Multiple Mutations and Bypass Mechanisms Can Contribute to Development of Acquired Resistance to MET Inhibitors

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Abstract

Therapies targeting receptor tyrosine kinases have shown efficacy in molecularly defined subsets of cancers. Unfortunately, cancers invariably develop resistance, and overcoming or preventing resistance will ultimately be key to unleashing their full therapeutic potential. In this study, we examined how cancers become resistant to MET inhibitors, a class of drugs currently under clinical development. We utilized the highly sensitive gastric carcinoma cell line, SNU638, and two related MET inhibitors PHA-665752 and PF-2341066. To our surprise, we observed at least two mechanisms of resistance that arose simultaneously. Both resulted in maintenance of downstream PI3K (phosphoinositide 3-kinase)-AKT and MEK (MAP/ERK kinase)-ERK signaling in the presence of inhibitor. One mechanism, observed by modeling resistance both in vitro and in vivo, involved the acquisition of a mutation in the MET activation loop (Y1230). Structural analysis indicates that this mutation destabilizes the autoinhibitory conformation of MET and abrogates an important aromatic stacking interaction with the inhibitor. The other cause of resistance was activation of the epidermal growth factor receptor (EGFR) pathway due to increased expression of transforming growth factor α. Activation of EGFR bypassed the need for MET signaling to activate downstream signaling in these cells. This resistance could be overcome by combined EGFR and MET inhibition. Thus, therapeutic strategies that combine MET inhibitors capable of inhibiting Y1230 mutant MET in combination with anti-EGFR–based therapies may enhance clinical benefit for patients with MET-addicted cancers. Importantly, these results also underscore the notion that a single cancer can simultaneously develop resistance induced by several mechanisms and highlight the daunting challenges associated with preventing or overcoming resistance. Cancer Res; 71(3); 1–11. ©2011 AACR.

Introduction

The emerging impact of targeted therapies as cancer treatments is promoting a paradigm shift in the field of oncology. Concomitant with the exciting progress in this field is the realization that the benefits associated with many of these therapies, although pronounced, are temporary. The emergence of resistance has limited the effectiveness of these therapies, and this observation has spurred efforts to understand how cancers become resistant to targeted therapies. The understanding of how resistance emerges should enable us to develop strategies to overcome or prevent resistance, thereby unleashing a greater therapeutic benefit for our patients. In the field of acquired resistance to kinase inhibitors, 2 major kinds of resistance mechanisms have begun to emerge: (i) mutations in the target kinase itself that abrogate the inhibitory action of the drug [e.g., T790M in epidermal growth factor receptor (EGFR) and T315I in ABL] or (ii) activation of other signaling events that bypass the continued requirement for the original target (reviewed in refs. 1, 2).

MET is the receptor tyrosine kinase (RTK) for hepatocyte growth factors (HGF), also called scatter factors (SF; refs. 3, 4). Although MET has been implicated in the metastases and migration of cancer cells (5, 6), recent studies have revealed that a subset of cancers are “addicted” to MET signaling. Such cancers include gastric carcinomas that harbor amplification of the MET oncogenes (7). In these cancers, MET inhibition dramatically reduces cell viability and invariably leads to down-regulation of the P13K (phosphoinositide 3-kinase)-AKT and MEK (MAP/ERK kinase)-ERK signaling pathways (7, 8). In addition, MET activation, via amplification or with a ligand, has been identified as an acquired resistance mechanism to EGFR inhibitors in EGFR mutant non–small cell lung cancers (8–11). In these cancers, concomitant inhibition of MET and EGFR leads to marked reduction of cell viability both in vitro and in vivo (8–11). These observations have increased enthusiasm for developing MET inhibitors as cancer therapeutics.
Although encouraging clinical data with MET are emerging (12, 13), experience with other RTK inhibitors suggests that resistance will develop even in the subset of cancers that initially derive clinical benefit. In addition, there is also the concern that a single cancer may develop multiple, distinct resistance mechanisms simultaneously. For example, in an autopsy of a lung cancer patient who became resistant to EGFR inhibitors, different resistance mechanisms were observed in distinct metastatic sites (8, 9). Indeed, the prevalence of simultaneous heterogeneous resistance mechanisms remains unknown, as does its potential impact on our ability to reprise remissions.

