Targeted Inhibition of the Molecular Chaperone Hsp90 Overcomes ALK Inhibitor Resistance in Non–Small Cell Lung Cancer

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ABSTRACT

EML4–ALK gene rearrangements define a unique subset of patients with non-small cell lung carcinoma (NSCLC), and the clinical success of the anaplastic lymphoma kinase (ALK) inhibitor crizotinib in this population has become a paradigm for molecularly targeted therapy. Here, we show that the Hsp90 inhibitor ganetespib induced loss of EML4–ALK expression and depletion of multiple oncogenic signaling proteins in ALK-driven NSCLC cells, leading to greater in vitro potency, superior antitumor efficacy, and prolonged animal survival compared with results obtained with crizotinib. In addition, combinatorial benefit was seen when ganetespib was used with other targeted ALK agents both in vitro and in vivo. Importantly, ganetespib overcame multiple forms of crizotinib resistance, including secondary ALK mutations, consistent with activity seen in a patient with crizotinib-resistant NSCLC. Cancer cells driven by ALK amplification and oncogenic rearrangements of ROS1 and RET kinase genes were also sensitive to ganetespib exposure. Taken together, these results highlight the therapeutic potential of ganetespib for ALK-driven NSCLC.

SIGNIFICANCE: In addition to direct kinase inhibition, pharmacologic blockade of the molecular chaperone Hsp90 is emerging as a promising approach for treating tumors driven by oncogenic rearrangements of ALK. The bioactivity profile of ganetespib presented here underscores a new therapeutic opportunity to target ALK and overcome multiple mechanisms of resistance in patients with ALK-positive NSCLC. Cancer Discov; 3(4); 430–43. © 2013 AACR.

INTRODUCTION

Non-small cell lung carcinoma (NSCLC) can be classified into distinct molecular subsets based on specific genomic alterations that drive tumorigenesis (1, 2). Approximately 3% to 7% of NSCLC tumors are characterized by rearrangement of the gene encoding anaplastic lymphoma kinase (ALK), most commonly with echinoderm microtubule-associated protein-like 4 (EML4; ref. 3), resulting in constitutively active kinase activity (3, 4). Crizotinib, a dual MET/ALK small-molecule tyrosine kinase inhibitor (TKI), was the first ALK-targeted agent evaluated clinically and was recently granted accelerated approval in the United States for the treatment of patients with ALK-positive (ALK+) NSCLC (5, 6). Among these patients, crizotinib therapy has been associated with improved survival over that in crizotinib-naive controls (7), thus providing clinical validation for targeting ALK in “oncogene-addicted” lung tumors with this genotype. Despite this success, however, durable responses to crizotinib therapy have been hampered by the development of drug resistance, a common feature of many TKI drugs (8). Relapses frequently occur owing to a spectrum of newly acquired secondary mutations within the ALK kinase domain (9, 10). Accordingly, considerable effort has been focused on the development of second-generation inhibitors designed to overcome this clinical challenge (11). Importantly, it has now emerged that other “ALK-independent” mechanisms, such as the activation of compensatory signaling pathways, may also confer resistance to targeted ALK agents (6). Strategies to counteract these types of acquired resistance in ALK-driven NSCLC have not yet been established.

Hsp90 is a molecular chaperone that plays a central role in regulating the correct folding, stability, and function of numerous “client proteins” (12). Inhibition of Hsp90 activity results in aggregation or proteasomal degradation of these clients, in turn promoting the simultaneous disruption of numerous oncogenic signaling pathways critical for tumor cell proliferation and survival (13, 14). Many of these proteins are kinases that have been shown to be oncogenic drivers in subsets of lung adenocarcinoma, including EGFR receptor (EGFR), BRAF, HER2, and, notably, the EML4–ALK fusion protein (15–20). Targeting the chaperone function of Hsp90, therefore, represents an alternative approach to direct kinase inhibition for therapeutic intervention in ALK-driven cancer. Furthermore, because of the coordinate impact on multiple signal cascades, pharmacologic blockade of Hsp90 may also overcome signaling redundancies and drug resistance mechanisms commonly seen in many cancers (21, 22).

Ganetespib is a novel triazolone inhibitor of Hsp90 with superior pharmacologic and biologic properties that distinguish it from other first- and second-generation Hsp90 inhibitors in terms of antitumor activity, potency, and safety (23). In human trials, ganetespib has shown promising efficacy in patients with advanced NSCLC, including robust...
single-agent activity in individuals with tumors harboring ALK gene rearrangement (24). Given the distinct mechanisms of action of ganetespib and crizotinib for inhibiting ALK, we have undertaken a comprehensive evaluation of the comparative sensitivity of ALK+ NSCLC to each of these treatment modalities. In combination with encouraging early clinical results in NSCLC, our data suggest that ganetespib may offer an alternative, and potentially complementary, strategy to targeted ALK inhibition for inducing substantial antitumor responses and overcoming acquired resistance in patients with ALK+ lung cancer.

