Establishing the Relationship between In Vitro Potency, Pharmacokinetic, and Pharmacodynamic Parameters in a Series of Orally Available, Hydroxyethylamine-Derived \(\beta\)-Secretase Inhibitors

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ABSTRACT

Sequential proteolytic cleavage of the amyloid precursor protein (APP) by \(\beta\)-site APP-cleaving enzyme 1 (BACE1) and the \(\gamma\)-secretase complex produces the amyloid-\(\beta\) peptide (\(\alpha\beta\)), which is believed to play a critical role in the pathology of Alzheimer’s disease (AD). The aspartyl protease BACE1 catalyzes the rate-limiting step in the production of \(\alpha\beta\), and as such it is considered to be an important target for drug development in AD. The development of a BACE1 inhibitor therapeutic has proven to be difficult. The active site of BACE1 is relatively large. Consequently, to achieve sufficient potency, many BACE1 inhibitors have required unfavorable physicochemical properties such as high molecular weight and polar surface area that are detrimental to efficient passage across the blood-brain barrier. Using a rational drug design approach we have designed and developed a new series of hydroxyethylamine-based inhibitors of BACE1 capable of lowering \(\alpha\beta\) levels in the brains of rats after oral administration. Herein we describe the in vitro and in vivo characterization of two of these molecules and the overall relationship of compound properties [e.g., in vitro permeability, P-glycoprotein (P-gp) efflux, metabolic stability, and pharmacological potency] to the in vivo pharmacodynamic effect with more than 100 compounds across the chemical series. We demonstrate that high in vitro potency for BACE1 was not sufficient to provide central efficacy. A combination of potency, high permeability, low P-gp-mediated efflux, and low clearance was required for compounds to produce robust central \(\alpha\beta\) reduction after oral dosing.

Introduction

Alzheimer’s disease (AD), a progressive neurodegenerative disorder, is the most common form of dementia that affects 4 to 8% of the elderly population worldwide. The neuropathological features of AD are the presence of extracellular amyloid plaques and intracellular neurofibrillary tangles in the hippocampus and cortical gray matter of the brain (Citron, 2010). The core constituent of the amyloid plaques, also known as senile plaques, is a small 4-kDa amyloid-\(\beta\) peptide (\(\alpha\beta\)). Assembly and aggregation of \(\alpha\beta\) into soluble oligomers and amyloid fibrils is believed by many to play a key role in the etiology of AD; therefore, strategies to lower \(\alpha\beta\) production have been pursued with great interest in the AD field over the past two decades (Hardy and Selkoe, 2002). \(\alpha\beta\) is a fragment of a much larger precursor protein, the amyloid precursor protein (APP). Sequential proteolytic cleavage of APP by \(\beta\)-site APP-cleaving enzyme 1 (BACE1) and the \(\gamma\)-secretase complex produces \(\alpha\beta\). The aspartyl protease

ABBREVIATIONS: AD, Alzheimer’s disease; \(\alpha\beta\), amyloid-\(\beta\) peptide; APP, amyloid precursor protein; BACE1, \(\beta\)-site APP-cleaving enzyme 1; CNS, central nervous system; CSF, cerebrospinal fluid; DMSO, dimethyl sulfoxide; ER, efflux ratio; FRET, fluorescence resonance energy transfer; HEA, hydroxyethylamine; HPMC, hydroxypropyl methyl cellulose; IS, internal standard; PD, pharmacodynamic; P-gp, P-glycoprotein; PK, pharmacokinetic; RLM, rat liver microsome; RLM CL\(_{int}\), intrinsic clearance in RLMS; QC, quality control.

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BACE1 catalyzes the rate-limiting step in the production of Aβ and is therefore considered to be an important target for drug development in AD (Vassar and Kandalepas, 2011).

The development of BACE1 inhibitor therapeutics has been challenging. The X-ray structure of BACE1 cocystalized with a transition-state analog inhibitor has been useful for the design of BACE1 inhibitors with good in vitro potency (Hong et al., 2000). However, the large active site of the enzyme has made it difficult to find small molecules that are both potent inhibitors of the enzyme and also possess the appropriate pharmacokinetic (PK) properties to enable inhibition of Aβ production in the brain (Hong et al., 2000; Turner et al., 2001; Stachel et al., 2009). Nonetheless, over the last decade, much progress has been made across industry and academia in designing BACE1 inhibitors with physiochemical properties that allow for effective Aβ lowering in vivo (Ghosh et al., 2012). In the last few years, a number of groups have succeeded in making potent BACE1 inhibitors capable of lowering central Aβ in APP transgenic mice, rats, guinea pigs, monkeys, and humans (Charrier et al., 2009; Sankaranarayanan et al., 2009; Chakrabarti et al., 2010; Fukumoto et al., 2010; Malamas et al., 2010; Truong et al., 2011; May et al., 2011). Using a rational drug design approach, we have designed and developed a new series of hydroxyethylamine (HEA)-based inhibitors of BACE1 that can potentely reduce Aβ levels in brain and cerebrospinal fluid (CSF) after oral administration (Dineen et al., 2012; Kaller et al., 2012; Weiss et al., 2012).

