Species Difference of (2R,4R)-2-(o-Hydroxyphenyl)-3-(3-Mercaptopropionyl)-4-Thiazolidinecarboxylic Acid (SA446) in Inhibition of Angiotensin Converting Enzyme

Katsuhiko NAKATA, Terumichi IWATANI, Masato HORIUCHI, Hirokazu KITO, Hideyasu YAMAUCHI and Tadashi ISO
Research Laboratory, Santen Pharmaceutical Co., Ltd., 3-9-19, Shimoshinjo, Higashiyodogawa-ku, Osaka 533, Japan
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Abstract—A marked species difference was observed both in vitro and in vivo in the activity of SA446, an orally active inhibitor of angiotensin converting enzyme (ACE), as compared with that of captopril in five different animal species. The activity of SA446 in vitro in inhibiting plasma ACE correlated with the activity in vivo as determined by inhibition of the pressor response to angiotensin I (AI). SA446 was more potent as an inhibitor of AI response in dogs, cats and rabbits than in guinea pigs and rats. Furthermore, ACE activity in whole blood in vivo was inhibited by SA446, and the activity of SA446 was also more potent in dogs than in rats. The concentration of SA446 in the ultrafiltrate of blood (free form) was significantly higher in dogs than in rats, while no difference was observed in level of SA446 in the whole blood (free and protein-bound form) between these two species after intravenous injection. The binding rate of SA446 to plasma protein of rats in vitro was more than twice as high as that of dogs. These results suggest that the difference in the protein binding rate of SA446 is reflected in ultrafiltrate level and is one of the important components in defining the species difference in SA446 action.

Materials and Methods

1. Animals and chemicals: Male Wistar rats (170–260 g), male Hartley guinea pigs (300–400 g), male albino rabbits (2.0–3.0 kg), and mongrel cats (2.5–5.5 kg) and dogs (10–16 kg) of either sex were used. SA446 and captopril synthesized at Santen Pharmaceutical Co., Ltd., Osaka, Japan (7) were dissolved in saline, and neutralized with NaOH immediately before use. Hippuryl-histidylleucine (Hip-His-Leu) and angio-
tensin I (Al) were purchased from the Protein Research Foundation, Osaka, Japan.

2. Inhibition of pressor response to Al: Rats, guinea pigs and rabbits were anesthetized with urethane (1 g/kg, i.v. or i.p.), and cats and dogs were anesthetized with pentobarbital sodium (30 mg/kg, i.v.). A polyethylene catheter was inserted into the right femoral artery, and the blood pressure was recorded with a pressure transducer (Nihon Kohden, MPU-0.5A) on the ink-writing polygraph (Nihon Kohden, RM-6000). Al was injected intravenously at the dose of 300 ng/kg through the catheter inserted into the right femoral vein at intervals of 10 min until the constant pressor response to Al was obtained. Then, SA446 or captopril was injected intravenously through the same catheter. The change in the pressor response to Al was monitored following the drug administration, and the doses which inhibit 50% of the pressor response (ID50) were calculated by using linear regression analysis.

3. Inhibition of plasma ACE in vitro: Fifty microliters of plasma from the 5 different species were incubated with SA446 or captopril and 5 mM Hip-His-Leu dissolved in 0.1 M potassium phosphate buffer (pH 8.3) containing 300 mM NaCl in a total volume of 250 μl (8). After incubation of 10 min at 37°C, the reaction was terminated by adding 0.75 ml of 3% metaphosphoric acid, and the mixture was centrifuged. The supernatant was applied in a volume of 20 μl to a high performance liquid chromatograph (Shimadzu, LC-3A) (9), and the enzyme activity was determined by measuring the quantity of hippuric acid liberated from the substrate. The concentration of the drug which inhibits 50% of the ACE activity (IC50) was calculated by linear regression analysis.

4. Inhibition of whole blood ACE by intravenous injection of SA446: Rats and dogs were anesthetized with urethane (1 g/kg, i.p.) and pentobarbital sodium (30 mg/kg, i.v.), respectively. Five minutes after the intravenous injection of SA446, blood was taken with a heparinized syringe, and ACE activity was assayed in 50 μl of whole blood by the same method as described in the inhibition of plasma ACE in vitro.

