ABSTRACT

Dual angiotensin-converting enzyme (ACE)/neutral endopeptidase (NEP) inhibitors, by decreasing angiotensin-II production and by preventing the degradation of atrial natriuretic peptide (ANP), may be useful for the treatment of hypertension and congestive heart failure. The thiol dipeptide CGS 30440 (prodrug of CGS 30008, IC$_{50}$: ACE/NEP = 19/2 nM) administered to rats (10 mg/kg p.o.) inhibited lung tissue ACE activity by 98% and 61% at 1 and 24 hr (P < .001) and inhibited the angiotensin-I pressor response by 75 to 90% for more than 6 hr. Renal tissue NEP activity was reduced by 80% at 1 hr and 73% at 24 hr (P < .001). In rats supplemented with exogenous ANP, CGS 30440 (1 mg/kg p.o.) elevated the concentration of circulating ANP (133%, P < .025) for 4 hr and increased the excretion of urine (300%, P < .001), sodium (194%, P < .025) and cyclic GMP (238%, P < .005). CGS 30440 (10 mg/kg p.o.) administered to hypertensive rats with aortic ligation between the renal arteries (mean arterial blood pressure, 209 ± 4 mm Hg) produced a 48 mm Hg blood pressure reduction (P < .001) within 4 hr. CGS 30440 given to cynomolgus monkeys at 2 mg/kg p.o. inhibited plasma ACE activity by 96% within 1 hr (P < .001) and this inhibition was maintained for 7 and 21 days in monkeys receiving the compound orally at 2.5 mg/kg b.i.d.. These studies demonstrate that CGS 30440 is an orally active agent which produces tissue ACE and NEP inhibition in rats and plasma ACE inhibition in primates and suggest that the compound may be useful in the treatment of hypertension and congestive heart failure.

During the past two decades ACE (EC 3.4.15.1) inhibitors have become first-line therapeutic agents for hypertension and important adjuncts for the treatment of CHF. Since the discovery of captopril in 1975, marked improvements in potency and duration of action have been achieved in many newly developed ACE inhibitors. However, a large population of patients still does not respond to these compounds and requires the use of diuretics or other types of antihypertensive agents (Williams, 1988; Heber et al., 1988). Similarly, several CHF studies suggest that ACE inhibitors are ineffective in the early stages of the disease (Richardson et al., 1987; Anand et al., 1990) when ANP and plasma adrenaline are increased but plasma renin activity is not yet elevated (Bailiss et al., 1986; Remes et al., 1991).

Since 1981 a new therapeutic approach to hypertension and cardiac failure aimed at increasing the circulating levels of the vasodilator and diuretic ANP has been conceived (de Bold et al., 1981). Intravenous infusion of this peptide reduces blood pressure while increasing urine volume and the urinary excretion of sodium and cyclic GMP (Janssen et al., 1989). The short half-life of ANP, however, has led to the development of inhibitors of NEP (EC 3.4.24.11) to prevent its degradation (Roques and Beaumont, 1990). Although the effects of NEP inhibitors in humans with hypertension are still controversial, some authors have observed mild natriuresis and borderline decreases in systolic and diastolic blood pressure during short-term administration of these agents (Richards et al., 1993a, b).

From an early stage in the development of selective ACE and NEP inhibitors it was recognized that some compounds moderately cross-reacted with both enzymes (Roques et al., 1982; Gordon et al., 1983; Gros et al., 1991). This finding led to the initiation of the search for compounds able to inhibit both ACE and NEP, and investigators at some pharmaceutical companies have obtained effective dual inhibitors (Seymour et al., 1991; Fournie-Zaluski et al., 1994a; French et al., 1994; Gonzales Vera et al., 1995). In our search for a new ACE/NEP inhibitor we found a series of dipeptide α-thiols...

Received for publication April 21, 1997.

ABBREVIATIONS: ACE, angiotensin-converting enzyme (EC 3.4.15.1); ANP, atrial natriuretic peptide; CHF, congestive heart failure; DOCA, deoxycorticosterone acetate; cGMP, cyclic GMP; MAP, mean arterial blood pressure; NEP, neutral endopeptidase (EC 3.4.24.11); SHR, spontaneously hypertensive rat.
which are long-acting, potent inhibitors for both enzymes and apparently possess good bioavailability. Thus, this report describes the characterization of the ACE and NEP inhibitory activity of the long-acting optimized lead CGS 30440 (Chatelain et al., 1996; Fink et al., 1996) in normal and hypertensive rats, as well as in cynomolgus monkeys.

Materials and Methods

CGS 30440, N-[[1-[2(S)-Acetylthio-3-methyl-1-oxobutyl]amino]-1-cyclopentyl carbonyl]-O-methyl-L-tyrosine ethyl ester (fig. 1; Fink et al., 1996), is a novel thromboxane-containing dual inhibitor of ACE (EC 3.4.15.1) and NEP (EC 3.4.24.11). It is a di-ester prodrug which is hydrolyzed to the active thiol carboxylic acid CGS 30008, N-[[1-[2(2S)-mercapto-3-methyl-1-oxobutyl]amino]-1-cyclopentyl carbonyl]-O-methyl-L-tyrosine. CGS 30440 can be synthesized in high yield in seven steps from readily accessible starting materials. It is a white crystalline powder with a melting point of 103 to 104°C and a solubility of 0.0516 mg/ml, pH 7.0 (Fink et al., 1996). Reference ACE inhibitors, NEP inhibitors and dual ACE/NEP inhibitors were synthesized in house (benazepril, candoxatril, glycopril, thiophan, SCH 42495 and MDL 100,240) or requested from other pharmaceutical companies. Captopril was obtained from Bristol-Myers Squibb (Princeton, NJ), enalapril from Merck, Sharp & Dohme (Rahway, NJ) and ramipril from Hoechst-Roussel Pharmaceuticals (Somerville, NJ).

