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

Synta Pharmaceuticals Corp., Lexington, MA

Running Title: Ganetespib activity in KRAS mutant NSCLC

Keywords: Hsp90 inhibition, ganetespib, non-small cell lung cancer, KRAS, cancer therapy

Abbreviations List: ALK, anaplastic lymphoma kinase; mTOR, mammalian target of rapamycin; NSCLC, non-small cell lung cancer; Hsp90, heat shock protein 90; EGFR, epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

Financial Support: All work was funded by Synta Pharmaceuticals Corp.

Corresponding author information: David A. Proia, Synta Pharmaceuticals, Corp., 125 Hartwell Avenue, Lexington, MA 02421. Phone: 781-541-7236; Fax: 781-274-8228; Email: dproia@syntapharma.com

Disclosure of Potential Conflicts of Interest: All authors are current or former employees of Synta Pharmaceuticals, Corp.

Word Count: 5500  Figures/Tables: 8 (6 Figures/2 Tables; after reviewer/editorial requests)
Abstract

Mutant KRAS is a feature of over 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 heat shock protein 90 (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 NSCLC, 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 co-treatment 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 anti-mitotic, 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.
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 NSCLC (2), although this approach has largely reached a plateau of effectiveness in improving overall survival (3, 4). Intensive research into the underlying biological and molecular basis of NSCLC 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 NSCLC has been revolutionized by agents that inhibit the epidermal growth factor receptor (EGFR) in tumors that harbor activating mutations in this receptor tyrosine kinase, including the tyrosine kinase inhibitors (TKIs) gefitinib and erlotinib (6). More recently, discovery of the anaplastic lymphoma kinase (ALK) gene rearrangement in a subset of NSCLC patients led to the rapid approval of the multi-targeted TKI crizotinib as another genotype-driven therapy (7). Despite this progress, however, treatment outcomes for NSCLC 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 NSCLC, 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 therapies (14).
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 heat shock protein 90 (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, pharmacological 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 presently undergoing evaluation in multiple human clinical trials, including patients with advanced NSCLC. 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. Further, 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 NSCLC. Taken together, the findings support a potential clinical utility for ganetespib in patients with KRAS mutant NSCLC.
Materials and Methods

Cell lines, antibodies and reagents

All cell lines were obtained from the ATCC (Rockville, MD) 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 (CST, Beverly, MA) with the exception of the C-RAF 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. AZD6244, AZD8055, gemcitabine, camptothecin and pemetrexed were purchased from Selleck Chemicals (Houston, TX); cisplatin from EMD Chemicals (Billerica, MA); docetaxel and BEZ235 from LC Laboratories (Woburn, MA); SN-38 from TCI America (Portland, OR); and oxaliplatin from Sigma Aldrich (St. Louis, MO). The chemical structures of all compounds are shown in Figure 1.

Cell viability assays

Cellular viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer’s protocol. KRAS mutant NSCLC 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 IC$_{50}$ 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 BEZ235 for 72 h and cell viability measured as above.

**Western blotting**

Following treatment, tumor cells were disrupted in lysis buffer (CST) 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 immunoblotted with the indicated antibodies and antibody-antigen complexes visualized using an Odyssey system (LI-COR, Lincoln, NE).

