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7-Bromo-1,5-Dihydro-3,6-Dimethylimidazo[2,1-*b*]Quinazolin-2(3H)-One (Ro 15-2041), a Potent Antithrombotic Agent that Selectively Inhibits Platelet cyclic AMP-Phosphodiesterase

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ABSTRACT

This study with the new analog Ro 15-2041 (7-bromo-1,5-dihydro-3,6-dimethylimidazo[2,1-*b*]quinazolin-2(3H)-one) confirms and substantially extends the activity spectrum of imidazoquinazolinones as potent platelet function inhibitors. Ro 15-2041 inhibited platelet aggregation induced by all common platelet agonists in platelet-rich plasma obtained from various species including man (IC_{50} = 1–3 μM). The compound potentiated platelet inhibition by prostacyclin, the prostacyclin-induced increase of intraplatelet cyclic (c) AMP levels and inhibited the collagen-induced release of serotonin and β-thromboglobulin. Ro 15-2041 reduced the increase and accelerated the normalization of cytosolic free Ca^{++} in thrombin-stimulated human platelets. Ro 15-2041 is a potent (IC_{50} = 70 nM) and selective inhibitor of platelet cAMP-phosphodiesterase activity. Whereas Ro 15-2041 caused complete inhibition of cAMP-phosphodiesterase activity in human platelet supernatants, breakdown of cAMP in cardiac homogenates was depressed to maximally 50%. In human brain and rabbit uterus Ro 15-2041 was at least 1000 times less potent. By comparison, papaverine fully inhibited phosphodiesterase activity in all four tissues with similar IC_{50} values of about 5 μM. Furthermore, Ro 15-2041 selectively inhibited cAMP-phosphodiesterase activity of a bovine calmodulin-independent but not of a calmodulin-dependent enzyme preparation. The compound exhibited significant p.o. activity in various *ex vivo* and *in vivo* platelet function tests. In Stumptail monkeys the ID_{50} was about 2.5 μmol/kg 2 and 4 hr after dosing. After 5 μmol/kg p.o. the platelet inhibitory effect persisted for more than 6 hr and was paralleled by an increase in cardiac rate. In rabbits, the compound prolonged the bleeding time and inhibited the platelet drop and the *in vivo* release of platelet specific proteins after i.v. injection of collagen fibrils. The results indicate that Ro 15-2041 inhibits platelet function by increasing cAMP levels through inhibition of cAMP-phosphodiesterase activity, thereby increasing the capacity of platelets to prevent and to reverse Ca^{++} transients.

Platelet adhesion to the vessel wall and subsequent aggregation are among the initial events (Fuster and Chesebro, 1981a) in the pathogenesis of arterial thrombosis, which usually develops in arteriosclerotic vessels under conditions of high blood flow (Turitto and Baumgartner, 1982). Therefore, and because the efficacy of anticoagulants in preventing arterial thrombosis is still controversial (Braunwald, 1980; Sixty Plus Reinfarction Study Research Group, 1980), agents that are capable of inhibiting platelet function have received a great deal of attention.

Among the many compounds that inhibit platelet function *in vitro* (Seuter and Scriabine, 1984), only a few have thus far been shown to have potential for clinical use in terms of safety, efficacy when taken p.o., sustained effect and therapeutic benefit in the prevention of thromboembolic events. Aspirin, sulfinpyrazone and dipyridamole are clinically the most extensively investigated. Such weak platelet function inhibitors, as they are *in vitro* and in laboratory models of thrombosis, have shown activity, but in selected clinical applications or have rare but severe side effects (Fuster and Chesebro, 1981b). Thus, more potent and specific inhibitors of platelet function could be more useful than the currently available agents in preventing thrombosis.

Fleming *et al.* (1975) and Fleming and Buyniski (1983) reported on two imidazoquinazolinone derivatives, namely BL-3459 and anagrelide, to be potent broad-spectrum platelet function inhibitors and to be effective in a number of animal thrombosis models. The activity profile and the potent inhibitory activity of imidazoquinazolinones on platelet cAMP phosphodiesterase (Tang and Frojmovic, 1980) suggested that the effect of members of this chemical class on platelets is mediated by cAMP, as all compounds known to increase intracellular cAMP concentration inhibit platelet function irrespective of the stimulus used (Mills, 1982).

Here we demonstrate that the new analog, 7-bromo-1,5-dihydro-3,6-dimethylimidazo[2,1-*b*]quinazolin-2(3H)-one (Ro

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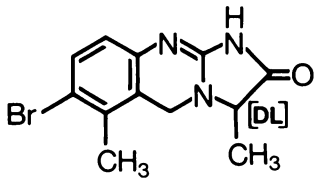
ABBREVIATIONS: cAMP, cyclic AMP; PRP, platelet-rich plasma; ASA, acetylsalicylic acid; PGI_2, prostacyclin; TG, thromboglobulin; PG, prostaglandin.

15-2041) (Kienzle *et al.*, 1982), is a potent *in vitro*, *ex vivo* and *in vivo* platelet function inhibitor which increases cAMP levels through inhibition of cAMP phosphodiesterase, thereby amplifying the capacity of platelets to maintain Ca^{++} homeostasis. The compound has platelet selectivity because it inhibits platelet cAMP phosphodiesterase preferentially over that of myocardium, brain and smooth muscle.

Materials and Methods

Drugs. Ro 15-2041, depicted as structure 1, was synthesized as the base by M. Chodnekar (F. Hoffmann-La Roche, Basel, Switzerland). Unless indicated, the compound was used as a microsuspension diluted with 0.9% NaCl. The microsuspension was prepared by grinding Ro 15-2041 in a solution of 8.5 or 15% modified gelatin with glass microspheres (0.5–0.75 mm) to an average particle size of < 0.5 μm.



Other drugs used in this study included papaverine hydrochloride and ASA. The respective vehicles from each experiment were used for control experiments. Ro 15-2041 is soluble in dimethyl sulfoxide. In water, at 37°C, the solubility is <1 and about 30 mg/100 ml at pH 7.5 and 1.2, respectively.

