BAK activation is necessary and sufficient to drive ceramide synthase-dependent ceramide accumulation following inhibition of BCL2-like proteins

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INTRODUCTION

One family of proteins that is a critical regulator of programmed cell death, or apoptosis, is the BCL2 family [1,2]. Since the initial discovery of BCL2 as the genetic driver mutation in follicular B-cell lymphoma, more than 20 additional family members have been identified on the basis of homology to a small amino acid motif, known as the BH3 (BCL2 homology 3) domain [3]. Most members of the family promote apoptosis, but six members have been shown to perform primarily anti-apoptotic functions [BCL2, BCLxL/BCL2L1, BCLw/BCL2L2, BFL1/BCL2A1, MCL1 (myeloid cell leukaemia 1) and BCLb/BCL2L10] [4]. The main function of each of the anti-apoptotic BCL2-like proteins is to antagonize the pore-forming functions of the pro-apoptotic BCL2-like proteins BAK and BAX in the mitochondrial outer membrane during the induction phase of apoptosis. However, there is evidence that these proteins can also perform additional functions, some of which may impinge indirectly on, or be completely unrelated to, the regulation of MOMP (mitochondrial outer membrane permeabilization) [5].

Mounting evidence indicates that inhibiting the action of the anti-apoptotic BCL2 proteins in multiple tumour types has significant efficacy in cell culture systems, animal models and in patients [6–13]. In fact, several small-molecule BH3 mimetics have been identified and are being tested in various clinical and pre-clinical trials. One of the most promising molecules, ABT-737, has a very high specificity for BCL2, BCLxL and BCLw (note that the orally bioavailable version of ABT-737 is ABT-263, which is used in the present study). ABT-263/ABT-737 has shown promise against many tumour types, including leukaemias and solid tumours, as a single agent and in combination therapeutic strategies respectively [6,14–17]. Pre-clinical studies have demonstrated that drug resistance to ABT-263/ABT-737, in lung and leukaemia samples, is commonly associated with increased expression of MCL1, which is not inhibited by either of these compounds [18–20]. A role for anti-apoptotic BCL2 family members in drug resistance is not limited to treatments that specifically target the BCL2 genes. Increased expression of BCL2, BCLxL, and BCLw has been observed in AML (acute myeloid leukaemia) cell lines resistant to high levels of busulfan treatment [21]. Increased copy numbers of the genomic loci encoding BCLw, MCL1 or BCLb were identified in various cell lines that were treated with, and became resistant to, etoposide, camptothecin or Ara-C (arabinofuranosyl cytidine) respectively [22]. Taken together, there is promise in targeting the BCL2 family in both primary and drug-resistant tumours, but as with all therapeutic strategies we must try to find combinatorial

Abbreviations used: BH3, BCL2 homology 3; BMK, baby mouse kidney; CerS, ceramide synthase; DMEM, Dulbecco’s modified Eagle’s medium; ESI–LC–MS, electrospray ionization–liquid chromatography–MS; ER, oestrogen receptor; FBS, fetal bovine serum; GFP, green fluorescent protein; HEK, human embryonic kidney; IRES, internal ribosomal entry sequence; KO, knockout; MCL1, myeloid cell leukaemia 1; MCS, multiple cloning site; MOMP, mitochondrial outer membrane permeabilization; MUSC, Medical University of South Carolina; MS/MS, tandem MS.

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strategies to limit tumour recurrence. Whether these combinational strategies include targeting individual BCL2 members or factors that regulate the BCL2 members is still to be determined.

Ceramides are known to regulate apoptosis at least in part through induction of MOMP. Ceramides have been shown to form channels in membranes capable of allowing the passage of proteins [23,24]. In addition, ceramide generation is upstream of, and has been reported to be important for, BAX activation in cells [25–27]. Indeed, in vitro data suggest that ceramide metabolites regulate MOMP induction via activation of BAX and BAK at mitochondrial membranes [28]. A previous study indicated that ceramide and BAX are capable of forming a novel channel with characteristics consistent with those required for MOMP induction [29]. Although several mechanisms have been proposed for ceramide induction of MOMP during apoptosis, most, if not all, of the literature suggests that it is via an interaction between ceramides (or one of its metabolites) and BCL2 proteins [30]. Thus, owing to the intimate interplay between BCL2-like family members and bioactive sphingolipids in apoptotic regulation, drugs that target both ceramide metabolism and anti-apoptotic BCL2 proteins may be good candidates for synergistic cancer therapies.

