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REVIEW

That which does not kill me makes me stronger; combining ERK1/2 pathway inhibitors and BH3 mimetics to kill tumour cells and prevent acquired resistance

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Oncogenic mutations in *RAS* or *BRAF* can drive the inappropriate activation of the ERK1/2. In many cases, tumour cells adapt to become addicted to this deregulated ERK1/2 signalling for their proliferation, providing a therapeutic window for tumour-selective growth inhibition. As a result, inhibition of ERK1/2 signalling by BRAF or MEK1/2 inhibitors is an attractive therapeutic strategy. Indeed, the first BRAF inhibitor, vemurafenib, has now been approved for clinical use, while clinical evaluation of MEK1/2 inhibitors is at an advanced stage. Despite this progress, it is apparent that tumour cells adapt quickly to these new targeted agents so that tumours with acquired resistance can emerge within 6–9 months of primary treatment. One of the major reasons for this is that tumour cells typically respond to BRAF or MEK1/2 inhibitors by undergoing a G1 cell cycle arrest rather than dying. Indeed, although inhibition of ERK1/2 invariably increases the expression of pro-apoptotic BCL2 family proteins, tumour cells undergo minimal apoptosis. This cytostatic response may simply provide the cell with the opportunity to adapt and acquire resistance. Here we discuss recent studies that demonstrate that combination of BRAF or MEK1/2 inhibitors with inhibitors of pro-survival BCL2 proteins is synthetic lethal for ERK1/2-addicted tumour cells. This combination effectively transforms the cytostatic response of BRAF and MEK1/2 inhibitors into a striking apoptotic cell death response. This not only augments the primary efficacy of BRAF and MEK1/2 inhibitors but delays the onset of acquired resistance to these agents, validating their combination in the clinic.

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Abbreviations

A1, BCL2-related protein A1; AP1, activator protein 1; APAF1, apoptotic peptidase activating factor 1; ARAF, v-raf murine sarcoma 3611 viral oncogene homologue; BAD, BCL-X_L/BCL2-associated death promoter; BAK, BCL2 homologous antagonist/killer; BAX, BCL2-associated x protein; BCL2, B-cell lymphoma 2; BCL-X_L, B-cell lymphoma extra large; BH3, BCL2 homology domain 3; BID, BH3 interacting domain death agonist; BIK, BCL2-interacting killer; BIM, BCL2-interacting mediator of cell death; BMF, BCL2-modifying factor; BRAF, v-raf murine sarcoma viral oncogene homologue B1; CCND1, cyclin D1; CDC25A, cell division cycle 25 homologue A; CDK, cyclin-dependent kinase; CIP1, CDK-interacting protein 1; CRAF, v-raf-1 murine leukaemia viral oncogene homologue 1; CREB, cAMP responsive element binding protein; DKO, double knockout; EGFR, epidermal growth factor receptor; CRC, colorectal cancer; ELK1, ETS-like gene 1; EMT, epithelial-mesenchymal transition; ETS, v-ets erythroblastosis virus E26 oncogene homologue; FOXO3, forkhead box O 3; G1, growth phase 1; GSK3, glycogen synthase kinase 3; HIV, human immunodeficiency virus; HRAS, v-Ha-ras Harvey rat sarcoma viral oncogene homologue; HRK, harakiri, BCL2

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interacting protein (contains only BH3 domain); KIP1, kinase inhibitory protein 1; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homologue; MCL1, myeloid cell leukaemia 1; MDM2, Mdm2, p53 E3 ubiquitin protein ligase homologue (mouse); MEK, MAPK or ERK kinase; MOMP, mitochondrial outer membrane permeabilization; MSK, mitogen- and stress-activated protein kinase; mTOR, mammalian target of rapamycin; MYC, v-myc myelocytomatosis viral oncogene homologue (avian); NRAS, neuroblastoma RAS viral (v-ras) oncogene homologue; NSCLC, non-small cell lung cancer; OMM, outer mitochondrial membrane; PDGFR, platelet-derived growth factor receptor; PI3K, phosphoinositide 3-kinase; PUMA, p53-up-regulated modulator of apoptosis; RAS, rat sarcoma virus oncogene; RB, retinoblastoma protein; RNAi, RNA interference; RSK, ribosomal protein S6 kinase; RTK, receptor tyrosine kinase; SCLC, small cell lung cancer; ZEB1, zinc finger E-box binding homeobox 1

Introduction

The RAS-regulated RAF-MEK1/2-ERK1/2 signalling cascade is frequently hyperactivated in human cancers due to mutations in *BRAF*, *RAS* or receptor tyrosine kinases (RTKs). Activating *BRAF* mutations, typically BRAF^{V600E}, are found in 60% of melanomas, 30% of thyroid cancers, 10% of colorectal cancers (CRCs) (Davies *et al.*, 2002; Xing, 2005) and almost all hairy cell leukaemias (Tiacci *et al.*, 2011). *RAS* is the most commonly mutated oncogene in human cancers, being detected in around 90% of pancreatic cancers, 40% of CRC, 20% non-small cell lung cancers (NSCLCs) and 15% of melanomas (Downward, 2003). Tumour cells with mutations that activate ERK1/2 frequently exhibit a high dependence upon, or addiction to, this signalling cascade for proliferation and tumourigenesis (Solit *et al.*, 2006; Davies *et al.*, 2007), and thus targeting components of this pathway is an attractive therapeutic strategy. Accordingly, numerous small molecule inhibitors of RAF and MEK1/2 have been developed. The most advanced of these is the RAF inhibitor vemurafenib (Zelboraf, PLX4032, RG7204), which has been approved for the treatment of melanoma (Bollag *et al.*, 2012). Vemurafenib is effective at inhibiting ERK1/2 in cells expressing mutant BRAF (typically BRAF^{V600E}); however, in cells expressing wild-type BRAF or mutant RAS, RAF inhibitors actually activate ERK1/2 signalling (Hatzivassiliou *et al.*, 2010; Heidorn *et al.*, 2010; Joseph *et al.*, 2010; Poulikakos *et al.*, 2010). Mutant BRAF^{V600E} signals as a RAF-inhibitor sensitive monomer, but conditions that promote RAF dimerization, such as RAS activation, induce ‘paradoxical activation’ of ERK1/2 signalling. Binding of one protomer to a RAF inhibitor within a RAF dimer has been postulated to allosterically transactivate the other protomer, resulting in activation of ERK1/2 (Hatzivassiliou *et al.*, 2010; Poulikakos *et al.*, 2010). This suggests that in BRAF^{V600E}-positive tumours, RAS activity is too low to support the formation of RAF dimers, and consequently BRAF^{V600E} signals as a RAF-inhibitor sensitive monomer (Lito *et al.*, 2012). This selectivity for mutant BRAF means that RAF inhibitors like vemurafenib have a broad therapeutic index but are limited to mutant BRAF-positive cancers. In addition, the activation of ERK1/2 signalling by vemurafenib in cells expressing wild-type BRAF is thought to be responsible for the frequent and often rapid development of previously unsuspected *RAS*-mutant cutaneous squamous cell carcinoma (SCC) in patients treated with vemurafenib (Su *et al.*, 2012a), as well as recently identified cases of *RAS*-mutant CRC, colonic adenomas, gastric polyps and leukaemia (Andrews *et al.*, 2012; Callahan *et al.*, 2012; Chapman *et al.*, 2012). These effects are not restricted to vemurafenib and have been observed with other BRAF and RAF inhibitors, such as dabrafenib and sorafenib (Anforth *et al.*, 2012; Arnault *et al.*, 2012). Consequently, ‘paradox breaker’ RAF inhibitors that do not induce paradoxical activation of ERK1/2 in cells with high RAS activity are in development (Ma *et al.*, 2011; Le *et al.*, 2013). One such inhibitor, PLX7904 (or paradox breaker 04), inhibited ERK1/2 as effectively as PLX4720 (a close structural analogue of vemurafenib) in *BRAF*-mutant melanoma cells, but unlike PLX4720 did not hyperactivate ERK1/2 in melanoma cells expressing mutant NRAS or SCC cells expressing mutant HRAS. In addition, PLX7904 was effective against *BRAF*-mutant melanoma cells with acquired resistance to vemurafenib mediated by secondary mutation in *NRAS* (Le *et al.*, 2013).

