The Amyloid-β Rise and γ-Secretase Inhibitor Potency Depend on the Level of Substrate Expression

Catherine R. Burton, Jere E. Meredith, Donna M. Barton, Margi E. Goldstein, Carol M. Krause, Cathy J. Kieras, Lisa Sisk, Lawrence G. Iben, Craig Polson, Mark W. Thompson, Xu-Alan Lin, JasonCorsa, Tracey Fiedler, MariaPierdomenico, YanGao, Joseph L. Cantone, Michael J. Ford, Dieter M. Drexler, Richard E. Olson, Michael G. Yang, Carl P. Bergstrom, Kate E. McElhone, Joanne J. Bronson, John E. Macor, Yuval Blat, Robert H. Graffstrom, Andrew M. Stern, Dietmar A. Seiffert, Robert Zaczek, Charles F. Albright, and Jeremy H. Toyn

The Amyloid-β (Aβ) peptide, which likely plays a key role in Alzheimer disease, is derived from the amyloid-β precursor protein (APP) through consecutive proteolytic cleavages by β-site APP-cleaving enzyme and γ-secretase. Unexpectedly γ-secretase inhibitors can increase the secretion of Aβ peptides under some circumstances. This “Aβ rise” phenomenon, the same inhibitor causing an increase in Aβ at low concentrations but inhibition at higher concentrations, has been widely observed. Here we show that the Aβ rise depends on the β-secretase-derived C-terminal fragment of APP (BCTF) or C99 levels with low levels causing rises. In contrast, the N-terminally truncated form of Aβ, known as “p3,” formed by α-secretase cleavage, did not exhibit a rise. In addition to the Aβ rise, low BCTF or C99 expression decreased γ-secretase inhibitor potency. This “potency shift” may be explained by the relatively high enzyme to substrate ratio under conditions of low substrate because increased concentrations of inhibitor would be necessary to affect substrate turnover. Consistent with this hypothesis, γ-secretase inhibitor radioligand occupancy studies showed that a high level of occupancy was correlated with inhibition of Aβ under conditions of low substrate expression. The Aβ rise was also observed in rat brain after dosing with the γ-secretase inhibitor BMS-299897. The Aβ rise and potency shift are therefore relevant factors in the development of γ-secretase inhibitors and can be evaluated using appropriate choices of animal and cell culture models. Hypothetical mechanisms for the Aβ rise, including the “incomplete processing” and endocytic models, are discussed.

Evidence suggests that the amyloid-β (Aβ)9 peptide plays a key role in Alzheimer disease. Aβ is generated by proteolytic processing of the amyloid-β precursor protein (APP) through consecutive cleavages by the β-site APP-cleaving enzyme (BACE) and γ-secretase. APP is cleaved by BACE to form a β-secretase-derived C-terminal fragment of APP (BCTF), which undergoes further cleavage by γ-secretase to form Aβ. In addition, APP is cleaved by α-secretase to form αCTF, which undergoes γ-secretase cleavage to produce an N-terminally truncated form of Aβ known as “p3.” Using the conventional amino acid numbering of Aβ, BACE cleavage leads to Aβ peptides with N-terminal ends at positions 1 and 11, whereas α-secretase leads to p3 peptides with an N-terminal end at position 17. Cleavage of BCTF and αCTF by γ-secretase produces a mixture of different C termini in the resulting Aβ and p3 peptides. For example, the predominant γ-secretase cleavage of BCTFs at position 40 produces Aβ-(1–40) and Aβ-(11–40), whereas other γ-secretase cleavage sites produce a range of less abundant Aβ peptides, such as the disease-associated Aβ-(1–42) (1, 2).

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The abbreviations used are: Aβ, amyloid-β; APP, amyloid-β precursor protein; BACE, β-site APP-cleaving enzyme; BMS, Bristol-Myers Squibb; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-2-hydroxy-1-propanesulfonate; BCTF, β-secretase-derived C-terminal fragment of APP; DAPT, N-[3,5-difluorophenacetyl]-l-alanyl-(S)-phenylglycine t-butyl ester; DMEM, Dulbecco’s modified Eagle’s medium; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; FAD, familial Alzheimer disease; HEK, human embryonic kidney; HEKsw, HEK293 cells stably transfected with APP Swedish variant; HEKwt, HEK293 cells stably transfected with APP wild type; LC/MS, liquid chromatography mass spectrometry; MES, 2-[N-morpholino]ethanesulfonic acid; PBS, phosphate-buffered saline; αCTF, α-secretase-derived C-terminal fragment of APP; HPLC, high pressure liquid chromatography; arbs, arbitrary units; bis-tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; E/S, enzyme to substrate.
Although γ-secretase cleavage can be fully inhibited in cell-based assays, some inhibitors cause an increase in the amount of Aβ at subinhibitory concentrations. This “Aβ rise” phenomenon, the same inhibitor causing an increase in Aβ at low concentrations but inhibition at higher concentrations, has been observed frequently (3–15). For peptide aldehyde inhibitors, some studies reported a rise that was specific for Aβ42 (3–6), whereas other studies reported a rise also for Aβ40 in addition to Aβ42 (7–10). However, these studies also differed as to the pharmacological target proposed to mediate the effects on Aβ; some authors considered only the protease calpain via an indirect effect on γ-secretase (7, 9, 10), whereas others proposed a direct effect on γ-secretase (3–6, 8). In one study, the Aβ rise was reported in isolated membrane preparations, suggesting a direct effect of peptide aldehydes on γ-secretase (6). Further evidence that γ-secretase can mediate the Aβ rise comes from studies with difluoroketone-based inhibitors, which are selective for γ-secretase and which cause a robust rise in Aβ42 both in cell culture (11–13) and in isolated membrane-based assays (6). Furthermore a rise in total Aβ, as well as Aβ42, in response to highly selective γ-secretase inhibitors has been observed in vivo in the plasma of guinea pigs (14) and in humans (15).

