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PHARMACOLOGICAL PROFILE OF A NON-PEPTIDIC DUAL INHIBITOR OF NEUTRAL ENDOPEPTIDASE 24.11 AND ENDOTHELIN-CONVERTING ENZYME

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CGS 26303 is a potent and structurally unique non-peptidic inhibitor of neutral endopeptidase (NEP) capable of protecting atrial natriuretic peptide (ANP) from enzymatic degradation. In addition, CGS 26303 displays modest endothelin-converting enzyme (ECE) inhibitory activity *in vitro*. Unlike CGS 24592, a potent but selective NEP inhibitor, CGS 26303 significantly blocks endothelin-1 production in rats after exogenous administration of big ET-1 and reduces the mean arterial pressure in spontaneously hypertensive rats during chronic administration. These results suggest that CGS 26303 represents a new class of therapeutic agents with potential benefits for the treatment of cardiovascular and renal disorders.

Endothelin-1 (ET-1), the potent 21-amino acid vasoconstrictor peptide secreted by the vascular endothelium (1), has been implicated in many cardiovascular and renal disorders. Infusion of ET-1 in animals and humans has caused, among other effects, an increase in mean arterial pressure (MAP) and reductions in renal blood flow and urinary sodium excretion (2-4). In addition, elevated ET-1 levels have been reported in patients with congestive heart failure, myocardial ischemia and acute renal failure (5-7). Furthermore, stimulation of vascular smooth muscle proliferation by ET-1 may reflect a possible involvement of this peptide in the development of atherosclerosis (8). The production of endogenous ET-1 results from the selective processing of a larger biosynthetic precursor, known as big endothelin-1 (big ET-1), by an endothelin-converting enzyme (ECE)(9). This conversion appears to be essential for full expression of the biological activity of the mature peptide. Therefore, inhibition of ECE might be expected to produce therapeutic benefits similar to those proposed for ET-1 receptor antagonists (10).

The biological effects of ET-1 contrast sharply with those of atrial natriuretic peptide (ANP), a 28-amino acid vasodilator produced by atrial myocytes (11). The natriuretic and antihypertensive effects of ANP have been demonstrated clinically (12). It has been shown that

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neutral endopeptidase (NEP, EC 3.4.24.11) is the major enzyme responsible for the inactivation of ANP *in vivo* and that NEP inhibitors can elicit ANP-like effects in animals and in patients (13-15). These observations suggest that antagonizing the action of ET-1 while potentiating endogenous ANP levels could offer a therapeutic benefit for the treatment of cardiovascular and renal diseases.

The cloning and expression of ECE has recently been reported (16), confirming that ECE is a zinc-metalloprotease (17) with some structural similarities and sequence homology to NEP. Accordingly, the potent NEP inhibitor phosphoramidon is also a modest inhibitor of ECE (18). However, the potential development of phosphoramidon analogs as orally-active drugs is limited by the presence of an acid-labile phosphorus-nitrogen bond. Other classes of potent NEP inhibitors (thiols, carboxylic acids, hydroxamic acids) have displayed weak inhibitory activity towards ECE (19). In this communication, we describe the pharmacology of a structurally unique non-peptidic phosphonic acid capable of protecting ANP from enzymatic degradation as well as significantly blocking the production of ET-1 from exogenously administered big ET-1 (20).

Materials and Methods

Synthesis of inhibitors. CGS 24592 (*(S)*-N-[2-(phosphonomethylamino)-3-(4-biphenylyl)-propionyl]-3-amino-propionic acid) was prepared according to the published procedure (21). The synthetic procedure for the preparation of CGS 26303 (*(S)*-2-biphenyl-4-yl-1-(1H-tetrazol-5-yl)-ethylamino-methyl phosphonic acid) will appear elsewhere.

Enzymes inhibition in vitro. The activity of NEP, obtained from washed kidney cortex membranes (22), was determined by hydrolysis of the synthetic substrate glutaryl-Ala-Ala-Phe-2-naphthylamide (GAAP) as reported previously (21, 23).

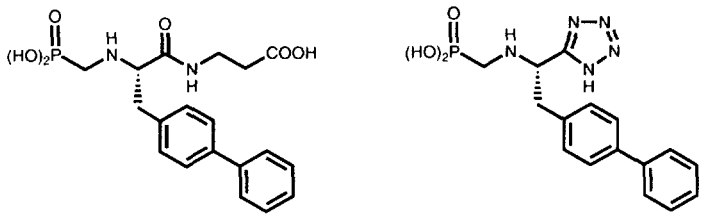
ECE was partially purified from porcine primary aortic endothelial cells by DE52 anion exchange column chromatography and its activity was quantified by radioimmunoassay (24).

