

RESEARCH PAPER

Oncocalyxone A inhibits human platelet aggregation by increasing cGMP and by binding to GP Ib α glycoprotein

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Background and purpose: Oncocalyxone A (OncoA) has a concentration-dependent anti-platelet activity. The present study aimed to further understand the mechanisms related to this effect.

Experimental approach: Human platelet aggregation was measured by means of a turbidimetric method. OncoA (32–256 μ M) was tested against several platelet-aggregating agents, such as adenosine diphosphate (ADP), collagen, arachidonic acid (AA), ristocetin and thrombin.

Key results: OncoA completely inhibited platelet aggregation with a calculated mean inhibitory concentration (IC₅₀- μ M) of 122 for ADP, 161 for collagen, 159 for AA, 169 for ristocetin and 85 for thrombin. The anti-aggregatory activity of OncoA was not inhibited by 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ). OncoA, at a concentration that caused no significant anti-aggregatory activity, potentiated sodium nitroprusside (SNP) anti-aggregatory activity (18.8 $\pm$ 2.9%-SNP vs 85.0 $\pm$ 8.2%-SNP + OncoA). The levels of nitric oxide (NO) or cAMP were not altered by OncoA while cGMP levels were increased more than 10-fold by OncoA in resting or ADP-activated platelets. Flow cytometry revealed that OncoA does not interact with receptors for fibrinogen, collagen or P-selectin. Nevertheless, OncoA decreased the binding of antibodies to GP Ib α , a glycoprotein that is related both to von Willebrand factor and to thrombin-induced platelet aggregation.

Conclusion and implications: OncoA showed anti-aggregatory activity in platelets that was associated with increased cGMP levels, not dependent on NO and with blocking GP Ib α glycoprotein. This new mechanism has the prospect of leading to new anti-thrombotic drugs.

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Abbreviations: AA, arachidonic acid; DMSO, dimethyl sulphoxide; ODQ, 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one; OncoA, oncocalyxone A; PDE5, phosphodiesterase 5; PGI₂, prostacyclin; PPP, platelet poor plasma; PRP, platelet rich plasma; sGC, soluble guanylate cyclase; SNP, sodium nitroprusside; TXA₂, thromboxane A₂

Introduction

Auxemma oncocalyx Taub. (Boraginaceae) known as 'pau-branco' is a tree native to the Northeast of Brazil. Pharmacological studies using a water-soluble fraction of an ethanolic extract obtained from the heartwood of *A. oncocalyx* was previously shown to inhibit human platelet

aggregation induced by several agonists such as ADP, collagen, thrombin, arachidonic acid and adrenaline in platelet rich plasma (PRP), in a reversible and concentration-related manner (Ferreira *et al.*, 1999). In addition, this extract was demonstrated to have antioxidant, analgesic and anti-inflammatory properties *in vivo* (Ferreira *et al.*, 2003, 2004). This fraction had no overt toxicity when administered orally to mice.

The phytochemical investigation of *A. oncocalyx* heartwood extracts has resulted in the isolation and characterization of several uncommon terpenoid quinones and hydroquinones with a C₁₆ backbone (Pessoa *et al.*, 1993,

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1995; Marques *et al.*, 2000). Two of these secondary metabolites, named oncalyxone A (OncoA) and C were obtained in significant amounts. Both compounds were shown to have antitumour activity (Leyva *et al.*, 2000; Pessoa *et al.*, 2000) possibly related to their antimetabolic effects (Lotufo *et al.*, 2002) and it was subsequently shown that, in primary cultures of human lymphocytes, OncoA is devoid of genotoxicity (Pessoa *et al.*, 2003).

More recently, we demonstrated that OncoA (Figure 1) is the active principle of *A. oncalyx* responsible for its platelet antiaggregatory activity and that this compound does not have hypotensive activity *in vivo* nor does it affect the perfusion pressure of the vascular mesenteric bed perfused at constant flow and precontracted with phenylephrine (Sousa *et al.*, 2002). The aim of the present study was to evaluate the mechanism of antiaggregatory activity in platelets induced by OncoA isolated from *A. oncalyx*.

Materials and methods

Isolation of OncoA from *A. oncalyx*

Auxemma oncalyx Taub was collected at Pentecoste City in the state of Ceará, which is located in Northeast Brazil. The species was identified by Professor Afrânio Gomes Fernandes from the Department of Biology of the Federal University of Ceará where a voucher was deposited at the Prisco Bezerra Herbarium under the registration number 18459.

OncoA was isolated as a deep red powder, mp 207–208 °C, from the ethanol extract of the heartwood of *A. oncalyx* as previously described by Pessoa *et al.* (1993). The structure of this secondary metabolite was unambiguously established by spectrometric techniques such as IR, MS and a combination of 1D and 2D NMR methods. The structure was deduced as *rel*-8 α -hydroxy-5-hydroxymethyl-2-methoxy-8 $\alpha\beta$ -methyl-7,8,8a,9-tetrahydro-1,4-anthracenedione, which was named as Oncocalyxone A.

