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# EFFECTS *IN VIVO* OF α-[*p*-(FLUOREN-9-YLIDENEMETHYL)PHENYL]-2-PIPERIDINEETHANOL (RMI 10,393) ON PLATELET AGGREGATION AND BLOOD COAGULATION

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**Abstract**—α-[*p*-(Fluoren-9-ylidenemethyl)phenyl]-2-piperidineethanol (RMI 10,393), previously found to inhibit platelet aggregation induced by ADP, thrombin, collagen, serotonin and epinephrine *in vitro*, was given orally to guinea pigs. The compound inhibited platelet aggregation induced by ADP infusion into a jugular vein of the guinea pig. In another procedure, in which blood was removed from the guinea pigs given RMI 10,393 orally, ADP-induced platelet aggregation *in vitro* was also inhibited, though larger doses were required for a significant inhibitory effect. Whole blood clotting time and bleeding time were not affected in the rat, but oral administration of 30 mg/kg for 4 days did produce a hemorrhagic tendency in dicumarolized rats. In general, effects on platelets and blood coagulation previously seen *in vitro* were also found in these experiments *in vivo*, but at much lower plasma and platelet concentrations of RMI 10,393. The results suggest that it may be possible to inhibit platelet aggregation *in vivo* without marked interference with hemostasis, provided the fibrin clotting system is intact.

THE COMPOUND, α[*p*-(fluoren-9-ylidenemethyl)phenyl]-2-piperidineethanol (RMI 10,393), was previously reported to have inhibitory activity against aggregation of human platelets *in vitro*.[1] Inhibition was found against aggregation induced by ADP, thrombin, collagen, epinephrine and serotonin; clot retraction was also inhibited. RMI 10,393 produced minimal activation of platelet factor 3 at plasma concentrations which altered platelet aggregation. A platelet-derived procoagulant effect was demonstrated in attenuated thrombin clotting systems, but not in the standard thrombin time test; this may have reflected slight activation of platelet factor 2.

This paper reports effects of RMI 10,393 on clotting and platelet parameters in the whole animal, specifically, effects on whole blood clotting time and bleeding time in the rat, and platelet aggregation and thrombin time in the guinea pig.

## MATERIALS AND METHODS

RMI 10,393 was used as the glycolate salt in all studies except where indicated Other materials used were: silicone, G.E. Dri-Film SC-87, 10% solution in toluene;· capillary tubes, 0·8 to 1·1 mm o.d., Scientific Products No. B4195; scalpel blades, size 10; dicumarol, Abbott Laboratories, North Chicago, Ill.; phenylbutazone, Geigy Pharmaceuticals, Ardsley, N.Y.; simplastin, Warner-Chilcott Laboratories, Morris Plains, N.J.; thrombin, topical, Parke-Davis & Company, Detroit, Mich. (purified, courtesy of Dr. L. McCoy, Wayne State University, Detroit, Mich.); adenosine diphosphate (ADP), trisodium salt, Cyclo Chemical Corp., Los Angeles, Calif.; cell

counter, Coulter counter, Model B, 70 $\mu$ aperture, thresholds at 5 and 50 for platelet count. The rats used were Sprague–Dawley derived, male, 150–200 g, from Charles River, North Wilmington, Mass. The guinea pigs were Hartley All White, male 550–700 g, from Camm Research, Wayne, N.J.

*Whole blood clotting time.* Whole blood clotting time was determined by a modified capillary tube method[2] in the rat. The capillary tubes were siliconized with G.E. Dri-Film. The rat was placed in a restraining cage with its tail protruding. The tail was dipped in a beaker of warm water and wiped clean and dry with a piece of cheesecloth. One of the veins was then pinched between the thumb and forefinger to block blood flow about two-thirds of the way from the base to the tip. The vein was sliced cleanly with a scalpel blade. The first drop of blood was taken into a siliconized capillary tube by mouth aspiration through a piece of polyethylene tubing. The capillary tube was checked every 30 sec for fluidity of the blood. When the blood no longer would flow due to gravity, the tube was broken at 30-sec intervals until a fibrin strand was seen between the broken ends. Second and third determinations were made by rewashing and clamping the tail vein at the site of the previous slice and slicing about 1 cm toward the tip.

