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INHIBITION OF PLATELET AGGREGATION BY 2-(*p*-CHLOROPHENYL)-4-THIAZOLEACRYLIC ACID (Wy-23,049)—COMPARISON WITH ACETYLSALICYLIC ACID

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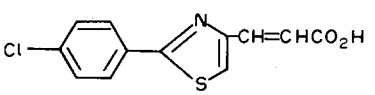
Abstract—2-(*p*-Chlorophenyl)-4-thiazoleacrylic acid (Wy-23,049) inhibited the first phase of adenosine diphosphate (ADP) and epinephrine-induced platelet aggregation, while acetylsalicylic acid (ASA) had little effect on the first but effectively inhibited the second phase. ASA was more effective than Wy-23,049 in inhibiting collagen-induced platelet aggregation. An *ex vivo* guinea-pig experiment demonstrated that Wy-23,049 inhibited the first phase and prevented the appearance of the second phase of ADP-induced platelet aggregation, while ASA inhibited the second phase. Oral administration of Wy-23,049 to rats was much more effective than ASA in protecting the animal against ADP-induced platelet loss. At an oral dose of 40 mg/kg, Wy-23,049 prolonged the Lee–White clotting time in rats; ASA did not prolong it.

THE MEDIATING effect of adenosine diphosphate (ADP) on platelet aggregation[1] has been recognized as an initiator of intravascular thrombosis.[2] The collagen-induced release of intrinsic ADP and other constituents from platelets (the release reaction) is inhibited by aspirin.[3] Two waves of platelet aggregation are observed when epinephrine is added to human platelet-rich plasma (PRP): the initial aggregation and the secondary one resulting from the release of ADP from the platelets. Aspirin inhibits primarily the second phase of platelet aggregation.[4,5] Extensive study of aspirin's capacity to inhibit aggregation has led to its clinical investigation as a potential antithrombotic agent. It has also raised the possibility that other compounds which inhibit aggregation might be more effective than aspirin as antithrombotic agents.

For a number of years we have been examining the platelet aggregation inhibitory activity of many compounds of varied structure. When aspirin emerged as a clinical candidate, we directed our attention to finding compounds that might better satisfy the requirements for an antithrombotic agent.

We present here the results we have obtained from an investigation of 2-(*p*-chlorophenyl)-4-thiazoleacrylic acid (Wy-23,049) as compared with aspirin (ASA). The results indicate that this new compound might have value as a practical antithrombotic agent.



Wy-23,049

MATERIALS AND RESULTS

Human blood was collected in siliconized 50-ml Vacutainers (Becton & Dickinson) fitted with 18-gauge needles using 3·8% sodium citrate as the anticoagulant (9 parts of blood to 1 part of sodium citrate). Platelet-rich plasma (PRP) was separated from the red blood cells by centrifugation at 900 *g* for 2 min at 5°. This centrifuge temperature was used to prevent heating of the blood during the centrifugation; the temperature of the blood after centrifugation did not go below 18·5°. PRP, the upper two-thirds of the supernatant, was carefully separated from the red cells using a siliconized Pasteur pipette. To obtain platelet-poor plasma (PPP) for standardization of the platelet aggregation module, the remaining portion of the plasma was centrifuged at 1000 *g* for 10 min at 25°. The number of platelets in the PPP was in the range of 3000–7000 platelets mm^3.

The effect of compounds on platelet aggregation *in vitro* was studied by a modification of the procedure of Born and Cross,[6] using a Payton Aggregation Module (Payton Associates, Inc.), which is based upon the instrument developed by Mustard *et al.*[7] In the assay, a siliconized cell containing 1·0 ml of PRP and a siliconized stirring bar were placed in the densitometer regulated at 37° with stirring at 1100 rev/min. The test compound was added in 0·2 ml of buffered saline (0·148 M NaCl–0·02 M Trizma, Sigma Chemical Co.) at pH 7·4 equilibrated with the PRP for 5 min. At 0 time either ADP (Sigma Chemical Co.), epinephrine (Mann Research Laboratories), or collagen (prepared from Sigma bovine achilles tendon by the procedure described by Packham *et al.*[8]) was added to the test system in 0·1 ml of buffered saline to induce platelet aggregation. Suitable controls without added compound were also run for each of these systems.

