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Title Page

Pharmacodynamics of Selective Inhibition of γ-Secretase by Avagacestat

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Running Title Page

Pharmacodynamics of γ-Secretase inhibition by avagacestat

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Abbreviations: ABEC, area between baseline and effect curve; A-β, amyloid beta-peptide; AD, Alzheimer's disease; AEs, adverse events; APP, amyloid precursor protein; AUC, area under the concentration-time curve; BACE, beta-site APP-cleaving enzyme; CSF, cerebrospinal fluid; GI, gastrointestinal; GSIs, gamma-secretase inhibitors; HES-1, hairy and enhancer of split homolog-1; IACUC, Institutional Animal Care and Use Committee; IRBs, Institutional Review Boards; LC/MS/MS, liquid chromatography/tandem mass spectrometry; LY-450139, semagacestat; NICD, Notch intracellular domain; PS, Presenilin.

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ABSTRACT

A hallmark of Alzheimer's disease (AD) pathology is the accumulation of brain amyloid β-peptide (Aβ), generated by γ-secretase-mediated cleavage of the amyloid precursor protein (APP). Therefore, γ-secretase inhibitors (GSIs) may lower brain Aβ and offer a potential new approach to treat AD. As γ-secretase also cleaves Notch proteins, GSIs can have undesirable effects due to interference with Notch signaling. Avagacestat (BMS-708163) is a GSI developed for selective inhibition of APP over Notch cleavage. Avagacestat inhibition of APP and Notch cleavage was evaluated in cell culture by measuring levels of Aβ and human Notch proteins. In rats, dogs, and humans, selectivity was evaluated by measuring plasma blood concentrations in relation to effects on cerebrospinal fluid (CSF) Aβ levels and Notch-related toxicities. Measurements of Notch-related toxicity included goblet cell metaplasia in the gut, marginal-zone depletion in the spleen, reductions in B cells, and changes in expression of the Notch-regulated hairy and enhancer of split homolog-1 from blood cells. In rats and dogs, acute administration of avagacestat robustly reduced CSF Aβ40 and Aβ42 levels similarly. Chronic administration in rats and dogs, and 28-day, single- and multiple-ascending–dose administration in healthy human subjects caused similar exposure-dependent reductions in CSF Aβ40. Consistent with the 137-fold selectivity measured in cell culture, we identified doses of avagacestat that reduce CSF Aβ levels without causing Notch-related toxicities. Our results demonstrate the selectivity of avagacestat for APP over Notch cleavage, supporting further evaluation of avagacestat for AD therapy.

Introduction

Alzheimer's disease (AD) is a progressive, neurodegenerative disorder characterized by memory loss and deficits in executive function—ultimately culminating in an impaired ability to perform activities of daily living, dementia, and death (Selkoe 2007; Desai et al., 2004). Although the etiology of AD remains unknown, the amyloid hypothesis contends that amyloid β-peptide (Aβ) is involved in the initiation and progression of AD symptoms (Hardy et al., 2002). Aβ peptides are formed by the sequential cleavage of amyloid precursor protein (APP) by β-secretase (β-site APP-cleaving enzyme; BACE) and γ-secretase, which catalyzes intramembrane cleavage of a C-terminal fragment generated by BACE (Steiner et al., 2008). Amyloidogenic processing of APP generates Aβ species of 36 to 43 amino acids due to different C-terminal γ-secretase cleavages (Steiner et al., 2008; Querfurth et al., 2010). Aβ40 is the most abundant species (80 to 90%); however, Aβ42 is considered largely responsible for the pathophysiologic events that occur in the AD brain (Walsh et al., 2004; Bergmans et al., 2010). Aβ42 has a propensity for aggregation and is the principal component of brain amyloid plaques that form in AD (Walsh et al., 2004; Bergmans et al., 2010). Thus, inhibiting γ-secretase activity by lowering brain Aβ levels, particularly Aβ42, is a potential AD treatment strategy (Bergmans et al., 2010).

A challenge in developing GSIs for AD therapy is to avoid effects on γ-secretase substrates other than APP (Bergmans et al., 2010; Barten et al., 2006). Various type I transmembrane proteins have been identified as γ-secretase substrates, including the Notch family of receptors (Notch 1-4) (Pollack et al., 2005). Notch proteins mediate cell-cell communication, regulating cell-fate decisions throughout development and in adult tissues (Kopan et al., 2009). γ-secretase cleavage releases the Notch intracellular domain (NICD), which

translocates to the nucleus and up-regulates gene expression (Kopan et al., 2009). As Notch proteins play a fundamental role in a wide range of tissues, GSI interference with Notch signaling can have adverse effects in several adult organ systems.

Subchronic dosing of GSIs can cause adverse effects in the gastrointestinal (GI) tract, thymus, spleen, skin, and hair in animals, likely resulting from reduced Notch signaling (Milano et al., 2004; Wong et al., 2004; Kumano et al., 2008; Prasad et al., 2007). In the GI tract, GSIs can cause decreased enterocyte differentiation, goblet cell metaplasia, and potential injury to the intestinal epithelium (Milano et al., 2004; Wong et al., 2004). These GSI-induced abnormalities in the gut resemble the Notch1-/Notch2-deficient murine phenotype (Riccio et al., 2008). In the thymus, GSIs alter T-cell maturation and cause thymic atrophy (Milano et al., 2004), mirroring loss of Notch1 (Radtke et al., 2004). In the spleen, GSIs alter B-cell maturation and cause atrophy of the marginal zone (Milano et al., 2004; Wong et al., 2004), mimicking loss of Notch2 (Saito et al., 2003). In the skin, GSI treatment of mice can cause epithelial cell hyperplasia reminiscent of the precancerous phase of squamous cell carcinoma (SCC) (Li et al., 2007a) seen when γ-secretase activity is reduced by genetic manipulations (Xia et al., 2001; Zhang et al., 2007). Encouragingly, manipulating components of the γ-secretase complex to achieve partial inactivation suggests that Notch-related toxicities are threshold-driven and not observed when γ-secretase activity is reduced by ≤30% (Li et al., 2007b). Studies with GSIs in mutant APP transgenic mice suggest that separation of pharmacologic activity and toxicity is possible (Hyde et al., 2006; Barten et al., 2005; Best et al., 2007). Furthermore, a recently identified mutation in APP protected against AD, while causing a 40% decrease in Aβ formation (Jonsson et al., 2012).