RESULTS

Loss of Viability and Client Protein Expression by Ganetespib in ALK+
NSCLC Cells

The H2228 NSCLC cell line expresses an E6a/b/A20 EML4–ALK fusion protein and, as shown in Fig. 1A, was comparatively more sensitive to the cytotoxic effects of ganetespib than crizotinib (IC50 values of 13 vs. 202 nmol/L, respectively). Importantly, these levels are within free (unbound), circulating Cmax levels observed at maximal clinical dosing for both drugs (∼200 nmol/L for ganetespib, ∼500 nmol/L for crizotinib; ref. 25). Ganetespib (Supplementary Fig. S1) treatment resulted in a robust and dose-dependent destabilization of EML4–ALK, as well as EGFR and MET, all established Hsp90 clients (Fig. 1B). Importantly, targeted degradation of these signaling proteins was accompanied by inactivation of downstream effectors [phosphorylated and total AKT, phosphorylated STAT3 (p-STAT3), and phosphorylated extracellular signal–regulated kinase (p-ERK)] and induction of BIM, an apoptotic marker. A concomitant increase in Hsp70 levels was observed, indicative of Hsp90 inhibition (Fig. 1B).

Next, we examined the comparative effects of ganetespib and crizotinib in the ALK-driven H3122 cell line, which is dependent on the E13/A20 EML4–ALK variant for growth and survival. Ganetespib was acutely cytotoxic to these cells and with 30 times greater potency than crizotinib (10 vs. 300 nmol/L; Fig. 1C). When expression changes in client proteins and signaling pathways were examined in this line (Fig. 1D), we found that ganetespib exposure at concentrations of 30 nmol/L or more resulted in the complete loss of phosphorylated EML4–ALK protein expression, as well as the active (phosphorylated) forms of STAT3, AKT, and ERK. Targeted degradation of EGFR and MET were seen at the same concentrations, as well as negative effects on the mTOR signaling pathway, evidenced by loss of p-p70S6K and p-4E-BP1 expression (Fig. 1D). Consistent with the potent cytotoxic activity of ganetespib, a robust increase in BIM and cleaved PARP expression were observed. In contrast, crizotinib displayed far weaker activity in terms of effector signaling and activation of apoptotic pathways. At least a 10-fold higher concentration of crizotinib (300 nmol/L) was required to significantly reduce phosphorylated EML4–ALK levels, and this was not complete until 500 nmol/L (Fig. 1D). Crizotinib at these same maximal concentrations achieved only relatively modest effects, compared with ganetespib, on the blockade of downstream ERK, AKT, and mTOR signaling, as well as apoptotic induction. Taken together, these data show that ganetespib displays greater in vitro potency than crizotinib in ALK+ NSCLC cells.

Ganetespib Suppresses Tumor Growth and Extends Survival in ALK+ NSCLC Xenografts

Ganetespib and crizotinib were highly efficacious in nude mouse models of ALK+ NSCLC, each inducing similar degrees of tumor regression (Supplementary Fig. S2). Crizotinib administered at its maximum tolerated dose (MTD) of 200 mg/kg 5 times per week orally over a 3-week cycle to mice bearing H3122 xenografts resulted in 24% tumor regression. Ganetespib treatment at its MTD of 150 mg/kg weekly resulted in a similar degree (27%) of tumor shrinkage. To more robustly evaluate potential differences in antitumor activity in vivo, we subsequently carried out experiments in tumor-bearing severe combined immunodeficient (SCID) mice. Animals bearing H3122 xenografts were dosed intravenously with ganetespib at 50 mg/kg once a week (Fig. 2A). This regimen resulted in significant tumor growth inhibition [treatment vs. control (T/C) value of 21%] over a 3-week period. Treating at MTD (data not shown) or splitting the dose into 2-consecutive-day dosing of 25 mg/kg each week resulted in a minor improvement in efficacy (T/C value of 10%) and disease stabilization (Fig. 2A). Importantly, all treatment regimens were well tolerated, with no toxicity or changes in body weight seen after 3 weeks of dosing (data not shown). In contrast, crizotinib was less efficacious in the same model. As shown in Fig. 2B, oral dosing of crizotinib 5 times per week at 50 mg/kg resulted in a T/C value of only 55%. When the crizotinib dose was doubled to 100 mg/kg, no substantial improvement in efficacy was seen; however, significant losses of body weight were more frequently observed. Thus, this dose was determined to be the MTD (data not shown). Representative images of H3122 xenograft tumors 50 days after treatment with ganetespib and crizotinib are shown in Fig. 2C.

To determine whether these tumor responses correlated with target modulation in vivo, we conducted pharmacodynamic analysis in additional mice bearing H3122 xenografts (Fig. 2D). Animals were treated with a single bolus injection of ganetespib at the effective 50 mg/kg dose and tumors harvested at 24, 48, 72, and 96 hours after treatment. For comparison, animals were treated with a single injection of vehicle or crizotinib at 50 mg/kg and tumors collected 24 hours later. EML4-ALK and downstream ERK signaling were degraded and deactivated, respectively, within 24 hours following ganetespib treatment. Importantly, these effects were sustained over time, as recovery did not occur until 72 hours after treatment. Similar kinetics were observed for the targeted destabilization of the Hsp90 clients EGFR and MET, as well as their effector signaling intermediates p-STAT3 and p-AKT. Loss of these signaling cascades was associated with a corresponding increase in BIM protein expression, indicative of intratumoral apoptotic induction. In stark contrast to ganetespib, single-dose crizotinib had negligible effects on ERK activity at 24 hours, nor effects on any of the other cascades. Overall, these data show that single-dose ganetespib exerts durable...
suppressive effects on ALK signaling in human tumor xenografts, destabilizing both the fusion kinase and its effectors for up to 72 hours.

