Ganetespib (STA-9090), a Non-Geldanamycin HSP90 Inhibitor, has Potent Antitumor Activity in In Vitro and In Vivo Models of Non-Small Cell Lung Cancer

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Several subsets of NSCLC are addicted to oncogenic kinases that are dependent on the HSP90 chaperone for conformational stability, stimulating evaluation of HSP90 inhibitors in this disease. The index geldanamycin compounds have biochemical and toxicity limitations that have prompted the development of non-geldanamycin compounds, including ganetespib (STA-9090), a resorcinol-containing triazolone. In *in vitro* assays and genotypically-defined NSCLC cell lines and xenografts, ganetespib is more potent and efficacious than 17-allylamino-17-demethoxygeldanamycin (17-AAG). Although favorable intratumoral pharmacokinetic parameters suggest that once-weekly dosing may be adequate, several clients, including mutant EGFR, are suppressed only transiently following ganetespib exposure, so that superior anti-tumor activity was observed with more frequent consecutive-day dosing. Ganetespib was active in a mutant ERBB2 YVMA-driven mouse lung adenocarcinoma model with expected pharmacodynamic effects. These results justify the continued development of ganetespib in NSCLC, including EGFR and ERBB2-mutant tumors, with assessment of schedules employing greater than once-weekly drug administration.
ABSTRACT

Purpose: We describe the anticancer activity of ganetespib, a novel non-geldanamycin heat shock protein 90 (HSP90) inhibitor, in non-small cell lung cancer (NSCLC) models.

Experimental Design: The activity of ganetespib was compared to that of the geldnamaycin 17-AAG in biochemical assays, cell lines and xenografts, and evaluated in an ERBB2 YVMA-driven mouse lung adenocarcinoma model.

Results: Ganetespib blocked the ability of HSP90 to bind to biotinylated geldanamycin and disrupted the association of HSP90 with its co-chaperone, p23, more potently than 17-AAG. In genomically-defined NSCLC cell lines, ganetespib caused depletion of receptor tyrosine kinases, extinguishing of downstream signaling, inhibition of proliferation and induction of apoptosis with IC50 values ranging 2–30 nM, substantially lower than those required for 17-AAG (20–3,500 nM). Ganetespib was also approximately 20-fold more potent in isogenic Ba/F3 pro-B cells rendered IL-3 independent by expression of EGFR and ERBB2 mutants. In mice bearing NCI-H1975 (EGFR L858R/T790M) xenografts, ganetespib was rapidly eliminated from plasma and normal tissues but was maintained in tumor with t1/2 58.3 hours, supporting once-weekly dosing experiments, in which ganetespib produced greater tumor growth inhibition than 17-AAG. However, after a single dose, re-expression of mutant EGFR occurred by 72 hours, correlating with reversal of anti-proliferative and pro-apoptotic effects. Consecutive day dosing resulted in xenograft regressions, accompanied by more sustained pharmacodynamic effects. Ganetespib also demonstrated activity against mouse lung adenocarcinomas driven by oncogenic ERBB2 YVMA.
Conclusions: Ganetespib has greater potency than 17-AAG and potential efficacy against several NSCLC subsets, including those harboring EGFR or ERBB2 mutation.
INTRODUCTION

Heat shock protein 90 (HSP90) is an ATPase-dependent molecular chaperone ubiquitously expressed in eukaryotic cells (1). HSP90 is essential for the post-translational conformational maturation and stability of client proteins, including protein kinases, steroid receptors and transcription factors, many of which are important for the proliferation and survival of cancer cells (2, 3). In contrast to normal tissues, in which HSP90 is found in a latent, uncomplexed state, tumor cells contain an abundance of catalytically active HSP90 found in multichaperone complexes, considered important for their survival in a hypoxic, nutrient-deprived and acidic microenvironment, and for the maintenance of overexpressed or mutant kinases to which they are addicted (4, 5). Relevant to non-small cell lung cancer (NSCLC) (6), where high HSP90 expression correlates with poor survival (7), mutant EGFR (8, 9), ERBB2 (10), MET (11), mutant B-RAF (12) and the EML4-ALK translocation product (13, 14) are all HSP90-dependent proteins, degradation of which leads to loss of tumor cell viability in the corresponding adenocarcinoma subset.

Most HSP90 inhibitors under development target the ATPase activity at the N-terminus (15). The most characterized agents comprise the geldanamycin class, including the benzoquinone ansamycin HSP90 inhibitor, 17-allylamino-17-demethoxygeldanamycin (17-AAG; tanespimycin) (16). Relatively poor physiochemical properties have prompted its modification, resulting in water soluble derivatives, including 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17-DMAG; alvespimycin) (17), and 17-allylamino-17-demethoxygeldanamycin hydroquinone hydrochloride (IPI-504; retaspimycin hydrochloride)
(18), all of which have demonstrated activity in a broad range of preclinical models as well as in phase 1 and 2 studies, particularly in ERBB2 (HER2)-positive breast cancer (19), acute myelogenous leukemia (20) and EML4-ALK-dependent NSCLC (21). Nonetheless, these agents are P-glycoprotein substrates (22), may require NAD(P)H:quinone oxidoreductase-1 (NQO1)-mediated reduction to a more active hydroquinone metabolite (23), and have caused gastrointestinal and hepatic toxicities in the clinical setting (15). These limitations have prompted the development of non-geldanamycin inhibitors of HSP90.

