Tumour cells typically exhibit a G1 cell cycle arrest in response to the MEK1/2 [mitogen-activated protein kinase/ERK (extracellular-signal-regulated kinase) kinase 1/2] inhibitor selumetinib, but do not die, and thus they acquire resistance. In the present study we examined the effect of combining selumetinib with the BH3 [BCL2 (B-cell lymphoma 2) homology domain 3]-mimetic BCL2 inhibitor ABT-263. Although either drug alone caused little tumour cell death, the two agents combined to cause substantial caspase-dependent cell death and inhibit long-term clonogenic survival of colorectal cancer and melanoma cell lines with BRAFV600E or RAS mutations. This cell death absolutely required BAX (BCL2-associated X protein) and was inhibited by RNAi (RNA interference)-mediated knockdown of BIM (BCL2-interacting mediator of cell death) in the BRAFV600E-positive COLO205 cell line. When colorectal cancer cell lines were treated with selumetinib plus ABT-263 we observed a striking reduction in the incidence of cells emerging with acquired resistance to selumetinib. Similar results were observed when we combined ABT-263 with the BRAFV600E-selective inhibitor PLX4720, but only in cells expressing BRAFV600E. Finally, cancer cells in which acquired resistance to selumetinib arises through BRAFV600E amplification remained sensitive to ABT-263, whereas selumetinib-resistant HCT116 cells (KRASG13D amplification) were cross-resistant to ABT-263. Thus the combination of a BCL2 inhibitor and an ERK1/2 pathway inhibitor is synthetic lethal in ERK1/2-addicted tumour cells, delays the onset of acquired resistance and in some cases overcomes acquired resistance to selumetinib.

Key words: acquired resistance, BCL2 homology domain 3 (BH3) mimic, BCL2-interacting mediator of cell death (BIM), BRAF, extracellular-signal-regulated kinase 1/2 (ERK1/2), RAS.

INTRODUCTION

The RAS-regulated RAF/MEK [mitogen-activated protein kinase/ERK (extracellular-signal-regulated kinase) kinase] 1/2/ERK1/2 signalling pathway is frequently deregulated in human cancers, due to mutations in BRAF, RAS or RTKs (receptor tyrosine kinases); consequently highly selective inhibitors of BRAF and MEK1/2 are in development. BRAFV600E is found in melanoma, thyroid cancer, CRC (colorectal cancer) [1] and hairy cell leukaemias [2]. Among several BRAF inhibitors, vemurafenib/PLX4032 is the most advanced [3] and only inhibits ERK1/2 signalling and proliferation in tumours with BRAFV600E [4]; in cells with wild-type BRAF, vemurafenib actually activates RAF and ERK1/2 signalling [5,6]. This provides a broad tumour-specific therapeutic index but limits the use of vemurafenib to tumour cells with BRAFV600E. In contrast, MEK1/2 inhibitors such as selumetinib (AZD6244/ARRY-142886) do not cause paradoxical ERK1/2 activation and can be used against tumours driven by BRAF, KRAS or RTK oncogenes. However, combination therapy is likely to be required to fully exploit their therapeutic effect.

Despite promising results in pre-clinical models and clinical trials, tumour cells invariably develop acquired resistance to BRAF or MEK1/2 inhibitors, leading to disease progression. Acquired resistance to BRAF inhibitors arises through multiple mechanisms, including alternative splice variants of BRAFV600E [7], a switch to ARAF or CRAF [8], a switch to alternative MEK1/2 activators [9], emergence of MEK1 mutations [10], emergence of RAS mutations [11] or RTK up-regulation [11,12]. Acquired resistance to MEK1/2 inhibitors may arise through emergence of MEK1 mutations [13] or through the amplification of BRAFV600E [14,15] or KRASG13D [15]. These mechanisms share the common theme of maintaining or reactivating the ERK1/2 pathway in the presence of a drug, underlining the degree to which these tumour cells are addicted to ERK1/2 signalling [16].

Tumour cells treated with BRAF or MEK1/2 inhibitors invariably exhibit increased expression of pro-apoptotic BH3 [BCL2 (B-cell lymphoma 2) homology domain 3]-only proteins such as BIM (BCL2-interacting mediator of cell death) or PUMA (p53 up-regulated modulator of apoptosis) [17–20]. Despite this, such cells do not die, because they exhibit increased expression of pro-survival BCL2 proteins such as BCL2 or BCL-XL (B-cell lymphoma-extra large) [21], which act to buffer BIM and PUMA. Instead, ERK1/2 pathway inhibition typically causes a G1 cell cycle arrest [14,15] and this may simply provide the tumour cell with the opportunity to re-model the ERK1/2 pathway to facilitate resistance [16]. Clearly strategies are required which harness the therapeutic opportunity provided by the ERK1/2 pathway in pre-clinical models and clinical trials, tumour cells invariably develop acquired resistance to BRAF or MEK1/2 inhibitors, leading to disease progression.

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Abbreviations used: BAD, BCL2/BCL-XL-antagonist, causing cell death; BAK, BCL2 homologous antagonist/killer; BAX, BCL2-associated X protein; BCL2, B-cell lymphoma 2; BCL-XL, B-cell lymphoma-extra large; BH3, BCL2 homology domain 3; BIK, BCL2-interacting killer; BIM, BCL2-interacting mediator of cell death; BMF, BCL2-modifying factor; CRC, colorectal cancer; ERK, extracellular-signal-regulated kinase; MCL1, myeloid cell leukaemia 1; MEK, mitogen-activated protein kinase/ERK kinase; NT, non-targeting; PARP, poly(ADP-ribose) polymerase; PI, propidium iodide; PI3K, phosphoinositide 3-kinase; PKB, protein kinase B; PUMA, p53 up-regulated modulator of apoptosis; RNAi, RNA interference; RTK, receptor tyrosine kinase; siRNA, small interfering RNA; TOR, target of rapamycin; mTOR, mammalian TOR.

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pathway addiction by targeting cell survival pathways to induce tumour cell death. Transforming cytostatic responses into cell death in this way may delay the onset of acquired resistance.

