Preclinical Development

Synergistic Effects of Foretinib with HER-Targeted Agents in MET and HER1- or HER2-Coactivated Tumor Cells

Li Liu1, Hong Shi1, Yuan Liu2, Amber Anderson3, John Peterson4, James Greger1, Anne-Marie Martin2, and Tona M. Gilmer1,5

Abstract

The HER and MET receptor tyrosine kinases (RTK) are coactivated in a subset of human tumors. This study characterizes MET and HER expression and signaling in a panel of human tumor cell lines and the differential susceptibility of these cell lines to single agents or combinations of foretinib, a multikinase MET inhibitor, with HER-targeted agents, erlotinib or lapatinib. Most MET-amplified tumor lines without HER1 or HER2 amplification are sensitive to foretinib, whereas MET-amplified lines with HER1 or HER2 amplification are more sensitive to the combination of foretinib with lapatinib or erlotinib. Interestingly, MET-overexpressing tumor cell lines with HER1 or HER2 amplification also exhibited reduced sensitivity to lapatinib or erlotinib in the presence of hepatocyte growth factor (HGF), indicating MET activation can decrease the effectiveness of HER1/2 inhibitors in some cell lines. Consistent with this observation, the effect of HGF on lapatinib or erlotinib sensitivity in these cells was reversed by foretinib, other MET inhibitors, or siRNA to MET. Western blot analyses showed that combining foretinib with erlotinib or lapatinib effectively decreased the phosphorylation of MET, HER1, HER2, HER3, AKT, and ERK in these cells. Furthermore, HER2-positive advanced or metastatic breast cancer patients treated with lapatinib who had higher tumor MET expression showed shorter progression-free survival (19.29 weeks in MET-high patients vs. 28.14 weeks in MET-low patients, \( P < 0.0225 \)). These data suggest that combination therapy with foretinib and HER-targeted agents should be tested as a treatment option for HER1- or HER2-positive patients with MET-amplified or -overexpressing tumors. Mol Cancer Ther; 10(3); 518–30. ©2011 AACR.

Introduction

MET is a receptor tyrosine kinase (RTK) whose frequent deregulation in human cancer is associated with poor prognosis and an aggressive cancer phenotype. MET activation noted in tumor cells includes amplification, rearrangement, mutation, and hepatocyte growth factor (HGF)-dependent autocrine or paracrine stimulation, resulting in cellular proliferation, migration, and invasion (1–3). Activation of the MET receptor promotes tyrosine phosphorylation of its intracellular domain and recruitment of protein complexes that stimulate downstream signaling including the Ras mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT pathways, contributing to the development of cancer (4).

The HER/ErbB protein family or epidermal growth factor receptor (EGFR) family includes 4 structurally related RTKs: EGFR/HER1 (ErbB-1), HER2/c-neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). Upregulation, amplification, and mutation of HER1 and HER2 occur in many human cancers and excessive HER1/2 signaling plays a critical role in the development of malignancy (5, 6).

Both the HER and MET families of kinases represent important targets for cancer treatment and are cooverexpressed in a subset of tumors (7–9). MET amplification confers resistance to gefitinib and erlotinib in lung cancer patients (7). Overexpression of MET is associated with poor prognosis in patients with HER2-positive breast cancer (8, 10). In addition, HGF, the ligand of the MET receptor, has been reported to be increased up to 2 ng/mL in the serum of metastatic breast tumor patients (8, 11). The coactivation of MET and HER in these tumors suggests a more effective approach of combining MET and HER target agents. Foretinib is an oral multikinase inhibitor targeting MET, RON, AXL, and VEGFR RTKs (IC_{50} values ≤8 nmol/L; refs. 12, 13) and is currently in phase II clinical trials. Lapatinib is a HER1/HER2-targeted agent approved in combination

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with capcitabine for the treatment of patients with advanced or metastatic breast cancer whose tumors overexpress HER2 and who have received prior therapy including an anthracycline, a taxane, and a trastuzumab (14) and with letrozole for the treatment of postmenopausal women with hormone receptor–positive metastatic breast cancer that overexpresses the HER2 receptor for whom hormonal therapy is indicated (15). Erlotinib is a HER1-targeted agent approved as a single agent for second and third lines of treatment of non–small cell lung carcinoma and in combination with gemcitabine for the treatment of pancreatic cancer. The purpose of this study was to determine the sensitivity and mechanism of action of foretinib as a single agent and in combination with HER-targeted agents, lapatinib or erlotinib, in tumor cell lines with different levels of expression/activation of HER1/HER2 and MET. The results suggest that foretinib and HER-targeted agents synergistically suppress growth of tumor cells with MET and HER1/2 amplification or overexpression. Therefore, combination therapy with foretinib and HER1/2-targeted inhibitors should be explored as a treatment option for subsets of patients with MET-amplified or -overexpressing tumors.