Evaluation of the effects of OncoA on platelet aggregation *in vitro*

The study was approved by the Butantan Institute Review Board and the Brazilian National Council. Platelets were isolated from the blood of healthy non-smoking human volunteers (seven men and 22 women), from 18 to 45 years of age (average of 25 years). The smokers were excluded because smoking is known to increase both platelet adhesiveness and aggregation (Law and Wald, 2003; Leone, 2005). Only volunteers who had not taken medicines at least 2 weeks before blood collection were included. Blood samples were taken from the antecubital vein and directly drawn into plastic tubes containing tri-sodium citrate (3.8%). Platelet rich plasma was prepared by centrifugation of blood samples at 180g for 10 min. After removal of the PRP, platelet poor plasma (PPP) was obtained by centrifugation of the remaining blood sample for 20 min at 500g. Platelets in PRP were counted by using a cell counter (System 9020 AX, Sero Diagnostics, Allentown, PA, USA), and the number adjusted to 3×10^8 platelets mL⁻¹ with homologous PPP. All the assays were performed immediately after venepuncture and processing of samples and all protocols were performed no more

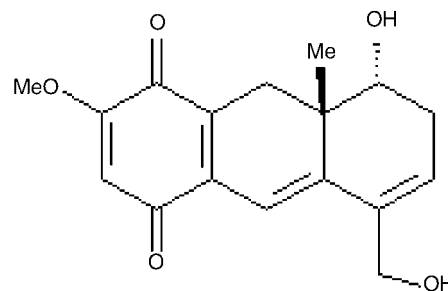


Figure 1 Chemical structure of oncalyxone A (OncoA).

than 3 h after blood collection to avoid loss of platelet sensitivity.

A platelet aggregation test was performed using the turbidimetric method described by Born and Cross (1963) on a lumi-aggregometer (modelo 400 VS, Chrono-Log, USA) with recording and analysis of data made by the Aggrolink software (Chrono-Log, USA). Aggregation was measured at 37 °C under continuous stirring at 1000 r.p.m. in 450 μ L aliquots of PRP or washed platelets with data being stored in the computer for analysis. Platelet aggregation was measured by light transmission, with 100% calibration as the absorbance of PPP and 0% calibration as the absorbance of PRP. The aggregation was measured by the height of aggregation curves after 10 min of incubation with specific agonists.

Platelets were stimulated in PRP with ADP (10 μ M), collagen (2 μ g mL⁻¹), AA (500 μ M) or ristocetin (1 mg mL⁻¹) and in suspensions of washed platelets with thrombin (5 nM) ($n = 6$ for each agonist). To determine the effect of OncoA on platelet aggregation, samples of PRP or washed platelets were incubated for 5 min with either vehicle (0.5% DMSO in saline; v/v) or OncoA (32, 64, 128 or 256 μ M) before addition of the specific agonist.

To test the reversibility of OncoA-induced platelet aggregation inhibition, PRP was incubated with the quinone (256 μ M) or vehicle at room temperature for 5 min and thereafter both aliquots were challenged with AA (500 μ M). After recording the inhibition induced by OncoA in PRP, the platelets were washed and incubated again with quinone or vehicle during the same time and then stimulated with AA.

Investigation of the role of thromboxane A₂ in inhibition of platelet aggregation by OncoA

In another set of experiments, the blood samples were collected from volunteers that had taken aspirin (500 mg) the night before to irreversibly block TXA₂ synthesis ($n = 4$). In these samples, aggregation was evoked by collagen (8 μ g mL⁻¹), which induced aggregation by a TXA₂-independent mechanism (Siess, 1989; Herman, 1998) in PRP and the curves of aggregation were compared between vehicle and OncoA (32, 64, 128 or 256 μ M).

Evaluation of the role of soluble guanylate cyclase (sGC) and PDE5 in the OncoA-induced inhibition of platelet aggregation
Cyclic GMP and some PDE5 inhibitors are known to be involved in the antiplatelet activity of several agents, for

example sodium nitroprusside (SNP) and dipyridamole (Geiger, 2001). Therefore, the potential involvement of sGC in the OncoA-induced platelet aggregation inhibition was investigated in PRP using ADP as an agonist ($n = 6$). The effects of SNP (80 μM) a NO-donor drug or OncoA (256 μM) were studied in the absence and in the presence of oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ) (10 μM), a specific inhibitor of the NO-sensitive site of sGC.

Samples of PRP (450 μL) were incubated at room temperature in the following conditions. In an initial experiment, PRP was incubated with OncoA for 5 min, SNP for 3 min or ODQ for 10 min before challenge with ADP (10 μM). In a second set of experiments, samples were treated first with ODQ (incubated for 10 min) and then treated with OncoA for 5 min or SNP for 3 min before ADP challenge. In a third set of experiments, a comparison was made between the effects of SNP (1 μM) on ADP-induced platelet aggregation in PRP either in the presence or absence of OncoA (32 μM) or sildenafil citrate (10 μM) ($n = 6$ for each protocol), a known specific PDE5 inhibitor. Here, samples of PRP were incubated at room temperature with OncoA for 5 min or sildenafil for 5 min or SNP for 3 min before challenge with ADP (10 μM). Finally, in a fourth set of experiments, samples were pretreated first with OncoA (5 min) or with sildenafil (5 min), then with SNP (3 min) before ADP challenge.