In this article, we describe the in vitro and in vivo characterization of two of these molecules and the overall relationship of compound properties (e.g., in vitro permeability, P-gp-mediated efflux, metabolic stability, and pharmacological potency) to the in vivo pharmacodynamic (PD) effect across the chemical series. We demonstrate that high in vivo potency for BACE1 was not sufficient to provide central efficacy. In fact, no single parameter on its own was able to provide an absolute in vitro surrogate for in vivo potency; compounds required a balanced PK profile that consisted of low metabolic clearance, high permeability, and low efflux to produce robust central Aβ reduction after oral dosing.

Materials and Methods

Human Recombinant BACE1 Enzymatic Assay in Fluorescence Resonance Energy Transfer Format. The extracellular domain of human BACE1 was expressed in CHO cells as described previously (Vassar et al., 1999). The enzymatic activity of recombinant BACE1 was measured by using a fluorescence resonance energy transfer (FRET) assay according to published methods (Yang et al., 2001). The substrate was a synthetic peptide that contained the BACE1 cleavage site (Turner et al., 2001) and fluorophore and acceptor BACE1 was measured by using a fluorescence resonance energy transfer format (FRET assay) as previously (Vassar et al., 1999). The enzymatic activity of recombinant domain of human BACE1 was expressed in CHO cells as described before oral dosing.

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Passive Permeability and Rat P-gp-Mediated Efflux Ratio. Transcellular transport was measured by using LLC-PK1 cell monolayers as described previously (Booth-Genthe et al., 2006) with slight modifications. LLC-PK1 cells were plated on porous (1.0 μm) polycarbonate 96-well membrane filters (Millipore Corporation, Billerica, MA) in a feeder tray of medium. The transport experiment was initiated on the sixth day after plating by replacing the buffer in each compartment with HEPES-buffered Hanks’ balanced salt solution containing 0.1% bovine serum albumin with and without 5 μM compound in triplicate. Transcellular transport of [3H]Hidigoxin at 0.5 μM was also evaluated as a positive control for P-gp. The monolayer integrity was also tested by using [14C]mannitol, a paracellular diffusion indicator. After 2 h, aliquots were taken from both apical and basolateral chambers and analyzed by liquid chromatography-tandem mass spectrometry on an API4000(Applied Biosystems, Foster City, CA) triple quadrupole mass spectrometer interfaced with turbo IonSpray, operated in positive mode by using Analyst 1.4.2 software (Applied Biosystems).

Efflux was measured in LLC-PK1 cells transfected with rat mdr1A/1B. Efflux ratio (ER) was calculated from the basolateral-to-apical permeability divided by the apical-to-basolateral permeability.

Microsomal Stability: Intrinsic Clearance in Rat Liver Microsomes. Compounds (1 μM) were incubated with rat liver microsomes (RLMs; 0.25 mg/ml in 67 mM phosphate buffer, pH 7.4) from rat at 37°C for 30 min with or without 1 mM NADPH in a total volume of 0.2 ml. The final concentration of DMSO in the incubation was <0.1%. Incubations were stopped by the addition of 200 μl of ice-cold acetonitrile containing 0.5% formic acid and an internal standard (IS; 500 ng/ml) followed by centrifugation at 3100 rpm for 20 min. The supernatants were analyzed directly (without any further sample clean-up) by high-performance liquid chromatography and mass spectrometric detection.

Plasma Protein Binding. Compounds were spiked into plasma to a final concentration of 5 μg/ml (0.1% DMSO final), gently mixed, and incubated at 37°C for 15 min. Aliquots of incubated plasma (0.5 ml) were transferred to polycarbonate tubes and centrifuged at 627,000 relative centrifugal force for 3 h at 37°C. The centrifugation process pellets 99.2% of plasma proteins. The supernatants (0.1 ml) were transferred into 0.1 ml of blank 4% plasma in plasma water. An additional aliquot of each compound-spiked plasma sample was diluted 1:50 in plasma water (Bioreclamation Inc., Hicsville, NY) and retained for analysis. Standard curve and analytical quality-control (QC) samples were constructed in 2% plasma in plasma water. Fifty microliters of internal standard (500 ng/ml in acetonitrile) was added to 50 μl of unknown samples, standards, and QCs. An additional 250 μl of acetonitrile ensured precipitation of protein with vortexing. Samples, standards, and QCs were centrifuged to pellet proteins, and a 250-μl aliquot was removed to a clean deep-well plate. Samples were either analyzed directly or after drying under nitrogen and reconstitution in 0.1 ml of methanol/water (1:1 v/v).

