Effects of α-(*p*-(Fluoren-9-ylidenemethyl)phenyl)-2-piperidineethanol (RMI 10,393) on Platelet Function[1] (35642)

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(Introduced by K. M. Brinkhous)

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Currently available anticoagulant drugs (heparin and the antivitamin K compounds) are ineffective, or nearly so, in prevention of thrombosis in the arterial system (1, 2). This is probably related to their ineffectiveness in controlling platelet aggregation (1, 2), since the platelet thrombus is of primary importance in the arterial system (3, 4). In this paper, we report the results of a series of *in vitro* tests in which a new synthetic compound was used that has inhibitory effects on platelet function. The structure of this new compound, α-(*p*-(fluoren-9-ylidenemethyl) phenyl)-2-piperidineethanol (RMI 10,393) is shown in Fig. 1.

Materials and Methods. Plasma isolation. Blood was collected by the two-syringe technique from the antecubital vein of human volunteers; a disposable 19-gauge needle[2] was used. The blood was collected in siliconized[3] centrifuge tubes containing 3.5% disodium citrate (analytical reagent), 1 part of citrate to 9 parts of blood, and was cooled in an ice-water bath to 15° and centrifuged for 10 min at 100*g*. The platelet-rich plasma (PRP) was carefully pipetted from these tubes and combined in a separate siliconized tube placed in ice water and maintained by centrifuging the blood residue at 1000*g* for 20 min. Platelet concentrations of PRP and PPP was determined by the method of Brecker *et al.* (5).

[1] Results of these studies were presented in part at the 54th Annu. Meet. Fed. Amer. Soc. Exp. Biol., Atlantic City, New Jersey, April 16, 1970.

[2] Monoject 200, Sherwood Medical Industries, Inc., Leland, Florida.

[3] Silicone used was G.E. Dri-Film SC-87, G.E. Silicones Div., Waterford, N.Y.; used to siliconize glassware and stirring bars.

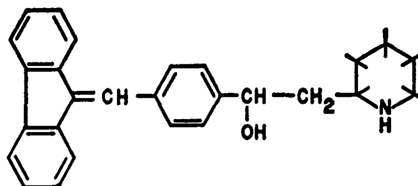

FIG. 1. Structure of α-[*p*-(fluoren-9-ylidenemethyl)-phenyl]-2-piperidineethanol (RMI 10,393).

Platelet aggregation. The method used for *in vitro* platelet aggregation was based on that reported by Mustard *et al.* (6). A constant platelet count of 400,000/mm^3, obtained by mixing PRP and PPP in calculated proportions, was used for all experiments.

Solutions of the test compound were prepared at concentrations that gave the desired final plasma concentration when 0.05 ml of the test solution was added to 0.5 ml of PRP. The PRP-compound mixture was incubated for 10–20 min at 37° before testing. Isotonic saline (0.86% NaCl) was used for control determinations on the same PRP. The free base of the test compound was solubilized by forming the acetate salt with one equivalent of acetic acid in aqueous isotonic solution (pH 4.9). A soluble glycolate salt was also used for some tests (pH 5.5). When these solutions were added to PRP, there was no detectable change in pH of the PRP.

All quantitative platelet aggregation measurements were made with a Bryston Platelet Aggregometer[4] (6) at 37°. The magnetic stirrer was set at 1300 rpm. The stirring bars were siliconized needle bearings,[5] 0.5/4.5 mm.

[4] Bryston Manufacturing, Scarborough, Ontario, Canada.

[5] Obtained from the Torrington Co., Torrington, Connecticut.

Adenosine diphosphate[6] (ADP), collagen,[7] thrombin,[8] epinephrine,[9] and serotonin[10] were used as aggregating agents. The challenging concentration of each aggregating agent was established by determining the dose–response relationship and using the concentration that produced about 50% of the maximum aggregation. Representative concentration ranges for aggregating agents were as follows: ADP (1–2 μg/ml of PRP), thrombin (0.1–0.3 units/ml of PRP), epinephrine (1 μg/ml of PRP), serotonin (2 μg/ml of PRP). Collagen preparations were made from human Achilles tendon.[7] Each preparation was standardized by biological activity in the platelet aggregation system. Control aggregations for each test system were determined on the same PRP sample within a few minutes of the test determination.