*Bleeding time.* The rats were prepared as described for the whole blood clotting time. The cut was made and the primary and secondary bleeding times were determined by the immersion method of Adelson and Crosby.[3]

*Thrombin time in the guinea pig.* Thrombin time was determined by the method of Poplawski and Niewiarowski.[4] Blood was collected into 3·5% disodium citrate, 1 part to 9 parts of blood, and PRP was isolated by centrifugation at 100 *g* for 10 min. PPP was isolated by recentrifuging the residual blood at 1500 *g* for 20 min.

*Activity* in vivo *in the guinea-pig as determined by the effect of ADP infusion.* RMI 10,393 was dissolved in water and given by stomach tube to guinea-pigs for 1 (single dose) or 5 days. The effect of ADP infusion on platelet count was determined 2 hr after completing the dosage schedule. The guinea pigs were fasted overnight for the single dose study and also for the last dose in multiple dose studies. The procedure as described by MacKenzie *et al.*[5] consists of infusion of ADP (0·2 mg/kg) for 1 min into the right jugular vein and removal of blood samples for the determination of platelet concentration from the right carotid artery at 3-min intervals. Platelet concentration is reduced through formation of aggregates, which are trapped in the lung capillaries. As these aggregates disperse (deaggregate), the single platelets reappear in the circulation and are counted.

*Activity* in vivo *in the guinea-pig as determined by ADP-induced platelet aggregation* in vitro. The compound was given to guinea-pigs by stomach tube as described in the procedure *in vivo* (above) for 1 or 4 days. Blood was removed by heart puncture 2 hr after the last dose and citrated PRP was isolated. The platelet count for each sample from both test and control groups was adjusted to 400,000/mm$^3$ by mixing appropriate quantities of PRP and PPP from the same blood sample. One-half ml of each adjusted PRP sample was placed in an aggregometer tube and ADP-induced aggregation measured at 37° in a platelet aggregometer as described by Mustard *et al.*[6] and by MacKenzie *et al.*[1] ADP was added to 0·8 $\mu$g/ml of PRP. This quantity produces a high incidence of secondary phase aggregation, usually with a good inflection between first and second phases. The area between the aggregation curve and baseline transmittance was measured for 5 min after ADP addition, using a planimeter. The average

area of the control samples was compared with the average area of the test samples and the per cent inhibition was calculated:

$$\frac{\text{Average control area} - \text{average treatment area}}{\text{Average control area}} \times 100 = \text{per cent inhibition.}$$

*Determination of RMI* 10,393 *concentrations in guinea-pig plasma.* The concentrations of the compound in citrated plasma, PRP and PPP were determined by the method of Lang.* This consists of extraction of the compound from basic solution into ether, re-extraction back into aqueous solution with sulfuric acid solution, oxidation with $KMnO_4$ in basic aqueous solution, and extraction of the resulting fluorenone into heptane for quantitation at 260 mμ in a spectrophotometer (Beckman model DB).

## RESULTS

*Activity* in vivo *in the guinea-pig as determined by the effect of ADP infusion.* The effect of ADP infusion on platelet concentration with time is shown in Fig 1. Figure 1 also reports the response obtained with RMI 10,393 when given orally to guinea-pigs at 30, 100 and 300 mg/kg as a single dose 2 hr prior to ADP infusion. The initial drop in platelet count was not significantly inhibited by single doses. The greatest effect of RMI 10,393 on platelet concentration was on the reappearance rate of platelets into the circulation (deaggregation).

