BH3-mimetic ABT-737 induces strong mitochondrial membrane depolarization in platelets but only weakly stimulates apoptotic morphological changes, platelet shrinkage and microparticle formation

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ABSTRACT

Background: Depolarization of mitochondrial inner transmembrane potential (ΔΨm) is a key biochemical manifestation of the intrinsic apoptosis pathway in anucleate platelets. Little is known, however, about the relationship between ΔΨm depolarization and downstream morphological manifestations of platelet apoptosis, cell shrinkage and microparticle (MP) formation.

Objectives: To elucidate this relationship in human platelets.

Materials and Methods: Using flow cytometry, we analyzed ΔΨm depolarization, platelet shrinkage and MP formation in platelets treated with BH3-mimetic ABT-737 and calcium ionophore A23187, well-known inducers of intrinsic platelet apoptosis.

Results: We found that at optimal treatment conditions (90 min, 37 °C) both ABT-737 and A23187 induce ΔΨm depolarization in the majority (88-94%) of platelets and strongly increase intracellular free calcium. In contrast, effects of A23187 and ABT-737 on platelet shrinkage and MP formation are quite different. A23187 strongly stimulates cell shrinkage and MP formation, whereas ABT-737 only weakly induces these events (10-20% of the effect seen with A23187, P < 0.0001).

Conclusions: These data indicate that a high level of ΔΨm depolarization and intracellular free calcium does not obligatorily ensure strong platelet shrinkage and MP formation. Since ABT-737 efficiently induces clearance of platelets from the circulation, our results suggest that platelet clearance may occur in the absence of the morphological manifestations of apoptosis.

Keywords: Apoptosis in platelets BH3-mimetic ABT-737 Calcium ionophore A23187 Mitochondrial inner membrane depolarization Platelet shrinkage Microparticle formation

Introduction

Depolarization of the mitochondrial inner transmembrane potential (ΔΨm) is a key manifestation of the intrinsic mitochondrial pathway of apoptosis in nucleated cells [1–4] and anucleate platelets [5–10]. Depolarization of ΔΨm in platelets has been shown to be triggered by multiple chemical stimuli [11] and very high shear stresses [6,12]. In platelets treated with calcium ionophore A23187, ΔΨm depolarization is associated with activation of caspase-9 of the intrinsic apoptosis pathway [13] and extra-mitochondrial apoptotic events, such as activation of caspase-8 of the extrinsic apoptosis pathway [13], apoptosis executioner caspase-3 [6,9,13] and phosphatidylserine (PS) exposure on the platelet surface [6,7,9,14]. Furthermore, ΔΨm depolarization in A23187-treated platelets and platelets subjected to very high shear stresses is associated with the downstream morphological manifestations of platelet apoptosis at the whole-cell level, i.e. platelet shrinkage and microparticle (MP) formation [6,9]. It has been also found that the mitochondria-targeted pro-apoptotic BH3-mimetic and anti-cancer drug ABT-737 [15] induces ΔΨm depolarization in platelets [16–18] together with activation of caspasas-9, -8 and -3 [13] and PS exposure [16,17,19,20]. However, whether ABT-737 is able to induce the cellular manifestations of platelet apoptosis, platelet shrinkage and MP formation, has not been reported.

In the current study, we demonstrate, for the first time, that BH3-mimetic ABT-737 triggers ΔΨm depolarization in the bulk of cells in platelet population, but the high level of mitochondrial membrane depolarization does not ensure a strong stimulation of platelet shrinkage and MP formation.

Abbreviations: PRP, platelet-rich plasma; ΔΨm, mitochondrial inner transmembrane potential; MP, microparticle; PS, phosphatidylserine; DMSO, dimethylsulfoxide; GPRP, Gly-Pro-Arg-Pro peptide; DiOC6(3), 3,3′-dihexyloxocarbocyanine iodide; FSC, forward scatter; SSC, side scatter.

