β-Amyloid Precursor Protein Mutants Respond to γ-Secretase Modulators**

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Pathogenic generation of the 42-amino acid variant of the amyloid β-peptide (Aβ) by β- and γ-secretase cleavage of the β-amyloid precursor protein (APP) is believed to be causative for Alzheimer disease (AD). Lowering of Aβ42 production by γ-secretase modulators (GSMs) is a hopeful approach toward AD treatment. The mechanism of GSM action is not fully understood. Moreover, whether GSMs target the Aβ domain is controversial. To further our understanding of the mode of action of GSMs and the cleavage mechanism of γ-secretase, we analyzed mutations located at different positions of the APP transmembrane domain around or within the Aβ domain regarding their response to GSMs. We found that Aβ42-increasing familial AD mutations of the γ-secretase cleavage site domain responded robustly to Aβ42-lowering GSMs, especially to the potent compound GSI-1, irrespective of the amount of Aβ42 produced. We thus expect that familial AD patients carrying mutations at the γ-secretase cleavage sites of APP should respond to GSM-based therapeutic approaches. Systematic phenylalanine-scanning mutagenesis of this region revealed a high permissiveness to GSM-1 and demonstrated a complex mechanism of GSM action as other Aβ species (Aβ11, Aβ14) could also be lowered besides Aβ42. Moreover, certain mutations simultaneously increased Aβ42 and the shorter peptide Aβ38, arguing that the proposed precursor-product relationship of these Aβ species is not general. Finally, mutations of residues in the proposed GSM-binding site implicated in Aβ42 generation (Gly-29, Gly-33) and potentially in GSM-binding (Lys-28) were also responsive to GSMs, a finding that may question APP substrate targeting of GSMs.

Alzheimer disease (AD)3 is the most common neurodegenerative disorder worldwide. The β-amyloid precursor protein (APP), a type I membrane protein, plays a central role in the pathogenesis of the disease (1). Sequential cleavage of APP by β- and γ-secretase generates the amyloid-β (Aβ) peptide, which deposits as plaques in the brain of affected patients and represents one of the principal pathological hallmarks of the disease (1). γ-Secretase is an intramembrane-cleaving protease complex, which cleaves the APP transmembrane domain (TMD) in a progressive, stepwise manner via cleavages at the ε-, ζ-, and γ-sites until it is sufficiently shortened to allow the release of Aβ from the membrane (2–4). Aβ peptides generated by γ-secretase cleavage differ in their C termini. The major product released is Aβ109 whereas Aβ18 and Aβ12 represent minor species (1). The highly aggregation-prone, neurotoxic Aβ42 is believed to be causative for AD by initiating a cascade of pathogenic events, which ultimately causes neurodegeneration and dementia (1). Increased production of Aβ42 underlies the vast majority of mutations associated with familial AD (FAD), which manifests with a very early disease onset. The majority of FAD mutations have been found in PS1, the catalytic subunit of γ-secretase (5), whereas only a few mutations were found in its homolog PS2. Few FAD-associated mutations were also found in APP, and those that affect γ-secretase cleavage toward an increased Aβ42 production localize to the C terminus of the APP TMD in the vicinity of the γ-secretase cleavage sites. Fluorescence resonance energy transfer-based studies suggest that changes in the generation of Aβ42 are due to alterations in the conformation of PS (6–10).

Inhibition of Aβ42 production is a major approach to therapeutically target AD. Selective Aβ42-lowering drugs, so-called γ-secretase modulators (GSMs) such as certain non-steroidal anti-inflammatory drugs (NSAIDs), have been identified as promising and attractive alternatives to inhibitors of γ-secretase, which target the active site and thus affect the processing of other physiologically important γ-secretase substrates, such as Notch1 (11). GSMs inhibit Aβ42 production with little effect on Aβ40 generation and the processing of other important γ-secretase substrates (12). Inhibition of Aβ42 by these compounds is accompanied by an increased production of Aβ38 (13). Because inverse modulators have also been identified (14), it was initially believed that the production of these peptides is...
interdependent, pointing to the possibility that Aβ42 might represent the precursor of Aβ38. Evidence has been presented that Aβ340 and Aβ42 derive from two different product lines by stepwise cleavage roughly every three residues at positions e49 – ω46 – γ43 – γ40 and e48 – ω45 – γ42 of the Aβ domain, from which cleavages occur further downstream to generate Aβ39, Aβ38, and Aβ37 (15, 16), with Aβ39 primarily originating from the Aβ42-generating product line (16). In addition, dimerization of the APP TMD mediated by its central GXXXG helix-interaction motif has been suggested to affect the formation of Aβ42 (17). Substitution of the glycine residues to reduce APP TMD dimerization was shown to lower the production of Aβ42, whereas increasing that of Aβ39 (17). Mechanistically, it was suggested that dimerization via the GXXXG motif imposes a sterical hindrance for γ-secretase to proceed with stepwise processing such that Aβ39 and Aβ42 are normally released as final products. Decreasing dimerization strength would resolve this sterical constraint, now allowing γ-secretase to efficiently proceed to more N-terminal cleavage sites, thus generating shorter Aβ species, mostly Aβ38 (17). Interestingly, the GXXXG sequence is part of a GSM-binding site mapped to residues Aβ29–36 (GAIIGLMV) in the APP TMD (18). This region is known to be critical for Aβ aggregation (19–21), and aggregation inhibitors interacting with this region also act as GSMS (18). However, a recent study demonstrated that dimerization per se might not be a factor that determines γ-secretase cleavage specificity (22), and whether GXXXG mutants inhibit dimerization is controversial (23). In addition, the lack of a consistent response to GSMS regarding an inversely correlated production of Aβ42 and Aβ38 by PS FAD mutants observed earlier argued against a strict precursor-product relationship between Aβ42 and Aβ38 (24, 25). Moreover, while this manuscript was in preparation, a biophysical study failed to demonstrate GSM-binding to the APP substrate, however, and suggested that the reported GSM-APP interaction (18) was unspecific (26).

