Geldanamycin disrupts platelet-membrane structure, leading to membrane permeabilization and inhibition of platelet aggregation

Sudawadee SUTTITANAMONGKOL[1], Adrian R. L. GEAR and Renata POLANOWSKA-GRABOWSKA
Department of Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA 22908, U.S.A.

Geldanamycin (GA), a benzoquinoid ansamycin antibiotic, has been used as a tyrosine kinase inhibitor and an anti-tumour agent and is known to bind to heat-shock protein 90. In the present study on human platelets we have found that GA inhibited platelet aggregation induced by ADP, thrombin and the thrombin-receptor-activating peptide and caused platelet plasma-membrane damage, detected by leakage of adenine nucleotides as well as serotonin. Scanning electron microscopy (SEM) revealed that platelet exposure to GA led to the formation of holes or fenestrations in the platelet plasma membrane, confirming GA's ability to initiate membrane damage. In addition, GA itself caused both the dephosphorylation and phosphorylation of proteins in resting platelets and prevented agonist-induced phosphorylation of pleckstrin, the 20-kDa myosin light chain and other proteins. Another ansamycin, herbimycin A, also inhibited platelet aggregation, but caused minimal membrane permeabilization, as detected by ^{3}H release from platelets labelled previously with [^{3}H]adenine, and much less membrane damage, revealed by SEM. Overall, GA is able to disrupt membrane structure and inhibit platelet aggregation, an ability which may be linked to alterations in the activity of protein kinases and phosphatases.

Key words: herbimycin A, heat-shock protein, tyrosine kinase inhibitor.

INTRODUCTION

Platelets are essential for haemostasis and normally circulate in a resting state. When exposed to agonists such as thrombin, ADP and collagen, they can aggregate, change shape and secrete granule contents within seconds [1,2]. Platelet activation by agonists leads to a change in the phosphorylation state of many platelet proteins on serine, threonine and/or tyrosine residues. Characteristically, pleckstrin (47 kDa) and myosin light chains (MLCs; 20 kDa), which are markers of activated protein kinase C and increasing $[Ca^{2+}]_i$, respectively, are phosphorylated [3–6]. In addition, non-receptor tyrosine kinases become activated, resulting in an increase in tyrosine phosphorylation levels of proteins including paxillin, talin, vinculin, focal-adhesion kinase, β_1-integrins, p21RasGAP (GTPase-activating protein), cortactin, Syk and Src homology region 2-domain phosphatase 1 (SHP-1) [7]. In resting platelets, the state of protein phosphorylation must be actively balanced and ready to respond to agonist stimulation [8].

Geldanamycin (GA), a benzoquinoid ansamycin antibiotic produced by *Streptomyces hygroscopocus* var. *geldanus*, is strongly cytotoxic against tumour cells, fungi and protozoa, as well as having weak anti-microbial activity [9]. Studies show that GA exerts inhibitory effects on cancer cells and transformed cells, including v-Src-induced transformation [10–12], and also suppresses c-Myc expression, inhibiting DNA synthesis [13]. Therefore, GA has been used as a tyrosine kinase inhibitor and an anti-tumour agent and is known to inhibit signalling by steroid hormone receptors, Src and Raf [14]. Similar to GA, herbimycin A (HA), another benzoquinoid ansamycin analogue [15], possesses anti-tumour activity and is a tyrosine kinase inhibitor [16–18]. Both HA and GA have been used in platelet signal-transduction studies involving tyrosine phosphorylation and fibrinogen receptors [19]. The mechanisms by which GA reverses cell transformation and inhibits tyrosine kinase activity are not well understood. GA has been reported to associate specifically with the ATP/ADP-binding domain of heat shock protein 90 (hsp90) [20], resulting in dissociation of complexes between hsp90 and its partners and accelerating the degradation of these proteins, including the glucocorticoid and dioxin receptors, Raf, erbB2 and mutated p53 [21–24]. In addition, GA blocks intracellular translocation of the glucocorticoid receptor, Raf and mutated p53 [25–27]. These results suggest that functions of hsp90 in signal transduction involving the stabilization and translocation of its partners can be inhibited by GA.

Recently, our laboratory reported that the heat-shock proteins hsp90 and heat-shock protein 70 (hsc70) are present in human platelets [28], being found in resting cells as members of a large phosphoprotein complex, which is dissociated following platelet adhesion to collagen. Therefore, we chose GA, which specifically acts on hsp90 and which can disrupt signalling pathways, to examine its effects on platelet function, specifically aggregation and signalling. We have found that GA inhibited platelet aggregation, induced disruption of the platelet plasma membrane and caused major changes in protein phosphorylation in resting platelets as well as inhibiting increased phosphorylation in activated platelets.

MATERIALS AND METHODS

Materials

GA was obtained from Dr. Leonard Neckers (National Cancer Institute, NIH, Rockville, MD, U.S.A.) and CalBiochem (La Jolla, CA, U.S.A.). Geldampicin (GP) was also a gift from Dr. Leonard Neckers. HA was from Gibco-BRL (Grand Island, NY, U.S.A.). [^{32}P]Orthophosphate, [^{14}C]serotonin and [^{3}H]adenine were purchased from Dupont De Nemours (Boston, MA, U.S.A.), Amersham (Arlington Heights, IL, U.S.A.) and ICN

Abbreviations used: GA, geldanamycin; HA, herbimycin A; GP, geldampicin; SEM, scanning electron microscopy; 6-CF, 6-carboxyfluorescein; TRAP, thrombin-receptor-activating peptide; MLC, myosin light chain; hsp90, heat-shock protein 90; hsc70, heat-shock protein 70; TTBS, Tween-20-containing Tris-buffered saline; ACD, acid-citrate dextrose; PRP, platelet-rich plasma.

[1] To whom correspondence should be addressed (e-mail ss2fn@virginia.edu).

(Costa Mesa, CA, U.S.A.), respectively. Polyclonal phosphotyrosine antibody was obtained from Transduction Laboratories (Lexington, KY, U.S.A.) and the Immuno-Lite Chemiluminescent (ECL) assay was from Bio-Rad (Hercules, CA, U.S.A.). Apyrase (grade VII) and other speciality chemicals were obtained from Sigma (St Louis, MO, U.S.A.). The Lumi-aggregometer and Chrono Lume luciferase reagent were from the Chrono-Log Corporation (Havertown, PA, U.S.A.).

