R68070, a Combined Thromboxane/Endoperoxide Receptor Antagonist and Thromboxane Synthase Inhibitor, Inhibits Human Platelet Activation In Vitro and In Vivo: A Comparison With Aspirin

By Bernard Hoet, Cristina Falco, Stefan De Reys, Jef Arnout, Hans Deckmyn, and Jos Vermeylen

We have investigated the effects of R68070 on platelet function in vitro and in vivo. The drug inhibits U46619-induced aggregation (IC₅₀ = 1.2 × 10⁻⁶ mol/L), blocks serum thromboxane formation (IC₅₀ = 1 × 10⁻⁷ mol/L), and increases serum prostaglandin (PG)E₂ and 6-keto-PGF₁α levels, indicating that it combines thromboxane receptor blocking and thromboxane synthase inhibiting properties. The thromboxane-dependent aggregation of blood platelets is blocked by R68070, whereas no inhibition of thromboxane-independent pathways occurs. A double-blind, randomized, cross-over study was performed on nine volunteers, comparing 400 mg placebo, 400 mg aspirin, and 400 mg R68070. Thromboxane-dependent aggregations were significantly inhibited by R68070 and by aspirin, the latter still having the most pronounced action. However, R68070 was clearly more powerful than aspirin (P < .0005) in prolonging the bleeding time. Serum TxB₂ formation was completely inhibited with both treatments, whereas serum 6-keto-PGF₁α and PGF₁α and intralesional 6-keto-PGF₁α were inhibited after aspirin and stimulated after R68070. We conclude that R68070 inhibits platelet thromboxane synthase and its thromboxane receptor both in vitro and in vivo: local reorientation of cyclic endoperoxide metabolism toward prostacyclin induces a stronger inhibition of hemostasis than that produced by aspirin.

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MATERIALS AND METHODS

Materials
R68070 ([E]-5[[[(3-pyridinyl)-3-(trifluoromethyl)phenyl]methylen]amino]oxo)pentanoic acid) (from Janssen Pharmaceutica, Beerse, Belgium) was dissolved to 10 mmol/L in NaOH 0.1 mol/L. Further dilutions were performed in 150 mmol/L NaCl. These stock solutions were kept frozen until use.

The anti-6-keto-PGF₁α antibodies were kindly provided by Dr. J. Beetsens (Janssen Pharmaceutica, Beerse, Belgium). The anti-TxB₂ antibodies were purchased from Dr. L. Levine (Brandeis University, Waltham, MA). BM13177 was kindly provided by Boehringer Mannheim (Mannheim, FRG) and CWS13080 by Ciba Geigy (Basel, Switzerland). SQ29548 was from ER Squibb & Sons (Princeton, NJ). TxB₂, 6-keto-PGF₁α, and the stable endoperoxide analogues, U46619 (9,11-dideoxy-9α,11-epoxymethano-PGF₂α) and U44069 (9,11-dideoxy-9α,11-epoxymethano-PGF₂α) were purchased from Upjohn Co (Kalamazoo, MI). [3H]-thromboxane B₂, [3H]-6-keto-prostaglandin F₁α, and carrier-free [32P]orthophosphate came from Amersham International (Amersham, England).

Collagen was obtained from Hormon-Chemie (München, West Germany), arachidonic acid sodium salt (AA), cyclic adenosine monophosphate (cAMP) and adenosine diphosphate (ADP) from Sigma Chemicals Co (St Louis, MO). Thrombin was from Roche.
(Brussels, Belgium), A23187 from Calbiochem-Behring Corp (San Diego, CA), and NaF from Baker Chemicals (Phillipsburg, NJ). Platelet-activating factor (1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine, PAF) and adenosine triphosphate (ATP) were from Boehringer Mannheim. \(^{[1]H}\)SQ29548, \(^{[2,8-\text{H}}\)cAMP, \(^{[8-\text{C}}\)eAMP, \(^{[2,8-\text{H}}\)adenine, and the kit for PGE\(_2\) radioimmunoassay (RIA) were from New England Nuclear (Boston, MA), and the luciferine luciferase reagent was from Chronolog Corp (Havertown, PA).

A stock solution of each chemical was prepared and frozen at -20°C until use, except collagen, which was stored at 4°C until use, and \(^{[32P]}\)orthophosphate and luciferine luciferase, of which a fresh solution was prepared immediately before each experiment.

