Targeted Inhibition of Multiple Receptor Tyrosine Kinases in Mesothelioma

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Abstract

The receptor tyrosine kinases (RTKs) epidermal growth factor receptor (EGFR) and MET are activated in subsets of mesothelioma, suggesting that these kinases might represent novel therapeutic targets in this notoriously chemotherapy-resistant cancer. However, clinical trials have shown little activity for EGFR inhibitors in mesothelioma. Despite the evidence for RTK activation in mesothelioma pathogenesis, it is unclear whether transforming activity is dependent on an individual kinase oncoprotein or the coordinated activity of multiple kinases. Using phospho-RTK and immunoblot assays, we herein demonstrate activation of multiple RTKs (EGFR, MET, AXL, and ERBB3) in individual mesothelioma cell lines but not in normal mesothelioma cells. Inhibition of mesothelioma multi-RTK signaling was accomplished using combinations of RTK direct inhibitors or by inhibition of the RTK chaperone, heat shock protein 90 (HSP90). Multi-RTK inhibition by the HSP90 inhibitor 17-allyloamino-17-demethoxygeldanamycin (17-AAG) had a substantially greater effect on mesothelioma proliferation and survival compared with inhibition of individual activated RTKs. HSP90 inhibition also suppressed phosphorylation of downstream signaling intermediates (AKT, mitogen-activated protein kinase, and S6); upregulated the p53, p21, and p27 cell cycle checkpoints; induced G2 phase arrest; induced caspase 3/7 activity; and led to an increase in the sub-G1 apoptotic population. These compelling proapoptotic and antiproliferative responses indicate that HSP90 inhibition warrants clinical evaluation as a novel therapeutic strategy in mesothelioma.

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Introduction

Malignant mesothelioma is a locally aggressive and highly lethal neoplasm in which the neoplastic proliferation originates from pleural, peritoneal, or, rarely, pericardial mesothelial cells [1]. Mesothelioma has been linked to asbestos exposure [1], and epidemiologic studies also show that mesothelioma risk increases after inhalation of the airborne mineral dust, erionite [2]. In addition, some investigations have implicated SV40 virus in the pathogenesis of a subset of mesotheliomas [3]. Mesothelioma histologic subtypes include epithelioid, spindle, or mixed (epithelioid and spindled) [1], of which the spindled subtype generally has the worst prognosis. Mesothelioma incidence increased after 1970, reflecting heavier worldwide occupational asbestos exposure after World War II [4]. An estimated 3000 new cases per year are diagnosed in the United States, and most

Abbreviations: 17-AAG, 17-allyloamino-17-demethoxygeldanamycin; AKT, v-akt murine thymoma viral oncogene homolog; EGFR, epidermal growth factor receptor; ERBB3, epidermal growth factor receptor 3; HSP90, heat shock protein 90; MAPK, mitogen-activated protein kinase; Meso, mesothelioma; MET, hepatocyte growth factor receptor; RTK, receptor tyrosine kinase

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2This article refers to supplemental material, which is designated by Table W1 and is available online at www.neoplasia.com.

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patients die within 1 year, with fewer than 5% of all patients cured, even when intensive combined modality therapeutic strategies are used. Conventional chemotherapies and radiation therapy have only limited efficacy against mesothelioma, and improved survival will presumably require development of novel and more effective pharmacological interventions. An improved understanding of mesothelioma biology—including key growth factor signaling pathways—might be useful in identifying biologically rational targets for novel therapies.