Whole-Cell Aβ Assay. Human embryonic kidney 293 cells stably transfected with APPK955 containing the Swedish familial Alzheimer’s disease mutation (APPswe,swend) were used to test compounds in a whole-cell assay. Cells were incubated 16 to 18 h with compounds at concentrations ranging from 0.0005 to 10 μM. Conditioned media was removed and analyzed for Aβ levels as described below.

Aβ40 Measurements and Data Analysis. Samples were analyzed for Aβ40 levels by sandwich immunoassay according to a previously published protocol (Best et al., 2005). In brief, 96-well avidin plates (MesoScale Discovery, Inc., Gaithersburg, MD) were coated with biotinylated-anti-Aβ antibody 4G8 (Covance Research Products, Princeton, NJ), which recognizes an epitope within the midregion of Aβ (amino acids 17–24). Samples were coincubated in the plate overnight at 4°C along with a ruthenium-labeled anti-Aβ antibody specific for the C-terminal region of Aβ40 (ConFab40; Amgen, Thousand Oaks, CA). Plates were then washed, 150 μl/well read buffer T (MesoScale Discovery, Inc.) was added, and plates were read immediately on a Sector6000 imager according to the manufacturer’s recommended protocol (MesoScale Discovery, Inc.). All samples were assayed in triplicate and analyzed by using Prism version 5.04
and centrifuged at 1900 × g for 5 min. Supernatant (250 μl) was transferred into a 96-well plate for sample analysis. Aliquots of CSF (25 μl) were combined with 25 μl of acetonitrile. An additional 150 μl of acetonitrile containing a structurally related IS was added to each sample, vortexed, and centrifuged at 1900g for 5 min. Supernatant (250 μl) was transferred into a 96-well plate for sample analysis. Brain tissue samples were homogenized in four volumes of water (volume/tissue weight ratio) by using a Covaris (Woburn, MA) acoustic homogenizer. Aliquots of 50 μl of homogenate were combined with 300 μl of acetonitrile containing a structurally related IS, vortexed, and centrifuged at 1900g for 5 min. Supernatant (250 μl) was transferred into a 96-well plate for sample analysis. For plasma, CSF, and brain homogenate, analytical standards were prepared in blank tissue or homogenate (for brain) and treated in an identical manner to the unknowns. Both standards and unknowns were measured by liquid chromatography mass spectrometry in an identical manner to the unknowns. Both standards and unknowns were prepared in blank tissue or homogenate (for brain) and treated in an identical manner to the unknowns. Both standards and unknowns were prepared in blank tissue or homogenate (for brain) and treated in an identical manner to the unknowns.

**Measurement of Plasma, CSF, and Brain Drug Concentration.** Aliquots of plasma (50 μl) were combined with 300 μl of acetonitrile containing a structurally related IS, vortexed, and centrifuged at 1900g for 5 min. Supernatant (250 μl) was transferred into a 96-well plate for sample analysis. Aliquots of CSF (25 μl) were combined with 25 μl of acetonitrile. An additional 150 μl of acetonitrile containing a structurally related IS was added to each sample, vortexed, and centrifuged at 1900g for 5 min. Supernatant (150 μl) was transferred into a 96-well plate for sample analysis.

**In Vivo Pharmacokinetics Studies.** Femoral catheterized male Sprague-Dawley rats (250–275 g) were purchased from Taconic Farms (Germantown, NY) and maintained on a 12-h light/dark cycle with unrestricted access to food and water until use. Rats were fasted 7 to 12 h before oral dose administration. The animals were dosed with compounds either as a solution or suspension in 2% HPMC and 1% Tween 80, pH 2 (10 mg/kg; 10 ml/kg dose volume) or intravenously (bolus) in DMSO at 2 mg/kg. Approximately 0.20 ml of whole blood was collected via the femoral vein catheter at 0.25, 0.5, 1, 2, 4, 6, 12, 16, and 24 h postdose. Blood withdrawal volumes were within the Institutional Animal Care and Use Committee Global Standards. Whole blood was centrifuged for plasma collection and stored at approximately −70°C until analysis.

**In Vivo Pharmacodynamics Studies.** Male Sprague-Dawley rats (175–200 g) were dosed orally with compounds in 2% HPMC and 1% Tween 80, pH 2 at a volume of 10 ml/kg. For a typical compound screen, tissue samples were collected at a single 4-h time point. Rats were euthanized with CO2 inhalation for 2 min, and the cisterna magna was quickly exposed by removing the skin and muscle above it. CSF (50–100 μl) was collected with a 30-gauge needle through the dura membrane covering the cisterna magna. CSF samples with visible blood contaminants were discarded. Blood was withdrawn by cardiac puncture, and plasma was obtained by centrifugation for drug concentration analysis. Brains were removed and, along with the CSF, immediately frozen on dry ice and stored at −80°C until use. The frozen brains were subsequently homogenized in 10 volumes (w/v) of freshly made 0.5% Triton X-100 in Tris-buffered saline containing a structurally related IS was added to each sample, vortexed, and centrifuged at 1900 × g for 30 min at 4°C. Supernatant (250 μl) was transferred into a 96-well plate for sample analysis.