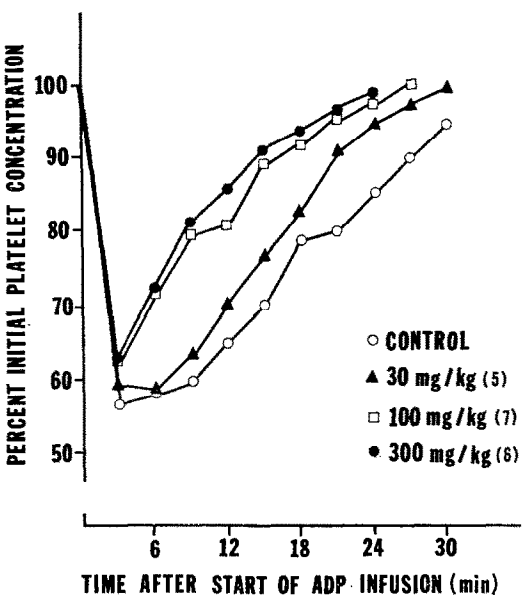


FIG. 1. Effect of RMI 10,393 on platelet aggregation in the guinea-pig. ADP, 0·2 mg/kg, was infused over 1 min, 2 hr after a single oral dose of compound. Number in parentheses is the number of determinations. There were 23 control animals. Initial platelet counts varied from 400,000 to 1,240,000/mm³ from different animals.

* J. F. Lang, private communication.

TABLE 1. EFFECT OF RMI 10,393 ON PLATELET AGGREGATION *in vivo* IN THE ADP INFUSION SYSTEM*

| Treatment (mg/kg) | Single dose | | | | | Multiple dose | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | No. of guinea-pigs | Initial response† | P | Total response‡ | P | No. of guinea-pigs | Initial response† | P | Total response‡ | P |
| 0 | | | | | | 6 | 43 ± 7 | | 299 ± 48 | |
| 10 | | | | | | 6 | 40 ± 9 | >0·05 | 186 ± 49 | >0·05 |
| 0 | 6 | 55 ± 4 | | 273 ± 48 | | 6 | 52 ± 4 | | 266 ± 18 | |
| 30 | 5 | 54 ± 2 | >0·05 | 209 ± 26 | >0·05 | 6 | 34 ± 3 | < 0·01 | 96 ± 24 | < 0·01 |
| 0 | 8 | 55 ± 3 | | 256 ± 35 | | 1 | 50 | | 280 | |
| 100 | 7 | 64 ± 6 | >0·05 | 137 ± 30 | < 0·05 | 1 | 30 | | 96 | |
| 0 | 9 | 52 ± 2 | | 277 ± 22 | | | | | | |
| 300 | 6 | 63 ± 2 | < 0·01 | 123 ± 16 | < 0·01 | | | | | |

* Values are expressed ± S.E.M.; P values were determined by Student's *t*-test.

† Per cent initial drop in platelet count, determined at 3 min after starting ADP infusion.

‡ Area between platelet count curves and baseline (100% in Fig. 1) for 30 min after start of ADP infusion; values calculated by trapezoid method [$\Sigma_{0-30}$(% change in platelet count)].

After daily administration of RMI 10,393 at doses of 10, 30 and 100 mg/kg for 5 days, ADP was infused 2 hr after the last dose. When subjected to pentobarbital anesthesia and ADP infusion, guinea-pigs receiving 100 mg/kg in this regimen exhibited erratic blood flow, so that blood sampling was inadequate in all but one animal. This effect is probably due to the sum of effects from anesthetic, RMI 10,393 and ADP. Data for this single animal are reported. The results of these experiments and the single dose experiments are reported in Table 1.

A single dose of 30 mg/kg produced only a slight, statistically insignificant change in total response to ADP. At 100 and 300 mg/kg, however, marked significant effects on platelet aggregation were found with single doses. In contrast to the single dose results, multiple dosing (5 days) considerably increased the anti-aggregation effect, with 10 and 30 mg/kg producing significant reductions in total response to ADP. A comparison of the effect of RMI 10,393 in a single dose and in a 5-day dose regimen is graphically presented in Fig. 2.

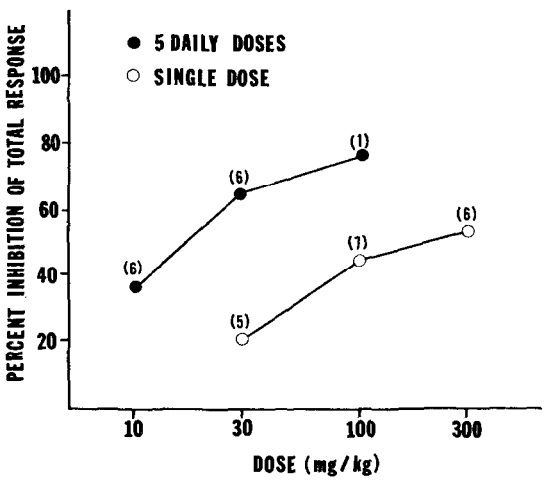


FIG. 2. Effect of RMI 10,393 on platelet aggregation in the guinea-pig. Dose response of a single dose and five daily doses; per cent inhibition of total aggregation response vs. dose. Number in parentheses is the number of determinations.