Various studies suggest that receptor tyrosine kinase (RTK) activation participates in the oncogenic progression of nonneoplastic mesothelial progenitor cells to malignant mesothelioma. For example, epidermal growth factor receptor (EGFR) is upregulated and tyrosine-phosphorylated in some mesotheliomas, resulting in downstream activation of the mitogen-activated protein kinase (MAPK) proliferation–associated signaling pathway [5,6], and implicating EGFR activation in asbestos-induced mesothelial mitogenicity and carcinogenesis. Furthermore, gefitinib, a specific EGFR small molecular inhibitor, inhibits EGFR-mediated AKT and MAPK phosphorylation, and inhibits proliferation in some mesothelioma cell lines [7]. The MET RTK protein and its ligand (hepatocyte growth factor) are co-overexpressed in mesothelioma compared with nonneoplastic mesothelial cells [8,9], and MET activation induces mesothelioma cell proliferation [9]. Furthermore, intragenic MET point mutations have been reported in a minority of mesotheliomas [10]. The MET inhibitor, PHA-665752, causes cell cycle arrest and decreased phosphorylation of MET, p70S6K, AKT, and MAPK in mesothelioma cell lines [11], and likewise MET inhibition by the small molecule inhibitor, SU11274, or by RNAi knockdown, inhibits mesothelioma cell line proliferation [10]. SV40 infection might contribute to autocrine mechanisms of MET activation in mesothelioma, thereby providing an intriguing connection between mesothelioma pathogenesis and potential drug targets [12]. In addition, the RTK EPHB4 is highly expressed in mesothelioma but not in normal mesothelial cells. EPHB4 knockdown is associated with inhibition of mesothelioma cell proliferation, migration, and invasion, accompanied by caspase-8–mediated apoptosis and down-regulation of the antiapoptotic protein Bcl-xl [13]. Vascular endothelial growth factor receptor 1/2 intratumoral tyrosine kinase activation, in addition to facilitating mesothelioma angiogenesis and lymphangiogenesis [14], might directly regulate mesothelioma cell proliferation [15]. Insulin-like growth factor 1 (IGF-1) and IGF-1R, although expressed at comparable levels in nonneoplastic and malignant mesothelial cells [16], might also contribute to mesothelioma development, as evidenced by the consequences of IGF-1R inhibition, which inhibits mesothelioma proliferation and tumorigenicity [17].

Despite the previously mentioned evidence for tyrosine kinase activation in mesothelioma pathogenesis, targeted tyrosine kinase inhibitor (TKI) therapies have not had dramatic clinical success in mesothelioma. This is surprising, given the many examples of tyrosine kinase inhibitor therapeutic successes in human cancers, including targeting of HER2 in metastatic breast cancer [18], KIT and platelet-derived growth factor receptor A in gastrointestinal stromal tumors [19], ABL in chronic myelogenous leukemia [20], and EGFR in non–small cell lung cancer [21]. In the present studies, we demonstrate coactivation of multiple RTKs—including EGFR, ERBB3, MET, and AXL—in individual mesothelioma cell lines. We also demonstrate that heat shock protein 90 (HSP90) inhibition inactivates these multiple RTKs in a given mesothelioma, thereby maximizing proapoptotic and antiproliferative effects compared with the consequences of inactivating any single RTK in a given mesothelioma.

**Materials and Methods**

**Antibodies and Reagents**

Monoclonal antibody to EGFR (immunoprecipitation), phospho-tyrosine (PY99), and p53 and polyclonal antibodies to EGFR, MET, and AXL were from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies to AKT and cleaved caspase 3 were from Cell Signaling Technology (Beverly, MA). Antibodies to MAPK and p21

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**Figure 1.** Coactivation of multiple RTKs in mesothelioma cell lines. (A) Total cell lysates (500 μg) from MESO924 cells grown for 2 hours in serum-containing (FBS+) or serum-free (FBS−) medium were analyzed by phospho-RTK array. Each RTK is spotted in duplicate, and the spots at each corner are positive controls, whereas eight spots at the lower right are negative controls. (B) Total cell lysates (500 μg) from normal mesothelial cells grown for 2 hours in serum-free medium analyzed by phospho-RTK array. (C) Immunoblot evaluations of EGFR, ERBB3, MET, and AXL in MESO924 versus nonneoplastic mesothelial cells. Actin stain is a loading control.
were from Zymed/Invitrogen Laboratories (Invitrogen life Technologies, Carlsbad, CA). Phospho-specific antibodies and monoclonal antibody to S6 were from Cell Signaling Technology. Antibodies to ERBB3, p27, and β-actin were from NeoMarkers (Fremont, CA), BD Transduction Laboratories (San Jose, CA), and Sigma-Aldrich (St Louis, MO), respectively. Human Phospho-RTK Array Kit was from R&D Systems (Minneapolis, MN). 17-Allyloamino-17-demethoxygeldanamycin (17-AAG) and gefitinib were obtained from LC Labs (Woburn, MA), and PHA-665752 was from Tocris Biosciences (St Louis, MO). DP-3975 was obtained from Deciphera Pharmaceuticals (Lawrence, KS). All inhibitors were reconstituted in DMSO. Protein A– and

