Overcoming Acquired BRAF Inhibitor Resistance in Melanoma via Targeted Inhibition of Hsp90 with Ganetespib

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Abbreviations List: Hsp90, heat shock protein 90.

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Abstract

Activating BRAF kinase mutations serve as oncogenic drivers in over half of all melanomas, a feature that has been exploited in the development of new molecularly-targeted approaches to treat this disease. Selective BRAFV600E inhibitors, such as vemurafenib, typically induce initial, profound tumor regressions within this group of patients; however durable responses have been hampered by the emergence of drug resistance. Here we examined the activity of ganetespib, a small molecule inhibitor of Hsp90, in melanoma lines harboring the BRAFV600E mutation. Ganetespib exposure resulted in the loss of mutant BRAF expression and depletion of MAPK and AKT signaling, resulting in greater in vitro potency and antitumor efficacy compared to targeted BRAF and MEK inhibitors. Dual targeting of Hsp90 and BRAFV600E provided combinatorial benefit in vemurafenib-sensitive melanoma cells in vitro and in vivo. Importantly, ganetespib overcame mechanisms of intrinsic and acquired resistance to vemurafenib, the latter of which was characterized by reactivation of ERK signaling. Continued suppression of BRAFV600E by vemurafenib potentiated sensitivity to MEK inhibitors after acquired resistance had been established. Ganetespib treatment reduced, but not abolished, elevations in steady-state ERK activity. Profiling studies revealed that the addition of a MEK inhibitor could completely abrogate ERK reactivation in the resistant phenotype, with ganetespib displaying superior combinatorial activity over vemurafenib. Moreover, ganetespib plus the MEK inhibitor TAK-733 induced tumor regressions in vemurafenib-resistant xenografts. Overall these data highlight the potential of ganetespib as a single-agent or combination treatment in BRAFV600E-driven melanoma, particularly as a strategy to overcome acquired resistance to selective BRAF inhibitors.
Introduction

Cutaneous melanoma ranks among the most aggressive and treatment-resistant human malignancies (1) and, at a time when the overall incidence and mortality rates for many cancer types are showing encouraging declines (2), the worldwide incidence of melanoma continues to increase (3). Mutational activation of the serine-threonine kinase BRAF, resulting in dysregulation of the RAF/MEK/ERK MAPK signaling cascade, is a feature of over half of all malignant melanomas (4, 5). The vast majority of BRAF mutations (>90%) are point mutations encoding for a valine-to-glutamic acid substitution at codon 600 (BRAF\textsuperscript{V600E}) (6). The high frequency of these genetic modifications underscores a critical role for BRAF mutation in melanoma oncogenesis (1), as well as providing an actionable target for molecular therapeutic approaches in this disease. Indeed, these considerations have been strikingly validated by the recent FDA approval of the first highly selective BRAF\textsuperscript{V600E} inhibitor, vemurafenib, for patients with metastatic melanoma (7). Despite this clinical success, however, durable responses to vemurafenib are rare and most patients invariably relapse with drug-resistant disease within 6-8 months (8). Strategies to counteract intrinsic and/or acquired resistance in mutant BRAF-driven melanoma have not yet been established, and this remains an ongoing clinical challenge for these cancers.

As is the case for a large number of oncogenes, the conformational stability of mutant BRAF is reliant on the activity of heat shock protein 90 (Hsp90) (9), a ubiquitously expressed molecular chaperone. Hsp90 is required for the maturation and function of numerous cellular client proteins (10), including others that have also been implicated in the pathogenesis of melanoma, such as CRAF, IGF-IR and AKT (11). Further, tumor cells can exploit the Hsp90 chaperone machinery as a biochemical buffer to protect mutated oncoproteins (such as BRAF\textsuperscript{V600E}) from targeted degradation, thereby facilitating aberrant cell survival and oncogene addiction (11, 12). Importantly, inhibition of Hsp90 activity targets its clients for proteasomal destruction to ultimately result in coordinate and simultaneous impacts on multiple signaling cascades (13-15). Targeting the chaperone function of Hsp90 therefore represents a rational and alternative approach to
direct BRAF<sup>V600E</sup> inhibition for potential therapeutic intervention in mutant BRAF-driven melanomas.

