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Different forms of cell death induced by putative BCL2 inhibitors

M Vogler[1], K Weber[1], D Dinsdale[1], I Schmitz[2], K Schulze-Osthoff[3], MJS Dyer[1] and GM Cohen*[,1]

Several inhibitors of BCL2 proteins have been identified that induce apoptosis in a variety of tumor cells, indicating their potential in cancer therapy. We investigated the specificity of six putative BCL2 inhibitors (obatoclax, gossypol, apogossypol, EM20-25, chelerythrine and ABT-737). Using cells deficient either for Bax/Bak or caspase-9, we found that only ABT-737 specifically targeted BCL2 proteins and induced apoptosis by activation of caspase-9, as only ABT-737 induced apoptosis was completely inhibited in cells deficient for Bax/Bak or caspase-9. Our data show that only ABT-737 is a specific BCL2 inhibitor and all other compounds investigated were not specific for BCL2 proteins. Furthermore, investigations of the effects of these compounds in primary chronic lymphocytic leukemic cells showed that all compounds induced certain biochemical hallmarks of apoptosis, such as release of cytochrome *c* and caspase cleavage. However, they all caused strikingly different ultrastructural changes. ABT-737 induced all the characteristic ultrastructural changes of apoptosis together with early rupture of the outer mitochondrial membrane, whereas obatoclax, chlelerythrine and gossypol induced pronounced mitochondrial swelling with formation of phospholipid inclusions. Therefore, we conclude that biochemical measurements used earlier to define apoptosis like mitochondrial release of cytochrome *c* and caspase cleavage, are insufficient to distinguish between classic apoptosis and other forms of cell death.
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The evasion of cell death is one of the hallmarks of cancer and contributes to tumor progression, resistance to chemotherapy and treatment failure.[1] Generally, cells can die primarily by apoptosis, a form of programmed cell death or after acute injury by cell and organelle swelling, disruption of cellular membranes and ultimately cell lysis/necrosis, which initiates an inflammatory response.[2] In addition to these two major forms of cell death, cells may also die by autophagic cell death, cathepsin- or calpain-mediated cell death, anoikis, or caspase-independent apoptosis.[3] Apoptosis can be triggered either at the cell surface (the extrinsic pathway) or at the mitochondria (the intrinsic pathway). In the intrinsic apoptotic pathway, cytochrome *c* is released from the mitochondrial intermembrane space into the cytosol and induces apoptosome formation, with activation of caspase-9 as the apical caspase.[4] Caspase-9 activates the effector caspases-3 and -7, which cleave several hundred cellular proteins, resulting in the characteristic biochemical and morphological features associated with apoptosis, including chromatin condensation, nuclear fragmentation and externalization of phosphatidylserine.[5] *In vivo*, apoptotic cells maintain their plasma membrane integrity and are rapidly phagocytosed in the absence of an inflammatory response.[2] However, *in vitro* at late times of incubation, apoptotic cells may undergo secondary necrosis, when the plasma membrane loses its integrity and increases its permeability. The release of cytochrome *c* from mitochondria is mediated by the multidomain B-cell lymphoma 2 (BCL2) proteins, BAX and BAK, which are regulated by antiapoptotic BCL2 proteins and BH3-only proteins. Antiapoptotic BCL2 proteins comprise BCL2 itself, BCL-X_L, MCL1, BCLw, BCL-B, and BCL2A1 and contain a hydrophobic groove, which is formed by their BCL2 homology 1, 2 and 3 domains. Both BAX/BAK and BH3-only proteins can bind into this hydrophobic groove and can thus be inhibited by antiapoptotic BCL2 proteins.[6,7]

Antiapoptotic BCL2 proteins are overexpressed in a variety of tumors and their expression often correlates with drug sensitivity.[7,8] Owing to their important function in regulating cell death, pharmacological inhibition of BCL2 proteins is a promising strategy for apoptosis induction or sensitization to chemotherapy. The first approach targeting BCL2 used antisense nucleotides, and subsequently, several small molecule inhibitors have been developed.[9,10] Most of these compounds have been identified by screening for binding to BCL-X_L, and the resulting compounds are pan-BCL2 inhibitors that bind antiapoptotic BCL2 proteins with affinities ranging from subnanomolar to micromolar concentrations. A noteworthy exception is the development of ABT-737 and its orally active analog ABT-263, which was the result of NMR-based structural design. As a result, ABT-737 and

[1]MRC Toxicology Unit, Hodgkin Building, University of Leicester, Leicestershire, UK; [2]Institute of Medical Microbiology and Hospital Hygiene, University of Duesseldorf, Duesseldorf, Germany and [3]Interfaculty Institute of Biochemistry, University of Tuebingen, Tuebingen, Germany
*Corresponding author: GM Cohen, MRC Toxicology Unit, University of Leicester, Hodgkin Building, PO Box 138, Lancaster Road, Leicester, Leicestershire LE1 9HN, UK. Tel: +44 116 2525601; Fax: +44 116 2525616; E-mail: gmc2@le.ac.uk
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Abbreviations: CLL, chronic lymphocytic leukaemia; DKO, double knock-out; FCS, fetal calf serum; MEFs, murine embryonic fibroblasts; MOMP, mitochondrial outer membrane permeabilization; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PARP, poly-(ADP-ribose) polymerase; PS, phosphatidylserine
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ABT-263 bind selectively to BCL2, BCL-X_L and BCLw but have low affinities for MCL1, BCL-B and BCL2A1.[11,12] ABT-737 induces apoptosis by binding to BCL2 and BCL-X_L and releasing proapoptotic binding partners.[13] In contrast, resistance to ABT-737 has been linked to high expression of MCL1.[14,15] Other BCL2 antagonists also bind MCL1, and obatoclax (GX15-070) disrupts BAK binding to MCL1.[16]

In this study, we investigated the mechanism of cell death induction by gossypol,[17] apogossypol,[18] chelerythrine,[19] obatoclax,[16] EM20-25,[20] and ABT-737.[11] Gossypol, isolated from cotton seeds and used as a male contraceptive, was recently recognized as binding and interacting with antiapoptotic BCL2 family members and inducing apoptosis.[17] Removal of the two reactive aldehyde groups in gossypol resulted in the synthesis of apogossypol, with similar binding kinetics to BCL2 and BCL-X_L but with reduced toxicity *in vivo*.[17,21] Initially described as a selective protein kinase C inhibitor,[22] chelerythrine, a naturally occurring benzophenanthridine alkaloid, was subsequently identified by high throughput screening as an inhibitor of BCL-X_L.[19] EM20-25, which binds to the BH3 domain of BCL2, is a derivative of HA14-1, but lacks its effects on mitochondrial respiration.[20] ABT-263, obatoclax and AT-101, the (−) enantiomer of gossypol, are in early clinical trials for lymphoid malignancies or solid tumors.[23–25]

However, despite the use of these inhibitors in preclinical mechanistic studies, proof for their specificity for BCL2 proteins is limited. Specific inhibitors of BCL2 proteins should induce apoptosis in a BAX/BAK-dependent manner with subsequent release of cytochrome *c* and activation of caspase-9. Although it has been shown that several BCL2 inhibitors might activate the intrinsic apoptotic pathway, there is little evidence that activation of this pathway is required for cell death induction. Our studies now show that only ABT-737, of the six inhibitors studied, induces cell death by inhibition of BCL2 and activation of the intrinsic pathway with caspase-9 as the apical caspase. All the other inhibitors seem to induce cell death primarily by damaging mitochondria and have additional cellular targets that may result in significant non-mechanism based toxicities.

