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Vemurafenib potently induces endoplasmic reticulum stress-mediated apoptosis in BRAFV600E melanoma cells

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Abstract

The V600E mutation in the kinase BRAF is frequently detected in melanomas and results in constitutive activation of BRAF, which then promotes cell proliferation by the mitogen-activated protein kinase (MAPK) signaling pathway. Although the BRAFV600E kinase inhibitor vemurafenib has remarkable antitumor activity in patients with BRAFV600E-mutated melanoma, its effects are limited by the onset of drug resistance. We found that exposure of melanoma cell lines with the BRAFV600E mutation to vemurafenib decreased the abundance of anti-apoptotic

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proteins and induced intrinsic mitochondrial apoptosis. Vemurafenib-treated melanoma cells showed increased cytosolic concentration of calcium, a potential trigger for endoplasmic reticulum (ER) stress, which can lead to apoptosis. Consistent with an ER stress-induced response, vemurafenib decreased the abundance of the ER chaperone protein GRP78, increased the abundance of the spliced isoform of the transcription factor X-box protein 1 (XBP1) (which transcriptionally activates genes involved in ER stress responses), increased the phosphorylation of the translation initiation factor eIF2 α (which would be expected to inhibit protein synthesis), and induced the expression of ER stress-related genes. Knockdown of the ER stress response protein ATF4 significantly reduced vemurafenib-induced apoptosis. Moreover, the ER stress inducer thapsigargin prevented invasive growth of tumors formed from vemurafenib-sensitive melanoma cells in vivo. In melanoma cells with low sensitivity or resistance to vemurafenib, combination treatment with thapsigargin augmented or induced apoptosis. Thus, thapsigargin or other inducers of ER stress may be useful in combination therapies to overcome vemurafenib resistance.

Introduction

The estimated median survival for patients with stage IV melanoma is 8 ± 2 months (1), with classical chemotherapy and immunotherapy regimens conferring little survival benefit. The mitogen-activated protein kinase (MAPK, also known as RAF-MEK-ERK) and the phosphoinositide 3 kinase (PI3K, also known as the PI3K-AKT-mTOR or AKT) signaling pathways play major roles in melanoma initiation, progression and therapy resistance (2-5). About 50% of melanomas have activating BRAFV600E kinase mutations. Although the BRAFV600E kinase inhibitor vemurafenib induces tumor regression and improves survival in the majority of individuals with BRAFV600E-mutant melanomas, nearly all responses are partial (6-8), with a subpopulation of patients showing primary resistance in metastases. Moreover, the acquisition of secondary resistance leading to relapse was observed in nearly every patient. The overall aim of the present study was to elucidate the mechanisms that underlie the antitumor activity of vemurafenib and to identify strategies to enhance its antitumor effects and overcome mechanisms of resistance.

The mechanisms of vemurafenib resistance are complex and multi-faceted. The available data suggests that resistance involves reactivation of the MAPK pathway or activation of other survival pathways, such as the AKT pathway, which may occur through several means: BRAFV600E amplification (9, 10), increased abundance of splice isoforms of BRAFV600E that dimerize in a RAS-independent manner (10), secondary mutations in NRAS (11) or MAPK kinase (MEK) (12), increased abundance of the mitogen-activated protein kinase 8 (MAP3K8 or COT) which activates the extracellular signal-regulated kinase (ERK) through MEK (13), switching among the three RAF isoforms (14) and increased abundance of receptor tyrosine kinases (14, 15).

Bim (Bcl-2 interacting mediator of cell death), a BH3-only proapoptotic Bcl-2 family member, plays a key role in BRAF inhibitor-induced apoptosis in BRAFV600E melanoma cells (16, 17). Bim is activated by endoplasmic reticulum (ER) stress and is essential for ER stress-induced apoptosis in various cell types (18). ER stress is caused by disturbances in the structure and function of the ER and can result from hypoxia, nutrient deprivation, calcium (Ca²⁺) imbalance, or perturbation of protein glycosylation, leading to the accumulation of unfolded proteins in the ER and activation of the unfolded protein response (UPR) pathway (19-21) (**Fig. S1A**). The UPR pathway is triggered through three sensors: activating transcription factor 6 (ATF6), PKR-like ER kinase (PERK), and inositol-requiring enzyme 1 (IRE1). Under normal conditions, these sensors are maintained in an inactive state bound to the chaperone protein GRP78 (glucose-regulated protein 78). Upon ER stress, misfolded and

unfolded proteins bind to GRP78, releasing it from the UPR sensors, which trigger the UPR by inducing the transcription of genes encoding proteins involved in the UPR, reducing global protein synthesis, and stimulating ER-associated protein degradation. These activities serve to restore normal ER function or, when normal ER function cannot be restored, trigger apoptosis (21-23). Δ^9 -tetrahydrocannabinol (THC) induces ER stress-mediated apoptosis in brain tumor cells through PERK-mediated phosphorylation and activation of the eukaryotic translation initiation factor eIF2 α and an increase in the abundance of ER stress-related proteins, including nuclear protein 1 (NUPR1; also known as p8), ATF3, ATF4, DNA-damage-inducible transcript 3 [DDIT3; also known as growth arrest and DNA-damage-inducible protein (GADD153), or CCAAT enhancer binding protein homolog (CHOP)], and Tribble 3 (TRB3) (24, 25) (**Fig. S1B**). In the present study, we demonstrate that vemurafenib-induced apoptosis in melanoma cells proceeds through the induction of ER stress. Furthermore, we show that vemurafenib resistance may be overcome by combination therapies that augment ER stress.