In this study we provide a comprehensive evaluation of the preclinical activity profile of ganetespib, a potent small molecule inhibitor of Hsp90 (16), in BRAF<sup>V600E</sup> melanoma lines in vitro and in vivo. Ganetespib was examined both as a single-agent as well as in combination with selective BRAF and MEK inhibitors in order to determine the comparative sensitivities of BRAF<sup>V600E</sup> melanoma cells to each of these treatment modalities. Significantly, ganetespib exposure could overcome mechanisms of intrinsic and acquired resistance to vemurafenib, and we further identify novel combinatorial approaches that may be particularly relevant in the context of acquired resistance to BRAF inhibitors. Ganetespib is presently undergoing evaluation in multiple human clinical trials, including patients with advanced and metastatic melanoma, and the data we are presenting here support the potential clinical utility of ganetespib in mutant BRAF-driven disease.
Materials and Methods

Cell lines, antibodies and reagents
Primary melanocytes and the A375, RPMI-7951, HT-144, A101D, SH-4, SK-MEL-2, SK-MEL-3, SK-MEL-24, SK-MEL-28, and C32 melanoma cell lines were obtained from the ATCC (Rockville, MD). IST-MEL1, MEL-HO, RVH-421 and COLO-679 were purchased from the DSMZ (Braunschweig, Germany). All were maintained according to suppliers instructions, authenticated by the routine company DNA profiling and were used within 6 months of receipt for this study. Vemurafenib-resistant A375 cells (A375-VR) were generated in continuous selective culture by serially increasing concentrations of vemurafenib (100nM to 5μM) over 8 weeks, with subsequent maintenance culture in 5μM. All primary antibodies were purchased from Cell Signaling Technology (Beverly, MA) with the exception of the BRAF, CRAF and GAPDH antibodies which were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Ganetespib [3-(2,4-dihydroxy-5-isopropylphenyl)-4-(1-methyl-1H-indol-5-yl)-1H-1,2,4-triazol-5(4H)-one] was synthesized by Synta Pharmaceuticals Corp. Vemurafenib, AZD6244 and TAK-773 were purchased from Selleck Chemicals (Houston, TX). The chemical structures of all compounds are shown in Fig. 1A.

Cell viability assays
Cellular viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturers’ protocol. BRAF mutant melanoma cell lines were seeded into 96-well plates based on optimal growth rates determined empirically for each line. Twenty-four hours after plating, cells were dosed with graded concentrations of ganetespib for 72 h. CellTiter-Glo was added (50% v/v) to the cells, and the plates incubated for 10 min prior to luminescent detection in a SpectraMax Plus 384 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Data were normalized to percent of control and IC50 values used to determine the sensitivity of each line. For the comparative analyses with BRAF and MEK inhibitors, A375, A375-VR and RPMI-7951 cells were treated with graded concentrations of
ganetespib, vemurafenib, or AZD6244 for 72 h and cell viability measured as above. A375-VR cells were also exposed to increasing concentrations of TAK-733 in the presence or absence of 5 μM vemurafenib. For the evaluation of apoptotic induction, caspase activity was assessed using the Caspase-Glo 3/7 assay (Promega) according to the manufacturers’ protocol. Melanoma cell lines were seeded into 96 well plates. Twenty four hours after plating cells were treated with graded concentrations of ganetespib, vemurafenib, or AZD6244 for 24 h. Caspase-Glo was added to cells (50% v/v) and the plates incubated for 1 h prior to luminescent detection.

Western blotting
Following respective treatments, tumor cells were disrupted in lysis buffer (Cell Signaling Technology) on ice for 10 min. Lysates were clarified by centrifugation and equal amounts of proteins resolved by SDS-PAGE before transfer to nitrocellulose membranes (Invitrogen, Carlsbad CA). Membranes were blocked with StartingBlock T20 blocking buffer (Thermo Scientific, Cambridge MA) and immunoblotted with the indicated antibodies. Antibody-antigen complexes were visualized using an Odyssey system (LI-COR, Lincoln, NE).

In vivo xenograft tumor models
Female immunodeficient CB.17 SCID and CD-1 (nude) mice (Charles River Laboratories, Wilmington, MA) were maintained in a pathogen-free environment, and all in vivo procedures were approved by the Synta Pharmaceuticals Corp. Institutional Animal Care and Use Committee. A375 melanoma cells (7.5 x 10^6) were subcutaneously implanted into the animals. SCID mice bearing established tumors (~150 mm^3) were randomized into treatment groups of 5 or 7 and i.v. dosed via the tail vein with vehicle, ganetespib formulated in 10/18 DRD (10% DMSO, 18% Cremophor RH 40, 3.6% dextrose) or p.o. dosed with vemurafenib or AZD6244 (formulated in a self-microemulsifying drug delivery system [SMEDDS] and DRD, respectively). Animals were treated with ganetespib at 50 mg/kg weekly, vemurafenib at 25 mg/kg 5 times a week BID, or AZD6244 at 3 mg/kg 5 times a week, either alone or in combination. Nude mice bearing A375 xenografts randomized into treatment groups of 6 were dosed with single agent or
combination treatment using ganetespib and TAK-733 (3 mg/kg, p.o. 5x/week, formulated in 0.5% methylcellulose in water). In a separate experiment, A375-VR cells (5 x 10^6) were subcutaneously implanted into nude mice. Animals were randomized into treatment groups of 5 and dosed with single agent or combination treatment using ganetespib (150 mg/kg) and TAK-733 (3 mg/kg, p.o. 5x/week). Tumor growth inhibition was determined as described previously (17).

