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Substrate-targeting γ-secretase modulators

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Abstract

Selective lowering of Aβ42 levels (the 42-residue isoform of the amyloid-β peptide) with small-molecule γ-secretase modulators (GSMs), such as some non-steroidal anti-inflammatory drugs, is a promising therapeutic approach for Alzheimer's disease[1]. To identify the target of these agents we developed biotinylated photo-activatable GSMs. GSM photoprobes did not label the core proteins of the γ-secretase complex, but instead labelled the β-amyloid precursor protein (APP), APP carboxy-terminal fragments and amyloid-β peptide in human neuroglioma H4 cells. Substrate labelling was competed by other GSMs, and labelling of an APP γ-secretase substrate was more efficient than a Notch substrate. GSM interaction was localized to residues 28–36 of amyloid-β, a region critical for aggregation. We also demonstrate that compounds known to interact with this region of amyloid-β act as GSMs, and some GSMs alter the production of cell-derived amyloid-β oligomers. Furthermore, mutation of the GSM binding site in the APP alters the sensitivity of the substrate to GSMs. These findings indicate that substrate targeting by GSMs mechanistically links two therapeutic actions: alteration in Aβ42 production and inhibition of amyloid-β aggregation, which may synergistically

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Author Contributions T.L.K., D.M.W., E.H.K. and T.E.G. conceived the project. T.L.K., T.B.L. and M.A.B. planned, performed and analysed the photoaffinity experiments and amyloid-β assays. P.C.F., W.Y., and M.S.W. performed photoaffinity experiments with purified γ-secretase and APP(C100)–Flag and Notch(C100)–Flag; T.L.K. wrote most of the paper. T.L.K., M.A.B., C.V. and R.W.P. performed and analysed mouse experiments. K.J.-W. cloned constructs and made stable cell lines. S.S., B.C., J.T. and E.H.K. made the APP-NOTCH chimaera construct and cell line and performed mass spectrometry analysis of amyloid-β. A.T.W. and D.M.W. analysed amyloid-β oligomers. D.Y. and C.E. screened and provided compound libraries. G.M.M., B.H., R.C., and A.F. synthesized fenofibrate and various photoprobes. J.E. characterized initial photoprobes. R.N. and B.S. synthesized flurbiprofen and related photoprobes. B.M., V.R., B.C. and T.L.R. performed mass spectrometry analysis of amyloid-β, APP-NOTCH amyloid-β and saturation binding experiments with X-34.

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reduce amyloid-β deposition in Alzheimer's disease. These data also demonstrate the existence and feasibility of 'substrate targeting' by small-molecule effectors of proteolytic enzymes, which if generally applicable may significantly broaden the current notion of 'druggable' targets[2].

We previously demonstrated that a subset of non-steroidal anti-inflammatory drugs (NSAIDs; that is, ibuprofen and sulindac) are examples of GSMs—agents capable of preferentially lowering Aβ42 *in vitro* and *in vivo*[3,4]. A signature of Aβ42-lowering GSMs is a reciprocal increase in shorter amyloid-β peptides. Further studies have identified tarenflurbil (formerly named *R*-flurbiprofen[5]) as a non-NSAID Aβ42-lowering GSM with potential therapeutic application in Alzheimer's disease[3,6]. In contrast to γ-secretase inhibitors, most GSMs do not inhibit γ-secretase-mediated release of the intracellular cytoplasmic domains of APP or other γ-secretase substrates such as Notch and ErbB4[3,4]. We have also identified other GSMs that selectively increase Aβ42 quantities, similar to mutations in presenilin 1 (PSEN1) and APP which cause familial Alzheimer's disease[7,8]. Although studies have suggested that GSMs target γ-secretase, the mechanism of amyloid-β modulation by GSMs has not been definitively established[3,9,10].

We synthesized two GSM derivatives for photoaffinity labelling studies to determine the molecular targets of the GSMs: fenofibratebiotin (Fen-B), a derivative of fenofibrate which is an Aβ42-raising GSM, and flurbiprofen-benzophenone-biotin (Flurbi-BpB), a derivative of tarenflurbil which is an Aβ42-lowering GSM (Fig. 1a). Each compound contains benzophenone (a preferred photoactive moiety for protein labelling[11]) and a biotin tag (for detection and affinity purification), and maintains the activity of the parent GSM (Supplementary Figs 2 and 3).

Initial crosslinking of Fen-B (1–100 μM) in lysates from human neuroglioma H4 cells overexpressing APP demonstrated that numerous proteins were labelled but that PSEN1 was not (data not shown). To determine whether this negative result was due to limited sensitivity, we investigated the ability of Fen-B to label a highly purified preparation of active γ-secretase[12]. Photolysis of purified γ-secretase (Fig. 1b) in the presence of Fen-B (300 μM) followed by precipitation with streptavidin did not label the core components of γ-secretase (PSEN1, nicastrin (NCSTN), anterior pharynx-defective 1 (APH1) or presenilin enhancer 2 (PSENEN, also known as PEN2)). Similar studies with biotinylated carprofen[13], an Aβ42-lowering GSM, did not detect labelling of γ-secretase (B.S., unpublished data). Having failed to label the subunits of γ-secretase, we turned our attention to the remaining member of an active γ-secretase 'complex': the substrate itself. We addressed whether a purified, recombinant Flag-tagged γ-secretase substrate derived from APP (APP(C100)–Flag, comprising the 99 C-terminal residues of APP plus a methionine at the N terminus, ref. [12]) could be labelled by Fen-B and Flurbi-BpB. Photoactivated crosslinking with concentrations of GSM photoprobes spanning the range over which they modulate Aβ42 led to increasing quantities of biotinylated APP(C100)–Flag (Fig. 1c). Labelling of APP(C100)–Flag by Fen-B was competed by Aβ42-raising and -lowering GSMs (Fig. 1d); however, non-GSM NSAIDs (aspirin, naproxen) did not compete (data not shown). The sulphone derivative of the GSM sulindac, which does not affect Aβ42 levels, did not compete for labelling, showing that small structural features influence the ability to modulate amyloid-β and compete for labelling of APP C-terminal fragments (CTFs; Fig. 1d). These results indicate that binding of GSMs to the APP substrate could mediate their ability to shift the location of γ-secretase cleavage.