Plasma ANP potentiation. The effect of the inhibitors on plasma ANP concentrations was measured by radioimmunoassay in Sprague-Dawley rats ([Tac:N(SD)fBR]) infused continuously with ANP(99-126) as described previously (14). The responses produced by CGS 24592 and CGS 26303 are expressed as a percent of those obtained in vehicle-treated animals.

Big ET-1-induced pressor response in the anesthetized rat. The effect of the inhibitors on the pressor response resulting from big ET-1 challenge was measured in Sprague-Dawley rats as described previously (25). Results are expressed as percent inhibition of the big ET-1-induced pressor response in rats treated with CGS 24592 or CGS 26303 as compared to vehicle.

Antihypertensive effect of CGS 26303 in spontaneously hypertensive rat. The effect of chronic administration (13 days) of CGS 26303 on blood pressure was measured in conscious spontaneously hypertensive rats (SHR, [Tac:N(SHR)fBR]). Each data point indicates a 24 hr average representing the change in mean arterial pressure (ΔMAP) from baseline for each group of rats as measured by a radiotelemetry system (26). Either vehicle (1 M $NaHCO_3$) or CGS 26303 was administered continuously for 13 days via an osmotic minipump implanted subcutaneously.

Effect of ECE inhibition on big ET-1-induced pressor response in conscious spontaneously hypertensive rat. Male SHR (16-18 weeks of age) were administered either CGS 26303 or vehicle (1 M $NaHCO_3$) via an osmotic minipump implanted subcutaneously. On day 5, femoral arterial and venous catheters were placed in anesthetized rats for the measurement of MAP and drug administration, respectively. After a 48-hr recovery period, MAP was recorded (day 7) through the arterial catheter connected to a pressure transducer. Blood pressure and heart rate were allowed to stabilize for 30 min before ganglion blockade was performed using chlorisondamine (10 mg/kg i.v.). Approximately 15 min later, a bolus dose of big ET-1 (0.25 nmol/kg i.v.) was administered to both vehicle- and CGS 26303-treated rats. The change in blood pressure in response to big ET-1 was then compared between the two groups of rats at 1, 5, 10, 15, 30 and 60 min after dosing using a two-way ANOVA.



CGS 24592 CGS 26303

Fig. 1. Structures of CGS 24592 and CGS 26303.

Results and Discussion

The most potent NEP inhibitors, including the phosphonomethyl dipeptide CGS 24592 (Fig.1), have usually been designed around a di- or tripeptide framework connected to a zinc-binding element (21). Recently, we have discovered a new type of phosphorus-containing dual inhibitor of NEP and ECE derived from a *single α-amino acid.* CGS 26303 is the prototype of this new generation of non-peptidic inhibitors (Fig.1). *In vitro*, CGS 26303 was found to be among the most potent NEP inhibitors reported, with an IC_{50} of 0.9 ± 0.1 nM (n=7), a value similar to that of CGS 24592. However, compared to CGS 24592 which inhibited ECE activity by only 20% at a concentration of 100 μM, CGS 26303 was a significantly more potent ECE inhibitor (IC_{50} = 1.1 ± 0.2 μM, n=3) (Fig. 2). It was also a slightly more potent dual inhibitor than phosphoramidon (IC_{50} = 39.9 ± 2.2 nM for NEP, IC_{50} = 3.5 ± 0.8 μM for ECE) (25).

