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The HSP90 inhibitor ganetespib synergizes with the MET kinase inhibitor crizotinib in both crizotinib-sensitive and crizotinib-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 anti-cancer 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, 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 TKI-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.

Introduction

The proto-oncogene product MET is a receptor tyrosine kinase whose ligand is 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 PI3K-AKT and MAPK-ERK 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 (TKIs) 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 II study of the dual MET/VEGFR2 inhibitor foretinib in patients with papillary renal cell carcinoma reported an overall response rate (using Response Evaluation Criteria in Solid Tumors 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 TKI is likely 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).

Heat shock protein 90 (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 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, 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, *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 RPMI 1640 (MKN45, H1993), or DMEM (HEK293) supplemented with 10% FBS. NIH3T3 cell lines stably expressing either wild type or mutant MET proteins (V1238I, H1112Y, Y1248H, L1213V, M1268T and V1110I) were kindly provided by Dr. Laura Schmidt (NCI, Bethesda, MD, USA). These cells 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), HSP90 (StressGen), V5 (Invitrogen), ubiquitin (Santa Cruz), 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 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 mM Hepes, 100 mM NaCl, 1 mM MgCl₂, 0.1% Nonidet P-40, 20 mM Na₂MoO₄, phosphatase and protease inhibitors. Immunoprecipitates or cell lysates were resolved by 4–20% SDS/PAGE, transferred to nitrocellulose membrane, and probed with respective antibodies.

Plasmid constructs. Human MET cDNA was kindly provided by Dr. Don Bottaro (NCI, Bethesda, MD, USA). Chimeric mutant MET/EGFR was generated using PCR and ligated into pcDNA vector (Invitrogen) in-frame with the C-terminal V5 tag. Point

mutation was made using QuikChange (Stratagene) according to the manufacturer's instructions.

Cell proliferation assay. Cells were seeded in 96-well plates at 5,000 per well and incubated for 24 h, followed by addition of drugs. After 48 h, MTT solution (Sigma) was added and plates were allowed to incubate at 37°C for 3 h. Optical density at 570 nm was determined by spectrophotometer (Bio-TEK). 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 h, followed by addition of drugs. After 48 h, cells were fixed with 70% ethanol for 4 h at -20°C, and then suspended in a solution containing 0.04% digitonin. Cells were incubated at 37°C for 1 h 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 > 0.1 mm were counted in 5 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 upper well in serum-free medium with indicated drugs. In the lower well, medium with 10% FBS was used as chemo-attractant. After 48 h, 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 DAPI. Invaded cells were fixed and

stained with Diff-Quik Stain kit (Dade Behring). The number of cells in 5 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 (1×10^6) were injected into the flank of female Nu/Nu mice (6 weeks of age, Taconic). After tumors reached a mean volume of 100 mm^3 , mice were administered crizotinib in water by oral gavage or ganetespib in 10/18 DRD (10% DMSO, 18% Cremophor RH40, 3.6% dextrose in water) by intraperitoneal injection. Tumor volume was calculated as the product of length $\times$ width² $\times$ 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 statistical significance of experimental data, we used the unpaired Student's *t*-test followed by Bonferroni post hoc test for multiple comparisons. Data represent the mean $\pm$ SD. All P values < 0.05 were considered statistically significant relative to control and designated with an asterisk (*). Statistical analysis was done with 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 overexpresses wtMET. As shown in Figure 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. Co-immunoprecipitation of HSP90 with MET clearly increased upon HGF stimulation and correlated with an increased population of activated (phosphorylated) MET (Figure 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 both total and activated MET protein in a dose- and time-dependent manner, but the activated MET fraction was most sensitive to HSP90 inhibition (Figure 1C). Loss of MET protein expression occurred much more rapidly in the presence of GA than following inhibition of protein synthesis with cycloheximide (Figure S1B, MKN45 panels), supporting Hsp90 inhibitor-mediated destabilization of activated MET. Next, we determined whether HSP90 inhibition promotes wtMET ubiquitination and degradation by the proteasome, a common fate of most HSP90 clients deprived of interaction with the chaperone (34). Co-treatment with the proteasome inhibitor MG132 reversed the impact of HSP90 inhibition on steady-state expression of activated MET (Figure 1D), while brief exposure of MKN45 cells to GA increased MET ubiquitination and this was further enhanced by co-treatment with proteasome inhibitor (Figure 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 wt MET or the constitutively active MET mutant Y1248H. HSP90 association with MET-Y1248H was markedly increased compared to wtMET (in the absence of exogenous HGF), in accordance with the different steady state activation states of these MET proteins (Figure 2A). Next, we compared the sensitivity of wtMET and the constitutively active MET-Y1248H mutant to GA. In stably transfected NIH3T3 cells, we found MET-Y1248H (both activated and total) to be more sensitive to Hsp90 inhibition compared to wtMET (Figure 2B).

Consistent with these data, activated MET-Y1248H is robustly protected from GA by co-treatment with the proteasome inhibitor MG132 (Figure S1A). Like activated wtMET, loss of activated MET-1248H protein expression occurred much more rapidly in the presence of GA than after protein synthesis inhibition (Figure 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 (Figure 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 α C- β 4 loop located in the MET kinase domain. 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 α C- β 4 loop. Replacement of the α C- β 4 loop in ErbB2 with that of the EGFR abrogated HSP90 binding, while replacing the EGFR α C- β 4 loop with that of ErbB2 conferred Hsp90 association and dependence (37). When we compared the sequence of the α C- β 4 loops in EGFR and MET, we found a divergence in 8 of 10 residues between amino acids 1132 and 1141 (numbering for MET kinase domain residues; highlighted in grey, Figure 2C). To investigate whether these residues are an important determinant of MET/HSP90 interaction, we replaced the 8 divergent residues in V5-tagged MET-Y1248H with those found in the α C- β 4 loop of the EGFR (V5-MET-Y1248H/EGFR, highlighted in red, Figure 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, co-immunoprecipitated 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 (Figure 2D), confirming that amino acid composition of the α C- β 4 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 to 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 α C- β 4 loop in MET-Y1248H nearly completely abrogated its constitutive phosphorylation (Figure 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 (Figure 2F, 'HGF -' lanes), both proteins are comparably activated by HGF (Figure 2F, 'HGF +' lanes). Further, we found MET-Y1248H/EGFR to be more sensitive to crizotinib compared to MET-Y1248H (Figure 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-Y1248D protein is antagonized by pre-incubation with purified Hsp90 prior to *in vitro* kinase assay. In contrast, inclusion of GA restores sensitivity to MET TKI *in vitro* (Figure 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 activated MET and, at least *in vitro*, appear to enhance sensitivity of activated MET to TKI. 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, addition of low-dose ganetespib dramatically enhanced the impact of crizotinib on the activation of all three kinases (Figure 3A). Although AKT itself is an HSP90 client, the lowest dose of ganetespib (10 nM) 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. 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 (Figure 3B), suggesting that combination of these two drugs might be synergistic. We confirmed this to be the case by calculating the combination index (CI) using the median-effect method of Chou and Talalay (29). CI values under 1 indicate synergy, whereas CI values over 1 reflect antagonism. Based on these data, ganetespib and crizotinib clearly synergize in MKN45 cells (CI = 0.6, Figure 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 (Figure 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 nM) and ganetespib (10 nM) alone had marginal effects on colony formation. However, combination of both drugs inhibited MKN45 colony formation by more than 90% (Figure 3E). Combination treatment also more effectively

inhibited MKN45 cell migration, compared with single agent treatment at equivalent concentrations (Figure 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). Since MET activity depends on association with HSP90, we speculated that HSP90 inhibitors might also retain activity towards 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-V1238I, 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 (Figure 4A and Figure S4). However, in contrast to crizotinib, ganetespib retained equivalent or greater inhibitory activity in TKI-resistant mutants compared to wtMET (Figure 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 below 0.5 μ M did not affect MET phosphorylation and downstream signaling in TKI-resistant MET-Y1248H expressing NIH3T3 cells, low concentrations of ganetespib (10 nM and

20 nM, see also Figure 4B) together with crizotinib (0.25 μ M) dramatically decreased pMET, pAKT and pERK1/2 when compared to these concentrations of ganetespib alone (Figure 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 nM) nor ganetespib (10 nM) individually affected colony formation, when combined they dramatically inhibited the anchorage-independent growth of these cells (Figure 5B). Indeed, with a calculated CI of < 1 (see Table S1), the combined activity of both drugs in this assay reflects synergy.

Combination therapy also significantly inhibited the invasive capability of MET-Y1248H expressing NIH3T3 cells at dose levels that were ineffective when administered separately (Figure 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), while 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) (Figure S5). In contrast, ganetespib was

effective in both xenograft models, although at the maximum dose used in this study (50 mg/kg administered 3x per week), somewhat greater activity was seen in wtMET xenografts (95% inhibition of tumor growth) compared to MET-Y1248H xenografts (82% growth inhibition) (Figure S5).

