Strengthening Context-Dependent Anticancer Effects on Non–Small Cell Lung Carcinoma by Inhibition of Both MET and EGFR

Yu-Wen Zhang, Ben Staal, Curt Essenburg, Steven Lewis, Dafna Kaufman, and George F. Vande Woude

Abstract

The MET and EGFR receptor tyrosine kinases (RTK) are often coexpressed and may cross-talk in driving the development and progression of non–small cell lung carcinoma (NSCLC). In addition, MET amplification is an alternative resistance mechanism for escaping EGFR-targeted therapy. To assess the benefits of combined targeting of MET and EGFR for treating NSCLCs, we investigated the activities of these two RTK pathways in NSCLC cell lines and evaluated their responses to SGX523 and erlotinib, the small-molecule kinase inhibitors of MET and EGFR, respectively. We showed that MET interacts with and cross-activates EGFR in MET-amplified or -overexpressed cells. The inhibition of both MET and EGFR results in maximal suppression of downstream signaling and of cell proliferation when their ligands are present. Furthermore, we showed that SGX523 plus erlotinib strengthens anticancer activity in vivo in a cellular context–dependent manner. The combination led to the regression of H1993 tumors by enhancing the suppression of proliferation and inducing apoptosis, whereas H1373 tumor growth was significantly reduced by the combination via suppression of proliferation without inducing apoptosis. SGX523 alone was sufficient to achieve near-complete regression of EBC-1 tumors; its combination with erlotinib strongly inhibited the viability of a population of insensitive cells emerging from an SGX523-treated EBC-1 tumor recurrence. Our data suggest that inhibition of both MET and EGFR can enhance anticancer effects against NSCLCs in a context-dependent manner and thus provide a strong rationale for combining MET and EGFR inhibitors in treating NSCLCs.

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Introduction

Receptor tyrosine kinases (RTK) are important cellular components in many normal developmental and physiologic processes, but their aberrant activation can cause serious pathologic outcomes by contributing to cancer development and progression (1–3). Activated RTK pathways can promote many hallmark cancer events such as cell proliferation, transformation, survival, invasion, metastasis, and angiogenesis (4). It is believed that targeting aberrant RTK pathways may reverse the course of or block cancer progression and provide effective therapeutic options for patients. Thus, drugs targeting RTKs have been developed or are under development for treating malignant cancers such as non–small cell lung cancer (NSCLC), among them inhibitors of EGF receptor (EGFR) and of MET.

NSCLC accounts for more than 80% of the cases of lung cancer, which is the leading cause of death among all human cancer casualties (5, 6). Advances in early diagnosis, therapy, and preventive measures have led to a substantial decline in lung cancer incidence and mortality over the years, but the prognostic outcome for advanced-stage lung cancer remains particularly poor because of a lack of effective therapeutic options. Because EGFR is overexpressed or mutated in many NSCLC tumors, 2 small-molecule kinase inhibitors targeting EGFR, erlotinib and gefitinib, have been approved for treating advanced and metastatic cases of NSCLCs (7, 8). However, the benefit of such treatment appeared to be limited to patients carrying particular EGFR mutations or amplifications (8, 9). The ineffectiveness of these treatments for most NSCLC tumors is probably due to availability of other pathways, such as that of MET, which can bypass EGFR inhibition.

Another problem of EGFR-targeted therapy is that patients initially sensitive to the treatment become unresponsive because of emerging drug-resistant tumor cell populations. While the T790M secondary mutation in EGFR is the major cause of resistance to erlotinib or...
gefitinib treatment, amplification of MET appears to be an important alternative resistance mechanism in NSCLCs (10–12). It has also been shown that both MET and EGFR regulate several microRNAs (miRNA) that play roles in modulating the signaling of these RTKs and in controlling gefitinib sensitivity in NSCLCs (13).

