Inhibition of Angiotensin Converting Enzyme by (R)-3-[(S)-1-Carboxy-5-(4-Piperidyl)pentyl]amino-4-Oxo-2,3,4,5-Tetrahydro-1,5-Benzothiazepine-5-Acetic Acid (CV-5975), a Non-Sulfhydryl Compound

Yoshiyuki INADA, Masao TANABE, Katsumi ITOH1, Hirosada SUGIHARA1 and Kohei NISHIKAWA

Biology Laboratories and 1Chemistry Laboratories, Central Research Division, Takeda Chemical Industries, Ltd., Juso, Yodogawa-ku, Osaka 532, Japan

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Abstract—CV-5975, (R)-3-[(S)-1-carboxy-5-(4-piperidyl)pentyl]amino-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepine-5-acetic acid, was found to inhibit rabbit lung angiotensin converting enzyme (ACE) activity with an IC50 of 3.1×10^-9 M and a Ki of 2.6×10^-9 M, inhibit the angiotensin I (A-I)-induced contraction of the guinea pig ileum with an IC50 of 1.3×10^-8 M, and augment the bradykinin (BK)-induced contraction of the ileum with an AC50 of 9.2×10^-10 M. The activity of CV-5975 was comparable to or slightly more potent than that of enalaprilat. The overall inhibition constant (Ki*), calculated from a steady-state analysis of enzyme reactions, was 4.4×10^-12 M for CV-5975; this indicates that the inhibition was about 5 times more potent than that of enalaprilat (2.0×10^-11 M). In rats, CV-5975 (0.03 and 0.3 mg/kg, i.v. and 3 and 10 mg/kg, p.o.) inhibited the A-I-induced pressor action more potently and for a longer period than did the corresponding doses of enalaprilat and enalapril. CV-5975 and enalapril (3 mg/kg, p.o.) augmented the BK-induced depressor action to a similar extent. In dogs, CV-5975 (0.3 and 1 mg/kg, p.o.) markedly inhibited the A-I-induced pressor action in a dose related manner, and the duration of this inhibitory activity was longer than with the corresponding doses of enalapril. These data provide evidence for the proposal that CV-5975 is a highly potent and long lasting ACE inhibitor.

The renin-angiotensin-aldosterone system plays an important role in the pathogenesis of hypertension (1, 2). Discovery of the orally active angiotensin converting enzyme (ACE) inhibitors, captopril and enalapril, led to the clinical use of agents which block the renin-angiotensin system, and hence provided treatment for patients with hypertension (3, 4). Captopril and enalapril are also effective in treating congestive heart failure (5). We reported the design and structure-activity relationships of (R)-3-amino-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepine-5-acetic acid derivatives for ACE inhibitory activity (6–9). We found that (R)-3-[(S)-1-carboxy-5-(4-piperidyl)pentyl]amino-4-oxo-2,3,4,5-tetrahydro-1,5-benzothiazepine-5-acetic acid, CV-5975 (Fig. 1), seemed to be the most promising ACE inhibitor (9). This agent has a potent and long lasting antihypertensive activity, as noted in various models of hyper-

CV-5975

![Chemical structure of CV-5975.](image-url)
tension in laboratory animals (10). We report here the ACE inhibitory activity of CV-5975, in vitro and in vivo, as compared with the findings on enalapril and enalaprilat.

Materials and Methods
1. Inhibition of angiotensin converting enzyme (ACE) in vitro
1.1. Rabbit lung ACE: Angiotensin converting enzyme (ACE) was prepared from the lung of albino male rabbits, as described (11). For the kinetic study of ACE inhibition, lung ACE was prepared according to Dorer et al. (12) but with some modification; the preparation was not purified further after fractionation with ammonium sulfate. The enzyme activity was determined using the spectrophotometric method of Cushman and Cheung (13), as described (11) using hippuryl-L-histidyl-L-leucine (HHL) (2 and 5 mM) as the substrate and incubating the reaction mixture at 37°C for 20 min. The IC50 value (the concentration of a drug required to inhibit ACE activity by 50%) was calculated. The rate of reaction and mode of inhibition were determined, and the value of Ki was calculated according to Dixon and Webb (14). The Ki* values for CV-5975 and enalaprilat were obtained from Henderson plots (15) of [I]/(1 - v/v0) vs. v/v0 at [S] << Km, where [I] is the concentration of total inhibitor, and v and v0 are initial velocities in the presence and absence of the inhibitor, respectively. The ACE activity was assayed using the synthetic substrate, furanacryloyl-L-phenylalanyl-glycyl-glycine (Fa-Phe-Gly-Gly), essentially as described by Holmquist et al. (16). Assay mixtures containing enzyme and inhibitor in 50 mM Hepes, 1 mM ZnCl2, and 300 mM NaCl had to be preincubated because of the extremely slow binding of the inhibitor to the enzyme. The reaction was initiated by adding the substrate (50 μM), and the ACE activity was monitored using a Hitachi U-3200 spectrophotometer. At [S] << Km, the initial velocity closely approximates the steady-state velocity, and the slope of the Henderson plot equals Ki*.