In this study, we have examined how cancers can become resistant to MET inhibitors. We examined resistance with the highly sensitive gastric carcinoma cell line SNU638. Acquired resistance was modeled in vitro and in vivo to 2 related MET inhibitors PHA-665752 and PF-2341066 (crizotinib). (crizotinib). Surprisingly, we observed that the single cell line, SNU638, simultaneously developed 2 distinct mechanisms to maintain downstream signals for cell survival.

Materials and Methods

Cell lines and reagents
The SNU638 cell line was characterized previously (8). The cell lines MKN45 and EBC-1 were provided by Dr. Jeffrey Settleman (Cancer Center, Massachusetts General Hospital, Boston, MA). Both cell lines were maintained in RPMI 1640 with l-glutamine (Cellgro; Mediatech Inc.) supplemented with 10% fetal bovine serum, 100 units/mL penicillin, and 100 units/mL streptomycin. PHA-665752 and PF-2341066 were obtained from Tocris and ChemieTek, respectively, and PF-00299804 was provided by Pfizer. Stock solutions were prepared in DMSO and stored at −20°C.

Antibodies against ERBB3 and AKT (Santa Cruz Biotechnology); p85 and GAB2 (Millipore); GAPDH (Chemicon); and actin (Sigma) were used per manufacturer’s directions. All other antibodies were purchased from Cell Signaling. The human phospho-RTK array kit, human transforming growth factor α (TGFα) immunousassay, and recombinant human TGFα were purchased from R&D Systems.

ShRNA and lentiviral infection
MET, ERBB3, and scrambled [(scRNA) control] short hairpin RNA (shRNA) constructs were described previously (8).

Immunoprecipitation and Western blot
Cells were treated with PHA-665752 for 6 hours and then lysed using lysis buffer (20 mmol/L Tris, 150 mmol/L NaCl, 1% NP40, 0.1 mmol/L EDTA, and protease and phosphatase inhibitors). Coimmunoprecipitations with the PI3K regular subunit p85 were carried out as previously described (14).

Xenograft studies
Nude mice (nu/nu; 6–8 weeks old; Charles River Laboratories) were carried out in accordance with the standards of the Institutional Animal Care and Use Committee at Massachusetts General Hospital. Mice were anesthetized by 2% isoflurane (Baxter) mixed with oxygen and inoculated with 5 × 10^6 SNU638 cells (in 0.2 mL PBS) subcutaneously into the lower left side of quadrant. Once the tumor size was ~500 mm^3, the mice were treated with either PF-2341066 (25 mg/kg/d) or vehicle by oral gavage. Mouse weight and tumor size were measured 3 times per week.

Results

Resistant clones maintain PI3K-AKT, MEK-ERK, and TORC1 signaling in the presence of MET inhibitors
SNU638 is a gastric carcinoma cell line that is addicted to MET signaling and thus highly sensitive to MET inhibitors (Fig. 1A; ref. 8). Not surprisingly, it expresses MET to levels comparable with cells harboring MET amplification (e.g., EBC1, HCC827 GR5, H1993; refs. 8, 15; Supplementary Fig. S1). We grew SNU638 cells in increasing concentrations of the PHA-665752 until cells were able to grow in medium containing 1 μmol/L PHA-665752, a dose previously shown to potently inhibit MET signaling and markedly decrease cell viability in cancers addicted to MET signaling but is not toxic to MET-independent lines (7, 8, 11). Subclones (A1, A2, A3, C1, C2, and B1) derived from single cells of the resistant cell line showed marked resistance (Fig. 1A). Clones A1 and C1 were utilized for further analyses.

