The HSP90 Inhibitor XL888 Overcomes BRAF Inhibitor Resistance Mediated through Diverse Mechanisms

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Abstract

**Purpose:** The clinical use of BRAF inhibitors is being hampered by the acquisition of drug resistance. This study shows the potential therapeutic use of the HSP90 inhibitor (XL888) in six different models of vemurafenib resistance.

**Experimental Design:** The ability of XL888 to inhibit growth and to induce apoptosis and tumor regression of vemurafenib-resistant melanoma cell lines was shown in vitro and in vivo. A novel mass spectrometry–based pharmacodynamic assay was developed to measure intratumoral HSP70 levels following HSP90 inhibition in melanoma cell lines, xenografts, and melanoma biopsies. Mechanistic studies were carried out to determine the mechanism of XL888-induced apoptosis.

**Results:** XL888 potently inhibited cell growth, induced apoptosis, and prevented the growth of vemurafenib-resistant melanoma cell lines in 3-dimensional cell culture, long-term colony formation assays, and human melanoma mouse xenografts. The reversal of the resistance phenotype was associated with the degradation of PDGFRβ, COT, IGFR1, CRAF, ARAF, S6, cyclin D1, and AKT, which in turn led to the nuclear accumulation of FOXO3a, an increase in BIM (Bcl-2 interacting mediator of cell death) expression, and the downregulation of Mcl-1. In most resistance models, XL888 treatment increased BIM expression, decreased Mcl-1 expression, and induced apoptosis more effectively than dual mitogen-activated protein–extracellular signal–regulated kinase/phosphoinositide 3-kinase (MEK/PI3K) inhibition.

**Conclusions:** HSP90 inhibition may be a highly effective strategy at managing the diverse array of resistance mechanisms being reported to BRAF inhibitors and appears to be more effective at restoring BIM expression and downregulating Mcl-1 expression than combined MEK/PI3K inhibitor therapy.

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Introduction

Despite the recent clinical success of BRAF inhibitors such as vemurafenib (PLX4032) and dabrafenib (GSK2118436) in BRAF mutant melanoma, most of the responses observed are transient, with relapse and resistance occurring in most cases (1, 2). The emerging data suggests that BRAF inhibitor resistance is complex, multifactorial, and results from intrinsic and acquired mechanisms. To date, the loss/inactivation of PTEN function, deletion of the retinoblastoma protein (RB), expression of the mitogen-activated protein kinase (MAPK) family member COT and amplification of cyclin D1 have each been shown to mediate intrinsic resistance by either diminishing the apoptotic response or allowing for cell-cycle entry when oncogenic BRAF is inhibited (3–6). Unlike the acquired drug resistance to imatinib seen in chronic myeloid leukemia and to epidermal growth factor receptor (EGFR) inhibitors in non–small cell lung cancer (NSCLC), resistance of melanoma cells to BRAF inhibitors does not result from secondary "gate-keeper" mutations in the BRAF kinase (7). Instead, acquired resistance is mediated through constitutive signaling by receptor tyrosine kinases (RTK; IGF1R and PDGFR-β), mutations in NRAS or MEK1, the increased expression of COT and as the result of BRAF truncations (4, 8–10). The apparent diversity of resistance mechanisms, and the likelihood that others exist is expected to complicate the design of future clinical trials to prevent or treat resistance to BRAF inhibitors. These observations led us to hypothesize that BRAF inhibitor
resistance may be best managed through broadly targeted strategies that inhibit multiple pathways simultaneously.

The HSP90 family of chaperones maintains the malignant potential of cancer cells by regulating the conformation, stability, and function of many RTKs and kinases required for oncogenic transformation (11, 12). Many proteins required for melanoma initiation and progression, including mutated BRAF, CRAF, IGF1R, cyclin D1, CDK4, and AKT are known to be clients of HSP90 (13, 14). The role of HSP90 in the stabilization of so many cancer-related proteins has made it an attractive target for therapeutic intervention. At this current time, more than 13 small molecule inhibitors of HSP90 are at various stages of preclinical and clinical development (12).

Although HSP90 inhibitors have shown only limited single-agent activity, more promising clinical efficacy has been shown when HSP90 inhibitors are combined with other agents. There is now good evidence that HSP90 inhibitors overcome trastuzumab resistance in breast cancer and potentiate the effects of bortezomib in treatment-refractory myeloma (15–18). In this study, we show that all of the signaling proteins implicated thus far in the escape from vemurafenib therapy are clients of HSP90. Inhibition of HSP90 using XL888 overcomes both acquired and intrinsic vemurafenib resistance by restoring the apoptotic response, which suggests that the combination of vemurafenib and an HSP90 inhibitor may be a strategy to delay and/or overcome BRAF inhibitor resistance.

