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Mechanisms involved in the antiplatelet activity of 8-methyl-4-(1-piperazinyl)-7-(3-pyridinylmethoxy)-2*H*-1-benzopyran-2-one (RC414)

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Abstract

The effect on human platelets of 8-methyl-4-(1-piperazinyl)-7-(3-pyridinylmethoxy)-2*H*-1-benzopyran-2-one (RC414) was tested *in vitro* by measuring aggregation induced by several agonists, cAMP and cGMP levels, cAMP phosphodiesterase and PKC activities and $[Ca^{2+}]_i$. The RC414 effect on nitric oxide production was also evaluated. RC414 in a dose-dependent manner inhibited aggregation both in platelet rich plasma and in washed platelets. It was particularly effective in platelets challenged by collagen, ADP and thrombin: IC_{50} values are 0.51 ± 0.12 μM, 0.98 ± 0.36 μM and 1.00 ± 0.15 μM, respectively. RC414 increased cAMP levels, through the specific inhibition of the cAMP high affinity phosphodiesterase ($IC_{50} = 1.73 \pm 0.35$ μM). RC414 reduced $[Ca^{2+}]_i$ transients and PKC activation induced by thrombin. In addition RC414 was able to increase nitric oxide formation involving the stimulation of constitutive nitric oxide synthase enzyme. In conclusion, RC414 exerts its powerful anti-platelet activity by increasing cAMP intracellular levels and nitric oxide formation.

Keywords: Benzopyran derivative; Antiplatelet activity; cAMP; cAMP phosphodiesterase; NO formation; NO synthase activity

1. Introduction

In the course of our studies on novel potential human platelet antiaggregating agents we synthesized and tested for their *in vitro* activity a number of N-substituted 2-aminochromones (**1**) [1–3], 4-aminocoumarins (**2**) [2–4], and their angular and linear benzo-fused derivatives [2,3]. Also numerous N-substituted 2-amino-4*H*-pyrido[1,2-*a*]-pyrimidin-4-ones (**3**) [3,5–7] and 4-amino-2*H*-pyrido[1,2-*a*]pyrimidin-2-ones (**4**) [6], isosteric analogues of compounds **1** and **2**, respectively, and their benzo-fused angular tricyclic derivatives [6] were prepared and their *in vitro* inhibitory properties on human platelet aggregation evaluated. Also some examples of compounds **5**, differing from **3** for proper modifications of the pyridine ring, were prepared and tested [3,7] (Fig. 1).

On the whole, the biological results of these studies suggest the following conclusions:

- In each structural class, the bicyclic compounds **1**–**4** were generally more active than their benzo-fused derivatives.
- The 1, 2-fused pyrimidine derivatives **3** and coumarin derivatives **2** appeared more active *in vitro* antiplatelet agents than their corresponding isomers **4** and **1**, respectively. Actually, some 2-(1-piperazinyl) substituted compounds **3**, **5** [3,6,7] and, in particular, some properly substituted 4-(1-piperazinyl) coumarins (**2**) recently prepared [3,4] proved to be the most interesting compounds synthesized by us and very potent *in vitro* antiplatelet agents.
- In each structural class 1-piperazinyl proved to be the most effective amino substituent among all those used. In the case of compound **4** such a substitution ( = ) was not possible.

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Abbreviations: BH_4, 5,6,7,8-tetrahydrobiopterin; cAMP-PKA, cAMP-dependent protein kinase A; DTT, dithiothreitol; FMN, flavin mononucleotide; cNOS, costitutive nitric oxide synthase; NO, nitric oxide; NOS, nitric oxide synthase; NO_x, nitrite + nitrate; PDE, phosphodiesterase; plts, platelets; PMSF, phenylmethylsulfonyl fluoride; PRP, platelet-rich platelets; WP, washed platelets.

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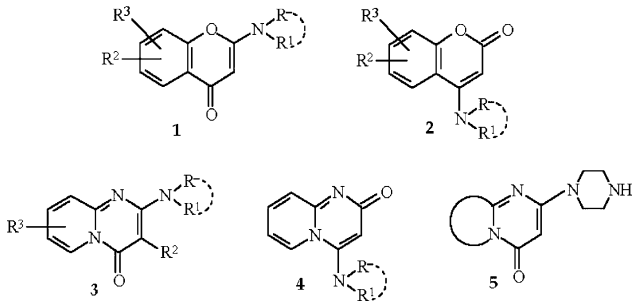
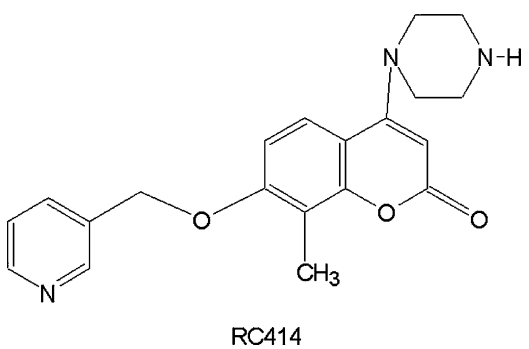