Thus, the biochemical mechanism of the Aβ rise has not been elucidated, and the experimental conditions required for this phenomenon have not been defined. Here we show that a rise in multiple Aβ species can be readily observed in cell cultures treated with γ-secretase inhibitors and that the key experimental requirement is a low level of BCTF or C99 expression. In addition, low substrate expression caused a shift in inhibitor potency that was independent of the Aβ rise. We also show that increased Aβ can occur in the brain following γ-secretase inhibitor dosing in rats, demonstrating the potential of γ-secretase inhibitors to cause the opposite of the intended effect in the target organ. Thus, the Aβ rise is a relevant issue in the development of γ-secretase inhibitors for Aβ-lowering therapy, and experimental conditions that exhibit the Aβ rise can be readily applied in cell culture models.

**EXPERIMENTAL PROCEDURES**

**Chemicals and γ-Secretase Inhibitors**—Compound E (16), DAPT (17), and L-685,458 (18) were purchased from Calbiochem (EMD Biosciences), BMS-299897 (19), BMS-433796 (20), and DPH-068455 (21) have been described previously. DPH-068455 contains a dimer of L-685,458 (18) and has been described previously. For HEK293 cells stably transfected with APP wild type (HEKwt) or Swedish variant (HEKsw) were derived essentially as described previously (26, 27). Growth media and supplements were obtained from Invitrogen. For HEKwt cells, growth medium was Dulbecco’s modified Eagle’s medium (DMEM) containing 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 1 mM sodium pyruvate, 400 μg/ml G418, and 5 μg/ml blasticidin. Growth medium for HEKsw cells was the same as for HEKwt cells but containing 200 μg/ml hygromycin B and lacking blasticidin. THP-1 cells were grown in roller bottles in RPMI 1640 medium containing 1-glutamine and 10 μM β-mercaptoethanol to a density of 1 × 10⁶/ml. Cells were harvested by centrifugation, and cell pellets were quick frozen in dry ice/ethanol and stored at −80 °C prior to use. Mouse embryonic fibroblasts were passaged twice per week in a 1:1 mixture of DMEM and F-12 nutrient mixture supplemented with 10% fetal bovine serum, penicillin, and streptomycin. HeLa cells were maintained in DMEM containing 10% fetal bovine serum, penicillin, streptomycin, and 2 mM L-glutamine. For inhibitor treatments, cell cultures were grown for 24 h, and the medium was replaced with DMEM containing high glucose, 0.0125% bovine serum albumin, non-essential amino acids, 2 mM L-glutamine, and 100 units/ml penicillin and streptomycin. Inhibitors were added in DMSO to a final concentration of 0.1% DMSO. Treated cells were incubated overnight at 37 °C in a humidified atmosphere containing 5% CO₂.

**Cell-free γ-Secretase Assay**—Assays were carried out based on a procedure described previously using C99 substrate expressed and purified from *Escherichia coli* (28). To prepare concentrated γ-secretase enzyme, lipid rafts were isolated based on methods described previously (29). Briefly THP-1 cell pellets were lysed in 4 volumes of lysis buffer (125 mM NaCl, 1% CHAPSO, 25 mM Na-MES, pH 6.5) containing a protease inhibitor mixture of 104 μM 4-(2-aminoethyl)benzenesulfonyl fluoride, 80 nM aprotinin, 2 μM leupeptin, 4 μM bestatin, 1.5 μM pepstatin A, and 1.4 μM E-64 (0.1% protease inhibitor mixture P8340, Sigma-Aldrich) using five passages through a 25-gauge needle, and the lipid raft fraction was isolated by discontinuous sucrose density gradient centrifugation (29). This procedure yielded a stock preparation of CHAPSO-solubilized proteins containing γ-secretase at a concentration of 3 nM as determined by saturation radioligand binding. γ-Secretase activity assays
were performed in assay buffer (100 nm NaCl, 0.25% CHAPSO, 50 mM HEPES, pH 7.0). The lipid raft preparation was mixed with C99 substrate at molar ratios of 1:1 and 1:100, corresponding to absolute concentrations of 0.3 nM:0.3 nM and 0.01 nM:1 nM, respectively, at a volume of 200 μl/reaction in 96-well polypropylene plates. After incubation for 3 h at 37 °C, Aβ-(1–40) was quantified by enzyme-linked immunosorbent assay (ELISA). Extensive experimentation to optimize assay conditions showed that the maximum concentration of γ-secretase that could be used in the assay was 0.3 nM because of inhibition of Aβ production at higher concentrations presumably due to inhibitory activities present in the raft preparation. Likewise C99 substrate at concentrations less than 0.03 nm yielded insufficient signal for reliable quantification of Aβ-(1–40). Thus, the highest enzyme to substrate ratio that could be utilized using this method was 1:1. Aβ-(1–40) was quantified by ELISA using the antibodies TSD953.2 and 26D6 described below.

**Radioligand Binding in Cell Homogenates—HEKsw, HEKwt, and mouse embryonic fibroblast cell pellets were homogenized in 10 ml of 50 mM HEPES with 0.1% protease inhibitor mixture (Sigma-Aldrich P8340) at pH 7.0 and 4 °C using a Dounce homogenizer. The homogenate was centrifuged at 48,000 × g for 20 min. Protein determinations were carried out using a Bradford based assay (Bio-Rad). The final pellet was resuspended in buffer to yield a protein concentration of 5 mg/ml. [3H]BMS-570479 binding was carried out in 50 mM HEPES, 0.1% CHAPSO, pH 7.0, at a protein concentration of 200 μg/ml. Binding assays were performed in polypropylene 96-deepwell plates (Beckman Coulter, Fullerton, CA) in a final volume of 0.25 ml containing 5% (v/v) DMSO. Assays were initiated by the addition of 25 μl of assay buffer containing radioligand to 12.5 μl of dimethyl sulfoxide containing various concentrations of unlabeled compounds followed by 212 μl of cell homogenate. Nonspecific binding was defined in the presence of 1 μM BMS-433796. After incubating at 25 °C for 1.5 h, bound radioligand was separated from free by filtration over GF/B glass fiber filters (Brandel, Gaithersburg, MD) presoaked in phosphate-buffered saline (PBS), pH 7.0, using a cell harvester (Brandel). Filters were washed four times with 1.0 ml of ice-cold PBS, pH 7.0, dried, and then assessed for radioactivity by liquid scintillation counting using a Wallac Microbeta Trilux (PerkinElmer Life Sciences). Equilibrium saturation data were analyzed using the Kell software package (Biosoft, Cambridge, UK).