When platelets aggregate, light transmission increases in the photometer. This relayed to the recorder causes a rise in the pen plotting a graph on the chart. The initial rise (slope) (which indicates the initial rate of aggregation) is one parameter that was measured (6). Another parameter is the maximum change in transmittance of light (ΔT); this reflects the degree of aggregation (6). The value of control for these two parameters was compared to the test and percentage of inhibition for the compound calculated by the equation:

$$\%\ \text{inhibition} = \frac{\text{value of control} - \text{value of test} \times 100}{\text{value of control}}.$$

In the ADP, epinephrine, serotonin, and thrombin systems, the most sensitive parameter is the maximum ΔT value. In the collagen system, slope is more sensitive. In the thrombin system, fibrin formation time was also noted.

In the kinetic studies of ADP aggregation, dose responses at various ADP levels were determined. This was repeated at two different levels of test compound, and the results were analyzed by Lineweaver-Burk plot (8), $1/\Delta$ OD vs 1/[ADP].

Clot retraction. The clot retraction determination was based on the procedure reported by Stefanini and Dameshek (9). Recalcified citrated PRP was used for the determinations. To sensitize the system, the platelet count was brought to 150,000/mm^3 with PPP to give the lowest platelet concentration that would give maximum clot retraction. Two ml of plasma and 0.2 ml of saline or compound in saline were mixed with 0.37 ml of 1.25% $CaCl_2$. This mixture was rapidly transferred to a special siliconized tube (made from a 5-ml graduate pipette, cut and sealed with a flat bottom at the 5-ml mark, and cut and fire polished just above the 2-ml mark) and a reading was taken of the total volume. The tube was incubated for 2 hr at 37°. At 30-min intervals, the progress of the retraction was observed to give an indication of rate. After 2 hr the clot was carefully removed with tweezers and the volume of remaining serum was measured.

$$\%\ \text{retraction} = \frac{\text{volume serum remaining}}{\text{initial plasma volume}} \times 100,$$

$$\%\ \text{inhibition} = \frac{\%\ \text{control retraction} - \%\ \text{test retraction}}{\%\ \text{control retraction}} \times 100.$$

Plasma clotting tests. The effects of the compound on plasma factors and platelet procoagulant factors were investigated by use of the prothrombin time, partial thromboplastin time, and a modified Stypven time system. Five-hundredths ml of compound solution at the appropriate concentration was added to 0.5 ml of plasma and incubated for 20 min at 37° prior to performance of coagulation tests.

The one-stage prothrombin time was used as originally reported by Quick *et al.* (10), but we used Simplastin[11] as the source of

[6] Adenosine diphosphate, purchased from Cyclo Chemical Corp., Los Angeles.

[7] Prepared by the Hovig procedure (7) using human Achilles tendon.

[8] Thrombin, Topical, purchased from Parke-Davis & Co., and Thrombin, purified, designated as 3.7 S thrombin, obtained through the courtesy of Dr. L. E. McCoy, Wayne State University School of Medicine. This purified thrombin is devoid of Factor X activity (W. H. Seegers, personal communication).

[9] As tartrate salt obtained from Winthrop Labs.

[10] As the creatine sulfate monohydrate salt, obtained from Nutritional Biochemicals Corp.

[11] Commerical thromboplastin from Warner-Chilcott.

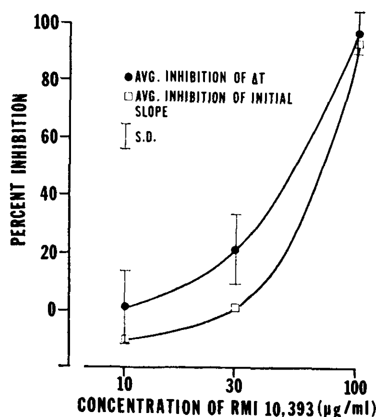

FIG. 2. Effect of RMI 10,393 on ADP-induced platelet aggregation: ADP added at 2 μg/ml of human PRP; average values of 6 determinations.

thromboplastin, with citrated instead of oxalated platelet-poor plasma.