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Materials and methods

Reagents and solutions

Bovine serum albumin (BSA), dimethylsulfoxide (DMSO), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), human α-thrombin and Gly-Pro-Arg-Pro peptide (GPRP) were purchased from Sigma (St Louis, MO, USA). ABT-737 was purchased from Selleck Chemical (Houston, TX, USA), A23187 was purchased from Calbiochem (San Diego, CA, USA). Green-fluorescent (FL1) cationic dye DiOC6(3) (3,3′-dihexyloxacarbocyanine iodide), green-fluorescent (FL1) calcium indicator Fluo-4 AM and phosphate-buffered saline (PBS) were purchased from Invitrogen (Carlsbad, CA, USA and Burlington, Canada). Green-fluorescent (FL1) fluorescein isothiocyanate (FITC)–conjugated anti-CD41 antibody (anti-CD41-FITC, clone P2) was purchased from Beckman-Coulter (Westbrook, ME, USA) and red-fluorescent (FL2) phycoerythrin (PE)–conjugated annexin V was purchased from BD Biosciences (San Jose, CA, USA). Buffer A was composed of PBS supplemented with 1 mM MgCl2, 5.6 mM glucose, 0.1% BSA and 10 mM HEPES, pH 7.4 and used as the buffer and control diluent for thrombin–treated platelets. Buffer B was composed of buffer A containing 0.1% DMSO and used as the buffer and control diluent for A23187– and A23187-treated platelets. Stock solutions of thrombin and GPRP were dissolved in buffer A and stock solutions of ABT-737 and A23187 were dissolved in 100% DMSO and stored at −80 °C.

Preparation and treatment of platelets

Venous blood from healthy volunteers was anticoagulated with 0.32% sodium citrate. Platelet-rich plasma (PRP) was obtained by centrifugation at 180 g for 15 min at room temperature (RT) and diluted with buffers A or B 10-fold (for determination of MP formation, platelet shrinkage, ΔΨm depolarization and intracellular calcium) or 60-fold (for determination of PS exposure).

Optimal final concentrations of 30 μM ABT-737 and 10 μM A23187 were employed in this study to ensure maximal apoptosis effects, based on the detailed dose titration study with 0.1–30 μM ABT-737 from Abbott Laboratories (Abbott Park, IL, USA) who developed and introduced ABT-737 [19] and the currently performed dose–response titration with 0.125–10 μM ABT-737 (Fig. S1). Time–response titrations with 30 μM ABT-737 and 10 μM A23187 performed by us previously revealed the optimal incubation conditions (90 min at 37 °C) for caspases-3, -8 and -9 activation [13], ΔΨm depolarization [18] and PS exposure [20]. Treatment of platelets for 90 min at 37 °C with 30 μM ABT-737 shows a stronger effect on ΔΨm depolarization and on the level of intracellular free calcium than treatment with concentration of 5 μM (Table S1). A23187 at concentration 10 μM does not induce platelet lysis determined as a loss of lactate dehydrogenase or β-glucuronidase [21].

For PRP with final concentrations of 10 μM A23187 and 30 μM ABT-737, 40 μl of diluted PRP aliquots were incubated for 90 min at 37 °C with 10 μl of 50 μM A23187 and 150 μM ABT-737 or 10 μl of buffer B as the control diluent. In some experiments, time–titration effects were analyzed by treatment of platelets for 15, 30, 60, 90 and 180 min at 37 °C with 10 μM A23187 and 30 μM ABT-737. Platelets were also treated with 1 U/ml thrombin [22] and diluent A; for this, PRP was diluted 10-fold with buffer A and 40 μl of PRP aliquots were incubated for 90 min at 37 °C with 10 μl of 5 U/ml thrombin or diluent A.

Determination of platelets, platelet-derived MPs and platelet shrinkage

Detection and enumeration of platelet-derived MPs is a difficult methodological issue since currently there is no specific single marker described that allows to distinguish platelets and MPs. In this study, we used three approaches for identifying platelets and platelet MPs: (i) calcium ionophore A23187 was employed as a potent platelet agonist known as a strong inducer of platelet-derived MP formation [9,23,24], (ii) staining of platelets with the anti-GPⅡbⅢa antibody was used for distinguishing platelet-derived MPs from small-size particles of non-platelet origin, and (iii) phosphatidylserine (PS) exposure on the surface of platelet MP was determined.

