0022-3565/09/3312-598–608$20.00
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS
Vol. 331, No. 2
152975/3523843
Printed in U.S.A.

JPET 331:598–608, 2009

Begacestat (GSI-953): A Novel, Selective Thiophene Sulfonamide Inhibitor of Amyloid Precursor Protein γ-Secretase for the Treatment of Alzheimer's Disease

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Wyeth Research, Departments of Discovery Neuroscience (R.L.M., H.Z., K.A., T.C., J.Z.X., X.G., M.J., S.A., C.G., M.M.Z., D.R.R., E.W., P.L., S.-C.S., J.S.-R., J.B., P.H.R., M.N.P., J.S.J.) and Chemical Screening Sciences (X.H., A.K., B.H., S.C.M., D.H., M.A.-G., R.M.), Princeton, New Jersey; Exploratory Drug Safety (K.A., M.W.L.), Andover, Massachusetts; Drug Safety and Metabolism (D.W.C.), Chazy, New York; and Drug Safety and Metabolism (A.O.), Early Development and Clinical Pharmacology (G.F., S.R.), and Clinical Translational Medicine (S.B.F., C.B., M.E.B., H.I.W.), Collegeville, Pennsylvania

Received March 6, 2009; accepted August 10, 2009

ABSTRACT

The presenilin containing γ-secretase complex is responsible for the regulated intramembraneous proteolysis of the amyloid precursor protein (APP), the Notch receptor, and a multitude of other substrates. γ-Secretase catalyzes the final step in the generation of $A\beta_{40}$ and $A\beta_{42}$ peptides from APP. Amyloid β-peptides (Aβ peptides) aggregate to form neurotoxic oligomers, senile plaques, and congophilic angiopathy, some of the cardinal pathologies associated with Alzheimer's disease. Although inhibition of this protease acting on APP may result in potentially therapeutic reductions of neurotoxic Aβ peptides, nonselective inhibition of the enzyme may cause severe adverse events as a result of impaired Notch receptor processing. Here, we report the preclinical pharmacological profile of GSI-953 (begacestat), a novel thiophene sulfonamide γ-secretase inhibitor (GSI) that selectively inhibits cleavage of APP over Notch. This GSI inhibits Aβ production with low nanomolar potency in cellular and cell-free assays of γ-secretase function, and displaces a tritiated analog of GSI-953 from enriched γ-secretase enzyme complexes with similar potency. Cellular assays of Notch cleavage reveal that this compound is approximately 16-fold selective for the inhibition of APP cleavage. In the human APP-overexpressing Tg2576 transgenic mouse, treatment with this orally active compound results in a robust reduction in brain, plasma, and cerebral spinal fluid Aβ levels, and a reversal of contextual fear-conditioning deficits that are correlated with Aβ load. In healthy human volunteers, oral administration of a single dose of GSI-953 produces dose-dependent changes in plasma Aβ levels, confirming pharmacodynamic activity of GSI-953 in humans.

This research was supported by Wyeth Research.
Article, publication date, and citation information can be found at http://jpet.aspetjournals.org.
doi:10.1124/jpet.109.152975.

ABBREVIATIONS: αAPPs, APP α-secretase-cleaved soluble fragment; Aβ, β-amyloid peptide; AD, Alzheimer's disease; APP, amyloid precursor protein; BBB, blood-brain barrier; βCTF, APP β-secretase-cleaved carboxy-terminal fragment; PS, presenilin; GS, γ-secretase; GSI, γ-secretase inhibitor; Amgen GSI, 4-fluoro-*N*-[(1*R*,2*R*,4*S*)-1,3,3-trimethylbicyclo[2.2.1]hept-2-yl]benzenesulfonamide; BMS GSI, (2*R*)-2-{[5-chloro-2-(hydroxymethyl)phenyl][(4-chlorophenyl)sulfonyl]amino}propylpropylcarbamate; DAPT, *tert*-butyl (2*S*)-({*N*-[(3,5-difluorophenyl)acetyl]-L-alanyl}-amino)(phenyl) ethanoate; GSI-953, 5-chloro-*N*-[(1*S*)-3,3,3-trifluoro-1-(hydroxymethyl)-2-(trifluoromethyl)propyl]thiophene-2-sulfonamide; GSI-953 analog, 5-chloro-*N*-[(1*S*,2*R*)-4,4,4-trifluoro-1-(hydroxymethyl)-2-methylbutyl]thiophene-2-sulfonamide; LY411575, N^2-[(2*S*)-2-(3,5-difluorophenyl)-2-hydroxyethanoyl]-N^1-[(7*S*)-5-methyl-6-oxo- 6,7-dihydro-5*H*-dibenzo[b,d]azepin-7-yl]-L-alaninamide; LY450139, (2*S*)-2-hydroxy-3-methyl-*N*-((1*S*)-1-methyl-2-{[(1*S*)-3-methyl-2-oxo-2,3,4,5-tetrahydro-1*H*-3-benzazepin-1-yl]amino}-2-oxoethyl)butanamide; L685458, *N*-{(2*R*,4*R*,5*S*)-2-benzyl-5-[(*tert*-butoxycarbonyl)amino]-4-hydroxy-6-phenylhexanoyl}-L-leucyl-L-phenylalaninamide; CFC, contextual fear conditioning; CSF, cerebral spinal fluid; NICD, Notch intracellular domain; SEAP, secreted alkaline phosphatase; ELISA, enzyme-linked immunosorbent assay; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonic acid; MED, minimal efficacious dose; MES, 4-morpholineethanesulfonic acid (systematic); WT, wild type; SP, single-positive; DP, double-positive; PK, pharmacokinetic; GI, gastrointestinal.

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized by amyloid β-peptide (Aβ) deposition and amyloidosis of the brain parenchyma and vasculature, as well as neuronal inclusions of hyperphosphorylated tau. Aβ is derived from the amyloid precursor protein (APP) by the sequential proteolysis of β-secretase and γ-secretase (GS) (Tanzi and Bertram, 2005). Aβ peptides aggregate to form soluble oligomers and insoluble fibrils and plaques. Although soluble oligomers are reported to be neurotoxic (Walsh et al., 2002; Klyubin et al., 2005), deposits of amyloid fibrils elicit reactive gliosis and neuroinflammatory responses, and they may compromise the integrity of the blood-brain barrier. Therefore, the reduction of Aβ levels by secretase inhibition is a widely pursued therapeutic strategy for AD (Tomita and Iwatsubo, 2004; Jacobsen et al., 2005).

GS is a membrane-associated complex containing presenilin (PS), a protease that belongs to a family of enzymes that mediate regulated intramembraneous proteolysis, and that includes rhomboid, signal peptide peptidase, and site 2 protease. PS is unique in this family of proteases in that it requires cofactors for enzymatic activity. Nicastrin, Aph-1, and pen-2 are additional essential components of GS and are required for reconstituted GS activity (Edbauer et al., 2003; Kimberly et al., 2003), whereas CD147 (Zhou et al., 2005) and TMP21 (Chen et al., 2006) are GS-associated regulatory proteins. Along with APP, more than 20 other GS substrates have been reported (Parks and Curtis, 2007) including the Notch receptors (DeStrooper et al., 1999). Although the consequences of inhibiting GS processing of many of these substrates are currently unknown, the inhibition of Notch processing is known to have specific adverse events (Geling et al., 2002; van den Brandt et al., 2004). GS cleavage of the Notch receptor is required for the release of the Notch intracellular domain (NICD) that is translocated to the nucleus where it regulates HES-driven gene transcription. PS knockout yields an embryonic lethal phenotype that closely resembles Notch 1 knockout (Shen et al., 1997). Peptidomimetic GS inhibitors (GSIs), such as DAPT (Dovey et al., 2001) and LY411575 (May et al., 2002), potently inhibit Notch processing and consequently alter Notch signaling, causing embryonic defects in zebrafish and altered thymocyte differentiation, gastroenteric disorders (mucoid enteropathy and goblet cell dysplasia), and skin lesions (Geling et al., 2002; van den Brandt et al., 2004; Wong et al., 2004).

There are several strategies to alter the generation of Aβ by GS while limiting the liabilities associated with inhibition of Notch processing. Cleavage site modulators of GS, such as flurbiprofen, can either affect the ability of a transition state analog to bind in the absence of substrate (Beher et al., 2004), or bind APP and alter the substrate cleavage site, resulting in reduced levels of the reportedly more toxic $A\beta_{42}$ isoform and increased levels of the nontoxic $A\beta_{38}$ isoform. Hence, modulators such as flurbiprofen may cause no net alterations in total levels of Aβ or other secretase products such as the NICD (Kukar et al., 2008). In addition, there are modulators of GS substrate selectivity that are based on the requirement of nucleotide binding to the GS enzyme complex for APP processing, but not for Notch processing. Some protein kinase inhibitors that bind and displace nucleotides from the GS complex can modulate APP binding to the GS enzyme, and thereby decrease Aβ production while leaving Notch processing unaltered (Fraering et al., 2005). Finally, several allosteric inhibitors of GS that have APP-selective properties, but do not alter Notch processing, have been reported (Barten et al., 2005). Strategies to identify safe therapeutic windows for these compounds are being investigated (Hyde et al., 2006; Choi and Norstrom, 2007).