In contrast to vemurafenib, MEK1/2 inhibitors, such as selumetinib (AZD6244; ARRY-142886), do not induce paradoxical activation of ERK1/2 signalling and are effective against a broader spectrum of tumours, including those driven by mutations in *BRAF*, *KRAS* or RTKs. However, the broader action of MEK1/2 inhibitors may result in a narrower therapeutic window when compared with RAF inhibitors that target mutant BRAF only. In contrast to the majority of kinase inhibitors, MEK1/2 inhibitors like selumetinib do not compete with ATP, but instead bind to an allosteric pocket within MEK1/2 (Davies *et al.*, 2007). This is thought to account for the exquisite selectivity of selumetinib for MEK1/2 in preclinical studies, in which ~40 other kinases (including the closely related MEK5) were not substantially inhibited at 10 μM (Yeh *et al.*, 2007).

Clinical trials have been conducted, or are ongoing, with several MEK1/2 inhibitors, including selumetinib. Phase II trials assessing the efficacy of selumetinib in patients with advanced NSCLC, CRC and pancreatic cancer who had failed first line therapy showed little or no advantage to selumetinib monotherapy versus standard treatment course (Hainsworth *et al.*, 2010; Bennouna *et al.*, 2011; Bodoky *et al.*, 2012). In addition, no significant difference in progression free survival was observed in patients with chemotherapy-naïve advanced melanoma undergoing treatment with selumetinib versus temozolomide (Kirkwood *et al.*, 2012). However, this study and a study of the efficacy of selumetinib in iodine-refractory papillary thyroid cancer indicated that future trials should focus on patients with *BRAF* mutations (Hayes *et al.*, 2012). Greater success has been achieved using selumetinib in combination with other agents. In a phase II trial of *KRAS*-mutant advanced NSCLC, selumetinib in combination with docetaxel improved median overall and progression free survival relative to placebo plus docetaxel by approximately twofold (Jänne *et al.*, 2012). There is also evidence that the clinical

sensitivity of *BRAF*-mutant metastatic melanoma is enhanced when selumetinib is combined with either conventional chemotherapeutics or targeted drugs (Patel *et al*., 2013). Thus, meaningful clinical responses to MEK1/2 inhibitors such as selumetinib are likely to depend on the development of effective combination therapies.

The enrolment criterion for phase II and phase III trials with the BRAF inhibitor vemurafenib included selecting patients with treatment-naïve *BRAF*-mutant metastatic melanoma. Both the phase II trial and interim analysis of the phase III trial comparing vemurafenib to dacarbazine reported confirmed clinical responses in around 50% of patients, compared with 5% for dacarbazine (Chapman *et al*., 2011; Sosman *et al*., 2012). Interim analysis of the phase III trial also reported superior median progression free survival with vemurafenib compared with dacarbazine (5.3 vs. 1.6 months). With these interim results and the conclusion of the phase II trial both demonstrating the substantial clinical efficacy of vemurafenib, the United States Food and Drug Administration recommended that the phase III trial be revised to assume the greater efficacy of vemurafenib over dacarbazine and independent data and safety monitoring board recommended patient crossover from the dacarbazine control arm to vemurafenib (Chapman *et al*., 2011). This rapid clinical development led to the approval of vemurafenib for the treatment of *BRAF*-mutant metastatic melanoma in the United States in August 2011 and in the European Union in February 2012.

Activating *BRAF* mutations occur in around 8–10% of CRCs and correlate with poor prognosis (Richman *et al*., 2009; Tol *et al*., 2009; Fariña-Sarasqueta *et al*., 2010). In comparison to the striking clinical responses seen in melanoma, the response to vemurafenib in a phase I study of patients with *BRAF*-mutant metastatic CRC was modest (Kopetz *et al*., 2010). This is consistent with reports that vemurafenib treatment does not lead to sustained suppression of ERK1/2 phosphorylation in $BRAF^{V600E}$-mutant CRC cells due to epidermal growth factor receptor (EGFR)-mediated reactivation of ERK1/2 (Corcoran *et al*., 2012; Prahallad *et al*., 2012).

Despite these advances, the efficacy of BRAF and MEK1/2 inhibitors is limited by the development of acquired resistance that typically results in disease progression 6–7 months after treatment initiation (reviewed in Little *et al*., 2013). Acquired resistance to BRAF inhibitors can arise through multiple mechanisms, such as switching to ARAF or CRAF (Montagut *et al*., 2008; Villanueva *et al*., 2010); up-regulation of alternative MEK1/2 activators (Johannessen *et al*., 2010); activating *MEK1* mutation (Wagle *et al*., 2011; Greger *et al*., 2012); expression of $BRAF^{V600E}$ splice variants that preferentially dimerize (Poulikakos *et al*., 2011); *BRAF* amplification (Shi *et al*., 2012); and activating *RAS* mutations (Nazarian *et al*., 2010; Greger *et al*., 2012; Su *et al*., 2012b) or up-regulation/activation of RTKs (Nazarian *et al*., 2010; Villanueva *et al*., 2010; Yadav *et al*., 2012; Girotti *et al*., 2013). Interestingly, mutations in *BRAF* itself, such as those encoding 'gatekeeper' mutations that block drug binding, have not been observed in cell lines or patients with acquired resistance to BRAF inhibitors, despite the observation that engineering such mutations within *BRAF* can confer resistance *in vitro* (Whittaker *et al*., 2010). Acquired resistance to MEK1/2 inhibitors may arise through amplification of $BRAF^{T1799A}$ (encodes $BRAF^{V600E}$) (Corcoran *et al*., 2010; Little *et al*., 2011), amplification of $KRAS^{G38A}$ (encodes $KRAS^{G13D}$) (Little *et al*., 2011) or mutations within *MEK1* or *MEK2* (Emery *et al*., 2009; Wang *et al*., 2011; Hatzivassiliou *et al*., 2012). The majority of these mechanisms serve to reinstate ERK1/2 signalling and cell proliferation in the presence of drug (reviewed in Little *et al*., 2013). Interestingly, we observed that acquired resistance to selumetinib resulting from *BRAF* amplification was reversible (Little *et al*., 2011). Culturing these resistant cells in the absence of drug for several months led to their complete re-sensitization to the anti-proliferative effects of selumetinib, with BRAF expression and phospho-ERK1/2 levels returning to those observed in selumetinib-naïve cells. The mechanisms underlying resistance reversibility are currently unclear and are under investigation, but these results nevertheless suggest that staggered treatment strategies may delay or overcome acquired resistance in this context. A recent study using primary human $BRAF^{V600E}$-positive melanoma cells that acquire resistance to vemurafenib through elevated BRAF expression has supported this hypothesis (Das Thakur *et al*., 2013). Dosing these cells intermittently with vemurafenib in xenograft models markedly stalled tumour growth relative to continual treatment. Together, these studies (Little *et al*., 2011; Das Thakur *et al*., 2013) suggest that such 'drug holiday' strategies with ERK1/2 pathway inhibitors may warrant evaluation in clinical trials.

