Targeting KRAS-Mutant Non–Small Cell Lung Cancer with the Hsp90 Inhibitor Ganetespib

Jaime Acquaviva, Donald L. Smith, Jim Sang, Julie C. Friedland, Suqin He, Manuel Sequeira, Chaohua Zhang, Yumiko Wada, and David A. Proia

Abstract

Mutant KRAS is a feature of more than 25% of non–small cell lung cancers (NSCLC) and represents one of the most prevalent oncogenic drivers in this disease. NSCLC tumors with oncogenic KRAS respond poorly to current therapies, necessitating the pursuit of new treatment strategies. Targeted inhibition of the molecular chaperone Hsp90 results in the coordinated blockade of multiple oncogenic signaling pathways in tumor cells and has thus emerged as an attractive avenue for therapeutic intervention in human malignancies. Here, we examined the activity of ganetespib, a small-molecule inhibitor of Hsp90 currently in clinical trials for NSCLCs in a panel of lung cancer cell lines harboring a diverse spectrum of KRAS mutations. In vitro, ganetespib was potently cytotoxic in all lines, with concomitant destabilization of KRAS signaling effectors. Combinations of low-dose ganetespib with MEK or PI3K/mTOR inhibitors resulted in superior cytotoxic activity than single agents alone in a subset of mutant KRAS cells, and the antitumor efficacy of ganetespib was potentiated by cotreatment with the PI3K/mTOR inhibitor BEZ235 in A549 xenografts in vivo. At the molecular level, ganetespib suppressed activating feedback signaling loops that occurred in response to MEK and PI3K/mTOR inhibition, although this activity was not the sole determinant of combinatorial benefit. In addition, ganetespib sensitized mutant KRAS NSCLC cells to standard-of-care chemotherapeutics of the antimitotic, topoisomerase inhibitor, and alkylating agent classes. Taken together, these data underscore the promise of ganetespib as a single-agent or combination treatment in KRAS-driven lung tumors. Mol Cancer Ther; 11(12): 2633–43. ©2012 AACR.

Introduction

Lung cancer is the leading cause of cancer death worldwide (1), and non–small cell lung cancer (NSCLC) accounts for 85% of all cases. Platinum-based combination chemotherapy represents the standard-of-care for individuals with advanced NSCLCs (2), although this approach has largely reached a plateau of effectiveness in improving overall survival (3, 4). Intensive research into the underlying biologic and molecular basis of NSCLCs has provided critical insights into essential oncogenic pathways that become dysregulated during tumorigenesis and, in turn, has identified important targets for drug development. These findings have subsequently translated into significant clinical advances due to the inclusion of molecularly targeted agents in novel therapeutic strategies for this disease (5). As an example, first-line therapy for NSCLCs has been revolutionized by agents that inhibit the EGF receptor (EGFR) in tumors that harbor activating mutations in this receptor tyrosine kinase, including the tyrosine kinase inhibitors (TKI) gefitinib and erlotinib (6). More recently, discovery of the anaplastic lymphoma kinase (ALK) gene rearrangement in a subset of patients with NSCLCs led to the rapid approval of the multi-targeted TKI crizotinib as another genotype-driven therapy (7). Despite this progress, however, treatment outcomes for NSCLCs are still considered disappointing (8).

KRAS is a member of the RAS family of oncogenes, a collection of small guanosine triphosphate (GTP)-binding proteins that integrate extracellular cues and activate intracellular signaling pathways to regulate cell proliferation, differentiation, and survival (9). Gain-of-function mutations that confer transforming capacity are frequently observed in KRAS, predominantly arising as single amino acid substitutions at residues G12, G13, or Q61 (9). Constitutive activation of KRAS leads to the persistent stimulation of downstream signaling pathways that promote tumorigenesis, including the RAF/MEK/ERK and PI3K/AKT/mTOR cascades (10). In NSCLCs, KRAS mutations are highly prevalent (20%–30%) and are associated with unfavorable clinical outcomes (11, 12). Mutations in KRAS appear mutually exclusive with those in EGFR in NSCLC tumors (13); more importantly, they have been linked to primary resistance to targeted EGFR-TKI

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therapies (14). As a therapeutic target, oncogenic KRAS has proven to be “undruggable,” and extensive efforts at developing specific anti-RAS agents have failed clinically (15, 16). Alternative strategies, such as blockade of RAS effectors are being evaluated; yet there remains an urgent unmet need for more effective therapies.

