Action of a Pyrimido-pyrimidine Compound on Platelet Behaviour in Vitro

C. D. FORBES,* M.R.C.P., M.R.C.P.ED., M.R.C.P.GLASG.; G. P. McNICOL,† MD., PH.D., F.R.C.P.ED., F.R.C.P.GLASG.
A. S. DOUGLAS,‡ M.D., F.R.C.P., F.R.C.P.ED., F.R.C.P.GLASG.

British Medical Journal, 1969, **2**, 483–485

Summary: A pyrimido-pyrimidine compound (RA433) was found in vitro to be a significantly more potent inhibitor of platelet behaviour than the previously available pyrimido-pyrimidine compound RA8—dipyridamole. In a turbidimetric system RA433 inhibits platelet aggregation induced by adenosine diphosphate, collagen, and noradrenaline; further, in a glass-bead-column technique it is a powerful inhibitor of platelet adhesiveness.

Introduction

One of the most promising compounds described in the search for agents which might be used to inhibit platelet aggregation in man is dipyridamole (Bunag *et al.*, 1964). Nevertheless, with dipyridamole it is difficult to achieve adequate concentrations in vivo without unacceptable side-effects. We here report on in-vitro studies with RA433 (2,4,6-trimorpholino-pyrimido(5,4-*d*)-pyrimidine), a synthetic dipyridamole analogue. In a number of test systems this analogue is shown to be a more powerful inhibitor of platelet aggregation and adhesiveness than dipyridamole.

The cause of thrombotic vascular occlusion is unknown, but as early as 1888 Eberth and Schimmelbusch showed that platelet masses were deposited at the sites of injury in mesenteric vessels. Whatever may be the mechanism concerned, platelet adhesion and aggregation on the inner surface of a vessel is undoubtedly an early and important event in thrombus formation. Moreover, platelet deposits may be precursors of atheromatous lesions (Duguid, 1955).

Many naturally occurring substances—for example, fatty acids, thrombin, collagen, and noradrenaline—increase the adhesiveness of platelets. Adenosine diphosphate (A.D.P.), which is present in normal red blood cells and also increases platelet adhesiveness (Hellem, 1960) has been studied intensively during recent years in relation to platelet aggregation and adhesiveness. Platelets are known to be rich in adenosine triphosphate (A.T.P.), which disappears during clotting (Born, 1958) or when platelets undergo viscous metamorphosis (Zucker and Borrelli, 1961). A.D.P., which is the first breakdown product of A.T.P., may be the factor promoting the formation of haemostatic platelet plugs and the white heads of vascular thrombi (Born, 1962). In animals infusion of A.D.P. can result in platelet aggregation (Born and Cross, 1963; Honour and Mitchell, 1963). There are also at least two agents present in the arterial wall which can cause platelet aggregation; one is probably collagen (Zucker and Borrelli, 1962) and the other A.D.P. (Mitchell and Sharp, 1964; Prentice *et al.*, 1966).

Various compounds have been found which inhibit platelet aggregation and reduce adhesiveness. Adenosine and 2-chloradenosine are competitive inhibitors of A.D.P.-induced platelet aggregation (Born *et al.*, 1964), but unfortunately both have unacceptable toxic effects. Adenosine is a powerful vasodilator and produces hypotension (Born *et al.*, 1965), whereas 2-chloradenosine produces respiratory arrest when given to animals (Born *et al.*, 1964). Dipyridamole, which slows the rate of disappearance of exogenous adenosine from whole blood in vitro (Bunag *et al.*, 1964), also inhibits thrombus formation at the sites of injury in rabbit vessels (Emmons *et al.*, 1965a) and A.D.P.-induced platelet aggregation in vitro, but not noradrenaline-induced aggregation (Emmons *et al.*, 1965b). This action of dipyridamole in inhibiting A.D.P.-induced platelet aggregation and in reducing adhesiveness in a glass-bead-column technique was also described by Gray *et al.*, 1969, who furthermore showed that when platelet-rich plasma was recalcified in a continuous rotating loop of plastic tubing (the Chandler tube technique) the initial stages of artificial thrombus formation were delayed in the presence of dipyridamole.