Figure 2. (A) Immunoblot analysis quantitations of EGFR, MET, and AXL expression in mesothelioma cells versus nonneoplastic mesothelial cells (HM3). The expression quantitations (lower panel) were normalized to the HM3 cells. (B) EGFR, MET, and AXL activation in mesothelioma cells was validated by immunoprecipitation of each kinase followed by phosphotyrosine immunoblot analysis.
protein G-Sepharose beads were purchased from Zymed Laboratories (Invitrogen Life Technologies).

**Novel Mesothelioma Cell Lines**

Ten mesothelioma cell lines were established from surgical materials in previously untreated patients. These studies were approved by the Brigham and Women’s Hospital Institutional Review Board, under a discarded tissues protocol. The MESO257 and MESO924 cell lines were established from epithelial-type mesotheliomas, whereas MESO296, MESO346, MESO507, MESO542, MESO647, and MESO1413 were from mixed-histology mesotheliomas, and MESO188 and MESO428 were from spindle cell mesotheliomas. Derivation of each cell line from the corresponding surgical specimen was corroborated by demonstrating persistence of unique clonal cytogenetic aberrations—as seen in the primary tumors—in each of the cell lines (data not shown).

The normal mesothelial cells in the phospho-RTK arrays were established in our laboratory, from a nonneoplastic pleural effusion. The normal mesothelial cells in the immunoblot analysis studies were established from nonneoplastic peritoneum, as described previously [22]. Mesothelioma cell lines were maintained in RPMI 1640 with 15% fetal bovine serum (FBS) containing penicillin/streptomycin and l-glutamine. Normal mesothelial cells were cultured in F10 containing 15% FBS, penicillin/streptomycin, l-glutamine, amphotericin, MitoTracker (BD Biosciences, Bedford, MA), and bovine pituitary extract.

**Phospho-RTK Array Analysis**

The Human Phospho-RTK Array Kit (R&D Systems) was used to determine the relative levels of tyrosine phosphorylation of 42 distinct RTKs, according to the manufacturer’s protocol. Briefly, mesothelioma cell lines were maintained in RPMI 1640 medium with 15% fetal bovine serum containing penicillin/streptomycin and l-glutamine. After incubating with DMSO versus 1 μM 17-AAG in serum-free medium for 6 hours, cell line lysates were prepared using lysis buffer (1% NP-40, 50 mM Tris-HCl pH 8.0, 100 mM

![Figure 3](image-url)

Figure 3. (A) Cell viability determined by the CellTiter-Glo ATP-based luminescence assay in mesothelioma cell lines after 72 hours of treatment with inhibitors of EGFR (1 μM gefitinib), MET (1 μM PHA-665752), AXL (1 μM DP-3975), and HSP90 (1 μM 17-AAG). Data were normalized to the DMSO control, and shown are mean values (±SD) of quadruplicate cultures. (B) Phospho-RTK arrays show concurrent inactivation of multiple RTKs in mesothelioma cell lines. After treatment with 17-AAG (1 μM) for 6 hours in serum-free medium, phosphorylation quantitations (lower panel) were normalized to the negative controls.
sodium fluoride, 30 mM sodium pyrophosphate, 2 mM sodium molybdate, 5 mM EDTA, 2 mM sodium orthovanadate) containing protease inhibitors (10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). The arrays were incubated with 500 μg of protein lysate overnight at 4°C after blocking for 1 hour with Array Buffer 1 (R & D Systems, Minneapolis, MN). The arrays were washed and incubated with a horseradish peroxidase–conjugated phospho-tyrosine detection antibody (1:5000). Detection was by chemiluminescence (Immobilon Western, Millipore Corporation, MA), and signals were captured using a FUJI LAS 1000-plus chemiluminescence imaging system. The intensity of each phospho-RTK array signal was quantitated by an image analyzer (FUJIFILM MultiGauge; Fuji Film, Tokyo, Japan), and relative intensities of the averaged signal from each pair of duplicated spots were determined in relationship to the negative control spots.