Many apoptotic stimuli are known to have pleiotropic effects on cells, making it difficult to de-convolute signalling pathways and apoptotic effectors. ABT-263 is a drug that specifically inhibits BCL2/BCLxL/BCLw and has been demonstrated to have a therapeutic window against cancer compared with normal cells [6,14–17]. ABT-263 specifically interacts with the hydrophobic groove of BCL2/BCLxL/BCLw, resulting in the release of bound pro-apoptotic BCL2-like proteins and activation of the pro-apoptotic multi-domain BCL2 proteins BAK and BAX. In the present study we utilize a variety of engineered human leukaemia and myeloma cell lines to demonstrate that ABT-263 is sufficient to induce C16-ceramide generation and increased in situ CerS (ceramide synthase) activity. ABT-263 is incapable of elevating CerS activity or C16-ceramide levels in human leukaemia and myeloma cells stably overexpressing MCL1. In vitro assays demonstrate that purified BAK is sufficient to activate CerS activity. In addition, activated BAK (via the addition of tBid) is a more potent activator of CerS than inactive BAK. Importantly, ABT-263 activates in vitro CerS activity in membranes isolated from wild-type BMK (baby mouse kidney) cells, but not in those isolated from Bak-KO (knockout) cells. Thus the results of the present study support a model in which activation of BAK induces elevated CerS activity and C16-ceramide generation. Furthermore, by combining the results of the present study with data from the literature, we present a new inclusive model by which BAK-induced ceramide accumulation potentiates a feed-forward mechanism to execute apoptosis.

**EXPERIMENTAL**

**Cell culture**

Human leukaemia and myeloma cell lines, U937, K562, RPMI8226 and MV411 were obtained from the A.T.C.C. and cultured in RPMI medium containing 10% (v/v) FBS (fetal bovine serum), 1% (v/v) L-glutamine and 1% (w/v) penicillin/streptomycin. Cells were maintained according to the manufacturer’s protocol and were not cultured for more than 30 passages. Cells were routinely assessed for mycoplasma using the MycoSensor PCR assay kit (#302108, Agilent Technologies) according to the manufacturer’s protocol. Both cell culture supernatant and cell lysates were tested for mycoplasma. In addition, cells were routinely examined for morphological characteristics and were tested for consistent IC50 to ABT-263, since each line has a distinguishable level of sensitivity to this drug.

Stably infected U937 or RPMI8226 cells were obtained by infecting parental cells with MIGRX replication-incompetent viral supernatant. MIGRX is a murine stem-cell-based retroviral vector that contains an MCS (multiple cloning site) followed by an IRES (internal ribosomal entry sequence) and GFP (green fluorescent protein). MCL1, BCLxL, or a fragment of the ER (oestrogen receptor; negative control) were inserted into the MCS. Retrovirus-containing supernatant was produced by transient transfection of HEK (human embryonic kidney)-293T cells with equal amounts of a packaging construct, amphotropic SV(PSi), and the respective MIGRX plasmid. The tissue culture supernatant was collected starting 72 h after transfection and used to infect U937 or RPMI8226 cells in the presence of 4 μg/ml polybrene during centrifugation (1000 g for 1.5 h at 30°C). At 48 h post-infection, GFP-positive cells were sorted using FACS. Stable cell lines were maintained as described for the parental cells.

BMK epithelial cells, wild-type and Bak-KO (gift from Dr E. White, Molecular Biology and Biochemistry, Rutgers University, New Brunswick, NJ, U.S.A.) were maintained in DMEM (Dulbecco’s modified Eagle’s medium), supplemented with 2 mM L-glutamine and 5% fetal bovine serum. Cells were routinely assessed for mycoplasma using the MycoSensor PCR assay kit. Both cell culture supernatant and cell lysates were tested for mycoplasma. In addition, cells were routinely examined for morphological characteristics. Cells were utilized for no more than ten passages. A total of 1 × 10⁶ cells were seeded into 10 cm² dishes. At 24 h following plating, the media was changed to that containing either vehicle or the indicated concentration of ABT-263. At the indicated time points cells were harvested for quantification of ceramides or alternatively in vitro CerS activity was measured as described below.

**In situ C17-sphingosine labelling**

Cells were labelled with C17-sphingosine (1 μM, 15 min, Avanti Polar Lipids), washed three times with ice-cold PBS, and collected via scraping and centrifugation. The reaction was stopped by the addition of 2 ml of extraction solvent containing ethyl acetate/2-propanol/water (60:30:10, by vol.) supplemented with an internal standard for ESI–LC–MS (electrospray ionization–liquid chromatography–MS) analysis. Lipids were extracted twice, dried under a stream of nitrogen, resuspended in 150 μl of 1 mM ammonium formate in 0.2% formic acid in methanol, and analysed at the MUSC (Medical University of South Carolina) Lipidomics Core Facility by ESI/LC/MS.

