Chemical Therapeutics

Inhibition of Wee1, AKT, and CDK4 Underlies the Efficacy of the HSP90 Inhibitor XL888 in an In Vivo Model of NRAS-Mutant Melanoma


Abstract

The HSP90 inhibitor XL888 is effective at reversing BRAF inhibitor resistance in melanoma, including that mediated through acquired NRAS mutations. The present study has investigated the mechanism of action of XL888 in NRAS-mutant melanoma. Treatment of NRAS-mutant melanoma cell lines with XL888 led to an inhibition of growth, G2-M phase cell-cycle arrest, and the inhibition of cell survival in three-dimensional spheroid and colony formation assays. In vitro, HSP90 inhibition led to the degradation of ARAF, CRAF, Wee1, Chk1, and cdc2 and was associated with decreased mitogen-activated protein kinase (MAPK), AKT, mTOR, and c-jun NH2 kinase (JNK) signaling. Apoptosis induction was associated with increased BIM expression and a decrease in the expression of the prosurvival protein Mcl-1. The critical role of increased BIM and decreased Mcl-1 expression in the survival of NRAS-mutant melanoma cell lines was shown through siRNA knockdown and overexpression studies. In an animal xenograft model of NRAS-mutant melanoma, XL888 treatment led to reduced tumor growth and apoptosis induction. Important differences in the pattern of client degradation were noted between the in vivo and in vitro studies. In vivo, XL888 treatment led to degradation of CDK4 and Wee1 and the inhibition of AKT/S6 signaling with little or no effect observed upon ARAF, CRAF, or MAPK. Blockade of Wee1, using either siRNA knockdown or the inhibitor MK1775, was associated with significant levels of growth inhibition and apoptosis induction. Together, these studies have identified Wee1 as a key target of XL888, suggesting novel therapeutic strategies for NRAS-mutant melanoma. Mol Cancer Ther; 12(6); 901–12. ©2013 AACR.

Introduction

Ras proteins are small molecular-weight (21 kDa) GTPases that localize to the plasma membrane and function as molecular switches linking cell surface receptor tyrosine kinase activity to downstream signaling pathways (1). Approximately 25% of all cancers harbor point mutations in Ras, with the most common being position 61 mutations leading to impaired GTPase activity and position 12/13 mutations that prevent GTPase domain deactivation. In its mutant, active state, Ras recruits a number of signaling cascades involved in tumor progression including the phosphoinositide 3-kinase (PI3K)/AKT pathway, the mitogen-activated protein kinase (MAPK) pathway, RAL-GDS, and phospholipase C (PLC)-e (1, 2).

Recent years have seen major advances in the therapeutic management of disseminated melanomas driven through activating mutations in the serine/threonine kinase BRAF (3, 4). Despite these successes, little progress has been made towards the development of targeted therapy strategies for the 50% of all melanomas that are BRAF wild-type (5). By far, the most well-characterized group of BRAF wild-type melanomas are the 15% to 20% of all melanomas that harbor activating position 61 mutations in NRAS (6). Emerging data show NRAS-mutant melanomas to be biologically distinct from BRAF-mutant melanomas with the typical NRAS-mutant patient with melanoma tending to be older (>55 years old) and more likely to have a chronic history of UV exposure (7, 8). The signaling of NRAS-driven melanomas also differs from that of BRAF-mutant melanomas in relying upon CRAF and phospho-diesterase IV activity to maintain MAPK signaling activity (9, 10). Unlike BRAF-mutant melanomas that are highly sensitive to BRAF and MEK inhibition, responses of NRAS-mutant melanomas to MEK inhibition are highly variable and it is likely that combination therapy strategies will be required (6, 11–14).
The HSP90 family of chaperones plays a key role in maintaining the malignant potential of cancer cells by regulating the conformation, stability, and function of many key receptors and kinases required for tumor initiation and maintenance (15, 16). A number of HSP90 client proteins, including CRAF, AKT, CDK4, ribosomal S6, and mutated BRAF, are known to be critical for melanoma progression, and there is now good evidence that the pharmacologic inhibition of HSP90 is an excellent strategy for targeting multiple oncogenic pathways simultaneously (15, 17, 18). XL888 is a novel, orally available HSP90 inhibitor with good selectivity for HSP90α and HSP90β (IC50, 22 and 44 nmol/L, respectively) and little activity against a panel of 29 diverse kinases (IC50, all >3,600 nmol/L; ref. 19). Recent studies from our laboratory showed XL888 to be highly effective at overcoming acquired BRAF inhibitor resistance in a panel of melanoma cell line models, including those in which resistance was mediated through mutant NRAS (20). In the current study, we show a requirement for CDK4, Wee1, and AKT inhibition in the antitumor effects of XL888 in NRAS-mutant melanoma. Of these, Wee1 is a checkpoint kinase implicated in the DNA repair response whose expression has been correlated with melanoma progression (21). Our studies support the further preclinical and clinical investigations of PI3K/AKT, CDK4, and Wee1 as well as HSP90 inhibitors in NRAS-mutant melanoma.