Like EGFR, MET is an RTK frequently activated in NSCLCs by overexpression, amplification, or mutation (14–18). Aberrant MET signaling can be driven by autocrine, paracrine, or ligand-independent mechanisms, and it leads to inappropriate activation of downstream signaling pathways such as RAS/MAPK and PI3K/AKT, most of which also mediate EGFR signaling. While normal MET activity—stimulated by its ligand, hepatocyte growth factor (HGF)—plays crucial role in mitogenesis, morphogenesis, and motility, its aberrant activation can result in uncontrolled tumor growth and metastasis. Therefore, the MET pathway is becoming another attractive target for cancer intervention, and diverse therapeutic approaches have been exploited to hinder the activation of this pathway (19, 20). Promising therapeutic outcomes have been observed in clinical trials of MET-targeted drugs (17, 20).

MET inhibition, like EGFR-targeted therapy, is also subject to drug resistance. One mechanism for escaping MET kinase inhibition in NSCLC cell line appears to be by switching to EGFR signaling for cell growth (21). Given that MET is often coexpressed with EGFR and acts as a crucial resistance mechanism for EGFR-targeted therapy, we reasoned that simultaneously targeting MET and EGFR may strengthen the anticancer effect and provide a better therapeutic option for patients with NSCLCs. The rationale behind combined MET- plus EGFR-targeted therapy is not only to prevent the emergence of acquired resistance tumor cell populations but also to mutually sensitize cells to the other targeted drug which is otherwise less effective as a single agent.

We have previously shown that the MET small-molecule kinase inhibitor SGX523 synergizes with the EGFR inhibitor erlotinib to suppress xenograft growth of the NSCLC cell line H596, which harbors a mutation in the juxtamembrane domain of MET (22). To further understand the benefit of a combined MET- and EGFR-targeted therapy against NSCLCs, we investigated the effects of SGX523 and erlotinib on in vitro signal transduction and cell proliferation and on in vivo tumor growth of NSCLC cell lines having distinct cellular properties. Here, we report that amplified or overexpressed MET interacted with and cross-activated EGFR in NSCLC cell lines. SGX523 effectively inhibited MET-dependent EGFR activation, whereas the SGX523 and erlotinib combination strengthened the inhibition of downstream extracellular signal–regulated kinase (ERK) and AKT and maximally suppressed cell proliferation in vitro when their ligands were present. Blocking both MET and EGFR in vivo resulted in enhanced suppression of H1373 tumor growth and in regression of H1993 tumors. The combination enhanced suppression of proliferation in both tumor models, but induced apoptosis only in the H1993 tumors. In contrast, EBC-1 tumors appeared to be addicted to MET signaling, as SGX523 treatment alone resulted in nearly complete tumor regression. However, SGX523 plus erlotinib reduced the viability of SGX523-insensitive cells derived from EBC-1 tumors that recurred after SGX523 treatment. Our data suggest that combined MET and EGFR inhibition can strengthen anticancer effects on NSCLCs in a context-dependent manner.

Materials and Methods

NSCLC cell lines

The A427, H358, H596, H1373, H1993, and SK-MES-1 cell lines were obtained from the American Type Culture Collection. The EBC-1 cell line was from the Health Science Research Resources Bank (Tokyo, Japan). The A549, EKVX, H23, H226, H322, H460, H522, and HOP92 cell lines were from the NCI-60 cell lines (NCI-Frederick). The H125, H157, H292, H441, H647, H661, H727, H838, H1264, H1299, H1334, H1725, H1944, H2122, and H2347 cell lines were from Drs. John Minna and Adi Gazdar (University of Texas Southwestern Medical School, Dallas, TX). The morphologic appearance of the cells was routinely monitored for authentication. All cell lines were cultured in RPMI-1640 medium (Invitrogen) supplemented with 10% FBS (Atlas Biologicals) plus 1% penicillin and 1% streptomycin.

