Renoprotective Effects of Combined Endothelin-Converting Enzyme / Neutral Endopeptidase Inhibitor SLV338 in Acute and Chronic Experimental Renal Damage

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SUMMARY

Background: Acute kidney injury (AKI) as well as chronic renal failure are associated with a huge mortality/morbidity. However, so far no drugs have been approved for the treatment of acute kidney failure and only a few for the treatment of chronic kidney disease (CKD). We analysed the effect of SLV 338, a neutral endopeptidase (NEP)/endothelin converting enzyme (ECE)-inhibitor in animal models of acute kidney failure as well as chronic renal failure.

Methods: Acute renal failure was induced in male Wistar rats by uninephrectomy and clamping of the remaining kidney for 55 minutes. SLV338 (total dose: 4.9 mg/kg) or vehicle was continuously infused for 2 hours (starting 20 minutes prior to clamping). Sham operated animals served as controls. Plasma creatinine was measured at baseline and day 2 and 8 after renal ischemia-reperfusion.

Hypertensive renal damage was induced in male Sprague Dawley rats by nitric oxide deficiency using L-NAME (50 mg/kg per day, added to drinking water for 4 weeks). One group was treated over the same time period with SLV338 (30 mg/kg per day, mixed with food). Systolic blood pressure was monitored weekly. At study end, urine and blood samples were collected and kidneys were harvested.

Results: Acute renal ischemia-reperfusion caused a 5-fold plasma creatinine elevation (day 2), which was significantly attenuated by more than 50 % in animals treated with SLV338 (p <0.05). Renal failure was accompanied by a 67 % mortality in vehicle-treated rats, but only 20 % after SLV338 treatment (p = 0.03 compared to sham controls).

Chronic L-NAME administration caused hypertension, urinary albumin excretion, glomerulosclerosis, renal arterial remodelling, and renal interstitial fibrosis. Treatment with SLV338 did not significantly affect blood pressure, but abolished renal tissue damage (interstitial fibrosis, glomerulosclerosis, renal arterial remodelling (p <0.05 versus L-NAME group in each case).

Conclusions: The dual ECE/NEP inhibitor SLV338 preserves kidney function and reduces mortality in severe acute ischemic renal failure. Moreover, combined ECE/NEP inhibition prevents hypertensive renal tissue damage in a blood pressure independent manner in L-NAME-treated rats.

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INTRODUCTION

Acute kidney injury (AKI) and chronic renal failure are disorders with high rates of morbidity and mortality [1,2,3,4]. To overcome these problems, new therapeutic strategies for tissue protection have recently emerged. Endothelin-1 (ET-1) is a potent proinflammatory and profibrotic mediator in the kidney [7]. It is cleaved from its biologically inactive precursor, Big-ET-1, by action of endothelin converting enzyme(s) (ECE) [8]. ET-1 has been implicated in several aspects of chronic kidney disease, including diabetic nephropathy [9,10]. Since endothelin receptor antagonists displayed a disappointing performance in some clinical trials; i. e. [11], which was probably due to complex systemic actions of these antagonists, research has now been focusing on ECE inhibiting compounds [12] as an antifibrotic strategy in cardiovascular target organ protection. An alternative approach is to enhance plasma or local levels of vaso-dilatory, anti-inflammatory, and antifibrotic mediators like the natriuretic peptides (ANP, BNP, CNP) [13] by inhibiting their degradation via the neutral endopeptidase (NEP). Thus, natriuretic peptides have been consistently shown to exert renoprotective effects in the setting of both acute and chronic renal diseases [14,15]. Interestingly, both principles (endothelin system blockade and preservation of natriuretic peptides) can be united in compounds with combined ECE/NEP inhibition. Those compounds reduce ET-1 production (ECE inhibition) and enhance the beneficial effects of natriuretic peptide signaling (NEP inhibition). Moreover, endothelin antagonist leads to salt and fluid retention due to the role of ET_{B} receptors in sodium handling in the kidney [16,17], which has been a major drawback in clinical studies. Combination with NEP inhibition and subsequent increase of natriuretic peptide action might be a feasible way to overcome this problem.

