



The dual endothelin converting enzyme/neutral endopeptidase inhibitor SLV-306 (daglutril), inhibits systemic conversion of big endothelin-1 in humans



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ABSTRACT

Aims: Inhibition of neutral endopeptidases (NEP) results in a beneficial increase in plasma concentrations of natriuretic peptides such as ANP. However NEP inhibitors were ineffective anti-hypertensives, probably because NEP also degrades vasoconstrictor peptides, including endothelin-1 (ET-1). Dual NEP and endothelin converting enzyme (ECE) inhibition may be more useful. The aim of the study was to determine whether SLV-306 (daglutril), a combined ECE/NEP inhibitor, reduced the systemic conversion of big ET-1 to the mature peptide. Secondly, to determine whether plasma ANP levels were increased.

Main methods: Following oral administration of three increasing doses of SLV-306 (to reach an average target concentration of 75, 300, 1200 ng ml⁻¹ of the active metabolite KC-12615), in a randomised, double blinded regime, big ET-1 was infused into thirteen healthy male volunteers. Big ET-1 was administered at a rate of 8 and 12 pmol kg⁻¹ min⁻¹ (20 min each). Plasma samples were collected pre, during and post big ET-1 infusion. ET-1, C-terminal fragment (CTF), big ET-1, and atrial natriuretic peptide (ANP) were measured.

Key findings: At the two highest concentrations tested, SLV-306 dose dependently attenuated the rise in blood pressure after big ET-1 infusion. There was a significant increase in circulating big ET-1 levels, compared with placebo, indicating that SLV-306 was inhibiting an increasing proportion of endogenous ECE activity. Plasma ANP concentrations also significantly increased, consistent with systemic NEP inhibition.

Significance: SLV-306 leads to inhibition of both NEP and ECE in humans. Simultaneous augmentation of ANP and inhibition of ET-1 production is of potential therapeutic benefit in cardiovascular disease.

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Introduction

The success of angiotensin converting enzyme (ACE) inhibitors as a treatment for chronic heart failure (CHF), myocardial infarction, hypertension and atherosclerotic disease has demonstrated the therapeutic value of neurohumoral manipulation using inhibitors of vasoactive enzymes (Garg and Yusuf, 1995; Flather et al., 2000; Yusuf et al., 2000).

Neutral endopeptidase (NEP) degrades natriuretic peptides and NEP inhibition increases plasma concentrations of atrial and brain natriuretic peptide (ANP and BNP) (Northridge et al., 1989). ANP and BNP have hemodynamic, neurohumoral and growth effects that might be of therapeutic benefit in CHF, hypertension and atherosclerosis (Chen and Burnett, 1999; Schirger et al., 2000). However, NEP

also metabolises angiotensin II and endothelin-1 (ET-1), effects that may counteract the potential benefits of increased natriuretic peptides (Ando et al., 1995; Richards et al., 1993).

Two distinct therapeutic strategies have emerged to block the unwanted action of endothelin-1 (ET-1) in pathophysiological conditions, receptor antagonists (Palmer, 2009; Davenport, 2002; Davenport and Maguire, 2006; Dhaun et al., 2007; Vachiéry and Davenport, 2009; Davenport and Maguire, 2011) and inhibitors of the endothelin converting enzymes (ECE-1, Xu et al., 1994 and ECE-2, Emoto and Yanagisawa, 1995), the major synthetic pathway in the human vasculature (Russell and Davenport, 1999). Using electron microscopy ECE has been localised primarily to the intracellular compartments of human endothelial cells (Russell et al., 1998a,b). However, some Big-ET-1 escapes intracellular conversion and circulates in plasma (Naruse et al., 1991). Water-soluble solutes such as transmitters have been shown to move across endothelial cells from the plasma through gap junctions to the underlying smooth muscle. However, big ET-1 does not bind to vascular ET receptors until cleaved to ET-1 by converting enzymes present on smooth muscle