Western blot analyses

Whole-cell lysates were prepared using radioimmuno-precipitation assay (RIPA) buffer (20 mmol/L Tris-HCl, pH 7.5, 150 mmol/L NaCl, 1% NP40, 0.5% deoxycholate, 0.1% SDS, 1 mmol/L EDTA, 50 mmol/L NaF, and 1 mmol/L sodium orthovanadate) containing proteinase inhibitor cocktail (Roche) and PhosSTOP (Roche). The lysates were quantified with a DC Protein Assay kit (Bio-Rad) and mixed with equal volume of 2× Laemmili Sample Buffer (Sigma). The proteins were separated in Mini-Protein TGX Gels (Bio-Rad) after electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Invitrogen). The proteins were detected with Amersham ECL Western Blotting Detection Reagents and exposed on Amersham Hyperfilm ECL (GE Healthcare) following primary and secondary antibody reactions. The anti-MET, anti-EGFR, and anti-ErbB3 antibodies were from Santa Cruz; anti-phospho-Met (Tyr1234/1235), anti-phospho-ERK1/2, anti-Akt, and anti-phospho-Akt (Ser473) from Cell Signaling Technology; anti-phospho-EGFR (Tyr1068) from Invitrogen; and anti-β actin from Sigma.

RNA preparation and RT-PCR

Total RNAs were isolated from each cell line using TRIzol reagent (Invitrogen). For reverse transcription (RT)-PCR, first-strand cDNA was first synthesized from 1 μg of RNA using SuperScript II reverse transcriptase (Invitrogen). Then, PCR was used to detect HGF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts using
GoTaq Green Master Mix (Promega). The primers used were: for HGF, 5'-AAACGCAAACAGGTTCTCAATG-3' (sense) and 5'-CTATGACTTGCTACCTTATAT-3' (antisense); and for internal control GAPDH, 5'-AAGGAT-TTGGTCTGATTGGGC-3' (sense) and 5'-GCTTACCAC-CTTCTTGATGTC-3' (antisense).

**Immunoprecipitation**

Cells were washed 3 times in ice-cold PBS, and the whole-cell lysates were extracted using lysis buffer consisting of 20 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP-40, 10% glycerol, 1 mmol/L sodium orthovanadate, 2 mmol/L EDTA, 1 mmol/L dithiothreitol (DTT), and proteinase inhibitor cocktail (Roche). Five hundred micrograms of total lysate was incubated with anti-EGFR immunoprecipitation-specific mouse monoclonal antibody (Cell Signaling) at 4°C overnight, followed by 2 hours of incubation with Protein G Sepharose (GE Healthcare) to precipitate the immune complexes. Following immunoprecipitation, Western blot analyses were conducted as detailed above to detect the immunoprecipitated proteins.

**[3H]-Thymidine incorporation assay**

The assay was conducted as previously described (22). Briefly, the cells were seeded in 96-well plate and, after 48 hours of serum starvation, treated with inhibitor and/or growth factor for 20 hours. Then, the cells were incubated with 1 μCi of [3H]-thymidine (GE Lifesciences) and/or growth factor for 20 hours. Then, the cells were treated with SGX523 were allowed to regrow following removal of the drug. After reaching certain sizes, the tumors were retreated with SGX523 (60 mg/kg, twice daily) for 2 to 4 weeks.

To establish cell lines, the tumors were harvested from euthanized mice and minced under aseptic condition. Then, the tumor explants were digested in growth medium containing 50 μg/mL of collagenase for overnight. After digestion, the cells were broken up by repeated pipetting, centrifuged to clear the debris, and cultured in RPMI supplemented with 10% FBS and 1% penicillin/streptomycin.

**Immunohistochemistry and immunofluorescence staining**

Immunohistochemical (IHC) staining of Ki67 in formalin-fixed, paraffin-embedded (FFPE) tumor sections was conducted using a Ventana IHC staining system (Ventana Medical Systems) and the anti-Ki67 rabbit polyclonal antibody, obtained from Abcam. For immunofluorescence, antigens were retrieved by immersing FFPE sections in boiled TE buffer containing 10 mmol/L Tris-HCl and 1 mmol/L EDTA (pH 9.0) for 30 minutes before staining. The tumor sections were incubated with anti-cleaved caspase-3 (Asp175) rabbit monoclonal antibody (Cell Signaling) overnight at 4°C and then were incubated with Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen) and 4',6-diamidino-2-phenylindole (DAPI) for 1 hour at room temperature. After staining, the slides were mounted with aqueous mounting medium (Biomedica) and images were recorded under a UV fluorescence microscope.

