Antianginal Effect of RS-5773, a Diltiazem Congener, in the Methacholine-Induced Anginal Model in Rats

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ABSTRACT—The antianginal effect of RS-5773 ((2S,3S)-3-acetoxy-8-benzyl-2,3-dihydro-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-1,5-benzothiazepine-4-(5H)-one hydrochloride), a newly developed benzothiazepine derivative, was evaluated in an angina model rat. Close-coronary artery injections of methacholine in anesthetized rats evoked ischemic electrocardiographic (ECG) changes (S wave elevation of about 0.6 mV). The ECG changes produced by methacholine were reproducible for as long as 6 hr. Intravenous and intraduodenal administration of RS-5773, diltiazem or clentiazem produced dose-dependent suppressions of the ischemic ECG changes. RS-5773 exceeded the other two agents both in the maximum suppressive effect on S wave elevation and in the duration of action after intravenous administration. The antianginal potency expressed as AUC (area under the curve), i.e., the percent suppression of S wave elevation integrated over time, revealed that RS-5773 was 16 times and 7 times more potent than diltiazem and clentiazem, respectively. A similar order of potency difference was observed after intraduodenal administration, and RS-5773 sustained its effect for about 6 hr at 3 mg/kg. In addition, RS-5773 did not cause excessive hypotension or depression of atrioventricular conduction. These results suggest that RS-5773 has a preferable profile as an antianginal agent.

Keywords: Methacholine, Antianginal effect, Benzothiazepine derivative, RS-5773

Diltiazem (Fig. 1), which has negative chronotropic and vasodilator effects, has been widely used for the treatment of variant angina pectoris (1) because of its selective action on coronary arteries (2). One of the shortcomings of the agent lies in its rather short duration of action because variant angina pectoris generally occurs from late in the night to early in the morning (3). To overcome this shortcoming, we searched for diltiazem congeners with more potent and long-lasting effect than diltiazem and found RS-5773 ((2S,3S)-3-acetoxy-8-benzyl-2,3-dihydro-5-[2-(dimethylamino)ethyl]-2-(4-methoxyphenyl)-1,5-benzothiazepine-4-(5H)-one hydrochloride) (Fig. 1) (4). This agent has vaso-relaxant action that is more potent and more slow in the onset of action than diltiazem, whereas its cardiodepressant action is comparable to that of diltiazem. The vaso-relaxant action of RS-5773 is characterized by its resistance to wash-out in an in vitro study (5).

To evaluate the antianginal efficacy of a drug, we need an in vivo animal model. There are very few reports on an animal model of angina induced by coronary spasms in which the drug efficacy can be assessed. Uchida et al. succeeded in producing coronary artery spasm by reduction of coronary flow in the dog (6). However, this method seems unsuitable for evaluating drug efficacy because the incidence of coronary spasm induced is low and the severity of the spasm is uncontrollable. Sakurai reported a swine model using methacholine to induce coronary spasm (7). Although they successfully induced coronary spasm in 83% of the pigs used, the method required so much time and labor that it was unlikely to be a practical model. Cereda et al. tried to evaluate drug efficacy after oral administration of drugs (8) using Sakai's in vivo angina pectoris model rat (9). In their study, however, the ST-segment elevation fluctuated widely with the lapse of time, suggesting that the original model reported by Sakai et al. was not suitable for evaluating duration of the antianginal action.

In the present study, we first modified Sakai's rat model and made it possible to evaluate drug efficacy for as long as 6 hr. Using this model, we examined the suppressive effect of RS-5773 on the elevation of S wave in electro-
cardiogram (ECG). Diltiazem and its congener, clen-
tiazem (Fig. 1), were used as standard drugs.

MATERIALS AND METHODS

Animals
Male SD rats (Charles River Japan, Atsugi), weighing 250–350 g, were fasted overnight. The experiment was performed after the animals were anesthetized with 100 mg/kg, i.p. of thiobutabarbital. In order to keep anesthesia constant, additional thiobutabarbital was given, if necessary. Following tracheal incision, the trachea was intubated with a polyethylene catheter to maintain respiration.

