BCL2/BCL-X<sub>1</sub>-inhibition induces apoptosis, disrupts cellular calcium homeostasis and prevents platelet activation

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Running Title: BCL2 inhibitors: platelet apoptosis and activation
Abstract

Apoptosis in megakaryocytes results in the formation of platelets. The role of apoptotic pathways in platelet turnover and in the apoptotic-like changes seen following platelet activation is poorly understood. ABT-263 (Navitoclax), a specific inhibitor of anti-apoptotic BCL2 proteins, which is currently being evaluated in clinical trials for the treatment of leukemia and other malignancies, induces a dose-limiting thrombocytopenia. In this study, the relationship between BCL2/BCL-XL-inhibition, apoptosis and platelet activation was investigated. Exposure to ABT-263 induced apoptosis but repressed platelet activation by physiological agonists. Notably, ABT-263 induced an immediate calcium response in platelets and the depletion of intracellular calcium stores, indicating that upon BCL2/BCL-XL-inhibition platelet activation is abrogated due to a diminished calcium signaling. By comparing the effects of ABT-263 and its analog ABT-737 on platelets and leukemia cells from the same donor, we show for the first time that these BCL2/BCL-XL-inhibitors do not offer any selective toxicity, but induce apoptosis at similar concentrations in leukemia cells and platelets. However, reticulated platelets are less sensitive to apoptosis, supporting the hypothesis that treatment with ABT-263 induces a selective loss of older platelets and providing an explanation for the transient thrombocytopenia observed upon ABT-263 treatment.
Introduction

All nucleated cells in multicellular organisms are genetically programmed to undergo apoptosis to remove unnecessary or damaged cells from the whole organism. This program has been recognized as the central mechanism of platelet production from megakaryocytes. However, the role of apoptosis in anuclear, mature platelets is less well characterized, with apoptotic-like changes seen in both aging platelets and in the formation of pro-coagulant microparticles following agonist stimulation.

Two main pathways lead to the execution of apoptosis; the extrinsic and the intrinsic, or mitochondrial, pathways. Both converge into the activation of caspases, which are proteases that cleave over 500 cellular targets and induce typical morphological changes associated with apoptosis in nucleated cells. A critical step in the intrinsic pathway is the loss of mitochondrial membrane potential (MMP) and the release of cytochrome $c$ into cytosol, where it triggers the activation of caspase-9. Therefore the release of cytochrome $c$ from mitochondria needs to be tightly regulated: a function that is fulfilled by the BCL2 protein family, which consists of pro-apoptotic and anti-apoptotic members that promote or block the release of cytochrome $c$, respectively. The pro-apoptotic family members BAX and BAK play an essential role in directly mediating the release of cytochrome $c$ by forming a pore in the outer mitochondrial membrane. Anti-apoptotic BCL2 proteins, including BCL2, BCL-XL, BCL-w, MCL1, and BCL2A1, prevent the activation of BAX and BAK. Besides their function in regulating mitochondrial cytochrome $c$ release, BCL2 proteins have also been implicated in the regulation of intracellular calcium homeostasis at the endoplasmatic reticulum (ER), possibly by interacting with inositol triphosphate receptors.

Owing to their key role the anti-apoptotic BCL2 proteins are attractive targets for anticancer therapy, with several small molecule inhibitors currently in preclinical testing or early clinical trials. Amongst these, the most promising and specific inhibitors are ABT-263 (Navitoclax) and ABT-737. ABT-737 shows promising antitumor activity in animal models of leukemia and lymphoma. A related compound, ABT-263, is metabolically more stable and currently in phase 1 and 2 clinical trials for leukemia and other malignancies. Both compounds have often been regarded as interchangeable because they bind with high affinity to BCL2, BCL-XL, and BCL-w but do not inhibit MCL1 or BCL2A1. Early results from the clinical trials with ABT-263, and from animal studies with ABT-737, show that thrombocytopenia is the major toxicity of these drugs. As this thrombocytopenia is reversible and platelet counts recover rapidly after cessation of treatment, it appears that these drugs induce platelet apoptosis directly without affecting megakaryocyte function.

In an intriguing study by Mason et al, ABT-737-induced thrombocytopenia was shown to be a mechanism-based toxicity explained by a dependence of platelets on BCL-XL for survival. Although some BCL2 proteins and caspases are expressed in platelets, their roles in platelet physiology are poorly understood. The view that apoptosis and phosphatidylserine (PS) externalization may play a
role in the clearance of senescent platelets by the mononuclear phagocyte system, receives some support from in vitro investigations that show a time-dependent induction of apoptosis in stored platelet preparations. Furthermore, during storage at 37°C the protein expression of BCL-XL decreases, further indicating a critical anti-apoptotic role for BCL-XL in platelets. Data from knockout mice confirm the importance of BCL-XL in platelet survival, since loss of BCL-XL, but not of BCL2, BCL-w or MCL1 resulted in thrombocytopenia.

The role of BAX and BAK in platelet apoptosis is less clear. Mason et al concluded that BAK is essential for platelet apoptosis and needs to be inhibited by BCL-XL to prevent apoptosis. During platelet aging, the levels of BCL-XL decrease owing to protein degradation, leading to a reduction in BCL-XL-mediated inhibition of BAK and resulting in apoptosis. The proposed model of the ratio of BCL-XL and BAK as a “molecular clock” may provide a useful explanation of how platelet life span is regulated in vivo. Support for an important role for BCL-XL in the regulation of platelet ageing was provided by the observation that platelets from BCL-XL mutant mice have a life span of 11 hours in vivo, compared with 68 hours in wild type control mice.