Having shown that ganetespib displayed greater antitumor activity than crizotinib in SCID mice bearing ALK+ NSCLC xenografts, we next extended the study time to measure overall survival, with an endpoint defined as animal death, cavitating tumors, or tumors larger than 1.5 cm. Animals were dosed with once-weekly i.v. ganetespib (50 mg/kg), crizotinib 50 mg/kg 5 times per week orally, or vehicle alone (Fig. 2E). Fifty percent of animals treated with vehicle died by day 40, whereas crizotinib extended survival of 50% of the animals.
to 54 days. After 75 days, all animals within the ganetespib group had survived.

**Distinct Actions of Ganetespib and Crizotinib Lead to Favorable Combinatorial Activity**

Given the distinct mechanisms of action of ganetespib and crizotinib on ALK inhibition, we examined whether combining the 2 compounds would lead to increased activity. Indeed, as shown in Fig. 3A, concurrent administration of low (IC<sub>50</sub>) doses of ganetespib and crizotinib to H3122 cells substantially increased cell death in vitro. Importantly, similar combinatorial benefit was observed when ganetespib was dosed in combination with the structurally unrelated ALK inhibitors ASP3026 and CH542802.

To evaluate whether the effects on cell viability seen in vitro translated to improved combinatorial efficacy in vivo, xenograft-bearing mice were treated with ganetespib and crizotinib, both as single agents and in combination. As shown in Fig. 3B, once-weekly administration of ganetespib at 25 mg/kg was comparable with dosing of crizotinib 5 times per week at its MTD, with each compound inducing a similar degree of tumor suppression (T/C values of 41% and 39%, respectively). Consistent with the in vitro findings, concurrent treatment with both drugs resulted in a significant enhancement of antitumor activity, inhibiting tumor growth by 93%. In addition, combination treatment was well tolerated, with no significant changes in body weights seen after 3 weeks of treatment (Supplementary Fig. S3). In fact, combination treatment seemed better tolerated than crizotinib monotherapy, strongly suggesting that no further toxicity is conferred by the addition of ganetespib to the regimen. Thus, ganetespib and crizotinib, when combined, displayed superior antitumor efficacy compared with monotherapy in H3122 NSCLC xenografts.

**Ganetespib Overcomes Acquired Crizotinib Resistance**

As has been the clinical experience with other TKIs, prolonged exposure to crizotinib may ultimately give rise to acquired resistance, thereby diminishing the efficacy of long-term treatment. An important consideration, therefore, was whether crizotinib-resistant NSCLC cells remained sensitive to ganetespib. To determine this experimentally, we generated crizotinib-resistant H3122 cells (H3122 CR1) by continuous selective culture in 1 μmol/L crizotinib. Endogenous expression of EML4–ALK was reduced in the resistant line compared with parental H3122 cells, yet the fusion protein remained sensitive to ganetespib-induced destabilization (Fig. 4A).

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**Figure 2.** Ganetespib suppresses tumor growth and extends survival in ALK<sup>+</sup> NSCLC xenografts. A, SCID mice bearing H3122 xenografts (n = 7 per group) were i.v. dosed with 50 mg/kg ganetespib once weekly, or on a weekly regimen of 2-consecutive-day dosing at 25 mg/kg, as indicated, for 3 weeks. %T/C values are indicated to the right of each growth curve, and the error bars represent the SEM. B, SCID mice bearing H3122 xenografts (n = 7 per group) were orally (p.o.) dosed with 50 mg/kg crizotinib 5×/wk over a 3-week cycle. C, representative images of tumors from vehicle-, ganetespib (2×/wk), or crizotinib (50 mg/kg)-treated animals at day 50. D, pharmacodynamic analysis of client protein modulation in H3122 xenografts. SCID mice bearing established H3122 tumors were treated with vehicle or a single bolus injection of ganetespib at 50 mg/kg at the indicated time points between 24 and 96 hours. Mice were also treated with a single bolus injection of crizotinib (50 mg/kg) for 24 hours. Tumors were resected and the levels of the indicated proteins determined by immunoblotting. E, Kaplan-Meier analysis of overall survival in the H3122 xenograft model. Beginning 12 days after tumor cell implantation, SCID mice bearing H3122 xenografts (n = 7 per group) were dosed with vehicle, 50 mg/kg ganetespib 1×/wk i.v., or 50 mg/kg crizotinib 5×/wk orally, and animal survival was monitored until day 75.
Figure 3. Enhanced activity of ganetespib in combination with ALK inhibitors in vitro and in vivo. A, H3122 cells were treated with the indicated concentrations of ganetespib, crizotinib, ASP3026, and CH5424802 either as single agents or in combination. Cell viability was determined at 72 hours. B, the combination of ganetespib and crizotinib induces enhanced antitumor efficacy in vivo. SCID mice bearing H3122 xenografts (n = 7 per group) were i.v. dosed with 25 mg/kg ganetespib once weekly, 100 mg/kg crizotinib 5×/wk orally, or the combination, as indicated. %T/C values are indicated to the right of each growth curve, and the error bars represent the SEM.