**Relative cell viability assays**

Exponentially growing human leukaemia or myeloma cells were seeded in 96-well dishes (5000 RPMI8226 cells, 5000 U937 cells, 7500 K562 cells or 7500 MV411 cells per well) and immediately treated with the indicated drug concentrations in a total volume of 100 μl per well. All treatments were done in triplicate. ABT-263 (catalogue number CT-A263) was obtained from Chemietek. Cells were incubated for 48 h and then 10 μl of Alamar Blue reagent (Invitrogen, catalogue number DAL1100) was added to each well. Plates were then incubated and the fluorescence of Alamar Blue was determined on a SPECTRAmax Gemini EM plate reader every 1 h until untreated wells were mid-linear, approximately 4000 arbitrary units. Wells containing only 100 μl of complete RPMI medium plus 10 μl of Alamar Blue were averaged and subtracted from all experimental readings. Drug
treatment regimens were then normalized to vehicle-treated cells. Each graph shown is a representative experiment of at least three biological replicates.

**In vitro CerS activity**

BMK cells were grown to 70% confluence, washed twice with ice-cold PBS, harvested by scraping in lysis buffer [20 mM Hepes, pH 7.4, 2 mM KCl, 2 mM MgCl₂, 250 mM sucrose and protease inhibitors (Sigma)], and lysed via 10 passages through a 28-gauge insulin syringe. Intact cells and nuclei were removed via centrifugation at 1000 g for 10 min, and the mitochondria-enriched fraction was obtained via centrifugation at 12000 g for 10 min. Microsomes were obtained from the supernatant of the 12000 g spin via a 100 000 g centrifugation for 1 h. CerS activity was measured in the microsomes or where indicated the mitochondria-enriched fraction (10–50 μg as indicated) as described previously [31]. Briefly, a reaction mixture (100 μl of final volume) containing 15 μM C₁₇₃-sphingosine and 50 μM C₁₆ fatty acyl-CoA in 25 mM potassium phosphate buffer (pH 7.4) was pre-warmed at 37°C for 5 min. The enzyme reaction was initiated via addition of the enzyme source (microsomes or mitochondria), and after 15 min at 37°C was terminated via the addition of 2 ml of extraction solvent containing ethyl acetate/2-propanol/water (60:30:10, by vol.) supplemented with internal standard for ESI–LC–MS analysis. Lipids were extracted twice, dried under a stream of nitrogen, and resuspended into 150 μl of 1 mM ammonium formate in 0.2% formic acid in methanol and analysed by ESI–LC–MS.

Where indicated, CerS activity was measured in the presence of added recombinant proteins. Recombinant BAX or BAK were obtained as described previously [32,33]. Where indicated, BAK activation was achieved via the addition of 5 ng of BAK and 10 ng of recombinant tBid (purchased from AnaSpec) at 37°C. Where indicated, recombinant proteins were added to microsomes 15 min before to the initiation of the enzymatic reaction (achieved via the addition of substrates).

**Sphingolipidomic MS**

Leukaemia or myeloma cells (5 × 10⁶) were seeded in each well of a six-well dish in 4 ml of total RPMI medium [containing 10% (v/v) FBS, 1% (v/v) penicillin/streptomycin and 1% (v/v) L-glutamine] and treated with the indicated amount of ABT-263 or DMSO, in biological triplicates. With the indicated times, cells were harvested, washed twice in 5 ml of ice-cold PBS and then snap frozen in liquid nitrogen. For BMK cells, 1 × 10⁶ cells were seeded in 10 cm² dishes and after 36 h were treated with ABT-10% of a six-well dish in 4 ml of total RPMI medium [containing 15% fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin and 1% (v/v) L-glutamine], and lysed via 10 passages through a 28-gauge insulin syringe. Intact cells and nuclei were removed via centrifugation at 1000 g for 10 min, and the mitochondria-enriched fraction was obtained via centrifugation at 12000 g for 10 min. Microsomes were obtained from the supernatant of the 12000 g spin via a 100 000 g centrifugation for 1 h. CerS activity was measured in the microsomes or where indicated the mitochondria-enriched fraction (10–50 μg as indicated) as described previously [31]. Briefly, a reaction mixture (100 μl of final volume) containing 15 μM C₁₇₃-sphingosine and 50 μM C₁₆ fatty acyl-CoA in 25 mM potassium phosphate buffer (pH 7.4) was pre-warmed at 37°C for 5 min. The enzyme reaction was initiated via addition of the enzyme source (microsomes or mitochondria), and after 15 min at 37°C was terminated via the addition of 2 ml of extraction solvent containing ethyl acetate/2-propanol/water (60:30:10, by vol.) supplemented with internal standard for ESI–LC–MS analysis. Lipids were extracted twice, dried under a stream of nitrogen, and resuspended into 150 μl of 1 mM ammonium formate in 0.2% formic acid in methanol and analysed by ESI–LC–MS.