Using preclinical models, we previously developed and screened a series of carboxamide-substituted sulfonamides as potential GSIs (Gillman et al., 2010). The compound designated

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avagacestat (BMS-708163) was selected for further evaluation based on potency, selectivity for effects on APP relative to Notch, oral bioavailability, and brain penetrance. In cultured H4-8Sw cells, avagacestat potently inhibited the formation of Aβ40 and Aβ42 with IC_{50} values of 0.30 and 0.27 nM, respectively (Gillman et al., 2010). Avagacestat exhibited a much weaker potency for inhibiting mouse Notch1 processing, with an IC_{50} of 58 nM. In vivo evaluation of avagacestat in dogs showed a plasma half-life that supports daily oral dosing, good brain penetration, and a correlation between reductions of Aβ40 levels in the brain and cerebrospinal fluid (CSF) (Gillman et al., 2010). Here, we evaluated the pharmacokinetic and pharmacodynamic properties of avagacestat, providing evidence for its selective effects on brain Aβ levels relative to effects on Notch signaling in rats, dogs, and healthy human subjects.

Methods

Avagacestat was supplied by Bristol-Myers Squibb Research and Development, and was prepared following the general procedures of Gillman et al., 2010.

Cellular Assay for Notch Signaling. Inhibition of Aβ40 and Aβ42 formation and mouse Notch1 signaling in cultured cells were performed as previously described (Gillman et al., 2010). Human Notch constructs (Notch 1-4) were generated by PCR and verified by sequencing as described for mouse Notch1 (Gillman et al., 2010). In contrast to mNotch1 ΔE, all 4 human Notch constructs contained intact cytoplasmic domains. Human Notch signaling assays were conducted as described for mouse Notch1 (Gillman et al., 2010).

Avagacestat Dosing Studies in Rats and Dogs. All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of Bristol-Myers Squibb, Inc. Details of acute and chronic dosing studies in rats and dogs, and the collection of tissues for analyses, are detailed in Supplemental Materials and Methods. Paraffin sections of gut and spleen were stained using the periodic acid Schiff's base and hematoxylin and eosin methods, respectively. Histopathology was evaluated by light microscopy.

Human Subjects. For human studies, all participants gave informed consent to participate. The local Institutional Review Boards (IRBs) approved the studies, which were conducted in accordance with the Helsinki Declaration. All subjects received compensation for participation. Details of the human subjects who participated in single- and multiple-ascending–dose studies, avagacestat dosing of these subjects, and CSF sampling are detailed in Supplemental Materials and Methods.

Measurement of CSF and Brain Aβ Levels. Collection of CSF, processing of brain tissue, and the measurement of Aβ levels are described in Supplemental Materials and Methods.

Plasma Avagacestat Analyses. The analyses of plasma samples for avagacestat were conducted by liquid chromatography/tandem mass spectrometry (LC/MS/MS) as described previously (Gu et al., 2010).

Measurement of Notch-regulated Hairy and Enhancer of Split Homolog-1(HES-1) Levels. HES-1 mRNA levels in blood were measured by qRT-PCR by standard techniques using an Applied Biosystems (ABI) 7700 system (Life Technologies, Carlsbad, CA). Blood from rats or dogs was collected in Paxgene® Blood RNA Tubes (Becton Dickinson, Franklin Lakes, NJ) and total RNA was isolated using Paxgene® blood RNA kit (Qiagen, Valencia, CA). GAPDH mRNA levels were used as a normalization control.

Results

Notch/APP Selectivity of Avagacestat in Cell Culture. As in our previous studies (Gillman et al., 2010), we used an assay for Notch signaling in Hela cells to evaluate the effects of avagacestat on the processing of each of the 4 human Notch proteins. These assays showed that avagacestat inhibited signaling of hNotch1, hNotch2, hNotch3, and hNotch4 with IC_{50} values of 41, 10, 35, and 38 nM, respectively. Using an IC_{50} value of 0.30 nM for Aβ40 (Gillman et al., 2010), the corresponding Notch/APP IC_{50} ratios were 137, 33, 117, and 127. The IC_{50} ratios demonstrate a much greater effect of avagacestat on APP cleavage relative to hNotch1, hNotch3, and hNotch4 processing in these cellular assays. Although the IC_{50} ratio for hNotch2 was less than for the other human Notch proteins, the results show a high degree of selectivity for APP relative to this isoform as well. These results extend our previous findings and show that avagacestat is a potent and selective inhibitor of APP processing relative to the signaling of human Notch proteins in cellular assays.

A range of Notch/Aβ IC_{50} ratios have been reported for avagacestat, from as low as 2.9 to as high as 193 (Martone et al. 2009; Gillman et al. 2010; Crump et al. 2012; Chavez-Guttierez et al. 2012; Mitani et al. 2012) (Supplemental Table 1). This range is partly due to differences in methodology, for example choice of enzymatic assays or different cell-based formats, and partly due to the increased potency of GSIs when substrate expression levels are higher (Burton et al. 2008). However, Notch/APP IC_{50} ratios also shift in a consistent fashion for other GSIs, such that the rank order of Notch/APP IC50 ratios remains unchanged. For example, avagacestat exhibits Notch/APP IC_{50} ratios that are consistently 15- to 43-fold greater than the corresponding ratios reported for semagacestat across multiple studies, as summarized in Supplemental Table 1 (Martone et al. 2009; Gillman et al. 2010; Chavez-Guttierez et al. 2012; Mitani et al. 2012). This

demonstrates that GSIs exhibit intrinsic and robust differences with respect to Notch/APP selectivity, and that avagacestat is an order of magnitude or more selective than semagacestat.

Effects of avagacestat on central Aβ levels in rats and dogs.