In the present study we have tested this hypothesis using the BH3 mimetic ABT-263/Navitoclax, a small molecule that disrupts BCL2/BCL-XL interactions with pro-death proteins such as BIM, leading to the initiation of apoptosis [22,23]. We show that, although ERK1/2 pathway inhibitors and ABT-263 alone cause little cell death, they synergize to cause a striking cell death response in tumour cells with BRAF or RAS mutations. This synthetic lethality depends on BAX (Bcl-2-associated X protein) and BIM, profoundly inhibits the onset of acquired resistance to MEK1/2 inhibitors and may even overcome established resistance driven by BRAF<sup>V600E</sup> amplification.

MATERIALS AND METHODS

Materials

Selumetinib (AZD6244) and AZ12321046 (AZ1046) have been described previously [15] and were provided by AstraZeneca. AZ12321046 is a mixed inhibitor of PI3K (phosphoinositide 3-kinase) and TOR (target of rapamycin); it exhibits an IC<sub>50</sub> of &lt;10 nM against the enzymes PI3K<sub>α</sub>, PI3K<sub>β</sub>, PI3K<sub>γ</sub>, PI3K<sub>δ</sub> and mTOR (mammalian TOR) in vitro, decreases phospho-PKB (protein kinase B; also known as Akt) abundance with an IC<sub>50</sub> of 4 nM in MDA-MA-468 cells, and inhibits proliferation of MDA-MB-468 cells with an IC<sub>50</sub> of 368 nM. ABT-263 was purchased from Santa Cruz Biotechnology. PLX4720 was purchased from Selleck Chemicals. Q-VD-OPH was purchased from Calbiochem. Antibodies specific for BAK (BCL2 homologous antagonist/killer) (AM03), cyclin D1 (CC12), NOXA (OP180) and p27<sup>kip1</sup> (NA35) were purchased from Calbiochem; antibodies specific for BAD (BCL2/BCL-XL-antagonist, causing cell death) (9239), BCL-w (2724), BCL-XL (2762), BID (BH3-interacting-domain death agonist) (2002), PARP [poly(ADP-ribose) polymerase] (9542), phospho-ERK1/2 (9106), PKB (9272), phospho-PKB Ser<sup>473</sup> (9271), phospho-PKB Thr<sup>308</sup> (9275) and phospho-Rb Ser<sup>308</sup> (9301) were from Cell Signaling Technology; anti-ERK1 antibody (610031) was from Cell Signaling Technology; anti-ERK1 antibody (610031) was from Cell Signaling Technology; anti-BCL-XL antibody (Cell Signaling Technology; 2762) and anti-MCL1 antibody (Santa Cruz Biotechnology; sc-819) using Protein A–Sepharose beads. For BIM immunoprecipitation, lysates were prepared in TG lysis buffer as described previously [26]. For BCL-XL and MCL1 immunoprecipitation, cells were lysed in TG lysis buffer as described previously [27] and immunoprecipitated with anti-BCL-XL antibody (Cell Signalling Technology; 2762) and anti-MCL1 antibody (Santa Cruz Biotechnology; sc-819) using Protein A–Sepharose beads. For BIM immunoprecipitation, lysates were prepared in TG lysis buffer and immunoprecipitated using the rat anti-BIM monoclonal antibody (14A8) from Merck Millipore, together with Protein G–Sepharose beads (GE Healthcare). Immunoprecipitated lysates were subjected to SDS/PAGE and Western blotting. For detection of some proteins, immunoprecipitates were run under non-reducing conditions in the absence of 2-mercaptoethanol, and the majority of primary antibodies were detected using Protein G–horseradish peroxidase (Bio-Rad Laboratories), to avoid heavy and light chain interference from the antibodies used in the immunoprecipitation reactions.

RNAi (RNA interference)

For transient siRNA (small interfering RNA), the following target sequences were used: BIM1, GACCGAGAAGGTAGACAA TT; and BIM2, GCAACCTTCTGATGTAAGT. PUMA and NT (non-targeting) siRNA were purchased from Dharmacon. For COLO205 transfection, 4 × 10<sup>4</sup> cells were resuspended in 100 μl of Nucleofector solution T (Lonza) and mixed with 3 μg of each BIM siRNA, or 6 μg of NT siRNA. Cells were transfected by electroporation with the Nucleofector Device (Amaxa) and protocol T-020. Cells were then plated out for subsequent experimental procedures. For HCT116 transfection, siRNA oligonucleotides were mixed with Opti-MEM<sup>®</sup> (Invitrogen), were provided by Judith Johnson (Institute of Immunology, University of Munich, Munich, Germany). Cells were cultured in medium comprising Dulbecco’s modified Eagle’s medium (HCT116, SW837 and A375 cells), RPMI 1640 (COLO205 and MelJuso cells) or McCoy’s 5A (HT29 cells) supplemented with glucose (4.5 mg/ml), penicillin (100 units/ml), streptomycin (100 mg/ml), 2 mM glutamine and 10% (v/v) [5% (v/v) for MelJuso cells] fetal bovine serum, and routinely tested for mycoplasma infection every 2–3 months (all tests were negative).

Preparation of cell extracts and Western blotting

Cells were lysed in ice-cold TG lysis buffer, assayed for protein content and fractionated by SDS/PAGE as described previously [24,25].

Flow cytometry

Distribution of cells in G<sub>1</sub>, S or G<sub>2</sub>/M and dead cells (sub-G<sub>1</sub>) was determined by PI (propidium iodide) staining and flow cytometry [25].

Colony formation assays

HCT116 and HT29 cells were seeded in 12-well plates at 200 cells per well, allowed to settle for 24 h and then treated as indicated in the Figure legends. The medium was replaced with fresh medium each week for the duration of the assay, after which colonies were stained with Crystal Violet to visualize colony growth and counted.

Immunoprecipitation

Immunoprecipitation of BAX from CHAPS lysates with the N-20 conformationally active BAX antibody has been described previously [26]. For BCL-XL and MCL1 immunoprecipitation, cells were lysed in TG lysis buffer as described previously [27] and immunoprecipitated with anti-BCL-XL antibody (Cell Signalling Technology; 2762) and anti-MCL1 antibody (Santa Cruz Biotechnology; sc-819) using Protein A–Sepharose beads.

