Inhibition of HSP90 by AT13387 Delays the Emergence of Resistance to BRAF Inhibitors and Overcomes Resistance to Dual BRAF and MEK Inhibition in Melanoma Models

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Abstract

Emergence of clinical resistance to BRAF inhibitors, alone or in combination with MEK inhibitors, limits clinical responses in melanoma. Inhibiting HSP90 offers an approach to simultaneously interfere with multiple resistance mechanisms. Using the HSP90 inhibitor AT13387, which is currently in clinical trials, we investigated the potential of HSP90 inhibition to overcome or delay the emergence of resistance to these kinase inhibitors in melanoma models. In vitro, treating vemurafenib-sensitive cells (A375 or SK-MEL-28) with a combination of AT13387 and vemurafenib prevented colony growth under conditions in which vemurafenib treatment alone generated resistant colonies. In vivo, when AT13387 was combined with vemurafenib in a SK-MEL-28, vemurafenib-sensitive model, no regrowth of tumors was observed over 5 months, although 2 of 7 tumors in the vemurafenib monotherapy group relapsed in this time. Together, these data suggest that the combination of these agents can delay the emergence of resistance. Cell lines with acquired vemurafenib resistance, derived from these models (A375R and SK-MEL-28R) were also sensitive to HSP90 inhibitor treatment; key clients were depleted, apoptosis was induced, and growth in 3D culture was inhibited. Similar effects were observed in cell lines with acquired resistance to both BRAF and MEK inhibitors (SK-MEL-28RR, WM164RR, and 1205LuRR). These data suggest that treatment with an HSP90 inhibitor, such as AT13387, is a potential approach for combating resistance to BRAF and MEK inhibition in melanoma. Moreover, frontline combination of these agents with an HSP90 inhibitor could delay the emergence of resistance, providing a strong rationale for clinical investigation of such combinations in BRAF-mutated melanoma. Mol Cancer Ther; 13(12); 1–12. ©2014 AACR.
address individual resistance mechanisms but are unlikely to target them all. In addition, multiple mechanisms of resistance have been observed in tumors from individual patients (16, 17, 22), further underscoring the need for therapeutics with broad spectral activity.

The BRAFV600E-mutant protein, a "client" of HSP90, relies on this molecular chaperone for its correct folding and stability (23, 24). Inhibitors of HSP90 have shown activity in preclinical models of melanoma, including those of vemurafenib resistance (25–27). In addition, the first-generation ansamycin HSP90 inhibitor, 17-N-allylamino-17-demethoxygeldanamycin (17-AAG), has shown some evidence of clinical activity in melanoma (28), despite major clinical limitations. As well as BRAFV600E, HSP90 clients include key components of cellular signaling pathways involved in BRAF inhibitor resistance such as CRAF, COT, PDGFR, IGF1R, and AKT. HSP90 inhibition has, therefore, been proposed as a potential approach to simultaneously inhibit multiple resistance mechanisms in melanoma (7, 26, 29).

AT13387 is a second-generation, fragment-derived HSP90 inhibitor, which is active in a number of in vitro and in vivo tumor models (30). It has been shown to be effective in kinase inhibitor-resistant diseases using preclinical imatinib-resistant gastrointestinal stromal tumor (GIST) models (31). AT13387 is currently in three phase II clinical trials [tumor types/ ClinicalTrials.gov identifiers: GIST/NCT01294202, anaplastic lymphoma kinase (ALK)–positive lung cancer/NCT01712217, prostate cancer/NCT01685268] in combination with targeted agents. Here, we demonstrated that AT13387 can overcome acquired resistance generated to BRAF inhibitors alone or to a BRAF/MEK inhibitor combination. In addition, combining AT13387 with a BRAF inhibitor in a sensitive model significantly delayed the emergence of BRAF inhibitor resistance. These data support the clinical testing of a frontline combination of an HSP90 inhibitor with a BRAF inhibitor alone or as a triple combination, including a MEK inhibitor.