Results

Cell death induction in MEFs deficient in Bax and Bak. To test the specificity of BCL2 inhibitors, we initially used murine embryonic fibroblasts (MEFs), either wild type (wt) or deficient in both Bax and Bak (double knock out - DKO). If cell death induction is specifically mediated by BCL2 proteins, Bax or Bak are required for release of cytochrome *c* and cell death execution. Therefore in cells deficient in Bax and Bak, cell death arising from BCL2 inhibition should be completely inhibited.[6,15] Amongst the BCL2 inhibitors tested, the most effective compound in MEFs was obatoclax, which was the only compound to induce cell death at submicromolar concentrations (Figure 1a). Using PS externalization as a measure of cell death, obatoclax was almost equally effective in killing both wt and DKO MEFs, showing that obatoclax is very effective in killing MEFs independently of Bax or Bak. To exclude any possible interference with this cell death assay owing to the strong inherent fluorescence of obatoclax, we also assessed the toxicity of obatoclax by two other methods, in which the assessment of toxicity was completely independent of any fluorescent signals. Firstly, viability was assessed using an MTT assay, which predominantly measures the ability of mitochondrial enzymes to reduce 3–4,5–dimethylthiazol-2–yl)-2,5–diphenyltetrazolium bromide (MTT) to formazan. Obatoclax induced a similar concentration-dependent inhibition of viability or metabolic activity in both wt and DKO MEFs, with the wt MEFs being slightly more susceptible (Supplementary Figure 1a). Furthermore, obatoclax (0.1 μM) inhibited colony formation of both wt and DKO MEFs to approximately similar extents (Supplementary Figure 1b). Taken together, these data show that obatoclax readily induces cell death in a Bax/Bak independent manner in MEFs.

Chelerythrine induced an almost identical concentration-dependent cell death in both wt and DKO MEFs (Figure 1b). Gossypol and apogossypol induced a concentration-dependent cell death in both wt and DKO MEFs but were clearly more potent in wt MEFs, indicating that Bax and/or Bak are involved in cell death induction (Figure 1c and d). However, at higher concentrations of apogossypol (30 μM), cell death was also induced in DKO MEFs, suggesting that at high concentrations, these compounds have other targets in addition to BCL2 family proteins. EM20-25 was a weak inducer of cell death in wt MEFs (Figure 1e). It is to be noted that, ABT-737 induced cell death in wt but not in DKO MEFs, even at much higher concentrations of ABT-737 (30 μM) (Figure 1f). These data show that only ABT-737, amongst the BCL2 inhibitors tested, is a specific inhibitor of BCL2. Cell death induced by obatoclax and chelerythrine seemed to be completely independent of BCL2 proteins, whereas cell death induction by gossypol and apogossypol seemed to be only partially due to their BCL2 inhibitory function.

Cell death induction in cells deficient in caspase-9. Next, we investigated whether cell death induced by BCL2 inhibitors occurs through activation of the intrinsic apoptotic pathway. To extend our studies from a nonmalignant murine cell line to human leukemic cells, we used Jurkat T-cells deficient in caspase-9 and the same cells reconstituted with caspase-9, as a control.[26,27] Caspase-9 is the initiator caspase in the intrinsic pathway and if apoptosis occurs through this pathway, it cannot proceed in the absence of caspase-9. Cell death was assessed by PS externalization for all the compounds except obatoclax. To exclude possible interference of the fluorescence of obatoclax, apoptosis was assessed by changes in the scatter properties of the cells (FSC/SSC). Both obatoclax (Figure 2a) and apogossypol (Figure 2d) induced cell death irrespective of caspase-9 expression, indicating that cell death did not occur as a consequence of activating the intrinsic apoptotic pathway. Upon exposure to chelerythrine (Figure 2b) or gossypol (Figure 2c), cell death was only slightly less in caspase-9 deficient cells as compared with caspase-9 expressing cells, indicating that both chelerythrine and gossypol also induced cell death mainly independently of caspase-9 and not primarily through the intrinsic apoptotic pathway. EM20-25 (1–30 μM) did not induce cell death in Jurkat cells

