Supporting Information

Discovery and Evaluation of BMS-708163, a Potent, Selective and Orally Bioavailable γ-Secretase Inhibitor

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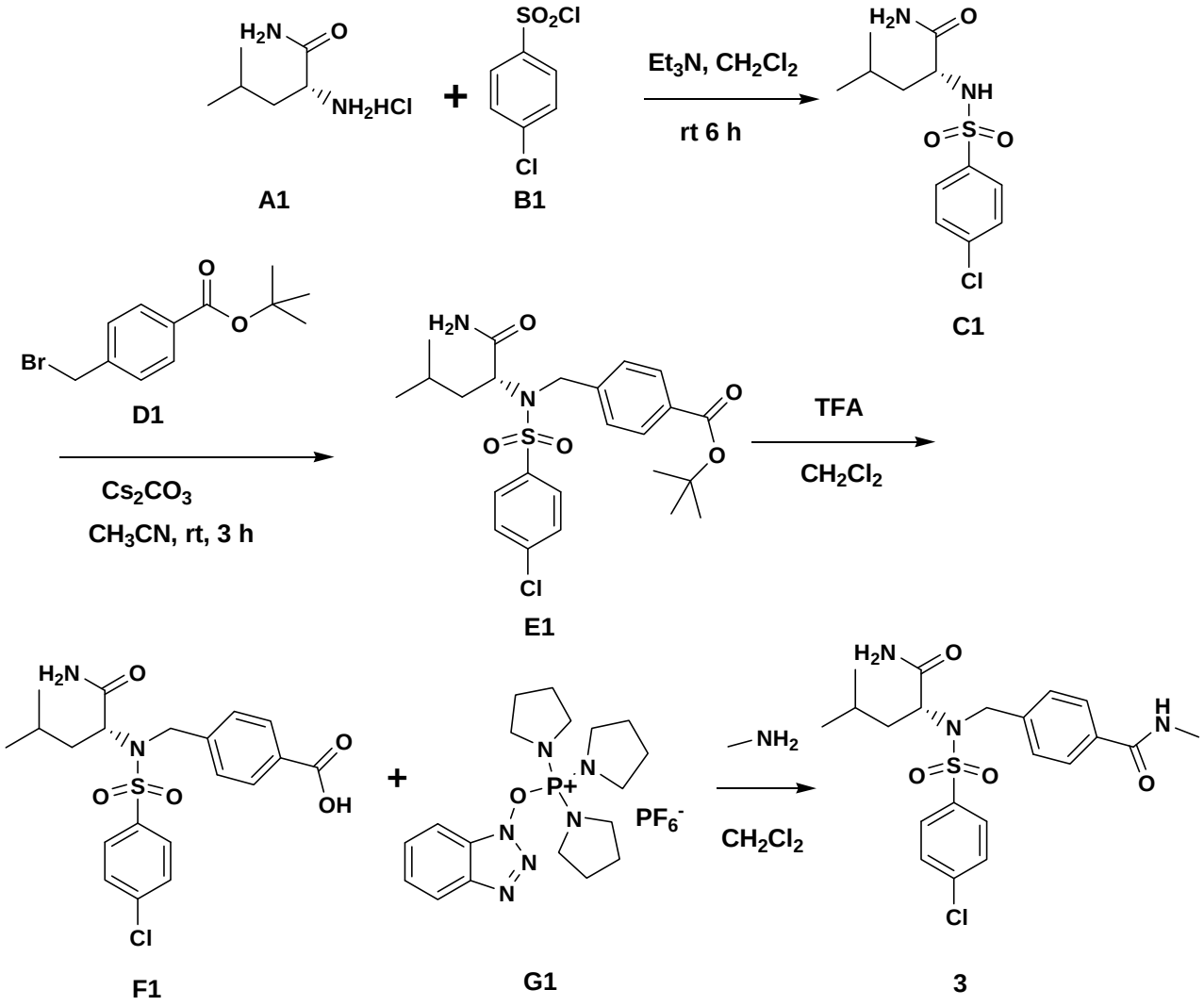
Table of Contents

Preparation of compounds 3, 4, 6-13

General Procedures. In the following examples, all temperatures are given in degrees Centigrade. Melting points were recorded on a Thomas Scientific Unimelt capillary melting point apparatus and are uncorrected. Proton magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker Avance 300, a Bruker Avance 400, or a Bruker Avance 500 spectrometer. All spectra were determined in the solvents indicated and chemical shifts are reported in δ units downfield from the internal standard tetramethylsilane (TMS) and interproton coupling constants are reported in Hertz (Hz). Multiplicity patterns are designated as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad peak; dd, doublet of doublet; br d, broad doublet; dt, doublet of triplet; br s, broad singlet; dq, doublet of quartet. Infrared (IR) spectra using potassium bromide (KBr) or sodium chloride film were determined on a Jasco FT/IR-410 or a Perkin Elmer 2000 FT-IR spectrometer from 4000 cm^{-1} to 400 cm^{-1}, calibrated to 1601 cm^{-1} absorption of a polystyrene film and reported in reciprocal centimeters (cm^{-1}). Optical rotations $[\alpha]_D$ were determined on a Rudolph Scientific Autopol IV polarimeter in the solvents indicated; concentrations are given in mg/mL. Low resolution mass spectra (MS) and the apparent molecular (MH^+) or $(M-H)^+$ was determined on a Finnegan SSQ7000. High resolution mass spectra were determined on a Finnegan MAT900. Liquid chromatography (LC)/mass spectra were run on a Shimadzu LC coupled to a Water Micromass ZQ. All reagents were from commercial suppliers and used without further purification. Representative procedures and physical properties for selected compounds are described.



(R)-2-(4-chlorophenylsulfonamido)-4-methylpentanamide (C1).