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

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

Based on comparison of expected and obtained fractional tumor volumes (FTV) in single agent-treated and combination-treated mice (30), crizotinib and ganetespib synergized to inhibit both wtMET-driven and TKI-resistant MET (Y1248H)-driven xenograft growth (Table S2). None of the drug regimens significantly affected animal body weight in either xenograft model (Figure 6A, 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 (Figure 6C, 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 appearance of drug resistant kinase mutations (40), as well 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). Likewise, 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 study reporting the combinatorial benefit of ganetespib, crizotinib and other TKI 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*.

While some of these effects may be due to 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), since 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). While 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 naïve MET-dependent cancers, as well in those patients whose cancers have developed resistance to MET TKI. While delaying or reversing MET TKI resistance, an effective MET/HSP90 inhibitor combination strategy may also require lower (and thus potentially less toxic) concentrations of both drugs.

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Figure legends

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 h, cells were lysed and subjected to immunoprecipitation (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 h (left panel), and incubated for increasing time intervals with 0.5 μ M GA (right panel). Cells were collected and subjected to immunoblotting. α -tubulin was used as loading control.

D, GA-stimulated wild type MET degradation is mediated by the proteasome. MKN45 cells were treated with the proteasome inhibitor MG132 (10 μ M) 1 h before treatment with 0.5 μ M GA for an additional 8 h. 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 μ M) 1 h before treatment with 0.5 μ M GA for an additional 4 h. Cells were collected, lysed and subjected to immunoprecipitation (IP) with anti-MET antibody followed by immunoblotting with anti-poly-ubiquitin and

anti-MET antibodies.

Figure 2. Activated MET depends on HSP90 association for its stability.

A, Constitutively active mutant MET strongly interacts with HSP90. NIH3T3 cells stably expressing wild type (wt) MET or constitutively active mutant MET (Y1248H) were lysed and subjected to immunoprecipitation (IP) with anti-MET antibody followed by immunoblotting with anti-HSP90, anti-MET and anti-p-MET antibodies.

B, Constitutively active mutant MET is more sensitive to HSP90 inhibition than is wtMET. NIH3T3 cells stably expressing wtMET or constitutively active mutant MET (Y1248H) were incubated in medium supplemented with serum including HGF and increasing concentrations of GA for 24 h. Cells were collected and subjected to immunoblotting with indicated antibodies.

C, Alignment of the amino acid sequence of MET and EGFR kinase domain α C- β 4 loops. Amino acid sequences (in one-letter code) of MET and EGFR in the α C- β 4 loops were aligned by using MacVector software. Star denotes identity, and dot denotes similarity. Residues that differ between MET and EGFR are shaded; MET/EGFR denotes MET with highlighted residues from EGFR. Amino acid residues in MET and EGFR are numbered from the starting methionine.

D, MET-Y1248H/EGFR loses association with HSP90. Expression vectors encoding V5-tagged MET-Y1248H, MET-Y1248H/EGFR or an empty vector (pcDNA, used as a control) were transfected into HEK293 cells. After 48 h, cells were lysed and subjected to immunoprecipitation (IP) with anti-V5 antibody followed by immunoblotting with anti-HSP90, anti-V5 and anti-p-MET antibodies.

E, Steady-state phosphorylation of MET-Y1248H/EGFR is reduced compared to MET-Y1248H. Expression vectors encoding V5-tagged MET-Y1248H,

MET-Y1248H/EGFR or an empty vector (pcDNA) were transfected into HEK293 cells.

After 48 h, cells were lysed and subjected to immunoblotting as shown.

F, MET-Y1248H/EGFR retains HGF sensitivity. HEK293 cells were transfected with V5-tagged empty plasmid, wtMET, or MET-Y1248H/EGFR (2 µg each). After 48 h, cells were serum-starved overnight and then treated for 20 min with 1 nM HGF.

Samples were then lysed in buffer (20 mM Hepes, 100 mM NaCl, 1 mM MgCl₂, 0.1% NP-40, 20 mM Na₂MoO₄, phosphatase and protease inhibitors). Equal amounts of total cell lysate were resolved by Western blotting and probed with indicated antibodies.

After normalization to total MET protein, HGF increased the activated fraction of wtMET 4.8-fold and the activated fraction of MET-Y1248H/EGFR 8.9-fold (by densitometric analysis).

G, HEK293 cells were transfected as in *F*, treated with HGF and exposed to increasing concentrations of crizotinib (16 h). MET-Y1248H/EGFR, which displays reduced interaction with Hsp90, is markedly more sensitive to the TKI compared to MET-Y1248H.

Figure 3. MET TKI and HSP90 inhibitor synergize in wild type MET-overexpressing cells.

A, HSP90 inhibitor enhances the efficacy of MET TKI on wtMET phosphorylation and downstream signaling. MKN45 cells (left panel) and H1993 cells (right panel) were treated for 16 h with increasing concentrations of crizotinib alone or in combination with the indicated concentrations of ganetespib, lysed and subjected to immunoblotting as shown.

B-C, Combination of crizotinib and ganetespib synergistically inhibits wtMET-driven cell growth. MKN45 cells were treated with increasing concentrations of crizotinib

alone or in combination with the indicated concentrations of ganetespib for 48 h and subjected to MTT assay (B). Data are expressed as mean $\pm$ SD of triplicate experiments. Combination index (CI) was calculated to examine the synergistic effect of these drugs (C). CI values < 1 indicate synergy, whereas CI > 1 indicates antagonism between two drugs.

D, Synergistic growth inhibition is due to G1 arrest. MKN45 cells were treated with the indicated concentrations of crizotinib alone or in combination with 10 nM ganetespib for 48 h and subjected to cell cycle analysis.

E, Combination treatment dramatically inhibits soft agar colony growth of wtMET overexpressing cells. MKN45 cells were plated in 0.4% soft agar with indicated drugs, either alone or in combination, and cultures were maintained for 3 weeks.

Representative images of colonies are shown for each condition (*Scale bar*, 0.1 mm); colonies with a diameter of > 0.1 mm were counted microscopically and graphed. Data are means $\pm$ SD of triplicate experiments. *, $P < 0.05$ vs. control (unpaired Student's *t*-test followed by Bonferroni test).

Figure 4. MET kinase domain mutants retain sensitivity to HSP90 inhibition.

A, Ganetespib inhibits MET phosphorylation and downstream signaling in TKI-sensitive MET mutants. NIH3T3 cells stably expressing TKI-sensitive MET mutants (V1238I and H1112Y) were treated for 16 h with increasing concentrations of either crizotinib or ganetespib, lysed and subjected to immunoblotting.

B, Ganetespib inhibits MET phosphorylation and downstream signaling in TKI-resistant MET mutants. NIH3T3 cells stably expressing TKI-resistant MET mutants (Y1248H and L1213V) were treated and analyzed as described above.

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 panel) or MET-L1213V (right panel) 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 mutant 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 upper and lower wells was coated with matrigel. Lower wells contained medium with 10% FBS as chemo-attractant. After 48 h, invaded cells (e.g., cells appearing on the lower surface of the separating membrane) were stained and counted microscopically. Data are shown as mean $\pm$ SD of triplicate experiments. *, $P < 0.05$ vs. control (unpaired Student's *t*-test followed by Bonferroni test).

Figure 6. MET TKI and HSP90 inhibitor synergistically inhibit tumor growth in MET-driven xenograft tumor models.

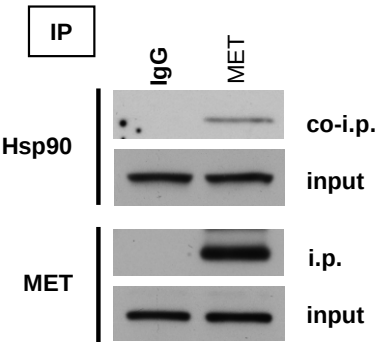
A-B, Combination of sub-optimal concentrations of crizotinib and ganetespib displays significantly greater efficacy compared to either drug alone in MET-driven xenograft tumor models. Athymic mice bearing established MKN45 xenografts (*A*), or NIH3T3 xenografts stably expressing TKI-resistant mutant MET-Y1248H (*B*) were administered crizotinib once daily and ganetespib three 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 $\pm$ SD (n=6/group). Percent tumor growth values were calculated on the final day of study by comparing tumor volumes in drug-treated and vehicle-treated mice. *, $P < 0.05$ vs. control (unpaired Student's *t*-test followed by Bonferroni test). Average body weight changes were measured over the course of the study (A-B, lower graphs).

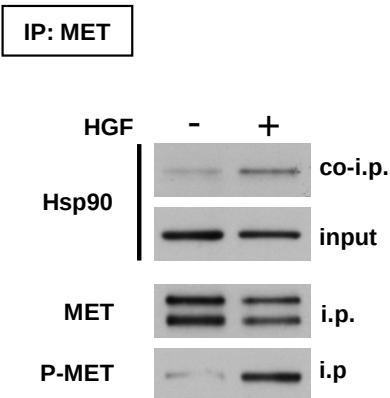
C-D, Pharmacodynamic assessment of the treatment regimens described in panels A and B. Tumors were resected on the final day of the study 6 h post drug administration, and subjected to immunoblotting as shown. Tubulin was used to demonstrate equal protein per sample.