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**Plasmid construction**

Total human Universal RNA (Clontech, catalogue number 636538) was used as a template to amplify BCLxl and MCL1 using the following oligonucleotides: 5 hu BCLxl atg RI, 5’-GC-GGCAATTCCACCATGTCTCAGAGCAACCAGGAG-3’; 3 hu BCLxl stop XhoI, 5’-GCGCGTCGACTTACATCGACTGAAG-AGTGGCCCG-3’; 5 hu MCL1 atg Bam, 5’-GGCCGAT-CCACCATGTCTCAGAGCAACCAGGAG-3’; and 3 hu MCL1 stop SalI, 5’-GGCGGTCGACTTACATCGACTGAAG-AGTGGCCCG-3’. PCR-amplified fragments were ligated into a TOPO blunt cloning kit (Invitrogen). Full-length BCLxL and full-length MCL1 were then digested with EcoRI and XhoI for BMCLxL (for BCLLxL) or BglII and XhoI (for MCL1). All clones were sequenced for accuracy. MIG-ER was developed by digesting pBABE-ER (described previously [35]) with BamHI and SalI and ligating the fragment into MIGR digested with BglIII and XhoI.

**Establishment of U937 and RPMI8226 stable cell lines**

Parental U937 and RPMI8226 cell lines were maintained as described above. The GFP-expressing infectious virus was produced by transiently transfecting HEK-293T cells with the MIGR–based plasmids described above and the packaging plasmid SV psi- and polyethyleneimine at a ratio of 1:2.5 DNA/PEI (polyethyleneimine). At 72 h post-transfection, the viral supernatant was harvested and incubated with U937 or RPMI-8226 cells in the presence of 4 μg/ml polybrene. This was centrifuged at 30°C for 1 h at 1000 g in a clinical tabletop centrifuge. At 72 h post-infection the cells were sorted at the James Graham Brown Cancer Center Flow Cytometry Core Facility using standard methods.

**RESULTS**

**ABT-263 induces generation of C₁₆-ceramide in leukaemia cells**

Our previously published results demonstrated a synergism between ABT-263 and drugs that modulate sphingolipid metabolism [36]. Specifically, the results indicated that the combination of ABT-263 with drugs that inhibit the metabolism of ceramide to pro-proliferative sphingolipids (e.g. sphingosine 1-phosphate or glucosylceramide) synergistically kill leukaemia cells [36]. Sensitivity to ABT-263 correlates with basal ceramide levels and the synergistic killing is not universal, but rather dependent on the accumulation of total ceramide [36]. Thus we hypothesized that ABT-263 regulates ceramide metabolism. To test this, we utilized three different human leukaemia cell lines with varying sensitivities to ABT-263, namely histiocytic lymphoma U937 cells, chronic myelogenous K562 leukaemia cells and myelocytic MV411 leukaemia cells. First, a dose–response curve of ABT-263 for 48 h was performed and viable cells were determined using the Alamar Blue assay to determine the appropriate concentration at which to administer ABT-263 for ceramide analysis (Figure 1A). The IC₀ values for ABT-263 were 7.5 μM, 0.4 μM and 0.03 μM for U937, K562 and MV411 cells respectively. Cells were treated for 8 or 24 h at their corresponding IC₀ for ABT-263 or vehicle control (DMSO) and individual ceramides were quantified via HPLC-MS/MS (tandem MS) as described in the Experimental section. In all cell lines, the only ceramide species that was significantly altered following ABT-263 treatment was C₁₆-ceramide (Figures 1B–1D). There were slight elevations in C₂₂- and C₂₂:1-ceramides (Figures 1B–1D). However, these were not statistically significant elevations and are very minor species in these leukaemia cells. In contrast, C₁₆-ceramide is a major species that is elevated in a statistically significant manner (Figures 1B–1D). These results indicate that
inhibition of anti-apoptotic BCL2-like proteins with ABT-263 causes accumulation of C16-ceramide.

