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Granzyme B triggers a prolonged pressure to die in Bcl-2 overexpressing cells, defining a window of opportunity for effective treatment with ABT-737

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Overexpression of Bcl-2 contributes to resistance of cancer cells to human cytotoxic lymphocytes (CL) by blocking granzyme B (GraB)-induced mitochondrial outer membrane permeabilization (MOMP). Drugs that neutralise Bcl-2 (e.g., ABT-737) may therefore be effective adjuvants for immunotherapeutic strategies that use CL to kill cancer cells. Consistent with this we found that ABT-737 effectively restored MOMP in Bcl-2 overexpressing cells treated with GraB or natural killer cells. This effect was observed even if ABT-737 was added up to 16 h after GraB, after which the cells reset their resistant phenotype. Sensitivity to ABT-737 required initial cleavage of Bid by GraB (gctBid) but did not require ongoing GraB activity once Bid had been cleaved. This gctBid remained detectable in cells that were sensitive to ABT-737, but Bax and Bak were only activated if ABT-737 was added to the cells. These studies demonstrate that GraB generates a prolonged pro-apoptotic signal that must remain active for ABT-737 to be effective. The duration of this signal is determined by the longevity of gctBid but not activation of Bax or Bak. This defines a therapeutic window in which ABT-737 and CL synergise to cause maximum death of cancer cells that are resistant to either treatment alone, which will be essential in defining optimum treatment regimens.
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Cytotoxic lymphocytes (CL), which include natural killer cells (NK) and cytotoxic T lymphocytes (CTL), protect the body from cancer or infection and also contribute to the anti-cancer immune response that can accompany chemotherapy.[1–3] CL primarily kill their targets by granule-induced death, a process that involves perforin (Pfp)-mediated delivery of granzyme B (GraB) to the target cell where it induces apoptosis.[4,5] Understanding why some cancers are resistant to granule-induced death should lead to strategies that restore CL-induced death when this process has failed.

We and others have shown that human GraB preferentially activates the mitochondrial pathway to apoptosis by cleaving the Bcl-2 family member Bid.[6–10] Truncated Bid (tBid) promotes mitochondrial outer membrane permeabilization (MOMP), which liberates cytochrome *c* (cyt *c*) to promote caspase activation.[11,12] This pathway is blocked by pro-survival Bcl-2 family members that are commonly elevated in cancer.[12,13] GraB can also initiate apoptosis by directly activating caspases. This pathway can be negatively regulated by X-linked inhibitor of apoptosis protein (XIAP), which is also elevated in many cancers.[14,15] However, MOMP also releases second mitochondrial activator of caspases (Smac), which can neutralise XIAP.[15] GraB can therefore activate two independent pathways to caspase activation that bypass anti-apoptotic proteins commonly elevated in cancer. However, blocking both pathways by overexpression of Bcl-2 together with XIAP can prevent granule-induced cell death by human NK (14) and these target cells continue to proliferate. Overexpression of these proteins may therefore be one way that cancer cells avoid CL-induced death and why some patients fail to respond to anti-cancer immunotherapy. These studies also suggest that restoring the mitochondrial pathway should be sufficient to re-sensitise these cancer cells to CL-induced death.

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Abbreviations: Cc, Caspase-cleaved; C20, Compound 20; cyt *c*, cytochrome *c*; CL, cytotoxic lymphocytes; CTL, cytotoxic T lymphocytes; gctBid, GraB-cleaved Bid; GraB, human granzyme B; MOMP, mitochondrial outer membrane permeabilization; NK, natural killer cells; Pfp, perforin
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Consistent with this, the small molecule antagonist of Bcl-2, ABT-737, has been shown to re-sensitise some cancer cells to CTL-induced death,[16] suggesting that it may have adjuvant activity for cell-based cancer immunotherapy. It is not clear how ABT-737 re-sensitises cancer cells to CL or GraB or how to use ABT-737 most appropriately in future clinical trials that combine drug- and immune-based therapies. We have therefore investigated the synergy between ABT-737 and GraB, and show that (1) GraB generates a persistent pro-apoptotic signal to die in cancer cells that overexpress Bcl-2 that can be released by ABT-737 even if GraB activity is subsequently blocked; (2) this signal is transduced by tBid but is blocked by Bcl-2 upstream of Bax and Bak, and neutralising Bcl-2 with ABT-737 then results in rapid activation of Bax and Bak and MOMP; and (3) the pro-apoptotic signal generated by GraB and blocked by Bcl-2 is surprisingly long lived and MOMP can be triggered by adding ABT-737 as long as 20 h after Pfp/GraB. This defines a 'window of opportunity' when ABT-737 and GraB can jointly kill cancer cells that overexpress Bcl-2, which will be important for defining treatment regimens to use ABT-737 most effectively in combination with immunotherapy.