1.2. Guinea pig ileum preparation: Segments of the terminal ileum (about 1.5 cm in length) isolated from male Hartley strain guinea pigs were suspended in an organ bath containing 20 ml of Tyrode’s solution saturated with 95% O2-5% CO2 gas at 37°C. A resting tension of 1 g was applied. After equilibration for about 1 hr, angiotensin I (A-I) or bradykinin (BK) was added. The contraction of the segments was monitored with an isotonic transducer (ME-4012, MEC, Japan) connected to a recorder (B-64, Rikadenki, Japan). ACE inhibitors (10^-9 to 10^-7 M) were added to the incubation medium, and 2 min later, A-I (3×10^-8 M) or BK (3×10^-9 M) was added. The ACE inhibitory activity was expressed as an IC50 value for A-I and an AC50 value for BK; i.e., the concentration of inhibitor necessary to produce a 50% inhibition or a 50% augmentation of contraction of the ileum in response to A-I or BK, respectively.

2. Inhibition of pressor response to A-I and augmentation of depressor response to BK
2.1. Rats: Male Sprague-Dawley rats (SD: Jcl), weighing 300–400 g, were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and a polyethylene cannula was placed in the abdominal aorta via the femoral artery (PE-10) to measure blood pressure and in the vena cava via the femoral vein (PE-50) for drug injection. Both catheters filled with saline containing heparin were passed subcutaneously and exteriorized at the dorsal neck. The animals were then placed in plastic cages for one night and allowed to move freely; tap water was given ad libitum, but solid food was withheld. The experiments were started in the next a.m. The aortic cannula was connected to a pressure transducer (Sanei 45277, Japan), and the mean blood pressure was recorded on a polygraph (Sanei 365, Japan). The pressor responses to A-I (300 ng/kg) and A-II (100 ng/kg) and the depressor response to BK (3 μg/kg), all of which were injected into the femoral vein, were measured twice, and the average response to each drug was used as the control for all calculations. After the blood pressure had reverted to pretreatment levels, ACE inhibitors were administered p.o. or i.v. Thereafter, A-I, A-II or BK were again given, and the inhibition of the pressor response to A-I or augmentation of the depressor response to BK was determined. The A-II challenge was used to correct for values of the inhibition
on the pressor response to A-I due to changes in vascular responsiveness during the course of the experiment. The values of % inhibition were calculated as follows: % inhibition = \(\frac{1-(A-I(\text{exp})\times A-II(\text{cont})\times A-I(\text{exp})\times A-II(\text{exp}))}{A-I(\text{cont})\times A-II(\text{cont})}\times 100\), where A-I(\text{cont}) and A-II(\text{cont}) are pressor responses (mmHg) to A-I and A-II in the control period, respectively, and A-I(\text{exp}) and A-II(\text{exp}) are pressor responses to A-I and A-II, respectively, at given times after the ACE inhibitors had been administered. The results shown in Figs. 3 and 4 represent the corrected values. For a precise quantification of the duration of action of the inhibitors, the upper area of the curve (AUC) was calculated by planimetry for Figs. 3 and 4; % inhibition of the pressor response to A-I was calculated for the time over 0 to 7 hr (i.v.) or 0 to 24 hr (p.o.). The dose that corresponds to AUC=50% was computed and used as ID50 values for the ACE inhibitors.

