Pharmacokinetic and pharmacodynamic properties of SOL1: A novel dual inhibitor of neutral endopeptidase and endothelin converting enzyme

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Aims: The pharmacological profile of the novel putative neutral endopeptidase (NEP) and endothelin converting enzyme (ECE) inhibitor SOL1 was examined.

Main methods: The enzyme inhibitory profile of SOL1 was established in vitro. The pharmacokinetic and pharmacodynamic profile was determined in rodents in vivo.

Key findings: In vitro, at neutral pH, 10 μM SOL1 inhibited NEP-1, NEP-2, and ECE-1 by 99%, 94% and 75%, respectively. The IC50s were 25, 25 and 3200 nmol/L, respectively. In anesthetized rats, SOL1 inhibited blood pressure (BP) responses to big-ET-1 and ET-1(1–31) with ED50s of 1.9 and 0.03 mg/kg, corresponding to plasma EC50s of 4.6 and 0.1 μmol/L, respectively. Pharmacokinetics of SOL1 were examined after single injections in mice and rats. In these species, the estimated clearance of SOL1 varied between 5 and 9 ml/kg/min. To 20 and 60 min. Steady state kinetics of SOL1 were examined after continuous s.c. infusions of SOL1 for 3 weeks at 50 mg/kg/day in DOCA-salt hypertensive rats. This treatment lowered BP by 22 mmHg. Steady state concentrations of SOL1 in plasma were 3.9 μmol/L. In heart, lung, and kidney the concentrations of SOL1 were 0.4, 1.8, and 20.5 μmol/kg, respectively. About 63% of the daily dose was retrieved unaltered in the urine.

Significance: These data indicate that SOL1 is primarily a NEP inhibitor in vitro as well as in vivo. Given the preferential renal accumulation and renal clearance of SOL1 additional ECE-1 inhibition in the kidney may have contributed to its chronic BP lowering effects in the DOCA-salt hypertensive rat model.

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Introduction

Endothelin (ET-1) is a pluripotent peptide that affects virtually all organ systems that regulate blood pressure (BP) and sodium homeostasis (Kohan et al., 2011). Soon after the discovery of ET-1 and its ETa and ETb receptors the potential therapeutic benefit of inhibiting the ET-axis was proven in several experimental animal models and clinical trials. ETa/b receptor antagonists have been developed to antagonize ET-1 related effects downstream of its synthesis. While these receptor antagonists are potentially effective in the treatment of several cardiovascular diseases, their clinical application is currently limited because of the high incidence of renal side effects leading to hemodilution and edema formation (Kirkby et al., 2008). This may be explained by blockade of the ET-1 mediated pro-natriuretic effects. Recent observations suggest that in addition to ETa receptors also ETb receptors may mediate sodium excretion (Kohan et al., 2011). One approach to overcome the side effects of ETa/b Receptor blockade is to further unravel the complexity of the intra-, auto- and paracrine functions of ET-1. Alternatively, as addressed in the present study, one may also target the formation of ET-1.

ET-1 can be formed by proteolytic cleavage of its inactive precursor big-ET-1 (ET-1(1–38)) by endothelin converting enzymes (ECE, Fig. 1). These metalloproteases are structurally related to neutral endopeptidase (NEP), an enzyme that converts not only chymase derived ET-1(1–38) to ET-1 but also degrades well-known vasoactivators such as atrial natriuretic peptide (ANP), bradykinin (BK) and calcitonin gene-related peptide (CGRP). Dual inhibitors of ECE-1 and NEP have been synthesized by a number of groups (Jeng et al., 2002) with varying selectivity for ECE-1 and NEP. These enzyme inhibitors are less advanced in clinical development than ET-receptor antagonists. In preclinical studies dual NEP/ECE inhibitors appear not as potent as ETa/b -antagonists in reducing elevated BP. Two recent studies (Kalk et al., 2011; Wengenmayer et al., 2011) suggest that their potential benefit may depend on their ability to alter the bioavailability of vasoactive peptides in an organ-specific manner rather than on their BP-lowering effect (Dhaun and Webb, 2011).