Miyajima et al., Figure 1

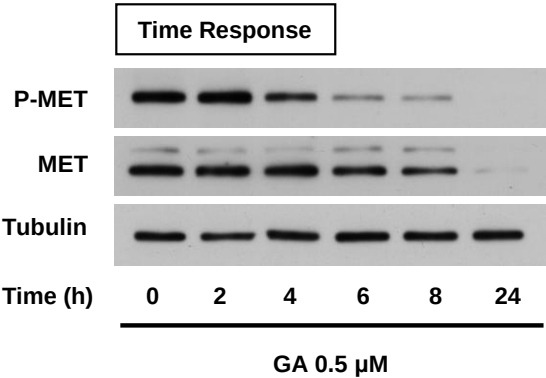
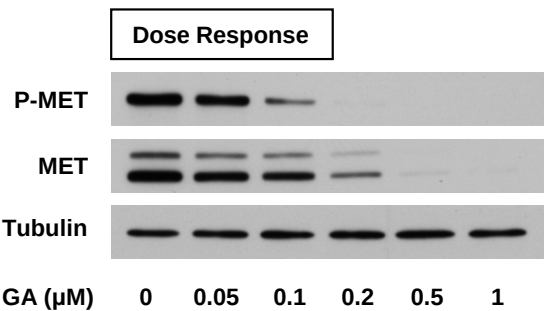
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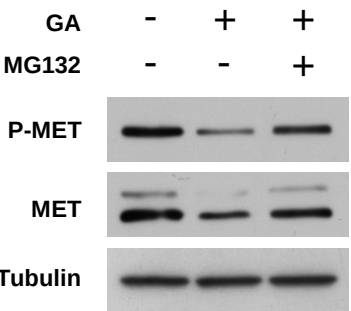
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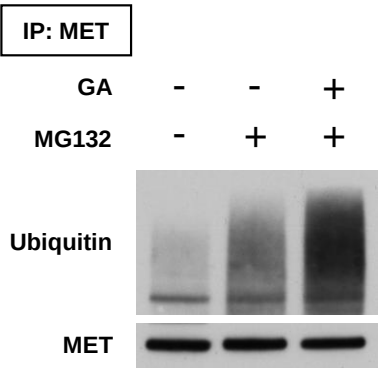
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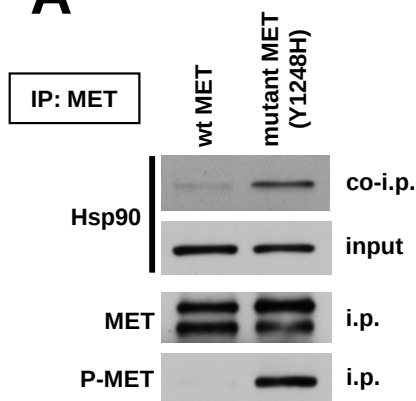


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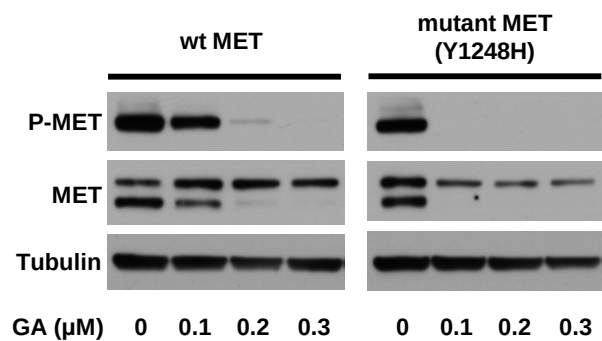


Miyajima et al., Figure 2

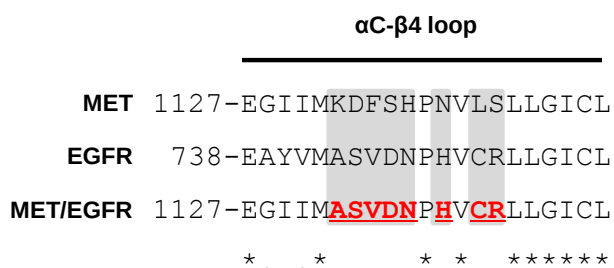
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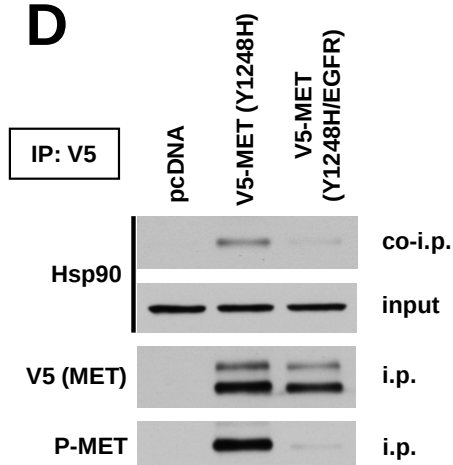
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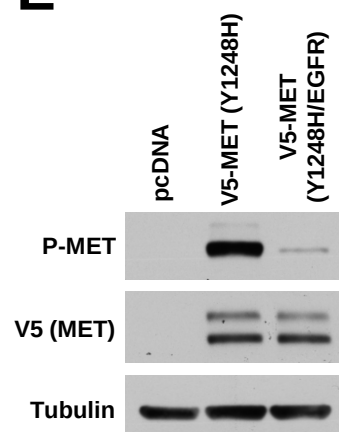
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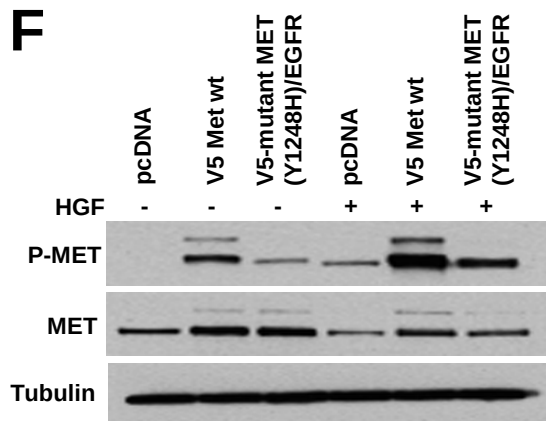
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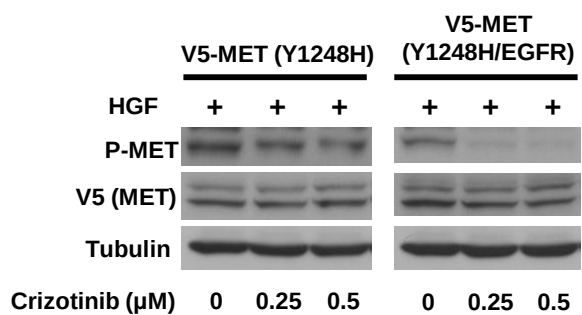
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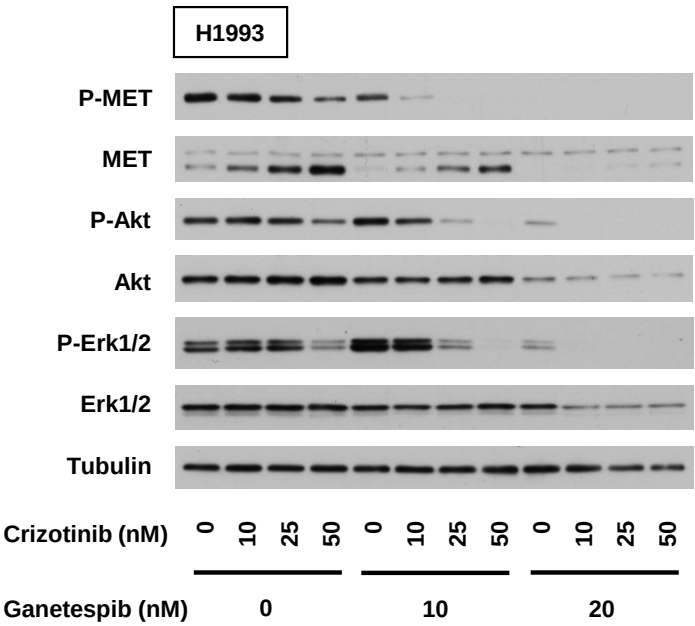
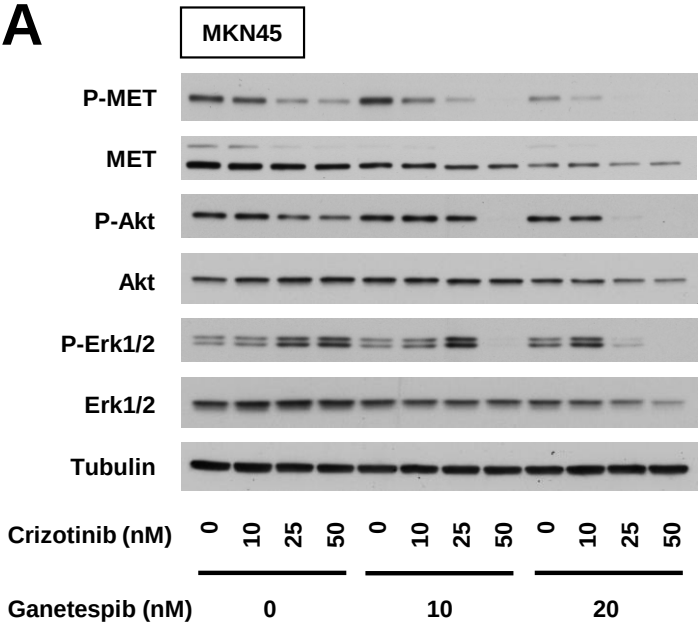