Materials and Methods

Materials

AT13387 was synthesized at Astex Pharmaceuticals as described by Woodhead and colleagues (32) and stored as a lyophilized powder. Vemurafenib (PLX4032) was purchased from Sequoia Research Products Ltd. or Selleck Chemicals. Selumetinib (AZD6244) was purchased from Selleck Chemicals. Dabrafenib and trametinib were from Chemie Tek. All other reagents were purchased from Sigma unless otherwise stated.

Cell culture and reagents

The human cell lines A375, SK-MEL-28, SK-MEL-2, SK-MEL-5, and WM266-4 were purchased from the ATCC. The A2058 human cell line was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. These cells lines were not passaged for more than 6 months since authentication by the cell banks [DNA fingerprinting and cytogenetic analysis or short-tandem repeat (STR) PCR]. The 1205Lu and WM164 melanoma cell lines were a kind gift from Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA; ref. 26). Their identity was validated by STR analysis (Biosynthesis) at 6 monthly intervals. All cells were grown in their recommended culture medium, supplemented with 10% FBS and maintained at 37°C in an atmosphere of 5% CO2 unless otherwise stated. All cell culture reagents were purchased from Invitrogen unless otherwise stated.

A375R cells were generated by culturing A375 cells in the presence of 2 μmol/L vemurafenib for 3 weeks. The resultant vemurafenib-resistant cell line was maintained in 2 μmol/L vemurafenib. SK-MEL-28R cells were derived as follows: A SK-MEL-28 xenograft tumor that had relapsed on chronic vemurafenib treatment (50 mg/kg orally twice daily × 5/everyday × 2 for 150 days, see below) was excised out of the SCID mouse on day 151 (tumor #7). The tumor was cut into small pieces and collagenase IV digested for 45 minutes at 37°C. The digested tumor cell suspension was passed through a 100 μm filter, washed and cultured in DMEM containing 10% FBS, penicillin/streptomycin and 1 μmol/L vemurafenib. All assays were performed in antibiotic-free medium at passage numbers 2 to 8. Dual BRAF and MEK inhibitor–resistant cell lines were established by chronically treating SK-MEL-28, WM164, and 1205Lu for 4 to 5 months with 1 μmol/L each of vemurafenib and selumetinib. Cell lines were maintained in 5% FBS in RPMI-1640 media with 1 μmol/L vemurafenib and 1 μmol/L selumetinib.

Viability assays

Viability studies were carried out using Alamar Blue (Invitrogen) as described previously (33) with slight modifications. Briefly, 5 × 10^5 cells were seeded in 200 μL of complete culture medium per well into flat-bottomed 96-well plates 1 day before the drug treatment. Cells were incubated with compound in 0.1% (v/v) DMSO for 3 or 6 days before viability was assessed by using Alamar blue. IC_{50} values were calculated using a sigmoidal dose–response equation (Prism GraphPad software).

Colony formation assays

A375 and SK-MEL-28 cells were seeded in 6-well plates at a density of 500 cells per well. Both cell lines were maintained in culture by feeding with 2-mL fresh complete culture medium. Cells were treated for 2 weeks with AT13387 (18 nmol/L) and vemurafenib (2 μmol/L) as single agents or in combination; fresh compound was added every 2 days. Colonies were fixed and stained with 0.1% crystal violet solution. Plates were scanned and colonies quantified using the GelCount software (Oxford Optronix).
Protein analysis

