mTOR Inhibition Potentiates HSP90 Inhibitor Activity via Cessation of HSP Synthesis

Jaime Acquaviva, Suqin He, Jim Sang, Donald L. Smith, Manuel Sequeira, Chaohua Zhang, Richard C. Bates, and David A. Proia

Abstract

Because of their pleiotropic effects on critical oncoproteins, inhibitors of HSP90 represent a promising new class of therapeutic agents for the treatment of human cancer. However, pharmacologic inactivation of HSP90 subsequently triggers a heat shock response that may mitigate the full therapeutic benefit of these compounds. To overcome this limitation, a clinically feasible method was sought to block HSP synthesis induced by the potent HSP90 inhibitor ganetespib. An immunoassay screen of 322 late-stage or clinically approved drugs was performed to uncover compounds that could block upregulation of the stress-inducible HSP70 that results as a consequence of HSP90 blockade. Interestingly, inhibitors of the phosphoinositide 3-kinase (PI3K)/mTOR class counteracted ganetespib-induced HSP70 upregulation at both the gene and protein level by suppressing nuclear translocation of heat shock factor 1 (HSF1), the dominant transcription factor controlling cellular stress responses. This effect was conserved across multiple tumor types and was found to be regulated, in part, by mTOR-dependent translational activity. Pretreatment with cycloheximide, PI3K/mTOR inhibitor, or an inhibitor of eIF4E (a translation initiation factor and downstream effector of mTOR) all reduced ganetespib-mediated nuclear HSF1 accumulation, indicating that mTOR blockade confers a negative regulatory effect on HSF1 activity. Moreover, combined therapy regimens with mTOR or dual PI3K/mTOR inhibitors potentiated the antitumor efficacy of ganetespib in multiple in vivo models.

Implications: Collectively these data identify a novel strategy to optimize the therapeutic potential of HSP90 inhibitors. Mol Cancer Res; 12(5); 703–13. ©2014 AACR.

Introduction

In recent years the advent of molecularly targeted therapy has revolutionized the landscape for cancer treatment. In contrast with traditional chemotherapeutic drugs, the current armamentarium of approved and investigational targeted agents offers increased selectivity, potency, and efficacy for a wide variety of human malignancies, and typically with more favorable toxicity profiles (1, 2). Their cellular targets include growth factor receptors, effector molecules of canonical signaling pathways, protein tyrosine kinases, cell-cycle regulators, and modulators of apoptosis—all of which are essential for normal cellular function but often become dysregulated as a consequence of oncogenic transformation. In this regard, pharmacologic inhibition of the molecular chaperone HSP90 (3) has emerged as a unique and promising strategy to simultaneously disrupt the activity of these and other molecules through one druggable target (4). As a consequence, HSP90 inhibitors currently rank amongst the most actively pursued classes of agents under clinical evaluation for human cancer (5).

HSP90 plays an indispensable role in normal cellular homeostasis by regulating the correct folding, stability, and function of its target substrates, known as ‘client’ proteins (3). During tumorigenesis, the chaperoning activity of HSP90 may become subverted by cancer cells, in turn conferring aberrant proliferative, survival, angiogenic, and/or metastatic potential (6, 7). Functional inhibition of HSP90 promotes the proteasomal degradation of its clients, resulting in the coordinate and simultaneous disruption of multiple oncogenic signaling pathways critical for tumor cell survival and proliferation. In this manner, HSP90 blockade can overcome signaling redundancies and drug resistance mechanisms commonly seen in many cancers (8, 9), and additionally may render tumor cells vulnerable to the cytotoxic activity of other targeted or chemotherapeutic agents (10). Importantly, a conserved feature of HSP90 inhibition is the induction of the cellular heat shock response, most notably characterized by upregulation of the stress-inducible molecular chaperone HSP70. HSP70 plays a critical role in the triage of damaged proteins following proteotoxic stress, in part facilitating a

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shift in chaperone activity from protein folding to degradation if the protein cannot be effectively renatured (11). In the clinical setting, HSP70 upregulation is commonly used as a pharmacodynamic biomarker for HSP90 inhibition (12, 13). However, in line with its cytoprotective cellular roles, HSP70 possesses strong antiapoptotic activity such that the increased expression of this chaperone can attenuate the cytotoxic effects of HSP90 inhibitors. Indeed, experimental evidence has shown that silencing HSP70 dramatically increases cancer cell sensitivity to targeted HSP90 inhibition (14). Thus, it has been proposed that realizing the full therapeutic potential of HSP90 inhibitors may require the concomitant inhibition of HSP70 induction and suppression of the heat shock response (5).