2.2. Dogs: Conscious normotensive beagle dogs of either sex, weighing 13 to 15 kg, were used. One to six weeks before the day of the experiment, right femoral arterial and venous catheters were inserted to measure blood pressure and to inject agonists, respectively. Both catheters filled with saline containing heparin were passed subcutaneously and exteriorized at the dorsal neck. After surgery, the animals were placed in individual cages and allowed to move freely. From the evening before each experiment, tap water was allowed ad libitum, but solid food was withheld. A-I (150 ng/kg) and A-II (100 ng/kg) were injected i.v. before and after the administration of the ACE inhibitor. The procedures used thereafter were the same as those used for rats.

3. Data analysis

All results in the text are expressed as the mean±S.E.M. Values for different groups were compared using the one way analysis of variance and Dunnett's test. P values less than 0.05 were considered to have a statistical significance.

4. Drugs

Drugs used in this study were angiotensin I, angiotensin II, bradykinin and hippuryl-L-histidyl-L-leucine (HHL) obtained from the Protein Research Foundation; hippuric acid from Sigma; heparin from Shimizu Pharmaceutical Co.; EDTA-2Na from Dojin Chemicals; and pentobarbital-Na (Somnopentyl) from Pitman Moore. Furancaryl-L-phenylalanlyl-glycyl-glycine (Fa-Phe-Gly-Gly) (16), CV-5975 (8), enalapril (MK-421) and enalaprilat (MK-422) were synthesized as described (17).

Results

1. Inhibition of ACE in vitro

1.1. Rabbit lung ACE: The IC50 value for CV-5975 was 3.1×10^{-9} M, a value comparable to that seen with enalaprilat (3.2×10^{-9} M) (Table 1). In the presence of HHL, CV-5975 competitively inhibited the reaction between rabbit lung ACE and HHL (Fig. 2a). The K_i values were 2.6×10^{-9} M for CV-5975 and 3.8×10^{-9} M for enalaprilat. Henderson plots for the steady-state analysis with CV-5975 were obtained using the substrate Fa-Phe-Gly-Gly. The plots were linear (Fig. 2b), and their slopes gave an apparent K_i value (K_i^*) of 4.4×10^{-12} M for CV-5975. The K_i^* value for enalaprilat was 2.0×10^{-11} M. CV-5975 was about 5 times as potent as enalaprilat in forming the steady-state enzyme-inhibitor complexes.

1.2. Inhibition of contractile response to A-I and augmentation of contractile response to BK in excised guinea pig ileum: In preliminary experiments, A-I at concentrations of 3×10^{-10} to 10^{-7} M induced a dose related contraction of the ileum. A submaximum concentration (3×10^{-8} M) of A-I was used to determine IC50 values for the agents. CV-

Table 1. Inhibitory effects of CV-5975 and enalaprilat on rabbit lung angiotensin converting enzyme (ACE)

| Compound      | IC50 (M)   | K_i (M)   | K_i^* (M) |
|---------------|------------|-----------|-----------|
| CV-5975       | 3.1×10^{-9} | 2.6×10^{-9} | 4.4×10^{-12} |
| Enalaprilat   | 3.2×10^{-9} | 3.8×10^{-9} | 2.0×10^{-11} |
5975 and enalaprilat at concentrations of $3 \times 10^{-9}$ to $10^{-7}$ M inhibited the contractile response of the ileum to A-I, in a dose-related manner. Table 2 shows the inhibitory effect of the two compounds on the contraction induced by A-I ($3 \times 10^{-8}$ M). The IC50 values were $1.3 \times 10^{-8}$ M for CV-5975 and $1.7 \times 10^{-8}$ M for enalaprilat; CV-5975 was as potent as enalaprilat.

Bradykinin (BK) given at $10^{-9}$ to $3 \times 10^{-6}$ M induced a dose-related contractile response in the ileum. The concentration of BK used to determine the AC50 was $3 \times 10^{-9}$ M, a dose which elicits one-third of the maximum response. Augmentation by CV-5975 and enalaprilat was dose-related from $10^{-9}$ to $10^{-7}$ M. As shown in Table 2, the AC50 values were $9.2 \times 10^{-10}$ M for CV-5975 and $2.4 \times 10^{-9}$ M for enalaprilat; CV-5975 was about 2.5 times as active as enalaprilat.