**In vivo tumor growth inhibition study**

Female immunodeficient 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. A549 NSCLC cells (7.5 x 10^6) were subcutaneously implanted into the animals. Mice bearing established tumors (100-200 mm^3) 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% DMSO, 18% Cremophor RH 40, 3.6% dextrose, 68.4% water) or p.o. dosed with BEZ235 formulated in PEG300/NMP (90% PEG300, 10% N-Methylpyrrolidone). Animals were treated with ganetespib at 50 mg/kg weekly or BEZ235 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 h prior to the addition of drug or vehicle to the culture medium. Drug
combinations were applied at a non-constant ratio over a range of concentrations for 72 or 96 hours. For each compound tested, a 7 point dose range was generated based on 1.5 fold serial dilutions using IC_{50} values set as the mid-point. Cell viability was assessed by either alamarBlue (Invitrogen, Carlsbad, CA) or CellTiter-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 nM) for 24 h. In the combination experiments, A549 cells were treated with ganetespib (25 nM) or BEZ235 (20 nM), either alone or in combination for 24 h. Lysates were then prepared as recommended by the Reverse Phase Protein Analysis 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 G61 variants (Table 1). Ganetespib potently reduced viability in all lines examined, with IC\textsubscript{50} values in the low nanomolar range. Representative viability curves for two lines, H727 and H441, are shown in Fig. 2A (left panels). The IC\textsubscript{50} values for these G12V mutant KRAS-expressing cells were 28 and 14 nM, respectively. KRAS itself is not a client protein of Hsp90 and was therefore unaffected by ganetespib treatment (Supplementary Table S1). However, since 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 two residues: serine 473 (Ser473) and threonine 308 (Thr308). Of note, phosphorylation of Thr308 has been correlated with activity in NSCLC cells (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, mammalian target of rapamycin (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). Since AKT Ser473 is regulated by mTOR complex-2 (mTORC2) (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 h, and then investigated the effects on client protein pathways at 24 h. Doses were determined empirically for each line, using the minimal concentrations required for client protein depletion. For comparison, we included a continuous 24 h exposure to ganetespib at the lowest relative dose (Fig. 2B). Continuous low dose ganetespib treatment (0.5 μM and 0.1 μM for A549 and H2030 cells, respectively) resulted in targeted degradation of phosphorylated and total EGFR, C-RAF, p-MEK, p-AKT and p-STAT3. However, even a 1 h exposure to ganetespib promoted a dose-dependent reduction in the levels of these same proteins. Although complete abrogation of downstream STAT3 signaling was not achieved, 1 h ganetespib treatment at 3 μM for A549 cells or 0.5 μM for H2030 cells was similarly effective as prolonged exposure at degrading p-EGFR, C-RAF and p-MEK levels in both cell lines (and p-AKT/AKT levels in H2030 cells) at the 24 h time point. Taken together, these data show that even brief exposure to the Hsp90 inhibitor was sufficient to efficiently induce client protein destabilization in KRAS mutant NSCLC lines.

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 NSCLC, 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 to 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\textsubscript{50} values ≥ 1000 nM in the four 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 four 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 up-regulation 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$_{50}$ or IC$_{20}$) 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 Figure 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 five mutant KRAS NSCLC lines when ganetespib was dosed in combination with BEZ235 (Fig. 4C). Selecting two 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 performed reverse phase protein array analysis using A549 cells treated with IC30 concentrations of each agent either alone or in combination (Table 2). Consistent with its mTOR inhibitory activity, BEZ235 exposure caused a ≥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 two 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. YB1 protein levels were unaffected by either compound, and only a minor reduction in phosphorylated YB-1 was seen following ganetespib exposure. However, when the two 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 non-severely toxic dose (HNSTD) of ganetespib on a weekly dosing regimen is 150 mg/kg (23). As shown in Fig. 5, weekly administration of a sub-optimal dose of ganetespib (50 mg/kg) and 5x/week 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 body weight seen after 3 weeks of dosing (data not shown). We also investigated the combination of ganetespib with AZD6244 (3 mg/kg, 5x/week) in the same model, however no significant improvement in efficacy over ganetespib alone was observed on this dosing regimen (Supplementary Fig. S2).

**Ganetespib sensitzes mutant KRAS NSCLC cells to standard of care chemotherapeutics**

Finally, we examined the potential for improved therapeutic benefit by combining ganetespib with standard of care agents used in the clinical treatment of lung cancer. These included chemotherapeutics of the antimitotic, antimetabolite, topoisomerase inhibitor and alkylating agent classes (Fig. 6). Combinatorial benefit was cell line specific; for example, in some lines ganetespib potentiated the cytotoxic effects of docetaxel (Fig. 6A), in agreement with an earlier study (31). When ganetespib was combined with either pemetrexed or gemcitabine enhanced cell killing was seen in H2030 and H2009 cells (Figs. 6B,C). Synergistic effects, most notably in the H2030 line, were also seen when the topoisomerase inhibitors camptothecin and SN-38 were used as co-treatment with ganetespib (Figs. 6D,E). Combinatorial benefit was also seen in Calu-1 cells when ganetespib was used in combination with either oxaliplatin or cisplatin (Fig. 6F). These findings indicate that, depending on the cellular context, ganetespib may enhance the anticancer activity of conventional chemotherapies.
Discussion

