The HSP90 Inhibitor Ganetespib Synergizes with the MET Kinase Inhibitor Crizotinib in both Crizotinib-Sensitive and -Resistant MET-Driven Tumor Models

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Abstract
The proto-oncogene MET is aberrantly activated via overexpression or mutation in numerous cancers, making it a prime anticancer molecular target. However, the clinical success of MET-directed tyrosine kinase inhibitors (TKI) has been limited due, in part, to mutations in the MET kinase domain that confer therapeutic resistance. Circumventing this problem remains a key challenge to improving durable responses in patients receiving MET-targeted therapy. MET is an HSP90-dependent kinase, and in this report we show that HSP90 preferentially interacts with and stabilizes activated MET, regardless of whether the activation is ligand-dependent or is a consequence of kinase domain mutation. In contrast, many MET-TKI show a preference for the inactive form of the kinase, and activating mutations in MET can confer resistance. Combining the HSP90 inhibitor ganetespib with the MET-TKI crizotinib achieves synergistic inhibition of MET, its downstream signaling pathways, and tumor growth in both TKI-sensitive and -resistant MET-driven tumor models. These data suggest that inclusion of an HSP90 inhibitor can partially restore TKI sensitivity to previously resistant MET mutants, and they provide the foundation for clinical evaluation of this therapeutic combination in patients with MET-driven cancers.

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Introduction
The proto-oncogene product MET is a receptor tyrosine kinase whose ligand is the hepatocyte growth factor/scatter factor (HGF/SF). HGF binding to MET induces receptor dimerization and trans-phosphorylation, and promotes activation of several signaling networks including phosphoinositide 3-kinase (PI3K)–AKT and mitogen-activated protein/extracellular signal–regulated kinase (MEK) pathways (1). Activating point mutations in the MET kinase domain are implicated in the etiology of hereditary papillary renal carcinoma and have also been detected in sporadic papillary renal carcinoma, lung cancer, and gastric cancer (2–5). Furthermore, amplification of the MET gene locus has been detected in patients with gastric and metastatic colorectal cancers (6, 7). Cell lines engineered to express high levels of wild-type MET or constitutively active mutant MET display a proliferative, motogenic, and invasive phenotype, and form metastatic tumors in nude mice (8–11). MET is a validated molecular target for cancer therapy, and MET tyrosine kinase inhibitors (TKI) represent a promising treatment modality. Crizotinib, an orally available ATP-competitive and selective small-molecule inhibitor of MET, exhibits marked antitumor activity in several MET-dependent xenograft models (12). A recent phase 2 study of the dual MET/VEGFR2 inhibitor foretinib in patients with papillary renal cell carcinoma reported an overall response rate (using the Response Evaluation Criteria in Solid Tumors version 1.0) of 13.5%, and the presence of a germline MET mutation was highly predictive of a response (13). However, recent studies suggest that primary (de novo) resistance to TKIs is likely to be encountered in tumors harboring certain activating MET mutations. Thus, although crizotinib can inhibit the activity of most MET mutants, some constitutively active mutants, including MET-L1213V and -Y1248H, are resistant to this inhibitor (12, 14). Additional studies have suggested that these mutations not only mediate primary resistance to MET inhibitors but may also play a role in acquired resistance to MET-TKI (15–17).

HSP90 is a molecular chaperone whose association is required for the stability and function of multiple mutated or overexpressed signaling proteins, including many kinases, that promote the growth and survival of cancer cells. Small-molecule HSP90 inhibitors cause HSP90 to dissociate from its

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clients, resulting in their destabilization and eventual degradation. HSP90 inhibitors have been validated in numerous preclinical tumor models, and have shown promising activity in several clinical trials (18–20).

HSP90 inhibitors have been reported to destabilize MET (21, 22), as well as several MET-activated downstream signaling proteins, including AKT and RAF (23–25). Based on these findings, HSP90 inhibitors are expected to effectively interdict MET signaling at multiple points to inhibit the proliferation of MET-driven cancer cells. Indeed, the HSP90 inhibitors ganetespib (STA-9090) and SNX-2112 have each shown potent activity in several preclinical MET-driven tumor models, including those resistant to MET-TKI (26, 27). However, a detailed study of the MET-HSP90 interaction and its role in supporting MET activation has not been reported, nor has the possible synergy between HSP90 inhibition and MET-TKI been rigorously examined.