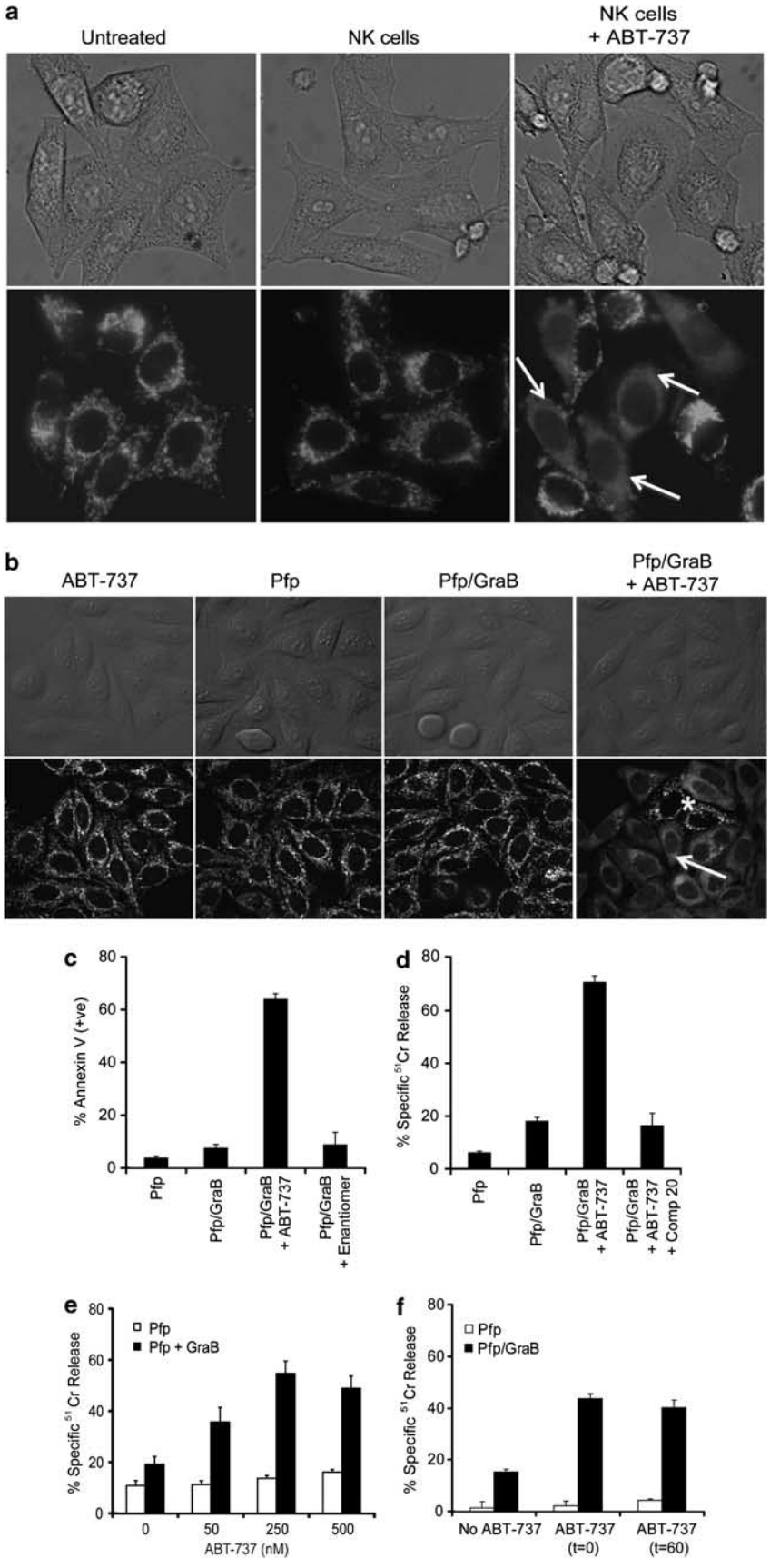
Results

GraB generates a persistent apoptotic signal that is rapidly released by ABT-737. As MOMP is the critical event in apoptosis that is regulated by Bcl-2 family members[12,15,17,18] we first used immunofluorescence staining for cyt *c* to investigate whether ABT-737 could restore MOMP in HeLa-Bcl-2 cells treated with Pfp/GraB or NK. HeLa-Bcl-2 cells treated with NK cells or Pfp/GraB alone showed punctate staining typical of intact mitochondria. However, staining became diffuse in HeLa-Bcl-2 cells treated with GraB- or NK in the presence of ABT-737, indicating that cyt *c* release had occurred (Figures 1a and b). Importantly, this effect was owing to restoration of the mitochondrial pathway, because the caspase-inhibitor zVAD-fmk was added in these assays to prevent any contribution of caspases directly activated by GraB. Consequently, ABT-737 did not restore apoptosis *per se* in these assays. To formally demonstrate that ABT-737 could restore GraB-induced apoptosis in the HeLa-Bcl-2 cells we used conditions that activate the mitochondrial pathway, but do not activate caspases directly.[7,14,18] As expected, Bcl-2 overexpressing cells were resistant to GraB-induced apoptosis under these conditions but apoptosis was restored by ABT-737 as determined by annexin V binding (Figure 1c) or release of ^{51}Cr from the targets cells (Figure 1d). Death was restored using low concentrations of ABT-737 (Figure 1e), but was not restored by an inactive enantiomer of ABT-737 that cannot neutralise Bcl-2 (Figure 1c) or if the cells were pre-treated with compound 20 (C20), which specifically blocks the activity of human GraB (14) (Figure 1d); thereby showing strong synergy between both GraB and ABT-737 to kill the Bcl-2 overexpressing cells.

Interestingly, ABT-737 was fully effective in restoring GraB-induced death of HeLa-Bcl-2 cells in these assays even if it was added 1 h after Pfp/GraB as shown by equivalent ^{51}Cr release (Figure 1f), or if GraB activity was blocked by adding C20 after 30 min ($t = 30$ min) followed by ABT-737 after 1 h (Supplementary Figure 1). This suggested that GraB rapidly generates a pro-apoptotic signal in Bcl-2 overexpressing cells (within 30 min) that is sustained for at least another 30 min even if GraB activity is blocked. This prompted us to investigate the nature of this pro-apoptotic signal. To investigate this we first used time-lapse microscopy to follow HeLa-Bcl-2 cells pre-treated with Pfp/GraB for 1 h followed by addition of ABT-737. Cyt *c* was located in the mitochondria of the Pfp/GraB-treated HeLa-Bcl-2 cells but rapidly translocated to the cytoplasm almost immediately after ABT-737 was added (within 15 min) and the cells subsequently showed classic signs of apoptosis including rounding and blebbing (Supplementary Movie 1). Similar experiments using flow cytometry to quantify cyt *c* release also revealed that ABT-737 triggered maximum cyt *c* release within 15 min in HeLa-Bcl-2 cells that had been pre-treated with Pfp/GraB for 1 h (Figure 2a). Immunoblot analysis also showed that although caspases were partially cleaved in HeLa-Bcl-2 cells treated with Pfp/GraB after 1 h, consistent with previous studies,[15] caspase-3, -7 and -9 were fully processed to their active forms within 20 min of adding ABT-737 (Supplementary Figure 2, long arrows). This confirmed MOMP was required for full caspase activation and that ABT-737 treatment replicated the death observed in cells expressing endogenous levels of Bcl-2. These experiments demonstrated that ABT-737 rapidly de-represses the anti-apoptotic effect of Bcl-2 to trigger cyt *c* release and caspase activation in a near-simultaneous manner in cells that had been pre-treated with Pfp/GraB.