Materials and Methods

Cell lines and reagents

Human breast carcinoma cell lines BT474, SK-BR-3, AU565, and HCC1954, head and neck squamous cell carcinoma line SCC15, gastric carcinoma cell lines SNU5, HS746T, and NCI-N87 (N87), lung carcinoma cell lines, NCI-H1993 (H1993), NCI-H1573 (H1573), NCI-H1648 (H1648), HOP-92, NCI-H596 (H596), NCI-H69 (H69), NCI-H2170 (H2170), NCI-H1975 (H1975), and epidermal carcinoma cell line A431 were obtained from the American Type Culture Collection. Esophageal carcinoma cell line OE33 was purchased from European Collection of Cell Cultures. Gastric carcinoma cell line MKN45 was purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. The LICR LON HN5 (HN5) head and neck carcinoma cell line was a gift from the Institute of Cancer Research, Surrey, UK. Both H1573 and H1648 were cultured in ACL-4 serum free medium containing 50:50 Dulbecco’s modified Eagle medium (DMEM) /F12, insulin transferrin selenium supplements, 50 nmol/L hydrocortisone, 1 mg/mL EGF, 0.01 mmol/L ethanolamine, 0.01 mmol/L phosphoethanolamine, 100 mmol/L triiodothyronine, 0.5% (w/v) bovine serum albumin (5 mg/mL), 2 mmol/L glutamine, 0.5 mmol/L sodium pyruvate, and 10 mmol/L HEPES. HN5 cells were cultured in DMEM containing 5% FBS. All remaining cell lines were cultured in RPMI 1640 containing 10% FBS media. All cell lines were characterized by genotypic and RNA expression analyses, using the Affymetrix 500K SNP chip and HG-U133Plus2 chip, respectively (Affymetrix, Inc.), and kept in culture for less than 3 months.

Recombinant human HGF was purchased from R&D Systems. Trastuzumab was purchased from AmersourceBergen. Lapatinib, erlotinib, foretinib, PF02341066, and PHA665752 were synthesized by GlaxoSmithKline (Fig. 1). Stock solutions of all drugs except trastuzumab were prepared in DMSO and stored at −20°C.

Cell growth inhibition assay and combination data analysis

Cell growth inhibition was determined via CellTiter-Glo (CTG) assay (Promega) according to the manufacturer’s protocol and methylene blue cell viability method (16). Approximately 24 hours after plating, cells were exposed to compounds with 2- or 3-fold serial dilutions alone or the combination of the two agents at a constant molar to molar ratio of 1:1 or as indicated. Cells were incubated with the compounds in culture medium containing either 5% or 10% FBS and in the presence or absence of 2 ng/mL HGF for 3 days. IC50 values were determined as described previously (16).

Combination effects on potency were evaluated using combination index (CI), which was calculated with the backinterpolated IC50 values and the mutually nonexclusive equation derived by Chou and Talalay (17):

\[ CI = \frac{Da}{IC50(a)} + \frac{Db}{IC50(b)} + \frac{Da \times Db}{IC50(a) \times IC50(b)} \]

where IC50(a) is the IC50 of inhibitor A; IC50(b) is the IC50 for inhibitor B; Da is the concentration of inhibitor A in combination with inhibitor B that inhibited 50% of cell growth; and Db is the concentration of inhibitor B in combination with inhibitor A that inhibited 50% of cell growth. In general, a CI value of less than 0.9, between 0.9 and 1.1, or greater than 1.1 indicates synergy, additivity, or antagonism, respectively. In general, the smaller the CI number, the greater is the strength of synergy.