Here, we report the pharmacological properties of a novel thiophene sulfonamide GSI, GSI-953 (Fig. 1A), an APP-selective GSI that is well tolerated in mouse and dog toxicity studies and has been advanced to human clinical trials.

Materials and Methods

APP Stable Cell Line. The characteristics of a CHO cell line stably expressing an APP reporter construct containing the Swedish KM/NL mutation (APP-Rep-751NL) with an amino-terminal deletion were reported previously (Jacobsen et al., 1994). Cells were plated in 96-well plates and allowed to adhere overnight in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% certified fetal bovine serum. For compound testing, compounds were diluted from stock solutions in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO) to yield a final concentration of 0.1% DMSO in media. Cells were treated for 24 h at 37°C.

Cellular Aβ Assay. Aβ levels in conditioned media were measured by sandwich $A\beta_{40}$ and $A\beta_{42}$ end-specific ELISA with use of monoclonal antibody 6E10 (Signet/Covance Labs, Dedham, MA) for capture, and rabbit C-terminal specific antibodies to $A\beta_{40}$ or $A\beta_{42}$ (Biosource International, Camarillo, CA) coupled with an alkaline phosphatase-conjugated anti-rabbit detection antibody and attophos (alkaline phosphatase substrate) for detection. ELISA plates were read using a Cytofluor fluorescence plate reader to determine the concentration of the fluorescent product of the alkaline phosphatase activity. Synthetic $A\beta_{40}$ and $A\beta_{42}$ peptides (AnaSpec, San Jose, CA) were used as standards. Reductions in Aβ levels were measured relative to control cells treated with 0.1% DMSO and expressed as a percentage inhibition. Data from five doses in triplicate were fitted to a four-parameter logistical model by use of LSW software (Life Sciences Workbench, MDL Information Systems, Inc., San Leandro, CA) to determine EC_{50} values. Cells were washed in phosphate-buffered saline and a CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS; Promega, Madison, WI) was used to assess cell viability.

Radiolabeled APP Cellular Assay. Compound activity was examined in radiolabeled APP cell assays. Cells were labeled with [^{35}S]methionine (PerkinElmer Life and Analytical Sciences) in methionine-free Dulbecco's modified Eagle's medium (Invitrogen) at 37°C/5% CO_2. After 3 h, the conditioned media were removed and clarified by centrifugation at 1000*g*. Immunoprecipitation buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.5% IGEPAL CA-630, 0.5% sodium deoxycholate) containing protease inhibitors (2 μg/ml pepstatin A, 50 μg/ml leupeptin, 10 μg/ml aprotinin, 250 μg/ml phenylmethylsulfonyl fluoride) was added, and SDS was added to a final concentration of 0.35%. Cells were lysed in immunoprecipitation buffer for 10 min on ice then centrifuged at 14,000*g*. The supernatant was collected, and SDS was added to a final concentration of 0.35%. Conditioned media and lysates were boiled briefly, cooled on ice, then immunoprecipitated overnight at 4°C with 6E10, an anti-Aβ monoclonal antibody linked to protein A Sepharose (Sigma-Aldrich) using a rabbit anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Samples were fractionated on 16.5% Tris-Tricine gels (Invitrogen), the gels were dried, and 6E10 immunoprecipitated proteins were visualized by autoradiography with a Storm 860 PhosphorImager (GE Healthcare, Little Chalfont, Buckinghamshire, UK).

Cell-Free γ-Secretase Cleavage Assay. The EC_{50} value for the reduction of $A\beta_{40}$ by GSI-953 was determined in a cell-free assay by incubating approximately 30 μg of solubilized GS prepared by use of published methods (Tian et al., 2002) with 2 μM recombinant peptide

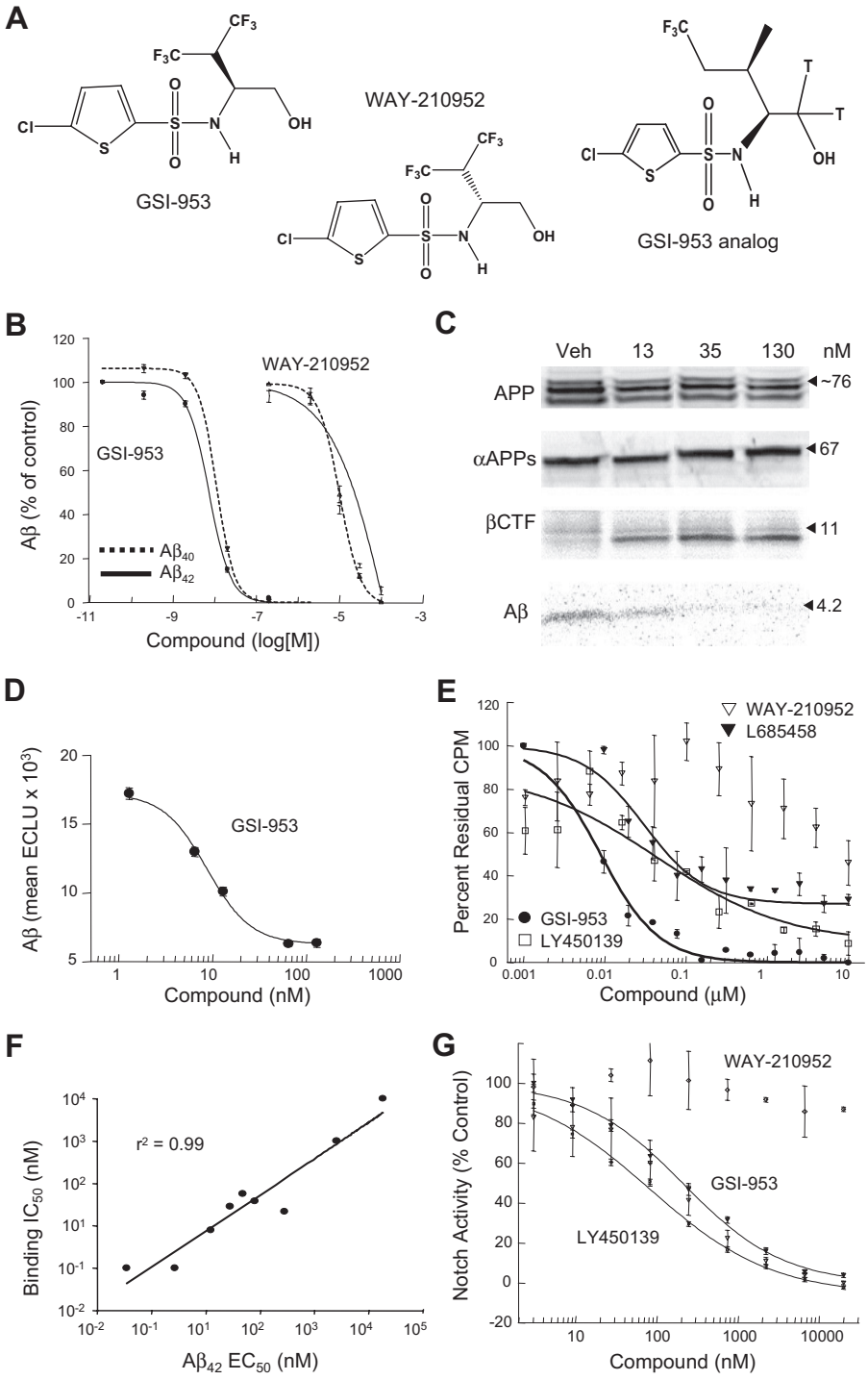

Fig. 1. A, structure of GSI-953, WAY-210952 (inactive enantiomer), and tritiated GSI analog. B, concentration-dependent inhibition of Aβ production by GSI-953 and WAY-210952 in a cellular assay. Levels of secreted $A\beta_{40}$ (dashed line) and $A\beta_{42}$ (solid line) are expressed as a fraction of Aβ levels in samples from vehicle-treated control cells (± S.E.M.). Levels of $A\beta_{40}$ and $A\beta_{42}$ are reduced to a similar extent by treatment with GSI-953. In contrast, WAY-210952, the inactive enantiomer of GSI-953, is >700-fold less potent. The EC_{50} values for Aβ inhibition of these and benchmark GSIs are presented in Table 1. C, effects of GSI-953 on APP processing. Cells overexpressing human APP were pulse-labeled with [^{35}S]methionine in the presence of vehicle or GSI-953 (13, 35, or 130 nM). Both cell lysates and conditioned media were immunoprecipitated with monoclonal antibody 6E10 to examine effects of compound on APP and βCTF (lysates), and secreted αAPPs and Aβ (conditioned media). A dose-dependent increase in βCTF and decrease in Aβ was observed. Molecular masses of the full-length APP reporter construct and associated fragments are presented in kilodaltons on the right. EC_{50} values for βCTF increases and Aβ reduction are presented in Table 2. D, concentration-dependent inhibition of Aβ production by GSI-953 in a cell-free GS assay. A recombinant peptide comprising the APP C-terminal 100 amino acids (APP-C100) was incubated with GS enzyme isolated from human SH-SY5Y cells as described, and levels of $A\beta_{40}$ measured by ELISA. The results are presented as mean electrochemiluminescence unit values (± S.E.M.). Treatment with GSI-953 caused a concentration-dependent reduction in AB_{40} generation resulting in an EC_{50} value of 16.5 nM. E, GSI-953 displaces a tritiated GSI-953 analog from GS. GSI-953 (●) and LY450139 (□), but not WAY-210952 (inactive enantiomer; △), displace [^{3}H]GSI-953 analog from GS prepared from isolated human SH-SY5Y cells in a concentration-dependent manner. L685458 (▲) only partially displaces the ligand. Results are presented as average percent residual CPM (± S.E.M.). IC_{50} values for GSI-953 and reference GSI are presented in Table 3. F, relationship between the binding of GSI to GS and the inhibition of GS activity. Plot of in vitro displacement of GSI (binding IC_{50} values, nM) versus