In this regard, targeting the molecular chaperone Hsp90 represents a promising avenue for therapeutic intervention. Hsp90 is required for the stability and maturation of numerous key signal transduction proteins, termed “client” proteins (17, 18). Many of these clients play indispensable roles in cell growth and survival and include such proteins as EGFR, RAF, and AKT. Of note, cancer cells exploit the Hsp90 chaperone machinery as a biochemical buffer to protect a variety of mutated and/or overexpressed oncoproteins from targeted degradation, thereby facilitating aberrant cell survival and oncogene addiction (18, 19). Importantly, inhibition of Hsp90 activity targets its clients for proteasomal destruction. Because of its coordinate and simultaneous impact on multiple signaling cascades, pharmacologic blockade of Hsp90 can therefore overcome signaling redundancies and drug resistance mechanisms commonly seen in many cancers (20–22). As such, Hsp90 has become an attractive molecular target for the development of novel anticancer agents.

Ganetespib (formerly STA-9090) is a small-molecule inhibitor of Hsp90 with pharmacologic and biologic properties that distinguish it from other first- and second-generation inhibitors in terms of superior antitumor activity, potency, and safety (23). In addition, ganetespib is presentely undergoing evaluation in multiple human clinical trials, including patients with advanced NSCLCs. Here, we evaluated ganetespib activity in a panel of KRAS-mutant NSCLC lines to determine the effects on signaling cascades responsible for KRAS-driven NSCLC cell survival. Furthermore, we examined the potential combinatorial activity of ganetespib with clinical agents targeting key nodal components of these pathways as well as standard-of-care therapeutics for NSCLCs. Taken together, the findings support a potential clinical use for ganetespib in patients with KRAS-mutant NSCLCs.

Materials and Methods

Cell lines, antibodies, and reagents

All cell lines were obtained from the American Type Culture Collection (ATCC) and maintained according to standard techniques. The cell lines were authenticated by the routine ATCC Routine Cell Biology Program using short tandem repeat (STR) analysis (DNA profiling) and were used within 6 months of receipt for this study. All primary antibodies were purchased from Cell Signaling Technology with the exception of the C-Raf and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies which were obtained from Santa Cruz Biotechnology, Inc. 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. AZD6244, AZD8055, gemcitabine, camptothecin, and pemetrexed were purchased from Selleck Chemicals; cisplatin from EMD Chemicals; docetaxel and BEZ2235 from LC Laboratories; SN-38 from TCI America; and oxaliplatin from Sigma-Aldrich. The chemical structures of all compounds are shown in Fig. 1.

Cell viability assays

Cellular viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s protocol. KRAS-mutant NSCLC cell lines were seeded into 96-well plates on the basis of optimal growth rates determined empirically for each line. Twenty-four hours after plating, cells were dosed with graded concentrations of ganetespib for 72 hours. CellTiter-Glo was added (50% v/v) to the cells, and the plates incubated for 10 minutes before luminescent detection in a SpectraMax Plus 384 microplate reader (Molecular Devices). Data were normalized to percentage of control, and IC50 values used to determine the sensitivity of each line. For the comparative analysis study with MEK and PI3K/mTOR inhibitors, A549, H2009, Calu-1, and H358 cells were treated with graded concentrations of ganetespib, AZD6244, or BEZ2235 for 72 hours, and cell viability measured as above.

