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Network analysis identifies an HSP90-central hub susceptible in ovarian cancer

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Abstract

Purpose—Epithelial ovarian cancer (EOC) is usually detected at advanced stage and frequently lethal. While many patients respond to initial surgery and standard chemotherapy consisting of a platinum-based agent and a taxane, most experience recurrence and eventually treatment-resistant disease. Although there have been numerous efforts to apply protein-targeted agents in EOC, these studies have so far documented little efficacy. Our goal was to identify broadly susceptible signaling proteins or pathways in EOC.

Experimental Design—As a new approach, we performed data-mining meta-analyses integrating results from multiple siRNA screens to identify gene targets that showed significant inhibition of cell growth. Based on this meta-analysis, we established that many genes with such activity were clients of the protein chaperone HSP90. We therefore assessed ganetespib, a clinically promising second-generation small molecule HSP90 inhibitor, for activity against EOC, both as a single agent and in combination with cytotoxic and targeted therapeutic agents.

Results—Ganetespib significantly reduced cell growth, induced cell cycle arrest and apoptosis in vitro, inhibited growth of orthotopic xenografts and spontaneous ovarian tumors in transgenic mice in vivo, and inhibited expression and activation of numerous proteins linked to EOC progression. Importantly, paclitaxel significantly potentiated ganetespib activity in cultured cells and tumors. Moreover, combined treatment of cells with ganetespib and siRNAs or small molecules inhibiting genes identified in the meta-analysis in several cases resulted in enhanced activity.

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Conflicts of interest: David A. Proia is an employee of Synta Pharmaceuticals Corp. The remaining authors have no conflicts of interest to declare.

Author Contributions

This study was designed by DCC and EAG; HL, FX, IGS, SWO, MM and IA performed experiments; HL, FX, IGS, LPM, DCC and EAG analyzed the data; DAP contributed the HSP90 inhibitor, helpful discussions and critical review of the manuscript. SL provided statistical analysis; EAG and DCC wrote the manuscript and all authors reviewed and edited the manuscript.

Conclusion—These results strongly support investigation of ganetespib, a single-targeted agent with effects on numerous proteins and pathways, in augmenting standard EOC therapies.

Keywords

HSP90; signaling network; ovarian cancer; ganetespib; combination therapy

Introduction

Epithelial ovarian cancer (EOC) is the most common form of ovarian cancer and occurs with few or no distinct symptoms. Because of this, most women are diagnosed when disease has spread beyond the ovaries to other organs in the abdominal cavity. After initial diagnosis, patients undergo aggressive surgery to remove all visible tumors and are treated with standard combination chemotherapy consisting of a taxane and a platinum-based agent. Most patients respond well to surgery and chemotherapy, but the majority experience disease recurrence. While additional chemotherapy may be effective for a time, recurrent disease ultimately becomes resistant to standard treatment. For these patients, there are few effective treatment options, underscoring the persistent unmet need to identify therapeutics that target pathways involved in tumor progression. Over the past two decades, significant effort has been devoted to identifying protein-targeted agents and evaluating these alone and in combination with standard cytotoxic chemotherapies.

Overexpression of individual cancer-associated proteins in patient tumors has been taken to suggest that targeting these proteins may have clinical efficacy. However, this surrogate biomarker strategy has not always been successful in clinical trials. For example, although EGFR is commonly overexpressed in EOC, numerous clinical trials with different classes of targeted inhibitors of this pathway have failed to demonstrate therapeutic efficacy in patients (1). Similarly, trials of single agents targeting HER2, RAF, c-KIT/PDGFR, mTOR, PKC and SRC have failed to show clinical efficacy (2–9). Among the reasons for the overall lack of success, EOCs differ between individual patients in their development, histologic subtype, genetic makeup, protein expression and pathway activation. Genomic analyses have revealed that high grade serous carcinomas, the most common type of EOC, are commonly characterized by overexpression and/or amplification of numerous (>30) growth stimulatory genes (10). High levels of genetic instability in these cancers may result in heterogeneity within tumors that contributes to escape from individual targeted therapeutic agents. These factors predict that monotherapy trials of agents targeting a single protein or pathway will remain unsuccessful; however, the ability to predict effective combinations of agents that will reliably inhibit EOC growth remains elusive.

Prior work from our group has shown the potential for using bioinformatics to develop target-centered signaling networks that can be used as a basis for siRNA screens designed to identify proteins regulating sensitivity to targeted therapies (11). Observations from this dataset regarding interactions of sensitizing proteins with catalytic partners that are the targets of existing drugs were useful in predicting therapeutic combinations that were effective in preclinical in vivo studies (11). Given the known challenges of treating EOC, and the urgent need for new treatment modalities, in the present study we have developed this initially productive strategy into a more comprehensive approach. We performed meta-analyses of five independent siRNA screens involving different combinations of cell lines and drugs to identify the most consistently sensitizing targets. We then modeled interactions among the sensitizing dataset to identify connections to therapeutic targets.

In this extended analysis, multiple proteins directly interacting with heat shock protein (HSP90) emerged as potent sensitizers of EOC cells to drug-induced cell death. HSP90 is an

ATP-dependent molecular chaperone protein that affects the maturation, stability and activation of a number of diverse client proteins (12). While abundantly expressed in normal cells, its overexpression in malignant cells promotes persistent activation of many cellular kinases and transcription factors and buffers cells from malignancy-induced cellular stresses (12). Because it mediates multiple target and pathway effects, HSP90 is an attractive therapeutic target. As an ATP-dependent chaperone, druggability of HSP90 was established in the mid-late 1990s with the natural products geldanamycin and radicicol. These agents exhibited selective toxicity for cancer cells (13), and although too toxic for clinical use, provided the chemical framework for development of additional agents. Among these, ganetespib is a particularly promising agent that does not suffer from the toxicity issues associated with earlier-generation HSP90 inhibitors and exhibits increased potency compared to first- and other second-generation agents (14–17). In our study, we demonstrate that ganetespib is a potentially valuable agent for augmenting the activity of cytotoxic therapies commonly used in EOC, both in vitro and in vivo, and that depletion of a group of proteins physically interacting with HSP90 sensitizes EOC cells to ganetespib, suggesting directions for future combination therapies.

