Combined EGFR/MET or EGFR/HSP90 Inhibition Is Effective in the Treatment of Lung Cancers Codriven by Mutant EGFR Containing T790M and MET

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Abstract

Tyrosine kinase inhibitors (TKI) that target the EGF receptor (EGFR) are effective in most non–small cell lung carcinoma (NSCLC) patients whose tumors harbor activating EGFR kinase domain mutations. Unfortunately, acquired resistance eventually emerges in these chronically treated cancers. Two of the most common mechanisms of acquired resistance to TKIs seen clinically are the acquisition of a secondary “gatekeeper” T790M EGFR mutation that increases the affinity of mutant EGFR for ATP and activation of MET to offset the loss of EGFR signaling. Although up to one-third of patient tumors resistant to reversible EGFR TKIs harbor concurrent T790M mutation and MET amplification, potential therapies for these tumors have not been modeled in vivo. In this study, we developed a preclinical platform to evaluate potential therapies by generating transgenic mouse lung cancer models expressing EGFR-mutant Del19-T790M or L858R-T790M, each with concurrent MET overexpression. We found that monotherapy targeting EGFR or MET alone did not produce significant tumor regression. In contrast, combination therapies targeting EGFR and MET simultaneously were highly efficacious against EGFR TKI–resistant tumors codriven by Del19-T790M or L858R-T790M and MET. Our findings therefore provide an in vivo model of intrinsic resistance to reversible TKIs and offer preclinical proof-of-principle that combination targeting of EGFR and MET may benefit patients with NSCLC. Cancer Res; 72(13): 3302–11. ©2012 AACR.

Introduction

Activating mutations in the kinase domain of EGFR receptor (EGFR) in non–small cell lung cancers (NSCLC) commonly arise as in-frame deletions in exon 19 and L858R exon 21 substitutions, and confer sensitivity to the reversible tyrosine kinase inhibitors (TKI) gefitinib and erlotinib (1–3). Despite initial responses, NSCLCs driven by EGFR activating mutations inevitably develop resistance to these TKIs. An acquired T790M mutation emerges in approximately 50% of EGFR-mutated patients with TKI resistance (4–9). The threonine to methionine change at the 790 amino acid “gatekeeper” residue in the EGFR kinase domain has been shown to confer resistance by increasing the affinity for ATP, compromising the potency of reversible TKIs (10). In contrast to the reversible TKIs, irreversible TKIs, including PF00299804 and BIBW2992, are thought to overcome T790M-mediated resistance because they do not compete with ATP, but rather covalently bind to the C797 residue of EGFR to irreversibly inhibit receptor tyrosine kinase activity (7, 11, 12). Irreversible EGFR TKIs, HKI-272 and BIBW2992, are modestly efficacious as single agents in a transgenic mouse model of lung adenocarcinoma driven by EGFR L858R-T790M (13, 14), and they do not fully extinguish downstream signaling, prompting their combination with inhibitors of mTOR preclinically and in clinical trials (15), and they do not fully extinguish downstream signaling, prompting their combination with inhibitors of mTOR preclinically and in clinical trials (16, 17). In contrast, an EGFR mutant-specific irreversible TKI (WZ4002) has been shown to be highly potent and efficacious in both EGFR L858R-T790M and EGFR exon 19 del-T790M-driven lung adenocarcinoma models, and molecules from this class are eagerly anticipated in clinical trials (18).

In addition to the secondary gatekeeper mutation, NSCLC patients whose tumors harbor sensitizing EGFR mutations and who initially respond to reversible EGFR TKIs may also acquire
resistance through activation of MET, via HGF ligand and MET gene amplification, which serves to reactivate the phosphoinositide 3-kinase (PI3K) signaling axis (6, 19, 20). The frequency of resistant cases with MET amplification ranges from 5% to 15% depending on the study (6, 9, 21). This mechanism was first shown in HCC827 (EGFR E746_A750del) cells rendered gefitinib-resistant in vitro. In TKI-resistant HCC827 cells, only simultaneous inhibition of both MET and EGFR was able to suppress downstream proliferation and survival pathways, required for antiproliferative effects in vitro and antitumor efficacy in vivo (6, 19). In contrast, NCI-H820 cells naturally harbor concurrent EGFR TKI-resistant EGFR mutation (E746_A750del, T790M) and MET amplification. In these cells, small molecule c-Met inhibition or siRNA-mediated MET depletion was sufficient to dephosphorylate ERBB3 and to compromise the cell viability, suggesting that resistant NCI-H820 rely more heavily on MET signaling for survival (16).

