Combined BRAF and HSP90 Inhibition in Patients with Unresectable BRAF<sup>V600E</sup>-Mutant Melanoma

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Abstract

**Purpose:** BRAF inhibitors are clinically active in patients with advanced BRAF<sup>V600E</sup>-mutant melanoma, although acquired resistance remains common. Preclinical studies demonstrated that resistance could be overcome using concurrent treatment with the HSP90 inhibitor XL888.

**Patients and Methods:** Vemurafenib (960 mg p.o. b.i.d.) combined with escalating doses of XL888 (30, 45, 90, or 135 mg p.o. twice weekly) was investigated in 21 patients with advanced BRAF<sup>V600E</sup>-mutant melanoma. Primary endpoints were safety and determination of a maximum tolerated dose. Correlative proteomic studies were performed to confirm HSP inhibitor activity.

**Results:** Objective responses were observed in 15 of 20 evaluable patients (75%; 95% confidence interval [CI], 51%–91%), with 3 complete and 12 partial responses. Median progression-free survival and overall survival were 9.2 months (95% CI, 3.8–not reached) and 34.6 months (6.2–not reached), respectively. The most common grade 3/4 toxicities were skin toxicities, such as rash (n = 4, 19%) and cutaneous squamous cell carcinomas (n = 3, 14%), along with diarrhea (n = 3, 14%). Pharmacodynamic analysis of patients' peripheral blood mononuclear cells (PBMC) showed increased day 8 HSP70 expression compared with baseline in the three cohorts with XL888 doses ≥45 mg. Diverse effects of vemurafenib-XL888 upon intratumoral HSP client protein expression were noted, with the expression of multiple proteins (including ERBB3 and BAD) modulated on therapy.

**Conclusions:** XL888 in combination with vemurafenib has clinical activity in patients with advanced BRAF<sup>V600E</sup>-mutant melanoma, with a tolerable side-effect profile. HSP90 inhibitors warrant further evaluation in combination with current standard-of-care BRAF plus MEK inhibitors in BRAF<sup>V600E</sup>-mutant melanoma. *Clin Cancer Res;* 24(22) November 15, 2018

See related commentary by Sullivan, p. 5496

**Introduction**

The discovery of activating mutations in the serine/threonine kinase BRAF and the appreciation of its role as a driver of melanoma growth and progression have transformed the treatment of disseminated BRAF-mutant melanoma (1, 2). Although the use of small-molecule BRAF inhibitors, such as vemurafenib and dabrafenib, frequently leads to rapid and impressive responses in patients with metastatic melanoma, resistance resulting in therapeutic escape is common, with median progression-free survivals (PFS) of 5.3 and 5.1 months demonstrated for vemurafenib and dabrafenib, respectively (1, 2). This therapeutic escape is frequently characterized by the recovery of signaling through the mitogen-activated protein kinase (MAPK) pathway, an effect that is mediated through multiple and diverse mechanisms including mutations in NRAS and MEK and BRAF splice mutations, and increased receptor tyrosine kinase (RTK) signaling (3–6). The continued dependence of resistant tumors upon MAPK signaling led to the development of strategies designed to vertically inhibit the pathway. Preclinical studies showed that dual BRAF–MEK inhibition abrogated therapeutic escape in vitro and delayed treatment failure in human melanoma mouse xenograft models (7, 8). In randomized clinical trials, the combination of a MEK inhibitor and a BRAF inhibitor (including vemurafenib and cobimetinib, dabrafenib and trametinib, and encorafenib and binimetinib) was associated with improved progression-free survival (PFS) and overall survival (OS) compared with BRAF inhibitor therapy alone (9–11).

