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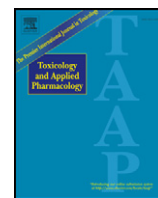
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The effect of angiotensin receptor neprilysin inhibitor, sacubitril/valsartan, on central nervous system amyloid- β concentrations and clearance in the cynomolgus monkey



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ABSTRACT

Sacubitril/valsartan (LCZ696) is the first angiotensin receptor neprilysin inhibitor approved to reduce cardiovascular mortality and hospitalization in patients with heart failure with reduced ejection fraction. As neprilysin (NEP) is one of several enzymes known to degrade amyloid- β (A β), there is a theoretical risk of A β accumulation following long-term NEP inhibition. The primary objective of this study was to evaluate the potential effects of sacubitril/valsartan on central nervous system clearance of A β isoforms in cynomolgus monkeys using the sensitive Stable Isotope Labeling Kinetics (SILKTM)-A β methodology.

The in vitro selectivity of valsartan, sacubitril, and its active metabolite sacubitrilat was established; sacubitrilat did not inhibit other human A β -degrading metalloproteases. In a 2-week study, sacubitril/valsartan (50 mg/kg/day) or vehicle was orally administered to female cynomolgus monkeys in conjunction with SILKTM-A β . Despite low cerebrospinal fluid (CSF) and brain penetration, CSF exposure to sacubitril was sufficient to inhibit NEP and resulted in an increase in the elimination half-life of A β 1-42 (65.3%; $p = 0.026$), A β 1-40 (35.2%; $p = 0.04$) and A β total (29.8%; $p = 0.04$) acutely; this returned to normal as expected with repeated dosing for 15 days. CSF concentrations of newly generated A β (AUC_(0-24 h)) indicated elevations in the more aggregable form A β 1-42 on day 1 (20.4%; $p = 0.039$) and day 15 (34.7%; $p = 0.0003$) and in shorter forms A β 1-40 (23.4%; $p = 0.009$), A β 1-38 (64.1%; $p = 0.0001$) and A β total (50.45%; $p = 0.00002$) on day 15. However, there were no elevations in any A β isoforms in the brains of these monkeys on day 16. In a second study cynomolgus monkeys were administered sacubitril/valsartan (300 mg/kg) or vehicle control for 39 weeks; no microscopic brain changes or A β deposition, as assessed by immunohistochemical staining, were present. Further clinical studies are planned to address the relevance of these findings.

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Abbreviations: A β , amyloid beta; ACE-1, angiotensin-converting enzyme 1; AD, Alzheimer's disease; AHU377, sacubitril; ANP, atrial natriuretic peptide; APP, amyloid precursor protein; ARB, angiotensin receptor blocker; ARNI, angiotensin receptor neprilysin inhibitor; AUC, area under curve; BID, twice daily; BNP, B-type natriuretic peptide; CNP, C-type natriuretic peptide; CNS, central nervous system; CSF, cerebrospinal fluid; ECE, endothelin-converting enzyme; FPPE, formalin-fixed paraffin-embedded; FLT, fluorescence lifetime; IC₅₀, half maximal inhibitory concentration; IDE, insulin-degrading enzyme; IHC, immunohistochemistry; LBQ657, sacubitrilat; LLOQ, lower limit of quantification; MW, molecular weight; NEP, neprilysin; NP, natriuretic peptide; QD, once daily; SILKTM, Stable Isotope Labeling Kinetics; TTR, tracer-to-tracee ratio.

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1. Introduction

Sacubitril/valsartan (previously LCZ696) is the first angiotensin receptor neprilysin inhibitor (ARNI) approved by the US FDA and European Medicines Agency shown to reduce cardiovascular mortality and heart failure hospitalizations by 20% (McMurray et al., 2014). Following oral administration, sacubitril/valsartan delivers systemic exposure to valsartan, an angiotensin receptor blocker (ARB) and sacubitril (also known as AHU377), an inactive prodrug which is subsequently metabolized by esterases to the active neprilysin (NEP) inhibitor sacubitrilat (also known as LBQ657) (Gu et al., 2010; Flarakos et al., 2016). This

results in simultaneous enhancement of the effects of biologically-active beneficial natriuretic peptides (NPs) via inhibition of their degradation by NEP and blockade of the detrimental effects of angiotensin II via ARB. Based on its unique mode of action, sacubitril/valsartan exhibits beneficial effects on cardiac, renal, and vascular function, which may underlie the improved mortality and morbidity observed in patients with heart failure and reduced ejection fraction in the PARADIGM-HF trial (McMurray, 2015; Gori and Senni, 2016).

NEP cleaves a number of physiologically relevant substrates, including NPs (atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), and to a lesser degree B-type natriuretic peptide (BNP)), enkephalins, tachykinins, chemotactic peptide, and adrenomedullin (Mangiafico et al., 2013; McMurray, 2015; Volpe et al., 2016). In mammals, NEP is expressed in several organs, including the kidney, lung, and brain, with the renal proximal tubule displaying the highest levels (Mangiafico et al., 2013).

In the brain, NEP is one of several enzymes involved in the degradation of amyloid- β (A β). A β is a peptide generated in the brain through sequential cleavage of amyloid precursor protein (APP) by β - and γ -secretases (Haass et al., 2012). The role of NEP in A β degradation is based on both in vitro and in vivo studies (Howell et al., 1995; Takaki et al., 2000; Iwata et al., 2000). In addition to NEP, there are several other A β degrading proteases, including NEP-2, insulin degrading enzyme (IDE), endothelin converting enzyme (ECE) and angiotensin-converting enzyme (ACE) (reviewed by Saido and Leissring, 2012). In addition to proteolytic degradation, A β is also cleared from the central nervous system (CNS) by non-enzymatic processes, including cell-mediated clearance and passive and active transport into the cerebrospinal fluid (CSF) and blood stream however, the relative contribution of each of these clearance pathways, including proteolytic degradation by NEP, has yet to be elucidated (Saido and Leissring, 2012). The amyloid cascade hypothesis posits that abnormal production and clearance of A β contributes to the formation of amyloid plaques, commonly found in the brains of patients with dementia due to Alzheimer's disease (AD) (Karran et al., 2011). Aggregation prone A β subtypes (A β 1-42 and A β 1-40) are found in senile plaques of the brains of patients with Alzheimer's disease (Glennier et al., 1984; Iwatsubo et al., 1994; Iwatsubo et al., 1995). However the exact role of A β in the subsequent pathophysiology of AD is still the subject of some debate (Sorrentino et al., 2014).

This study is the first reported use of SILK™-A β technology to assess changes in CSF A β associated with inhibition of A β clearance pathways by a neprilysin inhibitor. The cynomolgus monkey was selected as an appropriate preclinical model to assess the risk for changes in A β clearance and brain deposition based on 1) the complete homology of APP in cynomolgus monkeys and humans, and 2) shared similarity in progressive cerebral deposition of A β protein during normal aging not found in other species (Podlisny et al., 1991). Here, we report two separate studies carried out in this model investigating effects of sacubitril/valsartan on CSF A β clearance, A β concentrations in CSF, brain and plasma as well as A β brain deposition. The 2-week SILK™-A β study was an investigative study to evaluate the effect of sacubitril/valsartan treatment on A β concentrations and clearance in serial samples of CSF using the SILK™ technique. The SILK™-A β methodology has been previously used in monkeys to assess changes in CSF A β associated pathways of A β production by γ -secretase inhibition (Cook et al., 2010). This technique was selected because it provides the most reliable estimate of the clearance of newly generated A β peptides from the CSF compartment given constraints in CSF sampling volume and study sample size (Cook et al., 2010). The second study in cynomolgus monkeys treated for 39 weeks with sacubitril/valsartan assessed brain A β plaque formation by immunohistochemistry; this study is the longest duration nonclinical safety study in non-rodents required by ICH guidance (ICH M3 R2) (2009) to support human clinical trials marketing authorization among the regions of European Union, Japan and the United States. Localization of NEP in the cynomolgus monkey brain was also assessed by immunostaining using samples

from untreated animals. Finally, a separate set of experiments was performed to determine the in vitro potency and protease selectivity profile of sacubitril, sacubitrilat, and valsartan.

2. Materials and methods

2.1. In vitro potency and protease selectivity profile of compounds used

The in vitro potency and protease activity of sacubitril/valsartan analytes were assessed using fluorescence-based lifetime (FLT) assays as previously described for kallikrein 7 (Doering et al., 2009).

Recombinant human NEP enzyme was expressed in insect cells and purified to a final concentration of 5 pM. Sacubitril, sacubitrilat or valsartan were added at concentrations ranging from 0.0003 nM to 100 μ M for 60 min at room temperature in 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl and 0.05% (v/v) CHAPS. A similar protocol was followed for recombinant human NEP-2 with a final enzyme concentration of 3 pM.

Recombinant human endothelin-converting enzyme-1 (ECE-1) and recombinant human endothelin-converting enzyme-2 (ECE-2) were purchased from R&D Systems (Minneapolis, MN) with a final concentration of 0.3 pM and 2 pM, respectively. ECE-1 and ECE-2 were pre-incubated with sacubitril, sacubitrilat or valsartan at various concentrations for 60 min at room temperature in 50 mM Tris-HCl buffer, pH 7.4 containing 150 mM NaCl and 0.05% (w/v) CHAPS, and 50 mM MES-HCl buffer, pH 5.75, containing 125 mM NaCl and 0.05% (w/v) CHAPS, respectively.

Recombinant human angiotensin-converting enzyme-1 (ACE-1, expressed in insect cells and purified at a final concentration of 3.0 pM) was pre-incubated with sacubitril, sacubitrilat or valsartan at various concentrations for 60 min at room temperature in 10 mM sodium phosphate buffer, pH 7.4 containing 150 mM NaCl and 0.05% (w/v) CHAPS.