To a mixture of triethylamine (4.4 mL, 0.31.6 mmol) and (*D*)-leucinamide hydrochloride (**A1**) (1.667 g, 10.0 mmol) in CH_2Cl_2 (100mL) was added slowly 4-chlorobenzene-sulfonyl chloride (**B1**) (2.11 g, 10.0 mmol). The resulting mixture was stirred at rt for 6 hours, and then diluted with CH_2Cl_2 (200 mL) and washed with H_2O, and dried over Na_2SO_4. The product was purified by silica gel chromatography, eluting with a mixture of ethyl acetate and hexanes (2:1). The desired product **C1** was obtained as an orange solid (2.80g, 92 % yield). LC/MS MH^+ 305.13, ^1H NMR (DMSO-d_6) δ ppm 8.00 (1 H, d,

J=8.80 Hz), 7.77 (2 H, m), 7.62 (2 H, m), 7.31 (1 H, s), 6.91 (1 H, s), 3.65 (1 H, td, *J*=8.68, 5.87 Hz), 1.46 - 1.64 (1 H, m), 1.18 - 1.38 (2 H, m), 0.81 (3 H, d, *J*=6.60 Hz), 0.71 (3 H, d, *J*=6.60 Hz).

(R)-tert-butyl-4-((N-(1-amino-4-methyl-1-oxopentan-2-yl)-4-chlorophenylsulfonamido)methyl)benzoate (E1).

To a solution of **C1** (2.00 g, 6.56 mmol) and tert-butyl 4-(bromomethyl)benzoate (**D1**) (2.5 g (70% pure), 6.56 mmol) in CH_3CN (100 mL) was added Cs_2CO_3 (5.3 g, 16.5 mmol). The resulting mixture was stirred at rt for 3 hours. The precipitate was filtered and the solvent was evaporated. The product was purified by silica gel chromatography, eluting with a mixture of ethyl acetate and hexanes (2:1). The desired product **E1** was obtained as a white dry foam (2.8 g, 86% yield). MS (ESI), $(M+H)^+$ 495.17; LC/MS MH^+ 495.17, ^1H NMR (CD_3CN) δ ppm 7.90 (2 H, d, *J*=8.24 Hz), 7.80 (2 H, m, *J*=8.85 Hz), 7.56 (2 H, m, *J*=8.55 Hz), 7.50 (2 H, d, *J*=8.24 Hz), 6.27 (1 H, br. s.), 5.64 (1 H, br. s.), 4.74 - 4.82 (1 H, m), 4.63 - 4.71 (1 H, m), 4.39 (1 H, t, *J*=7.32 Hz), 1.60 (9 H, s), 1.53 (1 H, ddd, *J*=13.58, 7.02, 6.87 Hz), 1.35 - 1.41 (1 H, m), 1.26 - 1.35 (1 H, m), 0.84 (3 H, d, *J*=6.41 Hz), 0.63 (3 H, d, *J*=6.71 Hz).

(R)-4-((N-(1-amino-4-methyl-1-oxopentan-2-yl)-4-chlorophenylsulfonamido)methyl)benzoic acid (F1).

To a solution of (R)-tert-butyl-4-((N-(1-amino-4-methyl-1-oxopentan-2-yl)-4-chlorophenylsulfonamido) methyl)benzoate (**E1**) (1.0 g, 2.02 mmol) in CH_2Cl_2 (7 mL) was added trifluoroacetic acid (5 mL). The resulting mixture was stirred at rt for 3 hours. Evaporation of solvents provided the crude desired product (**F1**) as a white foam. (0.82 g, 92 % yield): LC/MS MH^+ 439.13.

(R)-4-((N-(1-amino-4-methyl-1-oxopentan-2-yl)-4-chlorophenylsulfonamido)methyl)-N-methylbenzamide (3).

To a mixture of (**F1**) (0.30 g, 0.68 mmol) and 1H-benzo[d][1,2,3]triazol-1-yloxy)tripyrrolidin-1-ylphosphonium hexafluorophosphate (**G1**) (0.51g, 0.98 mmol) in CH_2Cl_2 (7 mL) was added methylamine (2 M in THF, 1.0 mL, 2.0 mmol). The resultant mixture was stirred at rt for 2 hours. The product was directly purified by silica gel chromatography, eluting with a mixture of ethyl acetate and hexanes (2:1). The desired product **3** was obtained as a white solid. (0.25 g, 81% yield). Purity >95% as determined by parallel HPLCs: System A = 10%B to 100%B over 15 minutes; flow = 0.5 mL/min; solvent A = 0.1% TFA/95% water/5% acetonitrile; solvent B = 0.1% TFA/5% water/95% acetonitrile; 2 individual columns, Sunfire C18 3.5 um, 3.0 X 150 mm or Xbridge Phenyl 3.5 um, 3.0 X 150 mm; detection at 220 and 254 nm; System B = 10%B to 100%B over 15 minutes; flow = 0.5 mL/min; solvent A = 10 mm ammonium bicarbonate/95% water/5% methanol; solvent B = 10 mM ammonium bicarbonate/5% water/95% methanol; 2 individual columns, Xbridge C18 3.5 um, 3.0 X 150 mm or Xbridge Phenyl 3.5 um, 3.0 X 150 mm; detection at 220 and 254 nm; MS (ESI), $(M+H)^+$ 452.0; LC/MS MH^+ 452.26, ^{1}H NMR (CD_3CN) δ ppm 7.77 - 7.88 (2 H, m), 7.73 (2 H, m, *J*=8.07 Hz), 7.54 - 7.58 (2 H, m), 7.50 (2 H, m, *J*=8.07 Hz), 6.97 (1 H, br. s.), 6.26 (1 H, br. s.), 5.64 (1 H, br. s.), 4.73 - 4.84 (1 H, m), 4.61 - 4.73 (1 H, m), 4.23 - 4.43 (1 H, m), 2.89 (3 H, d, *J*=4.65 Hz), 1.45 - 1.56 (1 H, m), 1.29 - 1.40 (2 H, m), 0.84 (3 H, d, *J*=6.36 Hz), 0.60 (3 H, d, *J*=6.36 Hz).

5,5,5-trifluoro-2-(1-phenylethylamino)pentanenitrile (6).