### Translational Relevance

The impressive clinical response of melanoma patients to the BRAF inhibitor vemurafenib is limited by the onset of resistance. Resistance can be intrinsic or acquired; it is mediated through an array of mechanisms including acquired mutations in NRAS and MEK1, truncated BRAF, overexpression of COT, CRAF, PDGFR-β, cyclin D1, and IGF1R. This apparent diversity of resistance mechanisms, coupled with the phenotypic and cell signaling plasticity of melanoma cells, represents a considerable clinical challenge for which no management strategies currently exist. Here, we show that all of the signaling proteins implicated thus far in the escape from vemurafenib therapy are clients of HSP90. Inhibition of HSP90 using XL888 overcomes both acquired and intrinsic vemurafenib resistance by restoring the apoptotic response, which suggests that the combination of vemurafenib and an HSP90 inhibitor may be a strategy to delay and/or overcome BRAF inhibitor resistance.

### Materials and Methods

#### Cell culture and generation of BRAF inhibitor resistance

The parental 1205Lu, WM39, and WM164 melanoma cell lines were a gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA) and were genotyped as being BRAF V600E mutant in Smalley and colleagues (19). The M229, M229R, M249, and M249R were described in Nazarian and colleagues (8). The RPMI7951 melanoma cell line was purchased from American Type Culture Collection. The identities of all cell lines were confirmed by Biosynthesis Inc., through short tandem repeat validation analysis. Naive and intrinsically resistant lines were cultured in 5% FBS, RPMI. For all studies, all acquired resistant cell lines were maintained in 5% media with the addition of vemurafenib at the following concentrations: 1 μmol/L for M229R and M249R, 2 μmol/L for WM164R, and 3 μmol/L for 1205LuR.

#### Growth inhibition

Cells were plated at a density of 2.5 × 10⁶ cells per mL and left to grow overnight before being treated with increasing concentrations of vemurafenib or XL888 as described in Smalley and colleagues (19). Data show the mean of at least 3 independent experiments ± the SEM.

#### Western blotting

Proteins were extracted and blotted for as described in Smalley and colleagues (19). After analysis, Western blots were stripped once and reprobed for β-actin or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to show even protein loading. The antibodies to IGF1R, PDGFRβ, CRAF, phospho-AKT Ser473, total AKT, phospho-ERK (extracellular signal-regulated kinase), total ERK, cyclin D1, phospho-S6, total S6, phospho-BIM (Bcl-2 interacting mediator of cell death; Ser69), total BIM, HSP70, and MCL-1 were from Cell Signaling Technology. Anti-26S was purchased from Abcam whereas the antibody against COT was from Santa Cruz Biotechnology. For mouse xenograft studies, tumor samples were harvested and immediately placed into RNAlater solution (Invitrogen) before protein extraction.

#### Flow cytometry

Cells were plated into 6-well tissue culture plates at 60% confluency and left to grow overnight before being treated with either 300 nmol/L XL888, 3 μmol/L AZD6244, 3 μmol/L GDC-0941 (Selleck), or the combination of 3 μmol/L AZD6244 and 3 μmol/L GDC-0941 for 72 or 144 hours. In some studies, RPMI7951, WM793, and 1205LuR cells were treated with 300 nmol/L XL888 in the presence or absence of 3 μmol/L vemurafenib and harvested after 48 hours. Annexin V and TMRM staining was done as described in Parasio and colleagues (3).

#### Three-dimensional spheroid assays

Melanoma spheroids were prepared using the liquid overlay method (24). Spheroids were either treated for 144 hours with vehicle or 1 μmol/L XL888 or for 48 hours with vehicle, 1 μmol/L XL888, 3 μmol/L vemurafenib, or a combination of the 2 drugs before being washed and analyzed as described in Smalley and colleagues (24).

#### RNA interference

M229R and 1205LuR were plated at 1 × 10⁵ and left to grow overnight in RPMI complete media. Complete media was from Santa Cruz Biotechnology. For mouse xenograft studies, tumor samples were harvested and immediately placed into RNAlater solution (Invitrogen) before protein extraction.
was replaced with Opti-MEM (Invitrogen) and Mcl-1 or BIM (both 25 nmol/L; Cell Signaling Technologies) siRNAs in complex with Lipofectamine 2000 (Invitrogen) were added. In addition, scrambled siRNAs were added as nontargeting controls. A final concentration of 5% FBS in complete RPMI was added the next day. In the BIM studies, cells were transfected for a total of 48 hours before a 48-hour treatment with 300 nmol/L XL888. In the Mcl-1 studies, cells were treated for 96 hours.