For NEP, NEP-2, ECE-1, ECE-2, and ACE-1, the enzymatic reaction was started by the addition of a synthetic peptide substrate Cys(PT14)-Arg-Arg-Leu-Trp-OH (Product number BS-# 9288.1, Biosyntan, Berlin, Germany) to produce a final concentration of 0.7 μ M (0.8 μ M for ACE-1). Substrate hydrolysis led to an increase in the fluorescence lifetime (FLT) of PT14 measured by means of a FLT reader as previously described (Doering et al., 2009). The effect of each compound on the enzymatic activity was determined after 60 min incubation at room temperature. FLT measurements were conducted on an Ultra Evolution fluorescence lifetime reader (TECAN, Maennedorf, Switzerland) with an excitation light source of 405 nm wavelength and an emission wavelength of 450 nm through a bandpass filter and analyzed using instrument control software. The IC₅₀ values, corresponding to the inhibitor concentration showing 50% reduction of the FLT values measured in absence of an inhibitor, were calculated from the plot of percentage of inhibition vs. inhibitor concentration using non-linear regression analysis software.

Recombinant human insulin-degrading enzyme (IDE, R&D Systems, final concentration 0.2 nM) was pre-incubated with sacubitril, sacubitrilat or valsartan at various concentrations for 60 min at room temperature in 50 mM Tris/HCl buffer at pH 7.4, containing 1 M NaCl and 0.05% (w/v) CHAPS. The enzymatic reaction was started by the addition of a synthetic peptide substrate Mca Arg-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH (R&D Systems Europe Ltd., Abingdon, United Kingdom) to produce a final concentration of 2 μ M. Substrate hydrolysis led to an increase in fluorescence intensity measured by a monochromator-based fluorescence-reader at wavelengths of 320 nm and 405 nm taken for fluorescence excitation and emission acquisition, respectively. The effect of the compound on the enzymatic activity was determined after a 60-min incubation at room temperature. The IC₅₀ values, corresponding to the inhibitor concentration showing 50% reduction of the fluorescence intensity values measured in the absence of an inhibitor, were calculated as described above.

2.2. Study drug and formulation

Sacubitril/valsartan was administered in 0.5% (w/v) sodium carboxymethylcellulose, aqueous solution by either oral (2-week SILK™-A β study) or nasal gavage (39-week study).

2.3. Animals and housing

All primate experiments in the studies were reviewed and approved by the Institutional Animal Care and Use Committees of either Novartis, East Hanover, NJ or Charles River Laboratories, Inc. (Reno, NV or Montreal, Quebec, Canada) and conformed to the Guide for the Use and Care of Laboratory Animals (National Research Council of the National Academies, Institute for Laboratory Animal Research, 2011).

2.4. 2-week SILK™-A β study in cisterna magna ported monkey model

The study was conducted at the facility of Charles River Laboratories, Inc., Montreal, Quebec, Canada.

2.4.1. Cisterna magna ported cynomolgus monkey model

Thirty-six female cynomolgus monkeys of Chinese origin, aged between 2 and 4 years, were utilized in the 2-week SILK™-A β study for evaluating the effects of sacubitril/valsartan on CNS drug exposure and levels and clearance of A β in CSF and plasma. Animals were randomly assigned to either vehicle control or sacubitril/valsartan treatment groups to control bias. Randomization was by stratification using body weight as the parameter.

In-life technicians were not blinded to treatment group assignment; however, apart from assignment to vehicle control or sacubitril/valsartan treatment groups, all animals were treated as equally as possible.

Female monkeys were selected based on availability and general compliance with the surgical methodology. Surgically implanted cisterna magna catheter and port systems were employed for the serial collection of uncontaminated CSF samples as previously described (Cook et al., 2010). These monkeys were also implanted with a standard femoral vein silicone-based catheter system with the tip of the catheter approximately at the level of the vena cava for vascular infusion of $^{13}\text{C}_6$ -leucine. All animals were continuously infused with 0.9% sodium chloride injection, USP, at a rate of 2.0 mL/h until initiation of treatment and in between SILK™ infusions.

2.4.2. $^{13}\text{C}_6$ -leucine infusion and treatment initiation

In preparation for each $^{13}\text{C}_6$ -leucine infusion and SILK™-A β sampling, cynomolgus monkeys were restricted to a low leucine diet of vegetables and fruits as previously described (Cook et al., 2010). Intravenous infusion of $^{13}\text{C}_6$ -leucine was started at 8 pm and continued for 12 h. For A β analysis, CSF was collected at 4, 12, 16, 20, 24, 28, 32 and 36 h after the start of $^{13}\text{C}_6$ -leucine infusion. Analysis of labeled to unlabeled ($^{12}\text{C}_6$) leucine in proteins was measured by the tracer-to-tracee ratio (TTR) method (Bateman et al., 2009). Free leucine analysis in plasma samples (collected pre-infusion (0 min), as well as 6 min and 2, 4, 8, 12, 14 and 24 h after infusion) was conducted by Metabolic Solutions Inc. (Nashua, NH) using a validated gas chromatography/mass spectrometry (GC/MS) protocol.

The cisterna magna ported female cynomolgus monkeys received either vehicle control or 50 mg/kg/day sacubitril/valsartan treatment by oral gavage for up to 16 days, with SILK™-A β analyses occurring on days 1 and 15 of dosing; and tissue collections for assessment of A β concentrations in brain, CSF and plasma occurring at necropsy (2 hour post dose on day 16). The dose of 50 mg/kg/day was chosen as it results in plasma AUC_(0–24 h) and C_{max} sacubitrilat exposure similar to the clinical exposure delivered by a 400 mg QD dose of sacubitril/valsartan in healthy volunteers. The first dose of sacubitril/valsartan was administered 12 h after the initiation of $^{13}\text{C}_6$ -leucine infusion (Fig. 1). The final sample size was $n = 17$ for the control group and $n = 15$ for the sacubitril/valsartan treatment group due to the loss of port patency in

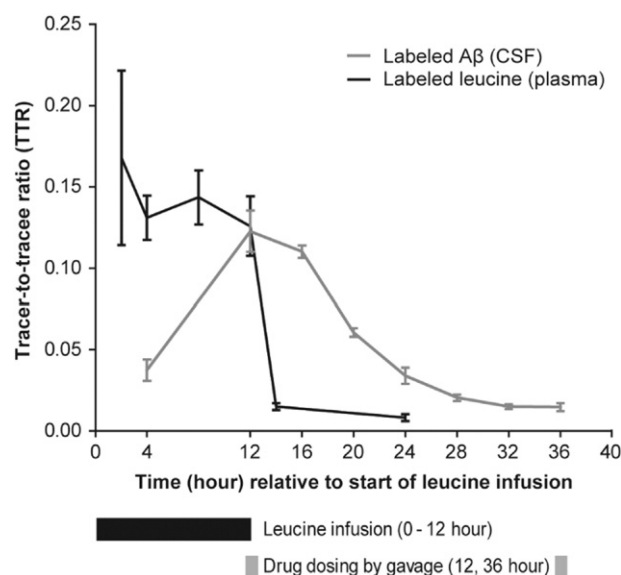


Fig. 1. 2-week SILK™-A β study: Stable Isotope Labeling Kinetics (SILK™) study design.

individual animals and was independent of, and occurred prior to, sacubitril/valsartan administration.

2.4.3. Pooling strategy for A β analysis

Plasma samples were analyzed for labeled and unlabeled free leucine as a measure and quality control for the leucine infusion. Due to the smaller size of the cynomolgus monkeys on the study, and in consideration of animal welfare, it was possible to collect only 200–225 μL of CSF per sampling timepoint during the study. Therefore, pooling of samples was necessary in order to provide sufficient sample volume for the assay. CSF samples from 3 animals were pooled in rank order based on individual leucine exposure (i.e. lowest to highest free plasma leucine levels) and analyzed for labeled and unlabeled A β 1–37, 1–38, 1–40, 1–42, and A β total (Table S1). This pooling strategy was selected based on modeling indicating this approach would provide the most accurate measure of the mean and result in an overestimate rather than underestimate of the actual standard deviation, relative to non-pooled samples. SILK™-A β samples were labeled as belonging to animals from one of two cohorts. Analysts at C2N Diagnostics were unaware of the treatment status of the two cohorts, and analysis of samples at C2N Diagnostics was performed blinded to the treatment group of the pooled animal samples.

2.4.4. A β immunoassay and immunoprecipitation/liquid chromatography-mass spectrometry

The TTR and concentration of A β was measured using a combination of immunoprecipitation and mass spectrometry (IP/MS). This method allowed the detection of newly synthesized (labeled) A β peptides as well as A β total (labeled and unlabeled) peptides (Table S1). The absolute quantitation of A β (A β 1–38, A β 1–40 and A β 1–42) in plasma samples from days 1 and 15 and in brain, CSF and plasma samples from 2 hour post dose on day 16 was determined by multiplex immunoassay (MesoScale Diagnostics Rodent/Human (4G8) A β 3-plex Ultra-Sensitive Kit (MesoScale Diagnostics, Rockville, MD)).

2.4.5. Calculation of elimination half-life and newly generated A β

Newly generated A β was calculated as previously described as the product of the percentage of labeled A β (LC-MS results) and the A β total concentration (LC-MS results) (Bateman et al., 2009). All statistical calculations were performed with GraphPad 5.04. A two-tailed Student's t -test was used for comparisons between treatment groups.