**CellTiter-Glo luminescent cell viability assay**

Cells were seeded on a 96-well plate (5,000 cells/well) in regular growth medium on day 1. The next day, the growth medium was changed to RPMI medium supplemented with 1% FBS. On day 3, the cells were treated with or without inhibitors in RPMI medium plus 1% FBS, with or without the addition of HGF and EGF. The assay was conducted on day 5 by lysing the cells in CellTiter-Glo reagent (Promega) according to the manufacturer’s instructions and measuring luminescence activity using a Wallace EnVision 2104 Multilabel Reader (PerkinElmer).

**Statistical analysis**

The Student t test was used to analyze the statistical significance of the data. The GraphPad Prism program was used to conduct IC50 analysis and Kaplan–Meier survival analysis. For synergistic/additive analysis, the CalcuSyn program based on the Chou–Talalay Method (24) was used.

**Results**

**MET interacts with and cross-activates EGFR in NSCLC cell lines**

Coexpression of MET and EGFR was detected in more than 75% of the NSCLC cell lines that we examined.
To determine the relationship between these RTKs, we examined their expression and tyrosine phosphorylation status in 4 NSCLC cell lines that have MET overexpression or amplification (Fig. 1B). We observed strong constitutive MET phosphorylation in the MET-amplified H1993 and EBC-1 cells (25) as well as in nonamplified H1373 cells (Fig. 1B). The constitutive phosphorylation of MET was not dependent on its ligand, because no HGF expression was detected (Fig. 1C). Constitutively phosphorylated EGFR was also observed in the 3 MET-activated cell lines (Fig. 1B). Surprisingly, EGFR phosphorylation in the H1993 and EBC-1 cells was strongly suppressed by the MET inhibitor SGX523 (26), but not by its own inhibitor, erlotinib, in the absence of its ligand EGF (Fig. 2C and D; and Supplementary Figs. S1–S3), indicating that the constitutive EGFR activation in these cells is, in part, MET-dependent. In H1373 cells, both SGX523 and erlotinib appear to significantly reduce EGFR phosphorylation (Fig. 2B). To determine whether the cross-activation of EGFR by MET is mediated by interaction between these 2 RTKs, immunoprecipitation analysis was conducted. We found that EGFR physically interacted with MET in the H1373, H1993, and EBC-1 cells (Fig. 1D), suggesting that MET may phosphorylate proximal EGFR via heteroreceptor dimerization.

**Blockage of both MET and EGFR enhances downstream signaling inhibition**

We next asked how MET and EGFR signaling might be affected by SGX523 and erlotinib in the absence or presence of their ligands. While there was MET-dependent activation of EGFR in the H1373, H1993, and EBC-1 cells, the presence of EGF induced more EGFR phosphorylation in these cells (Fig. 2B–D), indicating the existence of both ligand-dependent and -independent states. Constitutively phosphorylated ErbB3 in H1373 and H1993 cells also appeared to be dependent, in part, on MET kinase activity, as it was abolished by SGX523 (Fig. 2B and C). We are not clear at this point what role ErbB3 plays in these cells, even though it is known that activation of ErbB3 by amplified MET is a mechanism of resistance to EGFR-targeted therapy (10). Moreover, HGF and/or EGF stimulation induced higher activation of ERK and AKT (Fig. 2), the 2 major pathways downstream of these RTKs. In H358 and H1373 cells, HGF-induced ERK and AKT activation was inhibited by SGX523, whereas ERK activation induced by EGF was inhibited by erlotinib, provided there was only
one ligand present (Fig. 2A and B). It required both SGX523 and erlotinib to inhibit ERK activation when both HGF and EGF were present (Fig. 2A and B). In MET-amplified H1993 and EBC-1 cells, ERK and AKT appeared to be constitutively activated in a MET kinase–dependent manner because their phosphorylation was abolished by
SGX523 (Fig. 2C and D; and Supplementary Fig. S3); however, when EGF was present, their phosphorylation could only be abolished by the combination of SGX523 and erlotinib (Fig. 2C and D). These data suggest that combined inhibition of MET and EGFR is required to fully inhibit both ligand-dependent and -independent activation of these 2 RTKs in NSCLCs.