Preparation of platelet-rich plasma (PRP). Blood was obtained by antecubital venepuncture from human subjects and Stumptail monkeys (*Macaca arctoides*), by jugular venepuncture from dogs and from the ear artery of rabbits. The blood was drawn into syringes containing 10% of 108 or 90 mM Na-citrate and PRP was prepared by differential centrifugation 10 to 30 min after bloodletting.

Platelet aggregation *in vitro* and *ex vivo*. Prewarmed samples (500 μl) of PRP were incubated with Ro 15-2041, ASA or the vehicle (5 μl) at 37°C for 10 min. Platelet aggregation was induced by the addition of a submaximal concentration of the respective agonists and continuously measured by recording the light transmission in a Born aggregometer (Born, 1962). The maximum aggregation velocity was taken as a measure of the extent of platelet aggregation and expressed as percentage of light transmission increase per minute. The difference in light transmission between PRP and platelet-poor plasma was taken as 100%. The potency of the drug was expressed as the concentration that inhibited platelet aggregation velocity by 50% (IC_{50}).

In *ex vivo* studies, Ro 15-2041 or the vehicle was blended with half a banana and offered to female Stumptail monkeys weighing about 10 kg and which had been fasted overnight. The test meal was swallowed immediately and there was no evidence that part of it was retained in the cheek pouch. The animals received water *ad libitum* and their regular chow 8 and 25 hr after drug administration. Blood was obtained and PRP prepared as described above before and at various times after dosing. Platelet aggregation was induced by the addition of 3, 8, 20 or 50 μl ADP (10^{-4} M in 0.9% NaCl) or collagen (0.1 mg/ml) to 0.5 ml of PRP but was otherwise performed and evaluated as described above. The *ex vivo* potency of the drug was expressed as the dose that inhibited platelet aggregation induced by 1.6 μM ADP by 50% (ID_{50}).

For the study of synergism with PGI_2, Ro 15-2041 suspended in 8.5% modified gelatin was incubated in human PRP at 37°C for 9 min, then PGI_2 in 0.05 M TRIS-buffer, pH 9.0, was added. After a total of 10 min, platelet aggregation was induced with 1 μM ADP but was otherwise performed and evaluated as described above. From averaged dose-response curves at fixed concentrations of one agent while varying the concentration of the other, IC_{50} values were interpolated and plotted as an isobologram, in which each point represents a combination of Ro 15-2041 and PGI_2, which inhibited platelet aggregation by 50% (Loewe, 1953).

Serotonin and β-TG release *in vitro*. Platelets were labeled by incubating citrated human PRP with 0.8 μM [^{14}C]serotonin at 37°C for 10 min. Dilutions of microsuspensions of Ro 15-2041 or ASA dissolved in 8.5% modified gelatin were incubated in 1 ml of PRP at 37°C for 10 min. The diluent was 0.9% NaCl, the maximum volume added was 30 μl. Release of the platelet-bound [^{14}C]serotonin was induced with collagen fibrils (1.6 μg/ml). Collagen-induced release was stopped after 2 min by adding a 500-μl aliquot of an ice-cold antirelease cocktail (Stratton *et al.*, 1982) composed of 800 μl of PGE_1 (100 μg/ml in ethanol), 6.4 ml of ASA (1 M in ethanol) and 80 ml of acid-citrate-dextrose. After having been chilled further in ice-water, the plasma samples were centrifuged at 4°C. The [^{14}C]serotonin in the supernatant was determined by liquid scintillation counting and the [^{14}C]serotonin release was calculated after correction of uptake efficiency. β-TG, which is a releasable platelet-specific protein contained in α-granules (Kaplan *et al.*, 1979), was measured with a commercial β-TG radioimmunoassay. The extent of release in the presence of the respective vehicle controls was taken as 100%. The concentration of the compounds which inhibited release by 50% (IC_{50}) was interpolated from dose-response curves.

Potentiation of PGI_2-induced cAMP increase *in vitro*. Ro 15-2041 suspended in 0.9% NaCl was incubated without stirring for 10 min at 37°C in human PRP. PGI_2 in TRIS-buffer (50 mM, pH 9.0) was added after 10 min to a final concentration of 10 nM. Aliquots were removed 2.5 min after the addition of PGI_2, snap frozen and stored at −20°C. The enzymes were denatured by the addition of 10 to 100 volumes of 90°C H_2O, followed by incubation at 90°C for 5 min. The cell debris was then pelleted at 3000 × *g* for 10 min and the cAMP content in the supernatant measured with a commercial radioimmunoassay. The platelet cAMP content was calculated by subtracting from the PRP the platelet-free plasma control.

Collagen-induced platelet drop and platelet-specific protein

TABLE 1

***In vitro* inhibition of human platelet aggregation induced by various agonists**

Human platelet-rich plasma was incubated for 10 min with Ro 15-2041. Platelet aggregation was then induced with various agonists as indicated and measured in an aggregometer. Results with ASA are given for comparison. Mean ± S.E., *n* = number of blood donors.

Agonist	Final Conc.	*n*	IC_{50} 15-2041	IC_{50} ASA
			μM	
Collagen	0.8 μg/ml	4	1.1 ± 0.3	32
ADP	1 μM	4	2.9 ± 1.0	>300
Serotonin/epinephrine	2 μM/2 μM	4	2.8 ± 0.8	>300
Thrombin	0.3 U/ml	4	1.5 ± 0.3	>300
Na-arachidonate	1 mM	2	1.0 ± 0.4	9.2
Ca-ionophore A23187	1.2 μM	4	1.7 ± 0.7	>300
Platelet-activating factor	0.1 μM	4	2.4 ± 0.7	>300
Dithiothreitol	30 mM	2	>200	

TABLE 2

***In vitro* inhibition of collagen-induced platelet aggregation in various species**

Platelet-rich plasma of the respective species was incubated for 10 min with Ro 15-2041. Platelet aggregation was induced with a suspension of collagen fibrils at a concentration which induced submaximal aggregation. Mean ± S.E., *n* = number of blood donors.