The rapid release of cyt *c* by ABT-737 in these assays suggested that the mitochondria were 'primed' to initiate MOMP in these cells once Bcl-2 had been neutralised, and raised some important questions. The first was how long do the mitochondria of Bcl-2 overexpressing cells remain primed to release cyt *c*? This is important because NK disengage from their targets after they deliver GraB. Sensitivity of their targets to ABT-737 may therefore be limited. How long the GraB-treated cells remain sensitive to ABT-737 may therefore

Figure 1 ABT-737 restores cell death of HeLa-Bcl-2 cells treated with human NK cells or GraB. HeLa-Bcl-2 cells were treated with (**a**) human NK cells (stimulated with 25U IL-2 for 4 days), or (**b**) Pfp (1 nM) and GraB (25 nM) in the presence of zVAD-fmk (100 μM). ABT-737 (500 nM) was added at $t = 0$ and cells were fixed after 4 h. Images of morphology (DIC) and cyt *c* location (immunofluorescence) were taken using an Olympus CellR fluorescence microscope with a $\times$ 40 oil-immersion lens. Arrows indicate cells that have released cyt *c* and asterix are cells that have not. (**c**) HeLa-Bcl-2 cells were treated with Pfp (1 nM)/GraB (25 nM) in the presence or absence of ABT-737 (500 nM) or Enantiomer (500 nM). Cell death was determined by Annexin V binding. Data are the average $\pm$ S.E.M. for three independent experiments. (**d**) HeLa-Bcl-2 cells were treated with Pfp (1 nM)/GraB (25 nM) in the presence or absence of ABT-737 (500 nM) $\pm$ GraB inhibitor (C20; 10 μM) for 4 h. Cell death was determined by release of ^{51}Cr that had been pre-loaded into the target cells. Data are the average $\pm$ S.E.M. for three independent experiments. (**e**) HeLa-Bcl-2 cells were pre-labelled with ^{51}Cr and incubated with Pfp (1 nM)/GraB (12.5 nM) together with the concentration of ABT-737 indicated. Media containing 10% FCS was added at 1 h. Specific ^{51}Cr released from the target cells was measured at 4 h as an indication of cell death. Data are the average $\pm$ S.E.M. of five experiments. (**f**) ^{51}Cr release from HeLa-Bcl-2 cells treated with Pfp (1 nM)/GraB (12.5 nM) for 4 h. ABT-737 (500 nM) was either omitted (No. ABT-737) or added to the HeLa-Bcl-2 cells as indicated either at 0 min (ABT-737, $t = 0$) or after 60 min (ABT-737, $t = 60$)


a
Untreated
NK cells
NK cells
+ ABT-737
b
ABT-737
Pfp
Pfp/GraB
Pfp/GraB
+ ABT-737
c
% Annexin V (+ve)
Pfp
Pfp/GraB
Pfp/GraB
+ ABT-737
Pfp/GraB
+ Enantiomer
d
% Specific 51Cr Release
Pfp
Pfp/GraB
Pfp/GraB
+ ABT-737
Pfp/GraB
+ ABT-737
+ Comp 20
e
% Specific 51Cr Release
Pfp
Pfp + GraB
ABT-737 (nM)
f
% Specific 51Cr Release
Pfp
Pfp/GraB
No ABT-737
ABT-737
(t=0)
ABT-737
(t=60)

influence the design of treatment regimens using ABT-737 with immunotherapy. To investigate this, we delayed addition of ABT-737 to Pfp/GraB-treated HeLa-Bcl-2 cells for various times up to 24 h. At each time-point, cyt *c* release was assayed 15 min after adding ABT-737 (Figure 2b), sufficient time for ABT-737 to trigger maximal cyt *c* release in mitochondria primed to undergo MOMP (Figure 2a). The majority of cells released cyt *c* even when ABT-737 was added 16 h after Pfp/GraB. Approximately half were still responsive at 20 h but the death signal was finally lost by 24 h. The surviving cells were still sensitive to further treatment with Pfp/GraB and ABT-737 after 24 h (Figure 2c). Overall, these studies revealed that GraB generates a long but finite pro-apoptotic signal that defines a 'window of opportunity' during which the Bcl-2 overexpressing cells are sensitive to ABT-737 and that these cells are likely to respond to multiple rounds of combined therapy with NK and ABT-737.