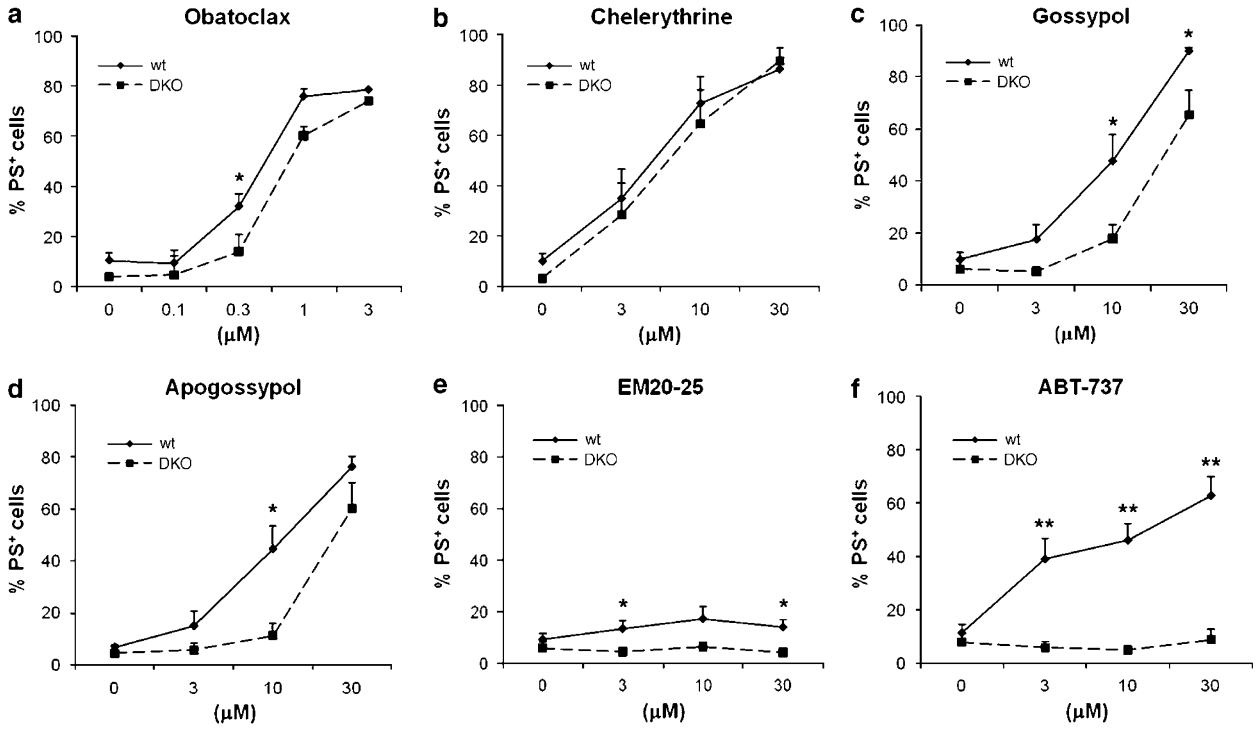

Figure 1 Cell death induced by putative BCL2 inhibitors is mainly independent of Bax and Bak. Wild type (wt) MEFs (solid lines) or Bax/Bak double knockout (DKO) MEFs (dotted lines) were exposed to the indicated concentrations of obatoclax (**a**), chelerythrine (**b**), gossypol (**c**), apogossypol (**d**), EM20-25 (**e**), or ABT-737 (**f**) for 48 h. In (**a** and **b**) cell death was assessed by phosphatidylserine (PS) externalization and binding of Annexin-APC. In (**c**–**f**) cell death was assessed by PS externalization, binding of Annexin-FITC and PI staining. Data are mean + S.E.M. of 5–8 experiments (*$P<0.05$, **$P<0.01$)

(Figure 2e). ABT-737 induced a concentration-dependent cell death in cells expressing caspase-9 (Figure 2f). In cells deficient for caspase-9, ABT-737 induced cell death was completely inhibited, showing that ABT-737 induces cell death selectively through the intrinsic apoptotic pathway, which is absolutely dependent on caspase-9. Taken together, the experiments in Figure 1 and 2 show that all of the putative BCL2 inhibitors tested, with the exception of ABT-737, induce cell death mainly in a non-specific way that requires neither Bax/Bak nor caspase-9.

ABT-737 is >100-fold more potent at inducing death of CLL cells than other BCL2 inhibitors. Irrespective of their additional cellular targets and their mode of killing, the BCL2 inhibitors investigated in this study, several of which are already in clinical trials, might be valuable for cancer therapy. To investigate which compound was most efficient in killing primary leukemia cells, we exposed freshly isolated chronic lymphocytic leukemia (CLL) cells from patients to the different BCL2 inhibitors. We have shown earlier that freshly isolated CLL cells express both BAX and BAK and BCL2 and MCL1 but do not express detectable levels of BCL-X_L.[28] Only ABT-737 and chelerythrine induced PS externalization of CLL cells after 4 h exposure (Figure 3a), whereas all the compounds induced PS externalization after 24 h (Figure 3b). ABT-737 induced cell death at low nanomolar concentrations after only 4 h of exposure (Figure 3a), whereas micromolar concentrations of chelerythrine were required to induce such rapid cell death. Obatoclax, gossypol, apogossypol and EM20-25 all required micromolar concentrations and longer incubation times for cell death induction (Figure 3b). For comparison, at 24 h the EC_{50} of ABT-737 was 9.4 nM, whereas the EC_{50} of obatoclax was $\sim 2\,\mu$M and of chelerythrine $\sim 1.1\,\mu$M. Owing to the concentrations used, it was not possible to calculate an EC_{50} for gossypol, apogossypol and EM20-25. In conclusion, we found that ABT-737 was >100-fold more potent at inducing apoptosis in CLL cells than any of the other putative BCL2 inhibitors (Figure 3).

Next, we investigated which form of cell death was induced by the BCL2 inhibitors in CLL cells. Owing to the higher potency of ABT-737 compared with the other BCL2 inhibitors, these experiments were carried out with 10 nM ABT-737 and 10 μM of the other BCL2 inhibitors. The release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol after mitochondrial outer membrane permeabilization (MOMP) is considered to be an essential component of the intrinsic apoptotic pathway[4] Release of cytochrome *c* from mitochondria to cytosol was induced by all BCL2 inhibitors tested (Figure 4a). It is to be noted that, chelerythrine induced a very rapid and extensive cytochrome *c* release within 30 min of exposure, whereas ABT-737 induced cytochrome *c* release within 2 h of exposure. In line with their kinetics of apoptosis induction, obatoclax, gossypol, apogossypol and EM20-25 required longer incubation times but release of cytochrome *c* was evident after 8 h of exposure.