Cells and cell culture

COLO205 and HT29 cells (both BRAF<sup>V600E</sup>) were obtained from the A.T.C.C. (Manassas, VA, U.S.A.). HCT116 (KRAS<sup>G13D</sup>) cells were provided by Bert Vogelstein and Richard Youle (NIH, Bethesda, MD, U.S.A.). SW837 (KRAS<sup>G12C</sup>) cells were provided by AstraZeneca. A375 (BRAF<sup>V600E</sup>) cells were obtained from Richard Marais (Institute of Cancer Research, Belmont, U.K.) and MelJuso (HRAS<sup>G12C</sup> and NRAS<sup>Q61L</sup>) cells were provided by Judith Johnson (Institute of Immunology, University of Munich, Munich, Germany). Cells were cultured in medium comprising Dulbecco’s modified Eagle’s medium (HCT116, SW837 and A375 cells), RPMI 1640 (COLO205 and MelJuso cells) or McCoy’s 5A (HT29 cells) supplemented with glucose (4.5 mg/ml), penicillin (100 units/ml), streptomycin (100 mg/ml), 2 mM glutamine and 10% (v/v) [5% (v/v) for MelJuso cells] fetal bovine serum, and routinely tested for mycoplasma infection every 2–3 months (all tests were negative).
and an equivalent volume of Opti-MEM® was combined with DharmaFECT2 reagent (Dharmacon) and incubated for 5 min. siRNA and DharmaFECT2 mixes were then combined and incubated for 20 min. siRNA–DharmaFECT2 complexes were then added to HCT116 cells with a final siRNA concentration of 12.5 nM (for each BIM siRNA) or 25 nM PUMA siRNA, and incubated for 48 h before subsequent experimental procedures.

RESULTS
Selumetinib drives expression of BIM, BMF and PUMA in ERK1/2-addicted tumour cells but fails to elicit cell death

Treatment of CRC or melanoma cells harbouring either BRAF or RAS mutations with selumetinib typically resulted in a G₁ cell cycle arrest (Figure 1A). In the CRC cell lines COLO205 (BRAF^{V600E}) or HCT116 (KRAS^{G13D}), this was accompanied by decreased expression of cyclin D1 and increased expression of p27^kip1 (Figures 1B and 1C). Selumetinib treatment increased the expression of the pro-apoptotic protein BIM<sub>EL</sub> in both cell lines (Figures 1B and 1C). In all experiments selumetinib caused a striking increase in the mobility of BIM<sub>EL</sub> on SDS/PAGE (a shift down the gel) commensurate with the inhibition of ERK1/2 signalling (Figures 1B and 1C, but see also Figures 5 and 6). Indeed, it is well known that ERK1/2 can phosphorylate BIM<sub>EL</sub> on at least three sites to promote its proteasomal degradation [24,26,27], so this increase in mobility reflects the dephosphorylation and stabilization of BIM<sub>EL</sub>. We also found, for the first time, that selumetinib treatment increased the expression of BMF in both COLO205 and HCT116 cells (Figures 1B and 1C). Finally, selumetinib treatment increased the expression of PUMA in HCT116 cells; this was not observed in COLO205 cells where PUMA was not detected. Despite the expression of these three pro-apoptotic BH3-only proteins, selumetinib caused little cell death (cells with sub-G₁ DNA) (Figure 1A). Indeed, selumetinib-resistant variants of COLO205 and HCT116 emerge that can circumvent this G₁ arrest owing to amplification of BRAF^{V600E} or KRAS^{G13D} [15]. In the present study we sought to validate a rational strategy for transforming this cytostatic response into a cell death response in the hope that it would delay the onset of acquired resistance to selumetinib.

Selumetinib and ABT-263 synergize to induce apoptosis of ERK1/2-addicted HCT116 tumour cells with KRAS<sup>G13D</sup>

Many tumour cells exhibit elevated expression of pro-survival proteins such as BCL2 and BCL-XL [21], which may provide excess buffering capacity for the BIM, BMF and PUMA that are induced by selumetinib, thereby preventing cell death. To test this hypothesis we combined selumetinib with ABT-263, a BH3-mimetic BCL2 inhibitor [22,23]. Treatment of HCT116 cells (KRAS<sup>G13D</sup>) with selumetinib or ABT-263 alone resulted in little cell death, but the combination was synthetic lethal, resulting in a striking synergistic increase in cell death (Figures 2A and 2B). This effect was dose-dependent and saturable for both selumetinib (Figure 2A) and ABT-263 (Figure 2B) with EC<sub>50</sub> values of 100–200 nM and 200 nM respectively. The broad spectrum caspase inhibitor Q-VD-OPH abolished cell death, indicating that selumetinib and ABT-263 synergized to induce apoptosis (Figure 2C). Finally, combined selumetinib and ABT-263 treatment severely inhibited the long-term clonogenic survival of HCT116 cells relative to treatment with either drug in isolation (Figure 2D). Thus the combination of selumetinib and ABT-263 was synthetic lethal for HCT116 cells.

Selumetinib and ABT-263 synergize to activate BAX and BAX-dependent apoptosis

To investigate the mechanism underpinning the synthetic lethality between selumetinib and ABT-263 we examined activation of BAX. We used a conformation–specific antibody that recognizes a C-terminal α-helix which is exposed during BAX activation to immunoprecipitate active BAX after mild lysis in CHAPS that conserved the structural integrity of BAX [26]. Harsher lysis conditions (Triton X-100), in which the C-terminal helix of BAX is fully exposed, were used as a positive control. In HCT116 cells, weak BAX activation was observed after treatment with selumetinib or ABT-263 alone, whereas their combination greatly increased BAX activation in a caspase-independent fashion (Figure 3A). Thus selumetinib and ABT-263 synergize upstream of caspases to activate BAX.

To determine whether BAX or BAK were required for cell death, we utilized isogenic clones of HCT116 lacking BAX, BAK or both (DKO) [28] (Figure 3B). BAK knockout in HCT116 cells was unable to protect from apoptosis in response to selumetinib, ABT-263 or the combination of the two. However, BAX-null
HCT116 cells (either BAX−/− or DKO) were almost completely resistant to cell death under these conditions (≤5% apoptosis) (Figure 3C), demonstrating for the first time that BAX was required for a second-generation MEK1/2 inhibitor, selumetinib, to synergize with ABT-263 to kill KRAS mutant tumour cells.

