Endothelin-Converting Enzyme/Neutral Endopeptidase Inhibitor SLV338 Prevents Hypertensive Cardiac Remodeling in a Blood Pressure–Independent Manner

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Abstract—Hypertensive heart disease is a major contributor to cardiovascular mortality. Endothelin is a potent vasoconstrictive and profibrotic mediator produced by the endothelin-converting enzyme (ECE), whereas natriuretic peptides, degraded by the neutral endopeptidase (NEP), have diuretic, vasodilatory, and antifibrotic properties. Thus, combined ECE/NEP inhibition may halt hypertensive cardiac remodeling. This study examined effects of SLV338, a novel ECE/NEP inhibitor, on cardiac protection in experimental renovascular hypertension (2-kidney, 1-clip [2K1C]). Male rats were allocated to 5 groups: sham-operated rats, untreated animals with 2K1C, 2K1C animals treated with oral SLV338 (30 and 100 mg/kg per day), and 2K1C animals treated with oral losartan (20 mg/kg per day). Treatment duration was 12 weeks. Blood pressure was assessed every 4 weeks. At study end, hearts were taken for histology/computer-aided histomorphometry/immunohistochemistry. Pharmacological properties of SLV338 are described. SLV338 is a dual ECE/NEP inhibitor, as demonstrated both in vitro and in vivo. In the 2K1C study, losartan lowered blood pressure by \( \leq 46 \text{ mm Hg} \), whereas both dosages of SLV338 had no effect. However, SLV338 (both dosages) completely normalized cardiac interstitial fibrosis, perivascular fibrosis, myocyte diameter, and media:lumen ratio of cardiac arteries, as did losartan. Cardiac transforming growth factor-\( \beta \) expression was significantly enhanced in untreated 2K1C rats versus controls, whereas treatment with SLV338 and losartan prevented this effect. Taken together, dual ECE/NEP inhibitor SLV338 prevents cardiac remodeling to the same extent as losartan, but in a blood pressure–independent manner, in a rat model of renovascular hypertension. This effect is at least partially mediated via suppression of cardiac transforming growth factor-\( \beta \) expression. \textit{(Hypertension. 2011;57:00-00.)}

Key Words: renovascular hypertension ■ cardiac remodeling ■ endothelin-converting enzyme ■ neutral endopeptidase ■ TGF-\( \beta 1 \)

Hypertension is responsible for 54% of stroke, 47% of ischemic heart disease, and 25% of other cardiovascular diseases worldwide; a total of 6.7 million deaths worldwide (\( \approx 13.5\% \) of all) are attributed to high blood pressure.\(^1\) Other than stroke or myocardial infarction, the development of heart failure is a major contributor to cardiovascular mortality. Hypertensive heart failure and death are the last steps of a cardiac remodeling process characterized by progressive left ventricular hypertrophy and fibrosis.\(^2\) Moreover, very recently the Action to Control Cardiovascular Risk in Diabetes Trial demonstrated that, even in a cardiovascular high-risk population of diabetic patients, there is no effect of further blood pressure lowering with regard to cardiovascular outcome.\(^3\) Thus, novel therapeutic strategies beyond blood pressure lowering to halt cardiac remodeling are vital.