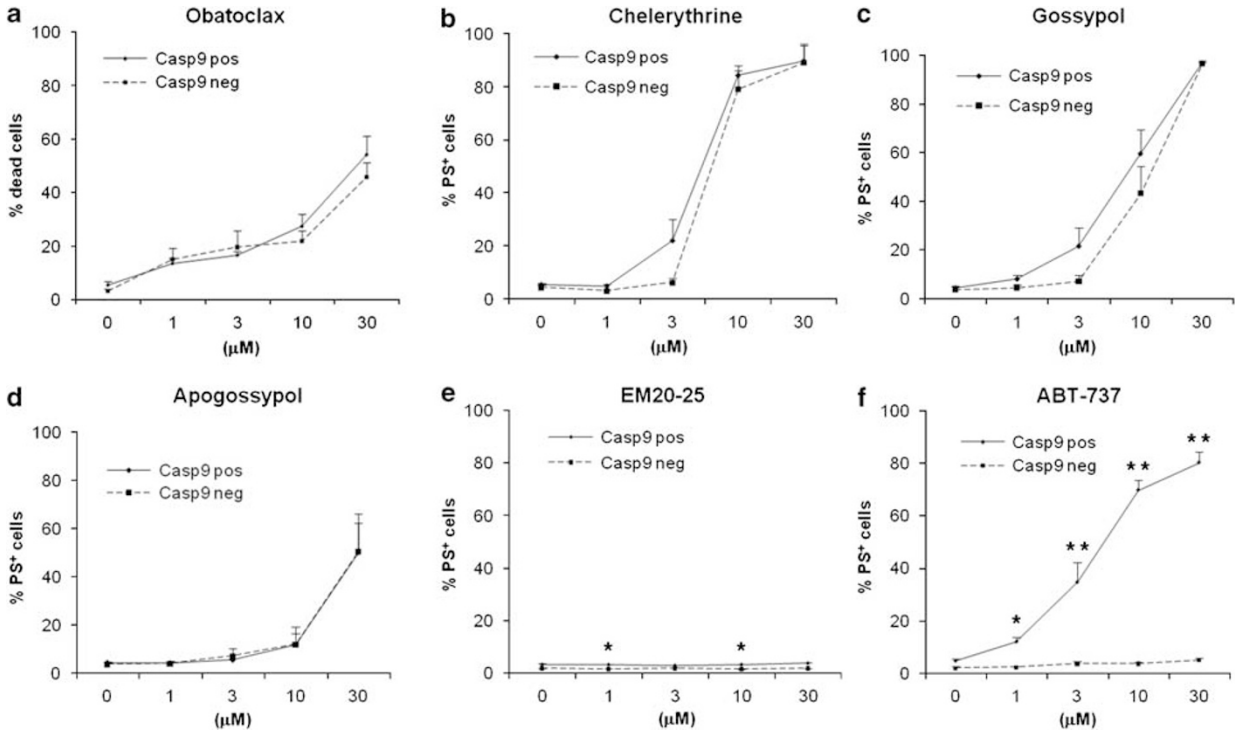

Figure 2 Cell death induced by putative BCL2 inhibitors is independent of caspase-9. Jurkat T-cells deficient in caspase-9 (dotted lines) or with reconstituted caspase-9 (solid lines) were exposed to the indicated concentrations of obatoclax (**a**), chelerythrine (**b**), gossypol (**c**), apogossypol (**d**), EM20-25 (**e**), or ABT-737 (**f**) for 24 h. In (**a**) cell death was assessed by FSC/SSC analysis, in (**b**) cell death was assessed by phosphatidylserine (PS)-exposure and binding of Annexin-APC, and in (**c**–**f**) cell death was assessed by PS-exposure, binding of Annexin-FITC and PI staining. Data are mean + S.E.M. of 4 experiments (*$P<0.05$, **$P<0.01$)

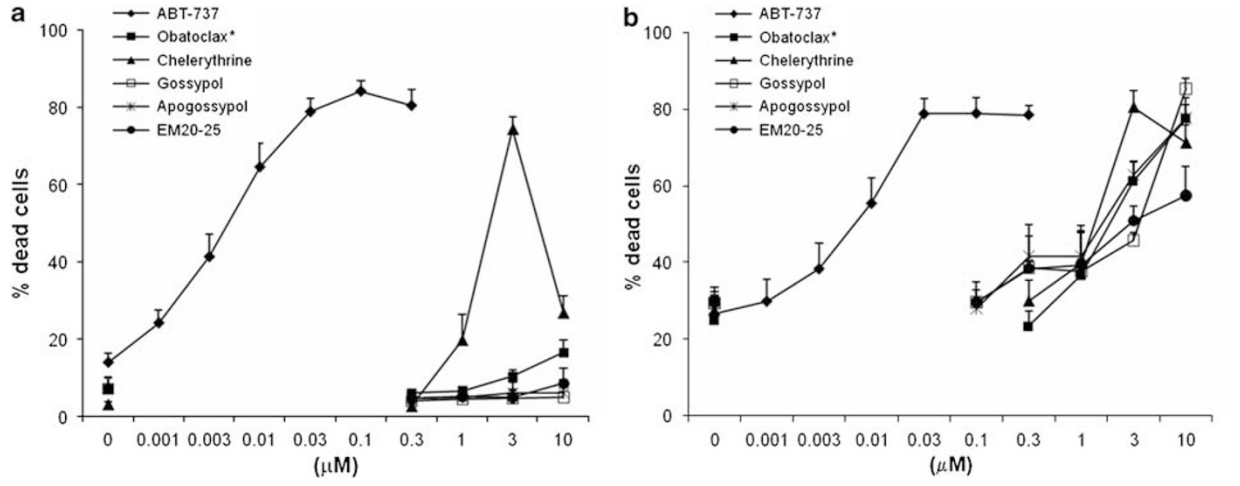

Figure 3 ABT-737 is most effective at killing primary CLL cells. CLL cells freshly isolated from the peripheral blood were exposed to the indicated concentrations of BCL2 inhibitors for 4 h (**a**) or 24 h (**b**). For ABT-737, gossypol, apogossypol and EM20-25, cell death was assessed by phosphatidylserine (PS)-exposure, binding of Annexin-FITC and PI staining. For chelerythrine, cell death was assessed by PS externalization and binding of Annexin-APC, and for obatoclax cell death was assessed by FSC/SSC analysis. Data are mean + S.E.M. of cells from 15 individual patients

Besides release of cytochrome *c*, the cleavage and activation of caspases is a hallmark of apoptosis. To this end, we analyzed cleavage of caspases after exposure of CLL cells to BCL2 inhibitors. All BCL2 inhibitors were able to induce a certain extent of caspase cleavage. Chelerythrine induced a rapid cleavage of caspase-3 and its canonical substrate poly-(ADP-ribose) polymerase (PARP), whereas caspase-9 cleavage was minor. ABT-737 induced cleavage of caspase-3, caspase-9 and PARP within 2 h of exposure, whereas caspases and PARP were cleaved by obatoclax, gossypol, apogossypol and EM20-25 only at later time points. In conclusion, these data show that in CLL cells, all BCL2