Materials and Methods

Cell culture

The NRAS-mutant cell lines WM852, WM1346, WM1361A, WM1366, and WMSbCl2 and the BRAF-mutant cell line 1205Lu were a gift from Dr. Meenhard Herlyn (The Wistar Institute, Philadelphia, PA). The NRAS-mutant cell lines M202, M207, M244, M245, and M318 and the BRAF-mutant cell line M229 were a gift from Dr. Antoni Ribas (Jonsson Comprehensive Cancer Center, UCLA, Los Angeles, CA). McI-1-overexpressing cell lines WM1361A-MCL1 and WM1366-MCL1 were a gift from Dr. Andrew Aplin (Kimmel Cancer Center, Philadelphia, PA). The Coriell Institute (Camden, NJ) cell identity mapping kit confirmed the identities of the Wistar cell lines. The UCLA cell line identity was confirmed by mitochondrial DNA analysis. All cell lines were verified in the past 6 months and were maintained in RPMI complete media. The body for p53 (OP43) was obtained from EMD Biosciences. The antibody for GAPDH was obtained from Sigma Aldrich.

Proliferation assay

Cells were plated in 96-well plates with 2.5 × 10^4 cells in 100 μL medium per well overnight before being treated with increasing concentrations of drug. Metabolic activity was assayed after incubation with XL888 for 72 hours (XL888) or 120 hours (PD032991, MK1775, and P1103), using Alamar Blue reagent according to manufacturer’s protocol (Invitrogen).

Cell-cycle analysis

Cells were plated in 10 cm dishes at 5.0 × 10^5 cells per dish and treated with 300 nmol/L XL888 the following day. After 24 hours, the cells were trypsinized, fixed with ethanol, stained with propidium iodide, and analyzed by flow cytometry.

Apoptosis

Cells were plated in 6-well plates at 2.0 × 10^5 cells per well. The cells were treated with 300 nmol/L XL888 for 24 to 72 hours before harvesting. Annexin V staining and flow cytometric analysis were conducted as described previously (22).

Western blotting

Proteins were extracted and blotted as described previously (23). For mouse xenograft studies, tumor samples were harvested and immediately placed into RNAlater solution (Invitrogen) before protein extraction. After analysis, Western blots were stripped once and reprobed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to confirm even protein loading. The following antibodies were obtained from Cell Signaling Technology: Akt (9272), phospho-Akt (4058), ARAF (4432), BAK (3814), BIM (2933), BRAF (9434), Cdc2 (9116), Cdc25A (3652), Chk1 (2360), CRAF (9422), p-CRAF-Ser338 (9427), ERK (9102), phospho-ERK (9101), HSP70 (4876), HSP90 (4877), Mcl1 (4572), phospho-p90RSK (9346), PARP (9542), Raf (4799), RB (9309), phospho-RB (9308), phospho-RSK2 (3556), S6 (2317), phospho-S6 (2215), and phospho-SAPK/JNK (4668). Antibodies for p21 (610233) and p27 (610241) were obtained from BD Biosciences. The antibody for p53 (OP43) was obtained from EMD Biosciences. The antibody for GAPDH was obtained from Sigma Aldrich.