The transcriptional induction of heat shock genes in response to proteotoxic stress is centrally orchestrated by the activity of heat shock factor 1 (HSF1; refs. 15, 16). In the latent state, HSF1 resides in a multichaperone complex with HSP90 and histone deacetylase (HDAC) 6 (17). Following cellular insult by a plethora of diverse chemical and physiologic stresses, monomeric HSF1 is released from this repressive complex and undergoes a homotrimerization process and extensive phosphorylation that promotes its translocation into the nucleus. In this active conformation, HSF1 gains the ability to bind to consensus sequence motifs within the regulatory regions of its target genes known as heat shock elements to subsequently drive the transactivation of numerous protective HSPs. Importantly, although this role as a master regulator of the stress-inducible heat shock response has been established for many years, more recently it has become apparent that HSF1 can also drive transcriptional programs that facilitate and maintain the malignant phenotype of cancer cells (18, 19).

Here, we report on our efforts to determine a clinically feasible method to overcome the HSP90 inhibitor-driven heat shock response. To achieve this, more than 300 late-stage or approved drugs were screened for their ability to block HSP70 upregulation in response to ganetespib, a potent triazolone inhibitor of HSP90 (20) currently under evaluation in multiple clinical trials. Among the most prevalent and effective modulators of the cellular stress response induced by ganetespib treatment were compounds targeting the phosphoinositide 3-kinase (PI3K)/mTOR signaling axis. mTOR is a highly conserved serine/threonine kinase that serves as a key regulator of protein synthesis and cell-cycle control, the major downstream targets of which are components of the cellular translational machinery (reviewed in ref. 21). A series of studies were performed to dissect the mechanism of HSP regulation by mTOR inhibitors at both the transcriptional and translational level in multiple tumor cell types. In addition, we explored the efficacy of dual ganetespib + PI3K/mTOR inhibitor treatment in vitro as a means to optimize the antitumor potency of HSP90 blockade. Our findings identify a promising new strategy to improve the antineoplastic properties of HSP90 inhibitors and, as such, are of considerable translational relevance for the future clinical development of this class of investigational agents as effective cancer therapeutics.

Materials and Methods

Cell lines, antibodies, and reagents

All cell lines were obtained from the American Type Culture Collection (ATCC). Each was maintained according to the supplier’s instructions, authenticated by routine company DNA typing, and used within 6 months of receipt. All primary antibodies were purchased from Cell Signaling Technology with the exception of the HSP70 antibody (Enzo Life Sciences) and the CRAF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies (Santa Cruz Biotechnology, Inc.). Ganetespib [3-(2,4-dihydroxy-5-isopropylphenyl)-4-(1-methyl-1H-1,2,4-triazol-5(4H)-one] was synthesized by Synta Pharmaceuticals Corporation. BEZ235 was purchased from LC Laboratories; temsirolimus, AZD8055, and XL765 from Selleck Chemicals; and 4EGI-1 from EMD Millipore.

In-cell Western inhibitor screen

A549 cells were seeded into 96-well plates at a density of 1.2 × 10^4 cells per well. Twenty-four hours after plating, cells were dosed with ganetespib (50 nmol/L) combined with drugs (1 µmol/L) from a chemical inhibitor library (Selleck Chemicals). After 24 hours drug exposure, cells were fixed and probed with HSP70 antibody according to standard In-cell Western (ICW) protocols for detection using an Odyssey system (LI-COR Biosciences). For quantification, HSP70 signal was normalized to the loading control DRAQ5. Selected hits from the library screen were validated by ICW for HSP70 by combining ganetespib (50 nmol/L) with graded concentrations of inhibitor.