To study the protective effect of the test compounds on aggregation induced by altered surface charge, poly-D,L-lysine[9] (Research Products Division, Miles Laboratories, Inc.) was added to the test system after the addition of the test compound.

To determine the effect of cyclic AMP build up on aggregation, 0·004 μg/ml of prostaglandin E_1 (PGE_1) was incubated with the compound before ADP was added.

In these systems *in vitro*, results are reported as compound concentrations that inhibit first phase aggregation by greater than 40 per cent. At concentrations where inhibition of the second phase of platelet aggregation occurred, it was always 90 per cent or greater.

The capacity to inhibit ADP-induced depletion of circulating platelets was determined by administering the test compound by gavage as a 1-ml aqueous suspension to male Charles River CD rats (4–6 per group). At 30 min after dosage, a 0·1-ml control blood sample was obtained by cardiac puncture, after which 15 mg/kg of ADP was injected into the leg vein. Cardiac blood samples were taken 20, 40 and 60 sec after the ADP injection. The control rats were treated in the same manner except that they received 1 ml of water. PRP was separated from the red blood cells by gravity sedimentation in a plastic tube. Three μl PRP was diluted with 18 ml Isoton (Coulter Electronics, Inc.) and counted in a Coulter counter according to the procedure of Bull *et al.*[10] The 20- and 40-sec time intervals were the most meaningful, because at 60 sec the rats were beginning to recover from the effects of the ADP, with a concomitant rise in circulating platelets in both the control and treated groups.

When guinea-pigs were used to determine the effect of Wy-23,049 and aspirin on

circulating platelets, they were fasted 17 hr prior to the experiment. Wy-23,049 or aspirin was solubilized in dimethylsulfoxide (DMSO, Reagent A.C.S. Matheson Coleman & Bell) at 150–200 mg/ml; 40 mg/kg was administered intraperitoneally. Comparable volumes of DMSO were injected into matched control animals and blood samples were taken after 1 hr, following the procedure used for the rat studies. Platelet counts for the most part fell within the range of 200,000–300,000 platelets/mm^3. PRP samples with platelet counts greater than 300,000 were diluted with PPP from the same guinea-pig so that the final count fell within the indicated range.

To test the effect on blood coagulability, the compounds were dissolved or suspended in water and administered by the oral route to fasted male rats, allowing various time intervals for absorption. A control group received the same volume of water alone. Blood was obtained from both the control and experimental groups by cardiac puncture for the determination of Lee–White clotting times using a three-tube system. Blood was obtained in this manner because of the necessity of getting a free flowing blood sample rapidly without resorting to any manipulative surgical procedure.

Wy-23,049 was prepared by Dr. K. Heatherington of John Wyeth & Brother, Taplow, England. The water-soluble K form of acetylsalicylic acid (ASA) was prepared by Dr. W. H. McGregor, Wyeth Research Division. Indomethacin (Indocin) was obtained from the Merck, Sharp & Dohme Division of Merck & Co., Inc., West Point, Pa. Prostaglandin E_1 was prepared by Dr. D. Strike of the Wyeth Research Division.

RESULTS

In the 2 μM ADP test system at 2·5 and 3·0 $\times$ 10^{-3} M, Wy-23,049 effectively inhibited the first phase of platelet aggregation and completely inhibited the second phase (Table 1). At 1·25 $\times$ 10^{-4} M, ASA effectively inhibited the second phase of platelet aggregation, but as has been indicated by other investigators,[5,11] it does not inhibit the first phase. The same situation was observed for epinephrine-induced aggregation where Wy-23,049 at 3·0 $\times$ 10^{-3} M inhibited the first and second phases, while ASA inhibited only the second phase but at the lower concentration of 2·5 $\times$ 10^{-4} M.

ASA inhibited collagen-induced platelet aggregation at 2·5 $\times$ 10^{-4} M, while 3·0 $\times$ 10^{-3} M Wy-23,049 was required to achieve a comparable level of inhibition.

When polylysine, an agent which alters the platelet surface charge, was added to human PRP to induce aggregation, a 3·7 $\times$ 10^{-3} M concentration of Wy-23,049 effectively inhibited aggregation, while a 5·0 $\times$ 10^{-3} M concentration of ASA was ineffective.

