Triple Vasopeptidase Inhibition Normalizes Blood Pressure in Conscious, Unrestrained, and Spontaneously Hypertensive Rats

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Background: CGS 35601 is a potent triple vasopeptidase inhibitor (VPI) of angiotensin-converting enzyme (ACE), neutral endopeptidase (NEP), and endothelin-converting enzyme (ECE). The aim of the study was to determine the effects of this VPI on the hemodynamic profile of conscious, unrestrained, spontaneously hypertensive rats (SHR), in comparison to selective inhibitors of ACE and ACE + NEP, than +ECE combined. Circulating plasma concentrations of vasoactive mediators and reactive oxygen species were measured.

Results: The lowest dose of CGS 35601 had no effect. Doses at 0.1, 1, and 5 mg/kg/d reduced mean arterial blood pressure by 10%, 22%, and 40%, respectively. Heart rate was unaffected in all groups. CGS 35601 decreased concentrations of angiotensin II (Ang II), endothelin-1 (ET-1), and pro-atrial natriuretic peptide (proANP), and increased those of big ET-1, atrial natriuretic peptide (ANP), bradykinin (BK), and hydrogen peroxide (H₂O₂) dose dependently.

Conclusions: The blood pressure-lowering effect of this triple VPI was superior to that of the other VPI in this preclinical rat model of hypertension. Further experiments are needed to assess triple VPI to other combinations in other models with regard to efficacy and angioedema. Only then it may constitute a first-in-class approach for the treatment of hypertension and other cardiovascular disorders.

Blood pressure (BP) regulation is the net result of complex interactions between vasoconstrictors (angiotensin II [Ang II]), endothelin-1 [ET-1]) and vasodilatators (natriuretic peptides [NPs]; Atrial natriuretic peptide (ANP), C-type natriuretic peptide (CNP), Brain natriuretic peptide (BNP), brady-kinin (BK) and nitric oxide [NO]) on endothelial and vascular smooth muscle cells. Their gene (receptor) expression, production, release, and degradation control vascular tone. Thus, inhibiting Ang II and ET-1 mediated activities, whereas potentiating those mediated by NPs and BK may prove beneficial in hypertension and in other cardiovascular complications. Consequently, simultaneous inhibition of the angiotensin, endothelin-converting enzymes (ECE),

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Combination of agents are used to treat hypertension, which involve different mechanisms of action such as the recent tentative development of dual vasopeptidase inhibitor (VPI) (angiotensin-converting enzyme [ACE] and neutral endopeptidase [NEP]),\(^5\)\(^6\) namely omapatrilat (BMS-186716), a heterocyclic dipeptide mimetic as one of the first dual VPI (IC\(_{50}\) of 6.0 and 8.9 nmol/L, respectively).\(^7\) This drug was shown to be effective in preclinical animal models of hypertension, which led to clinical trials where it was reported to be more effective than the single use of enalapril, a selective ACE inhibitor (ACEi), in preventing death and the worsening of heart failure in hypertensive patients. However, adverse effects such as angioedema were observed and halted its development.\(^8\)\(^9\)

The validation process of this novel class of agent as dual VPI (ACE/NEP) may have overlooked one fundamental aspect: this combination could increase the circulating plasma concentrations of peptidic ETs: potent vasoconstrictor\(^10\) and pro-mitogenic\(^11\) agents. The ETs also increase vascular permeability,\(^12\) which could lead to angioedema. The ETs are sensitive to degradation by NEP as demonstrated by candoxatril, a NEP inhibitor.\(^13\) Therefore, increased plasma concentrations of ETs were observed after the administration of omapatrilat in patients,\(^14\) which may limit the beneficial effects of omapatrilat, when ETs is added to NPs and BK toward angioedema. Because both NEP and ECE-1 are both located in the postcapillary endothelium, NEP inhibitors may drastically increase local concentrations of ETs and may 1) increase plasma extravasation,\(^12\) 2) cause vasoconstriction of the renal vascular bed mostly sensitive to this family of mediators,\(^15\) and 3) cause the release of NO through the activation of the endothelium-located ET\(_B\) receptor subtype.\(^16\) Thus, the concomitant modulation of the ET system through the additional inhibition of ECE-1 activity over dual ACE/NEP inhibition might offer a promising avenue to maintain BP homeostasis and prevent end-organ damage.\(^17\)