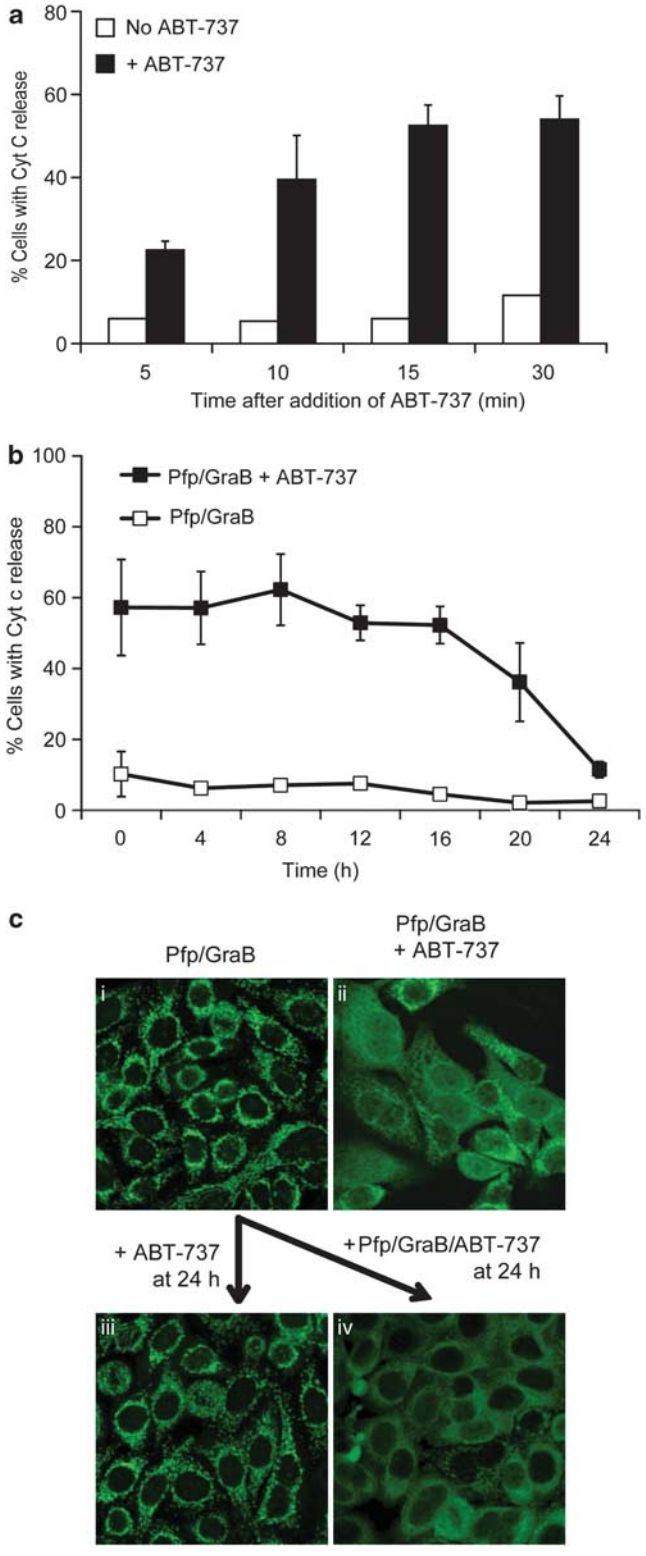


Figure 2 ABT-737 can trigger rapid and maximal cyt *c* release in HeLa-Bcl-2 cells up to 16 h after Pfp/GraB has delivered the apoptosis-inducing signal. (**a**) HeLa-Bcl-2 cells were pre-treated with Pfp (1 nM)/GraB (25 nM) for 1 h. ABT-737 (500 nM) was then added (designated as $t=0$) and cells were assayed for cyt *c* release by flow cytometry at 5, 10, 15 and 30 min after adding ABT-737. (**b**) HeLa-Bcl-2 cells were treated with Pfp (1 nM)/GraB (25 nM) and zVAD-fmk (100 μM). ABT-737 (500 nM) was added at the time points indicated. Cells were harvested after 15 min and analysed for cyt *c* release by flow cytometry. (**c**) HeLa-Bcl-2 cells were treated with Pfp (1 nM)/GraB (25 nM) and zVAD-fmk (100 μM) alone (Panel i) or together with ABT-737 (250 nM) and images of cyt *c*-GFP were obtained at 4 h. Similarly, cells were treated for 24 h then either ABT-737 alone (Panel iii) or ABT-737 together with Pfp (1 nM)/GraB (25 nM) and zVAD-fmk (100 μM) (Panel iv) was added. Images of cyt *c*-GFP were obtained at 28 h using a Leica-SP5 confocal microscope (North Ryde, NSW, Australia)

Bcl-2 blocks the pro-apoptotic signal initiated by GraB by binding to tBid upstream of Bax translocation and Bax/Bak activation. The second question raised by our observations was: what is the nature of priming that persists in GraB-treated HeLa-Bcl-2 cells to render them sensitive to ABT-737, and is persistent proteolytic activity by GraB required to sustain this pressure throughout the 'window of opportunity'? The molecular mechanism of GraB-induced MOMP involves direct cleavage of Bid at Asp75 and migration of tBid to the mitochondria where it activates the multimeric pro-apoptotic Bcl-2 family members (Bax or Bak) to cause MOMP.[15,19–22] Consistent with this we have previously demonstrated that Bid cleavage occurs upstream of Bcl-2 during GraB-induced death and that murine embryonic fibroblasts from both Bid$^{-/-}$ and Bax$^{-/-}$Bak$^{-/-}$ mice were resistant to GraB-induced death[18] (and data not shown). Bcl-2 has been shown to bind and block the active form of all three pro-apoptotic proteins via different mechanisms depending on the strength and nature of the stimulus applied.[22–27] We therefore investigated the role of Bid, Bax and Bak in maintaining the pressure to die in Bcl-2 overexpressing cells.

As Bcl-2 overexpressing cells were primed to undergo MOMP once ABT-737 was added at 1 h (Figure 1f, Figure 2), the pro-apoptotic signal must be active in these cells at this time. We therefore tested whether Bid, Bax or Bak were converted to their active conformation within 1 h of treatment with GraB. As expected, control HeLa cells treated with Pfp/GraB generated a large amount of tBid (GraB-cleaved (gc) tBid), which was largely localised in the mitochondrial pellet, and also produced smaller amounts of a slower-migrating form of tBid cleaved directly by caspases (caspase-cleaved

