Pharmacological Studies of N-(2-Mercapto-2-Methylpropanoyl)-L-Cysteine (SA96) (3)

Effects of SA96 on Experimental Allergic Reactions

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Abstract—The effects of an antirheumatic agent, N-(2-mercapto-2-methylpropanoyl)-L-cysteine (SA96), were investigated on allergic reactions in rats and guinea pigs. The effects of SA96 were compared with those of D-penicillamine (D-Pc). SA96 given twice orally at the doses of 10 to 50 mg/kg significantly caused inhibitions of 28%, 29% and 44% against passive cutaneous anaphylaxis (PCA), reversed cutaneous anaphylaxis (RCA) and reversed passive Arthus (RPA) reactions, which are classified as Type I, Type II and Type III allergic reactions, respectively. D-Pc also showed inhibitions of 30%, 23% and 18% on Type I, Type II and Type III reactions, respectively, and inhibitions on Type II and Type III reactions were not significant. On the other hand, SA96 (10 to 50 mg/kg twice) had no influence on the Type IV allergic reaction, delayed hypersensitivity, while D-Pc (20 mg/kg twice) showed an enhancement of 27% on the Type IV reaction. In the in vitro study, SA96 inhibited the hemolytic complement activity at 10^{-4} to 10^{-2} M and the macrophage migration at 1 \times 10^{-4} to 5 \times 10^{-3} M in a dose-dependent manner. These in vitro activities of SA96 were more potent than those of D-Pc. These results showed that SA96 had some different immunopharmacological properties on experimental allergic reactions as compared with those of D-Pc.

Immunomodulatory therapy, the treatment for autoimmune diseases such as rheumatoid arthritis by improving the immunological abnormality, has recently received a great deal of attention. Levamisole and D-penicillamine (D-Pc) are two representatives of immunomodulators that have shown their clinical efficacy in double-blind tests in rheumatoid arthritis (1, 2).

SA96, N-(2-mercapto-2-methylpropanoyl)-L-cysteine (Fig. 1), was shown to have low toxicity (3). SA96 was found to have prophylactic and therapeutic effects on adjuvant-induced arthritis in rats, although the compound had little effect on the various acute and subacute inflammation models (4). Furthermore, SA96 was shown to be a useful drug for the treatment of rheumatoid arthritis in a double-blind test (5). SA96 also had various immunopharmacological properties; that is, it potentiated the plaque forming cells (PFC) response of spleen in mice immunized with sheep red blood cell (SRBC) in vivo at 10 mg/kg, p.o. (4), and it inhibited the elevated PFC response to SRBC in the presence of 2-mercaptoethanol in vitro at 10^{-4} to 10^{-3} M, while the compound markedly enhanced the PFC response to DNP-Ficoll at 10^{-4} to 10^{-3} M (6). Thus, SA96 appears to possess an immunomodulator profile. It is therefore considered to interest

![Fig. 1. Chemical structure of SA96.](image-url)
to investigate the effects of the compound on immunologically induced inflammations.

The present study was undertaken to examine the effects of SA96 on four types of allergic reactions based on the classification by Coombs and Gell (7), the complement activity, and the macrophage migration as compared with those of D-Pc.

Materials and Methods

Animals: Male Wistar rats (160–300 g), male Hartley guinea pigs (300–400 g) and male albino rabbits (2.0–2.5 kg) were used.

Compounds and immune reagents: SA96 was synthesized at Santen Pharmaceutical Co., Ltd. (8). Other compounds and immune reagents were purchased from the following sources: D-Pc (Sigma), egg albumin (Sigma), Bordetella pertussis (Takeda), rabbit anti-egg albumin (Cappel Laboratory), 2,4-dinitrochlorobenzene (DNCB, Wako Pure Chemical), liquid paraffin (Wako Pure Chemical), fetal calf serum (FCA, Gibco), Eagle's MEM (Nissui Seiyaku) and hemolysin (Kitasato Institute).

SA96 and D-Pc were administered orally to rats and subcutaneously to guinea pigs. They were suspended in 0.5% tragacanth solution for oral administration, and they were dissolved in saline and adjusted pH to 7.0 with NaOH solution for subcutaneous administration. These compounds were administered twice at 0.5 and 18 hr before the provocation at three doses of 10, 20 and 50 mg/kg.