HSP PCR array

A549 cells were treated with 25 nmol/L ganetespib, 500 nmol/L BEZ235, or the combination of drugs for 6 hours. Total RNA was extracted from the cells, converted to cDNA, and analyzed by quantitative real-time PCR (qRT-PCR) using a human HSP PCR array (SABiosciences/Qiagen Inc.) according to the manufacturer’s instructions. Individual gene expression levels were normalized to the average of four housekeeping genes and fold changes calculated over dimethyl sulfoxide (DMSO) controls.

Western blotting

Following in vitro assays, tumor cells were disrupted in lysis buffer on ice for 10 minutes. Lysates were clarified by centrifugation and equal amounts of proteins resolved by SDS-PAGE before transfer to nitrocellulose membranes (Bio-Rad). Membranes were blocked with StartingBlock T20 blocking buffer (Thermo Scientific) and immunoblotted with the indicated antibodies. Antibody–antigen complexes were visualized using an Odyssey system (LI-COR Biosciences).

Combinatorial drug effect analysis

For combinational analysis, H3122, 22RV1, and H1703 cells were seeded in 96-well plates and incubated at 37°C, 5% CO_2 for 24 hours. Drug combinations were applied at a nonconstant ratio, using three 1.5-fold serial dilutions above...
and below the IC_{50} values for each compound. Cell viability was assessed 72 hours after drug addition by CellTiter-Fluor Cell Viability Assay (Promega) 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.

**In vivo xenograft tumor models**

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. A375 and H1703 (7.5 × 10^6) cells were subcutaneously implanted into female SCID mice and animals bearing established tumors (~150 mm^3) were randomized into treatment groups of 5 and 6, respectively. For the 22RV1 experiment, 5 × 10^6 cells were subcutaneously implanted into male animals and xenograft-bearing mice (~200 mm^3) were randomized into treatment groups of 5 to 6. For the BEZ233 combinatioral studies, mice were intravenously dosed with ganetespib (50 mg/kg) once weekly or orally dosed with BEZ233 (10 mg/kg) five times per week, either alone or in combination. Both compounds were formulated in DRD (10% DMSO, 18% Cremophor RH 40, and 3.6% dextrose), H1703 tumor-bearing animals were also dosed with single-agent or combination treatment using ganetespib and temsirolimus (0.2 mg/kg) or XL765 (10 mg/kg). Tumor growth inhibition was monitored by tumor volume measurements twice weekly. As a measurement of in vivo efficacy, the percentage of tumor growth inhibition ratio (T/C) value was determined from the change in average tumor volumes of each treated group relative to the vehicle treated. Statistical significance was determined using two-way ANOVA followed by Bonferroni posttests.

**Results**

**Inhibitor screen for HSP70 modulators**

Initially, we developed an ICW screening assay to interrogate a chemical library of 322 approved or late-stage drugs, primarily kinase inhibitors, for their ability to modulate ganetespib-induced HSP70 upregulation. Compounds (1 μmol/L concentrations) were screened using A549 nonsmall cell lung carcinoma (NSCLC) cells treated with 50 nmol/L ganetespib for 24 hours, and HSP70 protein levels were normalized to fluorescent nuclear staining to control for cell number. The degree of ganetespib-induced HSP70 expression was set at 100% and the comparative levels following cotreatment with the inhibitor compounds are plotted in Fig. 1A. From this analysis, a number of compounds were identified that effectively suppressed the heat shock response (>50% reduction in relative HSP70 levels), the majority of which were inhibitors of the PI3K/mTOR signaling pathway (Table 1). In addition, several other drugs augmented HSP70 induction, the most robust being HDAC and proteasome inhibitors (Table 1).

Compounds selected for their suppressive activity in the original screen were then evaluated in a titration assay, in which they were found to inhibit the ganetespib-driven induction of HSP70 expression in a dose-dependent fashion (Fig. 1B). These findings were further validated by immunoblotting data in the A549 cell line in which cotreatment of these drugs, along with additional PI3K and mTOR inhibitors (NU7441 and rapamycin, respectively), suppressed the upregulation of HSP70 (Fig. 1C). In contrast, and in agreement with the data in Table 1, cotreatment with bortezomib resulted in enhanced HSP70 expression over ganetespib alone (Fig. 1C). In subsequent experiments, we focused on the dual PI3K/mTOR inhibitor BEZ235, the most clinically advanced and, thus, translationally relevant of the PI3K/mTOR inhibitors listed in Table 1. Treatment of A549 cells with this agent effectively reduced HSP70 protein induction in response to ganetespib exposure (Fig. 1D).