Materials and Methods

Network analysis

Data for drug sensitization profiles for 638 genes encompassed in the siRNA library, corresponding to a receptor tyrosine kinase/cancer signaling network (detailed in (11)), were pooled from five independent screens of cancer cell lines. These data included sensitization of HCT116 to irinotecan or erlotinib (see Supplemental Methods and Data); A431 cells to irinotecan or erlotinib (11); and H1155 cells to paclitaxel (18). Validated sensitizing siRNAs were sorted by rank for each screen, and assigned a value from 638 (most sensitizing) to 1 (least sensitizing). Comparison of the rank across screens nominated 171 siRNAs that were among the 20% strongest sensitizers in 2 or more screens. The proteins depleted by these siRNAs were imported into Cytoscape (19) and a protein-protein interaction network constructed. The network was expanded using the MiMi plugin (20) to include nearest neighbors shared by at least two proteins in the initial gene set. Analysis in Ingenuity (<http://www.ingenuity.com/index.html>) and DrugBank (21) was used to identify drugs targeting genes in the expanded protein set. The cumulative group of 130 drug targets was queried against the original group of 171 sensitivity-regulating proteins, and topological parameters of the network calculated in Cytoscape. The degrees (the total interactions of each protein in this sub-network) of each node were used to calculate the number of connections of each of the 130 drug targets to the initial set of 171 most sensitizing genes. After HSP90 was identified as of particular interest, Ingenuity augmented by STRING (22) and manual inspection of data included in (23), and the database of the HSP90 machine interactome (24) and <http://www.picard.ch/Hsp90Int/index.php> to capture all known interactions. The prevalence of proteins from this extended dataset among the subsets of genes with varying sensitization levels was used to calculate the enrichment of HSP90 targets in each subset compared to the whole library, using hypergeometric distribution.

Antibodies and drugs used

Antibodies used and commercial sources are as follows: JAK2, pJAK2^{Y1007/1008}, STAT3, pSTAT3^{Y705}, STAT5, pSTAT5^{Y694}, pSRC^{Y416}, S6, pS6^{Ser235/236}, pCDK1^{Y15}, AKT, pAKT^{Ser473}, c-MYC, BCL-XL, EGFR, MCL-1, NF-κB p105/p50, PKC, PKC, PKC, PKC, MEK1, PI3K p100, FYN, CK2, RIP1, PDK1, N-WASP and caspase-3 (Cell Signaling); Cyclin D1, CDK1, HSC70 and -actin (Santa Cruz); SRC and p53 (EMD Millipore); -actin (Sigma-Aldrich); HIP1 and Ki-67 (Abcam); EGFR (BD Biosciences); HSP70 (Enzo Life Sciences) and PARP (RayBiotech). Drugs used and commercial sources

are as follows: ganetespib (Synta Pharmaceuticals); paclitaxel and cisplatin (Fox Chase Cancer Center (FCCC) Pharmacy); dasatinib, alisertib and ruxolitinib (Selleck Chemicals); erlotinib (LC laboratories); and GSK2334470 (Sigma-Aldrich).

Cell culture

Human OVCAR-5, OVCAR-8 and A1847 EOC cell lines were grown in RPMI (Life Technologies), with 10% FBS (Atlanta Biologicals), 2 mmol/L L-glutamine, penicillin/streptomycin (100 units/mL and 100 µg/mL respectively, Life Technologies (Invitrogen)) and 0.25 units/mL insulin (Novo Nordisk). SKOV-3 cells were grown in McCoy's 5A (Life Technologies) supplemented with 10% FBS, 2 mmol/L L-glutamine, penicillin/streptomycin and 0.25 units/mL insulin. OVCAR-5 and A1847 cells were transduced with a retroviral firefly luciferase construct (pWZL-Luc, a gift from Dr. Maureen Murphy) using standard methods (25) and selected in the presence of 75 µg/mL Hygromycin B (Life Technologies).

Cell viability, apoptosis and cell cycle assays

Cell viability was determined in ganetespib-treated cells (0.1 to 1000 nM) using the Cell Titer Blue Cell Viability Assay (Promega) according to manufacturer's instructions. Apoptosis was evaluated by Annexin-V staining (Guava Nexin® Reagent, Millipore) in cells treated with 0, 5, 25 and 50 nM ganetespib for 48 hours. Briefly, 1×10^5 cells were harvested and centrifuged at 300 xg for 5 minutes at room temperature. Cells were washed in PBS and suspended in 100 µl of serum-containing medium and 100 µl of Guava Nexin Reagent was added to each sample. The samples were stained for 20 minutes at room temperature in the dark and analyzed on the Guava EasyCyte PCA-96 system and the accompanying Cytosoft 3.6.1 software (EMD Millipore). Annexin V-PE (+)/7-AAD (−) cells were identified in the early stages of apoptosis and Annexin V-PE (+)/7-AAD (+) cells were identified in the late stages. For cell cycle analysis, cells seeded at 2.5×10^5 cells/well in a 6-well plate were exposed to 0, 5, 25 and 50 nM ganetespib or 100 µM etoposide as a positive control. After 24 and 48 hours, cells were harvested and stained with propidium iodide (Sigma-Aldrich), analyzed on the Guava EasyCyte System (EMD Millipore) according to manufacturer's instructions.

Drug synergy testing

Ganetespib, paclitaxel, cisplatin, dasatinib, erlotinib, GSK2334470, alisertib and ruxolitinib were tested individually or in combination. A1847 and OVCAR5 Cells were plated at 3000 cells per well in 96-well plates. After 24 hours of incubation, cells were treated with serial dilutions of individual drugs or combinations of two drugs at a constant molar ratio. After 72 hours of incubation, cell viability was measured with CellTiter-Blue (Promega) using an EnVision Plate Reader (Perkin Elmer). Combination Index (CI) values were established by the Chou-Talalay method (26, 27) calculated using the CompuSyn software package (ComboSyn).