**SGX523 plus erlotinib strengthens inhibition of cell proliferation in vitro**

To determine the effects of MET and/or EGFR inhibitors on NSCLC cell proliferation, we conducted [3H]-thymidine incorporation assays in vitro. SGX523 did not affect the basal proliferation of H358 and H1373 cells in the absence of ligands (Fig. 3A and B), but it strongly inhibited that of H1993 and EBC-1 cells (Fig. 3C and D). In the absence of HGF, the IC50 of SGX523 is 3 nmol/L for H1993 and 4 nmol/L for EBC-1, whereas in the presence of HGF it is 8 nmol/L for H1993 and 36 nmol/L for EBC-1, respectively (Fig. 3E). In contrast, erlotinib appeared to be more effective against H358 and H1373 proliferation, and a combination of the 2 inhibitors enhanced the inhibitory activity when both ligands were present (Fig. 3A and B). Regardless of HGF and or EGF presence, SGX523 strongly inhibited H1993 and EBC-1 cell proliferation, whereas erlotinib produced partial inhibition (Fig. 3C and D). Maximal inhibition of H1993 cell proliferation was achieved by SGX523 plus erlotinib (Fig. 3C), but SGX523 alone almost fully inhibited proliferation of EBC-1 cells (Fig. 3D), indicating that this cell line is addicted to MET activity for proliferation. For H1373 and H1993 cells, the effect of the combination (1 μmol/L SGX523 plus 5 μmol/L erlotinib) is synergistic (Supplementary Fig. S4). These data suggest that combined targeting of MET and EGFR may provide better antiproliferative activity against NSCLCs, although MET inhibitor alone might be sufficient for MET-addicted tumor cells.

**Context-dependent augmentation of anticancer effects by SGX523 and erlotinib combination**

We next evaluated the in vivo efficacies of SGX523 and erlotinib on NSCLC xenograft tumors raised in an hHGFtg-SCID mouse model that we engineered (23). This transgenic mouse strain produces human HGF, allowing paracrine activation of MET signaling—which is not activated by mouse HGF—in human xenograft tumors. Nontransgenic C3H-SCID mice were used as controls for comparing drug efficacy in the absence of HGF-dependent paracrine MET activity.

Even though SGX523 or erlotinib alone partially suppressed H1373 tumor growth, we found that the 2 inhibitors together significantly enhanced antitumor activity in the hHGFtg-SCID mouse (Fig. 4A). IHC staining of the proliferative marker Ki67 in the xenograft tumor sections revealed that SGX523 plus erlotinib markedly inhibited proliferation relative to the individual inhibitors alone (Fig. 4B). Also, the combination markedly reduces ERK phosphorylation in the H1373 tumors (Supplementary Fig. S5). The effect of the 2-inhibitor combination in the hHGFtg-SCID mice was much more impressive on H1993 tumors, than on H1373 tumors. In H1993 tumors, treatment with both inhibitors resulted in tumor regression (Fig. 5A). This synergistic effect appeared to be achieved not only by inhibiting the proliferation activity (Fig. 5B) but also by inducing apoptosis, as determined by the cleaved caspase-3 staining in the tumors (Fig. 5C). Apoptotic activity induced by SGX523 plus erlotinib was not found in H1373 tumors (Fig. 4C), indicating a context-dependent effect of the combination therapy. These data appear to be consistent with the observation that SGX523 and erlotinib combination strongly reduces the viability of H1993 cells but not of H1373 cells in vitro (Supplementary Fig. S6). A lesser drug efficacy was also observed in the control C3H-SCID mice with H1373 and H1993 tumors (Figs. 4A and 5A).