Results

Vemurafenib induces intrinsic mitochondrial apoptosis specifically in BRAFV600E melanoma cells

As expected, vemurafenib inhibited phosphorylation of ERK in BRAFV600E melanoma cells, but it did not affect phosphorylation of AKT (**Fig. 1A**). Vemurafenib caused greater growth inhibition in BRAF-mutated melanoma cells than in NRAS-mutated melanoma cells (**Fig. 1B**). Accordingly, vemurafenib caused a G1 cell cycle arrest in BRAF-mutated but not NRAS-mutated cells and likewise increased apoptosis only in BRAF-mutated cells, as indicated by a five to seven-fold increase in the percentage of cells in the subG1 fraction (**Fig. 1C**). Apoptotic cell death was confirmed by the enrichment of nucleosomes in the cytoplasm of BRAF-mutated melanoma cells using a cell-death detection ELISA (**Fig. S2**), and was caspase-dependent, because pre-treating cells with the pan-caspase inhibitor Q-VD-OPH abrogated the accumulation of subG1 cells (**Fig. S3**). Western blot analysis confirmed extensive processing of caspase-3 and its substrate PARP by 24 hours after treatment (**Fig. 1D**). Processing of the initiator caspase-9 (**Fig. 1D**) plus a decrease in the abundance of anti-apoptotic Bcl-2 and Mcl-1 proteins (**Fig. 1E**), indicated that activation of the intrinsic apoptosis pathway played a crucial role in vemurafenib-induced cytotoxicity.

Vemurafenib triggers ER stress

Both BRAF inhibitors and ER stress increase the abundance of the proapoptotic protein BIM (16, 18). Indeed, vemurafenib increased the abundance of the three BIM isoforms, BIM_S (12 kD), Bim_L (15 kD), and Bim_{EL} (23 kD), in BRAF-mutated melanoma cells (**Fig. 2A**). Bim knockdown by siRNA impaired vemurafenib-induced apoptosis demonstrating the relevance of Bim induction in this mechanism (**Fig. 2B**). We also evaluated other BH3-only pro-apoptotic Bcl-2 family members and found that vemurafenib also increased the abundance of PUMA and Noxa (**Fig. 2C**).

ER stress can be triggered by various stimuli, including disturbances in Ca²⁺ homeostasis. Like the classical ER stress inducer thapsigargin, vemurafenib treatment induced a morphology typical of ER stress in BRAF-mutated cells, particularly ER lumen swelling (**Fig. 2D**), and increased cytosolic Ca²⁺ concentration (26), whereas tunicamycin, another ER stress inducer, and the MEK inhibitor U0126 did not affect cytosolic Ca²⁺ concentration (**Fig. 2E**).

Upon ER stress, the sensor PERK is activated and phosphorylates eIF2 α (**Fig. S1A**). To determine the onset of ER stress in response to vemurafenib, we examined the

phosphorylation of eIF2 α by Western blotting and found that it was rapidly phosphorylated following vemurafenib treatment (**Fig. 2F**). IRE1 is another ER transmembrane protein that serves as an ER stress sensor and mediates the UPR (**Fig. S1A**). Activated IRE1 splices the mRNA for X-box binding protein 1 (XBP1) such that the encoded protein is a potent transcriptional activator. In addition to phosphorylation of eIF2 α , vemurafenib increased the abundance of the spliced form of XBP1 (XBP1s) in SKMEL19 and 451LU cells, as shown by Western blot analysis (**Fig. 2G**). Additionally, vemurafenib treatment increased the expression of the ER stress-related genes, including *p8*, *ATF4*, *ATF3*, *CHOP*, and *TRB3* (**Fig. S1B**) in BRAF-mutated melanoma cells, particularly in the expression of *p8*, which encodes a co-transcription factor, and *ATF4* (**Fig. 2H**).

The abundance of the ER chaperone protein GRP78 is increased in various cancers including melanoma (27, 28). GRP78 plays a key role in protecting cells against ER stress-induced apoptosis and its abundance is rapidly increased by ER stress inducers such as thapsigargin (29). However, vemurafenib treatment decreased the abundance of GRP78 in both cell lines after 72 hours (**Fig. S4, Fig. 2I**).

ER stress plays a role in vemurafenib-induced apoptosis

Because vemurafenib treatment led to increased expression of *p8* and *ATF4*, we next used siRNA-mediated knockdown to assess their roles in vemurafenib-induced apoptosis. Knocking down *p8* in BRAFV600E melanoma cells yielded conflicting results; we observed either diminished or no effect on the pro-apoptotic effects of vemurafenib. For example, in control-siRNA transfected SKMEL19 melanoma cells, vemurafenib treatment increased the abundance of *p8* by about 100-fold compared with untreated cells (**Fig. 3A**), whereas in *p8*-siRNA transfected cells, vemurafenib treatment increased the abundance of *p8* by about 40-fold, indicating that the *p8*-siRNA suppressed the vemurafenib-induced increase in *p8* abundance by only about 60% (**Fig. 3A**), suggesting that *p8* knockdown was insufficient in melanoma cells with high vemurafenib-induced *p8* expression. However, reduced *p8* expression did not translate into a reduced apoptotic response to vemurafenib (**Fig. 3B**).