Species	Final Collagen Conc.	*n*	15-2041 IC_{50}
	μg/ml		μM
Humans	0.8	4	1.1 ± 0.34
Monkey	1.6	7	0.27 ± 0.02
Dog	8.0	6	0.59 ± 0.14
Rabbit	3.2	5	0.92 ± 0.51

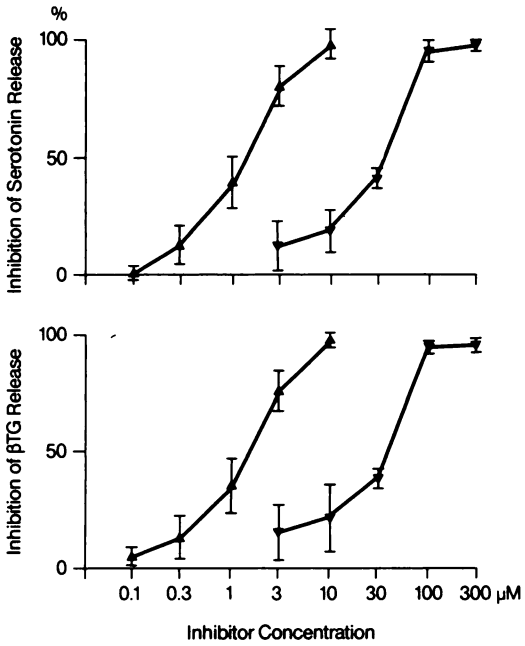


Fig. 1. Serotonin and β-TG release *in vitro*. Platelets loaded with [^{14}C] serotonin were incubated for 10 min with Ro 15-2041 (▲) or ASA (▼). Release induced by 1.6 μg/ml of collagen was stopped after 2 min with an antirelease cocktail. [^{14}C]Serotonin release was determined by liquid scintillation counting, β-TG release was measured by radioimmunoassay. The extent of release in the presence of the respective vehicle controls was taken as 100%. Mean ± S.E. of experiments with blood from three donors.

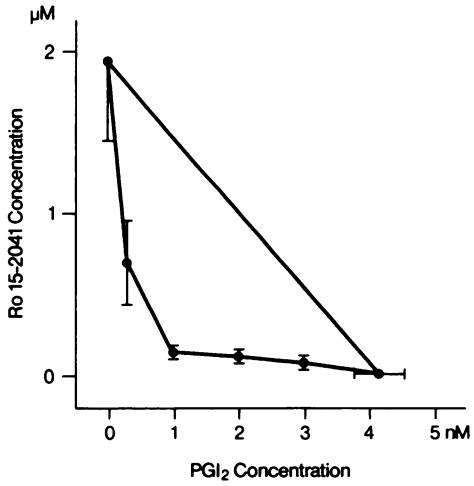


Fig. 2. Isobolographic representation of the effect of Ro 15-2041 on the inhibition of ADP-induced human platelet aggregation by PGI_2. Each point represents a combination of Ro 15-2041 and PGI_2 which inhibited platelet aggregation by 50%. In case of a pure additive effect the points would be on the diagonal. The deflection of the curve to the left of the line indicates superadditivity. Mean ± S.E. of experiments with blood from three donors.

release *in vivo*. Collagen reagent Horm was diluted 1:7 with phosphate-buffered saline (0.075 M NaCl, 0.068 M phosphate, pH 7.4) and 1.5 ml/kg (214 μg/kg) injected i.v. within 1 min into anesthetized rabbits 30 or 120 min after injection of 10 μmol/kg of Ro 15-2041 or ASA. Blood was collected from the Arteria carotis or A. femoralis into EDTA-coated tubes for platelet counting and into tubes containing an antirelease cocktail (see above) for the determination of a rabbit platelet-specific protein in plasma by radioimmunoassay (Muggli *et al.*, 1981). Platelet number and the concentration of rabbit platelet-specific protein were measured in blood taken before and 1 min after the end

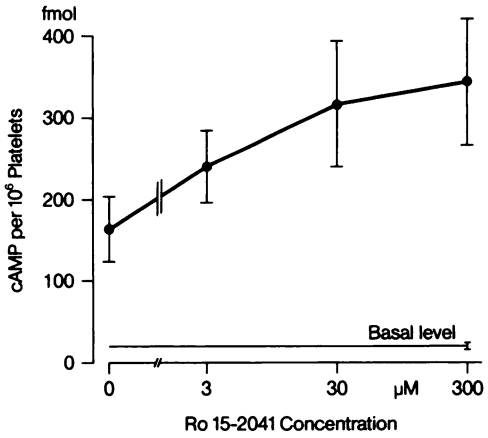


Fig. 3. Potentiation of PGI_2-induced cAMP increase *in vitro*. PRP was incubated for 10 min with Ro 15-2041 at concentrations as indicated, then 10 nM PGI_2 was added. cAMP was determined by radioimmunoassay in aliquots removed 2.5 min after the addition of PGI_2. The platelet cAMP content was calculated by subtracting the platelet-free plasma control from the PRP. The plasma cAMP concentration of the three individual donors was 17.9, 19.9 and 21.4 pmol/ml, respectively. Mean ± S.E. of experiments with blood from three donors.

of the collagen injection. The effect of drugs was assessed by comparing the inhibition of platelet drop and release of rabbit platelet-specific protein in drug-treated *vs.* vehicle-treated animals.

Bleeding time. A standardized incision was made on the inner side of the ear of anesthetized rabbits; large vessels were avoided. The ear was then floated on 0.9% NaCl at 37°C, and the threads of outflowing blood from 1 to 3 microvessels observed until cessation of bleeding. The bleeding time reported is the mean duration of bleeding of at least three threads per ear (if necessary a second or third incision was made). The effect of Ro 15-2041 and ASA was assessed by comparing the bleeding time before and 2 hr after i.v. administration on the right and left ear, respectively.

cAMP-phosphodiesterase assay. PRP was prepared from 90 ml of human blood donated by healthy donors, and the platelets were washed by centrifugation of the PRP at 3000 × *g* for 10 min at 4°C, resuspending the platelet pellet in 50 ml of buffer, pH 6.9, made up with 7 parts of Ringer's solution, 2 parts of 5% glucose and 1 part of 10% trisodium citrate H_2O, and repelleting at 3000 × *g*. Tissue samples of human heart and brain cortex, deep-frozen about 6 hr post mortem, were obtained from the Department of Pathology, University of Basel. Rabbit platelets were isolated and washed as described above. Rabbit tissues were taken from animals which had been perfused with about 1 liter of Krebs-Henseleit solution gassed with O_2 at room temperature.