Western blotting

Following treatment, tumor cells were disrupted in lysis buffer (Cell Signaling Technology) on ice for 10 minutes. Lysates were clarified by centrifugation and equal amounts of proteins resolved by SDS-PAGE before standard techniques. The cell lines were authenticated by the routine ATCC Routine Cell Biology Program using short tandem repeat (STR) analysis (DNA profiling) and were used within 6 months of receipt for this study. All primary antibodies were purchased from Cell Signaling Technology with the exception of the C-Raf and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies which were obtained from Santa Cruz Biotechnology, Inc. 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. AZD6244, AZD8055, gemcitabine, camptothecin, and pemetrexed were purchased from Selleck Chemicals; cisplatin from EMD Chemicals; docetaxel and BEZ2235 from LC Laboratories; SN-38 from TCI America; and oxaliplatin from Sigma-Aldrich. The chemical structures of all compounds are shown in Fig. 1.

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In vivo tumor growth inhibition study

Female immunodeficient CD-1 (nude) mice (Charles River Laboratories) 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. A549 NSCLC cells (7.5 × 106) were subcutaneously implanted into the animals. Mice bearing established tumors (100–200 mm3) were randomized into treatment groups of 8 and i.v. dosed via the tail vein with either vehicle, ganetespib formulated in 10/18 DRD [10% dimethyl sulfoxide (DMSO), 18% Cremophor RH 40, 3.6% dextrose, 68.4% water] or per os dosed with BEZ2235 formulated in PEG300/NMP (90% PEG300, 10% N-methylpyrrolidone). Animals were treated with ganetespib at 50 mg/kg weekly or BEZ2235 at 10 mg/kg 5 times a week, either alone or in combination. Tumor growth inhibition was determined as described previously (24).

Multiple drug effect analysis

For combinatorial analysis, cells were seeded in 96-well plates at a predetermined, optimum growth density for
24 hours before the addition of drug or vehicle to the culture medium. Drug combinations were applied at a nonconstant ratio over a range of concentrations for 72 or 96 hours. For each compound tested, a 7-point dose range was generated on the basis of 1.5-fold serial dilutions using IC50 values set as the midpoint. Cell viability was assessed by either alamarBlue (Invitrogen) or Cell-Titer-Glo assays and normalized to vehicle controls. For each combination study, the level of growth inhibition (fraction affected) is plotted relative to vehicle control. Data are presented as one relevant combination point and the corresponding single-agent data for each cell line tested.

Reverse-phase protein array analysis
For single-agent ganetespib profiling, H2009, A549, H538, and Calu-1 cells were treated with DMSO (control) or ganetespib (250 nmol/L) for 24 hours. In the combination experiments, A549 cells were treated with ganetespib (25 nmol/L) or BEZ235 (20 nmol/L), either alone or in combination for 24 hours. Lysates were then prepared as recommended by the Reverse-Phase Protein Analysis

Figure 1. Chemical structures of ganetespib and all compounds used in the study.
Core Facility at MD Anderson Cancer Center (Houston, TX). Serial diluted lysates were arrayed on nitrocellulose-coated FAST slides (Whatman) and probed for a standard list of antibodies as previously described (25, 26).

Results

Loss of viability and client protein expression by ganetespib in KRAS-mutant NSCLC cells