We further investigated the role of *p8* by transfecting BRAFV600E melanoma cells with *p8* tagged with green fluorescent protein (GFP) (**Fig. 3C**). FACS-sorted *p8*-GFP-positive melanoma cells (**Fig. 3C**) were treated with vemurafenib and subjected to cell cycle analysis. Overexpression of *p8* in melanoma cells did not induce apoptosis on its own but rather augmented the pro-apoptotic effects of vemurafenib, suggesting that high *p8* expression may increase the sensitivity of BRAFV600E melanoma cells to vemurafenib (**Fig. 3D**). In contrast, the siRNA-mediated knockdown of *ATF4* efficiently reduced both vemurafenib-induced increase in *ATF4* expression and apoptosis (**Fig. 3E,F**). In particular, at 24 hours after treatment with *ATF4*-targeted siRNA and vemurafenib, the reduction in apoptosis achieved was statistically significant (**Fig. 3F**). Thus, *ATF4* appeared to play a pro-apoptotic role in vemurafenib-induced, ER-stress mediated apoptosis.

Conventional inducers of ER stress induce apoptosis and inhibit invasive tumor growth of melanoma cells

Having shown that melanoma cells were sensitive to vemurafenib-induced ER stress-mediated apoptosis, we next asked whether conventional ER stress inducers, such as thapsigargin and tunicamycin, might elicit similar antitumor effects. Both thapsigargin and tunicamycin increased the expression of ER stress-related genes (**Fig. 4A**), inhibited growth (**Fig. 4B**), and induced apoptosis (**Fig. 4C**) in both BRAF- and NRAS-mutated melanoma cell lines. These effects were specific to melanoma cells because both thapsigargin and tunicamycin did not appear to affect the survival of human fibroblasts at concentrations that were pro-apoptotic in melanoma cells (**Fig. S5**).

We then tested whether ER stress inducers also affected invasive tumor growth of melanoma cells in a more physiological context by treating MV3 melanoma cells seeded onto human dermal reconstructs with thapsigargin. Thapsigargin reduced the number of proliferating melanoma cells and abolished invasive tumor growth of melanoma cells into the dermis compared with DMSO-treated controls (**Fig. 4D**). Impaired invasive tumor growth is likely due to the fact that melanoma cells undergo apoptosis in response to thapsigargin (**Fig. 2D**).

We then tested effects of thapsigargin on *in vivo* tumor formation and invasion using a chick embryo model (30) in which melanoma cells were injected into the rhombencephalic brain vesicle. The DMSO-treated control embryos showed massive solid tumor formation in the roof plate of the rhombencephalic brain vesicle and the adjacent mesenchyme (**Fig. 4E**), invasion of melanoma cells into the neural and mesenchymal tissues, as indicated by the melanoma-specific marker HMB45 (**Fig. 4F**), and a greater than 90% proliferation index in melanoma cells 96 hours after injection, as detected by immunohistochemistry with the Ki67-detecting antibody MIB1 (**Fig. 4G**). In contrast, the thapsigargin-treated embryos developed tumors comprised of loosely packed melanoma cells (**Fig. 4H**) which invaded into the mesenchyme surrounding the primary tumor to a lesser extent (**Fig. 4I**) and a lower than 10% proliferation index as detected by immunohistochemistry for Ki67 (**Fig. 4J**), demonstrating the effectiveness of thapsigargin in preventing the growth and invasion of melanoma cells with BRAFV600E.

We next determined whether the anti-tumor effects of vemurafenib were enhanced by ER stress inducers. In SKMEL19 melanoma cells, which were sensitive to vemurafenib, thapsigargin did not enhance the anti-proliferative and pro-apoptotic effects of vemurafenib. However, in 451LU cells, which were less sensitive to vemurafenib, combination treatment with thapsigargin significantly increased growth inhibition (**Fig. 4K**) and the percentage of cells undergoing apoptosis (**Fig. S6**). This indicated that ER stress might boost vemurafenib-mediated antitumor activity, and suggested that this combination approach may abrogate intrinsic resistance. In agreement with previously published studies we also noted that the heat shock protein 90 (HSP90) inhibitor XL888 also overcame intrinsic BRAF inhibitor resistance (31) (**Fig. S7A**).

ER stress inducers overcome acquired resistance to vemurafenib

We next explored whether melanoma cells with acquired vemurafenib resistance retained sensitivity to other ER stress inducers, such as thapsigargin. For this, we established vemurafenib-resistant cell lines by chronically treating BRAF inhibitor-sensitive melanoma cell lines with increasing concentrations of vemurafenib or DMSO (control) for 6 months. In the following experiments, we used the vemurafenib-sensitive parental cell line, SKMEL19-Vem-S and the vemurafenib-resistant cell line, SKMEL19-Vem-R.