ABT-263-induced C16-ceramide generation is inhibited by overexpression of MCL1

ABT-263 induces apoptosis via its ability to bind to and inhibit the activity of the anti-apoptotic BCL2 proteins BCL2, BCLxL, and BCLw. Thus we hypothesized that ABT-263-induced C16-ceramide generation is via its inhibition of one or more of these anti-apoptotic BCL2 proteins. To test this hypothesis, U937 cells were infected with a retrovirus expressing GFP and either an irrelevant control protein fragment (ER), BCLxL or MCL1, separated by an IRES [37]. Infected cells were sorted using FACS and the established cell lines were validated for GFP and the respective BCL2-like protein expression (Supplementary Figure S1 at http://www.biochemj.org/bj/452/bj4520111add.htm). The anti-apoptotic BCLxL protein is inhibited by ABT-263, whereas MCL1 is not inhibited by ABT-263 [38]. Interestingly, overexpression of either BCLxL or MCL1 in U937 cells does not dramatically alter their sensitivity to ABT-263 after 48 h of treatment. Ceramides were quantified following treatment (8 or 24 h) of U937-ER, U937-BCLxL or U937-MCL1 cells with ABT-263 or vehicle (DMSO), as described previously. Treatment of control U937-ER and U937-BCLxL cells with ABT-263 led to a significant increase in the level of the major ceramide species C16-ceramide at both time points examined (Figures 2A and 2B). In stark contrast, C16-ceramide was not elevated following treatment of U937-MCL1 cells with ABT-263 (Figure 2C). In addition to C16-ceramide, ABT-263 also induced elevations in the minor species C22- and C22:1-ceramide in the U937-ER cells and to a lesser extent in the U937-BCLxL cells that were not observed in the U937-MCL1 cells (Figures 2A and 2B). However, these are very minor species (Figure 1B) and are elevated mainly at 24 h, a time point after cleavage of caspase 3 (previously published to be as early as 2 h [36]). It is not known which enzymes are responsible for the generation of C22- and C22:1-ceramides, nor the location in the cell in which they are generated. It is possible that they are important to the apoptotic process and thus their generation should not be completely ignored. In summary, these data suggest that ABT-263-induced ceramide generation is via its inhibition of known target anti-apoptotic BCL2-like proteins.

Figure 1  Treatment of human leukaemia cell lines with the BCL2/BCLxL/BCLw inhibitor, ABT-263, causes accumulation of C16-ceramide

(A) U937, K562 and MV411 cells were incubated with increasing doses of ABT-263 (0.7 nM–45 μM) for 48 h and the relative numbers of viable cells were determined by reduction in Alamar Blue. Results are at least biological triplicates. (B) U937 cells were treated with 1.6 μM ABT-263 or vehicle (DMSO) for 8 and 24 h and the total amount of cellular ceramides was determined by HPLC-MS/MS. (C) K562 cells were treated with 150 nM ABT-263 or vehicle for 8 and 24 h and the total amount of cellular ceramides was determined by HPLC-MS/MS. (D) MV411 cells were treated with 50 nM ABT-263 or vehicle (DMSO) for 8 and 24 h and the total amount of cellular ceramides was determined by HPLC-MS/MS. Results are means ± S.D. ***P < 0.0005; **P < 0.005; *P < 0.05.
increase in C₁₆-ceramide observed in previous experiments, in situ CerS activity was significantly elevated in both U937-ER and U937-BCLxL cells following ABT-263 treatment (Figures 2D and 2E). However, there was no increase in in situ CerS activity in U937-MCL1 cells treated with ABT-263 (Figure 2F).

To determine whether the lack of ceramide generation in MCL1-overexpressing cells was specific to U937 cells, we utilized a human myeloma cell line, RPMI8226, also engineered to stably overexpress MCL1 (Supplementary Figure S2 at http://www.biochemj.org/bj/452/bj4520111add.htm). RPMI-MCL1 cells are resistant to ABT-263 as demonstrated by decreased conversion of C₁₇-sphingosine into C₁₆-C₁₇-ceramide, suggesting that CerS activity is greatly diminished (Figure 4A). To determine whether the presence of Bak is sufficient to activate CerS activity, we isolated mitochondria from Bak-KO BMK cells and added increasing amounts of soluble recombinant Bax or Bak (Figure 4B). In vitro CerS enzymatic activity was determined by monitoring the conversion of C₁₇-sphingosine into C₁₆-C₁₇-ceramide. Soluble Bak was sufficient to activate CerS enzymatic activity on Bak-KO mitochondria in a dose-dependent manner, whereas soluble Bax was not capable of increasing CerS activity.

BAK is sufficient to induce activation of CerS

ABT-263 inhibits anti-apoptotic BCL2-like proteins (BCL2, BCLxL, and BCLw) via binding to their hydrophobic groove and displacing pro-apoptotic BCL2 proteins, leading to the activation of BAK and/or BAX. MCL1 only binds BAK and ABT-263 is incapable of displacing BAK from MCL1 [41]. We previously reported that BAK is required for CerS-mediated long-chain ceramide generation during apoptosis [31]. Thus we hypothesized that ABT-263 activates CerS in a BAK-dependent manner. To test this hypothesis, we isolated mitochondria from either wild-type or Bak-deficient (Bak-KO) BMK cells and performed in vitro CerS enzyme activity assays. Increasing amounts of purified mitochondria, isolated from either wild-type or Bak-KO BMK cells, showed increasing CerS activity; however, this activity was significantly lower in the Bak-KO mitochondria, as demonstrated by decreased conversion of C₁₇-sphingosine into C₁₆-C₁₇-ceramide, suggesting that CerS activity is greatly diminished (Figure 4A). To determine whether the presence of Bak is sufficient to activate CerS activity, we isolated microsomes from Bak-KO BMK cells and added increasing amounts of soluble recombinant Bax or Bak (Figure 4B). In vitro CerS enzymatic activity was determined by monitoring the conversion of C₁₇-sphingosine into C₁₆-C₁₇-ceramide. Soluble Bak was sufficient to activate CerS enzymatic activity on Bak-KO microsomes in a dose-dependent manner, whereas soluble Bax was not capable of increasing CerS activity.

