Supporting Information

Application of the [1.1.1]-Bicyclopentane Motif as a Nonclassical Phenyl Ring Bioisostere in the Design of a Potent and Orally Active γ-Secretase Inhibitor

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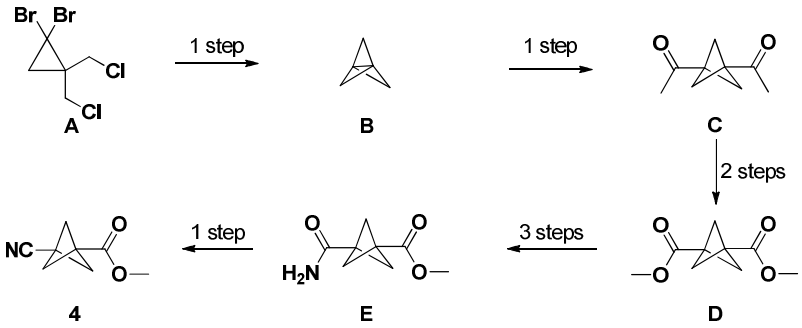
Synthesis of Compound 4



Tricyclo[1.1.1.0^{1,3}]pentane (B). To an oven-dried 500 mL 3-neck round bottom flask fitted with an overhead stirrer and a pressure-equalizing addition funnel were added 1,1-dibromo-2,2-bis(chloromethyl)cyclopropane (**A**, 50.0 g, 150 mmol) and pentane (50 mL). The reaction flask was cooled to –78 °C, methyllithium (1.6 M in diethyl ether, 200 mL, 320 mmol) was transferred by cannula to the addition funnel and then added drop-wise over a period of 60 min. The reaction mixture was stirred at –78 °C for 15 min, then at 0 °C for 1 h. The overhead stirrer was replaced with a magnetic stir bar and the reaction vessel was fitted with a short-path distillation apparatus (the condenser was cooled with a re-circulating chiller at –5 °C). The receiving vessel (1 L round bottom flask) was cooled with liquid nitrogen while intermittent vacuum was applied to the system to maintain boiling. Once boiling was established, the reaction vessel was warmed to 40 °C with a water bath. Compound **B** was obtained in a solid matrix of pentane/ether and carried forward without further isolation or purification.

1,1'-(Bicyclo[1.1.1]pentane-1,3-diyl)diethanone (C). The frozen solution (external liquid nitrogen bath) of **B** in pentane/ether (*vide supra*) was warmed to –10 °C and transferred to a photo reactor assembly in a –10 to –5 °C cooling bath (1:1 ethylene glycol/water), rinsing the flask with additional pentane (2 × 10 mL). 2,3-Butanedione (15.0 mL, 170 mmol) was added and the mixture was irradiated with a 450 W medium pressure mercury vapor UV lamp (quartz immersion cell). A re-circulating

chiller was used to maintain a –5 to +5 °C bath around the reaction mixture, and cold water was run through the immersion cell. The reaction was monitored by ^{1}H NMR (a 50 μL aliquot was removed, placed in an NMR tube and diluted with 0.50 mL $CDCl_3$) for the disappearance of tricyclo[1.1.1.0^{1,3}]pentane (δ 2.0 ppm) and 2,3-butanedione (δ 2.32 ppm), and the appearance of 1,1'-bicyclo[1.1.1]pentane-1,3-diyldiethanone (δ 2.25, 2.14 ppm). After 1.75 h, the reaction mixture was transferred to a 1 L round bottom flask and concentrated *in vacuo* to afford a wet-looking yellow granular solid. The crude product was purified by flash column chromatography using a 0 → 45% gradient of ethyl acetate/heptane to afford 17.2 g (75% yield over two steps) of **C** as an off-white solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 2.26 (s, 6H), 2.16 (s, 6H); GCMS *m/z* 152 (M+).