Platelet pellets or about 1 g of tissue were frozen in liquid nitrogen, thawed and homogenized at 4°C with 0.5 to 1.0 ml of phosphate-buffered saline (20 mOsm, pH 7.4) in an all glass tissue grinder. Rabbit tissues were sonicated for about 15 sec with 100 W (Sonifier B-12, Branson Sonic Power Company, Danbury, CN). The homogenate was centrifuged at 100,000 × *g* for 30 min at 4°C, and aliquots of the supernatant were stored at −60°C.

The phosphodiesterase assay was done as described by Davis and Daly (1979). The reaction mixture (0.1 ml) contained 0.05 nmol of [^{3}H] cAMP in TRIS-HCl (50 mM, pH 7.5), 10 to 45 μl of suitably diluted supernatant and 10 μl of additives as indicated. After 6 min the reaction was stopped by boiling. Unreacted cAMP was separated from 5′-AMP by affinity chromatography on a polyacrylamide-boronate gel (Affi-Gel 601). Blanks containing all buffer constituents except protein were run simultaneously. Conversion of cAMP to AMP was linear with time and with the amount of enzyme. Under control conditions, 10 to 20% of cAMP was hydrolyzed.

Intracellular free Ca^{++} assay. Quin2 is a calcium indicator that increases its fluorescence upon binding of calcium (Rink *et al.*, 1982). Its tetraacetoxymethylester (Quin2/AM) penetrates cell membranes

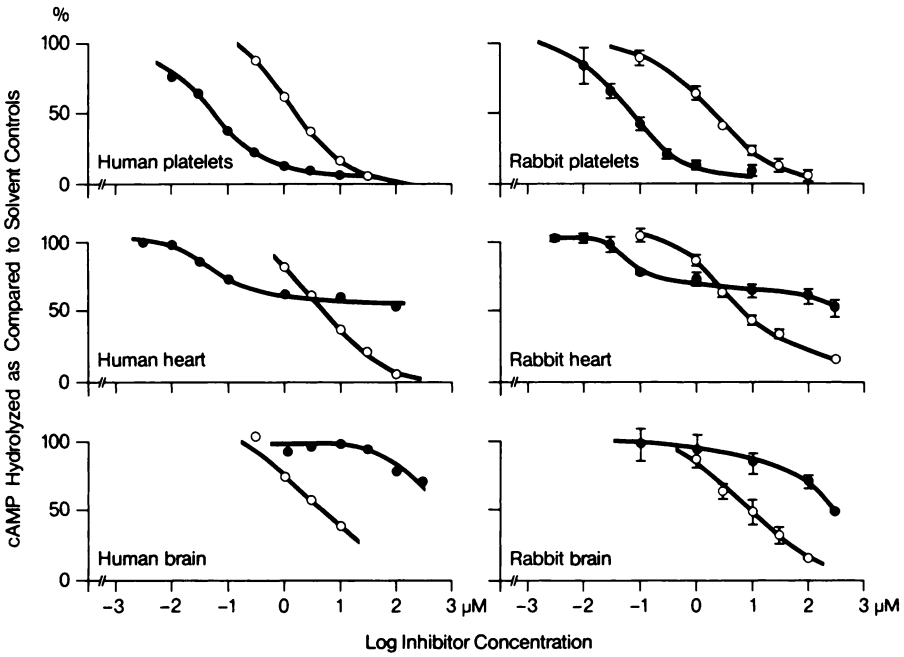


Fig. 4. Effect of Ro 15-2041 (●) and papaverine (○) on the cAMP-phosphodiesterase activity in supernatants of different human and rabbit tissues. Mean ± S.E. of experiments with tissues from three rabbits and two humans.

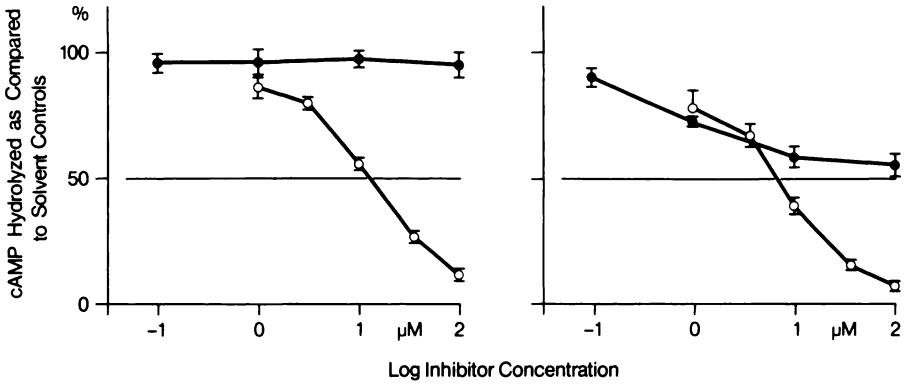


Fig. 5. Effect of Ro 15-2041 (●) and papaverine (○) on the cAMP-phosphodiesterase activity in commercially obtained bovine heart enzyme preparations. Left, calmodulin-dependent cAMP-phosphodiesterase in the presence of 10 μM Ca^{++} and 50 μg/ml of calmodulin. Right, calmodulin-independent cAMP phosphodiesterase. Mean ± S.E., $n = 3$.

readily and is trapped as Quin2 free acid after intracellular hydrolysis. This allows the measurement of cytosolic free calcium in small intact cells under nearly physiologic conditions.