*Activity* in vivo *in the guinea-pig as determined by ADP-induced platelet aggregation* in vitro. The results of these experiments are shown in Fig. 3. In this test system, RMI 10,393 did not show any inhibitory effect at 30 mg/kg, but did have an effect at 100 mg/kg in both single and 4-day dose regimens. Though the multiple dose regimen did show slightly greater effectiveness than a single dose, the difference was not as great as in the ADP infusion system *in vivo*. There was complete inhibition of platelet aggregation when 300 mg/kg was given for 4 days.

*RMI* 10,393 *levels in guinea-pig plasma.* The concentrations of RMI 10,393 in platelet-rich and platelet-poor plasma as a function of dose are given in Fig. 4A.* Concentrations associated with the platelets are shown in Fig. 4B.* These observations demonstrated that concentrations in plasma which reduced or reversed platelet

* These data were obtained in animals treated at the same time as those described in the ADP infusion experiments; blood was taken at 2 hr, the same time that ADP infusion was started.

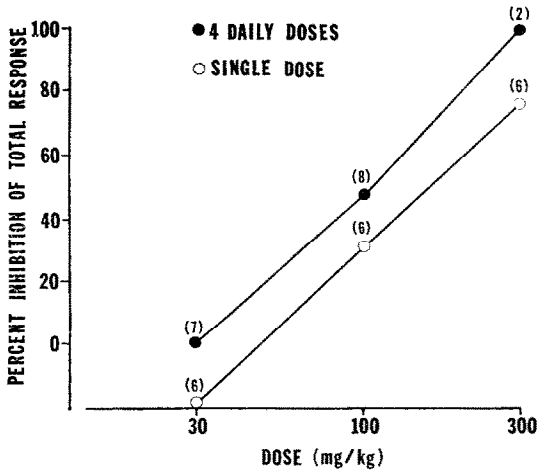


FIG. 3. Effect of RMI 10,393 dose on ADP aggregability of recipients' platelets determined *in vitro* Blood samples were obtained 2 hr after the last oral dose to guinea-pigs. ADP, 0·8 μg/ml of PRP, was added *in vitro*. Per cent inhibition of total response vs. dose. Number in parentheses is the number of determinations.

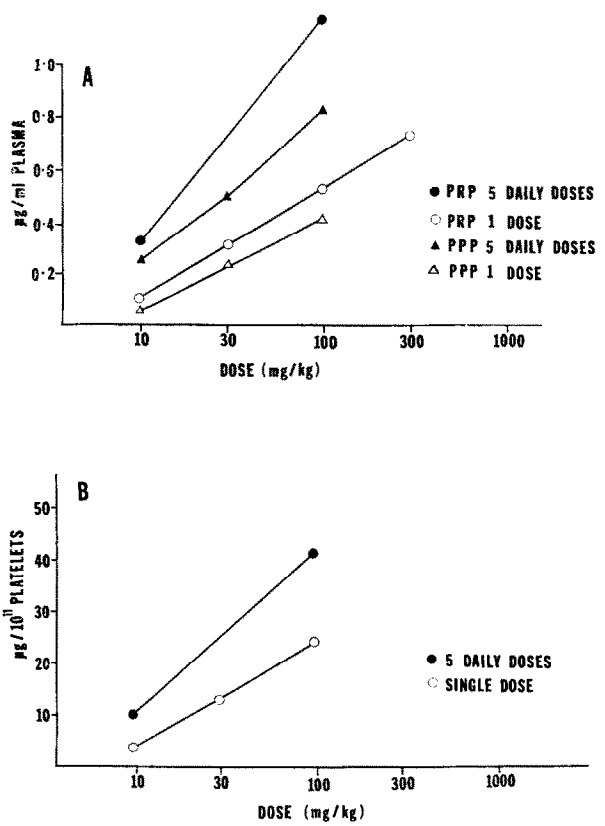


FIG. 4. Concentration of RMI 10,393 at 2 hr after oral administration in guinea-pig plasma (A) and guinea-pig platelets (B). Average values of four determinations.