Acute response. To investigate the effect of avagacestat on Aβ levels in vivo, the compound was administered to rats using a single oral dose of 2 or 10 mg/kg. In previous studies in rats and dogs, avagacestat administration produced plasma drug concentrations with half-lives that supported once-daily oral dosing (Gillman et al., 2010). In the present study, rat brain and plasma concentrations of avagacestat were similar, with a 1.1 brain/plasma ratio for mean area under the concentration-time curve (AUC). Brain Aβ40 and Aβ42 levels were significantly reduced at both doses relative to vehicle-treated animals in a dose-dependent manner in female rats. Female rats were used for the studies with Aβ measurements since avagacestat exposures in female rats were about 10-fold higher than in male rats. In particular, brain Aβ40 and Aβ42 levels were reduced up to 40 to 50% at 2 mg/kg and 75 to 85% at 10 mg/kg (Fig. 1A). Avagacestat also caused a dose-dependent decrease in CSF Aβ40 and Aβ42 levels similar to the effects in the brain (Fig. 1B). Aβ levels in both brain and CSF returned to pretreatment control levels by 50 h after dosing with no significant increase above pretreatment control levels (Supplemental Table 2). Brain and CSF Aβ levels were highly correlated (Aβ40, $r^2 = 0.6445$, $P < 0.0001$; Aβ42, $r^2 = 0.7410$, $P < 0.0001$; Fig. 1C).

The relationship between plasma drug concentrations and the time course of Aβ response in both rat and dog brain and CSF was evaluated using an indirect response model (Dayneka et al., 1993; Sharma et al., 1996; see Supplemental Material and Methods). The model recapitulates the observed reductions in Aβ levels reasonably well. The goodness-of-fit was determined by visual inspection, Akaike Information Criterion, Schwartz Criterion, examination of the

residuals, and the coefficient of variation of the parameter estimates. The EC_{50} values relating total plasma drug concentrations and Aβ levels were estimated using this model (Table 1). These results show that the pharmacodynamic effects of avagacestat on Aβ40 and Aβ42 levels were similar in rat brain and CSF. Moreover, EC_{50} values for brain and CSF Aβ40 levels in dogs indicate similar cross-species acute effects of avagacestat on central Aβ40 levels (Table 1). The EC_{50} values from these models (114 ng/ml - 206 ng/ml) were higher than the cellular IC_{50} values (0.14 - 3.5 ng/ml). The difference between the cellular IC_{50} and in vivo EC_{50} values likely results from the effect of substrate levels on absolute potency values and protein binding.

Response to subchronic dosing. In 3 studies, female rats were administered 3, 10, 30, 100, or 300 mg/kg/day for 4 days. Brain Aβ40 ABEC (Area between Baseline and Effect Curve) reductions were estimated to be 49 to 57% at 3 mg/kg/day and greater than 85% at doses of 10 mg/kg/day or higher (Supplemental Table 3). When dogs were administered doses of 0.5, 2, and 10 mg/kg/day for 14 days, the brain Aβ40 values 3-7 hours after the last dose were similar to those in previous experiments (Gillman et al., 2010), with significant Aβ40 reductions at all doses (28 to 80%) (Supplemental Table 4). Similarly, in 3-month and 6-month repeat-dose studies in dogs, significant decreases in brain Aβ40 (20 to 56% reductions) were seen 4-6 hours after that last dose at all doses tested (0.3, 1, 3 mg/kg/day) (Supplemental Table 4).

Dose-Dependent Effects of Avagacestat on Notch-Related Toxicities in Rats and Dogs.

GI tract. At the end of each subchronic dosing study, GI toxicity was assessed by microscopic evaluation of goblet cell metaplasia in GI tissue. In the 4-day study, histological evaluation of the duodenum from rats showed that none of the animals dosed at 3, 10, or 30 mg/kg/day had evidence of goblet cell metaplasia. In contrast, 5 of 7 rats dosed at 100 mg/kg/day

and 3 of 4 rats dosed at 300 mg/kg/day had mild goblet cell metaplasia (Supplemental Table 3). In a 1-month repeat-dose study, male and female rats were given avagacestat at 3, 30, or 100 mg/kg/day. In female rats, no effects were observed at 3 mg/kg, but 5 of 10 and 7 of 10 animals at 30 and 100 mg/kg/day, respectively, had minimal goblet cell metaplasia (Supplemental Table 5). Goblet cell metaplasia was found at lower doses and drug levels after 1 month of dosing compared to 4 days of dosing and will be discussed later. After a 1-month post-dose recovery period, 0 of 5 female rats at each dose had goblet cell metaplasia, indicating a reversal of the effect. Male rats at all doses did not show goblet cell metaplasia, consistent with much lower plasma drug concentrations in male vs female rats.

Subsequently, we evaluated the effects of avagacestat on goblet cell metaplasia in GI tissue in dogs. In dogs treated for 14 days, no GI abnormalities were found in the lower dose groups, while 3 of 5 dogs in the 10 mg/kg/day group had duodenal goblet cell metaplasia (Supplemental Table 4). Dosing over 3 and 6 months showed that 2 of 14 dogs at 3 mg/kg/day, but none of the dogs at 0.3 or 1 mg/kg/day, had goblet cell metaplasia (Supplemental Table 4). After a 2-month post-dose recovery period, 0 of 4 dogs had goblet cell metaplasia, consistent with the reversal effect seen in rats. In both rats and dogs, the goblet cell metaplasia observed was similar to that seen in other studies where Notch activity was reduced or eliminated either by GSIs or by genetic manipulations (Milano et al., 2004; Wong et al., 2004; Riccio et al., 2008). In these studies, goblet cell metaplasia preceded and/or was accompanied by the development of other changes in the GI tract, which are more variable and generally indicative of GI mucosal disruption and abnormal function.