Here we report the pharmacokinetic and pharmacodynamic properties of a novel dual NEP/ECE inhibitor named SOL1. Our preclinical studies support the concept that the efficacy of SOL1 depends on the renal specific accumulation of the compound and hence varying local degrees of NEP/ECE inhibition.
**Methods**

**Chemicals**

Benzazepine acid derivatives are well known NEP inhibitors (Brands et al., 2006; Jeng et al., 2002). The chemical name of SOL1 is 2-[[1-((3S)-1-(carboxymethyl)-2-oxo-2,3,4,5-tetrahydro-1H-1-benzazepin-3-yl)amino]carbonyl]cyclopentyl[methyl]-4-[[3-(methylamino)propyl]methyl]amino]-4-oxobutanoic acid. The dual NEP/ECE inhibitory profile was obtained by coupling a cyclopentyl group to benzazepine using a retrosynthetic strategy (Fig. 2). Details of the synthesis of this compound are given in the supplementary data. In the present study sodium salt batches of SOL1 were used.

**In vitro enzyme inhibition**

The enzyme inhibitory profile of SOL1 was determined in vitro using recombinant sources of NEP and ECE proteins (Innogenetics,
Ghent, Belgium). The potency of SOL1 to inhibit these enzymes was quantified by measuring the accumulation of a fluorescent big-ET-1 substrate analogue. Experiments were performed at pH 7.0. Since ECE is also located in endosomes (Padilla et al., 2007), and potentially active in more acidic conditions, inhibition of ECE-1 by SOL1 was also tested at pH 5.5. Concentrations up to 10 μM of SOL1 were used and the concentration of the inhibitor required to inhibit enzyme activity by 50% (IC50) was determined. Details of the procedures are provided in the supplement.

**Animals**

All animal studies were conducted in accordance with international guidelines and approved by the institutional animal care and use committees of Abbott Pharmaceuticals (Hannover) and the Maastricht University. Mice and rats were purchased from Charles River (l’Arbresle, France) or derived from institutional breeding stocks.

**In vivo enzyme inhibition**

An arterial and venous line were inserted via the femoral vessels of ketamine/xylazine anesthetized Sprague-Dawley (SD) rats to enable the recording of systolic arterial blood pressure (SBP) and i.v. injections of drugs. Changes in SBP were recorded after i.v. injections of big-ET-1 (as the ECE substrate) or ET-1(1-31) (as the NEP substrate) without or with prior administration (5 min. before) of SOL1. The same tests were performed after intraduodenal administration of SOL1. Changes in SBP were monitored for twenty minutes after injection and a blood sample was collected (at ~21 min.) to measure plasma SOL1 concentrations by mass spectrometry (see supplement). Using these data SOL1 concentrations were estimated at the peak response (for big-ET-1 at 11 min and for ET-1(1-31) at 8 min) using the pharmacokinetic properties given below. Finally, dose–response curves were generated and the effective concentration (EC50) and corresponding effect dose (ED50) of SOL-1 was determined to inhibit the pressor response by 50%.

In a separate group of rats we measured if SOL1 (30 mg/kg, i.v.) was able to prevent the breakdown of exogenously i.v. applied CGRP (3 μg/kg) in rats. SOL1 was given 5 min prior to CGRP. Twenty minutes after administration blood was sampled for CGRP measurements by a commercial radioimmunoassay kit (Phoenix Pharmaceuticals, Burlingame, CA, USA). Data were compared to those obtained in vehicle treated rats as well as animals treated with the selective ECE inhibitor SM-19712 (10 mg/kg).

**Pharmacokinetics of SOL1**

Pharmacokinetic characteristics of SOL1 were determined in conscious SD rats. SOL was administered as an i.v. bolus at 1 mg/kg or given orally at 10 mg/kg. Venous blood samples were collected regularly using the automated Culex® system (Bioanalytical Systems, Inc., West Lafayette, Indiana, USA) to determine plasma SOL1 concentrations. Data were fitted to a pharmacokinetic non-linear model (PKWinNonLin model) to derive the clearance rate and the plasma half-life T1/2. Kinetic parameters of SOL1 were also obtained in male adult C57BL/6 mice after oral (30 mg/kg) or s.c. (30 mg/kg) dosing. Plasma and organ concentrations of SOL1 were determined in mice sacrificed at 1, 7 and 24 h after SOL1 administration.