Dimethyl bicyclo[1.1.1]pentane-1,3-dicarboxylate (D). To a 1000 mL 3-neck round bottom flask cooled to 0 °C (external) and fitted with a thermocouple, mechanical stirrer and pressure equalizing addition funnel was added solid sodium hydroxide (41.5 g, 1.04 mol) and water (307 mL). When completely dissolved and cooled to 23 °C (internal), bromine (24.8 mL, 483 mmol) was added over a period of 4 min (internal exotherm to 27 °C) to the mechanically-stirred sodium hydroxide solution. Sodium chloride was added to the ice bath to lower the external temperature to –10 °C. A solution of **C** (9.68 g, 63.6 mmol) in dioxane (34 mL) was added drop-wise *via* an addition funnel over 45 min, maintaining an internal temperature of –3 °C to 3 °C. When the addition was complete, dioxane (6.0 mL) was used to rinse the vessel that initially contained 1,1'-bicyclo[1.1.1]pentane-1,3-diyldiethanone, and this was added over a period of 1 min to the reaction mixture *via* the addition funnel. The cold bath was replaced by an ice/water bath and the reaction was allowed to warm up to 23 °C over a period of 13 h. Solid sodium bisulfite (1.73 g, 14.4 mmol) was added to the reaction mixture in 4 portions and this mixture was stirred for 25 min. The reaction was extracted with chloroform (3 × 60 mL), and the upper aqueous phase was acidified to pH = 2 with concentrated hydrochloric acid. The aqueous phase was

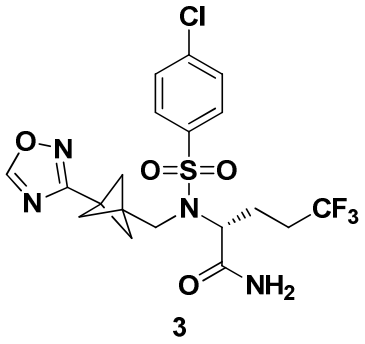
extracted with diethyl ether (3 × 400 mL) and the combined upper ether extracts were dried ($MgSO_4$), filtered and concentrated *in vacuo* until a white precipitate began to form (at ~ 100 mL of residual solvent). The solid was collected by filtration and dried *in vacuo* to afford 7.71 g of the intermediate di-acid as a white solid. The filtrate was further concentrated (to ~ 25 mL of residual solvent) and an additional 0.81 g of the intermediate di-acid was obtained as a pale tan solid, affording a total of 8.52 g (86% yield) of the intermediate di-acid, which was used in the next step without further purification. ^{1}H NMR [500 MHz, $(CD_3)_2CO$] δ 2.27 (s, 6H); GCMS *m/z* 155 (M–1). A 250 mL 3-neck round bottom flask equipped with a water-cooled condenser, calcium chloride drying tube, stopper and magnetic stir bar was charged with the intermediate di-acid (7.55 g, 48.4 mmol). Thionyl chloride (62.0 mL, 859 mmol) was added and the resulting magnetically stirred suspension was heated at reflux for 12 h. The resulting faint yellow solution was cooled to 23 °C, the condenser was removed and replaced with a short path distillation head fitted with a calcium chloride drying tube. The excess thionyl chloride was removed *via* distillation, and the flask containing the acid chloride was cooled to 0 °C using an ice/water bath. Methanol (100 mL) was cautiously added (internal exotherm to 40 °C), and the magnetically stirred reaction mixture was heated at reflux for 1 h. The reaction mixture was then cooled to 23 °C and concentrated *in vacuo* to afford 8.47 g (95% yield) of **D** as a white solid, which was used in the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.69 (s, 6H), 2.32 (s, 6H); GCMS *m/z* 183 (M–1).