Blood Collections

Blood, from healthy volunteers who denied having taken any drug in the past 10 days, was anticoagulated either with trisodium citrate 109 mmol/L, vol:vol/1:10 (citrated blood); with 270 mmol/L HEPES buffered trisodium citrate 109 mmol/L, pH 7.4, vol:vol/1:10 (HEPES buffered citrated blood); with 200 mmol/L EDTA, vol:vol/0.3:10 (EDTA blood) or with trisodium citrate 85 mmol/L, citric acid 71 mmol/L and dextrose 111 mmol/L, pH 4.5, vol:vol/1:6 (ACD blood). Platelet-rich plasma (PRP) was prepared by centrifuging the blood at 150 x g for 10 minutes with varying concentrations of R68070.

Comparison of R68070 with aspirin, CGS13080, a single TSI, and BM13177, a single TRA, on collagen-induced aggregation was performed on a computerized, four-channel aggregometer (Aggregocorder II, Menarini, Italy).

Whole blood aggregations measured by impedance technique were performed with a Chronolog whole blood aggregometer (Haverton, PA) as previously described.

Whole blood aggregations measured by counting residual single platelets were performed as follows: citrated whole blood samples were incubated at 37°C and stirred at 150 rpm. Collagen, 1 µg/mL or 5 µg/mL, was added, and 10 µL aliquots were taken before and 5 minutes after addition of the inducer. Earlier experiments showed that the maximal decrease in single platelets was reached at that moment. The 10 µL aliquots were immediately diluted with 10 mL counting buffer (Silos R; Baker, Allentown, PA) containing 0.47% formaldehyde. For basal counts, counting buffer without formaldehyde was used. Single platelets were counted, using a platelet counter Model 810 from Baker and expressed as percentage of the basal counts.

In Vitro Studies

Prostaglandin Measurement

The in vitro inhibitory activity of the drugs on prostaglandin production was determined in citrated whole blood and PRP. Whole blood samples were incubated in glass tubes containing varying concentrations of the drug for 15 minutes at 37°C and then recalculated with 1 mmol/L CaCl\(_2\), and activated with 20 U/mL thrombin to have full activation of the platelets. After 1 hour, the platelet count was collected and centrifuged for 2 minutes at 12,000 x g. The supernatant serum was stored at -20°C before assay.

Protein Phosphorylation

Protein phosphorylation in response to stimulation of platelets by U46619 was measured as \(^{32}\)P incorporation into proteins after labeling of the platelet metabolic phosphate pool with carrier-free \(^{32}\)PO\(_4\). platelets were isolated by differential centrifugation, as previously described, from ACD blood. The isolation was carried out at room temperature in isotonic phosphate-buffered saline, pH 6.5, containing 113 mmol/L NaCl, 4.4 mmol/L K\(_2\)HPO\(_4\), 4.3 mmol/L Na\(_2\)HPO\(_4\), 24.4 mmol/L NaHCO\(_3\), and 5.5 mmol/L glucose. The isolated platelets were resuspended in HEPES buffered saline, pH 7.4, containing 10 mmol/L HEPES, 145 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgSO\(_4\), 5 mmol/L glucose (resuspension buffer) and incubated for 20 minutes to deplete them of phosphate. For labeling, the platelets were pelleted and resuspended in the resuspension buffer with 0.8 mCi/mL \(^{32}\)PO\(_4\). for 90 minutes at 37°C. Thereafter they were again sedimented, washed once, and resuspended in the same buffer. The platelet count was adjusted to 2 x 10\(^9\)/mL.

Samples were incubated, while being stirred with or without R68070 (10\(^{-12}\) mol/L) or BM13177 (10\(^{-14}\) mol/L), for 10 minutes, and 10 µL U46619 was then added. The reaction was stopped after 10 minutes by 0.1 vol of 3 mol/L HClO\(_4\). The precipitated proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli buffer system. Gels were stained with Coomassie brilliant blue, destained, dried, and exposed to autoradiography. The intensities of each band were quantitated by densitometric tracing on the autoradiogram, using a Vitatron densitometer (Dieren, The Netherlands).