Inhibition of ACE and NEP in vitro. The ACE and NEP inhibitory activities of the test compounds initially were assessed in vitro. The inhibitory constants (Schulz et al., 1991) were determined from inhibition curves with the nonlinear regression curve fitting program of Research System/1 (RS/; Bolt, Beranek and Newman, Inc., Cambridge, MA) software package on a VAX 1/750 computer equipped with VMS operating system (Digital Equipment Corp., Marlboro, MA).

ACE was purified partially from the lungs of male (New Zealand White, Hare/Harlan Farms, Hewart, NJ) rabbits (1.8–2.2 kg), according to the method of Das and Sofer (1975) to the stage of Nonidet-P40 extract. The extract was frozen and stored at -70°C until use in the enzyme assay.

ACE activity was determined by the hydrolysis of hippuryl-L-histidyl-L-leucine. The resulting product, histidyl-leucine, reacts with o-phenaldaldehyde and is measured spectrophotometrically at 360 nm (Cushman and Cheung, 1981). The reaction mixture (250 μl) containing 300 mM NaCl, 100 mM KH2PO4, 2 mM substrate and 35 μg partially purified ACE was incubated at 37°C for 30 min. The reaction was terminated by the addition of 0.75 ml of 0.6 N NaOH, then 100 μl of o-phenaldaldehyde (2 mg/ml in methanol) were added and the reaction tubes were left at room temperature for 10 min. After adding 100 μl of 6 N HCl the tubes were centrifuged and the optical density of the supernatant was measured at 360 nm. The optical densities were converted to nanomoles of histidyl-leucine formed during the 30-min incubation by comparison with a standard curve.

NEP 3.4.24.11 activity was determined by the hydrolysis of the substrate glutaryl-Ala-Ala-Phe-2-naphthylamide by a modified procedure of Orloowski and Wilk (1981). The incubation mixture (total volume, 125 μl) containing 4.2 μg of protein from rat kidney cortex membranes (Maeda et al., 1983), 50 mM Tris buffer, pH 7.4 at 25°C, 500 μM substrate (final concentration) and leucine aminopeptidase M (2.5 μg), was incubated for 10 min at 25°C. After the incubation 100 μl of fast garnet (250 μg fast garnet/ml of 10% Tween 20 in 1 M sodium acetate, pH 4.2) were added and the enzyme activity was measured spectrophotometrically at 540 nm. One unit of NEP activity was defined as 1 nmol of 2-naphthylamine released per minute and NEP from rat kidney cortex membranes.

Fig. 1. Chemical structures of the prodrug CGS 30440 and the active compound CGS 30008.

Fig. 2. Concentration-response curves for the in vitro inhibition of ACE (IC50 = 19 nM) and NEP (IC50 = 2.2 nM) by CGS 30008. ACE was purified from rabbit lung and NEP from rat kidney cortex membranes.

Inhibition of ACE and NEP ex vivo. Determinations of tissue ACE and NEP were performed in the lung, kidney, aorta and, in some experiments, the adrenals of normotensive Sprague-Dawley rats after the oral administration of the compounds or the appropriate vehicle. The rats were sacrificed and the organs were removed rapidly, dissected free of adhering tissue, frozen in liquid nitrogen and stored at -70°C until assayed. Within 15 days the tissues were homogenized (Tekmar Tissumizer, Cincinnati, OH) and stirred on ice for 3 hr. Aliquots were used for determinations of enzyme activity and for protein measurements (Lowry et al., 1951). Plasma ACE was measured in blood samples obtained from monkeys, but tissue studies were not performed in these animals. Plasma and tissue ACE activities were determined by the methods described under “Inhibition of ACE and NEP in Vivo.”

NEP activity in tissues was determined by the hydrolysis of the substrate glutaryl-Ala-Ala-Phe-2-naphthylamide by a procedure differing slightly from that described above (Orloowski and Wilk, 1981). For the in vitro experiments, the enzyme source was rat kidney cortex membranes, whereas for the ex vivo studies the enzyme was from the particular tissue under study. The incubation mixture (total volume, 250 μl) contained 50 μl of tissue homogenate, 50 mM Tris buffer, pH 7.4 at 37°C, 500 μM substrate (final concentration) and leucine aminopeptidase M (1 mg/ml). The mixture was incubated for 10 min at 37°C and 1.0 ml of fast garnet (50 μl fast garnet/ml of 10% Tween 20 in 1 M sodium acetate, pH 4.2) was added. Enzyme activity was measured spectrophotometrically at 530 nm after 30 min at 37°C. One unit of NEP activity was defined as 1 nmol of 2-naphthylamine released per minute at 37°C.

Inhibition of pressor responses to angiotensin-I. The in vivo ACE inhibitory activity of CGS 30440 was studied by testing its ability to inhibit the pressor response evoked by intravenous injections of angiotensin-I. Sprague-Dawley rats (Hsd:SD) BR, Taconic Farms, Germantown, NY) were anesthetized with methohexital sodium and instrumented with catheters in the femoral artery and
vein to measure mean arterial pressure and administer angiotensin-I, respectively. The catheters were tunneled subcutaneously to exit from the lower back through a stainless steel spring and swivel system. On the next day, angiotensin-I (300 ng/kg i.v.) was injected four times at 15-min intervals to establish a reproducible control pressor response. CGS 30440 was administered orally before rechallenging with angiotensin-I at intervals ranging from 15 to 60 min during a 6-hr period. The pressor responses obtained before and after the administration of the compound were compared and the data expressed as a percent inhibition of the control response. Control animals received the vehicle alone (3% cornstarch p.o., 1 ml/kg).