However, the potential of combined ECE/NEP inhibition in renal tissue protection has not been fully explored so far. Thus, the present study aimed at examining the potential of SLV338, a novel ECE/NEP inhibitor, in renal tissue protection in rat models of both acute and chronic renal damage. Correspondingly, this study consisted of two parts: In a rat model of severe acute ischemic renal damage the effects of SLV338 on mortality and renal function were assessed, while the action of the compound on renal histology was investigated using a rat model of hypertensive kidney damage induced by chronic nitric oxide deficiency (using the nitric oxide synthase inhibitor N(G)-nitro-L-arginine methyl ester, L-NAME).

MATERIALS AND METHODS

Chemicals

Unless otherwise stated all reagents were of analytical grade and were purchased from SIGMA (Seelze, Germany), MERCK (Darmstadt, Germany), and ROTH (Karlsruhe, Germany).

SLV338 was synthesized by Solvay Pharmaceuticals, now Abbott Products GmbH (Hannover, Germany). The receptor binding affinities of SLV338 were evaluated in a broad panel of receptors and ion channels by Cerep (Celle L’essecault, France). For receptor binding assays the inhibition constants (Ki) were calculated from the Cheng-Pruhoff equation \( \text{Ki} = \frac{\text{IC}_{50}}{1 + \frac{L}{\text{Kd}}} \), where \( \text{IC}_{50} \) is the compound concentration producing 50 % radioligand displacement, \( L \) is the concentration of radioligand in the assay, and \( \text{Kd} \) the affinity of the radioligand for the receptor. Results were expressed as mean pKi values ± SD of at least 2 separate experiments done in duplicate. If no significant (i.e. >40 %) ligand displacement was found at a concentration of 10 µM, SLV338 was concluded to have no relevant binding activity (pKi <5.0).

Study design

All animal experiments were carried out in accordance with the German and French legislations on the use of laboratory animals. The acute renal failure study was performed at Phenos GmbH (Hannover, Germany, on behalf of Solvay Pharmaceuticals GmbH, now Abbott products GmbH) under the animal experimentation license 33-42502-06/1117 obtained from the Lower Saxony State Office for Consumer Protection and Food Safety (Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit, Hannover, Germany). The in vivo phase of the L-NAME-induced chronic kidney damage study was carried out by Pelvipharm (Gif-sur-Yvette, France) on behalf of Solvay Pharmaceuticals GmbH, according the Animal Care Regulations in force in France as of 1988 (authorization from competent French Ministry of Agriculture - Agreement No. 91-86, 7/20/2001).

Acute ischemic renal failure

Male Wistar rats were anesthetized with isoflurane via a nose mask and placed supine on a heating table to maintain body temperature between 36 and 37 °C. Midline laparotomy was made and the left renal pedical was clipped with a microaneurysm clip for 55 minutes. Simultaneously a right nephrectomy was performed. The duration of ischemia was chosen to maximize reproductibility of renal injury. SLV338 (or vehicle) was administered intravenously as a starting bolus of 0.9 mg/kg 20 minutes prior to the ischemia, followed by a continuous infusion at a rate of 33 µg/kg/minute for 2 hours (thus giving a total dose of 4.9 mg/kg). At the end of the treatment period, the rats were allowed to wake up from the anesthesia and were observed over 8 days. An additional group underwent right a nephrectomy, but was
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not subjected to renal pedicle clamping (sham controls). Blood samples were obtained and serum creatinine was analyzed before the operation (baseline) and after the operation on day 2 and day 8.

L-NAME-induced hypertensive renal damage

Male Sprague Dawley rats were exposed for 4 weeks to a L-NAME administration (50 mg/kg per day, added to drinking water; rats receiving no L-NAME were used as controls, n = 12). Animals were treated over the same time period without (n = 24) or with SLV338 (administered at a dose of 30 mg/kg per day, mixed with food; n = 12). Systolic blood pressure was monitored weekly using the tail-cuff method. After 3 weeks of treatment, creatinine clearance and urinary albumin excretion were assessed using metabolic cages to obtain urine samples (collected on ice over a 24 hour period, at the end of which blood samples were drawn). Creatinine clearance was calculated using the standard formula. Finally, kidneys were harvested to evaluate effects of SLV338 on L-NAME induced end-organ remodelling.