Immunoblot assays and analysis

Cells and tumor tissue were lysed in Mammalian Protein Extraction Reagent (MPER™) and Tissue Protein Extraction Reagent (TPER), respectively (Thermo Scientific). Lysis buffer was supplemented with Halt™ Phosphatase Inhibitor Cocktail (Thermo Scientific) and Complete™ Mini Protease Inhibitor Cocktail (Roche Diagnostics), and protein concentrations determined using the BCA assay (Thermo Scientific). Proteins were resolved on 4–12% gradient SDS-PAGE gels (Life Technologies) and transferred to PVDF membrane (EMD Millipore). Membranes were blocked in non-fat dry milk, incubated overnight at 4°C in primary antibody, followed by HRP-conjugated secondary antibody (GE Healthcare) and signal detected with SuperSignal West Pico Chemiluminescent Substrate

(Thermo Scientific). Immunoblots were quantified using Image J as described (28). Briefly, a rectangle region of interest (ROI) was drawn to outline each lane. The analyze gel function was used to create a plot of the average pixel intensity over the length of the ROI. A straight line was then used to close each peak and the area under the curve was measured and the density relative to α -actin was calculated for each band.

Mouse models and *in vivo* imaging

All procedures involving mice were approved by the FCCC Institutional Animal Care and Use Committee (IACUC). Female C.B-17 SCID mice (FCCC Laboratory Animal Facility) were used for intrabursal (i.b.) injections as described (29, 30). Mice were given unilateral i.b. (left side) injections of OVCAR-5-Luc or A1487-Luc cells (8×10^5) suspended in 5 μ g/ μ l final concentration of BD Matrigel™ Matrix High Concentration (BD Biosciences). Baseline BLI scans were acquired using the IVIS Spectrum (Perkin Elmer, Caliper Life Sciences) as described (29, 30) to confirm the presence of tumors. Transgenic TgMISIIR-TAg mice and *in vivo* magnetic resonance imaging and volumetrics analysis have been described (31–33). Ganetespib, 125 mg/kg formulated in 10/18 DRD (10% DMSO, 18% Cremaphore RH 40, 3.6% dextrose and 68.4% water), or 10/18 DRD (vehicle), was administered once weekly by tail vein injection. Paclitaxel was diluted in PBS and 5 mg/kg was administered once weekly by intraperitoneal (i.p.) injection. Mice with OVCAR-5-Luc and A1487-Luc xenografts were treated for three weeks and six weeks, respectively, and tumor growth monitored by weekly BLI. Briefly, regions of interest (ROIs) of identical size encompassing the luminescent signal were assigned, and the total flux calculated for each mouse using Living Image software (Perkin Elmer, Caliper Life Sciences). Statistical analyses were performed by subjecting pairs of data sets to the Wilcoxon two-sample test; $P < 0.05$ was considered significant.

ELISA assay

Levels of HSP70 and pSTAT3^{Y705} (activated STAT3) present in tumor protein lysates isolated 6 or 24 hours after vehicle- or ganetespib-treatment were assayed using an enhanced chemiluminescent ELISA assay (MesoScale Discovery™) according to manufacturers' instructions.

Reverse phase protein array (RPPA)

Triplicate samples of OVCAR-5 cells were treated with vehicle, 30 nM ganetespib, 1 nM paclitaxel, or 30 nM ganetespib + 1 nM paclitaxel for 24 hours. Following standard protocols of the RPPA Core Facility at MD Anderson Cancer Center (Houston, TX), cells were lysed on ice, and lysates cleared by centrifugation and denatured in SDS sample buffer, then submitted for analysis as described (34, 35). Data were visualized using the MultiExperiment Viewer (MeV) program (<http://www.tm4.org/mev/>) and analyzed by one-way analysis of variance and Tukey's Multiple Comparison Test using GraphPad Prism version 5.04.

Tissue preparation and immunohistochemistry

Mice were euthanized, necropsied and examined for the presence and location of primary tumors and tumor nodules. Reproductive tracts were removed and primary tumors were weighed and caliper measurements of length (l) and width (w) were made to determine tumor volume ($l \times w^2 \times 0.5$). Tumor nodules present in the abdomen were counted. Individual portions of tumors were snap frozen in liquid nitrogen for preparation of protein lysates, and fixed in 10% (v/v) neutral buffered formalin and paraffin embedded for staining with hematoxylin and eosin. Custom tumor tissue microarrays (TMAs) were constructed by arraying duplicate cores from primary OVCAR-5 and A1487 tumors isolated from mice at 6

and 24 hours after treatment with vehicle or ganetespib. Immunohistochemical staining was performed as described (30, 31) with the following antibodies at the indicated dilutions: Ki-67 (1:100), caspase-3 (1:300), PARP (1:100), STAT3 (1:400) and pSTAT3 (1:25). Stained TMAs were scanned and analyzed using the Vectra imaging system (Perkin Elmer, Caliper Life Sciences,). Images of IHC staining were acquired on a CCD camera and Nikon Eclipse E600 microscope with NIS-Elements D3.0 software (Nikon) at identical exposure times.

Sensitization testing for siRNAs

For the set of siRNAs defined in Results, sensitization to ganetespib was performed essentially as described in detail for library screening (Supplemental Methods). Two independent siRNA duplexes independently pre-validated for each target were used in A1847 and OVCAR5 cells, using optimized reverse transfection conditions to introduce siRNAs into 3000 cells arrayed in 96 well microtiter plates, in duplicate. Plates were treated with ganetespib at a previously established IC₃₀ concentration, or DMSO, after 24 hours, and viability assessed with CellTiter-Blue 96 hours after transfection, using an EnVision Plate Reader.

Results

Network analysis identifies HSP90 as a candidate for evaluation in EOC

To identify genes that consistently sensitized tumor cells to drug treatment, we performed meta-analysis of results from 5 independent siRNA drug sensitization screens that queried 638 genes in a signaling network enriched for many targets relevant to EOC pathology, including the previously assessed HER2, RAF, SRC, and mTOR; their physically interacting partners and downstream effectors; the TGF- β effector cascades, which have been associated with drug resistance and aggressive tumor phenotypes; and others ((11, 18); details of cell line selection and analysis are described in Supplementary Figure 1 and Supplementary Table 1). To identify siRNAs with the most consistent sensitizing activity, we sought those active in more than one cell line, and/or active against at least two of three drugs with different modes of activity: the topoisomerase inhibitor irinotecan, the microtubule-targeting agent paclitaxel, and the EGFR inhibitor erlotinib. By these criteria, 171 genes were identified as among the 20% scoring highest for mediating resistance to drug treatment, including 15 encoding proteins that are targets of drugs in preclinical development or clinical use (Figure 1A).