Methyl 3-carbamoylbicyclo[1.1.1]pentane-1-carboxylate (E). To a refluxing solution of **D** (1.48 mg, 8.04 mmol) in methanol (20 mL) at 23 °C was added drop-wise over a period of 10 min a solution of sodium hydroxide (324 mg, 8.10 mmol) in methanol (5.0 mL). The reaction mixture was refluxed for 1 h. The solvent was then removed *in vacuo*, water (40 mL) was added and the aqueous mixture was extracted with dichloromethane (3 × 50 mL) to afford 210 mg of recovered **D** as an off-white solid. The

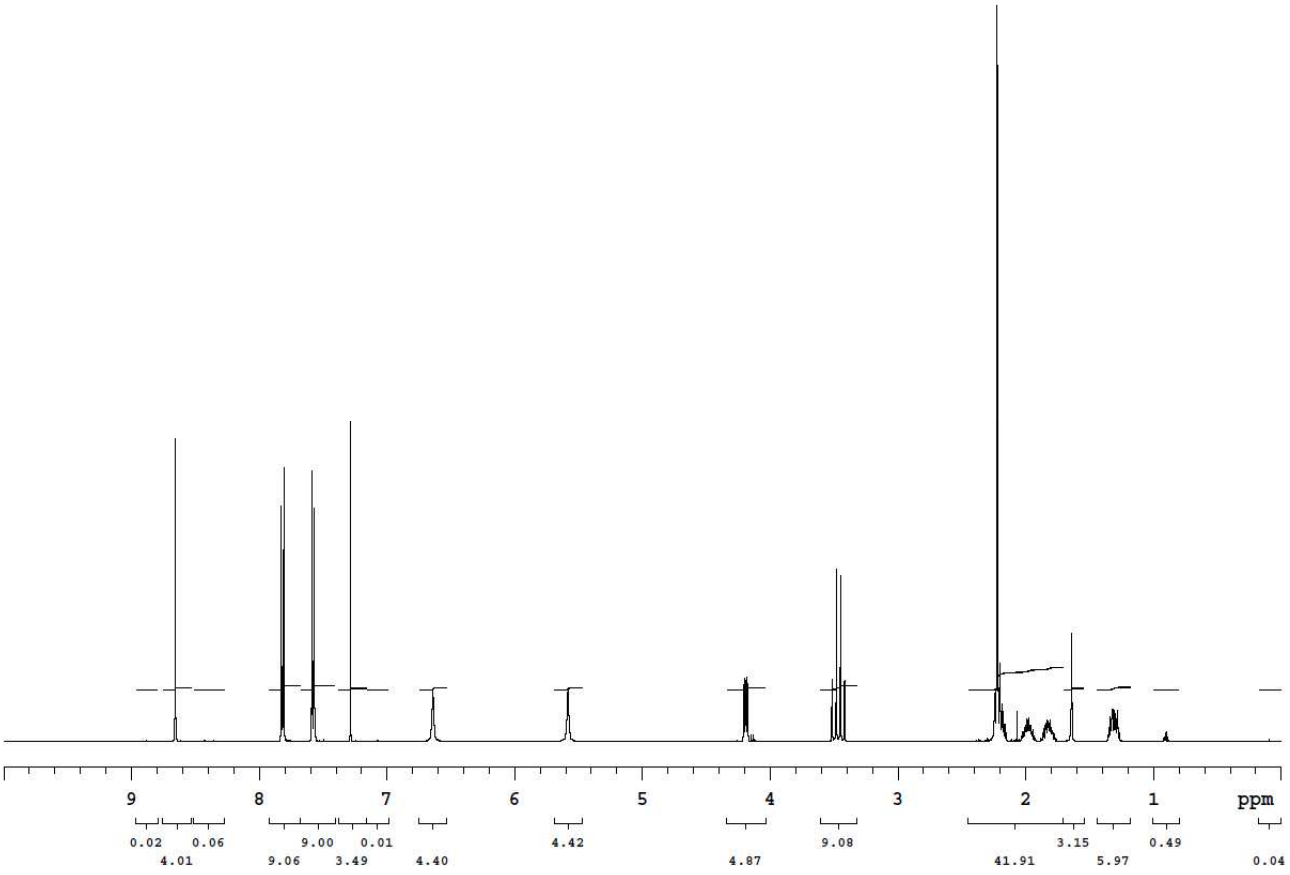
aqueous phase was acidified to pH = 2 using an aqueous solution of hydrochloric acid (3.0 M) and extracted with dichloromethane (4 × 20 mL). The combined organic phases were washed with brine, dried (Na_2SO_4), filtered and concentrated *in vacuo* to afford 1.02 g (75% yield) of the intermediate mono-acid as an off-white solid, which was used in the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.71 (s, 3H), 2.37 (s, 6H); GCMS *m/z* 169 (M–1). To a solution of the intermediate mono-acid (470 mg, 2.76 mmol) in diethyl ether (10 mL) at 23 °C was added oxalyl chloride (490 μL, 5.50 mmol) followed by dimethylformamide (20.0 μL, 0.26 mmol). The reaction mixture was stirred at that temperature for 30 min and then concentrated *in vacuo* to afford 500 mg (96% yield) of the intermediate acid chloride as an off-white solid, which was used in the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.72 (s, 3H), 2.46 (s, 6H). Ammonia gas was bubbled through a solution of the intermediate acid chloride (500 mg, 2.65 mmol) in dichloromethane (40 mL) at 23 °C for 45 min. The resulting white precipitate was removed by filtration, and the filtrate was concentrated *in vacuo* to afford 350 mg (78% yield) of **E** as a white solid, which was used in the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.49 (br s, 1H), 5.40 (br s, 1H), 3.71 (s, 3H), 2.32 (s, 6H); GCMS *m/z* 168 (M–1).