Endothelin (ET) 1 is a potent proinflammatory and profibrotic mediator in the heart\(^4,5\) that contributes to heart failure. It is derived from a biologically inactive precursor, Big-ET-1, by action of ET-converting enzyme(s) (ECEs).\(^6\) Because clinical trials with endothelin receptor antagonists in heart failure rendered disappointing results, which were probably attributable to complex systemic actions of these antagonists, research has now been focusing on ECE-inhibiting compounds.\(^7\) Another approach to curb hypertensive cardiac remodeling is to enhance plasma levels of vasodilatory, antihypertrophic, and antifibrotic mediators like the natriuretic peptides (ANP, BNP,
and CNP\textsuperscript{8} by inhibiting their degradation via the neutral endopeptidase (NEP). However, because NEP also degrades ET-1, sole NEP inhibition has been shown to cause a net vasoconstriction attributed to endothelin action prevailing over, that is, offsetting, natriuretic peptide action in resistance vessels in humans.\textsuperscript{9} Therefore, the rationale for creating combined ECE/NEP inhibitors is 2-fold: combined ECE/NEP inhibition reduces ET-1 production and unveils the beneficial effects of NEP inhibition via increased natriuretic peptide signaling without concomitant increase (or even with a decrease) of ET-1 levels. Moreover, blockade of the ET-system leads to salt and fluid retention because of the role of ET-1 in sodium handling in the kidney,\textsuperscript{10,11} which has been a major drawback in clinical studies. However, because salt and fluid retention are attributed to nonselective ET receptor blockade, it cannot be excluded that ECE inhibition, which inhibits action of ET-1 on both receptors, might experience similar clinical limitations. Combination with NEP inhibition and subsequent increase of natriuretic peptide action might be a feasible way to overcome this problem.

SLV338 is a novel combined ECE/NEP inhibitor. In this study we report the in vitro and in vivo features of this compound with special focus on the prevention of cardiac remodeling in a model of renovascular hypertension in comparison with standard end organ protection with losartan.

**Materials and Methods**

**Chemicals and Receptor Binding Assays**

Unless otherwise stated, all of the reagents were of analytic grade and were purchased from Sigma, Merck, and Roth. SLV338 was synthesized by Solvay Pharmaceuticals, now Abbott Products GmbH.

The receptor binding affinities of SLV338 were evaluated in a broad panel of receptors and ion channels by Cerep. For receptor binding assays, the inhibition constants were evaluated in a broad panel of receptors and ion channels by Cerep. For receptor binding assays, the inhibition constants were calculated from the Cheng-Prushoff equation. Results were expressed as mean p inhibition constant values of 2 separate experiments done in duplicate.

**In Vitro and In Vivo Assessment of ECE and NEP Inhibition by SLV338**

The potency of SLV338 to inhibit the enzymatic activities of NEP (E.C. 3.4.24.11) and ECE-1 (E.C. 3.4.24.71) was investigated in an assay using recombinant human enzymes and modified 17 amino acid truncated Big-ET-1 as a substrate. This peptide substrate contains a fluorescence label (7-methoxycoumarin-4-yl) at its N terminus, and the quencher (3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl) in its internal sequence. Because NEP and ECE-1 cleave this peptide, their activity leads to an unquenching and, thus, quantifiable increase of the 7-methoxycoumarin-4-yl fluorescence.

All of the animal experiments were carried out in accordance with the German and French legislation on the use of laboratory animals. To evaluate the efficacy and potency of SLV338 with regard to the compound’s ability to inhibit ECE in vivo, the effect of SLV338 to prevent the acute blood pressure response to Big-ET was examined in anesthetized male Sprague-Dawley rats, based on the notion that ECE inhibition reduces the conversion of Big-ET-1 to the active binding assays, the inhibition constants were calculated from the Cheng-Prushoff equation. Results were expressed as mean p inhibition constant values±SD of ≥2 separate experiments done in duplicate.

\textsuperscript{AQ:2}
from Bachem). Blood pressure and heart rate were recorded every 5 minutes for the next 15 minutes. The increase in blood pressure was expressed as the percentage of response to Big-ET-1 in vehicle-treated animals.

NEP inhibition by SLV338 in vivo was assessed using the degradation route for ANP via NEP. Accordingly, NEP inhibition increases ANP plasma levels and enhances ANP-dependent natriuresis and urinary cGMP excretion. Functional inhibition of NEP by SLV338 in vivo was examined using anesthetized male Sprague-Dawley rats. Catheters were placed in 1 jugular vein for vehicle or SLV338 administration, in the contralateral jugular vein for ANP infusion, and in the bladder for urine collection and assessment of natriuresis and urinary cGMP levels.