Bak is known to undergo activating conformational changes during apoptosis. ABT-263 treatment of cells induces activation of Bak. Thus we hypothesized that activated Bak is a more potent activator of CerS activity than non-activated Bak. Certain BH3-only pro-apoptotic proteins, such as cleaved Bid (tBid), are known to cause activation of Bak. Therefore we tested whether the addition of purified tBid could influence the ability of Bak to activate CerS activity on purified Bak-deficient microsomes.
a significant increase in the level of C\textsubscript{16}-ceramide (Figure 4D).

cells, treatment of wild-type BMK cells with ABT-263 caused quantified ceramide levels at 2 and 8 h. As in the human leukaemia vehicle (DMSO) for 1 h 45 min. C\textsubscript{17}-sphingosine (1 μM) was then added to the medium for an additional 15 min. Cells were immediately lysed and the amount of C\textsubscript{17}-ceramide in the cells was quantified by HPLC-MS/MS. (C) RPMI-ER cells were treated with 5 μM ABT-263 or vehicle (DMSO) for 2 and 8 h and C\textsubscript{16}-ceramide was quantified by HPLC-MS/MS. (D) RPMI-MCL1 cells were treated with 5 μM ABT-263 or vehicle (DMSO) for 2 and 8 h and C\textsubscript{16}-ceramide was quantified by HPLC-MS/MS. Lipids were normalized to total lipid phosphate and the results were expressed as a fold-change of the untreated control. All results are at least biological triplicates and are means ± S.D. ***<p>0.0005.

(Figure 4C). When a minimal amount of soluble Bak (5 ng) was added to microsomes isolated from Bak-KO BMK cells, we observed a slight increase in CerS activity. Purified tBid alone (10 ng) was not sufficient to alter CerS enzymatic activity in the absence of Bak. In contrast, when recombinant tBid was added with soluble Bak to microsomes, there was a dramatic increase in CerS enzymatic activity, indicating that activated Bak is a more potent inducer of CerS enzymatic activity.

**BAK is necessary for ABT-263-induced activation of CerS**

The results discussed above indicated that ABT-263 activates in situ CerS activity, leading to an increased accumulation of C\textsubscript{16}-ceramide. The ability of ABT-263 to induce C\textsubscript{16}-ceramide can be inhibited by the expression of MCL1. Thus we hypothesized that BAK is necessary for ABT-263-induced CerS activation and C\textsubscript{16}-ceramide generation. To formally determine the requirement of BAK on ABT-263-induced ceramide accumulation, we treated Bak-KO or wild-type BMK cells with 2.67 μM ABT-263 and quantified ceramide levels at 2 and 8 h. As in the human leukaemia cells, treatment of wild-type BMK cells with ABT-263 caused a significant increase in the level of C\textsubscript{16}-ceramide (Figure 4D).

In stark contrast, treatment of Bak-KO BMK cells with ABT-263 did not elevate C\textsubscript{16}-ceramide (Figure 4E). In vitro CerS activity assays were performed to determine whether ABT-263 can activate CerS activity in a cell-free system. ABT-263 induced a dose-dependent increase in conversion of C\textsubscript{17}-sphingosine into C\textsubscript{16}-C\textsubscript{17}-ceramide in heavy membranes isolated from wild-type BMK cells. However, ABT-263 did not elevate in vitro CerS activity in heavy membranes isolated from Bak-KO cells at any dose tested (Figure 4F). These results demonstrate that the ability of ABT-263 to cause C\textsubscript{16}-ceramide accumulation is dependent on CerS activation by Bak.

**DISCUSSION**

One key hallmark of cancer is the ability to evade apoptosis and one primary way in which cancers accomplish this is via up-regulation of anti-apoptotic BCL2-like proteins [18,19,41]. Cancer cells rely on anti-apoptotic BCL2-like proteins for survival and should in theory be highly sensitive to cytotoxic therapies that are aimed at their inhibition. Thus much effort has been devoted to designing small-molecule inhibitors that interfere with the pro-survival function of these oncoproteins [15].