3-(Hydroxymethyl)bicyclo[1.1.1]pentane-1-carbonitrile (4). A solution of **E** (350 mg, 2.07 mmol) in thionyl chloride (6.9 mL) was heated at reflux for 2 h. The reaction was cooled to 23 °C and the thionyl chloride was removed *in vacuo* to afford 280 mg (90% yield) of the intermediate nitrile-ester as off-white needles, which were used in the next step without further purification. ^{1}H NMR (500 MHz, $CDCl_3$) δ 3.70 (s, 3H), 2.52 (s, 6H); GCMS *m/z* 150 (M–1).

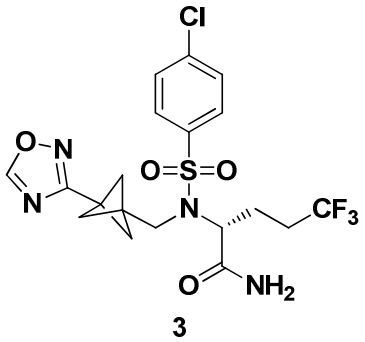
[1]H NMR spectrum of compound 3



3



^{13}C NMR spectrum of compound 3



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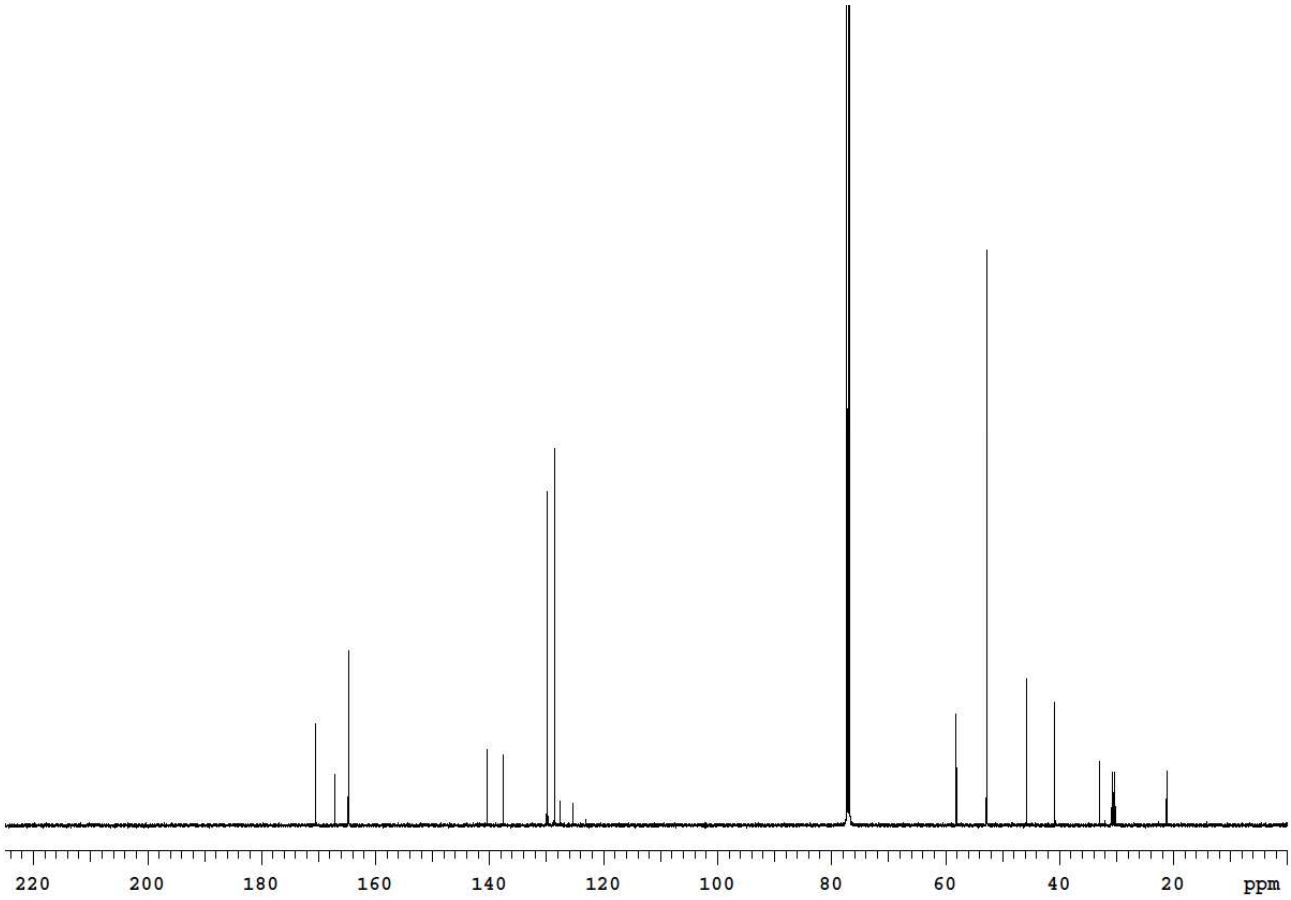

X-Ray crystallography report for compound 1 and 3

See Cambridge Structural Database # 858019 & 858020.

The crystal structure of compound **1** was solved at room temperature (296 K) in the triclinic crystal system in the P1 space group with a = 8.8278(4) Å, b = 8.9741(3) Å, c = 14.7343(6) Å with β= 96.090(2)°. The structure refined to an R value of 4.5 %, with Goodness-of-fit = 1.071 with Z=2.

The crystal structure of compound **3** was solved at room temperature (273 K) in the monoclinic crystal system in the $P2_1$ space group with a = 10.4592(4) Å, b = 9.2805(4) Å, c = 11.6488(5) Å with β= 99.591(2)°.

The structure refined to an R value of 4 %, with Goodness-of-fit = 1.056 with Z=2.