In the present study we tested CGS 35601, a triple VPI that simultaneously inhibits ACE, NEP, and ECE-1\(^18\) (Table 1) at lowering mean arterial BP (MABP) in spontaneously hypertensive rats (SHR), a normal renin-dependent and well-accepted model of human essential hypertension. This molecule was compared to combinations of selective inhibitors of ACE (benazepril)\(^19\) and NEP (CGS 24592),\(^20\) and ECE-1 (CGS 35066)\(^21\) (Table 1). Our results show that CGS 35601 dose-dependently reduced MABP, in correlation with the regulation of four natural surrogate markers: Ang II, ET-1, ANP, and BK, and was more effective than selective ACEi and subsequent combinations.

### Methods

All procedures were previously approved by the local Ethics Animal Care Committee and followed the guidelines on animal welfare established by the Canadian Council on Animal Care.

**Presurgical Setup, Anesthesia, Surgical Procedures, and Postoperative Care**

Adult male SHR (retired breeders; aged >36 weeks; 385 ± 12 g; Harlan Sprague-Dawley, Indianapolis, IN), adult male Wistar and Sprague-Dawley rats (aged 12 to 14 weeks; body weight matched; Charles River, St. Constant, Canada), were housed individually in modified metabolic cage (Nalgene, Rochester, NY), under a 12-h cycle of day/night, with free access to drinking water and fed ad libitum.

As described before,\(^22\) surgical procedures under inhaled anesthesia (2% isoflurane inhalation, Baxter Corp, Toronto, Canada) were performed in a strictly aseptic environment with sterilized materials. The urethane-coated antithrombogenic vascular catheters (PhysioCath, Data Sciences International, Saint Paul, MN) was inserted into the femoral artery up to the abdominal aorta and tunneled under the skin to the dorsal site at the neck, was connected to a low-flow peristaltic pump (Instech-Solomon, Plymouth Meeting, PA) through a stainless steel spring stock protector and swivel by an adjustable counterbalance lever arm (Instech-Solomon, Plymouth Meeting, PA). A constant infusion of sterile heparinized (4 U/mL) saline (250 \(\mu\)L/h or 6 mL/d) prevented the formation of blood clots within the catheter.

**Blood Sampling**

Arterial blood was collected twice daily through the catheter to minimize interference of the blood collection with

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**Table 1.** Potency (nmol/L) and selectivity of vasopeptidase inhibitors used in the present set of experiments

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>ACE EC 3.4.15.1</th>
<th>NEP EC 3.4.24.11</th>
<th>ECE EC 3.4.24.71</th>
<th>Selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACEi benazepril Lotensin (CGS 14824a)</td>
<td>2</td>
<td>ND</td>
<td>ND</td>
<td>ACE &gt;10,000-fold for NEP</td>
</tr>
<tr>
<td>NEP inhibitor (CGS 24592)</td>
<td>&gt;10,000</td>
<td>0.9</td>
<td>20,000</td>
<td>105-fold for ECE</td>
</tr>
<tr>
<td>ECE inhibitor (CGS 35066)</td>
<td>&gt;10,000</td>
<td>2300</td>
<td>22</td>
<td>11-fold for NEP</td>
</tr>
<tr>
<td>Triple VPI (CGS 35601)</td>
<td>22</td>
<td>2</td>
<td>55</td>
<td></td>
</tr>
</tbody>
</table>

ND = not determined. See text for other abbreviations.
metabolic functions of the rats, at 8 AM and 8 PM, in heparin-lithium (AM) and a K<sub>e</sub>EDTA (PM) vacutainers for biochemical analyses, and replaced by an equal amount of sterile saline.