Evaluation of effects of OncoA on the platelet release of ATP

The release of ATP from stimulated platelets was measured using the bioluminescence method described by DeLuca and McElory (1978) on a lumi-aggregometer (model 400 VS, Chrono-Log, USA) with Aggrolink package (Chrono-Log, USA). Briefly, the method is based on the formation of an enzymatic complex (luciferin-luciferase in the presence of O_2 and ATP) emitting luminescence, which is recorded in parallel to platelet aggregation. To calibrate the lumi-aggregometer the optical density was adjusted to 100% with PRP and to 0% with PPP.

The effect of OncoA (256 μM) on the release of ATP ($n = 4$ for each agonist) was determined in PRP, using as agonists ADP (2.5 μM), collagen (1 $\mu\text{g mL}^{-1}$) or AA (750 μM). The ATP release reaction was monitored by bioluminescence changes, with luciferin and luciferase (2 mg mL^{-1}) and the concentration of ATP calculated by comparison with an ATP (1.8 μM) standard.

NO determination in washed platelets

NO is a potent endogenous platelet antiaggregatory agent, which can be released by exogenous materials with anti-aggregatory effects. For instance, tetramethylpyrazine increases NO release and thus leads to subsequent antiaggregation (Sheu *et al.*, 2000). To test whether OncoA would increase NO release, the effect of OncoA on NO production was determined ($n = 4$) by chemiluminescence in a suspension of washed platelets as described by Sheu *et al.* (2000). Briefly, samples of washed platelets (10^9 platelets mL^{-1}) were incubated with vehicle, OncoA (64, 128 or 256 μM) or collagen (10 $\mu\text{g mL}^{-1}$) for 15 min at 37 °C. Afterwards, the samples were centrifuged at 328 g for 5 min and the supernatant was

separated and deproteinated with ice-cold 95% ethanol for 30 min at 4 °C. The mixture was then centrifuged at 283 g for 7 min and the supernatant kept at -80°C until the assay was performed.

The test was performed by adding 10 μL of the sample to a reaction chamber with 0.8% vanadium chloride in HCl (1 M) as a reducing agent to convert nitrate to NO, which itself was swept by a helium carrier gas and analysed in a Sievers NO Analyzer (Sievers 280 NOA, Sievers Inc., Boulder, CO, USA). The concentration of NO was calculated by comparison with standard sodium nitrate solutions.

Determination of platelet cAMP and cGMP levels

The intracellular levels of cAMP or cGMP were measured in PRP (2×10^8 platelets/sample) in both ADP (10 μM) activated and non-activated platelets. Non-activated platelets were incubated for 5 min with either vehicle or OncoA (256 μM) or prostacyclin (PGI_2 ; 0.1 $\mu\text{g mL}^{-1}$). PGI_2 was used as positive control because it increases intracellular cAMP. ADP-activated platelets were incubated for 5 min with vehicle or OncoA (256 μM).

Afterwards, EDTA (4 mM) was added to the sample, which was then centrifuged at 450 g for 2 min. The supernatant was disposed, and the remaining cell pellet was resuspended and incubated with 300 μL of HCl (0.1 M). After incubation, platelet lysis was confirmed by light microscopy (LABO PHOT, Nikon, Japan) and samples were centrifuged at 350 g for 10 min and the lysate was subsequently processed according to the manufacturer's instructions for immunoassay determination of intracellular cGMP or cAMP (Assay Designs, Ann Harbor, MI, USA).

Flow cytometry studies: interaction of OncoA with platelet receptors

The effect of OncoA on different receptors expressed on the surface of platelets and involved in platelet aggregation was also tested. Samples (20 μL) of PRP (3×10^8 platelets mL^{-1}) were incubated with vehicle or different concentrations of OncoA at 37 °C for 5 min ($n = 4$ for each protocol). Then, samples (3 μL) were transferred to a polystyrene tube containing 40 μL of fetal bovine serum 10% in phosphate-buffered saline pH 7.4 and 2.5 μL of monoclonal antibody. Monoclonal antibodies against the following antigens were used: CD42b (glycoprotein $\text{Ib}\alpha$ from GP Ib/V/IX complex, von Willebrand factor receptor), CD41a (glycoprotein IIb from GP IIb/IIIa complex, fibrinogen receptor), CD62P (P-selectin). Integrin $\alpha_2\beta_1$, a collagen receptor was marked with CD49b (α_2 -subunit of $\alpha_2\beta_1$) and CD29 (β_1 -subunit of $\alpha_2\beta_1$). After 30 min incubation at room temperature the reaction was stopped by addition of 1% paraformaldehyde (300 μL) and flow cytometry was performed using a FACScan cytofluorimeter Becton-Dickinson with CELL Quest package (Becton-Dickinson, Mountain View, CA, USA).

Statistical analysis

Data were expressed as mean $\pm$ s.e.mean. The concentration required to produce 50% inhibition of platelet aggregation

(IC₅₀) was calculated using non-linear regression by means of GraphPad 3.0 software and was expressed with its respective 95% confidence intervals. The data was analysed for statistical differences by using repeated measures one-way ANOVA with the correction of Tukey–Kramer as a *post hoc* test with 95% confidence. The statistical analysis for the platelet ATP-releasing protocol was done using a two-tailed paired Student's *t*-test with significance level set at 5%.

