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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 (Aβ), there is a theoretical risk of Aβ 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 Aβ isoforms in cynomolgus monkeys using the sensitive Stable Isotope Labeling Kinetics (SILK™)-Aβ methodology.

The in vitro selectivity of valsartan, sacubitril, and its active metabolite sacubitrilat was established; sacubitrilat did not inhibit other human Aβ-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 SILK™-Aβ. 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 Aβ1-42 (65.3%; $p = 0.026$), Aβ1-40 (35.2%; $p = 0.04$) and Aβtotal (29.8%; $p = 0.04$) acutely; this returned to normal as expected with repeated dosing for 15 days. CSF concentrations of newly generated Aβ ($AUC_{(0–24\ h)}$) indicated elevations in the more aggregable form Aβ1-42 on day 1 (20.4%; $p = 0.039$) and day 15 (34.7%; $p = 0.0003$) and in shorter forms Aβ1-40 (23.4%; $p = 0.009$), Aβ1-38 (64.1%; $p = 0.0001$) and Aβtotal (50.45%; $p = 0.00002$) on day 15. However, there were no elevations in any Aβ 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 Aβ deposition, as assessed by immunohistochemical staining, were present. Further clinical studies are planned to address the relevance of these findings.

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

Abbreviations: Aβ, 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; FFPE, formalin-fixed paraffin-embedded; FLT, fluorescence lifetime; IC_{50}, 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; SILK™, Stable Isotope Labeling Kinetics; TTR, tracer-to-tracee ratio.

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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 (Aβ). Aβ 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 Aβ 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 Aβ 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, Aβ 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 Aβ 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 Aβ subtypes (Aβ1-42 and Aβ1-40) are found in senile plaques of the brains of patients with Alzheimer's disease (Glenner et al., 1984; Iwatsubo et al., 1994; Iwatsubo et al., 1995). However the exact role of Aβ 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™-Aβ technology to assess changes in CSF Aβ associated with inhibition of Aβ clearance pathways by a neprilysin inhibitor. The cynomolgus monkey was selected as an appropriate preclinical model to assess the risk for changes in Aβ 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 Aβ 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 Aβ clearance, Aβ concentrations in CSF, brain and plasma as well as Aβ brain deposition. The 2-week SILK™-Aβ study was an investigative study to evaluate the effect of sacubitril/valsartan treatment on Aβ concentrations and clearance in serial samples of CSF using the SILK™ technique. The SILK™-Aβ methodology has been previously used in monkeys to assess changes in CSF Aβ associated pathways of Aβ 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 Aβ 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 Aβ 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 μM 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 μM (0.8 μM 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 IC_{50} 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-Pro-Gly-Phe-Ser-Ala-Phe-Lys(Dnp)-OH (R&D Systems Europe Ltd., Abingdon, United Kingdom) to produce a final concentration of 2 μM. 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 IC_{50} 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.