Next, we addressed whether the interaction between APP CTF and GSMs could be detected in cells. Direct exposure of H4 cells to ultraviolet and photoprobes was toxic (Supplementary Fig. 3); therefore, crosslinking of Fen-B and Flurbi-BpB was examined in solubilized membrane fractions from human neuroglioma H4 cells enriched for active γ-secretase and substrates (APP CTFs 83 and 99)[7]. Fen-B and Flurbi-BpB labelled an APP-derived protein of

~11 kDa; we assigned this protein as α-secretase-derived APP CTF (CTF83) on the basis of molecular weight, increased expression in APP-transfected cells and immunoreactivity with an APP C-terminal antibody (Fig. 1e and Supplementary Fig. 2), but not an antibody against Aβ1–16 which would recognize β-secretase-derived APP CTF (CTF99; not shown). Labelling of APP CTF was photoactivation dependent (Fig. 1e) and no binding to PSEN1 CTF, PSEN1 amino-terminal fragment (NTF) or nicastrin was observed in these experiments (data not shown). These data demonstrate that labelling of APP CTF by GSMs can occur in cellular membrane fractions, providing further evidence that this interaction may be responsible for the modulation of amyloid-β production.

GSMs have been reported to modulate the site of γ-secretase cleavage in other substrates such as Notch[14]. We previously reported that APP is more sensitive than Notch to shifts in the location of γ-cleavage induced by GSMs[15]. Consistent with the differential sensitivity of Notch to GSMs that we observe, we find that whereas Fen-B does label a recombinant substrate derived from mouseNotch (Notch(C100)–Flag), this reaction is less efficient than Fen-B labelling of the APP(C100)–Flag (Fig. 1f). Furthermore, the presence of purified γ-secretase does not prevent labelling of either substrate by Fen-B (Supplementary Fig. 4). These data suggest that a differential affinity of the GSM Fen-B occurs between APP and other γ-secretase substrates such as Notch, and further link substrate targeting to the GSM properties of these compounds.

Initial mapping experiments showed that Fen-B did not label the last 50 amino acids of APP (APP intracellular domain, CTF-γ), raising the possibility that it was binding the amyloid-β region of APP (Supplementary Fig. 5). To define precisely the binding site of GSMs, C-terminal-truncated versions of amyloid-β were irradiated in the presence of Fen-B. Fen-B efficiently labelled Aβ1–40 and Aβ1–36 but did not label Aβ1–28 (Fig. 2a), suggesting that a minimal binding site on APP(C100)–Flag corresponds to residues 29–36 (GAIIGLMV) of amyloid-β (Fig. 2a). Aβ1–36 can also be labelled by Flurbi-BpB (Fig. 2b), and both GSMs can label Flag-tagged Aβ25–36 (Fig. 2c). These residues represent the start of the predicted APP transmembrane domain (625–632 of APP695) that lies within the membrane; however, this region of APP is accessible to small molecules[16]. Because the putative binding region of GSMs is also found in full-length APP we looked for labelling of both APP fragments in cells. Using microsomal membrane fractions from H4 cells expressing wild-type APP, we observed that both Fen-B and Flurbi-BpB labelled full-length APP and APP CTFs (Supplementary Fig. 6). Labelling of APP CTF and APP by GSM photoprobes was competed by both Aβ28–36 and structurally diverse GSMs, further supporting the specificity of this interaction (Supplementary Fig. 7). Taken together, these data provide evidence that the minimal binding site for raising and lowering GSM is Aβ28–36. Notably, this region of Aβ and APP is important for amyloid-β aggregation[17] and has been implicated as a binding site for amyloid-β aggregation inhibitors[18]. Furthermore, we[19] and others[20] have shown that mutations in this region of APP can drastically change the spectrum of amyloid-β species, supporting the notion that binding of GSMs to substrate in this region could alter γ-secretase cleavage. Indeed, we find that a peptide (I1, NH_2-FEGKF-$CONH_2$) known to bind to this region of amyloid-β acts as a GSM[17]. I1 elevated Aβ42 production without lowering total amyloid-β amounts, similar to fenofibrate (Fig. 2d).

If GSMs target the amyloid-β region of APP CTF, then any compound that binds to Aβ is a potential GSM. To test this possibility, we examined 15 compounds for GSM activity that had previously been reported as amyloid-β binding compounds, amyloid-β aggregation inhibitors or amyloid-β binding agents (Supplementary Fig. 8). Six of these compounds did not act as GSMs (for example, melatonin, BTA-1), but the remainder all showed GSM activity. These included two Aβ42-raising (the kinase inhibitor DAPH (4,5-dianilinophthalimide) and the calmodulin inhibitor, calmidazolium) and seven Aβ42-lowering GSMs (for example, the

amyloid dye X-34 (1,4-*bis*(3-carboxy-4-hydroxyphenylethenyl)-benzene) and the hydrophobic probe Bis-ANS (4,4′-dianilino-1,1′-binaphthyl-5,5′-disulphonic acid); Fig. 3a). Immunoprecipitation and mass spectrometry analysis showed that the lowering of Aβ42 by X-34 and chrysamine G was accompanied by increased amounts of shorter amyloid-β, a characteristic signature of Aβ42-lowering GSMs (Supplementary Fig. 9). In cell-free γ-secretase assays, Congo red and chrysamine G lowered Aβ42 selectively, demonstrating that they are bona fide GSMs (Supplementary Fig. 10).