Fig. 1. Structures of the substituted amino-1-benzopyranones **1**, **2** and fused aminopyrimidinones **3**–**5** tested *in vitro* for their human platelet antiaggregating activity.

Both 2-(diethylamino)-7-hydroxychromone (**1**: $N(C_2H_5)_2$, R^2=7-OH, R^3=H) [8] and the very active 2-(1-piperazinyl)-4*H*-pyrido[1,2-*a*]pyrimidin-4-one (**3**: NH, R^2=R^3=H) [9], taken as examples of compounds **1** and **3**, respectively, proved to exert their antiplatelet activity by specifically inhibiting the activity of high affinity cAMP-PDE, thus increasing intracellular cAMP concentration. Moreover, cytosolic calcium increase and fibrinogen binding were also inhibited, confirming both that compounds **1**, **3** and their analogues inhibit platelet aggregation by a cAMP-mediated mechanism, and the bioisosteric feature of these compounds.

The structural peculiarity of compound **2**, as compared with **1** and **3**, has now prompted us to study the mechanism of action of 8-methyl-4-(1-piperazinyl)-7-(3-pyridinylmethoxy) coumarin (RC414) [4] (Fig. 2), as the most potent human platelet aggregation *in vitro* inhibitory agent until now synthesized by us.

The aim of the present work was to characterize the signalling pathways affected by RC414. We have studied the effect of RC414 on human platelets *in vitro* including assay of platelet aggregation induced by varying agonists, measurements of cAMP and cGMP, studies of cAMP-PDE activity, PKC activation and $[Ca^{2+}]_i$ transients evoked by thrombin. The RC414 effect on L-arginine/NO pathway was also tested.



RC414

Fig. 2. Chemical structure of RC414.

2. Materials and methods

2.1. Blood collection and preparative procedures

Human blood obtained from healthy volunteers was collected in 130 mM Na–citrate (9:1). PRP and WP were prepared as previously described [10]. Briefly PRP was obtained by centrifugation at 100 *g* for 25 min. Platelets, isolated from PRP centrifugation at 1000 *g* for 20 min, were washed once with pH 4.8 ACD solution (75 mM trisodium citrate, 42 mM citric acid and 136 mM glucose) and resuspended in pH 7.4 Hepes buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 10 mM glucose, 10 mM Hepes). The platelet concentration of PRP was always diluted to 2.0×10^8 plts/mL with platelet-poor-plasma.

For membranes preparation, washed platelets (1.0×10^9/mL) were resuspended in lysing buffer (5 mM pH 7.5 Tris–HCl, 5 mM EDTA) and chilled on ice for 30 min. The lysate was homogenized with a potter homogenizer (30 strokes) and centrifuged at 14,000 *g* for 20 min. The pellet was washed twice with lysing buffer and membranes were resuspended in 25 mM pH 8.0 Hepes, 1 mM EDTA and 0.1 mM DTT at the protein concentration of 10 mg/mL, prior to storage at −80°.

Protein concentration was assayed according to the Lowry method [11].

All chemicals, if not otherwise indicated, were from Sigma Chemicals Co.

2.2. Platelet aggregation

Aggregation, performed on an Aggrecoder PA-3210 Menarini aggregometer, was monitored according to Born's turbidimetric method [12] and quantified by the light transmission reached within 3 min. PRP and WP (2.0×10^8 plts/mL) were preincubated for 2 min at 37° with vehicle (dimethylsulfoxide, 5 μL) or drug solution before the addition of the platelet aggregation agent. Before each experiment the stock solutions of the agonists were diluted in saline.