After equilibration following initiation of anesthesia, 2 mL of 0.9% NaCl were slowly administered IV, followed by a continuous infusion of 62.5 μL of 0.9% NaCl per minute for 60 minutes. Thirty minutes after the start of this volume load, rats were treated with SLV338 by bolus injection of 1 mg/kg in 1 mL of vehicle per kilogram, followed by continuous intravenous infusion of SLV338 at a constant rate of 33 μg/kg per minute in 20 μL per minute until the end of the experiment. Fifteen minutes later, a continuous intravenous administration of rat ANP started, at a rate of 300 ng/kg per minute in 62.5 μL of 0.9% NaCl per minute for a total duration of 60 minutes, followed by continuous infusion of 62.5 μL of 0.9% NaCl per minute until the end of the experiment.

### In Vivo Assessment of Cardioprotective Properties of SLV338 in Renovascular Hypertensive Rats

#### Study Design

Rats were subjected to unilateral clipping of the renal artery to produce renovascular hypertension in a 2-kidney, 1-clip (2K1C) model. In brief, after a 2-week acclimatization period, male Sprague-Dawley rats (205 to 283 g) were anesthetized using ketamine/xylazine 100/10 mg/kg IM. A U-shaped silver clip (0.20-mm ID) was placed around the left renal artery through a dorsal flank incision. Sham animals underwent the same procedure without clipping. Afterward, animals were allocated to 5 groups: (1) sham surgery (sham; n=6); (2) untreated 2K1C (2K1C; n=12); (3) 2K1C plus SLV338 at 30 mg/kg per day (2K1C+SLV338 low dose; n=12); (4) 2K1C plus SLV338 at 100 mg/kg per day (2K1C+SLV338 high dose; n=12); and (5) 2K1C plus Losartan at 20 mg/kg per day (2K1C+LO; n=12). All of the animals received a standard diet with water ad libitum during the study period. SLV338 was administered orally (mixed with food at 30 and 100 mg/kg per day) for 12 weeks, starting 2 weeks (week 0) after left kidney clipping. The angiotensin II type 1 (AT1) receptor antagonist losartan (20 mg/kg per day) was administered under the same conditions. Systolic

**Figure 3.** Systolic blood pressure was assessed repeatedly during the course of the 2K1C study. Oral treatment with SLV338 (30 or 100 mg/kg per day, 2K1C+SLV low dose and 2K1C+SLV high dose, respectively) or losartan (20 mg/kg per day, 2K1C+LO) was started at week 0, that is, 2 weeks after the clipping. All of the values are given as mean±SEM. ***P<0.001, **P<0.01, and *P<0.05 vs all 2K1C groups; ††P<0.01 vs sham operated.

### Table: Body, Heart, and Kidney Weight and Heart Rate at Study End

<table>
<thead>
<tr>
<th>Parameter</th>
<th>2K1C</th>
<th>2K1C+SLV38 Low Dose</th>
<th>2K1C+SLV38 High Dose</th>
<th>2K1C+LO</th>
<th>Sham Operated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>521.5±15.02</td>
<td>551.7±8.85</td>
<td>560.33±13.39</td>
<td>580.5±12.54</td>
<td>539.17±11.5</td>
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<tr>
<td>Heart weight, % of body weight</td>
<td>0.72±0.04</td>
<td>0.65±0.03</td>
<td>0.6±0.03</td>
<td>0.58±0.02</td>
<td>0.52±0.01</td>
</tr>
<tr>
<td>Heart weight, % of body weight</td>
<td>0.5±0.03</td>
<td>0.45±0.02</td>
<td>0.41±0.02</td>
<td>0.4±0.02</td>
<td>0.33±0.01</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>444.7±26.6</td>
<td>451.7±19.38</td>
<td>438.7±19.36</td>
<td>420.9±11.5</td>
<td>402.16±14.5</td>
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<tr>
<td>Left kidney, mg/100 g of body weight</td>
<td>210±20</td>
<td>226±21</td>
<td>218±14</td>
<td>208±14</td>
<td>292±5</td>
</tr>
<tr>
<td>Right kidney, mg/100 g of body weight</td>
<td>412±18</td>
<td>369±24</td>
<td>391±33</td>
<td>422±20</td>
<td>304±7</td>
</tr>
</tbody>
</table>

Values are given as mean±SEM.