The cytotoxic activity of ganetespib was initially evaluated in a panel of 20 NSCLC cell lines selected for expression of known KRAS mutations, including G12, G13, and Q61 variants (Table 1). Ganetespib potently reduced viability in all lines examined, with IC₅₀ values in the low nanomolar range. Representative viability curves for 2 lines, H727 and H441, are shown in Fig. 2A (left). The IC₅₀ values for these G12V-mutant KRAS-expressing cells were 28 and 14 nmol/L, respectively. KRAS itself is not a client protein of Hsp90 and was therefore unaffected by ganetespib treatment (Supplementary Table S1). However, as targeted degradation of client proteins is a feature of Hsp90 inhibition, we examined expression changes in client and signaling pathway proteins associated with NSCLC progression. In H727 and H441 cells, ganetespib treatment resulted in a robust and dose-dependent decrease in EGFR and MET receptors, both established Hsp90 clients (Fig. 2A, right panels). The KRAS substrate C-RAF was potently destabilized in both cell lines and, as expected, only a relatively modest reduction in B-RAF protein levels was observed in H441 cells. Targeted degradation of these signaling intermediates was accompanied by inactivation of downstream effectors (p-MEK, p-ERK, p-AKT) and induction of apoptotic markers (cleaved PARP, increased BIM levels). AKT activation involves the phosphorylation of 2 residues: serine 473 (Ser473) and threonine 308 (Thr308). Of note, phosphorylation of Thr308 has been correlated with activity in NSCLCs (27), and p-AKT (Thr308) expression was acutely sensitive to ganetespib treatment in H727 cells. Potent destabilization of p-AKT (Ser473) was seen in both lines. Interestingly, mTOR signaling was affected by ganetespib in H441 cells, as evidenced by a reduction in levels of phosphorylated 4E-BP1 protein, a direct substrate of the mTOR-1 complex (mTORC1). Because AKT Ser473 is regulated by mTOR complex-2 (mTORC2; ref. 28), these data suggest that Hsp90 inhibition may coordinately impact cross-talk between the mTOR pathway and other KRAS-driven signaling cascades.

We have previously reported that even brief exposure of NSCLC lines to ganetespib in vitro results in sustained activity and potent effects on cellular viability (23). Therefore, we exposed A549 and H2030 cells to graded concentrations of ganetespib for 1 hour and then investigated the effects on client protein pathways at 24 hours. Doses were determined empirically for each line, using the minimal concentrations required for client protein depletion. For comparison, we included a continuous 24-hour exposure to ganetespib at the lowest relative dose (Fig. 2B). Continuous low-dose ganetespib treatment (0.5 and 0.1 μmol/L for A549 and H2030 cells, respectively) resulted in targeted degradation of phosphorylated total EGFR, C-RAF, and p-AKT (Ser473) and threonine 308 (Thr308). Of note, phosphorylation of Thr308 has been correlated with activity in NSCLCs (27), and p-AKT (Thr308) expression was acutely sensitive to ganetespib treatment in H727 cells. Potent destabilization of p-AKT (Ser473) was seen in both lines. Interestingly, mTOR signaling was affected by ganetespib in H441 cells, as evidenced by a reduction in levels of phosphorylated 4E-BP1 protein, a direct substrate of the mTOR-1 complex (mTORC1). Because AKT Ser473 is regulated by mTOR complex-2 (mTORC2; ref. 28), these data suggest that Hsp90 inhibition may coordinately impact cross-talk between the mTOR pathway and other KRAS-driven signaling cascades.

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Table 1. In vitro cytotoxicity values of ganetespib in KRAS-mutant NSCLC lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>KRAS mutation</th>
<th>Ganetespib IC₅₀, nmol/L</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1355</td>
<td>G13C</td>
<td>5</td>
</tr>
<tr>
<td>H157</td>
<td>G12R</td>
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<tr>
<td>H460</td>
<td>G61H</td>
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<tr>
<td>IA-LM</td>
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<tr>
<td>HOP-62</td>
<td>G12C</td>
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</tr>
<tr>
<td>H23</td>
<td>G12C</td>
<td>11</td>
</tr>
<tr>
<td>H2030</td>
<td>G12C</td>
<td>12</td>
</tr>
<tr>
<td>H441</td>
<td>G12V</td>
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</tr>
<tr>
<td>H2212</td>
<td>G12C</td>
<td>17</td>
</tr>
<tr>
<td>SK-LU-1</td>
<td>G12D</td>
<td>18</td>
</tr>
<tr>
<td>H2009</td>
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<td>19</td>
</tr>
<tr>
<td>H1792</td>
<td>G12C</td>
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<tr>
<td>COR-L23</td>
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<td>22</td>
</tr>
<tr>
<td>H727</td>
<td>G12V</td>
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</tr>
<tr>
<td>H1734</td>
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<td>28</td>
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<tr>
<td>H358</td>
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<tr>
<td>Calu-6</td>
<td>Q61K</td>
<td>64</td>
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Multimodal suppression of oncogenic signaling by ganetespib confers superior cytotoxic activity over selective MEK and PI3K/mTOR inhibitors