**Altered transcription contributes to the reduced heat shock response**

Having determined that PI3K/mTOR inhibition could overcome ganetespib-dependent HSP70 upregulation, we next examined the more global HSP gene response in A549 cells following ganetespib and/or BEZ235 treatment using qRT-PCR analysis (Fig. 1E). Single-agent ganetespib treatment exerted generally minor effects on gene upregulation across a panel of heat shock genes. An exception, and as expected following HSP90 inhibition, was the 20- to 25-fold increase in transcript levels of the two HSPA1A and HSPA1B homologs, which encode for HSP70 protein expression. This was associated with an approximate 7-fold induction of BAG3, the gene product for the HSP70-binding cochaperone protein Bcl-2–associated athanogene 3 (BAG3). Consistent with the data presented in Fig. 1D, cotreatment with BEZ235 robustly suppressed these responses. In addition, BEZ235 alone had minimally suppressive activity across the entire panel, although the two HSP70 genes were the most affected. When the kinetics of HSP70 gene regulation were examined (Supplementary Fig. S2), it was found that ganetespib-driven HSPA1A induction was detectable between 2 and 4 hours after treatment and continued to increase for at least 6 hours. In combination-treated cells, HSPA1A was also induced but suppression was evident by the 4-hour time point and relative expression levels declined thereafter.

**mTOR inhibitors attenuate the ganetespib-driven stress response across multiple tumor cell types**

Modulation of the stress response by targeted PI3K/mTOR inhibition was investigated in additional tumor cell lines. In A375 melanoma cells, ganetespib exposure induced robust upregulation of HSP70 protein expression, which was inhibited by cotreatment with BEZ235, or with the selective mTOR inhibitor temsirolimus (Fig. 2A). Interestingly, ganetespib exposure in this line induced similar fold increases in HSP27 protein levels; this response was correspondingly reduced by cotreatment with either BEZ235 or temsirolimus (Fig. 2A). Overall fold changes in HSP expression are quantitated in Fig. 2B.
Kinetics data are presented from another NSCLC line, H1975, in which increased HSP70 protein levels were detectable by 6 hours and continued to increase 24 hours following ganetespib treatment (Fig. 2C). In contrast, combination treatment with BEZ235 maintained HSP70 protein expression at near-basal levels over the entire time period. BEZ235-mediated suppression of HSP70 induction was additionally observed in LNCaP prostate cancer cells (Fig. 2D). Notably, combination BEZ235/ganetespib treatment resulted in significant elevations in cleaved PARP levels, a marker of apoptosis, in this line (Fig. 2D). Thus, combining BEZ235 with ganetespib promoted enhanced cellular death over single-agent treatment alone, suggesting that blocking HSP70 induction may improve the anticancer activity of the HSP90 inhibitor compound. Overall, these data suggest that PI3K/mTOR inhibitor-mediated attenuation of the HSP70 stress response was conserved across multiple tumor types.

**mTOR inhibition suppresses elevation of nuclear HSF1 expression**

Because HSP transcription is centrally regulated by HSF1, and because this transcription factor has recently been shown to be a direct substrate of mTOR (22), we examined inhibitor-induced effects on the nuclear translocation of this key regulatory molecule. As shown in Fig. 3A, BEZ235 treatment blocked the ganetespib-mediated entry and accumulation of HSF1 into the nucleus in H1975 cells. Exposure to 200 nmol/L ganetespib between 5 minutes and 1 hour initiated the translocation of HSF1 into the nucleus, with maximal accumulation occurring at the 6-hour time point. Nuclear
HSF1 expression subsequently declined by 24 hours after treatment (data not shown). BEZ235 alone (500 nmol/L) had no effects on nuclear HSF1 levels; however, when used as a cotreatment with ganetespib, it robustly suppressed nuclear HSF1 expression (Fig. 3A). In combination-treated cells, the highest levels of accumulated HSF1 were seen between 1 and 3 hours and declined thereafter. Interestingly, total HSF1 protein expression failed to exceed comparable levels induced within 1 hour of single-agent ganetespib exposure. To extend these findings, A375 cells were treated with ganetespib either alone or in combination with BEZ235, temsirolimus or the novel ATP-competitive mTOR inhibitor AZD8055 for 6 hours (Fig. 3B). Ganetespib treatment similarly induced a robust translocation of HSF1 from the cytoplasmic to the nuclear fraction, and this effect was potently reduced in the presence of all three mTOR inhibitor compounds.