We next used this data set to identify commonalities in signaling among the set of most sensitizing genes. Numerous studies of synthetic lethality have established that close physical interactions between proteins predict common functionalities that can be exploited for cell killing (36, 37). From the starting gene set, we constructed an interaction network in Cytoscape among their encoded proteins which we augmented to include additional “nearest neighbor” interactors shared by at least two proteins in the initial group of 171 proteins. In the resulting expanded network of 1391 proteins, 130 are drug targets (Supplementary Table 2). We then performed a topological analysis of the network, and extracted the number of direct connections between each of the 130 drug targets and the 171 proteins regulating sensitization. From this analysis, we identified a subset of drug targets as particularly densely connected to proteins in the sensitizing network (Figure 1B). Within the subset of targets of the top 10 drugs, we observed that the two subunits of HSP90 (HSP90AA1 and HSP90AB1) were among the most densely connected to proteins in the sensitizing set (Figure 1B, Supplementary Figure 2 and Supplementary Table 2). We also identified a statistically significant enrichment of HSP90-interacting proteins among the 20% most sensitizing siRNAs in 2 or more screens ($p=0.03$), and under-representation among the

group of siRNAs that were never among the most sensitizing 20% ($p=0.04$). Moreover, many of the drug targets densely connected to the sensitizing set were themselves clients or interactors of HSP90. These included STAT3, EGFR, ERBB2 (HER2), ESR1 (estrogen receptor- α) and multiple SRC family kinases, each of which is already implicated in EOC pathogenesis (Figure 1B) (38–48).

Ganetespib inhibits EOC cell viability and HSP90 clients *in vitro* and *in vivo*

HSP90 has been reported as the tumor-associated antigen targeted by antibodies in the ascites of patients with late stage EOC (49), while separate studies have shown that elevated HSP90 levels are common in peritoneal and pleural effusions of patients with advanced stage EOC (50). Based on these reports, and the strong connections of EOC to many HSP90 client proteins, we directly assessed ganetespib, a small molecule inhibitor of HSP90 (17), in commonly studied EOC cell lines, including OVCAR-5, OVCAR-8, A1847 and SKOV-3 cells. Ganetespib treatment resulted in dose-dependent inhibition of cell viability with IC_{50} values at 72 h ranging from 9–48 nM (Figure 2A). Treatment of cells within the IC_{50} range (e.g., 5–50 nM) of ganetespib for 48 h resulted in a significant increase in the percentage of apoptotic cells, while increasing the dose (10–100 nM) and duration (72h) of exposure increased the percentage of apoptotic cells further (Figure 2B). Exposure to ganetespib (25–50 nM) also resulted in the accumulation of cells in the G2/M phase of the cell cycle (Figure 2C). In addition, comparable concentrations of ganetespib (i.e., 25–50 nM), reduced the expression of canonical HSP90 clients including total and phosphorylated (p) proteins, including JAK2, pJAK2, pSTAT3 and pSRC (Figure 2D-E).

We next used orthotopic xenograft and transgenic mouse models of EOC to assess the *in vivo* efficacy of ganetespib monotherapy. Drug treatment was well-tolerated in both models, with no apparent toxicities. For the xenograft model, OVCAR-5-Luc cells were implanted by injection into the intrabursal space surrounding the ovary. Mice were monitored *in vivo* by longitudinal bioluminescent imaging (BLI) from the stably integrated luciferase (Luc) (Figure 3A-B). The BLI data indicated statistically significant inhibition ($p<0.01$) of xenograft growth rate, and endpoint assessments confirmed this observation, showing significantly decreased final tumor volume, weight, and dissemination of tumor nodules following three weeks of treatment with 125 mg/kg ganetespib (Figure 3B). In ovarian tumor-bearing transgenic mice, tumor growth was monitored and quantified by magnetic resonance imaging (29), and similarly showed decreased tumor growth rate in ganetespib-treated mice (Figure 3C).

In separate pharmacodynamic studies, mice with established orthotopic OVCAR-5 tumors were treated acutely with ganetespib and tumors collected 6 or 24 h later for evaluation of protein expression levels by immunoblot analyses of total and phosphorylated forms of > 25 proteins (antibodies listed in Methods). Among the proteins affected by ganetespib-treatment were many established HSP90 clients, with some more predominantly inhibited at 6h post-treatment (pJAK2, pSTAT3, total and pS6, pAKT), some at 24 h (pSTAT5, total and pCDK1 and AKT) and some inhibited at both time-points (total JAK2 and c-MYC) (Figure 4A-B). Consistent with the previously described HSF1-mediated induction of heat shock response elicited by HSP90 inhibitors, i.e., tanespimycin and radicicol derivatives (51), levels of HSP70 protein were increased in tumors 6 and 24 h after ganetespib treatment (Figure 4A-B). Independent ELISA analyses confirmed the significant induction of HSP70 (2-fold at 6h and 2.4-fold at 24h) and inhibition of pSTAT3 (2.6-fold decreased at 6h) in tumors from ganetespib-treated mice (Figure 4C-D). Immunohistochemical staining revealed no significant differences in Ki-67, caspase-3, PARP or total STAT3 levels at 6 or 24h post-dosing (not shown), but further confirmed significantly reduced levels of pSTAT3 present in tumor tissues 6h after ganetespib treatment (Figure 4E). These results suggested that there are differences in the timing and duration of client inhibition *in vivo*, and that the

mechanisms of tumor inhibition likely involve multiple signaling pathways with variable kinetics. The observed single agent activity of ganetespib in EOC cells, an orthotopic xenograft model and transgenic mice predicted that this agent may be promising for the treatment of patients, but also suggested that maximum clinical advantage might be gained by combining ganetespib with other therapeutic agents in standard use or development for EOC.