Inhibition of ERK1/2 signalling in tumour cells addicted to this pathway typically results in G1 cell cycle arrest (Davies *et al*., 2007; Sale and Cook, 2013). This reflects the pivotal role that ERK1/2 signalling plays in cell cycle progression (Figure 1). Through the activation of AP1 and ETS transcription factors, ERK1/2, in conjunction with ERK1/2-dependent ribosomal protein S6 kinase (RSK) activation, drives transcription of *CCND1* (cyclin D1) during the G1 phase of the cell cycle (Meloche and Pouysségur, 2007). CCND1 binds to and promotes activation of CDK4 and CDK6, which in turn phosphorylate and inactivate retinoblastoma protein (RB). RB inactivation alleviates repression of E2F-mediated transcription, thereby permitting expression of many genes important for entry into, and progression through, S phase (Cobrinik, 2005). In addition, ERK1/2-mediated phosphorylation stabilizes MYC (Sears *et al*., 2000), which in turn increases expression of cyclin D2, CDK4 and CDC25A, and may repress transcription of the cyclin-dependent kinase (CDK) inhibitor $p21^{CIP1}$, all of which act to promote progression into S phase (Galaktionov *et al*., 1996; Bouchard *et al*., 1999; Hermeking *et al*., 2000; Gartel *et al*., 2001). Finally, ERK1/2 and RSK may down-regulate the expression and/or promote inactivation of the CDK-inhibitor $p27^{KIP1}$ (Rivard *et al*., 1999; Delmas *et al*., 2001; Fujita *et al*., 2003). Thus, blockade of ERK1/2 signalling in tumour cells that are addicted to this pathway for proliferation typically promotes G1 cell cycle arrest through the down-regulation of positive cell cycle regulators and the accumulation of negative cell cycle regulators.

ERK1/2-mediated regulation of the BCL2 protein family

ERK1/2 signalling has been implicated in the regulation of many members of the BCL2 protein family. This regulation

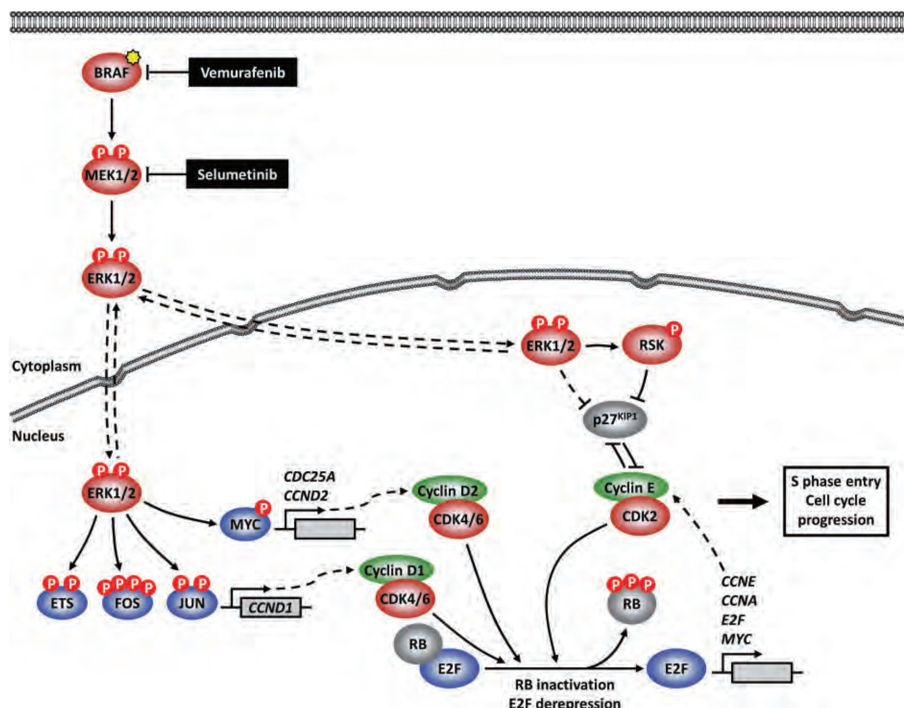

Figure 1

ERK1/2-mediated regulation of the G1-S phase transition. Nuclear ERK1/2 phosphorylates and stabilizes/activates members of ETS and AP1 transcription factor families, which can induce transcription of *CCND1* (cyclin D1). The transcription factor MYC is stabilized by ERK1/2-mediated phosphorylation, and MYC can up-regulate the expression of cell cycle regulators such as cyclin D2 CCND2 (cyclin D2) and CDC25A. CCND1 and CCND2 bind to and activate CDK4 and CDK6 (CDK4/6). Phosphorylation of RB by CDK4/6 frees the E2F transcription factors from RB-mediated repression, allowing E2F-induced transcription of genes such as *CCNE* (cyclin E), *CCNA* (cyclin A) and *MYC*. Newly synthesized CCNE binds and activates CDK2, which can also phosphorylate RB in a feedforward loop. $p27^{KIP1}$, an endogenous inhibitor of CDK2, is down-regulated and inactivated during cell cycle entry by a variety of mechanisms mediated by cyclin E-CDK2, ERK1/2 and RSK. CDK2, initially in complex with CCNE and later with CCNA, and the E2F transcription factors regulate many target factors to drive progression into, and through, S phase. ERK1/2 signalling is frequently hyperactivated in tumour cells as a result of mutations in RTKs, *RAS* or *BRAF* (shown, yellow star), providing validation for selective inhibitors of mutant BRAF (e.g. vemurafenib) or MEK1/2 (e.g. selumetinib).

typically promotes tumour cell survival through the up-regulation of pro-survival factors and down-regulation of pro-apoptotic BCL2 family members. Consequently, inhibition of ERK1/2 signalling using MEK1/2 or RAF inhibitors generally induces expression of pro-apoptotic BCL2 proteins in tumour cells.

Apoptosis is regulated by the BCL2 protein family

The mitochondrial pathway of apoptosis is regulated by members of the BCL2 protein family (Chipuk *et al*., 2010). Interactions between these factors ultimately control the integrity of the outer mitochondrial membrane (OMM), thereby determining whether a cell survives or commits to apoptosis. Pro-apoptotic signals converge to promote mitochondrial outer membrane permeabilization (MOMP), which allows the release of soluble proteins resident in the intermembrane space into the cytosol. Most notably, cytochrome *c* released from mitochondria binds to APAF1, promoting its oligomerization and assembly into the apoptosome. The apoptosome acts as a caspase activation platform by first recruiting pro-caspase-9 and promoting its activation. Active caspase-9 is then able to cleave and activate the executioner caspases, caspase-3 and caspase-7, which cleave a large number of cellular substrates resulting in apoptosis (Tait and Green, 2010).

BCL2 family members are classified as either pro-apoptotic or pro-survival. A1/BFL1, BCL2, BCL-w, BCL-X_L and MCL1 are the major pro-survival (or anti-apoptotic) members, and contain four BCL2-homology domains (BH1–4). They largely associate with the OMM and act to inhibit apoptosis by binding to pro-apoptotic factors (Chipuk *et al*., 2010). The pro-apoptotic members of the BCL2 family are further divided into the BH3-only proteins and the effector proteins. BH3-only proteins are induced by a variety of cellular stresses and include BAD, BID, BIK, BIM, BMF, HRK, NOXA and PUMA. Upon induction, these bind to and inhibit their target pro-survival BCL2 family members. Whereas BIM and PUMA are thought to bind to and inhibit all five major pro-survival factors, most other BH3-only proteins exhibit more restricted binding preferences (Chipuk *et al*., 2010). The pro-survival proteins are also able to bind and inhibit the effector proteins BAX and BAK. Thus, by binding to the pro-survival factors, BH3-only proteins can displace the effector molecules BAX and BAK, which are then free to undergo further activation events that lead to MOMP. In addition to inhibiting the action of pro-survival factors, certain BH3-only

proteins such as BIM and BID may directly activate BAX and BAK (Kuwana *et al.*, 2005; Chipuk *et al.*, 2010). Once activated, BAX and BAK induce MOMP by homo-oligomerizing to form pores within the OMM, thereby allowing the release of soluble factors such as cytochrome *c* and subsequent formation of the apoptosome (Tait and Green, 2010).