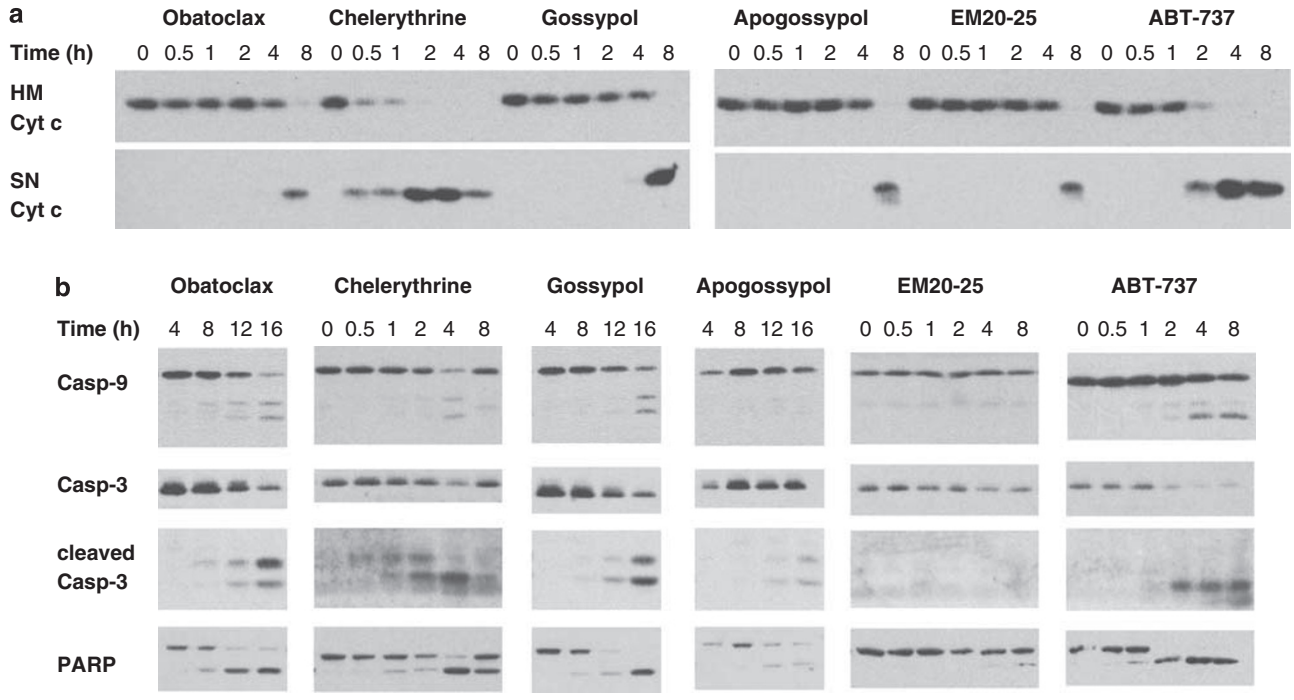

Figure 4 BCL2 inhibitors induce cytochrome *c* release and caspase cleavage. CLL cells freshly isolated from peripheral blood were exposed to 10 μM obatoclax, 10 μM chelerythrine, 10 μM gossypol, 10 μM apogossypol, 10 μM EM20-25 or 10 nM ABT-737 for the indicated time. (**a**) Release of cytochrome *c* from mitochondria into cytosol was assessed by fractional lysis with 0.05% digitonin and isolation of heavy membranes (HM) containing mitochondria and supernatant (SN)-containing cytosol. (**b**) Cleavage of caspases and the caspase substrate, PARP, was assessed by western blotting. Results shown are from one experiment representative of three

inhibitors tested induce classic biochemical hallmarks of apoptosis, such as mitochondrial cytochrome *c* release and caspase cleavage, albeit with very different kinetics and concentrations.

BCL2 inhibitors induce mitochondrial damage. In addition to biochemical analyses, we investigated the ultrastructure of CLL cells both untreated (Figure 5a) and upon exposure to BCL2 antagonists. Strikingly diverse ultrastructures were observed in CLL cells exposed to the putative BCL2 inhibitors with mitochondrial alterations being most prevalent (Figure 5). Obatoclax (10 μM) induced massive mitochondrial swelling and loss of cristae within 1 h of exposure but both inner and outer mitochondrial membranes remained intact and no detectable alterations in nuclear structure were observed (Figure 5b). Many mitochondria contained multilaminated phospholipid whorls, which probably originated from cristae membranes. At later times (8 h), some cells showed classical apoptotic nuclei with condensed chromatin (Figure 5c). Similarly, exposure of CLL cells to chelerythrine for only 1 h resulted in massively swollen mitochondria and inclusions showing concentric layers of phospholipid (Figure 5d). The mitochondrial changes were even more marked at 4 h (Figure 5e), when they were accompanied by outer mitochondrial membrane discontinuities and striking nuclear changes. The nuclear changes, which were particularly evident after exposure to lower concentrations (3 μM) of chelerythrine (Figure 5f), were characterized by the partial condensation of chromatin, similar to those we have described earlier involving the cleavage of chromatin into large kilobase pair fragments of 50 and 250 kbp in the absence of internucleosomal cleavage.[29] Signs of secondary necrosis, rare at 4 h, were common after exposure to chelerythrine for 8 h (data not shown). Gossypol required longer incubation times before ultrastructural changes were detected. After exposure to gossypol for 12 h, although most nuclei were unaffected, some apoptotic nuclei were clearly observed (Figure 6a). Gossypol also induced some mitochondrial swelling and loss of cristae accompanied by phospholipid inclusions, although this was much less common than with obatoclax and chelerythrine (Figure 6b). At this and later times, there was an increase in cells undergoing apoptosis and secondary necrosis. Taken together, these putative BCL2 antagonists all induced severe mitochondrial damage before cell death induction. Exposure to apogossypol for 12 h induced no significant mitochondrial swelling but interestingly caused a proliferation of the endoplasmic reticulum in localized clusters (Figure 6c and d). However some cells with pyknotic nuclei, which were often undergoing secondary necrosis, were also observed (Figure 6e). In line with our earlier data,[30] ABT-737 induced mitochondrial swelling and discontinuities in the outer mitochondrial membrane in addition to the characteristic ultrastructural changes of lymphocyte apoptosis (Figure 6f). No mitochondrial phospholipid inclusions were observed after exposure to ABT-737. Interestingly, although four compounds in this study induced swelling of mitochondria, only ABT-737 induced significant rupture of the outer mitochondrial membrane, as one of the earliest detectable lesions.

