Sacubitril is selectively activated by carboxylesterase 1 (CES1) in the liver and the activation is affected by CES1 genetic variation

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Abstract

Sacubitril was recently approved by the FDA for use in combination with valsartan for the treatment of patients with heart failure with reduced ejection fraction (HFrEF). As a prodrug, sacubitril must be metabolized (hydrolyzed) to its active metabolite LBQ657 in order to exert its intended therapeutic effects. Thus, understanding the determinants of sacubitril activation will lead to the improvement of sacubitril pharmacotherapy. The objective of this study was to identify the enzyme(s) responsible for the activation of sacubitril, and determine the impact of genetic variation on sacubitril activation. First, an incubation study of sacubitril with human plasma and the S9 fractions of human liver, intestine, and kidney was conducted. Sacubitril was found to be activated by human liver S9 fractions only. Moreover, sacubitril activation was significantly inhibited by the CES1 inhibitor bis-(p-nitrophenyl) phosphate (BNPP) in human liver S9. Further incubation studies with recombinant human carboxylesterase 1 (CES1) and carboxylesterase 2 (CES2) confirmed that sacubitril is a selective CES1 substrate. The in vitro study of cell lines transfected with wild type CES1 and the CES1 variant G143E (rs71647871) demonstrated that G143E is a loss-of-function variant for sacubitril activation. Importantly, sacubitril activation was significantly impaired in human livers carrying the G143E variant. In conclusion, sacubitril is selectively activated by CES1 in human liver. The CES1 genetic variant G143E can significantly impair sacubitril activation. Therefore, CES1 genetic variants appear to be an important contributing factor to interindividual variability in sacubitril activation, and have the potential to serve as biomarkers to optimize sacubitril pharmacotherapy.
Introduction

Entresto®, a combination of sacubitril, the first-in-class neprilysin inhibitor, and the angiotensin II receptor blocker valsartan, was approved by the FDA in July 2015 for the treatment of patients with heart failure with reduced ejection fraction (HFrEF). The randomized double-blind clinical trial PARADIGM-HF, which involved 8442 patients with class II-IV HFrEF, demonstrated that Entresto® significantly reduced rates of mortality and morbidity compared to the angiotensin-converting enzyme (ACE) inhibitor enalapril (e.g., first hospitalization for worsening heart failure (12.8% vs 15.6%), death from cardiovascular causes (13.3% vs 16.5%), and all-cause mortality (17.0% vs 19.8%)) (McMurray et al., 2014). Due to its established efficacy, Entresto® is expected to be widely used to treat patients with HFrEF, with annual sales estimated to reach as high as $8 billion by 2020. However, therapeutic outcomes varied significantly in patients treated with valsartan/sacubitril based upon previously published clinical studies (McMurray et al., 2014; Packer et al., 2015).

Sacubitril is an inactive ester prodrug that needs to be converted \textit{in vivo} to its active metabolite LBQ657 (Figure 1), a potent neprilysin inhibitor, in order to produce its intended pharmacological effects (Ksander et al., 1995). Thus, it is plausible to speculate that patients who cannot efficiently activate sacubitril may respond poorly to the treatment. To date, the enzyme(s) and organ(s) responsible for sacubitril activation have remained unknown. Significant interindividual variability in plasma concentrations of both sacubitril and its active metabolite LBQ657 was observed in clinical pharmacokinetic studies (Gu et al., 2010; Gan et al., 2015). However, the contributing
factors to the variation in sacubitril activation and therapeutic outcomes have not been explored. The study of sacubitril-activating enzyme(s) could lead to a better understanding of the interindividual variability in sacubitril pharmacokinetics, and has the potential to identify patients who will or will not benefit from sacubitril therapy.