Colony formation assay

Cells were plated in 6-well plates at 2 × 10^4 per well. Media with vehicle [dimethyl sulfoxide (DMSO)] or XL888 (10, 30, 100, or 300 nmol/L) were added the following day and replaced twice a week. After 4 weeks, the cells were harvested and immediately placed into RNAlater solution (Invitrogen) before protein extraction.

RNA interference

Cells were plated in 6-well plates at 2.0 × 10^5 cells per well in RPMI complete media. The following day, the media were replaced with 1 mL Opti-MEM (Invitrogen) and the cells were transfected with 0.5 mL complexes of Lipofectamine 2000 (0.5%, Invitrogen) and siRNA for either Mcl-1, BIM, BAK, Wee1, or nontargeting controls (40 nmol/L, Dharmacon On-Target Plus pools). Medium was replaced after 3 to 6 hours with 5% FBS in RPMI, and the cells were harvested 24 hours after transfection.
Real-time reverse transcription PCR

Cells were plated at 5.0 × 10^5 cells in 10 cm dishes and treated the following day with 300 nmol/L XL888. After 48 hours, total RNA was isolated using Qiagen’s RNeasy mini kit. The RNA levels were analyzed using TaqMan Gene Expression Assays primer/probes: Hs00197982_m1 (BIM), Hs01050896_m1 (MCL-1), P/N 4319413E (18S), and Hs99999905_m1 (GAPDH). The BIM and Mcl1 expression levels were normalized to 18S and GAPDH. Quantitative reverse transcriptase PCR (qRT-PCR) reactions were carried out as previously described (22).

Xenograft experiments

BALB SCID mice (Jackson Laboratory) were subcutaneously injected with 2.5 × 10^6 cells in 150 μL medium (L-15 with 10 μmol/L HEPES and 24% Matrigel). Tumors were grown to approximately 150 to 200 mm^3 before treatment. Mice were treated with 125 mg/kg XL888 or vehicle (10 mmol/L HCl) 3 times per week by oral gavage, according to the manufacturer’s protocol. The tumors were injected with 2.5 × 10^6 cells in 10 cm dishes and treated the following day with 300 nmol/L XL888. After 48 hours, total RNA was isolated using Qiagen’s RNeasy mini kit. The RNA levels were analyzed using TaqMan Gene Expression Assays primer/probes: Hs00197982_m1 (BIM), Hs01050896_m1 (MCL-1), P/N 4319413E (18S), and Hs99999905_m1 (GAPDH). The BIM and Mcl1 expression levels were normalized to 18S and GAPDH. Quantitative reverse transcriptase PCR (qRT-PCR) reactions were carried out as previously described (22).

Statistical analysis

Data show the mean of at least 3 independent experiments ± the SEM, unless stated otherwise. Statistically significant results were considered where P ≤ 0.05.

Results

XL888 induces apoptosis and cell-cycle arrest in NRAS-mutant melanoma cell lines

Treatment of a panel of 9 NRAS-mutant human melanoma cell lines with XL888 (Fig. 1) was associated with concentration-dependent decreases in cell growth (Fig. 2A). Four NRAS-mutant cell lines (M245, M318, WM1361A, WM1366) that were representative of the larger cell line panel (2 sensitive and 2 less sensitive) were used for all subsequent experiments. The growth inhibitory effects of XL888 were associated with the induction of a G2–M phase cell-cycle arrest (Fig. 2B) and robust levels of apoptosis by 72 hours (Fig. 2C). No colony formation was observed for any of the 4 cell lines following chronic XL888 (Fig. 2D), with complete eradication of all M318 cells being observed at XL888 concentrations as low as 10 nmol/L (Fig. 2D). The tumor selectivity of XL888 was confirmed through the lack of growth inhibitory/ proapoptotic activity upon normal human fibroblast cell lines (20).