Cells were seeded at 10^6 cells per well and incubated overnight at 37°C followed by treatment with AT13387 or other compounds. At the indicated time points, cells were harvested and lysed in ice-cold TG lysis buffer or RIPA buffer. Xenograft tumor samples were prepared by homogenizing snap-frozen tumors in ice-cold TG lysis buffer using a Precellys 24 homogenizer and incubating on ice for at least 15 minutes. After a freeze-thaw, all lysates were centrifuged at 14,000 rpm for 5 minutes at 4°C to remove debris. Protein concentrations were determined using BCA protein assay (Pierce) and normalized. For Western blot analysis, samples were resolved by SDS-PAGE on NuPage Novex Bis-Tris or Tris-Glycine gel systems, transferred to nitrocellulose or polyvinylidene difluoride membranes and incubated with primary antibodies specific for: HSP70 (Enzo Life Sciences), BRAF, COT (Santa Cruz Biotechnology), BRAF\textsuperscript{V600E} (Spring Biosciences), BRAF \textsuperscript{C} (BD Biosciences), phospho-AKT\textsuperscript{S473}, total AKT, phospho-ERK\textsuperscript{T202/Y204}, total ERK, phospho-S6\textsuperscript{S235/S236}, total S6, cleaved PARP, PDGFRβ, cKIT, IGFI, EGFR (Cell Signaling Technology), actin (Abcam), or GAPDH (Sigma; Supplementary Table S1). Blots were then incubated with either infrared-dye-labeled secondary antibodies and fluorescence detected on the Odyssey infrared imaging system (LI-COR Biosciences) or incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham) followed by detection with enhanced chemiluminescence (PerkinElmer).

Three-dimensional spheroid assays

Melanoma three-dimensional (3D) spheroids were prepared as described previously (34). Briefly, 50 μL of a 1.5% solution of agarose was added to each well of a 96-well tissue culture plate and allowed to solidify. Into each well, 5 × 10^5 cells in 200 μL of media (containing the respective compound that the cells are resistant to) were overlayed on the agarose bed and allowed to grow over 5 days. The resultant spheroids were implanted into rat tail collagen I and treated for 6 days with either 1 μmol/L vemurafenib and 1 μmol/L selumetinib alone or in combination with AT13387 (single-agent–resistant cell lines) or 1 μmol/L vemurafenib and 1 μmol/L selumetinib alone or in combination with AT13387 (dual-agent–resistant cell lines). Spheroids were then washed three times in media and combination with AT13387 (dual-agent–resistant cell lines) or 1 μmol/L selumetinib alone or in combination with AT13387. After the indicated treatment time, cells were harvested and stained for Annexin V as described previously (35).

Receptor tyrosine kinase array

Dual-inhibitor–resistant WM164RR and 1205LuRR cell lines were seeded at 50% confluency and grown overnight. Cells were then treated for 24 hours with 1 μmol/L vemurafenib and 1 μmol/L selumetinib (control) or 1 μmol/L AT13387, 1 μmol/L vemurafenib and 1 μmol/L selumetinib. Vehicle (DMSO) treated naive WM164 and 1205Lu cell lines were similarly plated and harvested. Global levels of RTK (receptor tyrosine kinase) tyrosine phosphorylation were determined using the R&D Systems Proteome Profiler Human Phospho-RTK Array Kit. Briefly, cells were lysed and proteins extracted with Lysis Buffer 17 supplemented with Cell Signaling Technology protease/phosphatase inhibitor cocktail. RTK array membranes were then incubated with 700 μg of protein lysate. All remaining steps were conducted per vendor instructions. Densitometric values of all positive signals on the RTK array were measured with Adobe Photoshop CS3. Corresponding levels of tyrosine phosphorylation were calculated by subtracting the average background values from the average values of duplicate RTK spots.