To determine whether the resistant clones had aberrant activation of RTKs, we assessed the activation status of multiple RTKs with human phospho-RTK arrays. In contrast to the parental sensitive cell line, the A1-resistant cells maintained MET and EGFR phosphorylation in the presence of PHA-665752. The C1 cells maintained only EGFR phosphorylation (Fig. 1B and C). In addition, unlike the parental sensitive cell line, drug treatment failed to substantially downregulate pAKT, pERK, or pS6 in either of the resistant clones (Fig. 1C).

TGFα-dependent activation of EGFR induces resistance
To determine how EGFR was being activated in the C1-resistant cells, we measured the expression levels of the EGFR ligands by quantitative reverse transcription PCR (RT-PCR). Of all the growth factors tested (including EGF, TGFα, amphiregulin, betacellulin, HB-EGF, and epiregulin), only TGFα RNA levels were dramatically increased (~13×; Fig. 2A, left, and Supplementary Fig. S2). There was also marked elevation of TGFα protein in the supernatant of resistant cells (Fig. 2A, right). To determine whether TGFα is sufficient to promote resistance, we added recombinant TGFα to parental SNU638 and MKN45 cells (an independent MET-addicted cancer cell line). We observed that exogenous TGFα was indeed sufficient to promote marked resistance to MET inhibition, but resistance was overcome by combined inhibition of MET and EGFR (Supplementary Fig. S3A). Although neither single-agent MET inhibitors nor single-agent EGFR inhibitors (gefitinib or PF-00299804) substantially blocked EGFR phosphorylation in C1 cells, combined EGFR and MET inhibition was more effective (Fig. 2B), suggesting that EGFR phosphorylation is due to both cross-talk with MET and TGFα-induced activation. Interestingly, EGFR inhibitors alone diminished ERK and
S6 phosphorylation, but not AKT phosphorylation, in C1 cells, suggesting that these cells undergo a “rewiring” in which EGFR signaling is the primary, independent driver of the ERK pathway. These findings were consistent with the observation that exogenous TGFα maintained phosphorylation of ERK and S6 in SNU638 and MKN45 cells treated with PHA-665752 but had only a modest effect on AKT phosphorylation (Supplementary Fig. S3B). Although EGFR inhibition alone had a moderate impact on C1 cell viability (possibly due to its regulation of ERK; Supplementary Fig. S4), EGFR inhibition potently re sensitized the cells to the effects of MET inhibition and overcame resistance (Fig. 2C, top).

**Somatic MET Y1230H mutation results in drug resistance in A1 cells**

Unlike the C1-resistant clone, the A1-resistant clone was not sensitive to combined EGFR and MET inhibition (Fig. 2C, bottom). Furthermore, they were resistant to 2 independent MET inhibitors, PHA-665752 (IC_{50} = 1.72 μmol/L vs. 13.2 nmol/L parental cells) and PF-2341066 (IC_{50} = 1.66 μmol/L vs. 2.42 nmol/L parental cells; Fig. 3A). Of note, the previous phospho-RTK arrays and Western blots revealed that a small amount of MET tyrosine phosphorylation persisted despite MET inhibitor treatment (Fig. 1B and C). Sequencing of the MET gene revealed the presence of a new mutation in the resistant cells (Fig. 3B). This mutation resulted in a change from a tyrosine to a histidine unit at position 1,230 (Y1230H). This mutation was further confirmed by sequencing individual bacterial colonies transformed with the MET RT-PCR product from the A1 cells (Supplementary Fig. S5). This mutation was not detectable in cDNA from parental cells (Fig. 3B).