Interestingly, lowering of Aβ42 by GSMS is not effective for the majority of the PS FAD mutants investigated so far. In particular, aggressive FAD mutants that manifest with a very early disease onset due to their strongly increased Aβ1–16 and polygonal antibody 3552 to Aβ1–40 were described previously (29, 30). Monoclonal antibody 4G8 against Aβ17–24 was obtained from Covance. The C-terminal specific anti-Aβ38 antibody was obtained from Meso Scale Discovery, and C-terminal specific anti-Aβ39 (BAP24) and anti-Aβ42 (BAP15) antibodies were a kind gift of Dr. Manfred Brockhaus (Roche).

cDNA Constructs—cDNA encoding APPsw-6myc (31) was recloned into pcDNA3.1/zeo (+) (Invitrogen). The given mutants were generated in this and in the C99-6myc construct (31) by QuikChange mutagenesis (Stratagene) using oligonucleotide primers encoding the respective point mutations. Likewise, QuikChange mutagenesis was used to generate C100-His6 mutant substrate constructs in pQE60:C100-His6. These were expressed in Escherichia coli and purified as described (32).

Cell Culture and cDNA Transfections—Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and penicillin/streptomycin on poly-l-lysine-coated plates. Cells were plated at a density of 200,000 cells/24-well plate or 1,000,000 cells/6-well plate, and the following day, cells were transiently transfected with the indicated APP cDNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Suspension-adapted HEK293S cells were cultivated in instrumented 10-liter bioreactors using a Roche Applied Science proprietary serum-free hydrolysate-containing culture medium.

Analysis of Secreted Aβ from Cultured Cells—Following transfection, HEK293 cells were incubated for 24 h before media change and overnight incubation for 16 h in the presence of sulindac sulfide, flurbiprofen, or fenofibrate (all Sigma), GSM-1 (kind gift of Dr. Karlheinz Baumann, Roche), or vehicle control (dimethyl sulfoxide (DMSO)). Conditioned media were then collected and immediately analyzed by sandwich immunnoassay to quantify Aβ species, or following immunoprecipitation, subjected to Tris-Bicine urea SDS-PAGE or mass spectrometry analysis. For Aβ quantitation, drug treatments were performed in triplicate, and all media samples were measured in duplicate for Aβ39, Aβ40, and Aβ42. For the analysis of modulation, data are always plotted as the percentage of change in the concentration of Aβ species from vehicle-treated cells, which are normalized to 100% for each cell line.
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FIGURE 1. Effect of GSM-1 on Aβ species of APP FAD mutants. A, schematic of the APP amino acid sequence encompassing the Aβ peptide and the transmembrane region of APP (underlined). Amino acids are numbered from position 1 of the Aβ peptide, and arrows indicate the positions of γ-secretase cleavage. The location of APP FAD mutations selected for analysis together with the corresponding mutant amino acids are indicated above the sequence. The amino acids subjected to phenylalanine scan are boxed in gray and contain the I45F and V46F mutants as additional FAD mutations. The reported GSM-binding site in APP is shown in italics. B, sandwich immunosassay of Aβ1–40, Aβ1–40, and Aβ1–42 species from conditioned media of cells overexpressing WT or the indicated APP FAD mutants. Aβ1–42 is increased for the three FAD mutants analyzed. Strikingly, in addition to a dramatic increase in Aβ1–42 for T43I, Aβ1–42 is also increased at the expense of Aβ1–40. Each species is plotted as a percentage of the total Aβ (Aβ1–40 + Aβ1–40 + Aβ1–42) measured for each cell line. Bars represent the mean of 3 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); ***, p < 0.001, **, p < 0.01, *, p < 0.05. D, Tris-Bicine urea SDS-PAGE analysis of Aβ species from cells expressing WT or the indicated APP FAD mutants upon 1 μM GSM-1 treatment. Aβ1–40 appears as a prominent species for T43I and V46F, and interestingly, this is also abolished along with Aβ1–42 upon GSM-1 treatment.

γ-Secretase in Vitro Assays—γ-Secretase was purified as described previously (28) except that HEK293S cells (kind gift of Georg Schmid and Elvira da Silva, Roche) were used as an enzyme source. In vitro γ-secretase activity was assessed as described using purified γ-secretase (Q-Sepharose eluate) and purified WT and mutant C100-His6 substrates in the presence or absence of GSM-1 or fenofibrate (28).

Quantification of Aβ—Secreted Aβ peptides in conditioned medium were quantified by a sandwich immunoassay using the Meso Scale Discovery SECTOR Imager 2400 as described previously (24). Quantification of total Aβ was done essentially the same procedure except that for detection, 4G8 mouse monoclonal and ruthenylated anti-mouse antibodies were used in combination. For more sensitive detection of Aβ species (for the Phe mutants), the Meso Scale Discovery Aβ triplex sandwich immunosassay was used. Here, Meso Scale Discovery C-terminal specific antibodies were instead prespotted into each well, and a ruthenylated 6E10 antibody was used as the detection antibody. Meso Scale Discovery Aβ peptide standards were used for the Meso Scale Discovery triplex immunosassay. Aβ peptides generated by γ-secretase in vitro assays were quantified using the Meso Scale Discovery sandwich immunosassay as described (28).

SDS-PAGE and Mass Spectrometry Analysis of Aβ—Secreted Aβ was analyzed from medium conditioned overnight for 16 h by combined immunoprecipitation/immunoblotting using antibodies 3552/2D8 followed by Tris-Bicine urea SDS-PAGE (33). To analyze Aβ by mass spectrometry, Aβ species were immunoprecipitated from conditioned media or from γ-secretase in vitro assays using antibody 4G8 and subjected to matrix-assisted laser desorption/ionization-time of flight mass spectrometry analysis as described previously (24, 28).