Elimination half-life was calculated using the following formula: $eT_{1/2} = \ln(2) / k$, where the elimination constant (k) is equal to the negative slope of the natural log transformed newly generated A β

concentration during the clearance portion of the metabolic study. For A β 1–40, A β 1–38, and A β total we included hours 16–32 for the log transformation, but for A β 1–42 the log transformation including the 32 hour time point was not possible since the concentrations measured at this time point were too close to the lower limit of quantitation (LLOQ).

2.4.6. Bioanalytical methods

Blood and CSF samples were collected at 0.5, 1, 4, 8, and 24 hour post dose on days 1 and 15 for analysis of sacubitril, sacubitrilat, and valsartan. Blood was processed for plasma using K₂EDTA (anticoagulant). CSF samples were pooled from the groups of three animals within each group (three aliquots of equal volume) and diluted 1:1 with blank (i.e. naive monkey) plasma. In addition, final plasma, CSF, and brain tissue samples were obtained at the scheduled necropsy on day 16 at approximately 2 hour post dose. All samples were frozen at or below –60 °C prior to analysis.

Toxicokinetic parameters were estimated using Watson LIMS. A non-compartmental approach consistent with the oral route of administration was used for parameter estimation. All parameters were generated from the concentrations in plasma and CSF from days 1 and 15 unless otherwise stated. Parameters were estimated using sampling times relative to the start of each dose administration. Plasma samples were processed by liquid-liquid extraction procedure using methyl t-butyl ether, followed by recovery of the organic portion of the extracts by drying down at 40 °C under nitrogen and reconstitution in methanol:water:formic acid (90:10:0.1). Samples were centrifuged and analyzed by LC-MS/MS using electrospray ionization (plasma sacubitril: precursor *m/z* 412.4 and product *m/z* 266.3; sacubitrilat: precursor *m/z* 384.3 and product *m/z* 266.3; valsartan: precursor *m/z* 436.4 and product *m/z* 291.3) with LLOQ of 10.0 ng/mL for the analytes using 0.025 mL of plasma. CSF samples were processed similarly as the plasma and measured against 1:1 CSF:plasma calibration standards. Brain tissue samples were homogenized in lysing solution to yield 0.2 mg/mL tissue homogenate and processed using protein precipitation with an acetonitrile/methanol mixture. Measurements were made against brain tissue homogenate calibration standards and the nominal concentrations were corrected by tissue mass (e.g., ng/g). Samples were centrifuged and analyzed by LC-MS/MS using electrospray ionization. The ionization outputs for CSF and brain homogenates were precursor *m/z* 412.3 and product *m/z* 266.2 for sacubitril, precursor *m/z* 384.3 and product *m/z* 266.2 for sacubitrilat, and precursor *m/z* 436.4 and product *m/z* 235.1 for valsartan with LLOQ of 0.1 ng/mL using 0.025 mL of CSF:plasma and 0.5 ng/mL using 0.030 mL of brain tissue homogenate, respectively, for the sacubitril/valsartan analytes.

2.5. 39-week repeated dose study with sacubitril/valsartan in cynomolgus monkeys: brain immunostaining for A β

A retrospective analysis of A β brain immunostaining was performed on brains from a 39-week toxicity study conducted at the facility of Charles River Laboratories, Inc., Reno, NV.

In this study, sacubitril/valsartan was administered to four groups (4/sex/group) of cynomolgus monkeys at daily doses of 0 (vehicle) 30, 100 and 300 mg/kg/day (dose volume of 5 mL/kg) via nasal gavage for at least 39 weeks. Additional groups of control and 300 mg/kg/day cynomolgus monkeys (2/sex) were included to serve as recovery populations. Animals were randomly assigned to either vehicle control or sacubitril/valsartan treatment groups to control bias. Randomization was by stratification using body weight as the parameter. In-life technicians were not blinded to treatment group assignment; however apart from assignment to vehicle control or sacubitril/valsartan treatment groups, all animals were treated as equally as possible.

Monkeys were of Chinese origin and aged between 2 and 4 years at the start of dosing. Animals were euthanized at the end of the 39-week dosing period (24 h after final dose) or at the end of a 4-week recovery period. Histopathology assessments were performed on routine formalin-fixed paraffin-embedded (FFPE) brain sections from all animals, and immunohistochemical analyses for A β were performed on control and 300 mg/kg/day main study and recovery phase animals.

2.6. Immunohistochemistry

Multiple brain regions, including the cerebral cortex, striatum, thalamus/hypothalamus, ventricular system, limbic system (including hippocampus), midbrain, cerebellum, pons region and medulla oblongata from animals receiving control and high-dose sacubitril/valsartan (39-week study) were evaluated by immunohistochemistry (IHC). The FFPE brain sections were stained with hematoxylin/eosin (H/E) and for A β using a rabbit monoclonal antibody (D12B2, Cat#9888, Cell Signaling Technology, Danvers, MA) at a 1:1600 dilution. The results were compared with sections from an A β 1–42-positive human brain affected with AD, which served as a positive control. In FFPE AD brain sections, this protocol stained both compact and diffuse plaques with minimal background of adjacent neuropil. An irrelevant rabbit IgG (Cat# 3900 Cell Signaling Technology) control slide was included for each section tested. Positive and negative controls served to minimize bias potentially introduced by the study pathologist, who was not blinded to treatment group assignments.

For NEP staining, brain sections from three naïve cynomolgus monkeys (two females and one male) of Chinese origin which were 4, 5, and 23 years old were utilized. These monkeys were used as vehicle controls in other experiments and did not show any neurological signs during the study. FFPE tissues were prepared as previously described (Pardo et al., 2012) and transverse brain sections cut for IHC assessment. These sections contained portions of the forebrain, multiple portions of the cerebrum, caudate putamen area, thalamus (rostral and caudal), and midbrain including the substantia nigra, pons, cerebellum, and

Table 1
In vitro potencies of sacubitrilat, sacubitril, and valsartan on selected human metalloproteases that degrade A β .

Metalloprotease	Abbreviation	In vitro IC ₅₀ - μ M (ng/mL)		
		Sacubitrilat	Sacubitril	Valsartan
Neprilysin (neutral endopeptidase)	NEP	0.0023 \pm 0.0004 (0.811 \pm 0.141)	16.7 \pm 2.3 (6870 \pm 946)	>100 (43550)
Neprilysin-2 (neutral endopeptidase-2)	NEP-2	84.7 \pm 2.8 (32,474 \pm 1074)	>100 (41150)	>100
Insulin-degrading enzyme	IDE	>100 (>38340)	>100	>100
Endothelin-converting enzyme-1	ECE1	>100	>100	>100
Endothelin-converting enzyme-2	ECE2	5.5 \pm 0.6 (2109 \pm 230)	>100	>100
Angiotensin-converting enzyme-1	ACE-1	>100	>100	>100

Other metalloproteases that degrade A β such as membrane metallo-endopeptidase-like protein (MMEL), matrix metalloproteinases (MMP2, MMP9, MMP14), cluster of differentiation (also known as extracellular matrix metalloproteinase inducer, CD147), serine proteases (e.g. plasmin), aspartyl proteases (e.g. cathepsin D), and cysteine proteases (e.g. cathepsin B) were not evaluated. Figures are represented as mean \pm standard deviation.

Molecular weight (MW) of sacubitrilat is 383.4; MW of sacubitril is 411.4; MW of valsartan is 435.5.

multiple portions of the medulla oblongata. Anti-NEP (CD10) rabbit monoclonal antibody (clone SP67) (Ventana Medical Systems Inc., Tucson, AZ) was used at 0.3 µg/mL.

Briefly, sections were cut at 5 µm and analyzed by the Ventana Systems Discovery XT (Ventana Medical Systems Inc., Tucson, AZ). IHC protocol was optimized by antigen retrieval, a primary antibody incubation with the concentrations indicated above, horseradish peroxidase (HRP) enzymatic amplification (OmniMap anti-RbHRP secondary antibodies for 4 min, and detection with Ventana Medical Systems). Stained slides were scanned with an Aperio slide scanner (Leica Biosystems, Vista, CA) at 20× magnification.

The NEP immunohistochemical analysis was scored by a pathologist (C. S.) on a semi-quantitative scale (0 to 4+, where 0 indicates no evidence of staining) by measuring the intensity and distribution of specific staining.

3. Results

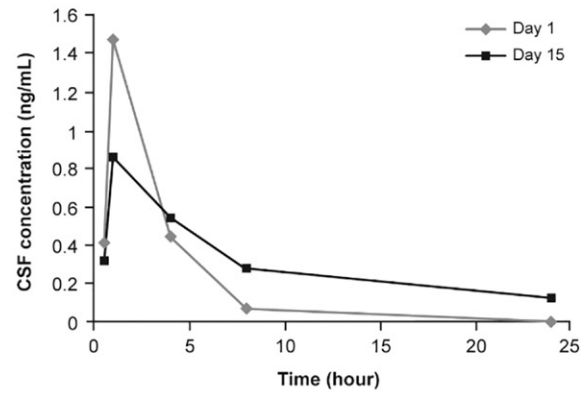
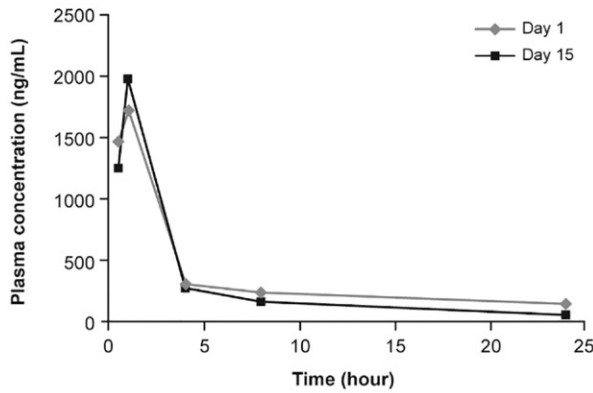
3.1. In vitro potency and protease selectivity profile of sacubitril, sacubitrilat, and valsartan

Following administration, sacubitril/valsartan dissociates into the inactive prodrug sacubitril, which is further metabolized to active NEP inhibitor sacubitrilat, and ARB valsartan. Sacubitrilat was selective for NEP. Sacubitril, sacubitrilat, and valsartan at physiologically relevant concentrations did not inhibit other metalloproteases that degrade Aβ in vitro, including ACE-1 (Table 1).