**γ-Secretase Inhibitor Binding Site Occupancy in Intact Cells—**HEKwt and HEKsw cells were seeded at a density of 125,000 cells in 500 μl of growth medium/well in Biocoat 24-well plates treated with poly-d-lysine and incubated for 24 h. After overnight incubation in assay medium in the presence or absence of compound, as described above, 275 μl was removed for assay of Aβ. To the remaining 225 μl, 25 μl of PBS, pH 7.0, containing [3H]BMS-570479 at 7.5 nM was added. Nonspecific binding was determined in the presence of BMS-433796 at a concentration of 1 μM. After incubation for 1 h at 37 °C in 5% CO₂, assay medium was removed, and the cells were rinsed gently three times in 1 ml of ice-cold PBS, pH 7.0. Cells were then incubated at room temperature for 30 min with rotary mixing in 200 μl of 0.5 N sodium hydroxide. The sodium hydroxide was then mixed into 3 ml of scintillation fluid and counted using a Microbeta scintillation counter (Wallac). The percentage of displacement of radioligand was then calculated, and an IC₅₀ value was determined by best fit to a four-parameter dose-response curve using GraphPad Prism (GraphPad Software, San Diego, CA). Because of the low concentration of radioligand present in these assays, we would expect a slight underestimation of occupancy for compounds with a competitive mechanism of radioligand displacement.

**Ex Vivo Inhibitor Occupancy—**Inhibitor occupancy of γ-secretase was quantified as described previously (30) except that a different radioligand, [3H]BMS-570479, was used. Briefly 20-μm coronal brain sections were incubated for 10 min in 50 mM HEPES buffer, pH 7.4, containing 5 nM [3H]BMS-570479. Nonspecific binding was determined by incubating adjacent sections in the presence of 500 nM BMS-433796. The sections were washed in PBS and dried, and bound radioligand was quantified by phosphorimage analysis.

**Handling of Rats—**Female Sprague-Dawley rats were obtained from Charles River Laboratories (Wilmington, MA) and orally dosed at 30 mg/kg, 4 ml/kg in polyethylene glycol 400, 1% Tween 80. Half of the forebrain was quick frozen on dry ice. All experimental procedures with rodents were authorized by and in compliance with the Bristol-Myers Squibb Animal Care and Use Committee.

**Quantification of Aβ Peptides by ELISA—**For Figs. 1, A and B, 3, and 5, ELISAs using an Aβ position 40 or position 42 epitope-specific antibody and a monoclonal antibody directed to Aβ residues 10–20 were used (16). For the remaining figures, Aβ-(1–40) was assayed using the monoclonal antibody TSD953.2, which is specific for the free C terminus of Aβ-(1–40), in combination with a peroxidase conjugate of 26D6, which is specific for an epitope within the 12 N-terminal amino acids of human Aβ-(1–40) (19), or with a peroxidase conjugate of 25Q6 (BIOSOURCE/InVitrogen), which is specific for an epitope within the 12 N-terminal amino acids of rodent Aβ-(1–40). Human Aβ-(1–x) was assayed using monoclonal antibody 26D6 in combination with a biotin conjugate of 4G8 (Signet/Covance, Berkeley, CA) and streptavidin-peroxidase conjugate (Zymed Laboratories Inc./Invitrogen). This antibody combination detects Aβ peptides containing the 1–24 amino acid region. Data from inhibitor-treated cell cultures were evaluated by non-linear regression using a four-parameter sigmoidal dose-response curve, and the derived IC₅₀ values represent the concentration of compound required to inhibit Aβ to 50% of vehicle-treated control.