**ABT-263 synergizes with selumetinib in cells with mutant BRAF or RAS, whereas synergy with PLX4720 is confined to cells with mutant BRAF**

Numerous other cell lines exhibited caspase-dependent cell death in response to selumetinib plus ABT-263 (Figure 4A and results not shown). For example, the CRC cell lines COLO205 and HT29 (both BRAFV600E) responded strongly to the combination as did the melanoma cell lines A375 (BRAFV600E) and MelJuso (HRASG12D and NRASQ61L) (Figure 4A). However, some cell lines (e.g. SW837) were intrinsically resistant to this combination (Figure 4A). We have previously shown that SW837 cells (KRASG12C) exhibit vanishingly low levels of phospho-ERK1/2, are not addicted to ERK1/2 signalling for cell proliferation and are therefore insensitive to selumetinib [29]. As with HCT116 cells, selumetinib plus ABT-263 treatment severely inhibited the long-term clonogenic survival of HT29 cells (Figure 4B). We also found for the first time that ABT-263 could greatly enhance tumour cell death in response to the BRAFV600E inhibitor PLX4720 [30] (a close analogue of vemurafenib), but only in those cell lines harbouning BRAFV600E (COLO205, HT29 and A375) and not in HCT116 cells (KRASG12D) (Figure 4C). A combination of PLX4720 with ABT-263 also inhibited clonogenic survival of HT29 cells (Figure 4D).

**BIM plays a pivotal role in apoptosis induced by selumetinib plus ABT-263 in COLO205 cells**

ERK1/2 signalling can regulate the expression, stability and activity of numerous members of the BCL2 protein family [31,32]. Treatment of COLO205 (Figure 5A) and HCT116 (Supplementary Figure S1 at http://www.biochemj.org/bj/450/bj4500285add.htm) with 1 μM selumetinib strongly
BH3 mimetics inhibit acquired resistance to MEK1/2 inhibitors

induced expression of the pro-apoptotic BH3-only proteins BIM and BMF (and PUMA in HCT116 cells), and reduced the expression of the pro-survival MCL1 protein (Figure 5B). More subtle effects on the expression of BCL2 family members were observed upon treatment with ABT-263, most notably the elevated expression of BCL2, BCL-XL and BCL-w, which has been previously reported in COLO205 cells (Figures 1B, 5A and 5C), consistent with profound increases in expression upon selumetinib treatment [33,34]. Since ABT-263 efficiently binds and inhibits the other major pro-survival family members (BCL2, BCL-XL and BCL-w), we reasoned that selumetinib-induced expression of BH3-only proteins that have high affinity for MCL1, such as BIM, might be crucial to the mechanism through which selumetinib and ABT-263 synergize to induce apoptosis. Indeed, BIM underwent a profound increase in expression upon selumetinib treatment in COLO205 cells (Figures 1B, 5A and 5C), consistent with its regulation by the ERK1/2 pathway [18,19,24,26]. siRNA-mediated knockdown of BIM in COLO205 cells reduced PARP cleavage in response to selumetinib plus ABT-263 (Figure 5C) and partially protected against apoptosis (50–70% reduction in apoptotic cells) (Figure 5D), demonstrating for the first time the pivotal role this BH3-only protein plays in the strong apoptotic response to selumetinib plus ABT-263.

To characterize further the mechanism through which BIM, and also potentially BMF, synergize with ABT-263 in COLO205 cells, we immunoprecipitated BCL-X and MCL1 to identify BH3-only protein binding partners (Figure 6A). Inhibition of

Figure 4 ABT-263 synergizes with selumetinib in cells with BRAFV600E or RAS mutations, whereas synergy with PLX4720 is confined to cells with BRAFV600E

(A) The indicated cell lines were treated with 1 μM selumetinib, 1 μM ABT-263 (0.2 μM ABT-263 for COLO205) or both (with or without the caspase inhibitor Q-VD-OPH) for 48 h before being fixed, stained with PI and assayed for sub-G1 DNA content by flow cytometry. The results are means ± S.D. of five independent experiments each performed with cell culture triplicates. (B) A total of 200 HT29 cells per well were treated with 1 μM selumetinib, 1 μM ABT-263 or both for 72 h. The cells were then allowed to grow out into colonies in drug-free medium for 2 weeks when they were visualized with Crystal Violet and counted. Representative results are shown together with pooled results which represent mean ± CoV (coefficient of variance) of five independent experiments each performed with cell culture triplicates. (C) Cell lines were treated with 1 μM PLX4720 (COLO205 and A375) or 5 μM PLX4720 (HT29, HCT116), 1 μM ABT-263 or both (with or without the caspase inhibitor Q-VD-OPH) for 48 h before being fixed, stained with PI and assayed for sub-G1 DNA content by flow cytometry. The results are the means ± S.D. of three experiments combined, each performed with cell culture triplicates. (D) A total of 200 HT29 cells per well were treated 5 μM PLX4720, 1 μM ABT-263 or both for 72 h. The cells were then allowed to grow out into colonies in drug-free medium for 2 weeks when they were visualized with Crystal Violet and counted. Representative results are shown together with pooled results which are the means ± CoV of three independent experiments each performed with cell culture triplicates. QVD, Q-VD-OPH; Sel, selumetinib.

Figure 5 Apoptosis induced by combined selumetinib and ABT-263 requires BIM in COLO205 cells

(A and B) COLO205 cells growing asynchronously in 10% serum were treated with 1 μM selumetinib, 0.2 μM ABT-263 or both (with or without the caspase inhibitor Q-VD-OPH) for 24 h. Whole cell lysates were fractionated by SDS/PAGE and immunoblotted with the indicated antibodies. (C and D) COLO205 cells were transfected with BIM-specific (siBIM) or NT siRNA. Control cells were left untransfected (UT). At 48 h after transfection cells were treated with 1 μM selumetinib, 0.2 μM ABT-263 or both. At 72 h post-transfection, BIM, phospho-ERK1/2, ERK1 and PARP cleavage levels were determined by Western blot analysis (C) and cell death was determined by PI staining and flow cytometry (D). The results are the means ± S.D. of three experiments combined, each with cell culture triplicates. A, ABT-263; C, control; P-, phospho-; S/Sel, selumetinib. Molecular masses in kDa are indicated to the left-hand side of the Western blots.