The combination effects on the response scale were quantified by both Excess Over Highest Single Agent (EOHSA) and Excess Over Highest Single Agent at Total Dose (EOHSATD), based on the concept of nonlinear blending as described in detail in the references (18, 19). EOHSA and EOHSATD values are defined as increases in improvement [here, in “percentage points” (ppt) difference] produced by the combination over the best single drug at its component dose level and at the same total dose as for the combination, respectively. Hence, EOHSATD is a stronger “synergy” measure on the response scale than EOHSA. In fact, it has been shown (for monotone dose–response curves) that EOHSATD > 0 implies that CI < 1 (when CI exists) for the mutually exclusive case (19). In practice, it is quite often the case that EOHSATD > 0 is associated with CI < 1 for the mutually nonexclusive case as well. The utility of the EOHSATD synergy measure over the CI measure is that it exists even if one or both of the IC50 values for the single-agent compounds do not exist.

We state that a combination exhibits a synergistic interaction, with regard to potency or on the response
scale, if the CI < 1 or the EOHSATD > 0 is achieved in a statistically significant way. A discussion of the statistical tests employed is described in the Supplement.

**Cell apoptosis assays: DNA fragmentation and caspase-3/7 activation**

Cell apoptosis was measured using Cell Death ELISAPlus kit (Roche) and Caspase-Glo 3/7 assay (Promega) according to the manufacturer’s instructions.

**Immunoprecipitation and Western blotting**

Protein extraction, immunoblotting, and immunoprecipitation were done as described previously (16). The antibodies used in immunoprecipitation included anti-HER1 (Ab13) and HER2 (Ab4) from Neomarkers; anti-HER3 from Santa Cruz Biotechnology; and anti-MET (25H2) from Cell Signaling Technology. The primary antibodies used for immunoblotting were anti-pMET (YY1234/1235), pMET (Y1003), pMET (Y1349), HER2 (44E7), pHER2 (YY1221/1222), pAKT (S473), pAKT (T308) (C31E5E), AKT (2H10), cyclin D1 from Cell Signaling Biotechnology; anti-HER1 (Ab12) from Neomarkers; anti-HER3, ERK1/2, pERK1/2 (T202/Y204) from Santa Cruz Biotechnology; anti-p-tyrosine (PT66) from Sigma; and anti-β-actin from Abcam. The membranes were developed with Odyssey Infrared Imaging System (LI-COR Biosciences).

**Quantitative reverse transcriptase PCR from patient samples and data analysis**

All procedures involving human subjects from EGF20009, an open-label, single-arm phase II trial of lapatinib in patients with HER2-amplified (FISH-positive), advanced, or metastatic breast cancer (20), were approved by the Institutional and Ethics Review Board. Human formalin-fixed, paraffin-embedded (FFPE) breast tumor samples were collected, and RNA was prepared according to a proprietary procedure of Response Genetics, Inc. FFPE tumor samples were microdissected to achieve a minimum of 90% tumor presence. MET and HER2 gene expression
levels were measured by quantitative reverse transcriptase PCR (qRT-PCR; Response Genetics, Inc.), using the following primer sets for MET and HER2. MET; MET_2-1862F1 CAGATCTGTCTCCTGCAACTC; MET_2-1954R TCCAGTTCGCCACGACACATA; MET_2-1908Tc 6FAM-TCTGGGTCTCCTTACAGGG. HER2: HER2-2705F CTGAACTCGGTTATGCGATTG; HER2-2787RTTCCGAGGCGCAAGTC; HER2-2745Tc 6FAM-TGTTGACGAGCCGCAATCC. β-Actin qRT-PCR primer set was used as a normalization control (ABI PRISM).

Statistical analysis of the results was done using a Student’s t test for unpaired samples and the values of P < 0.05 (2-sided) were considered statistically significant. Kaplan–Meier survival analyses for estimating the association between the expression levels of MET and the clinical outcome of progression-free survival (PFS) and receiver operator characteristic (ROC) analysis were performed using JMP software.

**Results**

**Foretinib is a potent MET inhibitor in human tumor cell lines in vitro**

The sensitivity of cell growth inhibition to foretinib was examined in a panel of human tumor cell lines with genetic mutations and variable levels of gene amplification and expression of MET, HER1, and HER2 (Fig. 2A, Supplementary Table S1 and Fig. S1). For the purpose of this study, a gain of 5 or more gene copy number was scored as positive amplification. MET RNA and/or protein expression were evaluated relative to BT474 cells as a reference. Cell lines that express higher MET RNA and/or protein than BT474 were considered MET-overexpressing cells, and those that express equal to or lower amounts were considered MET low-expressing cells.