Plasma drug exposure (as measured by AUC) was strongly related to goblet cell metaplasia in dogs and rats with an apparent threshold drug level required to cause GI toxicity

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(Fig. 2A). To determine this threshold level, a discriminant analysis method was used to differentiate between animals affected by toxicity from the ones not affected by toxicity. A discriminant analysis finds optimal cut points between groups that best differentiates the corresponding populations. In the case of the dogs, individual AUC values were used to predict group membership (toxicity affected/not affected). In the case of the rats, composite AUC values obtained from different sets of animals were used to predict group membership. Through the construction of a Mahalanobis distance statistic (Mahalanobis, 1936; Gnanadesikan et al., 1972), the distance of an observation from each group mean, taking into account the spread of the observations in each group was calculated. An optimal cut point was determined by assigning an observation to a group to which it had the smallest (Mahalanobis) distance. Prior to analysis, the AUC data were first converted into the $\log_{10}$ scale. After the analysis was completed, the results were transformed back to the original scale. This was done to meet assumptions of the statistical methods used. Using this analysis, the optimal AUC cut point separating the animals affected by GI toxicity from those unaffected was 31,623 ng•h/ml and 8710 ng•h/ml, in dogs and rats, respectively (Fig. 3A, C); cut point values correspond to the drug exposures which best define the threshold for toxicity. These threshold values were used to calculate therapeutic index values of 24 and 7 for a 25% brain Aβ40 ABEC reduction in rats and dogs, respectively, and values of 32 and 7 for a maximum reduction of 25% in brain Aβ40 levels in rats and dogs, respectively (Table 1). The plasma BMS-708163 AUC values targeting both 25% ABEC reduction and 25% maximum reduction were determined using the indirect response model for each species. The therapeutic index values of 7-32 were within the range of in vitro Notch/APP IC_{50} values (3 - 193).

Spleen. The effect of avagacestat on the spleen was examined in subchronic dosing studies in rats and dogs (Supplemental Tables 4 and 5). In dogs, plasma drug concentrations were strongly related to splenic, marginal-zone lymphoid depletion with an apparent threshold of 5012 ng•h/ml (Fig. 2B and Fig. 3B). The data from the 1-month dosing study in rats were consistent with a similar threshold, although data in the threshold region were too sparse for an accurate estimate. The composite AUC cut point separating affected rats from unaffected rats was ~3376 ng•h/ml (Fig. 3D). These threshold values yielded therapeutic index values of 3 and 4 for a 25% brain Aβ40 ABEC reduction and a maximum reduction of 25% in brain Aβ40 levels in rats and dogs, respectively (Table 1).

For both GI and spleen toxicity, the cut points determined for rats were based on composite AUCs obtained from groups of unequal sizes and are further limited by the small numbers of animals that experienced toxicity. Thus, cut points calculated for the 2 rat data sets may be less reliable than those for dogs.

Immune system. Avagacestat caused a concentration-dependent decrease in circulating B cells in dogs (Fig. 2C). The plasma drug concentrations required to decrease B cells below normal levels, defined as 2 standard deviations from the mean normal level (543 cells/μL, SD=272, range=21–1192, N=20), were 3000–4000 ng•h/ml (similar to the concentration that causes splenic pathology). To further evaluate the effects of avagacestat on Notch signaling, we determined changes in HES-1, a transcription factor whose expression is regulated by NICD (Ang et al., 2007). Avagacestat caused a concentration-dependent decrease in HES-1 mRNA levels in blood from rats and dogs (Fig. 2D). HES-1 reductions were related to the plasma drug concentrations of avagacestat with EC_{50} values of 7000 and 40,000 ng•h/ml in dogs and rats, respectively. Other potential Notch-dependent effects were also monitored in subchronic dosing

studies in rats and dogs. No changes in hair color, skin, or neuronal viability as assessed by histopathology were observed following avagacestat administration.

Effects of Avagacestat on CSF Aβ Levels and Notch Signaling in Healthy Human Subjects. Single- and multiple-ascending–dose studies in healthy human subjects showed that avagacestat has a good tolerability profile, with good pharmacokinetic properties (Tong et al., 2012a; Dockens et al., 2012). In particular, plasma levels of avagacestat exhibited linear pharmacokinetics up to a 200-mg single dose (and 150 mg multiple dose) with a T_{max} of 1 to 2 h, a typical biphasic profile, and a terminal half-life of approximately 40 h (Tong et al., 2012a). Based on these results, doses were chosen to study the effect of avagacestat on CSF Aβ levels.

Acute response. In a continuous CSF study, placebo or a single oral dose of 50 mg or 200 mg avagacestat was administered to human subjects with in-dwelling CSF catheters to allow repeated (i.e., hourly) sampling (Tong et al, 2012b). Several predose CSF samples were obtained and the Aβ values in these samples were used to normalize Aβ measurements for each subject. CSF Aβ values in the placebo group increased steadily during sample collection. The mechanism responsible for this placebo-induced increase is unknown, but similar results have been reported by others using similar CSF collection techniques (Bateman et al., 2009). To obtain a conservative estimate of Aβ reduction, only Aβ values below baseline were considered. Subjects dosed with 200 mg had median CSF Aβ40 concentrations at 63% of baseline at 12 h post-dose. The median time of maximal inhibition was 16 h at 200 mg, and Aβ40 values remained below baseline 24 h after dosing. For the 50-mg dose, the corresponding 12-h CSF Aβ40 levels (% of baseline) varied considerably and were within a range similar to those of the placebo group. Median time of maximal inhibition was 11 h at 50 mg.

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Response to subchronic dosing. In a 28-day, multiple-ascending–dose study, C_{trough} CSF Aβ levels in young healthy men, taken immediately before the last daily dose, were compared with the CSF Aβ levels prior to dosing (Dockens et al., 2012). This analysis showed that avagacestat caused dose-dependent reductions in C_{trough} CSF Aβ levels (Fig. 4A). In particular, the 100 mg and 150 mg doses caused approximately 30 and 60% reductions in CSF Aβ levels, respectively. Importantly, CSF Aβ40 and Aβ42 responses were similar. The mean concentrations of CSF Aβ40 and Aβ42 in the 15-mg and 50-mg dose groups were similar to the baseline levels, consistent with reduced drug levels in these dose groups at the C_{trough} sampling time. These results were consistent with acute response observed in the single-ascending–dose study, where CSF Aβ40 concentrations returned to baseline prior to the end of the dosing period.