2. Inhibition of pressor response to A-I in rats

2.1. I.v. administration: As shown in Fig. 3, a prompt and graded inhibition of the pressor response to A-I occurred after i.v. administration of 0.03 and 0.3 mg/kg of CV-5975 and enalaprilat. Each drug induced the maximum inhibition of the pressor response to A-I at 5 min; subsequently, the effects diminished gradually. With both doses, the inhibitory effect of CV-5975 lasted longer than that of enalaprilat. The duration of the inhibitory effects of CV-5975 was expressed as the time (T1/2) required for the 50% recovery of responses. The T1/2 for CV-5975 was about 5 hr at 0.3 mg/kg, i.v., and CV-5975 inhibited the pressor response to A-I with an ID50 of 0.07 mg/kg, i.v., over the 7 hr observation period. The ID50 of enalaprilat was over 0.3 mg/kg, i.v.

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Table 2. Inhibition of contractile response to angiotensin I (A-I) and augmentation of contractile response to bradykinin (BK) by CV-5975 and enalaprilat in excised guinea pig ileum

| Compound   | A-I ($3 \times 10^{-8}$ M) IC50 (M) | BK ($3 \times 10^{-9}$ M) AC50 (M) |
|------------|---------------------------------|---------------------------------|
| CV-5975    | $1.3 \times 10^{-8}$            | $9.2 \times 10^{-10}$           |
| Enalaprilat| $1.7 \times 10^{-8}$            | $2.4 \times 10^{-9}$            |

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Fig. 2. Determination of Kᵢ and Kᵢ* value for CV-5975. a) Dixon-Webb plot for inhibition of hippuryl-L-histidyl-L-leucine (HHL) hydrolysis by CV-5975, 2 mM (○) and 5 mM (●) HHL concentrations were used. A Kᵢ value of $2.6 \times 10^{-9}$ M was obtained. b) Henderson plot for inhibition of FA-Phe-Gly-Gly (50 µM) hydrolysis by CV-5975. A Kᵢ* value of $4.4 \times 10^{-12}$ M was obtained.

Fig. 3. Inhibitory effects of CV-5975 and enalaprilat on angiotensin I-induced pressor response in rats after the i.v. administration. (○) CV-5975, n=5; (▲) enalaprilat, n=5.
Table 3. Potentiating effects of CV-5975 and enalapril on bradykinin induced depressor response in rats

| Group   | Dose (mg/kg) | No. of rats | 0  | 1  | 3  | 5  | 7 hr |
|---------|--------------|-------------|----|----|----|----|------|
| Control | --           | 5           | -7.9±0.8 | -9.2±1.1 | -11.2±1.9 | -11.7±1.2 | -12.0±1.4 |
| CV-5975 | 3            | 5           | -7.4±0.4 | -31.6±2.6** | -32.0±2.7** | -28.6±2.8* | -24.8±2.4* |
| Enalapril | 3          | 5           | -8.0±1.6 | -36.8±4.4** | -34.2±5.3** | -32.6±5.3* | -28.2±4.2** |

Values represent the mean±S.E.M. *P<0.05, **P<0.01, compared with the control group using the Dunnett type rank sum test.

2.2. P.o. administration: CV-5975 and enalapril dose-dependently inhibited the pressor response to A-I in rats (Fig. 4). The ID50 was 1.8 mg/kg, p.o., for CV-5975 and 6.6 mg/kg, p.o., for enalapril; CV-5975 was 3.7 times more potent than enalapril over the 24 hr observation period. The duration of the inhibitory effects of CV-5975 and enalapril at 10 mg/kg, p.o., were over 24 hr and over 10 hr, respectively.

2.3. Augmentation of depressor response to BK: CV-5975 and enalapril at 3 mg/kg, p.o., potentiated the BK-induced vasodepressor responses to the same extent (Table 3).

3. Inhibition of pressor response to A-I in dogs

As shown in Fig. 5, CV-5975 at doses of 0.3 and 1 mg/kg, p.o., inhibited the pressor response to A-I by over 90%; the duration of the inhibitory action of CV-5975 was prolonged with the higher dose. Enalapril dose-dependently inhibited the pressor response to A-I. The maximum inhibition with enalapril was about 60% and 90% at 0.3 and 1 mg/kg, respectively. The inhibitory action of enalapril at 0.3 and 1 mg/kg, p.o., was no longer evident at 10 hr and 24 hr, respectively, after the administration. Thus, CV-5975 was more potent and longer lasting than enalapril.