**Multiple drug effect analysis**

For combinatorial analysis, A375 cells were seeded into the viability assay and combination treatments of ganetespib with vemurafenib or AZD6244 were performed at fixed, non-constant ratios. Drugs were added to cell cultures for 72 h and viability measured by alamarBlue assay (Invitrogen, Carlsbad, CA). The nature of the interactions were evaluated using the combination index (CI) method (18) and values generated using Median Effect analysis (Calcusyn Software; Biosoft, Cambridge, UK).

**In-Cell Western kinase screen**

A375-VR cells were maintained in culture media with 5μM vemurafenib and seeded into 96 well plates at a density of 2 x 10^4 cells per well. Twenty four hours after plating, cells were dosed with a library of kinase inhibitors (Selleck Chemicals) at a final concentration of 11.1 μM. After 24 h drug exposure, cells were fixed and probed with a phospho-ERK antibody (Cell Signaling Technology) according to standard In Cell Western protocols for detection using the Odyssey system. For quantification, the phosphorylated ERK signal was normalized to the control DRAQ5.
Results

Loss of viability and oncogenic signaling by ganetespib in BRAF mutant melanoma cells

The cytotoxic activity of ganetespib (Fig. 1) was initially evaluated in a panel of 15 melanoma cell lines harboring BRAF^{V600E} mutations (Table 1). Ganetespib potently reduced cell viability in all lines examined, with IC_{50} values in the low nanomolar range. Interestingly A2058 cells, which have lost their dependence on BRAF^{V600E} due to inactivation of PTEN and RB1 (19), remained acutely sensitive to ganetespib exposure. Next we examined expression changes in client and signaling pathway proteins using the A375 melanoma line. Ganetespib treatment resulted in a robust and dose-dependent decrease in levels of both BRAF and CRAF; this was accompanied by inactivation of downstream MAPK signaling, as evidenced by loss of the phosphorylated effectors p-MEK and p-ERK (Fig. 1B). Of note, Hsp90 inhibition resulted in the selective destabilization of mutated BRAF as also shown for SK-MEL-28 cells, in contrast to the wild type form of the protein expressed in normal melanocytes and the SK-MEL-2 melanoma line (Fig. 1C).

Ganetespib exposure confers superior cytotoxic activity over selective BRAF^{V600E} and MEK inhibitors in vitro

BRAF^{V600E} melanoma cell lines are sensitive to pharmacological inhibition by selective BRAF and MEK inhibitors that impact the RAF/MEK/ERK signaling axis and, as such, these agents have been shown to be of therapeutic relevance for BRAF mutant melanoma (6). Accordingly, we focused on vemurafenib, a specific BRAF^{V600E} inhibitor and AZD6244, an inhibitor of the MEK1/2 kinases (Fig. 1A). When the antiproliferative activity of these compounds was compared to that of ganetespib in A375 cells, it was found that ganetespib was four- to thirteen-fold more potent than AZD6244 or vemurafenib (19 vs. 81 and 255 nM, respectively; Fig. 1D). Caspase 3/7 activity was quantified in A375 cells following inhibitor treatment as a measure of apoptotic induction (Fig. 1E). The data showed that ganetespib, but not vemurafenib or AZD6244, caused a potent and dose-dependent elevation of activity at the 24 hour time point (Fig. 1E). When expression changes in
client protein and downstream signaling pathways were examined (Fig. 1F), ganetespib concentrations ≥50 nM resulted in robust degradation of CRAF protein expression as well as loss of the activated (phosphorylated) forms of MEK and ERK. Consistent with the cytotoxic activity of the compound, these effects were accompanied by a concomitant increase in cleaved PARP levels, another marker of apoptosis. In contrast, both vemurafenib and AZD6244 showed far weaker activity in terms of effector signaling blockade and activation of apoptotic pathways. Consistent with their respective modes of action, neither had any effects on CRAF protein levels. At least a ten-fold higher concentration of vemurafenib (500 nM) was required to significantly reduce phosphorylated MEK and ERK levels and, even at this maximal dose, did not lead to measurable apoptotic induction. MEK blockade by AZD6244 resulted in a dose-dependent loss of downstream ERK activity, but with no effects on cleaved PARP levels. Taken together, these results show that ganetespib displays greater in vitro potency than either vemurafenib or AZD6244 in mutant BRAFV600E melanoma cells.