*P<0.05 vs sham operated.

††P<0.01 vs sham operated.

††P<0.01 vs sham operated.

§P<0.05 vs 2K1C.

||P<0.01 vs 2K1C.
blood pressure (in millimeters of mercury) was measured via tail-cuff method (BP-2000, Visitech Systems) 2 weeks after surgery (week 0) and during weeks 4, 8, and 12 after the start of the treatment. Only 2K1C rats with systolic blood pressure ≥150 mm Hg at week 0 were included in the study protocol. At the end of the experiment (ie, in week 12), animals were euthanized under isoflurane anesthesia, and hearts were harvested for further studies.

**Histological Studies**

Tissue samples were all embedded in paraffin, cut into 3-μm sections, and subjected to Sirius Red, Elastica-van Gieson, and hematoxylin-eosin staining. Quantitative histomorphometry (ie, intima/media and lumen area of the arteries, interstitial fibrosis, and myocyte diameter) was analyzed using a computer-aided image analysis system as described previously.13,14

**Immunohistochemistry**

Paraffin-embedded sections fixed in 4% formaldehyde were dehydrated and rehydrated with xylene and alcohol series. For antigen unmasking, tissue sections were softly boiled in 10 mmol/L of citrate buffer. To quench endogenous peroxidase, all of the sections were incubated with 0.3% H₂O₂ in methanol. Nonspecific binding was blocked using 10% normal goat serum. Rabbit anti-transforming growth factor (TGF)-β1 antibody (dilution 1:50; Acris Antibodies GmbH) was applied overnight at 4°C, followed by biotinylated goat antirabbit secondary antibody (dilution 1:250; DAKO) and Vectastain Elite ABC kit (avidin/biotin/horseradish peroxidase system; Vector Laboratories). Visualization of peroxidase reaction was achieved using 3-amino-9-ethylcarbazole (DAKO) chromogen solution. Negative controls were prepared by leaving out the primary antibodies. TGF-β1 staining was quantified by image analysis as described for interstitial fibrosis measurement above.

**Statistical Analysis**

ANOVA was applied to detect any significant differences between groups; Student t test was used to detect significant differences between 2 groups of interest. Results were expressed as mean±SEM; differences were considered significant when the P value was <0.05.

**Results**

**In Vitro Selectivity Profile of SLV338**

In receptor binding experiments using cloned human receptors, SLV338 did not show relevant binding affinity (ie, p inhibition constant values were >5) toward a broad panel of...
The IC$_{50}$ of SLV338 for inhibition of ECE-1 is 5012 nmol/L.

channel, and potassium channels).

matergic, opioid, and a number of neuropeptide receptors and uptake sites. SLV338 did not show any interaction with ion channels (t-type calcium channel, voltage-gated sodium channel, and potassium channels).

The IC$_{50}$ of SLV338 for inhibition of NEP is 7.9 nmol/L.

in body weight in the 2K1C group treated with losartan versus both sham-operated and untreated 2K1C groups. However, this difference was <10% of body weight (versus sham controls, see Table). Regarding overall cardiac weight, 2K1C controls exhibited a significantly increased heart weight versus sham controls. Treatment with SLV338 (high dose) and with losartan significantly reduced cardiac weight versus untreated 2K1C animals, whereas treatment with low-dose SLV338 was accompanied by a nonsignificant trend to lower values. The same pattern was observed with left ventricular weight (Table). There was a significant decreased weight of the clipped kidney and a significant increased weight of the unclipped kidney in all of the 2K1C groups versus sham. There were no differences in kidney weight between the different treatment groups.