**Pharmacodynamics of SOL1**

Deoxycorticosterone (DOCA)-salt hypertensive male Wistar rats were chronically treated for 3 weeks with SOL1 (50 mg/kg/day s.c.) using osmotic minipumps (2ML4, Alzet®, Durect Corporation, Cupertino, CA). Treatment was started at the same time the DOCA (100 mg) pellet was implanted. Details on the rat model and surgical protocols can be found in the supplement. After 2 weeks of treatment, rats were maintained in metabolic cages to collect 24-h urine. After 3 weeks of treatment with SOL1, mean arterial pressure (MAP) was recorded in conscious unrestrained conditions and compared to values obtained in vehicle-treated DOCA-salt hypertensive rats. At the end of the study, SOL1 concentrations were determined in plasma, heart, kidney, lungs, and urine.

**Statistics**

Values are presented as means ± SEM. In the in vivo inhibition assays the effect of varying doses of SOL1 were expressed relatively to the values obtained in non-treated rats. Maximal inhibitory values were compared to the non-treated condition using a one-way analysis of variance (ANOVA). The BP-lowering effect between SOL1-treated and vehicle-treated DOCA-salt hypertensive rats were compared by a Student’s t-test.

Differences were considered statistically significant if P values <0.05.

**Results**

The enzyme-inhibitory selectivity of SOL1 is summarized in Table 1. In vitro and at neutral pH, the compound appeared to be primarily a selective NEP1 inhibitor with an IC50 between 10 and 50 nM and a maximal inhibitory effect of 90–95%. Inhibition of human ECE1 was observed in the μM range at an efficacy of 75%. The IC50 of SOL1 for rat ECE1 was even lower. However, when the latter assay was performed at pH 5.5 (as in endosomes) a reasonable IC50 of 250 nM and an inhibitory efficacy of 94% were obtained. In additional in vitro studies it was established that, at 10 μM, SOL1 did not inhibit the activities of angiotensin converting enzymes (ACE-1 and ACE-2), renin, the matrix metalloprotease MMP2, as well as dipetidylpeptidase (DPP4, data not shown).

Increasing doses of SOL1 inhibited the rise in SBP in response to i.v. injections of big-ET-1 or ET-1(1–31) as summarized in Fig. 3. Full inhibition of the acute rise in SBP in response to big-ET-1 and ET-1(1–31) was achieved at respective doses of 10 and 0.3 μmol/kg SOL1. This indicates an in vivo preference of the compound for the pressor responses to the small NEP substrate ET-1(1–31) over the larger ECE substrate ET-1(1–38). Plasma SOL1 levels were measured at the end of the in vivo inhibition assay (collected ~21 min post administration). Using the available kinetic information (Fig. 4, supplemental data), plasma concentrations of free SOL1 were estimated at the peak inhibitory response (for big-ET-1 at 11 min; for ET-1(1–31) at 8 min post administration) and compared to the IC50 concentrations obtained in vitro (Fig. 3C). From these curves we determined the in vivo plasma levels of SOL1 that inhibited the in vivo conversions of big-ET-1 and ET-1(1–31) by 50%. These EC50s averaged 2.6 and 0.044 ng/ml, respectively and were remarkably different from the observed IC50s in vitro. The EC50 values for ECE and NEP inhibition correspond to an i.v. ED50 of 1.9 and 0.03 mg/kg, respectively.