Individualized therapy is an emerging clinical reality for patients with advanced NSCLC. In recent years, an increasing array of treatment options has become available, based on an improved understanding of the biological 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 NSCLC. 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 as well as 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 (8/17) who harbored KRAS mutations in a recent Phase 2 monotherapy trial of ganetespib in advanced NSCLC, although no objective responses were seen (34). Here we have presented additional compelling evidence for the potential utility of ganetespib in KRAS-dependent NSCLC.

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 in order 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 physiological and pathological 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 h) 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 pro-survival 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 two 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 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 oncogene-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, down regulation of YB-1 would result in the loss of oncogenic and pro-survival 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 cancer patients. 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 2b/3 GALAXY trial, comparing standard-of-care docetaxel to 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.
Acknowledgments

We wish to thank Dr. Richard Bates who provided drafts and editorial assistance during preparation of this manuscript. We also thank the RPPA Facility at MD Anderson Cancer Center for their assistance in the reverse phase protein array analysis.

Grant Support

All work was funded by Synta Pharmaceuticals Corp. and no authors received any grant support.


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 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1355</td>
<td>G13C</td>
<td>5</td>
</tr>
<tr>
<td>H157</td>
<td>G12R</td>
<td>7</td>
</tr>
<tr>
<td>H460</td>
<td>Q61H</td>
<td>8</td>
</tr>
<tr>
<td>IA-LM</td>
<td>G12C</td>
<td>10</td>
</tr>
<tr>
<td>HOP-62</td>
<td>G12C</td>
<td>11</td>
</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>
<td>14</td>
</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>
<td>G12A</td>
<td>19</td>
</tr>
<tr>
<td>H1792</td>
<td>G12C</td>
<td>20</td>
</tr>
<tr>
<td>COR-L23</td>
<td>G12V</td>
<td>22</td>
</tr>
<tr>
<td>H727</td>
<td>G12V</td>
<td>28</td>
</tr>
<tr>
<td>H1734</td>
<td>G13C</td>
<td>28</td>
</tr>
<tr>
<td>H358</td>
<td>G12C</td>
<td>29</td>
</tr>
<tr>
<td>A549</td>
<td>G12S</td>
<td>43</td>
</tr>
<tr>
<td>H2122</td>
<td>G12C</td>
<td>53</td>
</tr>
<tr>
<td>Calu-1</td>
<td>G12C</td>
<td>58</td>
</tr>
<tr>
<td>Calu-6</td>
<td>Q61K</td>
<td>64</td>
</tr>
</tbody>
</table>
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>
<tr>
<th>Protein</th>
<th>Ganetespib (IC_{50})</th>
<th>BEZ235 (IC_{50})</th>
<th>Combination</th>
</tr>
</thead>
<tbody>
<tr>
<td>S6 (pS240/S244)</td>
<td>-1.28</td>
<td>-6.80</td>
<td>-9.07</td>
</tr>
<tr>
<td>S6 (pS235/S236)</td>
<td>-1.70</td>
<td>-6.86</td>
<td>-7.66</td>
</tr>
<tr>
<td>p70S6K</td>
<td>-1.38</td>
<td>-1.54</td>
<td>-1.72</td>
</tr>
<tr>
<td>4E-BP1 (pS65)</td>
<td>1.13</td>
<td>-1.54</td>
<td>-1.66</td>
</tr>
<tr>
<td>YB-1 (pS102)</td>
<td>-1.25</td>
<td>-1.03</td>
<td>-1.46</td>
</tr>
<tr>
<td>YB-1</td>
<td>1.02</td>
<td>-1.03</td>
<td>-1.41</td>
</tr>
<tr>
<td>BIM</td>
<td>1.14</td>
<td>1.00</td>
<td>+1.31</td>
</tr>
</tbody>
</table>
Figure Legends

Figure 1. Chemical structures of ganetespib and all compounds used in the study.