**Plasma ANP determinations.** Male Sprague-Dawley rats (Tac- onic Farms, Germantown, NY) were anesthetized with methohexitol sodium and instrumented with catheters in the femoral artery and vein to obtain blood samples and infuse ANP, respectively. The rats were tethered with a swivel system and were allowed to recover for 24 hr before being studied in the conscious, unrestrained state. On the day of the study, all rats were infused continuously with ANP at 450 ng/kg/min i.v. for the entire 5 hr of the experiment. Sixty minutes after beginning the infusion, blood samples for base-line ANP measurements were obtained (time 0). The rats were then randomly divided into subgroups and treated orally with different doses of the ACE/NEP inhibitor or the vehicle. CGS 30440 was administered at 0.3 mgEq/kg (0.351 mg/kg, which is equivalent to 0.3 mg/kg of the active inhibitor CGS 30008), at 1 mgEq/kg (1.17 mg/kg) and at 3 mgEq/kg (3.51 mg/kg). Additional blood samples were taken 30, 60, 120, 180 and 240 min after administration of the test compound. The responses produced by each dose of CGS 30440 were expressed as a percentage of those obtained in the vehicle-treated group (3% cornstarch, 1 ml/kg).

The plasma concentrations of ANP were determined by a specific radioimmunoassay. The plasma was diluted in buffer containing 19 mM sodium phosphate monobasic and 81 mM sodium phosphate dibasic (pH 7.4), 50 mM NaCl, 0.1% bovine serum albumin, 0.1% Triton X-100 and 0.1% NaN₃. One-hundred microliters of standards or ANP serum and incubated at 4°C for 16 hr. [125I]rANP (14,000 cpm) was then added to the reaction mixture which was incubated at 4°C for an additional 24 hr. Goat anti-rabbit IgG serum coupled to paramagnetic particles was added to the reaction mixture and bound [125I]rANP was pelleted by exposing the mixture to an attracting magnetic rack. The supernatant was decanted and the pellets counted in a gamma counter. All determinations were performed in duplicate.

**Studies of the renal effects of exogenous ANP.** These studies were performed in normotensive Sprague-Dawley rats which were fasted overnight. Under amobarbital anesthesia, catheters were placed in the jugular vein for infusion and in the bladder for urine collection. Isotonic saline was infused intravenously at 25 μl/min. This solution was supplemented with amobarbital to maintain the animals under anesthesia for the entire experimental period. After obtaining baseline urine samples for two consecutive control periods, CGS 30440 or vehicle was administered orally, and 15 min thereafter ANP (300 ng/kg/min) was added to the infusion. Urine samples were collected every 15 to 30 min. Urine volume was determined gravimetrically, sodium concentration by flame photometry and cGMP concentration by use of a commercially available enzyme-linked immunosorbent assay (Cayman Chemical, Ann Arbor, MI). Excretion rates for urinary sodium and cGMP were calculated by standard formulae.

**Blood pressure determination.** Experiments to study the effect of compounds on blood pressure were performed in outbred male Sprague-Dawley rats (Hsd:SDBR, Harlan Farms, Madison, WI). The animals were earmarked, housed in pending cages and given tap water and regular pelleted food ad libitum. They were maintained in an isolated room with constant temperature (74°F) and cycles of light and darkness lasting for 12 hr.

A complete aortic ligation was performed between the renal arteri-ies (Rojo-Ortega and Genest, 1969). Sham-operated animals underwent a similar surgical procedure by which the perirenal cavity was opened but the aorta remained untouched. After surgery, each animal received a subcutaneous injection of 200,000 U of Wycillin (sterile penicillin procaine suspension, Wyeth Laboratories, Inc., Philadelphia, PA). The evolution of the hypertension was studied in groups of aortic-ligated and sham-operated animals sacrificed between 3 and 30 days after operation. The elevated blood pressure reached a plateau 12 days after aortic ligation. In this period plasma renin activity was increased significantly, and hypertrophy and hyperplasia had developed in the vascular system; therefore, 12-day aortic-ligated rats were used in all the experiments. Direct blood pressure measurements were performed in conscious rats, mildly restrained in plastic cylinders, instrumented with an indwelling catheter (PE-50) implanted in the right carotid artery and connected to a Gould Statham physiological pressure transducer (Chatelain et al., 1990). This was coupled to a transducer amplifier (Gould, model 13–4615–50), and the blood pressure waves were displayed in a Gould 2400 S recorder, thermal writing model (Gould Inc., Cleveland, OH). After a period of stabilization, the compounds were administered orally and the changes in blood pressure and heart rate were monitored for a 3- or 4-hr period. Rats receiving ANP intravenously had an additional catheter (PE-10) implanted in the jugular vein. The responsiveness of the high blood pressure of aortic-ligated rats to ACE inhibitors and ANP to ANP was tested by use of captopril at 10 mg/kg p.o. and ANP at 30 μg/kg i.v.

