CGS 30440: A Dual Inhibitor of Angiotensin-Converting Enzyme and Neutral Endopeptidase 24.11

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INTRODUCTION

For nearly two decades, angiotensin converting enzyme (ACE) inhibitors have been used in the management of hypertension and as adjunctive therapy in the treatment of congestive heart failure (15,26,27). Clinical studies have demonstrated that ACE inhibitors decrease the morbidity and mortality following myocardial infarction (49) and prevent heart failure in patients with left ventricular dysfunction (50,63). Captopril, the first ACE inhibitor discovered in 1975, has been approved for the treatment of nephropathy associated with type 1 diabetes mellitus (38). Clearly, the success achieved with ACE inhibitors in the management of cardiovascular diseases demonstrates the important role that the renin-angiotensin system plays in the etiology of these conditions.

Upon the demonstration that neutral endopeptidase (NEP) 24.11 was the enzyme responsible for the degradation of the endogenous vasodilator and natriuretic hormone, atrial natriuretic peptide (ANP; 17,34,45), interest grew in the inhibition of this enzyme as a new therapeutic approach to hypertension and heart failure. Intravenous infusions of ANP reduce blood pressure while causing diuresis and increased urinary excretion of sodium and cGMP (32). Short-term studies of renal failure in rats with reduced renal mass have demonstrated some improvement in renal function with the use of phosphoramidon, a NEP inhibitor (36,72). As a result, several NEP inhibitors have been evaluated clinically for the treatment of hypertension and congestive heart failure (CHF). In one study involving patients with essential hypertension, substantial lowering of blood pressure was observed (44). However, most clinical trials have reported modest or no significant effects (7,43,54–57). Consequently, meaningful clinical improvements in the treatment of any cardiovascular disease with this class of compound have yet to be established.

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Increased activation of the renin-angiotensin system has been suggested as a factor limiting the expected antihypertensive effects of NEP inhibition (54,56). Furthermore, even though the natriuretic and diuretic effects of ANP are significantly inhibited in experimental and clinical CHF, treatment with an ACE inhibitor restores renal responsiveness to ANP (1,13,35,37,74). Moreover, several CHF studies suggest that ACE inhibitors are ineffective in the early stages of the disease when plasma ANP and epinephrine are increased but plasma renin is not yet elevated (2,6,53,58). As a consequence of these observations, it was hypothesized that blockade of the renin-angiotensin system with an ACE inhibitor coupled with the potentiation of ANP through NEP inhibition would provide therapeutic efficacy greater than that produced by inhibition of either enzyme alone. Combination therapy of a selective ACE inhibitor and a selective NEP inhibitor or treatment with single molecules containing both activities have confirmed this hypothesis by demonstrating additive or synergistic benefits in animal models of hypertension, CHF and chronic renal failure (14,40,51,61,62,67–69). Moreover, simultaneous administration of the ACE inhibitor captopril with sinorphan, a NEP inhibitor, to patients with essential hypertension conferred synergistic antihypertensive effects (21).

The reported synergistic actions of ACE inhibitors in combination with NEP inhibitors as well as the corroborating data with dual acting compounds (22,25,28,60,70) have led to the development of CGS 30440, a potent, orally active, long-acting inhibitor of both ACE and NEP. CGS 30440 has been examined extensively in a preclinical setting and was shown to possess excellent biochemical inhibitory activity against both enzymes. In various models of cardiovascular disease, CGS 30440 has shown potent, long-acting pharmacological efficacy. These data are detailed in this review.

**CHEMISTRY**

The chemical structure of CGS 30440, N-[[1-[(2(S)-acetylthio-3-methyl-1-oxobutyl)amino]-1-cyclopentyl]carbonyl]-O-methyl-L-tyrosine ethyl ester is shown in Figure 1. It is a white crystalline solid, m.p. 106°C, with a molecular weight of 492.64, and empirical formula of C$_{25}$H$_{36}$N$_{2}$SO$_{6}$. The solubility of CGS 30440 is 0.0516 mg/ml in pH 7.0 buffer, 0.059 mg/ml in simulated gastric fluid, and 0.0519 mg/ml in simulated intestinal fluid. The log of the distribution coefficient (log D) was determined experimentally to be 2.61 at pH 7.4. CGS 30440 is a thioacetyl-containing dipeptide, which is believed to be metabolized in vivo to its biologically active form CGS 30008. The chemical structure of CGS 30008, a free thiol-containing carboxylic acid is also shown in Figure 1.

![Chemical structures of the prodrug CGS 30440 and the active ingredient CGS 30008.](FIG. 1. Chemical structures of the prodrug CGS 30440 and the active ingredient CGS 30008.)
We first reported on a series of \( \alpha \)-mercaptodipeptides (8). In general, these compounds were found to be potent inhibitors of ACE and NEP activity \textit{in vitro} but were short-acting in the angiotensin-I induced pressor response in normotensive rats after intravenous dosing. In an attempt to improve their \textit{in vivo} efficacy, a strategy was developed to prepare molecules where the amino acids were replaced with non-natural amino acids that would not impair the \textit{in vitro} potency of these molecules but might improve their metabolic stability. This approach lead to a series of spirocyclic and gem-disubstituted dipeptides that were potent inhibitors of ACE and NEP activity \textit{in vitro} and \textit{in vivo} (23). The structure activity relationships of these compounds are outlined in Figure 2. In general an aryl or heteroaryl containing amino acid is preferred at the C-terminal end of the molecule as can be seen from the data in Table 1. At the central position (R\(_2\)), we investigated different spirocycles and the gem dimethyl substitution, compounds 1–6; all were found to be potent dual inhibitors \textit{in vitro}. Interestingly, the spiropentacyclo (2) and gem dimethyl (6) compounds were the longest acting \textit{in vivo}. A few modifications were made at the thiol region of the molecule. Compounds 15–17 had good \textit{in vitro} activity but were found to be short-acting in the angiotensin-I (A1) pressor response assay. We believe that the \( \alpha \)-thiol group is sterically more accessible in these molecules and, as a result, they are deactivated metabolically at a more rapid rate. Further optimization of compound 2, in particular by preparing a variety of carboxylic acid esters and acylation of the free thiol, led to compounds with improved \textit{in vivo} efficacy (22). CGS 30440 is the O-methyl, ethyl ester, S-acetate of compound 2. CGS 30440 can be prepared in seven steps from commercially available starting materials with an overall chemical yield of 27%.