We focused on X-34 to characterize its GSM properties further. Cellular dose-response studies showed that X-34 lowered Aβ42 (effector concentration for half-maximum response, EC_{50}, 13.7 μM) and Aβ40 at higher concentrations (EC_{50} 60.7 μM), but did not decrease total amyloid-β amounts (Fig. 3b). X-34 competes for binding of APP(C100)–Flag by Fen-B and Flurbi-BpB (Fig. 3c). We also found that X-34 fluorescence increased when incubated with monomeric Aβ42. This allowed saturation binding experiments to determine that X-34 bound to Aβ42 with a dissociation constant (K_d) of 12.1 ±4.7 μM (Supplementary Fig. 11). Thus, the affinity of X-34 for a peptide containing the putative GSM binding site is similar to concentrations at which it acts as a GSM in cells. Finally, we addressed whether X-34 modulated Aβ42 amounts in an APP transgenic mouse (Tg2576). We found that X-34 (100 mg kg^{-1}) decreased soluble Aβ42 (35%, P <0.01) in the brains of Tg2576 mice with no effects on Aβ40 (Fig. 3d). Tarenflurbil (50 mg kg^{-1}) in the same paradigm also lowered Aβ42 (25%, P <0.05) without a decrease in Aβ40 (123%, Fig. 3d). Collectively, these observations support the hypothesis that GSMs interact with substrate. Furthermore, the data show that selecting compounds on the basis of their ability to bind amyloid-β and APP is an efficient strategy to identify GSMs and suggest that screening compounds for binding to amyloid-β should be a simple and useful method to discover previously unknown GSMs.

On the basis of our previous findings which showed that GSMs bind a region in APP and amyloid-β that is involved in fibrilization[17], we reasoned that GSMs may also affect the aggregation of amyloid-β. Previous studies have shown that compounds with GSM activity, including certain NSAIDs[21], Congo red[22] and Bis-ANS[23], can inhibit the *in vitro* aggregation of synthetic amyloid-β. However, it is unknown if GSMs influence the concentration of secreted amyloid-β oligomers, such as those released by Chinese hamster ovary (CHO) cells expressing the APP V717F mutation (referred to as 7PA2 cells), which alter long-term potentiation and perturb the memory of learned behaviour when injected into rat brain[24,25]. To address this question we treated 7PA2 cells with two Aβ42-raising GSMs and a novel Aβ42-lowering GSM (Supplementary Fig. 12). Fenofibrate and FT-1 (ref. 7) increased amounts of Aβ42 whereas FT-9 lowered Aβ42, but all three GSMs decreased amounts of amyloid-β dimers and trimers. Extended dose experiments with fenofibrate and FT-9 show a consistent decrease in amyloid-β oligomers at doses where total amyloid-β amounts are not altered (Supplementary Fig. 12). In contrast to increased amyloid-β oligomer formation, when CHO cells are genetically manipulated to increase Aβ42 production by co-expression of APP and FAD-linked mutant PSEN1 proteins, we find that both Aβ42-lowering and -raising GSMs decrease oligomer formation[26]. Notably, amyloid-β oligomers in 7PA2 and neuronal cells are generated intracellularly before secretion[27]. Therefore, the finding that GSMs shift amyloid-β cleavage by targeting a region of substrate present in the amyloid-β cleavage product provides a mechanistic link between GSM activity and general anti-amyloid-β aggregation effects and also suggests that the binding of GSM to substrate and inhibition of amyloid-β oligomerization can occur in the same cellular compartment.

Our data suggest that mutation of the GSM binding site in APP should alter sensitivity to GSMs. To test this hypothesis we exchanged a portion of the GSM binding site in APP with the analogous region of human NOTCH (Fig. 4a, b and Supplementary Fig. 13). We used this chimaeric construct because in our hands the NOTCH transmembrane domain (TMD) is

resistant to GSM effects[15]. The APP-NOTCH TMD construct produces a spectrum of chimaeric amyloid-β species in conditioned cell media (Supplementary Fig. 13). Although the molecular weight and abundance of each chimaeric peptide differs from amyloid-β peptides from APP, the main species produced from the APP-NOTCH TMD construct have identical C termini to Aβ1–40 and Aβ1–42, and can be quantified by enzyme-linked immunosorbent assays (ELISAs; Supplementary Fig. 13). We therefore examined the relative sensitivity to GSMs of APP-NOTCH TMD versus APP in stably transfected cells. Cleavage of APP-NOTCH TMD was not significantly affected by either an Aβ42-lowering GSM or an Aβ42-raising GSM but remained sensitive to inhibition by treatment with the γ-secretase inhibitor L-685458 (Fig. 4a, b and Supplementary Fig. 13). Thus, exchange of residues in the putative binding site render the substrate much less sensitive to GSMs.

Several explanations for the GSM activity of NSAIDs have been proposed. It has recently been hypothesized that NSAIDs alter dimerization of APP[20]. Although plausible, there is no conclusive evidence that an APP dimer is the substrate for γ-secretase, and it is difficult to account for both Aβ42-raising and -lowering properties of structurally related GSMs with this model. Other studies propose that GSMs lower Aβ42 by shifting presenilin conformation through allosteric binding[28,29]. Our data implicating substrate targeting by GSMs is compatible with these previous findings (Supplementary Discussion). It is also possible that GSMs shift cleavage by changing the position of APP CTF in the plane of the membrane leading to shifts in the amyloid-β fragments produced[19]. This model is attractive as it accounts for the difference between the classes of GSMs: Aβ42-raising GSMs would ‘pull’ the substrate out of the membrane, whereas Aβ42-lowering GSMs allow the substrate to ‘sink’ further into the membrane.

Given the likelihood that GSMs exist that directly target the enzyme, we believe it is appropriate to refer to the GSMs we have identified as substrate-targeting GSMs. Substrate-targeting GSMs can, in theory, have two therapeutic consequences—alteration in Aβ42 production and inhibition of amyloid-β aggregation—that might synergistically benefit the Alzheimer’s disease phenotype (Supplementary Fig. 1). Future substrate-targeting GSMs could be developed that optimize the selective Aβ42-lowering and anti-aggregation properties. These data may also explain why substrate-targeting GSMs that raise Aβ42 may not be as risky as our data initially implied[7]: although they raise Aβ42, they may also inhibit amyloid-β aggregation. Finally, these data demonstrate a new means of modulating intramembrane cleaving proteases through substrate-binding small molecules, which may have broad therapeutic applications[2,30].