Sequencing of the *BRAF*, *NRAS*, and *C-KIT* genes in SKMEL19-Vem-S and SKMEL19-Vem-R cells did not reveal the acquisition of additional mutations in these genes (**Table S1**). Vemurafenib did not inhibit growth in SKMEL19-Vem-R cells, thus demonstrating the acquisition of drug resistance in these cells, whereas it inhibited growth in SKMEL19-Vem-S cells (**Fig. 5A**). Likewise, vemurafenib did not induce apoptosis in SKMEL19-Vem-R cells whereas it triggered a 6-fold higher rate of apoptosis in SKMEL19-Vem-S cells (**Fig. 5B**).

As expected, vemurafenib did not appear to affect the phosphorylation of ERK or AKT in SKMEL19-Vem-R cells (**Fig. 5C**), and the extent of basal phosphorylation of ERK and AKT in SKMEL19-Vem-R cells was greater than in SKMEL19-Vem-S cells (**Fig. 5C**). In contrast to its activity in SKMEL19-Vem-S cells (**Fig. 2I**), vemurafenib did not suppress the abundance of the ER chaperone protein GRP78 in SKMEL19-Vem-R cells after 72 hours

(**Fig. 5D**), induce the phosphorylation of eIF2 α (**Fig. 5E**), or increase the abundance of pro-apoptotic Bim isoforms (**Fig. 5F**).

We then investigated whether vemurafenib-resistant melanoma cells might be sensitive to apoptosis induction by other ER stress inducers. SKMEL19-Vem-S and SKMEL19-Vem-R cells treated with increasing concentrations of thapsigargin were growth inhibited (**Fig. 5G**) and underwent apoptosis (**Fig. 5H**). Thapsigargin increased phosphorylation of ERK in SKMEL19-Vem-S cells (**Fig. 5I**), whereas vemurafenib decreased phosphorylation of ERK in these cells (**Fig. 1A**). In SKMEL19-Vem-R cells, thapsigargin inhibited phosphorylation of AKT but not that of ERK (**Fig. 5I**), suggesting the two drugs operate through different mechanisms. Taken together, these data indicate that metastatic melanoma cells with acquired resistance to vemurafenib-induced ER stress-mediated apoptosis may remain sensitive to agents that induce ER stress-mediated apoptosis through a different mechanism. In agreement with previously published studies, we also observed that the HSP90 inhibitor XL888, which restores the abundance of Bim and suppresses the anti-apoptotic protein Mcl-1, also overcame acquired vemurafenib resistance (31) (**Fig. S7B**).

Discussion

Previous studies have demonstrated that some cancer types, including melanoma, may adapt to ER stress and become resistant to ER stress-induced apoptosis through the activation of pathways and expression of proteins that promote survival (32). In melanoma, it has been suggested that resistance to ER stress-mediated apoptosis may be mediated through the activation of the RAF-MEK-ERK or PI3K-AKT-mTOR pathways as well as increased abundance of the chaperone protein GRP78 and anti-apoptotic proteins, like Mcl-1 (32, 33). Previous studies show that inhibition of MEK signaling, either through siRNA knockdown or the MEK inhibitor U0126, sensitizes melanoma cells to ER stress-mediated apoptosis (33). In addition, inhibition of Mcl-1 function through the BH3 mimetic obatoclax also sensitizes melanoma cells to ER stress-mediated apoptosis (32). Consistent with these reports, we show here that the BRAFV600E inhibitor vemurafenib induces apoptosis by inhibiting the RAF-MEK-ERK pathway, decreasing the abundance of Bcl-2 and Mcl-1, and increasing the expression of ER stress-related genes. We propose a model for the mechanism of action of vemurafenib based on our findings (**Fig. 6**).

Our results showed that vemurafenib treatment led to a rapid increase in the concentration of cytosolic Ca²⁺ in a similar manner to thapsigargin, but unlike the MEK inhibitor U0126. The pan-RAF inhibitor sorafenib, which also inhibits the kinase activity of BRAFV600E, also increases the concentration of cytosolic Ca²⁺ through a MEK-independent mechanism that involves depletion of Ca²⁺ stores from the ER (34). Depletion of Ca²⁺ from the ER is a major factor in thapsigargin-induced ER stress and apoptosis (26). The mechanism that underlies sorafenib- or vemurafenib-induced Ca²⁺ mobilization remains to be determined.

Vemurafenib suppressed expression of the ER chaperone protein GRP78. Normally, upon ER stress expression of GRP78 is induced in an effort to restore normal ER function. Thus, the classical ER stress inducer thapsigargin rapidly induces GRP78 expression (34). However, in contrast to thapsigargin, the RAF inhibitor sorafenib and the MEK inhibitor PD184352 rapidly decrease the abundance of GRP78 (34). Thus, vemurafenib may induce ER stress and increase the abundance of GRP78 and simultaneously decrease the abundance of components in the MAPK pathway and GRP78. Diminishing the chaperone reserve and thus intensifying ER stress may contribute to the lethal actions of vemurafenib.