comprising the C-terminal 100 amino acids of APP (APP-C100) in MES buffer, pH 6.5, in the presence of GSI-953 for 2 h at 37°C. The reaction mix was immunoprecipitated overnight with an antibody to the C terminus of APP (Sigma-Aldrich, St. Louis, MO) bound to protein A Sepharose. The immune complexes were washed and denatured, and then supernatants were collected after filtration through 96-well Millipore filter plates (Millipore Corporation, Billerica, MA). $A\beta_{40}$ levels were determined by use of a 6E10 anti-$A\beta_{40}$ sandwich ELISA.

Radiolabeled γ-Secretase Binding and Displacement Assay. A tritiated analog of GSI-953 ([^{3}H]GSI-analog; Fig. 1A) was prepared as an 11.00 mCi/ml ethanol stock solution with specific activity of 26.6 Ci/mol and radiochemical purity of >99%. GS enzyme was prepared as CHAPSO-solubilized microsomes from human neuroblastoma SH-SY5Y cells. Filter plates were purchased from Millipore Corporation. Microscint-20 was purchased from PerkinElmer Life and Analytical Sciences. Polyethyleneimine solution (50% solution in water) was purchased from Sigma-Aldrich. A Multiscreen 96-well FB filter plate was prepared incubated with 200 μl of 0.6% (w/v) polyethyleneimine solution overnight at 4°C. The filter plate was vacuum filtered and washed twice with 200 μl of water and twice with 200 μl of assay buffer (25 mM MES, pH 6.5, 1 mM EDTA, 0.01% β-mercaptoethanol, 0.01% bovine serum albumin). Reactions were set up in Costar 96-well clear polypropylene plates (Corning Glassworks, Corning, NY) with the following addition sequence: 160 μl of γ-secretase-enriched microsomes diluted 1:10 in assay buffer, 20 μl of test compound serially diluted in 10% DMSO and 90% assay buffer and 20 μl of 250 nM [^{3}H]GSI analog in assay buffer. Each plate contained three test compounds and cold GSI analog (as positive control) in duplicate. The final concentrations in the reactions were 25 nM [^{3}H]GSI analog, 0 to 2 μM or 0 to 10 μM test compound, 1% DMSO, and ~92 pM GS. After a 1-h incubation at room temperature with shaking, the 180-μl reactions were transferred to the filter plate. The filter plate was then vacuum filtered, washed 4 times with 200 μl of wash buffer (5 mM Tris-HCl, pH 7.4) and vacuum dried for 5 min. The filter-plate bottom was removed, and the plate was blotted dry with a paper towel. Microscint-20 (30 μl) was added and ^{3}H radioactivity was counted on a Wallac Model 1450 Microbeta (PerkinElmer Life and Analytical Sciences). Data were analyzed in GraphPad Prism 4.0 with use of the following equation: Counts = $a + (b - a) \cdot IC_{50}{}^n/(Conc^n + IC_{50}{}^n)$, where n is the Hill number and IC_{50} is the compound concentration that results in 50% competition.

Notch Assay. To determine the effect of test compounds on Notch signaling, we generated a constitutively active (ΔE) mouse Notch construct containing the M1726V mutation as described previously (Kopan et al., 1996) and a reporter construct using secreted alkaline phosphatase (SEAP) driven by the HES1 promoter (pSEAP-Basic vector; Clontech, Mountain View, CA). CHO K1 cells were transiently transfected with both constructs by use of Polyfect (Qiagen, Valencia, CA) transfection reagent in Opti-MEM media. Cells were plated in 96-well plates and treated with compounds for 48 h. SEAP levels in the conditioned media were assessed by use of the Great EscAPe SEAP Chemiluminescence detection kit (Clontech) according to manufacturer's instruction. In brief, 15 μl of conditioned media was mixed with a dilution buffer and incubated at 65°C for 45 min. After cooling, assay buffer was added, and the samples were incubated with substrate. Luminescence was measured in a Wallac 1450 Victor luminescence counter.

Measurement of Aβ from in Vivo Samples. Twelve- to 20-week-old Tg2576 mice (n = 7–10/condition) received oral doses of GSI-953 in Phosal PG 50/Tween 80/H_2O (w/w/v, 10:2:88) and were sacrificed at the times indicated. Cerebral spinal fluid (CSF) was collected from the cisterna magna, and plasma was isolated from blood collected by cardiac puncture. Brain tissue was collected and extracted in guanidine. Plasma $A\beta_{40}$ and brain and CSF $A\beta_{40}$ and $A\beta_{42}$ measurements were conducted by sandwich ELISA as published (Jacobsen et al., 2006). All samples were analyzed in duplicate, and the average of results from two to three independent experiments are reported.

In studies in humans, plasma was obtained after a single oral administration of GSI-953 in healthy young subjects. Measurement of the $A\beta_{40}$ concentrations in EDTA plasma samples was performed by use of a validated ELISA as described by the manufacturer (Wako Chemicals, Richmond, VA).

Contextual Fear Conditioning Model. We have previously reported that Tg2576 transgenic animals exhibit an age- and Aβ-dependent deficit in contextual fear conditioning (CFC) (Comery et al., 2005). After acute dosing by oral gavage with 0, 2.5, 5, or 10 mg/kg GSI-953 or 30 mg/kg its inactive enantiomer WAY-210952 (Fig. 1A), 20-week-old Tg2576 mice (n = 11/genotype/treatment) were trained and tested on two consecutive days as described previously (Comery et al., 2005). Contextual freezing was analyzed by use of a two-way analysis of variance and post hoc pairwise comparison made by use of SAS Statistical Software (SAS Institute, Inc., Cary, NC). All data are presented as mean ± S.E.M.

Pharmacokinetic Parameters. Pharmacokinetic (PK) parameters for GSI-953 were established in transgenic Tg2576 or wild-type male mice after a single oral administration of compound. Corresponding exposures to various dose levels, expressed as C_{max} and $AUC_{0-\alpha}$ (area under the curve), are provided for brain and plasma of Tg2576 mice, and in brain, CSF, and plasma of wild-type mice (see Table 5).

Toxicity Studies in Vivo. GSI-953 was administered orally to Sprague-Dawley rats at dosages of 0, 200, 600, or 2000 mg/kg/day for 10 (5 males/group and 5 females at 600 mg/kg/day) or 28 (10/sex/group) consecutive days. The control groups of male and female rats received the vehicle (2.0% polysorbate 80 and 0.5% methylcellulose in water). Evaluations consisted of mortality, clinical observations, body weight, hematology, clinical chemistry, organ weights, and macroscopic and microscopic examinations. A full tissue list was examined in the toxicity studies, including the lymphoid tissues (gut-associated lymphoid tissue, spleen, mesenteric lymph node, mandibular lymph node, and thymus), and the digestive tract [tongue, esophagus, stomach (squamous and glandular), duodenum, jejunum, ileum, cecum, and colon]. Thymocytes were examined by use of flow cytometry for proportions of single-positive (SP) CD4+ or CD8+, and double-positive (DP) CD4+/CD8+ at the end of the 10-day study. Evaluation of peripheral blood CD4, CD8, and CD45 was conducted at the end of the 28-day study. The plasma pharmacokinetics values of GSI-953 were determined in satellite groups in both studies. In addition, GSI-953 or WAY-210952 was administered orally in gelatin capsules to beagle dogs (2/sex/group) at dosages of 0, 50, 150, or 500 mg/kg/day on consecutive days for up to 7 days, 14 days, 13 weeks, or 52 weeks. Clinical, macroscopic, and microscopic examinations were as for rat.