Interestingly, several studies have identified primary tumors genotypically similar to NCI-H820 cells, with concurrent T790M mutation and moderate MET amplification in 5% to 33% of NSCLC patients who become refractory to reversible T790M mutation further enhances the oncogenic potential of EGFRs carrying sensitizing mutations in vitro (24) and in vivo (13). However, the interaction of concomitant T790M mutation with MET amplification has only been studied in NCI-H820 cells to date, and has not been modeled in vivo. To clarify whether combined irreversible EGFR and MET inhibition is required for antitumor activity when both abnormalities are present, we have generated mouse lung cancer models expressing EGFR-mutant Del19-T790M or L858R-T790M, each with concurrent hMET overexpression. Using these models, we have assessed the efficacy of various monotherapies and combination treatments, providing a platform for development of clinical trials for patients whose tumors harbor multiple mechanisms of EGFR TKI resistance.

Materials and Methods

**Generation of the CCSP-rtTA/Tet-op-hMET mouse and the CCSP-rtTA/Tet-op-hMET/EGFR mouse**

A cohort of genetically engineered mice harboring a doxycycline-inducible human MET (hMET) oncogene was generated similar to mouse models we have previously described (13, 21). Briefly, we constructed a 6.5-kb DNA segment consisting of 7 direct repeats of the tetracycline (tet)-operator sequence, followed by human MET cDNA and β-globin polyA. The construct was injected into FVB/N blastocysts, and progeny were screened using a PCR strategy (25). Founders were identified and then crossed to Clara cell secretory protein (CCSP)-rtTA mice, harboring an allele that specifies transgenic expression of the reverse tetracycline transactivator protein (rtTA) in type II alveolar epithelial cells (26, 27) to generate inducible bitransgenic mouse cohorts harboring both the activator and the responder transgenes. Two tightly regulated hMET founders (#16 and #31) were identified by reverse transcriptase PCR (RT-PCR). CCSP-rtTA/MET mice were then crossed with CCSP-rtTA/EGFR exon 19 deletion/T790M (TD) mice (18) or with CCSP-rtTA/EGFR L858R/T790M (TL) mice (13) to generate animals expressing CCSP-rtTA/EGFR/TL/MET (TD/MET) or CCSP-rtTA/EGFR TL/MET (TL/MET), respectively. All mice were housed in a pathogen-free environment at the Harvard School of Public Health and were handled in accordance with Good Animal Practice as defined by the Office of Laboratory Animal Welfare.

**RT-PCR and quantitative PCR**

Total RNA samples were prepared as previously described (25) and then retro-transcribed into first-strand cDNA using the Superscript First Strand Synthesis System following the manufacturer’s protocol (Life Technologies). Quantitative real-time PCR was carried out by monitoring the increase in fluorescence of SYBR green, FAM, or VIC dyes in real-time (Qiagen) with the ABI 7700/StepOne Plus sequence detection system (Life Technologies). Primers used for RT-PCR and real-time PCR are as follows. Real-time PCR primers: Forward TTACGGACCCAAATCATGAC; Reverse ACTTCCGTGAATAGCCCAT; genotyping PCR primers: Forward CTGCGACCC-TAAAGCGGAAA; Reverse TCGTTATATTGTGAATTGTGATGC. Real-time primers and probes for human MET and EGFR were purchased from Life Technologies (Assay IDs: Hs01565584_m1 and Hs01076078_m1, respectively). Each sample was amplified in duplicate or triplicate for quantification of both transgenes and β-actin transcripts. Data were analyzed by relative quantitation using the ΔΔCt method and normalization to β-actin.