**Immunofluorescent staining**

M229R and 1205LuR cells were seeded at 50% confluency before being treated with 300 nmol/L XL888, 3 μmol/L AZD6244, 3 μmol/L GDC-0941, or AZD6244 + GDC-0941 in combination as previously described (3). Cells were stained with antibodies against BIM and FOXO3a followed by staining with secondary anti-rabbit AF488 and imaged with a Leica confocal microscope.

**Proteomics sample preparation**

Proteins were extracted as described for Western blotting and processed as described in Remily-Wood and colleagues (21).

**Liquid chromatography, multiple reactions monitoring mass spectrometry analysis**

Liquid chromatography, multiple reactions monitoring mass spectrometry (LC-MRM) was done as described in Remily-Wood and colleagues (21). Protein expression was determined using the ratio of peak area of the native peptide to corresponding internal standard; normalization of tissue results was done using GAPDH to control for cellularity (see Supplementary Table S1). Data were then normalized to the pretreatment (cell lines) or vehicle controls (tissue) and plotted to show the changes in expression after drug treatment.

**Human specimen procurement**

Patients scheduled to undergo surgical resection for metastatic melanoma were prospectively consented and accrued to an existing melanoma tissue procurement protocol approved by the Moffitt Cancer Center Scientific Review Committee and The University of South Florida Institutional Review Board. Following excision of the specimen in the operating room, fine needle tumor aspirates were taken using a 22-gauge needle for proteomic analysis of the resulting tumor homogenate.

**MCL-1–inducible cell line**

WM793TR MCL-1 cells were a kind gift from Dr. Andrew Aplin (Kimmel Cancer Center, Philadelphia, PA; ref. 22). Mcl-1 expression was induced by the addition of 100 ng/mL doxycycline for 72 hours before treatment with 300 nmol/L XL888 for an additional 72 hours.

**Quantitative real-time PCR**

Cells were treated for 48 hours with 300 nmol/L XL888, 3 μmol/L AZD6244, 3 μmol/L GDC-0941, or AZD6244 and GDC-0941 in combination before RNA isolation. Total RNA was isolated using Qiagen’s RNeasy mini kit. The following TaqMan Gene Expression Assays primer/probes were used: Hs00197982_m1 (BIM), Hs01050896_m1 (MCL-1), Hs00372937_m1 (BMF), P/N 4319413E (18S), and Hs99999905_m1 (GAPDH). The 18S + GAPDH data were used for normalizing BIM. Quantitative reverse transcriptase PCR (qRT-PCR) reactions were carried out as previously described (3).

**Colonies formation**

Cells (1 × 10⁶ per mL) were grown overnight before being treated with vehicle (dimethyl sulfoxide) or XL888 (300 nmol/L) for 4 weeks as described in Paraiso and colleagues (23), and relative colony density was determined by solubilizing the crystal violet dye in 10% acetic acid followed by measurement of absorbance at 450 nm.

**Xenograft experiments**

BALB severe combined immunodeficient (SCID) mice (The Jackson Laboratory) were subcutaneously injected with 2.5 × 10⁶ cells per mouse and grown to approximately 100 mm³ before dosing. Mice were treated with either 100 mg XL888/kg (n = 5) or an equivalent volume of vehicle (10 mmol/L HCl), 3 × per week by oral gavage. Mouse weights and tumor volumes (L × W²/2) were measured 3 × per week. Upon completion of the experiment, vehicle- and drug-treated tumor biopsies were processed for LC-MRM analysis (as above). Detection of apoptosis by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) staining (Millipore) was carried out according to the manufacturer’s instructions.

**Statistical analysis**

Data show the mean of at least 3 independent experiments ± the SEM, unless stated otherwise. Statistically significant results were considered in which P ≤ 0.05. Additional statistical analyses are described in the Supplementary Material.