Despite these improvements in efficacy, most patients eventually fail to respond to therapy, with similar mechanisms of resistance being reported for both single-agent BRAF inhibitor and BRAF–MEK inhibitor combination therapy (12). Clinical strategies to delay or prevent acquired BRAF and BRAF–MEK inhibitor resistance are complicated by the diversity of resistance mechanisms. Recent work from our group suggested that many of the proteins involved in developing resistance to BRAF and

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BRAF–MEK inhibitor therapy are dependent upon heat shock protein (HSP)-90 for their stabilities (13). Proteins stabilized by HSP90 (HSP90 client proteins) implicated in BRAF inhibitor resistance include CRAF, ARAF, cyclin D1, AKT, and CDK4, as well as multiple receptor tyrosine kinases (RTK) including c-MET, PDGFR, IGF1R, and ERBB3 (4, 14, 15). There are also preclinical data supporting the notion that HSP90 inhibitors can reverse BRAF and BRAF–MEK inhibitor resistance and delay the onset of BRAF inhibitor resistance in vivo (13, 16). XL888 (Exelixis) is a potent, orally administered small-molecular inhibitor of HSP90. In a phase I study of 33 patients with refractory solid tumors, the maximum tolerated dose (MTD) of XL888 was determined to be 135 mg twice weekly (b.i.w.) with diarrhea as a dose-limiting toxicity (DLT); the study did not include any patients with melanoma. In the current study, we performed a phase I escalation clinical trial of vemurafenib in combination with XL888 to determine an MTD and to evaluate the safety and potential efficacy of the combination in patients with advanced BRAFV600E/K-mutant melanoma.

**Patients and Methods**

**Study design and treatments**

This was an open-label, single-center phase 1 trial of escalating doses of XL888 in combination with vemurafenib in patients with unresectable or metastatic melanoma. Patients were enrolled using a modified 4+1 design (17) with the primary objective to determine the MTD of XL888 in combination with vemurafenib. There were four dose-level cohorts of XL888: 30 mg p.o. b.i.w., 45 mg b.i.w., 90 mg b.i.w., and 135 mg b.i.w., each given together with standard doses of vemurafenib (960 mg b.i.d., see Supplementary Fig. S1). Secondary objectives were to assess overall response rate (ORR), PFS and OS, in addition to assessing the biological activity and pharmacodynamics of XL888 utilizing proteomics-based biomarkers. Tumor responses were assessed using Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1, with radiologic assessments performed every 8 weeks. The study was approved by the institutional review board at the University of South Florida and was performed in accordance with recognized ethical guidelines [e.g., Declaration of Helsinki, Council for International Organizations of Medical Sciences (CIOMS), Belmont Report, U.S. Common Rule], with written, informed consent obtained from each patient.

**Inclusion and exclusion criteria**

Patients were ≥18 years of age, with cytologically or histologically confirmed unresectable (stage IIIC or IV) melanoma harboring a BRAFV600E/K mutation determined by a CLIA-certified assay. Patients had adequate hepatic, renal, and bone marrow function, along with an Eastern Cooperative Oncology Group (ECOG) performance status (PS) of 0 or 1. Treatment-naïve and previously treated patients were included, but patients could not have received prior BRAF inhibitors. Measurable disease defined by RECIST 1.1 was required. Patients were excluded if they had received systemic therapy or radiotherapy within 4 weeks of enrollment or if they had not recovered from adverse events (AE) caused by prior therapy. Untreated or uncontrolled central nervous system (CNS) metastases or evidence of leptomeningeal disease were also exclusions, although brain metastases treated with radiation and/or surgery were allowable if they had been stable for ≥4 weeks.