In the present study, we utilized several complementary in vitro experimental approaches to identify the enzyme(s) responsible for the activation of sacubitril and to determine the impact of genetic variation on sacubitril activation.
Materials and Methods

Materials

Sacubitril was purchased from MedKoo Biosciences (Chapel Hill, NC). The hydrolytic active metabolite of sacubitril, LBQ657, was obtained in our laboratory following incubation of 100 μM sacubitril with 50 ng/μl recombinant human carboxylesterase 1 (CES1) (R&D Systems Inc., Minneapolis, MN) at 37 °C for 2 h. Sacubitril was completely hydrolyzed to LBQ657 after incubation for 2 h as determined by LC-MS/MS analysis. Ritalinic acid, enalapril, fluorescein diacetate, fluorescein, bis-(p-nitrophenyl) phosphate (BNPP), LC–MS grade methanol, acetonitrile, and formic acid were all purchased from Sigma–Aldrich (St. Louis, MO). The enalapril hydrolytic metabolite enalaprilat dehydrate was purchased from Sellechchem (Houston, TX). Taq DNA polymerase with standard Taq buffer and deoxynucleotide (dNTP) solution mix were obtained from New England Biolabs (Ipswich, MA). Human intestine and kidney S9 fractions were purchased from XenoTech (Lenexa, KS). Recombinant human CES1 and carboxylesterase 2 (CES2) were obtained from R&D Systems Inc. (Minneapolis, MN). All other chemicals and reagents were of the analytical grade and commercially available.

A total of 53 individual normal human liver samples were obtained from the XenoTech LLC (Kansas City, KS), the Cooperative Human Tissue Network (Columbus, OH), and the Liver Tissue Cell Distribution System at the University of Minnesota (Minneapolis, MN). Two samples' demographic information was unknown. The other liver samples consisted of 25 males, 26 females with ages ranging from 1 to 81 years (57.6±15.3 years). The donors included 49 Caucasians and 2 African-Americans.
Preparation of S9 fractions from individual human livers and CES1 transfected cells

Individual human liver S9 fractions were prepared according to previous studies (Wang et al., 2015). Protein concentrations were determined using a Pierce BCA assay kit (Pierce, Rockford, IL). The Flp-In™ 293 cell lines stably expressing wild type (WT) CES1 and the G143E variant (rs71647871) were developed in a study previously published by our laboratory (Zhu et al., 2008). Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After reaching approximately 95% confluence, cells were washed and harvested in phosphate buffered saline (PBS) buffer. Afterward, cells were sonicated and then centrifuged at 9000g for 30 min at 4°C. The supernatant (S9 fractions) was collected and stored at –80°C until use.

Enzymatic Assays

An in vitro incubation study was conducted to determine the activation of sacubitril in human plasma, liver, intestine, and kidney S9 fractions, as well as in recombinant CES1 and CES2 enzymes. The incubations were carried out in 1.5 ml Eppendorf tubes with a final volume of 40 µl. Sacubitril, tissue S9 fractions, plasma, and recombinant CES1 and CES2 were all prepared in PBS buffer. Hydrolytic reactions were initiated by mixing 20 µl of sacubitril solution with equal volumes of the S9 fractions, plasma, recombinant CES1, or CES2. The final concentrations of sacubitril, S9 fractions, and recombinant CES1 and CES2 were 200 µM, 50 µg/ml, 5 ng/µl, and 5 ng/µl, respectively. After incubation at 37°C for 10 min, the reactions were terminated by the addition of 120 µl of acetonitrile containing the internal standard (IS) ritalinic acid (33 µM). The mixture was
vortexed for 5 min and centrifuged at 17,000g for 10 min to remove precipitated proteins. The supernatant was collected and quantified for LBQ657 concentrations by an LC-MS/MS assay described below. The hydrolysis rate of sacubitril was calculated according to the formation of LBQ657. Incubations of sacubitril with PBS were carried out under the same experimental conditions as a negative control to determine the non-enzymatic hydrolysis of sacubitril.

For the kinetic study, recombinant CES1 was prepared in PBS buffer at a concentration of 5 ng/μl. The hydrolysis reaction was initiated by mixing 20 μl of recombinant CES1 and 20 μl of sacubitril with the concentrations ranging from 31.25 μM to 2000 μM. The formation of LBQ657 was determined after incubation at 37 °C for 10 min.

