan Laboratories), 10 weeks old at the time of drug administration, were kept in conventional housing and fed standard (Harlan Laboratories), 10 weeks old at the time of drug administration, were kept in conventional housing and fed standard

**RESULTS**

**γ-Secretase-mediated Notch Processing Results in Many Different Notch β Peptides—**To investigate how different structural classes of GSMs affect Notch processing, we used human embryonic kidney cells (HEK293) stably expressing an N-terminally FLAG-tagged human Notch1 \(\Delta E\) (FLAG-\(\Delta E\)) variant (Fig. 1A). Our first goal was to confirm that the FLAG-\(\Delta E\) variant is a γ-secretase substrate by examining the well characterized S3 cleavage. Using \(\alpha\)-\FLAG M2 and neo-specific Val-1744 antibodies on immunoblotting, we obtained a robust expression of FLAG-\(\Delta E\), as well as of NICD, which was abolished in the presence of the GSI, L-685,458 (Fig. 1A, right panel). To study the production of secreted FLAG-tagged Notch-\(\beta\) peptides (F-\(\text{N}\beta\)), conditioned media from HEK/FLAG-\(\Delta E\) cells were immunoprecipitated using the \(\alpha\)-\FLAG M2 antibody and then subjected to MALDI-TOF MS analysis. The MS spectrum revealed a range of different M2-immunoprecipitated F-\(\text{N}\beta\) peptides (Fig. 1B), consistent with previous results (15). By culturing the cells in the presence of two different GSIs, L-685,458 and DBZ, we found that the majority of the F-\(\text{N}\beta\) peptides were generated in a γ-secretase-dependent manner (Fig. 1, C and D). Interestingly, the generation of some shorter peptides, below mass to charge \((m/z)\) 3400, were not inhibited by the GSIs and thus were probably generated through other enzymatic reactions (Fig. 1, C and D). Moreover, no peptides precipitated by M2-agarose were identified in the media of HEK293 cells that were lacking expression of FLAG-\(\Delta E\), which displayed the specificity of the precipitation protocol (Fig. 1E).

Next, we identified which peptide sequence corresponded to which peak in the MALDI spectrum. To confirm peptide identities, the immunoprecipitates were analyzed by MALDI-TOF/TOF. Given the natural cleavage site in the signal peptide, FLAG-\(\Delta E\), we matched peptides, starting with RGPR before

**Peptides**
the FLAG sequence, and identified 11 different peptides corresponding to a range of species between F-N\textsubscript{12} to F-N\textsubscript{25} (Fig. 1F). In addition, we could not observe peptides longer than F-N\textsubscript{25} (Fig. 1C), which is in line with data reported by Okochi et al. (15).

First Generation NSAID Class GSMs Affect \(\alpha\)\(\beta\) but Not N\(\beta\) Production—To explore how the NSAID class GSMs modulate the \(\gamma\)-secretase complex, we first studied their effect on APP processing by measuring the \(\alpha\)\(\beta\) peptide generation in HEK293 cells stably expressing the APPswe mutation, HEK/APPswe. Conditioned media were subjected to IP-MALDI-TOF MS analysis, resulting in detection of A\(\beta\)\textsubscript{37}, A\(\beta\)\textsubscript{38}, A\(\beta\)\textsubscript{39}, A\(\beta\)\textsubscript{40}, and A\(\beta\)\textsubscript{42} (Fig. 1G). Like F-N\textsubscript{25}, the generation of the A\(\beta\) peptides was abolished in the presence of either of two GSIs, L-685,458 or DBZ (Fig. 1, H and I). We treated the cells with two well characterized NSAIDs, R-flurbiprofen and sulindac sulfide or...
vehicle, and studied the Aβ peptide profile. Both GSMs are known to modulate γ-secretase activity by shifting the amino acid cleavage from positions 42 to 38 (19, 38), and as expected, we observed a decrease in Aβ42 accompanied by a relative increase in Aβ38 compared with untreated cells (Fig. 2, A–C). In line with previous findings, treatment with sulindac sulfide resulted in a relative decrease of Aβ39 (19). Interestingly, we also observed an increase in Aβ37 in cells treated with R-flurbiprofen, but not with sulindac sulfide. These results were confirmed by Aβ peptide analysis using MSD technology (Fig. 2D).