**Mouse drug treatment studies**

All animal treatment studies were reviewed and approved by the Institutional Animal Care and Use Committee at the Dana-Farber Cancer Institute. As previously described (12, 13, 25, 28–31), transgenic mice were exposed to a doxycycline-containing diet for 8 to 12 weeks and subjected to MRI to document tumor burden. After initial imaging, animals were subjected to treatment with WZ4002 formulated in 1% l-methyl-2-pyrrolidinone/90% PEG-300 by gavage at 50 mg/kg daily; crizotinib (PF-02341066) formulated in saline by gavage at 20 mg/kg daily, or 50 mg/kg daily; or 17-DIMAG (I.C laboratories) formulated in saline by intraperitoneal injection at 20 mg/kg daily. Cetuximab was used at 50 mg/kg (every 3 days). Drugs were administered as single agents or in combination.

**MRI imaging and tumor volume measurement**

Mice were imaged by MRI to determine the reduction in tumor volume after 2 weeks of treatment and then sacrificed for further histologic and biochemical studies. For pharmacodynamic studies, 2 doses of drugs were administrated within 24 hours, with the first dose on day 1 and the second dose on day 2, two hours before sacrifice and the harvesting of tumor nodules. MRI measurements were carried out as previously described (13, 25). Using the RARE sequence scans, tumor volume measurements were generated using in-house custom software. The MRI images were optimized for the mediastinal window setting and were saved on a computer as 8-bit gray-scale JPEG files. Analysis was carried out using ImageJ software (NIH). Consecutive 10 to 16 images (slice thickness, 1 mm; interslice gap, 1 mm) containing lung tumor were used for each.
mouse to measure tumor areas. Normal structures of the body, including chest wall, heart, liver, and normal mediastinal structures, were omitted manually from the images, so that lung fields were selected for further examination. High intensity areas above the automatic threshold of the software were selected and measured to calculate total tumor areas.

**Histology and immunohistochemistry**

Mice were sacrificed and the left lung was dissected and snap-frozen for biochemical analysis. The right lung was inflated with buffered 10% formalin for 10 minutes and fixed in 10% formalin overnight at room temperature. The specimen was washed once in PBS, placed in 70% ethanol, and embedded in paraffin, from which 5-µm sections were generated. Immunohistochemistry (IHC) and hematoxylin and eosin (H&E) stains were carried out in the Department of Pathology at Brigham and Women's Hospital; all antibodies were from Cell Signaling Technologies.

**Western blot analysis**

Snap-frozen tumor nodules were homogenized in radio-immunoprecipitation assay buffer supplemented with protease and phosphatase inhibitor cocktails (EMD Biosciences) and subjected to Western blotting using the antibodies

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**Figure 1.** Induction of hMET tyrosine kinase at both RNA and protein levels in bitransgenic mice from different founders. A, bitransgenic mice were fed a diet without (−) or with (+) doxycycline. After 12 weeks of doxycycline treatment, mRNA from the lungs from 2 founders (#31 and #16) was subjected to RT-PCR analysis to determine the transcriptional level of hMET. B, quantitative RT-PCR analysis of total MET transcript induction in bitransgenic mice fed a diet without (−) or with (+) doxycycline for 12 weeks. Each sample was amplified in triplicate for quantification of both total MET and β-actin transcripts. The endogenous mouse met level from normal mice was arbitrarily designated as 1. Data were analyzed by relative quantitation using the ΔΔCt method with normalization to β-actin. Error bars, SD. C, bitransgenic mouse founders were treated without (−) or with (+) doxycycline for 12 weeks, and lung lysates were subjected to Western blotting for total MET and β-actin. D, H&E images of normal lung from a CCSP-rtTA/MET bitransgenic mouse fed with a doxycycline-containing diet for 52 weeks. The right micrograph is a magnified view of the area indicated in the low magnification micrograph shown on the left. Scale bars indicate 1,000 µm (left) or 100 µm (right), respectively. There was no evidence of malignant tumor observed in 6 CCSP-rtTA/MET bitransgenic mice that were maintained on a doxycycline-containing diet for more than 40 weeks. E and F, quantitative RT-PCR analysis of total EGFR (E) and hMET (F) transcript in bi- or tritransgenic mice on a doxycycline-containing diet. Each sample was amplified in duplicate for quantification of EGFR, MET, and β-actin transcripts. Mean expression level of human MET or human EGFR from TD/MET mouse was arbitrarily designated as 1. Data were analyzed by relative quantitation using the ΔΔCt method and normalization to β-actin. Each bar represents the relative quantity of mRNA in one mouse.
indicated in the figures. A list of antibodies used is available in the Supplementary Methods.