Xenograft studies

The care and treatment of experimental animals were in accordance with the United Kingdom Coordinating Committee for Cancer Research guidelines (36) and with United Kingdom Animals (Scientific Procedures) Act 1986 (37). All animals were purchased from Harlan, UK. SK-MEL-28 and SK-MEL-28R xenografts were prepared by s.c. injecting 5 × 10^6 cells suspended in serum-free DMEM mixed 1:1 with Matrigel (BD Biosciences; ~10 mg/mL protein concentration) into the flank of each male BALB/c SCID mouse. A2058 xenografts were prepared similarly in BALB/c nude mice. Tumor burden was estimated from caliper measurements and by applying the formula for an ellipsoid. Mice were randomized into different treatment groups when the mean of the tumors reached 100 mm^3. AT13387 was suspended in an aqueous solution of 17.5% (w/v) (2-hydroxypropyl)-β-cyclodextrin and administered i.p. at 70 mg/kg, once (SK-MEL-28 and SK-MEL-28R) or twice (A2058) a week. Vemurafenib was suspended in 5% (v/v) DMSO and 95% (v/v) of an aqueous solution of 1% (w/v) methyl cellulose, and administered at 50 mg/kg twice daily on weekdays and once daily on weekends (twice daily × 5/everyday × 2) by oral gavage. All drugs were given at a dose volume of 10 mL/kg. Treated versus control (T/C) was calculated as 100 × mean treated tumor volume over mean control volume. Tolerability was estimated by monitoring body weight loss and survival over the course of each study.
Statistical analyses

All statistical analyses were performed using GraphPad Prism version 6.01. Effects of various drug treatments were compared using one-way ANOVA for comparing three or more groups, or the unpaired t test for two groups. Differences were deemed statistically significant when \( P < 0.05 \).

Results

**AT13387 prevents the emergence of vemurafenib resistance in vitro**

AT13387 is a potent (\( K_d \) 0.71 nmol/L) inhibitor of HSP90 (30, 32) with broad spectrum activity in tumor models. AT13387 potently inhibited the proliferation of a panel of melanoma cell lines, with different genetic backgrounds and sensitivity to the BRAF inhibitor, vemurafenib (Supplementary Fig. S1A; ref. 10) and depleted relevant client proteins (Supplementary Fig. S1B). Because HSP90 inhibitors, including AT13387, can overcome a variety of resistance mechanisms to BRAF inhibition once established, we investigated whether HSP90 inhibition could also have a delaying effect on the emergence of this resistance in models initially sensitive to vemurafenib.

A375 and SK-MEL-28 melanoma cell lines are both initially sensitive to vemurafenib treatment and their proliferation is also inhibited by AT13387 (Supplementary Fig. S1A). To investigate effects on the emergence of vemurafenib resistance, A375 and SK-MEL-28 cells were exposed to 2 µmol/L vemurafenib in vitro. After 2 weeks of treatment, vemurafenib-resistant colonies had appeared (Fig. 1A). However, significantly fewer colonies were generated when vemurafenib was combined with AT13387 (18 nmol/L) (Fig. 1A).

![Image](attachment:image.png)

**Figure 1.** AT13387 treatment delays the emergence of vemurafenib resistance in vitro. A375 (A) or SK-MEL-28 (B) cells were treated with 2 µmol/L vemurafenib and 18 nmol/L AT13387 as monotherapy or in combination. After 2 weeks, colonies were visualized by staining with 0.1% crystal violet and then quantitated. Graphs, average of three individual experiments for each cell line; \( * \), \( ** \), \( *** \), and \( **** \) indicate a significant difference from vemurafenib (2 µmol/L) or AT13387 (18 nmol/L; \( P < 0.05 \), \( P < 0.01 \), \( P < 0.001 \), and \( P < 0.0001 \), respectively).
18 nmol/L AT13387 (Fig. 1B), suggesting that the combination delays the emergence of resistant clones.

**AT13387 delays the emergence of vemurafenib resistance in vivo**

AT13387 inhibits the growth of both vemurafenib-sensitive and -resistant melanoma xenograft mouse models (Fig. 2A and Supplementary Fig. S1C). To further investigate the delay on emergence of resistance seen in vitro, we carried out a comparable experiment in a SK-MEL-28 in vivo model (Fig. 2A and B). AT13387 significantly inhibited the growth of SK-MEL-28 xenografts when dosed at 70 mg/kg i.p. once weekly. As expected, oral administration of vemurafenib at 50 mg/kg twice a day caused significant regression of the SK-MEL-28 tumors over the initial period of dosing and combination of vemurafenib and AT13387 showed similar initial regression, with no significant difference observed between the two treatment groups (Fig. 2A). However, over an extended dosing period (150 days) two tumors in the vemurafenib + vehicle group regrew (tumor #8 and #7, PFS of 94 and 147 days, respectively), whereas no such regrowth was seen in the combination group over the same time period (Fig. 2B). Combined with our in vitro data, this further suggests that a combination of AT13387 and vemurafenib in a vemurafenib-sensitive model can delay the emergence of resistance that arises to vemurafenib treatment alone. The combination of AT13387 and vemurafenib was well tolerated with no significant increase in toxicity observed (Supplementary Fig. S2).