Passive cutaneous anaphylaxis (PCA) in rats: Rat anti-egg albumin serum was prepared according to the method of Mota (9). Male rats were immunized by intramuscular injection of 1 mg of egg albumin and intraperitoneal injection of 1 ml of suspension of 2 × 10^10 killed Bordetella pertussis. Twelve days later, serum was obtained and pooled. The titer of the antiserum was 1:16 as estimated by the 48 hr PCA reaction.

The IgE-mediated 48 hr PCA reaction was done by the following procedure. Rats were passively sensitized by intradermal injection of 0.1 ml of 8-fold diluted antiserum. After a 48 hr latent period, the PCA reaction was induced by intravenous injection of egg albumin (25 mg/kg) and Evans blue (20 mg/kg) in saline. Thirty minutes later, the rats were sacrificed, and the skins were removed. Evans blue was extracted from the skin according to the method of Harada et al. (10) and assayed by a spectrophotometer at 620 nm.

Reversed cutaneous anaphylaxis (RCA) in rats: This experiment was carried out principally according to the method of Ungar et al. (11). The antiserum was prepared by intravenous injection of rat serum into rabbits twice a week for 4 weeks. Seven days after the last immunization, serum was obtained and dissolved in 1% Evans blue to make a 15% solution. This solution was injected intradermally at the volume of 0.05 ml into 3 sites in the left side of the back of the rats. The same volume of saline containing 1% Evans blue was injected at the right side. Two hours later, the animals were sacrificed, and the skins were removed. Skin samples taken from the inflammatory lesion using a punch with a 15 mm diameter were weighed. The intensity of inflammations (I) caused by RCA was calculated by the following formula:

\[ I = \frac{W_i - W_s}{W_s} \times 100, \]

in which \( W_i \) represents the weight of the inflamed lesion and \( W_s \) represents that of the lesion treated with saline.

Reversed passive Arthus (RPA) reaction in rats: This experiment was carried out according to the method of Gemmel et al. (12). Rats were injected into the plantar pad with 0.1 ml of 2-fold diluted rabbit anti-egg albumin serum. Immediately thereafter, 25 mg/kg of egg albumin was injected intravenously. The increase in the paw volume was measured by the water displacement 3 hr after the provocation.

DNCB-induced delayed type hypersensitivity (DTH) reaction in guinea pigs: This experiment was carried out principally according to the method of Weck et al. (13). Fifty microliters of 3% DNCB solution in acetone was applied to shaved dorsal skin of guinea pigs. Seven days later, 10 μl of 0.2% DNCB solution in acetone was applied to the dorsal skin. Twenty-four hours later, the degree of the hypersensitivity reaction was...
measured by the following criteria:

Severity of redness
0: none
1: faint redness
2: clear redness
3: redness with edema or necrosis

Hemolytic complement activity in vitro: The measurement was carried out according to the method of Lachmann and Hobart (14). Briefly, $5 \times 10^8$/ml SRBC sensitized with hemolysin was added to the mixture of 5.5 ml of gelatin veronal buffer and 1.0 ml of guinea pig serum, adjusted CH$_50$ to 1 unit, and then incubated at 37°C for 1 hr. Complement was previously incubated at 37°C for 30 min with the test compounds of the required concentrations. The reaction was stopped by cooling in an ice water bath. The mixture was centrifuged, and the complement activity of the supernatant was assayed by a spectrophotometer at 540 nm.

Macrophage migration in vitro: The measurement was carried out according to the method of George and Vaughan (15). Twenty milliliters of liquid paraffin was injected intraperitoneally to the guinea pig. Four days later, the guinea pig was sacrificed, and the peritoneal exudate cells (PEC) were collected. PEC were washed twice with Hanks’ solution and suspended in 10% FCS Eagle’s MEM. The PEC suspension was packed into a capillary tube sealed at one end and centrifuged at 800 r.p.m. for 8 min. The cell pellet was mounted in a tissue chamber containing 10% FCS Eagle’s MEM with or without the test compounds. After the chamber was cultured at 37°C for 48 hr, the migration area was measured by using a microscope.