**Statistical analysis**

Statistical analyses were carried out using Student t test (32). A P value less than 0.05 was considered statistically significant.

**Results**

**Tightly regulated expression of h**\textit{MET}** mRNA and protein in lung tissue**

We generated a cohort of genetically engineered mice, Tet-op-h\textit{MET}/CCSP-rtTA, harboring a doxycycline-inducible human \textit{MET} (h\textit{MET}) oncogene, similar to mouse models we have previously described (13, 25). RT-PCR with transgene-specific primers was carried out to determine the inducibility of h\textit{MET} RNA expression in the lungs of each potential founder before and after 12 weeks of doxycycline administration. The h\textit{MET} transcripts were undetectable from both nontransgenic and the bitransgenic mice without doxycycline administration, but 12 weeks of doxycycline administration robustly induced transgene expression (Fig. 1A and B).

Figure 2. Histologic comparison of the murine mutant EGFR lung tumors with or without coexpression of h\textit{MET}. Representative H&E staining of cross-sectional views of different transgenic mouse lungs showing no significant difference in histopathologic assessments of lung carcinoma. Picture on the right for each genotype is a magnified view of the area indicated in the picture with low magnification on the left. Scale bars, 100 \textmu m.

The induction of h\textit{MET} was verified at the protein level by Western blotting of lung lysates from the nontransgenic and the bitransgenic mice before and after doxycycline administration (Fig. 1C). Induction of h\textit{MET} protein was shown in samples from bitransgenic mice after doxycycline administration, but not in samples from bitransgenic mice before doxycycline administration or nontransgenic mice (Fig. 1C). The expression of h\textit{MET} alone was not sufficient to form tumors in the bitransgenic models (Fig. 1D).

Previously, we reported that the irreversible EGFR TKIs BIBW2992 and the EGFR mutant-specific irreversible EGFR TKI WZ4002 promote tumor regression in murine lung cancers driven by human EGFR L858R/T790M (TL; ref. 12) or human EGFR exon19 deletion/T790M (TD; ref. 18). To investigate whether the overexpression of the h\textit{MET} oncogene confers intrinsic resistance to irreversible EGFR TKI treatment in murine lung cancer expressing TD or TL, we crossed Tet-op-h\textit{MET}/CCSP-rtTA expressing animals with those expressing Tet-op-EGFR TD/CCSP-rtTA (18) or Tet-op-EGFR TL/CCSP-rtTA (13) to generate Tet-op-h\textit{MET}/EGFR TD/CCSP-rtTA (TD/MET) or Tet-op-h\textit{MET}/EGFR TL/CCSP-rtTA (TL/MET) cohorts. h\textit{EGFR} transcripts were undetectable from nontransgenic mice, but 12 weeks of doxycycline administration robustly induced transgene expression in all mouse models used in this study (Fig. 1E). The h\textit{MET} transcripts were abundantly expressed in tumors from TD/MET and TL/MET mice but virtually undetectable in lungs from nontransgenic mice, as well as in tumors from TD and TL mice (Fig. 1F). Expression of h\textit{MET} protein and its phosphorylated form in the TD/MET and TL/MET models was also verified by Western blot (Supplementary Fig. S1A and B). In both the TD/MET and TL/MET models, there were no significant differences in expression of the h\textit{MET} transgene between total lung and tumor nodules (Supplementary Fig. S2).
Overexpression of the hMET oncogene confers intrinsic resistance to irreversible EGFR TKI treatment in murine lung cancer models with mutant EGFR

After 8 to 12 weeks of doxycycline administration, mice were sacrificed and lung sections were stained with H&E (Fig. 2). TD and TL mice developed both parenchymal and bronchial adenocarcinomas as previously reported (13), and coexpression of hMET and TD or TL did not significantly change the histopathologic features of these lung tumors. Similarly, there was no significant increase in tumor burden in TL/MET mice because of the expression of hMET (Supplementary Fig. S3).