**Results**

Using a combination of rational design and classic iterative optimization, we have established a clear structure-activity relationship for BACE1 with a new series of potent, HEA-based inhibitors (Dineen et al., 2012; Kaller et al., 2012; Weiss et al., 2012). Two lead compounds, N-((2S,3R)-1-(benzoz[d][1,3]dioxol-5-yl)-3-hydroxy-4-(((S)-6’-neopentyl-3'-4'-dihydrospiro[cyclobutane-1,2'-pyrano[2,3-b]pyridin]-4'-yl)amino)butan-2-yl)-2-methoxyacetamide (compound 1) and (R)-N-((2S,3R)-3-hydroxy-4-(((S)-6’-neopentyl-3’,4’-dihydrospiro[cyclobutane-1,2’-pyrano[2,3-b]pyridin]-4'-yl)amino)-1-(3-(thiazol-2-yl)phenyl)butan-2-yl)-2-methoxypropanamide (compound 2) (Fig. 1), were potent inhibitors in a FRET-based BACE1 enzyme assay, with IC50 values of 5.4 and 5.5 nM, respectively (Table 1). These compounds also showed significant inhibition of Aβ production in a cell-based assay using human embryonic kidney 293 cells stably transfected with APP containing the Swedish familial Alzheimer’s disease mutation (APPsw). In this assay, compound 1 had an IC50 of 16.8 nM and compound 2 had an IC50 of 9.6 nM, resulting in cell/enzyme potency ratios of 3.1 and 1.8, respectively. To select appropriate candidates for in vivo PD studies in rats, all compounds were evaluated in a set of in vitro PK assays for passive permeability, rat P-gp mediated efflux, and metabolic stability in RLMs. Both compounds showed high passive permeability with values of 25 × 10⁻⁶ and 17 × 10⁻⁶ cm²/s for compounds 1 and 2, respectively. Compound 1 showed high efflux via P-gp with an ER of 16.2, whereas compound 2 showed little or no P-gp-mediated efflux with a ratio of 1.7. In addition, compound 1 was less metabolically stable than compound 2. The intrinsic clearance measured in rat liver microsomes (RLM CLint) was 140 μl/min/mg for compound 1 compared with 51 μl/min/mg for compound 2. In vivo, both compounds 1 and 2 displayed moderate clearance rates after intravenous dosing at 2 mg/kg in rats (1.25 and 0.87 l/h/kg, respectively). Oral dosing at 10 mg/kg in rats demonstrated that compounds 1 and 2 are readily absorbed with plasma half-lives of 2.0 and 1.0 h, respectively. The apparent bioavailability of both compounds was more than 100%, a property that is attributed to nonlinear pharmacokinetics (e.g., via saturable metabolism) at the 10

**Table 1**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Pharmacokinetic and pharmacodynamic properties of compounds 1 and 2</th>
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<tr>
<td></td>
<td>Compound 1</td>
</tr>
<tr>
<td>BACE1 FRET IC50, nM</td>
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</tr>
<tr>
<td>BACE1 cell IC50, nM</td>
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</tr>
<tr>
<td>Permeability, 10⁻⁶ cm²/s</td>
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<tr>
<td>Rat P-gp efflux ratio</td>
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<td>RLM CLint, μl/min/mg</td>
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<td>Rat intravenous clearance, l/h/kg</td>
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<tr>
<td>%F</td>
<td>&gt; 100</td>
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<tr>
<td>Rat oral T1/2, h</td>
<td>2.0</td>
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**Fig. 1.** Structures of compounds 1 (left) and 2 (right), HEA-derived BACE1 inhibitors.
mg/kg oral dose relative to the lower 2 mg/kg i.v. dose (Table 1). Maximal plasma concentrations of 4.5 and 8.9 μM were achieved at 2 and 5 h, respectively, for compounds 1 and 2 (data not shown). Combined with the fraction of drug unbound in plasma of 0.024 (compound 1) and 0.014 (compound 2), the unbound Cmax was 0.108 and 0.125 μM for compounds 1 and 2, respectively. The unbound plasma Cmax was 6- and 13-fold greater than the in vitro cell IC50 and 20-fold and 23-fold greater than the enzyme IC50 (Table 1) for compounds 1 and 2, respectively.