Parameters measured
The left femoral artery was cannulated with a poly-
ethylene catheter filled with heparinized saline (50 units/ml), and the other end of the catheter was connected to a pressure transducer (TP-200T; Nihon Kohden, Tokyo) and an amplifier (AP-630G, Nihon Kohden) to measure mean blood pressure (MBP). ECG was recorded on a thermal pen-writing recorder (Recti-Horiz-8K23; NEC San-ei, Tokyo) by apex-base lead using a bioelectric amplifier (AT-620G, Nihon Kohden). Heart rate (HR) was measured by means of a tachometer (AT-601G, Nihon Kohden) triggered by the R wave of the ECG. MBP and HR were recorded on another thermal pen-writing recorder.

Method for close-coronary artery injection of methacho-
line
We modified the method originated by Sakai et al. (9) to induce coronary constriction more effectively. A specially made metal cannula, which was about 5.5-cm-long and 0.4 mm in internal diameter, was used for close-corona-
ary artery injection of methacholine. About 3 mm of both ends of the cannula were bent at an angle of about 30 degrees in the opposite direction to each other. One tip of the cannula was covered with a 2-mm-long polyethyl-
ene tube in order not to injure the aortic lumen. The metal cannula was inserted through the right common carotid artery, and its tip was placed near the ostium of the left coronary artery. After several trials of methacholine injection, the cannula was fixed at the best position for inducing maximum ischemic changes in ECG; i.e., elevation of S wave (Fig. 2, left).

Induction of coronary artery constriction
Starting from 1.0 μg, the dose of methacholine was
decreased to 0.2 pg in each rat to determine the dose that evokes about a 0.6 mV elevation of S wave (Fig. 2, right). When 1.0 pg of methacholine could not induce an S wave elevation of more than 0.4 mV, the rats were not used for experiments. The volume of methacholine solution was fixed at 10 μl. The methacholine solution was loaded in the polyethylene tube with a microsyringe and then flushed with 50 μl of heparinized saline (50 units/ml). The time needed to inject methacholine varied among the animals from 1 to 5 sec, but it was kept the same in the same animal.

Schedule for drug administration and methacholine injection
Close-coronary artery injections of methacholine were repeated at least 3 times with an interval of 20 min. After blood pressure was stabilized and constant elevations of S wave was obtained, a test drug was administered intravenously or intraduodenally.

Intravenous administration study
RS-5773, diltiazem and clentiazem were dissolved in a volume of less than 0.1 ml and administered with 0.3 ml of physiological saline in about 10 sec through a cannula inserted in the right femoral vein. The following doses were used: 0.1, 0.3 and 1 mg/kg for RS-5773; 1 and 3 mg/kg for diltiazem; and 0.3 and 1 mg/kg for clentiazem. Methacholine challenges were repeated at 20-min intervals, before as well as after drug administration. The suppression of S wave elevation was evaluated at 2, 22, 42, 62, 82, 102 and 122 min after i.v. administration of the drug. The control group received 0.4 ml of physiological saline intravenously.

Intraduodenal administration study
A polyethylene cannula was inserted in the intestinal tract through a small incision on the surface of the duodenum. Using this cannula, RS-5773 (1 and 3 mg/kg), diltiazem (10 and 30 mg/kg) or clentiazem (1, 3 and 10 mg/kg) was intraduodenally administered, and their suppressive effect on the S wave elevation were evaluated. The volume of drug solutions was fixed at 0.1 ml, and the drug was flushed with 0.3 ml of physiological saline. Methacholine challenges were repeated at 10 and 20 min after drug administration and thereafter at 20-min intervals up to 6 hr after administration at the longest. The control group received 0.4 ml of physiological saline intraduodenally.

Drugs and the method for dissolution
RS-5773, diltiazem, clentiazem and thiobutabarbital were synthesized at the Sankyo Research Laboratories. Methacholine hydrochloride was purchased from Sigma Chemical Co. (St. Louis, MO, USA). RS-5773 was dissolved in deionized water. Diltiazem, clentiazem and methacholine were dissolved in physiological saline.

Statistics
All reported values represent the mean ± S.E. As there was no significant difference among the baseline values of
each group, the statistical analyses were carried out with the percentage values. After confirming that the variances of data in each group are homogeneous, the analysis of variance (ANOVA) and the multiple comparison by Dunnett's test or Student t-test at 5% P-value were performed.