aggregation after administration *in vivo* are much lower than concentrations required to be added *in vitro* to guinea-pig or human plasma to produce similar effects.[1]

In order to determine whether concentrations at the effective site (i.e. the platelet) were similar in the two experimental situations, an experiment was performed in which RMI 10,393 was added to human citrated PRP at 50 μg/ml (an effective anti-aggregation concentration[1]). $^{131}$I-labelled human serum albumin was also added as a tracer for the determination of plasma remaining with the platelet pellet. Platelet pellets from 1 ml PRP were isolated by centrifugation either immediately or after incubation at 37° for various intervals before centrifugation. Plasma was discarded and the platelet pellet washed with isotonic saline three times. The pooled washings and platelet pellets were analyzed for RMI 10,393 and radioactivity. The results are shown in Table 2. The saline washes from the platelets contained similar fractions of added RMI 10,393 and $^{131}$I-albumin, indicating that the washes simply removed adhering plasma from the platelets and did not leach out the compound or precipitate it. Since the concentration of RMI 10,393 in both plasma and platelets in this experiment *in vitro* (Table 2) was about 50 times the concentration (see Fig. 4) required for anti-aggregation effect in the ADP infusion experiment *in vivo* (25 vs. 1300 μg/$10^{11}$ platelets and 0·8 vs. 50 μg/ml of plasma), the difference between effective plasma concentrations of RMI 10,393 *in vivo* and *in vitro* cannot be explained by similar platelet concentrations.

*Effect of RMI* 10,393 *on thrombin time in the guinea-pig.* In a previous paper,[1] it was reported that a sensitized thrombin time was shortened in the presence of RMI 10,393. This was considered to be due to platelet procoagulant activation (platelet factors 2 and 3). In order to investigate whether such an effect can be found when the compound is given *in vivo* at levels that inhibit platelet aggregation, RMI 10,393 was given by stomach tube to guinea-pigs at 30 and 100 mg/kg for 5 days and thrombin time was determined. The results are shown in Table 3. A slight but significant shortening in thrombin time was found.

*Effect of RMI* 10,393 *on whole blood clotting time and bleeding time in the rat in the presence and absence of dicumarol.* Rats were given RMI 10,393, as the fumarate salt, or phenylbutazone at 30 mg/kg, with or without dicumarol at 9 mg/kg by stomach

TABLE 2. CONCENTRATION OF RMI 10,393 ON PLATELETS WHEN ADDED TO HUMAN CITRATED PRP AT 50 μg/ml

| Group incubation time (min) | Platelet concn (average total μg) | Concn of RMI 10,393* (μg/$10^{11}$ platelets) |
|---|---|---|
| 0 | 8·24† | 1030 |
| 10 | 9·5 | 1187 |
| 20 | 10·5 | 1312 |
| 60 | 10·8 | 1450 |
| 120 | 10·4 | 1300 |

* Platelet count was 820,000,000/ml PRP. The washes contained an average of 2·6% of the added RMI 10,393 and 3·2% of added $^{131}$I. Platelet $^{131}$I = 0·12% of total amount added.

† Average of duplicate samples.

TABLE 3. EFFECT OF ORALLY ADMINISTERED RMI 10,393 ON THROMBIN TIME IN THE GUINEA-PIG

| | Thrombin time (sec)† | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Topical thrombin | | | | Purified thrombin | | | |
| Treatment | 1 U/ml | P value | 0·1 U/ml | P value | 1 U/ml | P value | 0·1 U/ml | P value |
| Control | 21 ± 0·7 | | 57 ± 2·6 | | 21 ± 1·1 | | 66 ± 4·1 | |
| RMI 10,393 (30 mg/kg)‡ | 17 ± 0·7 | < 0·01 | 46 ± 1·3 | < 0·05 | 17 ± 0·6 | < 0·01 | 53 ± 1·7 | < 0·05 |
| RMI 10,393 (100 mg/kg) | 18 ± 0·7 | < 0·01 | 51 ± 0·3 | >0·05 | 18 ± 0·3 | < 0·02 | 58 ± 1·6 | >0·05 |

* Six animals per group.
† Values are expressed ± S.E.M.; P values were determined by Student's *t*-test.
‡ Compound given for 5 days.

tube. The dicumarol was given along with the other compounds in a 4-day study, or was given for 4 days and the other compounds were given as a single dose on the fourth day. Groups of six rats each were designated as control, dicumarol, test compound plus dicumarol, and test compound groups. Whole blood clotting time and bleeding time were determined for each group. The results are reported in Table 4.