To investigate the PD properties of BACE1 inhibitors in vivo, compounds were administered in a single oral dose to Sprague-Dawley rats, and Aβ40 levels were measured in CSF and brain tissue. Substantial Aβ40 lowering in both compartments was observed in a dose-dependent manner for both compounds 4 h after drug administration (Fig. 2). For compound 1, the total plasma EC50 values for CSF and brain Aβ40 lowering were 5.8 and 17.0 μM, respectively. For compound 2, CSF EC50 and brain EC50 values were 1.6 and 4.5 μM, respectively. Factoring in protein binding in plasma, the estimated unbound plasma EC50 for CSF Aβ40 lowering was 0.140 μM or approximately 8-fold higher than the BACE cell IC50 for compound 1 and 0.023 μM or approximately 2-fold higher than the BACE cell IC50 for compound 2. The EC50 values calculated for both CSF and brain Aβ40 lowering were greater for compound 1 than those observed for compound 2, consistent with the higher efflux ratio of compound 1. A time-course experiment was also conducted with compound 2 to further evaluate the PK-PD relationship for this compound (Fig. 3). Rats were dosed at 30 mg/kg, and samples were collected at the indicated time points and analyzed for both drug and Aβ40 levels. A 30 mg/kg dose was chosen because it was predicted to produce a transient, robust inhibition of CSF Aβ levels based on prior PK and PD data (data not shown). There was a clear relationship between unbound plasma drug concentrations, CSF drug concentrations, and Aβ lowering in the brain and CSF. Significant Aβ40 reduction was observed in CSF at 2 h and in brain 1 h after dosing (p < 0.01). The maximal reduction in Aβ40 levels were observed at 4 h in both CSF (86%) and brain (73%). Aβ40 levels in both CSF and brain returned to baseline 12 h after dosing. Note that the measurement of drug in CSF at the 12-h time point fell below the limit of detection (0.002 μM). The dynamic ranges of measurement for drug and Aβ40 in CSF, plasma, and brain homogenate, including limits of detection, are shown in Supplemental Table 2.

Assessment of >100 compounds that were analyzed in vivo provided insights into how various in vitro characteristics translated into PD effects in vivo. In vivo PD dose-response curves were generated for 48 compounds. A comparison of the

![Fig. 2. Compounds 1 (top) and 2 (bottom) demonstrated concentration-dependent lowering of CSF (●) and brain (▽) Aβ40 levels relative to vehicle controls when dosed orally to wild-type Sprague-Dawley rats. Each symbol represents a measurement from an individual rat (n = 5/dose group). Compounds were dosed at various concentrations in 2% HPMC and 1% Tween 80 at pH 2. Aβ and compound concentrations were measured 4 h postdose.](image)

![Fig. 3. Time-course experiment demonstrating PK-PD relationship for compound 2. Rats were dosed at 30 mg/kg, and samples were analyzed for both drug and Aβ40 levels at the indicated time points. A, PK-PD relationship between Aβ40 (●, black line) and drug levels in CSF (□, gray line). Note that the measurement of drug in CSF at the 12-h time point fell below our limit of detection (0.002 μM; see Supplemental Table 2). B, PK-PD relationship between Aβ40 (●, black line) levels in brain and unbound plasma drug levels (□, gray line). Data plotted are averages with S.D. (n = 5).](image)
CSF EC_{50} for Aβ40 reduction with the brain EC_{50} for these compounds (Fig. 4) displayed a linear relationship with good correlation between the Aβ40 reduction in the two compartments ($r^2 = 0.778; p < 0.0001; n = 48$). A plot of in vitro cell potency versus in vivo PD effect indicated no relationship between Aβ inhibition in vitro and in vivo ($r^2 = 0.008$; not significant; Fig. 5A). This is probably caused by the wide ranges of metabolic stability, permeability, and efflux ratios associated with these compounds. However, when in vivo exposure was factored in a clear relationship was observed. As the ratio of plasma drug levels relative to in vitro potency increased, the degree of CSF Aβ lowering increased (4-h time point after a single 30 mg/kg oral dose of each compound) ($r^2 = 0.26; p < 0.0001$) (Fig. 5B).