**Plasma ACE studies in primates.** The influence of CGS 30440 on plasma ACE was studied in trained cynomolgus monkeys (Macaca fascicularis) housed individually and fed a standard high-protein monkey diet. The study protocols were approved by the institutional Animal Care and Use Committee. Blood samples (2 ml) were drawn from the femoral vein with the animals in the fasting state and restrained in their cages by the pole and collar technique. CGS 30440 and benazepril were dissolved in vehicle (ethanol) and administered orally by adsorption in banana slices. Plasma samples were maintained at −70°C until assayed.

In an initial experiment, the acute change in plasma ACE produced by a single oral dose of CGS 30440 at 2 mg/kg was investigated in a group of females weighing 2.5 to 3 kg. Blood samples were obtained at base-line (0 time) and at 1, 3 and 24 hr after administration of the compound. In a second experiment, the study period was prolonged to 7 days. The effects of CGS 30440 (1 mg/kg b.i.d. or 6 mg/kg b.i.d.), benazepril (1 mg/kg b.i.d.) and vehicle alone were tested in three groups of male monkeys weighing 4.5 to 5 kg. Blood samples were drawn 2 hr after the last dose. In a third experiment, the effects of the long-term (21 days) administration of COS 30440 were studied in a group of males. They received an oral dose of CGS 30440 at 2.5 mg/kg b.i.d. and blood samples were obtained 18 hr after the last dose at 21 days. Plasma ACE activity was determined as described above.

**Statistical analysis.** Values are presented as mean ± S.E.M. The significance of differences between groups were analyzed by one-way analysis of variance followed by the Dunnett's test for comparison with control when necessary. The blood pressure changes were analyzed by one-way repeated measures analysis of variance. Comparisons between compound-treated and vehicle-treated groups were performed by unpaired t tests, whereas differences with their pre-treatment values were assessed by paired t test. Values of P < .05 were considered significant.

**Results**

**In vitro studies.** Of all the compounds tested, one of the most potent in the α-thiol series was CGS 30008 (fig. 1) with an IC₅₀ value of 2.2 nM for NEP and 19 nM for ACE (fig. 2).

**Ex vivo inhibition of ACE and NEP.** The activities of the prodrugs of CGS 30008 were tested by measuring the inhibition of ACE and NEP activity in lung, kidney, aorta
and adrenal tissue at 1 hr after oral administration of a 10 mg/kg dose. According to these criteria the most effective prodrug was CGS 30440 (fig. 1).

One hour after oral administration of CGS 30440 (10 mg/kg p.o.) lung ACE was inhibited by 98% (P < .010, fig. 3). The ACE reductions obtained in lung tissue with reference ACE inhibitors or with the dual ACE/NEP inhibitor MDL 100,240 (also administered at 10 mg/kg p.o.) are depicted in figure 3. Captopril, enalapril and MDL 100,240 inhibited lung ACE by 35, 92 and 61%, respectively, whereas benazepril and ramipril produced inhibitions amounting to 77 and 70% of control values (P < .010, fig. 3).

Twenty-four hours after administration of CGS 30440 (10 mg/kg p.o.) lung tissue ACE activity was reduced from 68.3 ± 3.9 nmol/min/mg protein in the vehicle-treated group to 26.9 ± 0.9 nmol/min/mg protein in CGS 30440-treated animals. This represented a 61% inhibition (P < .001).

NEP 24.11 also was inhibited significantly by CGS 30440 in renal tissue. One hour after oral administration (10 mg/kg), renal NEP activity was reduced by 80% compared with vehicle-treated animals (P < .010, fig. 4). Several reference compounds were tested in this assay at a higher dose. Thus, glycopril, candoxatril and SCH 42495 given orally at 30 mg/kg inhibited renal NEP by 40%, 26% and 92% (P < .010, fig. 4), whereas the dual ACE/NEP inhibitor MDL 100,240, which was tested at the same dose as CGS 30440 (10 mg/kg p.o.), produced a 21% reduction (P < .010, fig. 4). Thiorphan (administered intravenously at 30 mg/kg) also produced a statistically significant inhibition of renal NEP (42%, P < .010, fig. 4).

Twenty-four hours after administration of CGS 30440 (10 mg/kg p.o.), renal tissue NEP activity was reduced from 370 ± 34 nmol/min/mg protein in the vehicle-treated group to 100 ± 9.8 nmol/min/mg protein in CGS 30440-treated animals (73% inhibition, P < .001).

**Inhibition of the angiotensin-I pressor response.** The intravenous administration of angiotensin-I (300 ng/kg) produced a significant but short-lived increase in mean blood pressure averaging 58.9 ± 2.5 mm Hg, (P < .001). The oral administration of CGS 30440 resulted in a statistically significant inhibition of this response within 15 min regardless of the dose tested (1, 3 and 10 mg/kg p.o.). Thiorphan was given at 30 mg/kg i.v., CGS 30440 and MDL 100,240 were administered at 10 mg/kg p.o., The number of animals per group was 6 to 8.

![Fig. 3](image1.png)
**Fig. 3.** Inhibition of ACE activity in the lung of normotensive rats at 1 hr after oral administration of compounds at 10 mg/kg. ACE activity is expressed as nanomoles per minute per milligram protein. P refers to the level of significance for the difference between vehicle- and inhibitor-treated animals by Dunnett’s test (**P < .010). The number of animals per group was 8.