To a solution of (R)-phenethylamine (9.60 g, 79.4 mmol) and acetic acid (5.08 g, 79.6 mmol) in MeOH (150 mL) was added NaCN (3.88 g, 79.6 mmol). The reaction was cooled to 0 °C and a solution of 4,4,4-trifluorobutyraldehyde (**5**) (10.0 g, 79.6 mmol) in MeOH (50 mL) was added. The reaction was warmed to room temperature and stirred for 20 h. The reaction was diluted with water (400 mL) and extracted with CH_2Cl_2 (3 × 300 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under

vacuum to provide **6** (18.1 g, 89%, as a 4:1 mixture as isomers) as a pale yellow oil: ^{1}H NMR (300 MHz, CD_3OD) δ 7.38–7.27 (m, 5H), 4.15–4.02 (m, 1H), 3.69 (t, J = 7.5 Hz, 0.22H), 3.18 (t, J = 7.5 Hz, 0.78H), 2.48–2.26 (m, 1H), 2.25–2.03 (m, 1H), 2.01–1.86 (m, 2H), 1.39 (d, J = 6.5 Hz, 2.34H), 1.36 (d, J = 6.5 Hz, 0.66H); ESI MS m/z 257 [$C_{13}H_{15}F_3N_2$ + H].

(R)-5,5,5-trifluoro-2-((R)-1-phenylethylamino)pentanamide hydrochloride (8).

To a solution of aminonitrile **6** (18.0 g, 70.31 mmol) in CH_2Cl_2 (100 mL) was added H_2SO_4 (100 mL). The reaction was stirred at room temperature for 22 h, poured onto crushed ice and neutralized with NH_4OH. The mixture was extracted with EtOAc (3 × 500 mL). The combined organic layer was dried over Na_2SO_4 and concentrated under vacuum to provide the free base of **7** (18.94 g, 98%) as an orange oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40–7.18 (m, 5H), 6.78 (br s, 0.23H), 6.50 (br s, 0.77H), 6.00 (br s, 0.77H), 5.81 (br s, 0.23H), 3.82 (q, J = 6.5 Hz, 0.23H), 3.70 (q, J = 6.5 Hz, 0.77H), 3.14 (t, J = 6.0 Hz, 0.23H), 2.86 (t, J = 7.0 Hz, 0.77H), 2.35–1.86 (m, 2H), 1.84–1.64 (m, 2H), 1.39 (d, J = 6.5 Hz, 0.69H), 1.35 (d, J = 6.5 Hz, 2.31H); ESI MS m/z 275 [$C_{13}H_{17}F_3N_2O$ + H]. To a solution of the free base of **7** (11.9 g, 43.4 mmol) in Et_2O/MeOH (7:1, 40 mL) was added a solution of 1 N HCl in Et_2O (70 mL). The white precipitate formed was re-dissolved by heating the mixture and adding MeOH (to a final ratio of 4:1 Et_2O/MeOH). The solution was allowed to cool to room temperature and left to stand overnight. The aminoamide hydrochloride salt **8** was isolated as a single diastereomer (3.11 g, 23%) as a white solid: ^{1}H NMR (300 MHz, MeOD) δ 7.93 (br s, 1H), 7.69 (br s, 1H), 7.54–7.44 (m, 5H), 4.39 (q, J = 7.0 Hz, 1H), 3.50 (t, J = 6.5 Hz, 1H), 2.29–2.20 (m, 2H), 2.10–2.01 (m, 2H), 2.07 (d, J = 7.0 Hz, 3H); ESI MS m/z 275 [$C_{13}H_{17}F_3N_2O$ + H].

(R)-2-(4-chlorophenylsulfonamido)-5,5,5-trifluoropentanamide (10).

To a solution of **8** (3.10 g, 10.0 mmol) in EtOH (100 mL) was added $Pd(OH)_2$ (350 mg) and water (10 mL). The reaction mixture was hydrogenated (40 psi) for 4 h at 50 °C.

The reaction was filtered through celite and the filtrate was concentrated under vacuum to afford the intermediate amine **9** as a white solid. To a suspension of the amine **9** in CH_2Cl_2 (100 mL) was added DIPEA (5.25 mL, 30.0 mmol) and 4-chlorobenzenesulfonyl chloride (2.53 g, 12.0 mmol). The reaction was stirred at room temperature for 18 h. and diluted with EtOAc (200 mL), washed with $NaHCO_3$ (250 mL) and brine (250 mL), dried over Na_2SO_4, and concentrated under vacuum. Compound **10** (2.91 g, 84%) was obtained as a white solid by trituration of the residue with CH_2Cl_2/hexanes (2:1): ^{1}H NMR (300 MHz, MeOD) δ 7.84 (dt, J = 8.5, 2.0 Hz, 2H), 7.55 (dt, J = 8.5, 2.0 Hz, 2H), 3.85 (dd, J = 8.5, 5.0 Hz, 1H), 2.34–2.05 (m, 2H), 1.97–1.68 (m, 2H); ESI MS m/z 345 [$C_{11}H_{12}ClF_3N_2O_3S$ + H].

4-(bromomethyl)-3-fluorobenzonitrile (11).

To a solution of 3-fluoro-4-methylbenzonitrile (5.0 g, 0.23 mol) in 100 mL of carbon tetrachloride was added N-bromosuccinamide (4.97 g, 0.28 mol) and AIBN (100 mg, 0.61 mmol) was refluxed for six hours. The reaction was cooled and filtered. The filtrate was washed with water, dried over magnesium sulfate, filtered and the solvents were removed under vacuum to afford 5.44 g of **11** as an off-white solid. ^{1}H NMR indicated the presence of 20% starting material. Compound **11** would not ionize in the MS. ^{1}H NMR (400 MHz, CDCl3) δ 7. 54-7.30 (m, 3H), 4.83 (s, 2H).

(R)-2-(4-chloro-N-(4-cyano-2-fluorobenzyl)phenylsulfonamido)-5,5,5-trifluoropentanamide (12).