**Quantification of Aβ Peptides by Liquid Chromatography Mass Spectroscopy (LC/MS) —**Aβ peptides were concentrated from the cell medium by immunoprecipitation. 14 ml of cell medium was mixed for 1 h at 4 °C with 15 μg of 4G8 (Covance), 30 μg of 26D6 (Bristol-Myers Squibb), protease inhibitor mixture (Hoffmann-La Roche Ltd.), and 3.5 ng of synthetic [15N]Aβ-(1–40) peptide (rPeptide, Athens, GA) followed by the addition of 80 μl of protein G-agarose beads (Pierce) and continued incubation with mixing overnight at 4 °C. Samples were then centrifuged at 1000 × g for 3 min. The beads were washed three times by centrifugation with 1 ml of PBS and washed a final time with 1 ml of 10 mM Tris-HCl, pH 8.0. The Aβ peptides were eluted from the beads with 30 μl of 70%
acetonitrile, 0.1% formic acid. After a final 10,000 \( \times g \) centrifugation for 5 min, the supernatant was removed and stored on dry ice before LC/MS. The LC/MS system was comprised of a Leap Technologies (Carrboro, NC) CTC HTS PAL autosampler, an Agilent Technologies (Wilmington, DE) 1100 Capillary LC pump, and a Thermo Fisher (San Jose, CA) linear ion trap LTQ mass spectrometer. Sample injection carryover concerns were addressed with an injection port cleaning procedure based on the Clean LC\textsuperscript{TM} macro as part of the Leap Technologies autosampler operating software, Cycle Composer. The injection loop is taken off line during an LC run, and the loop, the injector needle, and the injector syringe barrel are then washed consecutively with 10 \( \mu \)L EDTA in 2\% acetonitrile and 10 \( \mu \)M ammonium hydroxide in acetonitrile. For HPLC/MS analysis, 3 \( \mu \)L of sample was injected onto an Agilent Technologies Zorbax Extend C\textsubscript{18} reversed-phase chromatography column (50 \( \times \) 1 mm, 3.5 \( \mu \)m) with a Zorbax Extend guard column (1 \( \times \) 1.7 mm, 3.5 \( \mu \)m). Mobile phase A was 100\% water (HPLC grade, J. T. Baker) containing 20 \( \mu \)M ammonium hydroxide (99.99\% purity, Sigma-Aldrich), and mobile phase B was 80:20 acetonitrile (HPLC grade, J. T. Baker)/water (v/v) also containing 20 \( \mu \)M ammonium hydroxide. The HPLC was performed at a flow rate of 50 \( \mu \)L/min with the following gradient: 10–40\% B at 10 min, 70\% B at 11 min, and a hold at 70\% B for 1 min. The mass spectrometer was operated in positive ion full-scan mode with the following ionization source conditions: spray voltage, 5 kV; capillary voltage, 30 V; tube lens voltage, 150 V; capillary temperature, 300 °C; sheath gas flow, 40 arbs; auxiliary gas flow, 5 arbs; and sweep gas flow, 2 arbs. Quantitative data were acquired in centroid mode with one microscan and 500-ms ion time by selectively monitoring the following ion current for \( 1 \) ng of \([15N]A\)-(1–40) the C100 samples and to serial dilutions of A\( \beta\)-(17–28) (American Peptide Co., Sunnyvale, CA) in water, acetonitrile, 0.1\% ammonium hydroxide. Low protein binding tubes (Protein LoBind Tube, Eppendorf AG, Hamburg, Germany) were utilized to prevent loss of analyte. The samples were concentrated to dryness in a nitrogen stream at 30 °C. To each tube 90 \( \mu \)L of 20 \( \mu \)M ammonium bicarbonate and 5 \( \mu \)g of trypsin (Trypsin Gold, MS grade, Promega, Madison, WI) were added. The samples were incubated at 37 °C, and after 3 h an additional 5 \( \mu \)g of trypsin was added following a 14-h incubation at 37 °C. The protein/peptide digestion was quenched with 1 \( \mu \)L of formic acid, and samples were analyzed by LC/MS. A Leap Technologies CTC HTS PAL autosampler, an Agilent Technologies 1100 Capillary LC pump, and a Thermo Fisher linear ion trap LTQ mass spectrometer was used as the LC/MS system. For HPLC/MS analysis, 10 \( \mu \)L of sample was injected onto a Phenomenex (Torrance, CA) Jupiter Proteo column (50 \( \times \) 1 mm, 4 \( \mu \)m, 90 Å). The mobile phases consisted of water/acetonitrile (98:2, v/v, mobile phase A) and acetonitrile/water (98:2, v/v, mobile phase B). The water in each of the solvent mixtures contained 0.1\% formic acid. The HPLC was performed at a flow rate of 57 \( \mu \)L/min and with the following gradient: a hold at 95\% A for 2 min, linear gradient to 30\% B over 8 min, linear gradient to 95\% B over 2 min, hold at 95\% B for 3 min, step gradient to 95\% A, and hold at 95\% A for 6 min. The mass spectrometer was operated in positive ion full-scan mode with the following ionization source conditions: spray voltage, 5 kV; capillary voltage, 35 V; tube lens voltage, 110 V; capillary temperature, 300 °C; sheath gas flow, 40 arbs; auxiliary gas flow, 5 arbs; and sweep gas flow, 5 arbs. Quantitative data were acquired in centroid mode with one microscan and 500-ms ion time by selectively monitoring the transitions (multiple reaction monitoring, 5-Da isolation window) of the most abundant signal in the doubly charged/proto-nated isotopic envelope of the molecular ions [M + 2H]\textsuperscript{2+} for...
**Aβ Rise and Potency Shift**

Aβ-(17–28) at m/z 663.3 to the product ions at m/z 557.3, 921.4, and 1113.5 (m/z 663.3 → m/z 557.3 + 921.4 + 1113.5) and for [15N]Aβ-(17–28) at m/z 670.4 to the product ions at m/z 563.4, 929.4, and 1125.5 (m/z 670.4 → m/z 563.4 + 929.4 + 1125.5). Recovery of Aβ-(17–28) from the trypsin digestion was found to be >90% based on the yield of [15N]Aβ-(17–28) recovered from digestion of [15N]Aβ-(1–40) under the same conditions.

**RESULTS**

The Aβ Rise Occurs in Cell Lines That Express Low Levels of Aβ—During initial studies with γ-secretase inhibitors, we found that subinhibitory concentrations of inhibitors caused a rise in Aβ in cell cultures expressing low levels of Aβ, such as the neuroblastoma cell line IMR-32 and cultured rat brain slices (not shown). To evaluate this effect in more detail, γ-secretase inhibitors were compared in HEKwt or HEKsw. The Swedish mutation of APP enhances β-secretase cleavage at amino acid position 1, thereby increasing production of the βCTF intermediate and Aβ-(1–x) where “x” denotes the range of different C-terminal end positions resulting from γ-secretase cleavage. Accordingly untreated control cultures of HEKwt typically accumulated 190 pg/ml of (44 pM)Aβ-(1–40) in the medium, whereas HEKsw accumulated 2,400 pg/ml of (550 pM)Aβ-(1–40) after overnight incubation in 96-well culture format. Cell cultures were treated with the γ-secretase inhibitor DAPT or the benzodiazepinone DPH-111122 at a range of concentrations, and the Aβ-(1–40) and Aβ-(1–42) secreted into the cell culture media were assayed by ELISA. Rises in both Aβ-(1–40) and Aβ-(1–42) were detected in the HEKwt cultures, whereas no rises were observed in the HEKsw cultures (Fig. 1, A and B). In addition, inhibitor potency was up to 10-fold greater in the HEKsw than in the HEKwt cell line. IC50 values are summarized in Table 1. In further experiments, we included an ELISA antibody combination, 26D6/4G8, which detects Aβ-(1–x), to evaluate whether the Aβ rise was unique to Aβ-(1–42) and Aβ-(1–40). A robust rise in Aβ-(1–x) was observed for DAPT and L-685,458 treatment in HEKwt but not HEKsw cell cultures (Fig. 1, C and D).