Ganetespib (STA-9090) is a non-geldanamycin resorcinol-containing triazolone compound that binds to the ATP-binding domain at the N-terminus of HSP90 and is currently in phase 1 and 2 clinical trials in both solid tumors and hematologic malignancies (24, 25). Preclinically, ganetespib and its derivatives have demonstrated activity with low nanomolar potency against KIT-dependent mast cell tumors (26), MET-dependent osteosarcoma cell lines (27), Wilms tumor 1 (WT1)-dependent myeloid leukemias (28) and hematologic malignant cells dependent on JAK/STAT signaling (29). Here, we have investigated the preclinical pharmacokinetics, pharmacodynamics and activity of ganetespib in NSCLC cells in comparison to 17-AAG. Ganetespib demonstrates efficacy in a variety of cell line, xenograft and genetically-engineered mouse models, including those driven by activated KRAS, mutant EGFR and mutant ERBB2. Although ganetespib displays prolonged intratumoral half-life, frequent dosing schedules are required to effectively suppress a subset of client proteins, including mutant EGFR, justifying the current development plan of a variety of treatment schedules.
MATERIALS AND METHODS

Cell lines and drug treatments

NSCLC cell lines were obtained from the American Type Culture Collection (ATCC). H3255 and DFCI-LU011 cells were provided by Drs. Bruce Johnson and Pasi Jänne (Dana-Farber Cancer Institute, Boston, MA). PC9 was a gift from Dr. Takashi Owa (Eisai Co., Ltd., Tsukuba, Japan). Cell lines were subjected to DNA profiling annually (Short Tandem Repeat Analysis) at the Dana-Farber Cancer Institute Molecular Pathology Core to confirm their authenticity (30). All cells were maintained in ATCC-specified growth medium. Ba/F3 cells stably expressing mutant EGFR or ERBB2 were established as previously described (31-33). Pooled stable cell lines transformed to IL-3 independence were used for drug sensitivity experiments. Ganetespib was provided by Synta Pharmaceuticals (Lexington, MA) and both ganetespib and 17-allylamino-17-demethoxygeldanamycin (17-AAG, LC Labs) were prepared as stock solutions in DMSO.

Cell proliferation assay

Cell proliferation assays were performed using the CCK-8 colorimetric assay (Dojindo) in at least duplicate samples according to the manufacturer's specifications. IC$_{50}$ values were calculated using Kaleidagraph or Graphpad Prism.

Western blots

Whole-cell lysates were prepared as previously described (9). Protein concentrations were determined and equivalent amounts (20 μg) were subjected to SDS-PAGE on 4-12% bis-tris
gradient gels (Invitrogen). The HSP27 antibody was from Enzo Life Sciences. p23 and HSP90α antibodies were from StressMarq. All other antibodies were from Cell Signaling Technology. Bands were quantified using ImageJ software (NIH).

**HSP90 binding assays**

Exponentially growing cells were processed in lysis buffer (20 mM HEPES, pH 7.4, 1 mM EDTA, 5 mM MgCl₂, 100 mM KCl) and incubated with increasing concentrations of 17-AAG or ganetespib for 30 min at 4°C, and incubated with biotin-GM linked to Dynabeads MyOne Streptavidin T1 magnetic beads (Invitrogen) for 1 h at 4°C. Beads were washed three times in lysis buffer and heated for 5 min at 95°C in SDS–PAGE sample buffer (Invitrogen). Samples were resolved on 4-12% Bis-Tris gradient gel and Western blots were performed using an anti-HSP90 antibody.

**Immunoprecipitation**

500 μg of whole cell lysate was immunoprecipitated with 2 μg of mouse anti-p23 monoclonal antibody (clone JJ6, StressMarq) conjugated with protein A Dynabeads (Invitrogen). Proteins bound to p23 were resolved on 4-12% bis-tris gradient gels and Western blot was performed with an anti-HSP90 antibody.

**Establishment and treatment of xenografts**

Female 7–8-week-old C.B-17 SCID mice (Charles River Laboratories) were maintained under pathogen-free conditions. All procedures were approved by the Synta Pharmaceutical Institutional Animal Care and Use Committee. NCI-H1975 or HCC827 cells were cultured as
above and 0.5 – 1×10^7 cells were mixed with 50% RPMI 1640/50% Matrigel (BD Biosciences) and subcutaneously injected into the flanks of SCID mice. For efficacy studies, animals with 100-200 mm³ tumors were then randomized into treatments groups of eight. Tumor volumes (V) were calculated by the equation V = 0.5236×L×W×T (Length, width, and thickness). Animals were treated by intravenous bolus tail vein injection at 10 ml/kg with ganetespib formulated in 10/18 DRD (10% DMSO, 18% Cremophor RH 40, 3.6% dextrose and 68.4% water). As a measurement of *in vivo* efficacy, the relative size of treated and control tumors [(%T/C) value] was determined from the change in average tumor volumes of each drug-treated group relative to the vehicle-treated group, or itself in the case of tumor regression. Body weights were monitored daily. For biomarker studies, mice bearing NCI-H1975 xenografts were treated with either a single dose of vehicle or ganetespib, or with 5 daily doses of vehicle or ganetespib, in groups of 3 or 8, and harvested at various time points. Tumors were excised and flash frozen in liquid nitrogen for preparation of protein lysates or fixed in 10% neutral buffered formalin for immunohistochemistry.

**Pharmacokinetic Analysis**

Female 7–8-week-old C.B-17 SCID mice bearing NCI-H1975 xenografts received a single intravenous (i.v.) dose slightly below the highest non-severely toxic dose (HNSTD, 150 mg/kg). At time points indicated, mice (n = 3/time point) were sacrificed and plasma and tissues (tumor, liver, and lung) were harvested. Concentrations of ganetespib in plasma and tissues were determined by isocratic reversed-phase high-performance liquid chromatography with electrospay ionization mass spectrometric (HPLC/MS-MS) detection.
Xenograft immunohistochemistry and image analysis. For Cabenda immunohistochemistry [EGFR, CD31, DiOC7(3), TUNEL, pimonidazole and BrdUrd], NCI-H1975 tumor xenograft-implanted SCID mice were treated with 125 mg/kg ganetespib for 6-72 h. At the end of the experiment mice were administered BrdUrd and pimonidazole to label S phase cells and hypoxic tumor regions and then 5 min prior to excision mice were administered DiOC7(3) to demarcate perfused vessels. Following tumor excision, cryosections were cut and sequentially immunostained to detect markers of tumor vasculature (CD31), proliferation (BrdUrd), apoptosis (TUNEL), as well as EGFR expression (see Supplementary Materials and Methods for details). Overall BrdUrd positive staining and average EGFR, TUNEL or pimonidazole intensity was calculated from images of entire tumor sections following removal of necrotic regions and tissue artifacts (folds, tears, debris etc).