The partial thromboplastin time was determined according to the method of Langdell *et al.* (11). Thrombofax[12] was used as the platelet substitute, with citrated platelet-poor plasma.

A modified Stypven test (12) was used to measure platelet factor 3 like activity. The general procedure was based on the reports of O'Brien (13) and Spaet and Cintron (14). After incubation of the compound solution or saline with PRP, a 1:10 dilution of the plasma was made with isotonic saline, Stypven reagent[13] + $CaCl_2$ (0.02 *M*) was added (1:1:1), and the coagulation time was determined. Dilutions of sonicated PRP were used to prepare a standard curve (activated PF-3 versus coagulation time, on the basis of 100% activation by sonication). Percentage activation in the unknown samples was then determined from the standard curve and the platelet count. Because of the 1:10 dilution, this procedure is more sensitive than the usual Stypven time procedure.

The standard thrombin time was determined by the method reported by Von Kaulla and Von Kaulla (15).

Results. Platelet aggregation systems. Figure 2 summarizes the inhibitory response to various concentrations of RMI 10,393 for ADP-induced aggregation. No inhibitory activity was found at 10 μg/ml; the compound at 30 μg/ml inhibited about 20%, while at 100 μg/ml, it inhibited almost completely.

An example of the inhibitory effects of RMI 10,393 in the collagen-induced platelet aggregation system is shown in Fig. 3. The slope (rate of change) and lag period are more susceptible to inhibition than total aggregation (total change in transmittance), the most sensitive parameter in the ADP-collagen system. This is probably due to the irreversible nature of collagen aggregation. In this system 10 μg/ml of compound did not effect lag time or initial slope. At 30 μg/ml, lag time and initial slope were affected; at 100 μg/ml, complete inhibition occurred.

The inhibitory effects at various concentrations of RMI 10,393 on aggregation induced by thrombin, epinephrine, and serotonin are shown in Fig. 4, 5, and 6, respectively.

The compound inhibited platelet aggregation in all 5 systems with about the same potency: there was little or no activity at 10 μg/ml, significant activity at 30 μg/ml, and almost complete inhibition at 100 μg/ml.

Kinetic studies. To investigate the mechanism of the inhibitory effect on platelet aggregation, we utilized a procedure similar to that of Born (16), and Skoza *et al.* (17) who showed that adenosine is a competitive inhibitor in the ADP-induced platelet aggregation system by Lineweaver-Burk plot analysis. We used a Lineweaver-Burk plot, $1/\Delta$ OD vs 1/[ADP] and confirmed the

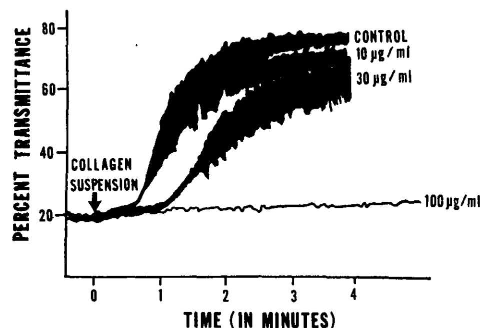

FIG. 3. Effect of RMI 10,393 on collagen-induced platelet aggregation.

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[12] Commercial partial thromboplastin manufactured by Ortho Diagnostics, Raritan, N.J.

[13] Stypven, Russell's viper venom from Burroughs-Wellcome & Co., Inc.