When a minimal concentration of PGE_1 (0·004 μg/ml) is added to PRP, a slight inhibition of ADP-induced platelet aggregation is observed. In this system, 2·0 $\times$ 10^{-3} M Wy-23,049 inhibited aggregation, while 5·0 $\times$ 10^{-3} M ASA had no effect.

Wy-23,049 was more active than ASA in protecting against ADP-induced platelet loss in rats. At an oral dose of 50 mg/kg, allowing 30 min for absorption, Wy-23,049 effectively protected the platelets at 20 and 40 sec after the ADP injection, while ASA did not (Table 2). At an oral dose of 10 mg/kg, Wy-23,049 was active at the 20-sec time interval but did not show a statistically significant difference from the control

TABLE 1. EFFECT OF ASA AND Wy-23,049 ON PLATELET AGGREGATION IN DIFFERENT TEST SYSTEMS*

System	ASA final concn ($M \times 10^3$)	Platelet aggregation (% Inhibition ± S.E.) First phase	Second phase	Wy-23,049 final concn ($M \times 10^3$)	Platelet aggregation (% Inhibition ± S.E.) First phase	Second phase
ADP (2 μM)	0·125	0	90 ± 12·2	2·5	52·7 ± 10·4	100
				3·0	59·7 ± 5·2	100
Epinephrine (5 μM)	0·25	6·7 ± 8·1	100	3·0	64·0 ± 7·1	100
Collagen (50 μl/ml)	0·25	73·0 ± 6·5		3·0	65·7 ± 8·0	
Polylysine (0·91 mg/ml)	5·0	5·3 ± 4·8		3·7	42·7 ± 1·6	
PGE_1-treated platelets (0·004 μg/ml) 3 μM ADP	5·0	6·0 ± 3·9		2·0	52·2 ± 11·2	
				2·5	61·0 ± 13·1	

* Values shown in this table represent the mean of three determinations run on different days using different platelet-rich plasmas. Results are recorded in the first phase column when only one phase is observed.

TABLE 2. COMPARISON OF THE PLATELET PROTECTIVE ACTIVITY OF Wy-23,049 WITH ASA AND INDOMETHACIN AFTER INTRAVENOUS ADMINISTRATION OF ADP

Compound*	Dose (mg/kg)		Platelet loss at stated time intervals after administration of ADP† (Mean % ± S.E.M.) 20 sec Control	20 sec Compound	40 sec Control	40 sec Compound	60 sec Control	60 sec Compound
Wy-23,049	50		60·6 ± 2·0 (10)‡	49·3 ± 2·7 (7)	45·8 ± 1·9 (8)	38·0 ± 1·6 (7)	22·3 ± 4·3 (8)	21·6 ± 6·3 (8)
		P	< 0·01		< 0·01		NS§	
	10		57·8 ± 2·3 (8)	44·7 ± 2·2 (9)	47·9 ± 2·6 (7)	39·9 ± 4·8 (7)	23·3 ± 7·9 (6)	25·3 ± 6·6 (7)
		P	< 0·01		NS		NS	
ASA	50		67·0 ± 2·7 (4)	63·0 ± 3·8 (6)	51·5 ± 6·4 (4)	48·0 ± 3·0 (6)	24·0 ± 4·4 (4)	28·8 ± 8·3 (5)
		P	NS		NS		NS	
Indomethacin	50		61·6 ± 1·5 (5)	49·6 ± 2·3 (5)	46·0 ± 2·7 (5)	37·8 ± 2·7 (5)	31·0 ± 8·3 (4)	25·0 ± 4·5 (5)
		P	< 0·01		NS		NS	

* Administered by the oral route allowing 30 min for absorption.
† Fifteen mg/kg.
‡ Number of animals in group is given in parentheses.
§ Not significantly different (Student's *t*-test).