**Nuclear accumulation of HSF1 requires mTOR-driven translational activity**

The mTOR signaling pathway serves as a master regulator of protein translation and, therefore, it was important to determine whether the reduced heat shock responses and corresponding effects on HSF1 localization identified here were under translational control. To do this, more detailed kinetic analyses of HSF1 expression following mTOR and protein synthesis inhibitor treatment were performed using A549 cells (Fig. 4). Nuclear accumulation of HSF1 was evident 1 hour after ganetespib.

### Table 1. Compounds identified through ICW screening that modulate ganetespib-induced HSP70 expression in A549 cells

<table>
<thead>
<tr>
<th>Drug</th>
<th>Target</th>
<th>% of HSP70 Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNS-032</td>
<td>CDK</td>
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</tr>
<tr>
<td>NVP-ADW742</td>
<td>IGF-1R</td>
<td>0</td>
</tr>
<tr>
<td>GSK1059615</td>
<td>PI3K/mTOR</td>
<td>0</td>
</tr>
<tr>
<td>YM155</td>
<td>Survivin</td>
<td>0</td>
</tr>
<tr>
<td>PK-75</td>
<td>PI3K/DNA-PK</td>
<td>5</td>
</tr>
<tr>
<td>WYE-354</td>
<td>mTOR</td>
<td>18</td>
</tr>
<tr>
<td>PI-103</td>
<td>PI3K/mTOR</td>
<td>18</td>
</tr>
<tr>
<td>KU-0063794</td>
<td>mTOR</td>
<td>26</td>
</tr>
<tr>
<td>Cytarabine</td>
<td>DNA polymerase</td>
<td>27</td>
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<tr>
<td>AZD8055</td>
<td>mTOR</td>
<td>30</td>
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<tr>
<td>TW-37</td>
<td>Bcl-2</td>
<td>32</td>
</tr>
<tr>
<td>Pelitinib</td>
<td>EGFR</td>
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</tr>
<tr>
<td>BEZ235</td>
<td>PI3K/mTOR</td>
<td>34</td>
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<tr>
<td>Fluvasin sodium</td>
<td>HMG-CoA reductase</td>
<td>35</td>
</tr>
<tr>
<td>Canertinib</td>
<td>EGFR/HER2</td>
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<tr>
<td>ZSTK474</td>
<td>PI3K</td>
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<td>Neratinib</td>
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<td>Obatoclax mesylate</td>
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<tr>
<td><strong>Ganetespib</strong></td>
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<td><strong>100</strong></td>
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<td>HDAC</td>
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<td>Indirubin</td>
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<tr>
<td>Vorinostat</td>
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<td>MLN2238</td>
<td>Proteasome</td>
<td>156</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>Proteasome</td>
<td>191</td>
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**NOTE:** This line is bold as it represents the degree of ganetespib-induced HSP70 induction set at 100%.
treatment and protein levels peaked at the 3-hour time point in this line (Fig. 4A). Similar to what was observed in H1975 cells, cotreatment with BEZ235 did not block the initial influx of HSF1 protein (1-hour time point), yet robustly attenuated the accumulation of HSF1 seen at 3 hours (Fig. 4A). Importantly, a 30-minute pretreatment with cycloheximide completely abrogated HSF1 nuclear translocation following ganetespib exposure at all time points, indicating that inhibition of protein biosynthesis could prevent this cellular response induced by HSP90 inhibition. Immunoblotting of the corresponding cytoplasmic cellular fractions (Fig. 4A, lower) confirmed the potent inhibitory effect of BEZ235 on mTOR activity, as evidenced by loss of phosphorylated eukaryotic initiation factor 4E-binding protein (p-4EBP1) expression. Of note, cycloheximide had no effect on the basal activation status of the mTOR pathway.