Vemurafenib increased the abundance of the spliced isoform of the transcription factor XBP1, a hallmark of ER stress, and triggered phosphorylation of the translation initiation

factor eIF2 α (**Fig. S1A**). Vemurafenib-induced ER stress was also associated with a marked increase in the expression of *p8* and *ATF4*. *p8* is a small DNA binding protein that acts as a co-transcription factor (35) through binding to proteins like p300, a transcriptional co-activator (36). *p8* has been implicated in various biological functions that appear to be dependent on the tumor type and microenvironment (37), because contradictory reports show that *p8* either promotes or suppresses tumor growth. In brain tumor cells, THC induces phosphorylation of eIF2 α and increases the abundance of *p8*, which induces apoptosis by increasing the expression of the ER stress-related genes *ATF4/3*, *CHOP*, and *TRB3* (24, 25). Furthermore, stimulation of the RAF-MEK-ERK pathway decreases the abundance of *p8* and promotes proliferation of pancreatic cancer cells (38). In the current study, siRNA knockdown of *p8* did not reliably affect vemurafenib-induced apoptosis. We hypothesized that *p8* was not sufficiently knocked down in the presence of high vemurafenib-induced *p8* expression, and indeed, *p8* overexpression enhanced vemurafenib-induced apoptosis. Alternatively, other ER stress-related factors may have compensated for the *p8* deficiency. *ATF4* knockdown significantly reduced vemurafenib-induced apoptosis. Consistent with our data, a report has shown that *ATF4* mediates ER-stress-induced cell death in neuroectodermal tumor cells treated with the chemotherapeutic agent, fenretinide, or the proteasome inhibitor, bortezomib (39). Together, these data indicate that *ATF4* plays a relevant pro-apoptotic role in ER stress-mediated apoptosis.

In melanoma cells, inhibition of the MEK-ERK pathway induces apoptosis and increases the abundance of Bim, a pro-apoptotic BH3-only member of the Bcl-2 family (40). Previous studies in breast carcinoma cell lines implicate Bim in the ER stress response. In these studies, ER stress increases the abundance of Bim through the induction of CHOP-mediated transcription and protein phosphatase 2A-mediated dephosphorylation, which prevents Bim ubiquitination and proteasomal degradation (18). Consistent with these studies, we found that vemurafenib inhibited phosphorylation of ERK, induced ER stress, and increased the abundance of all three Bim isoforms (Bim_S, Bim_L and Bim_{EL}) leading to increased apoptosis. The relevance of Bim induction was demonstrated by the impaired vemurafenib-induced apoptotic response after its knockdown by siRNA. In line with our observations, a study (16) shows that a key mechanism underlying apoptosis induction by the BRAFV600E inhibitor PLX4720 was induction of the short Bim isoform, Bim_S. Furthermore, we observed that vemurafenib increased the abundance of two other proteins of the BH3-only pro-apoptotic Bcl-2 family, PUMA and Noxa, both of which play a role in ER stress-mediated apoptosis (41).

Vemurafenib-resistant BRAFV600E melanoma cells exhibited enhanced basal phosphorylation of ERK and AKT compared to vemurafenib-sensitive cells. In resistant cells, vemurafenib did not inhibit phosphorylation of ERK, suppress the ER chaperone protein GRP78 after 72 hours, trigger phosphorylation of eIF2 α , increase the abundance of pro-apoptotic Bim isoform, inhibit growth, or induce apoptosis. We found that thapsigargin inhibited growth and induced apoptosis in vemurafenib-resistant melanoma cells. Thapsigargin is currently being tested in a phase I study for treating patients with advanced solid tumors (ClinicalTrials.gov Identifier: NCTT01056029). We found that thapsigargin inhibited activation of the AKT pathway, but not that of the ERK pathway. AKT exerts multiple inhibitory effects on apoptosis by directly targeting and inhibiting proapoptotic proteins including forkhead transcription factors, the BH3-only protein BAD (Bcl-2-associated agonist of cell death), and caspase-9 (42). Furthermore, AKT activates members of the IAP (inhibitor of apoptosis) family, including XIAP (X-linked inhibitor of apoptosis) and cIAP2 (cellular inhibitor of apoptosis protein 2), which inhibit ER stress-mediated apoptosis at the post-mitochondrial phase. (43). Thus, suppression of AKT activation may sensitize vemurafenib-resistant melanoma cells to ER stress-mediated apoptosis. As described in the introduction, resistance to vemurafenib is mediated through multiple

mechanisms, the diversity of which is a serious clinical challenge. Augmenting ER stress and blocking ER stress adaptation mechanisms may be one way to overcome vemurafenib resistance. Moreover, many signaling proteins involved in vemurafenib resistance are clients of HSP90 (31). Here, we showed that the HSP90 inhibitor XL888 overcomes both intrinsic and acquired resistance to vemurafenib.

Here, we demonstrate that vemurafenib is a potent inducer of ER stress in BRAFV600E mutant melanoma cells. Of clinical relevance, we found that the ER-stress inducer thapsigargin augmented or induced apoptosis in melanoma cells with low sensitivity or acquired resistance to vemurafenib. We suggest that the combination of vemurafenib with thapsigargin may be a strategy to manage both intrinsic and acquired resistance to BRAF inhibitors.

Materials and Methods

Isolation and culture of human cells

The use of human skin tissues was approved by the Medical Ethics committee of the University of Tuebingen. Experiments were performed in accordance with the Declaration of Helsinki. M. Herlyn kindly provided the metastatic melanoma cell lines 451LU, WM164 and 1205LU. SKMEL19 and MV3 metastatic melanoma cell lines were purchased (ATCC). Cell lines, keratinocytes, and fibroblasts were cultured and isolated as described previously (44-46).