BCL2 proteins also play a role in platelet activation by thrombin. The signaling pathways that distinguish platelet apoptosis and platelet activation are not clearly separated since both have been reported to involve a loss of MMP and externalization of PS. Some studies have addressed this question by comparing platelet activation and apoptosis. Here we investigated the effects of BCL2-inhibition on both platelet apoptosis and activation. We demonstrate that both processes are clearly distinguishable, since apoptosis occurs slowly over hours and results in biochemical and ultrastructural changes that are clearly different from those seen during the more rapid process of agonist-induced activation, which occurs within minutes. Furthermore, we found that inhibition of BCL2 proteins induces an immediate calcium response, which prevents the subsequent responsiveness of platelets to physiological agonists.
Material and Methods

Cells

Platelets were collected from healthy volunteers or from patients with chronic lymphocytic leukemia (CLL), with written informed consent in accordance with the Declaration of Helsinki and UK Home Office ethical authority approval. Blood was collected in 0.085 M tri-sodium citrate and 0.071 M citric acid. Platelet-rich plasma (PRP) was separated by centrifugation for 20 minutes at 140g. Washed platelets were isolated from PRP by centrifugation for 15 minutes at 600g in the presence of 200 ng ml⁻¹ prostacyclin (PGI₂; Sigma-Aldrich) and resuspended in HEPES-buffered saline (HBS: 10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgSO₄, pH 7.4).

Chemicals

ABT-737 and ABT-263 were purchased from Selleck Chemicals. Human serum albumin (HSA), A23187 and thrombin receptor activator peptide (TRAP) were from Sigma-Aldrich. Cross-linked collagen-related peptide (CRP-XL)²⁶ was provided by Richard Farndale, Department of Biochemistry, University of Cambridge, UK. The broad-spectrum caspase inhibitors z-VAD.fmk and Q-VD-OPh were from MP Biomedicals.

Analysis of apoptosis

To assess the differential toxicity of BCL2-antagonists on platelets and leukemia cells, citrated blood of CLL patients was incubated with different concentrations of ABT-737 or ABT-263 for 4 hours. Apoptosis of CLL cells was investigated in whole blood and apoptosis of platelets was investigated in plasma or on preparations of washed platelets incubated at 2 x 10⁷ cells ml⁻¹. PS externalization was assessed by flow cytometry following incubation for 15 minutes in HBS and 1.8 mM CaCl₂ (pH 7.4) with the pan-platelet antibody CD41/PE (Invitrogen) and annexinV/FITC.

Platelet activation assays

Calibrated automated thrombography was used to measure thrombin generation in platelets suspension essentially as described previously.²⁷ Washed platelets were pretreated for 2 hours at 37°C with or without ABT-737 or ABT-263, then stimulated for 10 minutes with CRP-XL (2 µg ml⁻¹), TRAP (1 x 10⁻⁴ M) or A23187 (1 x 10⁻⁵ M). Samples were mixed with an equal volume of autologous platelet poor plasma (PPP) that had been collected in the presence of 18.3 µg ml⁻¹ corn trypsin inhibitor (Enzyme Research) to inhibit contact phase activation, and filtered through a 0.2 µm
acrodisc filter (Pall Corporation). Samples were incubated with PPP reagent (1 pM tissue factor) and the fluorescent substrate ZGGA-AMC (both from Diagnostica Stago) analyzing fluorescence release for 60 minutes at 37°C in a fluorescent plate reader (Fluoroskan Ascent, Thermo Scientific) equipped with Thrombinscope software (Synapse BV). Surface exposure of P-selectin in response to CRP-XL (2 µg ml⁻¹) and TRAP (1x10⁻⁴ M) was assessed by flow cytometry using a P-selectin/FITC monoclonal antibody (R&D Systems) following incubation of washed platelets for 2 hours at 37°C in HBS with ABT-737 or ABT-263. Binding of fibrinogen and platelet aggregation in response to CRP-XL (2 µg ml⁻¹) and TRAP (1x10⁻⁴ M) were assessed in PRP following incubation of whole blood with drugs for 2 hours at 37°C. PRP was then isolated by centrifugation at 160g for 20 minutes and the platelet count adjusted to 1.5 x 10⁸ ml⁻¹ with autologous filtered PPP. For flow cytometry, 5 µl of PRP or platelet suspension were diluted 1:10 with HBS and incubated with 2 µl of fibrinogen/FITC (DAKO UK Ltd.) for 20 minutes before analysis. Aggregation in response to CRP-XL and TRAP was assessed in parallel in the ABT-737/-263-treated PRP samples using a PAP-8E aggregometer (Bio/Data Corporation®), measuring aggregation over 6 minutes at 37°C, with a stir speed of 1200 rpm.

**Calcium release assays**

Washed platelets were incubated for 2 hours in HBS with or without ABT-263 and z-VAD.fmk before staining with 1 µM Fluo4/AM (Invitrogen) for an additional 30 minutes. The intracellular calcium levels were assessed continuously at a FACScalibur after establishing a stable baseline fluorescence signal for 1 minute. A23187 or ABT-263 was added and fluorescence analyzed for additional 4 minutes.

**Thiazole-orange staining**

The proportion of reticulated platelets was assessed by staining with thiazole-orange as described previously.²⁸ Washed platelets were incubated in annexin-buffer (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, pH 7.4) plus 50 ng/ml thiazole-orange and ABT-263 for 2 hours before addition of annexin/APC and analysis by flow cytometry.