![Fig. 4](image2.png)
**Fig. 4.** Inhibition of NEP 24.11 in the kidney of normotensive rats at 1 hr after oral administration of compounds. NEP activity is expressed as nanomoles per minute per milligram protein. P refers to the level of significance for the difference between vehicle- and inhibitor-treated animals by Dunnett’s test (**P < .010). Glycopril, SCH 42495 and candoxatril were administered at 30 mg/kg p.o. Thiorphan was given at 30 mg/kg i.v., CGS 30440 and MDL 100,240 were administered at 10 mg/kg p.o., The number of animals per group was 6 to 8.

![Fig. 5](image3.png)
**Fig. 5.** Percent inhibition of the pressor response to angiotensin-I by CGS 30440. Angiotensin-I was injected four times at 15-min intervals to establish a reproducible control pressor response before the oral administration of CGS 30440. One-way ANOVA, Dunnett’s test and paired t tests were performed with the absolute values. Asterisks denote the first significant change from pretreatment values (P < .0001). The number of animals per group was 8 to 9.
receiving the larger dose (10 mgEq/kg) maintained a 75% inhibition for the duration of the study. Animals treated with the 3 mgEq/kg dose presented an 80% reduction of the response within 1 hr but showed a rapid recovery of the pressor response and the inhibition was 17% at the end of the study. There was no inhibition of the pressor effect of angiotensin-I in rats treated with vehicle alone (fig. 5).

**Blood pressure studies.** Twelve days after the operation the mean blood pressure of aortic-ligated rats was above the 200 mm Hg level in most of the animals. In the randomly selected group used to test CGS 30440, MAP averaged 209 ± 4 mm Hg. Oral administration of a 10 mg/kg dose produced a slow but steady decline in blood pressure which, at 1 hr, reached statistical significance from the pretreatment level (20 mm Hg, P < .010, fig. 6). The decline continued until the end of the 4-hr observation period at which time the blood pressure level was 161 ± 8 mm Hg (48 mm Hg decrease, fig. 6).

In the aortic-ligated group treated with captopril the preadministration blood pressure level was 205 ± 3 mm Hg. This compound produced a rapid MAP decrease which became significantly different from the pretreatment level at 5 min (20 mm Hg, P < .010). The maximal reduction was observed at 1 hr (41 mm Hg), and at 4 hr MAP was 169 ± 4 mm Hg (36 mm Hg decrease from the pretreatment level, fig. 6).

Intravenous administration of ANP produced a drastic MAP decrease at 5 min in a third group of aortic-ligated rats whose basal MAP averaged 203 ± 3 mm Hg. This reduction (36 mm Hg from the pretreatment level, P < .010) superseded the decrease obtained with captopril in the same period. The maximal ANP-induced MAP change occurred at 15 min (49 mm Hg change). After this time a steady recovery toward preadministration levels was evident, and at 3 hr MAP was 188 ± 4 (15 mm Hg decrease from the pretreatment level). MAP changes were not observed after intravenous administration of the vehicle alone (fig. 6).

**Elevation of plasma ANP.** In rats receiving an intravenous infusion of ANP the plasma concentration of this peptide averaged 5.45 ± 0.16 ng/ml (n = 6). Oral administration of CGS 30440 produced a statistically significant increase in the concentration of circulating ANP compared with values obtained in vehicle-treated controls (fig. 7). The largest dose tested in these experiments (3 mgEq/kg) produced a 172% elevation at 30 min which was sustained for the duration of the experiment and averaged 272% at 4 hr (fig. 7A). The 1 mgEq/kg dose increased plasma ANP 133% at 30 min, 182% at 2 hr and 95% at the end of the 4-hr observation period. In the group treated with the smallest dose of CGS 30440 (0.3 mgEq/kg) a slight increase was observed between the second and third hour after administration (36–66% increase, fig. 7), but this change was not statistically significant according to Dunnett’s test. In rats receiving vehicle, the plasma concentration of ANP did not change throughout the study, averaging 5.55 ± 0.79 ng/ml after 4 hr (fig. 7B).

**Enhancement of the renal effects of infused ANP.** The initiation of an intravenous infusion of ANP (300 ng/kg/min i.v.) to anesthetized normotensive control rats resulted in a significant increase in urinary output within 15 min with the first significant change occurring at 30 min (a 10-fold elevation from the beginning of the ANP infusion, P < .010, fig. 8A). The enhanced urinary excretion lasted for the

---

**Fig. 6.** Mean blood pressure changes in conscious 12-day aortic-ligated renal hypertensive rats after administration of CGS 30440, captopril, ANP or vehicle. CGS 30440 and captopril were administered orally at 10 mg/kg, whereas ANP was given intravenously as a bolus at 30 nmol/kg. Asterisks denote the first statistically significant difference from preadministration values by paired t test (P < .010). The number of animals per group was ANP, 4; CGS 30440, captopril and control, 6.

**Fig. 7.** Percent changes (A) and absolute changes (B) in plasma ANP concentrations after oral administration of CGS 30440. Rats received a continuous intravenous infusion of ANP (450 ng/kg/min) for the 4-hr experimental period. Asterisks indicate the first statistically significant difference from pretreatment levels by paired t test (P < .025). The number of animals per group was 3 to 6.
reached significance at 45 min after the initiation of ANP infusion (P < .025) but unlike urinary volume and sodium, the excretion of the nucleotide remained elevated during the entire observation period (fig. 8C).

An oral dose of CGS 30440 at 1 mg/kg enhanced the initial urinary volume increase above the values observed in vehicle-treated controls. The elevation produced by CGS 30440 first attained statistical significance from the pretreatment level at 30 min after administration (2-fold increase, P < .010, fig. 8A). The urinary volume increase in CGS 30440-treated animals superseded that of vehicle-treated rats at 75 min after administration (64% increase, P < .050). From this time forward the dual inhibitor-treated group showed a consistent and statistically significant elevation in urinary output. After 3.5 hr urinary volume in CGS 30440-treated animals remained increased by 300% greater than that in the vehicle-treated group (P < .025, fig. 8A).