To further study how NSAID class GSMs affect Nβ generation, we treated HEK/FLAG-NΔE cells with R-flurbiprofen,
Second Generation GMSs Modulate Both Nβ and Aβ Formation

Because we had found that the first generation GSM displays a clear differentiation with regard to Aβ and Nβ generation, we wanted to compare the effect of second generation GSM on the generation of these peptides. First, we analyzed the effect of AZ GMSs on Aβ peptide formation in HEK/APPsw cells using IP-MALDI-TOF MS. In line with MSD data (Fig. 3B), both AZ1136 and AZ4126 caused a differential reduction in Aβ40 and Aβ42 (2.3- and 6.4-fold decreases for Aβ40 and 8.3- and 16.5-fold decreases for Aβ42, respectively). The MALDI-TOF MS also revealed a unique Aβ pattern in response to the two AZ GMSs (Fig. 4, A–C): AZ1136 relatively increased both Aβ37 and Aβ39 (3.5 and 2.1 times, respectively), whereas Aβ38 was unaffected, consistent with previously published results (32). AZ4126 also increased Aβ37 but with a higher magnitude (4.8-fold), decreased Aβ39 (1.8-fold), and marginally induced the levels of Aβ38 (1.6-fold). Importantly, MSD analysis resulted in the same Aβ pattern as the MS results (Fig. 3B).

The effect of AZ GMSs on Nβ formation was studied using HEK/FLAG-NΔE cells and IP-MALDI-TOF. Interestingly, both AZ1136 and AZ4126 caused a relative decrease in F-Nβ24 (2.0 and 2.3 times, respectively) and F-Nβ25 (2.6-fold for both) levels, in comparison with untreated cells. However, we did not observe as distinct differences in the production of shorter F-Nβ, as observed for shorter Aβ peptides in response to the same compounds. AZ1136 increased F-Nβ18 1.7-fold, whereas AZ4126 increased F-Nβ21 only 1.3-fold. Examined together, these data suggest that second generation AZ GMSs modulate Nβ production, although to a lower extent compared with their effect on Aβ production (summarized in Fig. 4G).

Second Generation GMSs Do Not Modulate NICD Formation

We finally explored whether the Notch S3 cleavage, liberating the more biologically relevant NICD peptide, also was affected by second generation GMSs, because we observed that they modulate Nβ production. To address this question, we used a previously developed immunochemistry assay based on HEK/FLAG-ΔNΔE cells and the C20 antibody that recognizes total NICD (32). Here, we included an anti-NICD antibody that specifically recognizes NICD beginning at position Val-1744 (V1744-NICD). We performed dose-response experiments and analyzed the amount of V1744-NICD in the nucleus relative to untreated cells and confirmed that AZ GMSs do not inhibit NICD formation (Fig. 5A). L-685,458 inhibited V1744-NICD formation with similar potency (IC_{50,NICD} = 10.0 nM) as previously determined for its effect on total NICD formation (IC_{50,NICD} = 6.0 nM (32)). By calculating the ratio of the signal from both NICD antibodies, we could determine whether the relative amount of V1744-NICD to total NICD was affected. If GMSs modulate NICD formation, the amount of

FIGURE 2. The effect of first generation NSAID class GMSs on Aβ and F-Nβ formation. A, MALDI-TOF MS spectrum displaying the Aβ pattern in conditioned medium from HEK/APPsw cells treated with R-flurbiprofen, sulindac sulfide, or vehicle. The intensities of the highest peak were set to 100% in the spectrum. B, Aβ peak distribution under the influence of first generation GMSs. Each Aβ peak is plotted as a percentage of total Aβ (i.e., the sum of Aβ37–42). The bars represent the means of five or six experiments with error bars indicating S.D. C, scatter plots of the Aβ peptide distribution under the influence of first generation GMSs. The data are from five or six experiments and plotted as percentages of total Aβ (i.e., the sum of Aβ37–42). D, detection of secreted Aβ peptides in conditioned medium from HEK/APPsw cells treated with R-flurbiprofen, sulindac sulfide, or vehicle by MSD technology. Aβ peptide formation is determined as a percentage of total Aβ (i.e., the sum of Aβ37–42). The bars represent the means of two experiments with error bars indicating S.D. E, MALDI-TOF MS spectrum of F-Nβ using α-FLAG immunoprecipitated conditioned medium from HEK/FLAG-NΔE cells treated with R-flurbiprofen, sulindac sulfide, or vehicle. The intensities of the highest peak were set to 100% in the spectrum. F, F-Nβ peak distribution under the influence of first generation GMSs. Because only F-Nβ16–25 could be inhibited by GSIs and not the shorter F-Nβ12–15, the latter were excluded from peak analysis. Each F-Nβ peak is plotted as a percentage of total F-Nβ (i.e., the sum of F-Nβ16–25). The bars represent the means of five or six experiments with error bars indicating S.D. G, a summary of the effect of first generation GMSs on Aβ and F-Nβ peptide formation.
V1744-NICD and hence the ratio would change. None of the GSMs showed any change of V1744-NICD to total NICD ratio (Fig. 5B), suggesting that AZ GSMs do not modulate NICD formation. These results were also confirmed by Western blot analysis (Fig. 5C).