The Aβ Rise Occurs for β-Secretase-derived, but Not α-Secretase-derived, Peptides—To determine which species of Aβ peptides were involved in the Aβ rise, we carried out an analysis of medium from DAPT-treated HEKwt cultures by liquid chromatography/mass spectrometry. Both HEKwt and HEKsw cells secrete a range of Aβ peptides but in different relative concentrations. For example, in HEKwt medium the Aβ-(1–x) peptides predominate, whereas in HEKsw medium the Aβ-(17–x) peptides are more abundant (Fig. 2, A and B). When HEKwt cultures were treated with a range of concentrations of DAPT, a robust Aβ rise was exhibited by the BACE-derived Aβ peptides 1–37, 1–38, 1–40, 11–40, and 1–19 but not by the α-secretase-derived Aβ peptides 17–37, 17–38, 17–40, and 17–28 (Fig. 2, C–F). For the BACE-derived peptides, IC50 values were all in the 100–300 nM range, whereas the α-secretase-derived peptide IC50 values were in the 20–30 nM range consistent with the IC50 values for DAPT determined by ELISA (Table 1). Aβ-(1–42) was detected at low levels limiting its quantitation by this method.

**TABLE 1**

Summary of Aβ IC50 Values in HEKwt and HEKsw cultures

|          | HEKwt (nM) ± S.D. | HEKsw (nM) ± S.D. |
|----------|------------------|------------------|
| DAPT     |                  |                  |
| Aβ-(1–40)| 154 ± 21         | 17 ± 2           |
| Aβ-(1–42)| 129 ± 19         | 24 ± 10          |
| DPH-111122 |                |                  |
| Aβ-(1–40)| 58 ± 24          | 5 ± 0.7          |
| Aβ-(1–42)| 50 ± 21          | 8 ± 4            |

**Low Substrate Level, not BACE Cleavage, Is Required for the Aβ Rise**—To address both the role of substrate expression level and the potential need for BACE cleavage, experiments were performed by transient expression of BCTF, here referred to as “C99” because its formation does not require BACE cleavage. HeLa cell cultures were transfected with different quantities of C99 cDNA and then treated with a range of concentrations of DAPT, BMS-267593, or compound E for 16 h after which the Aβ-(1–40) secreted into the culture medium was determined (Fig. 3). All cultures exhibited a rise in Aβ-(1–40) at the lowest level of C99 transfection. For BMS-267593 and compound E, the two higher levels of C99 substrate exhibited no Aβ rise, but there was a potency shift despite the absence of an Aβ rise. Thus, the Aβ rise and potency shift were dependent on the low level of C99 or βCTF expression but independent of BACE and the Swedish mutation.

The Aβ Rise Is Not Readily Observed in Solubilized Cell-free Assays—Two observations suggested that it might be possible to recreate the Aβ rise in a γ-secretase enzyme assay: first, the
we therefore carried out cell-free secretase assays using defined enzyme to substrate (E/S) ratios. Concentration-response curves for Aβ(1–40) in the presence of DAPT and L-685,458 were determined at E/S ratios of 1:1 and 1:100. Although inhibition was observed in each case, an Aβ rise did not occur under any of the conditions tested (Fig. 4). Unexpectedly L-685,458 exhibited a 40-fold shift in potency with IC_{50} = 0.1 μM at an E/S ratio of 1:100 and IC_{50} = 4.1 μM at an E/S ratio of 1:1. In contrast, there was no significant difference in the IC_{50} values for DAPT under the same conditions that were 8.6 and 7.9 μM, respectively. A possible explanation for the IC_{50} shift for L-685,458 would be the presence of abundant quantities of signal peptide peptidases in the cell-free extracts that bind with high affinity to L-685,458 but with low affinity to DAPT (32). Under the conditions used at the E/S ratio of 1:1, L-685,458 at concentrations in the low nanomolar range would be mostly titrated by signal peptide peptidases (32) and so unavailable to inhibit γ-secretase. In conclusion, we could not reproduce the Aβ rise under solubilized enzyme assay conditions.

Many γ-Secretase Inhibitors Exhibit the Aβ Rise and Potency Shift—To evaluate the generality of the Aβ rise and potency shift, concentration-response curves for Aβ(1–42) and Aβ(1–40) in both HEKwt and HEKsw cell cultures were determined for 65 different compounds mostly with structures related to DPH-111122 and covering nearly a 1000-fold range in potency. The Aβ rise was observed only in the HEKwt cell line. In addition, the extent of the Aβ rise was compound-dependent and more pronounced for Aβ(1–42) than for Aβ(1–40) (Fig. 5A). The extent of the Aβ(1–42) rise correlated with the extent of the Aβ(1–40) rise for a given compound (Fig. 5B). Compound potency correlated with the Aβ rise, and a significant decrease (p = 0.0003) in the extent of the rise was observed among compounds of higher potency (Fig. 5C). Comparison of the IC_{50} values showed that Aβ(1–42) and Aβ(1–40) potencies were strongly correlated (Fig. 5D), but on average there was a 10-fold decrease in potency in the HEKwt relative to the HEKsw cultures (Fig. 5E). Surprisingly the Aβ rise and the extent of the potency shift were not significantly correlated (Fig. 5F), again suggesting that a significant part of the potency shift was independent of the Aβ rise and therefore not solely a secondary consequence of the rise in Aβ.