**Table 1**

Potency and selectivity of SOL1 to inhibit the in vitro enzyme activity of the specified ECE and NEP enzymes at 10 μM, n= number of observations (in duplicate). IC50 is the concentration of SOL1 in nM that was needed to inhibit 50% of the enzyme activity.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>n</th>
<th>% Inhibition at 10 μM</th>
<th>IC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ECE1</td>
<td>Human</td>
<td>3</td>
<td>75</td>
<td>7898 ± 4714</td>
</tr>
<tr>
<td>ECE1</td>
<td>Rat (pH 7.0)</td>
<td>2</td>
<td>38</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>ECE1</td>
<td>Rat (pH 5.5)</td>
<td>1</td>
<td>94</td>
<td>251</td>
</tr>
<tr>
<td>NEP1</td>
<td>Human</td>
<td>4</td>
<td>99</td>
<td>17 ± 7</td>
</tr>
<tr>
<td>NEP2</td>
<td>Human</td>
<td>6</td>
<td>94</td>
<td>34 ± 17</td>
</tr>
</tbody>
</table>
Both SOL1 (30 mg/kg) and SM-19712 (10 mg/kg) were able to attenuate the breakdown of exogenously applied CGRP. Compared to plasma levels of CGRP measured in vehicle-treated rats (2.8 ± 0.4 fmol/l, n = 4) CGRP levels after treatment with SOL1 (n = 4) and SM-19712 (n = 4) were significantly (p < 0.05) increased (7.7 ± 0.9 and 4.9 ± 1.0 fmol/l, respectively).

The pharmacokinetic behavior of SOL1 in the plasma of rats and mice is compared in Fig. 4. After i.v. injection in rats the estimated T1/2 of SOL1 was 1 h, whereas after oral dosing a T1/2 of 2.5 h was found. The oral bioavailability of SOL1 was limited to 3%. The volume of distribution (Vd) of SOL1 was 741 ml/kg. Given the good water solubility of SOL1 (>500 mg/ml), the Vd is indicative of distribution over the hydrophilic body compartments. The clearance was estimated at 8.8 ml/min.kg. Comparable clearance rates (~5 ml/kg.min) were derived from the steady state infusion experiments in DOCA-salt hypertensive rats.

In mice, SOL1 plasma and tissue concentrations were determined at 1, 7, and 24 h after a single bolus injection of the drug. The data in Fig. 5 indicate that the elimination rate of SOL1 is considerably slower in the kidney and lungs than in the heart or plasma. Similar findings were obtained in steady state conditions during continuous s.c. infusion of SOL1 for 3 weeks in DOCA-salt hypertensive rats. In these chronically SOL1-treated DOCA-salt hypertensive rats MAP was 22 mmHg lower than in vehicle-treated controls (128 ± 6 versus 151 ± 5 mmHg, n = 6). In the SOL1- and vehicle-treated rats no difference was found in 24-h urine volume (79 ± 7 versus 81 ± 9 ml) and urinary albumin concentrations (4.0 ± 1.5 versus 3.1 ± 0.7 mg/ml). Circulating concentrations of SOL1 in DOCA-salt hypertensive rats were far above the estimated IC50 level (from the in vitro studies) needed to inhibit NEP. Renal SOL1 concentrations also exceeded the estimated IC50 level required to inhibit rat ECE. Therefore, as summarized in Fig. 5, SOL1 behaves in vivo in the indicated organs as a NEP inhibitor and in the kidney as a dual NEP/ECE inhibitor.

Fig. 3. Panel A: in ketamine/xylazine anesthetized Sprague-Dawley rats, the responses of systolic blood pressure (SBP) to i.v. injections of big endothelin (big ET-1) were assessed without and after prior administration of increasing doses (1, 3, 10 μmol/kg) of SOL1. For reasons of clarity statistics are only shown for the peak responses: * p < 0.05, ** p < 0.01 compared to vehicle (0 μmol/kg). Panel B: SBP response to ET-1(1–31) without and after prior administration of increasing doses (0.03, 0.1, 0.3, 1.0 μmol/kg) of SOL1. For reasons of clarity statistics are only shown for the peak responses: * p < 0.05, ** p < 0.01 compared to vehicle (0 μmol/kg). Panel C: Comparison of the in vitro and in vivo inhibitory effects of SOL1. The full and open squares (average ± SEM) indicate the dose-dependent magnitude of enzyme inhibition by SOL1 in the ET-1(1–31) and big-ET-1 assays. The square and triangle marked in grey mark the relative inhibition observed after intraduodenal administration of SOL1 (30 μmol/kg). The in vitro IC50 estimates for the various neutral endopeptidase (NEP) and endothelin converting enzymes (ECE) (derived from Table 1) are indicated by the thin upward pointing arrows. SOL1 plasma concentrations were measured at the end of the in vivo experiments (taken ~21 min post administration) and SOL1 concentrations were estimated at the peak response (for big ET-1 at 11 min and for ET-1(1–31) at 8 min) using a PK WinNonLin model. Plasma levels of SOL1 were corrected for free drug concentration (rat FUU~96%). Averaged inhibition curves were constructed using a sigmoidal curve fitting program. From these curves the EC50 of SOL1 to inhibit NEP (dotted downward arrow (a)) and ECE (dotted downward arrow (b)) were derived. These values were 44 ng/ml for NEP and 2.6 μg/ml for ECE. These values correspond to an i.v. ED50 of 0.03 and 1.9 mg/kg, respectively.