Protein Lysate Preparations and Immunoblot Analysis

Immunoblot evaluations of signaling pathway expression and activation were performed after incubating cells for 6 hours with kinase inhibitors or 17-AAG in a serum-free medium. Immunoblot evaluations of apoptosis and cell cycle biomarkers were evaluated after drug treatments in serum-containing medium for 48 hours. Lysates were rocked overnight at 4°C, and the supernatants were collected after microcentrifuging at 14,000 rpm for 20 minutes at 4°C. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Electrophoresis and immunoblot analysis were performed as described previously [23], with hybridization signals detected by chemiluminescence (Amersham Pharmacia Biotechnology, Little Chalfont, UK) and captured using a FUJI LAS1000-plus chemiluminescence imaging system (Fuji Film, Tokyo, Japan).

Immunoprecipitation Evaluations of EGFR, MET, and AXL Activation

One milligram of protein lysate (500 μl) was preadsorbed for 30 minutes using 20 μl of protein G or protein A beads at 4°C. Sepharose–protein G beads were used for mouse monoclonal antibody and goat polyclonal antibody, whereas sepharose–protein A beads were used for rabbit polyclonal antibody. Two micrograms of EGFR, MET, or AXL antibody was added to the supernatants and rocked for 2 hours at 4°C. Twenty microliters of sepharose–protein G or protein A beads was added and rocked overnight at 4°C. Beads were collected by centrifugation in a microcentrifuge at 10,000 rpm for 2 minutes at 4°C, washed three times (25 minutes for each wash) with 750 μl of immunoprecipitation (IP) buffer, followed by one wash in 750 μl of 10 mM Tris-HCl buffer (pH 7.6). Twenty microliters of loading buffer was added to the beads and boiled for 5 minutes at 97°C. EGFR, MET, and AXL activation and expression were evaluated by immunoblot analysis.

Cell Proliferation and Apoptosis Assays

MESO924, MESO257, MESO296, and MESO428 cells were plated in a 96-well flat-bottomed plate (Falcon, Lincoln, NJ) and cultured for 24 hours before being treated with gefitinib (1 μM), PHA-665752 (1 μM), DP-3975 (1 μM), alone or in combination, and with 17-AAG (0.1, 0.25, 0.5, 0.75, and 1 μM). Cell viability and apoptosis were determined using the CellTiter-Glo and Caspase-Glo 3/7 assays, respectively (Promega, Madison, WI), and measured
using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA). Data were normalized to the control group (DMSO). All experimental points were set up in four replicate wells and independently performed in duplicate.

Cell Cycle Analysis
MESO924, MESO257, MESO296, and MESO428 cells in six-well plates were treated with inhibitors for 48 hours, then trypsinized and washed once with Hank’s balanced salt solution. For nuclear staining, a 4′,6-diamidino-2-phenylindole (DAPI)-containing solution (Nuclear Isolation and Staining Solution; NPE Systems, Pembroke Pines, FL) was added to the cells, and the cell suspension was immediately analyzed in a flow cytometer (NPE Quanta; NPE Systems) using ModFit LT software 3.1 (Verity Software House, Topsham, ME).

Results

Expression of Multiple Activated RTKs in Mesothelioma
Phospho-RTK array assays, in MESO924 malignant mesothelioma cells, demonstrated coordinated activation of RTKs EGFR, ERBB3, AXL, and MET, irrespective of whether the cells were evaluated in serum-containing or serum-free conditions (Figure 1A). However, IGF-1R was activated only in serum-containing conditions (Figure 1A). By contrast, phospho-RTK array of normal mesothelioma cells in serum-free conditions demonstrated apparent activation of EGFR, only, among the RTKs (Figure 1B), and this finding was not corroborated by immunoblot analysis (Figure 1C), indicating that the array phopsho-EGFR expression was a false-positive result. Likewise, we found, in validations of other human cancers, that the phospho-array EGFR activation signal was overly sensitive (data not shown). The immunoblot assays demonstrated that EGFR, MET, ERBB3, and AXL activation, in MESO924, resulted in part from a higher expression of each of these kinases, compared with the expression levels in the normal mesothelial cells (Figure 1C).