RESULTS

FAD Mutants in the γ-Secretase Cleavage Site Region Respond to GSMs—To address the question whether APP FAD mutants respond to GSM treatment, we introduced the Austrian T43I (T714I), Florida I45V (I716V), and London V46I (V717I) (34–36) mutations into APPsw-6myc, a well characterized and frequently used APP substrate (31), which was used as backbone for these and all other mutants of the APP TMD (Fig. 1A) analyzed in this study. The cDNA constructs were transiently transfected into HEK293 cells, and levels of secreted Aβ species generated by WT APP and the APP FAD mutants were analyzed by a highly sensitive specific Aβ immunoassay, which allows the detection and quantitation of Aβ1–38, Aβ1–40, and Aβ1–42.
species (24, 29). As expected, the ratios of Aβ42 to total Aβ (i.e., the sum of Aβ38, Aβ40, and Aβ42) were increased for all mutants (Fig. 1B). The strongest increase was observed for the T43I mutant. Unexpectedly, this mutant also showed a strongly increased Aβ38/Aβ42 ratio, whereas that of Aβ40/Aβ42 was reduced. This behavior was not observed for the I45V and V46I FAD mutants and appeared to be characteristic for the T43I mutant. We thus conclude that certain FAD mutants occurring in the APP TMD can increase Aβ42 and Aβ42 in parallel.

We next screened various GSMs to see whether the increased levels of Aβ42 produced from these mutants could be lowered. As shown in Fig. 1C, all GSMs were effective. GSM-1, a previously described GSM that is effective in the low micromolar range (24, 28), was overall the most potent compound with regard to modulation of the APP mutants. This GSM was capable of strongly reducing Aβ42 production from WT APP as well as from all three FAD mutants. The reduction of Aβ42 levels observed was ~90% as compared with the untreated controls. Consistent with previous results (24), GSM-1 increased the levels of Aβ38 produced from WT APP. Likewise, a robust increase of Aβ38 was also observed for the T43I, I45V, and V46I mutants. The NSAIDs sulindac sulfide and flurbiprofen were capable of reducing Aβ42 production for these mutants to a similar extent to WT APP, although flurbiprofen was more potent in this regard. Sulindac sulfide was more potent than flurbiprofen with regard to the modulation of Aβ38 levels, although all mutants responded by increasing Aβ38 upon treatment with either compound (Fig. 1C). As GSM-1 was the most effective modulator, we focused on this compound for the analysis of Aβ species by Tris-Bicine urea SDS-PAGE, which allows an effective separation of Aβ species. The modulatory effects were confirmed, and in addition, this analysis showed that the production of Aβ38 was reduced by GSM-1 treatment as well (Fig. 1D). Taken together, these data show that FAD mutants within the APP TMD that change the specificity of γ-secretase cleavage and thereby increase the production of Aβ42 are susceptible to different GSMs. GSM-1-mediated inhibition of Aβ42 generation is accompanied by an increased production of Aβ38, a strong inhibition of Aβ39, but has little effect on Aβ40. Since GSM-1 elicited the most potent effects with regard to both Aβ42 and Aβ38 modulation, this compound was used as the principal GSM for all subsequent modulation experiments in this study.

**Phenylalanine Mutants of the γ-Secretease Cleavage Site Region Respond to GSM-1**—To investigate whether mutations of the γ-secretase cleavage site region of APP are generally susceptible to GSM-1 as shown above for a subset of FAD mutants, we next analyzed previously described phenylalanine mutants, which span the region between the γ- and ε-cleavage sites and thus allow a systematic analysis of the GSM-1 response to mutants within this region (37, 38). These well characterized Phe mutants cover the sequence from Aβ43–51 of the β domain and include two other FAD mutants, the Spanish I45F (I716F) (39) and the V46F (V717F) Indiana mutation (40). Analysis of the profile of the Aβ species generated by these mutants by Tris-Bicine urea SDS-PAGE was entirely consistent with that of the previous reports (37, 38) (Fig. 2A). Each mutant affected the profile of Aβ species produced by γ-secretase in an individual and characteristic manner. In agreement with the previous results (37, 38), the most striking changes were observed for the V44F, I45F, I47F, and V50F mutants. The I45F mutant produced the lowest amount of Aβ38, and the highest amount of Aβ42, whereas the V50F mutant produced almost exclusively Aβ40. Similarly, the V44F and I47F mutants produced very little Aβ42, but interestingly, both gave rise to the production of an alternative Aβ species, which migrated somewhat slower than Aβ42 and apparently represented Aβ41 (37, 38). Consistent with the previous reports (37, 38), the T43F–V46F mutants produced higher levels of the shorter Aβ variants Aβ38 and Aβ39 than the T48F–V50F mutants. The highest level of Aβ38 was observed for the V44F mutant, which did not generate detectable levels of Aβ39. Only small amounts of Aβ41 were produced for WT APP and all of the Phe mutants.