Signaling pathway inhibitors and treatments

Signaling pathway regulators included the BRAFV600E kinase inhibitor, vemurafenib, (PLX4032, Plexxikon) and the MEK inhibitor, U0126, (Cell Signaling); ER stress inducers, thapsigargin (Sigma-Aldrich) and tunicamycin (Sigma-Aldrich); the selective Ca^{2+} ionophore ionomycin (Merck); the apoptosis inducer, staurosporine (Sigma-Aldrich); the caspase inhibitor, Q-VD-OPH and the inactive caspase inhibitor, Q-VD-OPH control (BioVision). The HSP90 inhibitor XL888 was provided by Exelixis. Inhibitors were dissolved in dimethylsulfoxide (DMSO), except for staurosporine (in water), and stored at -20°C . Inhibitors were added directly to the melanoma cell culture medium. Controls were incubated with culture medium or culture medium and DMSO.

Western Blotting

Total protein was extracted from cell pellets, separated by electrophoresis on 4-20% SDS-polyacrylamide gels (15-120 $\mu\text{g}/\text{lane}$), and blotted onto PVDF membranes (Roche). Blots were probed with antibodies against AKT, AKT phosphorylated at Ser⁴⁷³, ERK, phosphorylated ERK, cleaved caspase-3, Mcl-1, Bcl-2, Bim, PUMA, Noxa, eIF2 α , phosphorylated eIF2 α , GRP78, GFP, β -tubulin, β -actin (Cell Signaling), PARP (Alexis Biochemicals/Enzo Life Sciences), caspase-9 (S. Wesselborg, Institute of Molecular Medicine, Duesseldorf), caspase-3 (BD Transduction Laboratories), ATF4 (R&D), p8 (J. L. Iovanna, Centre de Recherche INSERM, Marseille), spliced XBP1 (Biolegend) and α -tubulin (Sigma). Proteins were visualized with secondary peroxidase-conjugated antibodies (Cell Signaling) and the ECL system (Amersham) or with IRDye[®] secondary antibodies and the LI-COR[®] infrared imaging system (LI-COR).

Cell viability assay

Viability was measured using the 4-methylumbelliferyl heptanoate assay as described previously (47).

Apoptosis

Cell cycle—Cell cycle analyses using propidium iodide staining was performed as described previously (47).

Apoptotic cell death—Apoptosis estimated with the cell death detection ELISA kit (Roche) was performed according to manufacturer's instructions, or with propidium iodide staining. Apoptosis measured by Annexin V staining was carried out as described previously (17) with cells seeded at 60% confluency in 6-well tissue culture plates and left to grow overnight before being treated.

Organotypic skin culture—Organotypic cultures of human skin and melanoma were established as described previously (46). Inhibitors were tested for effects against invasive melanoma growth. MV3 melanoma cells were seeded onto human dermal reconstructs, cultured for 4 days, and then treated with thapsigargin (60 nM). Controls were treated with culture medium or culture medium with DMSO.

Chick embryo model—Chick embryo incubation, fenestration, injection procedures, and immunohistochemistry were described previously (30).

Gene expression—Quantitative real-time PCR (RT-PCR) was performed with the LightCycler[®] 480 SYBR Green System (Roche). RNA extraction (MACHERY-NAGEL) and reverse transcription (Invitrogen) were performed according to manufacturer's instructions. 1 µg cDNA was used for PCR analyses. Primer sequences are listed in **Table S2**. The expression of the genes was normalized to the expression of TATA-binding protein (TBP).

Experiments were performed in triplicate. PCR product specificity was verified by melting curve analysis.

Transmission electron microscopy—Cells were fixed in Karnovsky's fixative, embedded in agarose, coagulated, cut into small blocks, and fixed again in Karnovsky's solution. Blocks were embedded in glycid ether, and sectioned (30 nm) with an ultra microtome. Sections were mounted on copper grids, and analyzed with a Zeiss LIBRA 120 transmission electron microscope.

Transfection experiments—Genes were knocked down with prevalidated, small interfering RNAs (siRNAs) against ATF4 (Qiagen, SI03019345), Bim (Cell Signaling, 6518) or p8 (Qiagen, SI02664326). The validated negative control siRNA was a non-silencing, luciferase-specific siRNA (Biomers). J. L. Iovanna (Centre de Recherche INSERM, Marseille) kindly provided the p8-GFP expression vector (pEGFP-C2-p8). For siRNA knockdown, $2\text{--}2.5 \times 10^5$ cells were plated in 6-well plates overnight. Then, 25 nM siRNA was transfected with Lipofectamine[™] RNAiMAX (Invitrogen) according to manufacturer's instructions. Cells were cultured in normal growth medium for 24 hours prior to drug treatment. Overexpression of p8 was achieved by transiently transfecting 1 µg of pEGFP-C2-p8 with Lipofectamine[™] 2000 according to manufacturer's instructions. Controls were transfected with empty vector (pEGFP-N1; Clontech).