Before treatment, TD, TL, TD/MET, and TL/MET mice were imaged with MRI to document the baseline tumor burden (Fig. 3 and Supplementary Fig. S4). Tumor-bearing mice were then treated with 50 mg/kg WZ4002 daily for 2 weeks, a previously established effective regimen in the TD and TL models (25). Quantification of MRI images revealed that all of the TD or TL mice showed significant tumor reduction by MRI (−79.6% and −71.2%, respectively, Fig. 4A). In contrast, the introduction of the MET oncogene confers intrinsic resistance to WZ4002 in TD/MET or TL/MET transgenic mouse models (Fig. 3 right, Fig. 4A). TD/MET and TL/MET mice showed significantly less tumor reduction compared with TD and TL mice (−23.2% vs. −79.6%; P < 0.001, and −17.9% vs. −71.2%; P < 0.001, respectively, Fig. 4A). To ensure the complete inhibition of EGFR activity, we also treated TD/MET mice with WZ4002 (50 mg/kg, daily) along with the anti-EGFR antibody cetuximab (50 mg/kg, every 3 days) for 2 weeks. The combination of WZ4002 and cetuximab did not improve the radiographic responses in TD/MET mice (data not shown).

Inhibition of MET alone does not result in tumor regression in murine lung cancer models with concurrent EGFR mutation and MET overexpression

Whereas lung cancer driven by EGFR TD or TL showed a significant tumor regression upon WZ4002 treatment, hMET overexpression rendered these cancers resistant to the same treatment. In NCI-H820 cells, which express mutant EGFR (E746_T751del, T790M) with the presence of MET amplification, MET inhibition alone was shown to be sufficient to compromise cell growth (16). On the basis of this finding, we evaluated whether TD/MET and TL/MET mice could respond to therapy with the ATP-competitive small-molecule MET inhibitor crizotinib (PF-02341066), which also inhibits ALK. Tumor-bearing TD/MET or TL/MET mice were treated orally with crizotinib (20 mg/kg) daily for 2 weeks. Crizotinib treatment failed to induce radiographic responses, not only in TD and TL mice but also in TD/MET or TL/MET mice (Fig. 3, Supplementary Fig. S4), confirmed by tumor volumetric analyses (Fig. 4B). TD and TD/MET mice were also challenged with the higher dose of 50 mg/kg daily for 2 weeks with no improvement in tumor reduction (Fig. 4B) despite the significant dephosphorylation of MET (Supplementary Fig. S1B).

Combined inhibition of EGFR and MET results in significant tumor regression in murine lung cancer models with concurrent EGFR mutation and MET overexpression

Because neither TD/MET nor TL/MET mice responded to single-agent EGFR or MET inhibition, we hypothesized that either receptor alone is insufficient to promote TD/MET and TL/MET tumor growth and survival. To test this hypothesis, tumor-bearing TD/MET or TL/MET mice were treated orally with concomitant WZ4002 (50 mg/kg) and crizotinib (20 mg/kg) daily for 2 weeks. For controls, we treated tumor-bearing TD or TL mice with the same combination therapy schedule. All 4 mouse models treated with this combination showed significant radiographic responses (Fig. 3, Supplementary Fig. S4), and quantification of tumor burden pre- and posttreatment confirmed the response in TD/MET mice (−69.5%) and TL/MET mice (−74.0%; Fig. 4C). Taken together with our previous experiments, these results indicated that concomitant treatment with EGFR and MET inhibitors produces greater shrinkage than treatment with either alone in tumors harboring compound EGFR mutations (TD or TL) along with MET overexpression.