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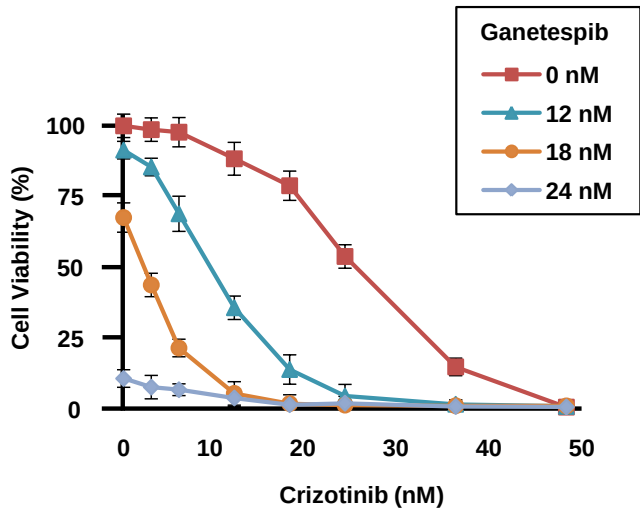


Miyajima et al., Figure 3

A



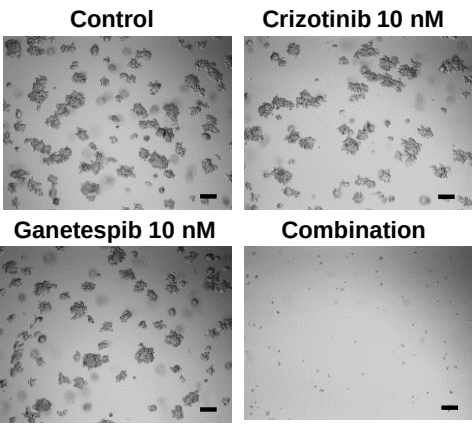
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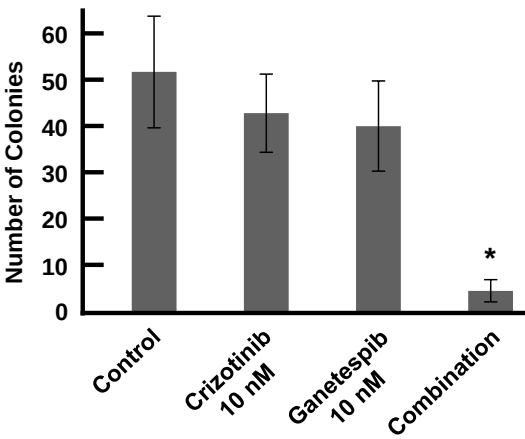
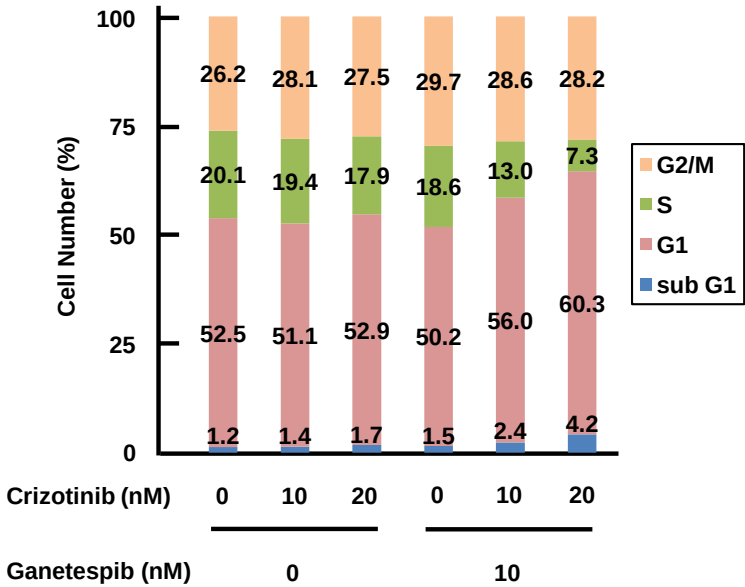
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Crizotinib	Ganetespib	Combination Index (CI)
24 nM	12 nM	0.60
36 nM	12 nM	0.61

E

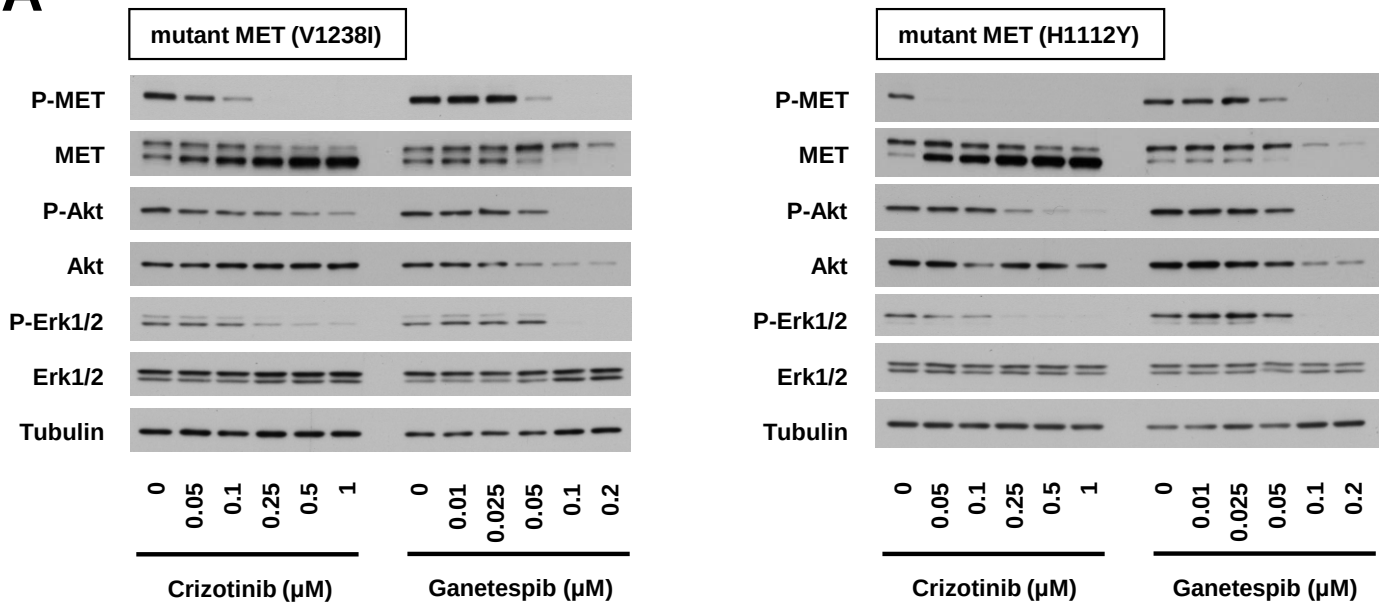


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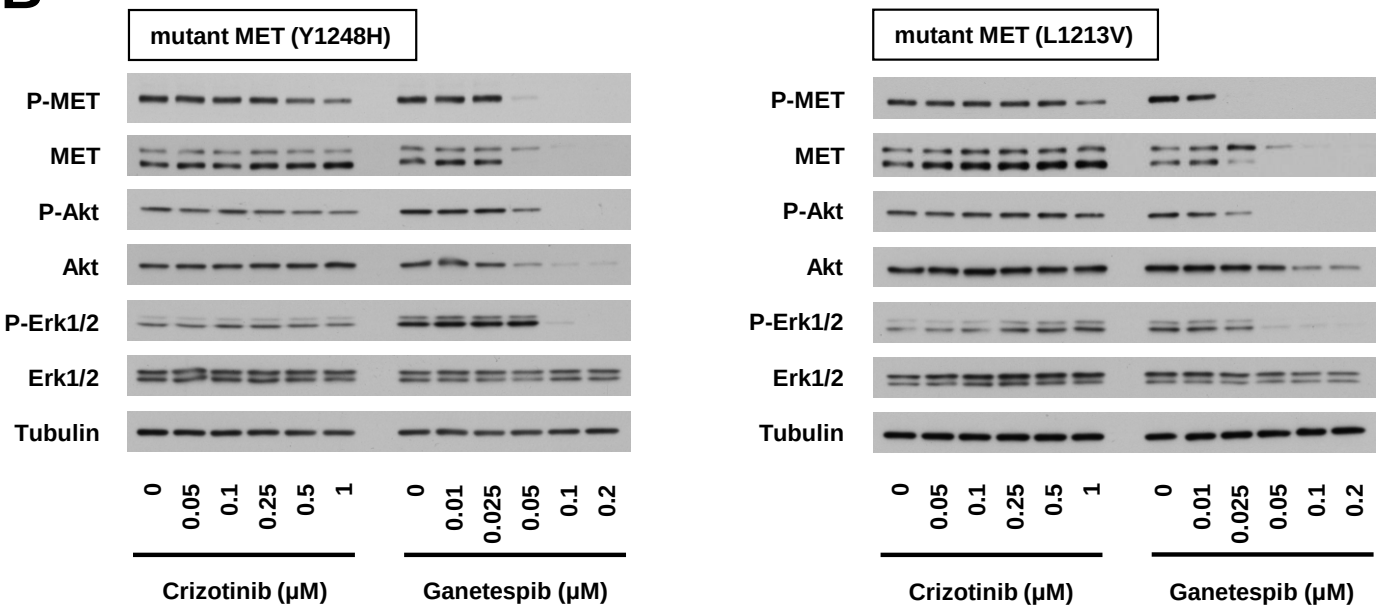


