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Different susceptibility of BNP and proBNP to neprilysin cleavage suggests a limited effect of neprilysin inhibition by LCZ696 on the level of immunoreactive BNP

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Introduction

The new FDA approved heart failure (HF) drug LCZ696 (Entresto™, Novartis), which combines the neprilysin inhibitor and the angiotensin II receptor inhibitor, has enhanced the interest of the cardiology community in neprilysin. This ubiquitous protease is responsible for the degradation of a number of important vasoactive peptides, including natriuretic peptides (NPs). In light of this, the inhibition of neprilysin activity for the augmentation of the endogenous NPs concentrations has been considered to be a potential therapeutic strategy in HF (see **Figure 1**). It was suggested that by increasing the circulating concentrations of B-type natriuretic peptide (BNP), LCZ696 could make BNP measurements ambiguous and misleading from a diagnostic perspective.

However, the predominant form of plasma BNP-immunoreactivity in HF patients (which is measured by commercial BNP immunoassays) is represented by its uncleaved precursor - proBNP, which differs from BNP due to the presence of the 76 amino acid N-terminal extension. Therefore, the inhibition of neprilysin in HF patients may only affect the level of immunoreactive BNP if proBNP is also a substrate of neprilysin. To the best of our knowledge, neprilysin mediated degradation of proBNP has never been analyzed. Furthermore, the susceptibility of different BNP epitopes to neprilysin-dependent proteolysis is unknown.

Hereby, the aims of the present study were:
(i) To compare the susceptibility of BNP and proBNP to cleavage by neprilysin;
(ii) To compare the susceptibility of two different epitopes within the central region of BNP and proBNP to cleavage by neprilysin.

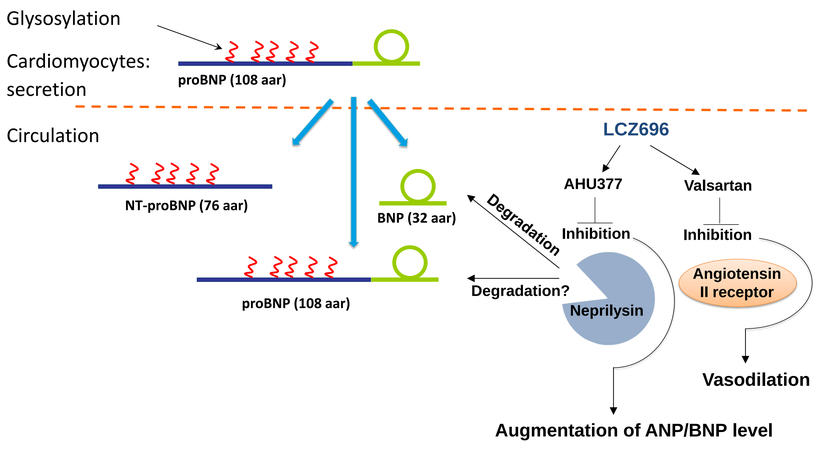


FIGURE 1. The mechanism of action of LCZ696 (Entresto™) and its influence on neprilysin-mediated BNP/proBNP degradation.

Methods

Synthetic BNP 1-32 (Bachem) as well as nonglycosylated (expressed in E. coli) and glycosylated (expressed in mammalian cells) forms of proBNP 1-108 (both from HyTest) were incubated with human recombinant neprilysin (R&D systems) for different time periods.
The susceptibility of two different epitopes of BNP and proBNP (within the ring structure of BNP) that are recognized by antibodies in commercial BNP assays to neprilysin cleavage was analyzed by utilizing two sandwich immunoassays. In the first assay, the mAb KY-BNP-II (epitope 14-21, Shionogi) was used as a capture antibody while the mAb 50E1 (epitope 26-32, HyTest) was used as a detection antibody labeled with stable europium chelate. Three commercially available BNP immunoassays utilize mAb KY-BNP-II: two come from Siemens (formerly Bayer and Dade Behring) and one comes from Shionogi.
The second assay was the Single Epitope Sandwich BNP assay (SES-BNP™) that is specific to the epitope 11-17 (capture mAb 24C5 and detection mAb Ab-BNP2, HyTest). This assay is implemented in the platform by ET Healthcare. A schematic representation of two BNP assays used in this study is shown in **Figure 2**.
Mass-spectrometry (MS) was applied to assess the sites of BNP cleavage by neprilysin.