In Vivo Studies

Pharmacokintic Studies in Female Rat: The in-life and bioanalytical portions of these studies were conducted at BioDuro, Pharmaceutical Product Development Inc. (Beijing, China). Female Sprague-Dawley rats (obtained from PUMC, China, 200–250g) underwent jugular vein cannulation surgery at BioDuro. Rats received either a 1 mg/kg intravenous (iv) dose or a 5 mg/kg oral (po) dose of compound **3**. The doses were prepared in 40% (w/v) hydroxypropyl-beta-cyclodextrin and delivered in a volume of 2 mL/kg (iv) or 10 mL/kg (po). Animals were sacrificed in a CO_2 chamber. Blood samples were collected in K_2EDTA treated tubes at designated times between 2 min and 24 hours via jugular vein cannula. Plasma was isolated after centrifugation. The plasma samples were stored at –80°C prior to analysis.

Single Acute Oral Dosing Time Course in Mice: Male 129/sve wild type mice (20–25 grams) were fasted 12 hours prior to dosing by oral gavage (p. o.) with vehicle or test compound, using a dosing volume of 10 mL/kg in phosal/tween vehicle. The formulation uses a stock vehicle comprised of 98% (v/v) Phosal 50 PG and 2% Tween (Polysorbate 80). The final formulation is comprised of 12% (by volume) of this mixture and the remainder of the volume (88%) is comprised of water. Mice (n = 5 for compound **3** and n = 8 for compound **1**) were sacrificed at 1, 3, 5, 7, 14, 20 and 30 h post dose for compound **3** and at 0.5, 1, 2, 4, 6, 8 and 18 h post dose for compound **1**. All procedures were carried out in compliance with the National Institute of Health Guide for the Care and use of Laboratory Animals (1985) under approval of an Institutional Animal Care and Use Committee (IACUC). Whole blood samples (0.5–1.0 mL) were collected by cardiac puncture into EDTA containing tubes and plasma separated by centrifugation (1500 × g for 10 min at 4 °C). The generated plasma was distributed into separate tubes for exposure measurements (50 μL) and Aβ analysis (remainder) and frozen at –80 °C until analysis. CSF samples (8–12 μL) were collected by cisterna magna puncture using a sterile 25 gauge needle and collected using a P-20 Eppendorff pipette. CSF samples were distributed into separate tubes for exposure measurements (5 μL) and Aβ analysis (50–100 μL) and frozen on dry ice. Whole brain was removed and bisected into left and right hemispheres (hemi brain), weighed, and frozen on dry ice. Cerebellum was removed, weighed, and frozen on dry ice for subsequent exposure analysis. All samples were stored at –80 °C prior to analysis.

Assay of $A\beta_{40}$ and $A\beta_{42}$ by ELISA

Configuration of the antibodies used in determining the level of $A\beta_{40}$ and $A\beta_{42}$ utilizes a common capture antibody (4G8) in combination with specific C-terminal antibodies for the 40 and 42 cleavage sites. The 4G8 epitope is on the C-terminal side of the α-secretase cleavage site. To distinguish from

other Aβ ELISA (enzyme-linked immunosorbent assay) configurations that may use a more N-terminal Ab antibody, the convention $A\beta_{x\text{-}40}$ and $A\beta_{x\text{-}42}$ specifically identifies the use of 4G8 as capture antibody. Frozen mouse hemi brains were homogenized (10% w/v) in 50 mM Tris buffer (pH 8.0) containing 5 M guanidine HCl, using a Qiagen TissueLyser. Each sample was homogenized with a 5 mm stainless steel bead, four times, at a shaking rate of 24 times/s for 90 s, then incubated at 23 °C for 3 h, and ultracentrifuged at 125,000 × g for 1 h at 4 °C. The resulting supernatant was removed and stored in a 96 well polypropylene deep well plate at –80 °C. The Aβ peptides were further purified through solid phase extraction using Waters Oasis reverse phase HLB 96 well column plates (60 mg). Column eluates in ammonium hydroxide from 800 μL of original brain supernatant were evaporated to complete dryness and stored at –80 °C until assay.

