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Preclinical Evaluation of MET Inhibitor INC-280 With or Without the Epidermal Growth Factor Receptor Inhibitor Erlotinib in Non–Small-Cell Lung Cancer

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Abstract

The MET inhibitor INC-280 restored sensitivity to erlotinib and promoted apoptosis in non–small-cell lung cancer models rendered resistant to erlotinib by hepatocyte growth factor.

Background: Although the epidermal growth factor receptor (EGFR) inhibitor erlotinib is initially effective in non–small-cell lung cancer (NSCLC) patients with tumors harboring activating mutations of *EGFR*, most subsequently develop acquired resistance. One recognized resistance mechanism occurs through activation of bypass signaling via the hepatocyte growth factor (HGF)-MET pathway. INC-280 is a small molecule kinase inhibitor of MET. We sought to demonstrate the activity of INC-280 on select NSCLC cell lines both as a single agent and in combination with erlotinib using exogenous HGF to simulate MET up-regulation. **Methods:** Four NSCLC cell lines (HCC827, PC9, H1666, and H358) were treated with either single-agent INC-280 or in combination with erlotinib with or without HGF. The activity of the drug treatments was measured by cell viability assays. Immunoblotting was used to monitor expression of EGFR/pEGFR, MET/pMET, GAB1/pGAB1, AKT/pAKT, and ERK/pERK as well as markers of apoptosis (*PARP* and caspase-3 cleavage) in H1666, HCC827, and PC9. **Results:** As a single agent, INC-280 showed minimal cytotoxicity despite potent inhibition of MET kinase activity at concentrations as low as 10 nM. Addition of HGF prevented erlotinib-induced cell death. The addition of INC280 to HGF-mediated erlotinib-resistant models restored erlotinib sensitivity for all cell lines tested, associated with cleavage of both *PARP* and caspase-3. In these models, INC-280 treatment was sufficient to restore erlotinib-induced inhibition of MET, *GAB1*, *AKT*, and *ERK* in the presence of HGF.

Conclusion: Although the MET inhibitor INC-280 alone had no discernible effect on cell growth, it was able to restore sensitivity to erlotinib and promote apoptosis in NSCLC models rendered erlotinib resistant by HGF. These data provide a preclinical rationale for an ongoing phase 1 clinical trial of erlotinib plus INC-280 in *EGFR*-mutated NSCLC.

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Keywords: Acquired resistance, AKT, Combination therapy, EGFR mutant, ERK

Introduction

Activating mutations in the epidermal growth factor receptor (EGFR), occurring primarily in non–small-cell lung cancer

(NSCLC) tumors with adenocarcinoma histology, promote cell growth, proliferation, and survival.¹⁻³ Patients whose tumors harbor these mutations have shown substantially improved outcomes when treated with EGFR tyrosine kinase inhibitors (TKIs).⁴⁻⁷ While EGFR TKIs block the catalytic domain of EGFR and initially prevent the activation of downstream signaling pathways, including PI3K/AKT and MEK/ERK, most patients eventually develop acquired resistance to EGFR therapy.⁸⁻¹² Other than a secondary *EGFR* mutation, one of the earliest identified mechanisms of EGFR TKI resistance involves activation of the MET receptor, leading to restored downstream signaling in both

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phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK)/extracellular signal-regulated kinase (ERK) pathways, independent of EGFR.^{13,14} MET may become aberrantly activated via gene amplification or ligand stimulation by hepatocyte growth factor (HGF) and, once active, is sufficient to bypass the antiproliferative and proapoptotic effects of EGFR inhibition.¹³⁻¹⁷ Although early studies with MET inhibitors in combination with EGFR TKIs have shown promising results in NSCLC, subsequent phase 3 trials have failed to demonstrate enhanced efficacy.¹⁸⁻²⁰ Thus, there is a need for more informative preclinical modeling of MET inhibition.

INC280 (INCB28060) is a novel orally bioavailable small molecule inhibitor of MET kinase activity. Highly potent and selective, INC280 has been shown to block MET-dependent tumor growth and migration in *in vitro* and *in vivo* models.^{21,22} Here, we investigated the effects of INC280 as a single agent and in combination with erlotinib on HGF-mediated erlotinib resistance in select NSCLC cell lines.