METHODS SUMMARY

Photoaffinity labelling

All experiments used the following general protocol. Samples (recombinant or synthetic peptides, cell lysates and membrane preparations) were exposed in borosilicate test tubes to ultraviolet light (350 nm) in a Rayonet Photoreactor R100 (RPR-3500 lamps, Southern New England Ultra Violet Company) in a cold room (4 °C) for 30 min or as otherwise noted. Crosslinked samples were analysed by ELISA for biotin incorporation, or analysed directly by SDS–PAGE (Criterion-XT, Bio-Rad), or after precipitation with strep-tavidin ultralink plus beads (Pierce). After immunblotting, proteins were detected using chemiluminesence (ECL Plus, GE Healthcare) or near-infrared fluorescence (LiCor Odyssey). Full-length APP and APP CTFs (CTF83, CTF99) were detected with CT20, a rabbit polyclonal antibody against the C terminus of APP. Biotin was detected with an affinity-purified rabbit polyclonal antibody (Bethyl).

Cell-based screens for amyloid-β modulation

Human H4 neuroglioma cells (American Type Culture Collection, ATCC) expressing wild-type APP695 protein or CTF105 fused to secreted alkaline phosphatase which is efficiently processed to APP CTFs (CTF83, CTF99) and produce high levels of amyloid-β, were used for the cell-based screens as previously described[7]. Cells were incubated for 5–6 h in the presence of the various compounds in Opti-Mem culture medium containing 1% fetal bovine serum. Compounds were dissolved in dimethyl-sulphoxide (DMSO; 0.5% final concentration) and diluted 200-fold. Media was analysed for various amyloid-β species (40, 42 and total) using ELISAs as described below and previously[7]. The EC_{50} values for changes in amyloid-β species were calculated by fitting sigmoid dose-response curves using nonlinear regression in Prism (GraphPad) and are shown as values ±s.e.m.

Statistical analysis

Data are presented as either percentage control or mean ± s.e.m. Results were analysed using Prism (Graph Pad) with *t*-tests or one-way analysis of variance analyses (ANOVAs) with Dunnett's post-hoc correction for comparison of multiple samples to a control. Statistical significance is shown as $P < 0.05$ (one asterisk), $P < 0.01$ (two asterisks) or $P < 0.001$ (three asterisks).

METHODS

Compounds and peptides

The following commercially available chemicals and peptides used in this work were purchased: BSB ((trans,trans)-1-bromo-2,5-bis-(3-hydroxycarbonyl-4-hydroxy) styrylbenzene), chrysamine G, FSB ((E,E)-1-fluoro-2,5-bis-(3-hydroxycarbonyl-4-hydroxy) styrylbenzene), half chrysamine G and amyloid-β peptides 1–40, 1–36 and 1–28 (Anaspec); APP-CTF50 (rPeptide); aspirin, BTA-1, Bis-ANS, clioquinol, Congo red, curcumin, DAPH, fenofibrate, melatonin, naproxen, sulindac sulphide and sulphone (Sigma); *R*-flurbiprofen (Cayman); NIAD4 (Nomadics). The compounds Fen-B, Flurbi-BpB, BpB, FT-1 and FT-9 were synthesized as described in Supplementary Information. AOI987, LY-411575 and X-34 were synthesized by the Mayo Clinic Laboratory of Synthetic Organic Chemistry following published references as described in Supplementary Information. The I1 peptide fragment (NH_2-FEGKF-$CONH_2$) and N-terminal Flag-tagged truncated amyloid-β fragments (Flag-Aβ1–12, 13–24 and 25–36) were synthesized by the Mayo Clinic MPRC Peptide Synthesis Facility. Aβ28–36 was synthesized by Elim Biopharmaceuticls; Celecoxib was a gift from Myriad Pharmaceuticals.

Peptide crosslinking

Amyloid-β peptides were diluted from DMSO stocks into PBS (5 μM final concentration), incubated with photoprobes and crosslinked. Flag–amyloid-β fragments (10 μM) were incubated with a GSM photoprobe (Fen-B, 100 μM; Flurbi-BpB, 40 μM) in PBS and crosslinked for 30 min. Recombinant peptides (APP(C100)–Flag and Notch(C100)–Flag; 1 μM final concentration) were diluted from stock into buffer (50 mM HEPES, pH 7.4, 0.25% CHAPSO) incubated with competitor (if required) at 37 °C for 15 min, spiked with photoprobe and crosslinked.

Membrane isolation

H4 cells overexpressing wild-type APP695 were lysed by nitrogen bomb cavitation at 700 p.s.i. for 1 h in PBS (4639, Parr instrument company), and then spun at 1000*g* for 10 min to remove unbroken cells and nuclei. Membranes were pelleted from the supernatant after centrifugation at 100,000*g*. Membranes were homogenized in sodium carbonate buffer (100 mM, pH 11.5)

by brief sonication or resuspension using a glass–teflon homogenizer to remove peripherally-associated membrane proteins and spun again. Membranes were then extracted with 0.25% CHAPSO in 50 mM HEPES, pH 7.4, and spun to remove insoluble proteins.

Mass spectrometry of amyloid-β

For matrix-assisted laser desorption/ionization–time of flight (MALDI–TOF) mass spectrometry analyses of amyloid-β peptides, the same H4 cell lines used in screens were treated with the indicated compounds as described[4]. Secreted amyloid-β peptides were analysed by minor modifications of the technique previously described[7]. Aβ1–x was immunoprecipitated from conditioned medium with antibody 9 covalently coupled to seize beads (Pierce), with synthetic Aβ40 with methionine 35 substituted by norleucine ([Nle35]-β-Amyloid(1–40); Anaspec) added as an immunoprecipitation control and mass standard. Proteins were eluted into 0.1% trifluoroacetic acid, 75% acetonitrile. Samples were mixed 1:1 with alpha-cyano-4-hydroxycinnamic acid matrix (6.2 mg ml^{-1}) in methanol:acetonitrile:water (36%:56%:8%; Agilent). Samples were analysed on a 4800 MALDI–TOF–TOF (Applied Biosystems).

In vitro γ-secretase assays

These assays were performed as previously described[3,7]. Briefly, buoyant cholesterol-rich fractions showing enriched γ-secretase activity were isolated from H4 cells stably overexpressing secreted alkaline phosphatase fused to the N terminus of APP(C105) that had been treated with IL-CHO, a reversible γ-secretase inhibitor, to accumulate APP CTF. Fractions 3–5 (1 ml each) were collected and activity was measured by incubation at 37 °C for 2 h and comparing amyloid-β and CTF-γ (AICD) amounts to a frozen sample. Amyloid-β concentrations were determined by sandwich ELISA and CTF-γ by western blotting with antibody CT-20.