ABT-263 is a potent inhibitor of three anti-apoptotic BCL2-like proteins, namely BCL2, BCLx\textsubscript{L} and BCLw [41]. The results of the present study indicate that ABT-263 treatment is sufficient to induce generation of C\textsubscript{16}-ceramide. Importantly, generation of ceramide was not specific to a particular cell type, as ABT-263 induced generation of C\textsubscript{16}-ceramide in multiple human leukaemia cell lines as well as in kidney epithelial cells. The results of the present study indicate that ABT-263-induced C\textsubscript{16}-ceramide generation is dependent on its ability to inhibit anti-apoptotic BCL2-like proteins. ABT-263 inhibits BCL2, BCLx\textsubscript{L} and BCLw by binding to their hydrophobic pocket and displacing bound pro-apoptotic BCL2-like proteins, including BAX and BAK [6]. Indeed, ABT-263 treatment of cells is sufficient to induce activation of BAX and/or BAK and induction of MOMP. ABT-263 has a low affinity for the hydrophobic pocket of MCL1 and thus is unable to inhibit the anti-apoptotic functions of MCL1. MCL1 binds to BAK, but not BAX. As ABT-263 has a low affinity for the hydrophobic pocket of MCL1, it is unable to induce the release of BAK. Thus cells overexpressing MCL1 should have lower levels of free BAK available for activation of CerS. Indeed, the results of the present study indicate that this is in fact the case. Overexpression of MCL1 in either U937 or RPMI8226 cell lines prevented the ability of ABT-263 to induce ceramide generation. If ABT-263 were indeed generating ceramide via a BAK activation of CerS, then following ABT-263 treatment of the parental cells CerS activity should be elevated. ABT-263 treatment of the control cells increased in situ CerS activity; this increase in activity was not observed in cells overexpressing MCL1. This is consistent with the model that overexpression of MCL1 binds to BAK and prevents it from activating CerS. Consistent with this model, purified recombinant BAK was sufficient to induce activation of CerS in an in vitro assay. This was specific to BAK as the seemingly functionally redundant protein BAX was incapable of activating in vitro CerS activity. Importantly, ABT-263 alone was also sufficient to induce activation of CerS in vitro. Again ABT-263 activation of CerS only occurred in the presence of BAK as no activation was observed when heavy membranes purified from Bak-KO cells were utilized as the enzyme source.

Cells that become resistant to ABT-263 often overexpress MCL1 and the results of the present study suggest that this resistance may be due, in part, to an inability of MCL1-expressing cells to generate long-chain ceramides. Thus one potential avenue of therapeutic exploration should be to identify drugs that can specifically inhibit the MCL1–BAK interaction, thereby leading to activation of CerS activity. Indeed, there are potential inhibitors that have recently been developed that do inhibit MCL1 and could be utilized in this capacity. However, many of these lack specificity and have off-target effects as shown by their ability to kill both wild-type and Bax/Bak double deficient cells. Thus more specific inhibitors are needed to increase the specificity of cancer treatments.
In addition, ABT-263 treatment is known to have negative side-effects in patients, such as thrombocytopenia [42]. We propose that combining ABT-263 with other clinically relevant drugs will be beneficial by limiting the possibility of drug resistance and by lowering the effective dose of ABT-263 required, which would in turn decrease the chances of patients developing detrimental side-effects. To this end, we have recently reported that drugs which inhibit the metabolism of ceramide to either of the pro-proliferative metabolites sphingosine 1-phosphate or glucosylceramide can be combined with ABT-263 to synergistically kill a variety of human leukaemia cell lines [36]. The combination of these drugs may allow it to be utilized at lower doses. Alternatively, these drug combinations may also prevent the recurrence of cancers.

Taken together, the results of the present study indicate that ABT-263-induced generation of C16-ceramide is dependent on BAK activation of CerS. Future work will aim to determine the specific CerS enzymes that are regulated by BAK. CerSs are key enzymes in the de novo and salvage pathways of ceramide generation. Six CerS isoforms are present in mammalian cells, each with its own fatty acyl-CoA preference [43]. Thus specific CerS isoforms generate ceramides of specific chain lengths, which have been shown to contribute differently to cellular responses to stress stimuli [44,45]. BAK-dependent CerS activity is restricted to particular long-chain fatty acyl-CoA substrates that are consistent with those preferred by CerS4, CerS5 and CerS6 (see above and [31]). Once we identify the CerS isoforms that are regulated by BAK, we can define a means for influencing specific CerS isoforms responsible for ceramide generation. This is not a trivial task, as CerS activity is in part regulated by heterodimerization with other CerS enzymes (for example CerS2 with either CerS5 or CerS6) and knockdown of the expression of one CerS affects the expression of the other CerS [46,47].