Methods

Cell Culture and Reagents

Four NSCLC cell lines were selected (Table 1), ranging in sensitivity to erlotinib therapy. The cell lines HCC827, H1666, and H358 were acquired from American Type Culture Collection (Manassas, VA). PC-9 cells were kindly provided by Reen Wu (University of California, Davis, CA). All cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum (JR Scientific, Woodland, CA), penicillin/streptomycin/L-glutamine, and minimum essential medium vitamin solution (Invitrogen, Carlsbad, CA), as previously published.²³ Cell line authentication for HCC827, PC-9, H1666, and H358 was performed by the University of Arizona Genetics Core on 2/3/14 comparing the autosomal STR profiles with reference databases. Erlotinib and INC-280 were purchased from Selleck Chemicals (Houston, TX). Both agents were diluted in dimethyl sulfoxide to a concentration of 10 mM. HGF was purchased from Peprotech (Rocky Hill, NJ) and reconstituted in 0.1% bovine serum albumin to a stock concentration of 10 µg/mL. Agents were stored at -20°C until use.

Proliferation Assay

Cell lines were plated at 1000 to 5000 cells per well in 96-well plates in the presence of media and were allowed to attach overnight prior to treatment. Plating density was determined based upon doubling

time of each cell line. All samples were performed in triplicate. For single-agent and drug interaction studies, the Cell Titer-Fluor Cell Viability Assay (Promega, Madison, WI) was performed according to manufacturer's specifications. Fluorescence was measured at 380 to 400 nm excitation/500 nm emission on a Tecan Safire fluorescent microplate reader with Magellan data analysis software (Tecan, San Jose, CA).

Immunoblotting

Cell lysates were prepared using a modified RIPA buffer containing 25 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, 1% NP-40, 10% glycerol, 2 mM Na₃VO₄, and 1× EDTA-free protease inhibitor cocktail tablets (Roche, Basel, Switzerland) and proteins blotted as previously described by Holland et al.²³ Protein concentration was determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL) according to the manufacturer's protocol. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed with 10 to 25 µg of protein loaded for each sample. Protein was transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and probed overnight at 4°C with the following primary antibodies: phospho-AKT (Ser473), AKT, phospho-ERK1/2 (Thr202/Tyr204), ERK1/2, EGFR (C74B9), phospho–growth factor receptor–bound protein

Figure 1 Growth Curves of Single-agent INC-280 in NSCLC Cell Lines and Immunoblot of Phospho-MET. (A) Growth Curves of Single-agent INC-280 in NSCLC Cell Lines. Cells Were Treated for 72 Hours Before Analysis. (B) Immunoblot of Phospho-MET (Tyr1234/1235) After 3 Hours of Treatment With INC-280 in H1666 Cell Line. HGF at 50 ng/mL Was Used to Stimulate MET Phosphorylation

Table 1 Panel of NSCLC Cell Lines Used in Study

Cell Line	Mutation Status of EGFR and KRAS	Erlotinib Sensitivity (IC50, µM)	Resistance Mediated by HGF
HCC827	19del/wt	0.005	Yes
PC-9	19del/wt	0.05	Yes
H358	wt/G12C	5	Yes
H1666	wt/wt	0.5	Yes

Abbreviations: EGFR = epidermal growth factor receptor; HGF = hepatocyte growth factor; IC50 = drug concentration causing 50% inhibition; KRAS = Kirsten rat sarcoma viral oncogene; NSCLC = non-small-cell lung cancer; wt = wild type.

Abbreviation: NSCLC = non-small-cell lung cancer.

2-associated binding protein 1 (GAB1) (Tyr627), phospho-MET (Tyr1234/1235), MET, cleaved caspase-3 (Cell Signaling Technology, Danvers, MA), GAB1 (EMD Millipore, Billerica, MA), phospho-EGFR (Tyr1068) (Invitrogen), poly(ADP-ribose) polymerase (PARP)-1 (Santa Cruz Biotechnology, Santa Cruz, CA), β -actin (Sigma-Aldrich, St Louis, MO). Blots were then incubated for 1 hour at room temperature with the horseradish peroxidase-conjugated secondary antibodies, anti-mouse IgG and anti-rabbit IgG (Promega), and visualized by chemiluminescence using Amersham ECL (GE Healthcare, Waukesha, WI).