Additional immunostaining on xenografts harvested from mice treated with vehicle or ganetespib at 150 mg/kg as a single dose or 25 mg/kg daily x 5 was performed as previously described (34); see Supplementary Materials and Methods for details. Rabbit anti-EGFR L858R (1:100 dilution, clone 43B2, Cell Signaling Technology), rabbit anti-S6 ribosomal protein (1:100 dilution, clone 5G10, Cell Signaling Technology), rabbit anti-phospho-S6 ribosomal protein ser235/236 (1:50 dilution, clone D57.2.2E, Cell Signaling Technology), mouse anti-HSP27 (1:1000 dilution, clone G31, Cell Signaling Technology), rabbit anti-HSP70 (1:2500 dilution, clone K-20, Santa Cruz Biotechnology), and mouse anti-Ki67 (clone MIB1, DAKO) antibodies were applied to individual slides in DAKO background-reducing diluent for 1 hour. Slides were washed in 50-mM Tris-Cl, pH 7.4, and detected with the species-appropriate Envision+ kit.
(DAKO) as per manufacturer’s instructions. After further washing, immunoperoxidase staining was developed using a DAB chromogen (DAKO) and counterstained with hematoxylin.

Stained slides were scanned at 200X magnification using an Aperio ScanScope XT workstation (Aperio Technology, Inc.). Images were then visualized and digitally annotated (as regions of interest, ROIs) using ImageScope software (version 10.0.35.1800, Aperio Technology). A pathologist (S.J.R.) verified that areas of viable tumor were included and dead/necrotic tumor excluded from the analysis. ROIs were analyzed using a standard analysis algorithm to quantitate the proportion of area positive for staining (color deconvolution v9.0, Aperio Technology), expressed as the percentage pixels within the ROI that are positive for staining. Staining of samples from ganetespib-treated mice was compared to that in the corresponding vehicle controls to derive the percent change in immunohistochemical staining for each time point after single and multiple-dose administration.

**Generation of the CCSP-rtTA/Tet-op- hHER2YVMA mouse cohorts, drug treatment, MRI scanning, tumor volume measurement, tissue histology and immunohistochemical analysis.**

Previously characterized bitransgenic mice with lung adenocarcinomas driven by ERBB2 YVMA were exposed to a doxycycline-containing diet for 6 to 10 weeks, and subjected to magnetic resonance imaging (MRI) to document tumor burden (35). Littermates were subsequently left untreated or treated with vehicle or ganetespib, formulated in DRD, and administered by i.v. injection at 25 mg/kg/daily. Mice underwent serial MRI imaging to assess reduction in tumor volume and were subsequently sacrificed for further histologic and biochemical studies. After Dana-Farber Cancer Institute Animal Care and Use Committee
approval, all mice were housed in a pathogen-free environment at the Harvard School of Public Health and were handled in accordance with Good Animal Practice as defined by the Office of Laboratory Animal Welfare. MRI scanning, image acquisition in coronal and axial planes, and assessment of tumor volume have been described previously (36, 37). H&E and immunohistochemical staining were performed on formalin-fixed paraffin sections using standard procedures (9). HER2 (A0485, DAKO), HSP27 (Cell Signaling), and phospho-S6 (Cell Signaling) were used. For immunohistochemical quantification, 100 to 200 cells were scored as 0, 1+, and 2+ over each of 2 to 4 high-power fields to determine the average percent strongly positive cells.

**Statistical Analyses**

For efficacy analyses, due to a non-normal distribution in the tumor volume datasets, statistical analyses of significance were performed using a Kruskal-Wallis one-way analysis of variance (ANOVA) on ranks, followed by the Tukey test multiple comparison procedure. Otherwise, analyses were performed using the two-tailed, unpaired Student's t test. In both cases, P values < 0.05 were considered statistically significant.

**RESULTS**

**Ganetespib shows greater affinity for HSP90 and induces HSP90-p23 dissociation more readily than 17-AAG.**

We first compared the binding affinities of ganetespib and 17-AAG to HSP90 in competitive binding assays using biotinylated-geldanamycin (biotin-GM) (Fig. 1A). NCI-1975 NSCLC
(EGFRL858R/T790M) cell lysates were used as a source of HSP90 and were incubated with either compound. Ganetespib inhibited the binding of HSP90 to biotin-GM at a concentration as low as 0.11 μmol/L, whereas 1 μmol/L of 17-AAG was required to inhibit binding to the same degree, suggesting greater affinity of ganetespib for HSP90.

HSP90 binds to co-chaperones, including p23, in an ATP-dependent manner and this assembly of the catalytically active complex is a prerequisite for efficient chaperone function (38). To further characterize the \textit{in vitro} activity of ganetespib in comparison to 17-AAG, we assessed the ability of these compounds to disrupt catalytically active HSP90-p23 complexes. Lysates from NCI-H1975 NSCLC cells were used for immunoprecipitation of p23 in the absence or presence of ganetespib or 17-AAG followed by Western blotting for HSP90. Both compounds resulted in a concentration-dependent decrease in the amount of HSP90 in complex with p23, with ganetespib requiring lower concentration to disrupt complex formation (Fig. 1B). Taken together, these experiments confirm the ability of ganetespib to bind and inhibit HSP90 and indicate biochemical superiority over 17-AAG.