In the current study, we show that HSP90 interacts preferentially with activated MET, regardless of whether activation depends on HGF engaging the receptor or is mediated by kinase domain mutation. Further, we show that the activated MET fraction is most sensitive to HSP90 inhibition. The HSP90 inhibitor ganetespib displays synergy, both in vitro and in vivo, with the MET-TKI crizotinib in cells overexpressing wild-type MET. Unexpectedly, low-dose ganetespib also partially restores crizotinib sensitivity, both in vitro and in vivo, to cells and tumors expressing TKI-resistant MET mutants. Our findings support the use of HSP90 inhibitors to overcome or delay the initiation of resistance to MET-TKI, and they provide the basis for clinical evaluation of this combination in patients with MET-driven cancers.

Materials and Methods

Cell lines

MKN45, H1993, and HEK293 cell lines (American Type Culture Collection) were maintained under 5% CO2 at 37°C in either RPMI1640 (MKN45, H1993), or Dulbecco’s Modified Eagle Medium (DMEM; HEK293) supplemented with 10% FBS. NIH3T3 cell lines stably expressing either wild-type or mutant MET proteins (V1238I, H1112Y, Y1248H, L1213V, M1268T, and V1110F) were kindly provided by Dr. Laura Schmidt [National Cancer Institute (NCI), Bethesda, Maryland]. These cell lines were cultured in DMEM with 10% FBS and 0.5 mg/mL G-418.

Antibodies and reagents

Antibodies to MET, phospho-Tyr1234/35 MET, Akt, phospho-Ser473 Akt, Erk1/2, phospho-Thr202/Tyr204 Erk1/2 (Cell Signaling Technology), HSP90 (StressGen), V5 (Invitrogen), ubiquitin (Santa Cruz Biotechnology), and α-tubulin (Calbiochem) were used for immunoprecipitation and/or immunoblotting. Geldanamycin was obtained from the Developmental Therapeutics Program, NCI. Ganetespib and crizotinib were obtained from Synta Pharmaceuticals. Mouse immunoglobulin G (IgG), MG132, and recombinant human HGF were purchased from Millipore, Sigma, and R&D Systems, respectively. FuGene6 (Roche) was used for transient transfection.

Immunoprecipitation and immunoblotting

These experiments were performed as previously described (28). Briefly, cells were lysed in buffer containing 20 mmol/L Hepes, 100 mmol/L NaCl, 1 mmol/L MgCl2, 0.1% Nonidet P-40, 20 mmol/L Na2MoO4, phosphatase, and protease inhibitors. Immunoprecipitates or cell lysates were resolved by 4% to 20% SDS–PAGE, transferred to a nitrocellulose membrane, and probed with respective antibodies.

Plasmid constructs

Human MET cDNA was kindly provided by Dr. Don Bottaro (NCI, Bethesda, Maryland). Chimeric mutant MET/EGFR was generated using PCR and ligated into a pcDNA vector (Invitrogen) in-frame with the C-terminal V5 tag. A point mutation was made by using QuickChange (Stratagene) according to the manufacturer’s instructions.

Cell proliferation assay

Cells were seeded in 96-well plates at a density of 5,000 cells per well and incubated for 24 hours, followed by addition of drugs. After 48 hours, MTT solution (Sigma) was added and plates were incubated at 37°C for 3 hours. The optical density at 570 nm was determined by a spectrophotometer (Bio-TEK). The combination index (CI) was calculated by the median-effect method of Chou and Talalay (29) using CalcuSyn software (Biosoft).

Cell-cycle analysis

Cells were plated at a density of 3 × 10^5 in 10-cm dishes and incubated for 24 hours, followed by the addition of drugs. After 48 hours, cells were fixed with 70% ethanol for 4 hours at −20°C, and then suspended in a solution containing 0.04% digitonin. Cells were incubated at 37°C for 1 hour in a solution containing RNase A (100 μg/mL; Novagen) and propidium iodide (50 μg/mL), and then analyzed with a FACSCalibur flow cytometer (Becton Dickinson).