**DISCUSSION**

The insights in the mode of action of first and second generation GSMs are not fully understood, even though they represent a prioritized therapeutic approach in AD drug discovery. However, a growing body of evidence suggests that many second generation GSMs have a mode of action that is different from first generation NSAID class GSMs, because the former appear to target γ-secretase instead of APP (21, 32–36). Even though the pharmacology of Aβ generation associated with GSMs has been thoroughly studied, less is known about their effect on other substrates, such as the Notch receptor. Here, we describe a head-to-head comparison of first and second generation GSMs affecting γ-secretase processing of APP and Notch.

Whereas both the first generation GSMs R-flurbiprofen and sulindac sulfide and the second generation GSMs AZ4126 and AZ1136 display Aβ modulation, only the second generations GSMs modify Nβ production. The modulatory effect of AZ4126 and AZ1136 on Aβ and Nβ differs both with regard to efficacy and profile, and the overall impact is more pronounced on Aβ generation compared with Nβ production. These data suggest that γ-secretase-targeting GSMs are not only modulat-
Second Generation GSMs Modulate Both N\(\beta\) and A\(\beta\) Formation

To compare the effect of GSMs on N\(\beta\) and A\(\beta\) formation, we established an antibody pulldown approach that we followed with MALDI-TOF MS analysis. This strategy enabled detection of the expected A\(\beta\) peptides, as well as 11 different F-N\(\beta\) species, ranging from F-N\(\beta\)12 to F-N\(\beta\)25, of which F-N\(\beta\)16–25 were generated in a \(\gamma\)-secretase-dependent manner. These observations are well in line with previously reported results (15). MALDI-TOF analysis of A\(\beta\) peptides revealed some clear commonalities but also differences in the pharmacological profile among the compounds examined. Both AZ1136 and

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

![Graph E](image5.png)

![Graph F](image6.png)

| AZ1136 | AZ4126 |
|--------|--------|
| A\(\beta\)37: 1.0 | F-N\(\beta\)25: 2.6 |
| A\(\beta\)38: 2.3 | F-N\(\beta\)21: 4.3 |
| A\(\beta\)39: 2.1 | F-N\(\beta\)23: 1.8 |
| A\(\beta\)40: 1.2 | F-N\(\beta\)21: 1.6 |
| A\(\beta\)37: 1.5 | F-N\(\beta\)20: 1.4 |
AZ4126 cause a very potent reduction in Aβ42 and Aβ40 and a parallel increase in Aβ37. In contrast, R-flurbiprofen and sulindac sulfide are associated with a clear relative reduction in Aβ42 but not Aβ40 and a concomitant increase of Aβ38, suggesting that these different classes of GSM compounds modulate Aβ production through different mechanisms. In line with our previous study, we also found that AZ1136 treatment increases the relative levels of Aβ39, whereas AZ4126 causes an increase in Aβ38 (32). The pharmacological profiles of R-flurbiprofen and sulindac sulfide display differently; R-flurbiprofen primarily increases Aβ37, whereas sulindac sulfide reduces Aβ39. Thus, besides the differences in Aβ modulation between the first and second generation GSMS, clear differences are found within each class of GSMS with regard to Aβ modulation. Further studies are needed to explain the mechanism that gives rise to these differences and thus shed more light on the pharmacology of these different GSMS with respect to Aβ modulation.

The fact that both AZ1136 and AZ4126, but neither R-flurbiprofen nor sulindac sulfide, affect Nβ production is interesting. Similar to their effect on Aβ40 and Aβ42, AZ1136 and AZ4126 reduce both F-Nβ24 and F-Nβ25, but the relative effects are not as discriminating. Strikingly, even though AZ4126 decreases Aβ40 and Aβ42 approximately twice as much as AZ1136 at their respective tested concentration, both compounds appear to have the same efficacy in reducing F-Nβ24 and F-Nβ25. Regarding the shorter F-Nβ peptides, we observed that most of these peptides were similarly unaffected by the AZ GSMS. In contrast to their very clear relative elevation of Aβ37, AZ41126 and AZ1136 do not cause a clear increase in the same F-Nβ peptide. However, AZ1136 increases F-Nβ18 slightly, whereas F-Nβ21 is elevated by AZ4126. Thus, it seems like both compounds share a general pharmacological profile on APP and Notch processing, specifically, a decrease in the longer Aβ and F-Nβ peptides (Aβ40/Aβ42 and F-Nβ24/F-Nβ25) and an increase in some of the shorter peptides, but that disparities do exist between the compounds, pertaining to the efficacy of the process and the specificity of which peptides are to be modulated. Our data therefore suggest that it is possible to generate γ-secretase targeting GSMS that are preselective for Aβ over Nβ production. γ-Secretase targeting is not restricted to these compounds, because there are several well characterized GSIs of different chemical classes that have been shown to bind to the γ-secretase complex and exhibit different potencies in APP and Notch processing. Such features are assigned the Notch-sparing GSIs, such as BMS-708163, PF-3084014, Begacestat, ELND-006, and Semagacestat, which differ in their magnitude of selectivity of APP toward Notch with 3–1473 times (see review in Ref. 18).