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(Maguire et al., 1997). ECE activity is increased in endothelium-denuded human vessels with atherosclerosis (Maguire and Davenport, 1998) suggesting that conversion of big ET-1 to ET-1 by smooth muscle ECE may contribute to increased plasma/tissue ET levels in disease. Conversion has been imaged *in vivo* by infusion of [^{18}F]-big ET-1 to quantify tissue specific conversion to [^{18}F]-ET-1 which bound to ET_A receptors on the vascular smooth muscle (Johnstrom et al., 2010). This was significantly reduced by phosphoramidon, an inhibitor of ECE and NEP, which does not cross the plasma membrane and therefore cannot inhibit endothelial cell ECE but is consistent with inhibition of enzyme conversion by vascular smooth muscle and subsequent reduction of [^{18}F]-ET-1 receptor binding (Johnstrom et al., 2010).

To date, orally active dual inhibitors of both NEP and ECE have been developed, rather than purely ECE selective compounds (Dive et al., 2009; Battistini et al., 2005). Combined NEP and ECE inhibition, leading to augmented natriuretic peptide concentrations, coupled with reduced ET-1 synthesis is an attractive therapeutic strategy in a range of cardiovascular diseases. Following oral administration, SLV-306 (daglutril) is hydrolysed to the active metabolite KC-12615 (Dickstein et al., 2004), a new chemical entity which is a mixed enzyme inhibitor of both ECE and NEP. The inhibitor has not been extensively studied but in diabetic rats, SLV-306 has been shown to reduce proteinuria and urinary albumin excretion (Thöne-Reinke et al., 2004). SLV-306 reduced pulmonary pressures in patients with chronic heart failure (Dickstein et al., 2004) and a Phase II trial has been completed for the treatment of essential hypertension and congestive heart failure (Bayes et al., 2003; Tabrizchi, 2003). The compound had no effect on albuminuria, but reduces day and night-time systolic blood pressure and night-time diastolic blood pressure on top of losartan in albuminuric type II diabetic patients (Van der Meer et al., 2011). The aim of the study was to characterise orally active, dual NEP and ECE inhibitor to demonstrate systemic ECE and NEP inhibition in healthy volunteers.

Materials and methods

SLV-306 and KC-12615

Following oral administration of SLV-306 it is quickly absorbed and hydrolysed to the active metabolite KC-12615 (Dickstein et al., 2004; Tabrizchi, 2003) which in enzyme assays is a potent NEP-inhibitor ($\text{IC}_{50} = 4.2 \text{ nM}$) with additional inhibitory activity on two endothelin-converting enzymes, ECE-1 ($\text{IC}_{50} = 1.5 \text{ }\mu\text{M}$, comparable with $0.3 \text{ }\mu\text{M}$ for phosphoramidon).

In vivo studies in healthy volunteers

Studies were approved by the hospital ethics committee and all volunteers gave written informed consent. There were two parts to this study.

Part 1 Owing to a high inter-subject variability in the plasma concentrations of SLV-306 and KC-12615, a concentration controlled design was used. Twenty nine male volunteers attended for the first stage of the study which involved taking two different doses of SLV-306 (400 mg ($n = 29$), and 600 mg ($n = 6$) or 800 mg ($n = 23$)) at least seven days apart. Blood samples were collected after dosing for measurement of plasma concentrations of KC-12615. Pharmacokinetic modelling was used to calculate the individual doses of SLV-306 needed to achieve average plasma concentrations over the first 6 h of KC-12615 of approximately 75 ng ml^{-1} , 30 ng ml^{-1} and 1200 ng ml^{-1} .

Part 2 Fifteen male volunteers (mean age 22, range 18–38 years) were selected from the initial screen and attended for the second part of the study which involved four further visits at least seven days apart. For these, volunteers attended the clinical laboratory at 08.00 h, having fasted from midnight. An intravenous cannula was placed in each forearm. After 1 h of supine rest, baseline