Although a detailed investigation into the mechanism of resistance of these tumors was not performed, one of the relapsed tumors (tumor #7) was found, by Western blot analysis, to have increased levels of PDGFRβ and EGFR compared with an untreated SK-MEL-28 tumor (SK-MEL-28; Fig. 2C). No changes in levels of cKIT, IGF1R, or cMET were detected (data not shown).

**AT13387 is active in models of acquired vemurafenib resistance**

Having demonstrated that AT13387 could delay the emergence of acquired vemurafenib resistance, we then continued to investigate the effect of AT13387 treatment once resistance had been acquired in these models. Two
cell lines (A375R and SK-MEL-28R) with acquired resistance to vemurafenib were generated using different methods. Acquired resistance in the A375R cell line was produced by culturing A375 cells in vitro with 2 μmol/L vemurafenib for 3 weeks, whereas the SK-MEL-28R cell line was created from cells recovered from one of the SK-MEL-28 xenograft tumors, which became resistant to vemurafenib after the extensive dosing described above (tumor #7). The proliferation of these cell lines was no longer inhibited by vemurafenib in vitro (IC50 values > 10 μmol/L; Supplementary Fig. S3) but was still potently inhibited by AT13387 with IC50 values in a similar range to those of the parental cell lines (22 vs. 19 nmol/L for A375 vs. A375R; 73 vs. 16 nmol/L for SK-MEL-28 vs. SK-MEL-28R; Supplementary Figs. S1A and S3). Treatment with AT13387 still brought about the depletion of client proteins and inhibition of signaling as in the parental lines (Fig. 3A and Supplementary Fig. S1B). Levels of PDGFRβ and EGFR, which were again upregulated in the SK-MEL-28R cell line, were also depleted by 24 hours treatment with 1 μmol/L of AT13387 in vitro (Supplementary Fig. S1B). Levels of cleaved PARP (Fig. 3A) and Annexin V (Fig. 3B) were increased on treatment of the SK-MEL-28R cell line with 0.1 or 1 μmol/L AT13387, indicating that apoptosis was being induced. Cell death and inhibition of growth were also observed when collagen-implanted 3D spheroids of the SK-MEL-28R cell line were treated with AT13387 (Fig. 3C).

Finally, the in vivo activity of AT13387 was investigated in a SK-MEL-28R xenograft model. Mice were s.c. injected with the vemurafenib-resistant SK-MEL-28R cells to form tumor xenografts and immediately treated with vemurafenib (50 mg/kg orally twice daily / everyday × 2), to maintain resistance. As expected, vemurafenib treatment did not inhibit tumorigenicity of these cells and xenograft tumors grew during dosing. When tumors reached a

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**Figure 3.** AT13387 treatment is still effective in in vitro models of acquired vemurafenib resistance. Cells with acquired vemurafenib resistance (A375R and SK-MEL-28R) were treated with varying concentrations of AT13387 or vemurafenib. Effects on cellular protein levels were measured by Western blotting with the indicated antibodies after 24 hours (A). SK-MEL-28R cells were treated with the indicated concentrations of AT13387 for 5 days in the presence of 1 μmol/L vemurafenib and apoptosis measured by Annexin V staining. Data, mean of three independent experiments each performed in duplicate (B). 3D spheroids were treated with the indicated concentrations of AT13387 for 6 days in the presence of 1 μmol/L vemurafenib. Red, dead cells; green, live cells; scale bar, 100 μm; bar graphs, average spheroid size as quantified by ImageJ analysis; *, significant difference in size compared with control (P < 0.05; C).
mean volume of approximately 100 mm³, AT13387 (70 mg/kg once weekly) was added to the vemurafenib treatment. This significantly ($P < 0.01$ from day 3 onward) inhibited the tumor growth (26% T/C on day 10), indicating that these vemurafenib-resistant tumors retained sensitivity to AT13387 treatment (Fig. 4A), although growth was not completely suppressed as it was in the upfront combination. Single-agent treatment with AT13387 also significantly ($P < 0.01$ from day 3 onward, 43% T/C on day 10) inhibited tumor growth compared with vehicle control (Fig. 4B).