TABLE 4. EFFECT ON WHOLE BLOOD CLOTTING TIME AND BLEEDING TIME IN THE RAT*

| Treatment | Dose (mg/kg) | Whole blood clotting time (sec) 0 hr | 2 hr | 5 hr | Bleeding time (sec) 2 hr |
|---|---|---|---|---|---|
| Control | 0 | 530 ± 16 | 575 ± 24 | 585 ± 23 | 85 ± 7 |
| Dicumarol | 9 | 2225 ± 215 | 2290 ± 170 | 2105 ± 124 | 110 ± 6 |
| Dicumarol + RMI 10,393 | 9 + 30 | 2025 ± 89 | 1070 ± 41 | 1110 ± 50 | 150 ± 13† |
| RMI 10,393 | 30 | 625 ± 27 | 685 ± 74 | 700 ± 30 | 92 ± 4 |
| Control | 0 | 440 ± 20 | 465 ± 8 | 475 ± 16 | 101 ± 10 |
| Dicumarol | 9 | 1920 ± 143 | 2035 ± 101 | 1825 ± 87 | 138 ± 8 |
| Dicumarol + RMI 10,393 | 9 + 30 × 4 | 2725 ± 517 | ‡ | ‡ | 178 ± 27† |
| RMI 10,393 | 30 × 4 | 485 ± 19 | 405 ± 17 | 495 ± 22 | 105 ± 14 |
| Control | 0 | 510 ± 8 | 500 ± 24 | 455 ± 18 | 91 ± 6 |
| Dicumarol | 9 | 1930 ± 53 | 1920 ± 57 | 1850 ± 55 | 151 ± 17 |
| Dicumarol + phenylbutazone | 9 + 30 | 2030 ± 105 | 1360 ± 234 | 1165 ± 149 | 185 ± 23† |
| Phenylbutazone | 30 | 560 ± 13 | 360 ± 29 | 300 ± 19 | 100 ± 6 |
| Control | 0 | 495 ± 20 | 480 ± 19 | 495 ± 14 | 85 ± 5 |
| Dicumarol | 9 | 1610 ± 141 | 1630 ± 125 | 1555 ± 112 | 150 ± 12 |
| Dicumarol + phenylbutazone | 9 + 30 × 4 | 1600 ± 118 | 1505 ± 56 | 1430 ± 124 | 168 ± 20† |
| Phenylbutazone | 30 × 4 | 410 ± 32 | 490 ± 34 | 475 ± 26 | 90 ± 3 |

* Values are expressed ± S.E.M.
† Compared to dicumarol group, P value >0·05 by Student's *t*-test.
‡ No blood samples could be obtained due to loss of blood.

Both RMI 10,393 and phenylbutazone caused a shortening of clotting time in dicumarolized rats in the single dose study; there was no effect in the animals not treated with dicumarol. Phenylbutazone was tested because Hovig *et al.*[7] used it in dogs that were either deficient in Factor IX or dicumarolized, and reported increased bleeding tendency. In the rat, no such effect was found. In the multiple dose study, phenylbutazone still showed no effect on bleeding time and a diminished effect on clotting time in the dicumarolized animals. RMI 10,393 showed a longer clotting time on the first determination on day 4; at 2 and 5 hr after the last dose, no blood could be obtained due to hemorrhage occurring at the site of the previous incision. This indicated that possibly the secondary bleeding time would be prolonged. When such a determination was made, the secondary bleeding time was more prolonged with RMI 10,393 in dicumarolized rats (178 ± 37 vs. 129 ± 31 sec), although the change was not statistically significant (P > 0·05).