cAMP Measurement

Citrated PRP, to which 9 mmol/L EDTA was added, was incubated with 15 µCi/mL \(^{3}H\)adenine for 30 minutes at room temperature. Labeling was terminated by centrifugation at 800 x g.
for 10 minutes and resuspension of the platelet pellet in an equal amount of citrated autologous plasma. Aliquots of this platelet suspension were preincubated for 10 minutes at 37°C with varying amounts of R68070 and stimulated with either 1 mmol/L arachidonic acid or an equal volume of 150 mmol/L NaCl. After 5 minutes, the proteins were precipitated with 0.5 mol/L trichloroacetic acid containing [14C] cAMP for assessment of the recovery. After thorough mixing, the precipitated proteins were removed by centrifugation at 12,000 × g for 5 minutes, and total [3H] and [14C] radioactivity in the supernatant was determined by liquid scintillation counting. cAMP was isolated from the trichloroacetic acid extract by ion exchange chromatography on a Dowex AG 50W-X4 column (Biorad, Richmond, CA) followed by ZnSO4/Ba(OH)2 precipitation of the non-cyclic adenine nucleotides as previously described.27

Samples were centrifuged at 12,000 × g for 5 minutes, and [3H] and [14C] radioactivity in the final supernatant was measured. Results were corrected for spillover and loss factors and expressed as percentage of basal [3H] adenine incorporation into cAMP.

[3H]/SQ29548 Binding Assays

Washed human platelets were obtained from EDTA blood. PRP from this blood was subjected to centrifugation (1,500 × g, 10 minutes), and the pellet was washed twice in phosphate buffer (6 mmol/L glucose, 113 mmol/L NaCl, 4 mmol/L K2HPO4, 4 mmol/L Na2HPO4, 24 mmol/L NaH2PO4, pH 6.5). Finally, the platelets were resuspended in Tyrode HEPES buffer (pH 7.4) supplemented with acetylsalicylic acid (ASA; 140 µmol/L).

Washed platelets (50 × 10⁶) were incubated with increasing concentrations (1 mmol/L to 20 mmol/L) of [3H]/SQ29548, a TRA with high specific binding, and a fixed concentration of R68070. Nondisplaceable binding was determined by adding 65 µmol/L SQ29548 dissolved in ethanol (final concentration: 0.5%). Incubations were done for 30 minutes at 37°C and terminated by dilution with 1 mL of 10 mmol/L HEPES buffer (pH 7.4) supplemented with 20 mmol/L MgCl2 at 4°C. The samples were filtered under reduced pressure through Whatman GF/C glass fiber filters, pre-treated with 0.3% polyethyleneimine for 1 hour. The filters were washed with 9 mL of the ice-cold buffer and transferred to scintillation vials. Radioactivity was determined by liquid scintillation counting.22

In Vivo Study: Comparison Between R68070, Aspirin, and Placebo

The studies were carried out according to the principles of the Declaration of Helsinki and were approved by the Ethical Committee of our institution.

Study Design

Nine healthy male nonsmokers participated after having given informed consent. They were free of drug intake for at least 2 weeks before the study and fasted overnight before the study days. Three oral treatments were studied: 400 mg R68070, 400 mg aspirin, and 400 mg placebo. These treatments were given according to a double-blind, randomized cross-over design in three separate sessions with an interval of 2 weeks between each treatment.

Assessments

Bleeding time. Ivy bleeding time (two cuts on each forearm) was performed before and 2 hours after drug administration using automated template devices (Simplate II; General Diagnostics, Morris Plains, NJ). The incisions were placed in the longitudinal direction on the volar surface of the upper forearm. The same operator carried out all the bleeding time determinations throughout the study. The bleeding time was expressed in seconds as mean of the four bleeding times.

Blood loss through the incisions. The Whatman filter papers used for removing the blood during the bleeding time determination were collected in 50 mL modified Drabkin solution (K,K,Fe[CN]6, 0.6 mmol/L; KCN, 1.1 mmol/L; KHPO4, 0.1 mmol/L, and Triton X-100, 1 mL/L) and shaken for 1 hour at room temperature. The light absorption of the solution was read at 540 nm in a Hitachi Model 100-40 Spectrophotometer (Japan) and compared with a hemoglobin cyanide reference solution from the Rijksinstituut voor de Volksgezondheid, Buitenhoven, The Netherlands. Preliminary experiments did not show differences between blood collected on Whatman filter papers and blood directly added to the modified Drabkin solution (data not shown).