\textbf{Ganetespib destabilizes HSP90 client proteins in NSCLC cells with greater potency than 17-AAG.}

We next examined the cellular effects of ganetespib, and its ability to deplete critical client proteins from NSCLC cells in comparison to 17-AAG. In both NCI-H1975 and HCC827 (EGFR delE746-A750) cells (Fig. 1C and Supplementary Fig. S1), exposure to ganetespib induced client protein depletion at lower concentration than 17-AAG. For example, both mutant EGFR and MET were degraded following exposure to 40 nmol/L of ganetespib, whereas concentrations >
120 and 370 nmol/L of 17-AAG were required to achieve similar levels of depletion of EGFR and MET, respectively. Treatment of NCI-H1975 or HCC827 cells with 120 nmol/L ganetespib resulted in complete depletion of IGF-IR, whereas 1,100 nmol/L of 17-AAG was required for a similar degree of degradation. As expected, both drugs also extinguished downstream signaling of the PI3K/mTOR and RAF/MEK/ERK pathways, with a lower concentration of ganetespib required to achieve decreased expression of phospho-S6 and phospho-ERK (Fig. 1C and Supplementary Fig. S1). Furthermore, depletion of mutant EGFR in HCC827 cells by ganetespib resulted in the upregulation of BimEL and its subsequent cleavage into the proapoptotic subtypes BimL and BimS (Fig. 1D). Induction of Bim is required for EGFR tyrosine kinase inhibitor (TKI)-induced apoptosis (39), indicating that cell death pathways mediated by TKIs or HSP90 inhibition in EGFR mutant NSCLC cells share common downstream effectors.

Ganetespib treatment of NSCLC cells also resulted in the depletion of other receptor tyrosine kinases more readily than 17-AAG, including the PDGFα receptor overexpressed in NCI-H1703 cells, as well as c-RET in HCC1883 cells and ERBB4 in NCI-H522 cells (Supplementary Fig. S2).

The comparative efficiency of client depletion by ganetespib and 17-AAG translates to the inhibition of cell proliferation in a panel of 24 NSCLC cell lines with defined genetic backgrounds (Supplementary Table S1). Ganetespib inhibited proliferation of these cell lines with IC_{50} values ranging 2-30 nmol/L (median IC_{50} = 6.5 nmol/L). In contrast, IC_{50} values for 17-AAG ranged from 20 -3,500 nmol/L (median IC_{50} = 30.5 nmol/L). The improved potency of
ganetespib occurred across genotypes, including *EGFR/ERBB2* mutant (n=11), *EGFR* wild-type (n=15), *KRAS* mutant (n=6), and *KRAS* wild-type (n=18) (Supplementary Table S2), with mean IC$_{50}$ values 5–7-fold lower for ganetespib. Finally, we also examined the relative antiproliferative effects of ganetespib and 17-AAG in Ba/F3 cells ectopically expressing various mutant EGFRs that render these cells IL-3-independent. In this isogenic system, ganetespib was also substantially more potent (Supplementary Table S3).

**Ganetespib accumulates in tumors relative to normal tissues and displays greater in vivo efficacy than 17-AAG without increased toxicity**

The pharmacokinetic parameters of ganetespib were evaluated *in vivo* using mice bearing NCI-H1975 xenografts. Ganetespib was administered as a single dose intravenously at 125 mg/kg (slightly below the highest non-severely toxic dose (HNSTD) of 150 mg/kg), and its elimination kinetics were determined in tumor, liver, lung and plasma over a 6-day time period (n = 3/time point) utilizing HPLC/MS-MS (Fig. 2A, B and Supplementary Table S4). Ganetespib is rapidly distributed from the bloodstream into tissues and has a short half-life of 3 hours in plasma. The half-life in normal liver and lung is 5-6 hours (Fig. 2A). In contrast, the half-life of ganetespib in tumor was 58.3 hours, 10–19-fold longer than that in normal tissues or plasma, respectively (Fig. 2B). Additionally, at 144 hr after dosing, the tumor concentration of ganetespib remained 215-fold higher than the median IC$_{50}$ of 6.5 nmol/L required for antiproliferative cytotoxicity against a broad NSCLC cell line panel (Supplementary Table S1).

The favorable intratumoral pharmacokinetics of ganetespib support evaluation of once-weekly dosing. We therefore compared the relative efficacy of ganetespib and 17-AAG administered on
a once per week schedule for three weeks against NCI-H1975 xenografts, utilizing a dose of 125 mg/kg ganetespib, and the HNSTD of 17-AAG of 175 mg/kg (Fig. 2C). Ganetespib displayed significantly greater efficacy than 17-AAG, with the relative size of treated and control tumors of 15% and 50%, respectively ($P < 0.05$; one-way ANOVA), with no significant weight loss (Fig. 2D). Similar results were obtained with the HCC827 xenograft model when ganetespib was administered once-weekly at the HNSTD (Supplementary Fig. S3).