2.3. Measurement of intracellular Ca^{2+} concentration

Intracellular Ca^{2+} concentration was measured as previously described [13]. WP (3.0×10^8 plts/mL), resuspended in Ca^{2+}-free pH 7.4 Hepes buffer, were incubated with 1 μM FURA 2/AM (Calbiochem-Novabiochem Corp.), for 45 min at 37°. PGE_1 (final concentration 2.0 μM) and EGTA (final concentration 1.0 mM) were added before centrifuging loaded platelets for 15 min at 1100 *g*. The pellet, resuspended at 2.0×10^8 plts/mL in pH 7.4 Hepes buffer, was preincubated at 37° for 2 min with saline or RC414, as indicated and then thrombin was added. FURA 2-loaded platelet fluorescence was followed at 37° in unstirred conditions for 15 min in a Perkin-Elmer fluorescence spectrometer model LS50B with excitations

at 340 and 380 nm and emission at 510 nm. The fluorescence of fully saturated FURA 2 (F_{max}) was obtained by lysing the cells with 50 μM digitonin in the presence of 2 mM Ca^{2+}, while F_{min} was determined by exposing the lysed platelets to 20 mM EGTA. The fluorescence was fully quenched with 5 mM Mn^{2+} to give the autofluorescence value. A software combined with the fluorescence spectrometer converted data into cytosolic Ca^{2+} concentration. The K_d value for FURA 2 and Ca^{2+} was 135 nM.

2.4. Cyclic nucleotides measurement in PRP

PRP was preincubated for 5 min at 30° with either saline or RC414 solution, then iloprost (final concentration 30 nM) was added when required. The reaction was stopped after 5 min by the addition of cold 2 M perchloric acid. The mixtures, sonicated for 5 s on ice, were centrifuged for 2 min at 12,000 *g*. Supernatants, neutralized with 2 M NaOH, were immediately analysed for cAMP or cGMP content by radioimmunoassay (RIA) kit (Amersham-Pharmacia Biotech).

2.5. cGMP measurement in WP

WP (1.0×10^9 plts/mL) resuspended in pH 7.4 Hepes buffer containing 2 mM $CaCl_2$ were prewarmed for 10 min at 37° with varying concentrations of RC414 and then incubated with 40 μM L-arginine for 15 min. The reaction was stopped by the addition of cold 2 M perchloric acid. cGMP content was quantified as above detailed by RIA kit (Amersham-Pharmacia Biotech).

2.6. Adenylate cyclase assay

Adenylate cyclase activity was measured in platelet membrane preparations according to Katada *et al*. [14].

2.7. Determination of cyclic nucleotide PDE activity

Platelet soluble cyclic nucleotide PDE activity was measured as previously reported [8] with several modifications. WP were resuspended at 2.0×10^9 plts/mL in assay buffer (1.3 M pH 8.0 Tris–HCl, 292 mM mercaptoethanol, 326 mM $MgCl_2$) in the presence of 20 μg/mL leupeptin, sonicated twice for 15 s on ice and centrifuged at 600 *g* for 20 min. Some experiments have been performed on soluble fraction obtained by 20,000 *g* for 20 min. Suitable aliquots of the supernatants were incubated for 10 min at 30° with increasing concentrations of RC414, 0.2 μCi/μL [^{3}H]cAMP or [^{3}H]cGMP and 1.3 μM cAMP or cGMP in assay buffer. After boiling for 90 s, the mixtures were added to 25 μg/mL 5′-nucleotidase (5′-ribonucleotide phosphohydrolase from *Crotalus atrox* venom; EC 3.1.3.5) and incubated for further 15 min at 30°. Reaction was stopped by the addition of EDTA (final concentration 2 mM). The samples were applied to 2 mL AG1 × 8 resin (200–400 mesh) (BIO-RAD pre-packed column), eluted with 50% ethanol. The eluates were collected in scintillation vials. The radioactivity of [^{3}H]adenosine or [^{3}H]guanosine was counted by liquid scintillation.

2.8. Assessement of PKC activation by measurements of 47 kDa phosphorylation

Platelets (2.5×10^9 plts/mL), resuspended in pH 7.4 Hepes buffer containing 1.0 mM EGTA and 5% platelet-poor-plasma, were incubated for 60 min at 37° with [^{32}P]phosphoric acid (250 μCi/mL), under gentle shaking, washed once and finally resuspended to 2.0×10^8 plts/mL in the same buffer containing 2 mM $CaCl_2$. Samples were preincubated for 10 min at 37° with saline or RC414 then thrombin or PMA were added. After 6 min at 37° activation was stopped by the addition of suitable aliquots of 2× Laemmli SDS reducing gel sample buffer containing 10% β-mercaptoethanol. Samples were boiled for 5 min and proteins separated by 10% SDS–PAGE. Running was performed in the presence of molecular weight markers. The gels were dried and [^{32}P] phosphorylated bands were observed by autoradiography by exposure to Amersham Hyperfilm-ECL.