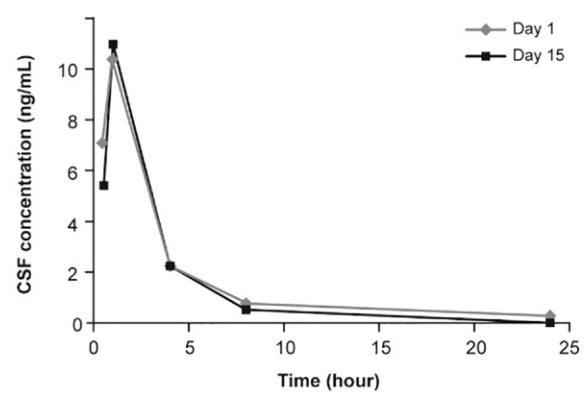
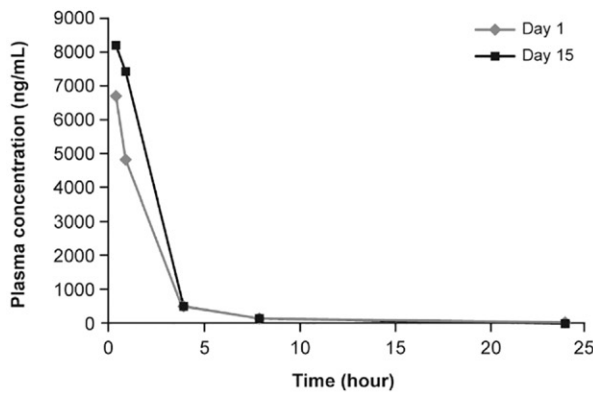
3.2. 2-week SILK™-Aβ study in cynomolgus monkeys

Daily oral administration of sacubitril/valsartan to female cynomolgus monkeys for 16 consecutive days at 50 mg/kg/day was well

A. Valsartan



B. Sacubitril



C. Sacubitrilat

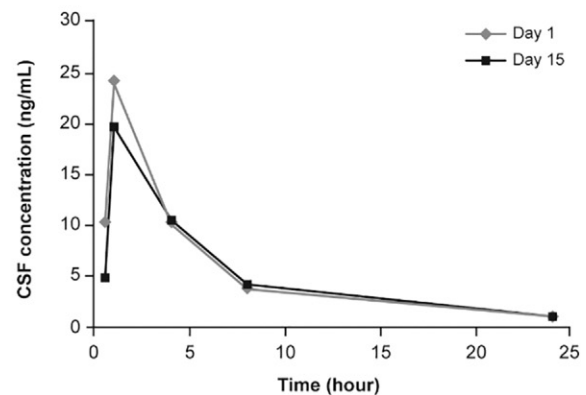
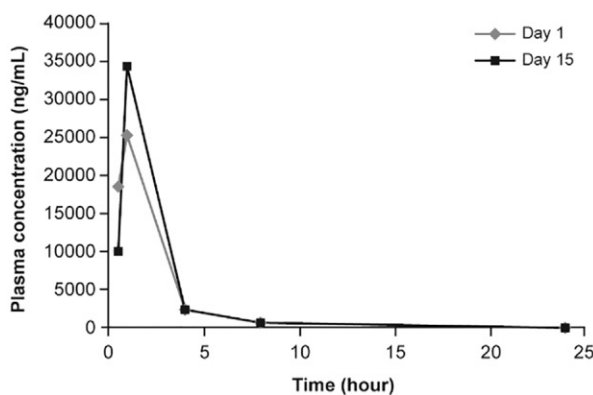


Fig. 2. 2-week SILK™-Aβ study: mean concentration-time plots of valsartan (A), sacubitril (B), and sacubitrilat (C) in female monkey plasma and CSF following administration of sacubitril/valsartan 50 mg/kg/day.

tolerated. No treatment-related changes were observed on clinical signs, body weight, food intake, body temperature, or macroscopic observations at necropsy.

All animals showed quantifiable amounts of valsartan, sacubitril, and sacubitrilat in both plasma and CSF (Fig. 2), with CSF concentrations of sacubitrilat exceeding the *in vitro* IC₅₀ for human NEP inhibition (2.3 nM) (Table 2). Concentrations of valsartan, sacubitril, and sacubitrilat observed in the CSF and brain were low (≤ 0.0044 CSF:plasma ratio; ≤ 0.0326 brain tissue:plasma ratio) when compared to the plasma concentrations at 2 hour post dose on day 16 (Table 2), consistent with the high plasma protein binding of sacubitril (93% protein bound) and valsartan (95.8% protein bound) in non-human primates.

Stable isotope kinetic assessments showed that the elimination half-life of newly synthesized A β was significantly increased in the 50 mg/kg sacubitril/valsartan dosing group relative to controls on day 1 for A β 1-42 (65.3%; $p = 0.026$), A β 1-40 (35.2%; $p = 0.04$) and A β total (29.8%; $p = 0.04$), but normalized by day 15 (Fig. 3).

The concentration of newly generated A β (AUC_(0–24 h)) was acutely increased compared to vehicle control for A β 1-42 on day 1 (20.4%; $p = 0.039$) and on day 15 (34.7%; $p = 0.0003$). Elevations were also noted after repeated daily dosing (day 15) for newly synthesized shorter forms including A β 1-40 (23.4%; $p = 0.009$), A β 1-38 (64.1%; $p = 0.0001$) and for newly synthesized A β total (50.45%; $p = 0.00002$) (Fig. 4 and Fig. 5). Similar trends were noted for total (labeled + unlabeled) CSF A β on day 15 (Fig. S1).

Plasma levels of A β 1-40 and A β 1-42 showed trends (by multiplex immunoassay) for increases in sacubitril/valsartan-treated animals at both 2 and 12 hour post-dose on days 1 and 15, with maximum increases observed at 12 hour post dose on day 15 (48.8% increase for A β 1-42 and 38.7% increase for A β 1-40, relative to concurrent controls) (Fig. 6A). Similar trends were observed for 2 hour post dose day 16 samples collected at necropsy (data not shown).

Brain levels of A β 1-42 or A β 1-40 (cerebral cortical or hippocampal) in sacubitril/valsartan-treated animals were not changed relative to controls at 2 hour post dose on day 16 (Fig. 6B). There were no statistically significant increases in the CSF levels of A β 1-42 or A β 1-40 in samples collected at 2 hour post dose on day 16, as assessed by a multiplex immunoassay (Fig. 6C).

Table 2

Concentration of sacubitrilat, sacubitril and valsartan in the plasma, brain and CSF at end of dosing and at necropsy (50 mg/kg/day dose).

	Analyte		
	Valsartan	Sacubitril	Sacubitrilat
Day 15 exposures			
Plasma (n = 17)			
AUC _(0–24 h)	6920 ± 2530	21,500 ± 8660	83,200 ± 25,100
C _{max}	2000 ± 1100	10,000 ± 6380	34,400 ± 12,600
CSF (n = 6 ^a)			
AUC _(0–24 h)	7.42 ± 3.18	36.5 ± 20.6	128 ± 41.1
C _{max}	0.862 ± 0.331	11.0 ± 6.98	19.8 ± 9.52
CSF:plasma			
AUC _(0–24 h)	0.00107	0.00170	0.00154
C _{max}	0.000431	0.0011	0.000576
2 h post dose, day 16			
Plasma (n = 9) (ng/mL)			
	1130 ± 1180	1730 ± 1850	14,900 ± 9260
Brain (n = 9) (ng/g)			
Cerebral cortex	20.7 ± 12.7	37.1 ± 40.3	147 ± 117
Hippocampus	36.9 ± 69.1	34.9 ± 32.0	114 ± 86.8
Brain:plasma			
Cerebral cortex	0.0183	0.0214	0.00986
Hippocampus	0.0326	0.0202	0.00763
CSF (n = 9) (ng/mL)			
	0.574 ± 0.670	7.61 ± 9.23	20.7 ± 11.8
CSF:plasma			
	0.000508	0.00440	0.00139

Units are: AUC_(0–24 h): ng * h/mL; C_{max}: ng/mL.

^a n = 6 pools, with 2 or 3 animals/pool with the exception of one sample which represents analyses from a single animal. All data are represented ± standard deviation.

3.3. 39-week study in cynomolgus monkeys with brain A β -immunostaining

Brain immunostaining for A β using an antibody which recognizes multiple isoforms of the protein (including A β 1-37, A β 1-38, A β 1-40, and A β 1-42) showed no evidence of sacubitril/valsartan-related increases in A β deposition or A β plaque formation following 39 weeks of dosing at 300 mg/kg/day (Fig. 7).