**Results**

**Inhibition of HSP90 overcomes resistance to vemurafenib resistance mediated through diverse mechanisms**

We first assembled a panel of BRAF V600E mutant melanoma cell lines with different mechanisms of intrinsic resistance and acquired vemurafenib resistance (see Supplementary Table S2). Treatment of matched BRAF inhibitor–naive and BRAF inhibitor–resistant melanoma cell lines with vemurafenib showed a statistically significant difference in the extent of growth inhibition (P = 0.02; t = –4.38; Fig. 1A, Supplementary Fig. S1) when resistance was mediated through increased PDGFRβ expression (M229R) and an acquired NRAS mutation (M249R), as well as 2 lines with uncharacterized mechanisms of resistance (WM164R and 1205LuR) (Fig. 1A). Cell lines with amplification of cyclin D1 (WM39) and overexpression of COT (RPMI 7951) showed signs of intrinsic resistance to...
Figure 1. The HSP90 inhibitor XL888 blocks the growth and survival of melanoma cell lines with diverse mechanisms of vemurafenib resistance. A, growth assay showing the response of matched pairs of vemurafenib-naive and vemurafenib-resistant melanoma cell lines and melanoma cell lines with intrinsic resistance. Left, cells were treated with increasing concentrations of vemurafenib (1 nmol/L–10 μmol/L; 72 hours) before being subject to the MTT assay. Right, cell growth assay showing the response of the cell line panel from (A) to the HSP90 inhibitor (1 nmol/L–10 μmol/L; 72 hours). B, cell-cycle effects of XL888 (300 nmol/L; 24 hours) upon vemurafenib-sensitive and vemurafenib-naive cell lines. Cells were fixed, stained with propidium iodide, and distributions analyzed by flow cytometry. C, XL888 induces apoptosis in every model of acquired vemurafenib resistance tested. Cells were treated for either 72 or 144 hours with XL888 (300 nmol/L; 24 hours) upon vemurafenib-sensitive and vemurafenib-naive cell lines. Cells were treated with 300 nmol/L XL888 for 4 weeks before being fixed and stained with crystal violet. Bottom: quantification of absorbance after 4 weeks of drug treatment.
vemurafenib (IC50 > 3 μmol/L). By contrast, treatment with the HSP90 inhibitor XL888 led to dose-dependent decreases in the growth of all the cell lines with no significant difference in IC50 values observed between the naive and resistance pairs of cell lines (t = 0.25, P = 0.82; Fig. 1A). The growth inhibitory effects of XL888 were associated with induction of either a G1-phase cell-cycle arrest (WM164, M229, M229R, M249, M249R, 1205Lu, and WM39) or a G2-M phase cell-cycle arrest (WM164R, 1205LuR, and RPMI7951; Fig. 1B). Treatment of all of the vemurafenib-resistant melanoma cell lines with XL888 (300 nmol) induced high levels (>66%) of apoptosis as shown by Annexin V binding, caspase-3 cleavage, and loss of mitochondrial membrane potential (TMRE) in every cell line tested (Fig. 1C and Supplementary Fig. S2). The cytotoxic effects of XL888 were durable with no signs of colony formation observed in any
of the cell lines (up to 4 weeks; Fig. 1D and Supplementary Fig. S3).

**Inhibition of HSP90 degrades all of the proteins identified as being critical for vemurafenib resistance**

We next asked whether XL888 treatment induced the degradation of all the signaling mediators implicated in acquired and intrinsic resistance (Supplementary Fig. S4 summarizes melanoma-relevant HSP90 clients). XL888 treatment (300 nmol/L, 48 hours) led to the degradation of IGF1R, PDGFRβ, ARAF, CRAF, and cyclin D1 and the inhibition of AKT, ERK, and S6 signaling in all of the cell lines with acquired BRAF inhibitor resistance (Fig. 2A). These effects were found to be time dependent with some sensitive proteins, such as pAKT being downregulated at 8 hours or earlier (Fig. 2A). In the intrinsically vemurafenib-resistant melanoma cell lines RPMI7951 and WM39, XL888 treatment was found to degrade both COT and cyclin D1, respectively (Fig. 2A). Because the microenvironment modulates the response of melanoma cells to targeted therapies (24), we next grew the panel of vemurafenib-resistant cell lines as collagen implanted 3-dimensional (3D) spheroids and noted that XL888 was effective at inducing cell death (Fig. 2B). In line with the observation that COT mediates resistance to vemurafenib (4), the combination of XL888 with vemurafenib significantly enhanced the level of apoptosis/cytotoxicity in 3D culture in RPMI7951 cells, compared with XL888 alone (Fig. 2C and D). A similar enhancement was noted when the vemurafenib ± XL888 combination was applied to 2 melanoma cell lines in which the primary resistance was mediated through PTEN loss (WM793 and 1205Lu; Fig. 2C and D).