ELISA

Amyloid-β species were detected with sandwich ELISAs using monoclonal antibodies as previously described[7]. Briefly, amyloid-β peptides were captured by C-terminal-specific antibodies for Aβ40 (antibody 40.1) or Aβ42 (antibody 42.2) that were coated on Immulon 4 HBX ELISA plates (Thermo Scientific) at 25 μg ml^{-1} in PBS. Captured amyloid-β was then detected by an HRP-conjugated antibody reactive to the N-terminal epitope 1–16 of amyloid-β (antibody 9). To measure incorporation of the photoprobes and corresponding biotin tag into peptides two strategies were used: (1) recombinant γ-secretase substrates (APP(C100)–Flag and Notch(C100)–Flag) or Flag-epitope tagged fragments of amyloid-β (Flag–Aβ1–12, 13–24 and 25–36) were captured on anti-Flag ELISA plates (M2 coated; Sigma) and biotin incorporation was detected with streptavidin- or NeutrAvidin-HRP (Pierce); and (2) C-terminal truncations of amyloid-β (1–28, 1–36 and 1–40) were captured at the N terminus by antibody 9, and again, biotin incorporation was measured by detection with streptavidin- or NeutrAvidin-HRP.

Production and purification of γ-secretase and recombinant substrates

The CHO γ-30 cell line expresses human PSEN1, Flag–PEN2 and APH1α2–HA. γ-secretase was purified from γ-30 using a multi-step purification as previously described[12]. For western blotting analysis of γ-secretase, samples were run on 4–20% Tris-glycine PAGE gels, transferred to polyvinylidene difluoride filter membranes and probed with antibody 14 (for PSEN1–NTF, 1:2,000, a gift from S. Gandy), antibody N19 (for PSEN1–NTF, 1:200, Santa Cruz), antibody 13A11 (for PSEN1–CTF, a gift from Elan Pharmaceuticals), antibody 3F10 (for APH1α2–HA, Roche), anti-Flag M2 antibody (for Flag–PEN2, 1:1,000, Sigma), R302 antibody (for NCSTN, 1:4,000, a gift from D. Miller and P. Savam), and guinea-pig anti-

NCSTN antibody (1:2,000, Chemicon). Recombinant γ-secretase substrates, APP(C100)–Flag and Notch(C100)–Flag (N100-Flag), were produced in *Escherichia coli* and purified over an anti-Flag M2 affinity column (Sigma) as previously described[12]. APP(C100)–Flag is derived from the C-terminal fragment of APP (CTF99), the endogenous precursor to amyloid-β, with the addition of an N-terminal methinone and a Flag epitope tag to facilitate detection and purification[12]. The Notch(C100)–Flag is derived from the analogous region of mouse Notch[12].

Animal studies

Female Tg2576 mice were treated with test compounds at 3 months of age before amyloid-β deposition. Compounds were mixed with vehicle (PEG-400 90%, DMSO 10%) and delivered by oral gavage (200 μl) and animals were killed 4 h later. Plasma, cerebellum and two hemi-brains were saved from each animal. One hemi-brain was extracted using RIPA buffer (1 ml per 150 mg) with complete protease inhibitors (Roche) and amyloid-β concentrations were assessed with Aβ40 and Aβ42 ELISAs. All animal studies were approved by the Mayo Clinic Institutional Animal Care and Use Committee.

Determination of X-34 affinity for Aβ1–42 by fluorescence titration

When X-34 was mixed with monomeric Aβ1–42 in 10 mM Tris-HCl (pH 8.0) and 10% DMSO, enhancement of X-34 fluorescence was noted at excitation wavelengths above 366 nm, the wavelength which gives maximum X-34 absorbance. Fluorescence was monitored on a Cary Eclipse Fluorescence Spectrophotometer (Varian Instruments) at 25 °C with excitation at 412 nm, emission at 502 nm, and excitation and emission slits at 2.5 and 10 nm, respectively. Stock solution A (10 μM Aβ1–42 in 10 mM Tris pH 8.0 and 10% DMSO) was titrated by sequential addition of solution B (10 μM Aβ1–42 plus varying amounts of X-34 (μM) in the same buffer). A parallel series of blank fluorescence values was obtained by omitting Aβ1–42 from solutions A and B. Data were fitted to the following equation by unweighted nonlinear regression analysis (SigmaPlot), with $[L]_{tot}$ as the independent variable and K_d and F_C as the fitted parameters[31]:

$$\Delta F = 0.5 F_{C-L}[D - (D^2 - 4[A\beta]_{tot}[L]_{tot})^{1/2}]$$

where F_C is the fluorescence of X-34 in the presence of Aβ1–42 and F_L is the fluorescence in its absence; $D = [A\beta]_{tot} + [L]_{tot} + K_d$, where $[A\beta]_{tot}$ and $[L]_{tot}$ are the total Aβ1–42 and X-34 concentrations, respectively, and K_d is the equilibrium dissociation constant; and F_{C-L} is the difference in fluorescence intensity coefficients for bound and free X-34. Because high concentrations of X-34 were used in the titration, the absorbance of X-34 was significant even at the 412 nm excitation wavelength ($\varepsilon_{412\ nm} = 850\ M^{-1}cm^{-1}$), observed fluorescence values were corrected for inner filter effects as described previously[32].