Having confirmed the characteristic Aβ profile for each mutant, we next investigated whether and how the mutants would respond to GSM-1 treatment. Following drug treatment, changes in Aβ42 and Aβ38 were assessed by the Aβ immunoassay. As shown in Fig. 2B, as compared with the untreated controls, GSM-1 potently lowered the levels of Aβ42 of WT APP and all the Phe mutants by ~70–80%, even for the V44F mutant, which produced only extremely small amounts of Aβ42. Strikingly, the Phe mutants behaved differently in their response to GSM-1 with regard to its potency in increasing the levels of Aβ38 (Fig. 2B). Although WT APP showed the expected robust increase of Aβ38, the T43F, V44F, and I45F mutants were less responsive to GSM-1 treatment with respect to Aβ38. The FAD-associated V46F as well as the M51F mutant responded to GSM-1 similarly to WT APP. In contrast, GSM-1 treatment induced the production of considerably higher levels of Aβ38 in the I47F, T48F, L49F, and V50F mutants, the latter mutant showing the maximal increase of Aβ38 among these mutants (~6-fold increase as compared with control). Interestingly, Tris-Bicine urea SDS-PAGE analysis revealed that GSM-1 effected a strong decrease of Aβ41 for the V44F and of Aβ39 for the V46F mutant, which were selected to analyze the modulation of alternative Aβ species (Fig. 2C). Consistent with the results above, Aβ38 was increased for both mutants in response to GSM-1, and Aβ42 was decreased by GSM-1 treatment for the V46F mutant (Fig. 2C). Mass spectrometry analysis confirmed these results (Fig. 2D). Taken together, all APP Phe mutants responded robustly to the Aβ42-lowering capacity of GSM-1, further supporting our notion that Aβ42 produced from APP mutant carriers harboring Aβ42-increasing mutations in the γ-secretase cleavage site domain can be expected to be targetable by GSMs. Furthermore, these data show that additional γ-secretase cleavages can be modulated, suggesting substantial flexibility in the modulation of γ-secretase cleavage specificity.

**Mutants of the GXXXG Motif in the APP TMD Respond to GSMS**—We next investigated whether mutation of the glycine residues of the GXXXG motif, which had been implicated in the production of Aβ38 and Aβ42 (17) and which lie within the proposed GSM-binding site of APP (18), would affect GSM-induced changes of γ-secretase cleavage specificity. APPsw-6myc constructs containing the G29A, the G33A, or the stronger G33I substitution described previously (17) were
transiently transfected into HEK293 cells, and the ratios of the secreted Aβ/H9252 species to total Aβ were examined by the Aβ immunoassay to assess changes in γ-secretase cleavage specificity. As shown in Fig. 3A, the G29A mutant showed a normal Aβ40/Aβtotal ratio but an increased Aβ38/Aβtotal ratio and a decreased Aβ42/Aβtotal ratio. The G33A mutant displayed an even higher Aβ38/Aβtotal ratio, but unlike the G29A mutant, it showed a normal Aβ42/Aβtotal ratio similar to that of the WT control. In further contrast to this mutant, a decreased Aβ40/Aβtotal ratio was immediately apparent for the G33I mutant, and this effect was observed even more strongly for the G33I mutant, whereas Aβ42 was almost undetectable. Thus, the G33I mutant showed a strong change in γ-secretase cleavage specificity.

FIGURE 2. Effect of GSM-1 on Aβ species of APP phenylalanine mutants. A, Tris-Bicine urea SDS-PAGE immunoblot of Aβ species from cells expressing WT or the indicated phenylalanine mutants of APP. For Phe substitutions from positions 43–46, γ-secretase appears to generate a broad spectrum of peptides, from Aβ37 to Aβ42. From positions 47–50, Aβ40 is the predominant species, and other species are greatly reduced. Notably, V44F and I47F produce predominantly Aβ41 instead of Aβ42. B, sandwich immunoassay showing the effect of 1 μM GSM-1 treatment on Aβ42 (upper panel) and Aβ38 (lower panel) in cells expressing WT or the indicated APP Phe mutants. The more sensitive Meso Scale Discovery triplex assay was used here to detect Aβ peptides with low abundance such as Aβ38 for the V44F mutant. All mutants respond to treatment by decreasing Aβ42 to a similarly dramatic extent. However, the increase in Aβ38 depends upon the amino acid position within the TMD, with a clear pattern of increasing response the further away the Phe mutation is from position 44. Bars represent the mean of 3 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); ***, p < 0.001, **, p < 0.01, *, p < 0.05. C, Tris-Bicine urea SDS-PAGE immunoblot of WT and V44F and V46F mutant Aβ species in the presence of GSM-1 or vehicle. For the V44F mutant, the species migrating as Aβ41 could be lowered upon GSM-1 treatment, as could the Aβ39 species produced by the V46F mutant. D, mass spectrometry of V44F and V46F mutant Aβ species in the presence of GSM-1 or vehicle. For the V44F mutant, the increased generation of Aβ41 in place of Aβ42 was confirmed, and this species could be lowered by GSM-1 treatment. The spectra for the V46F mutant also confirm that Aβ38 was indeed robustly increased upon GSM-1 treatment and that both Aβ39 and Aβ42 are lowered. The intensities of the Aβ40 peaks were set at 100% in the spectra. DMSO, dimethyl sulfoxide.
apparently at the expense of the normally major A\textsubscript{\beta}40 species. These results were confirmed using the C99-6myc substrate (31) to preclude any effects of dimerization of full-length APP via its ectodomain that may have affected its downstream processing and to rule out that the Swedish mutation at the \textbeta-secre-tase cleavage site of APP\textsubscript{sw}-6myc may have influenced the results (Fig. 3A). We next investigated whether the Gly mutants might also affect other A\textbeta species, which were not measured by our A\textbeta immunoassay. Tris-Bicine urea SDS-PAGE analysis was, however, hampered by an aberrant electrophoretic migration behavior of the mutant peptides, which precluded a clear assessment of the identity of the bands observed and revealed altered biochemical properties of the mutant peptides as compared with WT A\textbeta (data not shown). We therefore analyzed the profile of A\textbeta species by mass spectrometry, which confirmed the A\textbeta profiles of WT and the Gly mutants and additionally revealed substantial levels of A\textsubscript{\beta}39 and even shorter species for the G33I mutant (Fig. 3B) in full agreement with previous results (17). Thus, although overall the Gly mutants favor the production of shorter species, mostly A\textsubscript{\beta}38, they do not consistently change the cleavage specificity of \gamma-secretase toward a reduced production of A\textsubscript{\beta}42 but rather affect that of
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| Table 1 | Levels of the individual Aβ peptides (Aβ38, Aβ40, Aβ42) measured for mutants of the proposed GSM-binding site |
|----------------------|--------------------------------------------------|
| Cultured cells | | |
| WT | G29A | G33A | G33I | K28E |
| 2428 ± 164 | 4134 ± 159 | 6517 ± 6 | 2942 ± 20 | 2686 ± 40 |
| 14327 ± 64 | 8838 ± 49 | 8356 ± 76 | 326 ± 5 | 2112 ± 71 |
| 2432 ± 72 | 515 ± 10 | 1845 ± 14 | 52 ± 1 | 50 ± 6 |
| 3 | 3 | 3 | 3 | 3 |
| Cell-free assay | | | | | |
| WT | G29A | G33A | G33I | K28E |
| 1911 ± 227 | 4086 ± 453 | 3667 ± 292 | 1692 ± 222 | 1589 ± 180 |
| 9952 ± 776 | 5586 ± 348 | 7693 ± 635 | 2117 ± 24 | 6267 ± 471 |
| 1584 ± 98 | 2136 ± 93 | 2448 ± 87 | 403 ± 85 | 1904 ± 148 |
| 18 | 14 | 13 | 14 | 14 |