TABLE 3. EFFECT OF ABSORPTION TIME ON THE PLATELET PROTECTIVE ACTIVITY OF Wy-23,049 IN RATS AFTER INTRAVENOUS ADMINISTRATION OF ADP

Dose of compound* (mg/kg)		Absorption time (min)	Platelet loss at stated time intervals after administration of ADP† (Mean % ± S.E.M.) 20 sec Control	20 sec Compound	40 sec Control	40 sec Compound	60 sec Control	60 sec Compound
40		30	58·2 ± 3·2 (5)‡	46·2 ± 1·8 (5)	46·5 ± 3·9 (4)	31·0 ± 5·3 (4)	28·0 ± 5·0 (4)	31·5 ± 2·8 (4)
	P		< 0·02		< 0·05		NS§	
40		60	60·0 ± 2·3 (5)	49·8 ± 2·3 (5)	43·8 ± 3·8 (5)	31·6 ± 6·3 (5)		
	P		< 0·02		NS			
40		120	60·0 ± 2·3 (5)	39·0 ± 3·0 (5)	43·8 ± 3·8 (5)	29·8 ± 5·6 (4)	15·3 ± 7·7 (4)	10·3 ± 5·5 (3)
	P		< 0·01		NS		NS	
40		180	60·0 ± 2·3 (5)	50·5 ± 1·9 (4)	43·8 ± 3·8 (5)	40·3 ± 2·9 (4)		
	P		< 0·02		NS			

* Administered by the oral route.
† Fifteen mg/kg.
‡ Number of animals in group is given in parentheses.
§ Not significantly different (Student's *t*-test).

at 40 or 60 sec. At 50 mg/kg, indomethacin evinced platelet protective activity only at the 20-sec time interval.

To determine the duration of action of Wy-23,049, single oral doses of 40 mg/kg were administered to separate groups of rats 30, 60, 120 or 180 min prior to challenge with ADP. In the 30-min tests, Wy-23,049 had a platelet protective effect 20 and 40 sec after the ADP challenge (Table 3). When administered 60, 120 or 180 min in advance, it protected at the 20-sec interval. It was not effective in any experiment 60 sec after the injection of ADP. The protective activity of Wy-23,049 was diminished when it was administered 180 min before the ADP challenge.

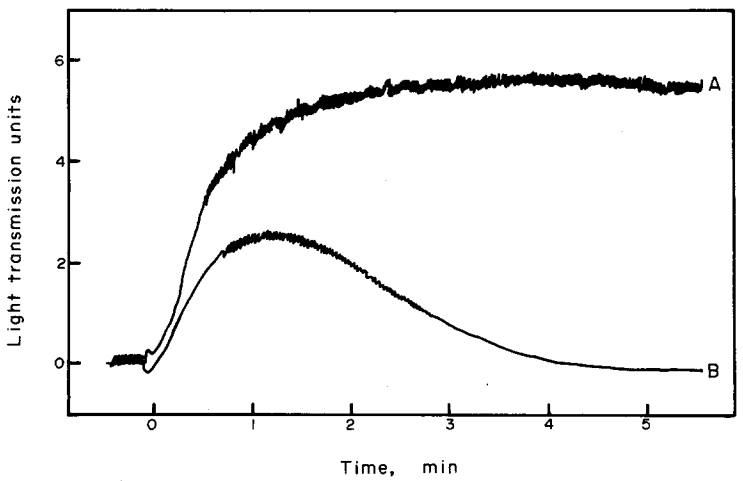


FIG. 1. Effect of an intraperitoneal dose of 40 mg/kg of Wy-23,049 on 1 μM ADP-induced platelet aggregation of the PRP obtained from the fasted guinea-pig 1 hr after compound administration. (A) Control guinea-pig; (B) Wy-23,049-treated guinea-pig.

Our examination of the effect of Wy-23,049 in preventing ADP-induced platelet loss in rats indicated that 40 mg/kg of Wy-23,049 was an effective dose that gave a good duration of activity. The effect of Wy-23,049 and ASA in guinea-pig PRP was, therefore, determined 1 hr after intraperitoneal administration, each at a dose of 40 mg/kg. Typical plots of ADP-induced platelet aggregation obtained from individual animals treated with these compounds in comparison with PRP obtained from untreated control animals are shown in Figs. 1 and 2. At this dose, Wy-23,049 effectively inhibited the first and second phases of platelet aggregation and ASA the second phase. Five control and six Wy-23,049-treated animals were used in this experiment, and the data were significant with a P value $< 0{\cdot}01$; six control and seven ASA-dosed experimental animals were used, and the results had a P value of $< 0{\cdot}05$ for second phase inhibition. Using the method described by Mackenzie *et al.*,[12] Wy-23,049 inhibits the first phase of platelet aggregation by 51·8 per cent, while ASA produced an 89·6 per cent inhibition of the second phase.