Construction of APP-NOTCH chimaera cDNA and selection of stable cell lines

APP695 was subcloned into the retroviral vector pLHCX (BD Biosciences/Clontech) to facilitate generation of stable cell lines. The retroviral expression vector for the APP-NOTCH TMD chimaera was generated by site-directed mutagenesis with the Stratagene Quick-Change kit according to the manufacturer's instructions. Primers were designed to insert the analogous sequence of human NOTCH1 and excise the corresponding sequence of APP (shown in Fig. 4). All new constructs were sequenced to assure accuracy. To generate a stable cell line, CHO cells were infected with retroviruses encoding APP-NOTCH TMD and selected with hygromycin B. GSMs did not consistently change the amounts of chimaeric Aβ X-40 or X-42 from the APP-NOTCH TMD line and thus an EC_{50} could not be calculated.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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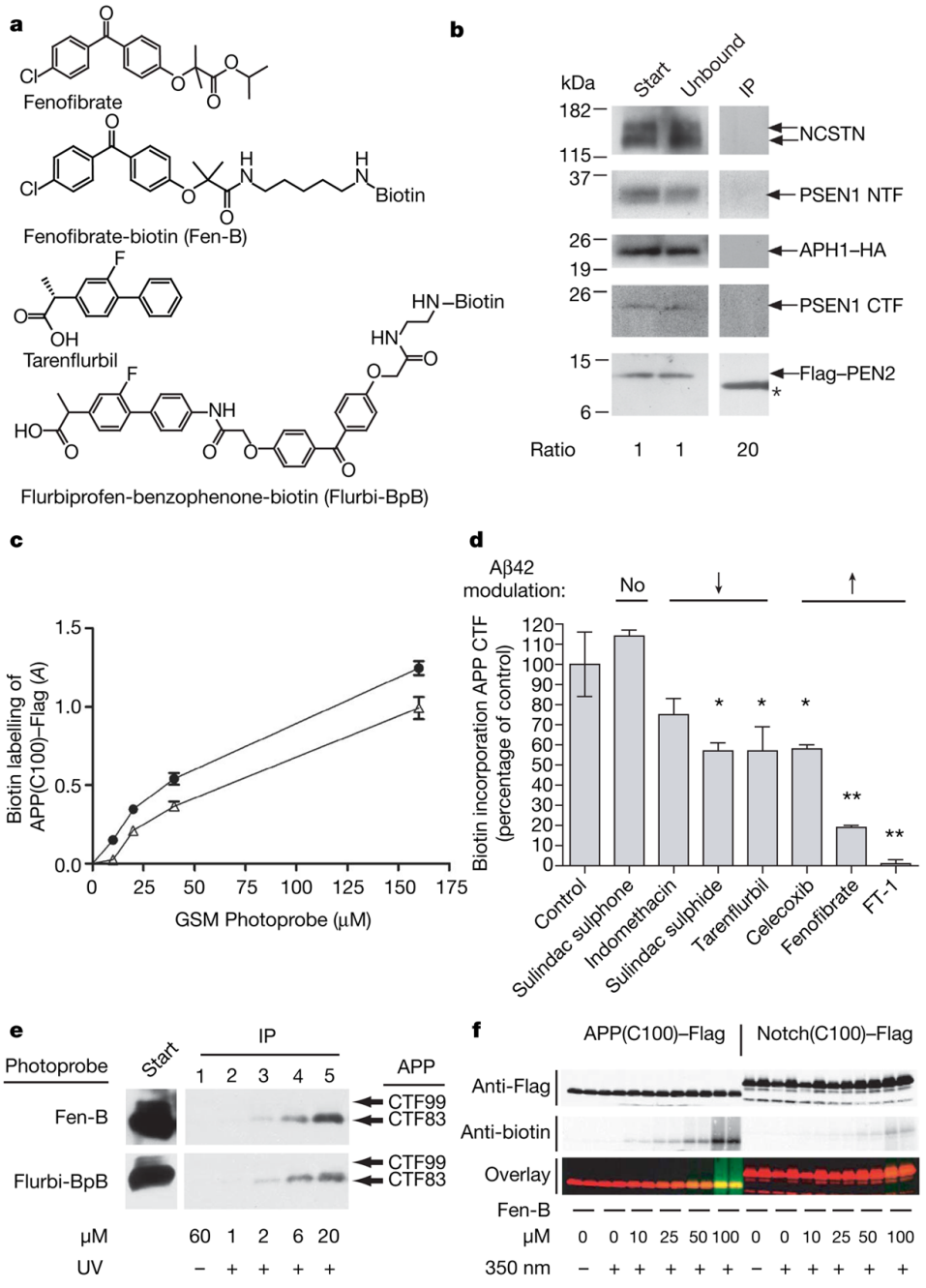

Figure 1. GSM photoprobes label APP CTF

a, Structures of the parent GSMs (fenofibrate and tarenflurbil) and photoprobe derivates (Fen-B and Flurbi-BpB) are shown. **b**, The absence of PSEN1, NCSTN, APH1 and PEN2 labelling by the GSM Fen-B in a purified γ-secretase preparation (from CHO γ-30 cells[12]) and immunoprecipitation with streptavidin. The ratios of sample relative to the starting material are shown. Start and unbound lanes contain 5% of the immunoprecipitated material (lane 3), therefore the ratios are 1, 1 and 20. Asterisk denotes nonspecific reactivity with streptavidin. **c**, GSM photoprobes (Flurbi-BpB, closed circles, and Fen-B, open triangles) label a recombinant APP γ-secretase substrate (APP(C100)–Flag) with similar potency. *A*, absorbance; data are mean ± s.e.m., $n = 2$. **d**, Labelling of APP(C100)–Flag by Fen-B (10 μM)

is competed by Aβ42-lowering and -raising GSMs (100 μM) but not by sulindac sulphone, a non-GSM NSAID. Data are presented as percentage control ±s.e.m., $n = 2$. Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; ANOVA with Dunnett's post-hoc analysis. **e**, GSM photoprobes label APP CTF from cells. CHAPSO solubilized membrane fractions from H4 APP-CTF–alkaline phosphatase cells were crosslinked with Fen-B and Flurbi-BpB (50 μM) and analysed by immunoprecipitation with streptavidin and immunblotting for APP (antibody CT20). Both GSMs label a fragment of APP that co-migrates with APP(C83). UV, ultraviolet. **f**, A GSM photoprobe preferentially labels a recombinant APP substrate (APP(C100)–Flag; left panel) relative to Notch (Notch(C100)–Flag; right panel). Samples were analysed by western blotting for incorporation of Fen-B. Green, biotin; red, Flag; yellow, dual reactivity; LiCor Odyssey.