Foretinib is a multikinase inhibitor with an IC50 value of 3 nmol/L against MET RTK. As shown in Fig. 2, foretinib showed the most potent antiproliferative activity in tumor cells with MET amplification, regardless of the presence of HGF in the media (MKN45, SNU5, HS746T, and H1993, cells with MET-amplification, or overexpression, and in the H596, HOP-92, and H1648 lines harboring MET mutations at the juxtamembrane domain (HOP-92, H69, H596) were less sensitive to foretinib with IC50 values ≥ 0.50 μmol/L. These effects were also seen with two more selective MET inhibitors, PF02341066 and PHA665752 (MET, IC50 = 9 nmol/L, >20-fold selective over RON and AXL, and >50-fold selective over VEGFR2; ref. 25; Fig. 2B and C and data not shown).

**Synergistic effects of foretinib in combination with erlotinib or lapatinib in tumor cells with MET amplification/amplification and HER1 amplification or HER1 ligand overexpression**

Lung and head and neck tumor cell lines that are MET-amplified or overexpress MET protein and are HER1-amplified or overexpress HER1 ligands were exposed to erlotinib or lapatinib alone, and the combination of foretinib with erlotinib or lapatinib. As summarized in Table 1, erlotinib alone was not an effective inhibitor of cell growth (IC50 values >1 μmol/L) in MET-amplified, MET-mutated, or MET-overexpressing lines with and without HGF. However, the combination of erlotinib with foretinib showed synergistic cell growth inhibition as shown by the CI values ranging from 0.25 to 0.37 or significantly greater than the best single agent by EOHSATD analysis (11–29 ppts) in tumor cells with MET amplification/amplification or overexpression and HER1 amplification or expressing high levels of HER1 ligands (Table 1). Specifically, the H1648 line, a MET-amplified lung tumor line that expresses high levels of HER1 ligands, TGF, and amphiregulin, was more sensitive to the combination of erlotinib and foretinib (IC50 = 0.27 μmol/L. without HGF; 0.12 μmol/L with HGF) than to either erlotinib (IC50 > 10 μmol/L) or foretinib alone (IC50 = 1.28 μmol/L, without HGF, 0.74 μmol/L with HGF; Table 1 and Fig. 3A). Similarly, H1573, a MET-amplified lung tumor line that is HER1 amplified and has a KRAS (G12A) mutation was also more sensitive to the combination of erlotinib and foretinib (IC50 = 0.53 μmol/L without HGF, 0.31 μmol/L with HGF) than to either of the single agents (Table 1). HER1-amplified and MET-overexpressing head and neck tumor lines SCC15 and HN5 became highly sensitive to the combination of erlotinib with foretinib in the presence and absence of HGF (IC50 values = 0.12–0.22 μmol/L; Table 1, Fig. 3A). Increased apoptosis as determined by caspase-3/7 activation and DNA fragmentation (Fig. 3B and C) was detected in the H1648 and SCC15 cells treated with foretinib and erlotinib. In the H1975 line containing HER1 L858R/T790M double mutation and MET overexpression, and in the H596, HOP-92, and H69 lines harboring MET mutations at the juxtamembrane domain, the combination of foretinib with erlotinib did not achieve EOHSATD (<0 ppts) however, the combination showed minimal to moderate enhancement of cell growth inhibition (~5–20 ppts EOHSATD ~18–37 ppts) was also observed in the HNS and SCC15 lines with and without HGF (Table 2).
Furthermore, the phosphorylation status of HER1, HER3, and MET RTKs, AKT, and ERK were determined for the H1648 and SCC15 cells (Fig. 3D and Supplementary Table S2). H1648 MET-amplified cells have high levels of MET phosphorylation independent of the presence of HGF. Erlotinib (1 μmol/L) alone had minimal effect on pMET, pHER1, pHER3, pAKT, and pERK (~25%), consistent with lack of cell growth inhibition.
whereas foretinib (0.1 μmol/L) alone significantly inhibited pMET and decreased pHER1, pHER3, pAKT, and pERK (Supplementary Table S2), and had little effect on cell growth inhibition (Fig. 3A) and apoptosis induction (Fig. 3B and C). siRNA to MET also reduced pHER1 and pHER3 in these cells (data not shown). However, combining foretinib with erlotinib further reduced pHER1, pHER3, pAKT, and pERK (Supplementary Table S2), sensitized cell growth inhibition, and increased apoptosis in H1648 cells. A more selective MET inhibitor, PF02341066, or siRNA to MET also enhanced cell growth inhibition by foretinib in H1648 cells (Supplementary Fig. S2A and B). Similarly, siRNA to HER1 enhanced cell growth inhibition by foretinib in these cells (Supplementary Fig. S2C). In addition, AXL was expressed at high levels whereas RON was expressed at very low levels in H1648 cells. Cell growth inhibition by erlotinib was moderately enhanced by siRNA to AXL but not by siRNA to RON (Supplementary Fig. S2C). In SCC15 HER1-amplified and MET-overexpressing cells, the addition of HGF increased the levels of pMET, pAKT, and pERK (Fig. 3D). Erlotinib (1 μmol/L) alone had moderate effects (>65%) on pHER3, pAKT, and pERK, less effect on pHER1 (28%), and a minimal effect on cell growth inhibition (IC_{50} >1 μmol/L). Foretinib (0.1 μmol/L) alone effectively inhibited pMET, decreased pERK in the presence of HGF, and did not decrease pHER1, pHER3, or pAKT (<25%). The addition of foretinib to erlotinib inhibited pMET, pHER3, and pHER1, decreased pAKT and pERK, and enhanced cell growth inhibition and apoptosis (Fig. 3A–D and Supplementary Table S2). HER2 and HER4 expression levels were very low or below the limit of detection by Western blotting or RT-PCR in both H1648 and SCC15 lines (data not shown). These results show that combining foretinib with HER1 or HER1/2-targeted agents more effectively inhibits cell signaling and growth in tumor cells that are MET amplified or overexpress MET and HER1 amplified or overexpress HER1 ligands.