Results

INC-280 Restores Erlotinib Sensitivity in HGF-mediated Resistance Models

We assessed the activity of INC-280 in 5 NSCLC cell lines previously assessed for erlotinib sensitivity and HGF-dependent erlotinib resistance (Table 1).²³ These included 3 *EGFR*-mutant cell lines (HCC827, PC9, and H1975), 1 Kirsten rat sarcoma viral oncogene (*KRAS*)-mutant cell line (H358), and 1 *EGFR* and *KRAS* wild-type cell line (H1666). As a single agent, treatment with INC-280 demonstrated minimal growth inhibition with an drug concentration causing 50% inhibition (IC₅₀) at 10 μ M or higher (Figure 1A). The lack of antiproliferative activity of single-agent INC-280 suggests that under standard growth conditions, these cell lines are not MET dependent, consistent with the absence of basal MET kinase phosphorylation observed in 4 of the 5 cell lines tested (with the exception being the HCC827 cells).²³

However, as shown in Figure 1B, MET phosphorylation stimulated by exogenous HGF was potentially inhibited by INC-280 at concentrations as low as 10 nM.

We previously demonstrated that the addition of exogenous HGF confers resistance to otherwise erlotinib-sensitive NSCLC cell lines.²³ To determine whether INC-280 could restore activity of erlotinib in cell lines rendered resistant by HGF, we assessed the growth inhibitory activity of INC-280 in cells cotreated with erlotinib and HGF. As a single-agent, erlotinib suppressed cell proliferation in a dose- and cell line-dependent manner in the 4 erlotinib-sensitive cell lines (Figure 2). H1975 cells, which harbor an *EGFR* T790M resistance mutation are refractory to erlotinib and were excluded for these experiments. Erlotinib-induced growth inhibition was abrogated when HGF was added to the erlotinib regimen. Treatment with INC-280 restored growth inhibitory activity to levels observed with single-agent erlotinib. While INC-280 had essentially no antiproliferative effects as a single agent on the cell lines at the doses (0.1 and 1.0 μ M) tested, it was nevertheless sufficient to override HGF-mediated resistance to erlotinib.

INC-280 Inhibits EGFR/MET Signaling Network in HGF-mediated Erlotinib-resistant NSCLC Cell Line Models

As a single agent, erlotinib potently down-regulated phosphorylation of EGFR and its downstream mediators of signaling including the docking protein GAB1, AKT, and ERK in the

Figure 2 Growth Response of NSCLC Cell Lines After 72 Hours of Treatment. Cells Were Treated With Erlotinib (E) at Indicated Dose, INC-280 (INC) at 0.1 or 1.0 μ M, and HGF at 50 ng/mL by Cell Titer-fluor Cell Viability Assay. Data Are Graphed as Percentage Growth Relative to Untreated Cells. White Columns, No Added HGF; Black Columns, Supplemented With HGF; Hatched and Dotted Columns, Supplemented With HGF and Treated With INC-280 at Indicated Concentrations

Abbreviations: HGF = hepatocyte growth factor; NSCLC = non-small-cell lung cancer.

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EGFR-mutant cell lines HCC827 and PC-9 (Figure 3A). *EGFR* inhibition in the *EGFR/KRAS* wild-type cell line H1666 primarily results in MEK/ERK down-regulation (Figure 3A, third panel). While MET protein is commonly expressed in NSCLC, we observe only limited MET phosphorylation in our panel of cell lines. The exception is the cell line HCC827 where basal phosphorylation is observed (Figure 3A, first panel, lane 1); however, it appears to be dependent on *EGFR* such that treatment with erlotinib, which ablates *EGFR* phosphorylation, also removes MET phosphorylation. Upon stimulation with HGF, erlotinib-induced inhibition of signal transduction activity was prevented in the 3 cell lines investigated. In this setting, INC-280 treatment at a dose of 0.1 μ M could reverse this effect, potentially abrogating the phosphorylation of GAB1, AKT, and ERK. Importantly, INC-280 is insufficient to knock down signaling (outside of directly targeting MET phosphorylation) in the cell lines absent erlotinib treatment. Furthermore, INC-280 treatment restored the cytotoxicity observed by erlotinib, as assessed by cleavage of caspase-3 and PARP, in our cell line panel (Figure 3B).