Miyajima et al., Figure 4

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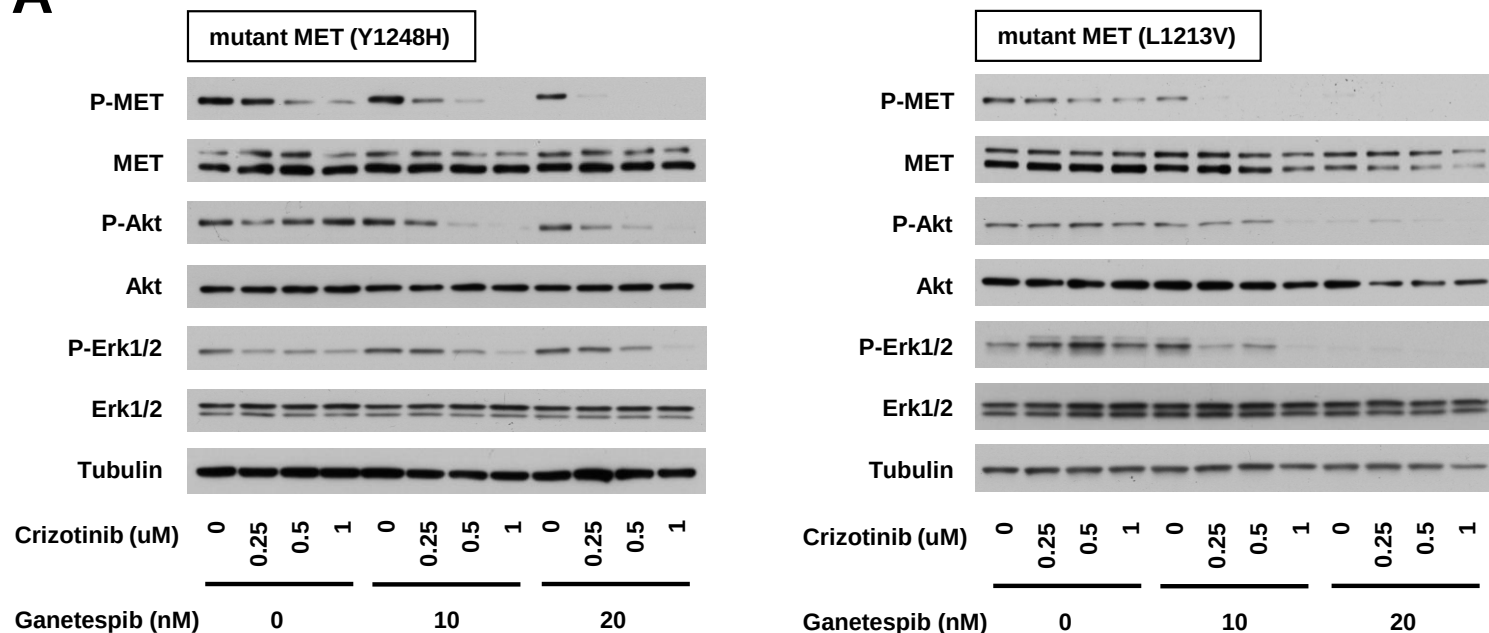


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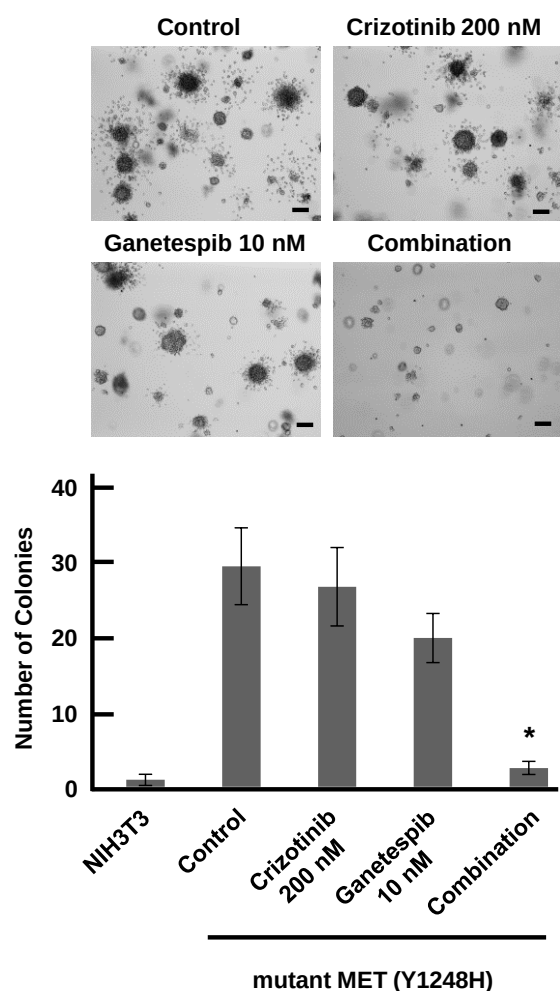


Miyajima et al., Figure 5

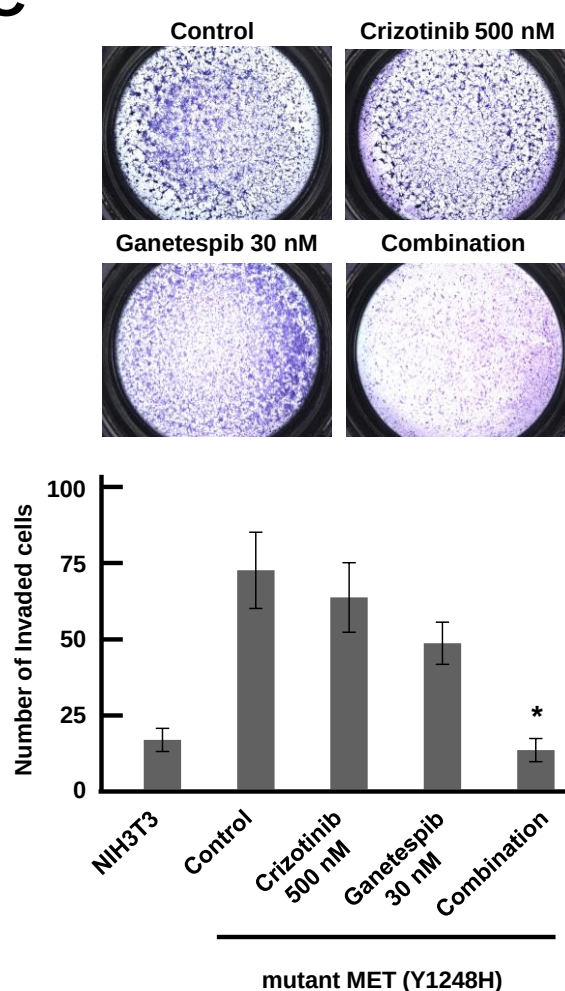
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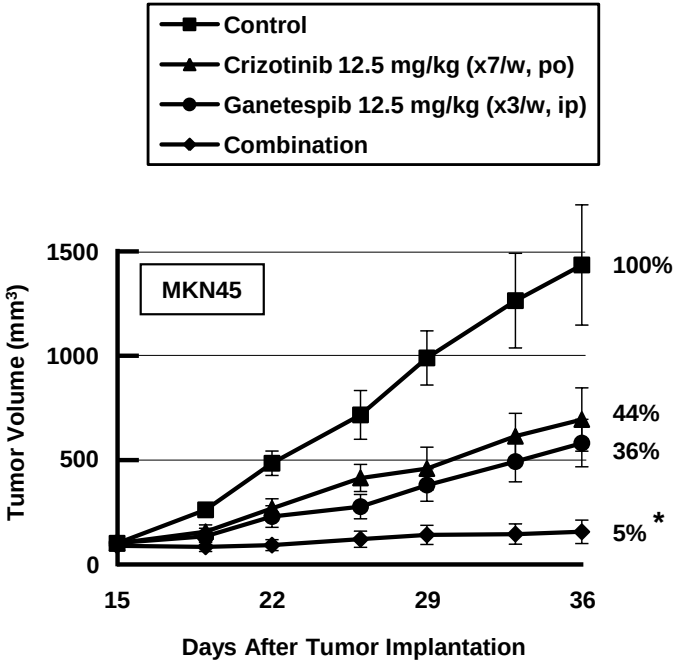