Aβ40. This was also reflected by the average amounts of the individual Aβ species measured in independent experiments (Table 1).

Having determined the changes in γ-secretase cleavage specificity for the Gly mutants, we next asked whether they would show a GSM response. Aβ42 levels could be robustly lowered for the G29A and G33A mutants by GSM-1 treatment (Fig. 3C). Concomitantly, GSM-1 treatment caused an increase of Aβ38 levels for the G29A and G33A mutants. Probably due to the higher Aβ38 starting levels of the mutants, the increase was attenuated as compared with WT APP. Given its favored production of shorter Aβ species such as Aβ39, the G33I mutant was tested for its ability to respond to the inverse GSM fenofibrate (Fig. 3D). This showed that fenofibrate treatment caused a dramatic increase of Aβ42 (Fig. 3D), which had almost been undetectable for this mutant at baseline (see above). Furthermore, Aβ38 could still be lowered, despite its baseline level for this mutant. Surprisingly, Aβ38 was increased in response to fenofibrate treatment as well (Fig. 3D), a modulation that, to our knowledge has so far not been observed for inverse GSMS. Thus, these data show that substitution of the glycine residues of the GXXXG motif does not interfere with the modulation of Aβ levels by GSMS.

APP TMD Mutants Respond to GSMS in a Cell-free γ-Secretase Assay Using Purified Components—A potential complication in the analysis of Aβ produced from Gly mutant APP is that these particular Aβ peptides are mutated within the Aβ domain itself and that their altered biochemical properties might potentially have an impact on the levels of Gly mutant Aβ peptides detectable in cultured cells downstream of production (e.g. altered secretion, enhanced degradation, or aggregation). In particular, the G33I mutant Aβ42 has been described as a highly aggregation-prone peptide (21). We therefore also carried out cell-free assays using purified recombinant APP C-terminal fragment-based C100-His6 substrates containing selected Phe mutants, the G29A, G33A, or G33I mutants, and purified lipid-reconstituted γ-secretase (28). We first monitored the production of Aβ peptides from C100-His6 substrates containing the I45F or V50F mutants, i.e. substrates that should generate high amounts of both Aβ40 and Aβ42 and low amounts of Aβ38 (I45F) or almost exclusively Aβ40 (V50F). Importantly, in contrast to the Gly mutants within the Aβ domain, Aβ species generated from these mutants are WT in sequence. Therefore, altered effects with respect to the cellular metabolism of Aβ do not apply to these mutations. As shown in Fig. 4A, the production of Aβ42, Aβ40 and Aβ42 peptides as assessed by Aβ immunoassay from WT, I45F, and V50F mutant C100-His6 substrates was consistent with the results obtained for these mutants in cultured cells and the previous reports by others (37, 38). We next monitored whether the I45F and V50F mutants would respond to GSM-1 treatment, which indeed proved to be the case (Fig. 4B). The responses of the two mutants to GSM-1 with respect to Aβ42 and Aβ38 generation were very similar to those observed in cultured cells, thus confirming the results described above and validating the cell-free assay for the analysis of APP TMD mutant substrates.

Analysis of the Aβ species generated in the in vitro assay revealed that the Gly mutants caused an increased relative production not only of Aβ38 but surprisingly also of Aβ27, whereas concomitantly reducing that of Aβ40 (Fig. 5A), although we noticed a greater variability of G33I mutant Aβ42 in this assay (Table 1). Mass spectrometry analysis of mutant Aβ peptides confirmed this unexpected result and revealed that G29A, and in particular the G33I mutant, addi-
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Figure 5. Profile of Aβ peptides generated in vitro from purified C100-His6 GXXXG mutants and their response to GSMs. A, sandwich immunoassay of Aβ38, Aβ40, and Aβ42 peptides that were generated in cell-free assays (28) from WT and the indicated Gly mutant substrates. Comparable with results from cultured cells (Fig. 3A), the increase in Aβ38 correlates with a decrease in Aβ42 for these mutants. However, unexpectedly and in contrast to the cell culture data, robust amounts of Aβ42 could be detected for all Gly mutants. Each species is plotted as a percentage of the total Aβ (Aβ38 + Aβ40 + Aβ42) measured for each substrate. Bars represent the mean of 13–18 experiments with error bars indicating the S.E. Mass spectrometry of Aβ species that were generated in cell-free assays (28) from WT and the indicated Gly mutant substrates. The data reflect the immunoassay measurements for Aβ38, Aβ40, and Aβ42, with the exception of low detectable amounts of Aβ42 for the G33I mutant, possibly due to reasons described under “Results.” In parallel with a decrease in Aβ42, a mild—robust increase in Aβ38 is observed for the Gly mutants in vitro. The intensities of the highest Aβ peaks were set at 100% in the spectra. A magnified part of the spectrum of the G33I mutant is shown on the right-hand side to better visualize the Aβ38 peak. C, sandwich immunoassay showing the effect of 2.5 μM GSM-1 treatment on Aβ38 and Aβ42 generated from WT and Gly mutant substrates in cell-free assays. In parallel with the observed increase in Aβ38 for the G29A and G33A mutants, the decrease of Aβ42 could be detected for all Gly mutants. Each species is plotted as a percentage of the total Aβ. Bars represent the mean of 13–18 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); *** p < 0.001, ** p < 0.01, * p < 0.05. D, sandwich immunoassay showing the effect of 150 μM fenofibrate treatment on Aβ38, Aβ40, and Aβ42 generated from WT and G33I mutant substrates in cell-free assays. Here, Aβ42 does not respond to modulation, whereas both Aβ38 and Aβ40 do. Bars represent the mean of 3 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); *** p < 0.001, ** p < 0.01, * p < 0.05. n.s. denotes not significant.