Prostaglandin measurement. Immunoreactive TXB2, 6-keto-PGF1α, and PGE2 were determined in serum before, 2 hours, and 24 hours after drug intake.

Intraluminal 6-keto-PGF1α was measured in the capillary blood emerging from the bleeding time wound as described.13 Briefly, two to four heparinized hematocrit capillaries (75 µL vol), that had been flushed with a mixture of 1:1 vol/vol sodium heparin 5,000 U/mL and lysine ASA 0.55 mol/L, were quickly filled with blood of the wound at least 30 seconds after the moment of incision. The tubes were immediately centrifuged at 6,000 × g for 5 minutes, broken at 0.5 cm from the plasma-to-erythrocyte interface, and the cell-free supernatant extruded and stored at −20°C until assayed. The RIA for 6-keto-PGF1α was performed in a final assay volume of 75 µL with 25 µL of unextracted samples as described.13 The cell-containing part of the capillaries was added to the Drabkin solution for the blood loss measurements.

Aggregations. Aggregation studies were performed immediately before and 2 hours after drug intake. All aggregations were carried out by the same operators under the same conditions and in the same time sequence. TACs for collagen, AA, ADP, and U46619 were determined in citrated PRP by light absorbance. In citrated whole blood, aggregation induced by 1 µg/mL and 5 µg/mL collagen was measured by the impedance technique and by the single platelet counting technique.

Hematological and biochemical safety parameters. Sodium, potassium, chloride, calcium, inorganic phosphate, total protein, albumin, cholesterol, triglyceride, BUN, creatinine, uric acid, total bilirubin, alkaline phosphatase, serum glutamic-oxaloacetic transaminase (SGOT), serum glutamate pyruvate transaminase (SGPT), lactate dehydrogenase (LDH), hemoglobin, hemoglobin, red blood cell count, white blood cell count, and platelet count were measured by the Leuven University Laboratories according to standard procedures before and 24 hours after drug intake.

Plasma levels of R68070. Plasma levels of R68070 were determined on lithium heparin anticoagulated plasma, by Janssen Pharmaceuticals using a high-pressure liquid chromatography (HPLC) method.30

Statistical evaluation. Results of in vitro experiments are expressed as mean ± SD or SEM as indicated in the stated number (n = 3) of experiments.

For the binding studies, a Schild plot was constructed by linear regression analysis. The statistical significance of the slope was elucidated by a two-tailed student's t test.

For in vivo and ex vivo experiments, statistical evaluation was performed by one- or two-way analysis of variance when the basal values were distributed normally, and by Friedman test when the basal values were not normally distributed.
RESULTS

In Vitro Study

Thromboxane Synthase Inhibition

R68070 inhibited in a dose-dependent manner the TxB₂ production after recalcification of citrated whole blood with 1 mmol/L calcium and stimulation with 20 U/mL thrombin (IC₅₀ 1 × 10⁻⁷ mol/L R68070). PGE₂ and 6-keto-PGF₁α production, on the contrary, was increased in a dose-dependent manner by R68070 (Fig 1A). After stimulation of citrated PRP with 0.5 mmol/L AA, the TxB₂ production was inhibited with an IC₅₀ of 5 × 10⁻⁷ mol/L R68070 (data not shown).

Thromboxane Receptor Antagonism

The TAC of U46619 (0.26 ± 0.04 μmol/L) and U44069 (0.62 ± 0.18 μmol/L) was inhibited by R68070 with an IC₅₀ of 1.2 and 1.5 × 10⁻⁶ mol/L, respectively (Fig 1B). Aggregations induced by native cyclic endoperoxides and TxA₂ (obtained by adding AA-stimulated, TxA₂-insensitive dog PRP to aspirinated human PRP) were inhibited with an IC₅₀ of 2.10⁻⁶ mol/L. This TxA₂ concentration, however, may not have been a TAC but instead a rather high amount of TxA₂, which could explain the higher IC₅₀.

Binding studies were performed with platelets from five normal volunteers with three different R68070 concentrations per donor. For each R68070-dose, five concentrations of SQ29548 were tested to obtain dose-response curves. R68070 provoked a concentration-dependent inhibition of specific SQ29548-binding. Analysis by Schild plot (Fig 2A) resulted in a slope (−0.94) not significantly different from −1.00 (n = 15, P > .95), suggesting that R68070 is a competitive inhibitor for specific SQ29548-binding to washed human platelets. With the slope constrained to −1.00, the inhibition constant (Kᵢ) was 7.00 μmol/L. The receptor antagonism was also shown to be competitive by a double-reciprocal plot of the concentration of U46619 versus the slope of the aggregation in the presence or absence of 10⁻⁴ mol/L R68070 (Fig 2B).