blood pressure and heart rate recordings were made and blood samples were taken (see below). Each subject then received either placebo or a single oral dose of SLV-306, as calculated individually from the pharmacokinetic analysis in the first part of the study. The selected dose of SLV306 varied from 44 to 258 mg (mean 165 mg) for the low target level, from 150 to 375 mg (mean 223 mg) for the medium level, and from 4504 to 1500 mg (mean 1862 mg) for the high level. A double-blind ascending dose protocol with random insertion of placebo was used to assign treatment. 160 min after dosing, a 20 minute infusion of $8 \text{ pmol kg}^{-1} \text{ min}^{-1}$ of big ET-1 was administered (Fig. 1). After a further 40 min a second 20 minute infusion of $12 \text{ pmol kg}^{-1} \text{ min}^{-1}$ of big ET-1 was given (i.e. between 220 and 240 min post-dosing). The doses of big ET-1 were chosen from pilot studies, as ones likely to lead to a rise in mean arterial pressure of approximately 20 mm Hg. The timing of the infusions was chosen to coincide with peak plasma concentrations of KC-12615 as demonstrated in the pharmacokinetic first stage of the *in vivo* studies.

Measurements of arterial pressure and heart rate

Heart rate and arterial pressure were measured using an automated blood pressure recorder (Dinamap 1846 SX, Critikon Inc., Tampa, Florida, USA). Measurements were made before the intake of study drug and frequently thereafter (Fig. 1).

Blood samples for neurohumoral measurements

Venous blood (11 ml) was collected from the contralateral forearm into chilled tubes at baseline and 160, 210, 240, 260, 280, 300 and 360 min after administration of study drug (Fig. 1). Sample handling and processing has been described previously as have the assays used to measure ANP (McDonagh et al., 1998), big ET-1 and the two cleavage products resulting from ECE activity, ET-1 and the C-terminal fragment (CTF) (Plumpton et al., 1995).

Statistical methods

The treatment groups were compared using a two-way Analysis of Variance (ANOVA) with factors for treatment group, study period and subjects. In addition, least square mean changes were calculated for each treatment group and the three, SLV-306 minus placebo differences were estimated. Each of the three comparisons was performed at the 5% significance level, without adjustment for multiple testing.

Results

In vivo studies in healthy volunteers

Of the 15 volunteers taking part in the big ET-1 infusion studies, 1 withdrew for personal reasons and 1 was withdrawn because of an excessive rise in blood pressure (30 mm Hg) and fall in heart rate ($-15 \text{ beats min}^{-1}$). No subjects experienced any significant adverse event.

Plasma concentrations of KC-12615

Maximum plasma concentrations of KC-12615 are achieved at about 3–4 h following oral dosing of SLV-306. The low, medium and high doses of SLV-306 resulted in clearly distinguishable plasma concentrations of KC-12615. The respective mean steady state concentrations were 26, 216 and 1435 ng ml^{-1} .

Arterial pressure

The rise in systolic, and diastolic arterial pressure following big ET-1 infusion is shown in Fig. 2. SLV-306 caused a concentration

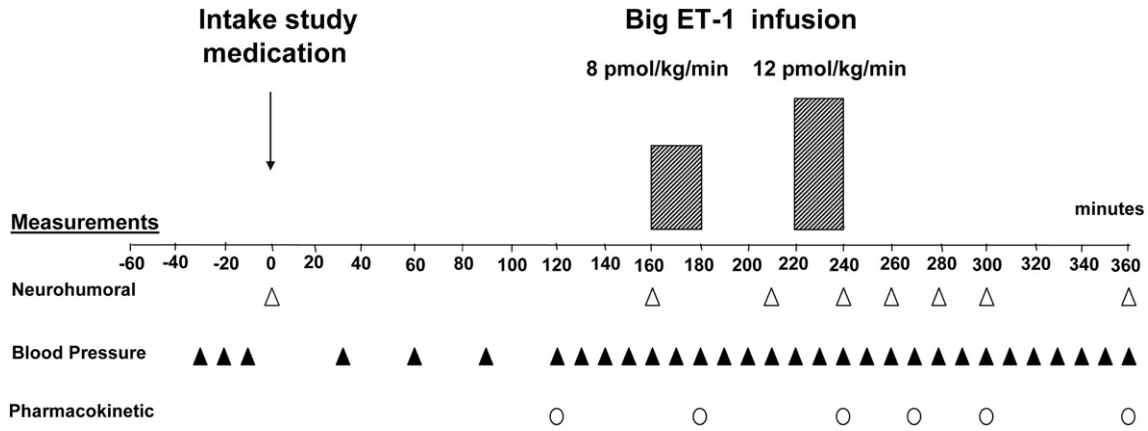


Fig. 1. In vivo study protocol. Subjects rested supine for 60 min before intake of study medication. The first big ET-1 infusion was commenced 160 min after dosing and the second after 220 min. Δ = neurohumoral, ▲ = blood pressure and ○ = pharmacokinetic measurements.