Recent preclinical studies have provided a convincing rationale for the combinatorial use of targeted MEK and PI3K/AKT inhibitors in NSCLCs, particularly in KRAS-driven lung tumors (29, 30). Accordingly, here we focused on AZD6244, an allosteric inhibitor of the MEK1/2 kinases, and BEZ235, a dual pan-PI3K and mTOR inhibitor, both of which are under clinical development. When the single-agent cell killing activity of these compounds was compared with that of ganetespib in a subset of mutant KRAS NSCLC lines (Fig. 3A), ganetespib was typically more potent at reducing cell viability than either
agent. AZD6244, in particular, was largely ineffective as a monotherapy, with IC$_{50}$ values $\geq 1,000$ nmol/L in the 4 lines examined.

We next examined modulation of the cellular signaling cascades targeted by these agents using Western blot analysis (Fig. 3B). As expected, MEK blockade by AZD6244 resulted in consequent loss of downstream ERK activity with no observed effects on upstream (B-RAF or C-RAF) AKT or mTOR signaling. However, in all of the lines, AZD6244 induced a significant accumulation of phosphorylated MEK protein, indicating that treatment with this inhibitor resulted in the activation of a feedback signaling loop. Induction of feedback signaling was also observed in each of the 4 lines following exposure to the PI3K/mTOR inhibitor BEZ235 (Fig. 3B). Consistent with its targeted activity, BEZ235 significantly suppressed p-AKT levels and completely abrogated mTOR activity, including complete loss of phosphorylated 4E-BP1 protein. Unexpectedly, BEZ235 treatment also increased p-MEK levels and this was associated with a robust upregulation of activated (i.e., phosphorylated) ERK expression (Fig. 3B). Therefore, despite direct effects on their molecular targets, both kinase inhibitors triggered compensatory signaling activity within the tumor cells.

In contrast, ganetespib treatment resulted in loss of C-RAF, p-MEK, p-ERK, total/p-AKT (and partial suppression of 4E-BP1 activity) across all lines and without concomitant upregulation of feedback signaling (Fig. 3B). In agreement with the cytotoxicity profiles shown in Fig. 3A, ganetespib treatment additionally manifested the highest pro-apoptotic activity, as shown by consistent elevations in cleaved PARP expression. These findings were supported by a more extensive reverse-phase protein array analysis of ganetespib exposure in the same cell panel (Supplementary Table S1). Besides leading to the expected downregulation of client protein receptors (including EGFR, MET, and HER2) and signaling intermediates of pathways regulated by these oncogenic drivers (Src, STAT3, GSK3), ganetespib treatment also selectively altered the expression of a number of proteins involved in mTOR signaling (S6, 4E-BP1, PDK1), cell-cycle regulation, and apoptosis. Taken together, these coordinate impacts on multiple signaling cascades conferred by Hsp90 chaperone inhibition underlies the superior...
therapeutic utility of ganetespib in KRAS-mutant lung cancer lines.

**Ganetespib suppresses feedback pathway activation and enhances the activity of MEK and PI3K/mTOR inhibitors in vitro**

To extend these observations, we subsequently tested ganetespib in combination with inhibitors of MEK and PI3K/mTOR. Concurrent administration of low (IC\textsubscript{50} or IC\textsubscript{20}) doses of ganetespib with AZD6244 substantially increased cell death in A549 and H2009 cells (Fig. 4A). This effect was not universal, however, as no additional benefit was seen in the Calu-1 or H358 lines when the drugs were combined at similar ratios (Fig. 4A). At the molecular level, single-agent ganetespib robustly inhibited the C-RAF/MEK/ERK signaling axis in A549 and H2009 cells (Fig. 4B). AZD6244 treatment alone reduced downstream p-ERK expression yet induced p-MEK accumulation in both cell types, consistent with the data shown in Fig. 3B. Importantly, co-treatment with ganetespib blocked this effect, suggesting that the feedback loop(s) responsible for promoting signaling redundancy in these NSCLC cell lines were sensitive to Hsp90 inhibition.