By utilizing computer modeling we were able to postulate binding modes of these new \( \alpha \)-thiol molecules to ACE and NEP (9). A composite template for ACE inhibitors was generated by using TFIND to superimpose known conformationally restricted inhibitors. For NEP binding, X-ray crystal structure data of the active site of thermolsyn (EC 3.4.24.4), a related zinc metalloprotease, was used to construct a model. The proposed structure-activity relationships for spiroalkyl \( \alpha \)-mercaptodipeptides are illustrated in Figure 2.

![FIG. 2. Structure-activity relationships for spiroalkyl \( \alpha \)-mercaptodipeptides.](image-url)
binding modes of CGS 30008 for ACE and NEP are shown in Figure 3; with the cyclopentyl substituent of the dual inhibitor fitting into the S1’ subsite of both ACE and NEP.

INHIBITION OF ACE AND NEP

The ACE and NEP inhibitory activities of CGS 30008 (compound 7 in Table 1), the active metabolite of CGS 30440, were initially assessed in vitro (12). ACE was purified from rabbit lung and NEP from rat kidney membranes. These experiments indicated that CGS 30440 had IC50 values of 19 nM for ACE and 2.2 nM for NEP (12,23). Under the conditions used in our in vitro assays, the selective ACE inhibitors captopril and enalaprilat exhibited IC50 values of 10 and 9 nM, respectively, whereas the selective NEP inhibitor thiorphan had an IC50 value of 5 nM (23). Furthermore, omapatrilat, a dual enzyme inhibitor that is currently in development, had IC50 values of 5 and 8 nM against ACE and NEP (59).

The efficacy of CGS 30440 to inhibit ACE and NEP in vivo was assessed by measuring the activity of both enzymes ex vivo in tissues and plasma of rats. Plasma and tissue ACE activities were determined by the method of Cushman and Cheung (16) and tissue NEP activity by the method of Orlowski and Wilk (46). The effect of the compound on lung ACE activity and renal NEP activity was initially evaluated in short-term experiments in Sprague-Dawley rats (12). The chronic inhibition of plasma and tissue ACE, as well as tissue NEP, was assessed in spontaneously hypertensive rats (SHR) and in their normotensive WKY controls (75).

<table>
<thead>
<tr>
<th>Compound</th>
<th>R1</th>
<th>R2</th>
<th>R3</th>
<th>IC50 ACE (nM)</th>
<th>IC50 NEP (nM)</th>
<th>% Inhibition of AI Pressor Response 4 hr (10 mg/kg, iv)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH3(p-OHPh)</td>
<td>(CH2)3</td>
<td>CH(CH3)2</td>
<td>15</td>
<td>5.0</td>
<td>30 ± 16</td>
</tr>
<tr>
<td>2</td>
<td>CH3(p-OHPh)</td>
<td>(CH2)4</td>
<td>CH(CH3)2</td>
<td>7.0</td>
<td>1.5</td>
<td>83 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>CH3(p-OHPh)</td>
<td>(CH2)5</td>
<td>CH(CH3)2</td>
<td>33</td>
<td>8.7</td>
<td>6 ± 6</td>
</tr>
<tr>
<td>4</td>
<td>CH3(p-OHPh)</td>
<td>((CH3)2O(CH2)3)</td>
<td>CH(CH3)2</td>
<td>11</td>
<td>6.4</td>
<td>4 ± 3</td>
</tr>
<tr>
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<td>1,2-(CH2)2Ph</td>
<td>CH(CH3)2</td>
<td>26</td>
<td>25</td>
<td>24 ± 8</td>
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<tr>
<td>6</td>
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<td>69 ± 3</td>
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<td>CH3(p-FPh)</td>
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<td>38 ± 6</td>
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<td>9</td>
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<td>CH(CH3)2</td>
<td>18</td>
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<tr>
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<td>CH3(3-thienyl)</td>
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<td>CH(CH3)2</td>
<td>11</td>
<td>2.6</td>
<td>87 ± 0</td>
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<td>CH3(2-pyr)</td>
<td>(CH2)4</td>
<td>CH(CH3)2</td>
<td>35</td>
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<tr>
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<td>CH(CH3)2</td>
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<td>10</td>
<td>2.1</td>
<td>86 ± 3</td>
</tr>
<tr>
<td>14</td>
<td>CH3CHPh</td>
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<td>CH(CH3)2</td>
<td>30</td>
<td>1.2</td>
<td>79 ± 6</td>
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<td>15</td>
<td>CH3(p-OHPh)</td>
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<td>H</td>
<td>58</td>
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<td>7 ± 2</td>
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<tr>
<td>16</td>
<td>CH3(p-OHPh)</td>
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<td>CH2Ph</td>
<td>22</td>
<td>3.0</td>
<td>43 ± 5</td>
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<tr>
<td>17</td>
<td>CH3(p-OHPh)</td>
<td>(CH2)3</td>
<td>-(CH2)4−</td>
<td>17</td>
<td>5.2</td>
<td>3 ± 7 (@1 h)</td>
</tr>
</tbody>
</table>

**TABLE 1.** In vitro ACE and NEP inhibitory activity and plasma inhibition of ACE activity in conscious rats.
In acute experiments, CGS 30440 was administered orally at 10 mg/kg to normotensive Sprague-Dawley rats. These animals and vehicle-treated control groups were sacrificed 1 and 24 h after dosing and their lungs processed for the determination of ACE activity. The kidneys were used to measure NEP activity. A single oral dose of CGS 30440 significantly reduced lung ACE activity by 98% at 1 h and inhibition was maintained for 24 h, at which time it averaged 61% (12). In the kidney, CGS 30440 reduced NEP activity by 80% after 1 h whereas at 24 h renal NEP activity was still significantly inhibited by 73% (12).