**Dual targeting of BRAFV600E or MEK with Hsp90 provides combinatorial benefit in vitro and in vivo**

Next we examined the potential for improved therapeutic benefit by combining ganetespib with either vemurafenib or AZD6244 (Supplementary Fig. S1A,B). First, A375 cells were concurrently treated with ganetespib and vemurafenib using fixed, non-constant ratios and combinatorial activity assessed using the Median Effect method. The results are summarized in the normalized isobologram shown in Supplementary Fig. S1A. At all concentrations and ratios of the ganetespib/vemurafenib combination, the two drugs synergized to produce increased growth inhibition. To determine whether these synergistic effects translated to improved efficacy in vivo, mice bearing A375 xenografts were treated with ganetespib and vemurafenib, either as single agents or in combination. We have previously determined that the maximally tolerated dose (MTD) of ganetespib on a weekly dosing regimen is 150 mg/kg (16), a level which inhibited tumor growth in this model by more than half (T/C [tumor/control] growth inhibition value, 46%;
Supplementary Fig. S2). As shown in Fig. 2A, weekly administration of a sub-optimal dose (one-third MTD) of ganetespib (50 mg/kg) and 5x/week BID dosing with vemurafenib (25 mg/kg) reduced tumor growth by 36% and 71%, respectively (T/C values of 64% and 29%). On this regimen, vemurafenib exposure induced some toxicity, as measured by >20% body weight loss (2/7 animals) and one death (1/7) in this cohort; thus this dose was determined to be the MTD for this agent. Consistent with the in vitro findings, concurrent administration of both drugs at the same dose levels resulted in enhanced antitumor activity, suppressing tumor growth by 92%. This represented a significant improvement in efficacy over single agent ganetespib alone (p=0.01).

Combination treatment also resulted in lower toxicity than vemurafenib alone (2/7 animals vs. 3/7 in the vemurafenib single agent arm) and mean body weights are shown in Supplementary Fig. S3A. Thus, ganetespib and vemurafenib, when combined, displayed superior antitumor efficacy compared to monotherapy in A375 melanoma xenografts.

Dual blockade of Hsp90 and MEK was subsequently evaluated using ganetespib and AZD6244. Isobologram analysis showed synergistic effects for combinations of the two compounds in A375 cells in vitro (Supplementary Fig. S1B), however only a modest improvement in antitumor efficacy over either agent alone was observed in A375 xenografts on an AZD6244 (3 mg/kg, 5x/week) plus ganetespib (50 mg/kg, 1x/week) dosing regimen (Fig. 2B). In contrast, single agent administration of the potent allosteric MEK inhibitor TAK-733 (Fig. 1A) at 3 mg/kg caused greater than 90% tumor growth inhibition. Importantly, even at this efficacious dose, co-treatment with ganetespib resulted in 22% tumor regression (Fig. 2C). Both combination treatments were well tolerated, with no toxicity or significant changes in body weight observed over the 3 weeks of dosing (Supplementary Fig. S3B,C).

**BRAF**V600E mutant melanoma cells with intrinsic vemurafenib resistance retain sensitivity to ganetespib
Despite the presence of the \( \text{BRAF}^{\text{V600E}} \) mutation, around 20-40\% of patients do not initially respond to vemurafenib because of intrinsic resistance to BRAF inhibition (20). One mechanism of intrinsic resistance involves overexpression of the MAPK family member COT, which activates ERK signaling cascades through MEK-dependent mechanisms that are not reliant on RAF (21). Accordingly, in cells with elevated COT expression, hyperactivation of MEK by COT is believed to mediate resistance to both BRAF and MEK inhibitors. Consistent with this, in the vemurafenib-resistant, COT-overexpressing RPMI-7951 melanoma cell line, vemurafenib and AZD6244 were ineffective at reducing cellular viability. In stark contrast, RPMI-7951 cells were acutely sensitive to ganetespib exposure with an IC\(_{50}\) value of 13 nM. Apoptotic induction following ganetespib exposure was confirmed by elevations in caspase 3/7 activity (Fig. 3A). At the molecular level, ganetespib potently abrogated MAPK and AKT signaling in these cells sufficient to account for its pro-apoptotic activity, whereas the other two agents had no effects on downstream ERK or AKT activity (Fig. 3B).