RESULTS

Angina model

Although we failed to find a suitable position of the cannula to evoke appropriate S wave elevation in about 30% of the rats used, stable and reproducible elevations of the S wave were successfully induced by methacholine injection in the other rats. In about 70% of these successful rats, the S wave elevations were not accompanied by any heart rate change, whereas a transient sinus bradycardia was observed in the other rats.

Influence of methacholine on various parameters

The methacholine injection lowered blood pressure transiently. In most rats, blood pressure decreased immediately after the injection (about 30–50 mmHg), followed by a gradual restoration. The magnitude of methacholine induced hypotension was attenuated when blood pressure was lowered by the Ca antagonists. Therefore, blood pressure never dropped below 50 mmHg even after methacholine injection. In rats in which transient bradycardia was observed or relatively high doses of methacholine was used, the decrease of blood pressure showed a triphasic pattern: a sharp decrease and a slight restoration followed by another decrease. There was almost no change in heart rate, but a few rats had transient sinus bradycardia. Elevations of the S and T waves, as shown in Fig. 2, appeared about 30 to 60 sec after induction of hypotension. At this time, blood pressure returned to near control levels. These ECG changes

![Fig. 3. Time course for hemodynamic and electrocardiographic changes induced by close-coronary artery injections of methacholine in eight anesthetized rats. MBP, HR and S wave represent the mean blood pressure, heart rate and S wave level of the electrocardiogram just before the methacholine injection, respectively. S and T wave elevations represent the maximal changes of the electrocardiogram in S and T waves induced by the methacholine injection, respectively. Each point represents the mean±S.E. There were no significant differences from baseline values.](image-url)
subsided within 2 min.

**Intravenous administration**

Mean blood pressure, heart rate and elevations of the S and T waves during repeated methacholine challenges were observed for 2 hr in 8 rats receiving physiological saline as the control (Fig. 3). Baseline values of MBP, HR and S wave elevation in the control group were 117±6 mmHg, 378±9 beats/min and 0.61±0.04 mV, respectively. There were no statistically significant differences in the baseline values of these parameters among the control group, RS-5773, diltiazem and clentiazem groups. MBP tended to decrease slightly, while HR and elevation of the S wave hardly changed up to 2 hr. The variations of the T wave elevation among the rats were greater than that of the S wave, and the T wave elevation varied with time even in the same rat. We, therefore, chose S wave elevation as an indicator of myocardial ischemia. Figure 4 shows typical ECG changes, recorded when 1.0 mg/kg of RS-5773 was intravenously administered. Although the intravenous administration of RS-5773 did not affect ECG directly, it suppressed the elevations of both S and T waves completely, and the effect lasted for 2 hr after administration.

Figure 5 summarizes MBP, HR and elevation of the S wave in 7 rats receiving RS-5773. At doses of 0.1, 0.3 and 1 mg/kg, RS-5773 decreased MBP in a dose-dependent manner. A significant decrease in MBP was observed even at 2 hr after 1.0 mg/kg of the agent. HR also decreased in a dose-related manner. The elevation of the S wave was significantly suppressed even at 0.1 mg/kg of RS-5773. Each dose exhibited maximum suppression at 2 min, and

*Fig. 4. A typical example of the effect of RS-5773 on ischemic electrocardiographic changes induced by methacholine injection.*

*Fig. 5. Effects of intravenous administration of RS-5773 on mean blood pressure (MBP), heart rate (HR) and S wave elevation in anesthetized rats. S wave elevations were induced by methacholine challenges with an interval of 20 min. Open circles indicate the control group (N=8). Closed circles, open squares and closed squares indicate the 0.1, 0.3 and 1 mg/kg RS-5773 groups (N=7), respectively. Each point represents the mean±S.E. *: Significantly different from the control group with P<0.05.*
the duration of these effects were dose-dependent. The suppressive effect by 1.0 mg/kg of RS-5773 lasted more than 2 hr.

At doses of 1 and 3 mg/kg, diltiazem markedly decreased MBP immediately after administration, but MBP returned near the pre-administration values 22 min after administration (Fig. 6). HR decreased in a dose-dependent manner, and a significant difference was noted even at 22 min after administration. In the 3.0 mg/kg group, a transient atrioventricular conduction block was observed in 3 out of 7 rats, and this disappeared within 2 min. The elevation of the S wave was suppressed at 2 min after administration by 51% and 64%, at low and high doses, respectively. The parameter returned to the pre-administration values at 42 min after administration in the 1.0 mg/kg group and at 62 min in the 3.0 mg/kg group.