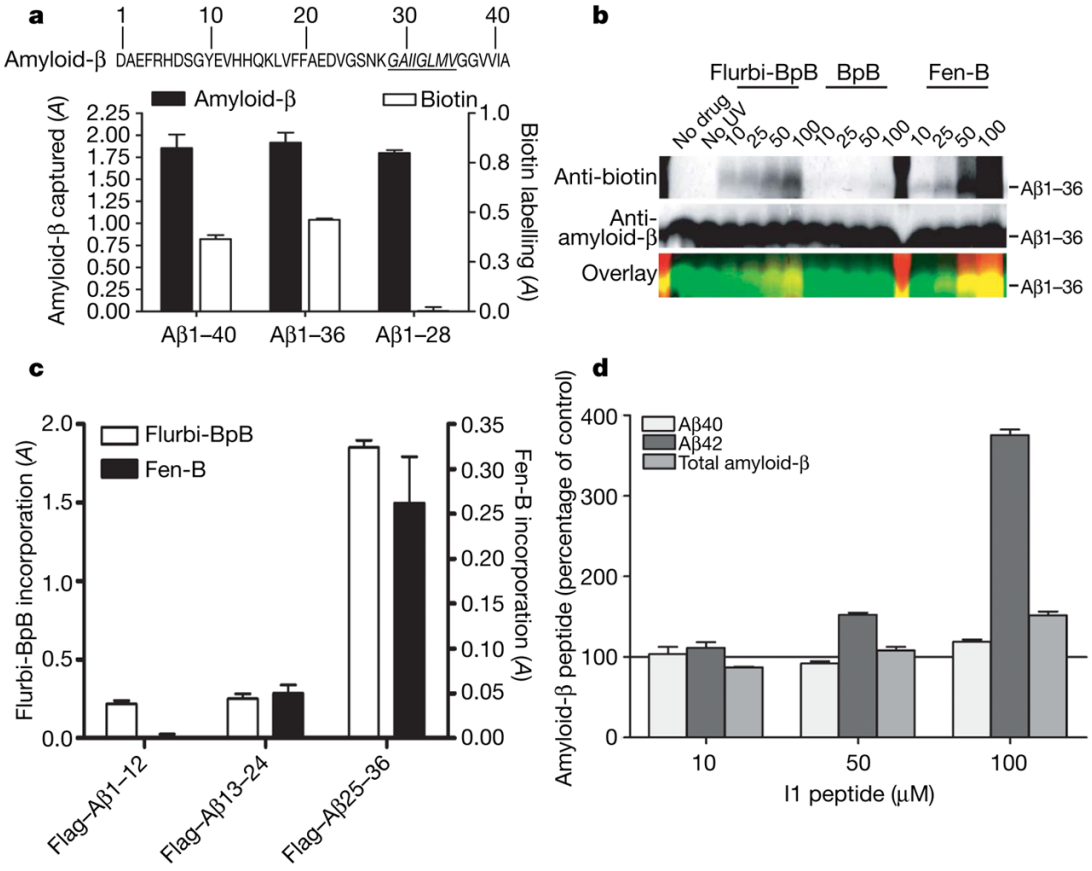

Figure 2. GSM photoprobes bind to the amyloid-β region of APP

a, Fen-B labels Aβ1–40 and Aβ1–36 but not Aβ1–28, suggesting that the binding site for Fen-B is located between residues 28 to 36 of amyloid-β, which are highlighted in italics. **b**, Flurbi-BpB and Fen-B label Aβ1–36 (biotin incorporation), whereas the photoaffinity tag alone (BpB) shows minor labelling. **c**, Flurbi-BpB and Fen-B preferentially label Flag-tagged Aβ25–36. Data are presented as biotin incorporation (absorbance, *A*) ±s.e.m., $n = 3$. **d**, The peptide fragment I1 (NH_2-FEGKF-$CONH_2$) increases Aβ42 in H4 cells expressing APP similar to the GSM fenofibrate.