**Development of a quantitative pharmacodynamic assay of HSP90 inhibition**

The clinical development of HSP90 inhibitors has been hampered by the lack of a good pharmacodynamic assay for quantifying target inhibition within the tumor (12). As inhibition of HSP90 typically leads to the increased expression of other HSP family members, which can be used as a surrogate for HSP90 inhibition, we developed a highly sensitive quantitative LC-MRM assay for the quantification of individual peptides in complex mixtures. Because the microenvironment modulates the response of melanoma cells to targeted therapies (24), we next grew the panel of vemurafenib-resistant cell lines as collagen implanted 3-dimensional (3D) spheroids and noted that XL888 was effective at inducing cell death (Fig. 2B). In line with the observation that COT mediates resistance to vemurafenib (4), the combination of XL888 with vemurafenib significantly enhanced the level of apoptosis/cytotoxicity in 3D culture in RPMI7951 cells, compared with XL888 alone (Fig. 2C and D). A similar enhancement was noted when the vemurafenib ± XL888 combination was applied to 2 melanoma cell lines in which the primary resistance was mediated through PTEN loss (WM793 and 1205Lu; Fig. 2C and D).
of 11 HSP family members (ref. 12; Fig. 3A). Treatment of cell lines that were naive, intrinsically resistant, and with acquired vemurafenib resistance with XL888 (300 nmol/L) led to significant levels of tumor regression ($P = 0.003$). Western blot experiments confirmed the XL888-dependent increases in HSP70 expression in every cell line evaluated (Fig. 3C). The potential clinical relevance of the LC-MRM assay was shown by the successful quantification of HSP70 and other chaperone proteins from fine needle aspirates (~2,000 cells) taken from 2 melanoma specimens (Fig. 3D).

XL888 treatment causes the regression of vemurafenib-resistant xenografts in vivo associated with increased intratumoral HSP70 expression

The relevance of HSP90 inhibition as a strategy to overcome BRAF inhibitor resistance in vivo was shown by the ability of XL888 (100 mg/kg, orally, 3 times a week) to significantly induce the regression of, or growth inhibition of established M229R and 1205LuR xenografts in SCID mice (Fig. 4A and Supplementary Fig. S5). Western blot experiments confirmed the XL888-dependent increases in HSP70 expression in every cell line evaluated (Fig. 3C). The potential clinical relevance of the LC-MRM assay was shown by the successful quantification of HSP70 and other chaperone proteins from fine needle aspirates (~2,000 cells) taken from 2 melanoma specimens (Fig. 3D).

**HSP90 inhibition restores nuclear localization of FOXO3a, upregulates BIM expression, and inhibits Mcl-1 expression in vemurafenib-resistant cell lines**

To determine the mechanism of XL888-induced apoptosis in the vemurafenib-resistant melanoma cell lines, we first focused upon Mcl-1. Whereas vemurafenib treatment increased expression of BIM in melanoma cell lines that were drug naive (3), the resistant cell lines suppressed their expression of BIM even in the continuous presence of vemurafenib (Fig. 5A). XL888 treatment reversed this and increased BIM expression, irrespective of resistance mechanism (Fig. 5A). It was noted that XL888 treatment increased the expression of BIM-EL, BIM-L, and BIM-S expression in the M229R, 1205LuR, RPMI7951, and WM39 cell lines, induced expression of BIM-L and BIM-S in the WM164R cell line, and BIM-EL in the M249R cell line (Fig. 5A). These effects were mediated in part through increased BIM protein stability as noted by decreased BIM phosphorylation at Ser69 in all of the cell lines tested apart from M249R (Fig. 5A). We next asked whether HSP90 inhibition also affected BIM expression at the mRNA level. In vemurafenib-naive cells, inhibition of BRAF leads to the nuclear accumulation of the transcription factor FOXO3a and...
increased BIM expression (3). In contrast, cell lines with acquired resistance to vemurafenib excluded FOXO3a from the nucleus and suppressed BIM protein and mRNA expression even in the continuous presence of vemurafenib (Fig. 5A and Supplementary Fig. S7). XL888 treatment reversed these effects and led to the nuclear accumulation of FOXO3a and an increase in BIM mRNA and protein expression (Fig. 5A, Supplementary Fig. S7). An increase in nuclear size following XL888 treatment was also noted. The importance of BIM expression in the XL888-mediated cell death response was shown by the significant inhibition of apoptosis observed when BIM expression was knocked down by siRNA (Fig. 5B).