To a solution of **10** (6.88 g, 20.0 mmol) and **11** (6.43 g, 30 mmol) in DMF (35 mL) was added anhydrous Cs_2CO_3 (19.56 g, 60 mmol). The resulting mixture was stirred at room temperature for 45 minutes, and then diluted with ethyl acetate (200 mL), washed with water (100 mL X 4) and dried over Na_2SO_4. The product was purified by a silica gel Biotage (40+M column, 3% to 80 % EtOAc in hexanes, 651 mL). Compound **12** was obtained as a white solid (6.50 g, 68.1% yield). ^{1}H NMR ($DMSOd_6$, 400 MHz) δ 7.80-

7.88 (m, 3H), 7.70-7.75 (m, 2H), 7.67 (d, 2H, J=8), 7.60 (s, 1H), 7.26 (s, 1H), 4.99 (d, 1H, J=16), 4.68 (d, 1H, J=16), 4.14 (t, 1H, J=8), 1.99-2.17 (m, 2H), 1.80-1.94 (m, 1H), 1.40-1.56 (m, 1H). LC/MS M+H 478.14.

(R)-2-(4-Chloro-N-(2-fluoro-4-(N'-hydroxycarbamimidoyl)benzyl)phenylsulfonamido)-5,5,5-trifluoropentanamide (13).

To a solution of **12** (6.5 g, 13.6 mmol) in EtOH (70 mL) was added NH_2OH (50% in H_2O, 2.6 mL. 40.8 mmol). Caution: Hydroxylamine is unstable upon concentration and elevated temperature and may spontaneously detonate. The resulting mixture was stirred at 80 ^{0}C under nitrogen for 1 h and then cooled to rt. The solvents were evaporated under reduced pressure. The residue was dissolved in EtOAc and washed with water and dried over Na_2SO_4. Evaporation of the solvent gave a white solid which was recrystallized from EtOAc and hexanes to afford the titled compound **13** as a white solid (6.93 g, 100%). ^{1}H NMR (300 MHz, DMSO-d_6) δ ppm 1.43 - 1.64 (m, 1 H) 1.77 - 1.93 (m, 1 H) 1.93 - 2.17 (m, 2 H) 4.41 (dd, *J*=8.48, 6.03 Hz, 1 H) 4.60 (d,*J*=17.14 Hz, 1 H) 4.94 (d, *J*=16.77 Hz, 1 H) 5.81 - 5.98 (m, 2 H) 7.19 - 7.27 (m, 1 H) 7.37 - 7.47 (m, 2 H) 7.52 (d, *J*=4.14 Hz, 2 H) 7.64 (d, *J*=8.67,Hz, 2 H) 7.85 (d, *J*=8.85 Hz, 2 H) 9.71 - 9.83 (m, 1 H). IR (KBr): 3491, 3379, 1680, 1651, 1592, 1433, 1343. Anal. Calcd. for $C_{19}H_{19}ClF_4N_4O_4S$ Calc. C, 44.66; H, 3.74; N, 10.96; S, 6.27; F, 14.87; Cl, 6.94. Found: C, 44.90; H, 3.91; N, 10.91; S, 6.41; F, 15.21; Cl, 6.95.

(2R)-2-[[(4-Chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide (4).

To a mixture of the amide oxime **13** (6.93 g, 13.6 mmol) and triethyl orthoformate (6.77 mL, 40.8 mmol) in dichloroethane (30 mL) was added BF_3•OEt_2 (0.17 mL, 1.36 mmol). The resulting mixture was stirred at 70 ^{0}C for 1 h and then cooled to room temperature. Chromatography (silica gel, biotage, 40+M column, 3% to 80% EtOAc in hexanes, 651 mL) provided the desired product as a white solid. (4.9 g, 69% yield). ^{1}H NMR, ($CDCl_3$, 300 MHz) δ: 1.40 - 1.58 (m, 1 H) 1.75 - 1.90 (m, 1 H) 1.92 - 2.07 (m, 1

H) 2.10 - 2.26 (m, 1 H) 4.37 (dd, *J*=8.67, 6.22 Hz, 1 H) 4.48 (d, *J*=15.64 Hz, 1 H) 4.64 (d, *J*=15.82 Hz, 1 H) 5.54 (s, 1 H) 6.33 (s, 1 H) 7.44 - 7.54 (m, 2 H) 7.62 (t, *J*=7.72 Hz, 1 H) 7.68 - 7.76 (m, 3 H) 7.85 (dd, *J*=7.91, 1.51 Hz, 1 H) 8.76 (s, 1 H). ^{13}C NMR, (DMSO-d_6, 75 MHz) δ: 170.34, 167.75, 165.80, 159.64 (d, *J* = 244.5 Hz, 1 C), 138.19, 137.64, 131.25 (d, *J* = 3.75 Hz, 1C), 129.31, 129.23, 129.05 (d, *J* = 14.25 Hz, 1C), 126.74 (q, *J* = 274.5 Hz, 1C), 126.91, 126.80, 123.12 (d, *J* = 3.75 Hz, 1C), 113.7 (d, *J* = 24.0 Hz, 1 C), 57.92, 41.38 (d, *J* = 4.5 Hz, 1 C), 30.04 (d, *J* = 30.0 Hz, 1 C), 22.90. ^{19}F NMR, ($CDCl_3$, 282 MHz) δ: -116.3, -66.5. IR (KBr): 3454, 334, 3286, 2952, 1705, 1432, 1325, 1260, 1167, 1084, 828 cm^{-1}. Anal. Calcd. for $C_{20}H_{17}ClF_4N_4O_4S$ Calc. C, 46.11; H, 3.29; N, 10.71; S, 6.15; F, 14.58; Cl, 6.80. Found C, 46.06; H, 3.24; N, 10.71; S, 6.25; F, 14.60; Cl, 6.88.