Preparation of blood platelets

After informed consent, human venous blood was collected and anti-coagulated with acid-citrate dextrose (ACD) for a final citrate concentration of 11.5 mM as described previously [29]. Platelet-rich plasma (PRP) was prepared by centrifugation at 350 ***g***, twice for 3 min and once for 5 min. The PRP was mixed with extra ACD to double its concentration, and apyrase (5 units/ml), prostaglandin I_2 (0.3 μg/ml) and indomethacin (1 μg/ml) and centrifuged at 620 ***g*** for 20 min. The platelet pellet was suspended in ACD (1/2 vol. of PRP), containing albumin (3 mg/ml) and apyrase (5 units/ml) and centrifuged at 620 ***g*** for 20 min, followed by resuspension in Eagle's/Hepes buffer [30] containing 0.5 mg/ml fibrinogen.

For ^{32}P labelling, the pellet from the first 620-***g*** centrifugation was resuspended in phosphate-free buffer (140 mM NaCl/5 mM KCl/0.05 mM $CaCl_2$/0.1 mM $MgCl_2$/0.01 g/ml albumin/16.5 mM glucose/15 mM Hepes, pH 7.35) and the platelet suspension was left at room temperature for 20 min in the presence of apyrase (5 units/ml) and indomethacin (1 μg/ml). [^{32}P]Orthophosphate (0.1 mCi/3 ml of platelets) was then added to the suspension, which was incubated for 90 min at 37 °C. ACD and prostaglandin I_2 were added and the suspension was centrifuged at 620 ***g*** for 20 min. The platelet pellet was next washed with ACD containing apyrase (5 units/ml), indomethacin (1 μg/ml) and albumin (3 mg/ml) and resuspended in Eagle's/Hepes buffer containing 0.5 mg/ml fibrinogen. These procedures are essentially identical to those described previously [28].

For [^{14}C]serotonin labelling, PRP was incubated with [^{14}C]serotonin (1.0 μCi/5 ml of PRP) for 20 min at 37 °C. The labelled platelet pellet was washed with ACD containing apyrase (5 units/ml) and indomethacin (1 μg/ml) and resuspended in Eagle's/Hepes buffer [31].

For ^{3}H labelling, the pellet from the ACD wash was suspended in a BSA-free modified Tyrode's Hepes buffer containing 100 μM $MgCl_2$ and 50 μM $CaCl_2$. The platelet suspension was left for 20 min at room temperature, mixed with [^{3}H]adenine (0.5 μCi/ml) and incubated for 60 min at 37 °C. The labelled platelet suspension was centrifuged and resuspended in Eagle's/Hepes buffer. This procedure was similar to that described by Simon and Gear [32].

Platelet aggregation

Two methods were employed to assess platelet aggregation. A test-tube stirring system was used for most of the aggregation experiments and other studies described below. Platelet suspensions were normally preincubated for 30 min at 37 °C with DMSO (control) or GA and then mixed with saline or agonists under stirring conditions (1000 revs./min). After 10 s, the reactions were quenched with an equal volume of glutaraldehyde (1 % final concentration) and aggregation assessed by using a resistive-particle counter from the loss of platelet singlets.

A quenched-flow method was used to assess platelet aggregation under arterial flow conditions [29]. Washed platelets and ADP were mixed by pumping through a narrow-diameter tube and the reactions were quenched by glutaraldehyde. Samples were then collected and aggregation assessed by single-particle counting.

For gel electrophoresis and Western blotting, the test-tube system was used and after collecting samples for aggregation assay, SDS-containing buffer [2 % (w/v) SDS/5 % (v/v) 2-mercaptoethanol/10 % (v/v) glycerol/0.002 % (v/v) Bromophenol Blue/62.5 mM Tris, pH 6.8] was added to the platelet suspension, followed by procedures described below.

Serotonin release

The [^{14}C]serotonin-labelled platelets were preincubated with DMSO (control) or GA and mixed with saline or agonists in the test-tube stirring system. After 10 s, the reactions were quenched with glutaraldehyde (1 % final concentration). The stimulated platelets were pelleted in a microfuge and supernatants were mixed with scintillation cocktail for analysis (multipurpose scintillation counter, Beckman LS6500). Serotonin release was determined by the following equation: $[(S_c - S_s)/(T_s - S_s)] \times 100$, where S_c is the counts in an aliquot of supernatant after GA treatment, S_s is the counts in the aliquot of supernatant without any treatment, and T_s is the counts in an aliquot of the total platelet suspension.

^{3}H release

The [^{3}H]adenine-labelled platelets were incubated with DMSO (control) or GA in the test-tube stirring system. After 10 s, the platelets were quenched with glutaraldehyde (1 % final concentration) and pelleted in a microfuge. Aliquots of the supernatant were mixed with scintillation cocktail for analysis (Beckman LS65000) with ^{3}H release calculated by the equation $[(S_c - S_s)/(T_s - S_s)] \times 100$, as for serotonin release.

Detection of ATP release by luciferase

Washed platelets were preincubated in an aggregometer (Lumi-aggregometer model 560) with the Chrono Lume luciferase reagent according to the manufacturer's directions and gain control set at 0.005× for ATP detection. DMSO or GA was added to the mixtures, and luminescence and light-scattering changes were recorded. At the end of each run, Triton X-100 was added to solubilize the platelet membranes, so that platelet ATP was released into the suspending buffer and detected, reflecting a total pool of ATP containing cytoplasmic and granule components.

Scanning electron microscopy (SEM)

Washed platelets were preincubated with DMSO or GA and aggregated in the test-tube stirring system. The platelets were fixed with 1 % glutaraldehyde, subjected to graded alcohol dehydration as described previously [33], and analysed by SEM (JSM-6400, JEOL).

Leakage of 6-carboxyfluorescein (6-CF) from lipid vesicles

Lipid vesicles were prepared and loaded with 6-CF, which fluoresces intensely when released into an aqueous medium [32]. The fluorescence intensity of vesicles alone was measured until it reached a baseline at 37 °C and then GA or GP was added. Recording of fluorescence intensity was performed for 10 min and then Triton X-100 was added to obtain the maximum fluorescence attainable. 6-CF leakage from the vesicles was assessed from the increase in fluorescence intensity, with the aim of assessing potential detergent properties of GA.