These findings suggested that a mutation in MET may have led to resistance, analogous to resistance mutations observed in EGFR and ABL when cancers become resistant to gefitinib/erlotinib and imatinib, respectively. To determine whether the resistant A1 cells still required MET expression for their resistance, we assessed the effects of MET knockdown on cell viability. Knockdown of MET with 2 independent shRNAs effectively reduced viability of the A1 cells in a manner similar
to that of the parental cells, showing their continued dependence on MET expression (Supplementary Fig. S6). In contrast, the C1 cells were not sensitive to MET knockdown (Supplementary Fig. S6). This was anticipated, as the C1 cells were resistant to MET inhibitors due to ligand-dependent activation of EGFR signaling. To confirm that the deleterious effects of MET shRNA on the A1 cells were specifically due to MET knockdown, MET expression was rescued with a lentivirus expressing an MET cDNA resistant to the knockdown induced by one of the shRNA constructs [which targets the 3’ untranslated region (UTR) of MET RNA]. As shown in Fig. 3 C and D, MET expression rescued the cells from the effects of MET shRNA. Furthermore, expression of the MET Y1230H mutant was capable of rescuing the parental cells from the effects of MET knockdown. Thus, the A1 cells are resistant to MET inhibitors but are sensitive to MET knockdown, consistent with the notion that resistance is driven by the newly identified MET mutation that results in incomplete inhibition of the MET kinase activity. In addition, the A1 cells were rescued by wild-type (wt) MET because the A1 cells rely on MET signaling for survival and this could be supplied by wt MET. As expected, wt MET was sufficient to rescue viability, as these experiments were not carried out in the presence of the MET inhibitor.
The MET Y1230H mutation is sufficient to cause resistance to MET inhibitors

To determine whether the MET Y1230H mutation is sufficient to cause drug resistance, we overexpressed wt MET or MET Y1230H in SNU638 cells (Fig. 4A). Cells expressing MET Y1230H were substantially more resistant to both PHA-665752 and PF-2341066 (Fig. 4B), but the control cells expressing wt MET were still sensitive to MET inhibitors. The cells...

Figure 3. PHA-665752 resistant A1 cells acquire a Y1230H mutation in MET. A, both SNU638 parental (P) cells and A1 cells were treated with increasing concentrations of the MET inhibitors PHA-665752 and PF-2341066 for 72 hours. Cell survival was measured by Syto60 assays as described in Fig. 1A. B, A1 cells harbor a MET mutation. The MET kinase domain was amplified by RT-PCR. The sequencing results from the parental (P) and A1 resistant cells (A1) are shown. Note the presence of a mutation of T→C in the A1 cells leading to an amino acid change of Tyr→His at amino acid 1,230. Arrowheads, the site of mutation. C, parental (P) cells were infected with lentivirus expressing GFP, wt MET, or MET Y1230H. The MET-expressing constructs were resistant to knockdown by the MET shRNA. The resulting cells were infected by lentivirus carrying scRNA or shRNA against ERBB3 for another control (ERBB3 KD) or targeting the MET 3′ UTR (MET KD) and then selected by puromycin. Expression of ERBB3, MET, and actin was detected by Western blot analysis. ERBB3 levels were measured as a control to show that MET knockdown did not have an off-target effect on other RTKs. Note that MET often appears as 2 bands. The upper band is pro-MET, the form before proteolysis (34). D, the resulting cells as in (C) were infected by lentivirus encoding control or MET shRNA, and cell viability was determined 4 days after infection by the Syto60 assay. Experiments were carried out in triplicate. The P values of Student’s t test are 0.0010 (P+GFP scRNA vs. MET KD), <0.0001 (A1+GFP scRNA vs. MET KD), <0.0001 (MET KD P+GFP vs. wt MET), 0.0010 (MET KD P+GFP vs. MET Y1230H), and 0.0028 (MET KD A1+GFP vs. wt MET).
expressing Y1230H maintained MET phosphorylation as well as downstream signaling in the presence of PHA-665752, indicating that the Y1230H is sufficient to induce resistance to the MET inhibitors.