γ-Secretase-mediated NICD formation is a key event in Notch receptor activation. Generating GSMS that do not affect NICD signaling is crucial toward developing tolerable AD therapeutics. The observation that many GSMS are Notch-sparing, that is, they do not affect the total amount of NICD generated, is applicable to the GSMS used in this study (Fig. 4A) (32). However, it may not be only the total amount of NICD generated that ought to be considered but also the specificity of the γ-secretase cleavage event at the S3 site. Indeed, Tagami et al. (39) recently reported that NICD exists in two distinct forms at the N terminus, which results in NICDs with quite different stabilities and thus different signaling properties. In this study we could not find evidence for modulatory effect by AZ1136 and AZ4126 on Notch S3 cleavage, suggesting that the AZ GSMS are selective for S4 cleavage modulation.

FIGURE 5. The effect of AZ1136 and AZ4126 on NICD formation and modulation. A, in vitro cellular V1744-NICD formation assay. Neither AZ1136 nor AZ4126 inhibit NICD formation. The GSI L-685,458 serves as positive control for inhibition. The curves represent the means of three experiments with error bars indicating S.D. B, the ratio of the S3 specific V1744-NICD/total NICD is unaltered by AZ GSMS compared with vehicle, as determined by measuring the fluorescent intensity from both Val-1744 and C20 antibodies. The curves represent the means of three experiments with error bars indicating S.D. Representative immunocytochemistry images of vehicle-, L-685,458-, AZ1136-, and AZ4126-treated cells are shown. C, Western blot analysis was performed to confirm the in vitro cellular NICD formation assay. The cells were treated with AZ1136, AZ4126, or vehicle and analyzed with the Val-1744 and α-GAPDH antibodies.
Second Generation GSMs Modulate Both Nβ and Aβ Formation

Our finding that second generation GSMs modulate both Aβ and Nβ supports the theory that the γ-secretase complex is the molecular target of second generation GSMs (21, 32–36). In contrast, there has been no consensus as to what is the binding site of NSAID-based GSMs. Some studies report that these compounds interact with APP-derived C99 fragment (25–27), whereas others have challenged this hypothesis (28–31). Our data, showing that R-flurbiprofen and sulindac sulfide affect Aβ but not Nβ production, indicate that APP is the target of NSAID-based GSMs. These data contrast with a previous report showing that sulindac sulfide decreases the ratio of Nβ25/(Nβ21 + Nβ25) (40). These discrepancies could be explained by differences in technical strategy. However, our results fit very well with a previous finding that NSAIDs such as sulindac sulfide interact and interfere with dimerization of the APP transmembrane domain, thereby affecting Aβ generation (27). APP forms dimers with the help of three different dimerization sites: two in the ectodomain and a third formed by three GXXXG motifs in the APP transmembrane domain. Recently, it was reported that a slight decrease in the dimerization strength of the GXXXG motifs gave rise to an enormous decline of Aβ42 formation (41). Although it has been claimed that Notch can dimerize through its epidermal growth factor repeats in the ectodomain, Vooijs et al. (42) also report that most surface Notch molecules are monomeric. Thus, it is plausible that NSAIDs interact with mechanisms affecting dimerization of APP, resulting in no modulation in Nβ. Further studies with competitive experiments and photo-probe labeling on NSAID-based GSMs are needed to fully understand the binding target of the NSAIDs.

Modulation by second generation GSMs may also affect other substrate-releasing Aβ-like peptides, such as APP-like proteins 1 and 2, CD44, and interleukin-1 receptor II (43–45). Therefore, it will be of great interest to monitor these peptides during the research development of this class of drugs. However, the biological relevance of the Aβ-like peptides is currently unclear. However, the APP-like protein 1 Aβ-like peptide, which is less amyloidogenic than Aβ42, is present in human cerebrospinal fluid and is purported to function as a surrogate marker for Aβ42 in response to γ-secretase-targeting drugs (46). Thus, the selectivity pattern of a given GSM should be a major consideration in biomarker development.

In summary, we report that second generation but not first generation GSMs affect Nβ production, although to a much lower level than they affect Aβ formation. However, second generation GSMs do not affect the Notch S3 cleavage site or the NICD formation, which is a crucial factor toward developing safe and tolerable AD therapeutics.

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