We further investigated the relationship between plasma drug concentrations at trough (i.e., 24 h after dosing, day 28) and CSF Aβ40 levels from the same time point in humans. As shown in Fig. 4B, there was a close relationship between plasma drug concentrations and CSF Aβ40. Data collected 24 h after 1 or multiple doses to rats and dogs were overlaid with the human data (Fig. 4C), showing that the pharmacodynamic effects of avagacestat on CSF Aβ levels are similar between rats, dogs, and humans. In contrast to findings in preclinical species, we did not detect significant decreases in the levels of B cells or HES-1 mRNA in humans after 28 days of dosing at exposures of up to 10,000 ng•h/ml (Fig. 5). GI AEs, which will be described in detail elsewhere, occurred at low frequency in young, healthy subjects. Most of the AEs, with the exception of mouth ulcer and flatulence, occurred in 1 subject.

Discussion

Several potent, small-molecule GSIs have been developed (Wu et al., 2009; Olson et al., 2008). Structure-function studies have shown that GSIs selected for in vivo use, i.e., azepines and sulfonamides, bind a common allosteric inhibitory site on PS, rather than the site targeted by GSIs in the isostere class—believed to interact with the active site of the enzyme (Steiner et al., 2008; Olson et al., 2008). Developing an effective and tolerable AD therapy that avoids negatively affecting γ-secretase substrates (other than APP) remains a challenge for GSIs. Evidence suggests that Notch is one of the most important among these off-target substrates because of its critical role in self-renewing cell differentiation in adult tissues (Pollack et al., 2005; Kopan et al., 2009; Milano et al., 2004; Wong et al., 2004; Kumano et al., 2008; Prasad et al., 2007; Riccio et al., 2008; Radtke et al., 2004; Saito et al., 2003; Li et al., 2007a). The binding of azepine and sulfonamide GSIs with an allosteric site on PS, rather than the catalytic site, raises the possibility of differential inhibitory interactions as PS processes different substrates, such as APP and Notch. Consistent with this hypothesis, in vitro studies have shown varying substrate differentiation for GSIs in the allosteric class, but not for GSIs in the isostere class (Olson et al., 2008; Martone et al., 2009; Basi et al., 2010; Lewis et al., 2003; Krefft et al. 2008; Chavez-Guttierez et al. 2012; Mitani et al. 2012). One GSI, LY-411575, was shown to reduce Aβ40 and Aβ42 levels in plasma and the brains of CRND8 transgenic mice, while causing intestinal goblet metaplasia and thymic atrophy (Wong et al., 2004). The most clinically advanced of the GSIs was semagacestat (LY-450139) (Henley et al., 2009). In a phase II study of patients with AD, semagacestat 30 mg/day for 1 week followed by 40 mg/day for 5 weeks significantly reduced *plasma* Aβ levels but not CSF Aβ levels (Siemers et al., 2006). Higher doses were then evaluated in AD patients (100 and 140 mg), but AEs affecting skin and hair occurred without a

significant effect on CSF Aβ levels (Fleisher et al., 2008); the drug was advanced to phase III trials. However, two 21-month phase III trials of semagacestat in patients with mild-to-moderate AD were terminated early (Imbimbo et al., 2011) due to its detrimental effects on clinical measures of cognition and activities of daily living. Semagacestat was also associated with increased rates of skin cancer, presumably related to "mechanism-based toxicities" (Imbimbo et al., 2011). Some investigators have proposed that both the toxicities and effects on cognition were related to interference with Notch signaling (Ross et al., 2010).

The results of the semagacestat trials underscore the importance of APP selectivity for a GSI ultimately used in AD patients. Our previous studies demonstrated that avagacestat is a highly potent inhibitor of Aβ40 and Aβ42 production in cell culture, with IC_{50} values of ~0.3 nM (Gillman et al., 2010). In the present study, we show that avagacestat has IC_{50} values of 41, 10, 33, and 38 nM for signaling inhibition of the human Notch 1-4 proteins, respectively. The corresponding Notch/APP selectivity ratios were 137, 33, 117, and 127. Although other groups have reported lower Notch/APP ratios for avagacestat, relative differences in the ratio between GSI were maintained. For example, avagacestat exhibits Notch/APP IC50 ratios that are consistently 15- to 43-fold greater than the corresponding ratios reported for semagacestat across multiple studies (Supplemental Table 1 [Martone et al. 2009; Gillman et al. 2010; Chavez-Guttierez et al. 2012; Mitani et al. 2012]). Thus, GSIs can exhibit intrinsic and significant differences with respect to Notch/APP selectivity. Furthermore, avagacestat is an order of magnitude or more selective than semagacestat.

Avagacestat selectivity for APP cleavage and its favorable pharmacokinetic properties support its use as an in vivo Aβ-lowering compound. In our previous studies, we showed that IV dosing of avagacestat in dogs resulted in low total body clearance (4.20 ml/min/kg), high volume

of distribution at steady-state (3.87 L/kg), and an oral bioavailability of 42% (Gillman et al., 2010). Furthermore, dosing achieved a high brain-to-plasma concentration ratio (>1), demonstrating good brain penetrance; the calculated half-life of 11.4 h and good oral bioavailability supported daily dosing of the drug (Gillman et al., 2010). Here, we showed that acute oral dosing of avagacestat in rats caused a rapid and robust decrease in brain and CSF Aβ levels, which were highly correlated and dose dependent (Fig. 1, Table 1). Chronic dosing studies with daily oral administration of avagacestat in rats and dogs also showed dose-dependent effects, and plasma concentrations were correlated with reductions in central Aβ levels (Fig. 4C). In rats and dogs, the estimated EC_{50} values relating plasma drug concentration and central Aβ responses ranged from 105 to 206 ng/ml.

