Effect of RA233 on Platelet Function in Vitro

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Summary: RA233, a new pyrimido-pyrimidine compound, is a powerful inhibitor of platelet function tested in vitro; it inhibits calcium and adenosine diphosphate (A.D.P.)-induced platelet aggregation, inhibits the retention of platelets by glass beads, decreases the release of platelet factor 3 by kaolin, and inhibits clot retraction. In some in-vitro systems RA233 is significantly more potent that its analogue RA433 in inhibiting platelet function.

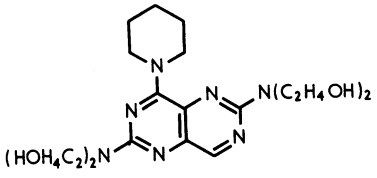
Introduction

The major contribution of platelets to the formation of intravascular thrombi has stimulated the search for agents capable of inhibiting platelet aggregation. The effect of the pyrimido-pyrimidine derivatives, dipyridamole and RA433, on platelet behaviour in vivo and in vitro has been extensively studied. Dipyridamole inhibits both platelet aggregation in vitro (Emmons *et al.*, 1965a; Gray *et al.*, 1968) and thrombus formation at sites of vascular injury in experimental animals (Emmons *et al.*, 1965b). Dipyridamole has also been shown to be of therapeutic value; Sullivan *et al.*, (1968) reported a significant decrease of thromboembolic incidents in patients with prosthetic heart valve replacement treated with combined dipyridamole and anticoagulant therapy, compared with those treated with anticoagulants alone. RA433 is a more powerful inhibitor of platelet aggregation in vitro than dipyridamole (Forbes *et al.*, 1969), but differs from dipyridamole in that thrombus formation in vivo in experimental animals is uninfluenced (Elkeles *et al.*, 1968). Clinical evaluation of RA433 is awaited.

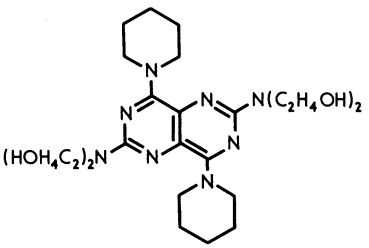
A new derivative of pyrimido-pyrimidine, RA233, is now available, and its effect on human platelet behaviour in vitro is reported in this study. The activity of RA233 is compared with that of its analogue RA433.

Chemistry

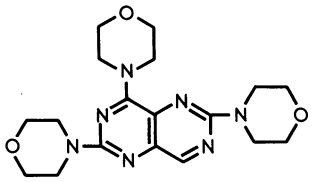
RA233 is 2,6-bis(diethanolamino)-4-piperidino-pyrimido-(5, 4-*d*) pyrimidine, with the following structural formula:



Its molecular weight is 421·5. It is insoluble in water, but soluble in dilute acids.



Dipyridamole



RA433

Materials

RA233 (Boehringer Ingelheim). Stock solution: 10^{-4}M RA233 in 0·025 N hydrochloric acid; stored at 4°C. Dilutions referred to in text were made in 0·877% saline.

RA433 (Boehringer Ingelheim). Stock solution: 10^{-4}M RA433 in 0·025 N hydrochloric acid; stored at 4°C. Dilutions referred to in text were made in 0·877% saline.

Adenosine 5-diphosphate (A.D.P.) (Sigma Chemical Company, St. Louis). Stock solution: 100 µg./ml. A.D.P. in barbitone/saline buffer, *p*H 7·2; stored at −20°C. Dilutions were made in barbitone/saline buffer.

Tubing. Chandler tube experiments. Transparent vinyl tubing (MT/13, Portland Plastics, Kent) and plastic adaptors (10M/634, Portex).

Glass bead columns. Transparent vinyl tubing (MT/13, Portland Plastics, Kent); translucent silicone tubing (Esco Rubber Limited, London).

Ballotini glass beads: 0·57 mm. diameter.

Calcium chloride: 0·25M : 0·025M.

Kaolin: 5% kaolin in imidazole buffered saline *p*H 7·2.

Russell viper venom (Stypven, Burroughs Wellcome Co.).

Citrated blood was collected by clean venepuncture in plastic syringes with 21-gauge needles; nine volumes of blood being mixed with one volume of 3·8% trisodium citrate in siliconized graduated centrifuge tubes maintained at room temperature. Platelet-rich plasma was obtained by centrifugation of citrated whole blood at 600 *g* for five minutes at room temperature. Siliconized glassware was used throughout (Siliclad, Clay-Adams, Inc., New Jersey).

Methods

The methods are described in detail by Forbes *et al.* (1969).