### Table 1. Cell growth inhibition by foretinib, erlotinib, and the combination

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HER and MET</th>
<th>HGF (2 ng/mL)</th>
<th>Single agent</th>
<th>Combination (erlotinib:foretinib = 1:1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IC_{50} (μmol/L)</td>
<td>EOHSA (ppts)</td>
</tr>
<tr>
<td>Lung</td>
<td></td>
<td></td>
<td>Tubulin</td>
<td>Tubulin</td>
</tr>
<tr>
<td>H1648</td>
<td>HER1 ligand-over, MET</td>
<td>–</td>
<td>&gt;10</td>
<td>1.28 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>&gt;10</td>
<td>0.74 ± 0.06</td>
<td>0.12 ± 0.05</td>
</tr>
<tr>
<td>H1573</td>
<td>HER1+, MET</td>
<td>–</td>
<td>&gt;10</td>
<td>1.26 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>&gt;10</td>
<td>1.36 ± 0.02</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>H596</td>
<td>MET-E14Del</td>
<td>–</td>
<td>&gt;10</td>
<td>1.21 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>&gt;10</td>
<td>0.83 ± 0.17</td>
<td>0.44 ± 0.02</td>
</tr>
<tr>
<td>HOP92</td>
<td>MET-T1010I</td>
<td>–</td>
<td>&gt;10</td>
<td>0.81 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>&gt;10</td>
<td>0.64 ± 0.16</td>
<td>0.34 ± 0.05</td>
</tr>
<tr>
<td>H69</td>
<td>MET-R988C</td>
<td>–</td>
<td>&gt;10</td>
<td>1.18 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>&gt;10</td>
<td>1.05 ± 0.18</td>
<td>0.78 ± 0.00</td>
</tr>
<tr>
<td>H1975</td>
<td>HER1-mut, MET-over</td>
<td>–</td>
<td>&gt;10</td>
<td>1.39 ± 0.33</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>&gt;10</td>
<td>1.22 ± 0.25</td>
<td>0.99 ± 0.10</td>
</tr>
<tr>
<td>Head and neck</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SCC15</td>
<td>HER1+, MET-over</td>
<td>–</td>
<td>1.67 ± 0.11</td>
<td>0.63 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>7.52 ± 2.26</td>
<td>0.61 ± 0.05</td>
<td>0.14 ± 0.02e</td>
</tr>
<tr>
<td>HN5</td>
<td>HER1+, MET-over</td>
<td>–</td>
<td>4.92 ± 2.99</td>
<td>0.65 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0.79 ± 0.39</td>
<td>0.22 ± 0.00</td>
<td>42.4 ± 5.4c</td>
</tr>
</tbody>
</table>

Abbreviations: mut, mutant; NA, not achieved; over, overexpressing; MET*, MET amplified; HER1*, HER1 amplified.