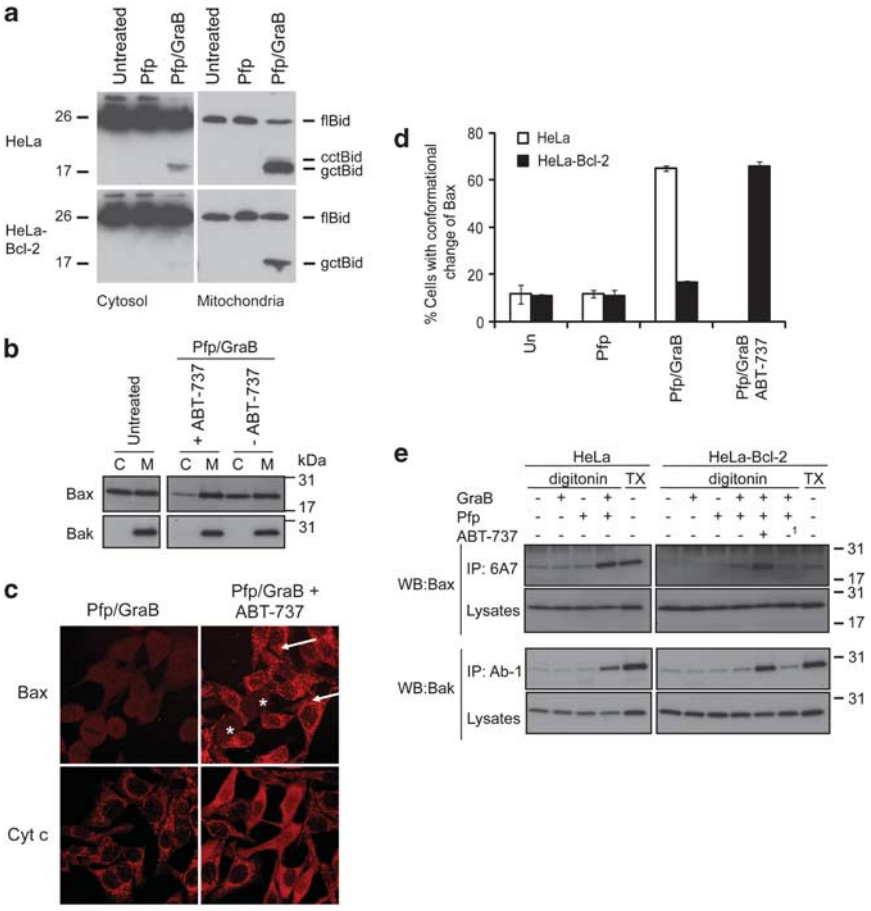


Figure 3 Bid cleavage by GraB and translocation of gctBid to the mitochondria is not blocked by Bcl-2 but Bax translocation and Bax/Bak activation are inhibited until after ABT-737 treatment. (**a**) Cells were treated with Pfp/GraB for 1 h. Cytosolic and membrane fractions were harvested and full length (fl) and tBid were detected by western blot analysis. Both GraB-cleaved tBid (gctBid) and caspase-cleaved tBid (cctBid) were detected in treated HeLa cells but only gctBid was detected in the HeLa-Bcl-2 cells. (**b**) HeLa-Bcl-2 cells were treated with Pfp/GraB for 1 h, ABT-737 was added for 30 min as indicated, before fractionation into cytosolic (C) and membrane (M) fractions and immunoblotting for Bax and Bak. Data are representative of three independent experiments. (**c**) HeLa-Bcl-2 cells were treated with Pfp (1 nM)/GraB (25 nM) and zVAD-fmk (100 μM). ABT-737 (500 nM) was added to the cells indicated after 1 h and incubated for a further 30 min before they were fixed and stained for Bax and cyt *c*. Images were obtained using an Olympus FV1000 confocal microscope. Cells marked with an arrow indicate cells with active Bax and those with an asterix indicate cells without active Bax. (**d**) HeLa and HeLa-Bcl-2 cells were treated with Pfp (1 nM)/GraB (25 nM) for 1 h. ABT-737 (500 nM) was added for a further 30 min. Flow cytometry was used to detect Bax activation using an antibody specific for the conformational-active form of Bax (clone 6A7). Data are average ± S.D. of four independent experiments. (**e**) Conformation change of Bax (upper panels) and Bak (lower panels) was determined by immunoprecipitation with conformation-specific antibodies against Bax (6A7) or Bak (Ab-1) (as described in the Materials and Methods). Lysis in Triton X-100 (TX) was used as a positive control for conformational change of Bax and Bak. [1]Cells were incubated for 30 min in the absence of ABT-737 after Pfp/GraB treatment (for 1 h). The experiment was performed twice

(cc) tBid) (Figure 3a). GctBid was also generated and was localised to the mitochondrial fraction of HeLa-Bcl-2 cells treated with Pfp/GraB, but cctBid was not observed as MOMP had not occurred. The presence of GraB-cleaved Bid (gctBid) at mitochondria in HeLa-Bcl-2 cells showed that the death signal had reached mitochondria in the absence of ABT-737 and gctBid accumulation at the mitochondria also provided a mechanism through which ABT-737 might potentially trigger rapid MOMP, even if GraB activity had ceased some time earlier.

We next examined whether Bcl-2 protects cells by sequestering gctBid or Bax/Bak that had been activated by gctBid, and whether ABT-737 liberated gctBid and/or activated Bax/Bak from Bcl-2 to induce MOMP. Bax is generally located in the cytoplasm and translocates to mitochondria to cause MOMP, whereas Bak is constitutively located at the mitochondria of healthy cells. Although the mechanism by which Bax and Bak promote MOMP remains to be fully elucidated, both molecules change conformation before oligomerising in the mitochondrial outer membrane. We therefore investigated the cellular location and conformational change of Bax and Bak in HeLa-Bcl-2 cells treated with Pfp/GraB in the presence and absence of ABT-737. As expected, Bak was located exclusively in the mitochondrial fractions of treated and untreated cells (Figure 3b). Bax was also predominantly in the mitochondrial fraction of cells after treatment with Pfp/GraB and ABT-737 (Figure 3b).

The conformational change in Bax exposes its N-terminus, which can then be detected with an antibody that binds specifically in this region (clone 6A7). Using flow cytometry, $>60\%$ of HeLa cells treated with Pfp/GraB alone were positive for the 6A7 epitope as were a similar proportion of HeLa-Bcl-2 cells treated with Pfp/GraB and ABT-737; background 6A7 binding occurred in the HeLa-Bcl-2 cells

treated with Pfp/GraB in the absence of ABT-737 (Figure 3c). This was confirmed by immunocytochemistry, which showed punctate Bax staining (cells with arrows) in HeLa-Bcl-2 cells treated with Pfp/GraB and ABT-737 indicating mitochondrial localisation, which contrasted with the faint diffuse staining when these cells were treated with Pfp/GraB alone (Figure 3d). The converse was observed for cyt *c*, located predominantly in mitochondria of HeLa-Bcl-2 cells treated with Pfp/GraB (punctate staining), and only released into the cytoplasm when ABT-737 was added (diffuse staining). Finally, immunoprecipitation with 6A7 showed activated Bax in HeLa-Bcl-2 cells treated with Pfp/GraB after ABT-737 addition but only background levels before (Figure 3e). Thus, Bax only became enriched at the mitochondria and acquired an active conformation if Bcl-2 was neutralised by ABT-737. With respect to Bak, immunoprecipitation with Ab-1 antibody showed Bak was activated in Pfp/GraB-treated HeLa cells or HeLa-Bcl-2 cells treated with Pfp/GraB and ABT-737, but not activated in HeLa-Bcl-2 cells treated with Pfp/GraB alone. Untreated cells lysed with Triton X-100 were used as a positive control to induce epitope exposure for both molecules.[28] We therefore concluded that Bak and Bax were maintained in their non-active conformations unless Bcl-2 was neutralised by ABT-737, and that the presence of gctBid must therefore be required to maintain the sensitivity of the GraB-treated cells to ABT-737.

To determine how Bcl-2 was acting, we used co-immunoprecipitation to determine whether gctBid, Bax and/or Bak was directly bound to Bcl-2. Bax did not co-precipitate with Bcl-2 following treatment with Pfp/GraB alone, but did following ABT-737 addition (Figure 4a). Bak did not associate with Bcl-2 either before or after ABT-737 (data not shown), although this may merely reflect the reduced ability of Bak to bind to the Bcl-2 overexpressed in these cells.[24,26] The observed Bax/Bcl-2 binding did not prevent translocation of Bax to the mitochondria or prevent MOMP as cyt *c* was still released in cells treated with Pfp/GraB and ABT-737. These data suggest that Bcl-2 does not block GraB by directly binding Bax (or Bak), and consequently that ABT-737 does not promote MOMP by displacing Bax or Bak from Bcl-2. In contrast, we found that gctBid did co-precipitate with Bcl-2 in cells treated with Pfp/GraB alone (Figure 4b), so this binding may prevent gctBid from activating Bax or Bak. Although addition of ABT-737 killed these cells, it did not displace large amounts of gctBid from Bcl-2. Rather ABT-737 generated cctBid, which could also associate with Bcl-2. In dying cells, the overexpressed Bcl-2 could therefore bind gctBid, cctBid, activated Bax and presumably ABT-737, but not sufficiently well to block apoptosis.