PRP was loaded with 50 μM Quin2/AM in a shaking waterbath at 37°C for 45 min. The loaded platelets were separated from remaining extracellular Quin2/AM by gel-filtration on a Sepharose CL-2B column (5 × 22 cm) at room temperature. The eluting buffer (pH 7.4) contained 145 mM NaCl, 5 mM KCl, 10 mM Tris-HCl, 1 mM $MgSO_4$, 0.5 mM NaH_2PO_4, 5 mM D-(+)-glucose and 0.1% bovine serum albumin. The platelet suspension was adjusted to 50,000/μl and extracellular calcium was restored to 1 mM. The platelets were kept at room temperature until used. The intracellular concentration of Quin2 was 1 to 1.5 mM. Two milliliters of the platelet suspension were incubated with 20 μl of Ro 15-2041 at various concentrations in dimethyl sulfoxide for 1 min followed by 10 μl of 0.3 U/ml of thrombin. For the study of the effect of Ro 15-2041 on re-establishment of free Ca^{++} concentrations, Ro 15-2041 was added 1 min after the thrombin challenge. The fluorescence (F) was monitored continuously in an Aminco SPF 5000 spectrofluorometer in quartz cuvettes (1 cm × 1 cm) and at 37°C (wavelengths: excitation, 339 nm; emission, 492 nm). The platelet suspension was not stirred during the measurement. The intracellular free calcium concentration was calculated from F as $[Ca^{++}]_i = K_d(F-F_{min})/(F_{max}-F)$ according to Rink *et al.* (1982). K_d is 115 nM. F_{min} and F_{max} were determined after the liberation of intraplatelet Quin2 by lysis of the cells. F_{min} is the minimal fluorescence of the free acid in the presence of an excess amount of ethylene glycol bis(aminoethyl ether)-N,N'-tetraacetic acid and F_{max} is the maximal fluorescence of Quin2 fully complexed with calcium.

Reagents. [8-^{3}H] [ammonium salt (24 Ci/mmol)], 5-hydroxy-[side chain 2-^{14}C]tryptamine creatinine sulfate (serotonin, 50–60 mCi/mmol), Quin2tetraacetoxymethyl ester and radioimmunoassay kits for β-TG were obtained from the Radiochemical Centre (Amersham, Bucks, England). Radioimmunoassay kits for cAMP were from New England Nuclear (Boston, MA). Collagen-reagent Horm was from Hormon-Chemie (Munich, FRG). The calcium ionophore A23187 was obtained as free acid from Calbiochem-Behring (San Diego, CA). ADP (sodium salt, Grade I, from equine muscle), arachidonic acid (99%, from porcine liver), serotonin (5-hydroxytryptamine as creatinine sulfate complex), DL-dithiothreitol, calmodulin-insensitive bovine heart 3′:5′-cyclic nucleotide phosphodiesterase (PO395) and calmodulin-sensitive 3′:5′-cyclic nucleotide phosphodiesterase (PO520) were purchased from Sigma Chemical Company (St. Louis, MO). Thrombin, papaverine hydrochloride and ASA were from F. Hoffmann-La Roche & Co., Ltd. (Basel, Switzerland). Platelet activating factor (1-O-alkyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) was prepared by G. Hirth and R. Barner (1982), PGI_2 was synthetized by F. Kienzle, F. Hoffmann-La Roche & Co., Ltd. Epinephrine (L-adrenalin-D-hydrogentartrate) and bovine brain calmodulin were obtained from Fluka (Buchs, Switzerland). PGE_1 was obtained from Ono Pharmaceutical Company (Osaka, Japan). Modified gelatin was from Laboratorien Hausmann (St. Gallen, Switzerland). Sepharose CL-2B was from Pharmacia Fine Chemicals (Uppsala, Sweden). Affi-Gel 601 was purchased from BIO-RAD Laboratories (Richmond, CA). Buffer salts were of analytical grade and were obtained from various commercial sources.

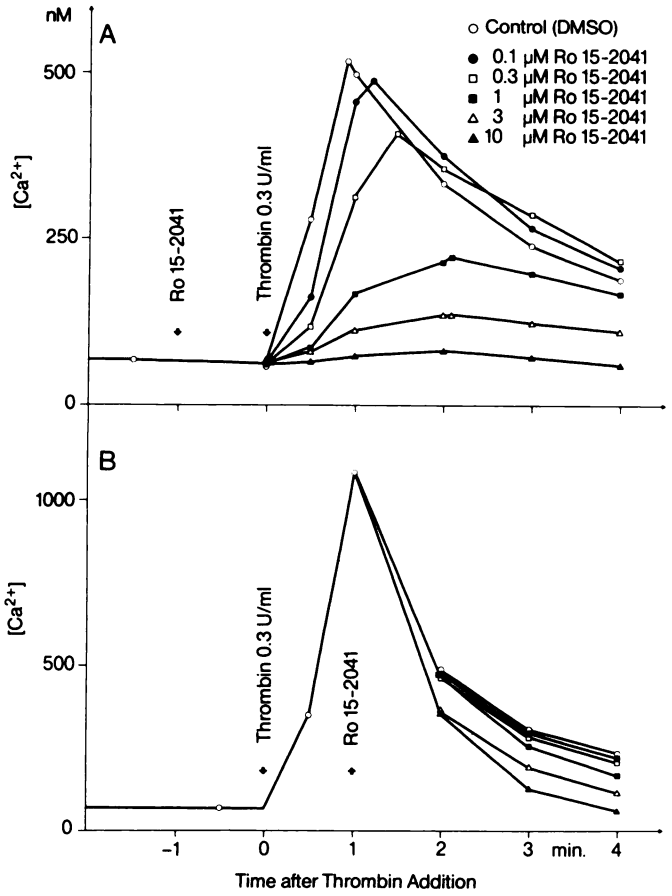


Fig. 6. Effect of Ro 15-2041 on the increase (A) and the rate of normalization (B) of cytosolic free Ca^{++} in thrombin-stimulated platelets. To washed human platelets, loaded with the calcium indicator Quin2, 0.3 U/ml of thrombin were added, and the intracellular free Ca^{++} concentration was monitored continuously by measuring the fluorescence intensity of the Ca^{++}-Quin2 complex. Ro 15-2041 at the concentrations as indicated was added 1 min before (A) or 1 min after (B) the addition of thrombin. Means of experiments with blood from three donors are given. DMSO, dimethyl sulfoxide.