2.9. L-Arginine uptake assay

L-Arginine uptake was measured as previously reported [15]. WP (2.0×10^8 plts/mL), prewarmed at 37° for 10 min in the presence of PBS or RC414, were incubated in the presence of 1.0 μCi/mL L-[2,3,4-^{3}H] arginine and 40 μM L-arginine. After 1 min aliquots of 1 mL were withdrawn, immediately filtered through a Titertek filter (Flow Laboratories) and washed twice with large volumes of cold PBS, containing 10 mM L-arginine. The radioactivity corresponding to the incorporated L-[2,3,4-^{3}H] arginine was directly measured by liquid scintillation counting of the filter in a Packard model TRI-CARB 1600 TR Liquid Scintillation Analyzer. The kinetic parameters of L-arginine transport were calculated by the Lineweaver-Burk plot.

2.10. Measurement of NO_x production

WP (1.0×10^9 plts/mL), resuspended in pH 7.4 Hepes buffer containing 2 mM $CaCl_2$, were prewarmed at 37° for 10 min with saline or various concentrations of RC414 and then incubated with 40 μM L-arginine for 15 min at 37°. Incubation was stopped by sonicating samples on ice. To measure NO_x content suitable aliquots of supernatant were added to equal volumes of pH 9.7 assay buffer (15 g/L glycine–NaOH) containing cadmium beds and incubated overnight at room temperature under horizontal shaking. Cadmium beds were activated immediately before each experiments by subsequent washings with 0.2 N H_2SO_4,

bidistilled water and assay buffer. NO_x accumulation, determined by the Griess reagent (1% sulphanilamide in 2.5% H_3PO_4, 0.1% naphtylenediamine dihydrochloride), was measured at 540 nm using a sodium nitrite calibration curve, as previously described [15].

2.11. cNOS assay in a cell-free system

WP (2.0×10^9 plts/mL) were sonicated twice for 15 s on ice, in the presence of 1.0 mM PMSF, 10 μg/mL leupeptin and 100 μM DTT and centrifuged at 600 *g* for 20 min. Suitable aliquots of the supernatants were mixed with 100 μM NADPH, 10 μM FAD, 10 μM FMN, 0.1 μM BH_4 and 1 mM $CaCl_2$ and various concentrations of the drug. Incubation was started by the addition of L-arginine (final concentration 40 μM). After 15 min at 37° the mixtures, added to equal volumes of pH 9.7 assay buffer (15 g/L glycine–NaOH), were incubated overnight at room temperature under horizontal shaking in the presence of activated cadmium beds and treated as above detailed.

2.12. Tests to assay platelet viability

To check up platelet viability upon drug treatment the efficiency of the glycolytic pathway was measured by the production of L-lactate according to Hohorst [16]. To verify the membrane damage the activity of lactic dehydrogenase released from platelets was quantified by the method of Vassault [17].

2.13. Statistics

Unless otherwise indicated all results are means ± SD of four experiments carried out in duplicate. In all cases similar findings were obtained in a minimum of three experiments. A control experiment in the presence of solvent was always carried out. Statistically significant differences were determined by using Student's two-tailed unpaired *t*-test.

3. Results

3.1. The RC414 effect on platelet aggregation

Incubation of PRP or WP for 2 min at 37° with RC414 (Fig. 2) produced inhibition of platelet aggregation. RC414 was a very powerful inhibitor in platelets challenged with collagen or ADP, the IC_{50} values being 0.51 ± 0.12 μM and 0.98 ± 0.36 μM, respectively. RC414 was lightly less effective in the presence of A23187: the IC_{50} values measured in PRP or in WP were 1.42 ± 0.43 μM or 1.50 ± 0.56 μM, respectively, indicating its low binding to serum proteins. In addition RC414 was a potent inhibitor of platelet aggregation induced by thrombin, the IC_{50} value being 1.0 ± 0.2 μM (Table 1). To exclude the direct involvement of the cyclooxygenase pathway in the effect of RC414, in some experiments PRP was preincubated with aspirin, known inhibitor of platelet cyclooxygenase enzyme. PRP was then challenged with collagen. The treatment with aspirin did not change significantly the IC_{50} value of RC414, that was 0.55 ± 0.10 μM.