Figure 7 summarizes the effects of 0.3 and 1 mg/kg of clentiazem. MBP decreased, and the duration of hypotensive effect was a little shorter than that of RS-5773. Heart rate tended to decrease. Although the elevation of the S wave was suppressed in a dose-dependent manner, the maximum suppressions were only 41% and 58%, and the suppression subsided 22 and 62 min after administration at low and high doses, respectively.

The antianginal potency of the three drugs were evaluated by using area under the curve (AUC) values for suppression of S wave elevation in the intravenous administration experiment. As an AUC value is the percent suppression of S wave elevation over time, it enables us to quantify the antianginal potency of a drug. Table 1 shows the mean AUC values with S.E. for RS-5773, diltiazem and clentiazem. Comparison of drugs at a 30% · hr AUC level revealed that RS-5773 was 16 times and 7 times more potent than diltiazem and clentiazem, respectively.
Table 1. The AUC values of suppression of S wave elevation for RS-5773, diltiazem and clentiazem in the intravenous administration study

|         | 0.1 mg/kg | 0.3 mg/kg | 1.0 mg/kg | 3.0 mg/kg |
|---------|-----------|-----------|-----------|-----------|
| RS-5773 | 18±5      | 59±18     | 109±14    |           |
| Clentiazem | 14±4      | 31±6      |           |           |
| Diltiazem | 20±3      | 35±8      |           |           |

An AUC value (%·hr) is the percent suppression of S wave elevation over the time. The entries are means ±S.E. of seven experiments for RS-5773, diltiazem or clentiazem.

Intraduodenal administration study

Figure 8 summarizes the effect of RS-5773 on MBP, HR and elevation of the S wave. In the control group, MBP gradually decreased about 10% at 6 hr after administration of saline, while the HR and S wave did not change. Baseline values of MBP, HR and S wave elevation in the control group were 117±3 mmHg, 379±9 beats/min and 0.64±0.04 mV, respectively (N=12). There were no statistically significant differences in the baseline values of these parameters between the control group and the RS-5773 group. In the RS-5773 group, MBP decreased by 13% and 25%, and the effects lasted 40 min and 3 hr after 1 and 3 mg/kg, respectively. The HR did not change throughout the 6-hr observation period. The suppression of S wave elevation was dose-related, and the maximum effects were 50% after 1 mg/kg and 90% after 3 mg/kg. S wave elevation returned to the pre-administration level 3 and 6 hr after 1 and 3 mg/kg, respectively.

Following diltiazem, the maximum hypotensions were observed at 20 min and MBP returned to the pre-administration value within 1 and 2 hr in the 10 and 30 mg/kg groups, respectively (Fig. 9). HR also maximally decreased 20 min after administration. The agent suppressed S wave elevation at 10 min after administration,
and the maximum suppression was observed at 20 min for 10 mg/kg and 10 min for 30 mg/kg. However, the duration of the action was very short: S wave elevation returned to the pre-administration value in less than 2 hr.

Clentiazem decreased MBP in a dose-dependent manner, and the durations of hypotensive action were 2, 4 and more than 6 hr for 1, 3 and 10 mg/kg, respectively (Fig. 10). The heart rate hardly altered at 1 and 3 mg/kg, but it decreased with time at 10 mg/kg. No significant suppression of S wave elevation was noted at 1 mg/kg, whereas 3 mg/kg of clentiazem produced a 63% suppression 20 min after administration, and the suppression lasted for about 3 hr. Clentiazem at 10 mg/kg suppressed the S wave for 5 hr, but the maximum effect was only 68%, which was almost the same level as that of 3 mg/kg.

Table 2 shows AUC values for the suppression of S wave elevation in the intraduodenal administration study. When the three drugs were compared at the 120% hr AUC level, RS-5773 exhibited 20 and 3 times more potent antianginal efficacy than diltiazem and clentiazem, respectively.