Mcl-1 is prosurvival BH3 family protein member that antagonizes the activity of BIM (22). Treatment of melanoma cell lines in which vemurafenib resistance was mediated through PDGFRβ, COT overexpression, and 2 melanoma cell lines with unknown resistance mechanisms with XL888 (300 nmol/L, 48 hours) led to a marked decrease in the expression of Mcl-1 (Fig. 5C). qRT-PCR experiments showed that XL888 treatment also blocked Mcl-1 expression at the mRNA level (Fig. 5C). The importance of Mcl-1 expression for the survival of vemurafenib-resistant melanoma cell lines was confirmed by the significant induction of apoptosis observed following siRNA knockdown of Mcl-1 expression (Supplementary Fig. S8). Further evidence for the role of Mcl-1 expression in the drug resistance phenotype came from overexpression studies in which induction of Mcl-1 expression following doxycycline treatment led to a significant reduction in the magnitude of XL888-induced apoptotic response (Fig. 5D).

HSP90 inhibition is more effective at inducing BIM expression and apoptosis than combined MEK+PI3K inhibition

The simultaneous targeting of mitogen-activated protein/extracellular signal-regulated kinase (MEK/ERK) and phosphoinositide 3-kinase (PI3K)/AKT signaling is being explored as a strategy for overcoming vemurafenib resistance. We next asked whether HSP90 inhibition was more...
effective than the MEK+PI3K inhibitor combination at restoring apoptosis in vemurafenib-resistant melanoma cells. Although both XL888 and the PI3K inhibitor GDC-0941 were highly efficient at increasing nuclear accumulation of FOXO3a (Fig. 6A), XL888 treatment led to a greater induction of BIM expression at both the protein and mRNA levels and significantly restored the apoptotic response (Fig. 6B and C). Similarly, XL888 treatment was also more effective than the MEK or PI3K inhibitor, alone or in combination, at downregulating the expression of Mcl-1 at both the mRNA and protein levels (Fig. 6B and C). This was in marked contrast to the responses observed in the parental M229 and 1205Lu cell lines, in which the MEK ± PI3K inhibitor combination was equally effective as XL888 at inducing BIM expression (Supplementary Fig. S9).

Although there is evidence that the BH3 protein family member BIM plays a role in the apoptotic response to BRAF inhibition (25), XL888 treatment only weakly induced BIM mRNA expression (Supplementary Fig. S10). In contrast, treatment of 2 vemurafenib-resistant cell lines with either the MEK inhibitor (M229R) or the MEK+PI3K inhibitor (1205LuR) led to a robust induction of BIM expression but induced less apoptosis than following XL888 treatment (Fig. 6D, Supplementary Fig. S10). As the phosphorylation of BIM by MEK/ERK leads to its proteasomal degradation and the 26S proteasome is an HSP90 client protein, we next determined the contribution of proteasome inhibition to the cytotoxic effects of XL888. Although XL888 treatment was observed to partly degrade the 26S proteasome, HSP90 inhibition had a considerably weaker effect upon proteasomal activity than either the MEK+PI3K inhibitor combination or the proteasome inhibitor (MG-132; Supplementary Fig. S11). In agreement with the marked effects of HSP90 inhibition on BIM and Mcl-1 expression compared with the MEK, PI3K, and MEK+PI3K inhibitor combination, XL888 was observed to induce significantly higher levels of apoptosis than each of the other drug combinations in cell lines in which resistance was mediated through amplification of COT, PDGFRβ overexpression and in 2 other models where the resistance mechanism is as yet unknown (Fig. 6D). The level of apoptosis induced by the MEK+PI3K inhibitor combination was equivalent to that of the HSP90 inhibitor when resistance was mediated through NRAS mutation or cyclin D1 amplification (Fig. 6D).

Discussion

This study addressed whether targeting multiple signaling pathways through the inhibition of HSP90 is sufficient to overcome intrinsic and acquired resistance to the BRAF inhibitor vemurafenib (PLX4032). XL888 is a novel, orally available HSP90 inhibitor with high selectivity for HSP90α and HSP90β (IC50 = 22, 44 nmol/L, respectively) and little activity against a panel of 29 other diverse kinases (IC50 all >3,600 nmol/L; ref. 20). XL888 inhibited the growth of, and promoted apoptosis in, melanoma cell lines in which vemurafenib resistance was mediated through NRAS mutations, PDGFRβ overexpression, COT overexpression, and cyclin D1 amplification. It was also proapoptotic in 2 melanoma cell lines with acquired vemurafenib resistance mediated through as yet unknown means. In all of the vemurafenib-sensitive cell lines, XL888 induced a G1-phase cell-cycle arrest and reduced the percentage of cells in S-phase. In some of the resistance models, XL888 treatment instead induced cell-cycle arrest in G2-M, perhaps suggesting an altered signaling dependency following the acquisition of drug resistance. In all cases, the responses to XL888 were highly durable with no resistant colonies emerging following 4 weeks of continuous drug treatment. The prolonged growth inhibition with XL888 was in marked contrast to that observed by our group and reported by others following vemurafenib (or PLX4720) treatment in which resistant colonies emerged in every case (8, 23, 26). As the lack of colony formation suggested that all the BRAF inhibitor resistance mechanisms present in our models were abrogated following HSP90 inhibition, we next confirmed that XL888 decreased the expression of the proteins implicated thus far in acquired and intrinsic vemurafenib resistance (IGF1R, cyclin D1, PDGFR-β, AKT, COT, ARAF, and CRAF; refs. 4, 5, 8, 9, 27). In each case, not only did XL888 reduce the levels of the RTK/kinases implicated in BRAF inhibitor resistance, it also blocked the signaling activity of the pathways (MEK, AKT, and mTOR/S6) involved in therapeutic escape (9, 23, 27, 28). Although IGF1R, COT, AKT, ARAF, MEK, and CRAF have previously been reported to be HSP90 clients and subject to proteasome-mediated degradation following HSP inhibition, this is the first report to potentially identify PDGFR-β as a client of HSP90 (a current list of HSP90 clients is maintained at http://www.picard.ch/downloads/Hsp90interactors.pdf).