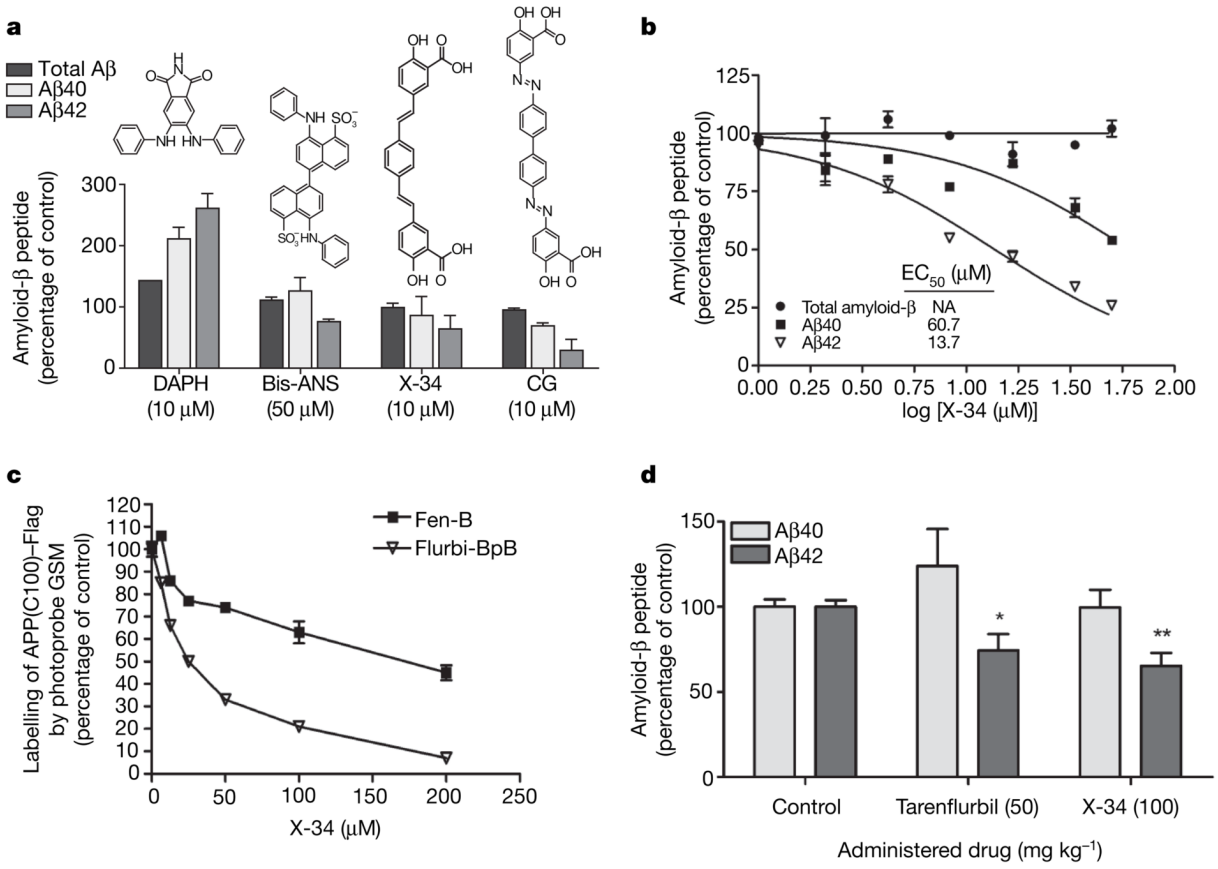

Figure 3. Compounds that bind Aβ are GSMs *in vitro* and *in vivo*

a, A cell-based screen of Aβ-binders identified molecules that increase Aβ42 (DAPH) or decrease Aβ42 (Bis-ANS, X-34 or chrysamine G (CG)). Data are mean ± s.e.m., $n = 3$. **b**, X-34 is an Aβ42-lowering GSM. Changes in amyloid-β peptide amounts after X-34 treatment are shown. Data are presented as percentage control ±s.e.m., $n = 3$. EC_{50} values were calculated as described in Methods. Total amyloid-β did not decrease. NA, not applicable. **c**, X-34 binds to APP(C100)–Flag and decreases labelling by GSM photoprobes. Biotin incorporation into APP by Fen-B and Flurbi-BpB is presented as percentage of control (peptide without X-34) ±s.e.m., $n = 2$. **d**, X-34 lowers Aβ42 in Tg2576 mice after 4 h. X-34 ($n = 7$) and tarenflurbil ($n = 5$) reduce Aβ42 selectively; control ($n = 7$). Data are presented as amyloid-β percentage of control relative to vehicle ±s.e.m. Animals per group (n). Asterisk, $P < 0.05$; two asterisks, $P < 0.01$; ANOVA with Dunnett's post-hoc analysis.

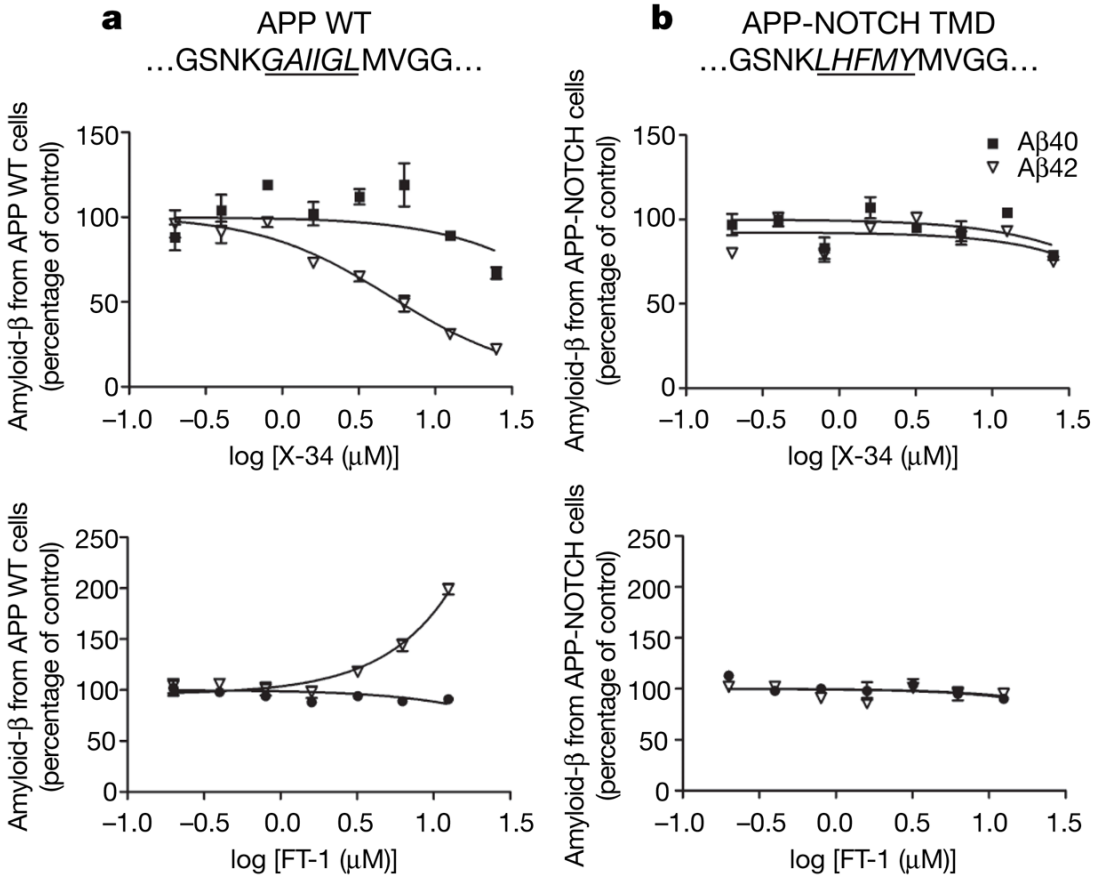

Figure 4. The ability of GSMs to shift Aβ42 amounts is sensitive to the amino acid sequence of the binding site on APP

a, b, The sequences of wild-type (WT; **a**) APP and the mutated substrate (**b**) containing the homologous region (italic, underlined) of the NOTCH transmembrane domain (TMD) in APP where GSMs are hypothesized to bind. Top row, X-34 lowers Aβ42 (EC_{50} 5.9 μM) from APP wild-type cells (**a**) but did not change either Aβ40 or Aβ42 concentrations in the APP-NOTCH TMD line (**b**). Bottom row, FT-1 (12.5 μM) raised Aβ42 200% in APP wild-type cells (**a**); however, in APP-NOTCH TMD cells (**b**), FT-1 caused minimal changes in the Aβ42 (95%) or Aβ40 (90%) signal. Data are presented as amyloid-β percentage of control ±s.e.m., *n*= 3.