HSP90 inhibition potentiates WZ4002 to inhibit tumor growth codriven by mutant EGFR and MET

Our results show that TD/MET and TL/MET are highly sensitive to combined EGFR and MET inhibition, suggesting codependence of these tumors on these receptors. To assess another strategy of depleting cancer cells of EGFR and MET activities, we treated mice with the HSP90 inhibitor 17-DMAG (20 mg/kg) daily. We and others have shown that...
mutant EGFR and MET are clients of HSP90 and are subject to degradation upon HSP90 inhibition (33, 34). We previously showed that TL mice respond to 17-DMAG, but only transiently; tumor regression was observed after 1 week, but substantial tumor regrowth was documented by 3 weeks (35). Consistent with these results, in the current experiments, we treated TD and TL mice with 17-DMAG for 2 weeks and did not observe significant radiographic response (Fig. 3 and Supplementary Fig. S4). Similarly, 2 weeks of 17-DMAG did not produce response in TD/MET and TL/MET mice (Fig. 4D).

Recently, using NCI-H1975 EGFR L858R/T790M xenografts, we have shown that depletion of mutant EGFR upon HSP90 inhibition is brief, lasting only 48 hours before expression is restored, despite persistent intratumoral drug levels (36). We therefore hypothesized that the addition of WZ4002 to 17-DMAG would serve to inhibit reexpressed EGFR, and that the 2 compounds together may be synergistic. We treated TD, TL, TD/MET, and TL/MET mice with the combination of WZ4002 (50 mg/kg, daily) and 17-DMAG (20 mg/kg, daily) for 2 weeks. For TD or TL mice, combination therapy did not afford advantage over treatment with 17-DMAG alone by MRI analyses (Fig. 3 and Supplementary Fig. S4), presumably because of the substantial single-agent activity of WZ4002 in these models. However, significant reductions in tumor volume were observed in TD/MET and TL/MET mice with the combination regimen (Fig. 4E), and the reductions in tumor size posttreatment were comparable with those observed with combined WZ4002 and crizotinib therapy (Fig. 4C).

To extend our findings to human lung cancer cells, we assessed the efficacy of similar treatments in mice bearing xenografts of gefitinib-resistant MET-amplified HCC827 cells (HCC827GR6; ref. 19) engineered to ectopically express EGFR E746_A750del/T790M (Supplementary Fig. S5). Here, partial tumor response was observed after monotherapy with 17-DMAG or WZ4002; however, the combination was more efficacious than either agent alone in suppressing tumor growth (Supplementary Fig. S5).

**Histologic confirmation of radiographic responses to combination treatments**

TD/MET and TL/MET tumors responded to combinations of WZ4002 and crizotinib or WZ4002 and 17-DMAG within 2 weeks. To confirm radiographic responses, we examined H&E stains from treated tumor. In TD mice, single-agent crizotinib or 17-DMAG failed to show reduction in tumor cell content,
although there was noticeable response to WZ4002 (Fig. 5A), consistent with our previous data (18). In contrast, single-agent WZ4002 (as well as crizotinib or 17-DMAG) failed to show a significant decrease in tumor cell content in the TD/MET model (Fig. 5B); however, in this model, tumor cell content was markedly decreased after combination therapy with WZ4002 and crizotinib or WZ4002 and 17-DMAG, with fibrosis evident in the tumor nests (Fig. 5B). Identical results were obtained in TL/MET mice, in which the superiority of combination treatment was also evident (Fig. 5C and D).

**Combination therapies promote efficient growth inhibition of tumors with concurrent expression of mutant EGFR and MET by suppressing both PI3K and MAPK pathways**

Because PI3K and mitogen-activated protein kinase (MAPK) pathways are downstream of mutant EGFR and MET (2, 3, 6, 13, 25), we used phospho-Akt and phospho-Erk as pharmacodynamic markers to evaluate the efficacies of targeted therapies in the mouse models. Mice were treated over 24 hours with 2 doses of vehicle, WZ4002 alone, crizotinib alone, crizotinib/WZ4002, or WZ4002/17-DMAG before sacrifice and harvesting of tumors for immunohistochemical analysis. In TD mice, WZ4002 alone resulted in EGFR dephosphorylation (Fig. 6A, left) and suppressed both Akt and Erk phosphorylation, although crizotinib treatment alone failed to suppress Akt and Erk phosphorylation (Fig. 6A middle and right panels). In contrast, WZ4002 alone failed to suppress phospho-Akt and phospho-Erk signals in TD/MET mice (Fig. 6B, middle and right panels), even though considerable suppression of EGFR phosphorylation was achieved (Fig. 6B, left).