Unlike the H1373 and H1993 tumors, the MET-addicted EBC-1 tumors were extremely sensitive to MET inhibitor; SGX523 alone resulted in a near-complete tumor regression in both hHGFtg-SCID and control C3H-SCID mice, although erlotinib alone displayed partial inhibitory activity (Fig. 6A). Despite this, it appeared that the SGX523-treated tumor cells were not completely eradicated, as most tumors regrew following removal of SGX523 (Fig. 6B). Interestingly, the relapsed EBC-1 tumors were still quite sensitive to SGX523 (Fig. 6B), indicating that the majority of the relapsed tumor cells mimicked the sensitivity of the parental population. Nevertheless, some insensitive populations seem to be established slowly, because the size of tumors remained measurable even after extended second round of SGX523 treatment (Fig. 6B). These tumors likely consisted of either preexisting, slow-growing cells that are primarily less sensitive to MET inhibitor or acquired resistant cells emerging from the initial SGX523-sensitive populations or both.

We established cell lines from the tumors remaining in the hHGFtg-SCID mice following the second round of SGX523 treatment and asked how these cells would respond to MET and EGFR inhibitors in vitro. Compared with the parental EBC-1 cells, EBC1-Sg3-H62 and EBC1-Sg3-H71 cells were relatively less sensitive to SGX523 (Supplementary Fig. S7), but their viability was significantly decreased by the addition of erlotinib to SGX523 treatment (Fig. 6C). A slightly higher EGFR phosphorylation can be observed in the EBC1-Sg3-H62 and -H71 cells, compared with that in EBC-1 cells in the presence of ligands (Supplementary Fig. S8). Also, a stronger p-EGFR level is left in the 2 EBC-1 derivatives after erlotinib treatment, compared with the parental cells in the presence of ligands (Supplementary Fig. S8). These data suggest that even with MET-addicted tumors, inhibition of both MET and EGFR can still provide significant benefit by preventing the emergence of a tumor cell population that is leaning toward the EGFR pathway for survival and/or growth.
Figure 3. SGX523 sensitized NSCLC cells to erlotinib in suppressing proliferation in the presence of MET and EGFR ligands in vitro. H358 (A), H1373 (B), H1993 (C), and EBC-1 (D) cells were treated with/without SGX523 (1 µmol/L) and/or erlotinib (5 µmol/L), with/without stimulation with HGF (200 U/mL) and/or EGF (50 ng/mL) for 24 hours before measuring proliferative activity. All assays were conducted in triplicate, and the error bars represent SD. The IC50 was determined using the GraphPad Prism program, as plotted on the right.
Figure 4. SGX523 plus erlotinib strengthened antiproliferation but did not induce apoptosis in H1373 tumors. A, enhanced suppression of H1373 tumor growth by SGX523 plus erlotinib. H1373 xenograft tumors in the hHGFtg-SCID or the control C3H-SCID mice were treated with/without SGX523 (60 mg/kg twice daily) and/or erlotinib (150 mg/kg once daily) for 14 days. B, the combination enhanced the suppression of cell proliferation in the tumors derived from the hHGFtg-SCID mice. Cell proliferation was determined by IHC staining of Ki67. Representative images and the quantification of Ki67-positive cells for each treatment are shown. The boxes in the graph represent the average number of Ki67-positive cells in a ×40 field, and the error bars indicate SD. C, the two-drug combination did not induce apoptosis in H1373 tumors. Immunofluorescence staining of cleaved caspase-3 (Asp175) was used to determine apoptotic cells. Representative images (merge of cleaved caspase-3 staining in red and DAPI staining in blue) for each treatment are shown. The statistical analysis was done by the Student t test and indicated as P value.
Discussion