**Drugs and Administration**

GS 35601, benazepril, CGS 24592, and CGS 35066 were kindly provided by Novartis. CGS 35601, a potent triple ACE, NEP, and ECE-1 inhibitor (L-tryptophan, N-{[(2S)-2-mercapto-4-methyl-1-oxopentyl] amino} cyclopentyl carbonyl) was dissolved at 10 mg/mL in sterile saline:NaOH (0.1 N) before its final dilution at 1 mg/mL in saline. Benazepril, a selective ACE inhibitor (CGS 14824a; (S)-(ethoxy-carbonyl-3-phenyl-(1S)-propyl)amino) 2,3,4,5-tetrahydro-2-oxo-1H-(3S)-benzazepine-1-acetic acid monohydrochloride) and the ECE-1 inhibitor (CGS 35066; (R)-α-[phosphonomethylamino]- dibenzofuran-3-propanoic acid) were readily soluble in saline at 1 mg/mL. The NEP inhibitor (CGS 24592; (S)-N-[2-(phosphonomethylamino)-3-(4-biphenyl)-propionyl]-3-amino propionic acid) was prepared at 1 mg/mL in buffered saline. Each drug solution was then sterilized through a 0.22-μm filter and kept frozen until its use. Seven days after surgery the rats were administered, through the vascular catheter, intra-arterially (i.a.) with CGS 35601 at 0.01, 0.1, 1, 5, mg/kg/d for 5 days at each dose or a selected combination of ACE/NEP/ECE inhibitors at 1 or 5 mg/kg/d for 5 days.

**End Point Parameters**

The hemodynamic profile was assessed each day at 1 PM on calm, resting, unrestrained and conscious SHR, through direct measurement by the implanted vascular catheter using a BP transducer (Harvard Apparatus, Montreal, QC, Canada) connected to a precalibrated computerized system (PowerLab, ADInstrument, Colorado Springs, CO). Heart rate (HR) was simultaneously derived from these data and recorded.

Biochemical assays for Ang II, ET-1, big-ET, ANP, proANP, BK, H<sub>2</sub>O, and NO metabolites were determined with various enzyme immunoassay (EIA) or chemiluminescent assay kits from Peninsula (San Carlos, CA) and Biomedica (Vienna, Austria), as described previously. A C18 Sep-Pak cartridge extraction procedure was conducted before Ang II, ANP, and BK determinations.

**HPLC Analysis of CGS 35601 in Plasma**

Arterial plasma concentrations of CGS 35601 were measured using a two-solvent gradient separating system (A: HPLC grade water [0.05% trifluoroacetic acid (TFA)]; B: acetonitrile [0.025% TFA]; flow rate: 1.0 mL/min; gradient at 0 min, 5% B; 2 to 25 min, 5% to 50% B; 50 to 95 min, 26% to 30% B and 35 to 40 min, 95% to 5% B) with a Zorbax 300SB-C18 analytical column (4.6 by 150 mm, 5 μm) on an HPLC system (Agilent model 1100 series, Wilmington, DE) using a diode array detector (Agilent model DE292916850). Absorbance at 252 nm was measured at a retention time of 15.2 min, and the plasma level of CGS 35601 was calculated according to freshly prepared calibration standards.

**Statistical Analyses**

Results are expressed as mean ± SEM. Because of the unavailability of some samples/data at a given time point, a repeated measurement analysis of variance was inapplicable to compare values obtained at different time periods. Thus, for comparisons within groups, a randomized block design was applied using two factors defined for the analysis: the subject effect and the time period effect.

Comparison between groups was performed by a three-way ANOVA with a blocking factor representing subjects. Interaction between the time period factor and one used to compare groups was added to the model. When interaction was significant for parameters, comparisons at different time period were analyzed using Student paired t tests. The normality and variance assumptions were met for almost all data. All analyses were conducted using the statistical package SAS (SAS Institute Inc., Cary, NC).