Development of GSIs as AD therapies has been limited by deleterious Notch-related effects on the GI tract, spleen, immune system, skin, and cognition (Milano et al., 2004; Wong et al., 2004; Kumano et al., 2008; Prasad et al., 2007; Imbimbo et al., 2011; Ross et al., 2010). However, studies in transgenic mouse models show that modest decreases in Aβ production by GSIs (15–30%) can reverse mutant APP-induced cognitive and synaptic deficits (Martone et al., 2009; Comery et al., 2005). Likewise, a recently identified mutation in APP was shown to have a protective effect against AD and cognitive impairment, while causing a 40% decrease in Aβ formation (Jonsson et al., 2012). By comparison, a 30% reduction of γ-secretase activity in mice ameliorated amyloid burden but had no adverse effects on cognition (Li et al., 2007b). Studies of partially inactivated γ-secretase complexes suggest that in mice, the threshold for GI and spleen toxicity and skin tumors corresponds to a 30–50% reduction in γ-secretase activity (Li et al., 2007b). These findings suggest that Notch-related toxicities may be threshold-driven, and that GSIs with better selectivity for APP over Notch cleavage may be able to lower central Aβ levels

enough to provide cognitive benefits without causing toxicities related to Notch-signaling interference.

Consistent with the murine Notch-toxicity thresholds, our studies in rats and dogs indicated that avagacestat caused threshold-dependent goblet cell metaplasia in GI tissue, marginal-zone lymphoid depletion in the spleen, and reductions in B cells. A reduction in the threshold for goblet cell metaplasia was observed when dosing was extended from 4 days to 1 month in rats, likely resulting from the system not being at steady state after 4 days with respect to both changes in the GI tissue and drug levels. However, thresholds for Notch toxicity in dogs were indistinguishable in the 14 day, 3- and 6-month dosing studies. The differences in the threshold for Notch toxicity between rats and dogs and between GI tissue and spleen are not understood.

Importantly, our studies suggest that Aβ levels can be significantly reduced without measurable toxicity. Chronic dosing studies in dogs identified doses of avagacestat that can achieve a ≤30% reduction in central Aβ levels without putative Notch-related toxicities in the GI tract or spleen (Supplemental Table 4). Avagacestat exhibited a therapeutic margin between Notch-dependent toxicities and Aβ reductions in rats and dogs (Table 1). In rats and dogs, avagacestat has a 24- and 7-fold margin, respectively, between a 25% reduction in brain Aβ ABEC and threshold levels for GI toxicity. We further observed a 3- and 4-fold margin, respectively, between a 25% reduction in brain Aβ ABEC and the threshold levels for spleen and B-cell changes.

Notably, avagacestat may also have a therapeutic margin in humans. Evaluation of the pharmacokinetic-pharmacodynamic properties of avagacestat was extended to single- and multiple-ascending dose studies in healthy human subjects. We showed that administration of

avagacestat to human subjects decreased CSF Aβ levels at C_{trough} in a dose-dependent manner (more than 50% at higher doses). In contrast to the biphasic response of plasma Aβ to avagacestat in humans (Tong et al., 2012a; Dockens et al., 2012) and animals (Gillman et al., 2010), human CSF Aβ had a typical monophasic concentration-response relationship. It is likely that the monophasic response of CSF Aβ and brain Aβ to avagacestat reflects the higher substrate levels in the brain compared to the peripheral tissues that contribute to plasma Aβ given the effect of substrate levels on Aβ response (Burton et al., 2008). The AEs observed in healthy subjects treated with avagacestat included low-frequency GI AEs (Dockens et al., 2012). While no changes in lymphocytes were observed following 28 days of dosing in humans at exposures that caused spleen changes in rats and dogs, it is possible that 28 days were not enough to observe such changes in humans. Unlike Notch-dependent GI toxicity, the effects of Notch-dependent hematopoietic toxicity on immune function are unknown and will need to be carefully monitored if such abnormalities are observed in humans. Subsequent studies in mild-moderate AD patients showed that the 100-mg and 125-mg doses were not well tolerated due to GI and dermatological AEs and trends for worsening of cognition (Coric et al., 2012). In contrast, the 25-mg and 50-mg doses had dose-dependent effects on CSF Aβ species in an exploratory analysis plus acceptable safety and tolerability to support future studies.

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Footnotes

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Figure Legends

Fig. 1. Acute effects of Avagacestat on brain and CSF Aβ in rats

Avagacestat reduced brain Aβ levels at 2 and 10 mg/kg in rats. Rats (n = 5/dose group) were euthanized at the indicated times (1 to 72 h after dosing) and CSF and brain tissue were collected. Brain (A) and CSF (B) Aβ40 and Aβ42 from the same animals were measured using ELISAs and normalized to vehicle-treated controls from the same time point. Values are mean ± SEM. For both Aβ40 and Aβ42, there was no significant difference between either dose group or vehicle-treated controls at 48 and 72 h ($P > 0.05$, See Supplemental Table 2). (C) Correlation between brain and CSF Aβ levels in these animals.

Fig. 2. Relationship between toxicity in animals and plasma drug levels

(A) Goblet cell metaplasia in the duodenum is related to plasma drug levels in both rats and dogs. (B) Marginal-zone lymphoid depletion in the spleen is related to plasma drug levels in both rats and dogs. (C) Circulating B-cell levels in dogs are related to plasma drug levels for dogs treated for 6 months with avagacestat. B cell values from vehicle-treated animals were used to determine the variability in B cells as indicated by the dotted lines, which show values 2 standard deviations above and below the mean from treated animals with normal spleen. Abnormal values from treated animals were identified as those that were more than 2 standard deviations below the mean from treated animals with normal spleen. In (A-C), values represent data from individual dogs, but rat data are based on composite AUCs obtained from groups of unequal sizes. (D) Blood HES-1 mRNA levels are related to plasma drug levels in rats and dogs. Data are from rats that were dosed for 1 month or dogs that were dosed for 3 or 6 months. Values from vehicle-treated animals were used to determine the variability as indicated by the dotted lines,

which show values 2 standard deviations above and below the mean from animals with normal spleen. Values are mean ± SEM for each dose group.