The lack of effective therapeutic options for advanced NSCLCs prevents us from improving the poor prognosis and high mortality rate of this disease (6). New therapies are greatly needed for patients with NSCLCs. Advances in our understanding of lung cancer development and
Figure 6. MET-addicted EBC-1 tumors were extremely sensitive to SGX523 in vivo. A, SGX523 treatment led to near-complete regression of EBC-1 tumors in both hHGFtg-SCID and control C3H-SCID mice. B, responses of the relapsed tumors to a repeated SGX523 treatment. The SGX523-treated tumors in the hHGFtg-SCID mice were allowed to regrow following removal of the drug. The relapsed tumors were retreated with SGX523 (60 mg/kg twice daily) for the second round: 2 weeks for round 2a (including #58, #6, #59, and #64) and 4 weeks for round 2b (including #62, #65, and #71). C, erlotinib sensitized SGX523 on reducing cell viability of SGX523-insensitive EBC-1 derivatives. EBC1-Sg3-H62 and EBC1-Sg3-H71 cells were established from tumor explants derived from the #62 and #71 hHGFtg-SCID mice, respectively, after the second round of SGX523 treatment. The cell viability was determined by CellTiter-Glo Luminescent Assay in the absence and presence of HGF (200 U/mL) and EGF (50 ng/mL). The Student t test P values are shown.
progression have led to the new application of targeted cancer therapies by inhibiting particular oncogenic driver pathway components such as EGFR or MET. Data from recent phase II clinical trials have shown that combining an MET inhibitor (MetMab, a neutralizing antibody against the MET extracellular domain; or AQR197, a small-molecule MET inhibitor) with erlotinib can improve the progression-free survival of patients with NSCLCs relative to erlotinib alone (27, 28). However, the response to the combination therapy seems to differ between patient subgroups and is greatly affected by the status of MET expression and/or other factors (27, 28).

Here, we evaluated the benefit of inhibiting both MET and EGFR in preclinical models and determined the mechanism of how the combination may strengthen anticancer effects on NSCLCs in different cellular contexts. We show that overexpressed or amplified MET can cross-activate wild-type EGFR via heteroreceptor interaction in H1373, H1993, and EBC-1 cells. This cross-activation seems to orient in one direction, from MET to EGFR, within the H1993 and EBC-1 cells, evidenced by the fact that SGX523 inhibits basal EGFR phosphorylation but erlotinib does not seem to affect MET tyrosine phosphorylation. However, in other cellular contexts, cross-activation between MET and EGFR can go the other way around, from EGFR to MET: for example, EGFR inhibitor significantly reduces MET tyrosine phosphorylation in HCC827 and H441 cells (10, 29). The difference in MET-EGFR cross-talk in different cellular contexts is likely determined by which receptor is more abundant on the cell membrane. Even in cell lines constitutively activated for MET or EGFR, the addition of their ligands can further activate their downstream signaling ERK and AKT. Strikingly, a combination of SGX523 plus erlotinib is required for full inhibition of their downstream signaling when MET and EGFR ligands are present, underlying the need for blocking both ligand-dependent and -independent activation to achieve an effective therapeutic outcome.

The key goals of many anticancer therapies are to suppress cell proliferation and/or to induce apoptosis, thereby reversing or slowing tumor progression. In vitro, it appears that different NSCLC cell lines show very different sensitivities to SGX523 and/or erlotinib. The MET-amplified H1993 and EBC-1 lines are very sensitive to SGX523, whereas non–MET-amplified H358 and H1373 cells are more sensitive to erlotinib. The presence of ligand greatly affects the suppressive activity of each inhibitor on proliferation. In general, both SGX523 and erlotinib are needed to achieve a maximal inhibition on downstream signaling and cell proliferation when the ligands are present, although EBC-1 cells appear to be an exception, as SGX523 alone can almost fully inhibit them. The activities of the drugs on proliferation in vitro seem to be in agreement with their activities on signal transduction. This is no surprise because both MET and EGFR can activate common downstream pathways, such as RAS-MAPK/ERK and PI3K-AKT, and via those pathways appear to trigger similar cellular activities including proliferation. Therefore, inhibiting either MET or EGFR alone would diminish the suppression when both RTKs are functioning in the same cells. This might explain why many patients with NSCLCs carrying wild-type EGFR do not respond well or are primarily resistant to erlotinib or gefitinib: EGFR activity might not be the dominant driver in such contexts and alternative pathways like MET may maintain tumor growth/survival.

Although the in vitro drug activity may provide a good indication of in vivo drug efficacy, they are by no means the same; in vivo systems are far more complicated and sophisticated than two-dimensional culture systems, in which many important factors are missing or ignored. Moreover, using an in vitro system that can truly reflect a drug’s efficacy in the human situation is also crucial. This is especially clear when it comes to assessing human MET activity in mouse xenografts because mouse HGF is incapable of activating human MET. We evaluated SGX523 in conjunction with other inhibitors in the hHGF<sup>tg</sup>-SCID mouse model, which produces human HGF and allows paracrine activation of MET in human tumors (23). We have shown the advantage of this transgenic mouse strain: only in hHGF<sup>tg</sup>-SCID mice do SGX523 and erlotinib synergize to inhibit the xenograft tumor growth of H596 NSCLC cells carrying an MET mutation (22).