**Microscopy**

For confocal microscopy, washed platelets were allowed to settle on glass coverslips for 30 minutes before fixing for 15 minutes with 4% paraformaldehyde in PBS. Platelets were permeabilized for 10
minutes using 0.5% Triton-X 100 in PBS with 3% bovine serum albumin, and incubated with mouse anti-cytochrome c, rabbit anti-active caspase 3 (both from BD Biosciences) or anti-tubulin antibody (Sigma-Aldrich) at 1:500 in PBS with 10% goat serum at 4°C overnight. Coverslips were washed and incubated with goat anti-mouse or rabbit secondary antibody/Alexa546 or Alexa488 (Invitrogen). For staining of actin, following antibody staining, platelets were incubated for 30 minutes with phalloidin/Alexa488 (Invitrogen). Coverslips were mounted and observed using a Zeiss LSM510 confocal microscope with a 63x oil immersion lens, using a 6x zoom. Ultrathin sections for electron microscopy were prepared as described previously and recorded using a Megaview 3 digital camera and iTEM software (Olympus Soft Imaging Solutions) in a Jeol 100-CXII electron microscope (Jeol UK Ltd.).

MMP and cytochrome c release

The loss of MMP was assessed by flow cytometry, staining with 50 nM tetramethylrhodamine ethyl ester (TMRE, Invitrogen) for 10 minutes at 37°C. For live cell imaging, platelets were incubated with 25 nM TMRE and 1:100 CD41/FITC during the 30 minute period of settling onto coverslips, and imaged immediately. To assess the release of mitochondrial cytochrome c, platelets were washed in cold PBS and resuspended at 1 x 10^8 in 30 μl of mitochondrial isolation buffer (250 mM sucrose, 20 mM HEPES, pH 7.4, 5 mM MgCl2 and 10 mM KCl) containing 0.05% digitonin. Cells were left on ice for 10 minutes followed by centrifugation at 13,000 rpm for 3 minutes. Subsequently, supernatant and pellets were analyzed by western blotting.

Western blotting and immunoprecipitation

For analysis of protein expression, 1x 10^8 platelets were lysed in 0.5% Triton-X 100, 150 mM NaCl, 20 mM TrisHCl, pH 8, and protease inhibitor cocktail (Roche). Western blot analysis was performed using mouse anti-gelsolin (BD Biosciences), mouse anti-fodrin (Millipore) and mouse anti-cytochrome c (BD Biosciences) rabbit anti-BCL-XL (BD Biosciences), mouse anti-BCL2 (DAKO UK Ltd.) rabbit anti-BCL2A1 (kindly provided by J. Borst, Netherlands Cancer Institute, Amsterdam), rabbit anti-MCL1 (Santa Cruz Biotechnology) rabbit anti-BAK or anti-BAX (Upstate Biotechnology), rabbit anti-BID (Cell Signaling Technology), rabbit anti-BIM (Calbiochem), mouse anti-NOXA (Calbiochem), rabbit anti-PUMA (Cell Signaling Technology), mouse anti-BAD (BD Biosciences) and mouse anti-α-tubulin (Merck). Enhanced chemiluminescence was used for detection (GE Healthcare). For immunoprecipitation, 1x 10^9 washed platelets were exposed to ABT-263 (100 nM)
and z-VAD.fmK (50 μM) for 2 hours before lysis in 1% CHAPS, 150 mM NaCl, 20 mM TrisHCl, pH 8, and protease inhibitor cocktail. Rabbit anti-BCL-X₇ (BD Biosciences) was crosslinked with ProtA-dynabeads using 20 mM dimethylpimelinediimidate (Sigma-Aldrich). Crosslinked antibodies were incubated with platelet lysate for 2 hours at 4°C. Beads were washed with lysis buffer containing 1% CHAPS before elution in SDS-loading dye and western blotting. Quantification of BAK binding to BCL-X₇ was performed using ImageJ software.

**Statistical Analysis**

The EC₅₀ (Figure 1) was calculated in GraphPad Prism. Comparison between two variables (Figures 1 and 7) was performed using paired t-test in Microsoft Excel. Comparison between multiple variables (Figures 3B-D) was performed using ANOVA and Bonferroni’s multiple comparison test in GraphPad Prism.
Results

Platelets are more sensitive to ABT-263 than to ABT-737

To investigate apoptosis induced by inhibition of BCL2 proteins, washed human platelets were incubated in HBS with ABT-263 or ABT-737. After 1 hour of exposure, only low levels of apoptosis were detectable as assessed by PS externalization (Figure 1A), but after 2 and 4 hours of exposure (Figures 1B-D) extensive platelet apoptosis was induced in a time- and concentration-dependent manner. These data indicate that the induction of apoptosis by BCL2-inhibition occurs much more slowly than platelet activation, which occurs within minutes of agonist exposure. The EC50 for ABT-737 and ABT-263 at 4 hours were in the low nanomolar range, demonstrating that platelets are intrinsically very sensitive to BCL2-inhibition. Notably, in clinical trials for ABT-263 plasma concentrations up to ~6 μM were achieved. At all time points measured, ABT-263 was more potent at inducing platelet apoptosis than ABT-737 (at 4 hours the EC50 for ABT-737 was 23 nM and 8 nM for ABT-263). To investigate the effects of BCL2-inhibition in a more physiological environment, whole blood of healthy volunteers was incubated with ABT-737 or ABT-263 before isolation of PRP and measurement of platelet apoptosis. In whole blood, micromolar concentrations of both drugs were required to induce platelet apoptosis, and surprisingly, ABT-263 was now less potent than ABT-737 (Figures 1C-D). We have previously shown that the presence of albumin decreases the efficacy of both compounds to induce apoptosis of CLL cells, and that ABT-263 has a higher affinity for albumin and is therefore more efficiently sequestered by this plasma protein. To investigate whether the binding of drug to albumin was responsible for the loss of sensitivity in whole blood, we incubated washed platelets in HBS containing 3% human serum albumin (HSA). The addition of albumin resulted in a loss of sensitivity to ABT-737 and ABT-263, confirming that albumin sequesters both drugs. Interestingly, in the case of ABT-737 (Figure 1C) the addition of albumin accounted for most of the reduced sensitivity in whole blood, while for ABT-263 (Figure 1D) the addition of albumin caused only a partial loss of sensitivity compared to whole blood. These data indicate that other factors in the blood besides albumin binding diminish the efficacy of ABT-263, but not of ABT-737, to induce platelet apoptosis.