An in vitro study was conducted to evaluate the effect of CES1 inhibition on sacubitril activation. The formation of LBQ657 was determined after incubation of sacubitril (200 μM) with pooled HLS9 (0.05 mg/ml) at 37 °C for 10 min in the presence of various concentrations of the CES1 inhibitor BNPP (0-100 μM).

The selective CES1 substrate enalapril and the selective CES2 substrate fluorescein diacetate were included in the in vitro incubation study as positive controls to measure CES1 and CES2 activity, respectively. The formation of enalaprilat, the hydrolytic metabolite of enalapril, was determined by an LC-MS/MS assay described previously (Wang et al., 2015). Fluorescein, the hydrolytic metabolite of fluorescein diacetate, was detected using a Synergy™ 2 Multi-Mode Microplate Reader (BioTek Inc., Winooski, VT) with an excitation wavelength of 485 nm and an emission wavelength of 525 nm as reported by Wang et al (Wang et al., 2011). All incubation experiments were performed...
in triplicate except for the study of individual human liver S9 fractions (HLS9) samples for which the experiment was duplicated for each sample.

**LC-MS/MS analysis of sacubitril and LBQ657**

The LC–MS/MS analysis was performed on a Shimadzu HPLC system (Shimadzu, Tokyo, Japan) coupled with an Applied Biosystems API 4000 triple quadrupole/linear ion trap (QTRAP) mass spectrometer (Foster City, CA, USA). Analytes were separated on a Shimadzu VP-ODS column (5μm, 150×2.0mm, Shimadzu, Tokyo, Japan). The quantification of sacubitril and its metabolite LBQ657 was performed based on a previously reported method with slight modifications (Gu et al., 2010). The mobile phase consisted of water containing 0.1% formic acid (v/v) (phase A) and acetonitrile containing 0.1% formic acid (v/v) (phase B), and was delivered at a flow rate of 0.2 ml/min. A gradient elution was applied for the separation with the time program set as follows: phase B was increased from 35% to 90% during the time period of 0 to 7 min, maintained at 90% for 1 min, then returned to 35% at 9 min, and maintained until the end of run (12 min). MS was operated on a positive ion mode using turbo electrospray ionization. The following transitions were monitored on a Multiple Reaction Monitoring (MRM) mode: sacubitril, m/z 412.7 > 266.7; LBQ657, m/z 384.7 > 266.7; IS, m/z 220.5 > 84.6.

Quantifications were performed based on the peak area ratios of analyte to IS. The regression coefficients of the calibration curves were greater than 0.99 for both sacubitril and LBQ657. Three quality controls representing low, medium, and high concentrations (100, 1000, and 5000 nM) of each analyte were included in every batch of analyzed
samples. Interday and intraday relative standard deviations were less than 10% for both sacubitril and LBQ657.

**CES1 genotyping study**

Genomic DNA was extracted from human liver samples using the PureLink® Genomic DNA Mini Kit (Life Technologies, CA) according to the manufacturer’s instruction. The extracted DNA was further purified using the PureLink® Genomic DNA Mini Kit. The DNA concentrations were determined by Qubit® dsDNA High Sensitivity Assay (Life Technologies, CA) using a Qubit® 2.0 Fluorometer (Invitrogen, CA). The PCR primers for the amplification of CES1 exon 4, where the CES1 variant G143E resides, were as follows: GACATCTTCTGAGTGCTCCCTG (forward); TGTGTCAACCTTCACCTGCTG (reverse). The PCR conditions involved initial denaturation at 95°C for 30s; 30 cycles of 95°C for 30s, 63°C for 45s, and 68°C for 50s; and a final extension for 5 min at 68°C. The PCR products were purified with the PureLink® PCR Purification Kit (Life Technologies, CA) and analyzed with agarose (2%) gel electrophoresis before being subjected to Sanger sequencing to determine the G143E genotypes.