Fig. 4. Average time-dependent changes in plasma SOL1 concentration after intravenous (iv), subcutaneous (sc) or oral dosing in rats and mice. Values are means of 3 observations in mice and 4 in rats. From these curves, kinetic parameters of SOL1 were derived using standard modeling programs. These data are presented in more detail in the supplement.
inhibitor. In the chronic dosing study, 63% of the infused amount of SOL1 was retrieved in an unaltered form in the urine. These data indicate that SOL1 excretion is predominantly renal.

Discussion

Here we report the synthesis and pharmacokinetic and pharmacodynamic properties of the novel dual NEP/ECE inhibitor SOL1. Using a retrosynthetic strategy, SOL1 was synthesized by the coupling of commercially available cyclopentyl and benzazepine building blocks. In vitro and at neutral pH, SOL1 acted as a potent NEP inhibitor but a rather weak ECE inhibitor with IC_{50} values of 17–34 nM and 7–10 μM, respectively. SOL1 did not inhibit the activity of any of the indicated proteases. This in vitro profile is comparable to the one reported for phosphoramidon. The latter compound has been widely used both in vitro and in vivo as a non-selective NEP/ECE inhibitor (IC_{50} for NEP 4–30 nM; IC_{50} for ECE 1–4 μM) (Brands et al., 2006; Jeng et al., 2002). It should be noted that these IC_{50} values are based on the use of recombinant enzymes in cell free conditions. In living organisms, NEP and ECE are membrane bound. Thus the in vivo inhibitory effects of such putative inhibitors also depend on their ability to reach these targets in a sufficiently high concentration. We found that SOL1 is highly water soluble (> 500 mg/ml) and that its volume of distribution is about 0.7 L/kg. These values are suggestive for a distribution of the drug over the full water compartment of the body.

Despite weak in vitro inhibitory effects on ECE, the in vivo potency of SOL1 against the big-ET-1-induced pressor response was quite remarkable. Following i.v. administration, SOL1 fully blocked the big-ET-1-induced rise in BP at a dose of 10 μmol/kg. The estimated ED_{50} of SOL1 was 1.9 mg/kg. This dose is comparable to those reported for the more selective ECE inhibitor SM-19712 (10 mg/kg i.v.) or indole-based compounds (3 mg/kg i.v.) (Brands et al., 2006). As indicated in Fig. 3c, the in vivo EC_{50} of SOL1 for rat-ECE is shifted by about 1 log scale to the left when compared to the in vitro IC_{50} estimate. In contrast, the in vivo EC_{50} for NEP inhibition was considerably higher than the in vitro estimates. These data suggest that the conversion of substrates by ECE and NEP may have quite a different in vivo efficacy. We attempted to quantify the in vivo ECE-inhibitory component of SOL1 by comparing big-ET-1 levels in the plasma of SOL1-treated and vehicle-treated DOCA-salt rats. Unfortunately, a conclusion could not be drawn because the levels of the peptide were below the detection limit. On the other hand, we could establish that SOL1 attenuated the clearance of exogenously applied CGRP to rats in a similar fashion as the selective ECE inhibitor SM-19712. Cumulatively these data suggest that SOL1, like phosphoramidon, does exhibit ECE-inhibitory effects in vivo, certainly at doses > 10 mg/kg, and that its effects are not entirely NEP mediated.