ABT-737 and ABT-263 lack selective toxicity in CLL

In initial clinical studies with ABT-263 as a potential anticancer agent, thrombocytopenia is the unwanted and dose-limiting toxicity. To compare directly the susceptibility of the therapeutic target (leukemia cells) with that of platelets, we incubated whole blood of patients with CLL in the presence of ABT-737 or ABT-263 and assessed apoptosis in the different cell types. ABT-737 induced similar levels of apoptosis in CLL cells and in platelets, whereas ABT-263 was less potent at inducing CLL
cell apoptosis than platelet apoptosis (Figure 1E-F), demonstrating that at least during the first treatment course ABT-263 would not be expected to display selective toxicity against leukemia cells.

**BCL2/XL-inhibition, but not platelet activation, induces apoptotic signaling**

There has been considerable discussion on the involvement of apoptotic signaling events in platelet activation. Therefore, we investigated apoptosis pathways following exposure to platelet agonists or BCL2-inhibition. Exposure to ABT-737 induced loss of MMP (Figures 2A,C), release of cytochrome c (Figures 2B-C), and caspase cleavage (Figure 2C-D), in agreement with activation of the intrinsic apoptotic pathway. Loss of MMP was also seen following exposure to the calcium ionophore A23187, but not the platelet agonists CRP-XL or TRAP. At the time points investigated, caspases-3 and -9 were clearly not activated by platelet agonists or by A23187 (Figure 2D). Apoptosis is defined as a caspase-dependent process that is blocked or delayed by caspase inhibitors. Addition of the caspase inhibitor z-VAD.fmk completely blocked the ABT-737-induced cleavage of the caspase substrates, gelsolin and fodrin (Figure 2D), and PS externalization (Figure 2E). Interestingly, ABT-737-induced loss of MMP was also severely inhibited by z-VAD.fmk (Figure 2A), indicating that, in contrast to many other cell types, the loss of MMP in platelets occurred primarily through a caspase-driven amplification process. Taken together these data demonstrate that caspases are essential for platelet apoptosis but not activation. Our data do not exclude that caspase activation or release of cytochrome c may occur at later times following platelet activation, but these events are clearly not required for the initiation of platelet activation.

**Platelet apoptosis triggers thrombin generation**

The externalization of PS is a common feature of both platelet activation and apoptosis. Since PS exposure on the platelet surface facilitates the formation of the prothrombinase complex, we investigated whether platelets exposed to ABT-263 or ABT-737 promote the generation of thrombin at time points when the PS exposure was clearly evident (2 hours). Although the PS externalization occurred much more slowly (Figure 1), both ABT-737 and ABT-263 induced the generation of thrombin at a faster rate (as indicated by shorter lag times and time to peak) and more strongly (as indicated by a greater level of peak thrombin) than CRP-XL or TRAP and nearly as strongly as A23187 (Figure 3A), confirming that the PS exposure during apoptosis triggers the formation of the prothrombinase complex.
Inhibition of BCL2 proteins prevents platelet activation

Incubation of platelets for 2 hours with ABT-263 did not induce surface expression of P-selectin (Figure 3B), binding of fibrinogen to activated αIIbβ3 (Figure 3C) or aggregation (Figure 3D), demonstrating that BCL2-inhibition did not contribute to platelet degranulation or activation of integrins. This contrasts with platelets treated with CRP-XL, TRAP or A23187 which demonstrated exposure of P-selectin, fibrinogen binding and platelet aggregation (Figure 3B-D). However, if platelets were pre-exposed to ABT-263 for 2 hours prior to incubation with CRP-XL, TRAP or A23187, there was a marked, concentration-dependent inhibition of all three measures of platelet activation, indicating that platelets in which BCL2 proteins have been inhibited have a diminished capacity to be activated via agonist receptor mediated pathways (CRP-XL and TRAP) or by a sustained rise in intracellular calcium (A23187). Comparable results were obtained with ABT-737 (data not shown). Taken together, these data show that despite PS exposure and thrombin generation, BCL2-inhibition prevents, rather than facilitates, platelet activation.