**Heterogeneous response of individual client proteins to HSP90 inhibition in vivo.**

To assess the pharmacodynamic effects of ganetespib compared to 17-AAG in NCI-H1975 xenografts, we documented the kinetics of client depletion over a 6-day period following a single intravenous administration at the HNSTD. The depletion of client kinases and the induction of HSP70 and HSP27 were monitored by Western blotting of xenograft lysates (Fig. 3A) and quantified by densitometry (Fig. 3B; n = 4/time point). Pharmacodynamic effects were uniformly more pronounced in response to ganetespib than 17-AAG. In this model, where EGFR depletion is critical, ganetespib depleted mutant EGFR (L858R/T790M) twice as effectively than 17-AAG; for both drugs, peak suppression occurred at 24 hours post post-dose (Fig. 3B). Surprisingly, recovery of EGFR expression was observed at later time points (Fig. 3B, 72 and 144 hours), despite the high intratumoral concentration of ganetespib (Fig. 2A). c-MET and CDK4 depletion followed similar kinetics, although the recovery of c-MET expression was slower than that of EGFR. In contrast, the depletion of other clients, such as c-RAF and AKT, was gradual, without evidence of restoration of expression. Because ERBB2 is known to be highly sensitive HSP90 client, the modest degree of depletion achieved in this experiment prompted us to examine earlier time points, demonstrating that ERBB2 was rapidly depleted by
6 hours, with a return to higher levels by 24 hours, although without restoration of baseline levels as shown by the longer time course. ERBB2 was the most extensively depleted client at the early time point (data not shown). The induction of the HSP70 and HSP27 chaperones in response to ganetespib was as expected, reaching high levels by 72 hours; HSP70 induction persisted until 144 hours, albeit with slight decline.

Immunohistochemical analyses of H1975 xenografts were also utilized to evaluate pharmacodynamic changes after a single dose of ganetespib (Fig. 4). Confirming the Western blot results (Fig. 3), a significant decrease in EGFR staining ($P < 0.0025$) was observed at 24 hours, but not at 6 hours, post-treatment (Fig. 4A). Additional multi-color staining, automated image analysis and quantification demonstrated reduced proliferation and induction of apoptosis at 24-48 hours post-dose, with recovery evident at 72 hours (Fig. 4B). In this mutant EGFR-driven model, the kinetics of reduced BrdUrd incorporation and increased TUNEL staining mirror those of EGFR depletion and recovery.

**More frequent dosing improves the efficacy of ganetespib against the NCI-H1975 xenograft model**

Despite the favorable intratumoral pharmacokinetics of ganetespib supporting once-weekly dosing, the depletion of mutant EGFR was not maintained through a 6-day period, suggesting that more frequent dosing may be superior. To determine if this was the case, we compared the schedules of 150 mg/kg administered once-weekly to 25 mg/kg administered five times weekly, both over a three-week period (Fig. 5). More frequent administration of ganetespib resulted in greater efficacy, with tumor regression achieved, rather than simply tumor growth inhibition. At
day 29, compared to vehicle control, the relative tumor volume was 15% with once-weekly
dosing (consistent with the prior experiment in Fig. 2B), and -28% with five times weekly dosing
\(P < 0.05;\) one-way ANOVA; Fig. 5A). Among the xenograft-bearing animals treated on the 5-
day schedule, all but one demonstrated tumor regression (Supplementary Fig. S4). Assessment
of body weight indicated that the once-weekly and 5-day schedules were equally well tolerated
(Fig. 5B).

Additionally, the pharmacodynamic effects of single dose and consecutive day dosing of
ganetespib were directly compared (Fig. 5C). Mice bearing NCI-H1975 xenografts were
administered a single dose of vehicle or ganetespib at 150 mg/kg, or alternatively vehicle or
ganetespib at 25 mg/kg x 5 consecutive days (with \(n = 3\)/timepoint for all vehicle or ganetespib
-treated animals). After a single dose of ganetespib, mutant EGFR is depleted at 24 hours, with
expression restored by 72 hours. Downstream signaling, assessed with phospho-S6
immunohistochemistry, is also reduced at 24 hours, but reversing by 72 hours and fully restored
at 144 hours. Reductions in Ki-67 staining were seen at 24 and 72 hours, but were not
statistically significant. In contrast, when xenograft-bearing mice treated with ganetespib for 5
consecutive days were compared with those treated with vehicle, reductions in expression of
mutant EGFR, phospho-S6 and Ki-67 were seen throughout the 120-hour time course, extending
to 168 hours. Although several doses of ganetespib at 25 mg/kg are required to cause the degree
of reduction of mutant EGFR and phospho-S6 achieved 24 hours after a dose at 150 mg/kg, the
sustained pharmacodynamic effects with consecutive day dosing translate to superior anti-tumor
activity. Increases in HSP70 and HSP27 expression were observed after ganetespib exposure on
both schedules, consistent with HSP90 inhibition (Supplementary Figure S5).
Ganetespib induces tumor regression in an ERBB2 YVMA–driven murine lung adenocarcinoma model.

ERBB2 is one of the few HSP90 client proteins that demonstrated rapid depletion without full re-expression after administration of a single dose of ganetespib (Fig. 3B). In isogenic Ba/F3 cells ectopically expressing ERBB2 harboring the YVMA exon 20 insertion activating mutation, the most common ERBB2 kinase domain mutation identified, ganetespib demonstrated superior activity compared with 17-AAG (Supplementary Fig. S6). These observations prompted us to test the efficacy of ganetespib in a transgenic murine lung adenocarcinoma model driven by ERBB2 YVMA (35). The no adverse effect level (NOAEL) dose was empirically determined at 25mg/kg three times per week in this model (data not shown). Compared to mice treated with vehicle (n = 2), in ganetespib-treated mice (n = 4), there was statistically significant tumor growth inhibition at 2 weeks, and reduction in tumor volume at 4 weeks (Fig. 6A), as demonstrated by MRI scans (Fig. 6B). Immunohistochemical staining performed directly after two doses 25 mg/kg ganetespib (administered every other day) demonstrated increased expression of HSP27, consistent with HSP90 inhibition, and reduced expression of ERBB2. At this early time point, phospho-S6 expression was also mildly decreased (Fig. 6C).