Results

Optimization of a phenylsulfonamide lead obtained from high-throughput screening from Wyeth and ArQule (Woburn, MA) compound libraries (Kreft et al., 2008) led to the identification of GSI-953 (Mayer et al., 2008; Fig. 1A).

reduction of cellular $A\beta_{42}$ by GSI ($A\beta_{42}$ EC_{50} values, nM) suggests that the binding of GSI to GS is directly linked to the inhibition of $A\beta_{42}$ activity in the cellular assay. See Table 3 for data. G, concentration-dependent inhibition of Notch gene transactivation. NICD generated by GS proteolytic activity on a constitutively active Notch construct was used to transactivate expression of the soluble alkaline phosphatase gene (SEAP) driven by the HES1 promoter. SEAP levels in conditioned media are expressed as the average fraction of vehicle-treated cells (± S.E.M.). The reference GSI LY450139, and to a lesser extent GSI-953, inhibit GS-dependent Notch signaling, whereas WAY-210952 (inactive enantiomer) has no effect on GS-mediated Notch signaling. EC_{50} values for inhibition of Notch function, as well as selectivity ratios for Notch versus Aβ inhibition are presented in Table 4.

Reduction of Aβ Production in a Cell-Based Assay. GSI-953 is a potent inhibitor of both $A\beta_{40}$ and $A\beta_{42}$ production in a cell line that stably expresses human recombinant APP. Average EC_{50} values of 14.8 and 12.4 nM were determined for the lowering of $A\beta_{40}$ and $A\beta_{42}$, respectively (Fig. 1B and Table 1). There was no cell toxicity (assessed by MTS assay) observed at concentrations up to 30 μM (data not shown). Treatment with WAY-210952 (Fig. 1A), the inactive enantiomer of GSI-953, displayed very low potency for Aβ reduction with EC_{50} values of ~10,000 and ~18,000 nM, respectively, for the lowering of $A\beta_{40}$ and $A\beta_{42}$ (Fig. 1B and Table 1). Several benchmark GSIs including DAPT (Dovey et al., 2001), LY411575 (May et al., 2002), LY450139 (May et al., 2004), L685458 (Shearman et al., 2000) and DuPont E (Seiffert et al., 2000) were evaluated to compare potency and are summarized in Table 1.

Alteration of APP Processing Is Consistent with the Inhibition of GS Activity. To demonstrate that lowering of Aβ levels in the cellular model is attributed to the inhibition of GS activity, we performed radiolabeled continuous-pulse cellular assays to assess compound effects on APP processing (Fig. 1C and Table 2). Treatment with GSI-953 demonstrated a dose-dependent reduction in total Aβ levels (EC_{50} value for Aβ lowering was 7.3 nM). Treatment with WAY-210952 (inactive enantiomer) resulted in no reduction of Aβ levels (data not shown). These compounds displayed little or no effect on levels of the full-length APP or the APP-soluble fragment that is secreted after cleavage by α-secretase (αAPPs). It was further observed that treatment with GSI-953, but not WAY-210952 (inactive enantiomer), caused a dose-dependent increase in levels of APP β-secretase-cleaved carboxyl-terminal fragment (βCTF), the substrate that is otherwise cleaved by GS (EC_{50} value for βCTF increase was 6.6 nM). Similar observations are reported with additional reference GSIs in Table 2. These observations suggest that the Aβ reductions in the cellular model are due to alterations in APP processing rather than changes in total protein synthesis, transport, or secretion, and that inhibition of GS activity results in both the accumulation of βCTF substrate and the reduction of Aβ production.

Inhibition of Enriched GS Activity in a Cell-Free System. To support binding and inhibitor displacement studies, we confirmed that GSI-953 binds GS in vitro by demonstrating the inhibition of GS proteolytic activity in microsome preparations. The inhibitory effects of GSI-953 in this cell-free system were demonstrated by the dose-dependent reduction of $A\beta_{40}$ generated by the cleavage of APP-C100, a βCTF-like recombinant protein (EC_{50} value for the reduction of $A\beta_{40}$ was 16.5 nM; Fig. 1D). This observation supports the conclusion that GSI-953 is an inhibitor of GS activity in both cell-free and cellular models, and confirms that GSI-953 readily permeates cell membranes.

TABLE 1
Comparison of EC_{50} values for the lowering of $A\beta_{40}$ and $A\beta_{42}$ levels in a cell-based assay

Compound	N^b	EC_{50} Values[a]	
		$A\beta_{40}$	$A\beta_{42}$
		nM	
GSI-953	3	14.8 ± 1.2	12.4 ± 0.8
WAY-210952	3	10,670 ± 1201	18,201 ± 1348
GSI-953 analog	6	100.3 ± 6.1	104.1 ± 16.4
DAPT	12	41.7 ± 9.5	27.7 ± 4.8
LY411575	6	0.32 ± 0.0	0.36 ± 0.0
LY450139	3	110.8 ± 4.5	79.4 ± 7.1
L685458	12	281.2 ± 34.2	303.1 ± 45.0
DuPont E	12	2.4 ± 0.1	2.2 ± 0.2

[a] EC_{50} values are stated as the mean ± S.E.M.
[b] N, number of individual determinations performed in triplicate.

TABLE 2
Aβ lowering and βCTF elevation in cell-based radiolabeled assay

Compound	N^b	EC_{50} Values[a]	
		Aβ	βCTF
		nM	
GSI-953	4	7.3 ± 1.0	6.6 ± 0.2
WAY-210952	1	>2500	>2500
GSI-953 analog	6	30.1 ± 4.2	12.7 ± 4.0
DAPT	5	21.7 ± 3.8	23.3 ± 3.9
LY411575	2	3.6 ± 0.2	6.0 ± 3.5
L685458	2	1622 ± 588.7	1027.0 ± 89.7
DuPont E	3	1.5 ± 0.1	2.5 ± 1.6

[a] EC_{50} values are stated as the mean ± S.E.M.
[b] *N*, number of individual determinations performed in triplicate.

In Vitro Binding and Displacement Studies. To determine whether GSI-953 binds to sites on GS, we performed displacement studies to compare the binding affinity of GSI-953 with reference compounds described previously (Fig. 1E and Table 3). The direct interaction of GSI-953 on GS was confirmed by displacement assays by use of a tritiated structural analog of GSI-953. GSI-953 displaced the [^{3}H]GSI analog (Fig. 1A) with an IC_{50} value of 8 nM (Fig. 1E). In contrast, WAY-210952 (inactive enantiomer) did not displace the [^{3}H]GSI analog (IC_{50} value, >10,000 nM). A total of 30 GSI-953 analogs and reference GSIs including DAPT, LY411575, LY450139, DuPont E, Amgen GSI (Rishton et al., 2000), and BMS GSI (Smith et al., 2000) were tested in the displacement assay. Unlike GSI-953, the transition state inhibitor L685458 only partially displaced the [^{3}H]GSI analog (Fig. 1E), suggesting that GSI-953 interacts with GS at a site removed from the catalytic domain (Tian et al., 2002). When IC_{50} values from displacement studies of these GSIs were plotted against the EC_{50} values for $A\beta_{42}$ reduction in the Aβ cellular assay, a linear relationship was demonstrated ($r^2 = 0.99$; Fig. 1F), suggesting that the binding of GSI to GS is directly linked to the inhibition of $A\beta_{42}$ activity in the cell-based assay.

Notch Selectivity Studies in Vitro. To demonstrate the selectivity of GSI-953 for inhibiting GS activity and the cleavage of different substrates, we compared the inhibition of APP processing (reduction of Aβ) to that of Notch processing (reduction of NICD). Consistent with its function as a GSI, high concentrations of GSI-953 were needed to inhibit Notch processing in a dose-dependent manner with use of a cellular assay that detects a chemiluminescent signal generated by

TABLE 3
Radioligand competition binding and $A\beta_{42}$ lowering for GSIs

GSI	IC_{50} (Competition Binding)	EC_{50} ($A\beta_{42}$)
	nM	*nM*
GSI-953	8	12.4
WAY-210952	≥ 10,000 (inactive)	>18,000 (inactive)
GSI-953 analog	15	101
DAPT	29	28
LY411575	~1	0.36
LY450139	38	79
L685458	22	281
DuPont E	~1	2.7
Amgen GSI	986	2634
BMS GSI	57	47

the cleavage of Notch substrate and the translocation of NICD into the nucleus (Fig. 1G and Table 4). However, GSI-953 was significantly more selective against Notch signaling in this assay than the reference GSI, DAPT, with EC_{50} values of 208.5 and 14.9 nM, respectively (Table 4). As a measure of selectivity, a ratio was calculated comparing the EC_{50} value for Notch inhibition with the EC_{50} value for reduction of $A\beta_{42}$. With use of this analysis GSI-953 is 16.8-fold selective (e.g., 208.5/12.4; Table 4) and preferentially inhibits APP processing by GS. In contrast, DAPT is 0.5-fold selective (e.g., 14.9/27.7) and inhibits Notch processing by GS. Both LY411575 and LY450139 are potent GSIs for $A\beta_{42}$ reduction, but lack Notch selectivity with ratios of 0.9 and 0.8, respectively (Table 4). These selectivity data suggest that GSI-953 may provide little or no alteration of Notch processing by the inhibition of GS activity in comparison with nonselective reference GSIs evaluated.