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MEK1/2 by selumetinib promoted the expression of BIM and BMF, which were found to bind to BCL-XL and MCL1. However, ABT-263 inhibited the selumetinib-induced binding of BIM and BMF to BCL-XL and instead promoted greater binding of these BH3-only proteins to MCL1 (Figure 6A). Performing the reciprocal experiment by immunoprecipitating BIM confirmed these results; ABT-263 disrupted selumetinib-induced binding of BCL-XL to BIM and promoted the accumulation of BIM–MCL1 complexes (Figure 6B). Thus, although ABT-263 cannot directly inhibit MCL1 [22,23], its combination with selumetinib resulted in indirect inhibition of MCL1 by inhibiting BCL-XL and promoting the transfer of BIM and BMF onto MCL1. This mechanism could in principle also free up more BIM to directly activate BAX [37,38]. Very similar results were observed in HCT116 cells (Supplementary Figure S2 at http://www.biochemj.org/bj/450/bj4500285add.htm). However, in these cells MEK1/2 inhibition also strongly induced PUMA expression (Supplementary Figure S2), which, like BIM and BMF, accumulated on MCL1 in the presence of the selumetinib plus ABT-263 combination. Knockdown of BIM and PUMA in HCT116 cells was unable to protect from apoptosis induced by selumetinib plus ABT-263, suggesting the involvement of other redundant BH3-only proteins induced by selumetinib in this setting (Supplementary Figure S3 at http://www.biochemj.org/bj/450/bj4500285add.htm).

ABT-263 delays acquired resistance to selumetinib and PLX4720

In the long term, tumour cells evolve to overcome the $G_1$ arrest induced by selumetinib and resistant derivatives emerge and prevail [14–16]. To assess whether ABT-263 could delay acquired resistance to selumetinib, we performed long-term colony-forming assays on HCT116 and HT29 cells, two cell lines that we have previously used to study acquired resistance [15]. In the first instance these treatments or a DMSO control were applied for 72 h and then replaced with selumetinib-only medium for 4 weeks (HCT116 cells) or 6 weeks (HT29 cells) to assess the frequency of selumetinib-resistant colonies. For both cell lines, 72 h treatment with selumetinib or ABT-263 had very little effect on the number and size of selumetinib-resistant colonies relative to the DMSO control (Figure 7A). In contrast, treatment with selumetinib plus ABT-263 for 72 h drastically reduced the frequency of selumetinib-resistant colonies (∼15 % of the DMSO control in HCT116 cells, ∼10 % in HT29 cells) (Figure 7A and Supplementary Figure S4A at http://www.biochemj.org/bj/450/bj4500285add.htm). Similarly, treating HT29 cells (BRAFv600E) with PLX4720 plus ABT-263 for 72 h greatly inhibited the formation of PLX4720-resistant colonies relative to DMSO control, PLX4720 or ABT-263 individual treatments (Figure 7B and Supplementary Figure S4B).

We next examined the clonogenic survival of HCT116 and HT29 cells treated continuously with selumetinib, ABT-263 or selumetinib plus ABT-263 (Figure 7C and Supplementary Figure S4C). After 2 weeks colonies were clearly apparent under control conditions, as well as in those cells treated continually with ABT-263 alone. Although ABT-263 had little effect on colony size (Supplementary Figure S4C), colony number was reduced to ∼60 % of control in HCT116 cells, and 75 % in HT29 cells. Continual treatment with selumetinib resulted in the outgrowth of even fewer colonies in HCT116 and HT29 cells (∼50 %). However, we observed a striking inhibition of clonogenic survival (∼5 % relative to control) in HCT116 and HT29 cells treated continually with selumetinib plus ABT-263. These results demonstrate that initial treatment with selumetinib plus ABT-263 can strongly inhibit the subsequent outgrowth of colonies with acquired resistance to selumetinib, and that continual treatment with this combination profoundly inhibits tumour cell survival and colony formation. Similar results were obtained when HT29 cells were continually treated with PLX4720 plus ABT-263, which resulted in a >95 % inhibition of clonogenic survival (Figure 7D and Supplementary Figure S4D).

Selumetinib-resistant COLO205 and HT29 cells are sensitized to ABT-263-induced apoptosis, whereas selumetinib-resistant HCT116 cells are not

COLO205 cells growing asynchronously in 10 % serum were treated with 1 μM selumetinib, 0.2 μM ABT-263 or 1 μM selumetinib plus 0.2 μM ABT-263. After 24 h, whole-cell extracts (input) were prepared and used for immunoprecipitation with antibodies against BCL-XL or MCL1 (A) or BIM (B). Input and immunoprecipitated (IP) samples were fractionated by SDS/PAGE and immunoblotted with the indicated antibodies. C, control; P-, phospho-; QVD, Q-VD-OPH; Sel, selumetinib. Molecular masses in kDa are indicated to the left-hand side of the Western blots.

Selumetinib-resistant COLO205 cells growing asynchronously in 10 % serum were treated with 1 μM selumetinib, 0.2 μM ABT-263 or 1 μM selumetinib plus 0.2 μM ABT-263. After 24 h, whole-cell extracts (input) were prepared and used for immunoprecipitation with antibodies against BCL-XL or MCL1 (A) or BIM (B). Input and immunoprecipitated (IP) samples were fractionated by SDS/PAGE and immunoblotted with the indicated antibodies. C, control; P-, phospho-; QVD, Q-VD-OPH; Sel, selumetinib. Molecular masses in kDa are indicated to the left-hand side of the Western blots.

Selumetinib-resistant COLO205 cells are sensitized to ABT-263-induced apoptosis, whereas selumetinib-resistant HCT116 cells are not
three independent experiments each performed with cell culture triplicates. Sel, selumetinib.

\[ B \approx \pm \] means was then assessed by visualizing with Crystal Violet and counting. In (A) well were treated with 5 \( \mu \)M D growth medium (1 strain) also exhibited hypersensitivity to ABT-263 in their normal resistant HT29 cells (HT6244-R, with enhanced BRAF expression) suppress ABT-263-mediated apoptosis. Similarly, selumetinib-263 except at high doses; thus hyperactivation of ERK1/2 can the ERK1/2 pathway [15] and this resulted in resistance to ABT-263 doses, relative to parental COLO205 cells (Figure 8A). The ERK1/2 pathway. Thus, even though C6244-R and HT6244-R selumetinib was withdrawn from HT6244-R cells to hyperactivate HT29 cells (Figure 8B); this priming event was again lost when selumetinib displayed ABT-263 or both for 72 h. The medium was then removed from all wells and replaced with medium containing 5 \( \mu \)M PLX4720. After 6 weeks selection in PLX4720, the frequency of selumetinib-resistant colonies was assessed by visualizing with Crystal Violet and colony counting.