The ability of CGS 30440 to produce a sustained enzyme inhibition in a chronic setting was studied in SHR and WKY rats and their respective controls (75). The compound was administered orally at 10 or 30 mg/kg/d for 8 weeks. After this treatment plasma samples were obtained for ACE determinations. The animals were sacrificed and the lungs and the kidneys were used to assess ACE and NEP activities. As described in Table 2, significant differences in the basal levels of both enzymes were observed between vehicle-treated WKY rats and SHR. Notwithstanding these inter-strain differences, 30 mg/kg of CGS

### TABLE 2. Effects of long-term administration of CGS 30440 on plasma ACE and on tissue ACE and NEP activities

<table>
<thead>
<tr>
<th></th>
<th>Wistar-Kyoto (WKY) rats</th>
<th>Spontaneously Hypertensive Rats (SHR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle (3% cornstarch)</td>
<td>CGS 30440 (30 mg/kg/day)</td>
</tr>
<tr>
<td><strong>ACE Activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>23.0 ± 2.2</td>
<td>4.0 ± 0.4a</td>
</tr>
<tr>
<td>Lung</td>
<td>1,543 ± 153</td>
<td>963 ± 26b</td>
</tr>
<tr>
<td>Kidney</td>
<td>35.0 ± 1.6</td>
<td>28.0 ± 1.3b</td>
</tr>
<tr>
<td><strong>NEP Activity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung</td>
<td>1,476 ± 321</td>
<td>222 ± 31a</td>
</tr>
<tr>
<td>Kidney</td>
<td>12,962 ± 2,513</td>
<td>1,368 ± 459a</td>
</tr>
</tbody>
</table>

All values expressed as the group mean ± SEM. Plasma ACE is expressed as nmol/min/ml and tissue ACE and NEP as nmol/min/organ. WKY, vehicle-treated, n = 10; CGS 30440 at 30 mg/kg/d, n = 10; SHR, vehicle treated, n = 10; CGS 30440 at 10 mg/kg/d, n = 10; at 30 mg/kg/d, n = 11; ACE, angiotensin converting enzyme; NEP, neutral endopeptidase. *p < 0.05 versus respective vehicle control. †p < 0.05 versus WKY.
30440 produced statistically significant reductions in all the parameters studied in both WKY and SHR. Thus, plasma ACE and lung and kidney ACE and NEP were significantly inhibited after 8 weeks of treatment in SHR as well as in WKY rats (75). In the SHR group receiving the lower dose of CGS 30440 (10 mg/kg/d), significant decreases in plasma and lung ACE activities were observed but this dose failed to maintain a chronic reduction in renal ACE activity. The lung and kidney NEP activities were significantly inhibited in the SHR treated for 8 weeks with the 10 mg/kg dose of CGS 30440.

**IN VIVO PHARMACOLOGY**

**Angiotensin-I Pressor Responses**

The degree of ACE inhibition produced by CGS 30440 in vivo was examined by determining its ability to block angiotensin-I-induced increases in mean arterial pressure (MAP; Figure 4). In rats treated with vehicle, the administration of angiotensin-I at 300 ng/kg i.v. was associated with increases in MAP that ranged from 63–69 mmHg over the course of 6 h. In contrast, treatment with CGS 30440 resulted in marked inhibition of the pressor effects of the vasoconstrictor peptide. During the first 2 h after dosing with the dual inhibitor, at 1–10 mgEq/kg p.o., angiotensin-I increased MAP only by 6–17 mmHg. Thereafter, the magnitude of inhibition diminished dose-dependently with 1, 3, and 10 mgEq/kg of CGS 30440 still decreasing angiotensin-I pressor responses by 29, 55, and 78% at 6 h. These studies suggest that CGS 30440 has a relatively long duration of action. In comparison, the dual ACE/NEP inhibitor, omapatrilat, was slightly less effective than CGS 30440 in blocking the pressor effects of angiotensin I (59).

**FIG. 4.** Inhibitory effect of CGS 30440 on the increase in mean arterial pressure produced by angiotensin-I in conscious rats. Angiotensin-I was injected i.v. at 300 ng/kg to establish a control value for its pressor response prior to treatment (time 0). Rats were then dosed with vehicle (3% cornstarch) or CGS 30440, at 1–10 mgEq/kg p.o., and rechallenged with angiotensin-I at various times over the course of 6 h. Values are mean ± SEM for 3 animals in the vehicle-treated group and 8–9 animals in the groups treated with CGS 30440. All responses to angiotensin-I in rats that received the dual inhibitor are significantly (p<0.05 by two-way ANOVA) less than those of vehicle-treated animals at all times between 15–360 minutes. One mgEq of CGS 30440 is the amount of prodrug containing 1 mg of the active metabolite CGS 30008.
Potentiation of Plasma ANP

The NEP 24.11 inhibitory activity of CGS 30440 was also assessed in vivo by determining the capacity of the dual inhibitor to increase plasma ANP immunoreactivity (irANP) concentrations during the infusion of exogenous ANP (Figure 5). In vehicle-treated rats infused with ANP (450 ng/kg/min i.v.) for 4 h, the area under the curve (AUC) of plasma irANP levels was 22.3 ± 1.1 ng · hr/ml. As expected, treatment with CGS 30440 was associated with a dose-dependent increase in plasma irANP, with 4-h AUCs of 32.1 ± 3.7, 50.8 ± 6.3 and 64.9 ± 10.1 ng · hr/ml in rats receiving 0.3, 1 and 3 mgEq/kg p.o., respectively.

Effects on Diuresis, Natriuresis and cGMP Excretion

The acute effects of CGS 30440 on urine volume and on the excretion of sodium and cGMP were assessed in normotensive rats with catheters in the jugular vein for ANP infusions, and in the bladder for urine collection (12). In chronic studies, the renal response to CGS 30440 was evaluated in two models of hypertension of 6 and 8 weeks duration, in which urine collections were performed in metabolic cages without previous bladder instrumentation or ANP supplementation (14,75).