Histological studies

In the study on L-NAME-induced hypertensive renal damage, kidneys were subjected to histological evaluation. Thus, renal tissue samples were embedded in paraffin, cut into 3 μm sections, subjected to Sirius Red, Elastica–van Gieson and periodic acid Schiff (PAS) staining. Quantitative histomorphometry (i.e. media and lumen area of the arteries, interstitial fibrosis) was analyzed using a computer-aided image analysis system as previously described [18]. In brief, the media/lumen ratio was evaluated after Elastica–van Gieson staining by means of computer-aided histomorphometry: microscopic pictures of renal arteries were transferred to a PowerMAC via a Hitachi-CCD-camera. The area values of media and lumen of arteries were measured using the ImageJ program (shareware from the NIH). Afterwards, the media/lumen ratio was calculated, serving as a marker for arterial wall thickening.

Interstitial fibrosis was evaluated in Sirius Red stained tissue slices by quantifying the percentage of Sirius Red positive tissue in randomly chosen pictures from each organ section by means of the above mentioned computer-aided histomorphometry devices. Perivascular fibrosis was evaluated after Sirius-Red staining using a semi-quantitative score by two independent investigators blinded to the groups to which the animals belonged. Glomerulosclerosis was likewise judged using a semi-quantitative score on sections in PAS staining.

Statistical analysis

Unless otherwise stated, ANOVA was applied in order to detect any significant differences between the groups; the Student’s t-test was used to detect significant differences between 2 groups of interest. Survival was analysed using the log-rank test. Results were expressed as mean ± standard error of the mean, and differences were considered significant when the probability error (p) was less than 0.05.

RESULTS

Ischemia-reperfusion induced acute renal failure

Survival of the animals after 55 minutes of renal ischemia and reperfusion is illustrated in Figure 1. After 8 days survival was 100 % in the sham group. Renal ischemia significantly (p <0.02) decreased survival to 33 % in the vehicle control group. Acute intravenous treatment with SLV338 during the ischemia-reperfusion procedure led to an 80 % survival, which was not (p = 0.3) different from sham controls. Serum creatinine measured at baseline, day 2, and day 8 is illustrated in Figure 2. There was no difference in baseline plasma creatinine between the study groups. However, on day 2 after renal ischemia-reperfusion, plasma creatinine was increased about 5-fold in vehicle treated animals, which is an indicator of a pronounced renal failure. This increase was significantly attenuated (by more than 50 % ) in animals treated with SLV338. Between surviving animals on day 8 no difference in plasma creatinine was detected.

The same pattern was observed with serum BUN (blood urea nitrogen): Baseline s-BUN levels were within normal ranges at the beginning of the study. S-BUN elevation at post-ischemia day 2 was significantly attenuated by treatment with SLV338 (30 ± 6.8 mmol/L, p <0.05 versus 70 ± 10 in vehicle-treated rats).

L-NAME-induced hypertensive renal damage

During 4 weeks of L-NAME administration blood pressure was assessed weekly. The results are illustrated in Figure 3. There was no difference in baseline blood pressure between all study groups. However, L-NAME administration significantly enhanced blood pressure by 30 - 40 mmHg compared to time-matched controls which did not receive L-NAME. Only in week 1 SLV338 treated animals exhibited a slightly decreased blood pressure versus the untreated L-NAME group. During the rest of the study there was no significant difference in blood pressure between SLV338 and untreated animals under L-NAME administration.

Body and kidney weight and functional renal parameters are summarized in Table 1. Regarding body weight, there was a statistically significant decrease in the group treated with SLV388 versus the other study groups. However, the absolute difference was about 20 g, which is only about 5 % of total body weight. No difference with respect to kidney weight was detected between the study groups. Regarding renal function, there was no difference in urinary output or creatinine clearance between all study groups. However, the urinary albumin/creatinine ratio (ACR) was significantly increased in both groups subjected to L-NAME administration. Treatment with SLV338 decreased mean
Table 1. Body and kidney weight, glomerular filtration rate, urinary output and albumin excretion in L-NAME hypertensive rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control (n = 12)</th>
<th>L-NAME (n = 12)</th>
<th>L-NAME + SLV338 (n = 11)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight (g)</td>
<td>431 ± 7</td>
<td>434 ± 8</td>
<td>411 ± 4*#</td>
</tr>
<tr>
<td>Kidney Weight (g)</td>
<td>2.9 ± 0.05</td>
<td>2.8 ± 0.06</td>
<td>2.8 ± 0.06</td>
</tr>
<tr>
<td>GFR (mL/min/100gBW)</td>
<td>0.55 ± 0.02</td>
<td>0.58 ± 0.05</td>
<td>0.58 ± 0.03</td>
</tr>
<tr>
<td>ACR (µg/mg)</td>
<td>15 ± 1</td>
<td>37 ± 7*</td>
<td>28 ± 5*</td>
</tr>
<tr>
<td>Urinary Volume (mL/24h)</td>
<td>13.5 ± 1</td>
<td>17.0 ± 2.4</td>
<td>15.2 ± 1.2</td>
</tr>
</tbody>
</table>