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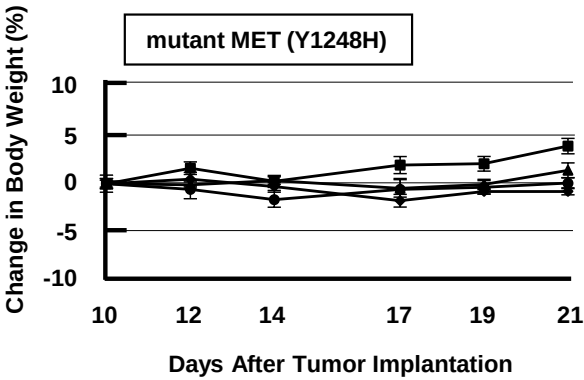
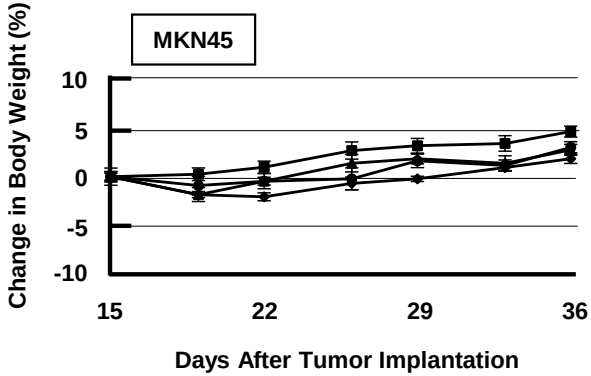
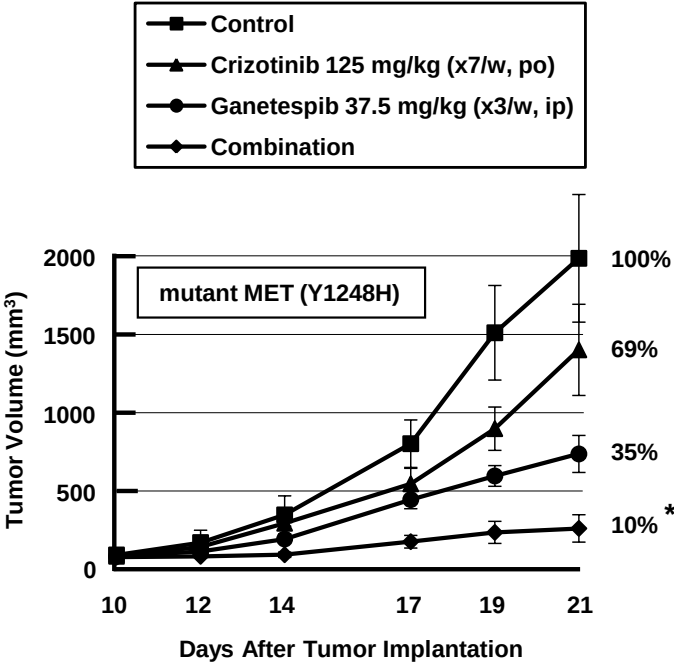


Miyajima et al., Figure 6

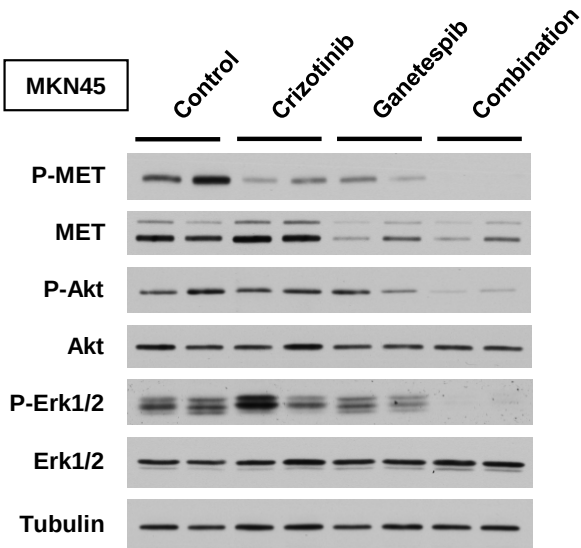
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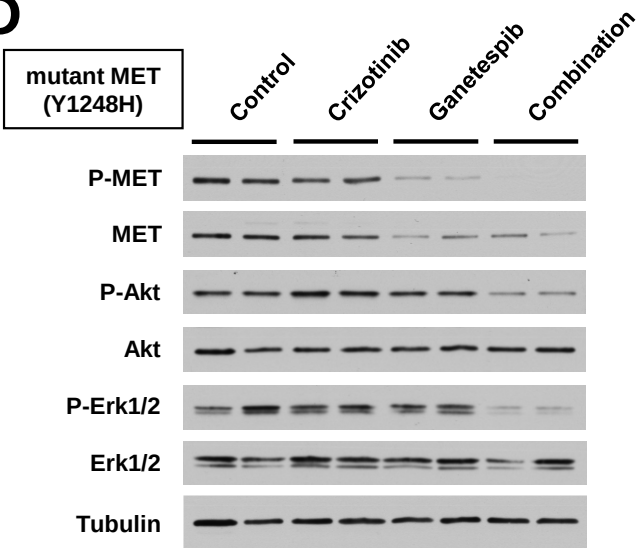
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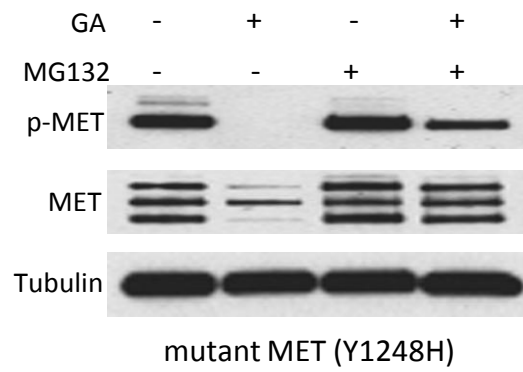
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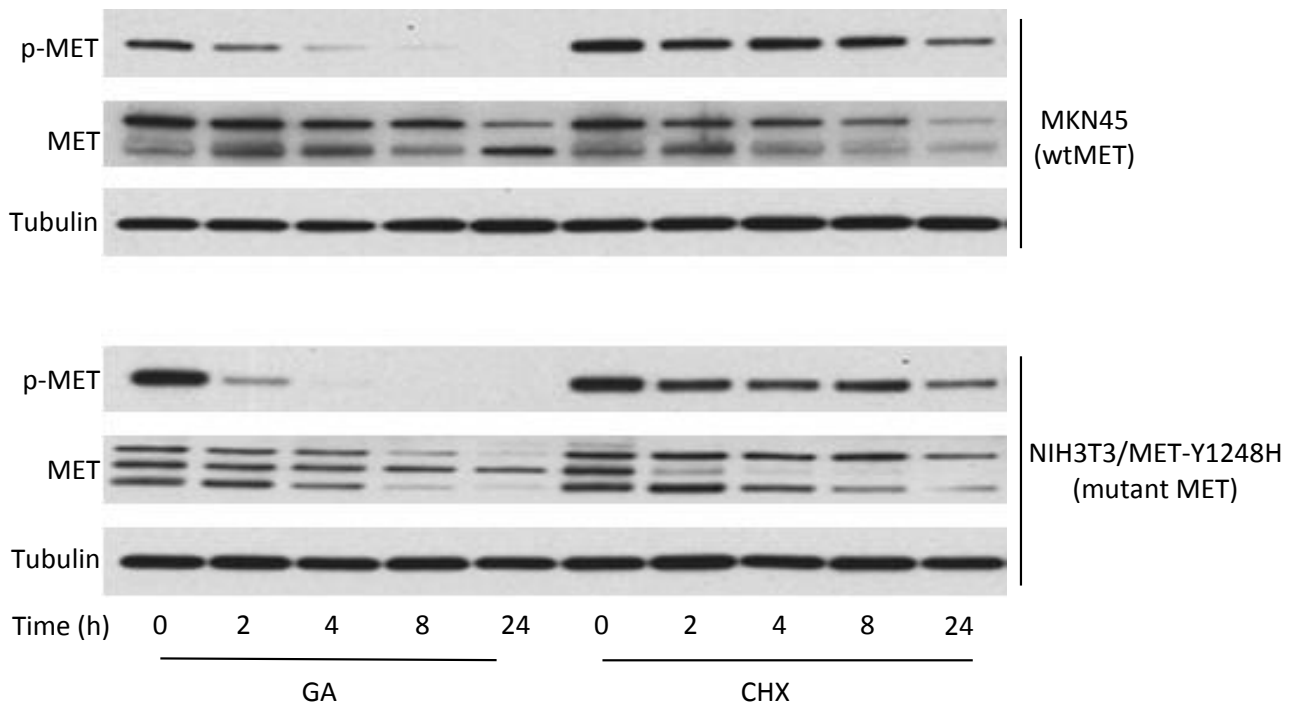
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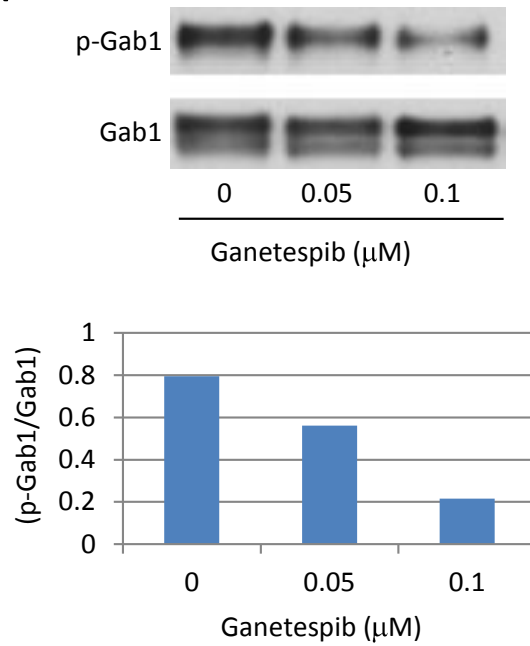
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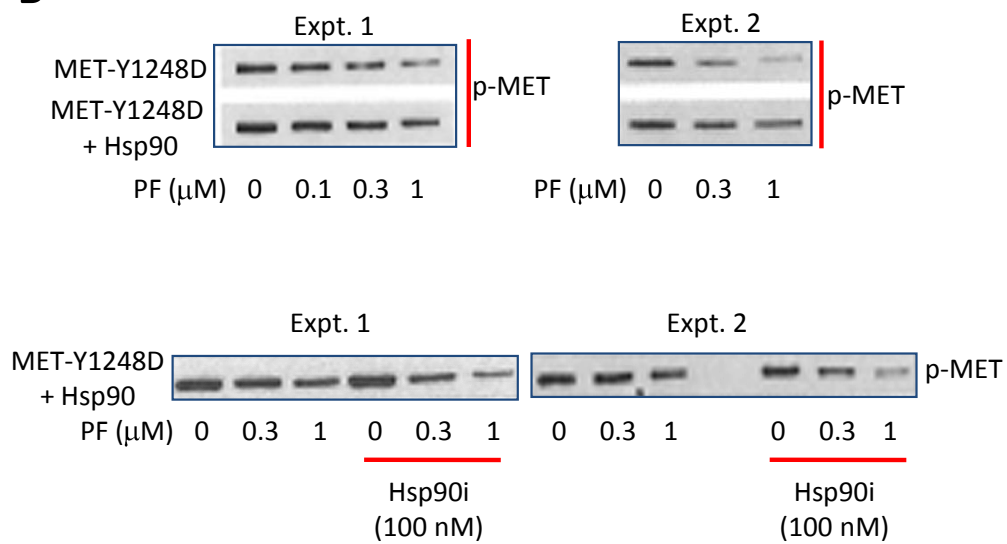
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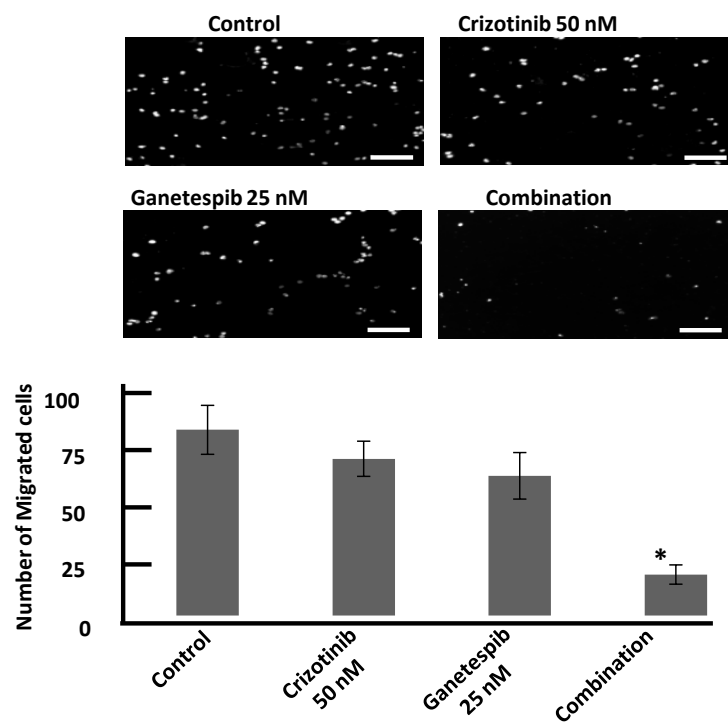