dependent attenuation of the hypertensive response to big ET-1. The mean peak (± standard error, SE) increases in systolic, diastolic and mean arterial pressure were 19.2 (2.1), 16.1 (1.5) and 15.9 (1.6) mm Hg after placebo pre-treatment. The increases after 75 ng ml⁻¹ were, 18.3 (3.0), 14.0 (2.1) and 15.0 (2.3) mm Hg, respectively. The respective increases after 300 ng ml⁻¹ were 15.5 (2.5), 13.3 (1.7) and 13.5 (1.9) mm Hg. Those after 1200 ng ml⁻¹ were 10.4 (2.8), 10.5 (2.0) and 9.0 (2.1) mm Hg, respectively. The differences between placebo and 1200 ng ml⁻¹ were all significant for systolic, diastolic, and mean arterial pressure.

Heart rate

SLV-306 caused an inhibition of the reflex bradycardia induced by big ET-1 infusion (Fig. 2). During big ET-1 infusion, the mean peak

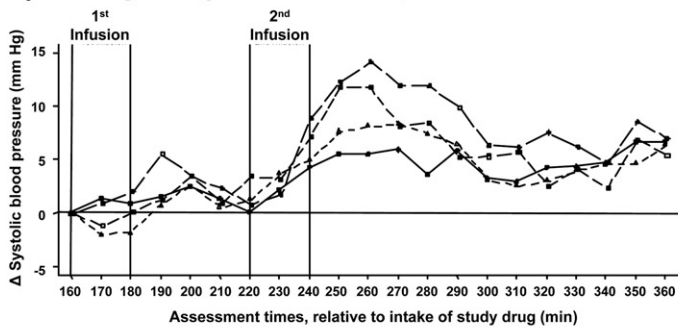
decreases in heart rate were 12.1 (1.1), 11.2 (1.5), 10.2 (1.2) and 7.9 (1.4) beats per minute after pre-treatment with placebo, 75, 300 and 1200 ng ml⁻¹, respectively.

Big ET-1, ET-1 and CTF

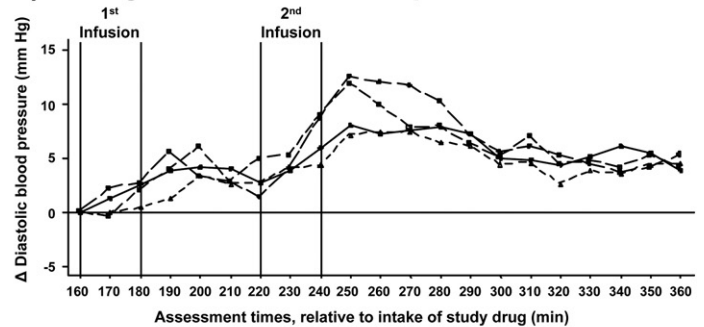
Basal levels of big ET-1 at time 0, before any infusions were 1.5 (0.4) for placebo and 3.0 (1.1), 2.1 (0.6) and 1.5 (0.4) pmol/l for 75, 300, and 1200 ng ml⁻¹ respectively.

SLV-306 caused a concentration dependent increase in plasma big ET-1 concentrations during and after the big ET-1 infusions (Fig. 3). The mean (SE) increases in plasma big-ET in the first hour following the completion of the big-ET infusions after treatment with placebo or 75, 300, and 1200 ng ml⁻¹ were 96.3 (18.3), 128.6 (25.8), 184.4 (21.6) and 216.1 (24.3) pmol l⁻¹. The between group differences