**Data analysis**

Data are presented as mean ± standard deviation (SD). $K_m$ and $V_{max}$ of CES1-mediated sacubitril activation were calculated using the Michaelis-Menten equation ($v = (V_{max} \times [S])/(K_m + [S])$). Apparent intrinsic clearance ($CL_{int}$) was determined by $V_{max}/K_m$. Mann–Whitney test was utilized to analyze the differences of CES1 expression and activity.
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between the wild type and the G143E genotype groups (Graphpad Prism software version 6.0; Graphpad Software, San Diego, CA, USA). A $P$-value less than 0.05 was considered statistically significant. The IC$_{50}$ value in the inhibition study was calculated by nonlinear regression using the 3-parameter Hill equation.
Results

Sacubitril is a selective CES1 substrate, and is activated in the liver

Sacubitril hydrolysis was determined after incubations with human liver, intestine, and kidney S9 fractions (HLS9, HIS9, and HKS9), as well as with human plasma. As shown in Figure 2, sacubitril was rapidly hydrolyzed to its active metabolite LBQ657 in HLS9 samples, whereas no appreciable hydrolysis was observed after incubation with human plasma, HIS9, or HKS9 samples.

To evaluate hydrolytic activity of the plasma, HLS9, HIS9 and HKS9 samples, enalapril, the selective CES1 substrate, and fluorescein diacetate, the selective CES2 substrate, were included in the study as positive controls under the same experimental conditions for sacubitril (Figure 2) (Wang et al., 2011; Wang et al., 2015). As expected, enalapril was efficiently hydrolyzed to its metabolite enalaprilat in HLS9 samples, but not in plasma, HIS9, or HKS9 samples. Fluorescein diacetate was readily hydrolyzed to its metabolite fluorescein in HLS9, HIS9, and HKS9 samples, where CES2 is present, but not in human plasma.

To further define the role of CESs in the hydrolysis of sacubitril, sacubitril and fluorescein diacetate were incubated with purified recombinant human CES1 and CES2. Sacubitril could only be hydrolyzed by CES1 (Figure 3A). In contrast, fluorescein diacetate could only be metabolized by CES2.

In addition, a kinetic study was carried out for sacubitril hydrolysis with recombinant human CES1. The data fit well according to Michaelis-Menten kinetics (Figure 3B).
Under our experimental conditions, the $V_{\text{max}}$, $K_{m}$ and $CL_{\text{int}}$ values were determined to be $557.5 \pm 18.1 \text{ nmol/mg CES1/min}$, $767.2 \pm 56.4 \text{ M}$ and $726.9 \pm 56.7 \text{ l/mg CES1/min}$, respectively.

The *in vitro* inhibition study demonstrated that the CES1 inhibitor BNPP can inhibit sacubitril hydrolysis in HLS9 in a concentration-dependent manner (Figure 4). Sacubitril activation was inhibited by approximately 90% when the concentration of BNPP reached 10 $\text{ M}$. The IC$_{50}$ was determined to be 0.6 $\text{ M}$ under the present experimental conditions.

**The CES1 polymorphism G143E is a loss-of-function variant for the hydrolysis of sacubitril, and sacubitril activation is impaired in human livers carrying the 143E allele**

Sacubitril was incubated with the cell S9 fractions prepared from the Flp-In$^{\text{TM}}$ 293 cells stably expressing WT CES1 and the variant G143E. Similar to the HLS9 samples, the S9 fractions of WT CES1 cells efficiently hydrolyzed sacubitril to its active metabolite LBQ657. However, no appreciable hydrolytic metabolite LBQ657 was observed after incubation with the S9 samples from cells expressing the 143E allele (Figure 5).