Colony formation assay

Cells were plated at a density of 1 × 10^4 in 60-mm dishes containing 0.4% top low-melting agarose and 0.5% bottom low-melting agarose medium, and cultured for 3 weeks. Colonies with a diameter of more than 0.1 mm were counted in five random high-power fields.

Migration and invasion assay

Twelve-well polycarbonate Transwell chambers with 8-μm pores (Corning) coated with Matrigel (BD Biosciences) were used. Cells (1 × 10^5) were added to the top well in serum-free medium with indicated drugs. In the bottom well, medium with 10% FBS was used as the chemoattractant. After 48 hours, cells on the upper surface of the filter were carefully removed with cotton pads. Migrated cells were fixed in 3.7% formaldehyde and stained with 4’, 6-diamidino-2-phenylindole (DAPI). The invaded cells were fixed and stained with Diff-Quik Stain kit (Dade Behring). The number of cells in five random high-power fields was determined.
Animal experiments

Animal experiments and procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. To establish tumor xenografts, cells (1 × 10⁶) were injected into the flank of female Nu/Nu mice (6 weeks of age; Taconic). After tumors reached a mean volume of 100 mm³, mice were administered crizotinib in water by oral gavage or ganetespib in the diluent 10/18 DRD [10% dimethyl sulfoxide (DMSO), 18% Cremophor RH40, 3.6% dextrose in water] by intraperitoneal injection. Tumor volume was calculated as the product of length × width² × 0.5. Fractional tumor volume (FTV) relative to untreated controls was determined as described previously (30). On the final day of the study, mice were humanely euthanized, and tumors were resected and pulverized using a homogenizer (Kinematica). Protein lysates were subjected to immunoblotting.

Statistical analysis

To determine the statistical significance of experimental data, we used the unpaired Student t test followed by the Bonferroni post hoc test for multiple comparisons. Data represent the mean ± SD. All P values less than 0.05 were considered statistically significant relative to control, and designated with an asterisk (*). Statistical analysis was done with the JMP software (SAS Institute).

Results

Wild-type MET is an HSP90-dependent kinase

We assessed the interaction of HSP90 and wild-type MET (wtMET) in the gastric cancer cell line MKN45, which over-expresses wtMET. As shown in Fig. 1A, endogenous HSP90 coprecipitated, albeit weakly, with wtMET. Previous reports by us and others have suggested that the activated states of some HSP90-dependent kinases have a greater dependence on HSP90 (31–33). To determine if this were the case for MET, we compared the degree of wtMET–HSP90 interaction in the presence and absence of the MET ligand HGF. Coimmunoprecipitation of HSP90 with MET clearly increased upon HGF stimulation and correlated with an increased population of activated (phosphorylated) MET (Fig. 1B). Next, we compared the sensitivity of total and activated wtMET protein to HSP90 inhibition. Exposure of MKN45 cells to the HSP90 inhibitor geldanamycin (GA) reduced the steady-state expression of total and activated MET (Fig. 1D), whereas brief exposure of MKN45 cells to geldanamycin increased MET ubiquitination, and this was further enhanced by cotreatment with a proteasome inhibitor (Fig. 1E).

HSP90 preferentially associates with activated MET

These data suggest that HSP90 interacts preferentially with and stabilizes the activated fraction of MET. In contrast to wtMET, which requires HGF binding for stimulation of its kinase activity, MET proteins with specific mutations in the kinase domain display constitutive activity in the absence of HGF. Therefore, we asked whether HSP90 also interacted robustly with a constitutively active MET mutant. To maintain a similar cell background, we immunoprecipitated MET from NIH3T3 cells stably expressing either wtMET or the constitutively active MET mutant Y1248H. HSP90 association with MET-Y1248H was markedly increased compared with wtMET (in the absence of exogenous HGF), in accordance with the different steady-state activation states of these MET proteins (Fig. 2A). Next, we compared the sensitivity of wtMET and the constitutively active MET-Y1248H mutant to geldanamycin. In stably transfected NIH3T3 cells, we found MET-Y1248H (both activated and total) to be more sensitive to Hsp90 inhibition compared with wtMET (Fig. 2B).