Determination of γ-Secretase and Cellular βCTF Concentrations—One hypothesis for the potency shift is that HEKwt cells contain an excess of γ-secretase enzyme relative to

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**FIGURE 3.** The C99 expression level affects the Aβ rise and inhibitory potency of γ-secretase inhibitors. A, HELa cell cultures were transfected with different amounts of the C99 DNA construct, and Aβ(1–40) secreted into the medium was quantified. Error bars, all smaller than symbols used, represent S.D. for 19 independent experiments. Linear regression indicated a goodness of fit, r^2, equal to 0.95. B, HEKwt cell cultures were transfected with 1.8 μg (○) or 36 μg (△) of C99 DNA construct and then treated with DAPT at a range of concentrations for 16 h. The level of Aβ(1–40) secreted into the culture medium is expressed as a percentage of Aβ(1–40) in vehicle-treated control cultures. Error bars represent S.D. for five independent experiments. C, HEKwt cell cultures were transfected with 0.9 μg (●), 1.8 μg (○), or 36 μg (△) of C99 DNA construct and then treated with γ-secretase inhibitor BMS-267593 at a range of concentrations for 16 h. The level of Aβ(1–40) secreted into the culture medium is expressed as a percentage of Aβ(1–40) in DMSO-treated control cultures. Error bars represent S.D. for four independent experiments. D, same experimental design as C except that the cell cultures were treated with γ-secretase inhibitor compound E (CPDE), and error bars represent S.D. for five independent experiments.

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**FIGURE 2.** LC/MS analysis of Aβ peptides secreted by HEKsw and HEKwt cell cultures and the Aβ rise in HEKwt cells. A, Aβ peptide species in HEKsw cell medium were quantified by LC/MS. The intensity of each peptide is expressed relative to the [15N]Aβ-(1–40) internal control. Error bars represent the S.D. for three measurements. B, Aβ peptide species in HEKsw cell medium were quantified by LC/MS as described in A. Panels C, D, E, and F, HEKsw cells were treated with a range of concentrations of DAPT, and Aβ species were quantified by LC/MS. Percentages are expressed relative to the quantity of each peptide in vehicle-treated control cultures except for Aβ-(1–19) for which the percentage is relative to the amount detected in the presence of 10 nM DAPT. C, Aβ-(1–37) (●) and Aβ-(17–37) (△); D, Aβ-(1–38) (●) and Aβ-(17–38) (△); E, Aβ-(1–40) (●), Aβ-(17–40) (△), and Aβ-(11–40) (▲); F, Aβ-(1–19) (●) and Aβ-(17–28) (△). Note that levels of Aβ-(1–42) were below the level of quantitation by this method.
substrate such that the amount of enzyme would not be rate-limiting for Aβ production, thus requiring considerable inhibition of enzyme before substrate turnover was affected. To quantify the level of γ-secretase enzyme in the HEKwt and HEKsw cells, saturation binding isotherms were determined in cell homogenates using the radiolabeled γ-secretase inhibitor [3H]BMS-570479. Analysis of the binding isotherms indicated single high affinity binding sites in both cultures with identical dissociation constants ($K_d$) of 0.6 nM and $B_{max}$ values of approximately 400 fmol/mg of protein (Fig. 6A). Based on the number of cells used to make the homogenate, the $B_{max}$ value of 400 fmol/mg is equivalent to 15,000 γ-secretase binding sites per cell for both HEKsw and HEKwt cells.

To quantify the amount of βCTF in HEKwt and HEKsw cells, quantitative Western blotting was carried out using recombinant C100 (equivalent to βCTF with an additional N-terminal methionine; purified from E. coli) as a calibration standard. In this method, the intensity of the Western blot was proportional to the amount of C100 loaded (Fig. 6B). The concentration of recombinant C100 used for Western blot calibration was determined by tandem mass spectroscopy of the Aβ(1–40) fragment in tryptic digests of the recombinant C100 calibrated against known amounts of synthetic Aβ(1–40). The linear response of synthetic Aβ(1–40) peptide by mass spectroscopy is shown (Fig. 6C). See “Experimental Procedures” for further details. In HEKwt cells, the concentration of βCTF was found to be 30 fmol/mg of homogenate protein, whereas in HEKsw cells there was 140 fmol/mg. Thus, subject to additional factors, γ-secretase has the potential to be present in molar excess with respect to βCTF substrate with the result that the enzyme may not be rate-limiting for Aβ production.

A High Level of Inhibitor Binding Site Occupancy Is Required for γ-Secrretase Inhibition in HEKwt Cells—A functional consequence of non-rate-limiting levels of enzyme would be the requirement for a high level of inhibitor binding site occupancy for inhibition to occur. To quantify inhibitor binding under conditions that produced the Aβ rise and potency shift, [3H]BMS-570479 binding was applied to living cells. HEKsw and HEKwt cultures were treated with the aryl sulfonamide inhibitor BMS-299897 and DAPT at a range of concentrations. Samples of the cell culture supernatants were then taken to measure Aβ(1–40), and [3H]BMS-570479 was added to the remaining cell cultures to determine binding site occupancy. As expected, the HEKwt cultures exhibited a robust rise in Aβ for both compounds, whereas the HEKsw cultures showed little or no Aβ rise. In contrast, binding site occupancy was not significantly different between the HEKwt and HEKsw cultures (Fig. 7). Thus, consistent with a functional excess of enzyme, a high level of occupancy was required for inhibition of Aβ in the HEKwt culture. In addition, the Aβ rise correlated with partial occupancy of inhibitor binding sites for both compounds consistent with a direct role for γ-secretase in the Aβ rise.

The Aβ Rise Occurs at Partial Binding Site Occupancy in Rat Brain—To examine whether rises in brain Aβ can occur in vivo, rats were dosed with BMS-299897, and brains were harvested between 1 and 12 h after dosing for determination of inhibitor binding site occupancy and Aβ. Brains from control animals were harvested 5 h after dosing with vehicle alone. Mean values of Aβ and radioligand-specific binding were then calculated relative to mean vehicle control. A rise in Aβ was observed starting at the 1-h time point and reached a maximum at 7 h (Fig. 8). Because the Aβ rise did not follow a period of Aβ inhibition in this experiment, this rules out the possibility that increased Aβ resulted from processing of accumulated APP βCTF intermediates. In contrast, in Tg2576 mice, Aβ lowering correlated with inhibitor occupancy, and there was no Aβ rise (28).