Crizotinib alone did not completely suppress phosphorylation of EGFR, Akt, or Erk in either the TD or the TD/MET models (Fig. 6A and B). In contrast, combination treatment with WZ4002 and crizotinib robustly suppressed expression of phospho-EGFR, phospho-Akt, and phospho-Erk in both of these mouse models (Fig. 6A and B). Similarly, the WZ4002/17-DMAG combination was also efficacious and substantially reduced phosphorylation of components of the signaling cascades dramatically in both TD and TD/MET mice (Fig. 6A and B).

To confirm the results from IHC, tumor lysates from TD and TD/MET mice similarly treated with vehicle, WZ4002 alone, crizotinib alone, crizotinib/WZ4002, or WZ4002/17-DMAG were subjected to Western blot (Supplementary Fig. S6). In tumors from TD mice, WZ4002 treatment alone was sufficient to downregulate phospho-EGFR, phospho-Akt, and phospho-Erk, whereas single-agent WZ4002 or crizotinib alone was not sufficient to inhibit the same phosphorylation events in TD/MET mice. In agreement with the immunohistochemical

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**Figure 5.** H&E staining of lung tumors shows that combination treatments are more efficacious than individual treatments against tumors harboring compound EGFR mutations and MET overexpression. Representative images of H&E staining of lung tumors from TD mice (A), TD/MET mice (B), TL mice (C), and TL/MET mice (D). All mice were sacrificed for pathologic analysis after 2 weeks of treatment with vehicle, WZ4002, crizotinib, 17-DMAG, WZ4002/crizotinib, or WZ4002/17-DMAG. Scale bars, 100 μm.
results (Fig. 6), Western blotting confirms that only the WZ4002/crizotinib and WZ4002/17-DMAG combinations can suppress the phosphorylation of EGFR, Akt, and Erk in tumors from TD/MET mice.

Discussion

To date, the 2 most common mechanisms of acquired resistance to gefitinib and erlotinib therapies are development of a secondary gatekeeper mutation T790M (~5%) and a focal amplification of MET (~5–20%; refs. 6, 19, 21). According to several studies, anywhere from 5% to 30% of instances of MET amplification occur concurrently with T790M in patients (4, 6, 16, 19, 22, 23, 37). It remains unclear whether EGFR T790M and amplified-MET coexist in a single tumor cell or whether these somatic genetic changes exist in distinct cells within the same tumor mass. However, in vivo models suggest that they can coexist in a single cell, as they are found naturally occurring together in the NCI-H820 cell line (16, 35).

Previous in vitro studies suggested that MET inhibition or MET depletion alone could suppress the growth and compromise the viability of the NCI-H820 cell line, suggesting that the presence of MET amplification is dominant and supersedes any dependence on EGFR carrying compound mutation with T790M. To test this further, we modeled similar genetic changes in vivo. Here, we have shown that mouse lung adenocarcinomas with concurrent TD or TL along with MET amplification clearly depend on both receptors, and that treatment with individual EGFR or MET inhibitors (i.e., WZ4002 or crizotinib) is ineffective. These results are different from those observed in the NCI-H820 model (16). However our experimental results in vivo are in line with the original report describing MET-amplified EGFR TKI-resistant cells as sensitive to concomitant suppression of EGFR and MET (6). The discrepancy may be explained by differences in how these models were derived or other differences in their underlying genetic composition that are yet to be defined.