Discussion

The angiotensin converting enzyme (ACE) not only hydrolyzes the biologically inactive decapeptide A-I to the vasopressor octapeptide A-II, but also degrades the vasodepressor BK to inactive fragments. Thus, ACE inhibitors can reduce blood pressure by inhibiting the formation of A-II and possibly by augmenting...
the vasodepressor activity of BK. Studies of ACE inhibition with rabbit lung homogenates (Table 1) and guinea pig ileum (Table 2) indicated that CV-5975 is a potent ACE inhibitor. The IC50 value of enalaprilat on the lung ACE was compatible with the published values (17, 18). CV-5975 augmented the BK-induced contraction of the guinea pig ileum more potently than did enalaprilat, but inhibited the A-I-induced contraction of the ileum to much the same extent as enalaprilat did (Table 2). These observations mean that CV-5975 inhibits more sensitively the inactivation of BK than it does the conversion of A-I to A-II. We reported that delapril, another ACE inhibitor, inhibits more sensitively the conversion of A-I to A-II than it did the inactivation of BK (9). One explanation for this dissociation of A-I conversion and BK-inactivation among the ACE inhibitors may relate to differences in the kinetic characteristics of ACE, depending on the substrates, and the difference in multiple binding properties of inhibitors to ACE (19).

The inhibition of the pressor response to A-I and augmentation of the depressor response to BK are currently used as indices of the ACE inhibitory activity in vivo. In rats, CV-5975 inhibited the A-I-induced pressor action more potently and for a longer time than did enalaprilat (i.v.) and enalapril (p.o.) (Figs. 3 and 4). The BK-induced depressor action was augmented to an extent similar to that seen with enalapril (Table 3). Thus, there are differences in the BK-inactivation of CV-5975 between in vitro and in vivo conditions. Difference in BK-inactivation was observed between delapril and captopril, in vitro and in vivo (9). Here, the ratio of the potencies between delapril and captopril was about 250 times, in vitro. In the case of CV-5975 and enalapril, because the ratio of the potencies of these drugs was only about 2.5 times in vitro, the different effects of these drugs were not evident in vivo.

The Dixon-Webb analysis of the interaction between CV-5975 and rabbit lung ACE in vitro (Fig. 2a) showed that CV-5975, enalaprilat (20) and captopril (21) all act as fully competitive inhibitors in this system. In general, enalapril and captopril are considered to be slow, tight-binding inhibitors of ACE (19). Evidence has been presented that this binding process involves at least two steps: first, the inhibitor rapidly binds to ACE to form an initial inhibitor-enzyme complex, and subsequently, the primary complex undergoes a slow rearrangement or isomerization reaction (19, 22). To determine the $K_i$ value (overall inhibition constant for the first and subsequent step) for CV-5975 and enalaprilat, the enzyme was preincubated with these inhibitors and the interaction was analyzed by the method of Henderson (15). The calculated values for $K_i$ were $4.4\times10^{-12}$ M for CV-5975 and $2.0\times10^{-11}$ M for enalaprilat. Like enalapril (19, 23), captopril (19) and ramipril (24), CV-5975 bound to ACE with a very high affinity, as was demonstrated by the difference between the $K_i$ and the $K_i^*$ values (Table 1). CV-5975 was about 5 times more potent than enalaprilat in forming steady state enzyme-inhibitor complexes, although the $K_i$ value of CV-5975 against rabbit lung ACE (Table 1) was comparable to that of enalaprilat. The kinetic properties of these inhibitors in the enzyme inhibition may determine, in part, their pharmacologic potency. Thus, the long-lasting ACE inhibitory action of CV-5975 may be attributable to its more potent binding characteristic to ACE; the high potency of CV-5975 required to form the enzyme-inhibitor complex seems to induce the long half-life for the ACE inhibitory action of CV-5975, in vivo. The onset of the ACE inhibitory action of these inhibitors may be largely determined by the rate of absorption from the digestive tract and the rate of hydrolysis of ester to active carboxylic acid (enalaprilat), in vivo (in the case of enalapril).

In conclusion, CV-5975 is a highly potent and long acting inhibitor of ACE activity and possesses a long-lasting antihypertensive action in animal models of hypertension (10).

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