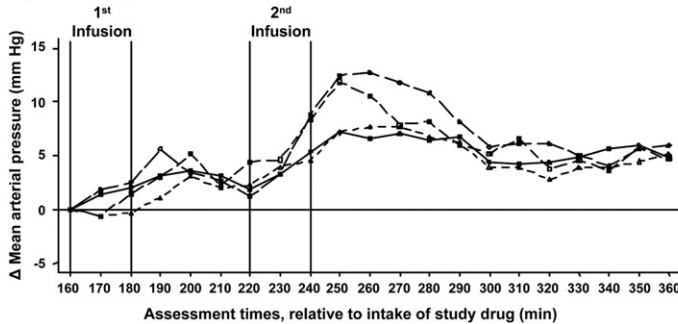
a) Change in systolic arterial pressure*



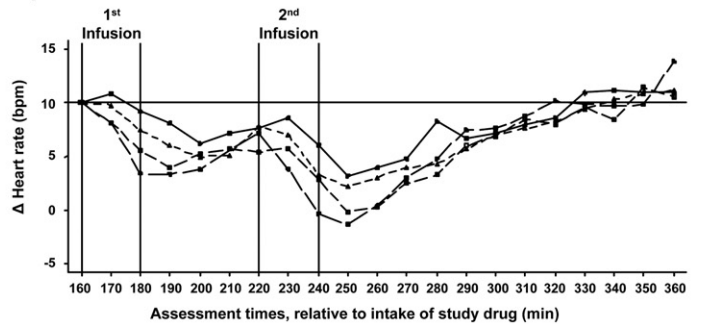
b) Change in diastolic arterial pressure*



c) Change in mean arterial pressure*



d) Change in heart rate*



Treatment: ■—■ KC-1200ng/ml ▲—▲ KC-300ng/ml □—□ KC-75ng/ml ○—○ placebo

* Corrected for baseline before the start of the 1st infusion (average of 140,150,160 minute measurements)

Fig. 2. Change in (a) systolic, (b) diastolic and (c) mean arterial pressures, from baseline, during and after big ET-1 infusion on each of the four study days i.e. after pre-treatment with placebo or SLV-306, to give KC-12615 plasma concentrations of approximately 75, 300 and 1200 ng/ml. "Baseline" was the average of the last 3 arterial pressure measurements made prior to commencement of the first big ET-1 infusion. Change in heart rate (d) is shown in the same way. In all graphs, the treatments are shown as follows: placebo (long dash and open circle); 75 ng/ml (medium dashed line and open square), 300 (short dashed line and open triangle) and 1200 ng/ml (solid line and solid squares).

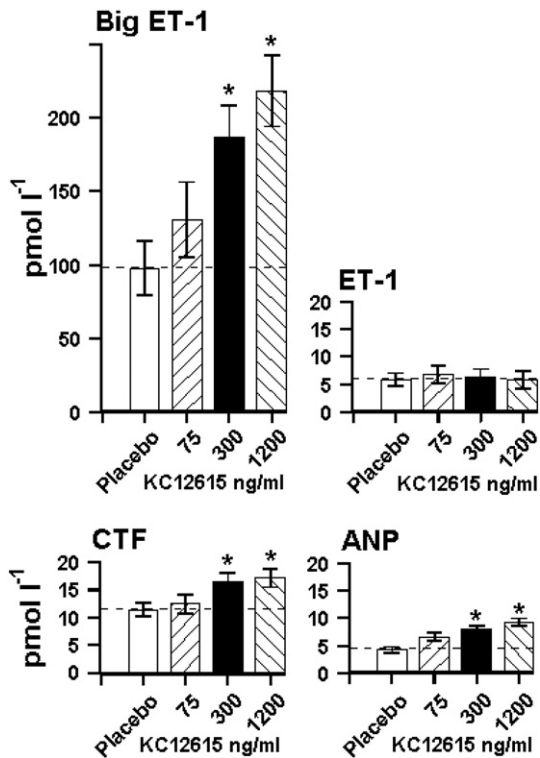


Fig. 3. Changes in plasma Big ET-1, ET-1, CTF and ANP peptide levels in response to increasing concentrations of KC-12615 (the active metabolite of SLV-306) following oral dosing with placebo or SLV-306 and subsequent infusion of big ET-1. Each value represents the mean increase in plasma levels (\pm standard error) measured in 13 volunteers in the first hour following completion of the big ET-1 infusion in either placebo (representing mean basal conversion indicated by horizontal line) and each of the increasing doses. There was a significant dose dependent rise in big ET-1 ($*p < 0.005$), (compared with placebo, two way analysis of variance, ANOVA, with adjustment for multiple comparisons, $p < 0.005$) consistent with inhibition of ECE, and a rise in ANP, consistent with inhibition of NEP activity. In the presence of the highest two doses of SLV-306, a small increase in the CTF was observed but ET-1 levels were unaltered.