In vitro assessment of ganetespib combination potential in EOC cells

Few targeted agents are effective as monotherapy in EOC. For example, erlotinib (inhibiting EGFR) and dasatinib (inhibiting SRC family kinases) have each been evaluated in EOC patients, but neither drug showed single agent activity (7, 52). However, given the close connection of the HSP90 clients EGFR and SRC to the sensitization network (Figure 1), we assessed erlotinib and dasatinib for combination with ganetespib. For this purpose, we performed Chou-Talalay analysis (53), combining each compound with ganetespib at different ratios in cultured cells. Ganetespib combined with either of these agents inhibited the growth of both A1847 and OVCAR-5 cells much more significantly than either drug administered independently (Supplementary Table 3). We next investigated the effect of combining ganetespib with paclitaxel and cisplatin, standard front-line cytotoxic agents used to treat EOC patients (54–56). Notably, the combination of ganetespib was synergistic with both cisplatin and paclitaxel at all ratios tested in A1847 cells, and at some ratios in OVCAR-5 cells (Supplementary Table 3, Supplementary Figure 3).

Combination of ganetespib with paclitaxel potently inhibits orthotopic ovarian xenograft growth *in vivo*

To confirm the *in vitro* findings showing ganetespib-mediated sensitization to paclitaxel, the effects of single agent and combination therapy with ganetespib and paclitaxel were evaluated in two human ovarian carcinoma xenograft models (Figure 3D-G and Supplementary Figure 4). Treatment of mice bearing orthotopic OVCAR-5-Luc cell xenografts with ganetespib or paclitaxel alone resulted in significantly ($p<0.001$) reduced *in vivo* tumor growth and final tumor volume and weight determined at necropsy (Figure 3D-F). The overall TGI observed was 57% and 61% for ganetespib and paclitaxel treated mice, respectively. Importantly, 85% TGI and 77% fewer tumor nodules were observed in mice treated with the combination therapy compared to vehicle treated mice. The observed TGI and reduction in tumor nodules was significantly greater ($p<0.01$) in mice treated with the combination therapy than with either drug used as a single agent (Figure 3D-G). Similarly, treatment of mice harboring orthotopic A1847-Luc xenografts with either ganetespib or paclitaxel significantly inhibited tumor growth rate and primary tumor volume and weight (Supplementary Figure 4A-C). As with OVCAR-5-Luc tumors, inhibition of several HSP90 client proteins (e.g., JAK2, pSTAT3, total and pS6, pAKT, c-MYC, cyclin D1 and survivin) was confirmed in mice bearing A1847-Luc tumors treated with ganetespib (Supplementary Figure 4D-F). Combination of ganetespib + paclitaxel was significantly better than ganetespib alone (77% TGI compared to 43% TGI, respectively). Mice with A1847 xenografts exhibited greater sensitivity to paclitaxel alone than mice with OVCAR-5 xenografts (72% versus 61% TGI); therefore, while combination therapy in A1847 xenografts resulted in 77% TGI, the difference between single agent paclitaxel and combination therapy was not significant ($p=0.12$) due to the potent effect of paclitaxel. Taken together, these data suggest that the combination of ganetespib with paclitaxel may be a promising clinical therapeutic strategy.

To identify potential mechanisms underlying the ganetespib-mediated sensitization to paclitaxel, we compared the effects of treatment of OVCAR-5 cells with ganetespib and paclitaxel as single agents or in combination by reverse phase protein array (RPPA)

analysis. Results of this experiment supported the immunoblot analyses (Figures 2D & 4A-B), and identified additional proteins that were significantly affected in cells treated with ganetespib alone or combined with paclitaxel (Figure 4F and Supplementary Table 4). This analysis showed significant depletion of AKT/mTOR and MAPK signaling pathway proteins, kinases and transcription factors, as well as increased levels of apoptotic proteins and E-cadherin (Figure 4F and Supplementary Table 4). However, the analysis did not reveal proteins that were significantly more affected by the combination of ganetespib and paclitaxel compared to either drug alone.

HSP90-interacting proteins sensitize EOC cells to ganetespib

In sum, the preceding data suggested considerable potential for supplementing standard paclitaxel regimens for EOC with ganetespib. As noted above, a number of members of the original group of HSP90-interacting proteins that led us to nominate HSP90 as a target have previously been linked to EOC pathogenesis, and in some cases been explored as drug inhibition targets in EOC. Among this group, some (including the genes PDPK1 (encoding PDK1), PRKCE (PKC ϵ), RIPK (encoding RIP1), HIP1, and PRKCD (PKC δ)) were rapidly degraded following treatment of EOC cell lines or tumors with ganetespib, (Figures 5A-B).

To gain additional insights into the functional relationship of these proteins in HSP90 activity, we assessed whether their depletion affected sensitization to ganetespib in OVCAR-5 and A1847 cells. This identified a group of 20 siRNAs, targeting RAF1, PDPK1, RIPK1, FGR, STAT3, AURKA, and others, that increased the sensitivity of cells to ganetespib in both cell lines (Figure 5C, Supplementary Figure 5 and Supplementary Table 5). We therefore directly tested whether drug inhibition of AURKA (with alisertib), JAK2 (an upstream activator of STAT3, with ruxolitinib) or PDK1 (with GSK2334470) enhanced ganetespib activity. Chou-Talalay analysis indicated significant synergy between each of these agents (alisertib, ruxolitinib and GSK2334470) and ganetespib at several different combination ratios in OVCAR-5 and A1847 cells (Table 1 and Figure 6). Collectively, these findings suggest the capacity of ganetespib to sensitize ovarian carcinoma cells to a broad range of cytotoxic and targeted therapeutic agents.

Discussion

Early efforts to target HSP90 with natural product antibiotics with anti-tumor activity such as geldanamycin and its analogs, including tanespimycin (17-AAG) and alvespimycin (17-DMAG), showed promising activity in clinical trials, particularly in cancers that are highly dependent on key HSP90 clients (e.g. HER2+ breast cancer) or that are sensitive to proteotoxic stress (e.g., multiple myeloma) (68, 69). Some assessments with these first-generation agents were performed in EOC cells or tumors (70, 71) and showed anti-proliferative and pro-apoptotic effects suggesting possible clinical benefit (71–74). In spite of these encouraging data, these first generation agents suffered from limitations related to hepatic toxicity, issues related to solubility and formulation, and consequently the inability to achieve sufficient doses required for sustained client depletion (12, 75), and clinical development of these agents ceased.