*IC_{50} is the concentration of erlotinib in the presence of equimolar quantity of foretinib that reduces cell growth by 50%.

{P} < 0.05 for both experiments.

{p} = 0.29 ± 0.02.

{t} = 0.25 ± 0.05.

{c} = 0.37 ± 0.22.
Figure 3. Combination effects of foretinib with erlotinib in H1648 and SCC15 cells. Cell growth inhibition (A), caspase-3/7 activation (B), and DNA fragmentation (C) were determined 72, 24, and 48 hours, respectively, after cells were exposed to foretinib, erlotinib, and the combination at a 1 to 1 molar ratio in the presence and absence of HGF (2 ng/mL) and 10% FBS. D, inhibition of RTKs and signaling pathways. Cells were exposed to foretinib (0.1 μmol/L), erlotinib (1 μmol/L), or the combination of foretinib with erlotinib for 2 hours before adding 2 ng/mL of HGF for an additional 0.5 hour in the presence of 10% FBS culture medium.
Synergism of MET- and HER-Targeted Agents

Table 2. Cell growth inhibition by foretinib, lapatinib, and the combination

<table>
<thead>
<tr>
<th>Cell line</th>
<th>HER and MET</th>
<th>HGF (2 ng/mL)</th>
<th>Single agent (IC50, µmol/L)</th>
<th>Combination (lapatinib:foretinib = 1:1)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lapatinib</td>
<td>Foretinib</td>
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<tr>
<td>Breast</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BT474</td>
<td>HER2⁺,</td>
<td>–</td>
<td>0.08 ± 0.00</td>
<td>4.74 ± 1.36</td>
</tr>
<tr>
<td></td>
<td>MET-low</td>
<td>+</td>
<td>0.06 ± 0.01</td>
<td>4.69 ± 1.00</td>
</tr>
<tr>
<td>SK-BR-3</td>
<td>HER2⁺,</td>
<td>–</td>
<td>0.22 ± 0.20</td>
<td>5.20 ± 0.59</td>
</tr>
<tr>
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<td>MET-low</td>
<td>+</td>
<td>0.25 ± 0.24</td>
<td>4.98 ± 0.55</td>
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<tr>
<td>AU565</td>
<td>HER2⁺,</td>
<td>–</td>
<td>0.12 ± 0.03</td>
<td>2.93 ± 0.94</td>
</tr>
<tr>
<td></td>
<td>MET-over</td>
<td>+</td>
<td>0.71 ± 0.12</td>
<td>1.88 ± 0.41</td>
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<tr>
<td>HCC1954</td>
<td>HER2⁺,</td>
<td>–</td>
<td>0.52 ± 0.06</td>
<td>1.83 ± 0.13</td>
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<tr>
<td></td>
<td>MET-over</td>
<td>+</td>
<td>4.70 ± 0.48</td>
<td>1.84 ± 0.35</td>
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<tr>
<td>Gastric</td>
<td>N87</td>
<td>HER2⁺,</td>
<td>0.05 ± 0.02</td>
<td>2.32 ± 0.12</td>
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<td></td>
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<td>MET-over</td>
<td>1.83 ± 0.09</td>
<td>2.06 ± 0.27</td>
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<tr>
<td>Lung</td>
<td>H2170</td>
<td>HER2⁺,</td>
<td>0.25 ± 0.11</td>
<td>0.68 ± 0.00</td>
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<td></td>
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<td>MET-over</td>
<td>6.03 ± 3.03</td>
<td>0.52 ± 0.14</td>
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<td>Esophageal</td>
<td>OE-33</td>
<td>HER2⁺,</td>
<td>3.69 ± 0.89</td>
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<td>MET⁺</td>
<td>+</td>
<td>5.00 ± 0.30</td>
<td>0.39 ± 0.19</td>
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<td>Head and neck</td>
<td>SCC15</td>
<td>HER1⁺,</td>
<td>1.05 ± 0.22</td>
<td>0.66 ± 0.20</td>
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<td></td>
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<td>MET-over</td>
<td>3.80 ± 0.23</td>
<td>0.66 ± 0.10</td>
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<td>HER1⁺,</td>
<td>2.46 ± 0.56</td>
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<td></td>
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<td>MET-over</td>
<td>3.53 ± 0.06</td>
<td>0.99 ± 0.09</td>
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</tbody>
</table>