GSI-953 Reduces Aβ Levels in the Brains of Transgenic APP Mice. In vivo efficacy of GSI-953 was first demonstrated in the transgenic APP (Tg2576) mouse model with the lowering of $A\beta_{40}$ and $A\beta_{42}$ levels in brain and CSF, and $A\beta_{40}$ levels in plasma (Fig. 2A). GSI-953 was orally administered at a single high dose (100 mg/kg, $n = 20$ per time point), and Aβ levels were measured in the brain, CSF, and plasma at 0, 2, 4, and 6 h after dosing. $A\beta_{40}$ was reduced ~88% in both CSF ($p < 0.001$; Fig. 2A, middle) and plasma ($p < 0.001$; Fig. 2A, bottom) by 2 h ($p < 0.001$), whereas brain $A\beta_{40}$ levels (Fig. 2A, top) reached a maximum reduction of ~60% at 6 h ($p < 0.001$).

Subsequently, a 24-h time course experiment was conducted after a single oral dose (30 mg/kg) to determine the optimal time of compound exposure for Aβ lowering (Fig. 2B). After compound administration, Aβ was reduced from 0.25 to 10 h ($p < 0.001$) in brain with maximal Aβ reduction of approximately 67% ($A\beta_{40}$) and 52% ($A\beta_{42}$) between 4 and 6 h. Efficacy and compound exposure (data not shown) were tightly correlated, and a 4-h terminal time point was used for subsequent studies. The finding that brain Aβ levels were significantly lowered within 15 min after the administration of GSI-953 demonstrated that the compound was rapidly absorbed, able to cross the blood-brain barrier (BBB), and inhibit de novo synthesis of Aβ.

A dose-response study to measure Aβ levels 4 h after a single oral administration of GSI-953 was performed (Fig. 2C). The minimal efficacious dose (MED) was determined to be 1 mg/kg with statistically significant lowering of $A\beta_{40}$ ($p < 0.01$); a significant lowering of both $A\beta_{40}$ and $A\beta_{42}$ was observed at 2.5 mg/kg ($p < 0.05$).

GSI-953 Reverses Contextual Fear Conditioning Deficits in Tg2576 Mice. To examine the ability of GSI-953 to reverse CFC deficits, we used CFC, a test of hippocampal-dependent learning and memory in young (preplaque) Tg2576 mice as described previously (Comery et al., 2005; Jacobsen et al., 2006). Contextual learning involves the association of an aversive stimulus (footshock) with a novel testing environment (context). In this model, memory is expressed as a context-dependent freezing behavior in the absence of the shock 24 h after learning. Vehicle-dosed Tg2576 mice displayed significant deficits compared with vehicle-treated WT control animals ($p < 0.05$; Fig. 3). However, treatment of Tg2576 mice with GSI-953 resulted in a dose-dependent reversal of these deficits when compound is orally administered 3 h before training. Significant deficits were observed after treatment with 2.5 mg/kg GSI-953 ($p < 0.05$), and there was some reversal of this at 5 mg/kg and full reversal at 10 mg/kg compared with vehicle-dosed Tg2576 mice ($p < 0.05$). WAY-210952 (inactive enantiomer of GSI-953) was not active at 30 mg/kg and did not reverse deficits in the CFC test (Fig. 3). Neither compound displayed effects on CFC performance in WT animals. These results demonstrate that GSI-953 is efficacious for improving CFC deficits in the APP transgenic mouse model, consistent with previous observations in Tg2576 mice, showing that administration of DAPT also completely reverses such deficits (Comery et al., 2005).

Pharmacokinetic Parameters and Efficacious Exposure. C_{max} and AUC exposures of GSI-953 concentrations achieved in mice at various dose levels are shown in Table 5. These PK parameters in brain, CSF, and plasma compartments suggest that GSI-953 readily penetrated the BBB and achieved a brain/plasma ratio of approximately 1 or more at all doses evaluated. GSI-953 exposure increased with a dose-proportional manner in all three compartments. There was no accumulation of compound based on a comparison of single or multiple daily dosing at 2.5 mg/kg. The MED, based on the lowering of brain $A\beta_{40}$ levels in Tg2576 mice (see above), was 1 mg/kg and resulted in a plasma C_{max} = 101 ng/ml ($AUC_{0-\alpha}$ = 246 h·ng/ml) and a brain C_{max} = 128 ng/g ($AUC_{0-\alpha}$ = 258 h·ng/g) at 4 h.

Lack of Notch-Related Toxicity in Rats and Dogs after GSI-953 Administration. GSI-953-related effects on thymic lymphocyte populations were evaluated in a 10-day rat study to demonstrate that thymocyte maturation was not inhibited (data not shown). A dosage-related trend of slightly lower percentages of SP CD4+ cells in males at all dosages (SP CD4+ cells = ~11% in controls compared with ~7% to

TABLE 4
Notch selectivity based on the ratio of EC_{50} values for Notch and $A\beta_{42}$ for GSI-953 and other reference GSIs in vitro

GSI	EC_{50} (Notch)[a]	N[b]	EC_{50} ($A\beta_{42}$)[a]	N	Notch/$A\beta_{42}$
	nM		*nM*		
GSI-953	208.5 ± 27.3	5	12.4 ± 0.8	3	16.8
WAY-210952	Inactive	2	18,201 ± 1348	3	N.D.
GSI-953 analog	1556 ± 9	2	104.1 ± 16.4	6	15.0
DAPT	14.9 ± 0.9	3	27.7 ± 4.8	12	0.5
LY411575	0.3 ± 0.1	3	0.36 ± 0.0	6	0.9
LY450139	62.9 ± 19.5	5	79.4 ± 7.1	6	0.8
L685458	372.3 ± 60.0	8	303.1 ± 45.0	12	1.2
DuPont E	3.0 ± 0.4	8	2.2 ± 0.2	12	1.4

[a] EC_{50} values are stated as the mean ± S.E.M.
[b] *N*, number of individual determinations performed in triplicate.