Figures 6 and 7. Aβ42 does not respond to modulation ranging from 0.5 to 25 μM fenofibrate. A, B, and C, sandwich immunoassay showing the effect of 0.5 and 2.5 μM GSM-1 treatment on Aβ38 and Aβ42 generated from WT and Gly mutant substrates in cell-free assays. In parallel with the observed increase in Aβ38 for the G29A and G33A mutants, the decrease of Aβ42 could be detected for all Gly mutants. Each species is plotted as a percentage of the total Aβ. Bars represent the mean of 13–18 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); *** p < 0.001, ** p < 0.01, * p < 0.05. D, sandwich immunoassay showing the effect of 150 μM fenofibrate treatment on Aβ38, Aβ40, and Aβ42 generated from WT and G33I mutant substrates in cell-free assays. Here, Aβ42 does not respond to modulation, whereas both Aβ38 and Aβ40 do. Bars represent the mean of 3 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); *** p < 0.001, ** p < 0.01, * p < 0.05. n.s. denotes not significant.
APP Mutants Respond to GSMs

A sandwich immunoassay of Aβ38, Aβ40, and Aβ42 species that were isolated from conditioned media of cells overexpressing WT and the K28E mutant of APP. This substitution results in a dramatic increase in Aβ38 and a decrease in Aβ40, and Aβ42, the latter being close to the detection limit of the immunoassay. Each species is plotted as a percentage of the total Aβ (Aβ38 + Aβ40 + Aβ42) measured for each cell line. Bars represent the mean of 3 experiments with error bars indicating the S.E. B, mass spectrometry of Aβ species from WT and the K28E mutant of APP. The spectra closely reflect the immunoassay measurements for Aβ38, Aβ40 and Aβ42, but similar to the G33I mutation, there is again a shift toward shorter Aβ peptides, with Aβ38, the predominant species and Aβ42, also becoming a major species. The intensities of the highest Aβ peaks were set at 100% in the spectra. Magnified parts of the spectra are shown on the right-hand side to better visualize the presence or absence of Aβ42 peaks. C, sandwich immunoassay showing the effect of 1 μM GSM-1 treatment on Aβ38 in cells expressing WT or the K28E mutant of APP. A decrease in Aβ38 could not be reliably determined for the K28E mutant given the low starting level for this mutant. However, Aβ40 could be robustly increased. Bars represent the mean of 3 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); **, p < 0.01. D, sandwich immunoassay showing the effect of 50 μM fenofibrate treatment on Aβ38, Aβ40, and Aβ42 in cells expressing WT or the K28E mutant of APP. Both WT and the K28E mutant respond to treatment by increasing Aβ42, and this increase is more pronounced for the K28E mutant. Strikingly, Aβ40 could be increased for K28E, similar to G33I, and Aβ42 could also be effectively decreased for both WT and K28E. Bars represent the mean of 3 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); ***, p < 0.001, **, p < 0.01, *, p < 0.05.

Despite differences in the relative amounts of each Aβ species generated.

The K28E APP Mutant Responds to GSMs—It has been speculated that GSMs may shift the position of the substrate in the membrane plane relative to the γ-secretase active site. Aβ42-raising GSMs and Aβ42-lowering GSMs would either pull the substrate out from the membrane or, respectively, sink it into the membrane (18). Interestingly, all so far described Aβ42-lowering GSMs have a carboxyl group, whereas Aβ12-raising GSMs lack this group. The positively charged lysine 28 residue of the Aβ domain located directly at the extracellular border of the membrane could potentially form a salt bridge with the carboxyl group of Aβ42-lowering GSMs, which as a consequence might position the γ42 site away from the γ-secretase active site. To address such a potential mechanistic contribution of Lys-28 in GSM binding, we substituted this residue by the negatively charged glutamate. As shown in Fig. 6A, assessment of the Aβ ratios revealed that Aβ38 was the major species detected for the K28E mutant (~60% of the total Aβ). The Aβ40/Aβ total ratio was strongly reduced, as was the Aβ12/Aβ total ratio, indicating a strong preference for γ-secretase cleavage at the γ38 site (see Table 1 for average Aβ levels measured in additional experiments). Mass spectrometry confirmed this result and additionally revealed a substantial increase of the shorter Aβ species Aβ37 and Aβ33 (Fig. 6B). Despite the already high Aβ38 levels, they could still be significantly increased by GSM-1 treatment (Fig. 6C). The levels of Aβ42 were too low such that changes were at the limit of detection, and no significant changes were observed for the levels of Aβ40 (data not shown). These data suggest that the proposed electrostatic interaction does not play an essential role for the mechanism of γ-secretase modulation. Finally, treatment of the K28E mutant with fenofibrate showed that Aβ42 could be tremendously increased, whereas Aβ38 could still be substantially lowered. Interestingly, fenofibrate caused an increase of Aβ40 as well (Fig. 6D). This result shows that the K28E mutant responds to GSMs of two different classes.