Although concurrent administration of BEZ235 and ganetespib was relatively ineffective at preventing the initial translocation of HSF1 into the nuclear compartment, we...
found that a 6-hour pretreatment with the PI3K/mTOR inhibitor significantly reduced the ganetespib-mediated nuclear increase of HSF1 seen 1 hour following treatment (Fig. 3B). Thus, prior inactivation of mTOR signaling was sufficient to prevent the initiation of the heat shock response, specifically HSF1 nuclear translocation, induced by ganetespib. Moreover, this effect was recapitulated by targeted blockade of the translation initiation factor eIF4E (eukaryotic translation initiation factor 4E), a specific downstream effector of mTOR. Hypophosphorylated 4E-BP1 binds to and inhibits eIF4E. However, upon phosphorylation by mTOR, 4E-BP1 releases eIF4E, which allows the protein to be recruited to a translation initiation complex that regulates cap-dependent translation (23). Pretreatment of A549 cells with the selective eIF4E inhibitor 4EGI-1 also reduced nuclear HSF1 protein levels within an hour of ganetespib treatment, and more effectively than when administered concurrently with the HSP90 inhibitor. Taken together, these data support an essential role for mTOR translational activity as an important mechanistic component of the attenuated heat shock response.

To further challenge the experimental system, we pretreated cells for 1 hour with ganetespib to trigger the heat shock response and evaluated whether subsequent mTOR inhibition by BEZ235 could reduce the further flow of HSF1 into the nucleus (Supplementary Fig. S3). Indeed, HSF1 levels were reduced compared with vehicle controls at both 2 and 4 hours after ganetespib exposure—further supporting the dependence of HSF1 on mTOR activity. Because of the initial influx of nuclear HSF1 following ganetespib pretreatment, BEZ235 had no impact on HSP70 expression levels versus control.

The antitumor efficacy of ganetespib is potentiated by PI3K/mTOR inhibition in multiple xenograft tumor models

The increased apoptotic response due to combined ganetespib/BEZ235 treatment shown in Fig. 2D suggested that blockade of mTOR could augment the in vitro cytotoxic activity of ganetespib. Therefore, we evaluated the combinatorial effects of ganetespib and BEZ235 on cell viability using A375 melanoma, 22RV1 prostate, and H1703 NSCLC tumor lines. In agreement with the data presented in Fig. 2, cotreatment with low (IC40 or IC20) doses of BEZ235 also attenuated ganetespib-driven HSP70 upregulation in the latter two lines (Supplementary Fig. S4). As shown in Fig. 5A, concurrent administration of low (IC40 or IC20) doses of BEZ235 and ganetespib substantially increased cell death in vitro in all lines. These data clearly demonstrated that BEZ235 could augment the cytotoxic effects of ganetespib. Moreover, we have previously reported that the anticancer activity of ganetespib could be potentiated by PI3K/mTOR inhibition...
In KRAS-mutant NSCLC models in vitro and in vivo (24). In light of these observations, we sought to evaluate whether the superior cytotoxic response to ganetespib treatment conferred by mTOR inhibition would translate to improved efficacy in vivo using multiple xenograft tumor models and targeted inhibitors of mTOR.

In the first set of experiments, mice bearing xenografts derived from the A375, 22RV1, and H1703 cell lines were treated with ganetespib and BEZ235, either as single agents or in combination. On the basis of our previous study (24), we selected a 50 mg/kg dose of ganetespib and 10 mg/kg for BEZ235 because these dose levels readily permit evaluation of potential combinatorial improvements in efficacy. Weekly administration of ganetespib and 5× per week treatment with BEZ235 at these doses to mice with established A375 xenografts (Fig. 5B) reduced tumor growth by 24% and 37%, respectively (T/C values of 76% and 63%). Consistent with the in vitro findings above, concurrent administration of both drugs resulted in enhanced antitumor activity, suppressing tumor growth by 56%. This represented a
significant improvement in efficacy over single-agent ganetespib alone \( (P = 0.01) \). In 22RV1 xenograft-bearing animals, ganetespib and BEZ235 were equally efficacious when used as monotherapy, both inhibiting tumor growth by 24\% (Fig. 5C). Combination treatment in this model was significantly more efficacious than either agent alone (71\% tumor inhibition; \( P = 0.001 \)). Similar therapeutic improvement was observed in mice with established H1703 tumors (Fig. 5D). Single-agent ganetespib and BEZ235 treatment had only modest effects on tumor growth (T/C values of 86\% and 81\%, respectively); however, when combined, tumor growth was suppressed by more than half (T/C value, 48\%; \( P = 0.001 \)).