To more clearly illustrate the impact of several key in vitro PK parameters on in vivo potency, compound PD effects were binned into three categories: inactive (CSF Aβ reduction ≤25%), moderately active (CSF Aβ reduction of 25–50%), and highly active (CSF Aβ reduction >50%). The percentage of compounds in each activity bin was examined as a function of in vitro PK parameters (Fig. 6). To mitigate the influence of pharmacological potency, this evaluation included only compounds with potencies of <30 nM in the cell-based assay ($n = 104$). Figure 6A shows a clear relationship between permeability and CSF Aβ reduction, with the most permeable compounds producing the largest reduction in CSF Aβ levels. In the set of compounds with permeability values >20 × 10^{-6} cm/s 48% were highly active and 26% were inactive. In contrast, 75% of compounds with passive permeability values <10 × 10^{-6} cm/s were inactive and none were highly active. The effect of rat P-gp efflux on in vivo potency was also pronounced (Fig. 6B). As the ER increased, the percentage of active compounds decreased and the percentage of inactive compounds increased. Nearly all compounds (97.6%) that showed no significant P-gp-mediated efflux (ER <3) demonstrated central nervous system (CNS) activity (≥ 25% Aβ reduction); 74% inhibited CSF Aβ by more than 50%. The majority of compounds (86.7%) that showed moderate P-gp-mediated efflux (ER = 3–10) were active in the CNS; 67% were highly active. The percentage of highly active compounds dropped significantly once efflux ratios exceeded 10. Only 9% of compounds with efflux ratios between 10 and 30, and no compounds with efflux ratios >30, were highly active. This efflux effect was also apparent in the moderately active compound group. A similar trend was observed for compound stability measured in rat liver microsomes (Fig. 6C). As RLM CL_{int} values increased, the percentage of active compounds decreased and the percentage of inactive compounds increased. Sixty-five percent of compounds with RLM CL_{int} values <100 μl/min/mg were highly active as opposed to only 14% of compounds with RLM CL_{int} values >100 μl/min/mg.

Within the three activity bins, average values for permeability, efflux ratio, and RLM CL_{int} were calculated and compared (Supplemental Table 1). As expected, an inverse relationship was observed between efflux ratio and CSF Aβ reduction. Mean efflux ratios ranged from 32.2 in the inactive bin to 10.3 in the moderately active bin to 2.8 in the highly active bin with mean differences between the inactive and highly active bins reaching statistical significance (adjusted $p = 0.0004$). Likewise, an inverse relationship was also observed for RLM CL_{int} values and CSF Aβ reduction. Mean RLM CL_{int} values ranged from 194.7 μl/min/mg in the inactive bin to 172.6 μl/min/mg in the moderately active bin to 92.9 μl/min/mg in the highly active bin. Mean differences between the inactive and highly active bins (adjusted $p < 0.0001$) and between the moderately active and highly active
bins (adjusted \( p = 0.0002 \)) reached statistical significance. With this set of compounds, the range of permeability values covered in these studies was relatively narrow, (mean permeability of the inactive group was \( 19.0 \times 10^{-6} \) cm/s compared with \( 22.5 \times 10^{-6} \) cm/s for the highly active group); however, overall, the data suggest that high permeability is necessary but not sufficient to drive A\( \beta \) reduction in the brain.

**Discussion**

One of the biggest challenges encountered with small-molecule BACE1 inhibitors is obtaining good passive permeability across the blood-brain barrier while avoiding P-gp-mediated efflux. BACE inhibitors that are subject to P-gp efflux would be predicted to deliver unbound drug levels in the brain significantly lower than unbound drug levels in the plasma based on basic physiological principles governing permeation of small molecules across the blood-brain barrier and supported by published data on marketed drugs (Mahar Doan et al., 2002). Indeed, reports show that BACE1 inhibitors that are also good P-gp substrates can effectively lower brain A\( \beta \) only in the presence of a P-gp inhibitor or in P-gp knockout mice (Hussain et al., 2007; Sankaranarayanan et al., 2009; Thompson et al., 2011). Much is now understood regarding the relationship of various physicochemical properties of small molecules (e.g., cLogP, polar surface area, number of hydrogen bond donors, etc.) to P-gp efflux, which has enabled medicinal chemists to engineer molecules with desirable efflux properties (Hitchcock, 2012). In addition to compound properties that permit good blood-brain barrier permeation, an ideal BACE1 inhibitor therapeutic would be orally bioavailable and have sufficiently low hepatic clearance to enable it to achieve and maintain adequate plasma concentrations. Indeed aspartyl protease inhibitors in general (e.g., HIV, BACE1, and renin) have tended to suffer from high in vivo clearance and poor in vitro metabolic stability in hepatic microsomal assays amended with the appropriate cofactors to support oxidative metabolism. This phenomenon probably can be attributed to the peptidomimetic nature of these aspartyl protease inhibitors. In this article, we have carefully explored the PK-PD relationship across a large series of novel, orally bioavailable, brain-penetrant BACE1 inhibitors.

Compounds 1 and 2 both are potent inhibitors of BACE1 in enzyme and cell-based assays. Both compounds have high in vitro permeability and moderate in vivo clearance in rat, and both have oral bioavailability >100% because of nonlinear pharmacokinetics and saturable metabolism. However, compound 2 has a low efflux ratio, whereas compound 1 has high efflux, allowing us to assess the effect of efflux on A\( \beta \)40 inhibition in the CSF and brain. As expected, the total plasma EC\(_{50}\) values for CSF and brain A\( \beta \)40 inhibition for compound 1 were 3.6- and 3.8-fold higher than the corresponding values for compound 2. Furthermore, after correction for plasma protein binding, the ratio between the in vivo unbound plasma EC\(_{50}\) for CSF A\( \beta \)40 inhibition and the in vitro cell IC\(_{50}\) was much higher for compound 1 than for compound 2 (8- versus 2-fold), consistent with a significant reduction in central efficacy caused by P-gp-mediated efflux.