Consistent with these data, activated MET-Y1248H is robustly protected from geldanamycin by cotreatment with the proteasome inhibitor MG132 (Supplementary Fig. S1A). Similar to activated wtMET, loss of activated MET-Y1248H protein expression occurred much more rapidly in the presence of geldanamycin than after inhibition of protein synthesis (Supplementary Fig. S1B). Taken together, these data suggest that Hsp90-mediated stabilization of activated MET is required for optimal MET kinase activity. To provide further support for this hypothesis, we examined the ability of constitutively active MET-Y1248H to phosphorylate its substrate Gab1 (35) in NIH3T3/MET-Y1248H cells treated with the Hsp90 inhibitor ganetespib (Supplementary Fig. S2A). Importantly, Gab1 is not an Hsp90 client, and total endogenous Gab1 expression was not affected by ganetespib treatment. Nonetheless, ganetespib caused a dose-dependent reduction of Gab1 phosphorylation in these cells.

To better understand where HSP90 interacts with MET and how it might affect its kinase activity, we examined the αC-β4 loop located in the MET kinase domain. The amino acid composition of this short loop was shown to be important for HSP90 binding to a number of kinases including ErbB2 (36, 37). We reported previously that although the kinase domains of HSP90-dependent ErbB2 and HSP90-independent EGFR (ErbB1) are highly homologous, they vary in sequence in the αC-β4 loop. Replacement of the αC-β4 loop in ErbB2 with that of the EGFR abrogated HSP90 binding, whereas replacing the EGFR αC-β4 loop with that of ErbB2 conferred Hsp90 association and dependence (37). When we compared the sequence of the αC-β4 loops in EGFR and MET, we found a divergence in eight of 10 residues between amino acids 1,132 and 1,141 (numbering for MET kinase domain residues; highlighted in gray, Fig. 2C). To investigate whether these residues are an important determinant of MET/HSP90 interaction.
interaction, we replaced the eight divergent residues in V5-tagged MET-Y1248H with those found in the C-b4 loop of the EGFR (V5-MET-Y1248H/EGFR, highlighted in red, Fig. 2C). After transient transfection into HEK293 cells, we immunoprecipitated the tagged MET proteins with antibody to V5 and we assessed the relative association of both MET-Y1248H and MET-Y1248H/EGFR with HSP90. Although V5 immunoprecipitation affinity-purified equivalent amounts of tagged MET proteins from cell lysates, coimmunoprecipitated HSP90 was present in only trace amounts in MET-Y1248H/EGFR immune pellets. This was in distinct contrast to the amount of HSP90 found associating with MET-Y1248H protein (Fig. 2D), confirming that the amino acid composition of the C-b4 loop in the MET-kinase domain determines HSP90 association.

We noticed that, concurrent with loss of HSP90 association, the activated fraction of immunoprecipitated MET-Y1248H/EGFR was dramatically reduced compared with MET-Y1248H. To confirm this result, we blotted equivalent amounts of protein lysate from the transfected cells with antibody to phospho-MET. Substitution of the C-b4 loop in MET-Y1248H nearly completely abrogated its constitutive phosphorylation (Fig. 2E). To ascertain whether reduced constitutive activation also reflected an inability to respond to ligand, we compared the ability of HGF to activate MET-Y1248H/EGFR and wtMET in transiently transfected serum-starved HEK293 cells. The data show that, although constitutive phosphorylation of MET-Y1248H/EGFR is even less than that of wtMET (Fig. 2F, "HGF/C0" lanes), both proteins are comparably activated by HGF (Fig. 2F, "HGF+" lanes). Further, we found