We next compared the activities of ganetespib and crizotinib, using the H3122 and H3122 CR1 lines (Fig. 4B). As expected, crizotinib treatment resulted in dose-dependent cytotoxicity in the parental line but had no effect on H3122 CR1 cells. In contrast, despite a small shift in IC₅₀ values, ganetespib retained full potency in both cell lines, irrespective of crizotinib resistance status. Indeed, H3122 CR1 cells remained several-fold more sensitive to ganetespib compared with the sensitivity of the parental line to crizotinib. Moreover, H3122 CR1 cells were insensitive to other ALK inhibitors (CH5424802, ASP3026, and TAE684), yet succumbed to Hsp90 inhibition by ganetespib (Fig. 4C).

Interestingly, the H3122 CR1 line exhibited a more fibroblastic morphology than the parental, typical of enhanced epithelial plasticity (Fig. 4D). We therefore conducted a reverse phase array comparing the expression of proteins between the H3122 and H3122 CR1 cells (Supplementary Fig. S4A). Epithelial markers such as E-cadherin and P-cadherin were downregulated, as well as other receptor tyrosine kinases, including insulin-like growth factor-I receptor (IGF-IR) and VEGF receptor 2 (VEGFR2). Concomitant upregulation of Snail, Notch 1, Caveolin, and Src was also seen. These changes, consistent with an epithelial–mesenchymal transition (EMT), were confirmed by Western blot analysis...
Ganetespib retains potency against crizotinib-resistant NSCLC tumor phenotypes. A, parental H3122 and crizotinib-resistant H3122 CR1 cells were treated with ganetespib at either 25 or 100 nmol/L for 24 hours and the levels of EML4–ALK protein determined by immunoblotting. B, H3122 and H3122 CR1 cells were treated with increasing concentrations of ganetespib or crizotinib, and cell viability was assessed after 72 hours. C, table of IC$_{50}$ cytotoxicity values in H3122 and H3122 CR1 cells in response to ganetespib or ALK inhibitor exposure. D, light micrographs of cellular morphology of H3122 and H3122 CR1 cells. Scale bar, 50 μm.

H3122 v25 100 25 100 Ganetespib
H3122 (crizotinib-sensitive) 10 nmol/L 40 nmol/L
H3122 CR1 (crizotinib-resistant) >3,000 nmol/L >3,000 nmol/L

Ganetespib Retains Activity against NPM-ALK-Transformed Cells Bearing Secondary ALK Mutations That Confer Crizotinib Resistance

One common mechanism leading to acquired resistance to ALK TKIs is the emergence of secondary point mutations within the kinase domain (9). To determine the potential impact of such mutational changes on ganetespib activity, we carried out experiments in BaF3 cells oncogenically transformed by engineered expression of the lymphoma-associated NPM-ALK fusion kinase. NPM-ALK–expressing BaF3 cells were exposed in culture to a variety of concentrations of crizotinib until the emergence of viable cell pools, which were then subjected to limiting dilution to isolate crizotinib-resistant clones. As shown in Fig. 5A, a spectrum of point mutations located in the ALK kinase domain and involving 15 different substitutions were associated with crizotinib resistance. The crizotinib-sensitive parental NPM-ALK/BaF3 cells used in these experiments showed a crizotinib IC$_{50}$ value of approximately 370 nmol/L. In contrast, the clones harboring the various ALK mutations exhibited varying degrees of resistance, with relative IC$_{50}$ values ranging from approximately 1.6-fold (E1408K, E1132K) to 4- to 5-fold (G1202R, L1196M) higher (Fig. 5A). We then examined whether crizotinib-resistant NPM-ALK/BaF3 cells showed sensitivity to Hsp90 inhibition. Crizotinib-sensitive NPM-ALK/BaF3 cells were also sensitive to ganetespib (IC$_{50}$ value of 21 nmol/L). Importantly, all of the crizotinib-resistant NPM-ALK/BaF3 clones retained high sensitivity to ganetespib; indeed, the IC$_{50}$ values were essentially identical to that of NPM-ALK/BaF3. Consistent with these observations, NPM-ALK protein degradation following ganetespib treatment showed similar dose-dependent responses regardless of the presence or identity of the crizotinib resistance mutation (Fig. 5B). The kinetics of protein degradation for NPM-ALK/BaF3 and NPM-ALK/BaF3 containing the L1196M gatekeeper mutation following exposure to ganetespib (250 nmol/L) is shown in Fig. 5C.