The potential use of HSP90 inhibitors in overcoming vemurafenib resistance was illustrated by the ability of XL888 to inhibit multiple, nonoverlapping resistance pathways in the same cell line model, for example, the inhibition of PDGFRβ, IGF1R, and COT in the COT-amplified cell line and PDGFRβ, IGF1R, and COT in the PDGFRβ-overexpressing cell line. The fact that melanomas express multiple...
RTKs and can flexibly switch between multiple signaling pathways suggests that individual melanoma cells may have a number of escape mechanisms at their disposal. Furthermore, effective strategies to manage resistance will need to concurrently target multiple oncogenic pathways (9). There is already evidence from other cancers that HSP90 inhibitors can overcome multiple drug resistance mechanisms. In preclinical studies of breast cancer, inhibitors of HSP90 abrogate diverse trastuzumab resistance mechanisms, including those mediated by PI3K mutations, truncation mutants of p95-HER2, and the upregulation of membrane associated mucin-4 (29–32). In NSCLC, inhibition of HSP90 prevents drug resistance associated with the oncogenic switch from EGFR to c-MET (32). HSP90 inhibitors have also proved effective at managing drug resistance in the clinic, with activity being reported against trastuzumab-resistant HER2+ breast cancer and bortezomib-resistant multiple myeloma (16, 17, 33, 34).

The measurement of HSP90 inhibition in vivo has proven to be challenging. Although it is known that HSP90 inhibition is well correlated with the increased expression of the cochaperone HSP70, which can be quantified in peripheral blood mononuclear cells, this does not correlate well with either intratumoral HSP90 inhibition or clinical activity (12). The high abundance of heat shock chaperone proteins makes them amenable to direct quantification by mass spectrometry with minimal processing (21). As patients with advanced melanoma typically present with accessible cutaneous lesions that can be biopsied or undergo fine needle aspiration, we developed a novel quantitative pharmacodynamic mass spectrometry–base assay for the quantification of HSP90 and its cochaperones. In agreement with previously published studies on other HSP90 inhibitors, XL888 treatment led to the consistent upregulation in the expression of HSP70 isoform 1 in every vemurafenib-sensitive and vemurafenib-naive cell line tested (21, 33, 35). Although there is evidence that increased HSP70 expression limits apoptosis in leukemic cells, the therapeutic relevance of this observation in melanoma is still under investigation (36). The in vivo use of the LC-MRM technique was shown by the robust increases in HSP70 expression observed in xenografts following XL888 treatment and the ability to quantify levels of HSP90 and its key cochaperones in small needle biopsies (fine needle aspirates) taken from fresh melanoma specimens. These results show the use of LC-MRM–based pharmacodynamic assays for measuring intratumoral HSP90 inhibition that can be incorporated into future clinical trials of these drugs.