Gel electrophoresis

The ^{32}P-labelled platelets were preincubated with DMSO or GA, then stimulated with the agonists in the test-tube stirring system and lysed with SDS-containing buffer. The platelet lysates were boiled for 5 min and normally loaded on SDS/polyacrylamide 12% gels. For autoradiography, gels were dried and exposed to autoradiograph film (Hyperfilm™ from Amersham) at −70 °C overnight.

Western blotting

Platelet lysates were prepared, subjected to SDS/PAGE and proteins transferred on to nitrocellulose membranes, which were blocked overnight at 4 °C with TTBS buffer (Tween-20-containing Tris-buffered saline) with 5% albumin. Then the blot was washed in TTBS, incubated with rabbit anti-phosphotyrosine antibody (P11230 from Transduction Laboratories) for 2 h, washed with TTBS, and incubated with anti-rabbit IgG (H + L) alkaline phosphatase conjugate (Bio-Rad) for 1 h. The blot was next developed by using an ECL assay (Immun-Star Substrate from Bio-Rad) and exposed to film.

Statistics

Student's *t* test was used to determine the statistical significance of the data where indicated. Results are expressed as means ± S.E.M.

RESULTS

GA inhibits platelet aggregation

Initial experiments involved the quenched-flow system. GA (18 μM) inhibited platelet aggregation induced by 10 μM ADP by about 60% (results not shown) with significant inhibition at the ADP-exposure times of 3, 4, 5 and 8 s ($P < 0.05$ for 3 and 4 s; $P < 0.01$ for 5 and 8 s).

To investigate further the inhibition of platelet aggregation by GA, we used a simpler system and studied three different agonists, ADP, thrombin and the thrombin-receptor-activating peptide (TRAP) at various concentrations of GA. The data (Figure 1) show that aggregation induced by these agonists was inhibited by GA in a dose-dependent manner. Inhibition of ADP, thrombin and TRAP-induced aggregation was statistically significant ($P < 0.01$) at 18 and 36 μM GA. In addition, ADP-induced aggregation was inhibited significantly ($P < 0.05$) by 9 μM GA. We also tested the alternative ansamycin GP, which does not bind to hsp90, but which still possesses the quinone- and ansa-ring structures [34]. GP at concentrations of up to 36 μM did not inhibit platelet aggregation induced by ADP, thrombin and TRAP (results not shown).

GA causes leakage of serotonin and adenine nucleotides

Since GA inhibited platelet aggregation (Figure 1), we expected that GA would also block agonist-induced secretion. Thrombin and TRAP induce serotonin release from platelets, but ADP does not. Surprisingly, GA did not inhibit agonist-induced serotonin secretion, but apparently induced leakage or release on its own without any evidence for blocking thrombin or TRAP-induced secretion. At the highest GA concentrations (18 and 36 μM) used, even more secretion or release of serotonin was noted than with thrombin or TRAP alone (Figure 2).

In view of these results, we hypothesized that GA might directly perturb platelet-membrane structure, causing granule-content release. Therefore, we carried out experiments using [^{3}H]adenine-labelled platelets to test for loss of low-molecular-mass compounds such as AMP, ADP and ATP. No release was observed following stimulation with any of the agonists (results not shown). However, incubation of [^{3}H]adenine-labelled platelets with GA caused a dose-dependent ^{3}H release (Figure 3,

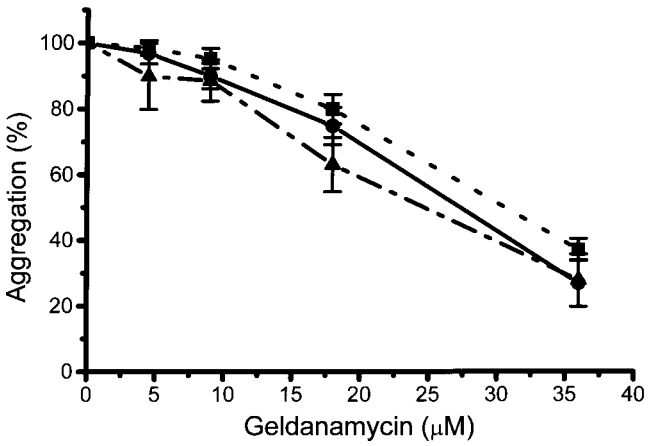

Figure 1 GA inhibits platelet aggregation

Washed platelets ($2.9 \pm 0.4 \times 10^8$ platelets/ml) were preincubated with DMSO (control) or GA (4.5, 9, 18 and 36 μM) for 30 min at 37 °C. The preincubated platelets were reacted for 10 s in the test-tube stirring system with 10 μM ADP (●), 1.0 unit/ml thrombin (■) or 10 μM TRAP (▲). Platelet aggregation was determined by the loss of platelet singlets. The data were normalized such that aggregation stimulated by ADP, thrombin or TRAP in the absence of GA was set at 100%. The absolute aggregations induced by 10 μM ADP, 1 unit/ml thrombin and 10 μM TRAP were 78 ± 2.2, 78.8 ± 2.1 and 60.8 ± 3.7%, respectively. The results are means ± S.E.M. from four different donors.

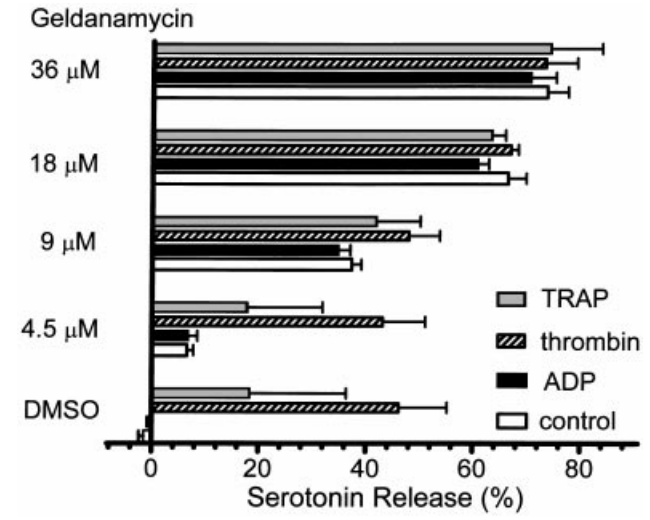

Figure 2 GA causes serotonin release from blood platelets

[^{14}C]Serotonin-labelled platelets were preincubated with 1% DMSO alone or DMSO with 4.5, 9, 18 or 36 μM GA for 30 min at 37 °C (DMSO was kept at 1% for all GA concentrations). The samples were then reacted with saline (white bars), 10 μM ADP (black bars), 1.0 unit/ml thrombin (hatched bars) or 10 μM TRAP (grey bars) for 10 s. Supernatants were analysed by scintillation counting. The data are presented as means ± S.E.M., with $n = 3$ different platelet preparations. The increases in serotonin release caused by 4.5 μM GA for control and ADP-treated platelets were highly significant ($P < 0.008$ and 0.006, respectively).