Here, we tested SGX523 and erlotinib efficacies on 3 different NSCLC models that are either overexpressing or amplifying wild-type MET and observed interesting context-dependent effects. The SGX523–erlotinib combination resulted in synergistic inhibition of H1993 tumor growth in the hHGF<sup>tg</sup>-SCID mice by enhancing antiproliferation activity and inducing apoptosis, leading to tumor regression. However, the combination led to an additive effect of inhibiting H1373 tumors, enhancing antiproliferation activity but not inducing apoptosis. In either scenario, we saw strengthened anticancer effects although by different mechanisms by simultaneously targeting MET and EGFR. Also, the combinatorial effect observed in the hHGF<sup>tg</sup>-SCID mice is more impressive than that in the control C3H-SCID, likely because both ligand-dependent and -independent MET activities are inhibited in transgenic mice, whereas only ligand-independent activity is inhibited in nontransgenic mice.

The efficacy observed in the MET-addicted EBC-1 tumors is completely different. In these tumors, SGX523 alone is sufficient to achieve nearly complete tumor regression, suggesting that MET is the dominant oncogenic driver here. However, although SGX523 treatment results in EBC-1 tumor disappearance, the tumor cells are not eradicated, and the tumors relapse following removal of the drug. Interestingly, the relapsed tumors are still quite sensitive to SGX523, but tumors of significant size still remained after an extended second round of SGX523 treatment. This indicates the emergence of drug-resistant tumor cells, either due to the accumulation of preexisting insensitive cells or acquired resistance by the initially sensitive cells. EBC-1–derivative cells established from the SGX523-treated tumors appeared to dodge SGX523.
inhibition in part by activating the EGFR pathway because erlotinib can work with SGX523 in reducing the viability of SGX523-insensitive tumor cells. This seems to be consistent with the resistance mechanism revealed by the in vitro selection of resistant EBC-1 cells after exposure to increasing concentrations of MET inhibitor (21). From a therapeutic prospective, we believe that even patients bearing tumors such as MET-addicted EBC-1 can still benefit from combined MET- plus EGFR-targeted therapy by preventing the emerging of drug-resistant/insensitive populations. Nevertheless, the concern that combining MET and EGFR inhibitors might also enhance the toxicity or side effect should not be overlooked, because we observed loss of mice in the erlotinib and SGX523 combination groups for both H1993 and EBC-1 studies (Supplementary Fig. S9). Practically, optimal dosages of combination might be sought out to minimize the potential toxicity or side effect.

Our results strongly suggest that the combined inhibition of MET and EGFR can strengthen anticancer effects against NSCLCs in diverse cellular contexts and that it may be an effective therapeutic option that is superior to targeting an individual pathway. This is crucial because we know that human cancers are heterogeneous and there is no one-for-all treatment solution (30). Understanding drug action modes under different cellular contexts and identifying the correct targets is the key to achieving a successful therapeutic outcome. Nevertheless, inhibition of both MET and EGFR in NSCLCs will not solve all problems, because there are other alternative resistance mechanisms related to each targeted therapy. In that sense, exploring other combined treatment regimens will also be vital in developing effective therapies for personalized medicine. For instance, combining inhibitors of MET and VEGF may lead to a synergistic antitumor activity, which was previously shown in a preclinical model (31).

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

Authors’ Contributions
Conception and design: Y.W. Zhang, G.F. Vande Woude Development of methodology: Y.W. Zhang, B. Staal, C. Eisenburg, S. Lewis Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y.W. Zhang, B. Staal, C. Eisenburg, S. Lewis Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y.W. Zhang Writing, review, and/or revision of the manuscript: Y.W. Zhang, G.F. Vande Woude Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): B. Staal, C. Eisenburg, D. Kaufman Study supervision: Y.W. Zhang, G.F. Vande Woude

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References


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