A total of 104 individual human liver samples were genotyped for the G143E variant. Five individuals were found to be the G143E heterozygotes while the others were non-carriers (Wang et al., 2015). Sacubitril activation was studied in the five carriers and 48 randomly selected non-carriers. As shown in Figure 6, both CES1 activity and expression varied markedly in the individual human liver samples with 29-fold and 7-fold differences in sacubitril hydrolysis rate and CES1 protein expression, respectively. The
CES1 activity on sacubitril activation was significantly correlated with CES1 expression in the tested individual human livers. The $R$-squared values were 0.53 and 0.64 for the total 53 samples and the 48 non-G143E-carriers, respectively. The activation rates of sacubitril were significantly lower in the carriers relative to that in the non-carriers (4.2 ± 1.0 vs 7.2 ± 4.0 nmol/mg protein/min, $P = 0.025$, Figure 7). CES1 expression in the carriers and non-carriers was determined in a previously published study (Wang et al., 2015), and no significant differences were found between the two groups ($P = 0.47$, Figure 7).
Discussion

Although hydrolases are expressed in many human tissues, the liver, intestines, kidneys, and plasma are responsible for the majority of hydrolytic activities. Besides playing an important role in physiological processes such as lipid metabolism, these enzymes are critical for the metabolism of many therapeutic agents. Human hydrolases are distinct in substrate selectivity and specific expression patterns. For example, both CES1 and CES2 are expressed in human liver, but CES1 is the primary hepatic hydrolase contributing to approximately 80-95% of hydrolytic activity in the liver (Imai, 2006). On the other hand, the intestines and kidneys have no CES1 expression, but instead express high levels of CES2 (Wang et al., 2015). In human plasma, the major hydrolases include paraoxonase and butyrylcholinesterase; neither CES1 nor CES2 is expressed here (Bahar et al., 2012).

Our *in vitro* incubation studies revealed that sacubitril is hydrolyzed by the liver, but not by the intestines, kidneys, or plasma, indicating that sacubitril is selectively activated by hepatic CES1. This conclusion is further supported by the observations that sacubitril was efficiently activated by purified recombinant CES1 but not CES2.

With regard to substrate specificity, in general, CES1 prefers substrates with small alcohol group and large acyl group, such as oseltamivir and methylphenidate, while CES2 recognizes substrates with large alcohol group and small acyl group, such as prasugrel and fluorescein diacetate (Sun et al., 2004; Shi et al., 2006; Hosokawa, 2008; Williams et al., 2008; Wang et al., 2011; Laizure et al., 2013). Sacubitril is an ester prodrug with a small alcohol group and a large acyl group (Figure 1). Thus, the finding
that sacubitril is metabolized by CES1 instead of CES2 is consistent with the substrate preferences of the two carboxylesterases.

The CL_{int} value for CES1-mediated sacubitril hydrolysis was determined to be 726.9 \mu l/mg CES1/min, which is higher than that of the established CES1 substrates d-methylphenidate (35.6 \mu l/mg CES1/min) and dabigatran etexilate (27.2 \mu l/mg CES1/min), suggesting sacubitril is a highly efficient CES1 substrate (Sun et al., 2004; Laizure et al., 2014).

Significant interindividual variability in CES1 expression and activity has been consistently demonstrated by our research group and many others (Hosokawa et al., 1995; Shi et al., 2006; Yoshimura et al., 2008; Hagihara et al., 2009; Yang et al., 2009; Zhu et al., 2009; Ross et al., 2012). CES1 variants are considered an important contributing factor to CES1 variability (Zhu et al., 2008; Laizure et al., 2013; Rasmussen et al., 2015). We have found that hepatic CES1 expressions were significantly correlated with the activity on sacubitril metabolism, and the \( R^2 \) was 0.53, indicating approximately half of the variability in sacubitril hydrolysis could be explained by CES1 expression variation. In addition, we further analyzed the correlation after excluding the samples carrying the G143E variant, and observed an increase of correlation with an \( R^2 \) value of 0.64. Thus, the G143E variant appears to contribute to approximately 10% variation of sacubitril activation in our samples.
The CES1 genetic variant G143E is a loss-of-function variant that was originally discovered in a poor metabolizer of methylphenidate, a CES1 selective substrate, in a clinical pharmacokinetic study (Zhu et al., 2008). The minor allele frequency of G143E ranges from 2% to 4% among different populations except in Asians in which the variant is very rare (Zhu et al., 2008). Our incubation studies with transfected cell lines revealed that G143E is a loss-of-function variant for the activation of sacubitril. Consistent with the results from the transfected CES1 cells, sacubitril activation was significantly impaired in human livers with the 143G/E genotype relative to those with the 143G/G genotype. Several clinical studies have consistently demonstrated that this variant was associated with significantly altered pharmacokinetics and/or pharmacodynamics of several CES1 substrate drugs including methylphenidate, clopidogrel, oseltamivir, and enalapril (Patrick et al., 2007; Zhu et al., 2008; Nemoda et al., 2009; Tarkiainen et al., 2012; Lewis et al., 2013; Tarkiainen et al., 2015a; Tarkiainen et al., 2015b). For example, attention deficit hyperactivity disorder (ADHD) patients with the G143E genotype required significantly lower doses of methylphenidate for symptom reduction (0.41 ± 0.13 vs 0.57 ± 0.15 mg/kg, \( P = 0.022 \)) (Nemoda et al., 2009). In another study, the AUC\(_{0-\infty}\) of clopidogrel and its active metabolite were found to be 123% (\( P = 0.004 \)) and 67% (\( P = 0.009 \)) larger in the subjects carrying the G143E variant relative to the non-carriers. Consistent with the pharmacokinetic data, the carriers exhibited significantly greater inhibition of P2Y12-mediated platelet aggregation (Tarkiainen et al., 2015a).