Similar to the increase in urinary volume, the excretion of sodium was elevated rapidly by the administration of CGS 30440. The first statistically significant difference versus the pretreatment level occurred at 45 min (4-fold increase, P < .025), and the first difference versus the vehicle-treated control group was observed at 60 min (66%, P < .050, fig. 8B). Therefore, when compared with vehicle-treated animals, the sodium change slightly preceded the volume elevation in CGS 30440-treated animals. Sodium excretion remained significantly elevated throughout the entire observation period and was 194% greater than the control group after 3.5 hr (P < .025, fig. 8B).

The most rapid urinary change evoked by CGS 30440 was an elevation in cGMP which was statistically significant already early as 30 min after administration (2-fold elevation from pretreatment values, P < .010, fig. 8C). In CGS 30440-treated animals, the cGMP increase became significantly higher than that of vehicle-treated rats at 45 min (103% increase, P < .050). The elevation of cGMP induced by CGS 30440 was maintained throughout the observation period and was 238% greater than values from vehicle-treated rats after 3.5 hr (P < .025, fig. 8C).

Thus, the administration of a 1 mg/kg dose of CGS 30440 to animals receiving a constant ANP infusion produced a sequence of changes initiated by a potentiation of the elevation of cGMP within the initial 45 min. At this time, when compared with values obtained in vehicle-treated animals, urinary volume and sodium were not yet enhanced. The potentiation of sodium excretion became significant after 60 min and was followed by an increase in urinary volume at 75 min. All these parameters remained significantly elevated until the end of the experiment 3.5 hr after CGS 30440 administration.

**Effects on plasma ACE in primates.** Basal levels of plasma ACE activity in monkeys averaged 31.6 ± 2.2 nmol/min/ml (n = 7). A single oral dose of CGS 30440 at 2 mg/kg reduced plasma ACE activity to 1.34 ± 0.6 nmol/min/ml at 1 hr (96% inhibition, P < .001) and to 1.73 ± 0.7 at 3 hr (94.5% inhibition, P < .001). At 24 hr after administration, plasma ACE activity was 6.2 ± 0.7 nmol/min/ml (80% inhibition, P < .001).

Oral administration of CGS 30440 for 7 days (1 mg/kg b.i.d.) reduced ACE activity to 3.9 ± 0.6 nmol/min/ml (n = 7, P < .001). In a second group of monkeys (n = 7) in which the selective ACE inhibitor benazepril was tested (1 mg/kg b.i.d.), a similar plasma ACE inhibition was observed (3.7 ±
0.4 nmol/min/ml). This change was not statistically different from the reduction observed in those animals treated with CGS 30440. In a group of monkeys treated with CGS 30440 for 21 days (n = 8, 2.5 mg/kg b.i.d.), plasma ACE was reduced to 9.5 ± 1.3 nmol/min/ml at 18 hr after the final dose of the compound.

Discussion

In previous experiments we observed that in the rat ACE activity is the highest in the lung and NEP activity is highest in the kidney (Chatelain et al., 1994; Odorico et al., 1995). Therefore, these organs were used to compare the inhibitory activities of CGS 30440 with those of other reference compounds. Selective ACE inhibitors, CGS 30440 and the dual ACE/NEP inhibitor MDL 100,240 were administered orally to rats which were sacrificed after 1 hr. This time was chosen because we and others have observed that nearly maximal inhibition of tissue ACE is obtained in 1 hr with most ACE inhibitors (Cushman et al., 1989). No attempts were made to adjust the dose of each compound to its in vitro potency, and a uniform 10 mg/kg dose was administered. The results show that CGS 30440 is able to inhibit lung ACE to a degree similar to the well known selective ACE inhibitors and the dual ACE/NEP inhibitor MDL 100,240.

One hour after administration of CGS 30440, ACE activity was reduced by 80% in the kidney. Comparisons with standard ACE inhibitors and with the dual ACE/NEP inhibitors glycopril and MDL 100,240 in the tissue ACE assay are less straightforward than the comparison performed with the standards in the tissue ACE assay. This is because the optimal time of NEP inhibition in the kidney was not investigated and because the issue of tissue bioavailability, when different routes of administration are used, was not addressed. (Thiorphan was administered intravenously because it is not orally bioavailable.) Nevertheless, our results suggest that, at the 1-hr time, the renal NEP inhibitory activity of CGS 30440 is significant and, notwithstanding differences in bioavailability, as good as that of other NEP or ACE/NEP inhibitors.

From the initiation of the study of ACE inhibitors, inhibition of the pressor response to intravenous injections of angiotensin-I has been used as an indirect measure of the generalized inhibition of plasma and tissue ACE. Oral administration of CGS 30440 produced a marked inhibition of this pressor response within 15 min even when a dose as low as 1 mg/kg was used with larger doses prolonging the inhibition for more than 6 hr.