Finally, we used mice bearing H1703 xenografts to evaluate the effects of additional targeted inhibitors of PI3K/mTOR signaling. As shown in Fig. 5E, low-dose treatment with the selective mTOR inhibitor temsirolimus (0.2 mg/kg) was minimally effective as a single agent (T/C value, 93\%), yet significantly improved the activity of ganetespib and to comparable levels seen with BEZ235 in this model. In the same experiment, 10 mg/kg dosing of another dual kinase PI3K/mTOR inhibitor, XL765, inhibited tumor growth by 39\%. Even at this more efficacious dose level, combining XL765 with ganetespib again resulted in superior tumor growth inhibition compared with the HSP90 inhibitor alone (Fig. 5F). Thus, in all cases examined, the addition of a selective mTOR or PI3K/mTOR inhibitor significantly improved the antitumor efficacy of ganetespib.

**Discussion**

It is well established that functional blockade of HSP90 activity elicits induction of the highly conserved and HSF1-directed cellular heat shock response (25). This in turn confers a level of protection to cells from the cytotoxic effects of small-molecule HSP90 inhibitors—a compensatory mechanism that is expected to contribute to drug resistance and lower sensitivity to targeted HSP90 inhibition. The results of the chemical screen used in this study identified novel drug combinations, involving targeted PI3K/mTOR inhibitors that effectively suppress the HSP90 inhibitor-induced heat shock response in tumor cells, as a means to overcome this limitation. Most importantly, this approach was validated as a viable therapeutic strategy as evidenced by the capacity of selective mTOR or dual PI3K/mTOR inhibitors to potentiate the antitumor efficacy of ganetespib in multiple in vivo xenograft models. Combinatorial benefit was observed in vitro when BEZ235 was used as a cotreatment with ganetespib in A375 melanoma, 22RV1 prostate, and H1703 NSCLC lines. Although combination BEZ235/ganetespib exposure in the A375 model showed a relatively modest benefit over BEZ235 monotherapy, the combination was significantly more efficacious than ganetespib treatment alone. In the two other models examined (22RV1 and H1703) combination treatment afforded highly significant improvements in efficacy over both single agents, a result that was recapitulated the mTOR inhibitor temsirolimus was used in combination with ganetespib in H1703 tumors.

Mitigation of HSP70 upregulation was used as a readout for suppression of the heat shock response, and it was shown at both the genomic and proteomic levels that this was readily achieved following functional blockade of the PI3K/mTOR signaling axis. Recently, it has been reported that pharmacologic inhibition of HSP70 (albeit at high concentrations) can result in synergistic improvements in the cytotoxic activity of the HSP90 inhibitor NVP-AUY922 in multiple myeloma cells (26). More significantly, the findings of Zaaour and colleagues are of direct relevance to the data we are reporting here (27). In that study, the authors similarly screened a chemical library, in part through evaluation of inducible HSP70 expression. Using this experimental approach, a group of structurally similar compounds were identified that inhibited the stress response and consequently sensitized tumor cells to the cytotoxic effects in both HSP90 and proteasome inhibitors. Furthermore, the enhanced sensitivity phenotype was recapitulated by siRNA-mediated knockdown of HSF1 expression in tumor lines (27).