Chandler Tube Technique.—The method used was that described by Chandler (1958) as modified by Cunningham *et al.* (1965).

Turbidimetric Method.—The turbidimetric method of Born

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(1962) was modified as described by Hassanein (1969) to assess the effect of RA233 and RA433 on calcium-induced platelet aggregation at room temperature. On recalcification of control platelet-rich plasma an initial fall in optical density occurred owing to dilution; there followed a lag phase in which no appreciable change in optical density was observed, then a slight increase in optical density, followed by a fall due to platelet aggregation; finally clot formation occurred. The results obtained using this system are expressed as follows:

Aggregation time: time taken for the initial fall in optical density, due to platelet aggregation, to occur.

Duration of platelet aggregation: time interval between the beginning of the initial fall in optical density due to platelet aggregation and clot formation.

Optical density fall: magnitude of the fall in optical density occurring as a result of platelet aggregation.

Clotting-time: time interval between recalcification and clot formation.

A.D.P.-induced Platelet Aggregation.—This was studied by the method of Born (1962) at room temperature. Indices of platelet aggregation and disaggregation are expressed as follows: 30-60 platelet aggregation, fall in optical density between 30 and 60 seconds after the addition of A.D.P.; Maximum aggregation, difference between the lowest recorded optical density and the constant arbitrary baseline of 0·600; percentage disaggregation, ratio between the increase in optical density occurring five minutes after the point of maximum aggregation and the maximum platelet aggregation.

Retention of Platelets by Glass Beads.—The glass bead column technique of Hellem (1960) as modified by Hirsh *et al.* (1966) was used.

Release of Platelet Factor 3 by Kaolin.—A modification of the technique of Spaet and Cintron (1965) was used; platelet-rich plasma was preincubated at 37°C. for 10 minutes with RA233 or RA433 or with corresponding solvent control.

Whole Blood Clot Retraction.—A modification of the method described by Dacie (1956) was used; whole blood was mixed in siliconized centrifuge tubes containing copper wire spirals with RA233, RA433, or solvent control before clot formation.

Plasma Clot Retraction with Added A.D.P.—Citrated platelet-rich plasma was added to siliconized centrifuge tubes containing copper wire spirals, mixed with RA233, RA433 or solvent control, and incubated at 37°C. for 10 minutes. A.D.P. (final concentration 5μg./ml.) was added and incubation continued for a further five minutes. Recalcification with 0·25M calcium chloride was carried out and clot retraction measured as described by Dacie (1956). Experiments without A.D.P. were performed and the results compared.

Platelet counts were performed with formal citrate as the diluting fluid (Dacie, 1956).

Results

Chandler Tube Technique.—The results obtained in seven experiments using RA233 and RA433 compared with solvent controls are shown in Fig. 1. Both drugs produced a significant prolongation of aggregation time (snow-storm effect).

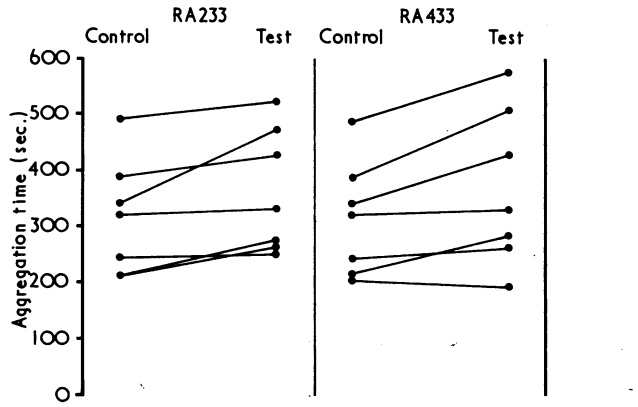

FIG. 1.—Effect of RA233 and RA433 in concentrations of 5 × 10^{-5}M on platelet aggregation time in the Chandler tube system. (RA233 P<0·02; RA433 P<0·02.)

Turbidimetric Method using Calcium.—The results of seven experiments and controls are shown in the Table. RA233 significantly prolonged the aggregation time, whereas with RA433, while there was a trend, with the small numbers conventional levels of statistical significance were not achieved. The duration of platelet aggregation and optical density fall were statistically significantly diminished by both drugs. Neither drug significantly influenced the clotting-time.

A.D.P.-induced Platelet Aggregation.—The mean results of seven experiments using RA233 and RA433 in two concentrations compared with solvent controls are shown in Fig. 2.