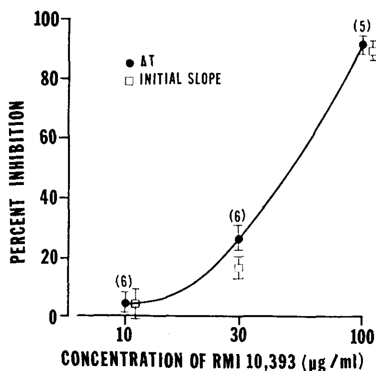

FIG. 4. Effect of RMI 10,393 on thrombin-induced platelet aggregation: thrombin added at 0.1–0.3 units/ml of human PRP. Number in parentheses is number of determinations.

pattern for competitive inhibition with adenosine. The procedure was then repeated, using two different concentrations of RMI 10,393. The results of the experiment with RMI 10,393 are shown in Fig. 7.

On the assumptions that platelets are equated to enzyme, that ADP is substrate, and that the aggregates are the product, the plot indicates, when interpreted according to Webb (18), a mechanism of mixed inhibition, or of competitive inhibition with respect to an activator of platelet aggregation. These kinetic studies indicate a mechanism or mechanisms different from that of adenosine, but do not define them precisely.

Clot retraction. The effect of RMI 10,393 on clot retraction is shown in Fig. 8. An average line has been drawn through the points, each of which is a determination. Many different human plasmas were used over a period of months. These data indicate that inhibition of clot retraction begins above 30 μg/ml and is almost complete at 100 μg/ml.

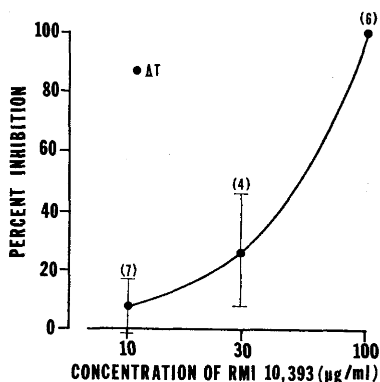

FIG. 5. Effect of RMI 10,393 on epinephrine-induced platelet aggregation: epinephrine added at 1 μg/ml human PRP. Numbers in parentheses is number of determinations.

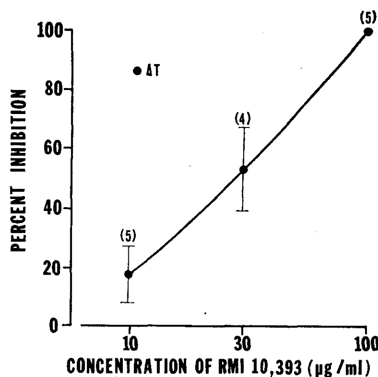

FIG. 6. Effect of RMI 10,393 on serotonin-induced platelet aggregation: serotonin added at 2 μg/ml of human PRP. Number in parentheses is number of determinations.

Plasma clotting tests. There was no effect on the partial thromboplastin time at levels that inhibit platelet function almost completely (100 μg/ml). There was little effect on the prothrombin time; only at 100 μg/ml

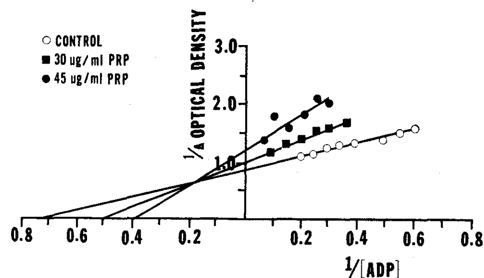

FIG. 7. Lineweaver-Burk plot of ADP-induced platelet aggregation inhibition by RMI 10,393.

was there a significant effect (+1.7 sec, which indicates about 70% of normal prothrombin level).

In the modified Stypven test, the compound caused a shortening of the clotting time, which indicates PF-3-like activity. When this activity was quantitated by comparison with various concentrations of sonicated platelet-rich plasma, the percentage of

TABLE I. Effect of RMI 10,393 on Thrombin Time.[a]

Conc of compound (μg/ml)	Thrombin time (sec)			
	Platelet-poor plasma	Platelet-rich plasma		
	Topical thrombin (0.125 U/ml)	Topical thrombin 3 U/ml	Topical thrombin 0.125 U/ml	Purified thrombin (0.125 U/ml)
0	106	12	98	90
30	104	—	68	92
100	102	12	48	65
300	105	—	50	50

[a] Thrombin time system: 0.5 ml of citrated plasma + 0.05 ml compound solution or saline were incubated for 20 min; then 0.1 ml of thrombin dissolved in 0.102 M $CaCl_2$ was added and time started; end point clot.

available PF-3 activated was very low (0.12% at 100 μg/ml). Though the activation is low, we did not know its significance. Therefore, the effect was compared to that of a standard breakfast.[14] The standard breakfast was found to produce the same activation of PF-3 (.12%) as 100 μg/ml of the compound (14).