Biological Protocols

Inhibition of Aβ formation in cultured cells

Compounds were assayed for Aβ40 or Aβ42 inhibition in cells using H4 APP751 SWE clone 8.20, developed at Bristol-Myers Squibb, an H4 neuroglioma cell line stably expressing the Swedish mutant of APP751. Cells were maintained in log phase through twice weekly passage at a 1:20 dilution. For IC_{50} determinations, 30 μL cells (1.5 x 10^4 cells/well) in DMEM media containing 0.0125% BSA (Sigma A8412) were plated directly into 384-well compound plates (Costar 3709) containing 0.1 μL serially diluted compound in DMSO. Following incubation for 19 hours in 5% CO_2 at 37°C, plates were briefly centrifuged (1000 rpm, 5 min). A 10 μL aliquot from each well was transferred to a second assay plate (Costar 3709) for Aβ40 measurements. Antibody cocktails were freshly prepared by dilution into 40 mM Tris-HCl (pH 7.4) with 0.2% BSA and added to assay plates. For Aβ42 measurements, antibodies specific for the Aβ42 neoepitope (565, developed at Bristol-Myers Squibb**;** conjugated to the Wallac reagent (Perkin Elmer)) and the N-terminal sequence of Aβ peptide (26D6, developed at SIBIA/Bristol-Myers Squibb; conjugated to APC (Perkin Elmer)) were mixed and 20 μL of the mixture was added to each well of the incubated cell plate yielding a final concentration of 0.8 ng/well

565 and 75 ng/well 26D6. For the Aβ40 measurements, antibodies specific for the Aβ40 neoepitope (TSD, developed at Bristol-Myers Squibb; conjugated to the Wallac reagent (Perkin Elmer)) and 26D6 as described above were mixed and 20 μL of the mixture was added to the 10 μL aliquots which had been removed previously from the cell plate yielding a final concentration of 1.6 ng/well TSD and 17.5 ng/well 26D6. Assay plates containing antibodies were sealed with aluminum foil and incubated overnight at 4°C. Signal was determined using a Viewlux counter (Perkin Elmer) and IC_{50} values determined using curve fitting in CurveMaster (Excel Fit based).

Inhibition of Notch signaling in cultured cells

A mouse Notch1 expression construct was generated by PCR using standard molecular biology techniques and verified by sequencing. This construct was generated in the pCDNA3.1+ Hyg vector (Invitrogen) modified to contain a N-terminal 20 amino acid signal sequence and a C-terminal 7X myc tag. The signal sequence was derived from mouse Notch1; the 7X myc-tag was created by using overlapping primers and subcloned into the HindIII/XhoI sites of the pCDNA3.1+ Hyg vector.

The mouse Notch1-ΔE construct contains the mouse Notch1 signal sequence and the M1727V mutation within the transmembrane domain to suppress internal translation initiation. Mouse Notch1-ΔE coding sequence from amino acid 1704 to 2193 was isolated from a Mouse Spleen Quick Clone cDNA library (Clontech) and subcloned into the modified vector containing the 7X myc-tag and the signal sequence as a XbaI/HindII fragment.

Hela cells were maintained in DMEM (GibcoBRL) containing 10% FBS (GibcoBRL), Penicillin/Streptomycin (GibcoBRL) and 2 mM L-glutamine (GibcoBRL). Cells were transiently transfected using TransIT-HelaMONSTER (Mirus) according to the manufacturer's directions. Hela cells (ATCC) were plated 16 hours before transfection at a density of 4 x 10^6 cells per T175 flask in Hela growth media (DMEM (high glucose with HEPES) with glutamine, penicillin, streptomycin and 10% fetal bovine serum). Cells were transfected in growth media with: 6 μg mouse Notch1-ΔE plasmid, 15.6 μg carrier plasmid (pCDNA3.1+ hyg), 14.4 μg CBF1 plasmid (luciferase reporter) using HelaMonster Transfection Reagent (Mirus). The CBF1-luciferase reporter construct consists of 4X copies of the CBF1 binding element upstream of the

SV40 promoter (pGL3-Promoter, Promega). The CBF1-luciferase reporter was generated using overlapping primers to generate the 4X CBF1 binding region. This fragment was subcloned into the NheI/BglII sites of pGL3-Promoter constructs. The integrity of this construct was confirmed by sequencing. DNA stocks were diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) for transfection. Five to six hours after DNA addition, cells were removed from the flask with Trypsin-EDTA and resuspended in defined media (DMEM (high glucose with HEPES) with glutamine, penicillin, streptomycin, 0.0125% bovine serum albumin and non-essential amino acids) at a concentration of 5 x 10^4 cells/mL. Cells were plated into 96 well black Clearview plates (Packard) at a volume of 200 μL/well (1 x 10^4 cells) and incubated at 37°C for 1.5 hours to allow cells to adhere to the plates. Test compounds were initially diluted in a 96 well polypropylene plate in 100% DMSO. The DMSO compound stocks were then diluted 47.6-fold by transferring into a plate containing defined media yielding a 2.1% DMSO concentration. Diluted compound solutions (10 μL) were added to the cell plate yielding a final 0.1% DMSO concentration. Cell plates containing compound were incubated overnight at 37°C. Following this overnight incubation, media was gently removed and 25 μL of phosphate buffered saline (with calcium and magnesium) was added to each well. Luc-Screen (Applied Biosystems) reagents A and B were mixed in equal proportions and 50 μL of the mixture was added to each well. The plate was incubated at room temperature for 10 minutes before black backing was attached to the bottom of the plate and the signal was read on a TopCount (Packard). Concentration response curves were then fit using nonlinear regression to determine IC_{50} values.

PXR Transactivation

Pregnane X receptor (PXR) is a nuclear hormone receptor principally responsible for the induction of cytochrome P450 (CYP) 3A4, which plays a major role in metabolizing many clinically prescribed drugs. It is well known that the induction of CYP3A4 can cause either drug-drug interaction by increasing the metabolic clearance of co-administered CYP3A4 substrates (Bertilsson, G. *et al*., *Proc. Nat. Acad. Sci. USA* (1998) 95: 12208-12213; Lehmann, J.M. *et al*., *J. Clin. Invest.* (1998) 102: 1016-1023)

or can cause loss of drug exposure due to autoinduction. Characterizing the induction potential of discovery or development drug candidates has become an important screen throughout the pharmaceutical industry. A PXR transactivation assay is used to assess the induction potential of CYP3A4, and a cytotoxicity assay of HepG2 cells is used to monitor the assay interference due to cytotoxicity.