Drugs and reagents

Arachidonic acid (AA), thrombin, dimethyl sulphoxide (DMSO), ATP, luciferin-luciferase, prostacyclin (PGI₂), SNP, 1H-[1,2,4] ODQ were obtained from Sigma Chemical (Saint Louis, MO, USA); ADP, collagen and ristocetin from Chrono-Log (Havertown, PA, USA); sildenafil citrate from Pfizer (New York, NY, USA); cAMPc and cGMP enzyme immunoassay kits from Assay Designs (Ann Harbor, MI, USA) and monoclonal antibodies to CD42b, CD41a, CD62P, CD49b, CD29 adhesion molecules from PharMingen (Franklin Lakes, NJ, USA).

The buffer solution (pH 6.5) used to wash platelets had the following composition (mM): NaCl 140.0, NaHCO₃ 10.0, KCl 2.5, Na₂HPO₄ 0.49, MgCl₂ 1.0, tri-sodium citrate 22.0, bovine serum albumin 52.2. The Tyrode/Ca²⁺ solution (pH 7.4) had the following composition (mM): NaCl 134.0, KCl 2.9, Na₂HPO₄ 0.34, MgCl₂ 1.0, NaHCO₃ 12.0, CaCl₂ 1.0 and HEPES 10.0.

All reagents and salts used were from analytical grade and obtained from Vetec (Rio de Janeiro, RJ, Brazil).

Results

Effects of OncoA on platelet aggregation in vitro

The mean aggregatory response of all agonists were between 70–90%. A typical example of aggregation, with AA (500 µM) is shown in Figure 2a. This Figure also shows a representative recording of the anti-aggregatory effect of OncoA on AA-induced aggregation. The reversibility of this effect is shown in Figure 2b.

Pre-incubation of platelets with OncoA (32, 64, 128 and 256 µM) inhibited, in a concentration-related manner, platelet aggregation induced by a range of agonists—ADP (10 µM), collagen (2 µg mL⁻¹), AA (500 µM), ristocetin (1 mg mL⁻¹) and thrombin (5 nM). The result from these experiments are summarized in Figure 2c. From these concentration-effect curves, the calculated IC₅₀ values (µM; mean, 95% con-

fidence intervals) for OncoA were: ADP, 122 [75–199]; collagen, 161 [100–259]; AA, 159 [139–181]; ristocetin, 169 [133–214] and thrombin, 85 [69–105].

Platelet aggregation induced by AA in platelets that were first incubated with OncoA and then washed was similar to