**Results**

**Plasma Concentrations of CGS 35601, a triple VPI**

The HPLC analysis of plasma from rats treated with CGS 35601 show a dose-dependent elevation in plasma concentration of the drug. CGS 35601 contains a thiol (R-SH) group (Fig. 1) that, under basic condition necessary to its dissolution, can be oxidized to disulfides (R-S-S-R). The resulting CGS 35601 homodimer (R-S-S-R) is inactive and represents 40% of the drug stock solution (data not shown). Therefore, both the reduced and oxidized forms of CGS 35601 were detected in the plasma of treated rats. Circulating plasma concentrations of CGS 35601 were not detected at 48 h after cessation of the treatment (Fig. 1).

**Hemodynamic Profile**

Conscious unrestrained SHR had elevated MABP of 156 ± 4 mm Hg compared to the 109 ± 9 and 98 ± 7 mm Hg during the experiment (0.01, 0.1, 1, and 5 mg/kg/d, continuous i.a. infusion, 5 d/dose) during 20 days followed by a 5-day washout period. The reduced form of CGS 35601 is the only active form. N = 5 samples per time point.
measured in normotensive Wistar and Sprague-Dawley rats, respectively (Fig. 2a). CGS 35601 at the lowest dose tested (0.01 mg/kg/d i.a.) did not affect the hemodynamic profile of treated SHR versus untreated SHR (Fig. 2a). Dosing at 0.1 mg/kg/d decreased the MABP by 10% after 5 days (a 5-day average of 156 ± 4 to 141 ± 7 mm Hg; Fig. 2a,c; P < .05). Higher doses at 1 and 5 mg/kg/d reduced the MABP by 22% (down to 122 ± 4 mm Hg; P < .01) and 40% (down to 94 ± 5 mm Hg; P < .001), respectively, compared to untreated SHR (Fig. 2a,c), without affecting the HR (Fig. 2b). The MABP gradually increased back to baseline after cessation of treatment (Fig. 2a).

In contrast, inhibition of ACE with the selective inhibitor benazepril was only 25% as effective as CGS 35601 against elevated BP in SHR at 1 mg/kg/d and twice as less at 5 mg/kg/d (Fig. 2d). Combination of the ACEi with a selective NEP inhibitor (CGS 24592) had no additional effect in this model, nor did the triple combination of selective inhibitors (ACEi + NEP inhibitor + ECE inhibitor) have increased efficacy at reducing MABP in SHR (Fig. 2d), although the doses of NEP and ECE inhibitors have been previously shown to be pharmacologically relevant.20 Triple inhibition with CGS 35601 was as effective at reducing both the systolic and diastolic BP (data not shown), suggesting that there was no noticeable effects, such as overload of the left ventricular function associated with a reduction in MABP, after 20 days of continuous treatment ranging from 0.01 to 5 mg/kg/d.