In other experiments, the oral administration of CGS 30440 also was accompanied by an increase in the concentration of circulating ANP when a continuous intravenous infusion of exogenous ANP was provided. The elevation in the concentration of ANP produced by low doses of CGS 30440 was statistically significant and was sustained for more than 4 hr. Also, in anesthetized normotensive rats supplemented with exogenous ANP, a 1 mg/kg dose of CGS 30440 significantly increased urinary volume, cGMP and sodium excretion for more than 3.5 hr. The increase in urinary cGMP concentration preceded the increase in sodium excretion which in turn preceded the increase in urinary volume. This pattern of changes is suggestive of NEP inhibition at the level of the proximal renal tubule and may be the result of the enzyme inhibition measured with the biochemical assay at the 1-hr time. The subject of the urinary changes produced by NEP inhibition has been the object of intensive investigation (Trapani et al., 1989; Webb et al., 1989; Sybertz et al., 1989), and detailed information about the effects of selective NEP inhibitors as well as ACE/NEP inhibitors is available (Seymour et al., 1989, 1991; Gros et al., 1991; French et al., 1994).

The antihypertensive effect of selective NEP inhibitors and dual ACE/NEP inhibitors have been well characterized in the DOCA-salt rat, a volume-dependent model of hypertension in which the renin-angiotensin system is suppressed. The high blood pressure of these animals is responsive to NEP inhibition (Sybertz et al., 1989; Seymour et al., 1991) but unresponsive to ACE inhibition. ACE inhibitors, on the other hand, are more effective in models in which the renin-angiotensin system is activated. For the evaluation of CGS 30440 we used the aortic-ligated rat, a renin-dependent model of hypertension responsive to ACE inhibitors but whose high blood pressure is also very responsive to a challenge with exogenous ANP (Lappe et al., 1987). Our experiments were performed in rats with hypertension of the benign type lasting for 12 days. At this time, plasma renin is still elevated but hypertrophy and hyperplasia have already taken place in the cardiovascular system (Chatelain et al., 1980; Chatelain, 1983). In these animals we confirmed the blood pressure-lowering effects of exogenous ANP and the selective ACE inhibitor captopril. CGS 30440 produced a blood pressure reduction similar to that of captopril and a more sustained decrease than a large intravenous dose of ANP, a vasodilator with a shorter half-life. There was a marked difference, however, in the initiation of the antihypertensive effect of captopril which was rapid and that of CGS 30440 which took 1 hr to reach statistical significance. The slow onset of the blood pressure reduction in aortic-ligated rats also contrasted with the more rapid responses evoked by CGS 30440 in some of the parameters studied in normotensive rats. Two reasons may account for these differences. In aortic-ligated rats we have observed a marked increase in ACE activity in the arterial wall, in the kidneys, lungs and adrenals (experiments not described in this report). On the other hand, CGS 30440 is a prodrug whereas captopril is in the active form and does not require a metabolic conversion to inhibit ACE. Thus, in aortic-ligated rats, CGS 30440 may need a longer time to achieve a physiologically meaningful ACE inhibition in the organs or systems involved in the maintenance of the elevated blood pressure, such as the ischemic kidney and the vascular system.

In our experiments with cynomolgus monkeys, oral administration of CGS 30440 produced a rapid inhibition of plasma ACE, which suggests that, similar to the initial observations in the rat, the compound may also have an appropriate oral bioavailability in primates.

The importance of angiotensin-II and ACE inhibitors in hypertension is well established. The role of ANP and NEP inhibitors in this disease, however, is less understood; because of their recent discovery, a significant body of clinical studies is not yet available. When tested in humans with hypertension some NEP inhibitors have produced significant decreases in blood pressure (Richards et al., 1993a, c). Although the antihypertensive effect may not have been as large as that achieved by other types of compounds, in some...
instances enhanced natriuresis and cGMP excretion were observed (Richards et al., 1993a, b). After prolonged NEP inhibition, however, elevations in plasma renin, aldosterone and catecholamines may have counteracted the potential beneficial effects of ANP (Richards et al., 1993a). Some authors have concluded that NEP inhibitors have a potent antihypertensive effect associated with an enhancement of endogenous ANP (Ogihara et al., 1994), which may be offset by an activation of the renin-angiotensin and sympathetic nervous systems (Richards et al., 1993c).

The discovery of selective NEP inhibitors has created the possibility of a new treatment for CHF with candesartanatril having been shown to increase diuresis and natriuresis in these patients (Northridge et al., 1989; Good et al., 1995). Although studies with other compounds have failed to confirm this finding, additional but modest beneficial effects have been described (Wilkins et al., 1993). The possibility exists that in CHF, despite a naturally occurring increase in the concentration of circulating ANP, the kidney does not respond to it in terms of vasodilation, diuresis and natriuresis. This unresponsiveness would impose a serious limitation to the therapeutic action of selective NEP inhibitors. Experiments in animals with CHF, however, suggest that dual ACE/NEP inhibition may offer some advantage over selective NEP inhibition. In dogs with CHF produced by rapid ventricular pacing, ACE inhibitors potentiated both the renal hemodynamic and excretory responses to NEP inhibitors (Margulies et al., 1991; Seymour et al., 1993). The advantages of the dual inhibitor BMS-182657 over selective ACE or NEP inhibition was demonstrated in hamsters with heart failure caused by genetic cardiomyopathy (Trippodo et al., 1995). In more recent studies performed with the same dual inhibitor, the importance of the ACE inhibitory arm in the hemodynamic response and of the NEP inhibitory arm in the renal response was demonstrated in non-human primates (Seymour et al., 1996a).