ERK1/2-mediated regulation of pro-apoptotic BH3-only proteins

ERK1/2 signalling is a prominent regulator of apoptosis, and influences the expression and/or activity of many members of the BCL2 protein family (Figure 2; Balmanno and Cook, 2009). At least six of the BH3-only proteins have been proposed to be regulated by ERK1/2 signalling. The potent BH3-only protein BIM, in particular the most abundant extra-long isoform, BIM_{EL}, is an important target of ERK1/2 signalling. Phosphorylation of BIM_{EL} on multiple sites by ERK1/2 targets it for ubiquitination and subsequent proteasome-dependent degradation (Ley *et al.*, 2003). In addition, *BIM* transcription is positively regulated by FOXO3 (Dijkers *et al.*, 2000), which is itself a target of ERK1/2. ERK1/2-mediated phosphorylation of FOXO3 promotes its MDM2-dependent ubiquitination and degradation by the proteasome, thereby repressing *BIM* transcription (Yang *et al.*, 2008). Phosphorylation of BIM_{EL} by ERK1/2 has also been shown to rapidly disrupt preformed BIM:BCL-X_L and BIM:MCL1 complexes, with dissociated BIM_{EL} then being more rapidly turned over (Ewings *et al.*, 2007). Thus, ERK1/2 activation represses expression of BIM_{EL} protein and mRNA, and promptly impairs its pro-apoptotic activity by preventing association with, and promoting dissociation from, pro-survival factors. Indeed, tumour cells with *BRAF* mutations are addicted to ERK1/2 signalling for repression of BIM (Wickenden *et al.*, 2008). Consequently, pharmacological inhibition of ERK1/2 signalling induces BIM expression in many contexts. Notably, pretreatment BIM expression levels may be predictive biomarkers for tumour cell responses to some kinase inhibitors (Faber *et al.*, 2011).

We recently demonstrated that inhibition of ERK1/2 in both *KRAS*- and *BRAF*-mutant CRC cells leads to a striking up-regulation of the BH3-only protein BMF (Sale and Cook, 2013). The mechanisms by which ERK1/2 signalling represses BMF expression are unclear, and form the focus of current efforts. ERK2 was recently shown to phosphorylate BMF on two sites; however, it is currently unclear how, or indeed whether, these phosphorylation events influence the function or properties of BMF (Shao and Aplin, 2012). In addition, ERK1/2 signalling can regulate expression of *BMF* mRNA and BMF localization (VanBrocklin *et al.*, 2009; Shao and Aplin, 2010).

Inhibition of MEK1/2 frequently leads to increased expression of the BH3-only protein PUMA (Wang *et al.*, 2007; Sale and Cook, 2013). *PUMA* transcription is known to be positively regulated by FOXO3 in response to growth factor or cytokine withdrawal (You *et al.*, 2006). Thus, ERK1/2-dependent modulation of FOXO3 expression may contribute to this up-regulation of PUMA.

Another BH3-only protein, BAD, is phosphorylated at three distinct sites, each acting to inhibit its pro-apoptotic activity. Growth factor-induced phosphorylation of Ser112 was initially demonstrated to be MEK1/2 dependent (Fang *et al.*, 1999; Scheid *et al.*, 1999), and subsequently found to be

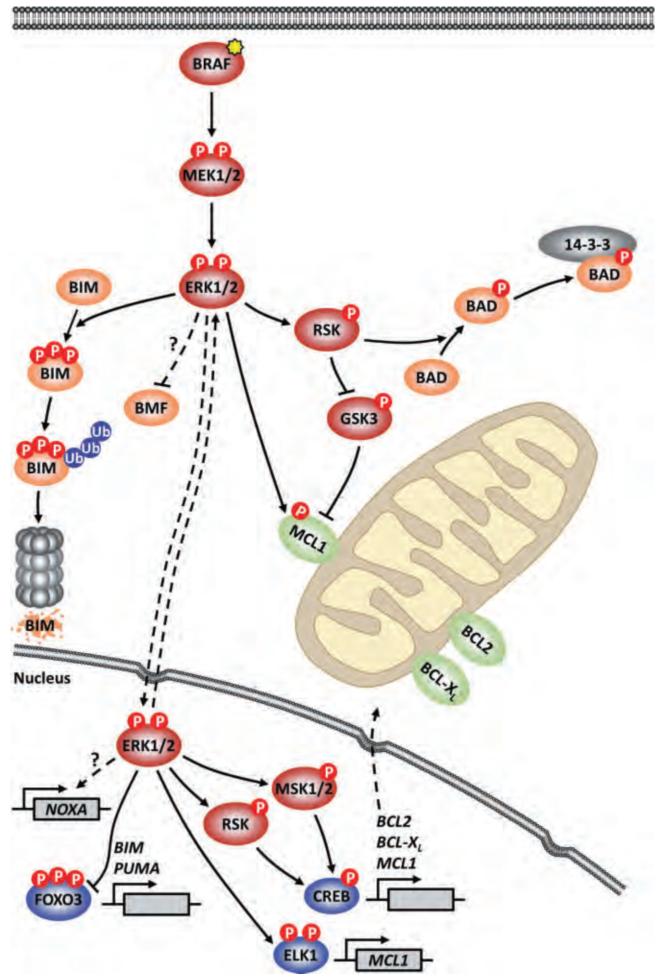

Figure 2

ERK1/2-mediated regulation of the BCL2 protein family. ERK1/2 activation by RTK signalling, mutant RAS or mutant BRAF (shown, yellow star) has pleiotropic effects on the expression and/or activity of BCL2 family members. The BH3-only protein BIM is phosphorylated by ERK1/2 on at least three sites, marking BIM for ubiquitination and subsequent degradation by the 26S proteasome. ERK1/2 also negatively regulates the expression and/or activity of BMF through incompletely understood mechanisms. Activation of RSK by ERK1/2 promotes BAD phosphorylation, which creates a 14-3-3 binding site and sequesters BAD away from the mitochondria. MCL1 stability is subject to reciprocal regulation by the actions of ERK1/2 and GSK3. Phosphorylation of MCL1 by ERK1/2 stabilizes MCL1, whereas GSK3-mediated phosphorylation promotes MCL1 degradation. In the nucleus, ERK1/2 influences the transcription of BCL2 family members. Activation of ERK1/2-dependent RSK and MSK1/2 activates CREB, which promotes transcription of the pro-survival genes *BCL2*, *BCL-X_L* and *MCL1*. ELK1 activation by ERK1/2 may also augment *MCL1* transcription. ERK1/2 promotes the degradation of FOXO3, thereby inhibiting FOXO3-dependent transcription of pro-apoptotic *BIM* and *PUMA*. In contrast to other pro-apoptotic BH3-only proteins, ERK1/2 signalling induces the expression of NOXA mRNA and protein. Thus, with the exception of NOXA, tumour cell ERK1/2 signalling typically promotes the expression of pro-survival factors, and represses the expression and/or activity pro-apoptotic BCL2 family members.

catalysed by the ERK1/2-regulated kinases RSK and MSK1 (Bonni *et al*., 1999; Tan *et al*., 1999; Shimamura *et al*., 2000; She *et al*., 2002). Phosphorylation of this site, as well as phosphorylation of Ser136 by PKB (also known as Akt) (Zha *et al*., 1996; Datta *et al*., 1997; del Peso *et al*., 1997), is proposed to inhibit BAD by facilitating binding to 14-3-3 proteins that sequester BAD in the cytosol away from pro-survival BCL2 proteins at the mitochondria (Zha *et al*., 1996; Tan *et al*., 1999).