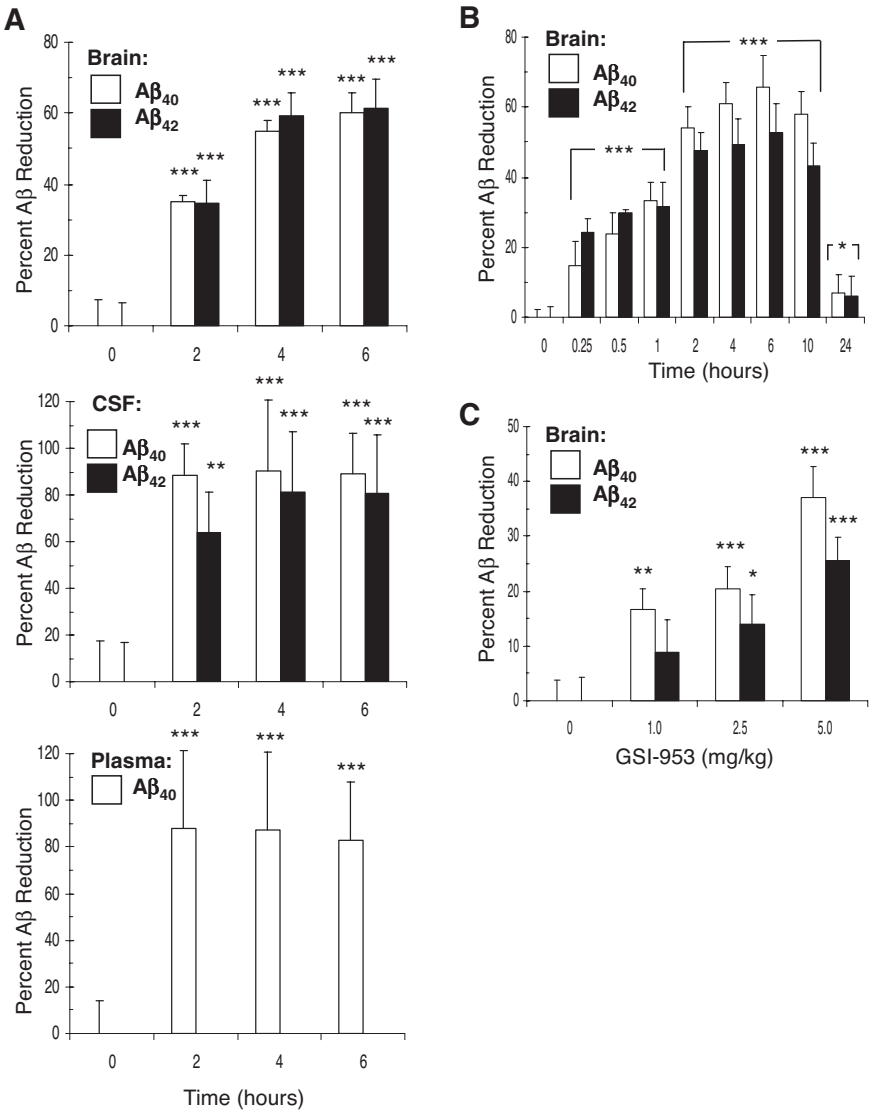

Fig. 2. GSI-953 is efficacious for Aβ reduction in Tg2576 transgenic mice. A, effects of 100 mg/kg oral dosage of GSI-953 on Aβ levels in the brain, plasma, and CSF of Tg2576 Mice. GSI-953- or vehicle-treated animals were sacrificed after dosing at times indicated. Brain (top), CSF (middle), and plasma (bottom) samples were collected and $A\beta_{40}$ (□) and $A\beta_{42}$ (■) levels were measured. Aβ levels are expressed as the percentage of levels measured in tissues from vehicle-treated control animals. Aβ levels in brain were reduced to a maximum ~60%. Aβ levels in CSF and plasma were rapidly reduced to near background levels. All Aβ reductions were highly significant compared with the 0-h control (***, $p < 0.001$), except as noted (**, $p < 0.01$). B, time course to determine the effects of oral dosage (30 mg/kg) of GSI-953 on Aβ levels in the brain of Tg2576 mice. GSI-953- or vehicle-treated animals were sacrificed after dosing at times indicated. Brain levels of $A\beta_{40}$ (□) and $A\beta_{42}$ (■) were significantly reduced at time points between 15 min and 24 h with maximum effects between 4 and 6 h. All $A\beta_{40}$ and $A\beta_{42}$ reductions between 0.25 and 10 h (***, $p < 0.001$) and at 24 h (*, $p < 0.05$) were significant. C, minimal efficacious dose for the reduction of Aβ levels in the brain of Tg2576 mice by GSI-953. Animals received dosages of GSI-953 as indicated and were sacrificed at the previously determined time point of 4 h. Brain levels of $A\beta_{40}$ (□) were significantly reduced at 1 mg/kg (**, $p < 0.01$) and at higher doses (***, $p < 0.001$). Brain levels of $A\beta_{42}$ (■) were significantly reduced at 2.5 mg/kg (*, $p < 0.05$) and at 5 mg/kg (***, $p < 0.001$).

~9% in GSI-953-dosed animals) and females at 2000 mg/kg/day (SP CD4+ cells = ~10% in controls compared with ~8% in GSI-953-dosed animals) was observed. These differences occurred concomitantly with a dosage-related higher proportion of DP CD4+/CD8+ precursor cells in males at all dosages (DP CD4+/CD8+ cells = ~80% in controls compared with ~84 to ~86% in GSI-953-dosed animals). These changes were statistically significant compared with the controls. However, differences in absolute counts of these thymocyte subtypes were not statistically significant between GSI-953-dosed and control groups. The percentage of thymic CD45+ (CD3) B cells were negligible for all groups in the study. The GSI-953-related alterations in relative lymphocyte populations occurred in the absence of changes in absolute cell numbers/g tissue. There were no microscopic correlates for the altered percentage of thymic lymphocyte populations. The toxicological significance of the changes in subtype proportions is not clear, but they are not consistent with Notch inhibition as reported (Hadland et al., 2001) where the inhibition of progression from immature double-negative CD4−/CD8− precursor cells to intermediate DP CD4+/CD8+ precursor cells was observed. In the 28-day rat study, no GSI-953-related effect on peripheral blood lymphocyte subtype counts or on the CD4/CD8 ratio was observed. GSI-953 was tolerated when administered to male and female rats at dosages of up to 2000 mg/kg/day for 10 or 28 days, and there were no dose-limiting effects.

Slight to mild GSI-953-related hypertrophy and hyperplasia of the mucous cells was observed in the 28-day study throughout the small and large intestines, and was more pronounced in the large intestine (data not shown). The mucous cell changes were present at all GSI-953 dosage levels in the cecum and colon, at 600 and 2000 mg/kg/day in the duodenum, and at 2000 mg/kg/day in the jejunum and ileum. Slight to mild colonic glandular dilation was seen at 600 and 2000 mg/kg/day. There were no clinical signs associated with the intestinal changes.

After 10 days, mean body weight gain was lower (21% and 29%, respectively) in groups of males at 600 and 2000 mg/kg/day, compared with controls; statistically significant lower final body weights were observed for these same groups compared with the control group (8% and 10%, respectively). Weight loss was not observed. After 28 days of dosing, mean body weights were lower (9%) in the group of males at 2000 mg/kg/day and in groups of females (4 and 8%,

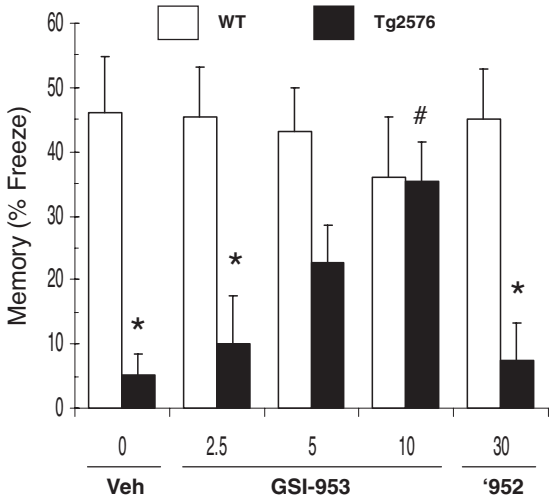

Fig. 3. GSI-953 reverses contextual memory deficits in Tg2576 transgenic mice. Twenty-week-old Tg2576 transgenic (■) or wild-type control (□) animals received oral dosages of GSI-953, WAY-210952 (inactive enantiomer; '952), or vehicle (Veh) 3 h before training. Initial contextual memory deficits observed in vehicle-treated Tg2576 mice compared with WT mice (*, $p < 0.05$) were reversed in a dose-dependent manner after treatment with GSI-953. Memory deficits are significant in Tg2576 mice treated with 2.5 mg/kg GSI-953 (*, $p < 0.05$), but are significantly reversed by treatment with 10 mg/kg GSI-953 compared with vehicle-treated Tg2575 animals (#, $p < 0.05$). Memory is not improved after treatment of Tg2576 mice with 30 mg/kg WAY-210952 (inactive enantiomer) and deficits are significant compared with WT-treated animals (*, $p < 0.05$).

respectively) at 600 and 2000 mg/kg/day, compared with control groups.

In the 10-day study, exposure to GSI-953 increased with increasing dosage in a less than dose-proportional manner in male rats. On day 10, the GSI-953 mean (± S.E.) *C*max values were 636 ± 208, 1981 ± 333, and 2100 ± 568 ng/ml in male rats given 200, 600, and 2000 mg/kg/day, respectively. The corresponding mean (±S.E.) AUC_{0-24} values were 4145 ± 453, 6991 ± 977, and 19,233 ± 4398 ng·h/ml, respectively. In female rats given 600 mg/kg/day, the mean (± S.E.), *C*max and AUC_{0-24} values were 5098 ± 2662 ng/ml and 22,102 ± 2608 ng·h/ml, respectively. The C_{max} was significantly higher (approximately three times) in female rats at 600 mg/kg/day (the only dosage evaluated) compared with male rats. All these values were much higher than the plasma C_{max} of 101 ng/ml ($AUC_{0-\alpha}$ = 246 h·ng/ml) after the MED dosage of 1 mg/kg in Tg2576 mice.

In the 28-day study, exposure to GSI-953 increased with increasing dosage in an approximately dose-proportional manner in male and female rats. On day 28, the mean (± S.E.) C_{max} values were 252 ± 85, 2050 ± 641, and 4646 ± 1178 ng/ml in male rats, and 2497 ± 537, 11,205 ± 6845, and 22,855 (n = 2) ng/ml in female rats. The corresponding mean (± S.E.) AUC_{0-24} values were 1073 ± 164, 5407 ± 551, and 13,074 ± 1268 ng·h/ml in males, and 4418 ± 501, 18,272 ± 4076, and 31,316 ± 10,510 ng·h/ml in females, respectively. Exposure was higher in females compared with males. In conclusion, GSI-953 was tolerated when administered to male and female rats at dosages of up to 2000 mg/kg/day for 10 or 28 days, and there were no dose-limiting effects. A maximum tolerated dose was not achieved and was considered to be >2000 mg/kg/day, the highest dosage tested.