Table 1
Inhibition of human platelet aggregation

Agonist	IC_{50} (μM)	
	PRP	WP
ADP (5 μM)	0.98 ± 0.36	ND
A23187 (20 μM)	1.42 ± 0.43	1.50 ± 0.56
Collagen (5 μg/mL)	0.51 ± 0.12	ND
Thrombin (0.1 U/mL)	ND	1.00 ± 0.15

PRP (2.0×10^8 plts/mL) or WP (2.0×10^8 plts/mL) were preincubated with solvent (dimethylsulphoxide) or RC414 solution for 2 min at 37° and then challenged with agonists for 3 min. Values are the mean ± SD of six separate determinations. ND: not determined.

3.2. The RC414 effect on cAMP levels and cAMP-phosphodiesterase activity

The compounds active against all agonists generate an increase in cAMP levels. To clarify the mechanism of action of RC414, we measured the cAMP levels in PRP in the presence or in the absence of RC414. RC414 increased in a dose-dependent manner the cAMP basal level in human platelets. Moreover, it potentiated the effect of iloprost, a prostacyclin analogue known adenylate cyclase activator, to increase cAMP (Fig. 3). However RC414, tested in the range 10–100 μM, did not modify platelet cGMP basal level: it was 1.0 ± 0.1 pmol/10^8 plts or 1.1 ± 0.1 pmol/10^8 plts in the presence or in the absence of 100 μM RC414.

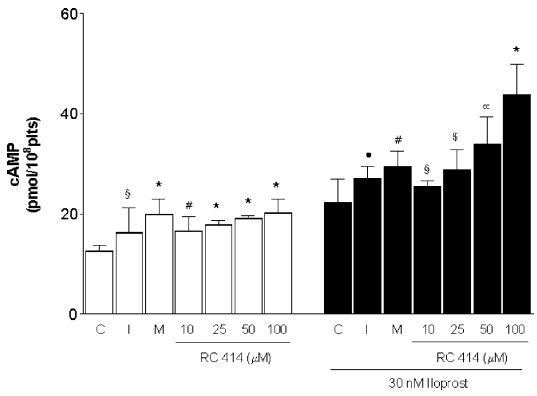


Fig. 3. Effect of RC414 on cAMP level in human platelets. PRP was incubated for 5 min at 30° with solvent or the drug, then iloprost (final concentration 30 nM) was added, when required. After 5 min at 30°, the reaction was stopped and cAMP measured. Each bar represents the mean ± SD of three experiments carried out in duplicate. C: control; I: 100 μM IBMX; M: 10 μM milrinone; $^{\$}P < 0.05$, $^{\bullet}P < 0.025$, $^{\#}P < 0.005$, $^{\propto}P < 0.0025$, $^{\$}P < 0.01$, $^{*}P < 0.0005$ vs. control.

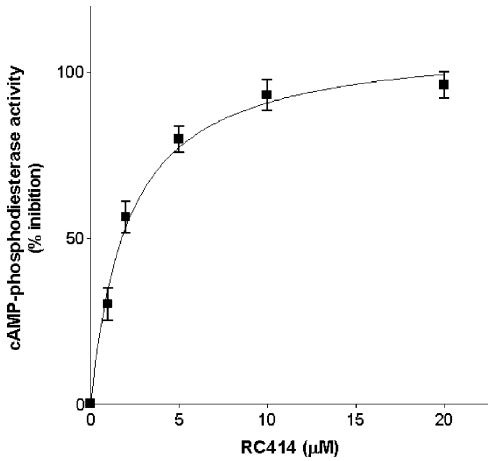


Fig. 4. Effect of RC414 on cAMP-PDE activity. Platelet crude extract was incubated for 10 min at 30° with 0.2 μCi/μL [^{3}H]cAMP, 1.3 μM cAMP and RC414 as indicated. The reaction was stopped by boiling for 90 s. After treatment with 5′-nucleotidase for 10 min at 30° and further addition of EDTA (final concentration 2 mM), the resulting nucleoside was separated by chromatography over AG1 × 8 (200–400) resins and eluted with 50% ethanol. Each point represents the mean ± SD of two paired samples from a total of three experiments.

The increase in cAMP is either due to adenylate cyclase activation or PDE inhibition. No effect on adenylate cyclase activity was shown: the activity measured in platelet membranes was 50 ± 9 pmol cAMP/mg protein/min and 53 ± 10 pmol cAMP/mg protein/min in the absence or in the presence of 100 μM RC414, respectively. Thus, it was reasonable to suppose that RC414 could likely be a PDE inhibitor. To verify this hypothesis we measured the RC414 effect on PDE activity of crude preparations or platelet soluble fractions: the IC_{50} values were 1.73 ± 0.35 μM or 1.66 ± 0.23 μM, respectively. Fig. 4 shows a dose–response curve of the inhibitory effect of RC414 on the high affinity cAMP-PDE of crude extracts, confirming the IC_{50} value above reported. On the contrary RC414 poorly inhibited cGMP-PDE activity: 10 μM RC414 or 50 μM RC414 inhibited cGMP-PDE by 3 or 10%, respectively.