* Registrar in Medicine, Royal Infirmary, Glasgow C.4.
† Senior Lecturer in Medicine, Royal Infirmary, Glasgow C.4.
‡ Professor of Medicine, Royal Infirmary, Glasgow C.4.

Materials

Citrated blood was collected by clean venepuncture, nine volumes of blood being mixed with one volume of 3·8% sodium citrate in a siliconized graduated centrifuge tube. The subjects studied were mostly colleagues or hospital patients suffering from various diseases, none of which is known to affect platelet function.

Platelet-rich plasma was obtained by centrifugation at 400 *g* for five minutes at room temperature. Siliconized glassware was used throughout (Siliclad Clay-Adams Inc.).

A.D.P. was used as the sodium salt (Sigma). A stock solution of 50 μg./ml. was made in a barbitone-saline buffer *p*H 7·2 and kept at −20° C. for use in all experiments. Appropriate dilutions were made from this stock solution.

R.A. 433.—A stock solution of 400 μg./ml. was prepared in 0·1 N hydrochloric acid and kept at −20° C. The various concentrations referred to in the text were made from this stock solution by the addition of 0·1 N hydrochloric acid.

Collagen was prepared from tendon by Sigma Chemical Company, 0·4 g. being mixed in 5 ml. of normal saline for 15 minutes at room temperature. The undissolved fibres were centrifuged out and the supernatant was stored at 4° C.

Noradrenaline (Bayer Products Ltd.).

Methods

Chandler Tube Technique.—The method was that described by Chandler (1958) as modified by Cunningham *et al.* (1965). Transparent vinyl tubing (Portland Plastics Ltd., code No. N/17), 12·3 mm. bore, 71 cm. long, was made into a loop by means of a Nylon adapter. The loop was washed in cold water and rinsed in 0·9% sodium chloride. Thirty minutes after venepuncture a 10-ml. sample of platelet-rich plasma, which had been kept at 37° C., was split into equal parts; to these was added 0·05 ml. of various dilutions of RA433 or control diluent and incubated for 10 minutes. The samples of plasma were then transferred to the loop, the volume of each having been made up to 15 ml. with 0·9% sodium chloride. The system was then recalcified with 1·5 ml. of 0·25 M calcium chloride and the loop rotated at 28·5 r.p.m. on the turntable of a blood cell suspension mixer (Matburn Ltd.) in a glass-fronted incubator at 37° C. Platelet aggregation was assessed by the length of time taken for the "snowstorm" of platelet aggregation to appear after recalcification.

Turbidimetric assessment of platelet aggregation was carried out as described by Born (1962). The apparatus used in this study consisted of an EEL titrator connected to a galvanometer

(EEL type 20, Evans Electroselenium Ltd.). A perspex cuvette is fitted on to the titrator above a magnetic stirrer and in the light path to the photoelectric cell. A 4-ml. plasma sample was divided into two equal parts and 0·02-ml. amounts of the dilutions of RA433 or control diluent were added. Both specimens were incubated at 37° C. for 10 minutes. The samples were placed in the cuvette together with a small stirring rod. With the stirring rod rotating at a uniform speed the optical density reading of the plasma sample on the galvanometer scale was adjusted to an arbitrary value of 0·600. Before adding the aggregating agent the plasma was stirred for at least 30 seconds to ensure a stable baseline; optical density was recorded at 30-second intervals over a period of 10 minutes from the time of adding the aggregating agent. Assessment of platelet aggregation was made following challenge with A.D.P. (0·5 μg./ml. of plasma), noradrenaline (1·5 μg./ml. of plasma), and collagen.

Platelet adhesion to glass beads was assessed in a glass-bead-column method as described by Hellem (1960) and modified by Hirsh *et al.* (1966). The column was made of a length of vinyl tubing (NT 13 Portland Plastics Ltd.) with 2·5-g. Ballotini glass beads (0·57 mm. diameter) to give a column 6 cm. in length. The glass beads are held in the column by a filter of fine Nylon gauze fitted at each end of the column. Citrated blood which had been standing at room temperature for 30 minutes was tested. Aliquots (0·02 ml.) of the appropriate dilution of RA433 or of control diluent were added to 2 ml. of blood and incubated at 37° C. for 10 minutes. Test and control samples were then drawn into 2-ml. graduated plastic syringes, which were fitted to an electrically operated pump, and the sample was pushed through the column at a constant rate so that the mean contact time between blood and glass was 30±1 seconds. Platelet counts were performed on the samples before and after passage through the column. The difference between the two counts was expressed as a percentage of the initial platelet count, and this value was taken as an index of platelet adhesiveness in the samples.