It should be noted that marked interindividual variability in sacubitril activation exists even in human livers without the G143E variant (Figure 7), indicating that other unknown genetic and/or non-genetic factors may affect sacubitril metabolism by CES1.
The CES1 gene is highly polymorphic with over 2000 genetic variants registered in NCBI dbSNP database. However, very few of these variants have been studied for their potential effect on CES1 expression and/or activity. In addition to genetic variation, non-genetic factors such as CES1 inhibitors may affect CES1 function as well (Laizure et al., 2013). For example, sacubitril activation can be significantly inhibited by the model CES1 inhibitor BNNP (Figure 4). Therefore, a wide range of genetic and non-genetic factors could affect CES1 activity and consequently alter the pharmacokinetics and the therapeutic outcomes of sacubitril.

In summary, our study has provided compelling evidence supporting the hypothesis that sacubitril is selectively activated by hepatic CES1 in humans, and that genetic variants of the CES1 enzyme can significantly affect sacubitril activation. Thus, patients who carry certain CES1 variants, such as the G143E, may not be receiving an adequate response to sacubitril due to the impairment of sacubitril activation. These patient groups may be unnecessarily exposed to a novel drug which may not successfully deliver the proposed therapeutic advantages of sacubitril therapy. Moreover, they may also find themselves with a considerable financial burden, as Entresto® costs approximately $4,000 – $5,000/year, whereas enalapril costs approximately $50 –$100/year. In order to improve its therapeutic outcomes, further basic and clinical research on CES1 pharmacogenetics is necessary to establish CES1 variants as valid biomarkers to optimize sacubitril pharmacotherapy.
Authorship Contributions

Participated in research design: Shi, Wang, Bleske, Zhu

Conducted experiments: Shi, Wang, Nguyen

Performed data analysis: Shi, Wang, Zhu

Wrote or contributed to writing of the manuscript: Shi, Wang, Nguyen, Wu, Bleske, Zhu
References


Footnotes

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Figure Legends

**Figure 1.** A scheme of the metabolism of sacubitril to its active metabolite LBQ657.

**Figure 2.** Hydrolysis of sacubitril (A), enalapril (B), and fluorescein diacetate (C) by pooled human plasma, and the S9 fractions prepared from human liver (HLS9), intestine (HIS9), and kidney (HKS9). The hydrolytic products were determined after the substrates were incubated with the enzymes at 37 °C for 10 min. The final protein concentrations of plasma, HLS9, HIS9, and HKS9 were 23.4, 0.05, 0.125 and 0.125 mg/ml, respectively. The concentrations of sacubitril, fluorescein diacetate and enalapril were 200, 100, and 100 μM, respectively. Data are expressed as the formation rate of hydrolytic products (Mean ± S.D., n=3).