Aggregation Induced by Other Agonists

R68070 inhibited in a dose-dependent fashion the aggregations of human platelets in PRP induced by TAC of AA (0.34 ± 0.23 μmol/L) and collagen (1.13 ± 0.33 μg/mL) and the second wave of TAC of ADP (16 ± 4 μg/mL), A23187 (3.2 ± 0.65 μmol/L), and PAF (24.5 ± 12.6 μmol/L) induced aggregation. The IC₅₀ of R68070 was between 1 and 2 × 10⁻⁶ mol/L. NaF (24 mmol/L)-induced aggregations were not influenced by R68070, even at 10⁻⁴ mol/L. The ATP secretion after TAC of collagen was inhibited in a way similar to the aggregation.

Protein Phosphorylation

Phosphorylation of the 40 Kd and 20 Kd proteins, observed after stimulation of blood platelets by 10 μmol/L U46619, was inhibited after pretreatment of these platelets by either 10⁻⁴ mol/L R68070 (78% and 100% inhibition, respectively) and by 10⁻⁴ mol/L BM13177 (98% and 100%, respectively).

cAMP Production

Basal cAMP production was not influenced by preincubation of the platelets for 10 minutes with 10⁻⁴ mol/L R68070. If these platelets were then stimulated for 5 minutes with different concentrations of arachidonic acid, cAMP production was increased in a dose dependent way (Fig 3).

Comparison Between R68070 and Other Drugs

In order to see whether a drug combining TSI and TRA properties is more potent than drugs with the single characteristics, a comparison was made between equipotent thromboxane receptor antagonistic concentrations of R68070 and a single TRA, BM13177, and between equipotent thromboxane synthase-inhibiting concentrations of R68070 and a single TSI, CGS13080, and the cyclo-oxygenase inhibitor ASA on collagen-induced aggregations.

Upon comparison of a concentration of R68070 and one of BM13177 inhibiting the TAC of U46619-induced aggregation by 95% (5 × 10⁻⁵ mol/L and 1 × 10⁻⁴ mol/L, respectively), R68070 proved to be more potent in inhibiting collagen-induced aggregation (EC₅₀ from 0.27 to 1.02 μg/mL) than BM13177 (EC₅₀ from 0.27 to 0.54 μg/mL) (Fig 4A).

R68070, CGS13080, or ASA, at concentrations inhibiting TxB₂ formation in 750 mmol/L AA-stimulated PRP by 90%...
(9 × 10^{-8} mol/L, 5 × 10^{-4} mol/L, and 1.4 × 10^{-4} mol/L, respectively), inhibited collagen-induced aggregation; ASA being more powerful than either R68070 or CGS13080. Since at these concentrations, R68070 only acts as a TSI, we next compared concentrations of R68070 up to 9 × 10^{-3} mol/L with CGS13080 up to 5 × 10^{-3} mol/L and aspirin up to 1.4 × 10^{-3} mol/L; R68070, now exerting its two activities, clearly was more powerful against collagen than CGS13080, but still was weaker than aspirin (Fig 4B).

**In Vivo Study**

**Dose Response**

In a preliminary study, we determined that 400 mg/orally was active in man on TxA2 synthase inhibition and TxA2/cyclic endoperoxide receptor blockade (inhibition of U46619 and collagen-induced aggregation), whereas 200 mg only inhibited the synthase activity. In the comparative study, the plasma levels of R68070 obtained 2 hours after intake were 41.93 ± 8.54 × 10^{-6} mol/L. This level fell to 2.29 ± 1.06 × 10^{-6} mol/L 24 hours after drug intake.

**Bleeding Time and Blood Loss**

The bleeding time increased significantly more (P < .0005) with R68070 (from 427 ± 130 seconds before drug intake to 753 ± 407 seconds 2 hours after drug intake) than with aspirin (from 440 ± 150 seconds to 636 ± 360 seconds) (Fig 5). R68070 treated volunteers lost much more hemoglobin through their wounds than placebo treated ones (P < .0005), but aspirin treated volunteers did not bleed significantly more than those receiving placebo (Fig 5).