There were no sacubitril/valsartan-related microscopic brain changes, increases in brain or cerebral vascular A β content, or plaque formation after 39 weeks of dosing as assessed by H/E and A β immunostaining. The 300 mg/kg dose resulted in a mean AUC_(0–24 h) sacubitrilat exposure of 614,000 ng * h/mL and mean C_{max} exposure of 143,500 ng/mL, which is approximately 4-fold (AUC_(0,7)) and 10-fold (C_{max}) the exposure associated with the 400 mg QD dose used in the healthy volunteer study (Langenickel et al., 2016). If one considers the recommended clinical dose (200 mg BID) for treating patients with heart failure, exposure is approximately 2-fold (AUC_(0–24 h)), and 9-fold (C_{max}) the exposure associated with this dose (AUC_(0–12 h)) of 151,611 ng * h/mL and C_{max} of 16,531 ng/mL).

3.4. Immunolocalization of NEP in cynomolgus monkey brain

The distribution and staining intensity of NEP was similar across all three animals (age ranging from 4 to 23 years).

NEP was predominantly localized in basal ganglia (caudate nucleus, putamen, and globus pallidus) with intense immunostaining within neuropil (perineuronal) and axon bundle (Fig. 8, Table S2). There was moderate NEP expression in some thalamic, pontine, cerebellar, and medullary nuclei. However, weak immunostaining was noted in hippocampus and cerebral cortex within neuropil and in glial cells. Notably, the choroid plexus did not show NEP immunoreactivity.

Immunolocalization of NEP in cynomolgus monkey brain was comparable with the human brain, with the exception of the choroid plexus (Table S3). The lack of NEP expression in the choroid plexus of cynomolgus monkeys contradicts published reports on human brain samples, wherein the human choroid plexus was shown to contain NEP, albeit using different methodology with potentially different sensitivities (e.g., immunoradiometric vs. IHC; specificity of antibodies – polyclonal vs. monoclonal antibodies) (Matsas et al., 1985).

4. Discussion

Sacubitril/valsartan has been shown to reduce cardiovascular mortality and morbidity by 20% in patients with reduced ejection fraction heart failure compared with the ACE inhibitor enalapril (McMurray et al., 2014). As NEP is one of several enzymes involved in the degradation of A β , there is a theoretical risk of accumulation of A β in the brain following inhibition of the enzyme by sacubitrilat. However, the role of A β clearance in the pathophysiology of AD is not well defined, with genes associated with the disease primarily involved in the production of the A β protein (Karran et al., 2011). Clinical data from the landmark PARADIGM-HF trial found no signal for cognitive impairment in over 4100 patients treated with sacubitril/valsartan for a median of 27 months. In the present studies, the non-human primate serves as an appropriate preclinical model to investigate the pharmacological effects of both short- and long-term administration of sacubitril/valsartan, attempting to replicate the human condition of A β production and clearance (Podlisny et al., 1991).

In vitro testing revealed that sacubitril and its active metabolite sacubitrilat are selective for human NEP and did not inhibit other metalloproteases known to degrade A β , such as ACE-1. Preclinical studies focused on the short-term effects of sacubitril/valsartan administration on the clearance of A β from the CSF of cynomolgus monkeys, as well as concentrations of A β in the CSF, brain and plasma. Another study evaluated the long-term effects of the drug on A β deposition in the brain of cynomolgus monkeys treated for 39 weeks.

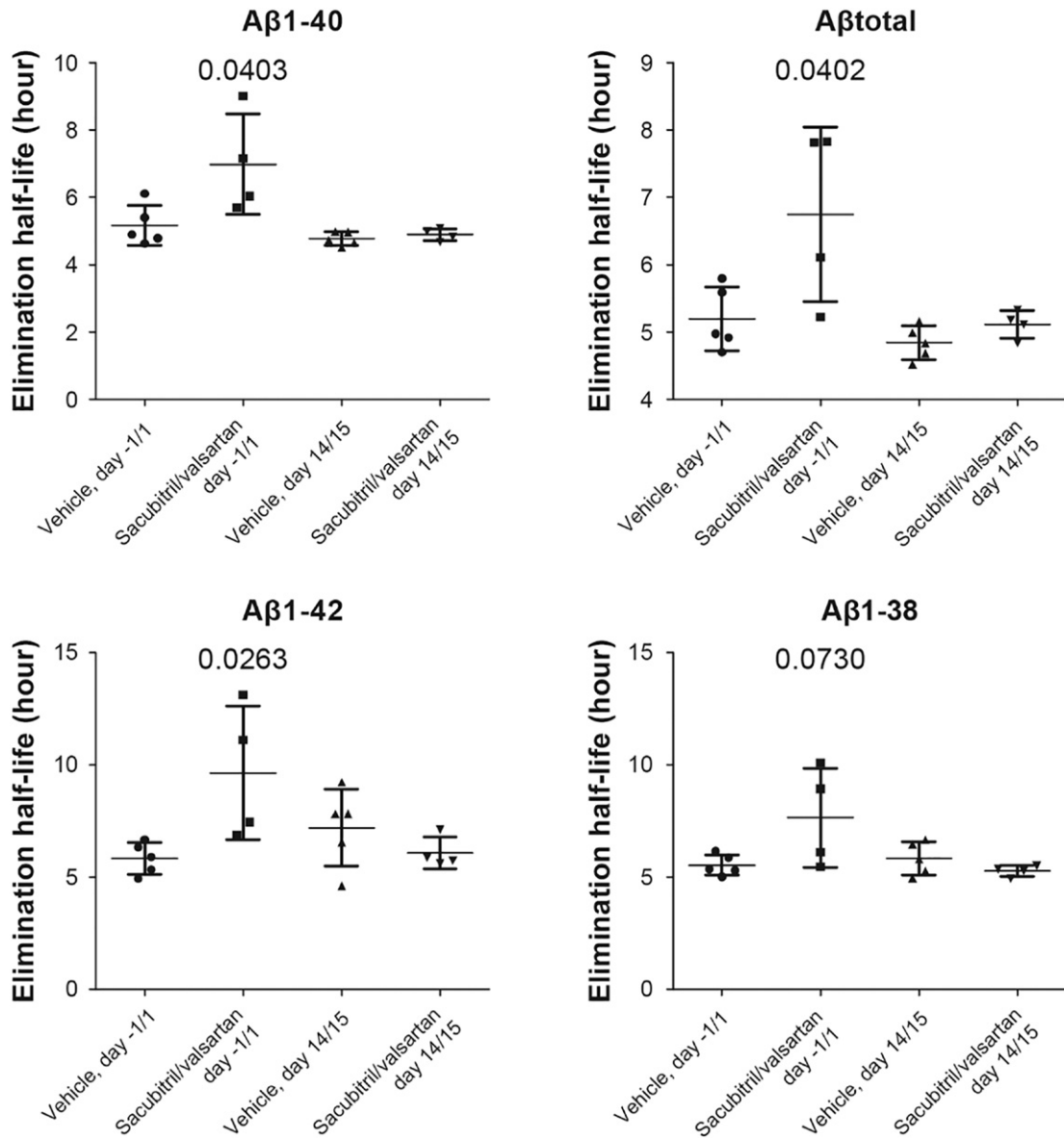


Fig. 3. 2-week SILK™-Aβ study: elimination half-life for newly generated Aβ isoforms in CSF. The p-values shown were derived from a t-test comparison of vehicle and sacubitril/valsartan groups.

Administration of sacubitril/valsartan to female non-human primates at a dose of 50 mg/kg resulted in clinically relevant exposure to sacubitrilat in plasma and CSF that was similar to that observed in healthy volunteers receiving a 400 mg once daily dose of sacubitril/valsartan. The C_{max} of sacubitrilat CSF exposure (19.8 ng/mL) on day 15 in cynomolgus monkeys was approximately equal to the C_{max} CSF exposure (19.2 ng/mL) in sacubitril/valsartan-treated healthy volunteers administered 400 mg sacubitril/valsartan QD (Langenickel et al., 2016). Plasma exposure to sacubitrilat in these animals was similar to [approximately 0.6-fold ($AUC_{(0-24\ h)}$) and 2.4-fold (C_{max})] the steady state exposure in the healthy volunteers (Langenickel et al., 2016). Despite very low CSF and brain penetration of valsartan, sacubitril, and sacubitrilat (≤ 0.0044 CSF:plasma ratio; ≤ 0.0326 brain tissue:plasma ratio), concentrations of sacubitrilat were sufficient to inhibit neprilysin.

We report the first application of SILK™-Aβ methodology to assess potential effects associated with inhibition of a CNS Aβ clearance pathway following oral administration of a neprilysin inhibitor. In a 2-week study, administration of sacubitril/valsartan resulted in acute decreases in CSF clearance rates of the more aggregable peptide Aβ1-42 as well as the shorter form Aβ1-40 and total Aβ as evidenced by an increase in the

elimination half-life; however, clearance rates had normalized by day 15. Although CSF concentrations of Aβ1-42, as well as the shorter isoforms Aβ1-40, Aβ1-38 and total Aβ were still increased at day 15, no elevations in brain Aβ1-40, Aβ1-42 or Aβ1-38 were detected 2 hour post dose on day 16 and no brain amyloid deposition was detected in the 39-week study. Young cynomolgus monkeys were utilized in this study rather than older monkeys due to the need to collect serial CSF samples from cisterna magna ported animals over an extended period. Perhaps of greater importance, younger animals were selected to reduce confounding effects of a decrease in CSF Aβ that accompanies age and hippocampal atrophy (Darusman et al., 2014). Thus, the interpretation of changes in CSF Aβ associated with pharmacologic inhibition of clearance of Aβ in the CNS by neprilysin could be more precisely defined.