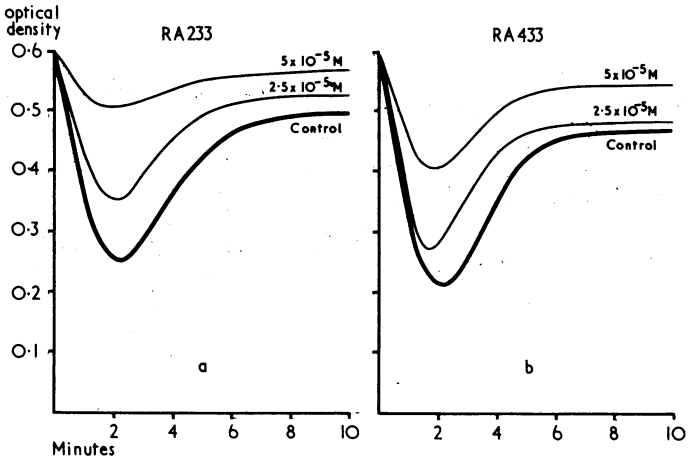

FIG. 2.—A.D.P.-induced platelet aggregation. The results of seven experiments using RA233a and RA433b, compared with solvent controls. A.D.P. concentration 0·25μg./ml. (5 × 10^{-5}M, RA233 P<0·001; RA433 P<0·001. 2·5 × 10^{-5}M, RA233 P<0·01; RA433 P<0·02.)

Effect of RA233 and RA433 on Calcium-induced Platelet Aggregation by the Turbidimetric Method

RA233. Final Concentration 5 × 10^{-5}M				Control				RA433. Final Concentration 5 × 10^{-5}M			
Aggregation Time (sec.)	Duration of Platelet Aggregation (sec.)	O.D. Fall	Clotting-Time (sec.)	Aggregation Time (sec.)	Duration of Platelet Aggregation (sec.)	O.D. Fall	Clotting-Time (sec.)	Aggregation Time (sec.)	Duration of Platelet Aggregation (sec.)	O.D. Fall	Clotting-Time
455	65	0·150	520	190	140	0·260	330	235	135	0·230	370
360	25	0·005	385	385	190	0·315	575	510	25	0·040	535
495	40	0·060	455	375	125	0·215	500	380	45	0·125	425
765	15	0·005	780	500	160	0·270	600	575	50	0·060	625
480	60	0·140	540	325	110	0·295	435	490	35	0·235	525
590	45	0·060	635	525	115	0·185	640	450	75	0·085	495
440	45	0·070	485	330	95	0·150	425	590	60	0·095	650
P<0·02	P<0·01	P<0·01	P>0·1					P>0·05	P<0·01	P<0·02	P>0·1

Both drugs produced statistically significant inhibition of both 30-60 and maximal platelet aggregation. At a concentration of 5×10^{-5}M RA233 caused greater inhibition than RA433 ($P<0.05$). Neither drug influenced platelet disaggregation in the test systems.

Retention of Platelets by Glass Beads.—The results of seven experiments with RA233 and RA433 are shown in Fig. 3. Both drugs significantly reduced platelet retention by glass beads. RA233 was significantly more potent than RA433 ($P<0.05$).

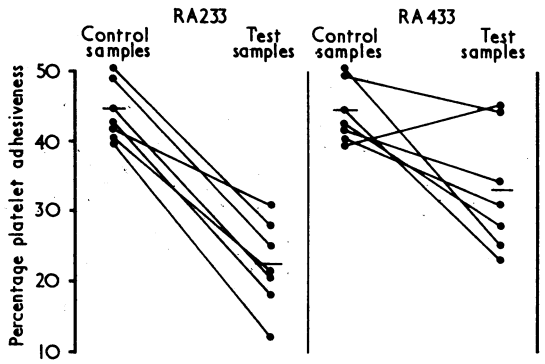

FIG. 3.—Effect of RA233 and RA433 (concentration 10^{-4}M) on platelet retention by glass beads. Citrated whole blood preincubated with RA233 and RA433 at room temperature for 10 minutes. (RA233 $P<0.001$; RA433 $P<0.05$.)

Release of Platelet Factor 3 by Kaolin.—RA233 and RA433 significantly inhibited the release of platelet factor 3 induced by kaolin (Fig. 4). Neither drug affected the clotting-times when platelet-poor plasma was used, indicating that they had no inhibitory effect on plasma coagulation factors.