**Prostaglandin Production**

Serum TxB2 was totally inhibited at 2 and 24 hours after drug intake by both R68070 and aspirin (Fig 6). Serum PGE1 and 6-keto-PGF1α production were also totally inhibited after 2 and 24 hours in aspirin-treated volunteers, whereas they were increased 2 and 24 hours after R68070 treatment (Fig 6).

The intralesional 6-keto-PGF1α formation after R68070 intake shows an even more important increase: from nearly undetectable (≤50 pg/mL) in placebo and aspirin-treated volunteers to 882.2 ± 98.5 pg/mL (mean ± SEM, n = 9) in R68070-treated volunteers.
R68070, A COMBINED TRA AND TSI 651

**Aggregation Study**

In PRP the U46619-induced aggregation was only inhibited \( P < .0001 \) after R68070 treatment. The AA and collagen-induced aggregation in PRP was inhibited by R68070 \( P < .0001 \), but it was more inhibited by aspirin. Similar findings were observed when looking at the second wave of ADP-elicted aggregation and at collagen-induced aggregation in whole blood followed by the impedance technique or by single platelet counting (Table 1).

**Safety**

The only other biochemical effect observed with R68070 was the lowering of uric acid from 5.8 ± 1.2 mg/dL to 4.4 ± 1.5 mg/dL. None of the other parameters measured were influenced by R68070 treatment.

**DISCUSSION**

In Vitro Studies

The present study confirms that R68070 has both TxA\(_2\) synthase inhibitory and TxA\(_2\)/cyclic endoperoxide receptor antagonizing activities. TxA\(_2\) synthase inhibition was determined as a drop in the formation of TxB\(_2\) in both thrombin-

**stimulated whole blood and AA-stimulated PRP. Furthermore, this inhibition was accompanied by an increase in the formation of immunoreactive PGE\(_2\) and 6-keto-PGF\(_1\alpha\) in whole blood stimulated with thrombin. These actions were observed at concentrations of the drug that were without effect on the TxA\(_2\) receptor and that had very little influence on TxA\(_2\)-dependent aggregations.**

Higher concentrations effectively blocked platelet aggregations induced by the TxA\(_2\)/cyclic endoperoxide analog U46619 as described, but also by U44069 and by authentic TxA\(_2\) and cyclic endoperoxides derived from AA-activated dog platelets. In addition, protein phosphorylation induced by U46619 was effectively prevented by R68070.

The receptor antagonistic action of R68070 is competitive as shown by the double reciprocal plot of the slope of the aggregation induced by various amounts of U46619 in the presence or absence of R68070. This was confirmed by direct binding studies of SQ29548, another specific TRA, to washed human platelets as analyzed by Schild plot (Fig 2).

Thromboxane-dependent pathways of platelet activation (AA, collagen, second wave of ADP, PAF, and A23187 aggregation) were inhibited, whereas the independent ones were not (NaF, first wave of ADP, PAF, and A23187). In

**Fig 4.** Comparison between R68070 and drugs with single TRA or TSI activities. (A) Effect of increasing concentrations of collagen on platelet aggregation in the presence of 10\(^{-5}\) mol/L BM13177; 5 \times 10\(^{-5}\) mol/L R68070, or vehicle (mean ± SEM, \( n = 3 \)). (B) Effect of increasing concentrations of collagen on platelet aggregation in the presence of 1.4 \times 10\(^{-5}\) mol/L ASA; 9 \times 10\(^{-1}\) mol/L R68070; 5 \times 10\(^{-1}\) mol/L CGS13080, or vehicle. The maximal amplitude was expressed in % of the difference in light absorption between PRP and PPP (mean ± SEM, \( n = 3 \)).

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**Fig 5.** Bleeding time (in seconds) and blood loss (in mg hemoglobin) before (hatched) and 2 hours after (open) placebo, 400 mg aspirin, or 400 mg R68070 (mean ± SEM; \( n = 9 \)). *, \( P < .05 \); **, \( P < .01 \); ***, \( P < .001 \); ****, \( P < .0005 \).
addition, R68070 did not affect basal cAMP levels, therefore excluding a direct action of the drug on adenylate cyclase or cAMP phosphodiesterase.