Fig. 3. GI and spleen toxicities with cut points in rats and dogs. (A) Plot of the dog GI data and the fitted separating line. The optimal cut point was estimated to be 8709.66 ng•h/ml. (B) Plot of the dog spleen data and the fitted separating line. The optimal cut point was estimated to be 5011.87 ng•h/ml. In (A) and (B), AUC values greater than the cut off were classified in the group affected by toxicity and lesser values were classified in the group unaffected by toxicity. (C) Plot of the rat GI data and the fitted line separating the toxicity-affected animals from the unaffected animals. The cut point was estimated to be 31622.78 ng•h/ml. (D) Plot of the rat spleen data and the fitted line separating the toxicity-affected animals from the unaffected animals. The cut point was estimated to be 3375.98 ng•h/ml. In (C) and (D), composite AUC values greater than the cut off were classified in the group affected by toxicity and lesser values were classified in the group unaffected by toxicity.

Fig. 4. Effects of avagacestat on CSF Aβ40/42 in humans

(A-B) In a 28-day study, multiple doses of avagacestat reduced CSF Aβ40/42 at C_{trough} (day 28) in healthy human volunteers. This decrease depended on dose (A) and plasma drug concentration (B). Values are mean ± SEM of 5 or 6 subjects. (C) CSF Aβ40 values are related to plasma drug levels at C_{trough} in humans, rats, and dogs in a similar manner.

Fig. 5. Relationship between B-cell levels and HES-1 mRNA levels to avagacestat plasma drug levels after multiple doses in healthy human volunteers. (A) B-cell levels were measured by FACs predose and day 28 postdose. Individual day 28 levels were normalized to predose levels

and then to the average of the placebo group. Variability of the placebo group is indicated by the dotted lines, which show values 2 standard deviations above and below the normalized mean (100). Values represent B-cell data and avagacestat plasma drug levels from individual subjects. (B) HES-1 mRNA levels in whole blood were measured by qRT-PCR by standard techniques; GAPDH mRNA levels were used as a normalization control. Individual day 28 HES-1/GAPDH levels were normalized to predose levels and then to the average of the placebo group. Variability of the placebo group is indicated by the dotted lines, which show values 2 standard deviations above and below the normalized mean (100). Values represent HES-1 data and avagacestat plasma drug levels from individual subjects.

Table 1. Summary of PK, PD, and toxicity cut points in rats and dogs

	Rat	Dog
Aβ40 - brain EC_{50}, ng/ml	114	205
Aβ40 - CSF EC_{50}, ng/ml	164	105
Aβ42 - brain EC_{50}, ng/ml	151	ND
Aβ42 - CSF EC_{50}, ng/ml	206	ND
BMS-708163 AUC (ng•h/ml) for 25% brain Aβ40 ABEC reduction	1336	1191
BMS-708163 AUC (ng•h/ml) for a maximum reduction of 25% in brain Aβ40	1002	1191
GI toxicity cut point, ng•h/ml	31623	8710
Spleen toxicity cut point, ng•h/ml	3376	5012
Ratio Aβ40 25% ABEC/GI toxicity cut point	24	7
Ratio Aβ40 25% ABEC/spleen toxicity cut point	3	4

Ratio Aβ40 25% maximum/GI toxicity cut point	32	7
Ratio Aβ40 25% maximum/spleen toxicity cut point	3	4

ND, not determined; PD, pharmacodynamic; PK, pharmacokinetic.

Figure 1

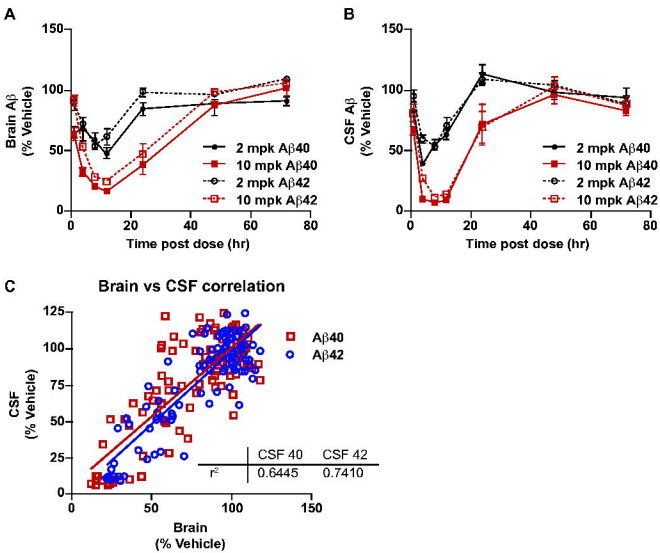

A
Brain Aβ
(% Vehicle)
150
100
50
0
0
20
40
60
80
Time post dose (hr)
2 mpk Aβ40
10 mpk Aβ40
2 mpk Aβ42
10 mpk Aβ42
B
CSF Aβ
(% Vehicle)
150
100
50
0
0
20
40
60
80
Time post dose (hr)
2 mpk Aβ40
10 mpk Aβ40
2 mpk Aβ42
10 mpk Aβ42
C
Brain vs CSF correlation
CSF
(% Vehicle)
125
100
75
50
25
0
0
50
100
150
Brain
(% Vehicle)
Aβ40
Aβ42
CSF 40
CSF 42
r²
0.6445
0.7410

Figure 2

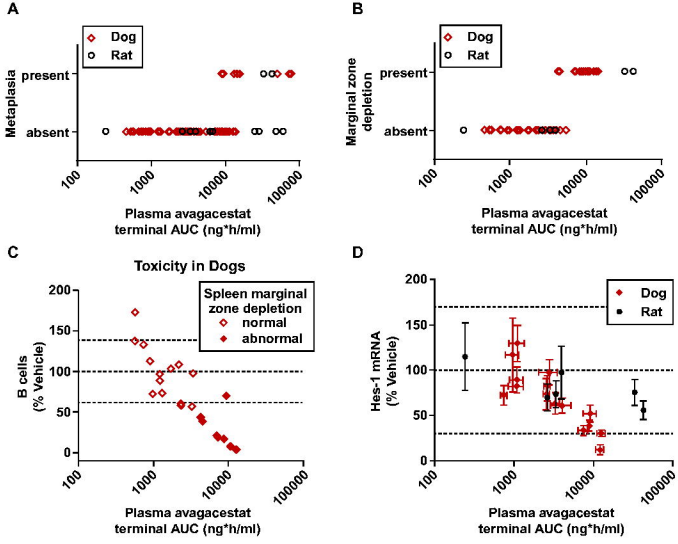

A
Dog
Rat
Metaplasia
present
absent
100
1000
10000
100000
Plasma avagacestat
terminal AUC (ng*h/ml)
B
Dog
Rat
Marginal zone
depletion
present
absent
100
1000
10000
100000
Plasma avagacestat
terminal AUC (ng*h/ml)
C
Toxicity in Dogs
Spleen marginal
zone depletion
normal
abnormal
B cells
(% Vehicle)
200
150
100
50
0
100
1000
10000
100000
Plasma avagacestat
terminal AUC (ng*h/ml)
D
Dog
Rat
Hes-1 mRNA
(% Vehicle)
200
150
100
50
0
100
1000
10000
100000
Plasma avagacestat
terminal AUC (ng*h/ml)