Although both compounds showed dose-dependent reduction of A\( \beta \) levels in brain homogenate and CSF after oral dosing, in both cases the apparent EC\(_{50}\) for brain effect was larger than the EC\(_{50}\) determined from CSF data, suggesting differential potency in the two CNS compartments. This apparent brain/CSF shift has been observed by others with both BACE1 and \( \gamma \)-secretase inhibitors (Sankaranarayanan et al., 2009; Hawkins et al., 2011; Lu et al., 2011; Dineen et al., 2012; Weiss et al., 2012). There are several possible explanations for this observation. The first is that each compartment has active BACE1 and generates A\( \beta \) at least partially independently from one another (Crossgrove et al., 2007). In this model it would be possible for different inhibitor potencies in vivo because of the different exposure of drug in the two compartments. However, evidence suggests that A\( \beta \) is produced primarily in brain tissue. After secretion from neurons, A\( \beta \) that is not enzymatically degraded is thought to drain through the extracellular space into CSF, presumably with some time lag. In studies using a single administration of compound, the amount of A\( \beta \) inhibition varies with drug concentration, as shown in Fig. 3. The amount of A\( \beta \) reduction observed in CSF may reflect the amount of A\( \beta \) reduction in brain at an earlier point in time. If A\( \beta \) is assessed at a point in time during which drug levels in the brain compartment are decreasing, the apparent inhibition in CSF will exceed that observed in brain tissue. If A\( \beta \) is measured at a
time during which drug levels are increasing in brain, the reverse would be true. Recent PK-PD modeling of Aβ reduction in brain and CSF after secretase inhibition supports this disparity in Aβ turnover kinetics between the two compartments (Lu et al., 2012).

Drug development programs often focus on trying to improve compound potency. Early HEA-based inhibitors showed good potency in biochemical and cell-based assays; however, they were not effective in vivo (Stachel et al., 2004; Fresskos et al., 2007; Kortum et al., 2007; Maillard et al., 2007). Our data show that although potency is clearly important, within a series of 134 compounds with potency <100 nM (and most important, within a series of 134 compounds with potency <50 nM) (and most important, within a series of 134 compounds with potency <100 nM), 46% of compounds with EC50 values for CSF Aβ40 inhibition in CSF, and only 25% demonstrated efficacy in clinical pharmacology model for identifying small-molecule γ-secretase inhibitors. J Med Chem 55:4877–4895.


Harding JC and Selkoe DJ (2000) Aβ in cerebral spinal fluid and brain Aβ levels. In contrast, 74% of compounds with efflux ratios over 30, and only 9% of compounds with efflux ratios between 10 and 30, showed more than 50% inhibition of CSF Aβ40 levels. In contrast, 74% of compounds with efflux ratios less than 3 produced more than 50% Aβ40 inhibition in CSF. Likewise, no compounds with permeability less than 10 × 10−5 cm/s produced efficacy more than 50% Aβ40 inhibition in CSF, and only 25% demonstrated efficacy in the 25 to 50% inhibition range. Finally, the importance of metabolic stability was also evident in this set of BACE1 inhibitors, because the fraction of highly active compounds with RLM CLint values <100 μL/min/μg (65%) was significantly greater than the percentage of highly active compounds with RLM CLint values >100 μL/min/μg (14%). The optimal BACE1 inhibitor based on this data set would have a cell IC50 value less than 30 nM, P-gp efflux ratio less than 3, and permeability more than 20 × 10−5 cm/s, and RLM CLint less than 50 μL/min/μg. Seven compounds met all four criteria, and they inhibited CSF Aβ40 levels by 61 to 80% 4 h after a single 30 mg/kg oral dose, with a mean reduction of 70%. The EC50 values for CSF Aβ40 inhibition for the same compounds ranged from 1.3 to 6.5 μM. A translation of this approach to guiding Aβ reduction in the clinic should be possible because human brain concentrations of all three in vitro PK assays described here are available.

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We thank Jessie Gu for statistical analysis.

Authorship Contributions

Participated in research design: Wood, Wen, Hitchcock, Citron, Zhong, Hickman, and Williamson.

Conducted experiments: Zhang, Zhu, Luo, Babu-Khan, Chen, Pham, and Esmay.

Contributed new reagents or analytic tools: Dineen, Kaller, Weiss, and Zhong.