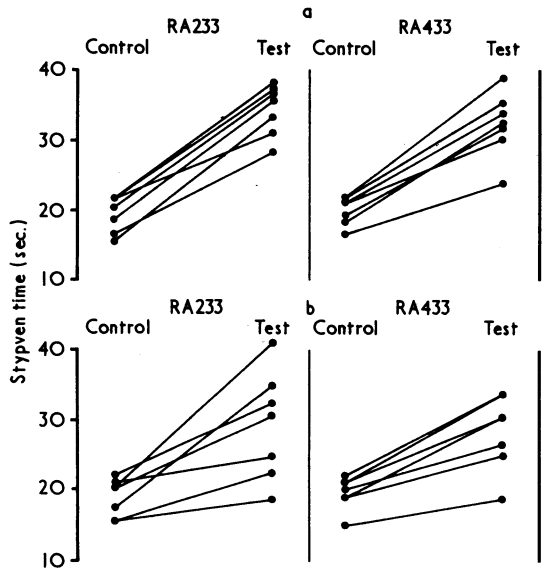

FIG. 4.—Effect of two concentrations of RA233 and of RA433 on the release of platelet factor 3 by kaolin. (Above, concentration 5×10^{-5}M, RA233 $P<0.001$; RA433 $P<0.001$. Below, concentration 10^{-5}M, RA233 $P<0.01$; RA433 $P<0.001$.)

Clot Retraction.—Whole blood clot retraction was diminished by both RA233 and RA433 (Fig. 5). There was no significant difference between the two drugs in this activity ($P>0.1$). Plasma clot retraction (platelet-rich plasma) was significantly diminished by both drugs (Fig. 6). The addition of A.D.P. before recalcification partially corrected the inhibition of clot retraction produced by RA233 ($P<0.02$) but had no significant effect in that produced by RA433 ($P>0.1$).

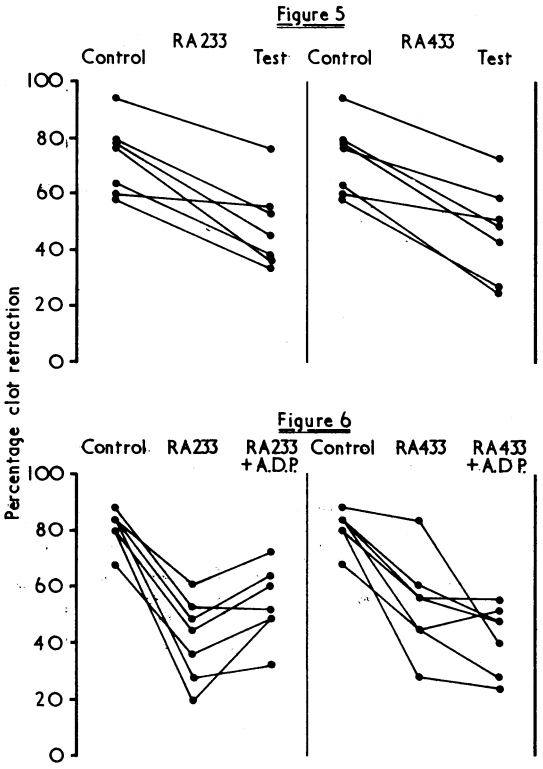

FIG. 5.—Effect of RA233 and RA433 in concentrations of 5×10^{-3}M on whole blood clot retraction. (RA233 $P<0.01$; RA433 $P<0.001$.)
FIG. 6.—Effect of RA233 and RA433 in concentrations of 5×10^{-3}M on platelet-rich plasma clot retraction, and effect of the addition of A.D.P. (concentration 0·5 μg./ml.). (RA233 $P<0.001$; RA433 $P<0.01$.)

Discussion

Interest in the pyrimido-pyrimidine group of compounds has been stimulated by the potential therapeutic value in occlusive vascular disease of substances which reduce platelet stickiness. There is considerable evidence that adherence of platelets to one another and to vessel walls at sites of endothelial injury is a fundamental step in the formation of intravascular thrombi (Mustard *et al.*, 1962). The initial aggregation of platelets is independent of blood coagulation (Jørgensen and Borchgrevink, 1964; Mustard *et al.* 1964) and may be brought about by the release of A.D.P. from the injured tissue (Honour and Mitchell, 1963; Born *et al.*, 1964). The release of additional A.D.P. and of platelet factor 3, and the subsequent generation of thrombin, leads to stabilization of the thrombus with fibrin formation.