Clinical Activity of Ganetespib in Crizotinib-Resistant NSCLC

In a recent phase II trial of ganetespib monotherapy in patients with advanced NSCLC (24), 8 patients (8%) were identified as harboring ALK gene rearrangements. All were crizotinib naive. Of these patients, 4 achieved an objective
Ganetespib Activity in ALK-Driven NSCLC

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Figure 5. Crizotinib-resistant NPM-ALK mutants retain sensitivity to ganetespib. A, ganetespib and crizotinib sensitivity was assessed in crizotinib-sensitive NPM-ALK BaF3 cells and crizotinib-resistant NMP-ALK mutants. Relative IC\textsubscript{50} values are plotted on the basis of the sensitivity of the parental NPM-ALK BaF3 line to each compound. Clinically relevant mutations in NSCLC are indicated by asterisks. B, dose–response analysis of ganetespib. Crizotinib-sensitive NPM-ALK/BaF3 cells (top left) together with NPM-ALK/BaF3 containing the indicated amino acid substitutions known to confer crizotinib resistance were incubated for 24 hours, with the range of ganetespib concentrations indicated, and the stability of the NPM-ALK protein expressed in each line was assessed by immunoblotting. C, kinetics of ganetespib-associated NPM-ALK protein degradation. The NPM-ALK protein degradation response of crizotinib-sensitive NPM-ALK/BaF3 and NPM-ALK/BaF3 containing the L1196M gatekeeper mutation to incubation in 250 nmol/L ganetespib for the indicated times is shown.

Figure 6. Response of ALK-rearranged, crizotinib-resistant NSCLC after one cycle of ganetespib. CT scans taken at (A) baseline and (B) after one cycle (200 mg/m\textsuperscript{2}, 1×/wk for 3 weeks) of ganetespib. Arrows indicate locations of tumor masses.

partial response (PR), 3 showed stable disease, and 1 experienced disease progression at 16 weeks. The median progression-free survival observed for the 4 patients with PR was 8.1 months, significantly better than for patients without ALK rearrangement. The 50% objective response rate combined with the overall 88% disease control rate within this subset, therefore, provides clinical validation for the therapeutic potential of ganetespib in ALK\textsuperscript{+} NSCLC. Further compelling evidence is provided by the computed tomography (CT) scans shown in Fig. 6. The images were obtained from a 24-year-old male with ALK\textsuperscript{+} NSCLC, who had progressed while on crizotinib after 12 months of therapy. Sequencing conducted upon rebiopsy confirmed the presence of a secondary G1269A mutation within the ALK domain sequence. Lung lesions were clearly apparent at baseline (Fig. 6A), and imaging revealed marked tumor shrinkage following one cycle on March 26, 2013. © 2013 American Association for Cancer Research. cancerdiscovery.aacrjournals.org Downloaded from Published OnlineFirst March 26, 2013; DOI: 10.1158/2159-8290.CD-12-0440
Ganetespib Is Active in Tumor Cells Driven by ALK Amplification and Additional Oncogenic Kinase Fusions

ALK gene amplification represents another event that can contribute to crizotinib resistance in NSCLC and, importantly, can coexist with ALK mutation (8). To examine targeted inhibitory effects in ALK-amplified tumor cells, we treated the NB-39-nu neuroblastoma cell line (which expresses 30–40 copies of the ALK gene per cell) with graded concentrations of ganetespib or crizotinib and found that these cells were acutely sensitive to ganetespib exposure (IC$_{50}$, 10 nmol/L) and comparatively less so to crizotinib (IC$_{50}$, 240 nmol/L; Fig. 7A). Ganetespib treatment resulted in a dose-dependent degradation of oncogenic ALK, as well as loss of downstream effector signaling (activated STAT3, AKT, and ERK) and concomitant induction of the apoptotic markers BIM and cleaved PARP (Fig. 7B). Ganetespib also reduced viability in NB69 neuroblastoma cells, which are wild type for ALK (no amplification, no mutation), with an IC$_{50}$ value of 21 nmol/L (data not shown). Thus, although ALK amplification itself was not the primary determinant of ganetespib sensitivity in neuroblastoma cells, the data clearly show that targeted Hsp90 inhibition results in potent destabilization of amplified ALK in cells dependent on this driver.

Chromosomal rearrangements involving the ROS1 receptor tyrosine kinase have recently been described in a subset of NSCLCs (26). Here, we examined the effect of ganetespib treatment on HCC78 NSCLC cells and U118-MG glioma cells that express ALK amplification and ALK fusion proteins, respectively (Fig. 7C). Both cell lines were highly sensitive to treatment on HCC78 NSCLC cells and U118-MG glioma cells, the data clearly show that targeted Hsp90 inhibition represents the predominant mechanism of acquired resistance to crizotinib in NSCLC.

Oncogenic gene rearrangements of ALK define a clinically relevant subset of human NSCLCs, and the success of crizotinib serves as a paradigm for molecularly targeted therapy in this malignancy. However, as is the case for many small-molecule TKIs, crizotinib produces responses that do not last, which highlights the ongoing challenge of discovering superior treatment options, particularly those that can overcome the invariable development of acquired resistance. EML4–ALK is a highly sensitive client protein of the molecular chaperone Hsp90 (19, 20), and preclinical evidence suggests that disrupting the chaperone function of this molecule can effectively overcome oncogenic ALK activity, including that in lines harboring ALK inhibitor–resistant mutations (8, 29). In addition to direct kinase inhibition, we and others have recently shown that patients with crizotinib-naïve ALK+ lung cancer can derive therapeutic benefit from targeted degradation of ALK via Hsp90 blockade, thereby confirming preclinical predictions (24, 30).