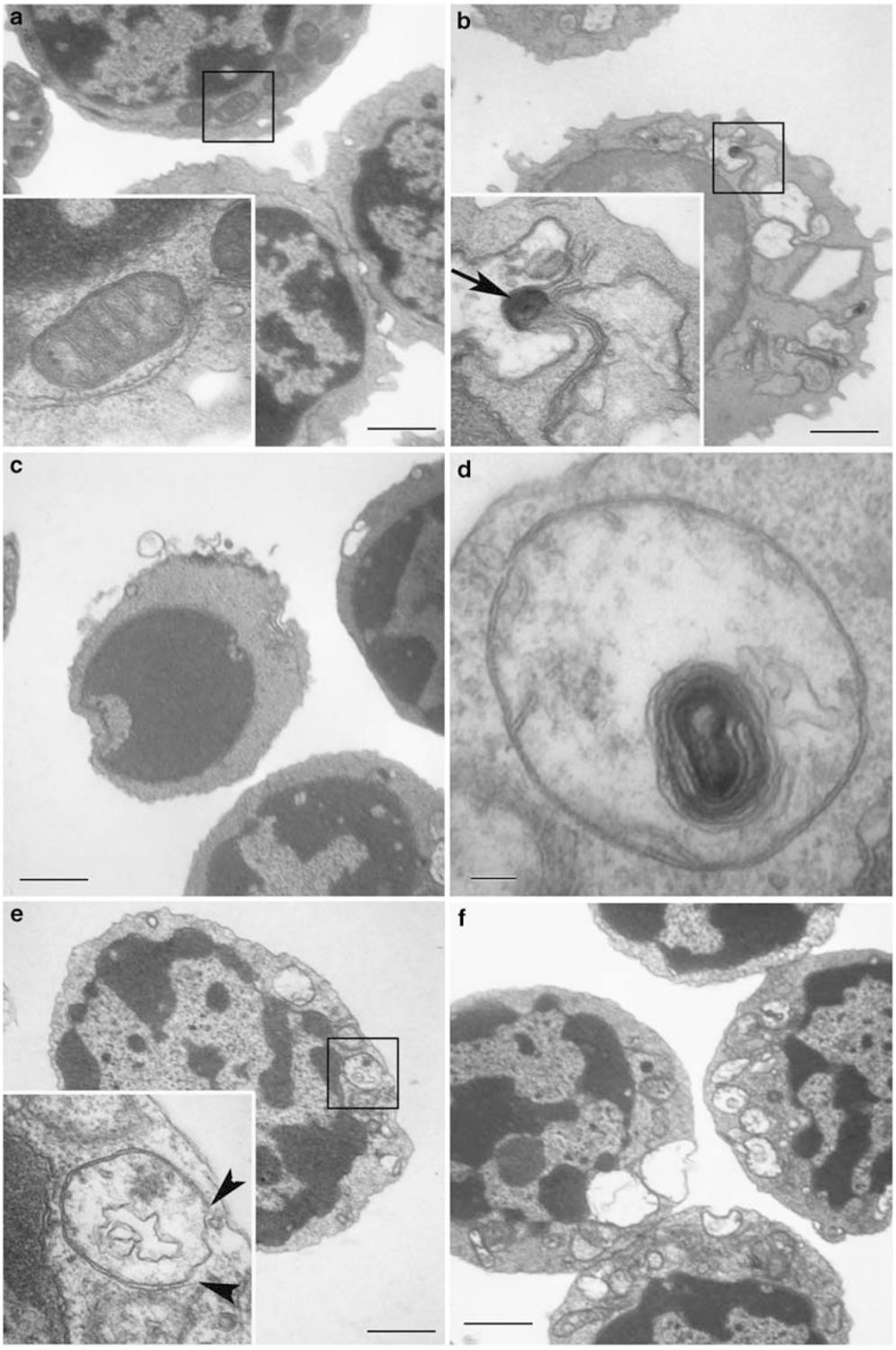

Figure 5 Obatoclax and chelerythrine induce mitochondrial damage. (**a**) Untreated CLL cells have mitochondria with well-defined transverse cristae. (**b**) Exposure to obatoclax (10 μM) induced massive mitochondrial swelling and loss of cristae after 1 h of exposure. The inner and outer mitochondrial membranes remained intact and no detectable alterations in nuclear structure were observed. A multilaminated phospholipid whorl (black arrow) is evident in one of the swollen mitochondria. (**c**) Exposure to obatoclax (10 μM) for longer times (8 h) also resulted in several cells exhibiting chromatin condensation characteristic of apoptosis. (**d**) Mitochondrial swelling, with loss of cristae but maintenance of mitochondrial membranes, occurred within 1 h of exposure of CLL cells to chelerythrine (10 μM). These changes were sometimes associated with the development of whorls of phospholipid within the mitochondrial matrix. (Bar = 100 nm). (**e**) After 4 h of exposure to chelerythrine (10 μM), mitochondrial changes were accompanied by outer mitochondrial membrane discontinuities (black arrowheads) together with partial chromatin condensation in the nucleus. (**f**) Exposure to lower concentrations of chelerythrine (3 μM) also induced mitochondrial and nuclear changes. In this Figure the scale bar = 1 μm, unless stated otherwise

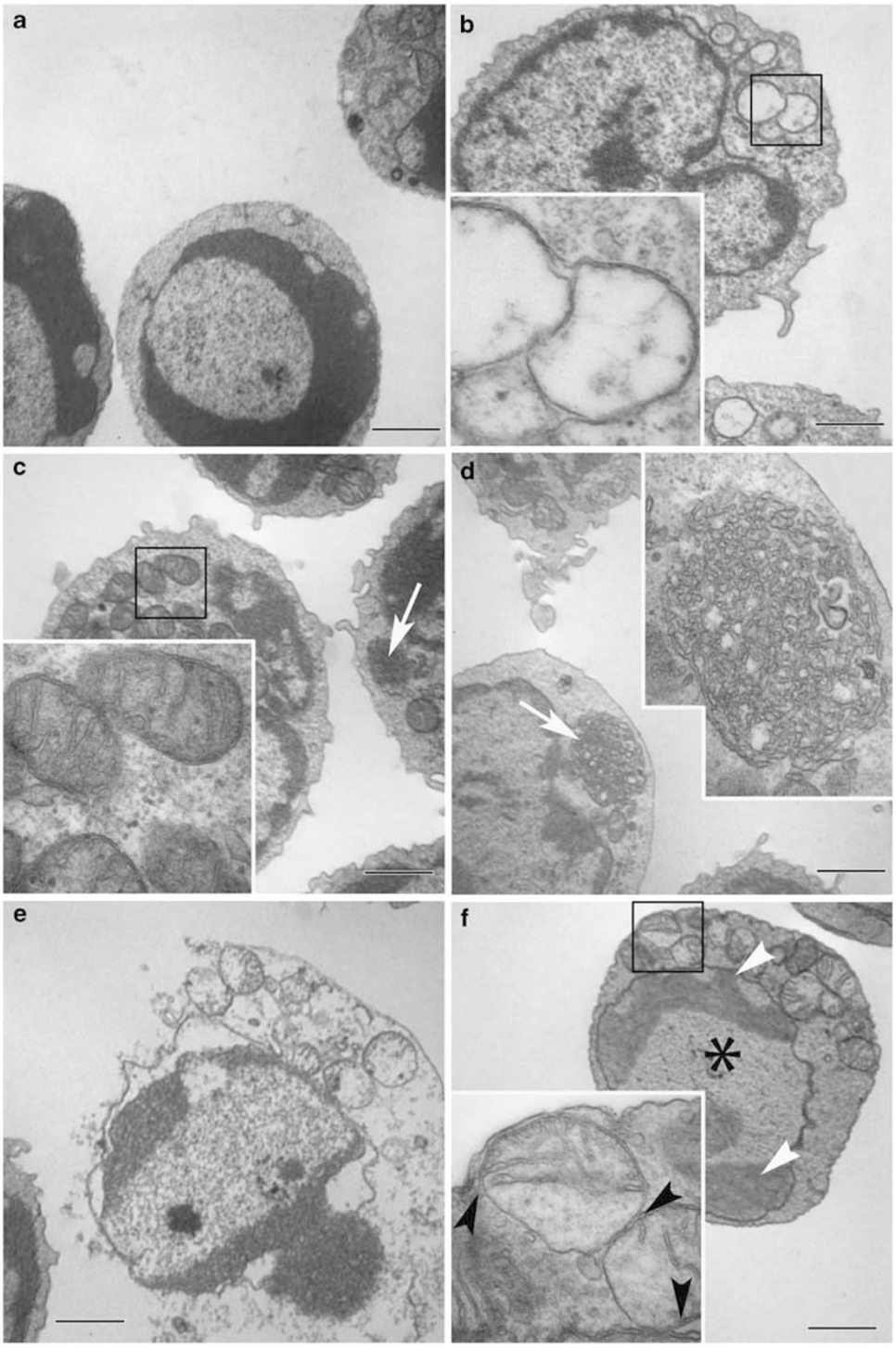

Figure 6 Different ultrastructural changes induced by gossypol, apogossypol and ABT-737. (**a** and **b**) Exposure to gossypol (10 μM), for 12 h, resulted in both the induction of characteristic apoptotic nuclei (**a**) and mitochondrial swelling and loss of cristae (**b**) in a small proportion of cells. (**c**) Mitochondria in cells exposed to apogossypol (10 μM), for 12 h, were indistinguishable from controls but localized clusters of endoplasmic reticulum (white arrow) were observed in many of these cells. (**d**) A higher magnification shows the proliferation of the endoplasmic reticulum in the apogossypol (10 μM) exposed cells. (**e**) In addition apogossypol (10 μM) resulted in some cells with pyknotic nuclei, which were often undergoing secondary necrosis. (**f**) Exposure to ABT-737 (10 nM) induced mitochondrial swelling and discontinuities in the outer mitochondrial membrane (black arrowheads) within 2 h. Apoptotic nuclear changes including chromatin condensation (white arrowheads) and nucleolar disintegration (asterisk) were also common but no phospholipid inclusions were found in any mitochondria. In this Figure, the scale bar = 1 μm

Breakdown of this membrane after chelerythrine treatment at 4 h and much later after the other agents probably results from the rapid onset of secondary necrosis. In conclusion, all the putative BCL2 antagonists induce strikingly different morphological changes, implying significant differences in the biochemical pathways by which they induce cell death.