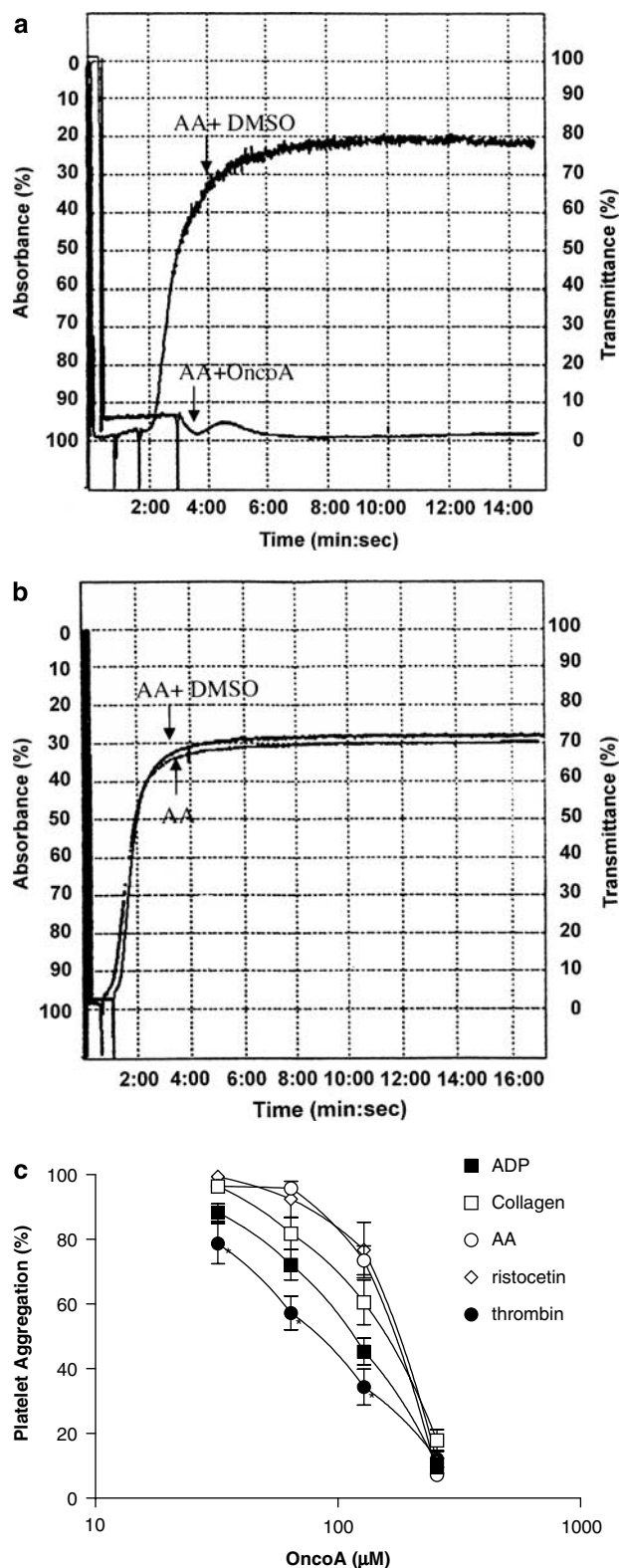


Figure 2 Representative recordings showing the effect of OncoA (256 µM) or DMSO on AA-induced platelet aggregation (a) and the reversal of this antiplatelet activity after washout (b). OncoA inhibits human platelet aggregation induced by ADP, collagen, AA, ristocetin or thrombin (c). Samples of PRP or washed platelets were incubated with vehicle or OncoA (32–256 µM) at room temperature for 5 min before addition of agonist. The heights of aggregation curves were measured at peak effect and expressed as percentage of the control. Results are the mean ± s.e.m. of six experiments (**P* < 0.05 vs collagen, acid arachidonic (AA) and ristocetin, ANOVA for repeated measurements followed by Tukey–Kramer for multiple comparisons).

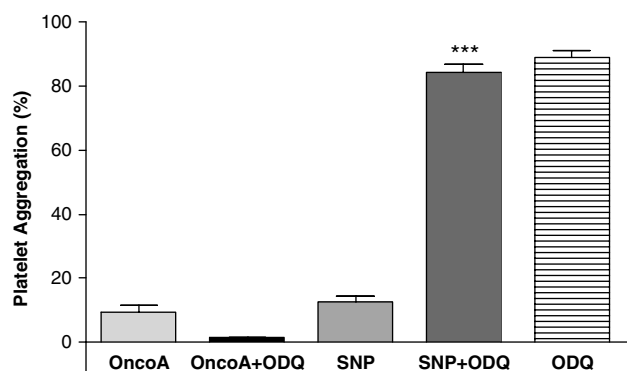


Figure 3 Role of sGC in the inhibition of ADP-induced platelet aggregation by OncoA. Samples of PRP were incubated at room temperature with OncoA (256 μ M; 5 min), SNP (80 μ M; 3 min) or ODQ (10 μ M; 10 min) before ADP (10 μ M) challenge. Other samples were treated first with ODQ (10 μ M for 10 min) and then treated with OncoA (5 min) or SNP (3 min) before ADP challenge. Data are expressed as mean $\pm$ s.e.m. of six experiments for each column (*** P < 0.001 vs SNP alone, ANOVA followed by Tukey–Kramer for multiple comparisons).

that obtained in control samples incubated only with vehicle. This indicates that the effect of OncoA is quite reversible and after washout of OncoA, platelets function normally. For instance, after incubation with OncoA (256 μ M) platelet aggregation was inhibited by $96 \pm 2.2\%$ (mean $\pm$ s.e.m. of 4 experiments). After washout, platelets were still able to aggregate by $97 \pm 2.4\%$ in relation to control, showing no functional damage induced by OncoA.

Role of thromboxane A_2 in the inhibition of platelet aggregation, mediated by OncoA

The inhibition of collagen-induced platelet aggregation by OncoA (32, 64, 128 and 256 μ M) was unaltered in platelets where TXA_2 synthesis was inhibited by aspirin. The percentage inhibition under these conditions was 16 ± 3.4 , 28 ± 4.3 , 52 ± 4.6 and $96 \pm 0.6\%$, respectively, with a calculated IC_{50} of 131 [65–265] μ M.

Role of sGC and PDE5 in OncoA-induced platelet aggregation inhibition

To analyse whether sGC or PDE5, were involved in the inhibitory effects of OncoA, specific inhibitors of these pathways were employed. Figure 3 shows that while the inhibition of ADP-induced platelet aggregation by SNP (80 μ M) was blocked by 82% in the presence of ODQ (10 μ M) (P < 0.001, n = 6), OncoA-mediated inhibition was not affected (P < 0.05, n = 6).

On the other hand, OncoA or sildenafil used in concentrations that alone were not able to induce significant inhibition of platelet aggregation (32 or 10 μ M, respectively) were both able to potentiate SNP-(1 μ M) induced anti-aggregatory activity (P < 0.001 with OncoA and P < 0.01 with sildenafil) (Figure 4).