Fig. 6. Effect of RC414 on pleckstrin phosphorylation. [^{32}P]-Labelled WP (2.0×10^8 plts/mL) were preincubated with saline or RC414 for 10 min at 37° then 0.1 U/mL thrombin or 1 μM PMA was added. After 6 min at 37° incubation was stopped by the addition to the samples of suitable aliquots of 2× Laemmli buffer. Proteins were separated by 10% SDS–PAGE and phosphorylated bands were localised by autoradiography. Results are representative of three similar experiments. Lane A: none; lane B: thrombin; lane C: 1 μM RC414 + thrombin; lane D: 5 μM RC414 + thrombin; lane E: PMA; lane F: 1 μM RC414 + PMA; lane G: 5 μM RC414 + PMA.

3.3. The RC414 effect on [Ca^{2+}] elevation induced by thrombin

The intracellular cAMP elevation may inhibit platelet function by reducing signal transduction and Ca^{2+} mobilization. We measured the effect of RC414 on thrombin-stimulated increase in cytosolic Ca^{2+} of FURA 2-loaded WP. RC414 was able to inhibit calcium elevation and aggregation induced by thrombin with identical potency. The IC_{50} values are 1.01 ± 0.04 μM and 0.92 ± 0.08 μM for platelet aggregation and calcium inhibition respectively (Fig. 5A and B).

3.4. The RC414 effect on PKC activation

Stimulation of platelets with thrombin induces a receptor-related activation of phospholipase C, which leads to the phosphorylation of several proteins, among which pleckstrin (47 kDa), indicative of protein kinase C activity (Fig. 6, lane B). So does PMA which directly stimulates protein kinase C producing pleckstrin phosphorylation (Fig. 6, lane E). Treatment of platelets with RC414 inhibited, in a dose-dependent manner, the thrombin-induced phosphorylation of pleckstrin (Fig. 6, lanes C and D), while no inhibition in PMA stimulated platelets was shown (Fig. 6, lanes F and G).

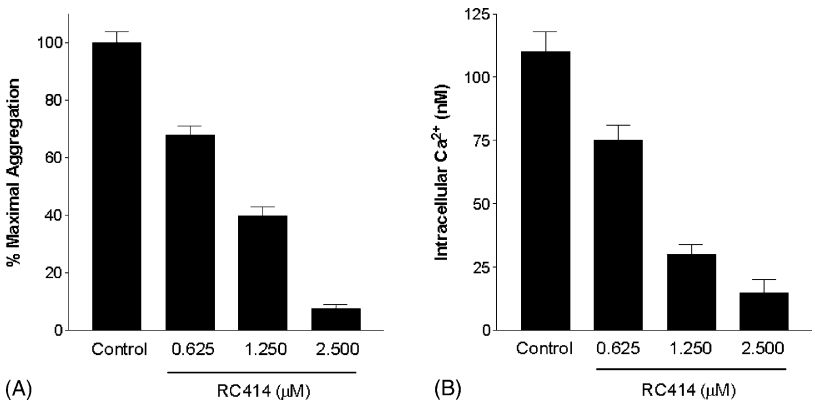


Fig. 5. Effect of RC414 on human platelet aggregation and on intracellular Ca^{2+} increase induced by thrombin. WP (2.0×10^8 plts/mL, panel A) or FURA 2-loaded platelets (2.0×10^8 plts/mL, panel B) were preincubated with RC414 for 2 min at 37° then challenged with 0.1 U/mL thrombin. Data are the mean ± SD of four experiments carried out in duplicate.