## DISCUSSION

Thomas *et al.*[8] reported that guinea-pig platelets are similar to human platelets in their responsiveness to ADP-induced aggregation. In an experimental survey of common laboratory species, we also came to similar conclusions. Consequently, the guinea-pig was chosen for our investigations.

Two procedures have been used for determining the effects *in vivo* of RMI 10,393 in the guinea-pig. In the first,[5] ADP was infused into control and treated animals, and the circulating platelet count was determined at intervals after starting the infusion. A precipitous fall in platelet count occurred after ADP, followed by gradual restoration of the count to pre-ADP levels. The initial fall is interpreted as removal of platelets from the circulation through aggregation and trapping of the aggregates in the lung. Restoration is believed to occur by deaggregation of the reversible ADP-induced aggregates. The restorative phase was shown to be the more sensitive to the pharmacological effects of RMI 10,393. Initial aggregation was also affected at the higher doses of the compound.

The second procedure involved removal of blood from the treated animal and measurement of the effect on ADP-induced platelet aggregation *in vitro*. This procedure proved less sensitive to the inhibitory action of RMI 10,393 than the ADP infusion procedure; however, RMI 10,393 was more effective in this test, since a dose-related response with complete inhibition at the highest dose was observed. The difference in results between these two methods cannot be explained at this time. Certainly there are many differences in these two systems. Other tissues, especially lung, play a role in the ADP infusion system, while in the procedure *in vitro*, isolation of citrated PRP can cause loss of platelet sensitivity,[9] especially at low inhibitory levels.

We have no explanation for the fact that RMI 10,393 is effective at much lower concentrations (in plasma or in platelets) *in vivo* than when added directly to plasma. In the latter case, there must be considerable binding of the compound at sites which do not affect platelet function. Platelet uptake of the compound occurs *in vivo* and *in vitro*. Plasma levels are dose related and increase with multiple administration, as does the inhibitory effect on platelets.

Phenylbutazone inhibits platelet "release reaction" and the second phase of aggregation, as well as adhesiveness of platelets in glass.[10] Hovig *et al.*[7] found that phenylbutazone had little effect on hemostasis in the presence of a normal fibrin clotting system, but caused a defect in hemostasis (prolonged bleeding time) in dogs with Factor IX deficiency. On the basis of this work, we gave rats dicumarol to reduce Factor IX and other members of the prothrombin complex, and challenged them with phenylbutazone or RMI 10,393. In the dicumarolized rat, neither phenylbutazone nor RMI 10,393 prolonged bleeding time significantly. In spite of this, rats treated with dicumarol and repeated daily doses of RMI 10,393 bled profusely from tail cuts when placed in their cages. This suggested a possible change in secondary bleeding time, but this measurement was only insignificantly prolonged. *In vitro*, RMI 10,393 inhibits clot retraction and it is possible that this effect, combined with defective fibrin clotting, reduced clot strength sufficiently to interfere with hemostasis during active movement of the animals about their cage but not enough to affect primary and secondary bleeding times. Phenylbutazone did not produce bleeding, with or without dicumarol.

In a previous paper,[1] it was reported that RMI 10,393, at high concentrations

*in vitro*, produced a shortening of thrombin time in an attenuated system. Data presented in the present paper also indicate a slight shortening of thrombin time when the compound was given orally to guinea-pigs at levels that inhibit platelet aggregation in the tests *in vivo*. Since both topical thrombin (containing active Factor X) and purified thrombin (devoid of Factor X) gave similar results, the interpretation is that PF-2-like activity, and not PF-3 activity, was present. These results obtained in thrombin time tests may be related to clotting time data obtained in rats (Table 4). In the normal rat, clotting time was not affected by RMI 10,393, but in the dicumarolized rat, at least after a single dose, the compound shortened clotting time. Similar results were obtained with phenylbutazone.

RMI 10,393 administered orally inhibited ADP-induced platelet aggregation in the guinea-pig. This effect alone did not produce obvious defects in hemostasis, but did so when combined with dicumarol. Clotting time was not affected measurably by the compound, unless the rate of fibrin formation was retarded below normal. These results suggest that it may be possible to control platelet aggregation, and thereby some common types of arterial thrombosis, without severe interference with hemostasis.

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