The results of our meta-analysis emphasizing the importance of HSP90 in EOC were well-timed to benefit from intensive efforts focused on the development of second generation small molecule synthetic inhibitors of HSP90 with favorable biological and clinical properties. Ganetespib (formerly STA-9090) is a highly promising anti-cancer agent (17). In preclinical studies, ganetespib exhibited potent *in vitro* cytotoxicity, degradation of client proteins, superior activity to tanespimycin and *in vivo* anti-tumor activity in several solid tumor models including NSCLC, melanoma, prostate and gastric cancers (17, 76–79). In the clinic, ganetespib has been given to over 700 patients and is well-tolerated, with the most

common side effects including fatigue, diarrhea, constipation, nausea, vomiting, anorexia and abdominal pain. Single agent clinical activity has been seen in patients with advanced breast cancer, NSCLC, GIST, CRC and melanoma [<http://www.syntapharma.com/PrdHsp90.aspx>]. Comparisons between ganetespib and other HSP90 inhibitors including 17-DMAG and AT13387 also emphasized the greater potency of ganetespib (14–17).

In our study, we show that ganetespib significantly reduced EOC cell viability and cell cycle progression, increased apoptosis and decreased client protein expression and stability *in vitro*. Ganetespib also significantly reduced tumor growth and dissemination *in vivo*, in the absence of any observed drug-related toxicities. Mechanistically, using both a candidate approach and RPPA-mediated screens, we found that ganetespib limited expression and/or activation of client proteins, including many linked to EOC pathogenesis, including JAK2, pSTAT3, EGFR, SRC, S6, AKT, mTOR, NF- κ B and c-MYC. Pharmacodynamic analysis performed both in vitro and in vivo showed ganetespib treatment resulted in depression of many targets for 24 hours; enough to interrupt the cycle of continuous utilization of proliferative pathways required for the viability of transformed cells, and to trigger an apoptotic response. The stronger responses to ganetespib observed in pure populations of cultured tumor cells than in tumors likely reflects the more heterogeneous cell population in the primary tumor, as well as the presence of drug-metabolizing enzymes: nevertheless, there was clear evidence for a significant depression of known HSP90 clients in tumor tissue.

As with most targeted therapeutics, there is concern over intrinsic or acquired resistance. Therefore, continued preclinical work directed at identification, analysis and validation of additional targets that sensitize EOC to ganetespib is warranted to understand mechanisms of resistance and potential ways to circumvent it. Our return to network analysis led us to investigate whether siRNAs and small molecule inhibitors of proteins from the original dataset that nominated HSP90 as a target were themselves sensitizing to ganetespib. Some of these interactors are known to be commonly activated and/or overexpressed in EOC, including AURKA and JAK2/STAT3, and we found both siRNA and small molecule inhibitors enhanced ganetespib activity (30, 41, 45, 46, 80–82). Others, such as the SRC-related kinase FGR, the inflammation associated kinase RIPK, and the PTEN/AKT pathway kinase PDK1, have been little studied in EOC. In this study, we found that both siRNA and a small molecule inhibitor of PDK1 enhanced ganetespib action, suggesting new directions for further evaluation of drug combinations for use in EOC.

The essential strategy of combining targeted therapeutics with front-line cytotoxic agents is to target different mechanisms of action and minimize potential for overlapping toxicity. Of particular importance for clinical practice, ganetespib potently sensitized EOC cells to the effects of standard cytotoxic chemotherapy agents used for EOC patients (e.g., cisplatin and paclitaxel) in vitro, suggesting potential benefit of combining ganetespib with standard therapy in patients. In vivo, sensitization to paclitaxel was confirmed in two independent orthotopic xenograft models. Although the underlying mechanism for this sensitization was not revealed by the RPPA analysis, we previously reported the synergistic activity of ganetespib with taxanes in NSCLC models (77). The observed synergy may be related to disruption of cell cycle checkpoints and spindle function and will require additional studies. These results are particularly promising, as patients with recurrent and platinum refractory disease are frequently treated with paclitaxel (83). These encouraging results established evaluation of the combination of ganetespib and paclitaxel in the clinical setting as an obvious next step.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Translational Relevance

Epithelial ovarian cancer (EOC) is typically diagnosed at advanced stage. Although many protein-targeted therapeutic agents have been evaluated in clinical trials, few have demonstrated efficacy. A likely reason for this is that advanced stage ovarian carcinomas exhibit a high degree of tumor heterogeneity and generally lack pronounced, drug-targetable oncogenic driver mutations. Our findings show that targeted inhibition of HSP90 results in broad inhibition of several oncogenic signaling proteins and/or pathways in EOC. In addition, our results suggest that targeted inhibition of HSP90 with ganetespib combined with chemotherapeutic and/or protein targeted agents may be an effective therapeutic strategy for treatment of ovarian cancer patients. Because it mediates the activity of multiple targets and pathways that are relevant to EOC, therapeutic targeting of HSP90 is predicted to be a more productive clinical strategy for treatment of highly heterogeneous advanced stage ovarian cancers.

Figure 1. Network analysis identifies HSP90 as a candidate for evaluation in EOC treatment

A, 638 genes assessed in 5 independent sensitization screens, subdivided in three tiers reflecting number of screen in which gene-targeting siRNAs fell among the 20% most potent drug sensitizers (in 0, 1, or 2–5 screens), and further sorted by rank (averaged for all 5 screens) within each subset. Genes encoding drug targets are shown in brown. **B**, Right, a network encompassing 171 proteins in the most sensitizing subset of the library (diamonds), augmented with 130 additional drug target proteins connected to 171 protein set by no less than two interactions (circles). Brown fill indicates drug target (130 drug targets total); blue, not drug target; purple outline, HSP90 interactors, including clients. Only the largest connected component, comprising 260 proteins, is shown. Left, bar graph indicates degree distribution (reflecting number of connections to most sensitizing subset of the library) for each drug target in the network. Drugs targeting proteins with the highest degree are indicated. Clients of HSP90 are indicated in brown.