A significant finding of this study was the capacity of ganetespib to overcome crizotinib resistance in ALK+ cancer, as was shown in multiple experimental models and also in the clinical setting. Unlike the case of EGFR, in which a secondary point mutation at the gatekeeper residue (T790M) represents the predominant mechanism of acquired resistance to erlotinib and gefitinib in NSCLC tumors (31, 32), crizotinib resistance arising from ALK mutation seems more analogous to the imatinib resistance seen in patients with chronic myeloid leukemia. In that case, multiple mutations within the kinase domain of BCR-ABL have been reported that are associated with the development of drug resistance, and mutational frequencies may even increase with disease progression (33). To date, a variety of ALK kinase domain mutations at different amino acid sites have been reported in patients with NSCLC who exhibited crizotinib resistance to erlotinib, G1152R, C1156Y, F1174L, L1196M (the ALK gatekeeper), G1202R, D1203N, and G1269A and a number of others that can mediate ALK TKI resistance have been identified through in vitro mutagenesis screens (reviewed in refs. 6, 11, 34). Importantly, our studies using NPM-ALK-expressing BAF3 cells that were rendered crizotinib resistant owing to these mutations revealed that ganetespib possessed robust cytotoxic activity irrespective of the mutational site or specific amino acid substitution present.

DISCUSSION

Oncogenic gene rearrangements of ALK define a clinically relevant subset of human NSCLCs, and the success of crizotinib serves as a paradigm for molecularly targeted therapy in this malignancy. However, as is the case for many small-molecule TKIs, crizotinib produces responses that do not last, which highlights the ongoing challenge of discovering superior treatment options, particularly those that can overcome the invariable development of acquired resistance. EML4–ALK is a highly sensitive client protein of the molecular chaperone Hsp90 (19, 20), and preclinical evidence suggests that disrupting the chaperone function of this molecule can effectively overcome oncogenic ALK activity, including that in lines harboring ALK inhibitor–resistant mutations (8, 29). In addition to direct kinase inhibition, we and others have recently shown that patients with crizotinib-naïve ALK+ lung cancer can derive therapeutic benefit from targeted degradation of ALK via Hsp90 blockade, thereby confirming preclinical predictions (24, 30). Here, we have provided a compelling rationale for the use of ganetespib as an alternative and potentially complementary strategy for patients with ALK-driven NSCLC.

A significant finding of this study was the capacity of ganetespib to overcome crizotinib resistance in ALK+ cancer, as was shown in multiple experimental models and also in the clinical setting. Unlike the case of EGFR, in which a secondary point mutation at the gatekeeper residue (T790M) represents the predominant mechanism of acquired resistance to erlotinib and gefitinib in NSCLC tumors (31, 32), crizotinib resistance arising from ALK mutation seems more analogous to the imatinib resistance seen in patients with chronic myeloid leukemia. In that case, multiple mutations within the kinase domain of BCR-ABL have been reported that are associated with the development of drug resistance, and mutational frequencies may even increase with disease progression (33). To date, a variety of ALK kinase domain mutations at different amino acid sites have been reported in patients with NSCLC who exhibited crizotinib resistance to erlotinib, G1152R, C1156Y, F1174L, L1196M (the ALK gatekeeper), G1202R, D1203N, and G1269A and a number of others that can mediate ALK TKI resistance have been identified through in vitro mutagenesis screens (reviewed in refs. 6, 11, 34). Importantly, our studies using NPM-ALK-expressing BAF3 cells that were rendered crizotinib resistant owing to these mutations revealed that ganetespib possessed robust cytotoxic activity irrespective of the mutational site or specific amino acid substitution present. This result was strikingly validated by the clinical observations seen in the patient with relapsed NSCLC after 1 year of crizotinib therapy. Despite the presence of a G1269A kinase domain mutation, a single cycle of ganetespib treatment resulted in a marked tumor response and discernible shrinkage of lung lesions, highlighting the therapeutic potential of the drug within this refractory population.