5. Blood concentration of SA446 after intravenous injection: SA446 was given intravenously to the urethane anesthetized rats and the pentobarbital anesthetized dogs at a dose of 3 mg/kg. Blood samples were taken with time after the administration of SA446, and a part of each blood sample, about 5 ml, was centrifuged at 2,500 rpm at 2°C for 5 min using a polycarbonate cell fitted with a molecular filtration membrane (Amicon Type CF25) to obtain the ultrafiltrate of the blood. The sample of whole blood (250 μl) or ultrafiltrate (200 μl) was added to the 0.2% acetonitrile solution of pentafluorobenzyl bromide, and the amount of SA446 was determined by a gas chromatograph (Shimadzu, GC-7A) equipped with a 63Ni electron capture detector according to the method of Iwatani et al. (10).

6. Plasma protein binding of SA446 in vitro: 35S-SA446 (specific radioactivity: 12 μCi/mg) dissolved in 0.5 ml of M/15 phosphate buffer (pH 7.4) at the concentration of 100 μg/ml was incubated with 4.5 ml of heparinized fresh blood from dogs or rats at 37°C for 30 min. The mixture was centrifuged at 3,000 rpm for 10 min, and 2 ml of the supernatant was applied to the chromatograph with the Sephadex G-200 column (1.6×61.0 cm) equilibrated with the buffer. Three ml of each fraction were collected, and a 0.1 ml aliquot was added to the scintillation cocktail (PPO: 5 g, dimethyl POPOP: 0.25 g, toluene: 1 l). The radioactivity was measured by a liquid scintillation counter (Aloka, LSC-700). The radioactivity of SA446 which was eluted with protein was regarded as the protein-bound drug. The plasma protein binding rate was shown as the percentage of radioactivity in protein fraction to the total radioactivity.

Results

1. Inhibition of pressor response to Al: The control pressor response to 300 μg/kg of Al varied with species as shown in Table 1. These pressor responses were inhibited by SA446 or captopril dose-dependently, and the maximum inhibition was observed 5 min after intravenous administration. Table 1 also shows that the ID50 values of SA446 which inhibit 50% of the pressor response to Al were distributed in a wide range of 10.1 to
64.9 μg/kg and that susceptibility to inhibition of the Al response by SA446 was higher in dogs, cats and rabbits than in guinea pigs or rats. On the other hand, ID50 values of captopril were within the range of 16.2 to 30.6 μg/kg. Thus, the marked species differences seen for SA446 in vivo was not observed with captopril.

2. Inhibition of plasma ACE in vitro: As shown in Table 2, ACE activity in plasma also varied with species, i.e., high in guinea pigs, moderate in rabbits and rats and low in dogs and cats. In these animals, the plasma ACE activity in vitro was inhibited by SA446 in a concentration-dependent manner, and the susceptibility to SA446 was also different among the species. However, the activity of captopril was almost same among these animal species, except in guinea pigs.

3. Inhibition of whole blood ACE by intravenous injection of SA446: Table 3 shows ACE activity in whole blood before and 5 min after the intravenous injection of SA446. The intravenous doses of SA446, 5 μg/kg in dogs and 30 μg/kg in rats, were fixed at about half of the ID50 values in the inhibition of pressor response to Al in these species. These doses were based on the fact

| Species          | Pressor response to AI (mmHg, mean±S.E.M.) | ID50 for pressor response to AI (μg/kg, i.v.) |
|------------------|-------------------------------------------|---------------------------------------------|
|                  |                                            | SA446                                      | Captopril                                  |
| Dog              | 29.0±2.9                                   | 10.1 (7.9–12.9)                            | 21.9 (17.2–28.0)                           |
| Cat              | 69.4±4.4                                   | 12.2 (10.4–14.5)                           | 23.5 (16.0–34.5)                           |
| Rabbit           | 13.9±1.7                                   | 12.7 (10.3–15.7)                           | 16.2 (11.9–22.0)                           |
| Guinea pig       | 39.7±1.0                                   | 53.9 (43.7–66.4)                           | 30.6 (24.9–37.4)                           |
| Rat              | 35.5±1.9                                   | 64.9 (36.2–116.3)                          | 31.1 (21.6–44.9)                           |

The intravenous dose of Al was 300 ng/kg. ID50 values are the means of 9 to 23 animals, and figures in parentheses indicate 95% confidence limits.

| Species          | Plasma ACE activity (mU/ml, mean±S.E.M.) | IC50 for plasma ACE activity (nM) |
|------------------|------------------------------------------|----------------------------------|
|                  |                                          | SA446                            | Captopril                           |
| Dog              | 10.5±2.0                                 | 4 (2–8)                          | 28 (15–51)                          |
| Cat              | 6.3±1.2                                  | 7 (5–11)                         | 28 (17–39)                          |
| Rabbit           | 62.9±7.6                                 | 10 (8–11)                        | 18 (11–30)                          |
| Guinea pig       | 843.3±18.9                               | 41 (37–46)                       | 193 (83–449)                        |
| Rat              | 50.7±6.2                                 | 18 (15–22)                       | 24 (17–33)                          |