Cytosolic Ca²⁺—Cytosolic Ca²⁺ was measured with Fluo3-AM (Invitrogen). Briefly, 5×10^6 cells were loaded with 2 µM Fluo3-AM for 20 min at room temperature, protected from light. Cells were washed, and then resuspended at 10^6 cells/ml in RPMI 1640 Medium, no Phenol Red (Gibco) with 10% fetal bovine serum. Changes in cytosolic Ca²⁺ concentrations

were measured on a LSRII FACS flow cytometer (BD) at an excitation wavelength of 488 nm.

Mutation analysis—DNA for PCR and sequencing was isolated by proteinase K digestion of cell pellets from SKMEL19-Vem-S and SKMEL19-Vem-R cell lines (DNeasy Blood & Tissue Kit; Qiagen). PCR primer sets were designed to amplify the BRAF codon 600, KIT exons 11, 13, 17 and 18, and NRAS codon 61. Primer sequences are listed in **Table S3**. Purified PCR products were directly sequenced (Quintara Bio Sequencing Service), and traces were analyzed with Mutation Surveyor V3.23 (Softgenetics, State College, PA).

Statistical analysis—Statistical analysis was performed with GraphPad Prism version 5.01 (GraphPad Prism Software Inc.). The two groups were compared using the nonparametric Mann-Whitney Test (95%CI). P-values <0.05 were considered statistically significant. N values represent the number of independent experiments unless stated otherwise.

Supplementary Material

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Figure 1. Vemurafenib induces intrinsic mitochondrial apoptosis in BRAF-mutant melanoma cells

(A) Western blot analysis for phosphorylated (p)-ERK, ERK, p-AKT, and AKT was performed on melanoma cells with BRAF (SKMEL19, 451LU) or NRAS mutation (MV3) that were treated with vemurafenib (Vem) or DMSO (control) for 24 hours (h). (B) Growth assessment using 4-methylumbelliferyl heptanoate of melanoma cells treated with vemurafenib for 72 h. One representative experiment of four is shown (mean $\pm$ SD). (C) Apoptosis (as assessed by the subG1 fraction) was quantified in melanoma cells treated with vemurafenib or DMSO for 72 h. Bar graphs show the quantification of results from 4 independent experiments (mean $\pm$ SD, Mann-Whitney test, 95% CI; * $P < 0.05$; ns=not significant). (D) Western blot analysis for caspase-9, caspase-3, cleaved caspase-3, and PARP was performed on melanoma cells treated with vemurafenib or DMSO for the indicated time periods or with staurosporine (Stauro, 1 μ M) for 15 h. Black arrow:

uncleaved protein. White arrow: cleaved protein. **(E)** Western blot analysis for Bcl-2 and Mcl-1 was performed on melanoma cells treated with vemurafenib or DMSO for 72 h. Western blots in **(A, D, E)** show a representative experiment ($n=3$); β -actin or α -tubulin served as the loading control.

melanoma cells treated with vemurafenib or DMSO for 24 h. **(D)** Electron microscopy of melanoma cells treated with vemurafenib, DMSO, or thapsigargin (TG) for 6 h. DMSO-treated cells show a normal-sized endoplasmic reticulum (arrows). Vemurafenib and thapsigargin-treated cells show dilation of the endoplasmic reticulum (arrows). Scale bar = 0.5 μm , $n=2$ experiments. **(E)** Melanoma cells loaded with Fluo3-AM were exposed to the indicated treatments and monitored by flow cytometry. Changes in fluorescence intensity over time reflect changes in cytosolic Ca^{2+} concentrations. As a positive control, melanoma cells were treated with ionomycin, a selective Ca^{2+} ionophore. One representative experiment is shown ($n=3$). **(F, G)** Western blot analysis for p-eIF2 α , eIF2 α , and spliced XBP1 (XBP1s) was performed at the indicated time points on BRAF-mutated (SKMEL19, 451LU) melanoma cells treated with vemurafenib or DMSO (D). **(H)** Real-time PCR to detect the expression of *p8*, *ATF4*, *ATF3*, *CHOP*, and *TRB3* relative to the DMSO control was performed in melanoma cells treated with vemurafenib or DMSO for 12 h. Mean $\pm$ SD; $n=3$ experiments. **(I)** Western blot analysis for GRP78 was performed on melanoma cells treated with vemurafenib or DMSO for the indicated times. **(A, B, C, F, G, I)** Western blots show one representative experiment ($n=3$); β -actin served as the loading control.