C6244-R cells; indeed, C6244-R cells maintained in 1 \( \mu \)M selumetinib displayed \( \approx \)1.5–2-fold sensitization to a range of ABT-263 doses, relative to parental COLO205 cells (Figure 8A). Withdrawal of selumetinib from C6244-R cells strongly activates the ERK1/2 pathway [15] and this resulted in resistance to ABT-263 except at high doses; thus hyperactivation of ERK1/2 can suppress ABT-263-mediated apoptosis. Similarly, selumetinib-resistant HT29 cells (HT6244-R, with enhanced BRAF expression [15]) also exhibited hypersensitivity to ABT-263 in their normal growth medium (1 \( \mu \)M selumetinib), with 4 \( \mu \)M ABT-263 killing \( \approx \)35% HT6244-R cells compared with \( \approx \)15% for parental HT29 cells (Figure 8B); this priming event was again lost when selumetinib was withdrawn from HT6244-R cells to hyperactivate the ERK1/2 pathway. Thus, even though C6244-R and HT6244-R cells have evolved to reinstate ERK1/2 activity and proliferation, they remain sensitive to ABT-263 and may even be somewhat ‘primed’ for death induced by the BH3 mimic.

HT116 cells acquire resistance to selumetinib (H6244-R) through amplification of their driving oncogene, KRAS\(^{G13D}\), which, as with C6244-R and HT6244-R cells, serves to reinstate ERK1/2 pathway signalling [15]. However, in contrast with C6244-R and HT6244-R cells, H6244-R cells were not primed for death induced by ABT-263; rather, these cells were cross-resistant to ABT-263 even at high doses of the BH3 mimic (Figure 8C). H6244-R cells with amplified KRAS\(^{G13D}\) also exhibit elevated PKB activity [15] (Figure 8D), a kinase that promotes cell survival. We therefore reasoned that the cross-resistance to ABT-263 in H6244-R cells was probably mediated by increased
PI3K/PKB-dependent signalling. However, even when H6244-R cells (in 2 μM selumetinib) were treated with a combination of ABT-263 and the PI3K/mTOR inhibitor AZ12321046 [15,29] (Figure 8D), this failed to overcome cross-resistance to ABT-263 (Figure 8C).

Thus, in addition to inhibiting the onset of acquired resistance to ERK1/2 pathway inhibitors, ABT-263 can overcome established selumetinib resistance in cells with BRAFV600E amplification, but this is not seen in cells where selumetinib resistance is driven by KRASG12D amplification.

DISCUSSION

The present study was prompted by our recent demonstration of rapid acquired resistance to selumetinib [15] and a previous study, which demonstrated that ABT-737 (a precursor of ABT-263) could co-operate with MEK inhibitors to kill BRAF-mutant tumour cells [19]. We have extended these studies to show for the first time that synthetic lethality: (i) between MEK1/2 inhibitors and BH3 mimetics is BAX- and BIM-dependent; (ii) is also seen in tumour cells with RAS mutations and (iii) also applies to BRAFV600E-selective inhibitors in tumour cells with BRAFV600E. Indeed, the synergy between PLX4720 and ABT-263 in HT29 and COLO205 cells is all the more significant since a recent study has demonstrated that CRC cells with BRAFV600E are intrinsically resistant to vemurafenib/PLX4032 when compared with BRAFV600E mutant melanoma [39]. Our results with the related molecule, PLX4720, show that its combination with ABT-263 effectively overcomes intrinsic resistance to BRAFV600E-specific inhibitors, resulting in 60–80% cell death of BRAFV600E-positive CRC cells. Thus combined inhibition of ERK1/2 signalling and Bcl-2 survival proteins is an attractive option in ERK1/2-addicted tumour cells with the choice of MEK1/2 inhibitor or BRAF inhibitor being determined by the nature of the driving oncoprotein.

ERK1/2 inhibition synergizes with ABT-263 to promote BIM-dependent apoptosis

Selumetinib caused a striking increase in expression of BIM in COLO205 and HCT116 cells; PUMA was also induced in HCT116 cells, indicating that these cells were addicted to ERK1/2 signalling to repress these pro-death proteins. In addition we observed a strong increase in BMF expression and its binding to MCL1 in both COLO205 and HCT116 cells following treatment with selumetinib. Although BMF is induced by TGF-β (transforming growth factor-β) [40,41], we believe this is the first time that inducible BMF expression has been demonstrated in response to selumetinib or any other second-generation MEK1/2-specific inhibitor that specifically inhibits ERK1/2 signalling. Of note, a recent study reported increased BIK expression upon ERK1/2 inhibition [42]; however, we repeatedly failed to observe any increase in BIK expression or increased BIK binding to Bcl-XL or MCL1 with selumetinib (Figures 5A and 6 and Supplementary Figure S1). We also noted that selumetinib reduced the expression of NOXA in both COLO205 (Figure 5A) and HCT116 (Supplementary Figure S1) cells, consistent with reports that this BH3-only protein is expressed rather than repressed by ERK1/2 signalling [43,44].

Although BIM, BMF and PUMA were the main BH3-only proteins induced by ERK1/2 pathway inhibition, their expression was not sufficient to induce apoptosis in several ERK1/2-addicted cell lines. However, selumetinib synergized strikingly with ABT-263 to kill cells with BRAFV600E or RAS mutations. Although the early-stage compound ABT-737 has previously been shown to co-operate with MEK inhibitors in BRAF mutant cells [19], this is the first demonstration that this mechanism also applies to CRC or melanoma cells with RAS mutations. Similar synergy was observed when we combined the BRAFV600E inhibitor PLX4720 with ABT-263, although this was confined to cells with BRAFV600E (COLO205, HT29 and A375). This represents the first demonstration that BRAFV600E-selective inhibitors can combine with BCL2 inhibitors to kill mutant BRAF CRCs and melanoma cells.