There was no effect of the compound in standard thrombin time test (see Table I). When the thrombin concentration was decreased in the thrombin time system, sensitizing the system to small changes in coagulation potential, the compound produced a shortening of clotting time. This shortening may reflect PF-3 activity in a topical thrombin system, since topical grade thrombin contains activated Factor X, the enzyme for which PF-3 acts as a cofactor. However, when we used purified thrombin which is devoid of Factor X,[8] there was still a shortening of the fibrin clotting time. Since only a slight difference was obtained in tests with topical and purified thrombin systems, PF-3 activity cannot explain all of the shortening that is found. The effect depends on the presence of platelets, however, since it was not obtained in platelet-poor plasma. One interpretation is that another platelet procoagulant such as PF-2 is also activated by RMI 10,393, since PF-2 can play a role in the thrombin–fibrinogen interaction.

[14] Standard breakfast contained 768 calories consisting of 39.5 g of protein, 42.8 g of fat, and 65.3 g of carbohydrate.

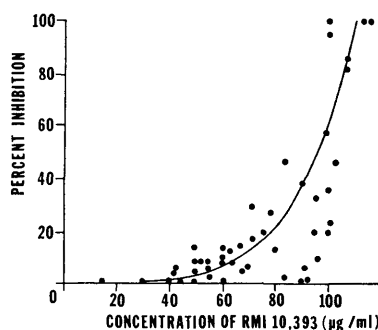

FIG. 8. Effect of RMI 10, 393 on clot retraction.

Discussion. RMI 10,393 is a new type of anticoagulant, with a mechanism different from those of the two classes of anticoagulant now in use, heparin and coumarin derivatives. RMI 10,393 inhibits platelet aggregation induced by ADP, collagen, thrombin, epinephrine, and serotonin, and also inhibits clot retraction (a function of platelets). A low level of platelet factor 3 activation was found at concentrations of the compound that completely inhibit platelet aggregation. This level was not considered excessive, as it was comparable with the PF-3 like activity produced by a standard breakfast. Another platelet-related procoagulant effect was detected in an attenuated thrombin time system to which RMI 10,393 had been added. This effect, which occurred with purified thrombin, may represent activation of platelet factor 2. It was not detectable in the usual thrombin time system, possibly because of the overwhelming effect of thrombin.

The levels of RMI 10,393 that inhibit

these platelet functions do not affect to any great extent partial thromboplastin time, prothrombin time, or standard thrombin time. Therefore the normal plasma coagulation factors are probably not influenced.

It is not possible to determine the effect of RMI 10,393 on the so-called release phenomenon using the platelet aggregometer. The compound inhibits primary aggregation due to ADP, and therefore can be expected to inhibit aggregation due to ADP released from the platelet. The fact that inhibitory concentrations for all aggregating agents tested were essentially identical is strong evidence for the anti-ADP effect as the common denominator. This type of effect, in a compound with low toxicity, could be useful in the prevention of thrombosis of several different origins.

Summary. RMI 10,393 is a new compound which inhibits aggregation of human platelets in plasma. It is effective against aggregation induced by ADP, thrombin, collagen, epinephrine, and serotonin. In the effective concentration range it also inhibits clot retraction, but does not cause activation of platelet factor 3. In an attenuated system activation of a platelet-derived procoagulant effect occurs, but this is not seen in the standard clotting system. RMI 10,393 has no effect on soluble clotting factors. A compound with such activity may have potential as an antithrombotic agent.

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