**DISCUSSION**

It is generally accepted that variant angina pectoris is caused by coronary spasm (10), but its mechanisms are still not clear (11, 12). Recently it has been reported that acetylcholine, which was once believed to be a coronary dilator, evoked coronary artery constriction in patients with variant angina pectoris, and the involvement of muscarinic receptors has been strongly suggested (13-16). On the other hand, Sakai demonstrated that methacholine, which is a more potent muscarinic agonist than acetylcholine, constricted the coronary artery of rats in an isolated donor-perfused heart preparation (17). They also succeeded in inducing ischemic ECG changes by injecting methacholine close to the coronary artery in anesthetized rats (9). These ECG changes, particularly elevations of the S and T waves, resembled the ST segment elevation in patients with variant angina pectoris. In addition, Ca antagonists, one category of popular medications for variant angina pectoris, were effective in this model, whereas dipyridamole, which is clinically ineffective, was not effective (9). The failure of dipyridamole in suppressing ST elevation in animal experiments as well as in clinical settings is attributed to its lack of vasodilator action on large coronary arteries. These facts support the notion that this model is suitable for human variant angina pectoris and that close coronary injection of methacholine induces constriction of large coronary arteries.

We modified Sakai’s model to obtain more stable and reproducible inductions of ischemic ECG change. We reduced the dose of methacholine, from 4-8 μg in the Sakai model to 0.2-1.0 μg. In spite of this reduction, we could induce marked ischemic change in ECG (elevation of the S and T waves). This seems to be due to the improvement of the cannula, which enabled us to perform more selective injection of methacholine into the left coronary artery. With this slight modification, we succeeded
in decreasing the incidence of transient sinus bradycardia, which was probably evoked by methacholine misled into the right coronary artery, hence to the coronary sinus. Although sinus bradycardia is inevitable in Sakai's method, we could limit it to about 30% of the rats in which coronary constriction was successfully induced. The transient hypotension was also lessened to 30–50 mmHg, which was almost less than half of that observed in Sakai's method. These improvements would contribute to the stability of the rat as well as to the reproducibility of the spasm induced. In addition, we used thiobutabarbital instead of pentobarbital as an anesthetic, which enabled us to maintain a more long-lasting stable condition.

The coronary constriction induced by methacholine evoked myocardial ischemia which was reflected in the elevations of the S and T waves. Although Sakai et al. used both S and T waves for the evaluation of a drug, we used only the elevation of the S wave as an index of ischemia, because individual variations among rats and time related changes were much less in the S wave than in the T wave. We selected the dose of methacholine so that the agent evokes an S wave elevation of around 0.6 mV for the following reasons: 1) The S wave elevation of 0.2 mV as used in the Sakai's study was too small to evaluate the anti-ischemic effect of an agent. 2) Methacholine that evokes an S wave elevation of more than 0.6 mV often produces severe sinus bradycardia.

Intravenous administration of RS-5773, diltiazem or clentiazem produced maximum suppression of S wave elevation immediately after the administration. However, there was a great difference among the three agents not only in the maximum suppression but in the duration of action. To express the difference in the potency among these agents, we calculated the AUC values, i.e., percent suppression of S wave elevation integrated over time. The calculated AUC values revealed that RS-5773 was 16 and 7 times more potent than diltiazem and clentiazem, respectively. On the other hand RS-5773 was almost the same as diltiazem and clentiazem in the potency for inducing hypotension and bradycardia. In addition, diltiazem produced an AV-block in 3 out of 7 rats at this dose, whereas RS-5773 and clentiazem never did. These results support the results of previous studies (5) that RS-5773 showed the greatest antianginal effect while it had the least hypotensive and negative chronotropic actions among the three drugs. From these observations, it is unlikely that the reduction of cardiac load is a primary reason. The second possibility is that the vasodilator effect of the three agents prevented the coronary vasoconstriction induced by methacholine injection. However, in view of the fact that dipyridamole, the selective dilator of coronary arterioles, failed to suppress methacholine induced S wave elevation both in Sakai's study (9) and in our study (data not shown), it is conceivable that diltiazem and its congeners antagonize vaso-constriction induced by methacholine in relatively large coronary arteries.

Provided that methacholine-induced coronary spasms represent variant angina pectoris in humans, RS-5773 seems to be a promising agent for the prevention or treatment of this type of anginal attack.

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