**Figure 3.** Hydrolysis of sacubitril and fluorescein diacetate by recombinant human CES1 and CES2 (A), and enzymatic kinetics of recombinant CES1-catalyzed sacubitril hydrolysis (B). The hydrolytic reactions were carried out at 37 °C for 10 min. The final concentration of recombinant CES1 and CES2 was 5 ng/μl. The concentrations of sacubitril and fluorescein diacetate were 200 μM and 100 μM, respectively (A), whereas the concentrations of sacubitril ranged from 31.25 μM to 2000 μM in the kinetic study (B). Data were presented as means ± S.D. (n=3).

**Figure 4.** Inhibition of sacubitril hydrolysis by CES1 inhibitor BNPP in pooled HLS9. The formation of LBQ657 was determined after incubation of sacubitril (200 μM) with pooled HLS9 (0.05 mg/ml) at 37°C for 10 min in the presence of various concentrations of the CES1 inhibitor BNPP (0-100μM). Hydrolytic activity was expressed as the
percentage of the activity determined when BNPP is absent. Data are presented as means ± S.D. (n=3).

**Figure 5.** Activation of sacubitril in cell S9 fractions prepared from cells stably transfected with wild type (WT) CES1 and the variant G143E. The formation of the active metabolite LBQ657 was determined after incubation of sacubitril with the S9 fractions at 37°C for 10 min. Final concentrations of sacubitril and cell S9 fractions were 200 μM and 0.05 mg/ml, respectively. Data are presented as means from three independent experiments with error bars representing S.D..

**Figure 6.** Correlation analysis of the hydrolysis rate of sacubitril with CES1 expression in 53 human liver S9 fraction (HLS9) samples. The final concentrations of HLS9 and sacubitril in the reaction system were 0.05 mg/ml and 200 μM. The formation of LBQ657 was determined after incubation with individual HLS9 at 37°C for 10 min. Data are means from two independent experiments. A: all samples (n=53); B: G143E non-carriers only (n=48).

**Figure 7.** Effect of the CES1 variant G143E on sacubitril activation (left panel) and CES1 protein expression (right panel) in human liver samples. The final concentrations of HLS9 and sacubitril in the reaction system were 0.05 mg/ml and 200 μM, respectively. LBQ657 formed from sacubitril hydrolysis was determined after incubation of the substrate with individual HLS9 at 37°C for 10 min. Data are mean values of two independent experiments. Horizontal bars represent mean values in each group. Mann–Whitney test was utilized to test the differences of CES1 expression and activity between
different G143E genotypes. A $P$-value less than 0.05 was considered statistically significant.
Figure 1

Sacubitril  \rightarrow \text{Activation} \rightarrow \text{LBQ657}
**Figure 3**

**A**

![Bar graph showing LBQ657 Formation Rate (nmol/mg protein/min) for rCES1 and rCES2.](image)

**Fluorescein Formation Rate (pmol/mg protein/min)**

- **Sacubitril**
- **Fluorescein**
- **Diacetate**

**B**

![Graph showing Formation rate of LBQ657 (nmol/mg CES1/min) vs Sacubitril Conc. (µM).](image)

\[ V_{max} = 557.5 \pm 18.1 \text{ nmol/mg CES1/min} \]

\[ K_m = 767.2 \pm 56.4 \mu\text{M} \]
Figure 4

Relative hydrolysis rate of sacubitril (%) vs. BNPP (µM)

IC$_{50}$ = 0.60 µM
Figure 5

LBQ657 Formation Rate (nmol/mg protein/min)

WT CES1

Vector

G143E CES1
**Figure 6**

A

Formation rate of LBQ567 (nmol/mg protein/min)

- $R^2 = 0.53, P < 0.0001$
- **●** G143E carrier
- **●** G143E non-carrier

B

Formation rate of LBQ567 (nmol/mg protein/min)

- $R^2 = 0.64, P < 0.0001$

CES1 Expression (ng/μg protein)
Figure 7

* $P = 0.025$

Formation rate of LBQ657 (nmole/mg protein/min)

CES1 Expression (ng/μg protein)

Activity

Expression