versus placebo were statistically significant for the 300 ng ml⁻¹ and 1200 ng ml⁻¹ doses: 88.1 (28.6) [$p = 0.004$] and 119.9 (29.6) pmol/l [$p = 0.0003$], respectively.

There was no evidence of any between group differences for plasma ET-1 levels during the same period.

Basal levels of ET-1 at time 0, before any infusions were 3.3 (0.6) for placebo and 2.8 (0.3), 4.0 (0.4) and 2.9 (0.6) pmol/l for 75, 300, and 1200 ng ml⁻¹ respectively. In the placebo group, at the end of the second infusion of big ET-1, plasma ET-1 immunoreactivity was increased more than three fold above basal to 11.4 (5.3). The ratio of big ET-1 to ET-1 increased in a concentration dependent manner consistent with systemic ECE inhibition preventing metabolism of the enzyme substrate (big ET-1) to its active metabolite (ET-1) (Fig. 3). The mean (SE) ratio of plasma big-ET-1/ET-1 concentration increased in a concentration dependent manner following the big ET-1 infusion: 21.6 (3.0), 20.1 (4.2), 34.1 (3.5), and 41.5 (3.9) after placebo, 75, 300 and 1200 ng ml⁻¹, respectively. The between group differences versus placebo were statistically significant for the 300 ng/ml and 1200 ng/ml doses: 12.5 (4.6) [$p = 0.011$] and 20.0 (4.8) [$p = 0.0002$], respectively.

In the placebo group, levels of the CTF increased an order of magnitude above basal confirming a proportion of the infused big ET-1 was being selectively converted as expected. In the presence of the highest two doses of SLV 306, a small increase in the CTF was observed, consistent with our previous studies with the dual NEP/ECE inhibitor, phosphoramidon (Plumpton et al., 1995) since CTF is also a substrate for metabolism by NEP and inhibition of this enzyme by SLV-306 may reduce proteolysis.

Atrial natriuretic peptide (ANP)

SLV-306 led to a concentration dependent increase in plasma ANP concentrations (Fig. 3), consistent with systemic NEP inhibition. The mean peak (SE) increases in plasma ANP after placebo, 75, 300 and 1200 ng/ml were 4.3 (0.5), 6.6 (0.7), 7.9(0.6) and 9.3 (0.7) pmol/l, respectively. The between-group differences for 300 ng/ml and 1200 ng/ml compared to placebo were statistically significant ($p < 0.05$).

Discussion

SLV-306 and its active metabolite have in vivo actions consistent with inhibition of both NEP and ECE. At the two highest concentrations tested, SLV-306 dose dependently attenuated the rise in blood pressure after big ET-1 infusion. There was a significant increase in circulating big ET-1 levels, compared with placebo, indicating that SLV-306 was inhibiting an increasing proportion of endogenous ECE activity. Plasma ANP concentrations were also significantly increased, consistent with systemic NEP inhibition.

NEP is also thought to metabolise ET-1 to biologically inactive fragments, therefore treatment with SLV-306 might be predicted to cause a rise in ET-1. Importantly, despite inhibition of NEP activity, there was no increase in levels of the mature peptide in these volunteers. This can be explained by our current model of conversion of big ET-1 (Fig. 4), where infused big ET-1 is converted by extracellular ECE, principally located on the smooth muscle cells and immediately binds to ET_A receptors to cause vasoconstriction. The lack of change of plasma ET-1 is likely to represent a combination of ECE inhibition reducing big ET-1 conversion, with any rise in ET-1 resulting from NEP inhibiting metabolism of the mature peptide balanced by clearing and internalisation from the circulation via ET_B receptors, mainly located on endothelial cells. Despite the increased big ET-1 infused into the control group, plasma levels of ET-1 are not increased but kept within a narrow range by these physiological mechanisms. These results suggest that in the presence of SLV-306, the biologically active peptide can continue to be removed beneficially from the circulation by ET_B clearing receptors, which may represent a therapeutic advantage