DISCUSSION

There is currently substantial interest in the development of HSP90 inhibitors for advanced NSCLC, since many oncogenic drivers defining groups of adenocarcinomas are dependent on HSP90 for conformational stability, including mutant EGFR, mutant ERBB2, EML4-ALK
mutant BRAF, c-RAF and CDK4 (40), the latter two clients possibly underlying the sensitivity of NSCLC cells carrying activating \(KRAS\) mutation (41, 42), demonstrated here with ganetespib and previously with 17-AAG (43). We have shown that ganetespib binds to the N-terminus of HSP90 and disrupts HSP90-p23 complexes, therefore resulting in inhibition of chaperone activity and client protein depletion, which occurs with greater potency than with 17-AAG both \textit{in vitro} and \textit{in vivo}. Among a large panel of genomically-defined NSCLC cell lines, including those harboring \(EGFR\) mutation, \(ERBB2\) mutation, \(ERBB2\) amplification and \(KRAS\) mutation, ganetespib routinely inhibited cellular proliferation with lower IC\textsubscript{50} than 17-AAG. Additionally, in ERBB-dependent xenograft and genetically engineered mouse models, ganetespib was well tolerated, with activity at the NOAEL. Early phase clinical trials of ganetespib have demonstrated that hepatic toxicity is substantially less common than with 17-AAG and its water soluble derivatives (24, 44, 45); therefore, ganetespib may have improved therapeutic index compared to agents in the geldanamycin class.

For NSCLC, the most mature HSP90 inhibitor studies have examined IPI-504 (retaspimycin hydrochloride) (21) and ganetespib (46) in genomically-defined subsets of patients; in the latter trial, ganetespib was used once weekly at the recommended phase 2 dose in cohorts of patients whose tumors harbored mutant EGFR, mutant KRAS or wild type forms of both proteins. As with IPI-504, the activity of ganetespib in the mutant EGFR arm was disappointing, with some patients achieving either minor regression or disease stability lasting 12-16 weeks, but without objective responses by response evaluation criteria in solid tumors (RECIST). The majority of patients treated had acquired erlotinib resistance; although tumors harboring secondary T790M mutation (9) or \(c-MET\) amplification (11) may be expected to respond, the activity of HSP90
inhibition against tumors acquiring resistance by other mechanisms, including the emergence of small cell histology or evidence of epithelial-mesenchymal transition has not been clarified (47).

In addition to the possible biological explanations for lack of response, our data suggest that the schedule of drug administration may be critical. The preclinical pharmacokinetic profile of ganetespib is typical of HSP90 inhibitors, demonstrating high penetrance and retention in tumor, with short half-life in normal organs (18, 48). Nonetheless, the expression level of mutant EGFR (L858R/T790M) in the NCI-H1975 xenograft model exhibits complete recovery by 5 days after single-dose exposure. These results suggest that once-weekly administration of ganetespib will not be adequate to effectively suppress mutant EGFR/T790M signaling, evidenced by the return of tumor cell proliferation and reversal of apoptosis that paralleled the re-expression of mutant EGFR. Therefore, the sustained reduction in client protein expression may be essential for efficient cell death in oncoprotein-driven NSCLC. Consistent with these data, ganetespib was more efficacious in the NCI-H1975 xenograft model with daily x5 dosing, which caused regressions rather than simply tumor growth inhibition. With consecutive day dosing, there was prolonged depletion of the mutant EGFR client, with consequent extinguishing of downstream signaling and proliferation. Importantly, an ongoing phase 1 trial of ganetespib administered more than once per week will soon establish recommended phase 2 doses of both twice-weekly and consecutive-day dosing schedules (45), with a plan to re-evaluate NSCLC patients with tumors harboring EGFR mutation with these more frequent administration schedules.
Another strategy could be the combination of HSP90 inhibition and with a small molecule inhibitor capable of suppression of the kinase activity of the reexpressed receptor. To date, irreversible EGFR inhibitors have had only modest activity against EGFRs carrying T790M, but may be adequate when combined with an HSP90 inhibitor.

The kinetics of c-MET and CDK4 depletion in response to ganetespib and 17-AAG in NCI-H1975 xenografts were similar to those of EGFR, with a return of expression despite persistent drug concentration in tumor, a phenomenon that has been observed with other HSP90 inhibitors as well (48). These results suggest that there is a poor correlation between intratumoral drug levels and the degree of HSP90 inhibition. The re-expression of these clients could therefore be related to diminution in HSP90 inhibitory activity over time, secondary to altered intracellular compartmentalization of drug, synthesis of new HSP90, or increased assembly of available HSP90 into an active high-affinity, co-chaperone bound complex. Induction of the HSP70 and HSP27 chaperones may also contribute to client re-expression.

However, not all clients are uniformly affected by such cellular changes. For example, in NCI-H1975 cells, c-RAF continues to demonstrate gradual depletion after 17-AAG or ganetespib exposure with lack of recovery of expression. Therefore, some clients may ultimately remain sensitive to degradation, even if cellular HSP90 activity recovers to some extent in the presence of drug.

Additionally, depending on cellular background, some clients exhibit exquisite sensitivity to decreases in HSP90 activity with more rapid and complete depletion than others. This is the case
with ERBB2 in NCI-H1975 cells, which was depleted by 6 hours; in addition, despite some restoration of expression by 24 hours, levels of protein remained below baseline for a 6-day period. A similar rapid decline of ERBB2 expression has been demonstrated with other HSP90 inhibitor compounds in ERBB2-amplified breast cancer cell lines and xenografts (48). Such may also be true of EML4-ALK, which has been shown to be readily depleted from ALK-translocated NSCLC cells by geldanamycins (13), to a greater degree than ERBB2 or EGFR are depleted from ERBB2-amplified or EGFR-mutated breast and lung cancer cells, respectively (14). After a single dose of IPI-504 administered to mice bearing ALK-translocated NCI-H3122 xenografts, EML4-ALK levels were depleted in tumor for at least 48 hours; although longer time periods were not examined, it is tempting to speculate that ALK would not be a client easily restored to full levels of expression. These results may in part explain some of the successes of HSP90 inhibitor compounds to date, including 17-AAG in ERBB2-amplified breast cancer (19) and IPI-504 and weekly ganetespib in ALK-rearranged NSCLC, where both drugs have produced durable partial responses (21, 46).