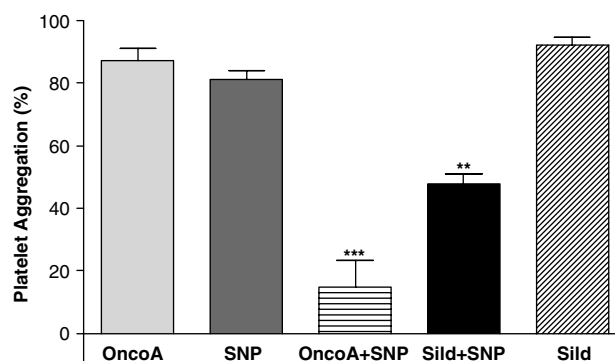


Figure 4 Comparison of the effects of sildenafil (Sild) or OncoA in combination with SNP in ADP-induced platelet aggregation. Samples of PRP were incubated at room temperature with OncoA (32 μ M; 5 min) or sildenafil (10 μ M; 5 min) or SNP (1 μ M; 3 min) before challenge with ADP (10 μ M). Other samples were pretreated first with OncoA (5 min) or with sildenafil (5 min), then with SNP (3 min) before ADP challenge. Data are expressed as mean $\pm$ s.e.m. of six experiments (*** P < 0.001 vs oncoA or SNP alone; ** P < 0.01 vs sildenafil or SNP alone, ANOVA followed by Tukey–Kramer for multiple comparisons).

Effect of OncoA on the platelet release of ATP

The release of ATP induced by ADP (2.5 μ M), collagen (1 μ g mL⁻¹) or AA (750 μ M) was strongly inhibited by OncoA (256 μ M). The percentage of inhibition was 100% for ADP, $93 \pm 4.6\%$ (P < 0.01) for collagen and $87 \pm 3.6\%$ (P < 0.05) for AA, respectively.

Effect of OncoA on NO production by platelets

The effect of OncoA on NO release in platelets was determined in suspensions of washed cells (10^9 platelets mL⁻¹). Release of NO on aggregation of washed platelets by collagen was more than double that released from resting platelets (Figure 5). This resting release was not altered by incubation with OncoA even at the highest concentration used (Figure 5).

OncoA raised cGMP but not cAMP levels

Exposure of platelets to PGI_2 increased platelet cAMP levels 8.6-fold compared to resting platelets (Figure 6a). However, OncoA was unable to modify cAMP concentration either in resting or ADP-activated platelets. On the other hand, OncoA increased cGMP production approximately 12-fold in resting and 11.6-fold in ADP-activated platelets (Figure 6b).

Flow cytometry studies: interaction of OncoA and platelet receptors

The possible interaction of OncoA with GP Ib α from the GP Ib/V/IX complex which is the von Willebrand's factor receptor, was investigated by using a specific monoclonal antibody directed against CD42b. OncoA induced a concentration-dependent inhibition of the binding of this antibody (Table 1). Note however that the same concentrations of OncoA did not affect binding of antibodies to a variety of

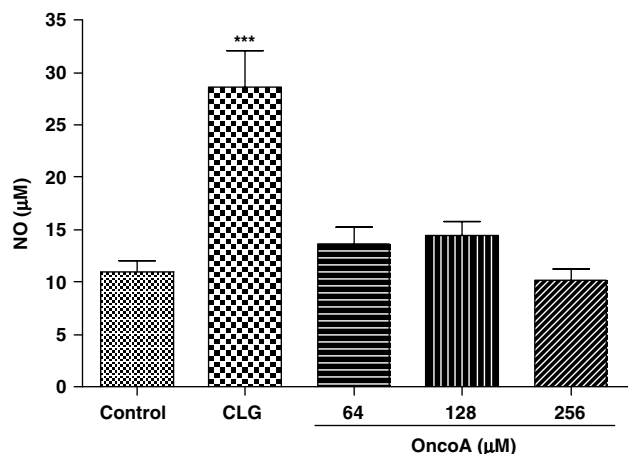


Figure 5 Effect of OncoA on NO production in washed platelets. Samples of washed platelets (10^9 mL^{-1}) were incubated at 37°C , for 15 min, with vehicle and OncoA (64, 128 or $256 \mu\text{M}$). Collagen ($10 \mu\text{g mL}^{-1}$) was used as positive control. The data are presented as mean $\pm$ s.e.m. ($n=4$). *** $P<0.001$ compared with resting platelets (ANOVA for repeated measures followed by Tukey–Kramer for multiple comparisons). CLG = collagen.

other platelet surface proteins (CD 41a, 62P, 49b or 29) (Table 1).

Discussion

The search for new antithrombotic agents has resulted in the isolation of many plant constituents with antiplatelet activity, including quinones (Guh *et al.*, 1995; Reis *et al.*, 1999; Wu *et al.*, 2001).

For instance, the anthraquinone Frangulin B isolated from *Rhammus formosana*, specifically inhibited platelet aggregation induced by collagen and was described as a putative antagonist of the collagen receptor (Teng *et al.*, 1993). Shikonin and other naphthoquinones isolated from *Arnebia euchroma*, a Chinese herb known for its antithrombotic properties, inhibited rabbit platelet aggregation induced by collagen, AA, platelet-activating factor and thrombin by blocking the hydrolysis of inositol phospholipids (Ko *et al.*, 1995). A synthetic naphthoquinone (NQ 301) inhibited human platelet aggregation induced by collagen, arachidonic acid, thrombin and the calcium ionophore A23187 by increasing intracellular levels of cAMP (Zhang *et al.*, 2001). Another synthetic naphthoquinone (J78) was shown to inhibit rabbit platelet aggregation by inhibition of TXA_2 synthesis, TXA_2 receptor blockade and suppression of calcium mobilization (Jin *et al.*, 2005).