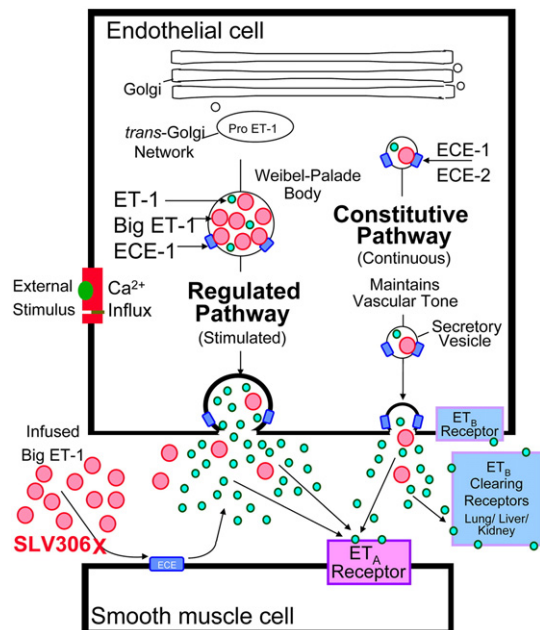


Fig. 4. Schematic diagram showing the synthesis of ET-1 by endothelin converting enzymes (ECE-1 and ECE-2) in the constitutive pathway and by ECE-1 in the regulated pathway within endothelial cells of the human vasculature. Some big ET-1 escapes conversion and is cleaved by smooth muscle cell ECE. It is proposed that following exogenous infusion of big ET-1, the precursor is converted to ET-1 principally by smooth muscle ECE which is the main target for inhibition by the active metabolite of SLV-306.

over mixed ET antagonists currently used in the clinic that block both sub-types.

Conversely, it might be expected that there would be a significant decrease in plasma ET-1 and CTF, particularly at the higher doses of inhibitor. There are a number of possible explanations, why this was not detected. Although the plasma concentration of the active metabolite (KC-12615) at the highest dose tested, would be predicted to inhibit most of the activity of extracellular smooth muscle ECE, based on EC₅₀ values in vitro, the compound may not have crossed the plasma membrane of endothelial cells at sufficiently high concentrations to inhibit the continuous formation of ET-1 from the constitutive intracellular pathway (Russell et al., 1998a).

A third possibility is that a proportion of the basal ET-1 was formed by an alternative synthetic pathway. Intriguingly, significant amounts of ET-1 were detectable in the ECE-1/ECE-2 double-knock-out mouse embryos suggesting other proteases are involved in ET-1 synthesis (Yanagisawa et al., 2000). One candidate is the serine protease chymase present in mast cells. This enzyme cleaves the Tyr³¹-Gly³² peptide bond of big ET-1 to generate ET-1_(1–31), which is in turn converted to ET-1 (Fecteau et al., 2005; D'Orleans-Juste et al., 2008). Importantly, ET-1_(1–31) was equipotent compared with big ET-1 in causing vasoconstriction in human isolated vessels, including coronary arteries, and this was associated with the appearance of measurable levels of ET-1 in the bathing medium, consistent with conversion to the mature peptide. Vasoconstriction was fully blocked by ET_A selective antagonists, reflecting the predominance of the ET_A receptor on vascular smooth muscle (Maguire et al., 2001). Mast cell chymase is associated with interstitial spaces with the potential to convert circulating big ET-1 and provide a further source of ET-1. In addition, chymase also generates the intermediate ET-2(1–31) which is in turn converted to the mature peptides by an as yet uncharacterised mechanism. Interestingly chymase inhibitors appear more efficient in blocking big ET-2 conversion at least in primates. The radioimmunoassay used in the study cross-reacted equally well with mature ET-2 and any peptide formed by this pathway would also be detected. The importance of this pathway for synthesis of either peptide is unclear although the number of mast cells increases with cardiovascular disease, for example in atherosclerotic lesions (Ling et al., 2012).