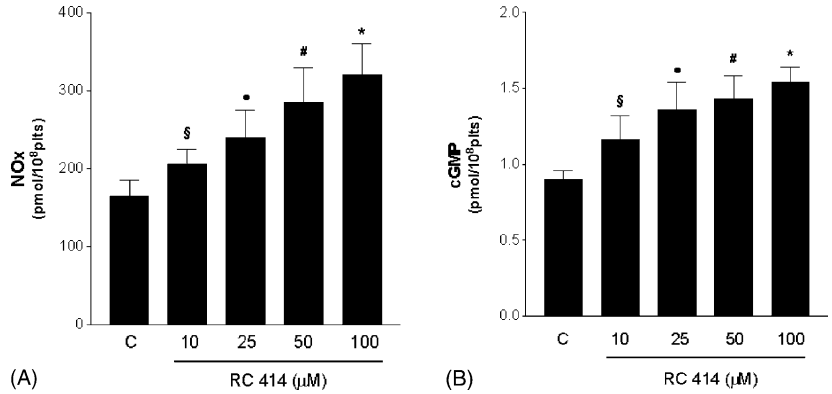


Fig. 7. Effect of RC414 on NO_x and cGMP accumulation. WP (1.0×10^9 plts/mL) were preincubated for 10 min at 37° in the presence of saline or RC414 as indicated, then L-arginine (final concentration 40 μM) was added. After 15 min at 37° the reaction was stopped and NO_x (panel A) or cGMP content (panel B) was quantified as detailed in Methods. $^{\S}P < 0.025$; $^{\bullet}P < 0.005$; $^{\#}P < 0.0025$; $^{*}P < 0.0005$ vs. control.

3.5. The RC414 effect on L-arginine/NO pathway

Furthermore we regarded it suitable to test the RC414 effect on NO cellular production. To evaluate whether RC414 was able to affect platelet NO formation some experiments have been performed incubating platelets in the presence of increasing concentrations of the drug and L-arginine. At the end of the incubation nitrite and nitrate accumulation was measured. As shown in Fig. 7A, RC414 was able to increase NO levels in whole platelets in a dose-dependent manner and the effect was significant at all concentrations tested. As additional evidence for the effect on NO production, the RC414 effect on cGMP levels was measured in platelets incubated in the presence of L-arginine. RC414 dose-dependently increased cGMP formation (Fig. 7B). cGMP levels correlate with NO_x accumulation ($y = 236.132874\,x + 58.777805$; $r^2 = 0.934609$).

Since previously [15,18] we have shown that in human platelets NO formation depends on L-arginine availability, we wanted to verify whether RC414 was able to affect the L-arginine transport across the membrane. Platelet treatment with various RC414 concentrations did not modify significantly neither the time-course of L-arginine transport nor the kinetic parameters (data not shown). Thus we tested the RC414 effect on the cNOS activity of cell-free extracts. Data of Fig. 8 show that RC414 produces a significant increase in the cNOS activity: a 5 μM RC414 solution stimulates the activity of about 70% upon basal level.

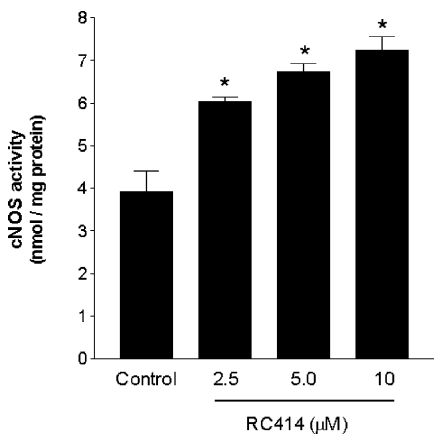


Fig. 8. Effect of RC414 on cNOS activity. Suitable aliquots of platelet cell-free system were incubated for 5 min at 37° in the presence of 40 μM L-arginine and RC414 as indicated. cNOS activity was determined as described in detail in Methods. $^{*}P < 0.0005$ vs. control.

3.6. Tests to assay platelet viability

Platelets treated with increasing concentrations of RC414 in the range 10–100 μM were not different from control platelets in the lactate produced or in the lactic dehydrogenase released during 15 min of incubation at 37°. Lactate produced was 22 ± 2.0 nmol/10^9 plts/min and 21 ± 1.5 nmol/10^9 plts/min in controls and in platelets treated with RC414, respectively. The leakage of lactate dehydrogenase that occurred during the incubation and centrifugation of the platelets corresponded to 8% of the total amount, both in the presence and in the absence of the drug (10 or 100 μM).

4. Discussion

The present work aimed to define the mechanism of action of a new benzopyran derivative (RC414) that was selected among a large number of compounds [4] for its powerful antiplatelet activity (Table 1). It is interesting that cilostazol, [19] usually administrated to patients with various thrombotic disorders [20], and RC414 are equally effective in inhibiting human platelet aggregation.

The action of compounds active against all agonists produces increase in cAMP levels. As shown in Fig. 3, RC414 by itself increases cAMP under basal level and potentiates the effect of iloprost, powerful activator of adenylate cyclase. The increase in cAMP was brought about by the specific inhibition of the low K_m cAMP-PDE (PDE3).