Together, the data clearly show that the GraB signal involves direct Bid cleavage. This signal is transduced as far as mitochondria, but is held in check by Bcl-2 upstream of Bax/Bak activation. We note importantly that gctBid (but not cctBid, Bak or Bax) was bound to Bcl-2 before ABT-737 addition. A small amount of displaced gctBid may then activate Bax/Bak, but this requires confirmation.

GctBid persists in cells during the 'window of opportunity' for sensitivity to ABT-737. For gctBid to maintain the pressure to die in GraB-treated cells, it must persist throughout the 'window of opportunity' during which GraB-treated cells were sensitive to ABT-737. We therefore determined using immunoblot analysis that gctBid is present after an extended time (17–18 h) and confirmed that cyt *c* was released once ABT-737 was added at this time, even if the GraB is no longer active (Figure 5). Pfp/GraB were added to HeLa and HeLa-Bcl-2 cells ($t=0$) and caspase activity was completely blocked with the inhibitor Q-VD-OPh. GraB inhibitor, C20, was added to HeLa cells at $t=0$ h to confirm that cleavage of Bid and loss of cyt *c* were completely blocked in HeLa cells under these conditions. To assess the need for on-going GraB activity in HeLa-Bcl-2 cells, the same concentration of C20 was added at $t=2$ h to block any further GraB-mediated Bid cleavage and ABT-737 addition was delayed for a further 15 h ($t=17$ h), as indicated. Finally, cells were fractionated 30 min later and assayed for the presence of gctBid and loss of cyt *c* from the mitochondrial fraction. Although the level of gctBid detected in the mitochondria was reduced in the Bcl-2 cells in which C20 was added (top panel,

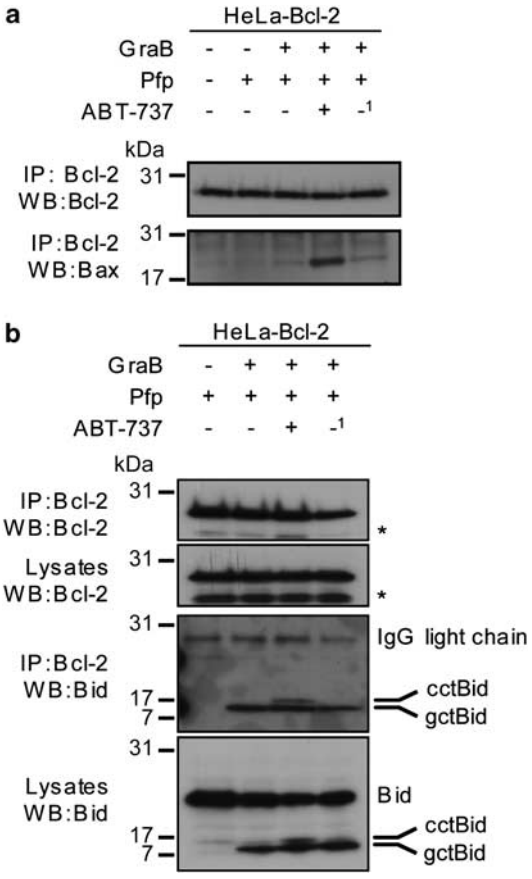


Figure 4 Bcl-2 sequesters gctBid in the absence of ABT-737 but only binds Bax after treatment with ABT-737. HeLa-Bcl-2 cells were treated with Pfp/GraB for 1 h and a further 30 min ± ABT-737 (500 nM) before lysis in 1% digitonin lysis buffer. (**a**) Bcl-2 was immunoprecipitated and immunoblotted for Bcl-2 (upper panel) or Bax (lower panel). (**b**) Similarly Bcl-2 was again immunoprecipitated and immunoblotted for Bcl-2 (top panel) or Bid (both the GraB (gc) and caspase (cc) cleaved forms; third panel). Whole-cell lysates were also immunoblotted for Bcl-2 (second panel) and Bid (bottom panel). [1]After Pfp/GraB treatment cells were incubated for an additional 30 min in the absence of ABT-737. *Is a non-specific band. The experiment was performed twice

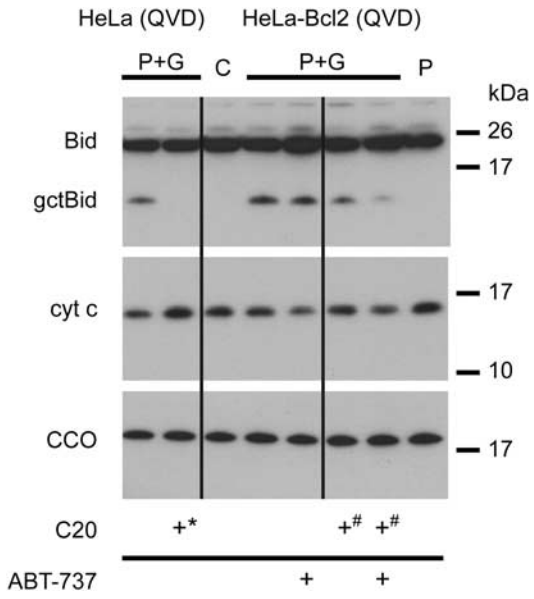


Figure 5 Prolonged maintenance of gctBid is associated with cyt *c* release after ABT-737 treatment up to 18 h after initiation of the GraB death signal. HeLa and HeLa-Bcl-2 cells were treated with Pfp/GraB in the presence of Q-VD-OPh (QVD; 20 μM). C20 (10 μM) was either added to HeLa cells (lane marked as C20*) together with the Pfp/GraB (at 0 h) or to the Bcl-2 cells after 2 h (lanes marked as C20$^{\#}$). ABT-737 (500 nM) was added to Bcl-2 cells as indicated after a further 15 h ($t = 17$ h) and the cells were fractionated at 17.5 h. Mitochondrial fractions were examined by Western blot analysis for the presence of gctBid (upper panel) and cyt *c* loss from the mitochondria (middle panel). cyt *c* oxidase (CCO) levels indicated equal protein loading (lower panel). Results are representative of three similar experiments

compare lanes 6 and 7 to lanes 4 and 5), the addition of ABT-737 still resulted in equivalent loss of cyt *c*, indicating that MOMP had taken place (compare lanes 7–5, middle panel). Reduced gctBid levels were detected in a similar experiment (Supplementary Figure 3) where the loss of mitochondrial cyt *c* was also associated with increased cyt *c* levels in the cytosol.