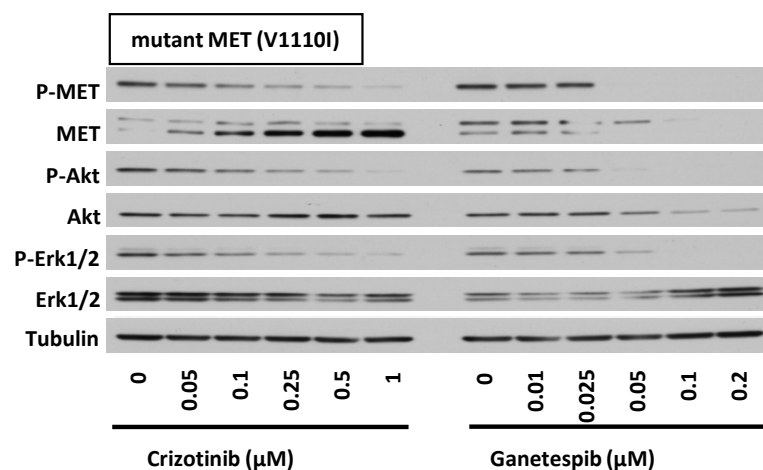
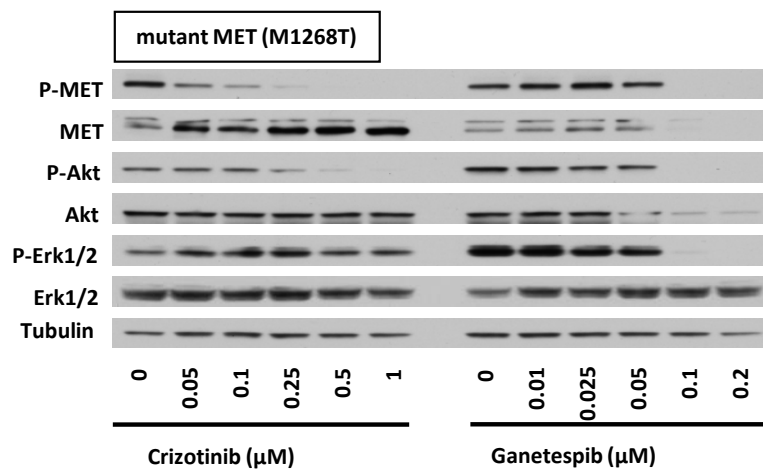
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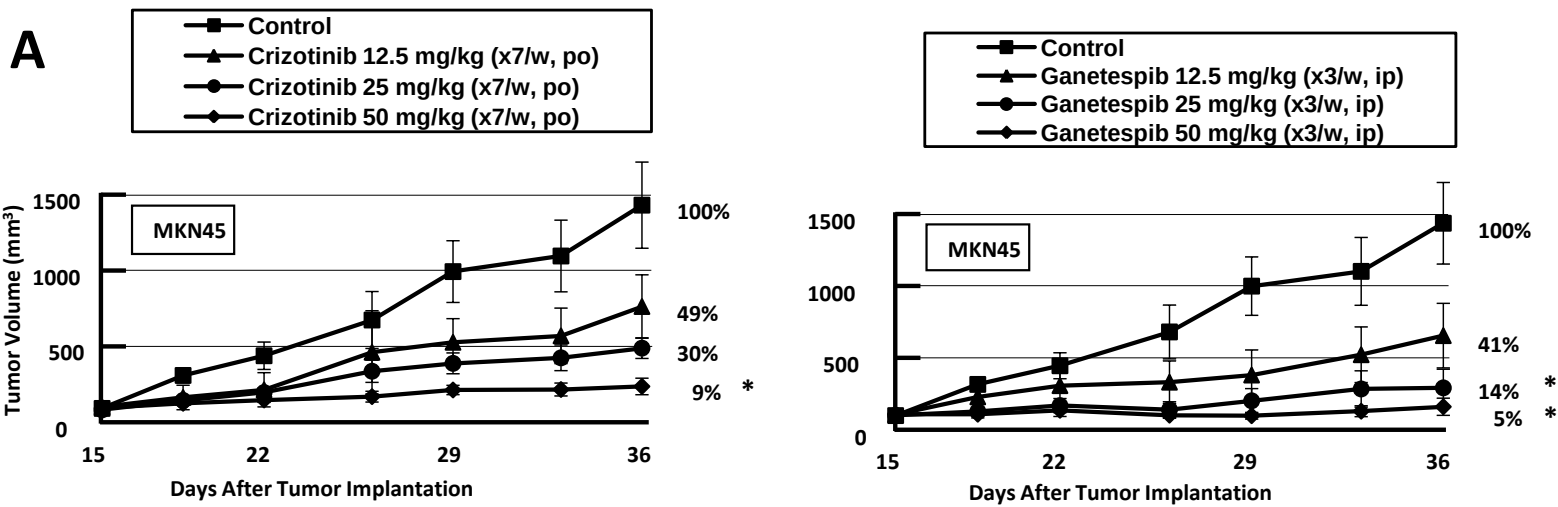
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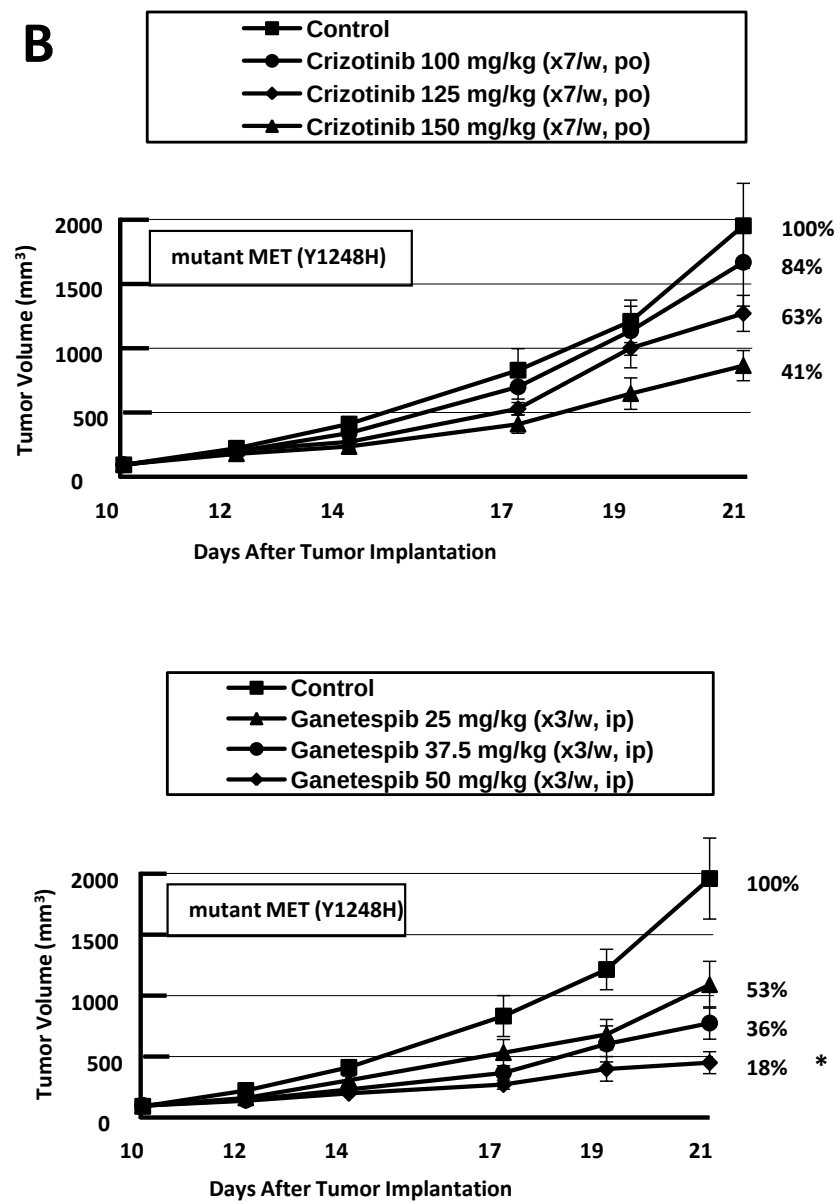