Figure 1. MET is a client protein of HSP90. A, interaction between endogenous wild-type MET and HSP90. MKN45 cells were lysed and subjected to immunoprecipitation (IP) with anti-MET antibody followed by immunoblotting with anti-HSP90 and anti-MET antibodies. Immunoprecipitation with immunoglobulin G (IgG) was used as a negative control. Input represents 5% of the total protein extract used for IP. B, phosphorylated wild-type MET preferentially interacts with HSP90. NIH3T3 cells stably expressing wild-type MET were incubated in medium supplemented with (-) or without (+) serum including HGF. After 24 hours, cells were lysed and subjected to IP with anti-MET antibody and blotted with indicated antibodies. C, dose and time response of wild-type MET and phosphorylated wild-type MET to HSP90 inhibitor. MKN45 cells were incubated with increasing concentrations of geldanamycin (GA) for 24 hours (left), and incubated for increasing time intervals with 0.5 μmol/L GA (right). Cells were collected and subjected to immunoblotting. α-Tubulin was used as the loading control. D, GA-stimulated wild-type MET degradation is mediated by the proteasome. MKN45 cells were treated with the proteasome inhibitor MG132 (10 μmol/L) 1 hour before treatment with 0.5 μmol/L GA for an additional 8 hours. Cells were collected, lysed, and subjected to immunoblotting. E, HSP90 inhibition results in enhanced wild-type MET ubiquitination. MKN45 cells were treated with MG132 (10 μmol/L) 1 hour before treatment with 0.5 μmol/L GA for an additional 4 hours. Cells were collected, lysed, and subjected to IP with anti-MET antibody followed by immunoblotting with anti-poly-ubiquitin and anti-MET antibodies.
MET-Y1248H/EGFR to be more sensitive to crizotinib compared with MET-Y1248H (Fig. 2G), supporting an inverse correlation between Hsp90 interaction and crizotinib sensitivity. This hypothesis is consistent with in vitro data showing that the TKI sensitivity of constitutively active, bacterially expressed MET-Y1248H protein is antagonized by preincubation with purified Hsp90 before in vitro kinase assay. In contrast, the inclusion of geldanamycin restores sensitivity to MET-TKI in vitro (Supplementary Fig. S2B).

The MET inhibitor crizotinib synergizes in vitro with ganetespib in wtMET-driven cells

Most MET-TKIs are ATP-competitive inhibitors and preferentially target the inactive form of MET (38). In contrast, the data presented here show that Hsp90 inhibitors appear to preferentially target the activated MET and, at least in vitro, appear to enhance the sensitivity of activated MET to TKIs. To determine whether a similar phenomenon occurs in cells, we first assessed the effect of the MET-TKI crizotinib and of the
second-generation HSP90 inhibitor ganetespib, alone or combined, on the activation state of wtMET, and on downstream signaling to AKT and ERK1/2 in the wtMET-overexpressing gastric carcinoma cell line MKN45 and the non–small cell lung carcinoma cell line H1993. In both cell lines, the addition of low-dose ganetespib dramatically enhanced the impact of crizotinib on the activation of all three kinases (Fig. 3A). Although AKT itself is an HSP90 client, the lowest dose of ganetespib (10 nmol/L) had minimal activity when used alone in either cell line.

To investigate whether enhanced inhibition of these signaling pathways correlated with enhanced cellular activity, we...
performed MTT assays using MKN45 cells. The addition of increasing concentrations of ganetespib to a fixed set of crizotinib concentrations shifted the dose–response curves to the left in a (ganetespib) concentration-dependent manner (Fig. 3B), suggesting that the combination of these two drugs might be synergistic. We confirmed this to be the case by calculating the CI using the median-effect method of Chou and Talalay (29). CI values of less than 1 indicate synergy, whereas CI values of more than 1 reflect antagonism. Based on these data, ganetespib and crizotinib clearly synergize in MKN45 cells (CI = 0.6; Fig. 3C). Cell-cycle analysis revealed an increase in G1 phase in ganetespib/crizotinib-treated cells (as well as a small increase in sub-G1), and this was not observed in cells treated with either drug alone (Fig. 3D).

Next, we determined the effect of combination therapy on the transformed phenotype of MKN45 cells by assessing anchorage-independent colony formation. Low-dose crizotinib (10 nmol/L) and ganetespib (10 nmol/L) alone had marginal effects on colony formation. However, a combination of both drugs inhibited MKN45 colony formation by more than 90% (Fig. 3E). In addition, combination treatment more effectively inhibited MKN45 cell migration, compared with single-agent treatment at equivalent concentrations (Supplementary Fig. S3).