Overall, we showed that GraB induces a long-lived (up to 20 h, Figure 2b and Figure 5) death signal that was held in check by Bcl-2 but could be de-repressed by ABT-737. The presence of gctBid at mitochondria many hours after GraB proteolysis had stopped suggesting that gctBid bound to Bcl-2 is the long-lived signal generated by GraB, which renders Bcl-2 overexpressing cells sensitive to ABT-737. After 24 h, the cells resumed their resistance to either stimulus (Pfp/GraB or ABT-737) alone, but remained sensitive to both stimuli in combination.

Discussion

ABT-737 is a small molecule antagonist of Bcl-2 being trialled alone or in combination with cytotoxic drugs.[29] Our previous studies showed that co-overexpression of Bcl-2 and XIAP confer resistance of cancer cells to NK-induced death[14] and this may explain why some patients do not respond or relapse following therapy. Our current study demonstrates that ABT-737 can restore the mitochondrial pathway in the target cells and validates the potential of ABT-737 to be effective in combination with cell-based immunotherapy. Although

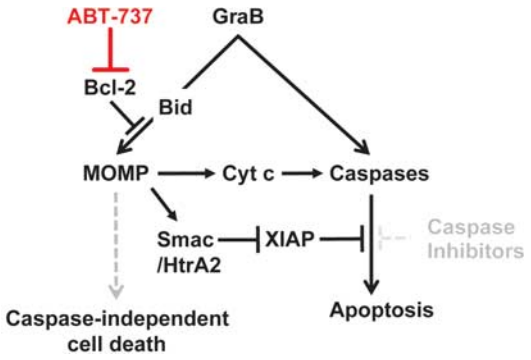


Figure 6 Restoration of cell death by ABT-737 in cancer cells that overexpress Bcl-2 and caspase activity is blocked. GraB can activate a mitochondria-dependent pathway to cell death by cleaving Bid (left), which triggers MOMP. This pathway can be blocked by overexpression of Bcl-2. GraB can also trigger apoptosis by cleaving caspases directly (right) and this pathway can be blocked by overexpression of caspase inhibitors, such as XIAP. Cell death can therefore be achieved by the complementary pathway if only one pathway is blocked. In cases where both pathways are blocked, neutralising Bcl-2 with ABT-737 is sufficient to restore the mitochondrial pathway. This results in apoptosis by activation of caspases, which can be achieved directly via cyt *c* or indirectly by Smac/HtrA2 that can neutralise some caspase inhibitors. MOMP can also cause caspase-independent cell death as a consequence of mitochondrial damage. Restoration of GraB-induced MOMP by neutralising Bcl-2 will therefore cause death of the cell even if caspase activity cannot be restored

ABT-737 can only restore the mitochondria-dependent pathway, MOMP also causes release of Smac/DIABLO to overcome XIAP. ABT-737 is therefore likely to restore NK-induced apoptosis in cancer cells that co-overexpress Bcl-2 and XIAP (Figure 6). Just as some viruses express caspase inhibitors that are not neutralised by Smac/DIABLO (e.g., CrmA), some cancer cells harbour defects that prevent them from executing mitochondria-independent pathways to apoptosis (e.g., MCF-7 breast cancer cells are deficient in caspase-3).[30] ABT-737 may not fully restore NK-induced apoptosis in these cells but Bax/Bak-mediated mitochondrial damage could still induce caspase-independent death. We therefore suggest that ABT-737 may be useful together with immunotherapy using CL to kill cancer cells, even those that harbour defects in caspase activation.

We showed that the pro-apoptotic signal generated by GraB is surprisingly long lived (up to 20 h) and that this signal depended on persistent gctBid even when GraB proteolytic activity had long been inhibited (Figure 5, Supplementary Figure 3). Importantly this suggests that timing of ABT-737 administration might be critical for the success of CTL/NK therapies. For example, delayed treatment with ABT-737, possibly 12–16 h after delivery of the NK cells, is likely to generate a large pool of cancer cells 'primed' for death and therefore sensitive to ABT-737. Further, NK can persist for many days in the tumour environment and a single killer cell can encounter multiple targets in succession. Therefore, sequential doses of ABT-737 12–16 h apart may be more effective than a single-dose delivered at the same time as immunotherapy. We also showed that target cells that survive ABT-737 following the initial treatment with Pfp/GraB are still sensitive to subsequent treatments with Pfp/GraB and ABT-737 (Figure 2c). Strategies that prolong the life of gctBid may also be worth exploring as degradation of

tBid is a control element in many apoptotic pathways and stabilisation of tBid, or proteosome inhibition can greatly amplify apoptosis.[31]

In addition to understanding the therapeutic potential of ABT-737 during cell-based immunotherapy our study elucidates how ABT-737 synergises with GraB to restore the mitochondrial death pathway. We found that gctBid translocated to mitochondria, but is bound to and held in check by Bcl-2 upstream of Bax/Bak activation. This block was rapidly neutralised by ABT-737 resulting in MOMP within 15 min. Interestingly, substantial amounts of gctBid remained bound to Bcl-2 after addition of ABT-737 even when cells were clearly destined to die. However, our data suggest that only partial release of gctBid (undetectable by immunoblot) from Bcl-2 may be required to activate Bax/Bak, a rapid and stochastic process that has been notoriously difficult to monitor, and the subject of intense study.[32] It is therefore likely that gctBid reduces the capacity of Bcl-2 to retain cell viability, as has been shown for cctBid[33,34] and that Bax and Bak only become involved downstream of the gctBid/Bcl-2 interaction.