XL888 decreases the expression of key NRAS-mutant melanoma signaling proteins

Mass spectrometry (LC-MRM) and Western blot studies showed the treatment of the NRAS-mutant melanoma cell line panel with XL888 to be associated with the rapid (<8 hours) increase in the expression of the chaperone HSP70 isoform 1 (Fig. 3A and B). XL888 also led to the decreased expression of many HSP client proteins including ARAF CRAF, CDK4, Wee1, CHK1, RB, and cdc2 (Fig. 3C and D). Together, the decreased expression of these HSP90 clients was associated with the abrogation of key melanoma signaling pathways such as decreased phosphorylation of CRAF, ERK, RSK2, and S6 and the increased expression (in some cell lines) of p27KIP1 (Fig. 3D). Little change in the expression of Ral, p53, or cdc25 was observed (Fig. 3C and D).

Increased expression of BIM is required for XL888-mediated apoptosis

In line with the ability of the HSP90 inhibitor to block pERK signaling (Fig. 3C), XL888 also increased BIM protein expression (Fig. 4A). qRT-PCR experiments revealed XL888 to increase expression of BIM mRNA in the M245, WM1361A, and the WM1366 cell lines but not the M318 (Fig. 4B). In the WM1361A and WM1366 cell lines, increased BIM mRNA expression was associated with nuclear relocalization of FOXO3a (Supplementary Fig. S1). The knockdown of BIM expression using siRNA significantly reduced the extent of XL888-mediated apoptosis induction in the M318 and WM1361A cell lines (Fig. 4C).

An LC-MRM screen identifies Mcl-1 as a key mediator of XL888-mediated apoptosis in NRAS-mutant melanoma cell lines

As BIM knockdown only partly reversed XL888-mediated apoptosis, an LC-MRM assay was used to quantify expression of 8 pro- and anti-apoptotic BH3 family proteins following HSP90 inhibition. These proteomic changes were measured using the SEQUEST algorithm to search against the HSP database, and proteins with a score of 3 were considered significant results were considered where P ≤ 0.05.
analyses, which were confirmed by Western blotting, revealed XL888 to increase the expression of the proapoptotic protein BAK in 3 of the 4 cell lines in our panel (M245, M318, and WM1366) and to inhibit the mRNA and protein expression of the anti-apoptotic Mcl-1 protein in all 4 cell lines (Fig. 5A and Supplementary Fig. S2). In contrast, little change was seen in the levels of BAD, BAX, BID, Bcl-2, BCL-w, and Bcl-XL. The role of increased BAK expression in XL888-mediated apoptosis was investigated by its siRNA knockdown followed by XL888 treatment (300 nmol/L, 48 hours). Although good levels of BAK knockdown were observed in all 4 NRAS-mutant cell lines, little effect was observed upon the level of HSP90-mediated apoptosis (Supplementary Fig. S3).

The role of Mcl-1 the survival of NRAS-mutant melanoma was confirmed in siRNA knockdown experiments, where depletion of the protein led to apoptosis in the M245, M318, and WM1366 cell lines (Fig. 5B). In contrast, knockdown of Mcl-1 was associated with very little apoptosis in 2 melanoma cell lines harboring BRAF.
V600E mutations (1205Lu and M229; Fig. 5B). We next addressed whether overexpression of Mcl-1 conveyed protection to XL888-mediated apoptosis by generating 2 isogenic NRAS-mutant melanoma cell lines that stably overexpressed Mcl-1 (WM1366 MCL-1 and WM1361A MCL-1). It was noted that while XL888 treatment degraded Mcl-1 in the parental cell lines, Mcl-1 expression was maintained in the overexpressing cell lines (Fig. 5C). The importance of Mcl-1 degradation in the cytotoxic activity of XL888 was shown by the ability of Mcl-1 overexpression to significantly attenuate levels of apoptosis and to enhance cell survival in a 3-dimensional (3D) collagen-implanted spheroid model (Fig. 5C and D). XL888 treatment also enhanced the effects of the BH3 family protein antagonist ABT-737 (which does not impact Mcl-1) in the WM1366 and WM1361A cell lines in both apoptosis and 3D spheroid assays (Supplemental Figs. S4 and S5).