Inhibition of BCL2 proteins results in a depletion of intracellular calcium

Next, we investigated whether the repression of platelet activation upon BCL2-inhibition was due to a caspase-mediated event. However, addition of the caspase inhibitors z-VAD.fmk or Q-VD-OPh to ABT-263 or ABT-737 did not restore the platelets ability to respond to agonist or calcium ionophore (Figure 4A-B), indicating that events upstream of caspase activation are responsible for the repression of platelet activation induced by BCL2-inhibition. BCL2 proteins have been implicated in the regulation of intracellular calcium homeostasis, and since the release of calcium from the ER is an important signaling event shared by platelet agonists and the calcium ionophore, we subsequently asked whether inhibition of BCL2 proteins by ABT-737 or ABT-263 has a direct effect on calcium homeostasis in platelets. Notably, exposure to ABT-263 induced an immediate increase in intracellular calcium (Figure 4C) in a concentration-dependent manner (data not shown). In addition, pretreatment with ABT-263 for 2 hours resulted in a depletion of intracellular calcium stores, as assessed using the response to A23187 (Figure 4D), suggesting an initial release of calcium from ER stores by ABT-263 and subsequent depletion of intracellular calcium in this virtually calcium-free environment. The depletion of intracellular calcium stores was not a caspase-mediated effect, since addition of z-VAD.fmk did not prevent the calcium depletion. Taken together, these data demonstrate that inhibition of BCL2 proteins in platelets has a direct effect on the intracellular calcium stores, thereby providing an explanation for the repression of agonist-induced platelet activation upon ABT-263 exposure.
Ultrastructural changes during platelet apoptosis

Since the ultrastructural changes during apoptosis of non-nucleated platelets have not been well characterized, we examined platelet morphology following exposure to ABT-737. Resting platelets displayed the typical discoid shape with a diffuse distribution of granules and organelles, whereas platelets exposed to ABT-737 were distended and displayed a peripheral distribution of most granules (Figure 5A), consistent with a disruption of the cytoskeleton. Following ABT-737 exposure, disorganized cytoskeletal structures also accumulated at the center of the cell. All ultrastructural changes induced by ABT-737 were mediated by caspases as they were inhibited by z-VAD.fmk (Figure 5A). Similar ultrastructural changes were observed upon exposure to ABT-263 (data not shown). By contrast, platelet activation by CRP-XL resulted in the characteristic opening of the surface connecting cannalicular system and a distinct morphology mainly characterized by filopodia and microparticle formation (Figure 5A). Further characterization of the ABT-737 induced cytoskeletal changes was performed on glass coverslips, which induced a spontaneous activation and spreading of the control platelets but not of ABT-737 treated platelets. Activated control platelets displayed a tubulin ring close to the cell center with actin fibres surrounding it (Figure 5B upper panels). Following exposure to ABT-737 a marked condensation of actin structures and inhibition of tubulin ring formation was observed in those cells that were apoptotic (Figure 5B lower panels). These changes, together with those observed by electron microscopy, indicate that caspases activated during apoptosis caused a dramatic change in cell morphology clearly distinct from the morphology observed during platelet activation.

BCL-XL sequesters BAK in resting platelets

To investigate which members of the BCL2 family were expressed in platelets, we analyzed platelet lysates from six different healthy donors (Figure 6A). Amongst the anti-apoptotic BCL2 proteins, only BCL-XL was expressed at detectable levels in platelets, suggesting that BCL-XL is the main target of ABT-737 and ABT-263 in platelets. BAX and BAK were both expressed, although to varying levels in the individual samples. Amongst the BCL2 homology domain 3 (BH3)-only proteins, BID was highly expressed, whereas NOXA, BAD and PUMA were not detectable. There was some reactivity with the BIM antibody, however, the resulting bands did not correspond to the BIM bands routinely detected in our studies with CLL cells. To elucidate the mechanism of ABT-263 induced apoptosis in platelets, we investigated the interaction of BCL-XL and pro-apoptotic BCL2 proteins by immunoprecipitation. To exclude any downstream effects mediated by caspases, this experiment was carried out in the presence of z-VAD.fmk. In resting platelets BAK was sequestered by BCL-XL and in some experiments (2/4) we detected minor displacement of BAK from BCL-XL upon treatment with ABT-263 (Figure 6B-C) under conditions that induced platelet apoptosis (2 hours...
of exposure to 100 nM ABT-263). Our data do not exclude that higher concentrations or longer incubation with ABT-263 might displace more BAK from BCL-X\textsubscript{L}. Notably, no interaction between BCL-X\textsubscript{L} and the BH3-only protein BID was detected (Figure 6B), despite high expression of BID (Figure 6A). Taken together, our data indicate that BCL-X\textsubscript{L} sequesters BAK in platelets, but whether displacement of BAK from BCL-X\textsubscript{L} may contribute to apoptosis induced by ABT-263 remains to be demonstrated.

**Reticulated platelets are resistant to ABT-263 induced apoptosis**

Treatment of mice with ABT-737 increased the number of reticulated “young” platelets, as assessed by staining with thiazole-orange,\textsuperscript{14} suggesting that ABT-737 selectively kills older platelets leaving younger platelets unaffected. Thus, prolonged exposure to ABT-263 might result in the generation of an overall younger platelet population. Co-staining with thiazole-orange and annexinV demonstrated that younger reticulated platelets were significantly more resistant to ABT-263 induced apoptosis (Figure 7), supporting the hypothesis that mature platelets are more sensitive to ABT-263 and providing an explanation for the generation of a less sensitive, and possibly younger, platelet population upon treatment with ABT-263.
**Discussion**

In this study we investigated the consequences of BCL2-inhibition in platelets. Owing to the shared characteristic of PS exposure, platelet apoptosis and activation are often considered related processes. In contrast to apoptosis induced by BCL2-inhibition, platelet activation induced by the thrombin receptor agonist TRAP, the collagen peptide-mimetic CRP-XL, or the calcium ionophore A23187 led to PS exposure and was accompanied by aggregation, increased fibrinogen binding and rapid exposure of P-selectin, but did not involve caspase activation or cytochrome c release (Figures 2 and 3). Our results indicate that mitochondrial changes and caspase activation are not required for platelet activation, although we cannot exclude that these may occur later during prolonged activation or as bystander effects. Apoptosis is defined as a strictly caspase-dependent process. Our data do not support an involvement of apoptosis in platelet activation but rather show that platelet apoptosis as induced by BCL2-inhibition is clearly distinct from platelet activation, in line with a previous report. Notably, PS externalization resulting from ABT-263- and ABT-737-induced apoptosis or from platelet activation both resulted in an increased thrombin generation (Figure 3A), confirming that PS exposure is directly linked to promotion of hemostasis irrespective of the pathways leading to PS externalization. However, the lack of P-selectin expression and the de-regulation of integrin-ligand interactions following BCL2-inhibition suggests that platelets would not become actively incorporated into a thrombus and therefore their thrombotic effect will be marginal in vivo.