Validation studies confirmed a strong expression of EGFR, MET, and AXL in a large panel of mesothelioma cell lines but not in normal mesothelial cells (Figure 2A). EGFR, MET, and AXL were co-expressed in most mesothelioma cell lines at levels two- to five-fold higher than in the normal mesothelial cells (Figure 2A). In addition, phosphotyrosine characterization demonstrated that EGFR, MET, and AXL were coordinately activated in the mesothelioma cell lines (Figure 2B).

Consequences of Single Versus Combination Tyrosine Kinase Inhibitor Treatments on Mesothelioma Viability
Inhibitors of EGFR (gefitinib), MET (PHA-665752), and AXL (DP-3975) were evaluated singly and in combination and were contrasted with HSP90 inhibitor (17-AAG) treatment, in novel low-passage mesothelioma cell lines (Figure 3A). In all mesothelioma cell lines, the greatest reduction in cell viability (>40%) was achieved by coordinated inhibition of EGFR, MET, and AXL, either through use of multiple kinase inhibitors or by HSP90 inhibition (Figure 3A). EGFR and MET inhibition, whether singly or in combination, had little effect on mesothelioma viability (Figure 3A). AXL inhibition resulted in 60% reduction of mesothelioma cell viability in MESO257, but with lesser effects in the other cell lines, whereas combined AXL and EGFR inhibition resulted in greater than 40% reduction in viability for all cell lines (Figure 3A).

Inactivation of Multi-RTKs and Downstream Intermediates by HSP90 Inhibition
The observation that individual RTK inhibitors have little effect on mesothelioma cell viability (Figure 3A) suggested that activation of any one RTK is insufficient to sustain mesothelioma growth and/
or survival. Therefore, we hypothesized that inactivation of multiple RTKs by HSP90 inhibition might inhibit mesothelioma growth and survival mechanisms, including phosphoinositide 3-kinase (PI3-K)/AKT and MEK/MAPK signaling [10,11]. This possibility is supported by published evidence that EGFR and MET are activated in many mesotheliomas [7,10,11] and evidence that HSP90 has crucial roles in maintaining conformation and stability of many activated RTKs, including EGFR and MET [24].

HSP90 inhibition by 17-AAG treatment in MESO924 and MESO428 inactivated EGFR, MET, ERBB3, AXL, EPHA7, and EPHB2, as shown by tyrosine kinase phosphorylation quantitations (Figure 3B). EGFR, MET, and AXL inactivation, in 17-AAG–treated mesothelioma cells, was confirmed by immunoprecipitation and phospho-tyrosine (PY99) immunostaining (Figure 4A), and EGFR, MET, and ERBB3 inactivation were further validated by phospho-immunoblot analysis of mesothelioma total cell lysates (Figure 4B). As expected, HSP90 inhibition induced degradation of these tyrosine kinases, as evidenced by the reduction of total EGFR, MET, and AXL expression after 48 hours of 17-AAG treatment (Figure 5A). AKT and S6 were substantially and dose-dependently inactivated in all

**Figure 4.** (continued).
mesothelioma cell lines after HSP90 inhibition, whereas MAPK was inactivated in two of the mesothelioma lines (MESO296 and MESO428; Figure 4B).

**HSP90 Regulation of Mesothelioma Proliferation and Survival**

Cell proliferation, as assessed using an ATP-based cell viability assay (CellTiter-Glo), was strongly inhibited in all mesothelioma cell lines after HSP90 inhibition (Figure 6A): 17-AAG half maximal inhibitory concentration (IC50) values in mesotheliomas with spindle-cell components, MESO296 and MESO428, were 69 and 72 nM, respectively, and in epithelioid mesotheliomas, MESO257 and MESO924, were 192 and 266 nM, respectively. These data suggest that HSP90 regulation of mesothelioma cell viability can be targeted effectively in poor-prognosis spindle-cell mesotheliomas.

HSP90 inhibition induced mesothelioma apoptosis, as evidenced by caspase 3 cleavage (Figure 5B), and heightened caspase 3/7 activity (Figure 6B). These 17-AAG effects were particularly prominent in MESO296, which also had the most nuclear fragmentation after 17-AAG treatment (Figure 6C and Table W1). HSP90 inhibition induced up-regulation of p27, p21, and p53 cell cycle checkpoint proteins (Figure 5B), which was accompanied by cell cycle arrest (Figure 6C and Table W1).