**Pharmacodynamic assessments**

HSP70 levels in peripheral blood mononuclear cells (PBMC) were analyzed as a marker of HSP90 inhibition (18, 19) since its inhibition leads to compensatory increases in the expression of the related chaperone protein HSP70 subtype 1. Serum samples were collected at treatment days 1 and 8 to evaluate PBMC HSP70 levels. Tumor biopsies on treatment days 1 and 8 were also obtained from consenting patients for analysis of HSP client protein levels. We utilized discovery proteomics and developed liquid chromatography-multiple reaction monitoring (LC-MRM) mass spectrometry assay panels for proteomics analysis of PBMC and biopsy specimens to validate increased HSP70 protein expression as a pharmacodynamic marker of HSP90 inhibition, and to determine the role of HSP90 inhibition in blocking the signaling pathways implicated in therapeutically resistant BRAF inhibitors (13, 18). Frozen PBMC samples were thawed using a water bath at 37 °C and spun at 500 × g for 5 minutes at 4 °C to pellet the cells. Pellets were washed with ice-cold PBS and then resuspended in aqueous 50% tetrafluoroethanol and sonicated (Branson 150). The lysates were reduced with tris (2-carboxyethyl)phosphine followed by alkylation with iodoacetamide. Protein samples were diluted 10-fold with 30 mmol/L ammonium bicarbonate and digested with trypsin prior to LC-MRM data acquisition. High abundance proteins, including HSPs, were quantified from ∼2,000 cells (~200 ng of total protein digest).

Tumor homogenates were resuspended in denaturing buffer (100 mmol/L ammonium bicarbonate with 8 mol/L urea), sonicated to maximize protein recovery (Branson 150), and clarified by centrifugation at 21,000 × g for 10 minutes. Protein concentrations were determined by Bradford assay and 50 μg of sample was fractionated by SDS-PAGE into five regions of 4% to 12% BisTris gels (Criterion XT, Bio-Rad), visualized with Coomassie Brilliant Blue G-250 (Sigma-Aldrich; see Supplementary Fig. S2) and excised as previously published. Gel regions were diced to ∼1 mm³ for processing. After destaining, disulfides were reduced with 2 mmol/L tris(carboxyethyl)phosphine and then cysteines were alkylated with 20 mmol/L iodoacetamide prior to overnight...
digestion with sequencing grade trypsin (Promega). The resulting proteolytic peptides were extracted with aqueous 50% acetonitrile, 0.01% trifluoroacetic acid and concentrated by vacuum centrifugation (SCC210A, Speedvac, Thermo Scientific). Peptides were reconstituted in 2% acetonitrile with 0.1% formic acid (loading solvent), containing the stable isotope-labeled internal standards (at 10 fmol/μL, so 50 fmol of each standard is injected for LC-MRM). The equivalent of 8.3 μm of total protein digest was analyzed in each LC-MRM experiment.

LC-MS/MS discovery proteomics was performed as described previously (19). LC-MRM analysis was performed in triplicate on a nanoLC interfaced with an electrospray triple quadrupole mass spectrometer (RSLCnano and Quantiva, Thermo Scientific). The following solvent system is used for LC-MRM analysis: solvent A is aqueous 5% acetonitrile with 0.1% formic acid, and solvent B is aqueous 90% acetonitrile with 0.1% formic acid. For each sample, an aliquot of the peptide mixture (5 μL, ~1/6 of the sample) was loaded onto the trap column at 6 μL/min and washed with loading solvent for 5 minutes. Then, a gradient of 5% B to 50% B was applied over 35 min prior to washing the column at 90% B and re-equilibrating over 10 minutes, for a total of 55 minutes for the LC experiment. Mass spectrometry instrument parameters included the following: 2,200 V spray voltage; 250°C transfer tube temperature; Q1 resolution 0.4 when transitions were monitored for the entire cycle; Q2–Q3 resolution 0.4 when transitions were monitored for the entire cycle, as previously optimized for this instrument by infusion of the standard peptides (see Supplementary Table S1).

Data analysis for protein quantification
Skyline version 3.7 was used for data evaluation (20). Peaks were evaluated by comparison of their elution time and fragment ion signal ratios to their matched internal standards. All transitions above 10% of the base peak were used for quantification. Data were exported to Excel for calculations of protein quantity, standard deviation, and coefficient of variation (%).