DISCUSSION

Although γ-secretase inhibitors can fully inhibit Aβ production, they often increase the production of Aβ at subinhibitory concentrations. Thus, a given inhibitor causes an Aβ rise at low concentrations but inhibition at higher concentrations. In this report, we describe a variety of novel observations about the Aβ rise. First, we confirmed that both the allosteric class and the active site-directed class of γ-secretase inhibitors can cause the Aβ rise, ruling out any allosteric activation mechanism. Second, the Aβ rise did not occur for p3 peptides or in the context of the APP Swedish variant. Third, the Aβ rise occurred only under conditions of low levels of C99 or βCTF expression. Fourth, cleavage of APP by BACE was not necessary for the Aβ rise because a rise could be observed even when βCTF was...
expressed ectopically. Fifth, Aβ exhibited a rise in the brains of rats dosed with a γ-secretase inhibitor, showing that a rise can occur in the therapeutically relevant organ.

The Potency Shift—In addition to the Aβ rise, low level C99 or βCTF expression caused a decrease in γ-secretase inhibitor potency. Two observations indicated that a significant part of this “potency shift” was independent of the Aβ rise. First, γ-secretase inhibitor potency varied as a function of C99 expression even when an Aβ rise did not occur (Fig. 3). Second, evaluation of a group of γ-secretase inhibitors showed that the extent of the Aβ rise was not correlated with the extent of the potency shift (Fig. 5), suggesting that the underlying mechanism of the rise and the potency shift were not identical. Thus, there were apparently two phenomena dependent upon the level of C99 or βCTF expression in the presence of γ-secretase inhibitors: the Aβ rise and a potency shift.

A simple hypothesis for the potency shift is based on the molar ratio between enzyme and substrate. To calculate this ratio in HEKwt and HEKsw cells, the quantity of γ-secretase enzyme was determined from saturation binding isotherms, and the quantity of cellular βCTF was determined by quantitative Western blotting calibrated by mass spectroscopy (see “Experimental Procedures”). In HEKwt and HEKsw cells, the total substrate to enzyme ratios were 0.075 and 0.36, respectively. Additional factors are expected to affect the functionally relevant ratio in cells, including the differential subcellular localization of enzyme and substrate molecules, the presence of other proteins that are γ-secretase substrates, and the separation of γ-secretase into lipid raft microdomains (33). Therefore, γ-secretase is potentially not rate-limiting for Aβ production such that a relatively high level of enzyme inhibition would be required before substrate turnover and Aβ production was affected. Indeed we observed that a high level of inhibitor binding site occupancy was required for inhibition of Aβ production in the HEKwt cells (Fig. 7). In addition, the observation that γ-secretase inhibitor binding was not affected by differences in the level of substrate expression suggested that an uncompetitive inhibition mechanism based on preferential binding to the enzyme-substrate complex was not involved. Thus the difference in substrate/enzyme ratio between the HEKwt and HEKsw cell lines could contribute to the difference in response of Aβ to γ-secretase in these cell lines. In support of this hypothesis, the Aβ rise and potency shift could be controlled by using transient transfection to vary βCTF expression levels in cells (Fig. 3).

The Aβ Rise as an Intrinsic Characteristic of γ-Secretase—A key question is whether or not the Aβ rise is mediated directly by inhibitor binding to γ-secretase itself or indirectly by inhibitor binding to a secondary target involved in Aβ turnover as suggested previously (5). Pharmacological evidence favors the
direct mechanism. First, a wide structural variety of γ-secretase inhibitors cause the Aβ rise, including both the allosteric and active site-directed classes of inhibitors. These diverse compounds have one obvious property in common: their inhibition of γ-secretase. Second, there are many inhibitors that cause an Aβ rise with potencies in the subnanomolar range. The indirect mechanism would require commensurate potency for the putative Aβ turnover target. Thus, in agreement with previous suggestions (6, 14), the Aβ rise is likely mediated through γ-secretase itself either by increased production of Aβ or decreased turnover of Aβ.

The Aβ Rise Is Not Consistent with the “Incomplete Processing” Hypothesis—Inhibition of γ-secretase-mediated turnover of Aβ appears to explain at least some cases of rises in Aβ, for example the increase in Aβ-(1–42) caused by presenilin familial Alzheimer disease (FAD) mutants. Ihara and co-workers (34–38) and Xu and co-workers (10, 39–41) have reported a stepwise mechanism of substrate cleavages by γ-secretase in which the shorter forms of Aβ are derived from longer forms of Aβ by successive proteolytic cleavages of the same substrate molecule. Some γ-secretase inhibitors cause the accumulation of longer forms of Aβ, which are γ-secretase processing intermediates that remain bound to the enzyme. Taken together, these studies show that longer forms, such as Aβ-(1–46) (40), accumulate because inhibitors cause incomplete processing to the shorter secreted forms of Aβ, such as Aβ-(1–40). Taking this idea further, it was proposed that the increase in Aβ-(1–42) caused by some presenilin FAD mutants results from incomplete processing of Aβ-(1–42) to shorter forms (42, 43). We therefore considered the possibility that γ-secretase inhibitors might cause incomplete processing of Aβ in the HEKwt cells. Using an ELISA that quantifies the combined Aβ-(1–x) species, we detected robust Aβ-(1–x) rises in response to DAPT and L-685,458 (Fig. 1), indicating that incomplete processing would have to involve Aβ peptides shorter than Aβ-(1–24) in this case. Mass spectrometry confirmed the rises in Aβ peptides with C termini at positions 37, 38, and 40 (Fig. 2). In principle, inhibition of shorter peptides that can be derived from C99 or βCTF but not from the N-terminally shorter αCTF could explain why rises were seen for Aβ but not for p3. However, shorter Aβ peptides appeared insufficiently abundant to account for an Aβ rise through incomplete processing. One shorter peptide itself, Aβ-(1–19), exhibited a rise in response to DAPT (Fig. 2F). Thus, we found no evidence for significant γ-secretase-mediated turnover of Aβ, although this does not rule out the possibility that turnover could result in abundant short peptide fragments that we did not detect in our assays. Furthermore there are several noteworthy differences between increased Aβ42 in presenilin FAD mutants and the inhibitor-mediated Aβ rise. First, the Aβ42 increase in FAD mutants does not require low substrate expression. Second, FAD mutants decrease total Aβ levels, whereas low concentrations of γ-secretase inhibitors can increase total Aβ (Fig. 1). Third, FAD mutants caused a change in presenilin conformation, whereas γ-secretase inhibitors did not (44, 45). Thus, it seems possible that the inhibitor-mediated Aβ rise may involve a mechanism different than that involved in the Aβ42 increase observed in presenilin FAD mutants.