Fig. 1 shows the flow cytometric analysis of platelets and platelet-derived MPs in a platelet population treated with control diluent buffer or calcium ionophore A23187. Platelets and MPs were detected by two methods. In the first, platelet-and MP-gating was performed by analyzing forward scatter – side scatter (FSC-SSC) dot plots (Fig. 1A, B). In the second, platelets were identified with anti-GPⅡbⅢa antibody, by adding aliquots (8.3 μl) of anti-GPⅡbⅢa-FITC antibody to 50 μl of control diluent-treated and A23187-treated platelets and incubating for 20 min at RT in the dark. Samples were diluted to 500 μl with buffer A and analyzed by a FACS Calibur flow cytometer. After acquisition of 20,000 events, light forward scatter – fluorescence 1 (FSC-FL1) dot plots were analyzed and platelets and MPs were identified in platelet- and MP-specific gates, respectively (Fig. 1C, D). The border between platelets and MPs was set to ensure the maximal number of MPs in the MP gate in A23187-treated versus diluent-treated platelet populations. MP formation was quantified in the MP-specific gate as the increase in number or the percentage of MPs. Platelet shrinkage was determined in platelet-specific gate and expressed as the decrease in the mean FSC (cell size). As shown in the Fig. 1A-D, both methods gave very similar results, indicating that the most small-size particles in the MP-gate are actually platelet-derived MPs. Thus, both methods can be used for distinguishing platelets and MPs, and platelet shrinkage using appropriate negative (diluent treatment) and positive (A23187 treatment) controls.

Platelet-derived MPs contain not only constitutive platelet surface glycophospholipid GPⅡbⅢa (Fig. 1C, D) but also exhibit PS exposure on the outer leaflet of plasma membrane (Fig. S2).
It should be noted, however, that since there are no platelet-derived MP-specific markers available, the composition of these MP fractions determined from FSC-SSC or FSC-FL1 (GPIIbIIIa) gating (Fig. 1), may contain not only MPs but also some amount of small-size platelets. While calibrator beads are useful for standardization of platelet-derived MP enumeration by flow cytometry [25], they also do not allow differentiation between MPs and small-size platelets. In this study, we used the term MP for definition of platelet-derived smaller particles which are characterized by FSC < 10^3, contain the constitutive platelet marker GPIIbIIIa on their surface and expose PS under appropriate stimulation.

**Determination of ΔΨm depolarization in platelets**

Following 15–180 min treatments at 37 °C, 50 μL of platelet samples were incubated for 20 min at RT in the dark with 10 μL of 600 nM DiOC6(3) to obtain a final concentration of 100 nM. Sampled solutions were then diluted to 500 μL with buffer A containing 2 mM CaCl2, 20,000 events were acquired and PS exposure was analyzed by the flow cytometry both in platelets and MPs. For this, platelets were primarily gated by FSC-SSC characteristics and then, ΔΨm depolarization was determined from FSC-DiOC6(3) fluorescence (FL1) dot plots and expressed as the percentage depolarized cells [22].

**Determination of PS exposure in platelets and MPs**

For assay of PS exposure, PRP was diluted 1:60 with buffer B and 30 μL aliquots were treated for 90 min at 37 °C with 10 μL of solutions containing 8 mM CaCl2 and either diluent B or 40 μM A23187 in diluent B and stained with 2.5 μL of annexin V-PE by incubation for 20 min at RT in the dark. Sampled solutions were diluted to 300 μL with buffer A containing 2 mM CaCl2, 20,000 events were acquired and PS exposure was analyzed by the flow cytometry both in platelets and MPs. For this, the entire platelet population, containing platelets and MPs, was primarily gated by FSC-FL1 dot plots and then PS exposure was determined from FSC-FL2 dot plots.

**Calculation of relative ABT-737 effects on platelet apoptotic responses**

Relative ABT-737 effects on ΔΨm depolarization, platelet shrinkage and MP formation were determined as the percentage of A23187 effects. For upregulated apoptotic parameters (% ΔΨm-depolarized cells and number of MPs in MP gate), the relative ABT-737 effects (ABT, %) were calculated by the formula:

\[
\text{ABT,} \% = \left( \frac{\text{ABT} - \text{Dil}}{\text{A23} - \text{Dil}} \right) 
\]

where ABT, A23 and Dil are the values of apoptotic parameters after the treatment of platelets with ABT-737 (ABT), A23187 (A23) and diluent control buffer B (Dil).