As above, to investigate modulation of γ-secretase cleavage by an independent assay, which assesses production in the absence of cellular metabolism, we also investigated the K28E mutant in the in vitro system using a C100-His2 K28E mutant substrate. In the cell-free assay, a slightly increased Aβ12/Aβ total ratio and a decreased Aβ40/Aβ total ratio were observed for this mutant, whereas the Aβ38/Aβ total ratio was similar to that of the WT substrate (Fig. 7A; see Table 1 for average Aβ levels of all experiments performed). Mass spectrometry confirmed this Aβ profile (Fig. 7B). Thus, unlike in cultured cells, the K28E mutant behaved as a comparably normal γ-secretase substrate in the cell-free assay. GSM-1 behaved as a modulator on the K28E mutant, lowering Aβ12 and increasing Aβ38 production. As compared with WT, the response of the K28E mutant to GSM-1 was attenuated, however (Fig. 7C). As shown in Fig. 7D, we also observed inverse modulation for the K28E mutant with fenofibrate. Taken together, despite the observed differences in the amounts of Aβ species detected in cultured cells versus the cell-free system, the K28E mutant was clearly responsive to GSMs both in cultured cells and in vitro using purified components, suggesting that Lys-28 of the Aβ domain is unlikely to play a major role for the action of GSMs.
APP Mutants Respond to GSMs

In this study, we have investigated the impact of GSMs on a variety of APP mutants to better understand their mode of action as well as the cleavage mechanism of γ-secretase. A summary of our principal findings is shown in Table 2. We establish the GSM-1 treatment on Aβ42, showing a slightly higher relative peak for the Aβ42 species that were generated in cell-free assays (28) from WT and K28E mutant substrates. The spectra closely reflect the immunoassay measurements for Aβ38, Aβ40, and Aβ42, showing a slightly higher relative peak for the Aβ42 species in the K28E mutant. Note also the higher relative peak of Aβ42 in this mutant in comparison with the WT spectrum. The intensities of the Aβ42 peaks were set at 100% in the spectra. C, sandwich immunoassay showing the effect of 2.5 µM GSM-1 treatment on Aβ42 and Aβ38 generated from WT and K28E mutant substrates in cell-free assays. Both Aβ38 and Aβ42 species could be significantly modulated for this mutant in vitro, but to a lower extent as compared with the WT. Bars represent the mean of 3 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); ***, p < 0.001, *, p < 0.05. D, sandwich immunoassay showing the effect of 150 µM fenofibrate on Aβ42 and Aβ38 generated from WT and K28E mutant substrates in cell-free assays. The K28E mutant is, like the WT, also susceptible to inverse modulation. Bars represent the mean of 3 experiments with error bars indicating the S.E. Statistical significance is calculated by paired Student’s t test (two-tailed distribution); ***, p < 0.001, **, p < 0.01.

DISCUSSION

In this study, we have investigated the impact of GSMs on a variety of APP mutants to better understand their mode of action as well as the cleavage mechanism of γ-secretase. A summary of our principal findings is shown in Table 2. We establish that naturally occurring pathogenic mutations of the γ-secretase cleavage site region in the APP TMD, which affect the precision of γ-secretase cleavage toward an increased production of Aβ42, respond to GSM treatment. These mutants even respond as strongly as WT APP following treatment with GSM-1, a well characterized potent GSM (24), which was selected as the principal GSM in this study. FAD patients with mutations in APP that affect γ-secretase cleavage should therefore be susceptible to GSM treatment. In addition, phyllaline-scanning mutagenesis analysis of the γ-secretase cleavage site region revealed responsiveness to GSM-1 for all mutants. We found that even very strong mutants among those, such as the I45F mutant that produces high amounts of Aβ42, responded to GSM-1. The I45F mutant represents the most aggressive APP FAD mutation identified so far with an extremely early disease onset of 31 years (39). Based on our data, we therefore conclude that treatment with GSMs might provide a successful therapeutic option also for these mutant carriers and others of the γ-secretase cleavage site domain with pathogenic Aβ42 production that may be identified in the future. Interestingly, our results contrast with those obtained recently for PS mutations (24, 25). Most of the PS FAD mutations investigated were not responsive to NSAIDs that act as Aβ42-lowering GSMs, such as sulindac sulfide. In particular, very strong and aggressive mutations producing high levels of Aβ42, such as PS1 L166P, and others, were not susceptible to the Aβ42-lowering capacity of sulindac sulfide. This mutant was also not responsive to the more potent GSM-1. Thus, our data indicate that the mode of action of GSMs is different for FAD mutants of APP and PS, i.e. different for substrate and protease. We also note that AD mouse models expressing human APP FAD mutants as transgene, such as some of the ones investigated here (e.g. V46I or V46F), should be more suitable for in vivo validation of GSMs than models that additionally express strong PS FAD mutations.

Recent data suggested that GSMs target the substrate rather than the protease by binding to the Aβ domain at residues 29–36 (18). Thus, it appeared possible that the modulatory capacity of GSMs could be different from that observed for the protease if they were targeting the substrate. Moreover, the GXXXG motif lying in this region was shown to determine Aβ42 and Aβ38 production in an inverse and independent manner via dimerization of the TMD. Our data do not support a mechanistic coupling of Aβ42 and Aβ38 production that may be identified in the future. Interestingly, our results suggested that GSMs target the substrate rather than the protease by binding to the Aβ domain at residues 29–36 (18). Thus, it appeared possible that the modulatory capacity of GSMs could be different from that observed for the protease if they were targeting the substrate.
found that also, this Aβ species could be lowered by GSM-1 treatment. Thus, not only Aβ₄₂ production, but also Aβ₄₉ and Aβ₄₁ production, can be lowered in response to a GSM. Taken together, these data are difficult to reconcile with the model that production of Aβ₄₂ and Aβ₄₈ is interdependent. The relationship between Aβ₄₂ and Aβ₄₈ is apparently more complex, and production of Aβ₄₈ from Aβ₄₂ (16) may in fact occur only in the WT situation.