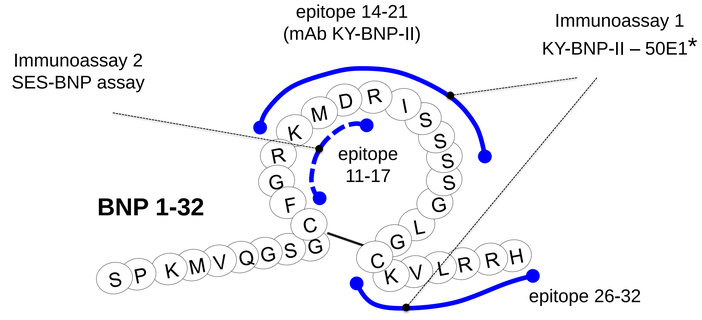


FIGURE 2. Epitopes recognized by BNP immunoassays used in the study.

Results

Neither form of proBNP - nonglycosylated or glycosylated - was degraded by neprilysin when measured by the KY-BNP-II – 50E1 assay or the SES-BNP assay (see **Figure 3**).
The SES-BNP assay (epitope 11-17) was found to be much less susceptible to the BNP cleavage by neprilysin as compared with the immunoassay that utilized the mAb Ky-BNP-II (epitope 14-21). The SES-BNP assay detected 62.4% of immunoreactive BNP after 4 h of incubation with neprilysin. Conversely, the immunoassay utilizing the mAb Ky-BNP-II detected just 7.4% (see **Figure 3**).
The sites of *in vitro* BNP cleavage by neprilysin as determined by MS are shown in **Figure 4**. Notably, proBNP remained intact even after 6 h of incubation with neprilysin as followed from MS analysis, which confirmed the results obtained by immunoassays.

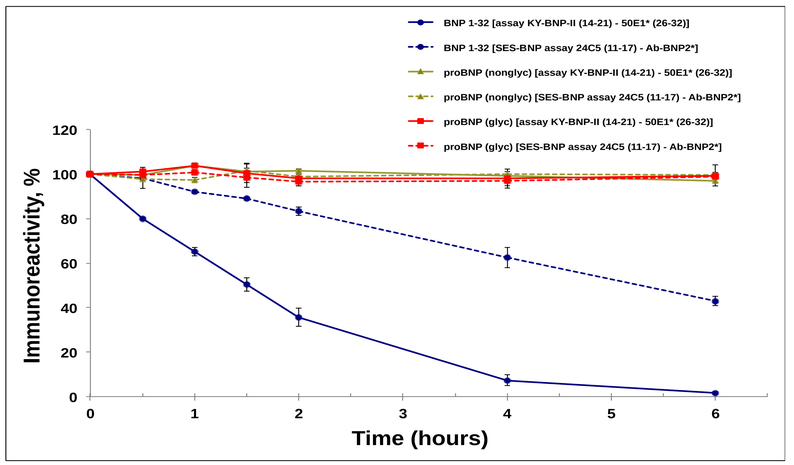
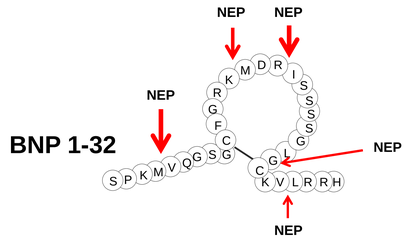


FIGURE 3. Degradation of BNP 1-32, nonglycosylated (expressed in *E. coli*) and glycosylated (expressed in mammalian cells) proBNP 1-108 by the action of neprilysin.



Incubation time	Bonds hydrolyzed
0.5 h	Met_4-Val_5
2 h	Met_4-Val_5, Arg_{17}-Ile_{18}
4 h	Met_4-Val_5, Arg_{17}-Ile_{18}, Lys_{14}-Met_{15}, Gly_{23}-Leu_{24}, Val_{28}-Leu_{29}

FIGURE 4. Sites of BNP cleavage by neprilysin (NEP) detected by MS analysis.

Conclusions

1. **Our findings demonstrate that the major BNP-immunoreactive form - proBNP - is not susceptible to neprilysin cleavage. On this basis, we suggest that modulation of neprilysin activity by specific inhibitors (e.g. LCZ696) may not greatly affect the circulating concentrations of immunoreactive BNP in HF patients.**
2. **BNP immunoassays that utilize antibodies with epitopes comprising the site Arg_{17}–Ile_{18} are expected to be more sensitive to proteolysis by neprilysin than immunoassays that utilize antibodies with other specificity.**

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