Figure 3. ER stress plays a role in vemurafenib-induced apoptosis

(A, B) BRAF-mutated melanoma cells (SKMEL19) transfected with control or *p8*-targeted siRNA were treated with vemurafenib (6 μ M) or DMSO. After 15 h, real-time PCR analysis (A) was performed to measure *p8* mRNA expression in vemurafenib-treated cells relative to DMSO-treated (control) cells. Abundance of *p8* protein were detected by Western blot analysis 18 h after treatment (one representative experiment of three is shown). 24, 48, and 72 h after treatment, apoptosis (subG1 fraction) was quantified (B). Bar graphs show the quantification of results from 4 independent experiments (mean $\pm$ SD). (C, D) Melanoma cells (SKMEL19) were transfected with Ctrl-GFP-plasmid (pEGFP-N1, 27kDa) or *p8*-GFP-plasmid (pEGFP-C2-*p8*, 37kDa). After 24 h, Western blot analysis for GFP (C) was performed (one representative experiment of three is shown). Apoptosis (subG1 fraction) was quantified in GFP-positive cells treated with vemurafenib (6 μ M) or DMSO for 48 h (D). Bar graphs show the quantification of results from 4 independent experiments (mean $\pm$ SD). (E, F) Real-time PCR to detect *ATF4* mRNA expression was performed in melanoma cells (SKMEL19) transfected with control or *ATF4* siRNA and treated with vemurafenib (3 μ M) or DMSO (E). Western blot analysis for *ATF4* was performed 48 h after treatment (one representative experiment of three is shown). 24, 48, and 72 h after treatment, apoptosis (as assessed by the subG1 fraction) was quantified (F). Bar graphs show the quantification of results from 4 independent experiments (mean $\pm$ SD). *P* values for (B, D, F) were determined by Mann-Whitney test, 95% CI; * *P* < 0.05; ns=not significant.

Figure 4. Inducers of ER stress trigger apoptosis and inhibit invasive tumor growth of melanoma cells

(A) Real-time PCR to detect *p8* and *CHOP* mRNA expression was performed in BRAF-mutated (SKMEL19, 451LU) and NRAS-mutated (MV3) melanoma cells treated with DMSO (control), 60 nM thapsigargin, or 595 nM tunicamycin for 12 h. One representative experiment of three is shown (means $\pm$ SD). (B) Growth assessment using 4-methylumbelliferyl heptanoate of melanoma cells treated with the indicated concentrations of thapsigargin or tunicamycin for 72 h. One representative experiment of three is shown (mean $\pm$ SD). (C) Apoptosis (subG1 fraction) was quantified in melanoma cells treated with thapsigargin or tunicamycin for 72 h. Bar graphs show the quantification of results from 3 independent experiments (mean $\pm$ SD). (D) NRAS-mutated melanoma cells (MV3) seeded onto dermal reconstructs were treated with DMSO (control) or thapsigargin. Red, Ki67 ($n=3$

experiments). Scale bar = 100 μm . **(E to J)** Melanoma cells were injected into the rhombencephalic brain vesicle of stage 13/14 HH chick embryos. After 48 and 72 h, embryos were treated with DMSO (control, top row) or thapsigargin (TG, bottom row). HE: hematoxylin and eosin stain at 5 $\times$ magnification (E and H). HMB45: immunohistochemistry with the melanoma-specific marker HMB45, at 40 $\times$ magnification (F and I). MIB1: immunohistochemistry for the proliferation marker Ki67 with the MIB1 antibody at 5 $\times$ magnification (G and J). $n=2$ experiments, 8 embryos per treatment. **(K)** Growth assessment of melanoma cells treated with the indicated concentrations of vemurafenib, thapsigargin, or both for 72 h. Bar graphs show the quantification of results from 3 independent experiments (mean $\pm$ SD, Mann-Whitney test, 95% CI; * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$).

Figure 5. ER stress inducers overcome acquired resistance to vemurafenib

(A) Growth assessment of SKMEL19-Vem-S and SKMEL19-Vem-R melanoma cells treated with the indicated concentrations of vemurafenib for 72 h. One representative experiment of 4 is shown (mean $\pm$ SD). (B) Apoptosis (subG1 fraction) was quantified in SKMEL19-Vem-S and SKMEL19-Vem-R cells treated with vemurafenib or DMSO for 72 h. Bar graphs show the quantification of results from 3 independent experiments (mean $\pm$ SD). (C, D, E, F) Western blot analysis for p-ERK, ERK, p-AKT, AKT, GRP78, p-eIF2 α , eIF2 α , and BIM isoforms was performed at the indicated time points in SKMEL19-Vem-S or SKMEL19-Vem-R cells treated with vemurafenib (6 μ M) or DMSO, β -tubulin or β -actin served as loading control. One representative experiment of three is shown. (C) The ratio of the amount of p-AKT to that of AKT for each lane from three experiments is indicated in the accompanying bar graph (mean $\pm$ SD, Mann-Whitney test, 95% CI; * $P < 0.05$). (G) Growth

assessment of SKMEL19-Vem-S and SKMEL19-Vem-R cells treated with the indicated concentrations of thapsigargin for 72 h. One representative experiment of 3 is shown (mean $\pm$ SD). **(H)** Apoptosis (subG1 fraction) was quantified in SKMEL19-Vem-R cells treated with vemurafenib, DMSO, or thapsigargin for 72 h. Bar graphs show the quantification of results from 3 independent experiments (mean $\pm$ SD). **(I)** Western blot analysis for p-ERK, ERK, p-AKT, AKT, and β -actin was performed in SKMEL19-Vem-S and SKMEL19-Vem-R cells treated with thapsigargin (TG) or DMSO for 24 h. One representative experiment of 3 is shown.

Figure 6. Proposed mechanism of vemurafenib action

Vemurafenib induces ER stress by increasing the concentration of cytosolic Ca^{2+} . eIF2 α is phosphorylated, thereby globally inhibiting translation. The expression of ER stress-related genes and the abundance of the pro-apoptotic protein Bim are increased. Furthermore, vemurafenib inhibits phosphorylation of ERK, which enhances downstream pro-apoptotic signaling. Dashed lines represent indirect effects.