The effect of ASA and Wy-23,049 on Lee–White clotting time in rats is shown in Table 4. Over a dosage range of 10–40 mg/kg, Wy-23,049 prolonged the clotting time, while aspirin shortened it. At 40 mg/kg, Wy-23,049 induced a statistically significant prolongation of the clotting time.

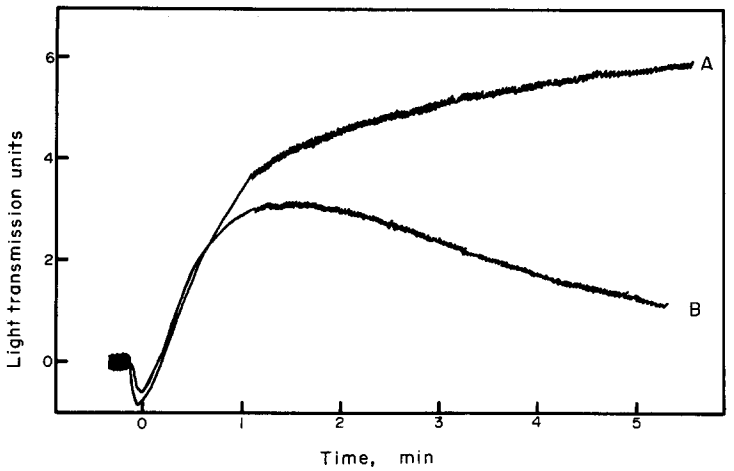


FIG. 2. Effect of an intraperitoneal dose of 40 mg/kg of ASA on 1 μM ADP-induced platelet aggregation of the PRP obtained from the fasted guinea pig 1 hr after compound administration. (A) Control guinea-pig; (B) compound-treated guinea-pig.

DISCUSSION

Acetylsalicylic acid has been reported to inhibit the second phase of ADP-induced platelet aggregation in human platelet-rich plasma.[5] Our experiments conducted with human PRP have indicated that $1{\cdot}25 \times 10^{-4}$ M ASA inhibits the second phase. Wy-23,049 markedly inhibits the first phase of aggregation and abolishes the second phase at $2{\cdot}5 \times 10^{-3}$ M concentration. It has been believed for some time that ADP is the physiological mediator of platelet aggregation,[2,13] and extensive research has demonstrated its importance as a potential mediator of thrombus formation. Therefore, a highly desirable attribute of an antithrombotic agent would be the capacity to inhibit the triggering effect of ADP on platelet aggregation without materially altering the balance of other factors in the clotting process. On this basis, a difference between ASA and Wy-23,049 emerges, that is ASA effectively inhibits the second phase of platelet aggregation as has been reported by O'Brien[5] but has little or no effect on the first phase, whereas at concentrations where Wy-23,049 effectively inhibits ADP-induced platelet aggregation it is effective against the first phase. This same

TABLE 4. EFFECT OF DIFFERENT DOSES OF Wy-23,049 AND ASA ON THE LEE–WHITE CLOTTING TIME IN RATS*

	Wy-23,049 (mg/kg)	No. of rats	Mean Lee–White C.T. at 1 hr (sec ± S.E.)	ASA (mg/kg)	No. of rats	Mean Lee–White C.T. at 1 hr (sec ± S.E.)
Control		9	485 ± 16·1		9	488·3 ± 14·1
	10	7	518·6 ± 10·8†	10	6	457·5 ± 27·7†
	20	9	505·0 ± 11·3†	20	8	470·6 ± 20·3†
Control		12	401·3 ± 13·9			
	40	8	459·4 ± 18·4‡	40	7	447·9 ± 8·2§

* The ASA study was completed in a single experiment, necessitating only one control value (488·3 ± 14·1).

† Not significantly different from control (Student's t-test).

‡ Significantly increased over control ($P < 0{\cdot}02$, Student's t-test).