Statistical and analytical methods

Sample size, safety, and DLT definitions. The trial was designed to enroll up to 36 patients but would be successfully concluded earlier if at least 15 patients were accrued at the dose determined to be the MTD, with no more than 4 DLTs using the modified Ji design. AEs were graded according to NCI CTCAE v4. Cohorts of 3 patients with possible expansions to 6, 9, 12, or 15 patients were treated at each dose level until the MTD was defined. The cohort size at each dose depended on the observed toxicity, as the design allowed the sufficient number of patients to be explored with targeting the toxicity at 18%. The modifications to the Ji design were to escalate the dose whenever the observed toxicity rate was below 17% and deescalate the dose when the toxicity rate exceeded 25%, and N ≥ 9 to account for the fact that the design was being used in cohorts of 3 rather than continuously as designed by Ji and colleagues (see Supplementary Fig. S3). Once the MTD of XL888 with vemurafenib was determined, 6 additional patients were to be treated at this dose to further define the safety and efficacy profile of the combination. If there was deescalation to a dose that had already accrued 15 patients, that dose was declared the MTD.

DLT was defined as any of the following occurring during cycle 1 (the first 8 weeks) of treatment: (i) nonhematologic grade 3/4 AE not easily managed or corrected by medical intervention; (ii) grade 4 neutropenia that did not resolve within 7 days, or any grade 3/4 febrile neutropenia, or any grade 4 thrombocytopenia; (iii) any treatment-related AE that in the investigator’s opinion warranted a dose reduction or where any further dose-escalation would expose patients to unacceptable risk; (iv) inability to take 75% or more of the planned XL888 doses in cycle 1 due to study-related AEs; and (v) failure to recover from any study-related AE within 14 days.

Efficacy analysis. The ORR and its 95% confidence interval (CI) were estimated using the exact binomial method. For PFS and OS, medians and 95% CIs were estimated using the Kaplan–Meier method. One-year PFS and OS rates were also estimated using the Kaplan–Meier method. PFS was defined from the start of treatment to progression or death from any cause. OS was defined from the start of treatment to death from any cause; patients still alive were censored at date of last follow-up.

Results

Safety and DLT
Twenty-one patients with advanced BRAFV600E/K-mutant melanoma were enrolled from July 2012 to May 2016. Fourteen (67%) patients had stage IV M1c disease, with 12 (57%) having an elevated baseline lactate dehydrogenase (LDH); 39% of all patients had prior systemic therapy (most commonly immunotherapy; Table 1). There were three patients each enrolled in cohorts 1 and 2, nine patients in cohort 3, and six patients in cohort 4. The most common grade 3 AEs were skin toxicities such as rash (n = 4, 19%), cutaneous squamous cell carcinomas (SCC; n = 3, 14%), and new primary melanomas (n = 2, 10%), along with diarrhea (n = 3, 14%), headache (n = 2, 10%), and fatigue (n = 2, 14%; Table 2). There were three grade 4 AEs noted: asymptomatic lipase elevation, AST elevation, and arthralgia. Skin events, including SCC and keratoacanthomas (KA), were reduced in cohorts 3 and 4 compared with cohorts 1 and 2, as previously

<table>
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<tr>
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reported (21). In the phase I study of XL888, blurred vision and visual impairment were reported in two of 33 patients. In this trial, 6 patients experienced grade 2 visual disturbances possibly or probably related to treatment, most commonly blurriness, and one patient had macular edema; no retinopathy was observed. Two patients had treatment interruption and subsequently resumed treatment with no recurrence of symptoms, while two patients discontinued study treatment. All visual symptoms resolved.

With enrollment of 3 patients to each of the first three cohorts without DLTs, six patients were enrolled onto the highest dose cohort of XL888 at 135 mg b.i.w. However, two DLTs (grade 3 diarrhea and pancreatitis) were observed, along with one additional patient receiving <75% of the planned XL888 dose in the first cycle of treatment. Therefore, with 3 DLTs observed in this dose cohort, the decision was made to deescalate to cohort 3 (90 mg b.i.w. of XL888 with vemurafenib), which was declared the MTD. Six additional patients were enrolled at this dose level; in total, two patients out of nine in this cohort experienced DLTs (one with grade 3 diarrhea and arthalgia, one patient had a grade 3 rash.)