In agreement with the cytotoxicity data (Fig. 4A), the increased PARP cleavage observed in H2009 cells highlights the more potent pro-apoptotic activity seen with the ganetespib–AZD6244 combination. Combinatorial benefit was also observed in 5 mutant KRAS NSCLC lines when ganetespib was dosed in combination with BEZ235 (Fig. 4C). Selecting 2 of these lines, we found that single-agent treatment of A549 and H2030 cells with either BEZ235 or another mTOR inhibitor, AZD8055, inhibited phosphorylation of 4E-BP1 as well as AKT (Ser473); thus, mTOR activation was effectively abrogated in these cells (Fig. 4D). However, cellular exposure to both mTOR inhibitors induced feedback pathway activation, again evidenced by an accumulation of p-MEK and p-ERK protein levels. Concurrent administration of ganetespib effectively suppressed this response in both lines (Fig. 4D). Of note, ganetespib treatment also suppressed feedback signaling induced by both AZD6244 and BEZ235 in Calu-1 and H358 cells (Supplementary Fig. S1). Calu-1 cells showed enhanced antiproliferative activity for the ganetespib + BEZ235 combination but not for ganetespib + AZD6244, and H358 cells were a line in which no benefit was observed for ganetespib with either inhibitor. Thus, while targeted Hsp90 inhibition could overcome compensatory signaling loops in mutant KRAS NSCLC lines, this effect was not the sole determinant of the improved therapeutic activity conferred by ganetespib treatment.

To examine potential mechanisms underlying the enhanced cytotoxicity seen with ganetespib + BEZ235 treatment, we conducted reverse-phase protein array analysis using A549 cells treated with IC\textsubscript{30} concentrations of each agent either alone or in combination (Table 2). Consistent with its mTOR inhibitory activity, BEZ235 exposure caused a \( \geq 6.8 \)-fold reduction in phosphorylated S6 protein levels and robust decreases in p70S6K and phospho-4E-BP1. Single-agent effects of ganetespib on these mTOR signaling intermediates were comparatively moderate. Notably, combination treatment promoted greater cellular loss of these proteins, suggesting that mTOR signaling represents a point of convergence between the 2 agents. Unexpectedly, this assay also identified Y-box protein 1 (YB-1), a translational repressor and AKT substrate, as a potential molecular effector linked to the pro-apoptotic response. YB-1 protein levels were...
unaffected by either compound, and only a minor reduction in phosphorylated YB-1 was seen following ganetespib exposure. However, when the 2 drugs were combined, a greater than 1.4-fold loss of YB-1 expression was observed. Increased BIM levels confirmed apoptotic induction by the combination (Table 2).

**The antitumor efficacy of ganetespib is potentiated by PI3K/mTOR inhibition in a KRAS-mutant xenograft model in vivo**

To evaluate whether the combinatorial benefits on cell viability observed in vitro translated to improved efficacy in vivo, mice bearing A549 xenografts were treated with ganetespib and BEZ235, both as single agents and in combination. We have previously determined that the highest nonseverely toxic dose (HNSTD) of ganetespib on a weekly dosing regimen is 150 mg/kg (23). As shown in Fig. 5, weekly administration of a suboptimal dose of ganetespib (50 nmol/L) and 5×/wk dosing with BEZ235 (10 mg/kg) each reduced tumor growth by 44% (T/C value 56%). Consistent with the in vitro findings above, concurrent treatment with both drugs at the same dose levels resulted in a significant enhancement of antitumor activity, inhibiting tumor growth by 79%. Combination treatment was well tolerated, with no significant loss of toxicity observed.