In anesthetized normotensive rats, the initiation of a 3.5 h intravenous infusion of ANP evoked significant increases in urinary volume, sodium, and cGMP excretion within 30 minutes. The increase in urinary volume and natriuresis lasted for 1 h, whereas the cGMP surge lasted for the entire observation period (12). Oral administration of CGS 30440 at a dose of 1 mg/kg significantly potentiated the response to ANP in all of the parameters under investigation and all of these changes were statistically significant (12). When compared to vehicle-treated controls, CGS 30440 produced more rapid and more marked

FIG. 5. Effect of CGS 30440 on plasma irANP concentration in conscious rats infused with exogenous ANP(99–126). All rats received ANP(99–126) at 450 ng/kg/min i.v. throughout the 5-h experiment. Sixty min after the ANP(99–126) infusion was started, blood samples were obtained to determine steady-state irANP levels. Rats then received CGS 30440 or vehicle (3% cornstarch) and blood samples were obtained over the course of the next 4 h. Each bar represents the AUC for plasma irANP concentration during the 4 h after dosing with CGS 30440 (n = 3–6) or vehicle (n = 10); values are mean ± SEM. The 1 and 3 mgEq/kg doses of the dual inhibitor significantly (*, p<0.05 by two-way ANOVA) increased the AUC of plasma irANP as compared to vehicle.
elevations in urine volume (300% increase at 3 h), sodium excretion (194%) and cGMP excretion (238%).

In SHR treated with CGS 30440 orally at 10 or 30 mg/kg/d, and in their normotensive WKY controls, urine output samples were obtained at weekly intervals for an 8 week study period (75). During the pre-dose period, urine output, sodium, and cGMP excretion were significantly greater in WKY than in age-matched SHR. These higher values, typical of WKY rats, persisted during the 8-week treatment with vehicle. When WKY rats were treated with CGS 30440 (at 30 mg/kg/d) an increase in urine volume was observed, although there were no long-lasting increases in sodium and cGMP excretion (75).

In SHR receiving a 10 mg/kg/d dose of CGS 30440, there were significant increases in urine volume, as well as in sodium and cGMP excretion lasting for the initial 5 weeks. However, the last 3 weeks of the experiment were characterized by a normalization in the urine and cGMP output but the sodium excretion remained elevated (75). At 30 mg/kg, CGS 30440 produced a renal response similar to that observed with the 10 mg/kg dose but the statistically significant elevation in urinary cGMP was sustained throughout the experiment (75).

In an experimental model of chronic renal failure, rats with reduced renal mass were treated with CGS 30440 delivered by subcutaneously implanted minipumps (14). Urine samples of animals receiving CGS 30440 at doses of 2.2 and 6.5 μmol/kg/d (molar equivalents of 1 and 3 mg/kg/d) or receiving the vehicle alone were collected at 2, 4, and 6 weeks after renal ablation. Urine output was increased in the treatment groups compared with the baseline pre-ablation levels but was not significantly different from the increases observed in the vehicle-treated group. A slight, but statistically significant, increase in cGMP excretion was observed at 2 weeks of treatment and this effect further increased to 190% and 600% above vehicle-treated controls at 4 and 6 weeks, respectively (14).

MODELS OF EXPERIMENTAL HYPERTENSION

Renal Hypertension

The antihypertensive effect of CGS 30440 was tested in a renin-dependent model of hypertension induced in rats by complete ligation of the aorta between the renal arteries (12). In aortic-ligated rats mean arterial pressure was measured in the conscious state and reached a plateau at 12 d after operation, at which time plasma renin activity was significantly elevated. Twelve-day aortic-ligated animals received an oral dose of CGS 30440 (10 mg/kg) and the blood pressure changes were monitored for 3 h (Figure 6). Additional groups of aortic-ligated animals were treated either with captopril (10 mg/kg p.o.) or with ANP (30 nmol/kg i.v. bolus).

In the randomly selected group of aortic-ligated rats used to test CGS 30440, the pre-treatment MAP averaged 209 ± 4 mmHg. CGS 30440 (10 mg/kg) produced a marked reduction in blood pressure which developed gradually (12). A statistically significant reduction of 20 mmHg was first observed after 1 h and the maximal decrease (40 mmHg) was recorded at 3 h (Figure 6).

In animals receiving captopril (10 mg/kg), the pre-treatment blood pressure level averaged 205 ± 3 mmHg. The antihypertensive effect of captopril (which is not a prodrug) developed more rapidly but was not as marked as that elicited by CGS 30440. At 1 h, a statistically significant 41 mmHg decrease was recorded but at the end of the 3 h study...
period the blood pressure was still reduced by 29 mmHg below pre-treatment levels (Figure 6).

As expected from an intravenously administered vasodilator, ANP produced a rapid and drastic reduction in blood pressure of 36 mmHg from the basal hypertensive level (203 ± 3 mmHg) within the first 5 minutes after administration. After 15 minutes, however, a steady recovery was observed and the blood pressure returned to the hypertensive pre-treatment level after 3 h. Vehicle administration had no effect on blood pressure (data not shown).

**Spontaneous Hypertension**

Oral administration of CGS 30440, given once daily to conscious, telemetered SHR, produced dose-related reductions in mean arterial pressure (Figure 7). These results confirm an earlier report demonstrating the oral activity of CGS 30440 in SHR (22) and extend these findings by proving the antihypertensive efficacy of once daily dosing. CGS 30440 decreased MAP in SHR treated once daily for 14 d by approximately 20, 25, and 40 mmHg at doses of 3, 10, and 30 mg/kg, respectively. These blood pressure effects represent 24 h averages which are comprised of 144 samples collected at 10 min intervals throughout the day. The data demonstrate that CGS 30440 produced sustained reductions in MAP during the entire 2-week course of study. Maximum antihypertensive effects were achieved after 4 d with doses of 3 and 10 mg/kg and after 9 d with a dose of 30 mg/kg p.o. The degree of blood pressure lowering produced by the highest dose of CGS 30440 was comparable to that reported with a high dose of omapatrilat using the tail-cuff method (71). The antihypertensive effects of CGS 30440 were accompanied by a dose-related tachycardia which persisted throughout the 2-week dosing period. In these same rats, a
single daily dose of CGS 30440 elicited antihypertensive effects that were sustained for over 24 h (Figure 8), thereby demonstrating the long duration of action of this compound. Representative hourly averages recorded over a 24 h interval between dosing on day 8 and day 9 revealed that the changes in mean arterial pressure in CGS 30440-treated rats remained below the values observed in vehicle-treated SHR at all time points throughout the 24 h sampling period.