Several observations suggest that selumetinib (or PLX4720) and ABT-263 are acting ‘on target’ in these studies. First, the cells that exhibited the most robust cell death (COLO205, HT29, A375 and HCT116) were ‘ERK1/2 addicted’ as judged by their sensitivity to selumetinib or PLX4720 (or vemurafenib) [15,18,29,45]; in contrast, SW837 cells, which are not addicted to ERK1/2 signalling [29], did not respond to the combination. Thus ERK1/2 pathway addiction underpins the synergy with ABT-263. Secondly, synergy between PLX4720 and ABT-263 was confined to cells with BRAFV600E [4]. Thirdly, we show for the first time that synergy between selumetinib and ABT-263 required BAX, which also suggests that cell death was apoptotic. Finally, the protection afforded by caspase inhibition defined cell death as being apoptotic. Thus the combination of ERK1/2 pathway inhibitors with ABT-263 is synthetic lethal for a range of tumour cell lines that are addicted to ERK1/2 signalling and is dependent upon BAX.

Knockdown of BIM can protect against tumour cell death in response to MEK1/2 inhibitors in the presence and absence of growth factors [18,19]. In the present study we demonstrate for the first time that BIM contributes significantly to apoptosis induced by selumetinib plus ABT-263. A previous study demonstrated that ABT-737 could co-operate with the first generation pan-MEK inhibitor U0126 to kill tumour cells, but failed to demonstrate a requirement for BIM [19], most likely reflecting off-target effects of U0126 [46,47]. In our experiments some apoptosis still occurred in the absence of BIM, suggesting that alternative BH3-only proteins, or the down-regulation of pro-survival factors, probably play a role. Indeed, siRNA-mediated knockdown of BIM and/or PUMA in HCT116 cells did not protect against apoptosis induced by selumetinib plus ABT-263. Given that this apoptosis was BAX-dependent, we suggest that there is substantial redundancy between the three BH3-only proteins (BIM, BMF and PUMA) induced by selumetinib that synergize with ABT-263 in HCT116 cells, whereas COLO205 cells (BIM and BMF) represent a somewhat simpler system.

Taken together, these results suggest that, although MEK1/2 inhibition may result in increased expression of BIM, BMF and PUMA and their binding to BCL-XL and MCL1, this results in little cell death, probably due to residual pro-survival activity, including BCL-XL and MCL1 (Supplementary Figure S5A at http://www.biochemj.org/bj/450/bj4500285add.htm). Our analysis of BCL-XL and MCL1 immunoprecipitates suggests that selumetinib plus ABT-263 causes a striking re-distribution of BIM, BMF and PUMA from ABT-263-sensitive BCL-XL to the ABT-263-resistant MCL1 (Supplementary Figure S5B). So although ABT-263 cannot directly inhibit MCL1 [22,23], its combination with selumetinib results in indirect inhibition of MCL1. These two events combine to result in greater inhibition of the cellular pool of pro-survival proteins (Supplementary Figure S5C). In addition, ABT-263 displaces BAX from BCL-XL more efficiently than selumetinib alone (Supplementary Figure S2), and thus under conditions of selumetinib plus ABT-263 BAX could potentially be directly activated by any residual BIM freed up from BCL-XL.
Combination with ABT-263 inhibits acquired resistance to selumetinib

Since tumour cells can acquire resistance to selumetinib [15], we investigated the effects of ABT-263 on the incidence of acquired resistance that had not previously been examined. The striking inhibition of clonogenic survival observed with selumetinib and ABT-263 translated into a strong inhibition of acquired resistance to selumetinib. Whether cells were treated with the selumetinib plus ABT-263 combination transiently followed by selumetinib selection (Figure 7A) or were selected continuously in the presence of selumetinib plus ABT-263 (Figure 7C), we observed a 90–95% reduction in the outgrowth of selumetinib-resistant colonies. This was seen regardless of the driving oncogene, in both HCT116 cells (KRASG13D) and HT29 cells (BRAFV600E) that otherwise rapidly adapt to become 100-fold resistant to selumetinib [15]. The simplest conclusion from these studies is that selumetinib plus ABT-263 delays acquired resistance by providing a much more stringent selection pressure, greatly increasing primary tumour cell death so that few cells remain viable to develop resistance. Thus the selumetinib plus ABT-263 combination may be an attractive clinical option both by increasing primary efficacy and by reducing or delaying acquired resistance. Notably, the same results were observed with PLX4720 and ABT-263 in HT29 cells (BRAFV600E) (Figures 7B and 7D), suggesting that combination with ABT-263 may also delay the onset of acquired resistance to BRAFV600E-selective inhibitors.

Selumetinib-resistant cells with BRAFV600E amplification remain sensitive to ABT-263, whereas amplification of KRASG13D confers resistance to ABT-263

In addition to unleashing selumetinib-resistant tumour cell killing and delaying selumetinib resistance, we found that ABT-263 could also kill tumour cells that had already developed acquired resistance to selumetinib. Indeed, selumetinib-resistant derivatives of COLO205 and HT29, (where resistance is driven by increased expression of BRAFV600E [15]) both exhibited 1.5–2-fold greater ABT-263-induced death than their parental counterparts, suggesting that they are somewhat ‘primed’ for ABT-263-induced death. The mechanism for this priming event is currently unclear and will form the focus of future work. The priming effect was lost when we withdrew selumetinib from these cells, probably as a result of the strong reactivation of ERK1/2 observed under these conditions [15]. Critically, this suggests that ABT-263 could potentially be used as part of an approach to treat cells that have already acquired selumetinib resistance through amplification of BRAFV600E.

In contrast with selumetinib-resistant cells with BRAFV600E amplification, selumetinib-resistant HCT116 cells (KRASG13D amplification) were cross-resistant to ABT-263 and this applied even in the presence of selumetinib and a PI3K inhibitor. We previously demonstrated that, although amplification of KRASG13D drives acquired resistance to selumetinib in H6244-R cells, combined inhibition of the ERK1/2 and PI3K pathways could not overcome this resistance [15]. These new results suggest that other KRAS-dependent effector pathways may also be involved in driving resistance to ABT-263 or that resistance is driven by some stable epigenetic change in these cells. Regardless, these results suggest that, although ABT-263 may be used as part of an up-front combination with selumetinib in cells with Ras mutations that are ERK1/2 addicted, it may be ineffective in treating established selumetinib resistance arising through KRAS amplification.