**Ganetespib overcomes acquired vemurafenib resistance**

The clinical experience with vemurafenib has also shown that the efficacy of long-term treatment for melanoma patients is hampered by the invariable development of acquired resistance to the drug. It was important, therefore, to determine whether \( \text{BRAF}^{\text{V600E}} \) mutant melanoma cells with acquired resistance to vemurafenib remained sensitive to ganetespib. To explore this experimentally, we generated vemurafenib-resistant A375 cells (A375-VR) by continuous selective culture. The activities of ganetespib and vemurafenib were then compared using parental A375 and A375-VR cells (Fig. 3C). As expected, vemurafenib treatment resulted in dose-dependent cytotoxicity in the parental line, but had no effect on A375-VR cells. In contrast, ganetespib retained full potency against both lines, irrespective of vemurafenib resistance status. Indeed, A375–VR cells remained several fold more sensitive to ganetespib compared to that of the parental line to vemurafenib. Importantly, these effects were recapitulated \textit{in vivo}, where...
ganetespib, but not vemurafenib, significantly inhibited tumor growth of A375-VR xenografts (Fig. 3D).

**Elevations in steady-state ERK signaling sensitize vemurafenib-resistant melanoma cells to MEK inhibition**

Next we investigated the molecular profiles of the vemurafenib-resistant and sensitive cell lines, and their response to inhibitor treatment (Fig. 4A). Compared to the parental line, A375-VR cells showed higher basal activation of ERK signaling, as well as increased MET receptor expression (Fig. 4A). These oncogenic signaling cascades, in particular the enhanced ERK activity, were maintained in A375-VR cells even following BRAF inhibition. Notably, while ganetespib treatment effectively destabilized BRAF, CRAF, MET, and p-MEK proteins in A375-VR cells, p-ERK levels were reduced, but not completely abrogated, by the compound.

Having identified a potential role for sustained ERK signaling in the vemurafenib-resistant phenotype of A375-VR cells, we sought to identify relevant agents that could overcome this activity. We screened a library of 194 kinase inhibitors using an In-Cell Western assay, performed in the continuous presence of 5 μM vemurafenib, to quantify levels of p-ERK. Compounds that reduced levels of p-ERK by >50% are listed in Supplementary Table S1. In agreement with the data presented in Fig. 4A, 18% of p-ERK levels remained following ganetespib treatment. As a class, MEK inhibitors showed the most consistent and potent activity, reducing ERK activity by ≥90%. These data suggested that MEK activity was essential for the elevated ERK phosphorylation status.

Based on these observations we selected a group of compounds for a dose-response analysis of ERK inactivation and cytotoxicity, including multi-kinase inhibitors and other drugs targeting upstream modulators that impinge on ERK activity (Table 2). Ganetespib and MEK inhibitors were again the most potent compounds tested, concomitantly depleting p-ERK and reducing
viability at low nanomolar concentrations. As a control, we included the BRAF inhibitors PLX-4720 and GCD-0879, which had no effect on survival or p-ERK levels. In addition, despite the increased expression of MET in A375-VR cells, the dual MET/ALK inhibitor crizotinib did not deplete ERK activity beyond 50% and was only weakly cytotoxic (IC\textsubscript{50}, 1734 nM).

**Inhibition of BRAF\textsuperscript{V600E} enhances the activity of MEK inhibitors in vemurafenib-resistant melanoma cells**

These findings are in concordance with recent reports that BRAF\textsuperscript{V600E} melanoma cells become dependent on reactivation of ERK signaling despite ongoing inhibition of mutant BRAF (22, 23). We therefore evaluated the antiproliferative activity of TAK-733 in A375-VR cells in both the presence and absence of the BRAF inhibitor (Fig. 4B). Consistent with the data in Table 2, A375-VR cells were acutely sensitive to MEK inhibition in the presence of vemurafenib however, upon removal of the BRAF antagonist, a greater than log-shift decrease in sensitivity occurred. A similar response was observed for AZD6244 (Supplementary Fig. S4). Thus continued suppression of BRAF\textsuperscript{V600E} appears important for promoting sensitivity to targeted MEK inhibition after acquired resistance to vemurafenib has been established. These data are in agreement with other models showing that MAPK reactivation predicts for MEK inhibitor sensitivity in the resistance setting (6, 24).