Cell culture medium used is DMEM. Lipofectamine 2000, PBS, trypsin-EDTA (0.25%), and penicillin-streptomycin were purchased from GIBCO/Invitrogen (Carlsbad, CA). Heat-inactivated fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO). Charcoal/dextran treated fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). HepG2 cells were obtained from ATCC (Manassas, VA). Human PXR-pcDNA3 and luciferase reporter containing CYP3A4 promoter, CYP3A-Luc, were generated at Bristol-Myers Squibb. Black standard 384-well plates were purchased from BD Biosciences (Lexington, KY). Luciferase substrate (Steady-Glo) was purchased from Promega (Madison, WI). Control compound rifampicin was purchased from Sigma (St. Louis, MO).

Culture of HepG2 cells is performed in T175 flasks using DMEM containing 10% FBS. The transfection mixture contains 1 μg/mL of PXR-pcDNA3 plasmid DNA, 20 μg/mL of Cyp3A-Luc plasmid DNA, 90 μL/mL of Lipofectamine 2000, and serum-free medium. After incubating at room temperature for 20 minutes, the transfection mixture (1 mL per flask) is applied to the cells in fresh medium (20 mL per flask), and flasks incubated at 37°C (5% CO_2) overnight.

Cells in each flask are washed with PBS and 4 mL of Trypsin-EDTA (0.25%) is added and incubated for one minute at room temperature. Trypsin is then aspirated off and flasks incubated for an additional five minutes at room temperature. The flasks are then tapped vigorously to break up cell aggregates. After the addition of 10 mL of DMEM containing 5% charcoal/dextran-treated FBS, the entire mixture is transferred to conical tubes. The pool of cells in suspension is further disaggregated by pipetting through a 1 mL pipet tip. Cells are then counted and diluted in media to 3.0×10^5 cells/mL. Fifty μL of cell mixture is added to each well of a black standard 384-well

plate containing 0.25 μL of test compound dissolved in 100% DMSO. The plates are incubated at 37°C (5% CO_2) overnight before 25 μL of luciferase substrate (Steady-Glo, Promega) is added to each well. After fifteen minutes, plates are read on a Viewlux (Perkin-Elmer) plate reader.

Rifampicin (10 μM), a well known agonist of PXR, is included in each plate as an internal standard and positive control. The data was then expressed as percent activation (%Act), where the total signal is the signal from the 10 μM rifampicin and the blank signal is that from the DMSO vehicle.

$$\%\text{Act} = \frac{\text{Compound signal - Blank signal}}{\text{Total signal} - \text{Blank signal}} \times 100\%$$

Compounds are tested at ten concentrations (50 μM – 2.5 nM, 1:3 serial dilution), and XL-Fit (IDBS, Inc.) used for curve-fitting. Concentrations of compound at which 20% and 60% activation occur (EC20A and EC60A, respectively) are reported.

The human pregnane-X receptor is principally responsible for the induction of CYP3A4, as well as CYP2B6, CYP2C8/9, Phase 2 enzymes such as UGT, and several transporters such as P-gp, MRP2, and OATP2. The above test demonstrated that (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide had an EC_{20A} (20% of the Rifampicin response at 10 μM) of 6.9 μM and an EC_{60A} (60% of the Rifampicin response at 10 μM) of greater than 16.7 μM in the hPXR transactivation assay. These results suggest that (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide has a significantly diminished PXR transactivation profile.

Metabolic Stability in Liver Microsomes

Mouse, rat, dog, human, and cynomolgus monkey liver microsomes were obtained from BD Gentest (Woburn, MA). The lot numbers were 13 (mouse), 8 (rat), 8

(dog), 19 and 26 (human), and 3 (cynomolgus monkey). The oxidative metabolism of (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide was studied in liver microsomes under three sets of conditions. The incubation mixtures for human and dog (total volume 3 mL, organic solvent content 0.3%) contained (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide (1 μM), microsomal protein (1 mg/mL), NADPH (1 mM), Tris chloride buffer (100 mM, pH 7.4), and magnesium chloride (3.3 mM). The reaction, conducted in triplicate, was initiated by the addition of NADPH followed by incubation at 37°C for 50 minutes. Aliquots of samples (0.25 mL) were taken at 0, 5, 10, 20, 30, 40 and 50 minutes and the reaction was quenched by the addition of 3 volumes of acetonitrile. The incubation mixtures for cynomolgus monkey and rat (total volume 3 mL, organic solvent content 0.3%) contained (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide (1 μM), microsomal protein (0.1 mg/mL), NADPH (1 mM), Tris chloride buffer (100 mM, pH 7.4), and magnesium chloride (3.3 mM). The reaction, conducted in triplicate, was initiated by the addition of NADPH followed by incubation at 37 °C for 50 minutes. Aliquots of samples (0.25 mL) were taken at 0, 5, 10, 20, 30, 40 and 50 minutes and the reaction was quenched by the addition of 3 volumes of acetonitrile. The incubation mixtures for mouse and cynomolgus monkey (total volume 3 mL, organic solvent content 0.3%) contained (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide (1 μM), microsomal protein (0.1 mg/mL), NADPH (1 mM), Tris chloride buffer (100 mM, pH 7.4), and magnesium chloride (3.3 mM). The reaction, conducted in duplicate, was initiated by the addition of NADPH followed by incubation at 37°C for 40 minutes. Aliquots of samples (0.25 mL) were taken at 0, 5, 10, 15, 20, 30 and 40 minutes and the reaction was quenched by the addition of 3 volumes of acetonitrile. For all studies, samples were analyzed immediately by an LC/MS method. Rate of parent disappearance was calculated from the peak area ratios of (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide at each time point .

The hepatic intrinsic clearance (CLh,int, mL/min/kg) of (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide in various species was estimated from liver microsome data using the method described by Houston JB., *Biochem Pharmacol* 1994;47:1469-1479; Iwatsubo, *et al*., *Pharmacol Ther* (1997) 73:147-171; and Obach *et al*., *J Pharmacol Exp Ther* (1997) 283:46-58.

The rate of in vitro metabolism was determined for (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide in the presence of NADPH-fortified liver microsomes of various species. The compound of the present invention (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide (1 μM) was metabolized at a rate of 690, 630, 40, 495 and 32 pmol/min/mg protein in the presence of mouse, rat, dog, cynomolgus monkey, and human microsomes, respectively. When the rates of (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide consumption were scaled to in vivo clearance, the predicted in vivo (serum) clearance values of (2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide were approximately 87, 52, 14, 39 and 8 mL/min/kg in mouse, rat, dog, cynomolgus monkey, and human, respectively.