For downregulated apoptotic parameter (mean FSC in platelet gate), characterizing platelet shrinkage, the relative ABT-737 effect (ABT, %) was calculated by the formula:

\[
\text{ABT,} \% = \left( \frac{\text{Dil} - \text{ABT}}{\text{Dil} - \text{A23}} \right) 
\]

**Statistical analysis**

Data are presented as means ± SEM. The statistical significance of the differences between different platelet groups was determined by Student’s t-test and one-way analysis of variance (ANOVA) with Dunnett’s multiple comparison test as appropriate. Differences were considered significant when P < 0.05.

**Results**

**Effects of ABT-737 and A23187 on ΔΨm depolarization, platelet shrinkage and microparticle formation**

As shown in Fig. 2, after treatment of platelets with calcium ionophore A23187 for 15 min at 37 °C, 80.4 ± 6.1% of cells contain mitochondria with depolarized inner membrane potential. Following a further 30–180 min treatment, the time-titration A23187 curve reaches the maximum of 90–95% ΔΨm depolarized cells. In contrast, during treatment of platelets with BH3-mimetic ABT-737, for reaching the maximal depolarization level of 88–94%, prolonged treatment for 90–180 min is required. Control diluent buffer causes only insignificant increase of ΔΨm depolarization during 15–90 min treatment (2.8 ± 0.4–6.5 ± 1.5%, P > 0.05) and significantly (P < 0.01) induces ΔΨm depolarization only in a minor platelet subpopulation of 12.2 ± 3.4% cells at 180 min treatment (Fig. 2).

Under conditions that assure an equally high maximal level of ΔΨm depolarization in ABT-737- and A23187-treated platelets (90 min at 37 °C), we analyzed the effects of these apoptotic triggers on platelet shrinkage and MP formation (Figs. 3A–F and 4A–D). Determination of these cellular morphological manifestations of platelet apoptosis in A23187-treated platelets compared to diluent-treated platelets (Fig. 3B, F), demonstrates that at very high level of mitochondrial membrane depolarization, when 92–94% cells have mitochondria with depolarized ΔΨm (Figs. 3E and 4A), A23187 strongly stimulates platelet...
Effects of ABT-737 and A23187 on intracellular free calcium in platelets

BH3-mimetic ABT-737 and calcium ionophore A23187 have very different mechanisms of triggering platelet responses. These agents, however, show equally strong effect on mitochondrial ΔΨm depolarization at optimal treatment conditions (Figs. 2–4), but ABT-737 has much weaker impact on platelet shrinkage and MP formation (Figs. 3–5). It can be hypothesized that the strong stimulation of cell shrinkage and MP generation by calcium ionophore and much weaker stimulation of these events by ABT-737 may be explained by a higher calcium overload in platelets treated with calcium ionophore in comparison with ABT-737-treated platelets. To check this hypothesis, we determined intracellular free calcium in ABT-737- and A23187-stimulated platelets. We found that both ABT-737 and A23187 significantly increased the level of free calcium in platelets in comparison with control diluent (Fig. 6, P < 0.01). However, free calcium level in ABT-737- and A23187-treated platelet groups does not differ significantly (Fig. 6, P > 0.05).

Taken together with previously reported data that ABT-737 and its analog ABT-263 disrupted calcium homeostasis in platelets [17,27,28], our results suggest that in ABT-737-treated platelets a high level of intracellulare free calcium (Fig. 6) does not ensure strong platelet shrinkage and MP formation (Figs. 3–5).

Activation, apoptosis, shrinkage and MP formation in platelets treated with physiological agonist thrombin

We further analyzed effects of physiological platelet agonist thrombin on biochemical manifestations of platelet activation (CD62 expression) and apoptosis (ΔΨm depolarization) as well as on MP formation and platelet shrinkage, and compared these effects with platelet responses induced by calcium ionophore A23187 (Table S2). For...
thrombin-treated platelets the predominant platelet response is activation as determined by the percentage CD62-positive cells (86%), whereas platelet apoptosis determined as ΔΨm depolarization accounts for only 11% cells. In contrast, in A23187-treated platelets the predominant platelet subpopulation consists of cells which have both mitochondria with depolarized ΔΨm and concurrently exposed CD62 on the cell surface (95% cells). Platelet responses induced by thrombin at the whole-cell level are not affected (platelet shrinkage, \( P > 0.05 \)) or only moderately affected (MP formation, \( P < 0.05 \)). For calcium ionophore, on the other hand, both MP formation and platelet shrinkage are strongly induced and differences versus control diluent and thrombin are highly significant (Table S2, \( P < 0.0001 \)).