A low concentration of R68070, only inhibiting TxA₂ synthase, and an equipotent concentration of a single TSI, CGS13080, were equally effective in inhibiting collagen-induced aggregations. At higher concentrations, R68070 was also active against the thromboxane receptor, and it then was more powerful in inhibiting collagen-induced aggregations than a single TSI. Furthermore, at this concentration it was also more active than a single TRA (BM13177, in an equipotent concentration versus U46619-induced aggregations). R68070 in vitro, however, appeared to be less powerful than aspirin. This may be due to the competitive nature of the blockade of the thromboxane receptor by R68070, which still can be overcome by increasing the concentration of the agonist.

In the presence of R68070, arachidonic acid stimulation produces a rise in cAMP by reorientation of the cyclic endoperoxide metabolism towards adenylate cyclase-stimulating prostaglandins, an effect which already had been shown using the combination of a single TSI and a single TRA.\\(^{15}\)

**In Vivo Studies**

After a preliminary dose-finding study and a small open study, a randomized double-blind, placebo-controlled, crossover human volunteer study was performed. From the ex vivo tests, it is clear that R68070 is active in humans both as a TSI and as a TRA, which results in inhibition of TxA₂, dependent aggregations.

As seen in the in vitro tests, aspirin had also ex vivo a significantly more powerful effect on blood platelet aggregation both in PRP and in whole blood. In contrast, the prolongation of the bleeding time and the blood loss were significantly more pronounced after R68070 than after aspirin administration. This apparent contradiction between aggregation results and bleeding time emphasizes the possible importance of antiaggregatory and vasodilatory prostaglandins formed by the vessel wall at the site of the lesion as a result of the redirection of cyclic endoperoxide metabolism by a TSI. This was corroborated by the measurement of increased 6-keto-PGF₁α in the blood emerging from the bleeding time wounds. Despite this, the prolongation of the bleeding time is still a rather mild event, and no spontaneous bleedings were observed in the treated subjects.

In conclusion, our study shows that R68070 is a thromboxane receptor antagonist and a thromboxane synthase inhibitor, active in humans and likely to be suitable for therapeutic evaluation. Although it is a less potent inhibitor of 

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**Table 1. Aggregation Before and Two Hours After 400 mg Drug Intake**

<table>
<thead>
<tr>
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<th>Placebo</th>
<th>Aspirin</th>
<th>R68070</th>
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<tr>
<td></td>
<td>Pre</td>
<td>Post</td>
<td>Pre</td>
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<tr>
<td><strong>PRP (TAC)</strong></td>
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<tr>
<td>U46619 (μmol/L)</td>
<td>0.65 ± 0.38</td>
<td>0.69 ± 0.22</td>
<td>0.64 ± 0.36</td>
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<tr>
<td>Collagen (μg/mL)</td>
<td>1.41 ± 1.48</td>
<td>1.20 ± 0.88</td>
<td>0.92 ± 0.56</td>
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<td>AA (mmol/L)</td>
<td>0.60 ± 0.25</td>
<td>0.60 ± 0.20</td>
<td>0.52 ± 0.16</td>
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<tr>
<td>ADP (μmol/L)</td>
<td>1.97 ± 2.00</td>
<td>2.21 ± 2.04</td>
<td>1.71 ± 0.68</td>
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<tr>
<td><strong>Whole blood impedance</strong></td>
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<tr>
<td>slope (Ω/min)</td>
<td>8.30 ± 0.80</td>
<td>8.40 ± 1.10</td>
<td>8.30 ± 1.80</td>
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<tr>
<td>Collagen (1 μg/mL)</td>
<td>9.70 ± 2.50</td>
<td>9.50 ± 1.70</td>
<td>10.6 ± 1.90</td>
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<tr>
<td>Collagen (5 μg/mL)</td>
<td>40 ± 26</td>
<td>53 ± 12</td>
<td>54 ± 17</td>
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<td>Whole blood single platelet count (%) initial count</td>
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<tr>
<td>Collagen (1 μg/mL)</td>
<td>10 ± 7</td>
<td>18 ± 24</td>
<td>19 ± 21</td>
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Values represent mean TAC ± SD.
platelet aggregation in PRP and whole blood than aspirin, it prolongs the bleeding time more than aspirin in humans, indicating a marked inhibition of the hemostatic mechanisms in vivo.

REFERENCES

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