**FIGURE 6.** Quantitation of γ-secretase enzyme and βCTF concentrations. A, saturation binding isotherms for [3H]BMS-570479 were determined in homogenates from HEKsw (□) and HEKwt (□) cultures. Error bars represent S.E. for three independent experiments. The dissociation binding constant, \( K_d \), was 0.6 ± 0.15 and 0.6 ± 0.2 nm in the HEKsw and HEKwt homogenates, respectively. The maximal number of binding sites, \( B_{max} \), was 410 ± 45 and 370 ± 70 fmol/mg of total protein in the HEKsw and HEKwt homogenates, respectively. B, the graph shows the linear response of recombinant C100 determined by Western blotting. The concentration of βCTF in HEKwt and HEKsw cells was determined by Western blotting from this linear calibration. C, the graph shows the linear response of synthetic Aβ-(17–28) peptide determined by LC/MS. The concentration of Aβ-(17–28) peptide derived from recombinant C100 digestion by trypsin was determined by LC/MS from this linear calibration.
The Aβ Rise May Depend on Dual Roles of γ-Secretase in the Endocytic Pathway—Production of p3 and Swedish APP-derived Aβ both occur predominantly in the late secretory pathway (46–49), and in both cases we found no rise in these peptides upon γ-secretase inhibitor treatment. In contrast, Aβ derived from wild type APP occurs predominantly in the endocytic pathway (47, 50, 51), suggesting that subcellular trafficking pathways determine the Aβ rise. This may be significant because inhibition of γ-secretase leads to accumulation of βCTF in the endocytic pathway (52). Potentially this could lead to increased exposure of βCTF to γ-secretase, which could increase Aβ production if the increased βCTF exposure exceeded the inhibition of γ cleavage. This circumstance might occur under conditions of low substrate expression where the enzyme would be present in relative excess consistent with our βCTF transfection studies (Fig. 3). A mechanism of this type would require intact endocytic membrane trafficking, hindering reproduction of the Aβ rise in solubilized γ-secretase assays (Fig. 4). Thus, the Aβ rise would be readily apparent only under conditions in which Aβ peptides are produced predominantly in the endosomal trafficking pathway where γ-secretase plays dual antagonistic roles in Aβ production.

Implications for Drug Discovery—We showed that a robust Aβ-(1–40) rise can occur in rat brain under conditions of partial occupancy of inhibitor binding sites, demonstrating that γ-secretase inhibitors have the potential to cause the opposite of the intended inhibitory effect in the target organ. In contrast to the rat, dosing of the Tg2576 mouse with the same γ-secretase inhibitor did not result in an Aβ rise (30) consistent with the results we obtained in cell cultures that overexpress the βCTF or APP Swedish mutant. This suggests that the Tg2576 mouse can exhibit a misleading degree of inhibitory activity for γ-secretase inhibitors. The potential influence of the Aβ rise site occupancy in the living cells was determined as described under “Experimental Procedures.” The percentage of γ-secretase inhibitor binding sites accessible to the radioligand are plotted. Error bars represent S.E. in groups of three animals.

The Aβ rise is shown to depend on dual roles of γ-secretase in the endocytic pathway. Production of p3 and Swedish APP-derived Aβ both occur predominantly in the late secretory pathway (46–49), and in both cases we found no rise in these peptides upon γ-secretase inhibitor treatment. In contrast, Aβ derived from wild type APP occurs predominantly in the endocytic pathway (47, 50, 51), suggesting that subcellular trafficking pathways determine the Aβ rise. This may be significant because inhibition of γ-secretase leads to accumulation of βCTF in the endocytic pathway (52). Potentially this could lead to increased exposure of βCTF to γ-secretase, which could increase Aβ production if the increased βCTF exposure exceeded the inhibition of γ cleavage. This circumstance might occur under conditions of low substrate expression where the enzyme would be present in relative excess consistent with our βCTF transfection studies (Fig. 3). A mechanism of this type would require intact endocytic membrane trafficking, hindering reproduction of the Aβ rise in solubilized γ-secretase assays (Fig. 4). Thus, the Aβ rise would be readily apparent only under conditions in which Aβ peptides are produced predominantly in the endosomal trafficking pathway where γ-secretase plays dual antagonistic roles in Aβ production.

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and potency shift in transgenic mice could therefore lead to misleading estimates of the exposure necessary for Aβ lowering in non-transgenic animals. This is important for a target such as γ-secretase where the therapeutic index is narrow and must be carefully monitored (53). As a consequence, γ-secretase inhibitors that do not cause an Aβ rise would be advantageous as drug candidates. The findings described here demonstrate that the Aβ rise and potency shift are relevant issues for the development of γ-secretase inhibitors and enable these phenomena to be studied under defined conditions.

In conclusion, the Aβ rise and potency shift are intrinsic characteristics of inhibitor binding to γ-secretase and depend on a low level of C99 or βCTF substrate expression perhaps because of a limitation in the amount of substrate trafficking within the endocytic pathway. The mechanistic details remain unresolved; however, our results show how the Aβ rise and potency shift phenomena can be taken into account for the purpose of drug discovery by making appropriate choices of animal and cell culture models.

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