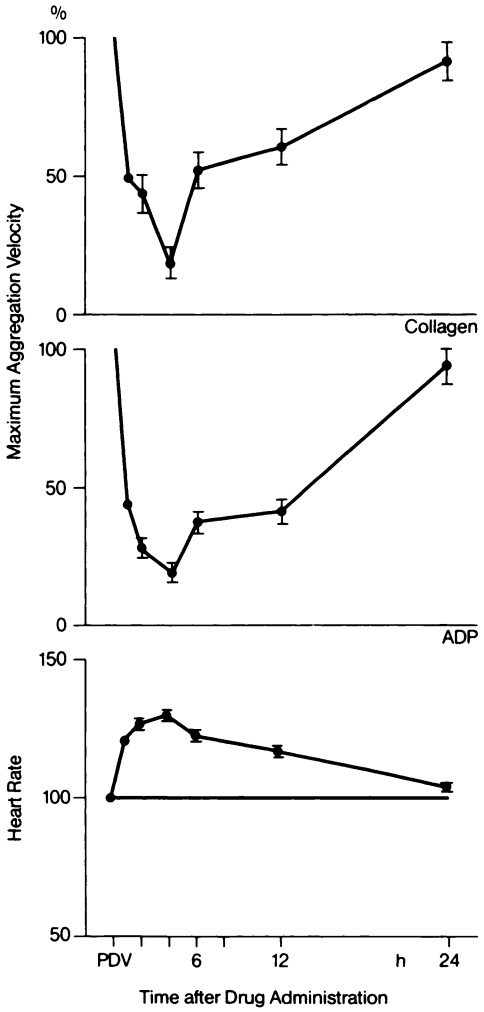


Fig. 7. Effect of Ro 15-2041, 5 μmol/kg p.o., on platelet aggregation and heart rate in Stumptail monkeys. Blood was obtained before and at various times after dosing, PRP was prepared, and platelet aggregation was induced with collagen (1.6 μg/ml) or ADP (1.6 μM). Heart rate was measured by auscultation. Values for maximum aggregation velocity and heart rate were expressed in percentage of the predrug value (PDV). Mean ± S.E. $n = 6$ (except $n = 2$ and 4 at 1 and 12 hr, respectively).

Results

Platelet aggregation *in vitro*. In comparison to ASA, Ro 15-2041 was a very potent inhibitor of human platelet aggregation, except for dithiothreitol-induced aggregation (table 1). IC_{50} values were between 1 to 3 μM. Similar strong inhibition of collagen-induced aggregation was observed in monkey, dog and rabbit with IC_{50} values of 0.27, 0.59 and 0.92 μM, respectively (table 2).

Serotonin and β-TG release *in vitro*. Ro 15-2041 potently inhibited collagen-induced serotonin and β-TG release in human PRP. IC_{50} values were 1.4 and 1.5 μM, respectively (fig. 1). ASA was about 30 times less potent.

Synergism with PGI_2 *in vitro*. Ro 15-2041 potentiated the inhibitory effect of PGI_2 on platelet aggregation (fig. 2). Platelet aggregation was inhibited by 50% with a combination in which PGI_2 and Ro 15-2041 were present in concentrations of one-fourth of their IC_{50} values.

cAMP metabolism. Whereas Ro 15-2041 alone had virtually no effect on the cAMP content in platelets, it potentiated the PGI_2-induced elevation of cAMP levels in a dose-dependent manner (fig. 3). The increase of cAMP over basal levels induced by 10 nM PGI_2 was 50% higher in the presence of 3 μM Ro 15-2041, a concentration close to the IC_{50} of Ro 15-2041 in platelet aggregation.

Whereas Ro 15-2041 caused a concentration-dependent and virtually complete inhibition of cAMP-phosphodiesterase activity in 100,000 g supernatants of human platelets, breakdown of cAMP in cardiac homogenates was depressed to maximally 50% only (fig. 4). The concentration of Ro 15-2041 causing half-maximal inhibition was 0.07 μM in both human heart and platelets. In human brain cortex, Ro 15-2041 was at least 1000 times less potent, inhibitory effects were observed only with concentrations of 100 μM and higher. By comparison, papaverine concentration-dependently inhibited phosphodiesterase activity in all three human tissues with similar IC_{50} values of about 5 μM. Qualitatively and quantitatively similar results were found in full homogenates (data not shown) and 100,000 × *g* supernatants (fig. 4) of rabbit platelets and freshly perfused rabbit myocardium and brain cortex. In rabbit uterus, which was investigated as a representative of a smooth muscle-rich tissue, a dose-inhibition curve similar to the one found in rabbit brain was obtained (data not shown).

When tested on commercial bovine cAMP-phosphodiester-

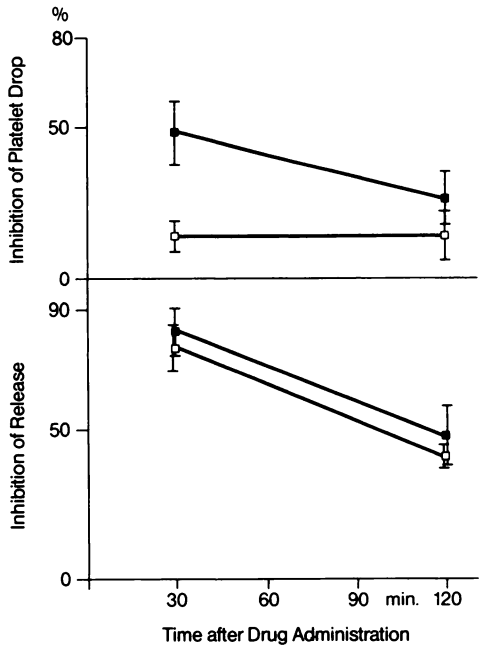


Fig. 8. Collagen-induced platelet drop and platelet-specific protein release *in vivo*. Rabbits were treated i.v. with 10 μmol/kg of Ro 15-2041 (■) or ASA (□). After the times indicated 214 μg/kg of collagen as a fibril suspension was injected i.v. Platelet number and the plasma concentration of the rabbit platelet-specific protein were determined 1 min after the collagen challenge. Rabbit platelet-specific protein was measured by radioimmunoassay. The effect of drugs was assessed by comparing the inhibition of platelet drop and platelet-specific protein release of drug-treated *vs.* vehicle-treated animals. Mean ± S.E., $n = 4$.