Figure 2. Ganetespib treatment affects cell viability, apoptosis, cell cycle distribution and HSP90 client proteins in EOC cells

A, OVCAR-5, OVCAR-8, A1847 and SKOV-3 cells were treated with increasing concentrations (0, 0.1, 0.5, 1, 5, 10, 50, 100, 500 and 1000 nM) of ganetespib for 72 hours and cell viability was assessed by Cell Titer Blue Assay. Data indicate the mean percent viability calculated from triplicate samples from three independent experiments ($\pm$ SE). B, OVCAR-5, OVCAR-8 and A1847 cells were treated with 0, 10, 25 or 50 nM ganetespib for 48 hours and analyzed for the presence of Annexin V-PE(+)/7-AAD(-) cells (early apoptosis) and Annexin V-PE (+)/7-AAD (+) cells (late apoptosis). Data shown are the mean values ($\pm$ SE) from three independent experiments. Statistically significant differences were determined by two-way ANOVA, followed by Bonferroni post-tests ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$). Analysis of OVCAR-5 and A1847 cells was extended to include a broader dose range (0, 10, 50, 80 and 100) and duration of the assay (72h) and shows increased apoptosis at higher concentrations of drug and after longer exposure. C, OVCAR-5, OVCAR-8 and A1847 cells were treated with 0, 10, 25 or 50 nM ganetespib for 24 or 48 hours, stained with propidium iodide and analyzed for cell cycle distribution. Data shown are the mean values ($\pm$ SE) from three independent experiments. D, Ovarian carcinoma cells (OVCAR-5, OVCAR-8, A1847 and SKOV-3) were treated with increasing doses of ganetespib for 24 hours and protein lysates were subjected to immunoblot analysis with the indicated antibodies. E, Densitometric analysis of the immunoblots (D) was performed using Image J 1.44 (NIH) to quantify pJAK, JAK, pSTAT3^{Y705}, STAT3, pSRC^{Y416} and SRC levels relative to -actin.

Figure 3. Ganetespiib exhibits single agent activity in an orthotopic xenograft and a transgenic mouse model of EOC

A, OVCAR-5-Luc cells were implanted as orthotopic xenografts in SCID mice (n= 15/ group) and mice were treated weekly with vehicle or 125 mg/kg ganetespiib for three weeks. **A**) The presence of tumors was confirmed in baseline bioluminescent imaging (BLI) scans ten days after tumor implantation, and tumor growth was monitored *in vivo* weekly thereafter by BLI (scans 1–3). **B**, Average radiance (photons/second) was measured weekly showing significant differences ($^{**}p < 0.01$) in ganetespiib-treated mice compared to vehicle-treated controls. Primary tumor volume, weight and the number of abdominal tumor nodules were determined at necropsy, and showed significant inhibition in ganetespiib-treated mice compared to vehicle-treated controls. **C**, A separate experiment evaluated the effects of ganetespiib in a transgenic mouse model of EOC, in which tumor growth rate was monitored and quantified by magnetic resonance imaging and volumetrics analysis. Mice treated weekly with 125 mg/kg ganetespiib exhibited significantly decreased tumor growth compared to vehicle (n=12) treated controls. Combination of ganetespiib with paclitaxel was evaluated in orthotopic OVCAR-5-Luc xenografts in SCID mice (n=12/group) treated weekly with vehicle, 125 mg/kg ganetespiib, 6 mg/kg paclitaxel or 125 mg/kg ganetespiib + 6 mg/kg paclitaxel. **D**, Tumor growth was monitored weekly *in vivo* by BLI (scans 1–3), showing significant differences ($^{****}p < 0.0001$) in mice treated with ganetespiib or paclitaxel as single agents or in combination compared to vehicle-treated controls. Average radiance was significantly lower in combination-treated mice compared to mice treated with ganetespiib or paclitaxel alone. **E**, Primary tumor volume, **F**, tumor weight, and **G**, tumor nodules. Differences among groups were compared by the Mann Whitney test with p values < 0.05 considered significant ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and $^{****}p < 0.0001$).

Figure 4. Ganetespiib-treatment inhibits HSP90 client protein expression and activation in tumors

A, Pharmacodynamic analysis was performed on tumors isolated from mice treated with vehicle or ganetespiib at 6 and 24 hours post-treatment ($n = 4$ mice/group/time point). Protein lysates were prepared and subjected immunoblot analysis with the indicated antibodies. **B**, Immunoblots were subjected to densitometric analysis using Image J 1.44 (NIH) to quantify each target protein levels relative to α -actin. Statistically significant differences were determined by two-way ANOVA, followed by Bonferroni post-tests ($*p < 0.05$, $**p < 0.01$, $***p < 0.001$, and $****p < 0.0001$). Detection of HSP70 (**C**) and pSTAT3^{Y705} (**D**) levels present protein lysates by enhanced chemiluminescent ELISA assay. **E**, Immunohistochemical detection of pSTAT3^{Y705} in tumors. Data are presented as the H-Score, considering both staining intensity and the percentage of positively staining cells with representative micrographs of pSTAT3^{Y705} staining in tumor tissue isolated 6 and 24 hr

after vehicle or ganetespib treatment (scale bar = 50 μ M). **F**, Heat map of RPPA analysis showing proteins with significantly decreased (blue) and increased (yellow) protein expression following treatment with ganetespib, paclitaxel, or ganetespib + paclitaxel.

Figure 5. HSP90-interacting proteins sensitize EOC cells to ganetespib
A. Western blot with indicated antibodies shows loss of HSP90 clients following ganetespib-treated A1847 and OV5CAR-5 cells in vitro, and in ganetespib-treated OV5CAR-5 xenograft tumors, 6 hours after administration of drug. **B.** Quantification of data in **A** indicates ratios of indicated proteins in ganetespib- versus vehicle-treated samples. Data reflects average of 3 independent experiments; error bars, standard deviations. **C.** Graphic representation of relative degree of sensitization to ganetespib by siRNA depletion of HSP90 interacting genes in A1847 (inner ring) and OV5CAR-5 (outer ring). More intense gray shading reflects greater sensitization; precise values are listed in Supplementary, Table 4.

Figure 6. Synergistic reduction of ovarian carcinoma cell viability with ganetespib (gan) in combination with alisertib or ruxolitinib

Cell viability in OVCAR-5 (A) and A1847 (B) treated with ganetespib (gan), alisertib (ali) or gan:ali; molar ratio 20:1, showing strong synergy. Cell viability in OVCAR-5 (C) and A1847 (D) treated with gan, ruxolitinib (rux) and gan:rux; molar ratio 10:1), showing strong synergy. Viability curves shown represent the average of three independent experiments; error bars, standard deviations.