§ Significantly decreased from control ($P < 0{\cdot}05$, Student's t-test).

relationship exists for epinephrine-induced platelet aggregation; ASA is an effective inhibitor of the second phase, and at a higher concentration Wy-23,049 inhibits the first phase of aggregation as well as the second phase.

ASA has been reported to be a good inhibitor of collagen (connective tissue preparation)-induced platelet aggregation. Our study indicated that it is a more effective inhibitor of collagen-induced platelet aggregation than Wy-23,049.

Polylysine induces rapid platelet aggregation,[9] presumably through alteration of the surface charge, without significant release of platelet constituents. Under the conditions used, Wy-23,049 was more effective than ASA in preventing polylysine-induced platelet aggregation.

In order to do a comparative study *in vivo* of Wy-23,049 with ASA, a system was developed in which the activities of these compounds were compared in rats by determining their capacity to prevent ADP-induced platelet loss. The starting point for the development of this system was the experiments of Nordoy and Chandler[14] in which they established the LD_{50} for intravenous administration of ADP to rats to be 166 mg/kg. If an ADP concentration could be found that would induce a significant platelet loss without killing the rats, the capacity of various compounds to ameliorate this loss could then be determined. This effect would only be transitory, as has been pointed out by Bosshardt and Howe,[15] and it was for this reason that cardiac puncture was used to obtain blood 20, 40 and 60 seconds after the i.v. administration of ADP. The optimal dose of ADP for use in this system was found to be 15 mg/kg.

In the experiments using this system *in vivo*, Wy-23,049 effectively protected against ADP-induced platelet loss at 50 mg/kg, while ASA was ineffective at this dose. Indomethacin was marginally effective at 50 mg/kg. The effective limit for platelet protection of a 40 mg/kg of Wy-23,049 was 180 min, at which time its platelet protective activity was greatly diminished. An oral dose of Wy-23,049 as low as 10 mg/kg gave partial platelet protective activity 30 min after administration. In this system *in vivo*, we are measuring the sum total of many events that alter the intravenous administration of ADP, and the effect of Wy-23,049 or ASA in mitigating platelet loss. These events are no longer defined by whether the orally administered compound exerts its principal activity on the first or second phase of platelet aggregation, but rather on how well the viability of the platelets is preserved in the presence of ADP. Under these conditions, Wy-23,049 is a more effective protective agent than ASA.

In a comparative experiment in which the activity of Wy-23,049 and ASA was measured in the guinea-pig after intraperitoneal administration, Wy-23,049 was shown to significantly inhibit the first and second phases of platelet aggregation, and ASA to significantly inhibit the second phase.

In order to bridge the gap between the effect of ASA and Wy-23,049 on platelets and clotting, their effect on the Lee–White clotting time in rats was determined. Wy-23,049 effectively prolonged blood clotting 1 hr after oral administration at a dose of 40 mg/kg. The effect of ASA was less consistent, but at this same dose and time interval it shortened the clotting time. It is difficult to obtain consistent results with the Lee–White clotting time in animals and, therefore, great care was taken in its performance, and a three-tube train system was employed to make it as accurate as possible. Blood was obtained from the heart so that a rapid free flow of blood could be obtained. Any other manipulative procedure that we might have used to obtain

blood we felt might affect the clotting time. Under these conditions, at an oral dose of 40 mg/kg, Wy-23,049 prolonged the clotting time and ASA tended to shorten it.

Hampton *et al.*[16] expressed the difficulty that investigators face in trying to evaluate antithrombotic activity of compounds based upon their effect on platelets. He indicated that despite extensive research with platelets we do not know the specific requirements for antithrombotic activity and thus the ultimate test of the efficacy of new compounds rests with man.

Our chief investigative emphasis has been on the activity *in vivo* of Wy-23,049 and ASA. In the systems employed, Wy-23,049 appears to be more active than ASA. Our *ex vivo* guinea-pig experiment re-emphasizes our observation *in vitro* that the primary activity of ASA is associated with inhibition of the second phase of platelet aggregation, while Wy-23,049 primarily inhibits the first phase.

Preliminary investigation has indicated that Wy-23,049 has a low acute toxicity and appears to be less irritating than ASA to the gastric mucosa. Detailed toxicity studies are currently in progress.

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