**Efficacy**

One patient on the trial switched to dabrafenib/trametinib after only 6 doses of XL888 prior to the first restaging scans (unrelated to toxicity) with a subsequent partial response. As this patient received a MEK inhibitor, they were excluded from the subsequent efficacy analysis. We observed objective responses to the vemurafenib–XL888 combination in 15 of 20 evaluable patients (ORR, 75%; 95% CI, 51–91%) with three complete (15%) and 12 partial (60%) responses (see waterfall plot, Fig. 1).

There was no clear correlation between level of response and XL888 dose cohort. Two patients with a partial response underwent resection of all residual disease, and both were found to have a pathologic complete response. At a median follow-up of 26.4 months, the median PFS was 9.2 months (95% CI, 3.8 months—not reached), with 1-year PFS of 45% (95% CI, 23%–65%; Fig. 2A). Among patients surviving beyond 2 years without progression, all of them had a normal LDH at baseline, as an elevated baseline LDH was significantly associated with worse PFS (HR = 1.969; P = 0.042). Median OS was 34.6 months (6.2 months—not reached), with 1-year OS of 60% (95% CI, 36%–78%, Fig. 2B). At the time of data cutoff, 9 of 21 patients were still alive. Of these 9 patients, 7 were without disease progression with a median follow-up of 35 months.

**Pharmacodynamics**

Our LC-MRM assay was used to quantify levels of the HSP70 chaperone protein in the blood of 10 patients from cohorts 2 to 4, and the expansion cohort (90 mg XL888), at baseline and following 8 days of therapy. These analyses showed increased HSP70 expression above baseline, indicating that XL888 was inhibiting HSP90 for most, but not all, patients (Fig. 3A).

We next analyzed the expression of 45 HSP client proteins across seven paired sets of pre- (day 0) and on-therapy (day 8) tumor biopsies from patients receiving vemurafenib-XL888 (30, 45, 90, and 135 mg of XL888) and from two other (nonprotocol) patients on vemurafenib alone (960 mg b.i.d.). Overall, the patterns of protein expression observed on vemurafenib and vemurafenib-XL888 were heterogeneous, with large patient-to-patient variability seen (Fig. 3B). Some trends were, however, noted. Expression of ERBB3, a known mediator of BRAF inhibitor resistance, increased in the day 8 samples of both patients receiving vemurafenib alone (Fig. 3B), but decreased in the day 8 samples of 4 of 7 patients receiving the vemurafenib–XL888 combination. The decreased ERBB3 expression was seen at all dose levels of XL888 evaluated in the proteomic studies. Decreased expression of the antiapoptotic protein BAD was also observed in 5 of 7 of the day 8 vemurafenib–XL888-treated samples, but not in those receiving vemurafenib alone (Fig. 3B). In general, however, similar trends in protein expression were noted for both the vemurafenib alone and vemurafenib–XL888-treated specimens with regard to multiple proteins, including Bcl-2, BIM, Bok, c-MET, and IRS1. To better understand some of the changes in HSP client protein expression at a systems level, we produced signaling maps of one patient (#14) who stayed on

**Figure 1.** Waterfall plot of best overall response (ORR) on therapy for 20 evaluable patients treated with vemurafenib/XL888 (ORR of 75%; 95% CI, 51–91%). Line delineates ~30% decreases in tumor size. *, Patient who had regression in target lesions but new subcutaneous brain metastases, therefore counted as progressive disease (PD). One patient who switched to another treatment prior to tumor assessment for non-toxicity-related reasons was considered unevaluable.
vemurafenib and 135 mg XL888 for 228 days, and one (#18) who was treated at the same dose of both drugs for 607 days (Figures 4A and 4B, respectively). The best response for both patients was partial response. These high-level overviews showed patient #14 to have decreased expression of multiple proteins involved in RTK signaling, the MAPK pathway, AKT signaling, components of the β-catenin pathway, and negative apoptosis regulators (Fig. 4A). Increases in some RTKs, such as PDGFR-α and VEGFR1, were also noted. Patient #18 showed decreases in multiple RTKs, including EGFR, c-MET, PDGFR-α, and VEGFR1, and increases in components of the MAPK and AKT pathways. In a similar vein to patient #14, patient #18 also demonstrated decreases in NFκB family transcription factors and the β-catenin signaling pathway (Fig. 4B). Together, these data illustrate that proteome-level responses to the BRAF–HSP90 inhibitor are diverse and heterogeneous, even among patients who respond to therapy. The relatively small cohort of patients being analyzed limited our ability to perform detailed statistical analyses of these trends in HSP90 client expression.