Recently, ERK1/2 was proposed to regulate the stability of the BH3-only protein BIK, in a manner analogous to BIM (Lopez *et al*., 2012). Direct phosphorylation of BIK on Thr124 by ERK1/2 was suggested to promote ubiquitination and subsequent proteasome-mediated degradation of BIK. Consistent with these observations, the authors demonstrated that MEK1/2 inhibition in tumour cells with *BRAF* and *RAS* mutations caused a striking up-regulation of BIK protein. However, we and others have observed little change in BIK expression upon perturbation of ERK1/2 signalling in tumour cells, and the reason for this discrepancy is currently unclear (Sheridan *et al*., 2008; Sale and Cook, 2013).

In contrast to the above examples, the BH3-only protein NOXA is induced rather than repressed by ERK1/2 signalling (Sheridan *et al*., 2010; Elgendy *et al*., 2011; Basile and Aplin, 2012). Inhibition of ERK1/2 down-regulates NOXA protein and mRNA expression, but the underlying mechanisms are unknown (Basile and Aplin, 2012). Why NOXA should exhibit this opposing reciprocal regulation by ERK1/2 signalling is unclear but it may be relevant to the onset of autophagy during oncogene-induced senescence. Strong ERK1/2 signalling induced by conditional overexpression of mutant HRAS increased NOXA expression, which appeared to be involved in the induction of autophagy under these conditions (Elgendy *et al*., 2011).

ERK1/2-mediated regulation of pro-survival BCL2 family members

The pro-survival factor MCL1 has a short half-life due to the presence of a PEST [peptide sequence that is rich in proline (P), glutamic acid (E), serine (S) and threonine (T)] domain. MCL1 turnover is regulated by phosphorylation of sites within the PEST domain. ERK1/2-mediated phosphorylation of Thr163 within this domain is proposed to stabilize MCL1 (Domina *et al*., 2004) whereas GSK3-catalysed phosphorylation within the PEST domain enhances MCL1 turnover (Maurer *et al*., 2006). Thus, inactivation of GSK3 by the ERK1/2-dependent RSK kinases is a further mechanism by which ERK1/2 may promote MCL1 expression. In addition, *MCL1* transcription is postulated to be positively regulated by ERK1/2-mediated activation of the transcription factor ELK1 (Townsend *et al*., 1999; Vickers *et al*., 2004; Booy *et al*., 2011).

cAMP responsive element binding protein (CREB) plays a central role in promoting transcription of *BCL2*, *BCL-X$_L$* and *MCL1* in response to ERK1/2 signalling (Wilson *et al*., 1996; Wang *et al*., 1999; Boucher *et al*., 2000). This is likely mediated by the ERK1/2-dependent kinases RSK and MSK, which can phosphorylate and activate CREB (Bonni *et al*., 1999).

Tumour cell responses to ERK1/2 pathway inhibitors are typically cytostatic

Broadly, with the exception of NOXA, tumour cell ERK1/2 signalling promotes cell survival by increasing expression of pro-survival proteins while repressing and inactivating pro-apoptotic proteins. Inhibition of ERK1/2 with MEK1/2 or BRAF inhibitors invariably promotes the expression or activation of multiple BH3-only proteins. However, despite strong induction of even the most potent pro-apoptotic proteins such as BIM and PUMA, the predominant response of tumour cells in culture or xenografts to ERK1/2 pathway inhibition is cytostatic rather than cytotoxic; that is, cells undergo G1 cell cycle arrest rather than apoptosis (Davies *et al*., 2007; Sale and Cook, 2013). This is exemplified by the observation that CCND1 overexpression alone can confer resistance to ERK1/2 pathway inhibitors (Smalley *et al*., 2008). Pro-survival BCL2 proteins are frequently up-regulated in tumour cells (Kirkin *et al*., 2004) and, in comparison to the regulation of pro-apoptotic factors such as BIM or BMF, ERK1/2 pathway inhibition often has only modest effects on pro-survival protein levels and/or activity (Sale and Cook, 2013). Thus, in this setting, pro-survival proteins may provide residual buffering capacity against the BH3-only proteins that accumulate upon ERK1/2 inhibition. This provides a rationale for co-targeting ERK1/2 signalling and the pro-survival BCL2 family members, in an attempt to push tumour cells over the threshold required for considerable (i.e. clinically relevant) apoptosis. Over the past 10–15 years, several agents have been developed that inhibit the pro-survival BCL2 family members, with varying degrees of success.

Targeting pro-survival BCL2 family members in cancer

Given that the deregulation of apoptosis in tumour cells typically occurs upstream of BAX and BAK, altering the balance between pro-apoptotic and pro-survival BCL2 family members is an attractive strategy for promoting tumour cell apoptosis. Several molecules have been developed along these lines (reviewed in Chonghaile and Letai, 2008) including the antisense oligonucleotide oblimersen that targets pro-survival BCL2 (Klasa *et al*., 2002), BH3 peptides, and small molecule mimics of the pro-apoptotic BH3 domain (BH3 mimetics). Oblimersen and the BH3 mimetics obatoclax (GX-15–070) and ABT-263 are the most clinically advanced.

Despite promising preclinical and early clinical trial data, the performance of oblimersen in phase III multiple myeloma and chronic lymphocytic leukaemia (CLL) clinical trials was unconvincing (Chanan-Khan *et al*., 2009; O'Brien *et al*., 2009b). These results have been attributed to the inability of oblimersen to reduce BCL2 protein expression in tumours *in vivo*.

Obatoclax is a putative BH3 mimetic small molecule that exhibits activity against all the pro-survival BCL2 family members, but has modest potency (Zhai *et al*., 2006). Obatoclax can induce cell death through various mechanisms, some of which are consistent with BH3 mimetic activity but some of which do not require BAX and BAK (Konopleva *et al*., 2008). Thus, the principal mechanism by which obatoclax induces cell death is uncertain. Obatoclax has shown modest clinical benefit in early stage trials of CLL and small-cell lung cancer (SCLC) (O'Brien *et al*., 2009a; Paik *et al*., 2010). However, a phase II study of obatoclax plus topotecan in

SCLC showed no improvement in overall response rate compared with that observed with topotecan monotherapy (Paik *et al*., 2011).

ABT-737 and ABT-263 (navitoclax) are small molecule BH3 mimetics developed by Abbott Laboratories (Abbott Park, IL, USA) which inhibit BCL2, BCL-X_L and BCL-w (Oltersdorf *et al*., 2005; Tse *et al*., 2008). Although used in the majority of preclinical studies, ABT-737 is not orally bioavailable and so the closely related molecule ABT-263 is being evaluated in clinical trials. ABT-263 is orally bioavailable and has similar affinities and specificities for the pro-survival proteins as ABT-737. In contrast to other putative BH3 mimetics, ABT-737/263-induced cell death absolutely requires BAX or BAK (van Delft *et al*., 2006), and ABT-737/263 can displace pro-apoptotic factors from pro-survival proteins (Del Gaizo Moore *et al*., 2007), indicating that ABT-737 and ABT-263 act 'on target'. In addition, these compounds exhibit 100–10 000-fold greater affinity for their target BCL2 family members than other small molecule inhibitors (Oltersdorf *et al*., 2005; Zhai *et al*., 2006; Tse *et al*., 2008). However, ABT-737 and ABT-263 have low affinity for MCL1, A1/BFL1 and BCL-B. Thus, although there are exceptions, these small molecules are typically most effective at killing cells expressing higher BCL2 and BCL-X_L, whereas tumour cells expressing higher MCL1 exhibit intrinsic resistance (Konopleva *et al*., 2006; van Delft *et al*., 2006). Consistent with this, acquired resistance to ABT-737 can arise through increased expression of MCL1 and/or A1 (Yecies *et al*., 2010).