Discussion

In this study, we investigated the specificity of BCL2 antagonists and their mechanisms of cell death induction. Although ABT-737 selectively binds BCL2, BCL-X_L and BCLw but does not bind MCL1, BCL2A1 or BCL-B,[11] all other inhibitors investigated here bind to all antiapoptotic BCL2 proteins with comparable affinities.[9,10] It is to be noted that, ABT-737 binds BCL2 and BCL-X_L with higher affinity than any of the other BCL2 inhibitors. The specificity of BCL2 inhibitors for BCL2 proteins can be investigated using cells that are deficient in Bax and Bak. Our data (Figure 1) strongly suggest that, with the exception of ABT-737, Bax or Bak are not necessary for cell death induction and most putative BCL2 inhibitors do not serve as BH3-mimetics or selective BCL2 inhibitors, which require Bak or Bax for cell death induction.[31] Similar experiments with Bax/Bak DKO MEFs had been carried out earlier for chelerythrine, gossypol and several other putative BCL2 antagonists with comparable results.[15,32,33] In one study obatoclax induced cell death was reported to be partially dependent on Bak expression.[32] Therefore it is possible that in some cell types there may be a small contribution of Bax or Bak to the toxicity of these compounds, as we observed a slightly lower sensitivity particularly to gossypol, apogossypol and obatoclax of Bax/Bak DKO compared with wt MEFs (Figure 1). Considering the higher potency of ABT-737 in CLL cells compared with the other inhibitors (Figure 3), it is possible that the development of analogs of gossypol, apogossypol and obatoclax with higher affinities for antiapoptotic BCL2 members will yield more potent and selective compounds, whose activity will also be more dependent on BAX/BAK. Furthermore, if BCL2 or BCL-X_L exerts functions independently of BAX/BAK, such as binding of the voltage-dependent anion channel (VDAC) or inositol-3 phosphate receptor, then it is possible that these various inhibitors might still target antiapoptotic BCL2 proteins and inhibit some of these other functions.[34,35] Therefore, we conclude that only ABT-737 is a true BH3-mimetic exerting its proapoptotic effect through BAX/BAK and most other BCL2 inhibitors must also target additional proteins outside the BCL2 family.

Furthermore, if these putative BCL2 antagonists bind specifically to antiapoptotic BCL2 proteins, they would be expected to induce cell death by releasing mitochondrial cytochrome *c*, formation of the Apaf-1 apoptosome and activation of caspases. Using cells deficient in caspase-9, the initiator caspase in the intrinsic apoptotic pathway, only ABT-737 but none of the other BCL2 inhibitors induced caspase-9-dependent cell death (Figure 2). These results imply that cell death induced by some of the other putative BCL2 antagonists may occur in a caspase-independent manner, in agreement with recent findings showing that cell death induced by gossypol and HA14-1 was not inhibited by the caspase inhibitor z-VAD.fmk.[36–39] In contrast, ABT-737-induced cell death was found earlier to require caspase activation.[30] Taken together, our data (Figures 1 and 2) indicate that of the inhibitors tested only ABT-737 is a true inducer of apoptosis by activation of the intrinsic pathway, requiring both Bax/Bak and caspase-9. We suggest that despite the known ability of many of these putative BCL2 antagonists to bind to BCL2 proteins, most of these inhibitors are non-specific compounds and should not be used for mechanistic studies related to the function of BCL2 proteins. Dependent on their potency and toxicity, the agents investigated in this study might be valuable for cancer therapy irrespective of their mechanism of cell death induction. Although their potency was comparable in Jurkat T-cells, in CLL cells ABT-737 was 2–3 orders of magnitude more potent than any of the other compounds tested, indicating that CLL cells are exquisitely sensitive to the mechanism-based cell death induced by ABT-737 (Figures 2 and 3). In line with our results, sensitivity of CLL cells to obatoclax or gossypol was reported earlier at 1.7 μM at 48 h[40] and $\sim$30 μM at 24 h of exposure,[36] respectively, whereas sensitivity to ABT-737 was observed at nanomolar concentrations.[11,13] Among the putative BCL2 inhibitors tested in this study, MEFs were most sensitive to obatoclax, which induced cell death at 100 nM (Figure 1a and Supplementary Figure 1). Thus it is evident that the efficacy of BCL2 inhibitors, such as obatoclax, is markedly dependent on cell type, possibly reflecting the presence of additional targets outside the BCL2 family.

We have shown that the putative BCL2 inhibitors investigated in this study, with the exception of ABT-737, induce cell death independent of BCL2 proteins and activation of caspase-9. However, somewhat surprisingly, all these BCL2 inhibitors induced release of cytochrome *c* and caspase cleavage (Figure 4), two commonly used biochemical indicators of apoptosis. Therefore, although most of these BCL2 inhibitors do not directly activate the intrinsic pathway of apoptosis, they nevertheless induce certain features of apoptosis, which could easily be mistaken as death by apoptosis. In this regard it was interesting to also consider the ultrastructural changes induced by these compounds (Figures 5 and 6). Obatoclax and chelerythrine induced rapid swelling of mitochondria with loss of cristae structure, which in the case of obatoclax occurred before induction of cell death. In line with these data, it has recently been shown that chelerythrine induces rapid release of cytochrome *c* and mitochondrial swelling.[33] With chelerythrine, obatoclax and gossypol, the mitochondrial damage was accompanied by phospholipid inclusions, which seemed to originate from the cristae membranes, raising the possibility that instead of specifically targeting BCL2 proteins, these compounds induce cell death by causing severe mitochondrial damage. In the present study, the appearance of these phospholipid inclusions was most evident in CLL cells exposed to chelerythrine for only 1 h (Figure 5d), when the concentric layers of phospholipid somewhat resembled the alterations in cristae observed in HeLa cells after loss of mitofilin.[41] Mitofilin is an inner mitochondrial membrane protein, which has been proposed to control cristae morphology. Thus it is possible that some of the putative BCL2 antagonists, including chelerythrine, obatoclax and gossypol, perturb mitochondria by affecting proteins that control cristae morphology.