To emphasize further the different pathways resulting in platelet activation or apoptosis our data demonstrate unequivocally that despite the increased thrombin generation (Figure 3A), apoptosis inhibits the subsequent response of platelets to their physiological agonists (Figure 3B-D). It is interesting to consider these findings in the context of observations made in stored platelets. In platelet concentrates stored for transfusion, apoptosis is increased over storage time. Simultaneously, platelets become less responsive to their physiological agonists, a phenomena referred to as the platelet storage lesion. Interestingly, the depletion of intracellular calcium observed upon BCL2-inhibition (Figure 4) provides a novel mechanistic explanation for the repressed platelet activation. Several BCL2 proteins like BCL-XL localize to the ER and have been implicated in calcium homeostasis in multiple ways. Notably, inhibition of BCL2 proteins has been previously shown to result in calcium depletion in pancreatic and leukemia cells. Since ABT-737-induced apoptosis in platelets was not affected by chelation of extracellular calcium, it is possible that the effect of BCL2/BCL-XL-inhibition on calcium is unrelated and independent of its apoptosis-inducing effects.

The ultrastructural characteristics of apoptotic platelets are not, as yet, well defined. Cytoplasmic condensation and budding, to yield microparticles, have been associated with apoptosis in aging platelets in stored concentrates, but these platelets have also undergone a degree of activation during their preparation, which increased during storage in parallel with their decline into an apoptotic-like
However, morphological changes similar to those observed in our study were also reported during apoptosis in platelets aged in vitro which was also accompanied by a reduced aggregatory response.39

Exposure of patients to ABT-263 results in thrombocytopenia during the first 4 weeks of treatment.12,13 In the current clinical studies, this problem is circumvented by giving the patients a low priming dose of ABT-263 (100 mg) for 7-14 days before starting with the full therapeutic dose (e.g. 250 mg). This priming dose is sufficient to induce a mild thrombocytopenia due to selective apoptosis of older platelets and supposedly results in the generation of an overall younger platelet population, more resistant to the drug thereby allowing dose escalation.12,14 In this study, we show for the first time that reticulated human platelets are significantly less susceptible to ABT-263 induced apoptosis (Figure 7), providing an explanation for the generation of an overall younger platelet population following treatment with ABT-263. Younger platelets are generally thought to be more active than older platelets,40 although direct evidence for this comes mainly from studies in animal models.41 It is possible that in patients treated with ABT-263 the lack of platelet numbers and the inhibition of platelet activation in apoptotic platelets might be compensated by a higher activation response in the remaining younger platelets.

The reduced susceptibility of young platelets may be due to an enhanced expression of the drug target itself, BCL-XL. However, younger platelets most likely show increased expression of multiple proteins that might contribute to the observed resistance. In nucleated cells, the most common resistance factor for ABT-737 or ABT-263 is the anti-apoptotic BCL2 protein MCL1, which can compensate for loss of BCL-XL function and is not targeted by either ABT-737 or ABT-263. Therefore, in nucleated cells overexpression of MCL1 or another related anti-apoptotic BCL2 protein, BCL2A1, protects cells from ABT-737- induced apoptosis.42-44 While neither MCL1 nor BCL2A1 were expressed at detectable levels in platelets from healthy donors (Figure 6A), these short-lived proteins might be expressed selectively in reticulated platelets and thus contribute to the resistance of young platelets.

To date little is known about the molecular mechanism of ABT-263- or ABT-737-induced apoptosis in platelets. We provide evidence for the interaction of BAK and BCL-XL in platelets, supporting the notion that BCL-XL ensures platelet survival by sequestering BAK. However, we detected only minor displacement of BAK following exposure to ABT-263 in 2/4 samples (Figure 6B-C), thus not convincingly demonstrating whether ABT-263-induced cytochrome c release is mediated by BAK displacement. Besides BAK, the pro-apoptotic protein BAX may mediate cytochrome c release.45 While BAX is clearly expressed, we found no evidence for a direct interaction between BAX and BCL-XL in platelets. Other likely candidates that could mediate ABT-263-induced apoptosis are the
BH3-only proteins, which have also been shown to be displaced from anti-apoptotic BCL2 proteins by ABT-263 or ABT-737. There is a great paucity of data on which, if any, BH3-only proteins are present in platelets. Our data suggests that BID (and possibly BIM) is the sole BH3-only protein expressed at detectable protein levels in platelets. This is reinforced by detection of transcripts for BID, but not BIM (BCL2L11) in both platelets and megakaryocytic cells, as well as transcripts for BCL-X\textsubscript{L} (BCL2L1), BCL-w (BCL2L2), BAK1 and BAX. Interestingly, recent experiments in mice suggest that neither BIM nor BID is important for the regulation of platelet life span or ABT-737-induced apoptosis.