Statistical analysis: The data were analyzed by Student’s $t$-test.

Results

Effects on PCA in rats: SA96 inhibited the PCA reaction in a dose-dependent manner, and it produced 28.3% inhibition at 50 mg/kg ($\times 2$). D-Pc also inhibited the response, but not dose-dependently, and the maximum inhibition of 30.0% was observed at 20 mg/kg ($\times 2$) (Table 1).

Effects on RCA in rats: Percent increase in weight of the skin lesion was 48.7±4.5 in the control. SA96 given at 20 mg/kg ($\times 2$) significantly caused a maximum inhibition of 28.5% on the RCA-induced swelling. D-Pc also showed an inhibition of 23.2% on the swelling at 20 mg/kg ($\times 2$), but not significantly (Table 2).

Effects on RPA in rats: Percent increase of paw volume was 35.8±3.2 in the control. SA96 significantly showed inhibitions of 36.3% and 44.4% on the paw swelling at 10 mg/kg and at 50 mg/kg ($\times 2$), respectively, and the inhibition was not dose-related, while D-Pc showed only 18.2% inhibition at 50 mg/kg ($\times 2$) (Table 3).

Effects on DTH in guinea pigs: SA96 had no effect on the hypersensitivity induced by DNCB at doses of 10 to 50 mg/kg ($\times 2$), while D-Pc significantly showed an enhancement of 27.3% at 20 mg/kg ($\times 2$) (Table 4).

Effects on hemolytic complement activity in vitro: The hemolytic complement activity was apparently inhibited by SA96 at the

| Compounds | Dose (mg/kg) | Extravasated dye (μg/site) | Inhibition (%) |
|-----------|-------------|---------------------------|---------------|
| Control   |             | 18.0±1.8                  |               |
| SA96      | 10×2        | 20.0±4.2                  | -11.1         |
|           | 20×2        | 14.9±3.4                  | 17.2          |
|           | 50×2        | 12.9±1.4*                 | 28.3          |
| D-Pc      | 10×2        | 13.5±2.3                  | 25.0          |
|           | 20×2        | 12.6±1.5*                 | 30.0          |
|           | 50×2        | 16.4±2.8                  | 8.9           |

Each value represents the mean±S.E. of six to seven rats. Compounds were administered orally 0.5 and 18 hr before the challenge. *: Significantly different from the control at $P<0.05$. 

Table 1. Effects of SA96 and D-Pc on passive cutaneous anaphylaxis (PCA) in rats
The inhibitions of SA96 at 10^{-3} M and 10^{-2} M were 55% and 98%, respectively. D-Pc also inhibited the complement activity, and the inhibitions of D-Pc at 10^{-3} M and 10^{-2} M were 23% and 48%, respectively (Fig. 2).

| Compounds | Dose (mg/kg) | Edema intensity (% swelling) | Inhibition (%) |
|-----------|--------------|-----------------------------|---------------|
| Control   | —            | 48.7±4.5                    | —             |
| SA96      | 10×2         | 50.7±7.4                    | 4.1           |
|           | 20×2         | 34.8±3.4*                   | 28.5          |
|           | 50×2         | 37.7±2.6                    | 22.6          |
| D-Pc      | 10×2         | 42.7±3.1                    | 12.3          |
|           | 20×2         | 37.4±3.7                    | 23.2          |
|           | 50×2         | 44.2±3.5                    | 9.2           |

Each value represents the mean±S.E. of six to seven rats. Compounds were administered orally 0.5 and 18 hr before the antiserum treatment. *: Significantly different from the control at P<0.05.

| Compounds | Dose (mg/kg) | Increase in paw volume (%) | Inhibition (%) |
|-----------|--------------|-----------------------------|---------------|
| Control   | —            | 35.8±3.2                    | —             |
| SA96      | 10×2         | 22.8±2.2*                   | 36.3          |
|           | 20×2         | 25.9±3.3                    | 27.7          |
|           | 50×2         | 19.9±2.4*                   | 44.4          |
| D-Pc      | 10×2         | 31.0±3.3                    | 13.4          |
|           | 20×2         | 29.5±2.3                    | 17.6          |
|           | 50×2         | 29.3±1.8                    | 18.2          |