Discussion

Although initial responses of melanoma patients to BRAF inhibitors are typically impressive, development of resistance is commonplace. Relapse is associated with recovery of signaling in the MAPK pathway in 52% of cases, PI3K/AKT signaling in 4% of individuals, and both the MAPK and PI3K/AKT pathways in 18% of cases (3). The mechanism of resistance in the remaining 26% patients is not well understood, but may result from nongenomic drug tolerance mechanisms that are associated with novel epigenetic states or immune-mediated effects, or other mechanisms of resistance that are as yet undefined (22, 23). Effective strategies to prevent the onset of BRAF–MEK inhibitor resistance in the clinic...
Figure 4.
Annotation of patient HSP client measurements on a simplified pathway diagram. Protein targets are grouped by pathway on a map created using GeneGO Metacore; icons in the key are derived from the GeneGO functions associated with each protein (see key). Each protein is labeled with a box showing the increase or decrease of protein expression between samples captured pretreatment and those collected after 8 days of treatment on a red to blue scale (white indicates that little change occurred, and the absence of the box indicates that the ratio could not be measured). Data are provided for patients 14 (A) and 18 (B) (both patients received 135 mg/kg XL888 and 960 mg of vemurafenib twice daily and had a partial response on treatment). Days on therapy are the total days that the patient remained on BRAF–HSP90 inhibitor therapy, although the on-treatment biopsies were done on day 8 of treatment.
are currently lacking, with the diversity of resistance mechanisms posing a formidable problem. The HSP90 family of chaperone proteins plays a critical role in regulating the stability of a great number of receptor tyrosine kinases, serine/threonine kinases, and other signaling molecules required to maintain the transformed state of cancer cells (24). Many of the key drivers of melanoma progression, including CRAF, CDK4, EGFR, IGF1R, mTOR, COT, and AKT, are known to be clients of HSP90 and are degraded following treatment with small molecule HSP90 inhibitors (13, 16). We reasoned that HSP90 inhibition, which has a broad “network level” of activity, was a promising strategy to overcome multiple mechanisms of BRAF inhibitor resistance. In preclinical studies, we and others demonstrated that HSP90 inhibition could overcome acquired BRAF and BRAF–MEK inhibitor resistance mediated through multiple mechanisms and, more importantly, that the BRAF–HSP90 inhibitor combination, when used upfront, prevented treatment failure in melanoma xenograft models (13, 16, 25). HSP90 inhibitors have also been demonstrated to reverse resistance to EGFR, KIT, and ALK inhibitors in triple-negative breast cancer, gastrointestinal stromal tumors, and ALK-mutant lung cancers, respectively (26–29). The purpose of the current study was to determine the safety and tolerability of the HSP90 inhibitor XL888 in combination with the FDA-approved BRAF inhibitor vemurafenib. The most thoroughly investigated HSP90 inhibitor in melanoma thus far in the single-agent setting is 17-allylamino, 17 demethoxygeldanamycin (17-AAG, tamesipimycin), a benzoquinone ansamycin with good preclinical activity against BRAF-mutant melanoma cell lines and xenografts (30). In both phase I and II clinical trials of patients with advanced melanoma, single-agent 17-AAG activity was modest (30–32). Some evidence for target engagement was observed in one phase I study, with decreased expression of CRAF and CDK4 and increased HSP70 expression reported (30). In a subsequent phase II clinical trial, 17-AAG was not found to alter BRAF expression or to inhibit phospho-ERK in melanoma biopsies, despite increases in HSP70 expression and decreased levels of intratumoral cyclin D1 being observed (32).