**AT13387 is active in cells with dual resistance to BRAF and MEK inhibitors**

Combined BRAF and MEK inhibition has been shown to extend PFS in the clinic but still ultimately leads to resistance in almost all cases. We therefore investigated whether HSP90 inhibition could also overcome resistance to a dual BRAF and MEK inhibitor combination. Three melanoma cell lines were generated with resistance to both BRAF and MEK inhibitors by prolonged (4–5 months, depending upon cell line) incubation with vemurafenib and selumetinib. The resulting cell lines (SK-MEL-28RR, WM164RR, and 1205LuRR) were found to be resistant to the vemurafenib and selumetinib combination as well as dabrafenib and trametinib (Supplementary Fig. S4A and S4B). All the resistant cell lines showed increased levels of phospho-ERK compared with the parental cell lines, and although there was a decrease in phospho-ERK in all of the resistant cell lines in the presence of vemurafenib and selumetinib, significant levels of signaling comparable with untreated parental cell lines were maintained (Fig. 5A). BRAF and MEK inhibitors also had little effect on the growth of collagen-implanted 3D spheroids of these cell lines (Supplementary Fig. S4C). In contrast, treatment of these cell lines with AT13387 inhibited MAPK signaling as well as AKT signaling (Fig. 5B), induced apoptosis (Fig. 5C), and inhibited the growth and survival of the resistant cell lines in a 3D spheroid assay (Fig. 5D).

Similar to the vemurafenib-resistant SK-MEL-28R cell line, Western blot analysis of the 1205LuRR, SK-MEL-28RR, and WM164RR cell lines demonstrated increased EGFR and/or PDGFRβ expression in the dual resistant cell lines (Supplementary Fig. S4D). We investigated whether other RTKs were upregulated in these cell lines using a phospho-RTK array (Fig. 6A). As RTKs are known to signal largely through the phosphorylation of tyrosine, this analysis also allowed us to measure relative changes in RTK activity. Comparison of the overall level of tyrosine phosphorylation of RTKs between the WM164RR and 1205LuRR cell lines and their respective parental lines revealed that overall tyrosine phosphorylation appeared to increase following acquired BRAF plus MEK inhibitor resistance (Fig. 6A and B) and that this increased level of phosphorylation was again decreased on treatment with AT13387 (1 μmol/L, 24 hours). In particular, the phosphorylated levels of PDGFRβ increased substantially in both resistant cell lines compared with very low levels in parental lines, and were depleted again after treatment with AT13387 (Fig. 6B).