**Discussion**

Magnitude and time-dependencies of apoptotic responses in A23187- and ABT-737-treated platelets

Pro-apoptotic BH3-only protein mimetic, ABT-737 specifically inhibits anti-apoptotic members of Bcl-2 family proteins (Bcl-2, Bcl-XL, and Bcl-w) by binding to the hydrophobic BH3-binding groove and shifting the balance between pro- and anti-apoptotic Bcl-2 proteins in favor of apoptosis [29]. ABT-737 and its analog Navitoclax exhibit anti-tumor activity and are under clinical trials for treatment of solid tumors and lymphoid malignancies [15,30]. ABT-737 induces thrombocytopenia in animal models [19,31] and different manifestations of apoptosis in platelets [13,17–20,31,32].

In the current study, we found that under optimal treatment conditions BH3-mimetic ABT-737 and calcium ionophore A23187 induce strong ΔΨm depolarization in human platelets (Table 1), i.e. with both kinds of treatment 88–94% cells have mitochondria with low inner transmembrane potential. Recently, we have shown that ABT-737 and A23187 also strongly stimulate downstream extra-mitochondrial apoptotic responses, resulting in caspase cascade activation, including activation of initiator caspase-9 of the intrinsic apoptosis pathway, initiator caspase-8 of the extrinsic pathway and apoptosis executioner caspase-3 (Table 1), when up to 80–93% cells contain these caspases in the active form [13]. Furthermore, it has been reported that both agonists cause strong apoptotic alterations in the platelet plasma membrane (Table 1), when 80–99% cells expose phosphatidylserine (PS) on the platelet surface [9,14,17,19,20]. However, the effects of A23187 and ABT-737 on cellular manifestations of platelet apoptosis are quite different: A23187 triggers strong stimulation of platelet shrinkage and MP formation, whereas ABT-737 only weakly induces these apoptotic responses (Table 1). This novel result indicates that a high level of ΔΨm depolarization in apoptotic platelets does not always ensure a strong stimulation of apoptotic manifestations at the whole-cell level.

We also demonstrated different time-dependencies for mitochondrial and cellular apoptotic responses in A23187-treated versus ABT-737-treated platelets. The maximal level of ΔΨm depolarization is reached after a short incubation of platelets with calcium ionophore, whereas much longer treatment is required for maximal stimulation of depolarization by mitochondria-targeted BH3-mimetic (Fig. 2). For cellular manifestations of platelet apoptosis, the maximal level of platelet shrinkage and MP formation is also reached after a short treatment with A23187 but only a weak stimulation of these cellular apoptotic

**Table 1**

Effects of calcium ionophore A23187 and BH3-mimetic ABT-737 on mitochondrial, extra-mitochondrial and cellular manifestations of platelet apoptosis.

<table>
<thead>
<tr>
<th>Apoptotic responses</th>
<th>Cellular localization</th>
<th>A23187 effect</th>
<th>ABT-737 effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔΨm depolarization</td>
<td>Mitochondrial</td>
<td>Strong</td>
<td>Strong</td>
</tr>
<tr>
<td>Caspases 9, 8, 3 activation</td>
<td>Extra-mitochondrial</td>
<td>Strong</td>
<td>Strong</td>
</tr>
<tr>
<td>PS exposure</td>
<td>Extra-mitochondrial</td>
<td>Strong</td>
<td>Strong</td>
</tr>
<tr>
<td>Platelet shrinkage</td>
<td>Whole-cell level</td>
<td>Strong</td>
<td>Weak</td>
</tr>
<tr>
<td>MP formation</td>
<td>Whole-cell level</td>
<td>Strong</td>
<td>Weak</td>
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The Table summarizes data presented in this study for ΔΨm depolarization, platelet shrinkage and MP formation (Figs. 2–5), as well as reported previously for caspases 9, 8 and 3 activation [13] and PS exposure [20].