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**Table 2.** Fold changes in protein expression following single-agent and combination treatment in A549 NSCLC cells using reverse-phase protein array analysis

<table>
<thead>
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<th>BEZ235 (IC30)</th>
<th>Combination</th>
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<td>S6 (pS240/S244)</td>
<td>−1.28</td>
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<td>p70S6K</td>
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</table>
heterogeneity of the disease and the development of new molecularly targeted agents. Indeed the clinical successes of EGFR and ALK inhibitors have ushered in a new era of drug development for NSCLCs. Of relevance, this particular malignancy is considered promising for the application of Hsp90 inhibitors (19), and evidence of clinical efficacy by small-molecule Hsp90 inhibition has recently been reported (32). At present, however, there are no effective therapies for KRAS-driven lung cancer and mutations in KRAS are associated with poor prognosis and resistance to both adjuvant therapy and targeted EGFR inhibitors (12). New preclinical data suggest that KRAS mutations may confer sensitivity to Hsp90 inhibition in lung adenocarcinoma (33), thereby suggesting a putative role for highly potent inhibitory agents targeting Hsp90 as novel treatment strategies for this subset of patients. In support of this premise, we observed tumor shrinkage in 47% of patients (8 of 17) who harbored KRAS mutations in a recent phase II monotherapy trial of ganetespib in advanced NSCLCs, although no objective responses were seen (34). Here, we have presented additional compelling evidence for the potential use of ganetespib in KRAS-dependent NSCLCs.

Targeted knockdown of KRAS in NSCLC cell lines has provided critical preclinical confirmation of the role of this driver in tumorigenesis, resulting in both the suppression of tumor growth and sensitization to inhibitors of other signaling pathways (35). However, in practical terms, KRAS itself has proven to be an intractable target for the development of therapeutics (16), and considerable effort has thus focused on inhibition of downstream effectors to perturb persistent activation of oncogenic signaling pathways. The canonical RAF/MEK/ERK kinase cascade is the primary mitogenic pathway stimulated by KRAS under both physiologic and pathologic conditions (9, 10). RAF serine/threonine kinases are direct substrates of the RAS family of proteins and, importantly, are also known Hsp90 clients. Here, we showed that C-RAF was more sensitive to destabilization by ganetespib in KRAS-mutant NSCLC lines than B-RAF, consistent with the observation that wild-type B-RAF is not dependent on Hsp90 for stability (36). While this result reflects differing sensitivities of individual client proteins to Hsp90 inhibition (37), it may additionally have important implications for lung cancer. Two elegant studies have recently provided convincing evidence that C-RAF, but not B-RAF, is essential for the initiation and development of KRAS-driven NSCLC using transgenic mouse models (38, 39). Therefore, the potent degradation and loss of C-RAF protein afforded by ganetespib treatment provides an effective means to uncouple aberrant KRAS signaling from downstream MEK and ERK activation in NSCLC cells and likely contributes to the antiproliferative activity of the compound.

With low nanomolar potency, ganetespib was cytotoxic to all mutant KRAS-bearing cell lines and even a brief exposure (1 hour) was sufficient to negatively impact Hsp90 activity, highlighting the durable response...
property of the drug. At the molecular level, Hsp90 inhibition by ganetespib resulted in blockade of the PI3K/AKT/mTOR signaling axis, which mediates prosurvival signaling downstream of mutant KRAS. Importantly, a feature of Hsp90 inhibition is the capacity to simultaneously block multiple growth and survival pathways, and concomitant inhibition of the PI3K/AKT/mTOR and RAF/MEK/ERK effector pathways represents an intrinsically intuitive strategy to counteract oncogenic KRAS activity. Indeed this approach has previously been explored using drug combinations. For example, Meng and colleagues (29) have shown that dual-agent combination therapy using AZD6244 and the AKT inhibitor MK2206 resulted in synergistic effects on cell viability in vitro as well as tumor growth in KRAS-driven NSCLC tumors in vivo. Our data revealed that ganetespib was more potent than AZD6244 in reducing viability when each agent was used as a monotherapy and combinatorial benefit was seen when the 2 compounds were used together in the A549 and H2009 cell lines. In another report, significant synergy in shrinking KRAS-driven lung tumors was observed when BEZ235 was combined with the MEK inhibitor ARRY-142886 (30), further supporting the potential benefit of coordinate blockade of the mitogenic and survival pathways activated by KRAS mutation. When ganetespib was used in combination with BEZ235, we observed superior cytotoxic activity in a greater number of KRAS-mutated cell lines than seen with the MEK inhibitor, as well as a significant enhancement of antitumor efficacy using the A549 xenograft model. These data are in agreement with an earlier study showing synergistic benefit by combining the mTOR inhibitor rapamycin with the Hsp90 inhibitor IPI-504 (40) and suggest that Hsp90 inhibition alongside mTOR blockade may represent an effective drug combination for the treatment of KRAS-mutant lung cancer.