As previously reported by Corder and Vane, exogenously applied big-ET-1 is rapidly cleared from the circulation (Corder and Vane, 1995). If conversion of big-ET-1 occurs mainly at the luminal site of the endothelium, then injections of big-ET-1 would be accompanied by ET_{A} mediated acute depressor responses, as is the case for intravenously administered ET-1. These are generally not observed. Most of the evidence thus far suggests that big-ET-1 is converted to ET-1 in the sub-endothelial matrix or at the plasma membrane of vascular smooth muscle cells (Corder and Vane, 1995; Hisaki et al., 1993). More recent observations showed that ECE located at plasma membranes can be internalized to intracellular endosomes. The ECE-dependent breakdown of CGRP occurred in these organelles more effectively at acidified (pH 5.5) than at neutral pH (7.4) (Padilla et al., 2007). These findings strengthen the possibility that despite its water-soluble characteristics, SOL1 together with ECE is internalized and inhibits the conversion of substrates more effectively at these locations than in the blood compartment. Clearly more work is needed to unravel the precise molecular mechanisms of action of such inhibitors.

From the pharmacokinetic studies, in both rats and mice, it appeared that SOL1 was rapidly cleared from the plasma. However, in the lungs and especially in the kidneys concentrations of SOL1 remained significantly higher than those found in plasma, both after a single administration in mice as well as during steady state conditions in rats. The preference of SOL1 to accumulate in these organs may be due to its binding to abundantly present NEP and ECE enzymes in these tissues (Goldie et al., 1996; Kohan et al., 2011). Alternatively, high renal concentrations may be due to the specific elimination pathway of the drug. In steady state conditions in rats, 63% of the daily infused amount of SOL1 was recovered in the urine. The renal concentration of SOL1 was sufficiently high to inhibit, besides NEP also ECE in this organ. Given the important role of ET-1 in renal sodium excretion (Kohan et al., 2011), additional ECE inhibition may have contributed to the chronic BP-lowering effect of SOL1 in DOCA-salt hypertensive rats. The present study was not designed to test the underlying mechanisms in detail. Because SOL1 is also a potent NEP inhibitor, the accumulation of endogenous vasoactive peptides like ANP, BK, and CGRP that are catalyzed by NEP may have also contributed to the BP lowering effect. Since C-fiber-derived peptides such as CGRP may be downregulated in salt-sensitive models of hypertension (Wang and Zhao, 2003) and CGRP selectively terminates the long-lasting binding of ET-1 to the ET_{A} receptor (Meens et al., 2010, 2011) we hypothesize that the accumulation of this particular peptide may play a crucial role in mediating the antihypertensive effects of SOL1 in this model. These aspects are currently under investigation (Nelissen et al., 2011).

In the present study SOL1 did not influence renal albumin excretion. This may be due to the fact that the extent of renal damage is still limited after 2 weeks of DOCA-salt treatment. In other rat models
The beneficial effects of combined NEP/ECE inhibitors may occur independent of their BP-lowering effects. The dual NEP/ECE inhibitor SLV338 reduced renal damage in chronically L-NAME-treated rats and preserved kidney function and reduced mortality in a rat model of severe acute renal failure (Sharkovska et al., 2011). SLV338 also prevented interstitial and perivascular fibrosis in the heart (Kalk et al., 2011) of 2-kidney 1-clip rats and reduced the incidence of stroke (Wengenmayer et al., 2011) in a BP-independent manner. These data strengthen the concept presented in this paper that the effects of NEP/ECE inhibition may vary throughout the body. Although NEP/ECE inhibitors are not potent BP-lowering agents, organ specific enzyme inhibition may add to their beneficial effect. As proposed recently, (Dhaun and Webb, 2011), the clinical potential of such NEP/ECE inhibitors should therefore not be judged only by their antihypertensive potential but should be reevaluated taking functional organ-specific parameters into account. The current dual NEP/ECE efficacy of SOL1 in renal tissue may make this drug applicable in conditions of chronic renal failure.

Conflict of interest statement

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.lfs.2012.01.015.