In Vivo Pharmacology

Abeta ELISAs

Aβ species from animals were measured using sandwich ELISA assays. A brief discussion of these assays is included here since the details of the epitopes for the individual antibodies determines the Aβ species that are detected. Mouse and rat Aβ share a common Aβ sequence that differs from human Aβ. As a result of these sequence differences, antibodies that recognize the N-terminal region of human Aβ, such as 26D6, bind weakly to rodent Aβ. Likewise, antibodies that bind tightly to rodent Aβ, such as 252, bind weakly to human Aβ. Two assays were developed for measuring rodent Aβ40:

252-TSD and 4G8-TSD. The TSD-4G8 assay can measure not only Aβ40, but other BACE-γ-secretase cleavage products (Aβ11-40) and α-secretase-γ-secretase cleavage products (P3). Table 1 summarizes the assays presented in this application and their use.

Table 1: Summary of Antibody Pairs Used to Assay In Vivo Samples

Antibody Pair	Tissues analyzed	Aβ species detected
252 - TSD	Rat brain	Aβx-40[a]
4G8 - TSD	Rat plasma, CSF	Aβx-40 & P3

[a] The exact location of "x" is unknown. While 252 recognizes the N-terminal region of Aβ, it is unknown whether amino terminal truncation of Aβ affects 252 binding. This uncertainty is unlikely to be an issue in rats since N-terminal truncation is rare.

Each of these assays was validated using several methods. First, varying amounts of synthetic Aβ were added to the biological matrix and the increase in signal was compared to that obtained with synthetic Aβ in buffer solution. Second, Aβ was depleted from the biological sample with anti-Aβ antibodies. Third, samples were assayed from animals that were treated with high doses of a γ-secretase inhibitor. A validated assay efficiently detected exogenously added Aβ (>80% recovery), had a greatly reduced signal after Aβ immunodepletion (>80% reduction compared to nonspecific controls), and had a signal reduced to values approaching or overlapping with the assay floor using samples from animals treated with high doses of a γ-secretase inhibitor. The optimized and validated Aβ assays still contained a small amount of the signal (5-20% of vehicle control) which could not be depleted by anti-Aβ antibodies or treatment with γ-secretase inhibitors. This signal is unlikely to be Aβ and is consequently referred to as the assay floor. The assay floor was not used to correct any of the Aβ measurements and consequently, the values reported are likely underestimates of the actual amount of Aβ inhibition.

Aβ40 was used as a surrogate for Aβ42 in vivo. Aβ40 is approximately 10-fold more abundant in biological samples than Aβ42. Aβ40 is a good surrogate for Aβ42 based on experiments in cultured cells where Aβ40 and Aβ42 were similarly inhibited by

(2R)-2-[[(4-chlorophenyl)sulfonyl][[2-fluoro-4-(1,2,4-oxadiazol-3-yl)phenyl]methyl]amino]-5,5,5-trifluoropentanamide and other γ-secretase inhibitors.

Rat Studies

In life

Female Harlan Sprague-Dawley rats (~200 – 250 g) were dosed daily by oral gavage with a dosing vehicle of 99% PEG-400, 1% Tween-80 at 4 mL/kg in the morning. Dosing solutions were made once at the start of the study. Heating at 56°C and sonication were used to solubilize compound in the dosing solution. All procedures were done in concordance with ACUC guidelines. Terminal blood samples were obtained by cardiac puncture after CO_2 euthanasia and collected in EDTA tubes. Plasma was obtained after centrifugation. Brain tissue was dissected, weighed and frozen on dry ice until analysis. CSF samples were centrifuged to remove cells or debris prior to dilution at 1:2 in 4% BSA and frozen for subsequent analysis. Histopathological samples were placed in neutral buffered formalin prior to processing. Samples collected for occupancy were coated in embedding matrix, and frozen at -25°C to -30^{o}C in a 2-methylbutane bath followed by dry ice. In life plasma samples were obtained using retro-orbital bleeding.

Brain Abeta40 assay

Rat brain (half a hemisphere) was homogenized using a polytron at 4 mL/g in PBS, pH 7.8, 2% CHAPS, complete protease inhibitors (Roche). Large debris was removed by centrifugation for 30 minutes at 20,800 x g and the resulting supernatant was diluted 1:2 in PBS, 2.5% BSA. White Microlite II ELISA plates (Thermo Electron) were incubated with 50 μg/mL TSD9S3.2 antibody in PBS for 1 hour at 37°C. Plates were blocked with 200 μL 5% bovine serum albumin (BSA; weight/volume prepared in PBS) for 2 hours at room temperature on a plate shaker and then washed 5 times with 500 μL/well of PBS, 0.05% Tween-20. Clarified brain homogenates were loaded in 6 replicates of 50 μL per well and incubated for 1 hour at room temperature. Plates were washed as before and then incubated with horse radish peroxidase (HRP)-conjugated 252 antibody (Biosource) diluted 1:2000 in PBS, 0.05% Tween, 0.1% BSA for 1 hour. Three

replicates contained the 252-HRP antibody only and three replicates contained the 252-HRP antibody with 1 μg/mL rat Aβ1-14 (Anaspec) which competed specifically bound antibody; this background signal was substrated from the total signal to yield the specific signal. The bound 252-HRP antibody was detected using Pierce Supersignal Pico Chemiluminescent substrate for 10 minutes and quantified a Packard TopCount. Samples were normalized to a brain homogenate reference placed on each plate. Based on Aβ40 standard curves, the LLQ was 10 pg/mL and the LLD was 20 pg/g tissue.