Hitherto, the main medical approach to the treatment of thromboembolic disease has been anticoagulant therapy, but the benefits to be gained by the use of anticoagulants in arterial thrombosis remain controversial after 20 years of large-scale clinical trial. The quest for drugs to reduce platelet adhesiveness is a logical step in therapeutic research. The pyrimido-pyrimidine derivatives, dipyridamole (Emmons *et al.*, 1965a, 1965b; Gray *et al.*, 1968) and RA433 (Elkeles *et al.*, 1968; Forbes *et al.*, 1969), have been shown to reduce platelet adhesiveness/aggregation in vivo and in vitro. They have, however, differing actions in the injured artery model in experimental animals. Dipyridamole inhibits thrombus formation whereas RA433 is ineffective (Elkeles *et al.*, 1968). Sullivan *et al.* (1968) showed that dipyridamole is therapeutically effective when combined with anticoagulants in reducing the number of thromboembolic incidents in patients with prosthetic heart valves compared with those treated with anticoagulants alone.

Dipyridamole and RA433 have the advantage that they are active after oral administration and that they are relatively free from side-effects (Elkeles *et al.*, 1968; Gray *et al.*, 1968).

RA233 is a new derivative of pyrimido-pyrimidine and its effects on platelet behaviour in vitro are reported and a comparison is made with its analogue RA433. RA233 inhibited calcium-induced platelet aggregation in the Chandler tube and also in the modified turbidimetric system; it decreased platelet aggregation as assessed by the optical density fall in the turbidimetric system and reduced the total duration of platelet aggregation. Despite these changes in platelet activity the clotting-time was uninfluenced in this system. In the Chandler tube system, both RA233 and RA433 significantly prolonged the platelet aggregation time, whereas in the assessment of calcium-induced platelet aggregation using the turbidimetric system, RA233 significantly prolonged platelet aggregation time whereas RA433 did not; both RA233 and RA433 reduced significantly the optical density fall and reduced the duration of aggregation, which are presumptive measures of the degree of aggregation of platelets. RA233 proved to be a more potent inhibitor than RA433 of A.D.P.-induced platelet aggregation and of platelet retention by glass beads. Neither drug affected platelet disaggregation. RA233 and RA433 decreased the release of platelet factor 3 induced by kaolin, but there was no significant difference in their activity.

RA233 and RA433 were effective in inhibiting clot retraction of whole blood and of platelet-rich plasma. The addition of A.D.P. before recalcification partially corrected the inhibition in clot retraction induced by RA233, but not that by RA433. This observation suggests that RA233 may have a combined effect on thrombasthenin, the contractile protein of platelets, and possibly on the generation of energy required for clot retraction. The concentrations of RA233 and RA433 used in the experiments on clot retraction are, however, unlikely to be attained in man in vivo.

The results show that RA233 has properties similar to dipyridamole and RA433 in altering platelet behaviour in vitro, but is significantly more potent when tested in some in-vitro models. RA233 is well absorbed by mouth and relatively non-toxic when administered to experimental animals (R. Kadatz, personal communication, 1969).

The hypothesis, however, of a therapeutic gain from reducing platelet stickiness in occlusive vascular disease remains to be proved, and once toxicity studies have been completed it would be of major importance to mount large-scale clinical trials with the pyrimido-pyrimidine group of compounds.

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Clofibrate, Serum Enzymes, and Muscle Pain

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Summary: Serum creatine kinas (C.K.), aspartate aminotransferase (G.O.T.), and alkaline phosphatase (A.P.) activities were measured in 211 men with serum cholesterol concentrations in the upper one-third of the normal distribution. Of these, 110 were receiving clofibrate and 101 were given identical capsules containing olive oil. These investigations were also carried out on 85 healthy men with low serum cholesterol levels not receiving clofibrate.

No differences were observed in C.K. and G.O.T. activities between any of these groups; A.P. was significantly lower in the clofibrate-treated group. No significant alterations in C.K. occurred during serial observations made in 15 patients with ischaemic heart disease over a period of five months. No instance of myalgia or muscle stiffness was recorded in 452 men who had received clofibrate for one year.

It is concluded that raised C.K. and G.O.T. concentrations and the occurrence of myalgia are uncommon accompaniments of clofibrate treatment.

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Introduction

Raised serum creatine kinase (C.K.) activities have been observed in 5 out of 60 patients with hyperlipoproteinaemia during treatment with clofibrate (Atromid-S), and severe myalgia and muscle stiffness developed in two patients (Langer and Levy, 1968). Because of these complications it has been suggested that frequent determinations of C.K. should be made on all patients receiving this drug (*Lancet*, 1968). Transient rise of serum aspartate aminotransferase (G.O.T.) and permanent depression of alkaline phosphatase (A.P.) have been reported to occur during clofibrate administration (Oliver, 1962; Hellman *et al.*, 1963).

The purpose of this paper is to report a study in which the serum levels of these enzymes (C.K., G.O.T., and A.P.) were