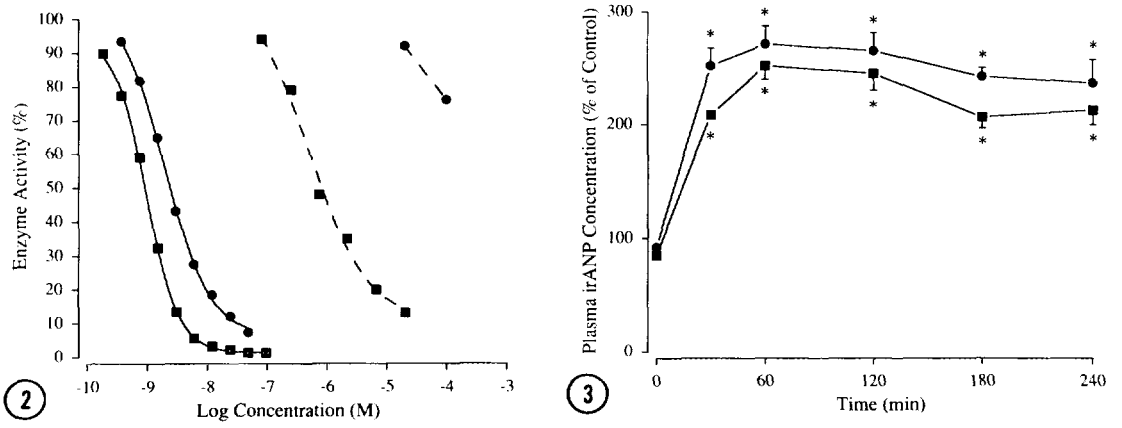


Fig. 2. *In vitro* inhibition profile of NEP (solid lines) and ECE (dashed lines) by CGS 24592 (●, IC_{50} = 2.6 ± 0.2 nM, n = 6 for NEP; IC_{50} > 100 μM for ECE) and CGS 26303 (■, IC_{50} = 0.9 ± 0.1 nM, n = 7 for NEP; IC_{50} = 1.1 ± 0.2 μM, n = 3 for ECE).

Fig. 3. Effect of CGS 24592 (●) and CGS 26303 (■) on plasma immunoreactive ANP (irANP) concentrations in conscious rats infused with exogenous ANP (99-126). Values represent mean ± SEM (n = 3). Asterisks indicate a significant ($p < 0.05$) difference in plasma irANP levels between vehicle- and drug-treated rats.

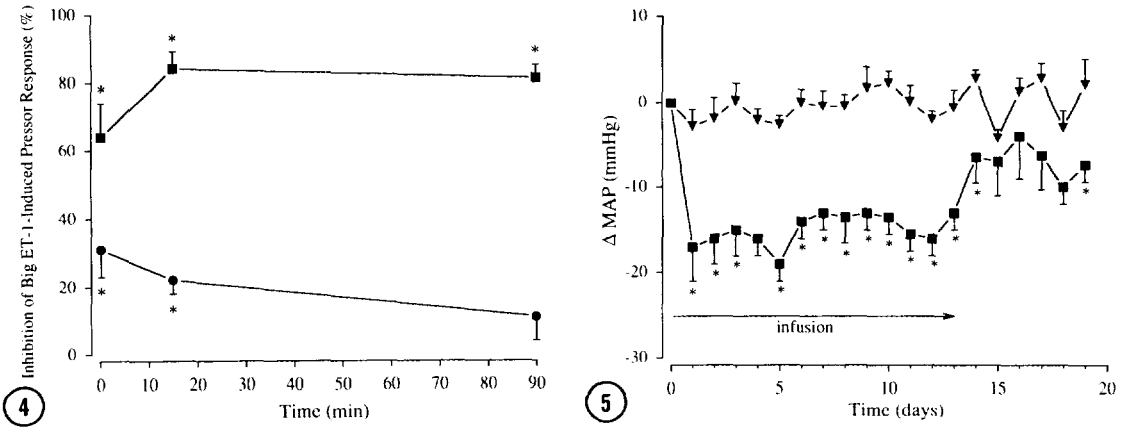


Fig. 4. Effect of CGS 24592 (●) and CGS 26303 (■) on the pressor response produced by big ET-1 in ganglion-blocked anesthetized rats. Values represent the mean ± SEM (n= 7). Asterisks indicate a significant ($p < 0.05$) difference in the pressor response inhibition between vehicle- and drug-treated rats.

Fig. 5. Effect of chronic infusion (day 1-13) of CGS 26303 (■, 5mg/kg/day) relative to vehicle (▼) on blood pressure in conscious SHR. Values are the 24 hr average change in MAP ± SEM (n= 7). Asterisks indicate a significant ($p<0.05$, ANOVA followed by Tukey's multiple range test) difference in blood pressure reduction between vehicle- and drug-treated rats.