It is interesting that, all the putative BCL2 antagonists induce strikingly different ultrastructural changes in CLL cells (Figures 5 and 6), implying significant differences in the biochemical pathways by which they induce cell death. Agents like ABT-737 may cause relatively specific MOMP that results in the release of cytochrome *c*, activation of caspases and

induction of biochemical and morphological changes characteristically associated with apoptosis. Other BCL2 antagonists, including obatoclax, chelerythrine and gossypol, may have additional targets and cause mitochondrial damage, which can be accompanied by release of cytochrome *c* and ultrastructural changes associated with either apoptosis or necrosis. It is to be noted that, the extensive mitochondrial damage and loss of cristae membranes induced by some of these agents will also severely compromise the ability of the cells to generate ATP by oxidative phosphorylation so also impairing the ability of the cells to undergo apoptosis, which is an ATP-requiring process.[42] Thus these different ultrastructures may be because of differing kinetics and extents of mitochondrial perturbation together with impaired ability to generate ATP so modifying the biochemical subroutines used by the dying cells. It is interesting that, our results highlight important limitations of solely using biochemical assays, including cytochrome *c* release, caspase activation and PARP cleavage, to define apoptosis or the mechanism of cell death. As all the compounds induced both cytochrome *c* release and caspase activation, in the absence of the ultrastructural studies, we would have been blissfully ignorant of the major cellular changes occurring including the phospholipid inclusions and the proliferation of the endoplasmic reticulum (Figures 5 and 6).

Besides their ability to induce cell death, it is also important to consider the potential *in vivo* toxicities of the BCL2 antagonists. The major toxicity due to ABT-263 is mechanism based and involves a dose-dependent induction of thrombocytopenia because of inhibition of BCL-X_L, which is critical for determining platelet survival.[43] For all other putative BCL2 inhibitors, non-mechanism based toxicity might occur *in vivo.* Gossypol induces the generation of reactive oxygen species, which may be responsible for the induction of cell death.[44] *In vivo* gossypol is known to cause male infertility, whereas obatoclax had been reported to induce both neurological symptoms in early clinical trials of patients with CLL as well as neuronal toxicity in mice,[25,45] which might be because of targets outside the BCL2 family. In this regard a recent study showed that obatoclax exerted growth inhibitory effects at lower concentrations than induction of apoptosis and suggested that obatoclax targeted other proteins in addition to BCL2 family members.[32]

In summary, we suggest that many of the putative BCL2 antagonists, including some of those currently undergoing clinical trials, are mitochondrial toxins that induce cell death primarily by massive mitochondrial damage rather than specific MOMP and activation of the intrinsic pathway of apoptosis. Owing to their nonspecific effects, the use of these putative BCL2 antagonists for mechanistic studies related to the function of BCL2 proteins should be viewed with caution. Of the inhibitors we have examined, only ABT-737 acts as a BH3-mimetic, inducing Bax/Bak-dependent activation of the intrinsic apoptotic pathway.

Materials and Methods

BCL2 inhibitors. Racemic gossypol (NSC# 19048) and racemic apogossypol (NSC# 736630) were kindly provided by Dr. VL Narayanan (Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, Bethesda, MD). EM20-25 (BCL2 inhibitor III) was from Calbiochem (Nottingham, UK) and chelerythrine from Sigma–Aldrich Ltd. (Dorset, UK). Obatoclax (GX15-070) and ABT-737 were kindly provided by Dr. Gordon Shore (GeminX Pharmaceuticals, Montreal, Canada) and Dr. Saul Rosenberg (Abbott Laboratories, IL, USA), respectively.

Cell culture. Wild type and Bax/Bak double knockout (DKO) MEFs were provided by Drs. A Strasser and D. Huang (The Walter and Eliza Hall, Institute of Medical Research, Melbourne, Australia), and cultured in DMEM medium supplemented with 5 mM L-glutamine and 10% fetal calf serum (FCS) (all from Life Technologies, Inc, Paisley, UK). Jurkat T-cells deficient in caspase-9 were cultured in RPMI 1640 medium supplemented with 10 % FCS and 5 mM L-glutamine (Life Technologies, Inc.).[26,27] Unlike some Jurkat cells, these caspase-9 deficient cells express Bax.[27] Peripheral blood samples from patients with CLL were obtained with patient consent and local ethical committee approval. Lymphocytes were purified and immediately cultured in RPMI 1640 medium supplemented with 10% FCS and 5 mM L-glutamine (Life Technologies, Inc.) as described earlier.[30]

Determination of cell death and cytochrome *c* release. Cell death was assessed either by phosphatidylserine (PS) externalization and staining with AnnexinV-FITC or AnnexinV-APC (Invitrogen, Paisley, UK) or by changes in scatter properties and FSC/SSC analysis. To assess release of cytochrome *c*, 10×10^6 CLL cells were washed in cold PBS and resuspended in 100 μl mitochondrial isolation buffer (250 mM sucrose, 20 mM Hepes, pH 7.4, 5 mM $MgCl_2$ and 10 mM KCl) containing 0.05% digitonin. Cells were left on ice for 10 min followed by centrifugation at 13 000 rpm for 3 min. Subsequently, supernatant and pellets were analyzed by western blotting.

Western blotting. Western blot analysis was carried out using mouse anti-cytochrome *c* Ab (BD Bioscience, San Diego, CA, USA), mouse anti-$\alpha-$tubulin Ab (Calbiochem), mouse anti-PARP Ab (Alexis Biochemicals, Nottingham, UK). Rabbit anti-caspase-9 and rabbit anti-caspase-3 Abs were kindly provided by Dr. Xiao-Ming Sun.[46] Enhanced chemiluminescence was used for detection (GE Healthcare, Bucks, UK).

Electron microscopy. The ultrastructure of 4–11 samples of CLL cells from at least 2 different patients was examined for all the inhibitors shown in Figures 5 and 6 except for ABT-737 where cells from 21 different patients have been examined. Cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) at 4°C overnight and postfixed with 1% osmium tetroxide/1% potassium ferrocyanide for 1 h at room temperature. After fixation, cells were stained en bloc with 5% aqueous uranyl acetate overnight at room temperature, dehydrated, and embedded in Taab epoxy resin (Taab Laboratories Equipment Ltd., Aldermaston, UK). Electron micrographs of ultrathin sections were recorded using a QICAM 12-bit Mono Fast 1394 digital camera and QCapture-Pro software (MAG, Pleasanton, CA, USA) in a Zeiss 902A electron microscope.

Statistics. To compare cell death in wt or DKO MEFS or caspase-9 deficient and expressing Jurkat cells, an unpaired *t*-test was used. For EC_{50} values at 24 h of drug exposure, the specific apoptosis was calculated as follows: (drug-induced death$-$spontaneous death) $\times$ 100/(100$-$spontaneous death). For calculation of the EC_{50} values, a nonlinear equation was used. All statistics were carried out in GraphPad prism.

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