The results of the cardiac histology are illustrated in Figures 4 to 7. Interstitial fibrosis was markedly increased in the heart of 2K1C animals versus sham animals; treatment with both dosages of SLV338 and with losartan completely prevented this effect (Figure 4). The same pattern was observed with perivascular fibrosis (Figure 5). Media:lumen ratio of cardiac arteries was significantly elevated in non-treated 2K1C animals compared with sham controls. This effect was completely prevented by both doses of SLV338 and by losartan (Figure 6). Cardiomyocyte diameter was enlarged in nontreated 2K1C animals compared with sham-operated rats, and this was completely suppressed by both doses of SLV338 and losartan (Figure 7).

**Immunohistochemistry (TGF-β1 Expression)**

In sham-operated animals, only weak staining of TGF-β1 was observed in the myocardium, whereas in untreated 2K1C animals there was a significant elevation of TGF-β1 expression versus sham controls. Treatment with SLV 338 or losartan completely prevented this effect. The results are illustrated in Figure 8.
Discussion

This study describes the pharmacological properties of the novel combined NEP/ECE inhibitor SLV338 and its protective effects on cardiac remodeling in a rat model of renovascular hypertension. We demonstrated that SLV338 is a potent ECE/NEP inhibitor in vitro and in vivo. Importantly, these investigations have revealed for the first time that combined NEP/ECE inhibition has blood pressure–independent protective effects on cardiac remodeling, that is, it prevents cardiac hypertrophy, fibrosis, and vascular remodeling in rats with renovascular hypertension without affecting the blood pressure. Of note, these cardioprotective effects are comparable to those produced by losartan. This observation is remarkable on 2 accounts: systemic pathological changes occurring in this renovascular model of hypertension are largely driven by the activation of the renin-angiotensin system, and at least part of the cardiac remodeling is considered to be secondary to the hypertension that results from the renal artery clipping. Hence, the organ protection afforded by the AT\(_1\) antagonist, which decreased blood pressure but also exerted specific beneficial effects via cardiac AT\(_1\) receptors, was expected, but less so the effects of SLV338, which does not primarily target the renin-angiotensin system and had no antihypertensive activity at all in this model. The lack of antihypertensive activity of SLV338 in the 2K1C rat is consistent with the absence of antihypertensive effects of ET receptor antagonists found by other groups in this model; thus, the 2K1C model has been termed an “endothelin-independent” model of hypertension. Moreover, our results are consistent with our previous study using selective ETA and ET\(_B\) receptor antagonists in the same animal model. We found no effect of both substances on blood pressure; moreover, we demonstrated with regard to tissue concentrations of ET-1, Big-ET-1, ET\(_A\), and ET\(_B\) that an overall activation of the cardiac endothelin system was not detectable in the 2K1C rat, which does not exclude an activated ET system in specific cell types, for example, cardiac fibroblasts. Of note, it is known that, despite an absence of overall vascular ET-1 overexpression in 2K1C rats, topographically specific ET-1 overexpression, that is, in the coronary endothelium, exists. In our

Figure 7. Myocyte diameter was quantified in hematoxylin-eosin–stained cardiac sections by means of computer-aided histomorphometry, magnification ×1000 (see representative microscopical pictures). All of the values are given as mean ± SEM; ***P < 0.001 vs sham group; †††P < 0.001 vs 2K1C.
above-mentioned previous study we further demonstrated that ETA antagonism reduced vascular remodeling, whereas ETB antagonism reduced cardiac fibrosis, which supports the findings in our present study.

In our present study we demonstrated via receptor binding experiments in vitro that SLV338 has no binding affinity for angiotensin receptors, so a direct interaction with these receptors cannot account for the observed antiremodeling effects of the compound. On the other hand, natriuretic peptides are known to functionally antagonize the renin-angiotensin system. In the present study, plasma renin activity, which was strongly increased (6.2-fold) in non-treated 2K1C animals, was only partially and nonsignificantly reduced by SLV338 (by 32%; data not shown), but other components of the renin-angiotensin system were not examined.