Several dual ACE/NEP inhibitors such as glycopril, ala-triopril, mixanapril and MDL 100,240 were able to decrease the activities of both ACE and NEP in the lung and kidney of mice and rats superseding the effects of thiorphan and selective ACE and NEP inhibitors on their respective target enzymes. These compounds as well as BMS-182657 (Trippodo et al., 1995; Seymour et al., 1996a) produced long-lasting inhibitions of the angiotensin-I pressor response and sustained increases in diuresis, natriuresis and cGMP excretion in several species (Gros et al., 1991; Fournier-Zaluski et al., 1994b; French et al., 1994). In experimental studies with models whose high blood pressure is sensitive to either NEP or ACE inhibitors, dual ACE/NEP inhibitors have been superior to their selective counterparts. SQ 28,133, a dual-acting compound, produced a blood pressure reduction in the SHR, which was not observed with selective NEP inhibitors, as well as in the DOCA-salt rats, a model unresponsive to selective ACE inhibitors (Seymour et al., 1991). The antihypertensive effect of mixanapril was demonstrated in the SHR, the DOCA-salt rat and the two-kidney, one-clip renal hypertensive rat (Fournier-Zaluski et al., 1994b), whereas BMS-182657 was effective in both sodium-depleted SHR, a model sensitive to ACE inhibitors, and in DOCA-salt hypertensive rats (Trippodo et al., 1995). These observations suggest that the dual inhibitors may be able to suppress or ameliorate a wider spectrum of hypertensive mechanisms than those evoked by the sole inhibition of ACE or NEP. In this regard, it has been speculated that dual inhibition may potentiate the action of bradykinin, substance P and brain natriuretic peptide which are also substrates for both ACE and NEP (Seymour et al., 1996b; Skidgel et al., 1987; Vanneste et al., 1990). Furthermore, the superiority of dual inhibition to selective inhibition recently has been demonstrated in humans with essential hypertension. In a group of these patients monotherapy with the NEP inhibitor sinorphan or with captopril failed to reduce blood pressure for a 24-hr period; however, a statistically significant decrease was observed when both agents were combined (Favrat et al., 1995).

The experiments performed with CGS 30440 indicate that this compound is able to produce physiological responses typical of an ACE inhibitor and of a NEP inhibitor. The vascular and renal responses evoked by CGS 30440 are long-lasting and have achieved statistical significance even at low doses. Our findings suggest that CGS 30440 has the potency and properties to merit further consideration in the treatment of both hypertension and CHF.

References


Rat brain enkephalinase: Characterization of the active site using mercapto-
propyl ammonium acid inhibitors, and comparison with angiotensin-converting en-

Gros C, Noel N, Souque A, Schwartz JC, Danvy D, Plaquevent JC, Duhamel P, 
Duhame L, Lecomte JM and Brelet J (1991) Mixed inhibitors of angiotensin-
converting enzyme (EC 3.4.15.1) and enkephalinase (EC 3.4.24.11): rational de-
sign, properties and potential cardiovascular applications of glycopil and alatri-

Heber ME, Bridgen GS, Caruana MP, Lahiri A and Raftery EB (1988) First dose 
response to SCH 42495, a neutral metalloendopeptidase inhibitor, ramipril. Am J
Cardiol 62:239–245.

Jansen WMT, de Zeeuw D and Gdalt K (1989) Antihypertensive effect of a five day 
infusion of atrial natriuretic factor in humans. Hypertension 12:640–646.

Lappe RW, Todt JA and Wendt RL (1997) Effects of atrial natriuretic factor on the 
vasoconstrictor actions of the renin-angiotensin system in conscious rats. Circ Res
61:134–140.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement 

Maeda T, Balakrishnan K and Mehdi SQ (1983) A simple and rapid method for the 
preparation of plasma membranes. Biochim Biophys Acta 731:115–120.

Margolies KB, Perrella MA, McKinley LJ and Burnett Jr JC (1991) Angiotensin 
inhibition potentiates the renal responses to neutral endopeptidase inhibition in 

Northridge DB, Alabaster CT, Connell JMC, Dilly SG, Lever AF, Jardine AG, 
Barclay PL, Dargie HJ, Findlay IN and Samsus GM (1989) Effects of UK 69,578: 

Oderici V, Chatelain RE, Schwartzkopf CD and Darzlik BN (1995) Reduced renal 
normal endopeptidase 24.11 (NEP) activity and elevated plasma ANP levels in 

tensive effects of the neutral endopeptidase inhibitor SCH 42495 in essential 


Richards AM, Crozier IG, Espiner EA, Yandle TG, Ikram H and Framp-
ton C (1993a) Systemic hemodynamics, renal function and hormonal levels during inhi-
bition of neutral endopeptidase 3.4.24.11 and angiotensin-converting enzyme in 
conscious dogs with pacing-induced heart failure. J Pharmacol Exp Ther
266:867–883.

Seymour AA, Asaad MM, Abbasi-Oefe BE, Smith PL, Rogers WL and Dorso CR 
(1996a) In vivo pharmacology of dual neutral endopeptidase/angiotensin-

Seymour AA, Asaad MM, Abbasi-Oefe BE, Smith PL, Rogers WL and Dorso CR 
(1996b) Determinants of in vivo activity of neutral endopeptidase 3.4.24.11 and 

(1989) Thiorphan, an inhibitor of endopeptidase 24.11, potentiates the natriuretic 

Degradation of atrial natriuretic peptide: pharmacological effects of protease EC

metabolism of brain natriuretic peptide in the rat involves endopeptidase 24.11 

Degradation of atrial natriuretic peptide: pharmacological effects of protease EC


Send reprint requests to: Ricardo E. Chatelain, M.D., Novartis Pharmaceu-
ticals Corporation, 556 Morris Avenue, Summit NJ 07901-1398.