Cell cycle analyses demonstrated dose-dependent G2 block with decreased S-phase population after HSP90 inhibition by 17-AAG (Figure 6C and Table W1). HSP90 inhibition induced apoptosis, as evidenced by nuclear fragmentation in all mesothelioma lines (Figure 6C: blue sub-G1 peak). Fragmented/apoptotic cells, in MESO924, MESO257, MESO296, and MESO428 treated with DMSO-only control versus 0.5 μM 17-AAG were 0.1% versus 8.0%, 1.7% versus 4.4%, 1.7% versus 20%, and 0.7% versus 2.6%, respectively (Figure 6C and Table W1).

**Discussion**

Available therapies, including surgery, radiation, and chemotherapy, have not substantially improved survival for patients with mesothelioma. Hence, there is an urgent need to validate novel and biologically rational therapies for this invariably lethal disease. Tyrosine kinase proteins regulate cell proliferation and survival in many human cancers [25], and there is increasing evidence that tyrosine kinase activation promotes biologic progression from nonneoplastic mesothelial progenitor cells to malignant mesothelioma. The studies reported here show that multiple RTKs are coactivated and coexpressed in an HSP90-dependent manner in individual mesothelioma populations, whereas these kinases are not strongly activated in normal mesothelial cells. The activated tyrosine kinases contribute collectively to mesothelioma PI3-K/AKT survival and proliferation signaling, suggesting that inhibitors of HSP90-mediated kinase chaperone functions might be useful therapeutic strategies in mesothelioma.

Our studies—using novel and histologically representative mesothelioma cell lines—are consistent with recent clinical evidence that inhibitors of individual activated tyrosine kinases generally do not induce major therapeutic responses in mesothelioma [26,27]. We
demonstrate activation and expression of multiple RTKs in individual mesotheliomas but not in normal mesothelial cells (Figures 1 and 2). These studies suggest that the coordinated activation of multiple RTKs (including EGFR, MET, ERBB3, and AXL) in mesotheliomas might enable resistance to drugs targeting single RTKs.

Hepatocyte growth factor/MET signaling has been implicated in mesothelioma pathogenesis, with this ligand/receptor pair coordinate overexpressed [8] and contributing to invasiveness and proliferation [9,28]. In addition, MET oncogenic mutations have been identified in a subset of mesotheliomas [10], and the MET inhibitors, SU11274 and PHA-665752, and MET knockdown by RNAi, exert antiproliferative effects in mesothelioma cell lines by inhibiting MET-dependent PI3-K/AKT and RAF/MAPK signaling pathways, culminating in cell cycle arrest [10,11]. In the present study, neither did a MET inhibitor, PHA-665752, substantially antagonize mesothelioma viability (Figure 3A) nor did PHA-665752 inhibition of viability correlate with baseline MET tyrosine phosphorylation in the mesotheliomas (Figures 1 and 2B). Similarly, only minor reductions in mesothelioma viability were observed after gefitinib-mediated EGFR inhibition, and these effects did not correlate with baseline EGFR tyrosine phosphorylation (Figures 1 and 2B), although EGFR was expressed strongly (Figure 2A) [5,7,29]. Our findings are in keeping with the lack of gefitinib activity in a phase 2 mesothelioma trial [26]. Although activation of EGFR and MET was completely inhibited by gefitinib and PHA-665752 (data not shown), antiproliferative effects after combined inhibition of EGFR and MET were no greater than those observed after inhibition of either of these kinases, individually (Figure 3A). Other recent studies have shown that combined EGFR and MET inhibition can suppress mesothelioma proliferation, although it is not clear that the high drug concentrations responsible for such effects can be achieved clinically [30].