XL888 is cytotoxic against NRAS-mutant melanoma cell xenografts through a mechanism involving the degradation of Wee1, CDK4 and decreased AKT/mTOR signaling

In a 3D cell culture, XL888 reduced the survival of all NRAS-mutant melanoma cell lines evaluated (Fig. 6A). The trend of sensitivity across the panel of 4 cell lines was similar to that observed in the apoptosis experiments, with M245 being less sensitive than the other cell lines (Figs. 2C and 6A). Of the 4 NRAS-mutant melanoma cell lines evaluated, only the M245 formed xenografts in severe combined immunodeficient mice. Treatment of the established M245 tumors with XL888 (125 mg/kg 3 times/wk) led to a significant slowing of tumor growth ($P = 0.017$) without any effect upon animal weights (Fig. 6A and B). Analysis of xenograft specimens by LC-MRM showed a marked increase in intratumoral HSP70 expression following XL888 treatment (Fig. 6C). Mechanistically, XL888 was noted to induce apoptosis, as shown by an increase in TUNEL staining (Fig. 6D). In vivo responses to XL888 are associated with degradation of Wee1 and CDK4 and inhibition of AKT/S6 signaling

In vivo, XL888 decreased expression of CDK4, S6, and Wee1 and inhibited pAKT and pS6 signaling (Fig. 7A). In contrast to the decrease of ARAF and CRAF expression and the suppression of MAPK signaling observed following XL888 treatment in the in vitro studies (Fig. 3C), little change was observed in any of these signaling molecules in the xenograft specimens (Fig. 7A). Inhibition of Weel expression using siRNA knockdown was associated with...
significant levels of apoptosis induction in 3 of the 4 NRAS-mutant melanoma cell lines (Fig. 7B). We next evaluated a panel of small-molecule inhibitors of CDK4 (PD0322991), PI3K/AKT/mTOR (PI-103), and Wee1 (MK1775) on the growth and survival of NRAS-mutant melanoma cell lines. Of these, MK1775 had the most potent growth inhibitory activity when used as a single agent, and the addition of PI-103 significantly enhanced these effects in all 4 cell lines (Fig. 8A). In addition, the combination of PI-103 and PD0322991 led to significantly more growth inhibition than either agent alone in all of the cell lines (Fig. 8A). With regard to apoptosis, only the Wee1 inhibitor MK1775 induced significant levels of cell death across all 4 cell lines when used as a single agent (Fig. 8B). Some heterogeneity of response was observed when the inhibitors were combined. The combination of MK1775 and PI-103 induced more apoptosis in the WM1366 cell line compared with either agent alone. In
the WM1361A cell line, the combination of PD0322991 and PI-103 was significantly more cytotoxic than each individual agent (Fig. 8B). In a 3D spheroid assay, the combination of PI-103, MK1775, and PD032291 reduced invasion and survival of M245 melanoma cells compared with each agent alone (Fig. 8B).

**Discussion**

The current study addresses the mechanism of action of the HSP90 inhibitor XL888 in NRAS-mutant melanoma. We observed that HSP90 inhibition decreased signaling through the Raf/MEK/ERK/CDK4 and PI3K/AKT pathways, 2 signal transduction cascades known to be

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**Figure 5.** Decreased Mcl-1 expression is required for XL888-mediated apoptosis. A, left, heatmap of LC-MRM screen shows XL888 to increase Bak expression and to decrease Mcl-1 expression in NRAS-mutant melanoma cell lines. Right, Western blotting validation of the decreased Mcl-1 expression and increased Bak expression following XL888 (300 nmol/L, 48 hours). B, siRNA knockdown of Mcl-1 increases apoptosis in NRAS-mutant melanoma cells but not 2 BRAF-mutant melanoma cell lines. Left, Western blotting shows the efficiency of the Mcl-1 knockdown. Right, Mcl-1 knockdown increases apoptosis in the M245, M318, and WM1366 NRAS-mutant melanoma cell lines. Apoptosis was measured by Annexin V binding and flow cytometry. C, Mcl-1 attenuates XL888-induced apoptosis. Left, Western blotting showing increased expression of Mcl-1 in WM1366 and WM1361A cell lines following lentiviral infection. Right, Mcl-1 overexpression attenuates XL888-mediated (XL) apoptosis as measured by Annexin V binding as compared with control (CT). * indicates significant difference from control, where P < 0.05. D, Mcl-1 prevents XL888-induced cell death in 3D culture. Control and Mcl-1-overexpressing WM1366 spheroids were treated with either vehicle or XL888 (300 nmol/L) for 5 days before being treated with Live/Dead stain. Green, live cells; red, dead cells.
important for the growth and survival of Ras-mutant cell lines and tumors (26). From a mechanistic standpoint, the MEK/ERK and PI3K/AKT pathways converge at the level of cell survival through the regulation of the proapoptotic proteins BAD and BIM as well as cap-dependent protein translation via 4E-BP1 signaling (20, 27–29). In NRAS-mutant melanoma, previous studies have shown that dual MEK and PI3K/mTOR inhibition, as well as concurrent short hairpin RNA (shRNA) knockdown of BRAF and PI3K, are associated with decreased cell growth and a delay in tumor formation in vivo (30, 31).