Although our results obtained for mutants of the GXXXG dimerization motif are to a large extent consistent with previously reported findings (17), we noted some differences. Although we observed an increase in Aβ₄₈ for all mutants, we did not detect a consistent concomitant change in Aβ₄₂ with respect to both Aβ₄₂/Aβ₄₂ total ratios and absolute levels of this species. As particularly evident for the G33A and the G33I mutants, the increase in Aβ₄₈ from Aβ₄₂ (16) may in fact occur only in the WT situation.

Because the GXXXG mutant substrates give rise to mutated Aβ peptides, whose altered biochemical properties may affect the levels detectable in cultured cells downstream of production, we also analyzed these using our recently described validated cell-free in vitro system consisting of purified γ-secretase and purified APP substrate (28). In this assay system, solely the production of Aβ is analyzed independent of e.g. altered secretion or degradation. As compared with cultured cells, similar results were obtained regarding the production of Aβ₄₈ and Aβ₄₀ in this system. In contrast, however, the rather substantial Aβ₄₂ production observed for all mutants in the cell-free system using purified components shows that γ-secretase can per se generate Aβ₄₂ from Gly mutant substrates. It is important to note that the cell-free assay system was fully validated by the I45F and V50F mutant substrates. These mutants represent two extremes of APP mutants regarding the production of Aβ₄₂ and Aβ₄₀ and, in contrast to the Gly mutants, generate Aβ₄₀ without internal mutations. Both mutants behaved exactly as in cultured cells, proving that the γ-secretase enzyme itself is in the correct conformation in the in vitro assay and thus further validating the cell-free assay used. It remains possible that the altered biochemical properties of Gly mutant Aβ₄₂ as compared with WT Aβ peptides may differentially affect the fates of this peptide in the two systems and thus account for the observed differences. Alternatively, it is also possible that slight conformational alterations may occur for
substrates carrying mutations within the Aβ domain regarding the γ42 site cleavage, such as that of certain Gly mutant substrates, in the in vitro system. In agreement with the modulation results from cultured cells, responsiveness to GSM-1 was also observed for the Gly mutants in the cell-free system, although differences regarding the respective GSM response were noticed for the G33I mutant.

An interesting residue that might contribute to GSM binding in the APP TMD is lysine 28. This residue, which lies directly adjacent to the GSM-binding site in the APP TMD, might form a salt bridge between the positively charged ε-amino group of the lysine side chain and the negatively charged carboxyl group of GSMs, which is essential for their Aβ42-lowering activity (41). This ionic interaction might change the position of the APP TMD relative to the active site of γ-secretase, thus mediating a change in its cleavage specificity. However, our data show that the K28E mutant was susceptible to GSM-1 treatment, suggesting that an ionic interaction mediated by Lys-28 does not contribute to a potential GSM-APP interaction. The K28E mutant was also responsive to the inverse GSM fenofibrate, which lacks the carboxyl group and thus is apparently effective in the absence of an ionic interaction. Interestingly, mass spectrometric analysis revealed Aβ33 and Aβ37 as major Aβ species (species that are not detected by our Aβ immunoassay), possibly indicating that the K28E mutant Aβ might be turned over to shorter Aβ peptides in cultured cells. Analysis of the K28E mutant substrate in the cell-free system, however, i.e. in the absence of cellular metabolism, revealed that this mutant is normally processed by purified γ-secretase with only minor changes in the profile of Aβ species as compared with the WT APP control. Importantly, GSM-1 was also effective on the K28E mutant in this system, further suggesting that the membrane-flanking lysine residue does not play a major role for the mode of action of carboxyl group-containing GSMs.

With respect to the mechanism of γ-secretase cleavage, our data show an uncoupling of Aβ39 and Aβ42 generation for APP mutations located at different sites in the APP TMD, including mutations at or within the proposed GSM-binding site. All mutants allow a change of γ-secretase cleavage specificity with respect to the generation of Aβ39 and Aβ42 in response to GSMs. GSM-mediated modulation of γ-secretase cleavage specificity was shown to occur largely independent of the glycine residues of the GXXXG motif within the proposed GSM-binding site of APP, which were implicated in the generation of Aβ42 (17). Although these data do not entirely exclude GSM binding to this site, they suggest that the glycine residues are unlikely to play an essential mechanistic role for the mode of action of GSMs, irrespective of the current controversy regarding the GSM-APP interaction (18, 26). The GSM response of APP TMD mutants shown here, irrespective of their site and the amounts of Aβ42 generated, may favor a substrate-independently targetting mechanism of GSMs. Binding studies with more potent high affinity GSMs rather than the currently existing low affinity compounds (18) will provide important answers by clarifying whether GSMs target the enzyme, which was initially suggested by several previous studies (6, 27, 42–44). Mechanistically, NSAIDs that lower Aβ42 were suggested to allosterically alter the conformation of PS (6, 10, 44), and conformational changes of PS opposite to that induced by such NSAIDs were also observed for PS FAD mutants (6, 7, 10). It is thus conceivable that many aggressive PS FAD mutations are locked in a conformation that makes the PS-substrate interaction refractory to the Aβ42-lowering capacity of GSMs (24, 25).

Clearly, as shown in this study, mutations in the APP substrate are permissive to GSMs, suggesting that the substrate is conformationally more flexible than the γ-secretase enzyme, allowing APP substrate positioning such that the γ42 site is less exposed to the active site of γ-secretase. Thus, unlike the situation for PS FAD mutants, AD mouse models carrying APP FAD mutant transgenes should be useful for the in vivo evaluation of GSMs, and APP FAD mutant carriers are expected to be susceptible to GSM-based therapeutic strategies for AD treatment.

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