In the chronic study, cynomolgus monkeys were treated with high doses of sacubitril/valsartan (300 mg/kg/day) for 39 weeks that resulted in higher sacubitrilat exposure as compared to 200 mg BID clinical dose in humans ($AUC_{(0-24\ h)}$ exposure approximately 2-fold and C_{max} exposure approximately 9-fold); however, these animals displayed no sacubitril/valsartan-related microscopic brain changes or Aβ deposition (as assessed by immunostaining using an antibody that cross-reacts

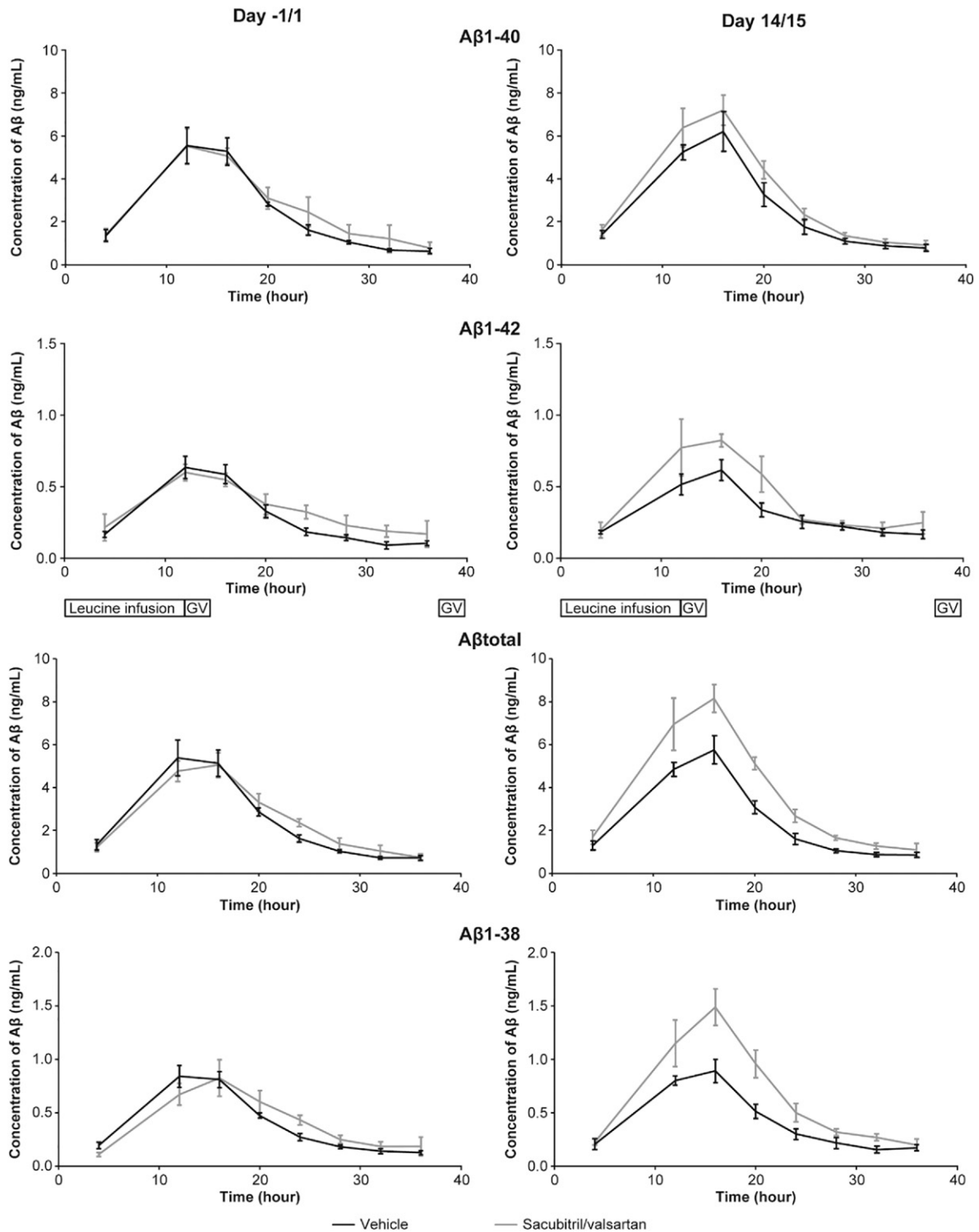


Fig. 4. 2-week SILK™-Aβ study: concentration of newly generated Aβ in the CSF. Data shown are averages by group and day ± standard deviation (error bars). Leucine infusion (0–12 h) and drug administration (12 h and 24 h gavage (GV)) are marked below the Aβ1-42 data plot.

with several isoforms including Aβ1-37, Aβ1-38, Aβ1-40, and Aβ1-42). These data are consistent with the absence of elevation in brain concentrations of Aβ1-40 and Aβ1-42 in the 2-week SILK™-Aβ study. Although it is reassuring that there was no evidence of Aβ plaque formation by immunohistochemistry after 39-weeks of treatment with sacubitril/valsartan, it is acknowledged that we cannot rule out the potential for Aβ deposition and plaque formation following longer term treatment.

The relationship between CSF and brain levels of Aβ is currently not well understood but a recent study in humans determined that direct

transport of Aβ across the blood-brain barrier may account for 25–50% of Aβ clearance (Roberts et al., 2014). The reasons why sacubitril/valsartan-associated Aβ increases occurred in CSF but not in the brain are not clear. One possible explanation for this disparity may include that Aβ pools in the brain and CSF are differentially affected by NEP inhibition. The elimination half-life of Aβ isoforms was significantly slowed on day 1, but not altered on day 15. Normalization of the elimination half-life by day 15 is expected and reflects Aβ clearance reaching a steady-state in the CSF with a larger pool size, thus not affecting a

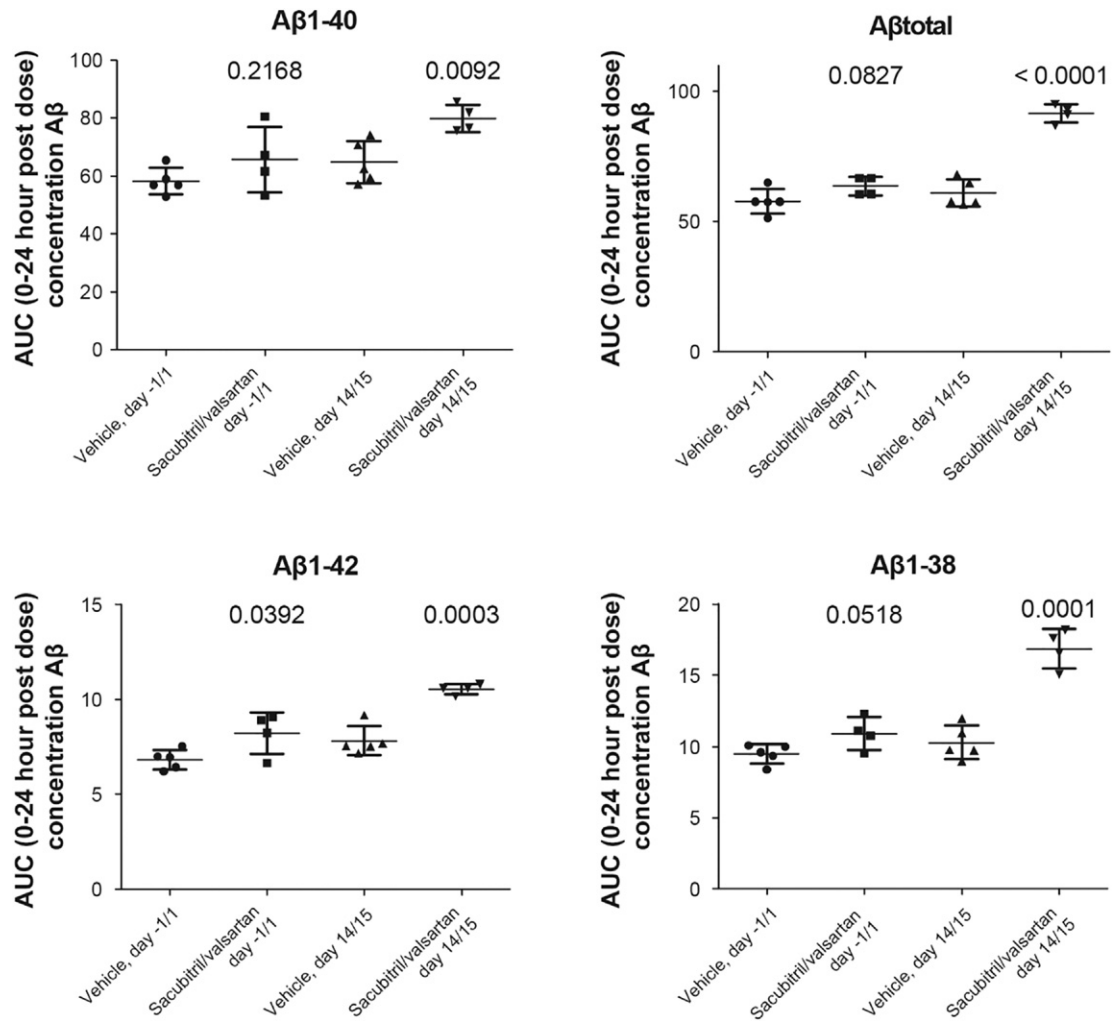


Fig. 5. 2-week SILK™-Aβ study: AUC for the newly generated Aβ for the various Aβ isoforms in CSF. Similar results were noted for the total labeled and unlabeled Aβ isoforms in the CSF (See Fig. S1). The *p*-values shown were derived from a *t*-test comparison of vehicle and sacubitril/valsartan groups.

relative clearance rate. Upregulation of Aβ clearance from the brain tissue by alternative pathways, or maintained clearance of Aβ through the CSF by active or passive transport, are possibilities following longer-term exposure.