For the $A\beta_{40}$ assay, a 384 well black Nunc Maxisorp plate (VWR, 62409-062) was coated with 15 μL/well (4 μg/mL) proprietary capture antibody (Rinat 1219) in 0.1 M sodium bicarbonate coating buffer, pH 8.2. For the $A\beta_{42}$ assay, 15 μL/well (8 μg/mL) proprietary capture antibody (Rinat 10G3) was used. The plates were sealed and incubated at 4 °C overnight. Plates were washed with phosphate buffered saline containing 0.05% Tween-20 (PBS-T), and blocked with 75 μL of blocking buffer (1% BSA in PBS-T) for 2 h at 23 °C. After washing the plates with PBS-T, the rodent $A\beta_{40}$ (California Peptide, 642-10) or $A\beta_{42}$ (California Peptide, 642-15) standard was serially diluted in blocking buffer and 15 μL was applied to the plate in quadruplicate. Dried brain samples were reconstituted in 120 μL of blocking buffer, which corresponds to a 6.67-fold concentration. Then 15 μL of undiluted brain sample was added to the $A\beta_{42}$ assay plate in triplicate, and 15 μL of a 1:2 diluted brain sample was added to the $A\beta_{42}$ assay plate in triplicate. Plates were incubated with sample for 2 h at 23 °C. The plates were washed with PBS-T and 15 μL of detection antibody (4G8-Biotin, Covance 9240-10),

200 ng/mL in blocking buffer, was added to each well, incubating for 2 h at 23 °C. The plates were then washed with PBS-T, and 15 μL of europium-labeled Streptavidin (Perkin Elmer 1244-360), 50 ng/mL in blocking buffer was added for a 1 h incubation in the dark at 23 °C. The plates were washed with PBS-T, and 15 μL of Perkin Elmer Enhancement solution was added to each well with 20 minute incubation at RT. Plates were read on an Envision plate reader using DELFIA time resolved fluorimetry (Exc340/Em615). Data represent mean +/- s.e.m. (n = 5 for compound **3**, n = 8 for compound **1**).

Generic Liquid Chromatography Tandem Mass Spectrometry (LC-MS/MS) Assay for Exposure Measurements in Plasma, Brain and CSF: Plasma, brain and CSF were collected as described above and frozen at –20 °C until analysis by LC-MS/MS. Standard curves were prepared in respective matrix via serial dilution at a concentration of 0.49–2000 ng/mL (plasma and CSF) or 0.49–2000 ng/g (brain). For plasma, a 50 μL aliquot of sample was precipitated with 200 μL of acetonitrile containing an internal standard. Samples were vortexed for 1 min, then centrifuged at 3000 rpm for 10 min. The supernatant (120 μL) was transferred to a 96-well plate, then equal part water (120 μL) was added. Frozen brain tissue was weighed and an isopropanol:water (60:40) volume equivalent to 4 times the mass was added before homogenization in a bead beater (BioSpec Products Inc., Bartlesville, OK). For brain and CSF, a mixed matrix approach was employed. To generate the brain standard curve, a 50 μL of blank brain homogenate matrix was added to a 50 μL aliquot of each point in the plasma curve. Likewise, 50 μL of blank plasma was added to 50 μL of each brain homogenate sample. For CSF, 5 μL of blank artificial CSF matrix was added to a 5 μL aliquot of each point in the plasma curve to generate the CSF standard curve. Likewise, 5 μL of blank plasma was added to 5 μL of each CSF sample. These brain and CSF samples were then processed as described above for plasma. LC-MS/MS analysis was carried out for **3** using a high-performance liquid chromatography system consisting of tertiary

Shimadzu LC20AD pumps (Shimadzu Scientific Instruments, Columbia, MD) with a CTC PAL autosampler (Leap Technologies, Carrboro, NC) interfaced to an API 4000 LC-MS/MS quadrupole tandem mass spectrometer (AB Sciex Inc., Ontario, Canada). Compound **3** and a structurally similar internal standard were separated on a MacMod Halo C18, 30x3.0 mm, 2.7 μm column by gradient elution using a flow rate of 1.0 mL/min. A 10 μL of sample was injected on the above column. The mobile phase consisted of solvent A (10 mM ammonium formate in 0.1% formic acid) and solvent B (Acetonitrile). The gradient was as follows: solvent B was held at 5% for 0.5 min., linearly ramped from 5% to 90% in 1.5 min., held at 90% for 0.5 min. and then ramped to 5% over 0.5 min. The mass spectrometer was operated using negative electrospray ionization. The ion pairs monitored for **3** were 491/174. All raw data was processed using Analyst Software ver. 1.4.2 (AB Sciex Inc., Ontario, Canada).