Furthermore, it is conceivable that ET-1 mediates some of the effects of angiotensin II (downstream of the AT1 receptors). There are indeed examples of ET-1–dependent angiotensin/AT1-induced mechanisms. If this is true, one would expect that both drugs act via the same downstream effector pathways. To further clarify this point we investigated cardiac TGF-β1 expression by means of computer-aided immunohistochemistry, because TGF-β1 is not only a well-known profibrotic mediator, but moreover has been shown to be effected by both the renin-angiotensin system
and the endothelin system (for review of this topic see Khan et al). Indeed, we demonstrated in our study that the elevated cardiac TGF-β1 expression in untreated 2K1C rats was completely prevented by both SLY338 and losartan, thus strengthening the hypothesis that both drugs might be involved in the same pathway. Moreover, it is known that ET-1, angiotensin II, and TGF-β1 are also closely intertwined with connective tissue growth factor and platelet-derived growth factor forming a signaling network that is vital both in normal tissue repair and maladaptive fibrotic remodeling (for review see Leask). Additional studies targeting all of the components of this network alone or in combination are urgently needed to uncover an optimal therapeutic approach in maladaptive cardiac remodeling.

There are limited data on cardiac organ protection by ECE/NEP inhibition. In a model of salt-sensitive hypertension it was shown that dual ECE/NEP blockade decreased cardiac fibrosis and led to improvement of cardiac function. This effect was comparable to the effect of angiotensin-converting enzyme inhibition in the same study. However, because this model is based on salt overload, ECE/NEP inhibition is expected to curb the net salt accumulation via natriuretic peptide elevation. In line with this, ECE/NEP inhibition led to a decrease of blood pressure by ~50 mm Hg, comparable to the effect of the angiotensin-converting enzyme inhibitor. Another study by Mulder et al demonstrated in a postinfarction model of heart failure that ECE/NEP inhibition improves cardiac function and decreases collagen accumulation in the viable part of the left ventricle. However, the myocardial remodeling in that model is not attributed to hypertension but is secondary to cardiac ischemia, which produces a 27-fold upregulation of cardiac ET-1 one week after myocardial infarction, that is, the time point at which Mulder et al started treatment. Nonetheless, a significant lowering of blood pressure by ECE/NEP inhibition was observed in this postinfarction heart failure model. A study by Tikkanen et al also showed significant blood pressure reduction by ECE/NEP inhibition in diabetic rats and concomitant reduction in heart/body weight ratio, but no cardiac histology was performed. Interestingly, in none of the above cited studies dealing with ECE/NEP inhibition was there a significant beneficial impact of sole NEP inhibition on cardiac target organ protection. Thus, we did not include sole NEP inhibition in our study. Nevertheless, because we cannot exclude a significant contribution of NEP inhibition to the cardiac protection seen in our study, this clearly limits interpretation of our results.

**Perspectives**

Our study is the first to demonstrate blood pressure–independent cardioprotective effects of ECE/NEP inhibition, which encompassed several aspects of cardiac remodeling, that is, cardiomyocyte hypertrophy, interstitial fibrosis, and perivascular fibrosis, as well as coronary vascular remodeling in a rat model of renovascular hypertension. These cardioprotective effects are at least partially mediated via suppression of cardiac TGF-β1 expression. Because sole ET-1 receptor antagonism is severely hampered by concomitant fluid overload in clinical trials, combined ECE/NEP inhibition, which enhances natriuretic and diuretic action via natriuretic peptide elevation, might be a feasible way to unveil the clinical potential of endothelin antagonistic compounds in cardiovascular disease.

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**Disclosures**

K.H. and Y.F. are employees of Abbott Products, which owns the investigated compound. Parts of the histological evaluations were funded by Solvay Pharmaceuticals. B.H. is a former employee of Solvay Pharmaceuticals, now Abbott Products.

**References**


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