Each value represents the mean±S.E. of six rats. Compounds were administered orally 0.5 and 18 hr before the provocation. *: Significantly different from the control at P<0.05.

| Compounds | Dose (mg/kg) | Grade of redness | Augmentation (%) |
|-----------|--------------|------------------|-----------------|
| Control   | —            | 2.2±0.2          | —               |
| SA96      | 10×2         | 2.0±0.3          | — 9.1           |
|           | 20×2         | 2.0±0.3          | — 9.1           |
|           | 50×2         | 2.2±0.3          | 0.0             |
| D-Pc      | 10×2         | 2.0±0.4          | — 9.1           |
|           | 20×2         | 2.8±0.2*         | +27.3           |
|           | 50×2         | 2.0±0.4          | — 9.1           |

Each value represents the mean±S.E. of six guinea pigs. Compounds were administered subcutaneously 0.5 and 18 hr before the challenge. *: Significantly different from the control at P<0.05.

Table 4. Effects of SA96 and D-Pc on DNCB-induced delayed type hypersensitivity (DTH) reaction in guinea pigs.

Random migration of macrophages from the capillary tube was inhibited by SA96 at the final concentrations of 1×10^{-4} to 5×10^{-3} M in a concentration-related manner. The
Inhibitions of SA96 at 1 × 10^{-3} M and 5 × 10^{-3} M were 34% and 74%, respectively. D-Pc also inhibited the macrophage migration, and the inhibitions of D-Pc at 1 × 10^{-5} M and 5 × 10^{-5} M were 18% and 37%, respectively (Fig. 3).

**Discussion**

The present study showed that SA96 caused inhibitions against RCA and RPA reactions, which are classified as Type II and Type III allergic reactions, respectively, and that SA96 caused an inhibition of hemolytic complement activity in vitro. D-Pc also showed inhibitions on Type II and Type III reactions, but not significantly. The Arthus reaction, a Type III reaction, is accepted to involve the activation of complement (16). Since there was a correlation between the inhibitory effects of SA96 or D-Pc on the Type III reaction and the complement activity, the anti-allergic effect of SA96 may be due in part to the anti-complement activity. These results supported the view that the RPA reaction was a sensitive model for the assessment of anti-complement agents (12). On Type II and Type III reactions, dose-dependency of SA96 was not observed. With respect to this point, it was already reported that SA96 had an optimal dose in the efficacies on adjuvant arthritis in rats and PFC response in mice (4).

In addition to the Type II and Type III reactions, a Type I reaction, the PCA reaction, was inhibited by SA96. The mode of action on PCA by SA96 is unclear. However, it is not considered that SA96 shows its anti-PCA action by anti-mediator action since SA96 has no anti-histaminic action (17).

SA96 was shown to inhibit the macrophage migration in vitro. This activity was more potent than that of D-Pc. It is clear that macrophages participate in both host defense and expression of immune based chronic inflammatory diseases such as rheumatoid arthritis (18). The formation of immune complex has been also known to result in the activation of the complement system, and the generation of chemotactic stimuli such as C5a which attracts both granulocytes and macrophages to sites of inflammation (18). Therefore, it may be considered that the inhibitory effect of SA96 on macrophage migration is related to the anti-allergic effect and plays a partial role in its efficacy on adjuvant arthritis. In vivo efficacies of SA96 on macrophage migration as well as complement activity remain to be determined.

A difference of drug efficacy on a Type IV reaction, the DTH reaction, was observed; that is, SA96 had no influence on DTH, while D-Pc enhanced it. The reason why SA96 had no effect on this Type IV reaction is not clear. With respect to the effect on the DTH reaction, different authors with different antigens have obtained conflicting results. Tarayre and Laressergues (19) reported that D-Pc inhibited the DTH induced by picryl chloride, while Arrigoni-Martelli and Bramm (20) reported that D-Pc enhanced the DTH induced by *Bordetella pertussis*. SA96 appears to possess a different mechanism from
D-Pc on reactions involving cellular immunity such as the DTH model. The effect of SA96 on other DTH models remain to be determined.

In conclusion, SA96 was shown to have some different immunopharmacological properties on experimental allergic reactions as compared with those of D-Pc.

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