Studies in which CGS 30440 was given orally once daily for a period of 8 weeks to SHR at doses of 10 and 30 mg/kg/d confirmed the chronic antihypertensive effects of the compound (75). Moreover, this chronic reduction in blood pressure was associated with
a significant dose-related regression of left ventricular hypertrophy. Left ventricular mass to body weight ratios were 1.97 ± 0.08 mg/g for untreated WKY rats and 2.91 ± 0.09 mg/g for SHR, demonstrating a significant degree of left ventricular hypertrophy in untreated genetically-hypertensive rats. Chronic once daily dosing with CGS 30440 for 8 weeks regressed SHR left ventricular hypertrophy to 2.45 ± 0.08 and 2.26 ± 0.07 mg/g at doses of 10 and 30 mg/kg/d, respectively (75). These results are consistent with previous studies demonstrating regression of left ventricular hypertrophy in the SHR treated with either ACE inhibitors (39,42) or with a selective inhibitor of NEP (41). However, the results obtained with CGS 30440 in the SHR do not allow the assignment of a specific mechanism, that is, NEP or ACE inhibition, as the predominant underlying factor responsible for the observed reduction in left ventricular mass.

The 8 week CGS 30440 treatment regimen also evoked a significant reduction in aortic mass (aorta wet weight/body weight, mg/g) in SHR (75). The aorta/body weight ratio was 0.31 ± 0.01 mg/g in vehicle-treated SHR and was reduced to 0.25 ± 0.01 mg/g by treatment with 10 mg/kg/d of CGS 30440. Interestingly, this dose of CGS 30440 normalized aortic mass since comparable aorta to body weight ratios were recorded in untreated, normotensive WKY control rats (0.26 ± 0.01 mg/g). Although regression of vascular hypertrophy has not been reported previously with chronic administration of a
selective inhibitor of NEP, it has been shown that chronic ACE inhibition with captopril effectively reduced aortic medial hypertrophy (47). Therefore, the CGS 30440-induced regression of aortic hypertrophy is likely due to the inhibition of angiotensin-converting enzyme.

MODELS OF RENAL DISEASE

Stroke Prone Spontaneously Hypertensive Rats

Oral administration of CGS 30440 (10 mg/kg/d) prevented the progressive rise in mean arterial pressure in a model of malignant hypertension, the salt-loaded, stroke-prone SHR (SHRsp; Figure 9). Mean arterial blood pressure was continuously recorded in these animals by means of radiotelemetric implants. MAP began to rise rapidly in SHRsp with the introduction of 1% NaCl as drinking fluid, commencing at 9.5 weeks of age in vehicle-treated animals. In contrast, mean arterial pressure remained at pre-drug levels throughout the study period in SHRsp, receiving daily doses of CGS 30440. The CGS 30440-treated rats were also maintained on 1% NaCl as drinking fluid. Baseline heart rates (7 d average prior to the start of treatment) were 335 ± 5 and 336 ± 7 beats per min in vehicle, and CGS 30440-treated SHRsp, respectively. Although heart rates varied during the course of the study, they remained at or near pre-dose values at all times.

A dramatic improvement in survival was also evident in SHRsp treated chronically with CGS 30440 (Figure 10). A precipitous decline in survival was seen in vehicle-treated rats at 9.5 weeks of age shortly after the animals began drinking 1% NaCl. Upon termination of the study when rats had reached twenty weeks of age, only one vehicle-treated SHRsp had survived. In contrast, all CGS 30440-treated animals (10 mg/kg p.o.) completed the full course of study. A similar survival benefit has been reported consistently by others using numerous selective ACE inhibitors (64,65,73). While the effect of a selective NEP

FIG. 9. Continuous mean arterial blood pressure measurements determined in telemetered SHRsp. Each point represents a 24 h average ± SEM. CGS 30440 was administered orally, once daily at 10 mg/kg. The overall blood pressure curves were significantly different between drug-treated and vehicle-treated rats using a two-way ANOVA with repeated measurements (p<0.05).
inhibitor on survival in the SHRsp has not been reported, preliminary data from our laboratory clearly indicate that chronic administration of a specific inhibitor of NEP provides no survival benefit in this animal model of malignant hypertension (unpublished observations).

Varying degrees of end-organ dysfunction ultimately develop in the salt-loaded SHRsp with uncontrolled hypertension. Serious hypertension-induced histopathological changes can also be detected in most organ systems and these alterations worsen with time resulting eventually in hypertensive crisis and death. The presence of protein in the urine serves as an early surrogate marker of renal dysfunction. Elevated levels of urinary protein excretion can be detected in SHRsp shortly after replacing drinking water with 1% NaCl. In our studies, by thirteen weeks of age, urinary protein excretion was 149 ± 40 and 11 ± 1 mg/dl in vehicle- and CGS 30440-treated SHRsp, respectively. Long-term administration of CGS 30440 at a dose of 10 mg/kg/d prevented this progressive rise in urinary protein excretion and afforded significant histopathologic protection to the kidney (Table 3), especially in the glomeruli and arterioles. In the heart, interstitial fibrosis and myocardial degenerative changes were also markedly reduced. These results are consistent with other investigations and confirm that blockade of the renin-angiotensin system provides an extremely effective means of reducing the hypertensive complications seen in SHRsp (10,24,64,65,73,76).