ABT-263 has shown efficacy in early stage clinical trials, particularly for the treatment of CLL. In a phase I study of relapsed or refractory lymphoid malignancies (including CLL) 22% of patients had partial responses (Wilson *et al*., 2010), and in a phase I study of relapsed or refractory CLL 35% of patients exhibited partial responses (Roberts *et al*., 2012). In contrast, the results of trials targeting SCLC and other solid tumours have been modest. In a phase II trial assessing the efficacy of ABT-263 against advanced or recurrent SCLC, 1 patient of 39 had a partial response (Rudin *et al*., 2012). This suggests that, for solid tumours at least, ABT-263 may be best employed as combination therapy with other agents.

Harnessing the apoptotic potential of ERK1/2 pathway inhibitors using BH3 mimetics

Response duration to targeted cancer monotherapies is often short, with acquired resistance rapidly emerging. This is reminiscent of early strategies that targeted rapidly mutating or heterogeneous infectious diseases such as human immunodeficiency virus (HIV) or tuberculosis, in which responses to monotherapies were transient and quickly gave rise to resistant variants (Bock and Lengauer, 2012; Glickman and Sawyers, 2012). However, the use of targeted combination therapies has changed this: antibiotic combinations cure tuberculosis, and treatment of HIV with anti-viral combinations indefinitely suppresses the virus to a chronic low level (Fox *et al*., 1999; Clavel and Hance, 2004). HIV combination therapy works by rapidly and synergistically reducing viral load relative to single agent therapies, thereby minimizing the pool of viruses from which resistance can develop (Clavel and Hance, 2004; Bock and Lengauer, 2012). In the case of tuberculosis, similar initial responses are achieved regardless of whether treated with a single antibiotic or combination (Fox *et al*., 1999); importantly, however, combination therapy halts the emergence of resistance that occurs with single agents. Clearly, human cancer biology is far more complex than that of HIV and tuberculosis, but based on this experience, combinations of targeted cancer therapeutics that achieve rapid tumour elimination and suppress the development of acquired resistance will be required for durable clinical responses.

ABT-263 synergizes with ERK1/2 pathway inhibitors to induce substantial tumour cell apoptosis

As discussed above, despite the induction of potent pro-apoptotic BH3-only proteins such as BIM, BMF and PUMA, MEK1/2 and BRAF inhibitors typically induce minimal apoptosis with the predominant response being a G1 cell cycle arrest. However, the BH3-only proteins induced by ERK1/2 inhibition may prime tumour cells for apoptosis. Thus, combining an ERK1/2 pathway inhibitor with other agents that take advantage of this priming event may tip the balance of pro-apoptotic and pro-survival factors in favour of commitment to apoptosis. One such approach is to combine an ERK1/2 pathway inhibitor, such as selumetinib or vemurafenib, with the BH3 mimetic ABT-737 or ABT-263. Scott and colleagues first demonstrated the potential for MEK inhibitors to combine synergistically with BH3 mimetics and induce tumour cell apoptosis (Cragg *et al*., 2008). Recent studies have now extended these findings using the MEK1/2-selective inhibitor selumetinib in both *BRAF*- and *RAS*-mutant cancers, and the BRAF selective inhibitor PLX4720 in *BRAF*-mutant cancers. Results from our work demonstrated that while treating a variety of CRC tumour cells with selumetinib or ABT-263 alone, induced little cell death (typically < 20% at 48 h post-treatment), strong synergistic cell death and inhibition of clonogenic survival were observed upon treatment with combined selumetinib and ABT-263 (~60–80% cell death at 48 h). We observed similar results in melanoma cell lines expressing mutant RAS or BRAF, and others have demonstrated that combined MEK1/2 and BCL2/BCL-X_L inhibition is frequently effective against *KRAS*-mutant lung and pancreatic tumour cells (Corcoran *et al*., 2013; Tan *et al*., 2013). In all cases examined, cell death required caspase activity and was confined to tumour cells addicted to ERK1/2 signalling; tumour cells with high PI3K-PKB signalling known to be intrinsically resistant to MEK1/2 inhibitors also exhibited resistance to this combination (Cragg *et al*., 2008; Balmanno *et al*., 2009; Corcoran *et al*., 2013; Sale and Cook, 2013). Several reports have demonstrated that PI3K/mTOR inhibitors can also interact synergistically with BH3 mimetics to induce apoptosis in certain contexts (Qian *et al*., 2009; Spender and Inman, 2012; Rahmani *et al*., 2013). Thus, combining such agents may be a valid approach for tumour cells with high PI3K-PKB activity and intrinsic resistance to MEK1/2 inhibitors. Furthermore, a recent study has shown that while the novel MEK1/2 inhibitor G-963 synergized to promote apoptosis in the majority of NSCLC and pancreatic

cancer cell lines tested, these effects were enhanced by the addition of the PI3K inhibitor GDC-0941 (Tan *et al*., 2013).

Engelman and colleagues observed that sensitivity to selumetinib plus ABT-263 correlated with the expression of markers of epithelial differentiation, such as E-cadherin (Corcoran *et al*., 2013). Furthermore, knock-down of ZEB1 in the mesenchymal *KRAS*-mutant lung cancer cell line A549 promoted expression of epithelial markers and sensitized these cells to both ABT-263 and selumetinib plus ABT-263. This is consistent with many reports demonstrating that epithelial-mesenchymal transition (EMT) promotes resistance to various apoptotic stimuli (Vega *et al*., 2004; Robson *et al*., 2006; Arumugam *et al*., 2009), which has frequently been attributed to PKB activation (Escrivà *et al*., 2008; Tiwari *et al*., 2012).

The BRAF inhibitor PLX4720, a close preclinical analogue of vemurafenib (PLX4032), also combined synergistically with ABT-737/263 to induce caspase-dependent cell death and inhibit clonogenic survival of colorectal and melanoma tumour cell lines (Sale and Cook, 2013; Wroblewski *et al*., 2013). This combination was also effective against cells established from patients with BRAF inhibitor-naïve melanoma (Wroblewski *et al*., 2013). In addition to these observations in solid tumour types, ERK1/2 pathway inhibitors have also been shown to combine synergistically with ABT-737 to induce the death of acute myeloid leukaemia cells (Zhang *et al*., 2008; Konopleva *et al*., 2012).

Combining a MEK1/2 inhibitor and BH3 mimetic also shows potent *in vivo* efficacy. Xenograft models of the *KRAS*-mutant CRC cell lines HCT116, SW620 and SW1463 yielded similar results: selumetinib treatment in isolation slowed tumour growth considerably relative to control but did not induce regressions; ABT-263 treatment had little effect on tumour expansion relative to control but combination of selumetinib with ABT-263 caused marked tumour regressions that were sustained for the entirety of the 21–27 day experiments (Corcoran *et al*., 2013). In addition, generating true *in vivo* tumours using a genetically engineered KRAS-driven lung cancer mouse model revealed that while selumetinib or ABT-263 led to average tumour regressions of around 30–40% after 2 weeks of treatment, the combination caused 70–80% tumour regression (Corcoran *et al*., 2013).