Inhibition of BRAF, either by siRNA knockdown or small molecule inhibitors of BRAF or MEK, induces apoptosis in BRAF V600E mutant melanoma cells through the proapoptotic proteins BIM, BMF, and BAD (25, 37–39). BIM is a BH3 family protein member that plays a key role in the induction of cell death by binding to and antagonizing the prosurvival proteins Bcl-2, Bcl-w, Bcl-XL, and Mcl-1 (40, 41). Vemurafenib resistance (both intrinsic and acquired) is characterized by a diminished apoptotic response and impaired BIM expression in the continuous presence of drug. The observation that BIM is regulated both transcriptionally and posttranscriptionally through many pathways including ERK, AKT, JNK, and p38 MAPK, led us to hypothesize that XL888 may overcome vemurafenib resistance by upregulating BIM expression at both the mRNA and protein levels through the simultaneous targeting of multiple signaling pathways (42, 43). Regulation of BIM mRNA is mediated by the transcription factor FOXO3a, which is inactivated following its phosphorylation by AKT at T32, S253, and S315, leading to its nuclear exclusion and localization to the cytoplasm (3, 44). BIM levels are controlled posttranslationally through phosphorylation of the protein at a number of sites (including S69) by MEK/ERK signaling, with the phosphorylation of BIM leading to its polyubiquitination and proteasomal degradation (39). Our previous studies showed that vemurafenib increased nuclear FOXO3a localization and BIM expression in drug-naive cells leading to increased apoptosis (3). Here we noted that vemurafenib resistance was associated with suppression of nuclear FOXO3a and BIM expression in the continued presence of drug that was reversed upon addition of XL888. Interestingly, XL888 treatment was more effective at restoring the expression of BIM at the mRNA and protein levels and inducing apoptosis than dual inhibition of MEK and PI3K, perhaps suggesting the involvement of other (as yet unidentified) pathways that are also HSP90 clients. Although expression of BIM is regulated both through 26S ubiquitin-dependent and 20S polyubiquitin–independent proteasomal mechanisms and the 26S proteasome is a known HSP90 client, we were unable to show a role for downregulation of the 26S proteasome in the recovery of BIM expression following HSP90 inhibition (45, 46). A number of recent studies have suggested a role for increased BMF (Bcl-2 modifying factor) expression in mediating the apoptotic response of melanoma cells treated with inhibitors of BRAF and MEK (25, 38). Here, we observed that XL888 treatment was a relatively weak inducer of BMF expression in the vemurafenib-resistant melanoma cell lines compared with that seen following MEK or PI3K + MEK inhibition, suggesting that BMF is relatively dispensable in overcoming BRAF inhibitor resistance in our models.

The decision between survival and apoptosis is regulated through the balance of pro- and antiapoptotic Bcl-2 family proteins. Survival of melanoma cells is controlled in part by the antiapoptotic protein, Mcl-1, whose stability is regulated by the BRAF/MEK/ERK pathway (22). A potential role for Mcl-1 in the tolerance of BRAF inhibition was suggested by the studies showing that acquired vemurafenib resistance led to the recovery of MAPK signaling, whereas resistant cells maintained their Mcl-1 expression in the presence of vemurafenib, and that the forced overexpression of Mcl-1 decreased the vemurafenib-induced apoptotic response (22, 23). Inhibition of HSP90 led to the degradation of Mcl-1 protein and reduced Mcl-1 expression at the mRNA level. XL888 was more effective at reducing Mcl-1 mRNA levels than inhibitors of MEK, PI3K, and the MEK + PI3K inhibitor combination. It therefore seems likely that the
induction of BIM in concert with Mcl-1 downregulation plays a key role in the induction of XL888-mediated apoptosis.

Current preclinical and clinical strategies for managing vemurafenib resistance in melanoma are centered upon combining vemurafenib with inhibitors of the MEK and PI3K/AKT/mTOR pathways (9, 28). Although our study supports use of the MEK+PI3K inhibitor combination when resistance is mediated through NRAS mutations or cyclin D1 amplification, it appears suboptimal when resistance is mediated by increased COT expression, PDGFRβ overexpression, and in other cell line models with undetermined resistance mechanisms. These findings suggest either that other pathways (that also happen to be HSP90 clients) are required for therapeutic escape or that vertical inhibition of the same pathway at multiple points (e.g., ARAF/CRAF/MEK or IGF1R/AKT/S6) simultaneously may be a more effective way of shutting down a signal transduction pathway.

In summary, we have shown for the very first time that all of the signaling proteins implicated thus far in intrinsic and acquired BRAF inhibitor resistance are clients of HSP90 and that inhibition of HSP90 can restore sensitivity to vemurafenib-mediated cell death by upregulating expression of BIM and inhibiting expression of Mcl-1. These studies provide the rationale for the dual targeting of HSP90 and BRAF in BRAF mutant melanoma as a strategy to limit the therapeutic escape seen with single-agent vemurafenib therapy.

**References**

34. Arteaga CL. Why is this effective HSP90 inhibitor not being developed in HER2+ breast cancer? Clin Cancer Res 2011;17:4919–21.
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## The HSP90 Inhibitor XL888 Overcomes BRAF Inhibitor Resistance Mediated through Diverse Mechanisms


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