Data are the means of 3 to 6 experiments, and figures in parentheses indicate 95% confidence limits.

| Species | Dose (μg/kg, i.v.) | ACE activity in whole blood before SA446 (mU/ml) | ACE activity in whole blood 5 min after SA446 (mU/ml) | Inhibition (%) |
|---------|-------------------|--------------------------------------------------|------------------------------------------------------|----------------|
| Dog     | 5                 | 7.2±0.4                                         | 2.1±0.2                                              | 71.4±2.6*      |
| Rat     | 30                | 28.4±3.6                                        | 11.6±1.5                                            | 59.2±1.4       |

Data are the means±S.E.M. of 4 experiments. *: Significantly different from rats by Student's t-test.
that the inhibition of enzyme activity by ACE inhibitors is generally more potent than that of pressor response to Al (11). The enzyme activity was inhibited by SA446, and the activity in rats was significantly lower than in dogs, even at the 6 times higher dose.

4. Blood concentration of SA446 after intravenous injection: Figure 1 shows the concentrations of SA446 in the whole blood and ultrafiltrate of blood after the intravenous injection of 3 mg/kg of SA446 to dogs (—●—) and rats (—○—). Each point represents the mean±S.E.M. of 5 animals. *: Significantly different from rats by Student’s t-test, P<0.001.

![Figure 1](image)

**Table 4.** Plasma protein binding of 35S-SA446 in vitro

| Species | Radioactivity found in protein fraction (%) |
|---------|------------------------------------------|
| Dog     | 36.4                                     |
| Rat     | 85.3                                     |

Discussion

A clear species difference was observed in the sensitivity to SA446 estimated by the inhibition of pressor response to Al and of ACE activity in whole blood by intravenous injection. The sensitivity of SA446 in the inhibition of plasma ACE activity in vitro also differed with species, and the order of the activity in vitro was in good agreement with that in vivo in 5 different animal species, that is, dogs, cats, rabbits, guinea pigs and rats. The sensitivity to SA446 showed a tendency to increase in species having lower initial level of ACE activity and to decrease in species having higher ACE activity in blood. Thus, the sensitivity to SA446 seems to depend on the initial levels of ACE activity in the blood. However, the sensitivity in vitro or in vivo to captopril was not markedly affected by the initial level of ACE activity, suggesting that the initial level of ACE activity in blood does not always determine the sensitivity to inhibition by all ACE inhibitors.
On the other hand, since a species difference was observed in the activity of SA446 in vitro and in vivo as described above, another set of experiments for SA446 was performed in dogs and rats to clarify the reason of the species difference. The concentration of SA446 in the ultrafiltrate of blood (free form) after the intravenous injection was significantly higher in dogs than rats, although no difference was observed in the whole blood level (free and protein-bound form). The difference in ultrafiltrate levels of SA446 in dogs and rats, can be explained by the finding that the binding rate of the drug to plasma protein of rats is more than twice as high as that of dogs. Thus, the ultrafiltrate level of SA446 is determined by its binding to plasma in vitro in dogs and rats. As for captopril, Koike et al. (11) showed that the inhibition of the pressor response to Al closely correlated with the blood concentration of free unchanged captopril in dogs. Our data, as well as Koike’s, suggest that the concentration of the free form of these drugs in blood determines the degree of inhibition of ACE and that differences in the amount of free form of SA446 found in different animal species is determined by plasma protein binding. The protein binding rate of SA446 to human plasma was 55.6%, although the data was not shown in Table 4. The protein binding rate to human plasma was the intermediate value between that of dogs and rats, suggesting that the effect of SA446 in inhibiting ACE activity in humans was also intermediate between dogs and rats. Although the reason why there was a difference in plasma binding rate of SA446 to dog, rat and human plasma is not clear, the difference in plasma protein binding may be due to characteristics in the chemical structure of SA446.

The factor of anesthetics could be excluded in the species difference of SA446, because the activity of SA446 in inhibiting the pressor response to Al did not differ between the rats anesthetized with urethane and pentobarbital sodium (data not shown).

In conclusion, our present data suggest that the difference in the binding rate of SA446 to plasma protein is reflected in the ultrafiltrate level and is one of the important components in the definition of species difference in SA446 action.

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