ERK1/2 pathway inhibitors combine with ABT-263 to induce BAX- and BIM-dependent apoptosis

The interplay between the pro-survival BCL2 proteins and pro-death BH3-only proteins ultimately serves to regulate the activation of the pro-death effector proteins BAX and BAK. Using isogenic HCT116 cells lacking *BAK*, *BAX* or both [double knockout (DKO)] (Wang and Youle, 2012), we found that those lacking *BAX* ($BAX^{-/-}$ and $BAK^{-/-}BAX^{-/-}$ DKO) were almost completely resistant to apoptosis induced by selumetinib, ABT-263 or selumetinib plus ABT-263, whereas those lacking only *BAK* remained sensitive. Thus, at least in HCT116 cells, apoptosis in response to these agents absolutely required *BAX*, whereas *BAK* was dispensable (Sale and Cook, 2013). Consistent with this, selumetinib and ABT-263 combined to synergistically activate BAX (Sale and Cook, 2013).

Analysing the expression of upstream regulators of BAX in COLO205 cells revealed that selumetinib (alone or in combination with ABT-263) strongly induced the expression of the pro-apoptotic BH3-only proteins BIM and BMF. In these cells, RNAi-mediated knock-down of BIM inhibited apoptosis induced by selumetinib plus ABT-263 by 50–70%, demonstrating that cell death in response to this combination was in large part BIM dependent (Sale and Cook, 2013). However, in other cell types, including HCT116 ($KRAS^{G13D}$), the picture was more complex. As with COLO205 cells, MEK1/2 inhibition in HCT116 cells strongly induced BIM and BMF expression, but in this case PUMA expression also increased and robust knock-down of BIM and/or PUMA did not inhibit apoptosis induced by selumetinib and ABT-263 (Sale and Cook, 2013). Given that cell death was absolutely dependent on BAX, it is likely that alternative BH3-only proteins, such as BMF or BAD, or the reduced expression of pro-survival factors acts in a redundant fashion to promote apoptosis. Knock-down of BIM in the $BRAF^{V600E}$-positive melanoma cell lines Mel-RMu and SK-MEL-28 inhibited apoptosis induced by the combination of PLX4720 plus ABT-263 by 50–70% (Wroblewski *et al*., 2013). In these cells, PLX4720 plus ABT-263 also promoted PUMA expression and altered the abundance of pro-survival factors. These effects, in addition to possible up-regulation of BMF, which was not assessed, may contribute to apoptosis in these melanoma cells.

ABT-263 promotes redistribution of selumetinib-induced BH3-only proteins from BCL-X_L to MCL1 resulting in stronger inhibition of pro-survival BCL2 proteins

To further define the mechanisms by which selumetinib and ABT-263 synergize to induce apoptosis, we immunoprecipitated BCL-X_L and MCL1 from cells treated with selumetinib, ABT-263 or the combination to assess their interaction with BH3-only binding partners. In COLO205 cells, MEK1/2 inhibition strongly promoted accumulation of BIM and BMF and their binding to both BCL-X_L and MCL1 (Sale and Cook, 2013). However, in the presence of the BH3 mimetic ABT-263, BIM and BMF no longer bound to BCL-X_L, but instead exhibited greater binding to MCL1. This is consistent with the high affinity of ABT-263 for BCL-X_L preventing binding of BIM and BMF to BCL-X_L, but not MCL1. Very similar effects were observed in HCT116 cells, except in this case the combination of selumetinib and ABT-263 promoted the redistribution of PUMA as well as BIM and BMF onto MCL1 (Sale and Cook, 2013). Thus, while ABT-263 alone can efficiently inhibit BCL-X_L but not MCL1, in the presence of selumetinib it promotes the redistribution of the BH3-only proteins BIM, BMF and PUMA from BCL-X_L to MCL1, resulting in greater overall inhibition of the pro-survival BCL2 proteins (Figure 3). In addition, while selumetinib only induced partial displacement BAX from BCL-X_L (we did not observe BAX binding to MCL1), ABT-263 robustly disrupted this interaction. Thus, in the presence of selumetinib plus ABT-263, BAX may undergo more efficient direct activation by any residual BIM freed up from BCL-X_L.

ABT-263 can delay acquired resistance to ERK1/2 pathway inhibitors

Tumour cells chronically exposed to ERK1/2 pathway inhibitors evolve to circumvent the G1 arrest induced by