**Circulating Plasma Concentrations of Vasoactive Mediators**

Circulating plasma concentrations of key vasocontractile mediators (Ang II, ET-1) and vasodilators (ANP, BK, and NO), and some of their precursors, big ET-1 and proANP, were measured before and on day 5 after each dosing (when MABP had stabilized), and after washout. CGS 35601 dose-dependently decreased the concentrations of Ang II and ET-1 (Fig. 3a,b), whereas it increased the concentrations of BK and ANP (Fig. 4a,b). Concentrations of Ang II were reduced by 39% at the end of the highest dose of 5 mg/kg/d (P < .05) and bounced back to 1.72-fold above baseline (P < .05) 5 days after removal of the drug (Fig. 3a). A similar profile was observed for ET-1 (−56% at 5 mg/kg/d of CGS 35601; Fig. 3b). The plasma concentrations of BK and ANP dose dependently increased (Fig. 4a,b) by 1.82- and 1.88-fold, respectively, (P < .05) on day 5 of the 5 mg/kg/day dose, and returned toward baseline values upon removal of the drug (Fig. 4a,b). Interestingly, big ET-1 and proANP precursors presented opposite patterns to each other and to their respective mature active peptides. Triple VPI increased big ET-1 concentrations by 2.5-fold (Fig. 3c), whereas it decreased proANP in a dose-dependent manner by up to 50% (Fig. 4c). Finally, the plasma concentrations of NO were dose dependently decreased up to 45% at the highest dose tested of the triple VPI (Fig. 5a). In contrast to the peptidic mediators, the circulating plasma concentrations of NO remained below baseline up to 5 days after cessation of treatment. The ratio of mature peptides to their respective precursors confirmed that the conversion of big ET-1 into active ET-1 was inhibited by CGS 35601 (Fig. 3d). The increase in the ANP/proANP ratio by a factor of 2.2 reflected the accumulation of ANP, resulting from the inhibition of NEP combined with a downregulated synthesis and release of proANP (Fig. 4d). Circulating plasma concentration of H2O2 was measured as a marker of oxidative stress. The concentration of H2O2 showed a constant decrease (from 7.57 ± 0.67 to 5.45 ± 0.37 μmol/L at the end of the 5 mg/kg/d dosage) (Fig. 5b).

**Circulating Plasma Concentrations of Toxicologic Markers**

Triple VPI CGS 35601 showed no increased in various hepatic and renal toxicologic markers over the dose and time range (data not shown). A detailed pharmacotoxicologic profile of CGS 35601 in these SHR will be published elsewhere. In this study, an increase in the plasma concentrations of potassium was observed between 12 and 22 days after surgery. However, there were no differences in SHR whether treated or not with CGS 35601 (Fig. 6a). Selective ACEi, or combination with a NEP inhibitor and then an ECE inhibitor, also did not reveal any difference (Fig. 6b).

**Discussion**

Vasopeptidase inhibition presented a novel therapeutic principle beyond selective ACEi, combining ACE and NEP inhibition. Dual VPI simultaneously modulates peptidic vasoconstrictors and vasodilators, decreasing vascular tone and BP.5 Donckier et al.24 noticed that ET blockade caused an additional hypotensive effect compared to single ACEi in hypertensive dogs. Here we have shown that the single molecule triple VPI CGS 35601 is more effective at reducing elevated MABP in old SHR with more than 5 days of treatment at 1 and 5 mg/kg/d than dose-matched with a single ACEi (although benazepril is an 11 times more potent ACEi than CGS 35601; Table 1) or an ACEi + NEP inhibitor combination. Triple VPI reduced MABP to, and slightly below, baseline values measured in normotensive Wistar or Sprague-Dawley rats using the same set-up (Fig. 2a).

The ACEi was reported to inhibit the stimulated release of ET-1 from human endothelial cells,25 but failed earlier on to modulate the ET system in patients with congestive heart failure (CHF),26 until more recently when it decreased circulating plasma concentrations of ET-1 in patients with CHF27 and in post-myocardial infarcted rats.28 Here, the antihypertensive effect of CGS 35601 is associated with a reduction in circulating plasma concentrations of two vasoconstrictors: Ang II and ET-1. The effect of the triple VPI was greater than dose-matched single ACEi or
FIG. 2. In vivo pharmacologic effects of CGS 35601 and other combinations of vasopeptidase inhibitor on the hemodynamic profile. (a) Mean arterial blood pressure (MABP; mm Hg) in instrumented, unrestrained, and conscious control Wistar (saline, □, n = 9) and Sprague-Dawley (saline, ●, n = 10), and spontaneously hypertensive rats (SHR) (saline, group 1, ○, n = 10) and SHR administered (group 2, ●, n = 13 to 18) with increasing doses of the vasopeptidase inhibitor CGS 35601 (starting 7 days after surgery, renamed time 0; 0.01, 0.1, 1, and 5 mg/kg/d, continuous i.a. infusion, 5 d/dose) during 20 days after a 5-day washout period. (b) Heart rate (beats/min) in instrumented, unrestrained, and conscious control SHR (saline, group 1, ○, n = 10) and SHR administered (group 2, ●, n = 13 to 18) with increasing doses of the vasopeptidase inhibitor CGS 35601 (starting 7 days after surgery, renamed time 0; 0.01, 0.1, 1, and 5 mg/kg/d, continuous i.a. infusion, 5 d/dose) during 20 days followed by a 5-day washout period. Percentage of MABP reduction in instrumented, unrestrained, and conscious SHR treated with (c) CGS 35601 (0.01, 0.1, 1, and 5 mg/kg/d) (n = 13 to 18) or (d) selective ACEi, NEP inhibitor, and ECEi inhibitor in combinations at 1 and 5 mg/kg/d (open square and black square, respectively) (n = 7) versus dose-matched CGS 35601. *P < .05 to .001, significantly different between untreated group 1 and CGS 35601-treated group 2 over time; **P < .05 to .001, significantly different between CGS 35601 treated and other dose-respective treated combination groups; #P < .001, significant variation within the same group over time. ACEi = angiotensin-converting enzyme inhibitor; NEPi = neutral endopeptidase inhibitor; ECEi = endothelin-converting enzyme inhibitor.
combined ACEi/NEP inhibitor in old SHR. CGS 35601 distinctively blocked big ET-1 conversion into mature ET-1.