Figure 3

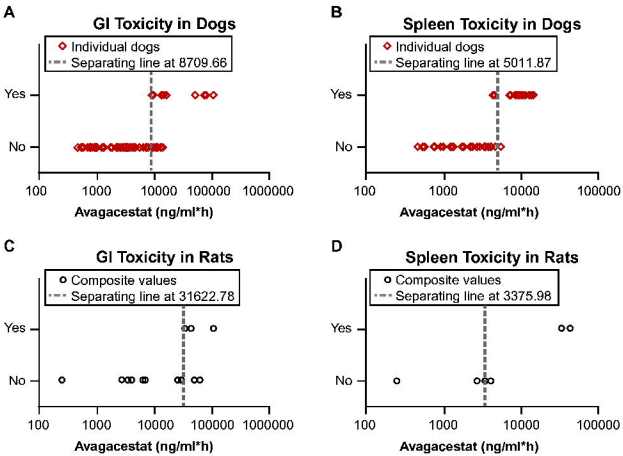

A
GI Toxicity in Dogs
Individual dogs
Separating line at 8709.66
Yes
No
100
1000
10000
100000
1000000
Avagacestat (ng/ml*h)
B
Spleen Toxicity in Dogs
Individual dogs
Separating line at 5011.87
Yes
No
100
1000
10000
100000
Avagacestat (ng/ml*h)
C
GI Toxicity in Rats
Composite values
Separating line at 31622.78
Yes
No
100
1000
10000
100000
1000000
Avagacestat (ng/ml*h)
D
Spleen Toxicity in Rats
Composite values
Separating line at 3375.98
Yes
No
100
1000
10000
100000
Avagacestat (ng/ml*h)

Figure 4

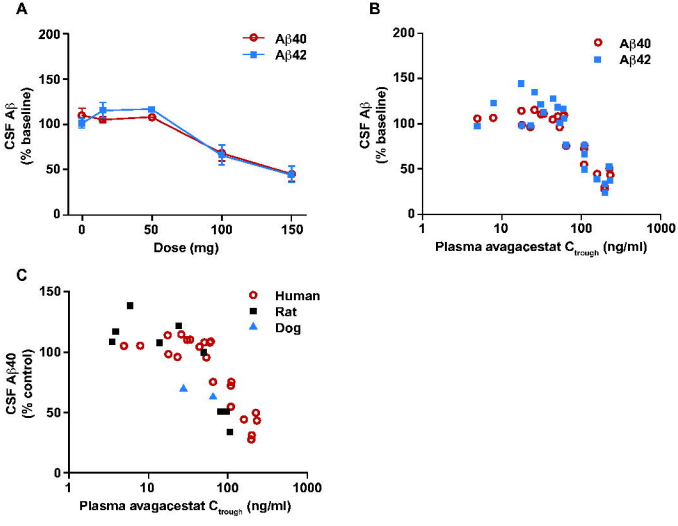
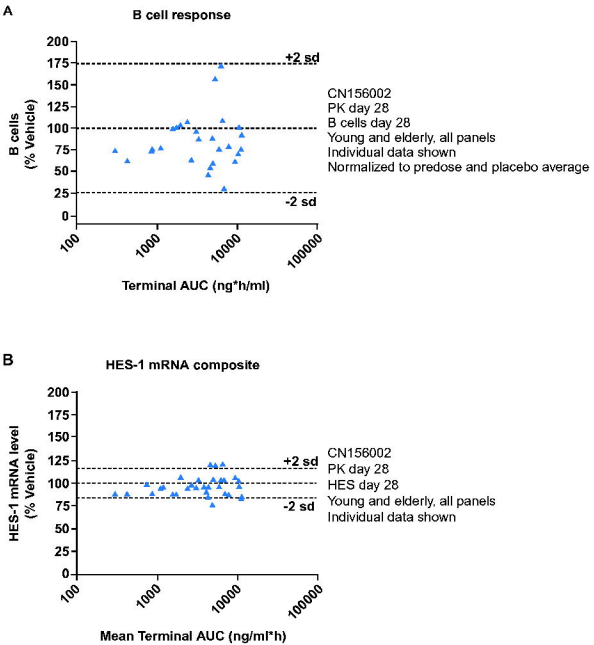

A
CSF Aβ (% baseline)
Aβ40
Aβ42
Dose (mg)
B
CSF Aβ (% baseline)
Aβ40
Aβ42
Plasma avagacestat C trough (ng/ml)
C
CSF Aβ40 (% control)
Human
Rat
Dog
Plasma avagacestat C trough (ng/ml)

Figure 5


A
B cell response
200
175
150
125
100
75
50
25
0
B cells
(% Vehicle)
+2 sd
-2 sd
100
1000
10000
100000
Terminal AUC (ng*h/ml)
CN156002
PK day 28
B cells day 28
Young and elderly, all panels
Individual data shown
Normalized to predose and placebo average
B
HES-1 mRNA composite
200
175
150
125
100
75
50
25
0
HES-1 mRNA level
(% Vehicle)
+2 sd
-2 sd
100
1000
10000
100000
Mean Terminal AUC (ng/ml*h)
CN156002
PK day 28
HES day 28
Young and elderly, all panels
Individual data shown