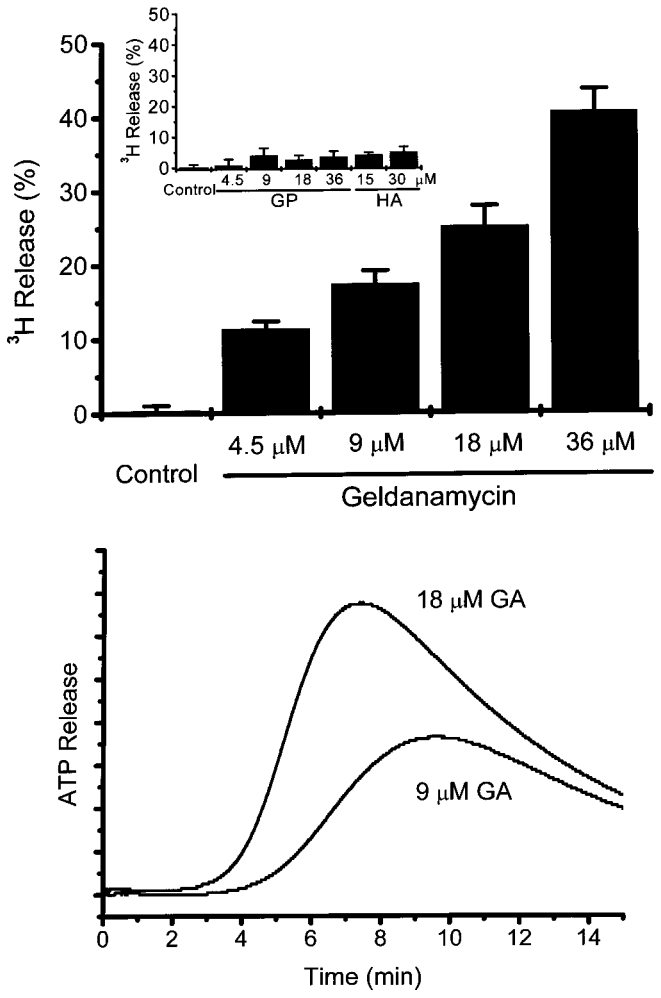

Figure 3 GA causes membrane leakage, evidenced by [^{3}H]adenine-labelled nucleotide (upper panel) and ATP (lower panel) release

(Upper panel) [^{3}H]Adenine-labelled platelets were preincubated with 1% DMSO (Control), GA (4.5, 9, 18 or 36 μM), GP (4.5, 9, 18 or 36 μM) or HA (15 and 30 μM) for 30 min at 37 °C and then stirred for 10 s, and ^{3}H release analysed by scintillation counting as described in the Materials and methods section. The data are means $\pm$ S.E.M., $n = 3$. The inset shows the effects of GP and HA on ^{3}H release. (Lower panel) Washed platelets (450 μl) were preincubated with 50 μl of the luciferase reagent for 2 min in the Lumi-aggregometer. GA was added to the mixture (9 and 18 μM) and ATP release was detected by luminescence (arbitrary units). Gain control for sensitivity was set at 0.005 $\times$. Data representative of three experiments with different donors are presented.

upper panel), which was significant ($P < 0.01$) even at 4.5 μM GA. This result is consistent with the hypothesis that GA causes membrane damage and leakage of cell contents.

As an alternative approach to using [^{3}H]adenine, we observed that GA caused ATP leakage from platelets with a delay of about 2–6 min (Figure 3, lower panel). When lower concentrations of GA were used, the lag phase of ATP leakage was longer. Additions of Triton X-100 or thrombin (1 unit/ml) caused an immediate, large increase in ATP detected by the luciferase reagent (results not shown). When untreated platelets were activated by thrombin, there was a very large luminescence signal reflecting ATP secretion, as expected, severalfold greater than that detected after GA treatment.

In addition to GA, the ansamycins HA and GP were used to test if they caused any ^{3}H release. HA, which has a similar hsp90-binding activity to GA [34], induced little ^{3}H release (Figure 3, upper panel, inset). GP also caused minimal adenine leakage (Figure 3, upper panel, inset).

GA can cause membrane damage

Since our results revealed that significant platelet-membrane leakage occurred in response to GA, but that little happened after HA or GP treatment, we examined effects of GA and HA on platelet-membrane structure by using SEM. This analysis (Figure 4) showed that platelet exposure to GA resulted in plasma-membrane damage, even at low doses of GA (4.5 μM). Formation of holes or fenestrations with varied sizes occurred and the high dose of GA produced both more and larger holes or fenestrations. Some of the platelets treated with 4.5 μM GA still appeared smooth and discoid and they changed shape normally, forming aggregates when stimulated with ADP. At the high dose of GA (36 μM), aggregation was strongly inhibited (Figure 1) and virtually all platelets exhibited major membrane disruption with cells rounding up and often appearing highly fragmented (Figure 4e). No pseudopodia were formed in response to ADP (Figure 4f).

Since the ansamycin HA is known to possess similar effects to GA and has been shown recently to inhibit exposure of fibrinogen receptors [19], we also used this compound to examine whether HA caused platelet-membrane damage. HA induced some formation of holes at both concentrations used (15 and 30 μM, Figures 4g–4j) and inhibited platelet aggregation, but to a lesser extent than GA (36.5 ± 0.1% inhibition of aggregation by 30 μM HA, results not shown). In contrast to GA-treated platelets, HA-treated platelets were still able to produce pseudopodia in response to ADP stimulation (Figures 4h and 4j).