Figure 2. Ganetespib effects on cell viability and Hsp90-dependent signaling in mutant KRAS NSCLC cell lines. **A**, H727 and H441 NSCLC cells were treated with ganetespib over a broad range (0.1-1000 nM) and cell viability assessed after 72 h (**left panels**). For signaling (**right panels**), H727 (**upper**) and H441 (**lower**) cells were exposed to graded concentrations of ganetespib (0-100 nM) for 24 h and cell lysates immunoblotted with the indicated antibodies. GAPDH was included as a loading control. **B**, A549 cells were exposed to 0, 0.5, 1, 2 or 3 μM ganetespib and H2030 cells to 0, 0.1, 0.25 or 0.5 μM ganetespib for 1 h. A549 and H2030 cells were also treated with ganetespib at 0.5 and 0.1 μM, respectively, continuously for 24 h. At the 24 h time point, cell lysates were immunoblotted with the indicated antibodies.

Figure 3. Cellular viability and pathway modulation in mutant KRAS cell lines following Hsp90, MEK and PI3K/mTOR inhibition. **A**, A549, H2009, Calu-1 and H358 cells were treated with increasing concentrations of ganetespib, AZD6244 or BEZ235 and cell viability was assessed after 72 h. **B**, A549, H2009, Calu-1 and H358 NSCLC cells were exposed to 500 nM ganetespib, 1000 nM AZD6244 or 500 nM BEZ235 for 24 h and cell lysates immunoblotted with the indicated antibodies.

Figure 4. Combinations of ganetespib with MEK and mTOR inhibitors in mutant KRAS NSCLC cells. **A**, A549, H2009, Calu-1 and H358 cells were treated with the indicated concentrations of ganetespib or the MEK inhibitor AZD6244 either alone or in combination. Cell viability was assessed at 72 h. **B**, A549 and H2009 cells were treated with 100 nM ganetespib or 1 μM AZD6244 either alone or in combination for 24 h. The levels of C-RAF, p-MEK, p-ERK, cleaved PARP and GAPDH were determined by immunoblotting. **C**, A549, H2030, Calu-1, H2122 and H441 cells were treated with ganetespib or the PI3K/mTOR inhibitor BEZ235 at the indicated
concentrations either alone or in combination. Viability was assessed at 72 h. D, A549 and H2030 cells were treated with 100 nM ganetespib either alone or in combination with the BEZ235 (500 nM) or the mTOR inhibitor AZD8055 (500 nM) for 24 h. The levels of p-MEK, p-ERK, p-AKT p-4E-BP1, cleaved PARP and BIM proteins were determined by immunoblotting.

**Figure 5.** *In vivo* activity of ganetespib in combination with BEZ235 in a mutant KRAS NSCLC xenograft model. Mice bearing established A549 xenografts (n = 8 mice/group) were i.v. dosed with ganetespib (50 mg/kg) once weekly and BEZ235 (10 mg/kg) administered p.o. 5x/week, either alone or in combination, as indicated (arrowheads). % T/C values are indicated to the right of each growth curve and the error bars are the SEM. The combination of ganetespib and BEZ235 displayed significantly greater efficacy than either agent alone (*, p<0.05).

**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 h (96 h for pemetrexed and gemcitabine).
Figure 2

Author Manuscript Published OnlineFirst on September 25, 2012; DOI: 10.1158/1535-7163.MCT-12-0615
Figure 3

(A) Graphs showing the viability of A549, H2009, Calu-1, and H358 cells treated with Ganetespib, AZD6244, and BEZ235 at different concentrations.

(B) Western blots for A549, H2009, Calu-1, and H358 cells treated with Ganetespib, AZD6244, and BEZ235, showing the expression levels of B-RAF, C-RAF, p-MEK, MEK, p-ERK, p-4E-BP1, p-AKT (Ser473), AKT, cleaved PARP, and GAPDH.
Figure 4
Targeting KRAS Mutant Non-Small Cell Lung Cancer with the Hsp90 Inhibitor Ganetespib

Jaime Acquaviva, Donald L Smith, Jim Sang, et al.

*Mol Cancer Ther* Published OnlineFirst September 25, 2012.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-12-0615

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2012/10/15/1535-7163.MCT-12-0615.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.