**Crizotinib-resistant MET mutants retain sensitivity to HSP90 inhibition**

*De novo* or acquired resistance to MET-TKI as a consequence of MET mutation remains a key therapeutic challenge to the clinical utility of these drugs. Several germline and somatic mutations in the tyrosine kinase domain of MET have been identified in hereditary papillary renal cancers, and these tumors are MET-driven (5, 13). A majority of these MET mutants are constitutively active, and some demonstrate de novo resistance to MET-TKI (9, 39). Because MET activity depends on association with HSP90, we speculated that HSP90 inhibitors might also retain activity toward a range of MET mutants, including those that are TKI resistant. To examine this possibility, we compared the inhibitory activity of single-agent crizotinib with that of single-agent ganetespib in six MET mutants—four that retain TKI sensitivity (MET-Y1248H, MET-H1112Y, MET-M1268T, and MET-V1110I) and two that are resistant to MET inhibitors (MET-Y1248H and MET-L1213V). For TKI-sensitive mutants, both MET phosphorylation and the activity of downstream signaling pathways (pAKT and pERK) were equally or more potently inhibited by ganetespib than by crizotinib (Fig. 4A and Supplementary Fig. S4). However, in contrast to crizotinib, ganetespib retained equivalent or greater inhibitory activity in TKI-resistant mutants compared with wtMET (Fig. 4B).

Given these data and in light of the synergy we observed for wtMET, we investigated next whether combining crizotinib and ganetespib might provide a therapeutic benefit in MET-TKI-resistant cells. Indeed, although crizotinib alone at a dose less than 0.5 µmol/L did not affect MET phosphorylation and downstream signaling in TKI-resistant MET-Y1248H–expressing NIH3T3 cells, low concentrations of ganetespib (10 nmol/L and 20 nmol/L, see also Fig. 4B) together with crizotinib (0.25 µmol/L) dramatically decreased pMET, pAKT, and pERK1/2 when compared with these concentrations of ganetespib alone (Fig. 5A). We observed similar results for TKI-resistant MET-L1213V–expressing NIH3T3 cells, suggesting that low-dose ganetespib can partially re-sensitize MET-TKI–resistant cells to crizotinib. To further examine this hypothesis, we assessed colony formation using MET-Y1248H–expressing NIH3T3 cells. Although at the concentrations chosen neither crizotinib (200 nmol/L) nor ganetespib (10 nmol/L) individually affected colony formation, when combined they dramatically inhibited the anchorage-independent growth of these cells (Fig. 5B). Indeed, with a calculated CI of less than 1 (see Supplementary Table S1), the combined activity of both drugs in this assay reflects synergy. In addition, combination therapy significantly inhibited the invasive capability of MET-Y1248H–expressing NIH3T3 cells at dose levels that were ineffective when administered separately (Fig. 5C). These *in vitro* data suggest that combining ganetespib with crizotinib provides at least an additive therapeutic benefit in MET-TKI-resistant cells.

**HSP90 inhibition synergizes in vivo with MET-TKI, even in MET-TKI–resistant xenografts**

In order to determine whether there may be therapeutic benefit in combining ganetespib with crizotinib *in vivo*, we employed two distinct xenograft models. MKN45 xenografts (expressing wtMET) were sensitive to MET-TKI (91% inhibition of tumor growth 36 days after tumor inoculation in mice treated daily with 50 mg/kg crizotinib) in agreement with previous reports (12, 27), whereas MET-Y1248H NIH3T3 xenografts were predictably MET-TKI resistant (59% inhibition of tumor growth at 21 days after daily treatment with 150 mg/kg crizotinib; Supplementary Fig. S5). In contrast, ganetespib was effective in both xenograft models, although at the maximum dose used in this study (50 mg/kg administered 3 times per week), somewhat greater activity was seen in wtMET xenografts (95% inhibition of tumor growth) compared with MET-Y1248H xenografts (82% growth inhibition; Supplementary Fig. S5).