Notably, treatment with vemurafenib or TAK-733 alone did not effectively block ERK reactivation in A375-VR cells, although p-ERK levels were abrogated when the two inhibitors were combined (Fig. 4C). Moreover, when TAK-733 was dosed with ganetespib, the addition of the MEK inhibitor was sufficient to overcome the activated ERK signal. Indeed this combination provided the most robust inhibition of MAPK and AKT signaling in A375-VR cells, with concomitant induction of apoptosis (Fig. 4C), highlighting a superior combinatorial benefit over vemurafenib plus TAK-733. This finding was strikingly validated *in vivo* where TAK-733 displayed minimal single-agent efficacy in vemurafenib-resistant A375-VR xenografts, however the combination of ganetespib...
with TAK-733 induced tumor regression (38%) (Fig. 4D). Thus, the addition of ganetespib potentiated the activity of TAK-733 in this model. Taken together, these data support a rationale for co-targeting BRAF V600E, either with a selective inhibitor or through Hsp90 blockade, and MEK to overcome vemurafenib resistance in melanoma cells.
Discussion

Targeted interference of BRAF<sub>V600E</sub> in melanoma cells has provided critical confirmation, both in experimental models and the clinical setting, of the role of this driver in melanoma oncogenesis. The development of selective inhibitors of mutated BRAF, such as vemurafenib and dabrafenib (25), has resulted in promising improvements in survival for melanoma patients. However, the invariable development of acquired resistance to these agents represents a significant clinical obstacle to their long-term efficacy. Here we show that ganetespib has potent activity in melanoma cells driven by BRAF<sub>V600E</sub>, in agreement with the findings of a recent study using cell lines established from patient samples (26). This report extends those observations, providing the first evidence of in vitro and in vivo activity of this investigational agent, both as a single agent and as part of novel combinatorial strategies, in multiple models of BRAF-inhibitor sensitive and resistant melanoma. Mutated BRAF is a client of Hsp90 (9) and our data reveal that ganetespib treatment results in the simultaneous destabilization of BRAF<sub>V600E</sub> as well as CRAF, AKT and the canonical RAF/MEK/ERK signal cascade that is stimulated by mutant BRAF activation. This broader spectrum of biological activity conferred by targeted Hsp90 inhibition accounts for the superior potency and efficacy of ganetespib over vemurafenib.

In light of the exquisite dependence on oncogenic MAPK signaling for proliferation and survival, pharmacological inhibition of MEK has also emerged as an important strategy for therapeutic intervention in mutant BRAF-driven melanoma (27). In accordance with preclinical predictions, the clinical evaluation of small molecule inhibitors of MEK has shown encouraging results, with superior response rates and outcomes compared to chemotherapy in this population (8, 28). However, response rates for MEK inhibitors are typically lower than those seen with selective BRAF inhibitors - thus the role of MEK inhibitor monotherapy in mutant BRAF melanoma, given the advent of approved BRAF-targeted agents, remains to be determined (8). A number of combination trials investigating the dual blockade of mutant BRAF and MEK are currently underway, and early evidence suggests that this strategy may not only improve the efficacy over
single agent treatments alone (29), but may also be an effective approach to prevent or delay the onset of resistance due to ERK reactivation (30, 31). Similar to what was found for vemurafenib, ganetespib was considerably more potent than AZD6244 in terms of MAPK pathway modulation and cytotoxic activity in BRAF^{V600E}-driven melanoma cells. In vitro, strong synergistic activity was seen when ganetespib was used as a co-treatment with both vemurafenib and AZD6244 and these effects were more robustly recapitulated in vivo with the ganetespib + vemurafenib combination. Thus, our data strongly suggest that combining the modalities of Hsp90 inhibition with either selective BRAF or MEK targeting warrants further investigation as a potential avenue of therapeutic intervention in melanoma cells displaying oncogenic addiction to BRAF^{V600E}.

A significant finding of this study was that ganetespib could overcome both intrinsic and acquired vemurafenib resistance in melanoma lines. Similar activity has been reported for another Hsp90 inhibitor XL888 (32). Unlike the case for a number of mutant kinase-driven malignancies treated with small molecule inhibitors, to date there is no evidence of secondary ‘gate-keeper’ type mutations in BRAF that account for a resistant phenotype (8). Instead, a variety of mechanisms have been identified that allow for either bypass or reactivation of MAPK signaling (reviewed in (1, 6, 28)), with the vast majority leading to reactivation of ERK activity despite the presence of the inhibitor. For example, Lito and colleagues recently showed that an elevated state of ERK-dependent feedback potently suppresses ligand-dependent signaling by growth factors and upstream RAS activity in BRAF^{V600E}-driven melanoma cells (22). Treatment with vemurafenib, which inhibits BRAF monomers but not homo- or heterodimers, potently relieves this ERK-dependent feedback and creates a permissible environment for reactivation of ligand-dependent signaling. This in turn promotes a rebound in RAS and ERK activity such that in the new steady-state melanoma cells become inhibitor resistant but still reliant on ERK (22). In another model, repression of mutant BRAF by inhibitor resulted in a kinase switch in which the addicted melanoma cells continued to rely on the MAPK pathway for maintenance of the malignant phenotype (33). Moreover, it has also been shown that vemurafenib-resistant xenografts displaying ERK reactivation may become drug-dependent for their continued proliferation and...
survival (23). Consistent with these reports, we observed elevated basal ERK activity in our model of acquired resistance generated by chronic exposure to vemurafenib (A375-VR). It is reasonable to suggest that the complexity of such resistance mechanisms to selective BRAF inhibitors may be overcome by the simultaneous targeting of multiple signaling nodes that is afforded by Hsp90 inhibition. In support of this, ganetespib retained full single-agent potency against vemurafenib-resistant cells and, indeed, they remained more sensitive to ganetespib compared to the parental line to vemurafenib.