Quinones are thought to rapidly deplete reduced glutathione levels in various tissues (Di Monte *et al.*, 1984; Brown *et al.*, 1991; Tzeng *et al.*, 1994) and this effect is thought to be related to their antiaggregatory activity (as a primary manifestation of cytotoxicity) (Kim *et al.*, 2001). However, OncoA itself has antioxidant activity both *in vitro* and *in vivo* (Ferreira *et al.*, 2001, 2003) and has no pro-oxidant activity (data not published).

In this study, we demonstrated that OncoA inhibited, in a concentration-dependent and reversible manner, at least for

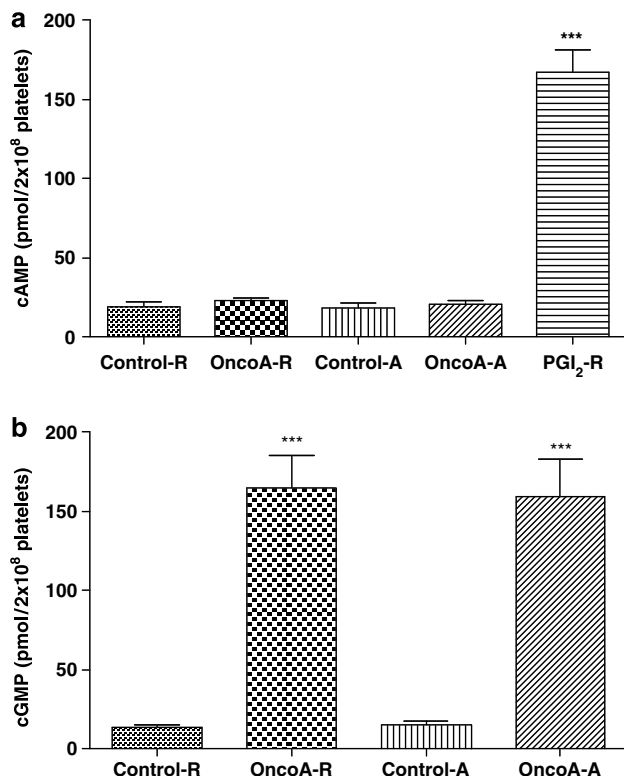


Figure 6 Effect of OncoA on platelet cAMP (a) or cGMP (b) intracellular concentration. PRP ('resting platelets') was incubated with vehicle, OncoA ($256 \mu\text{M}$) or PGI_2 ($0.1 \mu\text{g mL}^{-1}$) at room temperature for 5 min. Other samples ('ADP-activated platelets') were incubated with vehicle or OncoA before ADP ($10 \mu\text{M}$) stimulation. The reaction was stopped by HCl (0.1 M) and cAMP or cGMP measured by immunoassay in triplicate. Results are the mean $\pm$ s.e.m. of four experiments (*** $P<0.001$ vs control, ANOVA for repeated measures followed by Tukey–Kramer for multiple comparisons). R=value obtained in resting platelets; A=value obtained in ADP-activated platelets.

arachidonic acid, the platelet aggregation induced by ADP, collagen, arachidonic acid, ristocetin and thrombin. The finding that OncoA inhibited aggregation induced by different agonists suggested that OncoA might interfere with a common step of platelet activation. OncoA also inhibited ATP release showing that this quinone impaired secretion of platelet granule content.

The synthesis of TXA_2 is a key step in the platelet response to several agonists and this eicosanoid is one of the most powerful agonists for platelet activation (Siess, 1989; Jin *et al.*, 2005). The inhibition of TXA_2 synthesis appears not related to the mechanism of OncoA-induced inhibition of human platelet aggregation, since this quinone inhibited collagen-induced aggregation despite the irreversible blockade of cyclo-oxygenase enzyme induced by aspirin. For this assay, we chose collagen as an agonist because at the given concentration it produces platelet aggregation by TXA_2 -independent mechanisms (Siess, 1989; Herman, 1998). Even in this condition, OncoA was able to inhibit platelet aggregation in a dose-related manner and we therefore did not pursue this pathway further.

An increase in cytosolic levels of cAMP or cGMP is the most potent endogenous mechanism for the inhibition of

Table 1 Effect of OncoA on GP Ib α (CD 42b), P-selectin (CD62P), GP IIb (CD41a) and GP Ia (CD49b) and GPIIa (CD29)