To evaluate whether combination therapy was beneficial in the MKN45 model, we chose crizotinib (12.5 mg/kg) and ganetespib (12.5 mg/kg) dosing that provided approximately 50% growth inhibition when used singly (Supplementary Fig. S5). Treatment with this drug combination proved to be significantly more potent (95% growth inhibition) compared with single-agent treatment (56% growth inhibition for crizotinib alone and 64% growth inhibition for ganetespib alone; Fig. 6A).

In mice bearing TKI-resistant MET-Y1248H xenografts, we similarly observed that combination therapy with ganetespib and crizotinib (37.5 and 125 mg/kg, respectively) inhibited tumor growth by 90%, which was significantly greater than the inhibition achieved by treatment with either drug alone (65% growth inhibition for ganetespib and 31% growth inhibition for crizotinib; Fig. 6B).

Based on a comparison of expected and obtained FTVs in single agent- and combination-treated mice (30), crizotinib
and ganetespib synergized to inhibit both wtMET-driven and TKI-resistant MET (Y1248H)-driven xenograft growth (Supplementary Table S2). None of the drug regimens significantly affected animal body weight in either xenograft model (Fig. 6A and B). In agreement with these tumor growth data, the impact of combination therapy on tumor MET activation status and downstream signaling pathways was greater than single-agent treatment in both xenograft models, recapitulating the in vitro data described earlier (Fig. 6C and D).

**Discussion**

Although small-molecule kinase inhibitors have demonstrated clinical efficacy in cancer, patients who initially respond to such targeted therapy frequently will develop resistance. Mechanisms of resistance include the growth advantage provided by the appearance of drug-resistant kinase mutations (40), as well as a process termed "oncogene switching," whereby TKI-treated cells utilize an alternative kinase to drive shared downstream signaling pathways (41). Recent studies have revealed that HSP90 inhibition can overcome both forms of kinase inhibitor resistance. For example, HSP90 inhibition suppresses EGFR activity and downstream signaling in erlotinib-resistant EGFR/T790M-expressing cells (42), and suppresses ALK activity and signaling in cells expressing crizotinib-resistant EML4-ALK/L1196M (43). Similarly, targeting HSP90 prevents escape of ErbB2-driven breast cancer cells from chronic ErbB inhibition and escape of MET-amplified tumor cells from MET-TKI (44, 45).

TKI-resistance–conferring MET kinase domain mutations, including Y1248H and L1213V, have been identified in hereditary papillary renal cell carcinomas (14), and long-term exposure to MET TKI in vitro leads to acquisition of these mutations in MET-expressing gastric cancer cells (15, 16). Such kinase-activating mutations are thought to interfere with ATP-competitive TKI binding, and they likely contribute to both primary and acquired drug resistance in MET-dependent cancers. We have shown that a panel of MET proteins (both TKI-sensitive and TKI–resistant) retains dependence on HSP90 and remains sensitive to HSP90 inhibition. Importantly, we show that the active state of MET displays the strongest dependence on HSP90, whether activation is induced by HGF or is a consequence of kinase domain mutation (ligand-independent).

Because of the preference of HSP90 for activated MET, these data suggest the possibility that HSP90 inhibition might synergize with MET-TKI. Recent preclinical evaluation of the effects obtained upon simultaneously exposing acute myelogenous leukemia cells expressing activated FLT3 tyrosine kinase to both a FLT3-directed TKI and a HSP90 inhibitor lends support to this possibility, as does a recent...
Figure 5. MET TKI and HSP90 inhibitor synergize in TKI-resistant MET mutant-expressing cells. 

A, NIH3T3 cells stably expressing TKI-resistant MET-Y1248H (left) or MET-L1213V (right) were exposed to increasing concentrations of crizotinib in the presence or absence of defined concentrations of ganetespib, lysed, and subjected to immunoblotting. 