**Discussion**

Inhibition of HSP90 has been proposed as a treatment option for BRAF-mutant melanoma both because the driver, mutant BRAF, is a client for HSP90 and because other HSP90 clients play key roles in melanoma...
progression and resistance to BRAF inhibitors. HSP90 inhibitors such as 17-AAG, PF-4470296, XL888, and gane-tespib have been shown to be effective in a number of melanoma models, including those with BRAF inhibitor resistance (25–27, 29, 38). Here, we demonstrated that the HSP90 inhibitor, AT13387, is active not only in models of acquired vemurafenib resistance, but also in models with acquired resistance to both BRAF and MEK inhibitors. AT13387 treatment of melanoma cells with acquired resistance to BRAF inhibitors led to inhibition of growth, depletion of HSP90 clients, and inhibition of the MAPK and AKT signaling pathways. In addition, we showed similar effects in cell lines with acquired resistance to a dual BRAF and MEK inhibitor combination. Furthermore, although prolonged exposure of sensitive melanoma models to vemurafenib both in vitro (A375 and SK-MEL-28 cells) and in vivo (SK-MEL-28 xenograft) led to the emergence of resistance, combining AT13387 with a BRAF inhibitor delayed the emergence of resistance in both cases. Together, these data suggest that HSP90 inhibition has potential use not only in disease resistant to BRAF monotherapy, but also in disease resistant to the
Figure 6. HSP90 inhibition blocks RTK activation. WM164RR and 1205LuRR cell lines were treated for 24 hours with 1 μmol/L AT13387 before probing for phosphorylation. RTK array dot blots showing global changes in RTK tyrosine phosphorylation between naive, resistant, and AT13387-treated resistant cells (A). The relative intensities of each RTK with a positive phospho-signal were quantified by densitometry (B).

**Legend:**

- **Naive**
- **Resistant**
- **Resistant treated 24 h 1 μmol/L AT13387**
BRAF/MEK inhibitor combination and moreover, that use of an HSP90 inhibitor in an upfront combination with these agents may delay the appearance of resistance. Although these data confirm that HSP90 inhibition could be used to treat BRAF inhibitor refractory melanoma, this is the first time that frontline use of an HSP90 inhibitor to prevent the development of resistance has been demonstrated.

Although we did not investigate in detail the mechanisms of acquired resistance to either single-agent BRAF inhibitor or the BRAF/MEK inhibitor combination here, PDGFRβ and EGFR were found to be upregulated in one of the vemurafenib-relapsed tumors and a significant increase in the levels of phospho-PDGFRβ was observed in the BRAFi/MEKi dual resistant cell lines. Upregulation of PDGFRβ has been previously identified as a clinical mechanism of resistance (5), and there is evidence that increased RTK signaling, following that the relief of feedback inhibition may be an important adaptive mechanism involved in the escape from BRAF inhibition (5, 39, 40). Upregulation is, therefore, a potential resistance mechanism in our models. AT13387 treatment depleted the levels of phospho-PDGFRβ and EGFR and also brought about the depletion of overall phospho-RTK levels in the dual resistant cell lines (5, 7–10). The MAPK and AKT signaling pathways were inhibited in all cell lines with acquired resistance, indicating that potential resistance mechanisms were likely to be disabled by the inhibition of HSP90 afforded by AT13387 treatment. Combining BRAF inhibition with AT13387 treatment prevented the emergence of resistance, suggesting that the multiple effects of HSP90 inhibition on signaling pathways are sufficient to block the potential escape routes of the cell to BRAF inhibition.

Dabrafenib and trametinib have recently been FDA approved as single agents for the treatment of BRAF-mutant metastatic melanoma. Although phase II clinical trials of the BRAF/MEK inhibitor combination have shown increased PFS compared with BRAF inhibitor alone, resistance is still widespread (12, 18–20). There is already good evidence of cross-resistance between BRAF and MEK inhibitors, suggesting similar mechanisms of resistance (13–17). Further combinations to bring about dual inhibition of the MAPK and AKT pathways, such as BRAF or MEK inhibitors with AKT, PI3K or mTOR inhibitors, have been tested preclinically and shown to be effective (10, 19, 34). However, such combinations target only a few of the many mechanisms of resistance that have been identified to BRAF and MEK inhibition. HSP90 inhibition not only targets multiple signaling pathways and so can be used to address many of the diverse MAPK-dependent and -independent mechanisms of resistance simultaneously, but is also required for stabilization of mutant proteins that accumulate as resistance develops. This may explain why the upfront combination is more effective than the use of the same combination after resistance has arisen, because generation of mutant proteins is suppressed by HSP90 inhibition allowing vemurafenib to remain active.