TABLE 3

Effect of Ro 15-2041 on bleeding time in rabbits

Ro 15-2041 was administered as microsuspension, ASA as a solution in 0.9% NaCl. Bleeding times were measured before (BT_1) and 2 hr (BT_2) after injection on the right and left ear, respectively. Mean ± S.E., n = number of animals.

Treatment	Dose	n	Bleeding Time		
			BT_1	BT_2	$BT_2 - BT_1$
	kg^{-1}		sec		
Control	1 ml	5	127 ± 16	144 ± 18	17 ± 7
Ro 15-2041	5 μmol	7	146 ± 10	217 ± 21	71 ± 16*
ASA	5 μmol	5	113 ± 11	138 ± 13	25 ± 11

* Significant prolongation according to Student's t test, $2P < .05$.

ase preparations, Ro 15-2041 had no effect on the calmodulin-dependent form, whereas the calmodulin-independent enzyme was maximally inhibited to 50%, half-maximal inhibition was observed at 0.9 μM (fig. 5). By comparison, papaverine inhibited the calmodulin-dependent and calmodulin-independent bovine phosphodiesterase with similar potencies, IC_{50} values were 12.0 and 6.4 μM, respectively.

Platelet Ca^{++} homeostasis. Thrombin (0.3 U/ml) increased free Ca^{++} from 69 ± 2 nM ($\bar{x}$ ± S.E., $n = 15$) in resting platelets to over 500 nM after 60 sec (fig. 6). Ro 15-2041 dose-dependently prevented the increase and accelerated the normalization of cytosolic free Ca^{++} in thrombin-stimulated platelets: the increase of Ca^{++} from 69 to 336 ± 36 nM 120 sec after stimulation with 0.3 U/ml of thrombin was blunted to 133 ± 22 nM by preincubation with 3 μM Ro 15-2041 for 1 min (fig. 6A). The same concentration of Ro 15-2041, added 1 min after thrombin, reduced the free Ca^{++} within 120 sec from a peak of 1075 ± 196 to 196 ± 17 nM compared to 311 ± 28 nM in solvent controls (fig. 6B).

Platelet aggregation *ex vivo*. The *ex vivo* potency of Ro 15-2041 in platelet aggregation was determined in Stumptail monkeys. Two and 4 hr after dosing, ID_{50} values for ADP-induced aggregation (1.6 μM) were 2.6 and 2.4 μmol/kg, respectively.

Duration of inhibitory effects in collagen- and ADP-induced aggregation after a single p.o. dose of 15-2041, 5 μmol/kg, is shown in figure 7. Effects were maximal (80% inhibition) 4 hr after dosing and were still significantly below predose values after 12 hr. Predose values were reached 24 hr after dosing. The concomitantly measured heart rates increased in parallel with the degree of platelet inhibition.

Collagen-induced platelet drop and platelet-specific protein release *in vivo*. Thirty seconds after i.v. injection of collagen into untreated rabbits, the average platelet count fell from 505 to 110 nl^{-1}. This platelet drop was inhibited by i.v. administration of 10 μmol/kg of Ro 15-2041 (fig. 8). The extent of inhibition was 48 and 26% in animals which were challenged with collagen 30 and 120 min, respectively, after dosing. The concomitant release of rabbit platelet-specific protein in control animals was documented by a rise of its plasma concentration from 37 to over 2890 ng/ml 60 sec after the collagen challenge. Thirty and 120 min after dosing, the release of rabbit platelet-specific protein was inhibited by 82 and 48%, respectively. Although ASA inhibited the release of rabbit platelet-specific protein, it had very little effect on the collagen-induced drop of the number of circulating platelets.

Bleeding time. Ro 15-2041 significantly prolonged the bleeding time in rabbits after i.v. dosing of 5 μmol/kg (table 3). The effect was more pronounced than with an equimolar dose of ASA.

Discussion

The results of this study characterize Ro 15-2041 as a potent inhibitor of platelet function in various species. *In vitro*, Ro 15-2041 inhibited the collagen-induced serotonin and β-TG release and inhibited platelet aggregation with a variety of agonists at concentrations of about 1 μM. This activity spectrum of Ro 15-2041 is similar to PGI_2 but differs from ASA and other nonsteroidal antiinflammatory agents which inhibit aggregation induced by agonists that activate the arachidonate pathway only.

Unlike PGI_2, whose instability and short biological half-life restricts its clinical use to continuous i.v. administration (Dusting *et al.*, 1978; Szczeklik *et al.*, 1978), the potent activity of Ro 15-2041 observed *in vitro* carried over to the *ex vivo* and *in vivo* situation after p.o. administration. Doses of about 2.5 μmol/kg inhibited platelet aggregation in monkeys by 50%. From the time-effect curves after a single p.o. dose of 5 μmol/kg of Ro 15-2041 in monkeys, a half-time for inhibition of aggregation of 5 hr can be estimated. In rabbits, 5 μmol/kg significantly prolonged the bleeding time.

Additional evidence for the *in vivo* activity of Ro 15-2041 is its inhibition of the collagen-induced platelet drop and of the release of platelet-specific proteins in rabbits. Quite unexpectedly we found ASA to inhibit only the *in vivo* release but virtually not to normalize the platelet count. Apparently, the two parameters measure two different functional aspects of platelets. On the one hand, the plasma concentration of rabbit platelet-specific protein is a measure of the platelet release reaction which can be inhibited by ASA. On the other hand, the collagen-induced platelet drop is a measure for platelet

consumption presumably mainly by adhesion of platelets to collagen fibrils. Because platelet adhesion is not affected by ASA (Baumgartner, 1979), the platelet drop is not inhibited by ASA.