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**Fig. 5.** A23187 much stronger than ABT-737 stimulates platelet shrinkage and microparticle formation over the entire treatment period. Platelets were treated for 15–180 min at 37 °C with 30 μM ABT-737, 10 μM A23187 and diluent buffer, and platelet shrinkage (A) and MP formation (B) were determined. Data are presented as means ± SEM for 8–9 independent experiments in each platelet group; SEM bars are shown when a bar size is larger than a symbol size. Note that for all treatment-times effects of A23187 on platelet shrinkage and MP formation are significantly higher than time-matched ABT-737 effects; "***" P < 0.001.

**Fig. 6.** Effects of BH3-mimetic ABT-737 and calcium ionophore A23187 on intracellular free calcium in platelets. Platelets were treated for 90 min at 37 °C with 30 μM ABT-737, 10 μM A23187 and diluent buffer B and loaded with Fluo-4 AM. Data are presented as means ± SEM for 3 independent experiments in each platelet group; SEM for diluent-treated group is too small to be visible. The differences between ABT-737- and A23187-treated platelets versus diluent-treated platelets are significant (** P < 0.01), whereas the difference between ABT-737- versus A23187-treated platelets is not significant (P > 0.05).
events could be achieved with ABT-737 even after prolonged treatment (Fig. 5).

Putative mechanisms of clearance of A23187- and ABT-737-treated platelets

In animal models, it has been reported that treatment of platelets with A23187 and ABT-737 induces clearance of platelets from the circulation. Treatment of rabbit platelets in vitro with A23187 followed by platelet infusion into recipient rabbits results in a rapid platelet clearance [7]. Similarly, injection of ABT-737 into dogs [19] and mice [15,31] induces platelet clearance and causes acute thrombocytopenia. Taken together with our current finding that A23187 strongly stimulates platelet shrinkage and MP formation, whereas ABT-737 induces these apoptotic events only weakly (Table 1), the data suggest two putative scenarios for the clearance of apoptotic platelets from the circulation. In the first, when platelets are treated with calcium ionophore A23187, the cellular manifestations of apoptosis, platelet shrinkage and MP formation, may be involved in the platelet clearance. The second scenario occurs when platelets are exposed to BH3-mimetic ABT-737. In this case, platelet removal from the circulation takes place without participation of cell shrinkage and MP generation (i) by stimulation of different platelet caspases, such as caspases-9, -8 and -3 [13], which cleave a broad spectrum of vital cell proteins [33,34] and/or (ii) by a strong PS exposure on the surface of apoptotic platelets [9,14,17,19,20], which may serve as an "eat-me" signal for recognition and removal of apoptotic cells by macrophages [35,36].

Platelet responses induced by thrombin

Exposure to thrombin resulted in a 28-fold stimulation of CD62 exposure versus control diluent, but only a moderate 1.7-fold stimulation of MP formation and no stimulation of platelet shrinkage (Table S2). In contrast, when platelets were treated with calcium ionophore A23187, the majority (95%) of cells are both apoptotic and activated, undergoing concurrent ΔΨm depolarization and CD62 exposure, with strong MP formation and platelet shrinkage (Table S2).

Platelet-derived MPs are cell fragments, exposing PS (Fig. S2). Platelet-derived MPs are cell fragments, exposing PS (Fig. S2). Platelet shrinkage may occur as the result of lost volume due to shed MPs. When the process of MP formation is strongly induced, as during treatment with A23187, platelet shrinkage is also strongly induced and easily detectible (Table S2). However, if MP formation is only moderately induced, e.g. by thrombin, platelet shrinkage is not appreciable (Table S2). These data suggest that when platelets are treated with weak or moderate stimuli, MP formation is a more sensitive morphological marker of platelet apoptosis than is platelet shrinkage.

Platelet shrinkage and microparticle formation: novel observations

Shedding of membrane-enclosed MPs, apoptotic bodies, and cell shrinkage are the terminal points of no return in apoptosis of nucleated cells [4]. By investigating the processes of MP formation and cell shrinkage in anucleate platelets, a number of novel observations were obtained in the current study.