In contrast to a study in healthy human volunteers, where elevations of Aβ1-38, but not Aβ1-40 and Aβ1-42, were observed in the CSF following sacubitril/valsartan treatment (Langenickel et al., 2016), we report elevations in the more aggregable form Aβ1-42, in the shorter forms Aβ1-40, Aβ1-38, as well as Aβtotal in the CSF of cynomolgus monkeys at day 15. This may reflect species-specific differences in the clearance of Aβ or assay-specific differences in the methods used (multiplexed immunoassay vs. SILK™ method, respectively). Indeed, there were no statistically significant increases in CSF levels of Aβ1-42 or Aβ1-40 in the samples collected at 2 hour post dose on day 16, as assessed by a multiplex immunoassay (Fig. 6C); however, large inter-animal variability in CSF levels may also have confounded the interpretation of detectable changes in these single timepoint evaluations, given the relatively small-sized sample set. Given that CSF concentrations of Aβ1-40 and Aβ1-42 were unaltered in healthy volunteers administered sacubitril/valsartan (400 mg QD for 2 weeks), this may suggest a limited contribution of NEP to the Aβ degrading capacity and/or a possible up-regulation of redundant pathways for Aβ clearance in humans, which may differ in non-human primates.

Across species, there is weak to no expression of NEP in the regions associated with Aβ plaque formation, namely the cerebral cortex and hippocampus (Matsas et al., 1985; Matsas et al., 1986; Pollard et al.,

1987; Bourne et al., 1989; Gaudoux et al., 1993; Akiyama et al., 2001; Takeuchi et al., 2008; Chambers et al., 2010), potentially reinforcing the importance of other Aβ clearance pathways in these areas; immunostaining for Aβ in the cynomolgus monkey brain showed no evidence of Aβ accumulation in these regions. The brain regions with the highest NEP expression included the basal ganglia (caudate nucleus, putamen and globus pallidus) and some thalamic, pontine, cerebellar, and medullary nuclei. While soluble Aβ levels were not assessed in these brain regions, immunostaining for Aβ in the primate model showed no evidence of Aβ accumulation in these regions.

Our studies demonstrate that NEP was not expressed in the choroid plexus of cynomolgus monkeys; this is in contrast to demonstrated expression of NEP in the human choroid plexus (Matsas et al., 1985). The significance of this apparent species difference with regards to Aβ is unclear. However, the absence of NEP expression in the primate choroid plexus would suggest that elevations of CSF Aβ are not related to inhibition of NEP in the choroid plexus. It is of interest to note that the epithelial cells of the choroid plexus form the blood-CSF barrier and mediate both secretion of proteins and processes that clear substances from the CSF and blood. Choroid plexus dysfunction has been associated with impaired Aβ clearance (Gonzalez-Marrero et al., 2015).

The relative contribution of different proteases, including NEP, to Aβ metabolism in different species has not been entirely elucidated (Carson and Turner, 2002); nevertheless, NEP appears to play a role in Aβ degradation in rodents and primates (Nalivaeva et al., 2014). In addition to redundant proteolytic clearance pathways, Aβ is also cleared

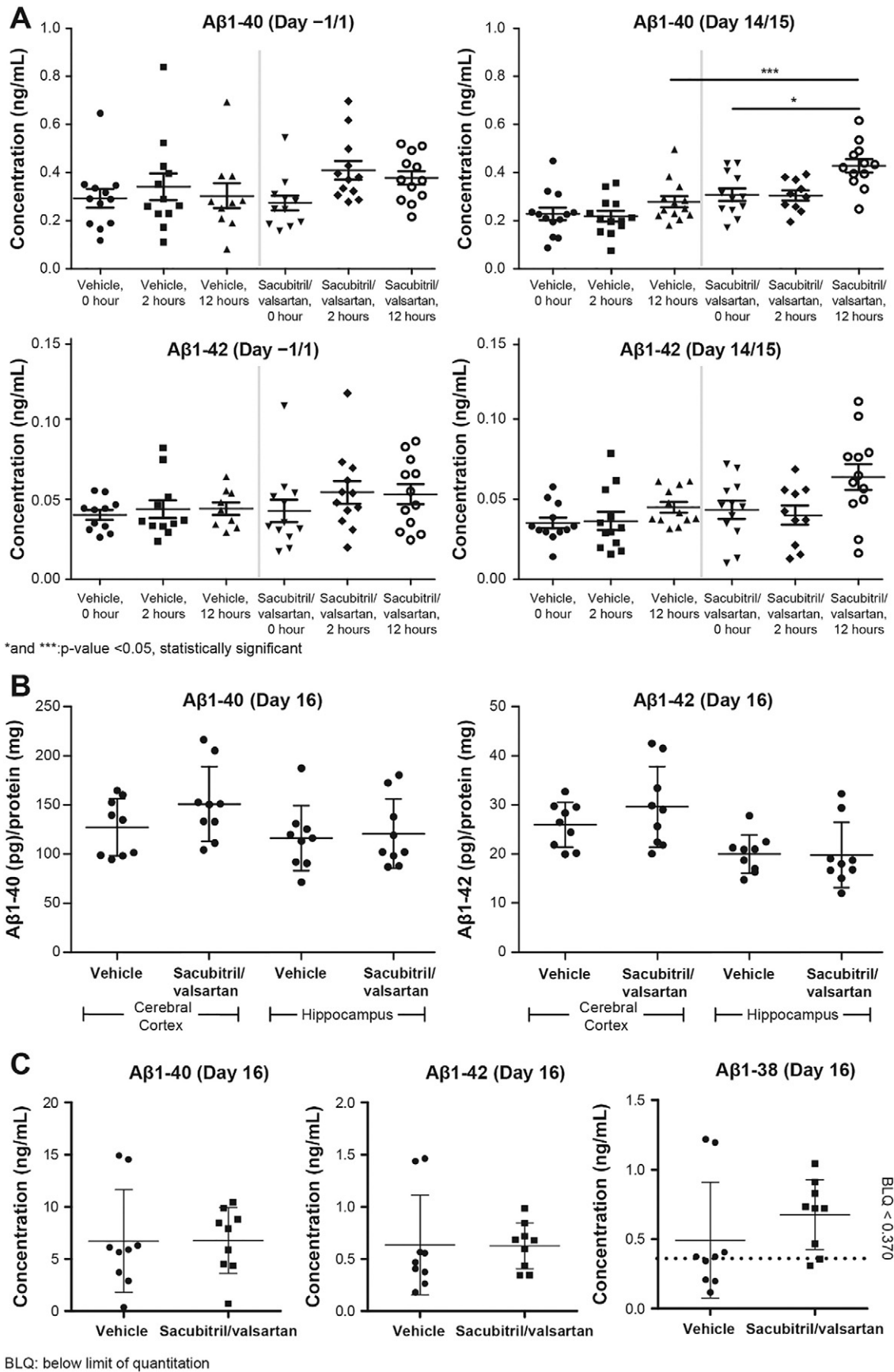


Fig. 6. 2-week SILK™-Aβ study: concentration of Aβ1-40 and Aβ1-42 in plasma at 0, 2 and 12 hour post dose on day 1 and day 15 (A); concentration of Aβ1-40 and Aβ1-42 in the (B) cortex and hippocampus and (C) CSF at necropsy (day 16, 2 hour post dose).