To determine whether MET Y1230H activates PI3K by the same molecular mechanisms as wt MET, we conducted PI3K immunoprecipitations that identify the adaptors leading to PI3K membrane recruitment and activation (8, 14, 16). We found that the parental and MET-overexpressing cells utilized ERBB3 and GAB2, but unlike the control cells and those overexpressing wt MET, the MET Y1230H cells maintained interactions with GAB2 and ERBB3 despite treatment with PHA-665752 (Supplementary Fig. S7A), consistent with the inability of the MET inhibitor to fully inhibit MET and downregulate PI3K-AKT signaling in these cells (Fig. 4C). Of note, we observed that exogenous expression of the Y1230H mutant was sufficient to induce resistance to two other MET addicted cell lines, EBC1 (non-small cell lung cancer) and MKN45 (gastric; ref. 7; Supplementary Fig. S8).

Development of resistant mutations in vivo

We also determined how SNU638 cells developed resistance to MET inhibition in vivo. SNU638 cells were subcutaneously injected into nude mice. Once the tumors were ~500 mm³, PF-2341066 was administered daily by oral gavage. Compared with the control mouse treated with vehicle alone, PF-2341066 resulted in tumor regression for 3 to 4 weeks before resistance developed (Fig. 5A). This resistant tumor was harvested at day 46 of treatment and used for establishing the cell line M1 (Mouse1).

We observed that the M1 cells maintained resistance to PHA-665752 and PF-2341066 in vitro (Fig. 5B). MET phosphorylation
Figure 5. Xenograft models of acquired resistance to PF-2341066 develop point mutations in amino acid Y1230. A, SNU638 parental cells were inoculated subcutaneously into nude mice. Placebo (water) or PF-2341066 was administered by oral gavage daily, and tumor size was measured 3 times weekly. B, a cell line derived from the resistant tumor (termed M1) was assessed for sensitivity to MET inhibitors in vitro by Syto60 assay as in Fig. 4B. C, cell lines derived from untreated (vehicle) and treated (resistant) mouse tumors (M1) were treated with 1 μmol/L PHA-665752 for 6 hours and the resulting lysates were analyzed by Western blots with the indicated antibodies. D, RNA from the resistant tumor and derived M1 cell line was extracted. The kinase domain was amplified by RT-PCR and sequenced. Note that the resistant tumor shows 2 nucleotide mutations impacting codon 1,230 (indicated by arrowheads). E, the kinase domain of the individual cell clones from the resistant M1 cell line reveals a heterogeneous population of cells. The majority of the resistant cells have acquired mutation leading to either Y1230H or Y1230C codon changes. No clones had both mutations.
(as well as intracellular PI3K, ERK, and TORC1 signaling) was maintained in the M1 cells after treatment with 1 μmol/L PHA-665752 (Fig. 5C) similar to the A1 cells described earlier. Furthermore, these cells maintained the association between PI3K and ERBB3 and GAB proteins despite treatment with the MET inhibitor similarly to the cells overexpressing MET Y1230H (Supplementary Fig. S9A and B).

Assessment of both the in vivo resistant tumor and the derived M1 cell line identified mutations in Tyr1230 (Fig. 5D) that were not detected in the parental cell line and untreated xenograft tumors. Assessment of single clones of cDNA isolated from the M1 cell line showed 2 different mutations in Tyr1230 in the resistant cancers Y1230H (TAT → CAT) and Y1230C (TAT → TGT). We derived cell lines from single-cell clones from the M1 cell line and assessed 15 of the derived clones. Three clones had no mutations in MET (wt/wt), 9 harbored MET Y1230H mutations, and 3 harbored Y1230C mutations (Fig. 5E). All of the clones harboring mutations in MET maintained resistance to PHA-665752 in vitro (Supplementary Fig. S9A).