These SEM pictures were further analysed quantitatively (Table 1). Platelets were counted and differentiated according to their morphology, from normal to damaged, and low (1+) to high (4+) levels of damage. GA at 4.5 μM caused membrane damage in > 20% of cells. Most of the damaged cells had small holes with smooth surfaces (1+), as in Figure 4(c). However, more severe effects were induced by 36 μM GA with > 50% of cells exhibiting a sponge-like appearance (3+ and 4+). In the case of HA, we observed much less cell damage.

GA alters phosphorylation of ^{32}P-labelled platelet proteins

Since GA inhibited platelet aggregation and perturbed platelet-membrane structure and permeability, we investigated potential changes in protein phosphorylation in GA-treated platelets and found a number of changes in phosphorylation status (Figure 5). GA treatment of resting platelets under stirring conditions caused dephosphorylation of protein bands at molecular masses of about 62, 70, 85 and 90 kDa as well as of higher-molecular-mass proteins of about 120, 130, 140 and 150 kDa (*, GA control, Figure 5a) and of about 175, 208 and 220 kDa (†, GA control, Figure 5a). These phosphorylation changes were dose-dependent and observed at concentrations of GA as low as 9 μM (results not shown). We also observed an increase in pleckstrin phosphorylation (Figure 5a) and a marked increase in phosphorylation of 25-, 28- and 38-kDa unidentified protein bands (Figure 5b), compared with untreated, resting platelets.

In agonist-stimulated platelets (ADP, thrombin and TRAP), there was the 'normal' activation of protein kinase C and Ca^{2+}/calmodulin-dependent MLC kinase, seen as phosphorylation of the 40–47-kDa protein pleckstrin and the 20-kDa protein MLC, respectively. In addition, there was increased phosphoryl-

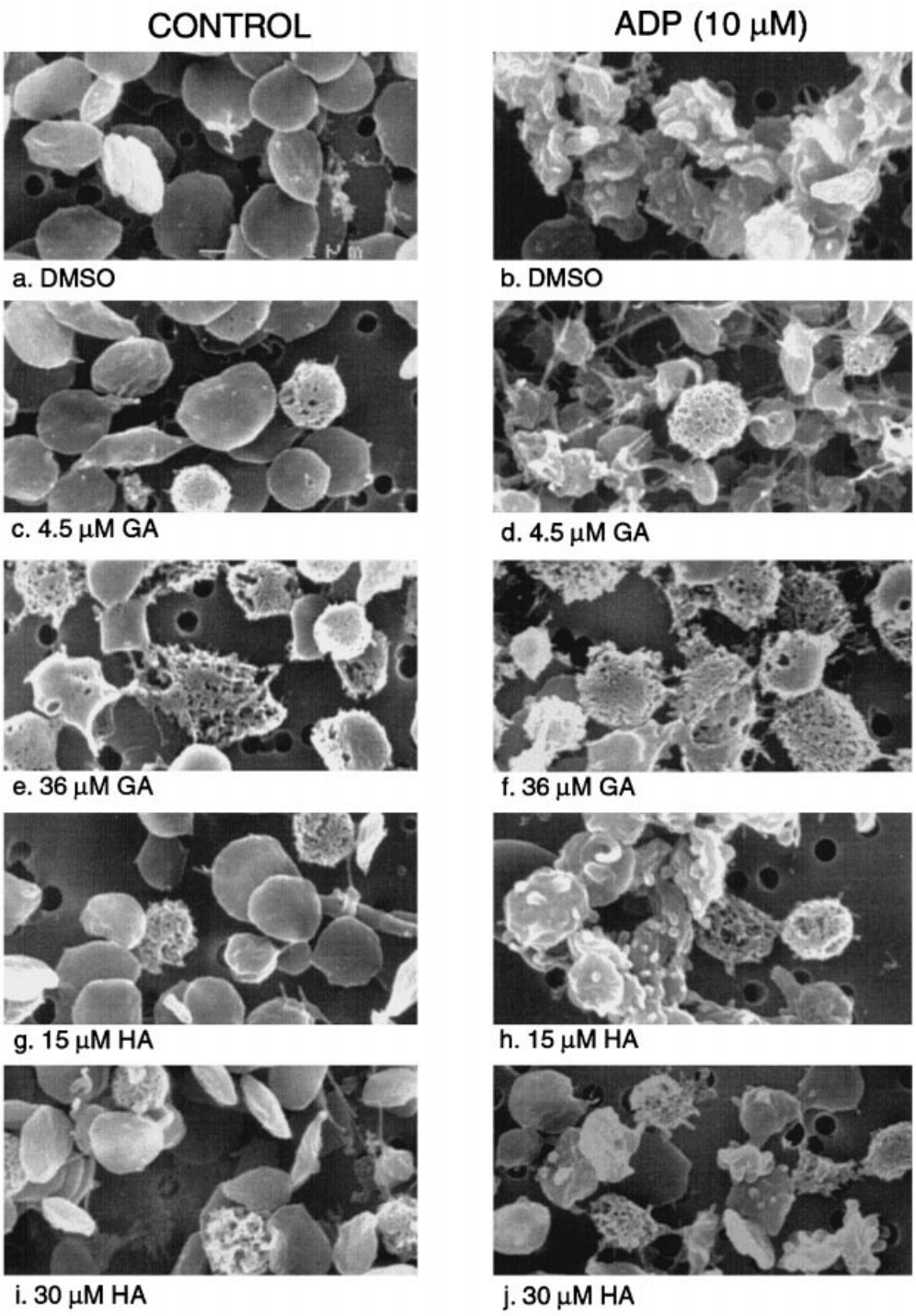

Figure 4 GA disrupts platelet membranes, as seen by SEM

Washed platelets were preincubated with 1% DMSO (**a**, **b**), GA at 4.5 (**c**, **d**) and 36 (**e**, **f**) μM, or HA at 15 (**g**, **h**) and 30 (**i**, **j**) μM. Preincubated platelets were stimulated with saline (**a**, **c**, **e**, **g** and **i**) or ADP (10 μM; **b**, **d**, **f**, **h** and **j**) for 10 s in the test-tube stirring system. The white scale bar in **a** represents 1 μm.

ation of protein bands of about 56, 62, 70, 85 and 90 kDa (Figure 5a). However, GA completely inhibited the phosphorylation of pleckstrin stimulated by ADP, but only partially inhibited it when thrombin and TRAP were used (Figure 5a), and the phosphorylation of MLC was partially prevented by GA treatment (Figure 5b). GA also completely blocked the extra phosphorylation of the 56-, 62-, 70-, 85- and 90-kDa protein bands induced by the agonists.