Table 1

Coefficient of interaction (CI) between ganetespib and targeted therapeutic agents alisertib, ruxolitinib and GSK2334470 in OVCAR-5 and A1847 cells.

Cell line	Molar ratio	Coefficient of interaction, CI* ($\pm$ S.D.)		
		ED50	ED75	ED95
OVCAR-5	ganetespib:alisertib			
	80:1	0.24 $\pm$ 0.23	0.16 $\pm$ 0.23	0.11 $\pm$ 0.16
	40:1	0.49 $\pm$ 0.08	0.39 $\pm$ 0.42	0.57 $\pm$ 0.87
	20:1	0.42 $\pm$ 0.16	0.38 $\pm$ 0.43	0.57 $\pm$ 0.81
	5:1	0.79 $\pm$ 0.27	1.11 $\pm$ 0.82	1.10 $\pm$ 0.85
	1:1	1.04 $\pm$ 0.38	1.22 $\pm$ 0.31	1.70 $\pm$ 0.76
	1:5	0.60 $\pm$ 0.22	0.64 $\pm$ 0.40	1.32 $\pm$ 1.14
	1:20	0.94 $\pm$ 0.32	0.85 $\pm$ 0.18	0.80 $\pm$ 0.22
	1:40	1.15 $\pm$ 0.19	0.64 $\pm$ 0.31	0.15 $\pm$ 0.06
	1:80	2.46 $\pm$ 1.17	0.82 $\pm$ 0.53	0.14 $\pm$ 0.11
	ganetespib:ruxolitinib			
	50:1	0.46 $\pm$ 0.06	0.43 $\pm$ 0.05	0.38 $\pm$ 0.04
	10:1	0.46 $\pm$ 0.06	0.45 $\pm$ 0.13	0.47 $\pm$ 0.30
	1:1	0.95 $\pm$ 0.13	0.85 $\pm$ 0.25	0.75 $\pm$ 0.39
	1:5	0.82 $\pm$ 0.14	0.60 $\pm$ 0.14	0.36 $\pm$ 0.12
	1:50	0.93 $\pm$ 0.12	0.92 $\pm$ 0.44	1.18 $\pm$ 1.04
	1:100	0.95 $\pm$ 0.17	1.09 $\pm$ 0.30	1.85 $\pm$ 0.93
	ganetespib:GSK2334470			
	100:1	0.21 $\pm$ 0.15	0.08 $\pm$ 0.05	0.03 $\pm$ 0.02
	10:1	0.20 $\pm$ 0.11	0.07 $\pm$ 0.04	0.03 $\pm$ 0.02
	1:1	0.79 $\pm$ 0.24	0.77 $\pm$ 0.18	0.73 $\pm$ 0.31
	1:5	1.03 $\pm$ 0.33	0.87 $\pm$ 0.35	0.65 $\pm$ 0.27
	1:20	1.42 $\pm$ 0.42	1.34 $\pm$ 0.44	1.22 $\pm$ 0.38
	1:50	7.39 $\pm$ 2.10	5.48 $\pm$ 1.88	3.32 $\pm$ 0.89
A1847	ganetespib:alisertib			
	80:1	0.46 $\pm$ 0.01	0.47 $\pm$ 0.27	0.49 $\pm$ 0.01
	40:1	0.52 $\pm$ 0.03	0.80 $\pm$ 0.04	1.75 $\pm$ 0.40
	20:1	0.44 $\pm$ 0.27	0.53 $\pm$ 0.06	0.80 $\pm$ 0.10
	5:1	0.68 $\pm$ 0.16	0.70 $\pm$ 0.35	0.85 $\pm$ 0.82
	1:1	1.01 $\pm$ 0.35	1.37 $\pm$ 1.03	2.95 $\pm$ 3.06
	1:5	0.30 $\pm$ 0.10	0.41 $\pm$ 0.18	1.74 $\pm$ 1.69
	1:20	0.39 $\pm$ 0.08	0.59 $\pm$ 0.09	2.80 $\pm$ 1.88
	1:40	0.62 $\pm$ 0.26	0.60 $\pm$ 0.13	1.16 $\pm$ 0.85
	1:80	0.91 $\pm$ 0.46	0.71 $\pm$ 0.06	0.98 $\pm$ 0.68
	ganetespib:ruxolitinib			
	50:1	0.45 $\pm$ 0.07	0.50 $\pm$ 0.29	0.77 $\pm$ 0.92
	10:1	0.47 $\pm$ 0.05	0.47 $\pm$ 0.08	0.49 $\pm$ 0.24

Cell line	Molar ratio	<u>Coefficient of interaction, CI* ($\pm$S.D.)</u>		
		ED50	ED75	ED95
ganetespib:GSK2334470	1:1	0.86 $\pm$ 0.27	0.79 $\pm$ 0.37	0.74 $\pm$ 0.56
	1:5	0.74 $\pm$ 0.17	0.70 $\pm$ 0.29	0.72 $\pm$ 0.63
	1:50	1.05 $\pm$ 0.15	1.22 $\pm$ 0.41	1.83 $\pm$ 1.15
	1:100	1.04 $\pm$ 0.01	1.05 $\pm$ 0.20	1.15 $\pm$ 0.58
	100:1	0.71 $\pm$ 0.32	0.69 $\pm$ 0.28	0.65 $\pm$ 0.29
	10:1	0.64 $\pm$ 0.25	0.61 $\pm$ 0.33	0.56 $\pm$ 0.20
	1:1	0.53 $\pm$ 0.24	0.40 $\pm$ 0.19	0.26 $\pm$ 0.15
	1:5	1.18 $\pm$ 0.38	1.23 $\pm$ 0.31	1.33 $\pm$ 0.40
	1:20	1.28 $\pm$ 0.31	0.91 $\pm$ 0.41	0.51 $\pm$ 0.39
	1:50	3.06 $\pm$ 1.08	2.75 $\pm$ 0.79	2.32 $\pm$ 0.88

* Values indicate: CI >1 antagonism, CI=1 additive effects, CI<0.9 synergy and CI<0.5 strong synergy