Neither 13-week nor 52-week repeat-dose toxicity studies in dogs revealed microscopic changes in the gastrointestinal (GI) tract (data not shown). Peak plasma exposure of GSI-953 was 13 μM, a plasma exposure which is ~50-fold higher than the plasma C_{max} for an efficacious Aβ-lowering dosage of GSI-953 (1 mg/kg is 258 nM) and ~65-fold higher than the plasma C_{max} for the in vitro EC_{50} for Notch inhibition (208.5 nM). However, in a short-term, 7-day repeat-dose toxicity study in dogs, potential Notch-related effects were observed in the GI tract, including fecal alterations (e.g., soft, liquid, mucoid feces), with slight to moderate crypt abscess and dilated mucosal glands observed microscopically in the duodenum and colon, respectively, which were considered adverse at dosages that are above those that are clinically relevant. Microscopic changes in the GI tract of dog were observed at dosages with peak plasma concentrations at ~23 μM, a plasma exposure that is ~100-fold higher than the plasma C_{max} for both an efficacious Aβ-lowering dosage of GSI-953 and the in vitro EC_{50} for Notch inhibition. In a 7-day dog study comparing GSI-953 and WAY-210952 (an inactive enantiomer of GSI-953 which has >1000-fold *less* activity toward γ-secretase and Notch), similar clinical and microscopic changes described for the 14-day study were observed for GSI-953, but not the inactive enantiomer, indicating that the GI effects may be related to either γ-secretase or Notch inhibition (data not shown).

GSI-953 Demonstrates Target Engagement in Human. After performing dose-ranging and investigational new drug enabling toxicity studies in mice, rats, and dogs, and demonstrating the lack of Notch-related adverse events in vivo, we initiated a first-in-human single ascending dose study with healthy subjects to investigate safety and tolerability of GSI-953 (to be published elsewhere). After single oral doses of GSI-953 (3–600 mg) to healthy young subjects (ages 18–55 years), plasma samples were collected over a 24-h period and assayed for $A\beta_{40}$ levels (Fig. 4 and Table 6). The lowering of plasma $A\beta_{40}$ levels, as measured by the mean

TABLE 5

Mean GSI-953 pharmacokinetic parameters for brain, CSF, and plasma in wild-type male mice after single oral administration

At a dosage of 1 mg/kg, the plasma terminal half-life was of moderate duration ($t_{1/2}$ = 4 h) and the BBB penetration, taken as the brain/plasma ratio, was approximately 1.1 to 1.3, based on C_{max} or $AUC_{0-\alpha}$ data, respectively. The evaluation of PK parameters at higher oral doses (10 and 30 mg/kg) suggests that exposure (AUC) increases with dose, but in a less than dose-proportional manner in plasma, and a greater than dose-proportional manner in brain. There is no accumulation of compound based on a comparison of single or multiple daily dosing at 2.5 mg/kg.

Compartment	Dose	C_{max}	Exposure ($AUC_{0-\alpha}$)[a]
	mg/kg	*ng/g* or *ng/ml*	
Brain	1.0[b]	128	258
	2.5[b,c]	258	1083
	2.5	742	1258
	10.0	1370	9338
	30.0	7509	37,313
CSF	1.0[b]	N.D.	N.D.
	2.5	28.9	130
	10.0	68.2	350
	30.0	283.0	1161
Plasma	1.0[b]	101	246
	2.5[b,c]	236	936
	2.5	552	1352
	10.0	1107	5951
	30.0	4414	19,443

N.D., not determined.
[a] Units are h·ng/g or h·ng/ml.
[b] Tg2576 male mice.
[c] Single daily administration for 5 days.

percentage of change from baseline, was observed in a dose-dependent manner. Transient reductions of plasma $A\beta_{40}$ levels were observed at 1 h for the 10- to 600-mg doses, whereas more sustained reductions were observed at 2 h for the 150- to 600-mg doses, and at 4 h for the 600-mg dose. This initial reduction was followed by a subsequent increase in plasma $A\beta_{40}$ levels (data not shown).

Discussion

GS is widely regarded as a viable target to achieve therapeutically relevant reductions of Aβ in AD, and multiple classes of GSIs have been reported including peptidomimetics and sulfonamides (for review, see Imbimbo, 2008). Here, we report the preclinical pharmacological and safety profile, as well as clinical biomarker data for GSI-953, a novel thiophene sulfonamide GSI that was observed to selectively inhibit the cleavage of APP over Notch. We have shown that GSI-953 is a potent inhibitor of Aβ production in vitro, and in the transgenic APP Tg2576 mouse model, is orally active and displays a robust reduction in brain, plasma, and CSF Aβ levels, and reverses contextual fear conditioning deficits that are correlated with Aβ load. In healthy volunteers, oral administration of a single dose of GSI-953 produced dose-dependent changes in plasma Aβ levels, confirming target engagement of GSI-953 in humans.

GSI-953 was shown to inhibit Aβ production in a cellular assay with low nanomolar potency ($A\beta_{40}$ EC_{50} = 14.8 nM, $A\beta_{42}$ EC_{50} = 12.4 nM) with a corresponding increase in cellular βCTF levels, a property shared by other benchmark GSIs. The inhibition of cleavage of recombinant GS substrate in a cell-free microsome system coupled with the ability to displace a tritiated analog of GSI-953 that was established to bind to GS, and the linear relationship between GS binding and the reduction of Aβ levels, confirms that GSI-953 inhibits its GS activity. The treatment of transgenic APP Tg2576 mice with GSI-953 caused a rapid dose-dependent reduction of $A\beta_{40}$ and $A\beta_{42}$ levels in the brains of these mice that correlated with changes in both CSF and plasma Aβ levels. GSI-953 time course studies revealed significant reductions in brain Aβ levels as early as 15 min after administration of compound, indicating rapid compound absorption, robust inhibition of GS, and turnover of Aβ in the central nervous system. Brain Aβ reductions extended for 24 h, with maximum inhibition observed at 4 to 6 h. We performed additional GSI-953 dose-response studies, collected tissue samples at 4 h, and observed a significant reduction of brain $A\beta_{40}$ levels after a single oral dosage of 1 mg/kg. Similar observations of dose-dependent reductions in brain or CSF Aβ levels have been demonstrated for other GSIs, including LY411575 in Tg2576 mice (Lanz et al., 2004) and rats (Best et al., 2005), and for LY450139 (Lanz et al., 2006) in guinea pig.

TABLE 6
Plasma $A\beta_{40}$ changes after single oral dose administration of GSI-953 to healthy young subjects

GSI-953 Dose	Mean Percentage Change from Baseline (± S.E.M.)		
	1 h	2 h	4 h
mg	%	%	%
3	5.8 (5.4)	13.7 (6.3)	8.5 (4.3)
10	−4.6 (3.2)	17.2 (6.6)	15.3 (5.3)
30	−10.1 (2.5)	36.7 (7.2)	41.0 (6.2)
60	−16.1 (4.5)	8.7 (4.0)	70.1 (4.0)
100	−17.5 (4.0)	0.1 (7.3)	81.4 (4.7)
150	−21.4 (2.6)	−11.4 (2.3)	75.3 (9.4)
200	−20.1 (1.9)	−25.3 (4.0)	34.4 (7.5)
300	−26.1 (4.6)	−33.6 (3.4)	47.1 (13.9)
450	−20.9 (4.4)	−30.8 (3.6)	12.3 (12.2)
600	−30.3 (3.3)	−39.8 (5.7)	−13.7 (11.2)

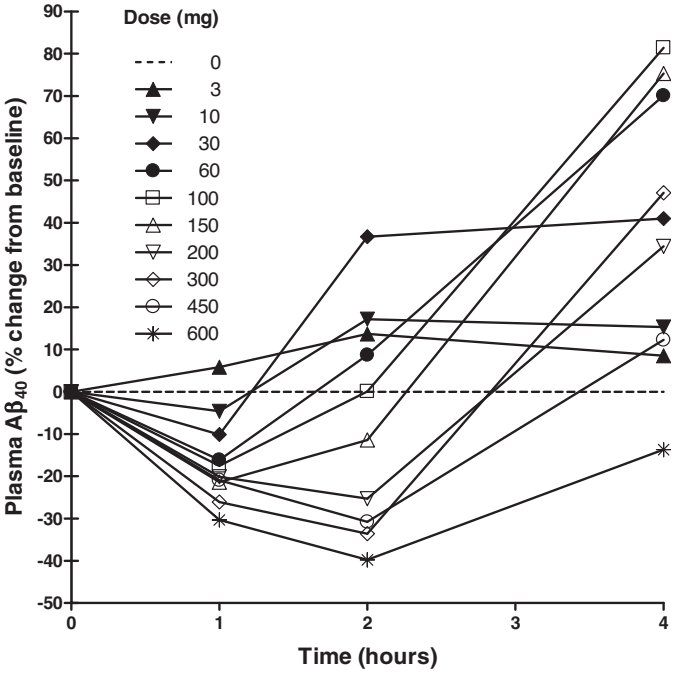

Fig. 4. GSI-953 alters plasma $A\beta_{40}$ levels in human subjects after acute dosing. Human subjects received 3- to 600-mg oral doses of GSI-953 in a single ascending dose study. Plasma samples were collected over a 24-h period and assayed for $A\beta_{40}$ levels (expressed as percentage of change from baseline; data from the 0–4 h period is shown). Transient reductions of $A\beta_{40}$ in plasma at 1 h were observed for 10- to 600-mg doses. Sustained reductions of $A\beta_{40}$ in plasma at 2 h were observed for subjects treated with 150- to 600-mg doses; or reductions at 4 h treated with 600 mg. Plasma $A\beta_{40}$ reductions were followed by reversible increases in plasma $A\beta_{40}$ levels at all doses. Doses (mg) are 0 (vehicle, no symbol), 3 (▲), 10 (▼), 30 (♦), 60 (●), 100 (□), 150 (△), 200 (▽), 300 (◇), 450 (○), and 600 (∗).