Melanoma cells are known to be particularly resistant to cell death and typically show increased expression of antiapoptotic proteins such as Bcl-2 and Mcl-1 as well as...
having constitutive activity in many signaling pathways that downregulate the expression of proapoptotic proteins such as BIM (32–35). In BRAF-mutant melanoma cells, signaling through the MAPK pathway downregulates BIM expression through MEK/ERK mediated phosphorylation at Ser69, leading to its proteasome-mediated destruction (33). Inhibition of BRAF through use of small-molecule inhibitors and siRNA knockdown induces cell death in melanoma cells, in part, through the binding of BIM to Bcl-2, Bcl-w, Bcl-XL, and Mcl-1, which antagonizes their prosurvival function (22, 34). In line with the known role of the MAPK pathway in regulating BIM expression, XL888 treatment led to the degradation of ARAF/CRAF expression, the inhibition of the MAPK pathway, and increased BIM expression.

Mcl-1 is a prosurvival protein first identified in myeloma that exerts its antiapoptotic activity by binding to BIM-EL and through the inhibition of proapoptotic Bak/Bax (36, 37). In melanoma, Mcl-1 has been implicated in the protection from anoikis; its overexpression also confers resistance to cytotoxic chemotherapy agents, radiation, and the proteasome inhibitor bortezomib (38–40). Expression of Mcl-1 is regulated both at the transcriptional level through the PI3K/AKT signaling pathway and posttranslationally through MEK/ERK-mediated phosphorylation at Ser163, which prevents its degradation by the proteasome (36, 41). An important role for Mcl-1 downregulation in the observed cytotoxic activity of XL888 was suggested by the ability of Mcl-1 siRNA knockdown to induce cell death across our NRAS-mutant melanoma cell line panel and the observation that Mcl-1 overexpression reverses XL888-induced apoptosis. Further studies also showed XL888 to enhance the proapoptotic activity of ABT-737 (a Bcl-2/Bcl-XL antagonist with little activity against Mcl-1) in 2 NRAS-mutant melanoma cell lines (42). Taken together, our results suggest that the simultaneous increase in BIM and inhibition of Mcl-1 expression underlies the potent long-lasting antitumor activity seen following XL888 treatment in our adherent culture system.
The ability to downregulate Mcl-1 expression while increasing BIM expression would seem to be a prerequisite for any future therapy developed for NRAS-mutant melanoma. Further studies will be required to determine whether NRAS-mutant melanomas are uniquely dependent upon the prosurvival activity of Mcl-1.