Within the resistance setting, MAPK reactivation predicts for MEK inhibitor sensitivity (24). As shown by our results using allosteric MEK inhibitors, continued suppression of BRAF<sub>V600E</sub> may be important for treating tumors with acquired resistance to vemurafenib since inactivation of mutant BRAF by kinase inhibition enhanced the antiproliferative activity of both TAK-733 and AZD6244 in the resistant cell line. However, in contrast to the parental line which requires BRAF<sub>V600E</sub> for growth, the addition of vemurafenib did not augment ganetespib-induced cytotoxicity in A375-VR cells. This suggests that, within the context of inhibitor resistance, the direct contribution of BRAF<sub>V600E</sub> to cell growth is diminished and that maximal efficiency is afforded by Hsp90 inhibition. Importantly, single-agent ganetespib treatment diminished, but not abolished, ERK reactivation activity in A375-VR cells and our profiling studies clearly showed that only the addition of a MEK inhibitor could completely abrogate elevations in steady state ERK levels. By extension, the ganetespib + TAK733 combination provided superior apoptotic induction and inhibition of MAPK and AKT signaling compared to dual vemurafenib + TAK733 treatment. Moreover, these effects were recapitulated in vemurafenib-resistant tumors in vivo, where combination treatment induced significant tumor regression. These data provide a compelling rationale for combining ganetespib with targeted MEK agents as a promising approach for treating tumors with acquired resistance to BRAF inhibitors.

In summary, here we have shown that the Hsp90 inhibitor ganetespib exhibits robust cytotoxic activity and antitumor efficacy in preclinical models of BRAF<sub>V600E</sub> melanoma and can readily
overcome mechanisms of intrinsic and acquired resistance to selective BRAF inhibitors. These data suggest that ganetespib may offer an alternative, and potentially complementary, strategy for therapeutic intervention in mutant BRAF-driven disease. In light of these findings further evaluation of the therapeutic utility of this agent, both as a single agent and/or combinatorial partner, is warranted.
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References


Table 1. *In vitro* cytotoxicity values of ganetespib in BRAF mutant melanoma lines.

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†A375-VR cells were maintained in 5 μM vemurafenib throughout the assay.
* IC₅₀ values determined at 72 h; ** EC₅₀ values determined at 24 h
Figure Legends

**Figure 1.** Ganetespib potently destabilizes mutant BRAF and MAPK signaling in BRAF<sup>V600E</sup> melanoma cells leading to loss of viability. **A**, Chemical structures of the inhibitor compounds used in this study. **B**, A375 mutant BRAF<sup>V600E</sup> melanoma cells were exposed to vehicle (V), 10, 50, or 100 nM ganetespib for 24 h. Cellular extracts were immunoblotted with antibodies against BRAF, CRAF, phosphorylated MEK (p-MEK), total MEK, phosphorylated ERK (p-ERK) and total ERK as shown. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was included as a loading control. **C**, Primary melanocytes, SK-MEL-2 (both wild-type BRAF) and SK-MEL28 melanoma (BRAF<sup>V600E</sup>) cells were treated with vehicle (V), 10, 50 or 200 nM ganetespib for 24 h and BRAF protein levels examined by immunoblotting. **D**, A375 cells were treated with ganetespib, AZD6244 or vemurafenib over a broad range (1-1000 nM) and cell viability assessed after 72 h. **E**, A375 cells were treated with graded concentrations of ganetespib, vemurafenib or AZD6244 for 24 h and caspase 3/7 activity quantitated by luminescence (RLU: relative luminescence units). **F**, A375 cells were exposed to graded concentrations of ganetespib, vemurafenib or AZD6244 for 24 h and cell lysates immunoblotted with the indicated antibodies.