Platelet counts were performed with formal citrate as the diluting fluid (Dacie, 1956). Freshly filtered diluting fluid (10 ml.) was delivered to a clear glass test-tube and to this was added 0·1 ml. of blood. The tube was inverted gently until the contents were thoroughly mixed. A sample was run on to a Neubauer improved counting chamber and allowed to stand in a damp atmosphere for 20 minutes before the platelets were counted.

Results

Chandler Tube Experiments

Table I shows the mean values and standard deviations of the times for the "snowstorm" effect to appear in seven experiments at various concentrations of RA433 and also with seven controls. There is no statistical difference between controls and tests.

TABLE I.—*Effect of Addition of RA433 in Various Concentrations on the "Snowstorm" Effect Produced by Recalcifying Platelet-rich Plasma in the Chandler Tube. (Mean of Seven Experiments Compared with Controls)*

RA433 Concentration (μg./ml.)	Time of "Snowstorm" Effect (Seconds)		*t*	P
	Control	Test		
10	591±195	592±218	0·11	>0·1
20	511±173	637±260	2·1	>0·05
30	681±212	714±291	1·0	>0·1
80	618±276	713±266	1·6	>0·1

Turbidimetric Method

Fig. 1 shows the effect of RA433 in various concentrations (10–80 μg./ml.) on A.D.P.-induced platelet aggregation. Each graph shows the mean of seven tests with RA433 and seven controls. RA433 produced a concentration-dependent inhibition of platelet aggregation in this system. Statistical evaluation of the difference between test and control experiments was carried out on the observations made at 2 and at 10 minutes; significant differences ($P<0·02$) were found with RA433 concentrations of 20 μg./ml. and higher.

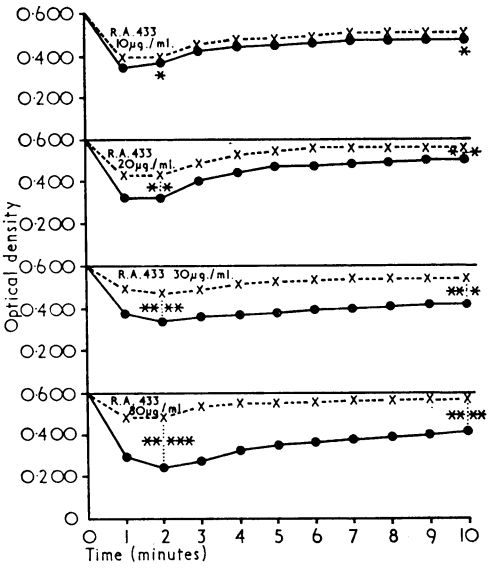

FIG. 1.—Comparison of effects of RA433 in various concentrations on A.D.P.-induced platelet aggregation. Statistical differences between tests and controls at 2 and 10 minutes are as follows: * $P<0·1$, ** $P<0·05$, *** $P<0·02$, **** $P<0·01$, ***** $P<0·001$. × - - - - × Mean of seven observations with addition of RA433. ●——● Mean of seven control observations.

The results of experiments with collagen as the aggregating agent are shown in Fig. 2, which shows the mean of seven controls and seven tests with RA433 present at a concentration

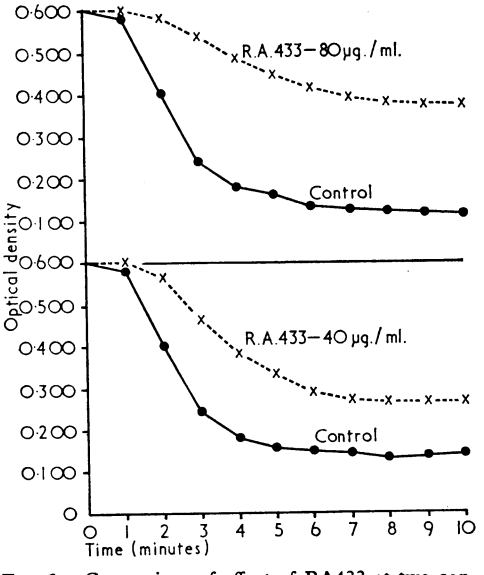

FIG. 2.—Comparison of effect of RA433 at two concentrations in inhibiting collagen-induced aggregation in a turbidimetric system. × - - - × Mean of seven observations with addition of RA433. ●——● Mean of seven control observations.

of 40 and 80 μg./ml. of plasma. At both concentrations there is significant inhibition of platelet aggregation ($P<0·001$) at both 2 and 10 minutes.