A



B



Supplemental Tables and Figure Legends

Supplemental Table 1. Combination index (CI) was calculated (as in Figure 3C) to examine whether crizotinib and ganetespib synergized to inhibit soft agar colony growth of NIH3T3 cells stably expressing TKI-resistant MET-Y1248H. A CI value < 1 indicates synergy.

Crizotinib (nM)	Ganetespib (nM)	Combination Index (CI)
150	10	0.81
200	10	0.73

Supplemental Table 2. Crizotinib and ganetespib synergistically reduce growth of wild-type and mutant MET-driven tumor xenografts. Relative tumor volumes of control and treated groups on the final day of the study shown in Fig. 6A, B were determined. Combination therapy had a greater than additive effect on tumor growth inhibition in both MKN45 (wild-type MET) and NIH3T3/MET-Y1248H xenografts. ^a FTV, fractional tumor volume, calculated as mean tumor volume experimental/mean tumor volume control. ^b Mean FTV of crizotinib-treated mice x mean FTV of ganetespib-treated mice. ^c Obtained by dividing the expected FTV by the observed FTV. A ratio > 1 indicates synergy; a ratio < 1 indicates a less than additive effect of the drug combination.

Fractional tumor volume (FTV) relative to untreated controls^a

Xenograft	Crizotinib	Ganetespib	Expected ^b	Observed	Ratio expected FTV/observed FTV ^c
MKN45	0.52	0.45	0.23	0.12	1.86
NIH3T3/MET (Y1248H)	0.71	0.36	0.25	0.15	1.61

Supplemental Figure Legends

Supplemental Figure 1. *A*, NIH3T3/MET-Y1248H cells were treated with MG132 (10 μ M) 1h before treatment with 0.5 μ M GA for an additional 8 h. Cells were collected, lysed in TNES buffer (50 mM Tris-HCl pH 7.5, 1% NP-40, 2 mM EDTA, 100 mM NaCl, plus protease and phosphatase inhibitors) and subjected to immunoblotting. *B*, MKN45 and NIH3T3/MET-Y1248H cells were treated with either 0.5 μ M GA or 100 μ g/ml cycloheximide for the indicated times and then lysed in TNES buffer. Equal amounts of total protein from each sample were subjected to immunoblotting.

Supplemental Figure 2. *A*, NIH3T3/MET-Y1248H cells were treated with indicated concentrations of ganetespib (16 h), then lysed in TNES buffer and subjected to immunoblotting. *B*, Inclusion of bacterially purified Hsp90 protein (50 nM) with bacterially purified, constitutively active MET-Y1248D protein (50 nM, Millipore) inhibits sensitivity to crizotinib ('PF') in an *in vitro* kinase assay (top 2 panels); pre-incubation with GA (100 nM) restores sensitivity to crizotinib (bottom 2 panels). Briefly, proteins were resuspended in kinase buffer (50 mM Tris-HCl pH 7.5, 10 mM MgCl₂, 0.1 mM EDTA, 2 mM DTT). The kinase reaction was initiated by adding 0.2 mM ATP and incubating at 30°C for 15 min. Kinase activity was assessed by visualizing MET autophosphorylation with appropriate antibodies.

Supplemental Figure 3. Combination of crizotinib and ganetespib inhibits migration of wtMET-overexpressing cells. MKN45 cells in serum-free medium containing indicated drugs were added to upper wells of transwell chambers. After 48 h, migrated cells (e.g., cells appearing on the lower surface of the uncoated separating membrane) were stained and counted microscopically. *Scale bar*, 0.1mm. Data are graphically displayed as mean $\pm$ SD of triplicate experiments. *, $P < 0.05$ vs. control (unpaired Student's *t*-test followed by Bonferroni test).

Supplemental Figure 4. Ganetespib inhibits MET phosphorylation and downstream signaling in NIH3T3 cells expressing the TKI-sensitive MET mutants M1268T or V1110I. Cells were treated, lysed and analyzed as described in Figure 4.

Supplemental Figure 5. *A*, A dose range study of single agent crizotinib or ganetespib in wtMET-driven xenografts. Athymic mice bearing MKN45 tumors were administered either crizotinib or ganetespib at the indicated dose and schedule for 3 weeks. Tumor volume was measured as described in Figure 6. Data are shown as mean $\pm$ SD (n=5/group). *, P < 0.05 vs. control (unpaired Student's *t*-test followed by Bonferroni test). *B*, A dose range study of single agent crizotinib or ganetespib in TKI-resistant MET-Y1248H-driven xenografts. Athymic mice bearing NIH3T3 tumors stably expressing MET-Y1248H were administered either crizotinib or ganetespib at the indicated dose and schedule for 2 weeks. Tumor volume was measured as described in Figure 6. Data are shown as mean $\pm$ SD (n=5/group). *, P < 0.05 vs. control (unpaired Student's *t*-test followed by Bonferroni test).