Although our mouse model facilitated the evaluation of drugs designed for human kinases in the presence of human transgenes, these models carry some limitations because they do not completely simulate the human disease. First, these models
might not express MET at the level observed in human EGFR TKI-resistant tumors with MET amplification. However, as most EGFR TKI-resistant tumors are analyzed for MET amplification but not for MET overexpression, the exact level of MET expression required in these models is unclear. Second, our mouse model does not account for the impact of HGF on overexpressed MET. However, it has been shown that overexpression of HGF is able to confer resistance in vivo that is also overcome with simultaneous inhibition of both EGFR and MET (6).

Despite these caveats, we propose that simultaneous inhibition of mutant EGFR carrying T790M and MET is likely necessary in tumors in which both genetic abnormalities are observed. Our EGFR/MET-mutant mouse models provide novel platforms to preclinically evaluate compounds in vivo to suppress EGFR and MET signaling simultaneously. Our data suggest that the combination of an EGFR T790M mutant-selective kinase inhibitor such as WZ4002, combined with a MET inhibitor such as crizotinib, should be a high priority once T790M mutant-selective compounds are evaluated on their own.

EGFR and MET are both client proteins of HSP90 (39–35, 38). To date, however, HSP90 inhibitors have had limited efficacy in our preclinical TD and TL models, in which regressions are short-lived, as well as in clinical trials in the EGFR mutant population (39, 40). This may be in part because of very transient suppression of the EGFR-mutant client protein upon HSP90 inhibition (36). It was therefore not a surprise that 17-DMAG alone performed poorly in the TD/MET and TL/MET models. Of note, it is possible that regression occurred over the first week of treatment that we did not capture with an assessment only at the 2-week mark. However, the combination of 17-DMAG and WZ4002 did cause substantial regression in these models, presumably because WZ4002 was capable of inhibiting the activity of EGFR TD and TL after expression reemerges. Of note, these results also suggest that HSP90 inhibition is capable of prolonged suppression of MET expression, so that codelperation of both activities is accomplished by the combination. Further studies will be required to assess the relative sensitivities of MET and mutant EGFR to degradation in response to HSP90 inhibition. Of note, the combination of WZ4002 and 17-DMAG was similar to that of WZ4002 and crizotinib in the clearance of TD/MET and TL/MET tumor cells, as assessed by H&E staining (Fig. 6 and Supplementary Fig. S6).

Previous studies suggested that MET can couple with ERBB3 to activate downstream signals mediated by Akt that bypass EGFR and MET signaling simultaneously. Our data suggest that the combination of an EGFR T790M mutant-selective kinase inhibitor such as WZ4002, combined with a MET inhibitor such as crizotinib, should be a high priority once T790M mutant-selective compounds are evaluated on their own.

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Previous studies suggested that MET can couple with ERBB3 to activate downstream signals mediated by Akt that bypass the inhibited EGFR (6). In addition, MET could activate PI3K to activate downstream signals mediated by Akt that bypass EGFR signaling is inhibited. Therefore, combination therapies (WZ4002 and crizotinib or WZ4002 and 17-DMAG) most efficiently suppressed PI3K and MAPK signaling in all the models tested (Fig. 6B and Supplementary Fig. S6). The suppression of critical downstream signaling resulted in radiographic and histologic responses, overcoming resistance to EGFR inhibition conferred by MET overexpression.

In conclusion, we have developed in vivo models of intrinsic resistance to reversible EGFR TKIs conferred by both EGFR mutants containing T790M and MET overexpression to identify efficacious combination therapies against these genetically defined lung cancers. Our data support the design of appropriate combination clinical trials for patients with EGFR mutant NSCLCs carrying compound TKI resistance mechanisms.

Disclosure of Potential Conflicts of Interest
P.A. Janne is a consultant and an advisory board member of Astra Zeneca, Boehringer Ingelheim, Pfizer, Roche, and Genentech and received royalty payments on a LabCorp patent. J.A. Engelman has ownership interest in Ventana/Roche and is a consultant and an advisory board member of Amgen, Astra Zeneca, Bristol Myers Squibb, Genentech, Glaxo Smith Kline, Roche, Sanofi Aventis.

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