In the current study of the BRAF inhibitor vemurafenib in combination with escalating doses of the HSP90 inhibitor XL888, PFS, and OS rates compared favorably with previously published data on single-agent vemurafenib with a median PFS of 5.3 to 6.2 months, with results similar to the median PFS observed with vemurafenib/cobimetinib of 9.9 months (9). The response rate of 75% experienced by the vemurafenib–XL888-treated patients in our study also compares favorably with the 48% response rate seen in single-agent vemurafenib and is similar to the 68% response rate reported with vemurafenib/cobimetinib (1, 33). There were also differences in the side-effect profile seen when compared to single-agent vemurafenib. The most frequent off-target effect seen with the vemurafenib–XL888 combination (when all cohorts were combined) was the development of proliferative skin lesions including SCCs, KAs, melanomas, and verruca vulgaris (VV). These are known side effects of treatment with vemurafenib monotherapy, where up to 26% of patients developed either SCC or KA (34). In the case of vemurafenib monotherapy, secondary skin lesions are known to arise following the paradoxical activation of MAPK signaling in clones of skin keratinocytes that harbor preexisting HRAS mutations (35, 36). Of interest, no SCCs or KAs were observed in patients at the higher XL888 doses (cohorts 3–4). Although it is tempting to speculate that this resulted from XL888 blocking the paradoxical activation of MAPK (as previously shown by our group in preclinical studies; ref. 21), the limited number of patients treated in each cohort of the present study makes this difficult to determine. The addition of higher dose (90–135 mg) XL888 to vemurafenib, as experienced by patients in cohorts 3 and 4, did not reduce the incidence of VV, suggesting these may have a different etiology than that underlying SCC or KA development (21). Two patients developed new primary stage 1 melanomas that were resected in entirety; the association of new primary melanomas with single-agent vemurafenib has been well documented (37).

Measuring HSP90 inhibitor activity in a clinical setting has proven to be challenging. The most commonly used method has been the quantification of co-chaperone HSP70 expression in PBMCs. Approaches used to quantify HSP70 expression clinically have included ELISA assays and semi-quantitative assessment of limited numbers of client proteins (typically 2–4) using immunohistochemistry (IHC) or Western blot (30, 38, 39). HSP chaperone proteins are known to regulate the stability of >200 clients, making these limited pharmacodynamic assessments insufficient to determine the breadth of target engagement. To address these issues, and to provide mechanistic insights into the effects of HSP90 inhibition, we developed a mass spectrometry–based proteomic assay for the quantification of HSP70 expression in PBMCs and HSP90 client proteins in tumor samples that allows the changes in the expression of multiple HSP client proteins to be determined in pre- and posttreatment melanoma biopsies (18). These assays showed HSP70 expression to be robustly increased in PBMC from most patients on vemurafenib–XL888 therapy.