Though NEP inhibition alone appeared as a possible therapeutic approach to hypertension and CHF, clinical experience with candoxatril and similar agents was disappointing (Ando et al., 1995; Richards et al., 1992b, 1993; Favrat et al., 1995; O'Connell et al., 1992; Kentsch et al., 1996). Consistent reductions in blood pressure were not obtained in healthy volunteers and patients with hypertension (Ando et al., 1995; Richards et al., 1992b, 1993; Favrat et al., 1995; O'Connell et al., 1992; Kentsch et al., 1996). Infusion of thiorphan into the human forearm, leading to local NEP inhibition, causes vasoconstriction at least in part reversed by a co-administration of an ET receptor antagonist Haynes and Webb, 1994; Love et al., 1996; Ferro et al., 1998). Candoxatril also increases plasma ET-1 concentrations (Ando et al., 1995; McDowell et al., 1997). Both these observations suggest that NEP may also contribute to degradation of ET-1 (Russell et al., 1996). Local vascular accumulation of ET-1 may counteract the blood pressure lowering effect of an increase in plasma natriuretic peptide concentrations after NEP inhibition, at least in healthy subjects and hypertensives (Ando et al., 1995; Richards et al., 1992a; Ferro et al., 1998; McDowell et al., 1997). NEP may also degrade angiotensin II and this too may counteract the hypotensive effect of natriuretic peptides (Richards et al., 1992b, 1993). NEP inhibition alone seemed to be more promising in CHF, compared to hypertension, possibly because of the much greater effect on circulating natriuretic peptide concentrations in this condition (Newby et al., 1998; Northridge et al., 1999). Even in CHF, however, a vasoconstrictor response to systemic NEP inhibition has been observed (Kentsch et al., 1996).

Combined NEP and ECE inhibition is a logical pharmacological approach. Phosphoramidon is not orally active and has not been

given systemically in humans. When infused into the human forearm brachial artery, however, phosphoramidon causes an increase in forearm blood flow, in striking contrast to the vasoconstrictor action of local NEP inhibition alone (Ferro et al., 1998; Haynes et al., 1995; Hand et al., 1999). This demonstrates that the vasodilator effect of inhibition of ET-1 production overwhelms the vasoconstrictor action of decreased ET-1 and angiotensin II degradation when both ECE and NEP are inhibited simultaneously.

We employed a similar experimental approach to that used to demonstrate the actions of ACE inhibitors (and dual NEP and ACE inhibitors) to confirm any ECE inhibiting action of SLV-306 in vivo (Buchwalder-Csajka et al., 1999; Rouso et al., 1998). As discussed earlier, NEP inhibition alone might have been expected to augment the hypertensive effect of big ET-1 infusion. We also found that plasma concentrations of big ET-1 increased significantly more on the SLV-306 days than on the placebo day. Furthermore, there was no significant change in plasma ET-1 concentration during big ET-1 infusion. Moreover, the big ET-1/ET-1 ratio significantly increased consistent with reduced conversion of big ET-1 to ET-1.

We were also able to confirm that SLV-306 is an NEP inhibitor in vivo. SLV-306 caused a dose dependent increase in plasma ANP concentration almost identical to that obtained with candoxatril in healthy volunteers (i.e. an approximate doubling of plasma ANP concentrations with the highest dose of inhibitor) (Northridge et al., 1989; Jardine et al., 1990).

Conclusion

In conclusion, the results suggest SLV-306 functions as a NEP inhibitor, augmenting circulating natriuretic peptide production and as an ECE inhibitor, reducing ET-1 synthesis, which is an attractive therapeutic option in cardiovascular disease. Such an agent overcomes the limitations of a sole NEP inhibitor, especially if used in combination with an ACE inhibitor or angiotensin II receptor antagonist.

Conflict of interest statement

This study was funded by Solvay Pharmaceuticals B.V. Netherlands. HE and HJV are employees of Solvay.

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