Firstly, although the effects of proapoptotic BH3-mimetic ABT-737 on platelets have been studied previously by us [13,18,20] and others [17,19,27,28,31,32,37], in the current study we, for the first time, demonstrated that ABT-737 has very weak effects on the cell shrinkage and MP formation, in contrast to the calcium ionophore A23187, which is strongly induced these apoptotic responses (Figs. 3-5). These quite different effects of ABT-737 and A23187 on platelet shrinkage and MP formation were obtained under optimal treatment conditions when the mitochondrial manifestation of platelet apoptosis ΔΨm depolarization was equally strongly stimulated in ABT-737- and A23187-treated platelets (Figs. 3 and 4).

Furthermore, we investigated the cellular apoptotic events in platelets treated with the physiological platelet agonist thrombin, which preferentially induces platelet activation, as determined by CD62 exposure, in contrast to ABT-737, which preferentially induces ΔΨm depolarization [18], and to A23187, which induces both ΔΨm depolarization and CD62 exposure (Table S2). Our data demonstrate that, in thrombin-treated platelets, the cellular apoptotic events are not stimulated (platelet shrinkage) or are significantly more weakly stimulated (MP formation), in comparison to A23187-treated platelets (Table S2), indicating that shedding of platelet-derived MPs and platelet shrinkage are not classical manifestations of platelet activation.

Finally, for the first time, we showed that the lack of these terminal cellular apoptotic responses in ABT-737-treated platelets occurs under the conditions when intracellular free calcium is highly stimulated by ABT-737, resulting in elevation to a level which is not significantly different from that induced under calcium overloading conditions by calcium ionophore A23187 (Fig. 6), indicating that a high level of intracellular free calcium is not sufficient to ensure strong platelet shrinkage and MP formation.

Taken together, these results suggest that preferential stimulation of platelet apoptosis (as in the case of ABT-737 treatment) or platelet activation (thrombin treatment) are not enough by themselves for inducing strong platelet shrinkage and MP formation, and only simultaneous stimulation of both apoptosis and activation (A23187 treatment) is able to cause these terminal cellular manifestations of platelet apoptosis.

Biomedical significance and potential clinical applications

In the current and previous studies, we demonstrated that, depending on different triggering stimuli, platelets may undergo or not undergo platelet shrinkage and shedding of MPs, as well as biochemical manifestations of platelet apoptosis (ΔΨm depolarization, caspases-9, -8 and -3 activation and PS exposure) and activation (CD62 exposure, GPIIIa up-regulation and GP Ibα downregulation) [6,9,13,18,20,22,38,39]. These data provide an experimental basis for investigating the role of specific manifestations of platelet apoptosis and activation in hemostatic function, phagocytosis and clearance of platelets in vitro and/or in vivo, in animal (and human) models of thrombosis, atherosclerosis, inherited and autoimmune thrombocytopenias, cancer and platelet storage defect. Whether the different manifestations of apoptosis relate to differing initiating mechanisms or to severity of disease or are of prognostic significance remains to be determined.

Increased or impaired platelet-derived MP formation [40-42] and platelet size abnormalities [43-45] have been demonstrated in various platelet-associated diseases. Investigation of conditions and the role of apoptotic and activation processes in platelet MP shedding and platelet shrinkage may contribute to pharmacological manipulation of these cellular platelet responses and clinical diagnostics.

Further clinical studies are required to elucidate how administration of ABT-737 to cancer patients, which may be associated with a strong stimulation of ΔΨm depolarization, caspase activation and PS exposure in platelets and weak stimulation of platelet activation, shrinkage and MP formation, will contribute to platelet-associated clinical settings, such as (i) magnitude and duration of thrombocytopenia, (ii) potential bleeding complications associated with ABT-737 administration, (iii) cancer-associated thrombosis and cardiovascular diseases and (iv) efficiency of platelet transfusion in ABT-737-treated cancer patients.

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Author contributions

AVG and VL designed the research study. AVG and DJA performed the research. AVG, DJA, AM, JF and VL analyzed the data and wrote the paper.
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Conflict of interest statement
The authors have no conflict of interests.

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