Legend: Values are given as mean ± SEM. *: p <0.05 vs. Control; #: p <0.05 vs. (untreated) L-NAME.
ACR: Urinary albumin/creatinine ratio; GFR: glomerular filtration rate (measured as creatinine clearance).

Figure 1. Survival after renal ischemia.
Survival analysis after renal pedicle clamping for 55 minutes and contralateral nephrectomy. *: p <0.02 vs Sham. IR = ischemia-reperfusion.

ACR by about 25%. However, due to the large variation in both L-NAME groups this did not reach statistical significance (see Table 1). L-NAME administration significantly increased the degree of renal fibrosis whereas this effect was abolished by concomitant SLV338 treatment (see Figure 4). The results regarding glomerulosclerosis, perivascular fibrosis, and media/lumen-ratio of renal arteries are illustrated in Figure 5 A-C. L-NAME administration caused a relatively small, but significant increase in glomerulosclerosis. Treatment with SLV338 completely prevented this effect. The same pattern was observed with regard to perivascular fibrosis and media/lumen ratio, therefore, SLV338 significantly suppressed the L-NAME-dependent increases seen in non-treated animals. Finally, 21% of the animals died in the untreated L-NAME group during the 4 week period of the study, while only 8% died in the SLV338-treated group. However, this difference was not statistically significant (all control animals without L-NAME survived).
Figure 2. Plasma creatinine during acute ischemic renal failure.

Shown are mean values ± SEM 1 day before (baseline) and at different time points after the ischemia-reperfusion challenge (n = 6 -10). #: p <0.05 vs. vehicle group. IR = ischemia-reperfusion.

Figure 3. Systolic blood pressure during L-NAME study.

Legend: Systolic blood pressure was assessed weekly during the course of the L-NAME study. Oral treatment with SLV338 (30 mg/kg/d) was started at week 0. All values are given as mean ± SEM; ***/**/#: p<0.001/0.01/0.05 vs Control group; #: p <0.05 vs. (untreated) L-NAME group. n = 12 – 24.
Figure 4. Effect of oral SLV338 on renal interstitial fibrosis in L-NAME hypertensive rats.

Legend: Interstitial fibrosis was quantified in Sirius Red stained renal sections by means of computer-aided histomorphometry, magnification 200x (see representative microscopic pictures). Results were calculated as percentage of Sirius-Red-positive area in randomly chosen pictures from renal sections; all values are given as mean ± SEM; **: p <0.01 vs Sham-group; #: p <0.05 vs (untreated) L-NAME group (see graph).
Figure 5. Effect of SLV338 on (A) glomerulosclerosis, (B) perivascular fibrosis, and (C) media-to-lumen ratio of renal arteries in the L-NAME study.

Legend: All values are given as mean ± SEM; */***: p <0.05/0.001 vs Sham-group; #: p <0.05 vs (untreated) L-NAME group.