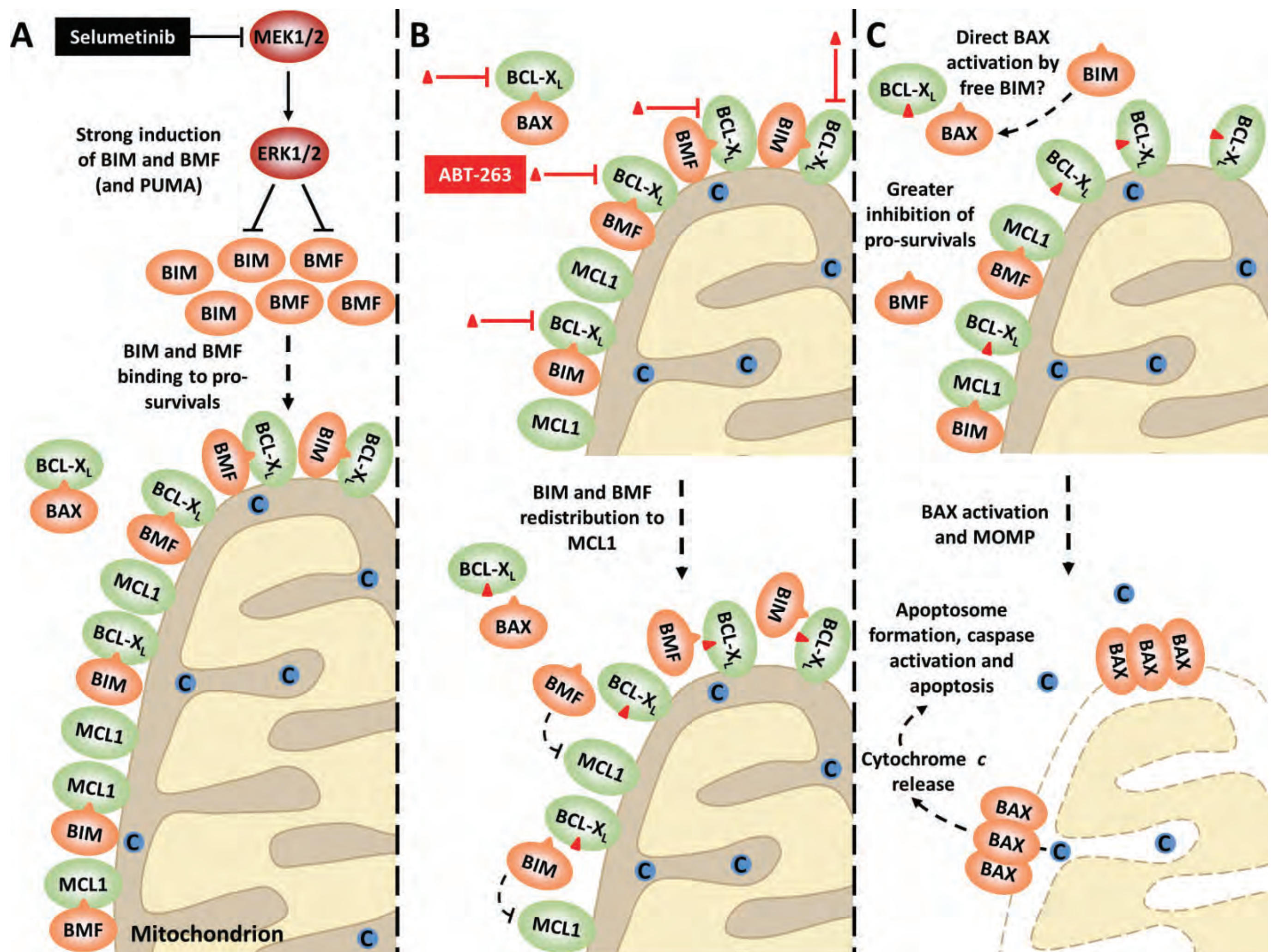

Figure 3

Selumetinib and ABT-263 synergize to inhibit pro-survival BCL2 family proteins and activate BAX in ERK1/2-addicted tumour cell lines. Treatment of tumour cells with a MEK1/2 inhibitor such as selumetinib invariably induces strong expression of BH3-only proteins such as BIM and BMF (A, top). PUMA expression may also be induced. These BH3-only proteins then bind to pro-survival factors such as BCL-X_L and MCL1 (A, bottom). Despite this, little cell death occurs with MEK1/2 inhibition alone, likely due to residual pro-survival activity, including BCL-X_L 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-X_L to 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-survivals (C, top). In addition, whereas selumetinib only resulted in partial displacement of BAX from BCL-X_L, ABT-263 efficiently disrupted this interaction. BAX could then potentially be directly activated by any residual BIM freed up from BCL-X_L (C, top). Activated BAX can subsequently oligomerize and insert into the outer mitochondrial membrane, resulting in MOMP (C, bottom). This allows cytochrome *c* release from the intermembrane space, followed by apoptosome formation, caspase activation and consequent apoptosis. (Reproduced with permission from Sale and Cook, 2013, The Biochemical Journal, 450, 285-294 © the Biochemical Society)

these agents, thereby acquiring resistance to their anti-proliferative effects (Little *et al.*, 2013). We reasoned that the weak cell death responses to ERK1/2 pathway inhibitors may simply allow tumour cells greater opportunity to adapt and evolve acquired resistance. Thus, using ABT-263 to boost primary cell death responses to ERK1/2 pathway inhibitors might minimize the residual population of cells from which resistant clones could emerge and/or increase the number of mutations required before a strong selective advantage is gained, in a manner analogous to drug combinations that rapidly reduce HIV viral load and inhibit acquired resistance (Clavel and Hance, 2004; Bock and Lengauer, 2012).

We tested this hypothesis in CRC cells with *RAS* and *BRAF* mutations. Treating CRC cells transiently (72 h) with selumetinib plus ABT-263 inhibited the frequency of colonies that subsequently developed acquired resistance to selumetinib over 4–6 weeks by 90–95% (Sale and Cook, 2013). Continual treatment with selumetinib plus ABT-263 over a period of 2 weeks similarly inhibited the frequency of resistant colonies by 95%. This was observed in both *KRAS*- and *BRAF*-mutant tumour cells that otherwise rapidly

develop 100-fold resistance to selumetinib (Little *et al.*, 2011). These results are consistent with the *KRAS*-mutant CRC xenograft models discussed above, in which selumetinib plus ABT-263 induced sustained tumour regressions over the course of these 3–4 week experiments (Corcoran *et al.*, 2013). In addition, durable regressions of up to 7 weeks were observed in mutant KRAS-driven lung cancer mouse models (Corcoran *et al.*, 2013).

Thus, these studies provide substantial support for the clinical use of ERK1/2 pathway inhibitors in conjunction with ABT-263. This combination has the potential to augment primary responses and delay acquired resistance, while importantly continuing to harness tumour cell addiction to ERK1/2 signalling.

ABT-263 can overcome established acquired resistance to ERK1/2 pathway inhibitors in some cases

We also examined the effect of ABT-263 treatment on *BRAF*- or *KRAS*-mutant CRC cells that had already acquired resistance to selumetinib. In these cells, acquired resistance had arisen through an amplification of the driving oncogene, which acts to reinstate ERK1/2 signalling in the presence of drug (Little *et al.*, 2011). Under their normal growth conditions (in the presence of selumetinib), selumetinib-resistant COLO205 and HT29 cells (driven by *BRAF* amplification) remained sensitive to ABT-263. In fact, these resistant cells exhibited greater sensitivity to ABT-263 than parental COLO205 or HT29 cells by ~1.5–2-fold, suggesting they may be 'primed' for ABT-263-induced apoptosis. In the absence of selumetinib, however, these cells were resistant to ABT-263 treatment relative to parental cells. Selumetinib removal results in a rapid and sustained hyperactivation of ERK1/2 (Little *et al.*, 2011) which appears to abolish the priming event and diminish sensitivity to ABT-263. In contrast, HCT116 cells with acquired resistance to selumetinib (*KRAS* amplification) were cross-resistant to ABT-263, regardless of whether selumetinib was present. *KRAS* amplification in these cells activates PI3K-PKB signalling (Little *et al.*, 2011), which could potentially provide survival signals to promote ABT-263 cross-resistance. However, even when PI3K-PKB and ERK1/2 signalling in selumetinib-resistant HCT116 cells was inhibited to levels seen in parental HCT116, these cells remained less sensitive to ABT-263 than parental HCT116 cells; thus, *KRAS* amplification conferred resistance to ABT-263 under all conditions. This is consistent with our observations that alternative pathways contribute to acquired resistance in this setting (Little *et al.*, 2011) and underline the challenge faced by *KRAS* amplification as a mechanism of resistance.

Using melanoma cell lines established from patients pre- and post-vemurafenib treatment, Wroblewski *et al.* demonstrated that while vemurafenib-naïve cells underwent apoptosis in response to PLX4720 plus ABT-263, cells harvested post-treatment were refractory to this combination. It is currently unclear why some tumour cells with acquired resistance to ERK1/2 pathway inhibitors remain sensitive to ABT-263, while others are resistant. Nevertheless, ABT-263 has the potential to overcome acquired resistance to ERK1/2 pathway inhibitors in some settings.

Conclusions

Oncogene addiction and the therapeutic window that this can provide for tumour selective intervention holds a great promise for the development of new targeted anti-cancer agents. This has been most evident in the success of agents such as imatinib for the treatment of chronic myelogenous leukaemia (CML) (Capdeville *et al.*, 2002), gefitinib and erlotinib, for the treatment of NSCLC with mutant EGFR (Sequist and Lynch, 2008) and vemurafenib, and for the treatment of $BRAF^{V600E}$-positive melanoma (Chapman *et al.*, 2011). However, acquired resistance to these therapies is a major problem; indeed, the exquisite selectivity of some new targeted agents may actually provide a strong and very focused selection pressure for the rapid emergence of resistance through pathway remodelling (Little *et al.*, 2013). This may be exacerbated by a general failure to sufficiently engage or activate pro-death signalling pathways, including the BCL2 proteins. Indeed, the predominant response to BRAF and MEK1/2 inhibitors is cytostatic rather than cytotoxic. However, addiction to ERK1/2 signalling provides one arm of a synthetic lethal pair that, when combined with BH3 mimetics, results in a strong synergistic tumour cell death that is only observed in ERK1/2-addicted cells. This greatly improves primary efficacy and inhibits and delays the onset of acquired resistance. This strategy is also effective in tumours addicted to breakpoint cluster region/Abelson murine leukaemia viral oncogene and EGFR (Kuroda *et al.*, 2006; Cragg *et al.*, 2007), and so may merit more general consideration as a drug combination that can harness oncogene addiction and transform it into tumour cell-specific cell death.

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Conflict of interest

Payments from AZ were confined to laboratory consumables and associated overheads. Neither S. C. nor M. S. received any personal financial remuneration of any sort from AstraZeneca.

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