B, soft agar colony growth of NIH3T3 cells stably expressing TKI-resistant MET-Y1248H was assessed and analyzed as described in Figure 3E. C, combination treatment dramatically inhibits invasion of TKI-resistant MET-expressing cells. NIH3T3 cells stably expressing TKI-resistant mutant MET-Y1248H in serum-free medium containing indicated drugs were added to upper wells of Transwell chambers. The membrane separating top and bottom wells was coated with Matrigel. The bottom wells contained medium with 10% FBS as the chemoattractant. After 48 hours, invaded cells (e.g., cells appearing on the lower surface of the separating membrane) were stained and counted microscopically. Data are shown as mean ± SD of triplicate experiments. *; P < 0.05 vs. control (unpaired Student t test followed by Bonferroni test).

study reporting the combinatorial benefit of ganetespib, crizotinib, and other TKIs in the context of EML4-ALK–driven non–small cell lung cancer (46, 47). Our data show that simultaneous treatment with MET-TKI and HSP90 inhibitor causes a true synergistic inhibition of cell growth in wild-type MET-expressing MKN45 gastric cancer cells. Synergy of this drug combination in these cells was reflected by enhanced inhibition of downstream signaling pathways, significantly greater inhibition of colony growth and cell motility, and by significantly greater growth inhibition of MKN45 xenografts in vivo.

Unexpectedly, we found that HSP90 inhibition also partially restored crizotinib sensitivity to two TKI-resistant MET mutants. Combination treatment dramatically inhibited MET signaling, colony growth, cell invasion, and xenograft growth in vivo. Although some of these effects may be due to the inhibition of additional HSP90 clients functioning within or parallel to MET-driven signaling pathways, the data we have presented here indicate that, at least for MET, HSP90 inhibition directly affects MET sensitivity to a TKI. Our model is in general agreement with a recent study proposing that TKI binding stabilizes HSP90-dependent kinases and obviates the need for HSP90 interaction (48), because we have shown that it is the activated state of MET that interacts most strongly with and is most dependent on HSP90. Another report has suggested that TKI binding promotes kinase degradation by denying access to HSP90 (49), although in the case of MET it appears that this would be the case only if the TKI bound to the active conformation of the kinase, promoted dissociation of HSP90, and limited a return to the inactive conformation. The unexpected combinatorial benefit we have shown for TKI-resistant MET highlights the complex influence of HSP90 on kinase conformation, especially in the context of activating mutations, and our model provides a rationale for the increased dependence of other constitutively active kinase mutations on HSP90. For example, others have shown that HSP90 inhibitor synergizes with a Bcr-Abl TKI in TKI-resistant chronic myelogenous leukemia cells (50). Although the benefit of simultaneous treatment with TKI and HSP90 inhibitor should be evaluated on a case-by-case basis, a rationale for such a strategy clearly exists in certain settings. Specifically, our data suggest that a MET/HSP90 inhibitor combination regimen is a viable strategy to be explored in patients with
naive MET-dependent cancers, as well as in those patients whose cancers have developed resistance to MET-TKI. When delaying or reverse MET-TKI resistance, an effective MET/HSP90 inhibitor combination strategy may also require lower (and, thus, potentially less toxic) concentrations of crizotinib and ganetespib displays significantly greater efficacy compared with either drug alone in MET-driven xenograft tumor models. A and B, combination of suboptimal concentrations of crizotinib and ganetespib displays significantly greater efficacy compared with either drug alone in MET-driven xenograft tumor models. A, or NIH3T3 xenografts stably expressing TKI-resistant mutant MET-Y1248H (B) were administered crizotinib once daily and ganetespib 3 times per week, either as single agents or concurrently for 3 weeks (A) or 2 weeks (B). Tumor volume was measured using calipers on indicated days; data are shown as mean ± SD (n = 6 per group). Percentage tumor growth values were calculated on the final day of the study by comparing tumor volumes in drug- and vehicle-treated mice. *P < 0.05 vs. control (unpaired Student t test followed by Bonferroni test). Average body weight changes were measured over the course of the study (A and B, bottom graphs). C and D, pharmacodynamic assessment of the treatment regimens described in (A) and (B). Tumors were resected on the final day of the study 6 hours after drug administration, and subjected to immunoblotting as shown. Tubulin was used to demonstrate equal protein per sample.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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