A desirable property for any anticancer agent is selective toxicity exhibited against the tumor cells compared to the cells/tissue displaying dose-limiting toxicity. In this regard, no difference in sensitivity of CLL cells and platelets to ABT-737 was observed, whereas platelets were clearly more sensitive to ABT-263 than CLL cells (Figure 1E-F). However, this apparent lack of selectivity is in part overcome by the administration of a low priming dose of ABT-263 to patients as discussed above. In CLL cells, the main target of ABT-737 and ABT-263 is BCL2, since circulating CLL cells display very little BCL-X\textsubscript{L} but overexpress BCL2. In platelets however, the main target of these drugs is BCL-X\textsubscript{L}, since BCL2 and BCL-w are not expressed at detectable levels (Figure 6A). Hence a selective antagonist of BCL2 that does not inhibit BCL-X\textsubscript{L} could potentially circumvent thrombocytopenia and be used at higher doses, which might be more effective in leukemia therapy.
Acknowledgements

This work was supported by funding from the Medical Research Council (UK) and Leukaemia and Lymphoma Research (UK). HAH was supported by a studentship from the Saudi Cultural Bureau. We thank R. Walewska, A. Majid and M. Ahearne for helping to acquire blood samples from healthy volunteers and M. Mahaut-Smith for helpful discussions of the calcium experiments.

Authorship Contribution and Disclosure of Conflicts of Interest

MV has performed most experiments and written the manuscript. HAH has carried out the platelet activation assays and XMS and RTS have helped with the flow cytometric analysis. EDT and DD have performed the microscopic analysis. MJSD, AHG and GMC designed the study, analyzed data and have written the manuscript. The authors declare no conflict of interest.
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Figure Legends

Figure 1. ABT-263 and ABT-737 induce platelet apoptosis

(A,B) Washed human platelets were incubated in HEPES-buffered saline (HBS) with different concentrations of ABT-737 (open boxes) or ABT-263 (filled boxes) for 1 hour (A) or 2 hours (B). Apoptosis was assessed by PS externalization and staining with annexinV/FITC before analysis by flow cytometry (*= P <0.05; **= P <0.01). (C,D) Washed platelets were incubated in HBS with (♦,◊) or without (■,.box3) 3% human serum albumin (HSA) and different concentrations of ABT-737 (C, open symbols) or ABT-263 (D, filled symbols) for 4 hours. Alternatively, whole blood (▲,Δ) was incubated with different concentrations of ABT-737 (C) or ABT-263 (D) for 4 hours before analysis of platelet apoptosis by PS externalization and staining with annexinV/FITC. Data shown are mean + S.D. (n=6). (E,F) Citrated blood of patients with chronic lymphocytic leukemia (CLL) was incubated with different concentrations of ABT-737 (E) or ABT-263 (F) for 4 hours. Apoptosis of CLL cells (♦) was investigated in whole blood by staining with CD5/CD19 antibodies and annexinV/APC. Apoptosis of platelets (box3) was investigated in plasma by staining with CD41 antibodies and annexinV/FITC. Data shown are mean + S.D. (n=4) (*= P <0.05; **= P <0.01).

Figure 2. Platelet apoptosis but not platelet activation depends on caspase activation.

Washed human platelets were incubated in HEPES-buffered saline with ABT-737 (100 nM) for 2 hours or A23187 (10 μM), CRP-XL (2 μg/ml) or TRAP (100 μM) for 15 minutes with or without the caspase inhibitor z-VAD.fm (50 μM). (A) Loss of mitochondrial membrane potential was assessed by staining with TMRE (50 nM) before analysis by flow cytometry. Data shown are mean + S.D. (n=4). (B) Release of mitochondrial cytochrome c was assessed by lysis in 0.05% digitonin and differential centrifugation. The heavy membrane fraction (HM) containing mitochondria and the supernatant (SN) containing cytosol were analyzed for cytochrome c content by western blotting. (C) Release of mitochondrial cytochrome c and loss of mitochondrial membrane potential following exposure to ABT-737 (100 nM) for 2 hours was assessed by immunohistochemistry and staining with TMRE (red, upper panels) and anti-CD41/FITC (green, upper panels), or anti-cytochrome c (green, lower panels) and anti-active-caspase-3 (red, lower panels) (scale bar = 5 μm). Note that control cells display spreading on glass slides while ABT-737 treated cells do not. (D) Cleavage of caspases and caspase targets (fodrin, gelsolin) was analyzed by western blotting (cl=cleaved caspase fragments). (E) Apoptosis was assessed by PS externalization and staining with annexinV/FITC before analysis by flow cytometry. Data shown are mean + S.D. (n=4).

Figure 3. ABT-263 induces thrombin generation but inhibits platelet activation and aggregation.
(A) Washed human platelets were incubated for 2 hours at 37°C in HEPES-buffered saline (HBS) with ABT-263 (1 μM), ABT-737 (1 μM), or for 10 minutes with CRP-XL (2 μg/ml), TRAP (100 μM) or A23187 (10 μM). Thrombin generation over time was measured by calibrated automated thrombography. Data are shown from one representative donor typical of 3 examined. (B) Washed platelets were incubated in HBS with ABT-263 (0, 0.1, or 1 μM) for 2 hours before exposure to the platelet agonists CRP-XL, A23187 or TRAP for 10 minutes. Platelet activation was assessed by exposure of P-selectin and flow cytometry. (C,D) Whole blood was incubated with ABT-263 (0, 1, 3 or 10 μM) for 2 hours. Platelet rich plasma count was adjusted with autologous filtered platelet poor plasma to 1.5 x 10⁷/ml and either (C) fibrinogen binding was assessed by flow cytometry or (D) aggregation was measured using an aggregometer. Data shown are mean + S.D. (n=3 except 3 μM ABT-263 in (D) when n=2). Statistical significance was tested using ANOVA and comparison of “no ABT-263” with “ABT-263 (0.1)” and “ABT-263 (1)” in (B) and with “ABT-263 (1)” and “ABT-263 (10)” in (C) and (D) (* =P<0.05).