Total Exposure and Aβ Values for Figure 3

Post dose time (h)	Treatment	Brain exposure nM	Brain Aβ40 ng/gm	Brain Aβ42 ng/gm	Plasma exposure nM	Plasma Aβ40 ng/ml	CSF exposure nM	CSF Aβ40 ng/ml
1	Veh		4.313	0.988		0.144		1.855
	10 mg/kg	1086.4	2.187	0.866	1699.8	0.079	17.59	0.909
	30 mg/kg	5595.4	1.813	0.811	6480	0.059	56.625	0.676
3	Veh		4.807	1.059		0.176		1.726
	10 mg/kg	255.8	2.836	0.888	434	0.169	3.2	1.591
	30 mg/kg	900.8	2.055	0.811	1197.2	0.095	9.01	0.743
5	Veh		5.184	1.064		0.150		1.546
	10 mg/kg	86.38	2.906	0.801	133.72	0.178	1.79	1.809
	30 mg/kg	493	1.871	0.737	792.2	0.094	6.95	1.110
7	Veh		4.026	0.935		0.146		1.522
	10 mg/kg	65.94	3.013	0.860	111.4	0.210	0.636	1.753
	30 mg/kg	101	2.352	0.704	138.8	0.179	0.9175	2.284
14	Veh		3.905	0.980		0.148		1.286
	10 mg/kg	55.84	3.956	0.943	BLQ	0.148	BLQ	1.424
	30 mg/kg	4	3.816	0.983	4.9	0.159	0.31	1.263
20	Veh		3.747	0.941		0.139		1.737
	10 mg/kg	16.14	4.117	0.914	BLQ	0.145	0.234	1.603
	30 mg/kg	BLQ	3.694	0.983	BLQ	0.159	BLQ	1.654
30	Veh		3.816	0.882		0.149		1.426
	10 mg/kg	BLQ	3.769	0.753	BLQ	0.158	BLQ	1.699
	30 mg/kg	5.28	3.994	0.942	BLQ	0.130	BLQ	1.584

Total Exposure and Aβ Values for Figure 4

Post dose time (h)	Treatment	Brain exposure nM	Brain Aβ40 ng/gm	Brain Aβ42 ng/gm	Plasma exposure nM	Plasma Aβ40 ng/ml	CSF exposure nM	CSF ng/ml Aβ40 ng/ml
0.5	veh		3.032	0.781		0.054		1.573
	30 mg/kg	1935.25	2.236	0.722	1239.75	0.024	10.7775	0.978
1	veh		3.355	0.708		0.050		1.361
	30 mg/kg	2076.25	1.846	0.521	1408.75	0.023	11.525	0.597
2	veh		2.966	0.705		0.056		1.332
	30 mg/kg	1073.75	1.401	0.472	645.5	0.035	5.5825	0.459
4	veh		2.727	0.701		0.056		1.352
	30 mg/kg	355.5	1.276	0.410	254.5	0.052	2.17	0.929
6	veh		2.823	0.629		0.055		1.559
	30 mg/kg	125.5	1.694	0.447	67.1	0.073	BLQ	1.409
8	veh		3.062	0.828		0.051		1.402
	30 mg/kg	91.8	1.806	0.571	57.3	0.091	BLQ	1.447
18	veh		3.313	0.621		0.059		1.480
	30 mg/kg	BLQ	3.009	0.617	3.955	0.088	BLQ	1.419