Our findings are consistent with a previous study that used plasmid transfection to deliver different levels of tBid, which mimicked cctBid. Cells primed to die with lower levels of tBid underwent MOMP faster (0–20 min) than cells with higher levels of tBid (40–120 min) following ABT-737 addition.[27] This model predicted that Bcl-2 blocked lower levels of tBid by a mechanism designated mode 1, but higher levels of tBid activated Bax and Bak, blocked by a different mechanism designated mode 2. Bcl-2 appears to block GraB-induced death by mode 1 in our model (direct antagonism of gctBid) as the signal was rapidly released by ABT-737 (<20 min) and did not involve prior activation of Bax or Bak. This questions whether mode 2 inhibition can be achieved when Bid is activated under physiological conditions. Our model, which is more physiological and therapeutically relevant, should be useful for future studies aiming to unravel the complex sets of interactions between pro- and anti-apoptotic Bcl-2 family members ultimately leading to MOMP, and thereby influence drug design to restore the mitochondrial pathway in cancer cells where MOMP is blocked.

Materials and Methods

Cell culture and reagents. HeLa cells transfected with cyt *c*-GFP (clone 2H18) and also transfected with human Bcl-2[9] were maintained in DMEM (supplemented with 2 mM glutamine and 10% FBS). Human NK cells were generated as described previously and maintained in supplemented RPMI-1640 containing rhIL-2.[14] Caspases in target cells were blocked using either zVAD-fmk (100 μM, Enzyme Systems Products, Dublin, CA, USA) or Q-VD-OPh (20 μM, Calbiochem, La Jolla, CA USA). Human GraB was inhibited by either pre-incubation of target cells with C20 (10 μM; SYN/THESIS Med Chem, Shanghai, China)[14] for 30 min at 37 °C or the addition of the inhibitor after treatment of cells with Pfp/GraB. The Bcl-2 inhibitor ABT-737, or its enantiomer (Abbott Laboratories, Abbott Park, IL, USA) were added to cells at a final concentration of 250–500 nM. Recombinant GraB was produced in *Pichia pastoris* [35] and recombinant mouse Pfp was produced in a baculovirus expression system.[14]

Cell death assays. Cell death induced by GraB (12.5-25 nM) and Pfp (1 nM) was quantitated by chromium (^{51}Cr) release, annexin V binding and cyt *c* release as previously described.[13,14,18] Death of target cells induced by human NK was assessed by release of cyt *c*. Cells were fixed and stained using a specific antibody as previously described.[14]

Immunocytochemistry. The translocation of Bax from the cytosol to the mitochondria and the release of cyt *c* from the mitochondria to the cytosol was determined by immunocytochemistry, as described previously,[14] using antibodies specific for either cyt *c* (clone 6H2.B4) or Bax (6A7) followed by an anti-mouse Alexa 594 secondary antibody (Life Technologies, Mulgrave, VIC, Australia). Cells were plated overnight in 8-well chamber slides before treatment with GraB (12.5 nM), Pfp (18 nM), zVAD (100 μM) and ABT-737 (500 nM). Cells were visualised on an Olympus FV1000 confocal microscope (Mount Waverley, VIC, Australia).

FACS. Cells with active Bax were detected by flow cytometry (BD Biosciences, BD FACS Canto II, Franklin Lakes, NJ, USA), using an antibody, specific for the conformation change (6A7). The antibody was used on fixed and permeabilised cells, as described previously,[36] at a 1 : 100 dilution and detected with an anti-mouse Alexa 594 secondary antibody.

Western blot analysis. Typically $1–2 \times 10^6$ cells were treated with 7.5-15 μM GraB and 2.2 nM Pfp for 45–60 min. ABT-737 (500 nM) (20–30 min, 37 °C) was added as indicated. Whole-cell lysates (in 0.1% NP40) were separated on either 12 or 15% acrylamide gels and probed using specific monoclonal antibodies to caspase-3 (BD Transduction Laboratories, San Jose, CA, USA) caspase-7 and caspase-9 (BD Pharmingen, San Diego, CA, USA), and Bid (clone no. 2D1)[37] as previously described.[7]

Immunoprecipitation. Following treatment with Pfp/GraB and ABT-737 as described above, cells were lysed in lysis buffer (20 mM Tris-HCl pH 7.4, 135 mM NaCl, 1.5 mM MgCl2, 1 mM EGTA, 10% glycerol) containing 1% digitonin or 1% TX-100 supplemented with Complete protease inhibitors (Roche, Basel, Switzerland) for 30 min on ice. Insoluble debris was pelleted and lysates were pre-cleared with a 1 : 1 slurry of sepharose in lysis buffer for 1 h at 4 °C with constant agitation. Immunoprecipitation was performed for 2 h at 4 °C with anti-Bcl-2 (Bcl-2-100, BD Biosciences), anti-Bax (6A7, BD Biosciences) or anti-Bak (Ab-1, Calbiochem). Immune complexes were captured with Protein G/sepharose for 1 h at 4 °C, washed four times with lysis buffer and eluted by boiling in SDS-PAGE sample buffer. Immunoprecipitates and lysates were run on SDS-PAGE and immunoblotted for Bcl-2 (clone no. 7, BD Biosciences), Bax (N-20, Santa Cruz Biotechnologies, Santa Cruz, CA, USA), Bak (B5929, Sigma-Aldrich, Castle Hill, New South Wales, Australia), or Bid (clone no. 2D1).[37]

Subcellular fractionation. To investigate the cellular location of Bax and Bak, cells were harvested and resuspended in permeabilization buffer (20 mM HEPES/KOH pH 7.5, 100 mM sucrose, 2.5 mM $MgCl_2$, 50 mM KCl, 0.025% digitonin and complete protease inhibitors (Roche Applied Science, Castle Hill, New South Wales, Australia) for 10 min on ice. Permeabilized cells were centrifuged at 13 000 *g* for 5 min at 4 °C and the supernatant (cytosol, C) and pellet (membranes, M) were separated and boiled in reducing SDS-PAGE sample buffer. Fractions were run on SDS-PAGE and immunoblotted for Bax (N-20, Santa Cruz Biotechnologies) or Bak (B5929, Sigma, St. Louis, MO, USA). The cellular location of tBid and cyt *c* following Pfp/GraB, with and without ABT-737 was also determined by western blot analysis using antibodies to Bid (clone no. 2D1) and cyt *c* (clone 7H8.2C12). Cells were fractionated into membrane and cytosolic fractions, as previously described.[15] Equal loading was assessed using antibodies to either β-actin (cytosolic) (AC-74, Sigma-Aldrich) or bovine cyt *c* oxidase, subunit IV (mitochondrial) (Molecular Probes, Leiden, The Netherlands).

Conflict of Interest

The authors declare no conflict of interest.

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