Supporting evidence that the effect of Ro 15-2041 is mediated by cAMP was provided by experiments in which the combined effect of Ro 15-2041 and PGI_2 was tested. Inasmuch as cAMP is formed by adenylate cyclase from ATP and is degraded by cAMP phosphodiesterase, the only enzyme known to metabolize cAMP, increased levels of cAMP in platelets can be achieved by stimulation of adenylate cyclase or by inhibition of cAMP phosphodiesterase. Thus, the combination of a phosphodiesterase inhibitor with the adenylate cyclase stimulator PGI_2 (Tateson *et al.*, 1977; Gorman *et al.*, 1977) is expected to give supra-additive effects. Indeed, Ro 15-2041 potentiated the PGI_2-induced elevation of cAMP levels, and ADP-induced platelet aggregation was inhibited by 50% with a combination in which PGI_2 and Ro 15-2041 were present in concentrations of one-fourth their IC_{50} values. This is a highly desirable property for any antithrombotic agent as it strengthens the natural defense mechanism of the vessel wall against platelet deposition by maintaining elevated intracellular platelet cAMP levels at lower concentrations of PGI_2. Platelet adhesion and mural platelet thrombus formation have been shown to be inversely related to the production of PGI_2 by the vessel wall (Tschopp and Baumgartner, 1981). That the antithrombotic effect of PGI_2 can be potentiated by the concomitant administration of a phosphodiesterase inhibitor has been demonstrated in the dog by Romson *et al.* (1983) and in the rabbit by Baumgartner *et al.* (1982).

The contention that the broad activity spectrum of Ro 15-2041 on platelet function is mediated by cAMP is strengthened further by the new observation that the compound inhibited the increase and accelerated the normalization of cytoplasmic free Ca^{++} in thrombin-stimulated platelets. Various agonists such as collagen, ADP, thrombin, platelet-activating factor, arachidonate, thromboxanes and PGs activate platelets through partly different pathways. These pathways ultimately converge, in that most agonists lead to an increase of the cytosolic free Ca^{++} concentration (Gerrard *et al.*, 1981; Rink *et al.*, 1982), primarily by releasing Ca^{++} from intracellular stores. cAMP is believed to amplify the capacity of cells to maintain Ca^{++} homeostasis by increasing the rate of free Ca^{++} uptake into the dense tubular system and to facilitate the efflux of Ca^{++} across the plasmalemma (Feinstein *et al.*, 1981; Käser-Glanzmann *et al.*, 1978). Therefore, compounds that increase the level of cAMP in platelets are expected to be general platelet function inhibitors.

Although Ro 15-2041 may have hitherto unrecognized other effects besides being a phosphodiesterase inhibitor, our observations are fully compatible with the notion that cAMP is responsible for its mechanism of action. Dithiothreitol-induced aggregation, which is not inhibited by the adenylate cyclase stimulator PGE_1 (MacIntyre and Gordon, 1974; Zucker and Masiello, 1984), was likewise not affected by Ro 15-2041. Even though Ro 15-2041 does not increase cAMP levels at concentrations which affect platelet function, this does not argue against the pivotal mediatory role of cAMP. Haslam and Davidson (1982) contend that an elevation of cAMP of only 50%, an increase which is difficult to measure reliably, suffices for the inhibition of platelet function. It is possible that the degradation of cAMP by phosphodiesterases is regulated separately in different compartments, so that a fraction of cAMP, which may be too small to measure, might regulate the inhibition of aggregation.

New and with possible important general implications for the development of phosphodiesterase inhibitors as antithrombotic agents is the pronounced platelet selectivity of Ro 15-2041 with respect to cAMP-phosphodiesterase inhibition. The effect appears to be independent of the species, as it was observed in human as well as in rabbit tissues. It is not confined to soluble forms of cAMP phosphodiesterase or is an artefact caused by tissue-dependent variations of isozyme distribution during the preparation of supernatants, as virtually identical dose-response curves were obtained with full homogenates.

cAMP is an important intracellular messenger in stimulus-reaction coupling probably in all cells. Thus, compounds which indiscriminately prevent the breakdown of cAMP in all tissues will *in vivo* not only affect platelets but other organ functions as well, and undesirable side-effects, *e.g.* such as tachycardia and hypotension, are likely to be associated with inhibition of platelet function in antithrombotic therapy with phosphodiesterase inhibitors. For the purpose of preventing thrombosis it is thus imperative to find compounds with a sufficient degree of platelet selectivity.

There is ample evidence for multiple forms of cAMP phosphodiesterases (for references see Weiss and Hait, 1977; Hidaka *et al.*, 1977; Strada and Thompson, 1978) and their selective inhibition by pharmacologic agents. These isozymes differ with respect to substrate specificity and affinity, kinetic constants and dependency on Ca^{++}-calmodulin complex and other cofactors. Whereas there is no general agreement on their exact number and biochemical-biophysical characteristics, it is believed that the pattern and ratio of these multiple molecular forms of phosphodiesterase vary with the specific tissue and cell type (for references see Weiss and Hait, 1977). Thus, selective inhibition of one of several phosphodiesterase isozymes can lead to a tissue selective increase of cAMP and, consequently, to a physiological response in the target tissue only.

Lugnier *et al.* (1983) and Hidaka *et al.* (1984) have shown that two cyclic nucleotide mediated processes, vascular relaxation and inhibition of platelet aggregation, can be dissociated by selective inhibitors of cyclic nucleotide phosphodiesterase. Similarly, in line with its selective inhibition of platelet phosphodiesterase *in vitro*, Ro 15-2041 may preferentially affect platelet function *in vivo*. The selectivity of Ro 15-2041, however, is not absolute as phosphodiesterase activity in human and rabbit heart was also partially inhibited which probably reflects the presence of two phosphodiesterase populations, one sensitive the other insensitive to Ro 15-2041. It is tempting to speculate that the sensitive forms are responsible for the tachycardic effects observed in monkeys. Further studies are required to determine which ones among the several myocardial phosphodiesterase isozymes (Thompson *et al.*, 1979) are inhibited by Ro 15-2041 and whether they are involved in the regulation of heart rate.

In summary, Ro 15-2041 is a potent *in vitro*, *ex vivo* and *in vivo* platelet function inhibitor which potentiates the inhibitory effect of PGI_2. We believe that the compound acts by increasing the platelet cAMP level through inhibition of the cAMP phosphodiesterase, thereby improving the ability of platelets to maintain Ca^{++} homeostasis. Its platelet selectivity in inhibiting the breakdown of cAMP may possibly reduce the severeness of

side effects commonly associated with agents which increase cAMP levels.

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