**Figure 4. ABT-263 depletes intracellular calcium stores.**

(A) Washed platelets were incubated in HEPES-buffered saline (HBS) with ABT-263 (1 μM) and caspase inhibitors (z-VAD.fm, 100 μM, Q-VD-POh, 50 μM) for 2 hours before exposure to the platelet agonists CRP-XL (2 μg/ml), A23187 (10 μM) or TRAP (100 μM) for 10 minutes. Platelet activation was assessed by exposure of P-selectin and flow cytometry. (B) Whole blood was incubated with ABT-737 (10 μM) and caspase inhibitors (z-VAD.fm, 100 μM, Q-VD-POh, 50 μM) for 2 hours. Platelet rich plasma count was adjusted with autologous filtered platelet poor plasma to 1.5 x 10⁷/ml and aggregation was measured using an aggregometer. Data shown are mean + S.D. (n=3). (C) Washed human platelets were stained with Fluo4/AM in HBS for 30 minutes. Intracellular calcium levels were continuously monitored by flow cytometry. After establishing a baseline fluorescence signal for 1 minute, ABT-263 (10 μM) was added and the calcium response was monitored for an additional 4 minutes. Left panel: representative graph is shown, right panel: mean + SD of the geometric mean fluorescence (MFI) (n=3). (D) Washed human platelets were incubated with or without ABT-263 (1 μM) and z-VAD.fm (100 μM) in HBS for 2 hours before staining with Fluo4/AM for an additional 30 minutes. After establishing a baseline fluorescence signal for 1 minute, A23187 (5 μM) was added and the calcium response was monitored for an additional 4 minutes. Data are mean + SD of the geometric mean fluorescence (n=3).

**Figure 5. Platelet apoptosis is characterized by ultrastructural changes distinct from platelet activation.**

(A) Washed human platelets incubated in HEPES-buffered saline (HBS) retained their normal discoid shape and ultrastructure whereas treatment with ABT-737 (100 nM) for 2 hours resulted in their
Figure 6. **BCL-X<sub>L</sub> directly interacts with BAK in resting platelets**

(A) Lysates from washed platelets (20 μg) from 6 healthy volunteers were analyzed by western blotting for expression of BCL2 proteins. A lysate from chronic lymphocytic leukemia (CLL) cells cultured for 24 hours on CD154-expressing fibroblasts, as well as lysates from Jurkat or HeLa cells, were used as positive controls. (B) Washed platelets from a healthy volunteer were exposed to ABT-263 (100 nM) in the presence of z-VAD.fmk (50 μM) for 2 hours before lysis and immunoprecipitation of BCL-X<sub>L</sub>. Interaction of BCL-X<sub>L</sub> with BAK or BID was assessed by staining with anti-BAK or anti-BID antibody, respectively. (C) Binding of BAK to BCL-X<sub>L</sub> was quantified using densitometry on lanes 5 and 6 of western blots as shown in (B) and expressed as a ration of BAK/BCL-X<sub>L</sub> in four individual samples together with the mean (square bar).

Figure 7. **Reticulated platelets are resistant to ABT-263 induced apoptosis**

Washed platelets of healthy volunteers or CLL patients were incubated in annexin-buffer plus 50 ng/ml thiazole-orange and different concentrations of ABT-263 for 2 hours. Apoptosis was assessed in the thiazole-orange positive (reticulated) or negative (non-reticulated) platelet population by staining with annexinV/APC and gating by flow cytometry. Data shown are mean ± S.D. (n=4) (*= P <0.05).
Figure 2

A

% TMRE low cells

ABT-737  A23187  CRP-XL  TRAP

w/o inhibitor  + zVAD.fmk

B

z-VAD.fmk  ABT-737  A23187  CRP-XL

Cyt c HM

Cyt c SN

control

ABT-737

C

proform Casp-3

proform Casp-9

Gelsolin

Fodrin

β-actin

D

z-VAD.fmk  ABT-737  A23187  CRP-XL

proform

Casp-3

Casp-9

Gelsolin

Fodrin

β-actin

E

% Annexin + cells

w/o inhibitor  + zVAD.fmk

ABT-737  A23187  CRP-XL  TRAP
Figure 5

A

- control
- ABT-737
- ABT-737 + z-VAD
- CRP-XL

B

- actin
- tubulin
- merge

control
Figure 6

A

BCL-X\(_L\)
BCL2
BCL2A1
MCL1
BAX
BAK
BID
BIM
NOXA
PUMA
BAD
β-actin

anti-apoptotic

multidomain pro-apoptotic

BH3-only pro-apoptotic

B

Lysate
IP
SN

Beads
BCL-X\(_L\)
Beads
BCL-X\(_L\)

ABT-263
BAK
BID
BCL-X\(_L\)

C

Ratio BAK/BCL-X\(_L\)

control
ABT-263
Figure 7

A

% Annexin+ platelets

ABT-263 (nM)

non-reticulated

reticulated

*
BCL2/BCL-XL-inhibition induces apoptosis, disrupts cellular calcium homeostasis and prevents platelet activation

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