In addition to ganetespib, several other non-geldanamycin compounds are under active development. Currently, ganetespib is distinguished from several of these compounds since it lacks ocular toxicities, with more favorable retinal distribution and elimination (44, 45, 49-51). AT13387, discovered with a fragment-based discovery approach, has also been characterized in NCI-H1975 NSCLC cells (52). In vitro, a 7-hour exposure resulted in depletion of mutant EGFR lasting in excess of 168 hours. Following single dose exposure to mice bearing NCI-H1975 xenografts, there was rapid clearance from blood with prolonged intratumoral retention of drug to 240 hours; however, similar to ganetespib, depleted mutant EGFR expression with
downstream signaling was restored by 72 hours (52). An administration schedule on days 1, 4, 8, 12 and 16 led to similar tumor growth inhibition as a once-weekly schedule, neither producing clear regressions, raising the possibility that consecutive day dosing schedules may be optimal in this model as well as in trials in which NSCLC patients with tumors harboring \textit{EGFR} mutation are evaluated. To this end, once-weekly, twice-weekly and consecutive day dosing administration schedules of AT13387 are all under evaluation in Phase 1 trials (49, 53).

NVP-AUY922, an isoxazole resorcinol, has been studied in multiple preclinical models. A wide variety of NSCLC cell lines, including those harboring \textit{EGFR} mutation, are highly sensitive to NVP-AUY922, with low nanomolar IC\textsubscript{50}’s in 72-hour MTS assays (54). \textit{In vivo}, AUY922 is also preferentially retained in tumor over plasma (55). In ERBB2-dependent BT-474 breast cancer xenografts, ERBB2 depletion occurred by 6 hours after a single dose, with restoration of expression by 48 hours (56). More sustained regression was noted with three times per week compared to once-weekly administration, at the expense of significantly greater toxicity, manifesting with animal weight loss. Because substantial tumor growth inhibition was still noted with once-weekly dosing, NVP-AUY922 has been evaluated clinically with this schedule (56). Interestingly, in a Phase 2 NSCLC trial, confirmed partial responses were noted in patients with ALK-positive tumors, but also among 5 of 35 patients with tumors harboring \textit{EGFR} mutation (57). The kinetics of EGFR depletion in response to NVP-AUY922 in preclinical EGFR-dependent NSCLC models will therefore be of substantial interest in order to explain the preliminary efficacy of once-weekly dosing in this subset.
The efficient and prolonged depletion of ERBB2 in xenografts following HSP90 inhibitor exposure, and the substantial superiority of ganetespib over 17-AAG against Ba/F3 cells transformed to IL-3 independence by ERBB2 carrying an exon 20 YVMA activating insertion mutation, prompted us to evaluate ganetespib in a mouse model of lung adenocarcinoma driven by the same mutation. Previously, we showed that these tumors demonstrate only partial sensitivity to a dual EGFR-ERBB2 tyrosine kinase inhibitor that is augmented by mTOR inhibition, which further extinguishes the ERBB2-driven signaling pathway (35). In this model, single agent ganetespib demonstrated anti-tumor activity with depletion of mutant ERBB2 after initial exposure, translating to tumor growth inhibition predominating after 2 weeks of treatment, followed by tumor regressions after 4 weeks of drug exposure. These results suggest potential efficacy of ganetespib against NSCLCs driven by mutant ERBB2, a group not yet represented among ganetespib-treated patients whose tumors express wild type EGFR and KRAS.

In summary, ganetespib displays improved preclinical potency compared to 17-AAG with potential for activity in several NSCLC subsets defined by their addiction to individual oncoproteins. In particular, the current results justify further clinical evaluation of ganetespib in ERBB family member-driven tumors. Optimization of dosing schedules and integration with tyrosine kinase inhibitor based therapy, both of which may vary depending on the targeted client, remain important research avenues that will move the HSP90 inhibitor field forward.
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**FIGURE LEGENDS**

**Figure 1.** Ganetespib binds to HSP90, disrupts HSP90-p23 complexes, depletes client proteins and suppresses signaling cascades with greater potency than 17-AAG in NSCLC cell lines. (A) NCI-H1975 lysates were treated with DMSO (0 μmol/L) or the indicated concentrations of 17-AAG or ganetespib and then incubated with biotinylated geldanamycin (biotin-GM) linked to Streptavidin-containing beads. The amount of HSP90 bound to biotin—GM in the absence or presence of drug was determined by Western blot. (B) NCI-H1975 lysates from cells treated with DMSO or the indicated concentrations of 17-AAG or ganetespib for 2 hours were subjected to immunoprecipitation with anti-p23 antibody followed by Western blotting with the indicated antibodies. (C) Exponentially growing NCI-H1975 (EGFR L858R/T790M), cells were treated with DMSO (0 μmol/L) or the indicated concentrations of 17-AAG or ganetespib for 24 h, and lysates were subjected to Western blotting with the indicated antibodies, demonstrating depletion of receptor tyrosine kinases and suppression of downstream signaling in response to HSP90 inhibition. (D) HCC827 cells were treated with DMSO (0 μmol/L) or the indicated concentrations of ganetespib for 24 h. Lysates were subject to Western blotting with indicated antibodies, demonstrating induction of pro-apoptotic Bim.