Because Aβ is believed to have a detrimental effect on cognition (Klyubin et al., 2005; Jacobsen et al., 2006), we predicted that reduction in brain Aβ levels mediated by GSI-953 inhibition of GS would have cognitive enhancing effects. We observed that CFC deficits in the Tg2576 transgenic model were partially reversed 3 h after a single oral dosage of GSI-953 at 5 mg/kg, a dose that lowered parenchymal Aβ levels by 25 to 35%, but were completely reversed 3 h after the administration of GSI-953 at a dosage of 10 mg/kg. It is possible that the pharmacokinetic and pharmacodynamic relationship for demonstrating the lowering of brain Aβ levels may not optimally translate for improvement in CFC deficits. For example, more robust reversal of these deficits at lower doses of GSI-953 may be achieved by maintaining reduced Aβ levels over an extended period of time either before or after training in the CFC model. The inhibition of Aβ fibril formation is not likely to account for the reversal of CFC deficits because these animals are only 5 months old and, hence, of preplaque deposition age. In contrast, WAY-210952, an inactive enantiomer of GSI-953 that did not lower Aβ levels in the cellular assays, also did not improve CFC deficits in vivo. These observations confirm that efficacy associated with GSI-953 can be attributed to the inhibition of GS activity.

A key property of GSI-953 is that cellular assays detecting

TABLE 7

Expected in vivo APP/Notch selectivity window based on peripheral and central C_{max} exposure after a single oral administration of GSI-953 (1 mg/kg)

The ratio of C_{max} to EC_{50} values of either $A\beta_{40}$ or Notch calculated by use of the total GSI-953 (i.e., protein-bound and free) in brain or plasma (taken from Table 5 and converted into nanomolar values), or the 17% fraction of GSI-953 that remains free.

Compartment	GSI-953 (Total)				GSI-953 (17% Unbound)			
	C_{max}	EC_{50}		Ratio	C_{max}	EC_{50}		Ratio
		$A\beta_{40}$	Notch			$A\beta_{40}$	Notch	
	nM					*nM*		
Brain	327	12.4		26.4	55.6	12.4		4.5
Plasma	258		208	1.24	43.9		208	0.21

APP and Notch cleavage demonstrate this compound to be selective for the inhibition of APP cleavage. At high concentrations GSI-953 inhibited processing of a Notch reporter construct (EC_{50} = 208.5 nM), consistent with a mechanism involving the inhibition of GS. However, when EC_{50} values for the Notch reporter were expressed as a ratio to EC_{50} values for Aβ reduction, GSI-953 was found to have greater than a 16-fold selectivity in vitro for the preferential inhibition of APP processing by GS. This selectivity is in sharp contrast to that of DAPT, which displayed a selectivity ratio <1, suggesting greater potency in vitro for the inhibition of Notch processing by GS. Using similar analysis, other benchmark GSIs tested also lacked Notch selectivity in vitro. Likewise, a ratio comparing the maximum plasma drug concentration (C_{max}) with the Notch EC_{50} values determined in vitro was used to estimate Notch selectivity in vivo. For example (assuming that drug is unbound), the plasma C_{max} for an efficacious dosage of LY411575 at 1 mg/kg is 47.2 nM for Aβ lowering (taken from Table 1 in Lanz et al., 2006) and the in vitro EC_{50} for Notch inhibition is 0.3 nM (Table 4). Therefore, at concentrations required to lower Aβ in the brain, plasma drug exposures will be approximately 157 times the Notch EC_{50}. This suggests that LY411575 may likely modulate peripheral Notch processing at these concentrations. In contrast, the plasma C_{max} for an efficacious Aβ-lowering dosage of GSI-953 at 1 mg/kg is 258 nM and the in vitro EC_{50} for Notch inhibition is 208.5 nM. It is therefore unlikely that GSI-953 drug exposure in the plasma will reach sufficiently high levels to completely inhibit Notch processing (Table 7). Furthermore, in vitro protein-binding studies simulating plasma or brain environments (data not shown) suggest that only 17% of GSI-953 is free and not protein-bound (i.e., 83% is protein-bound). If the plasma C_{max} is corrected for the amount of free GSI-953, the potential for inhibiting peripheral Notch processing becomes less likely as the corrected plasma drug exposure is only 0.2 times the Notch EC_{50} value (Table 7). This is important because partial inhibition of Notch is unlikely to affect the physiological influences of NICD (Barten et al., 2005). This is further supported by the lack of Notch-related changes in thymic lymphocyte subpopulations or peripheral blood lymphocyte populations in rats in studies of up to 28 days in duration and achieving plasma concentrations up to 11.8 μM, or Notch-related gastrointestinal changes in dogs in studies of up to 13 weeks in duration at plasma concentrations up to ~7.4 μM. These peak plasma concentrations are approximately 56- and 36-fold the Notch EC_{50} value, respectively, and are total concentrations of GSI-953 without correction for plasma protein binding. However, in a 7-day study in dogs, Notch-related adverse crypt abscess and dilated mucosal glands were observed microscopically in the duodenum and colon, respectively, and occurred at plasma concentrations of GSI-953 higher than 23 μM (100-fold the Notch EC_{50}). These Notch-related adverse microscopic events were not observed with use of the inactive enantiomer, WAY-210952, as a comparator at similar exposures.

It was recently reported (Kukar et al., 2008) that GS modulators such as flurbiprofen actually bind to APP substrate, rather than GS as originally thought, and thereby reduce the generation of $A\beta_{42}$ *only* while not altering Notch processing. Furthermore, the recent report announcing the failure of a phase 3 clinical trial evaluating Flurizan (*R*-flurbiprofen), Myriad Genetics, Inc. (Salt Lake City, UT) (2004), raises doubt as to the mechanism of action and therapeutic utility for a γ-secretase modulator. Thus, a GSI that selectively inhibits APP processing and reduces brain levels of both $A\beta_{42}$ and $A\beta_{40}$ without inhibiting Notch may potentially achieve the therapeutic objectives of a γ-secretase modulator, but with superior safety. As far as we are aware, BMS-708163 (Albright et al., 2008) and GSI-953 are the only GSIs currently undergoing clinical evaluation that selectively inhibit APP processing.[1] This selectivity may offer an advantage over other compounds currently in clinical development for AD that display little or no Notch selectivity in vitro.

During first-in-human single ascending dose studies in healthy young subjects, we assessed the pharmacodynamic effects of GSI-953 and observed dose-dependent changes of plasma $A\beta_{40}$ levels after single oral doses of compound. The initial decrease in plasma $A\beta_{40}$ levels at early time points after all doses and subsequent increases in plasma $A\beta_{40}$ levels at later time points are similar to what has been described in preclinical (Burton et al., 2008) and clinical (Siemers et al., 2005, 2007) studies with other GSIs. This effect on a pharmacodynamic biomarker has provided preliminary evidence for target engagement in humans, but additional studies of GSI-953 PK and pharmacodynamic parameters in human CSF are required to understand target engagement in the central nervous system. The plasma drug exposures obtained in the first-in-human study reached and exceeded the plasma exposure in rodents where brain Aβ reduction was observed. Taken together with the dose-dependent plasma Aβ lowering observed at these doses, this initial PK/pharmacodynamic results provides rationale for advancing such dose levels to further clinical testing. Although the amyloid hypothesis is an important driver of therapeutic strategies, clinical evaluation of this and other

[1] Subsequent to the submission of this manuscript, we synthesized and tested BMS-708163 in-house as a comparator to GSI-953. BMS-708163 displayed an EC_{50} of 4.5 nM in the cell-based assay, Notch selectivity of 26-fold, and reduced brain levels of $A\beta_{40}$ and $A\beta_{42}$ by 43 and 33%, respectively, in Tg2576 mice following a single oral administration (10 mg/kg) of compound.

GSIs in longer studies in AD patients will provide critical testing of this hypothesis.

In summary, the preclinical data for GSI-953 demonstrating potent Aβ lowering and in vitro selectivity against Notch processing, robust in vivo efficacy for the lowering of brain, CSF, and plasma Aβ levels, reversal of Aβ-dependent cognitive deficits in Tg2576 mice, and the lowering of plasma Aβ levels in humans, provides evidence supporting that GSI-953 (begacestat) treatment has the potential for disease modification in the development of AD.

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