Plasma Abeta40 assay

Plates were coated with TSD antibody and washed as for the brain Aβ40 assay. Plasma samples were diluted 1:3 in PBS buffer, pH 7.8, 0.25% nonidet P40, 2.5% BSA. Samples were loaded in 6 replicates of 50 μL per well and incubated for 1-2 hours at room temperature. Samples were detected using 4G8-biotin (Signet) diluted 1:8000 in PBS, 0.05% Tween, 0.1% BSA for 1 hour. Three replicates had the 4G8-biotin antibody only and three replicates had the 4G8-biotin antibody with 1 μg/mL Aβ17-24 which competed the specific signal and thereby established a background value for each sample. Following washing as above, plates were incubated with streptavidin-HRP (Zymed) diluted 1:50,000 in PBS, 0.05% Tween, 0.1% BSA for 10 minutes. Detection and quantification were as for brain Aβ40 assays. Samples were normalized to a plasma reference placed on each plate. Based on Aβ40 standard curves, the LLQ was 7.5 pg/mL and the LLD was 23 pg/mL plasma.

CSF Abeta40 assay

Plates were coated with TSD antibody and washed as for the brain Aβ40 assay. CSF samples were diluted 1:10 in PBS, pH 7.8, 0.1% Tween-20. At time of collection, CSF was previously diluted 1:2 in 4% BSA in water. Samples were loaded in 3 replicates of 50 μL per well and incubated for 1-2 hours at room temperature. Samples were detected using 4G8-biotin (Signet) diluted 1:8000 in PBS, 0.05% Tween, 0.1% BSA for 1 hour. Because the background was low, it was not necessary to run replicate samples with a competing peptide for this assay. Bound 4G8-HRP was detected and quantified as for the plasma Aβ40 assay. Samples were normalized to a CSF reference

placed on each plate. Based on Aβ40 standard curves, the LLQ was 20 pg/mL and the LLD was 400 pg/mL CSF.

Dog Studies

In life

Dog studies at BMS were performed using the ACUC approved protocol, ATM-405-142K9 with 7- 10 month old naïve, grade II beagles from Marshall Laboratories. Dogs were observed at least twice daily for clinical signs. BMS-708163 was dosed at 1 ml/kg in 94% labrafil-1944, 5% ethanol, 1% tween-80 by oral gavage. Blood samples were obtained via the saphenous or cephalic veins. Plasma samples in EDTA were obtained for Aβ40 measurements, compound levels and hematology, and serum was used for clinical chemistry and proteomics samples. Blood was also collected in Paxgene blood RNA tubes from Becton Dickinson (VWR Cat #77776-026) for the preparation of RNA from white blood cells. Dogs were euthanized using an overdose of pentobarbital. CSF was centrifuged to remove cells or debris prior to aliquoting and freezing.

Brain Aβ40 assay

Dog brain tissue (cortex, hippocampus or cerebellum) was homogenized using a polytron at a volume of 6.5 ml/g in ice cold 4% Tween-20, 20 mM Tris-HCl, pH 8.5, complete protease inhibitors (Roche Diagnostics). Homogenates were incubated on ice for 15 minutes before centrifugation for 1 hour at 135,000 x g at 4° C. The resulting supernatants were removed, placed into clean microcentrifuge tubes, and centrifuged at 220,000 x g for 2 hours at 4° C. The supernatants were frozen in liquid nitrogen and stored at -80° C. White Microlite II ELISA plates (Thermo Electron) were incubated with 50 μg/ml TSD antibody in phosphate buffered saline, pH 7.5 (PBS) for 1 hour at 37° C. Plates were washed with 500 μl per well for 5 times with PBS containing 0.05% Tween-20 before 5% bovine serum albumin (BSA; w/v prepared in PBS) was added as a blocking agent. Plates were blocked for 1 to 2 hours at room temperature on a plate shaker before being washed again. Dog brain supernatants were thawed and diluted 1:1 in 0.1% BSA in PBS before addition to the ELISA plate (brain extracts were assayed at a volume of 13 ml/g in a final buffer of 2% Tween-20, 0.05% BSA, 10 mM Tris-HCl, complete protease inhibitors in PBS). Synthetic human Aβ1-40 peptide (Bachem) was diluted to 260 pg/ml in curve diluent (2% Tween-20, 0.05% BSA, 10 mM Tris-HCl in PBS) and added to plates yielding final concentrations of 260, 130, 65, 32.5, 16.25, 8.13, 4.06 pg/ml. ELISA plates containing dog brain extracts and human Aβ40 standards were

incubated for 2 hours at room temperature while shaking. Plates were then washed and biotinylated 4G8 antibody was added to a final concentration of 0.5 μg/ml. Biotinylated 4G8 was diluted in PBS containing 0.05% Tween-20 and 0.1% BSA. Plates containing biotinylated 4G8 were incubated for 1 hour at room temperature while shaking. Plates were then washed and 25 ng/ml streptavidin-HRP (Zymed) in PBS containing 0.05% Tween-20 and 0.1% BSA was added. Plates containing streptavidin-HRP were incubated for 15 minutes at room temperature while shaking. Plates were then washed and chemiluminescent substrate was added (Pierce Chemical). Plates containing chemiluminescent substrate were incubated for 15 minutes at room temperature while shaking and then quantified using a TopCount (Packard).

CSF Aβ40 Assay

White Microlite II ELISA plates (Thermo Electron) were incubated with 50 μg/ml TSD9S3.2 antibody in phosphate buffered saline, pH 7.5 (PBS) for 1 hour at 37° C or 4^{o} C overnight. All subsequent incubations occur at room temperature with shaking. Plates were blocked with 200 μl 5% bovine serum albumin (BSA; w/v prepared in PBS) for 1 to 3 hours . Plates were washed 5 times with of 500 μl/well of PBS, 0.05% Tween-20. CSF samples were diluted 1:20 and/or 1:40 in PBS buffer, pH 7.8, 0.1% Tween-20. Samples were loaded in 3 replicates of 50 μl per well and incubated for 1-2 hours at room temperature. Samples were detected using 26D6-HRP diluted 1:50,000 in PBS, 0.05% Tween, 0.1% BSA for 1 hour. Detection and measurements were as for the brain Aβ40 assay. Samples were normalized to a CSF reference placed on each plate. Based on Aβ1-40 standard curves, the LLQ was 80 pg/ml and the LLD was 1.6 – 3.2 ng/ml CSF.