GA induces changes in tyrosine phosphorylation

To investigate whether GA also alters levels of tyrosine

Table 1 Effect of GA and HA on platelet morphology

Washed platelets were treated as described in Figure 4 and platelet morphology was evaluated from the scanning electron micrographs. Cell morphology represents the degree of membrane damage: 1+, platelets with small holes and a smooth surface; 2+, platelets with larger holes and a rough surface; 3+, sponge-like platelets, but retaining cell shape; 4+, sponge-like platelets without a defined cell shape. Total indicates the total number of cells counted. Data were derived from two independent preparations.

		GA		HA	
Cell morphology	Control	4.5 μM	36 μM	15 μM	30 μM
Normal	87%	65%	21%	77%	71%
1+	13%	31%	17%	18%	15%
2+	0%	1%	8%	0%	0%
3+	0%	3%	34%	5%	7%
4+	0%	0%	20%	0%	6%
Total	93	55	69	160	84

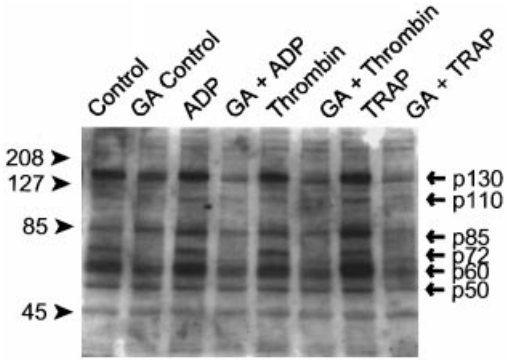

Figure 6 GA affects tyrosine phosphorylation in blood platelets

Platelet proteins were analysed as described in the Materials and methods section, and blotted with a rabbit phosphotyrosine antibody. A blot representative of three experiments is shown.

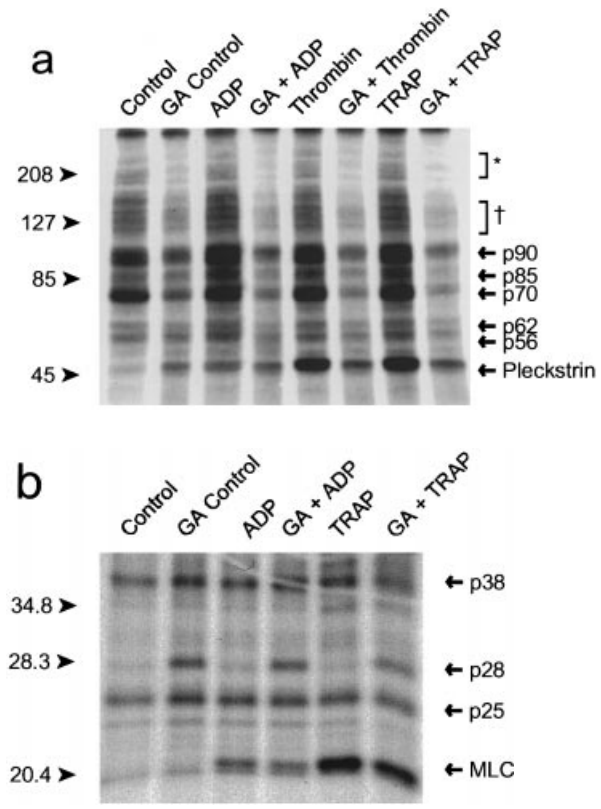

Figure 5 GA alters protein phosphorylation in blood platelets

^{32}P-Labelled platelets were preincubated with 1% DMSO (control) or GA (36 μM) for 30 min at 37 °C, exposed for 10 s in the test-tube stirring system to saline, 10 μM ADP, 1.0 unit/ml thrombin or 10 μM TRAP, and then analysed by SDS/PAGE. (**a**) Autoradiograph from a 12% minigel. * represents bands at 175, 208 and 220 kDa and † is for bands at 120, 130, 140 and 150 kDa. (**b**) Autoradiograph from a 12% full-length gel. The molecular masses of standard proteins are indicated on the left, and detected phosphoprotein bands on the right. An autoradiogram of a gel representative of three experiments is shown.

phosphorylation, we carried out Western blots on total platelet lysates. GA itself caused the dephosphorylation of protein bands with molecular masses of 60, 72 and 130 kDa (Figure 6), with the 72-kDa protein band being almost completely dephosphorylated. Increased tyrosine phosphorylation of an 85-kDa protein band was noted. After stimulation by ADP, thrombin and TRAP, levels of tyrosine phosphorylation increased for many proteins, notably for bands of 60, 72, 85, 110 and 130 kDa. However, when platelets were preincubated with GA before exposure to the agonists, phosphorylation of these bands of 60, 72, 85, 110 and 130 kDa was prevented.

DISCUSSION

GA inhibits platelet aggregation and disrupts membrane structure

GA was discovered in 1970 [9] and possesses anti-tumour activity as well as being a tyrosine kinase inhibitor. How it exerts these effects is not well understood, but there is good evidence that GA's ability to bind to hsp90, causing disruption of hsp90 complexes, may be involved. Thus GA has inhibitory effects on signal transduction, involving steroid hormone receptors, Src, Raf and mutated p53 [14]. In the present study, we have examined the effects of GA on platelet function. Platelet aggregation was inhibited by GA, but it apparently did not block dense-granule secretion, but rather caused serotonin, ATP and ^{3}H leakage (Figures 2 and 3), resulting from membrane damage, reflected by our SEM images (Figure 4). In addition to GA, the ansamycin HA also inhibited platelet aggregation (results not shown), but caused little ^{3}H leakage. GP, a negative ansamycin control [34], neither inhibited platelet aggregation nor caused significant ^{3}H leakage. Treatment with GA and HA resulted in the formation of holes or fenestrations, as seen in the SEM images (Figure 4 and Table 1). GA, however, was about three times more destructive than HA. GP did not influence normal platelet morphology (results not shown).