Discussion

EGFR TKI resistance facilitated by MET activation, characterized by MET copy number abnormalities or elevated HGF production has been well documented.^{13,17,24-26} HGF expression has been shown to mediate resistance to both reversible and irreversible *EGFR* TKIs as well as the monoclonal antibody cetuximab in NSCLC cells.^{16,27,28} Here, we show that inhibition of MET signaling, using the orally bioavailable MET inhibitor INC-280, could restore sensitivity to erlotinib in our models of acquired resistance. Importantly, we utilized HGF stimulation to simulate MET-mediated acquired resistance to erlotinib. These results are consistent with prior reports on the role of the HGF-MET axis in mediating erlotinib resistance in NSCLC.^{28,29}

The HCC827, PC-9, and H1666 cell lines had the greatest growth inhibitory responses to single-agent erlotinib and, following HGF-mediated resistance, showed the greatest restoration of erlotinib activity when treated with INC-280. It should be emphasized that, as a single agent, INC-280 had almost no observable effects on cell growth, signal transduction or apoptosis. The only context in

Figure 3 Immunoblotting Analysis After 24 Hours of Treatment. Cells Were Treated With Erlotinib (0.5 μ M) and INC-280 (0.1 μ M) With or Without HGF (50 ng/mL). (A) Expression of Total and Phosphorylated *EGFR*, *MET*, *GAB1*, *AKT*, and *ERK*. (B) Analysis of PARP Cleavage and Cleaved Caspase-3 (CC3). HCC827 and PC-9 Cells Were Treated With Lower Doses of Erlotinib (0.05 μ M)

Abbreviations: AKT = protein kinase B; *EGFR* = epidermal growth factor receptor; ERK = extracellular signal-regulated kinase; GAB1 = growth factor receptor-bound protein 2-associated binding protein 1; HGF = hepatocyte growth factor; PARP = poly(ADP-ribose) polymerase.

which INC-280 showed efficacy was in restoring activity of erlotinib in cells rendered resistant by HGF. Thus, the mere presence of MET protein (abundant in all the cell lines tested), appears to not be a reliable indicator of MET dependency, at least in culture.

The work described here has important clinical implications. The success of EGFR TKIs such as erlotinib in treating *EGFR*-mutant NSCLC represents one of the true breakthroughs in therapeutic oncology; nevertheless, emergence of resistance is universal.³⁰ With US Food and Drug Administration approval of the third-generation EGFR TKI osimertinib, which successfully targets the T790M “gatekeeper” mutation, future resistance mechanisms may increasingly utilize bypass pathways such as human epidermal growth factor receptor 2 (HER-2) or MET activation to evade EGFR inhibition. This underscores the need to identify selective and potent agents that can be deployed in combination with EGFR TKIs. Our results suggest that in the appropriate clinical context, MET-directed therapy with INC-280 can overcome erlotinib resistance mediated by activation of HGF-MET signaling.

Based in part on the results of these preclinical studies, a phase 1B clinical trial of INC280 in combination with erlotinib has been initiated at the UC Davis Comprehensive Cancer Center. This trial will assess the tolerability, safety, and preliminary efficacy of this combination in patients with MET activated tumors including NSCLC. The results of this study will be used to design a formal phase 2 trial evaluating the efficacy of this doublet in MET-positive NSCLC.

Clinical Practice Points

- EGFR TKIs such as erlotinib have been successful in treating *EGFR*-mutant NSCLC; however, emergence of resistance is universal.
- US Food and Drug Administration approval of the third-generation EGFR TKI osimertinib, which successfully targets the T790M “gatekeeper” mutation, means that future resistance mechanisms may increasingly utilize bypass pathways such as HER-2 or MET activation to evade EGFR inhibition.
- Selective and potent agents must be identified that can be deployed in combination with EGFR TKIs.
- In the appropriate clinical context, MET-directed therapy with INC-280 can overcome erlotinib resistance mediated by activation of HGF-MET signaling.

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Disclosure

The authors have stated that they have no conflict of interest.

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