Reduced Renal Mass

In chronic renal insufficiency, rescue of renal function has been difficult to achieve with traditional drug therapy and dietary management. In various animal models of chronic kidney disease, including rats with reduced renal mass, ACE inhibitors have demonstrated
efficacy (5,19,20,52,66,77). The model of renal mass reduction (five-sixths renal ablation) is representative of infarctive renal disease, resulting in progressive renal failure. Male Sprague-Dawley rats undergo a unilateral nephrectomy and, through ligation of 2–3 branches of the renal artery, the remaining kidney’s blood supply is severely reduced. Uremia in this model results from hypertrophy of the remnant kidney, increased intra-glomerular pressure and hyperfiltration (18,29). The resultant disease is characterized by severe hypertension, reduction in glomerular filtration (as determined by creatinine clearance), proteinuria and increased fractional excretion of sodium (30). Histopathologically, focal segmental glomerulosclerosis, tubular dilatation and degeneration, granulomatous vasculitis and interstitial fibrosis are observed. Angiotensin II, acting as a constrictor of the efferent arteriole, causes increased intraglomerular capillary pressure further exacerbating and damaging the remaining functional nephrons (3,4,48). ACE inhibition has been reported to reduce proteinuria, improve glomerular function, and normalize blood pressure in this model (31,33).

Inhibitors of NEP 24.11, on the other hand, have not shown efficacy in rats with reduced renal mass when assessing reduction of chronic renal deterioration. Short-term studies with NEP inhibitors such as thiorphan and phosphoramidon have demonstrated some improvement in renal function (36,72). However, these studies involved treatment of rats under high-salt diets or short-term drug administration to anesthetized animals. Conversely, the efficacy observed with ACE inhibitors was the result of long-term therapy. Regardless, NEP inhibition did exert action at the renal tubular level as indicated by improvements in the fractional excretion of sodium. Since ACE inhibitors provide benefit at the level of the glomerulus and NEP inhibition, with its attendant elevation of renal ANP levels, may provide tubular protection, the question arose as to whether the combination of both these activities would provide greater protection in rats with reduced renal mass than ACE inhibition alone. Consequently, CGS 30440 was compared to the ACE inhibitor, benazepril, in this model. Previous studies determined that benazepril, administered subcutaneously at 21.7 μmole/kg/d (10 mg/kg/d) initiated one week following 5/6 renal ablation, provided maximum protection in reducing systemic hypertension.

### TABLE 3. Comparative histopathological changes in the kidney and heart of stroke-prone SHR treated chronically with CGS 30440

<table>
<thead>
<tr>
<th>Microscopic Observations</th>
<th>Vehicle (n = 12)</th>
<th>CGS 30440 (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Kidney</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glomeruli: GS</td>
<td>2.0 ± 0.2</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>Arterioles: PV</td>
<td>2.4 ± 0.2</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>Tubules: proteinaceous casts</td>
<td>1.7 ± 0.1</td>
<td>0.3 ± 0.1*</td>
</tr>
<tr>
<td>dilatation</td>
<td>1.8 ± 0.1</td>
<td>0.5 ± 0.2*</td>
</tr>
<tr>
<td>degen/regeneration</td>
<td>1.9 ± 0.1</td>
<td>0.5 ± 0.2*</td>
</tr>
<tr>
<td><strong>Heart</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial fibrosis</td>
<td>1.3 ± 0.3</td>
<td>0.2 ± 0.1*</td>
</tr>
<tr>
<td>Myocardial degeneration</td>
<td>0.7 ± 0.2</td>
<td>0*</td>
</tr>
<tr>
<td>Inflammation</td>
<td>0.3 ± 0.2</td>
<td>0 N.S.</td>
</tr>
<tr>
<td>Arterioles: PV</td>
<td>1.9 ± 0.1</td>
<td>0.4 ± 0.1*</td>
</tr>
</tbody>
</table>

Abbreviations: GS, glomerulosclerosis; PV, proliferative vasculitis and/or hyaline degeneration; degen, degeneration; N.S., not significant. *p < 0.05 Mann-Whitney Rank Sum test. CGS 30440 was administered once daily by oral gavage in 3% cornstarch at a dose of 10 mg/kg/d. Kidneys were graded from 1 through 4 with 4 being the most severe lesion, 3 moderate, 2 mild, and 1 minimal.
and improving renal function (data not shown). Therefore, a dose of benazepril, 2.2 μmole/kg/d (1 mg/kg/d), which provided minimal renal protection, was compared to the same molar dose of CGS 30440. Both treatments were initiated one week following ablation and the drugs were administered subcutaneously by osmotic minipumps for a six week period. Systolic blood pressure, proteinuria, creatinine clearance (Crcl), percent fractional excretion of sodium (%FE_{Na}) and urinary cGMP levels, an indicator of renal ANP activity, were determined. At the end of the studies, remnant kidneys were evaluated histologically without prior knowledge of the treatment group from which each kidney was derived.

After 6 weeks on drug, benazepril provided little functional protection nor histological improvement. Systolic blood pressure, proteinuria, creatinine clearance and %FE_{Na} were similar to that of vehicle-treated rats. Some significant (p<0.05) improvements in proteinuria at two weeks on drug and %FE_{Na} at 4 weeks on drug were noted. Benazepril treatment did reduce the extent of vascular degeneration and glomerulosclerosis. In contrast, CGS 30440 greatly improved all parameters studied. Systolic blood pressure (Figure 11) did not increase throughout the study. At 7 weeks after renal ablation, Cr_{cl} was reduced by 81% in vehicle-treated animals and declined to a similar extent in the benazepril-treated group. CGS 30440 treatment resulted in a Cr_{cl} reduction of 57%; proteinuria (Figure 11) was significantly (p<0.05) reduced as was %FE_{Na} (Figure 12). The reduction in %FE_{Na} is noteworthy in that it indicates less tubular damage allowing for greater reabsorption of filtered sodium. Vehicle-treated rats had 64% mortality, 79% mortality occurred in the benazepril-treated group and there was no mortality in the CGS 30440-treated group. Urinary cGMP, an indicator of renal ANP levels, was significantly elevated in the CGS 30440-treated animals. In contrast, cGMP declined over time in both the vehicle-treated and benazepril-treated groups (Figure 12).