Using phosphotyrosine immunopurification, we have demonstrated AXL tyrosine kinase activation in mesotheliomas (Ou and Fletcher, unpublished observations). AXL has transforming activity in other human cancers [31,32], and herein we show that AXL inactivation inhibited mesothelioma viability by 20% to 50%, whereas the combination of EGFR, MET, and AXL inactivation inhibited viability by 40% to 60% (Figure 3A).
Many activated RTKs are dependent on HSP90-mediated chaperone functions, and we therefore hypothesized that HSP90 inhibition might interfere with the multi-RTK activation in mesothelioma. HSP90 optimizes and maintains folding and localization for many activated tyrosine kinases and also prevents their proteasomal degradation [24]. HSP90 is an abundant protein in eukaryotic cells, comprising up to 1% to 2% of the total cellular protein, and has key roles in regulating cell proliferation, differentiation, and apoptosis [33,34]. The HSP90 inhibitor 17-AAG, a geldanamycin derivative [35], binds a conserved ATP interaction pocket in the HSP90 NH2-terminal domain [36] and has activity against various human cancers in preclinical models, where it can be highly selective in degrading HSP90-client oncoproteins [37,38]. Whereas 17-AAG clinical applications have been hampered by its low water solubility, IPI-504, a 17-AAG derivative, exhibits improved aqueous solubility while maintaining the biologic HSP90-inhibitory properties of 17-AAG [39].

Furthermore, clinical trials are ongoing, in various cancer types, with new-generation synthetic HSP90 inhibitors. Additional studies are needed to determine which HSP90 inhibitors are most effective as mesothelioma multi-RTK inhibitors and to determine whether the compelling in vitro mesothelioma responses reported herein are achievable using in vivo models.

HSP90 inhibition by 17-AAG reduced cell viability dramatically (Figure 6A) in all mesothelioma cell lines and was associated with AKT and S6 inhibition (Figure 4B). Notably, 17-AAG inhibition of mesothelioma viability has also been reported, recently, in a different panel of mesothelioma cell lines [40], although biochemical correlates for 17-AAG treatment were not evaluated. In the studies performed herein, HSP90 inhibition was associated with concurrent AKT and MAPK inactivation in MESO296 and MESO428, whereas in the other mesothelioma lines, MAPK was not inactivated despite AKT inhibition (Figure 4B). These observations suggest that MAPK activation might not be HSP90-dependent in all mesotheliomas, or alternately, different HSP90 clients might both activate and inactivate MAPK, such that HSP90 inhibition results in little net change in MAPK phosphorylation. In keeping with these signaling perturbations, dose-dependent 17-AAG inhibition of the EGFR, MET, and ERBB3 RTKs was demonstrated by staining Western blots with phospho-specific antibodies and showed coordinate inactivation of EGFR, MET, and ERBB3 in all mesotheliomas (Figure 4B).

The effects of 17-AAG on AXL, EGFR, and MET activation were also evaluated by phospho-RTK array assays and by immunoprecipitation of each of these kinase proteins, followed by immunoblot analysis for phosphotyrosine (Figures 3B and 4A). These studies confirmed coordinate AXL, EGFR, and MET inactivation, after HSP90 inhibition, in all mesothelioma cell lines (Figure 4A). HSP90 inhibition reduced cell viability dramatically in each mesothelioma cell line (Figure 6A). Forty-eight-hour treatments with 17-AAG, in serum-containing medium, were associated with AXL, EGFR, and MET degradation in all cell lines (Figure 5A), accompanied by up-regulation of the cell cycle checkpoint inhibitors p21, p27, and p53 and caspase 3 cleavage (Figure 5B). Notably, higher levels of p21 and p27 expression are associated with improved survival in mesothelioma [41,42].

In conclusion, these studies demonstrate that mesothelioma cell proliferation and survival require coactivation of multiple RTKs, including EGFR, MET, and AXL. Signaling pathways dependent on these collective activated kinases can be antagonized by treatment with HSP90 inhibitors, which inactivate the kinases or block downstream survival/proliferation signaling. Hence, HSP90 inhibition, whether as a single agent or combination therapy, warrants evaluation as a novel therapeutic strategy in mesothelioma.

References


Table W1. Cell Cycle Analyses (%), as Shown in Figure 6C, after 17-AAG Treatment.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>MESO924</th>
<th>MESO257</th>
<th>MESO296</th>
<th>MESO428</th>
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<td></td>
<td>$G_{1/0}$</td>
<td>$G_2$</td>
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<td>10</td>
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A indicates apoptosis before $G_1$ peak.