HSP90 inhibitors have been previously investigated for melanoma in the clinic. The first-generation, ansamycin HSP90 inhibitor, 17-AAG, showed some evidence of clinical activity in melanoma, but this class of compounds was limited by toxicity and formulation issues. A clinical trial in melanoma of a compound in this class, IPI-504, was terminated and this compound has shown unacceptable toxicity in the clinic (41). There is, therefore, a need for improved, second-generation HSP90 inhibitors to test in melanoma. Compounds such as ganetespib and XL-888 are currently being investigated in melanoma as single agents or in combination with vemurafenib, but no clinical data are yet available on the activity of these compounds. Our preclinical data, showing HSP90 inhibition is still effective in BRAF/MEK inhibitor–resistant models, suggest further options for use of HSP90 inhibitors in melanoma such as in combination with both BRAF and MEK inhibitors. In addition, because AT13387 in combination with a BRAF inhibitor delays the emergence of resistance, we would predict that a frontline triple combination of an HSP90 inhibitor with BRAF and MEK inhibitors could delay the emergence of resistance even further and potentially prolong PFS. Indeed, given that regrowth of resistant tumors was not completely inhibited by AT13387, an upfront combination may be the best setting for use of an HSP90 inhibitor in melanoma. These data provide a rationale for the clinical investigation of such a combination in BRAF-mutant melanoma.

Melanoma is not the only disease in which targeted therapy, such as kinase inhibition, is effective but limited by the emergence of resistance. Combinations of HSP90 inhibitors with BRAF or MEK inhibitors may also have potential use in other tumor types. Approximately 15% of colorectal cancers have BRAF mutations, but BRAF inhibitors have shown limited responses to date. Intrinsic resistance appears to derive from upregulation of EGFR activity in response to BRAF inhibition, resulting in sustained activation of the AKT pathway (42, 43). Combined treatment with BRAF and EGFR or PI3K/mTOR inhibitors has a synergistic effect and is being tested as a therapeutic strategy for BRAF-mutant colorectal cancers in the clinic (42–44). HSP90 inhibition offers an alternative method of targeting upregulated EGFR and achieving simultaneous inhibition of the MAPK and AKT pathways, suggesting that an HSP90/BRAF inhibitor combination could also be an effective approach in BRAF-mutant colorectal cancer.

Resistance to targeted agents, such as imatinib in GIST, and crizotinib, gefitinib, and erlotinib in non–small cell lung cancer (NSCLC), has also been widely reported and often involves multiple resistance mechanisms. HSP90 inhibition has been shown to be effective against many of these resistance mechanisms (31, 45) and so it is tempting to speculate that, as demonstrated here, combining an HSP90 inhibitor with a targeted kinase inhibitor in sensitive disease may reduce or delay the emergence of resistance to these targeted agents too, provided such a combination is tolerated. AT13387 is currently being
tested in a phase II trial in ALK-dependent NSCLC in combination with crizotinib (ClinicalTrials.gov identifier NCT01712217). Moreover, these effects may not be limited to combinations with kinase inhibitors, because HSP90 inhibitors are also known to be effective at overcoming trastuzumab resistance in patients with breast cancer whose tumors overexpress HER2 as well as potentiating the effects of proteasome inhibitors in individuals with treatment refractory multiple myeloma (46, 47).

Overall our data suggest a number of scenarios in which HSP90 inhibitors, such as AT13387, could be used for melanoma treatment in the clinic: In patients with intrinsic resistance and so refractory to BRAF inhibitors alone, in patients whose resistance has been acquired to prior BRAF/MEK inhibition or upfront in combination with BRAF or BRAF/MEK inhibitors to delay the emergence of resistance and prolong response rates. These approaches may not be limited to melanoma but may also be applicable to other tumors in which intrinsic or acquired resistance to targeted agents limits their effectiveness.

Disclosure of Potential Conflicts of Interest
V.K. Sondak is a consultant/advisory board member for Merck, Novartis, and BMS. No potential conflicts of interest were disclosed by the other authors.

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