Although the precise mechanisms are yet to be fully elucidated, our data add to the growing body of evidence that HSF1 activation is under translational control. In the case of the novel compounds that inhibited the heat shock response described above, the inhibitors did not affect the transcriptional activity of HSF1, nor the rate of HSP mRNA or protein degradation, suggesting that they exerted a specific regulatory effect on HSP translation (27). We cannot discount the possibility of a direct role for HSP70 itself contributing to the regulation observed in the present study, because it has been reported that HSP70 can block HSF1 activation through stabilization of a HSF1–HSP70 complex (28). However, in a recent elegant study, Santagata and colleagues provided compelling evidence that regulation of HSF1 transcriptional activity was intimately linked to translational flux (29). When protein translation was blocked in breast cancer cells, the strongest transcriptome changes included a category of genes with promoters containing consensus HSF1-binding sites. Similar to what was observed following BEZ235 treatment in our study, the inducible HSP70-encoding *HSPA1A* gene was among the 10 most downregulated transcripts. Furthermore, using a gene signature-based genetic screen, they showed that the perturbations that most positively correlated with the HSF1 activation gene signature were highly enriched for translation inhibitors (such as cycloheximide) and compounds targeting signal pathways that regulate protein translation, including PI3K/mTOR (29). mTOR controls protein synthesis through two distinct but interconnected downstream pathways: via activation of the 70 kDa ribosomal protein S6 kinase 1 (p70^{S6K)}) and its substrate ribosomal protein S6, and through phosphorylative inactivation of 4EBP1, a repressor of translation initiation (30). Here, we showed that selective inhibition of the 4EBP1 pathway, using a specific inhibitor of e4fE, was sufficient to suppress levels of nuclear HSF1 accumulation in response to ganetespib exposure, thus implicating direct involvement of cap-dependent translation in this process. However, when mTOR kinase
activity was blocked by BEZ235, and both effector pathways were inhibited in parallel, almost complete attenuation of HSF1 nuclear localization occurred. Taken together, these data indicate that both downstream arms play a contributory role in HSF1 regulation, and identify mTOR signaling as a central mediator of this cellular mechanism.

The findings of this study have clear implications for the continuing development of HSP90 inhibitors as anticancer therapeutics. HSP90 is an actively pursued target in oncology; however, no selective agents have yet been approved for human therapeutic use. HSP90 inhibitors, including ganetespib, have shown encouraging single-agent efficacy in early-stage clinical trials, most commonly in molecularly defined subgroups of tumors that are oncogenically "addicted" to specific client proteins, for example, ALK-rearranged NSCLC and HER-amplified breast cancer (5, 31, 32). More typically, however, the results obtained for HSP90-directed monotherapy in unselected patient populations have proven less definitive (33). In light of the observations presented here, it is reasonable to suggest that superior responses might be achieved by the addition of a PI3K/mTOR inhibitor to exploratory HSP90 inhibitor-based treatment regimens, thus providing a strategy to improve therapeutic benefit and accelerate the drug approval process for safe and efficacious agents. Importantly, a number of selective mTOR inhibitor drugs, including temsirolimus and everolimus, are already approved for a variety of solid malignancies and other investigational agents, such as BEZ235, are undergoing late-stage clinical evaluation (34, 35). The results of two clinical trials currently underway evaluating the safety and activity profiles of the HSP90 inhibitor IPI-504 in combination with everolimus in mutant KRAS NSCLC and the HSP90 inhibitor NVP-AUY922 in combination with a novel PI3K inhibitor BYL719 in metastatic gastric cancer (NCT01427946 and NCT01613950, respectively) are likely to be informative in this regard.

In summary, this proof-of-concept study provides convincing evidence that concomitant blockade of the PI3K/mTOR signaling axis can attenuate the HSF1-driven cellular heat shock response elicited by pharmacologic inhibition of HSP90. Importantly, these findings provide a molecular framework for the investigation of novel combinatorial strategies designed to enhance the antineoplastic activity of targeted HSP90 inhibitors. Over the longer term, such approaches are likely to have a substantial impact on the clinical efficacy of this class of agents for the management of multiple human malignancies.

Disclosure of Potential Conflicts of Interest
R.C. Bates is a senior scientific writer at Synta Pharmaceuticals Corporation. D.A. Proia is a director of cancer biology and has ownership interest (including patents) in Synta Pharmaceuticals Corporation. No potential conflicts of interest were disclosed by the other authors.

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References
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