Leakage of labelled adenine compounds is usually interpreted as indicating loss of ATP and ADP primarily and of AMP to a lesser extent, since these normally cannot traverse the plasma membrane [35,36]. However, if metabolic ATP levels drop significantly, then hypoxanthine may be formed and potentially cross an undamaged plasma membrane to the extracellular environment [36]. Therefore, some of the apparent leakage of adenine as nucleotides may represent loss of hypoxanthine. The direct measurement of ATP via luciferase and, importantly, the visualization via the scanning electron micrographs, provide good evidence of significant membrane damage induced by GA.

Similarly, the serotonin-transport system of serotonin storage granules is sensitive to the electrochemical proton gradient across

the granule membrane [37,38]. GA-induced platelet-membrane damage (Figure 4) may result in dissipation of this gradient, such that serotonin cannot be retained in the granules and leaks out into the cytoplasm and extracellular space (Figure 2). Therefore, serotonin release caused by GA may not involve granule secretion, but may rather be a result of membrane damage.

GA and phosphorylation of platelet proteins: mechanisms and specificity of effects

Resting platelets exhibit a number of ^{32}P-labelled phosphoprotein bands, including ones at 56, 62, 70, 85 and 90 kDa, as well as at higher molecular masses (Figure 5a). GA caused changes (both increases and decreases) in protein-phosphorylation status of several of these bands, suggesting an altered balance between protein-kinase and protein-phosphatase activities in resting platelets. GA apparently did not activate the calcium-dependent MLC kinase, since the 20-kDa band remained unphosphorylated. Interestingly, although pleckstrin phosphorylation increased following platelet exposure to GA, the drug prevented phosphorylation of pleckstrin in response to ADP, but only partially for thrombin and TRAP.

Based on molecular masses and the fact that the heat-shock proteins hsc70 and hsp90 are phosphorylated in resting cells [28], it is likely that the 70- and 90-kDa protein bands contained hsc70 and hsp90, respectively. GA is known to bind to hsp90 [34], resulting in dissociation of hsp90 complexes, and that hsp90 forms a complex with hsc70 in platelets [28]. In the experiments reported here, GA caused dephosphorylation of these protein bands as well as inhibiting additional phosphorylation of the 70- and 90-kDa bands in response to agonists (Figure 5a). It is possible that these proteins are present in these bands and are involved in platelet signalling, such that GA-dependent inhibition of their phosphorylation by agonists impairs subsequent platelet responses.

In the present study, we demonstrated specificity of GA by two series of experiments using GP, which is an ansamycin derivative with no hsp90-binding activity. First, GP neither caused ^{3}H leakage nor induced membrane damage and did not inhibit platelet aggregation. Second, we carried out a series of 'leakage' tests on phospholipid vesicles loaded with 6-CF. The aim of these experiments was to assess whether GA or GP might exert 'detergent'-like effects on lipid bilayers without involvement of cellular proteins [32]. Neither GA nor GP caused 6-CF leakage from the lipid vesicles. These results suggest that the damage to platelet membranes by GA is caused by a specific mechanism rather than by non-specific detergent-like effects on membranes.

Detergents or amphipathic compounds, including octyl glucoside, SDS, Triton X-100, dilauroylphosphatidylcholine, dilauroylphosphatidylserine and chlorpromazine can induce changes in platelet morphology, causing filopodium formation, short blebs, rounding-up of the cells, as well as unusual elongated or even ring forms [39,40]. C_2-Ceramide may even act as a detergent since it has an amphipathic structure, and after only 1 min of exposure will cause thin filopodium formation and significant disruption of the membrane surface [32]. However, these various 'detergent' effects on platelet morphology [32,39,40] are distinct from those seen after GA exposure (Figure 4). Here, few elongated filopodia were formed, and affected platelets often exhibited major disruption or breakdown of the membrane surface, together with appearance of large holes or fenestrations.

There are several reports about the effects of amphipathic compounds on the patterns of ^{32}P-phosphorylation of platelet proteins. Opstvedt et al. [41] showed that chlorpromazine in non-lytic conditions does not alter phosphorylation patterns. C_2-Ceramide under conditions which cause nearly complete platelet lysis [32] has little effect on protein phosphorylation, except for causing about a 50 % decrease in a 67-kDa band. On the other hand, GA induced a significant dephosphorylation of high-molecular-mass bands, while increasing phosphorylation of several low-molecular-mass bands (Figure 5).

Uses of GA and HA: relationships to other studies

GA and HA have usually been used to study signal-transduction pathways involving tyrosine kinases as well as hsp90, which can participate in signalling complexes, associating with tyrosine kinases and other signalling enzymes. These ansamycins have been tested in a wide variety of cell types and under different incubation conditions. Concentrations of GA and incubation times have varied between studies, with concentrations generally being lower than used in the present study (4.5–36 μM), whereas incubation times were usually much longer (hours) compared with 30 min in our experiments.

It should be noted that choice of cell type may represent an important factor with regard to drug sensitivity, with particular importance as to membrane permeability of GA and subsequent intracellular concentrations. For example, Czar et al. [25] used L929 mouse fibroblasts to study the assembly of the hsp90–glucocorticoid-receptor complex and its involvement in glucocorticoid-receptor signalling. They incubated L929 cells with 10 μM GA for 2 h at 37 °C and observed about 50 % dissociation of the hsp90–glucocorticoid-receptor complex. Moreover, Schulte et al. [23] found that incubation of NIH 3T3 cells with 2 μM GA for 16 h resulted in destabilization of Raf. In the case of blood platelets, Hers et al. [19] used HA and GA to study roles of tyrosine kinases in the exposure of integrin $\alpha_{IIb}\beta_3$ on the cell surface after platelet stimulation. They observed that preincubation of platelets with 30 μM HA and 10 μM GA for 5 min completely inhibited integrin exposure in response to thrombin under stirring conditions. This study suggests that higher concentrations of the drugs may be needed for blood platelets. In addition, Mimnaugh et al. [22] treated mycoplasma-free SKBr3 human breast carcinoma cells with 3 μM GA for only 15–60 min and detected polyubiquitination of erbB2 and loss of erbB2. This indicates that varied cell types respond with different sensitivities to the drug.

We used GA at 4.5, 9, 18 and 36 μM and HA at 15 and 30 μM. At these concentrations and after 30 min of incubation at 37 °C both drugs inhibited platelet aggregation and caused membrane damage. The effect of HA was less than that of GA, particularly with regard to leakiness of the plasma membrane (Figure 3). Otherwise, their potencies were relatively similar to the results reported by Hers et al. [19].