ABT-263 has shown some efficacy as a single agent in chronic lymphocytic leukaemia [48], but limited single-agent activity against advanced and recurrent small-cell lung cancer [49], suggesting that it will be best employed in combination with other agents. The results of the present study show that the ERK1/2 pathway inhibitors selumetinib and PLX4720 cause a strong increase in expression of the BH3-only proteins BIM, BMF, and PUMA, but this is not sufficient to promote tumour cell death. However, it lowers the threshold of sensitivity to ABT-263 so that the combination is synthetic lethal, resulting in a striking increase in cell death and delaying the onset of acquired resistance to these ERK1/2 pathway inhibitors. This validates the use of an ERK1/2 pathway inhibitor and ABT-263 as an up-front combination in BRAFMut or RASMut tumour cells addicted to ERK1/2 signalling. In addition, ABT-263 may be effective in the treatment of tumour cells with established selumetinib resistance driven by BRAFV600E amplification, but this will not apply in cases of resistance driven by amplification of KRASMut.

AUTHOR CONTRIBUTION

Matthew Sale and Simon Cook conceived and designed the study. Matthew Sale performed all of the experiments, analysed all of the results and prepared the Figures. Matthew Sale and Simon Cook co-wrote the paper.

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SUPPLEMENTARY ONLINE DATA

The BH3 mimetic ABT-263 synergizes with the MEK1/2 inhibitor selumetinib/AZD6244 to promote BIM-dependent tumour cell death and inhibit acquired resistance

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Figure S1 Selumetinib induces BIM, BMF and PUMA expression in HCT116 cells

HCT116 cells growing asynchronously in 10% serum were treated with 0, 0.1, 0.5 or 1 μM ABT-263 in the absence or presence of 1 μM selumetinib (with or without 10 μM Q-VD-OPH) for 24 h. Whole-cell lysates were fractionated by SDS/PAGE and immunoblotted with the indicated antibodies. P-, phospho-; QVD, Q-VD-OPH; Sel, selumetinib. Molecular masses in kDa are indicated to the left-hand side of the Western blots.

Figure S2 Combined selumetinib and ABT-263 induces greater binding of BIM, BMF and PUMA to MCL1

HCT116 cells growing asynchronously in 10% serum were treated with 2 μM selumetinib, 1 μM ABT-263 or 2 μM selumetinib plus 1 μM ABT-263. After 24 h, whole-cell extracts (input) were prepared and used for immunoprecipitation (IP) with antibodies against BCL-XL or MCL1. Input and immunoprecipitation samples were fractionated by SDS/PAGE and immunoblotted with the indicated antibodies. C, control; P-, phospho-; Sel, selumetinib. Molecular masses in kDa are indicated to the left-hand side of the Western blots.

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Figure S3 Knockdown of BIM and PUMA does not prevent apoptosis induced by combined selumetinib and ABT-263 treatment in HCT116 cells

HCT116 cells were either left untreated (UT), treated with vehicle only (FECT2), or transfected with non-targeting (NT), BIM-specific (siBIM), PUMA-specific (siPUMA), or BIM-specific and PUMA-specific (siBIM + siPUMA) siRNA. At 48 h after transfection, cells were treated with 2 μM selumetinib, 1 μM ABT-263 or both. At 72 h post-transfection, cell death was determined by PI staining and flow cytometry (A) and BIM, PUMA, ERK1 and PARP cleavage levels were determined by Western blot analysis (B). Results were taken from a single experiment which was representative of two experiments. A, ABT-263; C, control; S/Sel, selumetinib. Molecular masses in kDa are indicated to the left-hand side of the Western blots.

Figure S4 Combination of selumetinib or PLX4720 with ABT-263 greatly inhibits the subsequent outgrowth of selumetinib- and PLX4720-resistant colonies

(A) A total of 200 HCT116 cells per well were treated with 2 μM selumetinib, 1 μM ABT-263 or both for 72 h. These treatments were then removed and the medium was replaced with medium containing 2 μM selumetinib. After 4 weeks selection in selumetinib, the frequency of selumetinib-resistant colonies was assessed by visualizing with Crystal Violet and colony counting. A representative image is shown of ten independent experiments. (B) A total of 200 HT29 cells per well were treated with 5 μM PLX4720, 1 μM ABT-263 or both for 72 h. The medium was then removed from all wells and replaced with medium containing 5 μM PLX4720. After 6 weeks selection in PLX4720, the frequency of PLX4720-resistant colonies was assessed by visualizing with Crystal Violet and colony counting. The results shown are a representative image of three independent experiments. (C) A total of 200 HCT116 cells plated per well were treated with 2 μM selumetinib, 1 μM ABT-263 or both for a total of 2 weeks. After this period colonies were visualized with Crystal Violet and counted. A representative image is shown of four independent experiments. (D) A total of 200 HT29 cells plated per well were treated with 5 μM PLX4720, 1 μM ABT-263 or both for a total of 2 weeks. Colony formation was then assessed by visualizing with Crystal Violet and counting. Results shown are a representative image of three independent experiments. Con, control; Sel, selumetinib.
BH3 mimetics inhibit acquired resistance to MEK1/2 inhibitors

Figure S5  A model describing how selumetinib and ABT-263 may synergize to promote cell death in ERK1/2-addicted tumour cell lines

Treatment of tumour cells such as COLO205 and HCT116 with the MEK1/2 inhibitor selumetinib strongly induces expression of the BH3-only proteins BIM and BMF (A, top). PUMA expression may also be induced, as observed in HCT116 cells. Immunoprecipitation of Bcl-xL and MCL1 demonstrates that BIM and BMF (and PUMA) induced by selumetinib bind to both of these pro-survival factors (A, bottom). Despite this, little cell death occurs with MEK1/2 inhibition alone, probably due to residual pro-survival activity, including BCL-XL and MCL1 (A, bottom). Addition of the BH3 mimetic ABT-263 (red triangle; B, top) causes a redistribution of selumetinib-induced BIM and BMF from ABT-263-sensitive BCL-XL to the ABT-263-resistant MCL1 (B, bottom). Thus, although ABT-263 cannot directly target MCL1, its combination with selumetinib results in indirect inhibition of MCL1 and consequently greater inhibition of pro-survival proteins (C, top). In addition, whereas selumetinib only resulted in partial displacement of BAX from BCL-XL in HCT116 cells, ABT-263 efficiently disrupted this interaction. BAX could then potentially be directly activated by any residual BIM freed up from BCL-XL (C, top). Activated BAX can subsequently oligomerize and insert into the outer mitochondrial membrane, resulting in mitochondrial outer membrane permeabilization (MOMP) (C, bottom). This allows cytochrome c release from the intermembrane space, followed by apoptosome formation, caspase activation and consequent apoptosis.

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