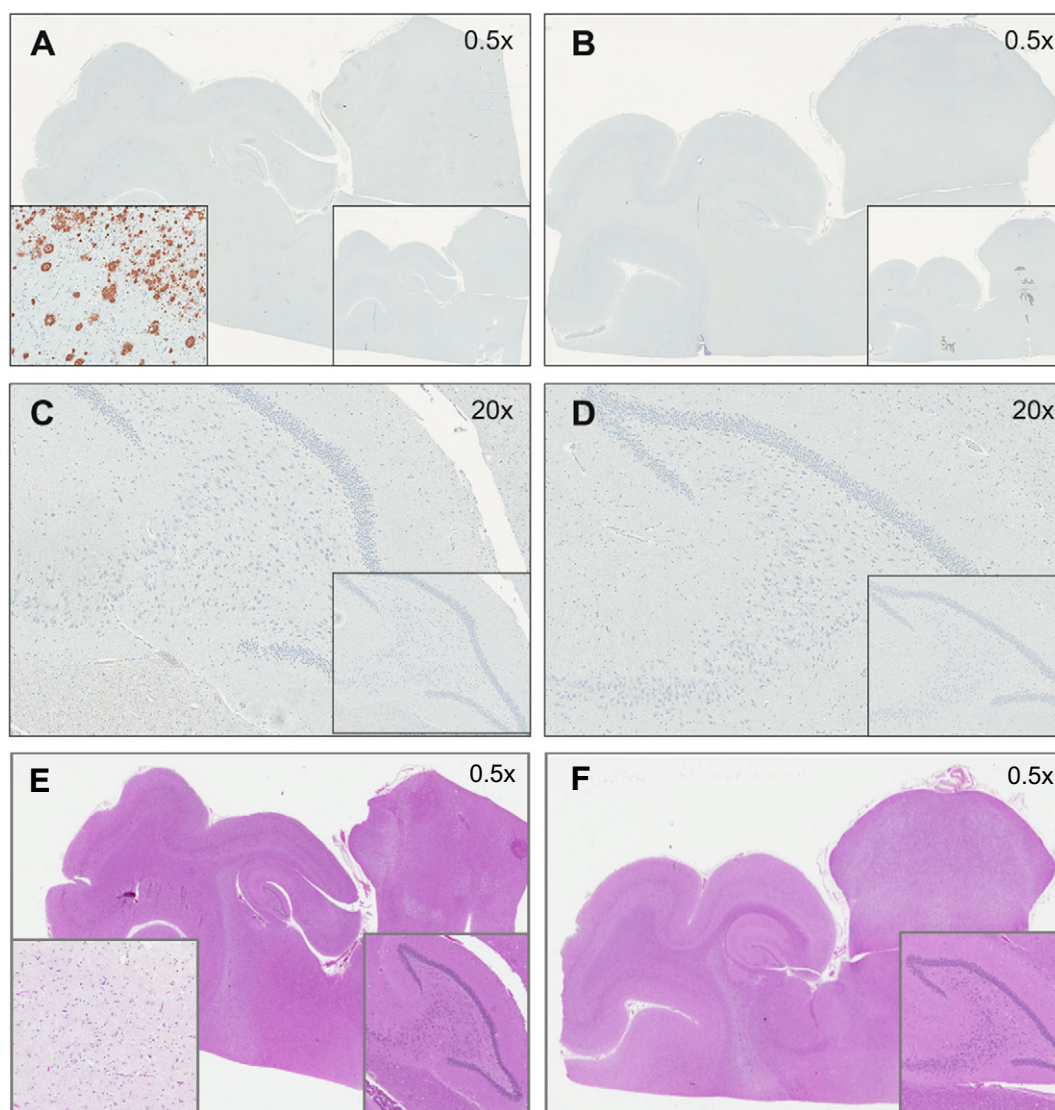


Fig. 7. 39-week study: localization of A β in cynomolgus monkey brain. Immunohistochemistry utilizing rabbit monoclonal antibody D12B2 was performed on control (A and C) and sacubitril/valsartan-treated (B and D) animals and did not detect A β (left insert positive control brain, right insert irrelevant antibody negative control). Lower magnification views of transverse sections of brain containing cerebral cortex and midbrain were shown in A and B. Representative images from hippocampus are shown in C and D. Standard H/E stained sections were also evaluated from control (E) and sacubitril/valsartan treated (F) animals (left insert positive control brain stained with H/E).

from the CNS by non-enzymatic processes, including cell mediated clearance and passive and active transport (Wang et al., 2006; Leissring, 2008; Saido and Leissring, 2012; Tarasoff-Conway et al., 2015). There are also no human genetic data suggesting that alteration in enzymes that remove A β are involved in the development of AD. In a meta-analysis of genome wide association studies totaling 74,064 individuals, neither NEP nor other enzymes implicated in A β enzymatic clearance were associated with late-onset AD (Lambert et al., 2013). Furthermore, persons with a complete loss of NEP protein have not been reported to display overt early onset impairment of cognitive function, although no brain imaging studies or specific cognitive tests were reportedly carried out (Debiec et al., 2004).

Treatment with ARNI, sacubitril/valsartan, resulted in an overwhelming benefit of sacubitril/valsartan on morbidity and mortality in patients with heart failure with reduced ejection fraction (McMurray et al., 2014) leading to early study termination due to efficacy and expedited review and approval by the US Food and Drug Administration and

the European Medicines Agency. The theoretical mechanistic concern of NEP inhibition impacting A β metabolism is being further characterized in ongoing and planned clinical studies.

Study limitations

Limitations of this study included the age and health status of animals used, given that young (2–4-year old) healthy cynomolgus monkeys are not representative of the older heart failure population that will be prescribed sacubitril/valsartan. Furthermore, spontaneous A β plaque formation has not been observed in cynomolgus monkeys under the age of 15 years and non-human primates do not display dementia and neurodegenerative changes present in human AD which develop over decades of life (Heuer et al., 2012). Although A β deposition was evaluated in animals chronically treated for 39 weeks (i.e. the maximum nonclinical study duration required by ICH guidance (ICH M3(R2)) to support human clinical trials and marketing authorization) at dose levels providing sacubitrilat

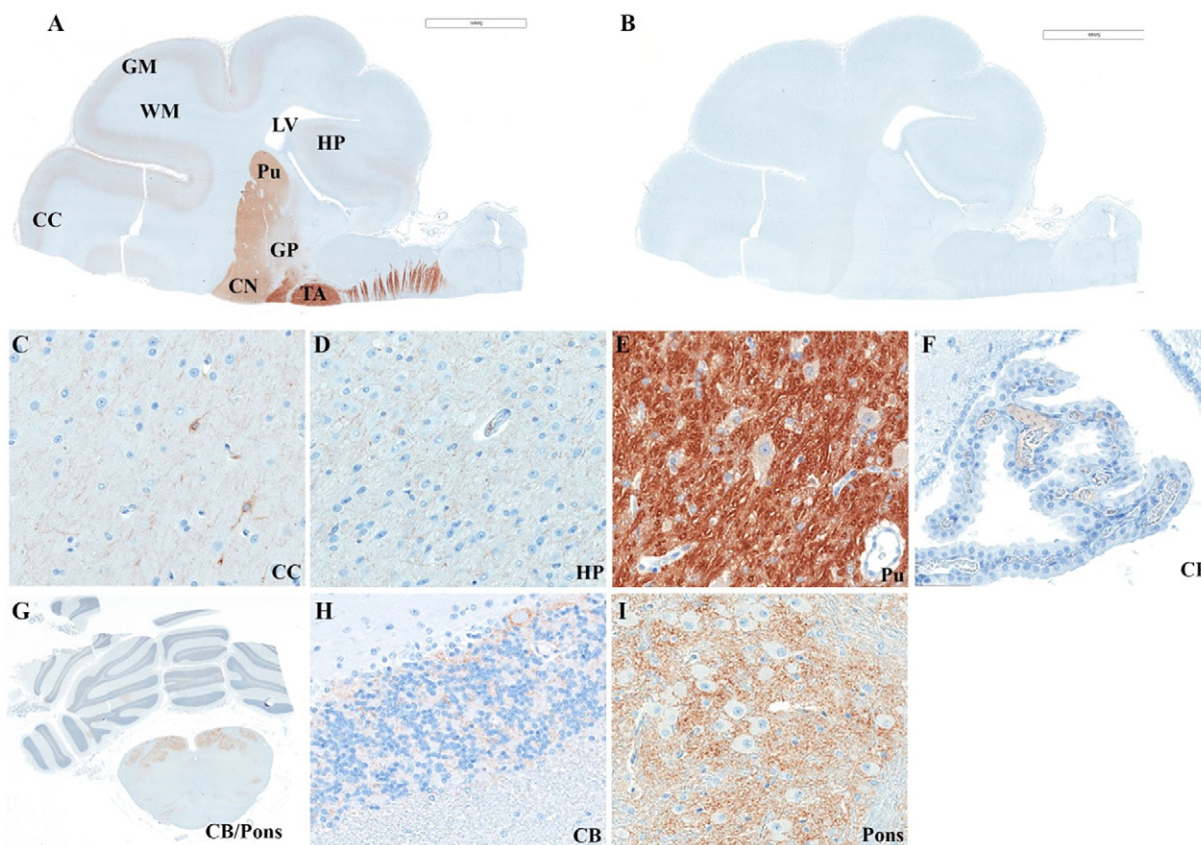


Fig. 8. Localization of NEP protein in cynomolgus monkey brain. Representative brain sections from a single animal are shown. Distribution of NEP in a transverse section of brain at the level of hippocampus is shown (A). Note that NEP is predominantly expressed in basal ganglia (caudate nucleus, putamen, and globus pallidus) and in some thalamic nuclei. NEP is weakly expressed in hippocampus and cerebral cortex. Irrelevant (rabbit IgG1) antibody control did not show immunoreactivity (B). Higher magnification images from this section of brain (A) are shown in images C–F. Note strong NEP immunostaining in striatum (E) with weaker staining in cerebral cortex (C) and hippocampus (D) and no signal in choroid plexus (F). NEP immunostaining in brain stem and cerebellum are shown in images G–I. Note that some pontine, cerebellar, and medullary nuclei express NEP. CB, cerebellum; CC, cerebral cortex; CN, caudate nucleus; GM, gray matter; GP, Globus pallidus; HP, hippocampus; LV, lateral ventricle; Pu putamen; TA, thalamus; and WM, white matter.

exposure that exceeded clinical exposures, it is acknowledged that this period of time may still not be sufficient to detect the full effects of long-term chronic treatment with sacubitril/valsartan on plaque deposition.

In the 2-week SILKTM-A β study, differences were observed in the elimination half-life between day 1 and day 15 and may be expected based on the day 1 non-steady state condition and the day 15 steady state condition. As a result, only the stable label clearance phase was impacted by NEP inhibition on day 1 whereas both the synthesis phase and clearance phases were impacted by NEP inhibition on day 15. The CSF pooling strategy employed to provide sufficient sample volume for assaying both A β concentration and drug concentration was also not equivalent for pharmacokinetic and A β assessments and so pharmacokinetic/pharmacodynamic comparisons between animals could not be conducted.

5. Conclusion

In conclusion, these data show sacubitril/valsartan acutely impaired the clearance of A β isoforms from the CNS of cynomolgus monkeys, but clearance normalized by day 15 as expected due to steady state kinetics. A β levels in the hippocampus and cerebral cortex of sacubitril/valsartan-treated animals were similar to controls 2 hour post dose on day 16 and concentrations of sacubitrilat in the brain were low. These data potentially indicate that compensatory mechanisms may be in place to restore A β clearance with chronic NEP inhibition. The relationship between A β concentrations in the CSF and brain are not well understood. Brain immunostaining of primates treated for 39 weeks revealed no evidence of A β deposition. In view of the absence of A β accumulation

in the brain, the clinical relevance of acutely-impaired clearance of A β from the CSF by NEP inhibition is unclear. Further clinical studies assessing cognitive function in patients with heart failure are ongoing.

Transparency document

The [Transparency document](#) associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.taap.2017.03.014>.

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