The results of the present study are consistent with previous reports of cross-talk between sphingolipids and BCL2-like proteins in the induction of MOMP. They suggest a feed-forward model by which ABT-263, and probably other chemotherapeutic drugs, lead to BAK-dependent activation of CerS (Figure 5, stage 1). CerS activation, in turn, leads to ceramide-induced inhibition of anti-apoptotic BCL2-like proteins (Figure 5, stage 2) and synergistic channel formation by ceramide (or one of its metabolites) and BAX/BAK (Figure 5, stage 3). Indeed, in vivo data from the Kolesnick laboratory demonstrate that ceramide production is required for radiation-induced apoptosis [40]. Furthermore, they also show that CerS activation of ceramide production and co-localization of ceramide with BAX in platforms in the outer mitochondrial membrane to induce apoptosis [27]. Colombini and co-workers indicated that ceramide alone is capable of forming large and stable channels in planar phospholipid membranes in the absence of proteins as well as in mitochondrial outer membranes of isolated mitochondria [48]. Thus BAK activation could lead to the activation of CerS and generation of sufficient ceramide to form channels large enough to induce MOMP (Figure 5, stage 4). Studies from the Colombini laboratory also indicated that these ceramide channels are regulated by BCL2-like proteins [29,30,49,50].
Anti-apoptotic proteins like BCLxL and the BCL2 homologue Ced-9 inhibit ceramide channels [49]. Indeed, BCLxL binds ceramide in the same hydrophobic pocket that it binds to pro-apoptotic BCL2-like proteins and ABT-263 is capable of displacing bound ceramide [30]. Thus ABT-263 could effectively prevent BCLxL or BCL2 inhibition of ceramide channels. Alternatively, ceramide binding to the hydrophobic pocket of BCL2-like anti-apoptotic proteins may prevent their interaction with pro-apoptotic BCL2-like proteins (Figure 5, stages 2 and 5). Thus ceramide could, in effect, behave as a pleotropic version of ABT-263 that is capable of binding more promiscuously than ABT-263 to anti-apoptotic BCL2-like family members, such as MCL1, to displace BAK and further potentiate the production of ceramide (Figure 5, stage 5). Thus ABT-263 functions at least in part by increasing the concentration of BAK available to activate CerS and induce ceramide generation that would function in a positive-feedback loop to: (i) form ceramide channels in mitochondrial outer membranes; (ii) activate BAX and BAK to form channels; (iii) induce the formation of ceramide-BAX hybrid channels; and (iv) bind to the hydrophobic pocket of BCLxL and release bound pro-apoptotic BCL2-like proteins. Interestingly, we also published recently that ceramide metabolites are capable of inducing the activation of BAK and BAX [28]. Although these data might seem contradictory, it actually supports the notion of a positive feed-forward process whereby the BAK-induced ceramide is further metabolized to species that activate BAX and BAK. The activated BAK would lead to further activation of CerS and increased ceramides (and its metabolites) that would induce MOMP through the multiple potential pathways outlined above. The sphingolipid species that initially leads to the activation of BAK and synergizes with BAK and BAX to induce MOMP most likely depends on both the cell type as well as the apoptotic stimulus.

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**REFERENCES**


**AUTHOR CONTRIBUTION**

Levi Beverly and Leah Siskind conceived the experiments and wrote the paper. Lauren Howell, Maria Hernandez-Corbacho, Lavona Casson, Levi Beverly and Leah Siskind performed the experiments. Jerry Chipuk provided critical reagents and contributed to writing the paper.

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SUPPLEMENTARY ONLINE DATA

BAK activation is necessary and sufficient to drive ceramide synthase-dependent ceramide accumulation following inhibition of BCL2-like proteins

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Figure S1 Characterization of U937 cells stably expressing GFP, BCLxL and MCL1

(A) Following infection and sorting of U937 cells (as described in the Experimental section of the main text), cells were analysed by flow cytometry to determine the percentage of GFP-expressing cells. U937 cells stably infected with empty virus (MIG-ER), BCLxL (MIG-BCLxL) or MCL1 (MIG-MCL1) were analysed by the intensity of GFP on the x-axis against an empty channel (FL3) on the y-axis. Gates were determined by analysing non-GFP-expressing cells. (B) Whole-cell extracts were prepared from U937 stable cell lines and analysed by Western blot analysis with anti-BCLxL, anti-MCL1 or anti-tubulin antibodies. (C) ABT-263 dose–response curves for U937 cells stably expressing ER, BCLxL or MCL1. Results are means±S.D.

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Figure S2  Characterization of RPMI8226 cells stably expressing GFP and MCL1

(A) Following infection and sorting of RPMI8226 cells (as described in the Experimental section of the main text), cells were analysed by flow cytometry to determine the percentage of GFP-expressing cells. RPMI8226 cells stably infected with empty virus (MIG-ER) or MCL1 (MIG-MCL1) were analysed by intensity of GFP on the x-axis against an empty channel (FL3) on the y-axis. Gates were determined by analysing non-GFP-expressing cells. (B) Whole-cell extracts were prepared from RPMI8226 stable cell lines and analysed by Western blot analysis with anti-MCL1 or anti-tubulin antibodies. (C) ABT-263 dose–response curves for RPMI8226 cells stably expressing ER or MCL1. Results are means±S.D.