DISCUSSION

This study investigated the renoprotective potential of combined ECE/NEP inhibition in models of acute and chronic renal diseases. As far as we know, there is currently no literature investigating survival with combined ECE/NEP inhibition in acute ischemic renal failure. Our study is the first to demonstrate a significant improvement of combined ECE/NEP inhibition on survival in this model. Considering the lower plasma creatinine levels in the animals treated with SLV338, this effect on survival is most likely due to a better preserved kidney function after the injury. These results are supported by others solely using ECE blockade in similar experimental settings: Matsumura et al found a significant improvement in renal function and less tubular necrosis 24 hours after the ischemic insult in animals subjected to 45 minutes of renal ischemia if an ECE inhibitor was given prior to ischemia [19]. However, they did not examine any effects on mortality in this short-duration study. They also observed an increase in tissue ET-1 concentration in the postischemic kidney which underlines the well-known role of ET-1 in ischemic conditions in the kidney [20] or the heart [21]. Similar observations were made by others using phosphoramidon as ECE inhibitor [22],...
which exerted better kidney protection than an ET₁ receptor antagonist in the same study. Moreover, Vemula-palli et al [23] likewise showed a protective effect of phosphoramidon in acute ischemic renal failure, whereas a NEP inhibitor had no effect in the same study, thus suggesting that the main renoprotective effect in this setting was derived from the inhibition of ET-1 formation.

The demonstration of a decrease of serum creatinine after 48 hours mimics a potential phase II clinical trial where the read out is the change of biomarkers from baseline to 48 hours describing kidney function. The Phase III registration trial will be a morbidity/mortality trial (see www.clintrial.gov). This is reflected in the survival data. Thus our data can be most likely translated to successful clinical development. It is important to keep in mind that many drugs for AKI in clinical development show promising effects just on creatinine levels but not on mortality, neither in the preclinical nor in the clinical setting.

Chronic effects of SLV338, and more specifically, structural renoprotective effects, were examined in a model of nitric oxide deficiency (chronic L-NAME-induced hypertensive renal damage) in which the endothelin system is thought to play a less dominant or even minor role [24]. In this model chronic oral treatment with SLV338 totally prevented kidney tissue damage as indicated by the compound’s capacity to abolish L-NAME-induced glomerulosclerosis, fibrosis, and arterial wall thickening. Moreover, although not statistically significant due to large variation, SLV338 tended to decrease albuminuria by about 25 %. Interestingly, blood pressure was not significantly lowered during most of the study period. We therefore conclude that the renal tissue protection afforded by SLV338 is likely to occur at the tissue level, i.e., independently of blood pressure. Similar results were observed for the ECE/NEP inhibitor SLV 306, which reduced renal interstitial fibrosis without exerting an antihypertensive effect in rats with streptozotocin-induced diabetes. Moreover, these effects were comparable to those of angiotensin converting enzyme inhibition [25]. However, as the present study was designed as a relatively short-term (4 weeks) investigation focusing on renal tissue injury, further long-term studies are warranted to determine if SLV338 would also slow progression of renal dysfunction and preserve GFR, which was not yet impaired in this experimental protocol. One interesting finding is also the significantly lower body weight of animals treated with SLV338 when compared with the other groups. We suggest that this might be potentially due to a natriuretic effect of this drug, since NEP/ECE inhibitors also block the degradation of BNP. However, this hypothesis that elevated BNP levels due to treatment with ECE/NEP inhibitors promote diuresis after acute renal failure needs to be confirmed. It is interesting that functional protection of the kidney by ECE/NEP inhibition has been demonstrated in the context of diabetes mellitus: thus, Tikkanen et al investigated the effects of ECE/NEP inhibition versus sole NEP inhibition, dual endothelin receptor antagonism, angiotensin converting enzyme (ACE) inhibition, and dual ACE/NEP inhibition in a rat model of diabetes (streptozotocin-induced diabetes) [26]. Although blood pressure was reduced similarly by all drugs (except for the NEP inhibitor), only ECE/NEP and ACE/NEP inhibition reduced albuminuria. Similar results were obtained with another ECE/NEP inhibitor (SLV306) in the same diabetic model, in which this compound likewise reduced albuminuria as well as renal matrix protein accumulation [25]. In a different kind of model, purinergic-induced nephropathy in rats, Feldman et al demonstrated a beneficial effect of chronic treatment with an ECE inhibitor on renal histology [27]. Taken together, these data support the notion of a blood pressure independent renoprotective potential of ECE/NEP inhibition.

In summary, the present work is the first to show that ECE/NEP inhibition reduces mortality in acute renal failure. Moreover, chronic oral treatment with SLV338 was found to abolish structural renal damage in a blood-pressure independent manner.

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Declaration of Interest:
Yvan Fisher is an employee of Abbott Products GmbH, Hannover, Germany.

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