The mTOR pathway was also shown to be a critical point of convergence for combined ganetespib–BEZ235

![Figure 6. Combinatorial activity of ganetespib with standard-of-care chemotherapeutics in vitro. NSCLC cell lines were treated with the indicated concentrations of ganetespib either alone or in combination with docetaxel (A), pemetrexed (B), gemcitabine (C), camptothecin (D), SN-38 (E), or the platins (oxaliplatin and cisplatin; F). Cell viability was assessed after 72 hours (96 hours for pemetrexed and gemcitabine).](https://aacrjournals.org/mct/2012/11/12/2641-F.png)
treatment. Interestingly, reverse-phase protein analysis also implicated a role for the translational and transcriptional factor YB-1 in the enhanced cytotoxic activity of the combination. Notably, YB-1 has been identified as an essential regulator of proliferation and gene expression in KRAS oncopogene–transformed cancer cells (41) and also plays a role in mediating drug resistance (42). Phosphorylation at serine 102 by AKT attenuates the translational repressor activity of the molecule (43) and also weakens its mRNA cap–binding capability, in turn, facilitating activation of silenced mRNA species (44). We found that total and phosphorylated YB-1 protein levels were unaffected by single-agent exposure but were robustly reduced following combination treatment. In this context, downregulation of YB-1 would result in the loss of oncogenic and prosurvival signals, in turn, promoting apoptosis, as has been reported in breast carcinoma (45). Studies confirming the role of YB-1 in the enhanced therapeutic benefit conferred by ganetespib–BEZ235 co-treatment are underway.

One interesting observation to arise from this study involved the drug-induced activation of compensatory feedback signaling pathways in mutant KRAS NSCLC lines following exposure to either MEK or mTOR inhibitors. This response typically arises due to relief of negative feedback pathways that maintain cellular homeostasis, and this mechanism has recently been proposed to contribute to adaptive resistance to therapeutic agents (46). Ganetespib treatment potently suppressed these signaling loops, even in NSCLC lines that did not show combinatorial benefit. Therefore, while attenuation of this feedback signaling activation did not appear to account for the improved therapeutic index of ganetespib in combination with MEK or mTOR inhibition, the more universal response suggests that Hsp90 inhibition may provide a means to overcome adaptive resistance to targeted agents, and this possibility is currently under investigation.

Finally, we showed that ganetespib sensitized mutant KRAS cell lines to standard-of-care therapeutics currently in clinical use for NSCLC treatment. Again, benefit was not observed in all lines, further highlighting the complexity of treating this particular subset of patients with cancer. It is reasonable to suggest that the heterogeneous response may simply reflect the cellular context, additional mutational status, and/or signaling redundancies present within the cell lines. Overall, however, the ability of ganetespib to potentiate the cytotoxic effects of chemotherapeutic agents in the KRAS-mutant setting provides a strong rationale for combinatorial approaches as a potentially useful therapeutic strategy. In this regard, we and others have previously shown that Hsp90 inhibition potentiates the activity of taxanes in preclinical NSCLC models (31, 47, 48). Perhaps, more importantly, from our ongoing phase Ib/II GALAXY trial, comparing standard-of-care docetaxel with the combination of ganetespib plus docetaxel in the second-line advanced NSCLC treatment setting, we have observed encouraging activity with ganetespib in the mutant KRAS population. Taken together, the data we are presenting here suggest that ganetespib may offer considerable promise for patients suffering from these generally untreatable cancers.

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

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