Of interest, clones without mutant MET maintained sensitivity to PHA-665752, suggesting that, in vivo, they may have been resistant via non–cell autonomous mechanisms. Of note, we measured TGFα by RT-PCR in –they may have been resistant via non–cell autonomous mechanisms. Three clones had no mutations in clones from the M1 cell line and assessed 15 of the derived resistant tumor harbored a mutation in the unique activation loop conformation, as its hydroxyl is involved in a hydrogen-bonding network with Ala1226 and the side chain of Lys1110, which is also positioned to hydrogen bond with Asp1228. One explanation for the diminished inhibitory activity of PHA-665752 toward the Y1230H mutant MET is that the substitution of histidine for tyrosine at residue 1,230 results in decreased binding of PHA-665752 because of a weaker stacking interaction of the smaller histidine imidazole ring (vs. the tyrosine phenol) with the dichlorophenyl ring of PHA-665752 (Fig. 6B). Tyr1230 also seems to be an important residue in stabilizing the unique activation loop conformation, as its hydroxyl is involved in a hydrogen-bonding network with Ala1226 and the side chain of Lys1110, which is also positioned to hydrogen bond with Asp1228. One explanation for the diminished inhibitory activity of PHA-665752 toward the Y1230H mutant MET is that the substitution of histidine for tyrosine at residue 1,230 results in decreased binding of PHA-665752 because of a weaker stacking interaction of the smaller histidine imidazole ring (vs. the tyrosine phenol) with the dichlorophenyl ring of PHA-665752 (Fig. 6B). Loss of direct favorable interactions with PHA-665752 and other class I inhibitors may be even

**Figure 6.** Crystal structure reveals basis for resistance conferred by the Y1230H mutation. A, crystal structure of PHA-665752 bound to MET kinase domain (PDB ID = 2WKM). The protein backbone fold is depicted in gray with the exception of the activation loop, which is shown in magenta. Specific atom coloring is nitrogen (blue), oxygen (red), sulfur (yellow), chlorine (orange), and PHA-665752 carbon atoms (green). B, Y1230 compared with modeled H1230 interactions in the MET + PHA-665752 complex. The protein backbone fold is depicted in gray with the exception of the activation loop, which is shown in cyan. Specific atom coloring is nitrogen (blue), oxygen (red), sulfur (yellow), chlorine (orange), PHA-665752 carbon atoms (green), and modeled H1230 carbon atoms (magenta).

**The crystal structure of the MET tyrosine kinase domain bound to PHA-665752 reveals the role of Y1230**

A crystal structure of PHA-665752 bound to the kinase domain of MET (PDB entry = 2WKM) was determined. PHA-665752 binds to an autoinhibitory conformation of MET in which the beginning of the kinase activation loop forms a turn that is inserted between α-helix C and the N-terminal domain β-sheet (Fig. 6A). In this conformation, α-helix C is displaced from a catalytically competent orientation and the position of the activation loop prevents the binding of substrates. As bound to MET, the conformation of PHA-665752 is C-shaped, as has been observed for other class I MET inhibitors including PF-2341066 (17, 18). Activation loop residue Tyr1230 makes an aromatic stacking interaction with the dichlorophenyl ring of PHA-665752 (Fig. 6B). Tyr1230 also seems to be an important residue in stabilizing the unique activation loop conformation, as its hydroxyl is involved in a hydrogen-bonding network with Ala1226 and the side chain of Lys1110, which is also positioned to hydrogen bond with Asp1228. One explanation for the diminished inhibitory activity of PHA-665752 toward the Y1230H mutant MET is that the substitution of histidine for tyrosine at residue 1,230 results in decreased binding of PHA-665752 because of a weaker stacking interaction of the smaller histidine imidazole ring (vs. the tyrosine phenol) with the dichlorophenyl ring of PHA-665752 (Fig. 6B). Loss of direct favorable interactions with PHA-665752 and other class I inhibitors may be even
greater for the Y1230C mutation than for the Y1230H mutation due to the nonaromaticity and smaller size of the sulfydryl side chain.