**Figure 2.** Combinations of ganetespib with BRAF and MEK inhibitors in mutant BRAF<sup>V600E</sup> melanoma cells. **A**, Mice bearing established A375 xenografts (n=7 mice/group) were i.v. dosed with ganetespib (50 mg/kg) once weekly and vemurafenib (25 mg/kg) administered p.o. BID 5x/week, either alone or in combination, as indicated (arrowheads). Numerical T/C values (%) are indicated to the right of each growth curve and the error bars are the SEM. The combination of ganetespib and vemurafenib displayed significantly greater efficacy than ganetespib alone (*, p=0.01); ns, not significant. **B**, Mice bearing established A375 xenografts (n=5 mice/group) were i.v. dosed with ganetespib (50 mg/kg) once weekly and AZD6244 (3 mg/kg) administered p.o. 5x/week, either alone or in combination, as indicated (arrowheads). **C**, Mice bearing established A375 xenografts (n=6 mice/group) were i.v. dosed with ganetespib (50 mg/kg) once weekly and TAK-733 (3 mg/kg) administered p.o. 5x/week, either alone or in combination, as indicated.
The combination of ganetespib and TAK-733 was significantly more efficacious than ganetespib alone, inducing tumor regression (*, p=0.0015).

Figure 3. Ganetespib overcomes intrinsic and acquired vemurafenib resistance in BRAFV600E melanoma cell lines. A, RPMI-7951 cells were treated with ganetespib, AZD6244 or vemurafenib over the range of concentrations indicated for 24 h and caspase 3/7 activity was quantitated by luminescence (RLU: relative luminescence units). B, RPMI-7951 cells were exposed to graded concentrations of ganetespib (10-200 nM), AZD6244 (10-200 nM) or vemurafenib (0.1-10 μM) for 24 h. The levels of BRAF as well as total and phosphorylated MEK, ERK, and AKT were determined by immunoblotting. C, Parental A375 or vemurafenib-resistant A375-VR cells were treated with ganetespib or vemurafenib over a broad range of concentrations and cell viability assessed after 72 h. D, Nude mice bearing established A375-VR xenografts (n=8 mice/group) were i.v. dosed with ganetespib (150 mg/kg) once weekly and vemurafenib (75 mg/kg) was administered p.o. BID 5x/week. Numerical T/C values (%) are indicated to the right of each growth curve and the error bars are the SEM. Ganetespib, but not vemurafenib, significantly inhibited tumor growth in this resistant model (*, p=0.001; ns, not significant).

Figure 4. Inhibition of BRAFV600E enhances the activity of MEK inhibitors in vemurafenib-resistant melanoma cells. A, A375 and A375-VR cells were treated with graded concentrations of ganetespib or vemurafenib for 24 h. The levels of MET, BRAF, CRAF, RAS, as well as total and phosphorylated MEK and ERK were determined by immunoblotting. B, Vemurafenib-resistant A375-VR cells were treated with the MEK inhibitor TAK-733 over a broad range of concentrations either in the presence or absence of 5 μM vemurafenib and cell viability assessed after 72 h. C, A375-VR cells were treated with vemurafenib, TAK-733 and ganetespib either as single agents or in combination, as indicated. After 24 h the levels of BRAF, total and phosphorylated MEK, ERK and AKT, and cleaved PARP were determined by immunoblotting. D, Mice bearing established A375-VR xenografts (n=5 mice/group) were i.v. dosed with ganetespib (150 mg/kg) once weekly and TAK-733 (3 mg/kg) administered p.o. 5x/week, either alone or in combination, as indicated (arrowheads). Numerical T/C values (%) are indicated to the right of each growth curve and the
error bars are the SEM. The addition of ganetespib to TAK-733 was significantly more efficacious than the MEK inhibitor alone in these vemurafenib-resistant tumors, inducing tumor regression (*, $p=0.0015$; ns, not significant).
Figure 1
Figure 2

A. Average tumor volume (mm$^3$) over days after tumor implantation for different treatments:
- Vehicle
- Ganetespib (50 mg/kg, 1x/wk)
- Vemurafenib (25 mg/kg, BID 5x/wk)
- Ganetespib + Vemurafenib

B. Average tumor volume (mm$^3$) over days after tumor implantation for different treatments:
- Vehicle
- Ganetespib (50 mg/kg, 1x/wk)
- AZD6244 (3 mg/kg, 5x/wk)
- Ganetespib + AZD6244

C. Average tumor volume (mm$^3$) over days after tumor implantation for different treatments:
- Vehicle
- Ganetespib (50 mg/kg, 1x/wk)
- TAK-733 (3 mg/kg, 5x/wk)
- Ganetespib + TAK-733

Dosing schedules:
- i.v. dosing (1x/wk)
- p.o. dosing (BID 5x/wk)
- p.o. dosing (5x/wk)
Figure 3
Figure 4
Molecular Cancer Therapeutics

Overcoming Acquired BRAF Inhibitor Resistance in Melanoma via Targeted Inhibition of Hsp90 with Ganetespib

Jaime Acquaviva, Donald L Smith, John-Paul Jimenez, et al.

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