Similar results were obtained with noradrenaline as the aggregating agent. Fig. 3 shows the mean of seven controls and tests with RA433 present at a concentration of 80 μg./ml. There is a significant difference ($P<0.001$) between test and control experiments at 2 and at 10 minutes.

A comparison of the action of RA433 at a concentration of 20 μg./ml. and various concentrations of dipyridamole is shown in Fig. 4. The experiments were all carried out with aliquots from a single sample of platelet-rich plasma kept at room temperature. The control line in the graph is the mean of eight control experiments carried out in the course of one and a half hours. In this comparison it is the ability of RA433 to inhibit aggregation which is the striking feature; RA433 is shown to inhibit A.D.P.-induced platelet aggregation at a much lower concentration than dipyridamole.

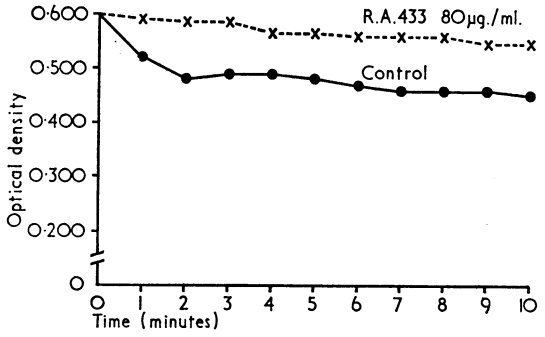

FIG. 3.—Effect of RA433 in inhibiting noradrenaline-induced platelet aggregation in a turbidimetric system. × - - - × Mean of seven observations with the addition of RA433. ●——● Mean of seven control observations.

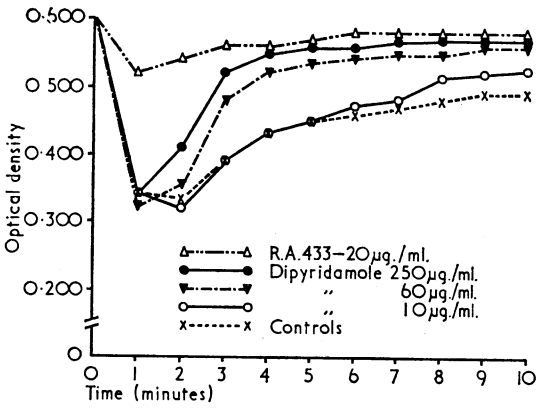

FIG. 4.—Comparison of dipyridamole and RA433 in inhibiting A.D.P.-induced platelet aggregation in a turbidimetric system.

Glass-bead-column Technique

The mean and standard deviations of seven controls and seven tests with various concentrations of RA433 are shown in Table II. A significant difference in platelet adhesiveness is present when the concentration of RA433 is 30 μg./ml. of plasma and above.

TABLE II.—*Effect of Addition of RA433 in Various Concentrations on Platelet Adhesiveness (Modified Hellem Technique) Compared with Controls. (Mean Values and Standard Deviations of Seven Experiments are Shown in Each Case)*

Concentration of RA433 (μg./ml. Plasma)	Per cent. Adhesiveness		*t*	P
	Control	Test		
10	68±6	67±6	0·65	>0·1
30	52±16	36±15	5·94	0·01>P>0·001
80	56±18	23±10	15·7	>0·001

Discussion

We have shown that RA433 at levels which might well be achieved following administration by mouth to man inhibits platelet aggregation induced by A.D.P., collagen, and noradrenaline, and also significantly reduces platelet adhesiveness. These findings are complementary to the observations of Elkeles *et al.* (1968), who found that RA433 inhibits A.D.P.-induced and noradrenaline-induced platelet aggregation. These workers also showed that RA433 had a striking effect on the changes in electrophoretic mobility induced by A.D.P. and noradrenaline in addition to being some 40 to 100 times more active than dipyridamole in aggregation and electrophoretic studies.