At day 8, decreased client protein expression was frequently seen in pathways implicated in melanoma progression and adaptation to BRAF inhibitor therapy (3, 7, 40, 41). Increases in some client proteins were also observed. Proteomic responses were heterogeneous between patients, with different client proteins being degraded or increasing in expression in different pairs of matched tumor specimens. This variation in response likely reflects the high level of genomic and phenotypic diversity and heterogeneity of melanoma (42). Although the limited numbers of patients enrolled on this trial, and the small numbers of paired samples available for analysis, made it difficult to ascertain the link between HSP client degradation and clinical response, possible indicators of HSP90 inhibitor activity were observed. ERBB3 is an RTK implicated in BRAF inhibitor resistance, whose expression increases as an adaptation to BRAF inhibitor therapy (15). ERBB3 is known to be an HSP client protein, with multiple studies showing that HSP90 inhibitors lead to its degradation and a decrease in its signaling activity (43, 44). In agreement with published data, we noted that ERBB3 expression was increased in the day 8 biopsies from two patients receiving vemurafenib alone. In contrast, 4 of 7 patients receiving vemurafenib–XL888 experienced a decrease in ERBB3 expression at day 8. Heterogeneous responses were also seen with regard to c-MET (another RTK implicated in BRAF inhibitor resistance; refs. 45, 46), with 4 of 7 patients showing increased expression at day 8 and 3 of 7 showing decreased expression. CRAF (or RAF1) is another HSP90 client implicated in BRAF inhibitor failure (47, 48). Analysis of CRAF expression levels showed decreased expression in 3 of 7 day 8 samples from patients receiving vemurafenib–XL888. Our results herein agree with previous Western blot studies in which patients responding to the HSP90 inhibitor 17-AAG frequently demonstrated decreased expression of the HSP90 client RAF-1 after drug dosing (30). Unexpectedly, increased KRAS expression...
was seen in 6 of 7 posttreatment samples from patients on the BRAF–HSP90 inhibitor combination, which was not observed in 2 patients on BRAF inhibitor alone. It was the evident that XL888 was impacting pathways other than the MAPK pathway, supporting our preclinical findings that HSP90 inhibitors also impact RTK signaling, AKT, mTOR etc. [13, 16]. At the same time, there was also significant overlap between the proteins impacted by vemurafenib and vemurafenib–XL888; these included robust induction/degradation of both antiapoptotic (Bcl-2 and Bak) and proapoptotic proteins (BIM, Bad, and Bok). The complexity of these results illustrates the difficulties associated with predicting responses from the measurement of individual proteins. We therefore believe that a systems-level analysis of the HSP clientome performed on greater numbers of patients will be required to determine which combination of clients must be degraded for robust clinical responses to be observed and/or maintained. Recent preclinical studies have demonstrated that HSP90 inhibitors also improve antitumor immunity through upregulation of interferon response genes, and that this can potentiate responses to anti–CTLA-4 and PD-1 therapy (49). This raises the possibility that the vemurafenib–XL888 combination could also positively impact the tumor microenvironment.

Besides XL888, other HSP90 inhibitors such as AT13387 and ganetesib are also in clinical testing; trials studying the combination of BRAF and MEK inhibitors with both XL888 and AT13387 are ongoing in patients with BRAFV600E mutated advanced melanoma (NCT022721459 and NCT02097225). Results of these trials will provide further insight into the role of HSP90 inhibitors in treatment of these patients. BRAF–MEK inhibitor combinations have now replaced single-agent BRAF inhibitor therapy as a standard-of-care targeted therapy for advanced melanoma. As with single-agent BRAF inhibition, patients on BRAF–MEK inhibitor therapy also show signs of resistance; there is evidence that escape from BRAF–MEK inhibitor therapy is also dependent upon HSP client proteins and that HSP90 inhibitors can reverse this (16). We are currently investigating a triplet combination of vemurafenib–cobimetinib–XL888, with the goal of further improving the PFS and OS seen with vemurafenib–cobimetinib. However, given the toxicity profile seen with vemurafenib–XL888, it is possible that alternative doses of vemurafenib and cobimetinib may need to be explored when combined with XL888.

Disclosure of Potential Conflicts of Interest

Z. Eroglu is a consultant/advisory board member for Compugen. G.T. Gibney reports receiving speakers bureau honoraria from Genentech and Merck, and is a consultant/advisory board member for Incyte, NewLink Genetics, and Novartis. J.S. Weber is a consultant/advisory board member for Genentech. N.I. Khushalani reports receiving speakers bureau honoraria from Bristol-Myers Squibb, and is a consultant/advisory board member for Bristol-Myers Squibb, EnMD Senso, Genentech, and Regeneron. J.L. Messina is a consultant/advisory board member for Castle Bioscience. V.K. Sondak is a consultant/advisory board member for Array, Bristol-Myers Squibb, Genentech/Roche, Merck, Novartis, Oncoly, Pfizer, and Polynoma. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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5523

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