Histological evaluation of the remnant kidneys revealed that CGS 30440 treatment significantly improved tubular structure with less degeneration and dilatation as compared to the benazepril group (Table 4). Kidneys from CGS 30440-treated animals had less glomerular disease than the benazepril group and both groups were significantly improved as compared to vehicle.

From these data, the benefit of the addition of NEP inhibition with ACE inhibition is clear. At the same doses, the dual inhibitor CGS 30440 conferred functional and structural protection to the remnant kidney. Based on the reductions in proteinuria, improved histology and %FE_{Na}, the added protective effects of CGS 30440 are most prominent in the glomerulus and proximal tubule. However, the mechanisms underlying the dramatic improvements in renal function and pathology resulting from CGS 30440 treatment are difficult to explain. Clearly the combination of ACE inhibitor with NEP inhibitory activity provided better renal protection than an equal dose of a selective ACE inhibitor. These results are consistent with experimental data demonstrating significant additive or synergistic effects of an ACE inhibitor and a NEP inhibitor in experimental models of congestive heart failure and hypertension (40,51,61,62,67,68). Elucidation of the specific mechanism(s) underlying these effects though still remains to be resolved.

**PHARMACOKINETICS AND METABOLISM**

The pharmacokinetics and metabolism of CGS 30440 were examined in rats after administration of a single, oral dose of [14C]CGS 30440 at 10 mg/kg (as a suspension in
fortified 3% cornstarch) for periods of up to 7 weeks post-dose. The concentrations of radioactivity in blood and plasma peaked within 2 h. The concentrations of radioactivity in blood and in most tissues showed a slow subsequent decline, but generally remained above the limit of detection throughout the 7 week study period. The apparent terminal half-life of radioactivity was examined in plasma and in selected tissues and was estimated to be approximately 2 weeks or longer. Recovery from bile and urine ranged from 6–8% and approximately 30%, respectively. These values suggested that absorption ranged from approximately 20–40% after an oral dose of 10 mg/kg of \[^{14}C\]CGS 30440. The identity of the drug-derived substances in bile was not characterized. Fecal elimination accounted for the remainder of the administered dose.

The tissue distribution of radioactivity was studied by both quantitative whole-body autoradiography (QWBA) in male, pigmented rats and by tissue dissection, followed by combustion, in Sprague-Dawley rats. In the QWBA study, radioactivity was widely distributed at 2 h post-dose. Concentrations were highest in organs associated with excretion,
such as the contents of the large and small intestine, bile, renal medulla and liver. Levels in most other tissues were slightly greater than, or equal to those in blood. By 24 h post-dose, the levels of radioactivity generally declined in most tissues. In Sprague-Dawley rats, qualitatively similar results were observed. At early time-points, the highest levels of radioactivity (excluding the gastrointestinal tract) were in the kidney and liver. Levels in most other tissues were comparable to, or slightly greater than those found in plasma. Notable exceptions were the brain and testis, in which minimal radioactivity was detected. The time course of radioactivity in pancreas was particularly unusual. The concentrations of radioactivity were successively higher at later sampling times, not reaching their highest levels (0.89 μg-equivalents/g) until 2 weeks post-dose. However, the subsequent decline in concentrations had an estimated half-life of 3 weeks, comparable to the half-life estimated in the other tissues. A qualitatively similar time course was observed in certain other tissues (e.g., eye and thymus), but their levels of radioactivity

**FIG. 12.** Comparison of the effects on % fractional excretion of sodium (%FE\textsubscript{Na}, top) and urinary cGMP excretion (bottom) induced by CGS 30440 and benazepril in rats with reduced renal mass. Rats were administered either drug, at a dose of 2.2 μmole/kg/d, or vehicle subcutaneously by osmotic minipump starting one week following surgery and continuing for a period of 6 weeks. Groups sizes were: vehicle, n = 40; CGS 30440, n = 16; benazepril, n = 13. All values are expressed as mean ± SEM. One-way analysis of variance was used to test for statistical significance amongst treatment groups. All pairwise multiple comparison procedures were carried out using the Student-Newman-Keuls method. * p<0.05 from the vehicle group; ▲ p<0.05, CGS 30440 vs. benazepril.
were substantially lower than in the pancreas. The mechanism responsible for this highly delayed redistribution and elimination is unknown.

Overall, radioactivity was rapidly absorbed and widely distributed after an oral dose of $[^{14}C]$CGS 30440 to rats, and the pattern of distribution was consistent with a moderate volume of distribution in the central compartment. Radioactivity was persistent in virtually all tissues, especially in the pancreas.

CGS 30440, a diester prodrug of CGS 30008, requires two hydrolytic reactions in order to express its enzyme inhibitory activity. Hydrolysis of the ethyl ester was found to occur very rapidly in vitro in any medium in which significant carboxyesterase activity was present (e.g., rat plasma or simulated intestinal fluid). The reaction was much slower in media in which esterase activity was low or absent (e.g., dog plasma or heat-inactivated rat plasma).

Disulfide-linked dimers were also observed in all preparations that contained free thiols, but it is not known whether such disulfides would be present in significant concentrations in vivo or were primarily artifacts produced during sample incubation and preparation. Consistent with expectations arising from the results in vitro, no parent CGS 30440 was detected at 2 hr post-dose in plasma or liver of rats in vivo. CGS 30008 was detected in both biomatrices, but at low concentrations. Additional metabolites observed in vivo included products of two reactions, i.e., S-methylation and the free amide.

Since thiol methylesters were found in vivo but not in vitro preparations, CGS 30440 and the active inhibitor, CGS 30008, were incubated in vitro with rat liver microsomes and a NADPH generating system, containing 120 mM S-adenosylmethionine spiked with 0.2 mCi of $[^{14}C]$-S-adenosylmethionine. The reduction of the prodrug occurred readily in vitro in the presence of rat hepatic 12,000 × g homogenate, cytosolic and microsomal proteins. In the presence of $[^{14}C]$-S-adenosylmethionine, the methylation product of CGS 30008 and des-tyrosyl-S-methylated CGS 30008 were observed.