**Figure 2.** Ganetespib accumulates in tumor relative to normal tissues and displays greater *in vivo* efficacy than 17-AAG. (A) Mice bearing NCI-H1975 xenografts received a single intravenous dose of 125 mg/kg ganetespib and concentrations were determined in tumor, liver, lung and plasma over a 24 hr time period. (B) Similar analysis over of ganetespib concentrations after a single intravenous dose over a 144 hr time period. In (A) and (B), N =
3/time point; error bars represent +/- SEM. (C) Mice bearing NCI-H1975 xenografts were treated intravenously 1X/week for 3 weeks (arrowheads) with vehicle, 17-AAG (175 mg/kg) or ganetespib (125 mg/kg) Tumor volumes were assessed over the one-month time course. Error bars +/- SEM. (D) For mice treated in (C), cumulative average changes in body weight were followed over the one-month time course. Error bars +/- SEM.

Figure 3. Depletion of HSP90 clients in vivo in response to 17-AAG and ganetespib.

(A) Mice bearing NCI-H1975 xenografts model, received a single dose of vehicle, 17-AAG (175 mg/kg) or ganetespib (125 mg/kg). At 24, 72 and 144 hrs, lysates from harvested xenografts (N = 4/group) were subjected to Western blotting with the indicated antibodies. (B) Western blots were quantified using ImageJ and expression levels are presented as a percentage of expression in vehicle-treated tumors. Error bars +/- SEM.

Figure 4. Ganetespib inhibits proliferation and induces apoptosis in parallel with EGFR depletion in NCI-H1975 xenografts. (A) Mice bearing NCI-H1975 xenografts were treated with a single intravenous dose of vehicle or 125 mg/kg ganetespib. Six and 24 hours post-treatment, cryosections of harvested xenografts (N = 8/group) were subjected to immunohistochemistry for EGFR. (Left) Representative images. (Right) Quantification of EGFR staining at 6 and 24 hrs post-treatment. Error bars ± SEM. (B) Mice bearing NCI-H1975 xenografts were treated as in A; cryosections from tumors (N = 8/group) harvested at 6, 24, 48 and 72 hours after treatment were subjected to immunohistochemistry for markers of proliferation (BrdU), apoptosis (TUNEL), vascular density (CD31), hypoxia (pimonidazole) and
perfusion (DiOC7(3)). (Left) Representative images. (Right) Quantification of BrdU and TUNEL staining at the indicated time points post-treatment. Error bars ± SEM.

**Figure 5. Comparison of once-weekly and five times per week dosing of ganetespib.**

(A) Mice bearing NCI-H1975 xenografts were treated with vehicle, 150 mg/kg ganetespib once weekly (white arrows) or 25 mg/kg ganetespib five times per week (black arrows) over a three-week period. % T/C values are shown. *P < 0.05. Errors bars ± SEM. (B) For mice treated in (A), the percent change in body weight was followed over the treatment time course. Errors bars ± SEM. (C) Mice bearing NCI-H1975 xenografts were treated with vehicle or ganetespib at 150 mg/kg x 1, with xenografts harvested 24, 72 and 144 hours after dosing (V1-24, V1-72, V1-144; G1-24, G1-72 and G1-144, for vehicle and ganetespib-treated samples, respectively). Alternatively, mice were treated with vehicle or ganetespib at 25 mg/kg/day x 5 days, with xenografts harvested 24, 72, 120 and 168 hours after the first dose (i.e. the 120- and 168-hour time points were 24 and 72 hours after the last dose; V5-24, V5-72, V5-120, V5-168; G5-24, G5-72, G5-120 and G5-168 for vehicle and ganetespib-treated samples, respectively). For each time point and treatment, xenografts were subjected to immunohistochemistry with the indicated antibodies and quantified as described in the Materials and Methods. (Left) Graphs represent the percent change in average IHC positivity of ganetespib-treated xenografts (n = 3) relative to the corresponding vehicle controls (n =3). Bars, Standard Error. *P < 0.05 for the comparison of ganetespib-treated xenografts compared to the corresponding vehicle control. (Right) Representative immunohistochemically stained sections, demonstrating more sustained reductions in expression of mutant EGFR, phospho-S6 and Ki-67 with consecutive day dosing. Magnification, 100X. Bar, 100 μm.
Figure 6. Ganetespib induces tumor regression in a mouse lung carcinoma model driven by ERBB2YVMA. (A) ERBB2 YVMA mice were treated with doxycycline for 4 weeks and imaged before treatment (baseline) and after every other day treatment with vehicle (V) or 25 mg/kg ganetespib (G) at 25 mg/kg for two or four weeks, after which tumor volume was calculated, demonstrating tumor growth inhibition at 2 weeks and regression at 4 weeks in ganetespib treated mice. (Left) Quantification of tumor volumes in individual mice. (Right) Results averaged over the entire vehicle or gantespib-treated group. * P < 0.05 compared to vehicle-treated contol. (B) Representative MRI scans performed at baseline and at 2 and 4 weeks post-treatment, demonstrating substantial reduction in tumor burden in two of the ganetespib–treated mice. (C) HER2 YVMA tumor-bearing mice were left untreated, or treated with vehicle or ganetespib (25 mg/kg every other day for two doses) and sacrificed for histology and immunohistochemical analyses. Representative results for the peripheral compartment are shown. Magnification 400x. Scale bars 50 μm. (D) Immunohistochemical quantification for showing percentage of positive cells. Error bars ± SEM. * P < 0.001.
Figure 1
Figure 2

**A**

- NCI-H1975
- Brain
- Tumor
- Liver
- Lung

Graph showing the change in tumor volume (mm$^3$ ± SEM) over time.

**B**

Graph showing the plasma concentration (µM) of compounds over time.

**C**

Graph showing the change in body weight (% compared to baseline) over time.

**D**

Graph showing the increase in tumor volume over time for different treatments.
Figure 3
Figure 4
Ganetespib (STA-9090), a Non-Geldanamycin HSP90 Inhibitor, has Potent Antitumor Activity in In Vitro and In Vivo Models of Non-Small Cell Lung Cancer

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