It is remarkable that the rank order of inhibitory potency of RC414 vs. PDE was the same as that obtained when the compound inhibited platelet aggregation. Platelets contain two cAMP phosphodiesterases which regulate intracellular cAMP levels, the cGMP-inhibited PDE (PDE3) and the cGMP-stimulated PDE (PDE2) [21]. The PDE3 has been shown to account for over 80% of the total PDE activity in platelet extracts and therefore represents a suitable target for the design of antiplatelet agents. Recent data indicate that inhibitors of PDE3 may be more effective antiplatelet agents than inhibitors of PDE2 [22]. Early development of PDE3 specific inhibitors was focused on the design of structural analogs of specific nucleotides or xanthine derivatives, such as IBMX and theophylline. Subsequent work has identified more potent and specific inhibitors such as the bipyridines (milrinone and amrinone) [23], the flavonoids [24], and the imidazoquinolone derivatives (anagrelide) [25,26]. In general, these agents do not increase significantly the intracellular cAMP basal level in resting platelets, although they do potentiate the ability of PGI_2 or other adenylate cyclase agonists to increase cAMP.

Considerable evidence suggests that the inhibition of platelet function generated by cAMP is mediated by cAMP-dependent protein kinase A (cAMP-PKA). It is known that an increase in cAMP of about two times upon basal level potentiates significantly cAMP-PKA activity [9,27,28] producing the phosphorylation of various proteins involved in platelet inhibition by cAMP. One of the most important steps is the inhibition of receptor-mediated phosphoinositide hydrolysis by phospholipase C activation. The inhibitory effect of cAMP on protein kinase C activation, Ca^{2+} mobilisation, fibrinogen-receptor exposure, myosin light chain phosphorylation, actin polymerisation and cytoskeletal assembly are believed to be consequence of the inhibition of receptor-mediated phospholipase C activation [29]. Therefore we examined the effect of RC414 on rise in cytosolic Ca^{2+} and on phosphorylation of pleckstrin (47 kDa) in whole platelets challenged with thrombin. It was shown that RC414 was able to inhibit Ca^{2+} elevation induced by thrombin. The dose-response curves for both inhibition of calcium mobilisation and platelet aggregation were similar: IC_{50} values were 0.92 ± 0.08 μM and 1.01 ± 0.04 μM, respectively (Fig. 5). In addition RC414 in a dose-dependent manner inhibited pleckstrin phosphorylation induced by thrombin, while RC414 was ineffective on platelets challenged by PMA. These results clearly indicate that RC414 affects one or more steps in the very early activation of G protein-coupled receptor in the activation of phospholipase C by thrombin.

As the elevation of cGMP by NO could lead to the concomitant increase of intracellular cAMP through the inhibition of cAMP breakdown by the PDE3, the involvement of RC414 in the platelet L-arginine/NO pathway was investigated. Results of these studies show that RC414 increases NO and cGMP intracellular levels in platelets incubated in the presence of L-arginine. Infact NO activates guanylate cyclase to produce cGMP from GTP, thus NO and cGMP levels are strictly correlated [15,18]. To obtain further insight into the mechanisms leading to the increase in NO production associated with RC414 platelet treatment, we studied the effect of the benzopyran derivative on L-arginine uptake and on cNOS activity of cell-free extracts. Results obtained allowed us to exclude an effect at the level of L-arginine transport, a very important regulatory step for platelet NO production [15]. On the contrary a dose-dependent stimulatory effect of RC414 on cNOS activity was shown. We can suggest that this effect could be due to a direct interaction between the drug and cNOS. Otherwise RC414 could improve the availability of substrate and/or cofactors to the enzyme. Nevertheless we haven't experimental evidence. Experiments are in progress to clarify the action mechanism of RC414 on cNOS in platelets.

The cAMP and cGMP elevation would lead to activation of cAMP (protein kinase A) and cGMP (protein kinase G) protein kinases, which could be partially responsible for some of the observed effects of NO on platelet activation.

In conclusion the simple linear model of independent cAMP and cGMP action seems to be unlikely in platelets. The cGMP-inhibited PDE (PDE3) represents a major mediator for "cross talk" between cGMP and cAMP mediated signalling pathways. Thus interactions between cAMP and cGMP are likely to have very important consequences for the inhibition of platelet function by physiological mediators and drugs that increase platelet cyclic nucleotide levels.

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