Performed data analysis: Wood, Wen, Luo, Babu-Khan, Chen, Pham, Esmay, Hitchcock, Hickman, and Williamson.

Wrote or contributed to the writing of the manuscript: Wood, Wen, Pham, Esmay, Dineen, Kaller, Weiss, Hitchcock, Zhong, Hickman, and Williamson.

References


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Supplemental Data

Title: Establishing the relationship between in vitro potency, pharmacokinetic and pharmacodynamic parameters in a series of orally available, hydroxyethylamine-derived \(\beta\)-secretase inhibitors

Authors: Stephen Wood, Paul H. Wen, Jianhua Zhang, Li Zhu, Yi Luo, Safura Babu-Khan\(^1\), Kui Chen, Roger Pham, Joel Esmay, Thomas A. Dineen, Matthew R. Kaller, Matthew M. Weiss, Stephen A. Hitchcock\(^2\), Martin Citron\(^3\), Wenge Zhong, Dean Hickman, and Toni Williamson\(^4\)

Journal: The Journal of Pharmacology and Experimental Therapeutics (JPET)
Supplemental Table 1

Compound PD effects were binned into 3 categories: inactive (CSF Aβ reduction ≤ 25%), moderately active (CSF Aβ reduction of 25-50%) and highly active (CSF Aβ reduction > 50%). Averages and standard deviations were calculated for permeability, efflux ratio and RLM CL_{int} for the 3 activity bins and these averages were compared in a pair-wise fashion (Group 1 vs. Group 2) to assess statistical significance using both ANOVA (p-value) and Tukey’s test (p-value adjusted for multiple comparisons).

<table>
<thead>
<tr>
<th>PK parameter</th>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>Group 1</th>
<th>Group 2</th>
<th>p-value</th>
<th>Adj. p-value (Tukey)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Efflux Ratio</strong></td>
<td>≤ 25%</td>
<td>32.2</td>
<td>30.2</td>
<td>&gt; 50%</td>
<td>≤ 25%</td>
<td>0.0001</td>
<td>0.0004</td>
</tr>
<tr>
<td></td>
<td>25-50%</td>
<td>10.3</td>
<td>11.7</td>
<td>&gt; 50%</td>
<td>25-50%</td>
<td>0.0537</td>
<td>0.1292</td>
</tr>
<tr>
<td></td>
<td>&gt; 50%</td>
<td>2.8</td>
<td>3.0</td>
<td>≤ 25%</td>
<td>25-50%</td>
<td>0.0239</td>
<td>0.610</td>
</tr>
<tr>
<td><strong>Permeability</strong></td>
<td>≤ 25%</td>
<td>19.0</td>
<td>8.2</td>
<td>&gt; 50%</td>
<td>≤ 25%</td>
<td>0.007</td>
<td>0.0191</td>
</tr>
<tr>
<td>(10^{-6} cm/s)</td>
<td>25-50%</td>
<td>23.6</td>
<td>8.2</td>
<td>&gt; 50%</td>
<td>25-50%</td>
<td>0.7189</td>
<td>0.9308</td>
</tr>
<tr>
<td></td>
<td>&gt; 50%</td>
<td>22.5</td>
<td>6.0</td>
<td>≤ 25%</td>
<td>25-50%</td>
<td>0.0076</td>
<td>0.0205</td>
</tr>
<tr>
<td><strong>RLM CL_{int}</strong></td>
<td>≤ 25%</td>
<td>194.7</td>
<td>162.3</td>
<td>&gt; 50%</td>
<td>≤ 25%</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>(µl/min/mg)</td>
<td>25-50%</td>
<td>172.6</td>
<td>105.3</td>
<td>&gt; 50%</td>
<td>25-50%</td>
<td>0.0001</td>
<td>0.0002</td>
</tr>
<tr>
<td></td>
<td>&gt; 50%</td>
<td>92.9</td>
<td>90.0</td>
<td>≤ 25%</td>
<td>25-50%</td>
<td>0.9297</td>
<td>0.9957</td>
</tr>
</tbody>
</table>
**Supplemental Table 2**

The dynamic ranges of measurement for drug and Aβ40 in CSF, plasma and brain homogenate are shown. The low end of each range represents the Lower Limit of Detection (LLOD). Assays for the quantification of drug and Aβ40 levels in biological samples are described in the materials and methods section.

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Drug (Dynamic Range)</th>
<th>Aβ40 (Dynamic Range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSF</td>
<td>1 – 5,000 ng/ml</td>
<td>35 – 8,500 pg/ml</td>
</tr>
<tr>
<td>Plasma</td>
<td>1 – 5,000 ng/ml</td>
<td>NA</td>
</tr>
<tr>
<td>Brain Homogenate</td>
<td>5 – 25,000 ng/g</td>
<td>35 – 8,500 pg/g</td>
</tr>
</tbody>
</table>