Furthermore, systemic resistance to ALK inhibitors can arise in the absence of secondary ALK kinase domain mutations (10). Although the mechanisms remain to be fully elucidated, it seems that ligand-mediated activation of secondary and/or separate oncogenic signaling pathways—in particular,
Figure 7. Ganetespib displays potent cellular activity in models of ALK amplification, ROS1 kinase fusion, and RET kinase fusion. A, NB-39-nu neuroblastoma cells were treated with increasing concentrations of ganetespib or crizotinib, and cell viability was assessed at 72 hours. B, NB-39-nu cells were exposed to graded concentrations of ganetespib (1–500 nmol/L) for 24 hours and cell lysates immunoblotted with the indicated antibodies. v, vehicle control. C, HCC78 NSCLC and U118-MG glioma cells were treated with increasing concentrations of ganetespib, and cell viability was assessed at 72 hours. D, HCC78 cells were treated with ganetespib at 5, 10, 50, or 100 nmol/L for 24 hours and the levels of phosphorylated and total SLC34A2-ROS1 fusion protein determined by immunoblotting. E, TPC1 thyroid carcinoma cells were treated with increasing concentrations of ganetespib, and cell viability was assessed at 72 hours. F, TPC1 cells were treated with increasing concentrations of ganetespib, and cell viability was assessed at 72 hours.
EGFR and HER2 (35–37)—is one process that may bypass the dependency of tumor cells on ALK signaling and contribute to a resistant phenotype. Moreover, the use of ALK-selective inhibitors with increased potency is unlikely to provide any clinical impact on these forms of resistance. In this regard, the broader spectrum of biologic activity afforded by Hsp90 inhibition represents a promising strategy to counteract such compensatory mechanisms. These driver kinases are established Hsp90 clients, and our data show that ganetespib exposure results in the simultaneous destabilization of EML4–ALK as well as receptor kinases, such as EGFR and MET in ALK+ NSCLC lines both in vitro and in vivo, with concomitant loss of multiple downstream effector signaling pathways. These effects were distinct from those of crizotinib, and this multimodal activity of ganetespib accounts for its superior potency and antitumor efficacy. Although the underlying basis of resistance in the H3122 CR1 line was not identified, sequencing analysis of 12 clones failed to identify any acquired ALK mutations (D. Proia; unpublished data), and amplification of the ALK gene was not present. It is interesting that these cells manifested a morphology and molecular profile consistent with having undergone EMT, and this transition has been associated with activation of the EGFR pathway (38, 39). The mechanistic nature of the resistance exhibited by these cells, one that retains sensitivity to ganetespib, is currently under investigation.

Combination treatment of crizotinib with other antineoplastic agents represents a potential approach for inducing durable remissions in patients with ALK+ NSCLC, and a number of clinical trials are currently ongoing (40), including a phase I study of crizotinib with the dual EGFR/HER inhibitor PF299804 (NCT01121575). This consideration therefore prompted an investigation of combining the 2 modalities of Hsp90 inhibition and selective ALK targeting. In vitro, combinatorial benefit was seen when ganetespib was used as a cotreatment with crizotinib as well as the structurally unrelated inhibitors ASP3026 and CH542802, both of which have been reported to display tropic effects of Hsp90 inhibition on both ALK itself and other client proteins provide more complete and durable responses compared with direct kinase inhibition. In light of its select advantages over ALK-specific TKIs and maturing clinical profile, these findings are likely to provide a framework for the optimal design of ganetespib-based therapies in the future management of advanced NSCLC.

**METHODS**

**Cell Lines, Antibodies, and Reagents**

The H2228 and U118-MG cell lines were obtained from the American Type Culture Collection, and the HCC78 and BaF3 cells were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ). All were maintained according to the suppliers’ instructions, authenticated by the routine company DNA typing, and used within 6 months of receipt for this study. H3122 cells were a kind gift from Dr. John Minna (University of Texas Southwestern Medical Center, Dallas, TX). The NB-39-nu line was obtained from the National Cancer Center Research Institute (Tokyo, Japan), and TPC1 cells were a kind gift from Drs. Hiroshi Sato (Kanazawa University Cancer Research Institute, Kanazawa, Japan) and Rebecca Schewpe (University of Colorado, Aurora, CO). No authentication of the lines obtained as gifts was carried out by the authors. All primary antibodies were purchased from Cell Signaling Technology, with the exception of Hsp70 (Enzo Life Sciences), claudin (Invitrogen), and GAPDH and cyclin E antibodies (Santa Cruz Biotechnology Inc.). Ganetespib [1H-1,2,4-triazol-5(4H)-one] was synthesized by Synta Pharmaceuticals Corp. Crizotinib, ASP3026, CH5424802, and TAE684 were all purchased from Active Biochem.

**Cell Viability Assays**

Cellular viability was assessed using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) according to the manufacturer’s protocol. Tumor 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 or crizotinib for 72 hours. CellTiter-Glo was added (50% volume for volume) to the cells, and the plates were incubated for 10 minutes before luminescent detection in a SpectraMax Plus384 microplate reader ( Molecular Devices). Data were normalized to percentage of control, and IC50 values were determined using XLFit software.

**Western Blotting**

Following in vitro assays, tumor cells were disrupted in lysis buffer (Cell Signaling Technology) on ice for 10 minutes. For the pharmacodynamic analysis, xenograft tumors (average volume of 100–200 mm3) were excised, cut in half, and flash frozen in liquid nitrogen. Each tumor fragment was lysed in 0.5 mL of lysis buffer, using a FastPrep-24 homogenizer and Lysing Matrix A (MP Biomedicals). Lysates were clarified by centrifugation and equal amounts of proteins resolved by SDS-PAGE before transfer to nitrocellulose membranes (Invitrogen). Membranes were blocked with Starting Block T20 Blocking Buffer (Thermo Scientific) and immunoblated
with the indicated antibodies. Antibody–antigen complexes were visualized using an Odyssey system (LI-COR).