Another contributing factor to the inhibitor resistance of the Y1230H/C mutations may be that the substitutions at position 1,230 destabilize the autoinhibitory conformation of the activation loop and change the protein conformational equilibrium in the direction of a catalytically active conformation. Modeling of histidine or cysteine at position 1,230 reveal that they would not be able to form the same stabilizing hydrogen bonding network observed with Tyr1230 (Fig. 6B). Loss of this hydrogen bonding network as well as the impact of the smaller side chains not completely filling the space of the tyrosine likely destabilize the autoinhibitory conformation. It is therefore likely that acquired resistance mutations at position 1,230 may also be discovered with other class I MET inhibitors that bind to this autoinhibitory conformation of MET and make a direct interaction with Tyr1230.

Discussion

The deflating realization that cancers become resistant to effective targeted therapies has spurred great interest in determining how cancers become resistant so that we can identify more effective strategies to induce more durable remissions. In this study, we examined resistance to MET tyrosine kinase inhibitors (TKI). To our surprise, using a single cell line, SNU638, we observed multiple mechanisms by which these cells became resistant to MET inhibitors. Some clones became resistant by activating the EGFR through autocrine production of ligand, whereas other clones acquired novel mutations in amino acid 1,230 that conferred resistance. These results were recapitulated by developing resistance models in vivo as well. The finding that a single plate of 1 million cells and a small subcentimeter tumor in vivo can simultaneously develop multiple mechanisms of resistance highlights the notion that patients with cancers consisting of billions to trillions of cells have the capacity to simultaneously develop a wide array of resistance mechanisms. This will continue to challenge our capacity to strategically reinduce remissions.

Resistance to other targeted therapies including EGFR and ABL inhibitors has been associated with the development of secondary mutations that abrogate TKI inhibition. The most common mutation that develops after treatment with EGFR kinase inhibitors is EGFR T790M (19, 20), and a common one after treatment with imatinib is ABL T315I (21–23). Both mutations are located in an analogous position in the kinase domain and have been termed “gatekeeper” mutations. In this study, we identified mutations in Y1230 as an acquired resistance mechanism to class I MET inhibitors. The occasional existence of MET Y1230 mutations in pretreatment cancers (24) is analogous to the observations that some lung cancers and leukemias harbor EGFR T790M and ABL T315I, respectively, prior to treatment (25, 26). In the case of MET, this is likely related because of increased MET activity conferred by the Y1230 mutation. Indeed, the structural analyses (Fig. 6) suggest that mutation destabilizes the autoinhibitory confirmation. This is supported by the finding that MET Y1230H has increased catalytic activity in vitro (18) and has transforming activity in vivo (27).

The MET Y1230H mutation is located in the activation loop of the enzyme. Structural analyses suggest that the substitution of Y1230 with histidine or cysteine has a lower affinity with PF-2341066 and PHA-665752. Indeed, these results are supported by previous in vitro kinase assays showing that these compounds have decreased inhibitory activity toward MET Y1230H as compared with wt MET in enzymatic and cellular assays (18). Although these and other class I MET inhibitors seem to have decreased activity against MET Y1230H (28), there have recently been reports of class II MET inhibitors that can potently inhibit Y1230H (29, 30). Theoretically, such inhibitors will effectively treat these Y1230 mutant resistant cancers. Moreover, these inhibitors might prevent the acquisition of Y1230 mutations as a resistant mechanism.

Recent studies suggest that “pulse dosing” may permit one to overcome resistance and effectively treat oncogene-addicted cancers with targeted therapies (31). Indeed, we observed that very high levels of PF-2341066 (10 μmol/L) could potently suppress MET in Y1230 mutant cells (Supplementary Fig. S10A). Although this dose was capable of inhibiting growth of SNU638 parental cells after only 1 hour of exposure, the resistant M1 cells required 24 hours of high-dose exposure (Supplementary Fig. S10B). Of note, previous studies found that mice could tolerate 50 mg/kg dosage level and plasma levels achieved concentrations of 2 μmol/L (32). Although it remains unknown if mice, or more important, humans, could tolerate doses required to provide sufficient target inhibition of Y1230 mutants, the marked decrease in potency against the resistant mutant (300- to 1,000-fold) suggests that newer MET inhibitors that can effectively target Y1230H may ultimately be a more effective clinical strategy.