Treatment of melanoma cells grown in vitro and in vivo with XL888 was associated with increased expression of HSP70 isoform 1, a protein often used as a biomarker of HSP90 inhibition (20). Although increased HSP70 expression has been implicated in reduced HSP90 inhibitor-mediated apoptotic responses in leukemic cells, its role in melanoma has not yet been established (43). Analysis of HSP90 client levels by Western blotting revealed distinct patterns of degradation between the adherent cell culture and animal experiments. Under in vivo conditions, XL888 treatment was not associated with decreased expression of ARAF or CRAF, and little inhibition of MAPK signaling was observed. Instead, it was noted that XL888 treatment led to the reduction of CDK4 and Wee1 expression and the inhibition of AKT and S6 signaling. The differences in XL888-mediated client protein degradation between the in vitro and in vivo experiments are not currently well understood. Expression of ARAF and CRAF, in particular, was not reduced despite being recently described as ‘strong’ HSP90 clients (the sixth and eleventh strongest HSP90 interactors, respectively; ref. 44). Although there is known to be some correlation between the strength of the HSP90/kinase interaction and the likelihood of client degradation following HSP90 inhibition (e.g., strong clients are more likely to be degraded than weak clients), this is not universal and some strong clients are not degraded following HSP90 inhibitor-mediated chaperone dissociation (44). One possible explanation for the observed differences in client degradation between adherent cell culture and the in vivo model is the marked changes in the patterns of intracellular signaling seen in melanoma cells grown under different microenvironmental conditions. Previous studies from our laboratory have shown the MAPK signaling pathway to be differentially regulated between the same BRAF-mutant melanoma cell lines grown as either 2D or 3D cell cultures (12). Whether these focal differences in signaling are reflected in altered interactions between ARAF/CRAF and the HSP chaperones remains to be determined. These observations clearly illustrate the need to screen large panels of HSP client proteins when investigating the mechanisms of action of HSP90 inhibitors.

The validity of cotargeting CDK4 and PI3K/AKT signaling simultaneously in NRAS-mutant melanoma cell lines was confirmed by the significant increases in growth inhibition observed following treatment with the PI3K/mTOR inhibitor PI-103 in combination with the CDK4 inhibitor PD0322991 compared with either agent alone. Previous work has already indicated that BRAF- and NRAS-mutant melanoma cells are sensitive to inhibition of CDK4/6 and undergo senescence when treated with PD0322991 (45). A potential role for PI3K/AKT signaling in regulating the entry of melanocytes from BRAF-induced senescence has also been recently shown (46). These data suggest that combined PI3K/CDK4 inhibition could be one strategy to induce a maximal senescence response in melanoma cells. Interestingly, the CDK4/PI3K inhibitor combination induced only limited apoptosis in 3 of the 4 NRAS melanoma cell lines, suggesting that other mechanisms were responsible for the XL888-induced cytotoxicity observed in our xenograft model.

Wee1 is a tyrosine kinase and HSP90 client protein that negatively regulates the G2–M checkpoint. Previous studies have shown that abrogation of Wee1 function enhances the antitumor activity of DNA-damaging agents such as radiation and some chemotherapy drugs (47, 48). A small-molecule inhibitor of Wee1, MK1775, has cytotoxic activity in a number of tumor types including sarcoma, where it induces apoptosis through inappropriate mitotic entry (48, 49). In melanoma, Wee1 expression levels increase with tumor stage, with high levels of Wee1 being correlated with greater rates of ulceration and a poorer disease-free survival (21). In BRAF-mutant melanoma cell lines, siRNA knockdown of Wee1 leads to a DNA damage response and apoptosis (21). Our data provide the first evidence that Wee1 inhibition, through siRNA knockdown or Wee1/HSP90 inhibition, leads to growth inhibition and apoptosis in NRAS-mutant melanoma cell lines. We further show that Wee1 is a highly sensitive HSP90 client in melanoma cells grown both in vitro and under tissue culture conditions, and that its degradation may underlie the antitumor activity seen in XL888.

Emerging evidence suggests that NRAS-mutant melanomas show a greater diversity of response to targeted therapy agents than their BRAF-mutant counterparts, and many possible combination therapy strategies have been suggested (11, 14). There is already evidence of synergy between MEK and CDK4 inhibitors in mouse and human models of NRAS-mutant melanoma, although it is not yet clear what percentage of these tumors will show susceptibility to this combination (50). In the current study, we show the potential use of Wee1 inhibition and combined CDK4 and PI3K/AKT/mTOR inhibition in NRAS-mutant melanoma. It is hoped that the development of rational combination therapy strategies for NRAS-mutant melanoma will yield similar successes to those that have been seen in BRAF-mutant melanoma.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References

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