**Combinatorial Drug Effect Analysis**

For combinatorial analysis, H3122 cells (7.5 × 10⁴) were seeded in 96-well plates and incubated at 37°C, 5% CO₂ for 24 hours. Drug combinations were applied at a nonconstant ratio, using 3 1:5-fold serial dilutions above and below the IC₅₀ values for each compound. Cell viability was assessed 72 hours after drug addition by Cell Titer-Glo 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.

**Xenograft Tumor Models**

Female CD-1 nude and CB.17 (SCID) mice (Charles River Laboratories) at 7 to 12 weeks of age were maintained in a pathogen-free environment, and all in vivo procedures were approved by the Synta Pharmaceuticals Corporation Institutional Animal Care and Use Committee. H3122 NSCLC cells (7.5 × 10⁴) were subcutaneously implanted into the animals. Mice bearing established tumors (∼200 mm³) were randomized into treatment groups of 7 and dosed with vehicle, ganetespib (intra-venous), or crizotinib (orally), the latter 2 agents formulated in DRD [10% dimethyl sulfoxide (DMSO), 18% Cremophor RH 40, 3.6% dextrose], using the schedules indicated. Tumor volumes (V) were calculated by caliper measurements of the width (W), length (L), and thickness (T) of each tumor, using the following formula: V = 0.5236(L)(W)². Tumor growth inhibition was determined as described previously (46). Statistical analyses were conducted using a Kruskal–Wallis one-way ANOVA on ranks, followed by the Tukey test. For the survival analysis, H3122-implanted SCID mice were randomized into groups of 7 after 12 days, and then dosed with vehicle, 50 mg/kg ganetespib once per week, or 50 mg/kg crizotinib 5 times per week. Animal survival was assessed for 75 days, with the endpoints defined as animal death, cavitating tumors, or tumors larger than 1.5 cm. Overall survival is presented using the Kaplan–Meier method.

**Selection of NPM-ALK/BAF3 Cells for Crizotinib Resistance and Characterization of Ganetespib Responsiveness of Crizotinib-Resistant NPM-ALK/BAF3**

Baf3 cells engineered to stably express NPM-ALK were used to select for crizotinib resistance, and screening for inhibitor-resistant colonies was conducted as previously described (47). No chemical mutagenesis (e.g., with ethylnitrosourea) was used to accelerate the emergence of crizotinib-resistant clones. Clonally derived, crizotinib-resistant NPM-ALK/BaF3 lines were isolated by limiting dilution, and sequence analysis of the ALK kinase domain was conducted to identify the resistance mutations. Cytotoxic IC₅₀ determinations were conducted using an XTT Cell Viability Assay Kit (Cell Signaling Technology) after a 72-hour incubation of crizotinib with NPM-ALK/BaF3 clones bearing each of the identified inhibitor-resistant mutations. Furthermore, each putative mutation was confirmed to confer crizotinib resistance by engineering into NPM-ALK cDNA, generating clonal NPM-ALK/Baf3 cell lines to express the mutation, and determining IC₅₀ cytotoxicity values for crizotinib in the clonal lines. Ganetespib cytotoxicity IC₅₀ values for crizotinib-resistant NPM-ALK/BaF3 cells were determined following incubation with graded concentrations of the compound for 72 hours. Immunoblotting to assess the degradation of NPM-ALK in response to ganetespib exposure was conducted using an ALK 11 rabbit polyclonal antibody (48) at a dilution of 1:2,000.

**Ganetespib Trial**

This study includes preliminary data of the clinical response in one 24-year-old male patient enrolled in a phase II multicenter trial of ganetespib monotherapy in advanced NSCLC (NCT01031225). The patient harbored an EML4 exon 6-ALK translocation and had failed previous crizotinib therapy; subsequent direct ALK exon sequencing identified the presence of a secondary G1269A mutation. The trial was conducted in accordance with the Declaration of Helsinki and was approved by the ethics committee at each participating institution; patients were required to provide written informed consent before enrollment.

**Disclosure of Potential Conflicts of Interest**

J. Sang and J. Acquaviva are employed as Scientist II by Synta Pharmaceuticals Corp. C. Zhang is employed as Research Associate II by Synta Pharmaceuticals Corp. C.M. Lovly is a consultant/advisory board member of Abbott. J.-P. Jimenez is employed as Assoc. Scientist by, and has ownership interest (including patents) in, Synta Pharmaceuticals Corp. A.T. Shaw is a consultant/advisory board member of Daiichi Sankyo and Novartis. R.C. Doebele has commercial research grants from Pfizer, Eli Lilly, and ImClone; has honoraria from Speakers Bureau of Abbott Molecular; and is a consultant/ advisory board member of Boehringer Ingelheim and Pfizer. S. He is employed as a Scientist by Synta Pharmaceuticals Corp. R.C. Bates is employed as Senior Science Writer by Synta Pharmaceuticals Corp. S.W. Morris has ownership interest (including patents) in Insight Genetics, Inc. I. El-Hariry is Vice President of Clinical Research and D.A. Proia is Director of Cancer Biology at Synta Pharmaceuticals Corp. No potential conflicts of interest were disclosed by the other authors.

**Authors’ Contributions**


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Targeted Inhibition of the Molecular Chaperone Hsp90 Overcomes ALK Inhibitor Resistance in Non–Small Cell Lung Cancer

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