The differences between dipyridamole and RA433 are not only quantitative; RA433 inhibits platelet aggregation induced by noradrenaline, while this is not a feature of the action of dipyridamole (Emmons *et al.*, 1965a). Conversely dipyridamole prevents the formation of white bodies in injured cerebral arteries in the rabbit (Elkeles *et al.*, 1968), whereas RA433 does not. Furthermore, in previous studies it was shown that dipyridamole delayed the initial stages of artificial thrombus formation of a Chandler tube system (Gray *et al.*, 1968), but RA433 in the present investigation did not have this effect.

The mechanism whereby RA433 inhibits platelet adhesiveness and platelet aggregation in response to A.D.P., collagen, and noradrenaline is not known. In view of these findings RA433 clearly has potential therapeutic importance in the prevention of thrombus formation, and a clinical pharmacological investigation should be started in man. In animal toxicity studies the drug appears to be relatively non-toxic and free from vasomotor side-effects (J. H. Shelley, personal communication, 1967).

We are grateful to Professor E. M. McGirr for his interest in this work and to the Medical Research Council for financial support. We thank Dr. J. H. Shelley, of Boehringer Ingelheim Ltd., for the supply of RA433. We gratefully acknowledge the technical help provided by Mrs. E. Cunningham.

REFERENCES

Born, G. V. R. (1958). *Biochemical Journal*, **68**, 695.
Born, G. V. R. (1962). *Nature*, **194**, 927.
Born, G. V. R., and Cross, M. J. (1963). *Journal of Physiology*, **168**, 178.
Born, G. V. R., Haslam, R. J., Goldman, M., and Lowe, R. D. (1965). *Nature*, **205**, 678.
Born, G. V. R., Honour, A. J., and Mitchell, J. R. A. (1964). *Nature*, **202**, 761.
Bunag, R. D., Douglas, C. R., Imai, S., and Berne, R. M. (1964). *Circulation Research*, **15**, 83.
Chandler, A. B. (1958). *Laboratory Investigation*, **7**, 110.
Cunningham, G. M., McNicol, G. P., and Douglas, A. S. (1965). *Lancet*, **1**, 729.
Dacie, J. V. (1956). *Practical Haematology*, 2nd ed. London, Churchill.
Duguid, J. B. (1955). *British Medical Bulletin*, **11**, 36.
Eberth, C. J., and Schimmelbusch, C. (1888). *Die Thrombose nach Versuchen und Leichenbefunden*. Enke, Stuttgart.
Elkeles, R. S., Hampton, J. R., Honour, A. J., Mitchell, J. R. A., and Prichard, J. S. (1968). *Lancet*, **2**, 751.
Emmons, P. R., Harrison, M. J. G., Honour, A. J., and Mitchell, J. R. A. (1965a). *Nature*, **208**, 255.
Emmons, P. R., Harrison, M. J. G., Honour, A. J., and Mitchell, J. R. A. (1965b). *Lancet*, **2**, 603.
Gray, G. R., Wilson, P., and Douglas, A. S. (1968). *Scottish Medical Journal*, **13**, 409.
Hellem, A. J. (1960). *Scandinavian Journal of Clinical and Laboratory Investigation*, **12**, Suppl., p. 51.
Hirsh, J., McBride, J. A., and Dacie, J. V. (1966). *Australasian Annals of Medicine*, **15**, 122.
Honour, A. J., and Mitchell, J. R. A. (1963). *Nature*, **197**, 1019.
Mitchell, J. R. A., and Sharp, A. A. (1964). *British Journal of Haematology*, **10**, 78.
Prentice, C. R. M., McNicol, G. P., and Douglas, A. S. (1966). *Journal of Clinical Pathology*, **19**, 343.
Zucker, M. B., and Borrelli, J. (1961). In *Blood Platelets*, edited by S. A. Johnson, R. W. Mouto, J. W. Rebuck, and R. C. Horn, jun. Boston, Little and Brown.
Zucker, M. B., and Borrelli, J. (1962). *Proceedings of the Society for Experimental Biology and Medicine*, **109**, 779.