We believe that our findings are novel since there are apparently no data indicating that GA induces membrane damage and permeabilization in other cell types. As discussed earlier, detergents exert effects on platelets that appear to be distinct from those caused by GA. Several minutes are required for GA to induce lysis in intact platelets and this failed to lyse lipid vesicles, unlike detergents and C_2-ceramide, which do so with no delay. In conclusion, the effects of GA on platelets are relatively specific since the non-specific ansamycin GP failed to induce any of the changes caused by GA. Binding of GA to hsp90 could well be responsible, with subsequent modulation of protein kinase and phosphatase activities. The mechanisms involved will require further study.

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REFERENCES

1 Gear, A. R. L. (1993) Can. J. Physiol. Pharmacol. **72**, 285–294
2 Gear, A. R. L. and Burke, D. (1982) Blood **60**, 1231–1234
3 Wallace, W. C. and Bensusan, H. B. (1980) J. Biol. Chem. **255**, 1932–1937
4 Daniel, J. L., Molish, I. R. and Holmsen, H. (1981) J. Biol. Chem. **256**, 7510–7514
5 Tyers, M., Haslam, R. J., Rachubinski, R. A. and Harley, C. B. (1989) J. Cell Biochem. **40**, 133–145
6 Naka, M., Nishikawa, M., Adelstein, R. S. and Hidaka, H. (1983) Nature (London) **306**, 490–492
7 Jackson, S. P., Schoenwaelder, S. M., Yuan, Y., Salem, H. H. and Cooray, P. (1996) Thromb. Haemost. **76**, 640–650
8 Levy-Toledano, S., Gallet, C., Nadal, F., Bryckaert, M., Maclouf, J. and Rosa, J. (1997) Thromb. Haemost. **78**, 226–233
9 DeBoer, C., Meulman, P. A., Wnuk, R. J. and Peterson, D. H. (1970) J. Antibiotics **23**, 442–447
10 Sasaki, K., Yasuda, H. and Onodera, K. (1979) J. Antibiotics **32**, 849–851
11 Brunton, V. G., Steele, G., Lewis, A. D. and Workman, P. (1998) Can. Chemother. Pharmacol. **41**, 417–422
12 Uehara, Y., Hori, M., Takeuchi, T. and Umezawa, H. (1986) Mol. Cell Biol. **6**, 2198–2206
13 Yamaki, H., Suzuki, H., Choi, E. C. and Tanaka, N. (1982) J. Antibiotics **35**, 886–892
14 Pratt, W. B. (1998) Proc. Soc. Exp. Biol. Med. **217**, 420–434
15 Omura, S., Iwai, Y., Sadakane, N., Nakagawa, A., Oiwa, H., Hasegawa and Ikai, T. (1979) J. Antibiotics **32**, 255–261
16 Uehara, Y., Hori, M., Takeuchi, T. and Umezawa, H. (1985) Jpn. J. Can. Res. **76**, 672–675
17 Garcia, R., Parikh, N. U., Saya, H. and Gallick, G. E. (1991) Oncogene **6**, 1983–1989
18 Uehara, Y. and Fukazawa, H. (1991) Methods Enzymol. **201**, 370–379
19 Hers, I., Donath, J., van Willigen, G. and Akkerman, J. W. (1998) Arteriosclerosis Thromb. Vascular Biol. **18**, 404–414
20 Stebbins, C. E., Russo, A. A., Schneider, C., Rosen, N., Hartl, F. U. and Pevletich, N. P. (1997) Cell **89**, 239–250
21 Chen, H., Singh, S. S. and Perdew, G. H. (1997) Arch. Biochem. Biophys. **348**, 190–198
22 Mimnaugh, E. G., Chavany, C and Neckers, L. M. (1996) J. Biol. Chem. **271**, 22796–22801
23 Schulte, T., An, W. G. and Neckers, L. M. (1997) Biochem. Biophys. Res. Commun. **239**, 655–659
24 Whitesell, L., Sutphin, P., An, W. G., Schulte, T., Blagosklonny, M. V. and Neckers, L. M. (1997) Oncogene **14**, 2809–2816
25 Czar, M. J., Galigniana, M. D., Silverstein, A. M. and Pratt, W. B. (1997) Biochemistry **36**, 7776–7785
26 Schulte, T. W., Blagosklonny, M. V., Ingui, C. and Neckers, L. M. (1995) J. Biol. Chem. **270**, 24585–24588
27 Dasgupta, G. and Momand, J. (1997) Exp. Cell Res. **237**, 29–37
28 Polanowska-Grabowska, R., Simon, Jr, C. G., Falchetto, R., Shabanowitz, J., Hunt, D. F. and Gear, A. R. L. (1997) Blood **90**, 1516–1526
29 Gear, A. R. L. (1982) J. Lab. Clin. Med. **100**, 866–886
30 Jones, G. D. and Gear, A. R. L. (1988) Blood **71**, 1539–1543
31 Geanacopoulos, M. and Gear, A. R. (1988) Thromb. Res. **52**, 599–607
32 Simon, Jr., C. G. and Gear, A. R. L. (1998) Biochemistry **37**, 2059–2069
33 Gear, A. R. L. (1984) Br. J. Haematol. **56**, 387–398
34 Whitesell, L., Mimnaugh, E. G., De Costa, B., Myer, C. E. and Neckers, L. M. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 8324–8328
35 Murakami, M. and Odake, K. (1971) Thromb. Diathesis Haemorrhag. **25**, 223–233
36 Holmsen, H., Day, H. J. and Setkowsky, C. A. (1972) Biochem. J. **129**, 67–82
37 Fishkes, H. and Rudnick, G. (1982) J. Biol. Chem. **257**, 5671–5677
38 Carty, S. E., Johnson, R. G. and Scarpa, A. (1981) J. Biol. Chem. **256**, 11244–11250
39 Shiao, Y.-J, Chen, J.-C. and Wang, C.-T. (1989) Biochim. Biophys. Acta **980**, 56–68
40 Ferrell, Jr., J. E., Mitchell, K. T. and Huestis, W. H. (1988) Biochim. Biophys. Acta **939**, 223–237
41 Opstvedt, A., Rongved, S., Aarsaether, N., Lillehaug, J. R. and Holmsen, H. (1986) Biochem. J. **238**, 159–166

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