In addition, we observed that activation of EGFR induced resistance to MET inhibitors. Of note, we had previously observed the reciprocal finding that MET activation is one mechanism of resistance in EGFR mutant lung cancers treated with EGFR TKIs (8, 11). In this study, we found that SNU638 cells adapt to MET inhibition by overexpressing the EGFR ligand TGFα to promote resistance. Similarly, another study showed that exogenous addition of other growth factors (EGF and heregulin) rescued MET-driven cells (GTL-16 and MKN45) from MET inhibition (33); however, that report did not identify upregulation of ligand as a naturally occurring resistance mechanism. Both the C1-resistant cells and the cells treated with exogenous TGFα show that ligand-dependent activation of EGFR strongly maintained ERK signaling, but its effects on PI3K signaling were more modest. Importantly, EGFR inhibition re sensitized these cells to MET inhibition. Because tumor stroma can secrete TGFα in vivo, cancers may acquire resistance by autocrine- or paracrine-derived sources.

In addition to SNU638 cell line, we also aimed to determine how other MET-addicted cancer models would develop resistance. We recently developed resistant clones from EBC1 cells in vivo (designated 1A, 7A, and 4B) by the same procedure that generated the SNU638-resistant cells (Supplementary
Fig. S11A). These resistant clones do not seem to share the same resistance mechanisms identified in the SNU638 cells. Unlike the C1 cells, they were not sensitive to PHA-665752 plus gefitinib combination treatment (Supplementary Fig. S11B). There were also no observed resistant mutations in the kinase domain (Supplementary Fig. S11C), MET phosphorylation was fully suppressed by MET inhibitors (Supplementary Fig. S11D), and they were insensitive to MET knockdown by MET shRNA (Supplementary Fig. S11E). Although the mechanism is unknown, these studies do reveal that there will be additional mechanisms of acquired resistance to MET inhibitors. However, the Y1230I/C point mutations identified in the SNU638 cells may ultimately prove to be a highly prevalent resistance mechanism to class I MET inhibitors. Indeed, acquired point mutations in drug targets have been a commonly observed resistance mechanism in other targeted therapy paradigms as well (e.g., EGFR T790M and ABL T315I).

In summary, our data suggest that even a single cell line in vitro can develop more than one type of mechanism to become drug resistant. Indeed, we find evidence of both acquired mutations in MET and the upregulation of EGFR ligand to promote resistance. As cancers become resistant to the "C-shaped" MET inhibitors in the clinic, it will be important to assess for these resistance mechanisms in patients. Indeed, the therapeutic strategies that combine MET inhibitors capable of inhibiting Y1230 mutant MET in combination with anti-EGFR–based therapies may translate into enhanced clinical benefit for patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments
We thank Dr. Jeff Settleman and his laboratory, Dr. Anthony Faber, and Dr. Hiromichi Ebi for helpful discussions.

Grant Support
This study is supported by NIH K08 CA120060-01 (J.A. Engelman, P.A. Janne), B01CA120060-01 (J.A. Engelman, P.A. Janne). B01CA140594 (J.A. Engelman). R01CA114465-01 (P.A. Janne), B01CA125257-01 (P.A. Janne, J.A. Engelman). National Cancer Institute Lung SPORE P50CA096756 (J.A. Engelman, P.A. Janne), P50 CA127003 (J.A. Engelman). AACR (J.A. Engelman), the V Foundation (J.A. Engelman), ACS RSG-06-102-01-CCU (P.A. Janne, J.A. Engelman), Hazel and Samuel Bellin research fund (P.A. Janne), and the Ellison Foundation Scholar (J.A. Engelman).

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Received May 11, 2010; revised October 4, 2010; accepted October 14, 2010; published OnlineFirst January 25, 2011.


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Cancer Res  Published OnlineFirst January 25, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-10-1623

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/01/25/0008-5472.CAN-10-1623.DC1

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