As stated above, radioactivity was rapidly absorbed and widely distributed after an oral dose of $[^{14}C]$CGS 30440 to rats. The half-life of circulating radioactivity was prolonged, as a result of the presence of circulating metabolites. The metabolism in vitro and in vivo was similar with a number of primary metabolites of the prodrug and active moiety being identified.

### Table 4. Results of the histological analyses of the remnant kidneys of rats treated chronically with CGS 30440 or benazepril

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Gv/hd</th>
<th>Gs</th>
<th>Dg/rg</th>
<th>Tub dil</th>
<th>Casts</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.1 ± 0.2</td>
<td>2.4 ± 0.2</td>
<td>2.8 ± 0.1</td>
<td>3.0 ± 0.2</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>CGS 30440</td>
<td>0.3 ± 0.3a</td>
<td>0.6 ± 0.4a</td>
<td>1.1 ± 0.5ab</td>
<td>1.0 ± 0.5ab</td>
<td>1.4 ± 0.4</td>
</tr>
<tr>
<td>Benazepril</td>
<td>0.9 ± 0.2a</td>
<td>1.5 ± 0.5a</td>
<td>2.9 ± 0.1</td>
<td>2.4 ± 0.4</td>
<td>1.9 ± 0.3</td>
</tr>
</tbody>
</table>

Histological grading values from kidneys that underwent 2/3 arterial ligation. Kidneys were removed 7 weeks following ablation (6 weeks on drug). Parameters assessed were granulomatous vasculitis/hyaline degeneration (Gv/hd), glomerulosclerosis (Gs), tubular degeneration/regeneration (Dg/rg), the extent of tubular dilation (Tub dil) and proteinaceous casts (Casts). CGS 30440 and benazepril (BZ) were administered for 6 weeks at 2.2 μmole/kg/day s.c. Group sizes were: vehicle, n = 40, CGS 30440, n = 16, benazepril, n = 13. Kidneys were graded from 1 through 4 with 4 being the most severe lesion, 3 moderate, 2 mild, and 1 minimal. Values are the mean ± SEM of the histological grading. Analysis of variance (ANOVA) was used to test for statistical differences amongst treatment groups. All pairwise multiple comparison procedures were carried out using the Student-Newman-Keuls Method. a p < 0.05 vs. vehicle-treated group. b p < 0.05, CGS 30440 vs. benazepril.
SUMMARY

The renin-angiotensin system and ANP have been implicated as important mediators in the pathophysiology of hypertension, congestive heart failure and renal failure of many etiologies. By virtue of its vasoconstrictive and proliferative properties, angiotensin II plays a harmful role in the progression of these conditions. Although not as well established, direct administration of ANP or potentiation of its activity through NEP inhibition has the potential to provide protection to the cardiovascular system (32,36,44,72). CGS 30440, a dual ACE/NEP inhibitor prodrug, combines potent inhibition of both angiotensin-converting enzyme and neutral endopeptidase 24.11 in one molecule. In vitro, CGS 30008 (the active metabolite of CGS 30440) blocked ACE and NEP with IC\textsubscript{50} values in the low nanomolar range. In vivo, CGS 30440 reduced plasma and lung ACE activity and kidney NEP activity in Sprague-Dawley rats for 24 h following a single administration. Furthermore, this enzyme inhibition was sustained during chronic eight week administration. CGS 30440 blocked angiotensin-I pressor responses and increased plasma ANP immunoreactivity during the infusion of exogenous ANP to Sprague-Dawley rats. CGS 30440 administration caused diuresis, natriuresis and elevated urinary cGMP levels in SHR and WKY rats. Mean arterial blood pressure was reduced by CGS 30440 treatment in renal hypertensive rats and SHR. Moreover, in SHR, CGS 30440 regressed left ventricular hypertrophy; an observation consistent with previous studies in SHR treated with ACE inhibitors (39,42) or selective NEP inhibitors (41). In two models of renal disease, salt-loaded stroke prone-SHR and rats with reduced renal mass, CGS 30440 markedly reduced blood pressure, proteinuria and mortality and conferred structural renal protection as evidenced by reduced renal histopathology. In rats with reduced renal mass, the efficacy observed with CGS 30440 was much greater than that of an ACE inhibitor given at an equivalent dose. Pharmacokinetic analysis revealed that CGS 30440 and its metabolites have a moderate volume of distribution and long half lives consistent with pharmacological findings.

The question remains, however, as to the benefit of a dual-acting compound over an ACE inhibitor alone. Much of the efficacy of CGS 30440 in the models of renal disease reported herein can be reproduced with ACE inhibitors given at high doses. In rats with reduced renal mass, similar efficacy has been observed with benazepril when administered at a dose 10-fold higher than CGS 30440 (data not shown). Possibly the enhanced efficacy of a dual acting agent like CGS 30440 rests in its ability to increase the levels of and potentiate the actions of bradykinin. Bradykinin has vasodilatory activity, and its systemic concentrations are increased in the presence of ACE inhibition, which blocks extrarenal degradation (78). Several groups have shown that ACE inhibitors increase bradykinin-induced release of nitric oxide (NO) from the endothelium (11,78,79), which may be beneficial in many organ systems. In addition, in the kidney, NEP is the primary route of bradykinin breakdown (72). Consequently, a combined ACE/NEP inhibitor, like CGS 30440, could augment endogenous bradykinin levels to a greater extent than a selective inhibitor of either enzyme alone. In summary, CGS 30440 has the potential to affect the progression of cardiovascular diseases by mechanisms that involve the renin-angiotensin, bradykinin and atrial natriuretic peptide systems. Additional studies will be required to elucidate which mechanism(s) affords therapeutic benefit in various cardiovascular conditions. Regardless of the mechanism, CGS 30440 has demonstrated pharmacological
efficacy in several disease models and could prove efficacious in states where ACE inhibition alone is not adequate.

REFERENCES


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