In contrast to phosphoramidon, both CGS 24592 and CGS 26303 achieved high and sustained plasma levels in rats after i.v. administration at 10 mg/kg. The concentrations of unbound CGS 26303 and CGS 24592 were about 6-9 μM at 10 min after dosing and were still maintained between 0.8 and 1.3 μM four hours later (data not shown). Such levels were expected to efficiently protect exogenously administered ANP from degradation by NEP *in vivo*. Indeed, both CGS 26303 and CGS 24592 (3 mg/kg i.a.) produced a 100-130% increase ($p<0.05$) in plasma immunoreactive ANP (irANP) levels which were sustained for 4 hr (Fig. 3). Furthermore, the high plasma levels of the dual inhibitor, CGS 26303, were also expected to be sufficient to inhibit the conversion of big ET-1 to ET-1. Indeed, the big ET-1-induced pressor response was markedly inhibited (64-84%, $p<0.05$) following the administration of CGS 26303 at 30 mg/kg i.v. and remained unchanged between 15 and 90 min after dosing (Fig. 4). By comparison, phosphoramidon had only a transient effect, showing 72 ± 5% and 22 ± 6% inhibition at 15 sec and 90 min, respectively (data not shown). CGS 24592 produced a modest inhibition of the big ET-1-induced pressor response initially, but its effect also rapidly diminished with time (Fig. 4).

The antihypertensive effects of CGS 26303 and CGS 24592 were determined in SHR using telemetry. Although this model is not responsive to selective NEP inhibitors, including CGS 24592 (27) (maximum decrease in MAP was 2 ± 1 mmHg during a 13-day infusion of CGS 24592 at 30 mg/kg/day), the dual inhibitor CGS 26303 produced a sustained reduction in MAP when infused continuously (5 mg/kg/day for 13 days, Fig. 5). A depressor response of approximately 15-20 mmHg occurred on day 1 and persisted for the remainder of the study.

Blood pressure in CGS 26303-treated rats returned to values not significantly different from those in vehicle-treated animals within 1 day following the removal of the osmotic minipump.

To assess the degree of ECE inhibition under these conditions, SHR were continuously infused with CGS 26303 (5 mg/kg/day) or vehicle and challenged with big ET-1. CGS 26303 significantly inhibited the maximum big ET-induced increase in MAP by 45% (peak value) or 64% (area under the curve) when compared to vehicle (Fig. 6). These data are consistent with a functional blockade of ECE during the chronic administration of CGS 26303. It is unlikely that the observed fall in arterial pressure is due to NEP inhibition since the structurally related but selective NEP inhibitor CGS 24592 was ineffective under identical conditions. Previously, acute studies with the shorter-acting dual inhibitor phosphoramidon have led to conflicting conclusions about the contribution of endogenous ET-1 to the hypertensive state in SHR (28, 29), thereby raising questions about the potential of ECE inhibition as a viable antihypertensive therapy (30). However, our results with long-term administration of a more effective ECE inhibitor, CGS 26303, support a role for endothelin in the tonic regulation of blood pressure in the SHR. Furthermore, the magnitude of the antihypertensive effect during chronic ECE inhibition is similar to that elicited by acute injections of the ET_A antagonist BQ123 in conscious SHR (29).

In conclusion, we have discovered a structurally unique dual NEP/ECE inhibitor, CGS 26303, capable of potentiating ANP levels, inhibiting the big ET-1-induced pressor response and lowering arterial pressure in conscious SHR. This non-peptidic and long-acting phosphonic acid exhibits an improved antihypertensive effect as compared to the similarly potent but selective NEP inhibitor CGS 24592. Our results suggest that these differences result mainly from the inhibition of ECE and that this new class of inhibitors can regulate both ANP and ET-1 levels *in vivo*, thereby holding promise as novel agents for the treatment of cardiovascular and renal diseases.

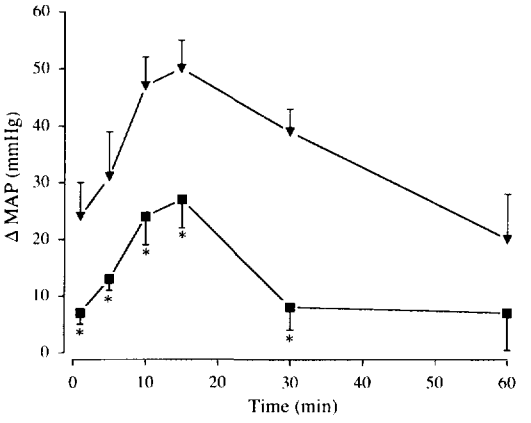


Fig. 6. Effect of CGS 26303 (■, 5 mg/kg/day) compared to vehicle (▼) on the change in MAP in response to exogenous administration of big ET-1 (0.25 nmol/kg, i.v.) in conscious SHR. Values represent the mean ± SEM (n= 5). Asterisks indicate a significant ($p < 0.05$) difference in reduction of the big ET-1-induced pressor response in drug-treated rats as compared to those receiving vehicle only.

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