Monoclonal antibody	Fluorescence intensity				
	Control	OncoA (μ M)			
		41	82	164	328
Anti-CD42b	193.8 $\pm$ 19.8	167.0 $\pm$ 13.6 [#]	125.9 $\pm$ 12.5*	104.2 $\pm$ 2.1*	107.0 $\pm$ 1.7*
Anti-CD41a	340.4 $\pm$ 26.5	333.7 $\pm$ 26.3	349.8 $\pm$ 31.1	338.5 $\pm$ 24.0	337.4 $\pm$ 30.0
Anti-CD62P	17.2 $\pm$ 1.3	17.6 $\pm$ 1.1	18.1 $\pm$ 0.1	20.4 $\pm$ 1.6	18.0 $\pm$ 1.5
Anti-CD49b	31.6 $\pm$ 0.8	31.6 $\pm$ 0.8	30.8 $\pm$ 0.8	29.8 $\pm$ 0.2	30.0 $\pm$ 1.5
Anti-CD29	211.4 $\pm$ 9.6	215.2 $\pm$ 3.7	216.7 $\pm$ 2.76	222.9 $\pm$ 6.0	217.8 $\pm$ 6.9

Samples of PRP (3 μ L) were incubated at 37 °C, for 5 min, with vehicle or OncoA. Then, 40 μ L of 10% bovine fetal serum in phosphate-buffered saline solution, pH 7.4 was added with the specific antibody. After 30 min incubation at room temperature, the reaction was terminated by 1% paraformaldehyde (300 μ L) and the samples analysed by flow cytometry. Results are the mean $\pm$ s.e.m. of four experiments.

[#] $P < 0.05$ vs control; * $P < 0.001$ vs control, ANOVA for repeated measures followed by Tukey–Kramer for multiple comparisons.

platelet activity (Herman, 1998; Schwarz *et al.*, 2001). OncoA was unable to induce an increase in cAMP levels of either resting platelets or ADP-activated platelets, yet this quinone specifically increased cGMP levels, whether platelets were stimulated or not. Increases in platelet cGMP levels could explain the inhibition of both granule secretion and platelet aggregation mediated by OncoA (Haslam *et al.*, 1999).

The effect on cGMP levels observed is not dependent on a NO mechanism since OncoA did not increase NO production in human platelets. In addition, the blockade of the NO-dependent activation of soluble guanylate cyclase by ODQ (Moro *et al.*, 1996) did not affect the antiplatelet activity of OncoA. These data suggest that this quinone might act downstream of the activation of sGC or, alternatively, that it activated sGC by interaction with a different site from that for NO. NO-independent activators of sGC such as YC-1 and BAY 41–2272 have already been described (Ko *et al.*, 1994; Stasch *et al.*, 2001).

Both compounds, that is, YC-1 (Galle *et al.*, 1999) and BAY 41–2272 (Mullershausen *et al.*, 2004), were also shown, besides activation of sGC, to inhibit PDE5 activity. Antiplatelet activity could be derived from a synergistic mechanism of reduced degradation and increased production of cGMP. However, sildenafil, a known PDE5 inhibitor, up to 100 μ M had no direct antiaggregatory activity in ADP-activated platelets in our observations. Wallis *et al.* (1999) showed that sildenafil has no direct effect on the platelet function but potentiated SNP antiaggregatory activity in both rabbit and human platelets. In this regard, we also found that OncoA, used at concentrations with no significant platelet antiaggregatory activity, potentiated the inhibitory action of SNP in human platelet aggregation with an effect quite similar to sildenafil. Hence, like YC-1 and BAY 41–2272, OncoA may directly increase cGMP levels in platelets by a synergistic mechanism, combining increased production and reduced degradation of cGMP.

The glycoprotein complex Ib-V-IX is the receptor involved in the adhesion of non-activated platelets to von Willebrand factor. In this receptor, the GP Ib α subunit is the most important in terms of mass and functional sites. The interference of OncoA in the binding of a specific monoclonal antibody to GP Ib α , as shown by flow cytometry, would explain the inhibition of ristocetin-induced platelet

aggregation (Ward *et al.*, 1996). In addition, this receptor is also related to thrombin-induced platelet activation (Jackson *et al.*, 2003) and the interference of antibody-specific binding promoted by OncoA, associated with the induction of cGMP synthesis, clarifies why OncoA is a potent inhibitor of thrombin-induced platelet aggregation. In addition, the binding of OncoA to GP Ib α could evoke downstream effects connecting to activation of sGC at an alternative site to NO, as already noted for YC-1 or BAY41–2272.

Platelet activation and aggregation is essential for primary haemostasis, but is also involved in thrombosis, the leading cause of myocardial infarction and stroke (Ruggeri, 2002). Antiplatelet drugs such as ticlopidine (ADP receptor antagonist) or aspirin were shown to reduce the incidence of stroke in high-risk patients (Hass *et al.*, 1989). Nevertheless, recent reports have shown that adequate antiplatelet effects are not achieved in 5–45% of patients taking aspirin and in 4–30% patients taking clopidogrel (Gum *et al.*, 2001; Eikelboom *et al.*, 2002; Gurbel *et al.*, 2003; Serebruany *et al.*, 2005). These patients are at increased risk of stent thrombosis and cardiovascular complications (Gum *et al.*, 2003; Gurbel *et al.*, 2003; Matetzky *et al.*, 2004; Cuisset *et al.*, 2006). OncoA might be a promising lead compound for antithrombotic drug development, aimed particularly at agents to overcome aspirin and clopidogrel resistance. Synergism or summation of the anti-platelet activity of these drugs is quite possible and deserves further investigation.

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Conflict of interest

The authors state no conflict of interest.

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