The dual ACEi/NEP inhibitor omapatrilat increased circulating plasma concentrations of ET-1 in DOCA-salt rats and in patients with CHF. This may explain in part why a triple VPI could be more effective than a dual VPI, while blocking ET-1-mediated peripheral renal vasoconstriction (mostly sensitive to ET-1), cardiac dysfunction, and elevation of vascular permeability (edema). The ET-mediated plasma extravasation is mediated, at least in part, through endothelial ETB receptor subtypes releasing both prostacyclin and NO.

Here, triple VPI, as demonstrated for NEP inhibitor, elevated plasma concentrations of peptidic vasodilators such as ANP and BK through decreased NEP activity. The concentrations of ET-1 were not increased, as its synthesis is already suppressed by the inhibition of ECE by the triple VPI. Single ACEi or dual (ACEi/NEP inhibitor) VPI increased the concentrations of BK in SHR and post-myocardial infarcted rat. The distinct and relative contribution of selective ACEi and NEP inhibitor into the release of ANP and BK in SHR remains to be evaluated. Adrenomedullin (ADM), another peptidic vasodilator, and other autacoids such as prostacyclin, could also be altered by triple VPI.

Triple VPI also decreased the circulating plasma concentrations of NO, another vasodilator. This observation is paradoxical, as NO is released in part through the activation...
of ANP and BK, which are significantly increased by CGS 35601. Dual ACEi/NEP inhibitor was reported to increase ANP, BK, and NO.32 It is known that ET-1 stimulates the release of NO through endothelial ETB receptors. In the present study CGS 35601 inhibited the release of ET-1. Therefore, ET-1-mediated NO release appears to be a key component in SHR BP regulation as triple VPI significantly and dose-dependently decreased NO, and still concomitantly reducing MABP.

Triple VPI also attenuated the circulating H2O2, a key reactive oxygen species (ROS). It has been reported that stimulated renin-angiotensin (RAS) and ET systems can increase the production of superoxide anions (·O2−) and H2O2, contributing to vascular oxidative stress. Vice versa, ROS upregulate both RAS and ETs (positive feedback loop).34 Here, triple VPI reduced Ang II, ET-1, and NO concentrations, which resulted in less H2O2. Thus, the reduction of NO, which as yet to be reported with another triple VPI35, may be beneficial as the action of dual VPI omapatrilat is plagued by incidences of angioedema40 and plasma extravasation in other organs to further assess the potential of this new class of VPI (triple) in comparison to other classes of VPI before entering safety and efficacy trials toward treating hypertension, myocardial infarction, and CHF. CGS 35601’ pro-drug (CGS 37808) is available18 for long-term preclinical experiments.

Acknowledgments
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