Abbreviations: NA, not achieved; low, low expressing; over, overexpressing; MET⁺, MET amplified; HER1⁺, HER1 amplified; HER2⁺, HER2 amplified.

aIC50 is the concentration of lapatinib in the presence of equimolar quantity of foretinib that reduces cell growth by 50%.
bP < 0.05 for both experiments.

Synergistic effects of foretinib with lapatinib in tumor cells with MET amplification/overexpression and HER2 amplification

The role of MET signaling in response to a HER2-targeted agent was examined by treating HER2-amplified cell lines with various levels of MET expression with lapatinib and/or foretinib in the presence or absence of HGF. The HER2-amplified/MET low-expressing breast cancer cell line BT474 served as a control. BT474 was sensitive to lapatinib with and without added HGF (IC50 values ≤0.08 µmol/L) and was relatively insensitive to foretinib (IC50 values ≥4.5 µmol/L). Furthermore, the sensitivity to the combination is similar to that of lapatinib alone (Table 2, Fig. 4A). Similar to BT474, SK-BR-3 cells were sensitive to lapatinib regardless of the presence of HGF, although the combination of lapatinib with foretinib showed some synergy based on CI values of 0.58–0.80 at the IC50, but it did not achieve synergy based on EOHSATD (Table 2). In contrast, AU565, HCC1954, N87, and H2170 (HER2 amplified/MET overexpressing) cells responded to lapatinib in the absence of HGF (IC50 values of 0.05–0.52 µmol/L) but showed a 6- to 36-fold decreased sensitivity to lapatinib (IC50 values of 0.71–6.03 µmol/L) in the presence of HGF (Table 2 and Fig. 4A). This relative insensitivity to lapatinib in the presence of HGF was reversed by foretinib. The combination was highly synergistic with CI values of 0.04–0.17 and EOHSATD of 17–39 ppts in the presence of HGF. The esophageal cell line OE-33, which is HER2 amplified/MET amplified, was not sensitive to lapatinib alone (IC50 values >3.5 µmol/L with or without HGF), moderately sensitive to foretinib alone (IC50 values ~0.40 µmol/L with or without HGF), and highly sensitive to the combination of lapatinib and foretinib (IC50 values ≤0.07 µmol/L, CI ≤0.22, EOHSATD ≥24 ppts with and without added HGF; Table 2, Fig. 4B). These results suggest that...
MET amplification or overexpression of MET in the presence of ligand reduces the effectiveness of lapatinib in HER2-amplified cancer cells and that foretinib can restore lapatinib sensitivity in these cells.

We further characterized the effects of foretinib and lapatinib on MET and HER2/3 RTKs and downstream MAPK and PI3K/AKT pathways in response to these agents. As shown in Fig. 4A, MET protein expression
was low in BT474 and high in AU565, HCC1954, and N87 cells, consistent with the levels of MET mRNA expression (Supplementary Table S1). HGF increased pMET in AU565 cells and sustained pERK and/or pAKT in the presence of lapatinib in AU565, HCC1954, and N87 cells but not in BT474 cells, suggesting a MET-dependent effect. Foretinib inhibited pMET and restored the inhibition of both pAKT and pERK by lapatinib in AU565, HCC1954, and N87 cells in the presence of HGF (Fig. 4A). Two more selective MET inhibitors, PF02341066 and PHA665752, and siRNA to MET (Supplementary Fig. S3A and B, and data not shown) also restored lapatinib sensitivity in HCC1954 cells in the presence of HGF. In contrast, siRNA to RON did not restore lapatinib sensitivity (Supplementary Fig. S3C), whereas siRNA to HER2 enhanced cell growth inhibition by foretinib in HCC1954 cells (Supplementary Fig. S3D). Furthermore, in OE-33 cells, in which HER2 and MET are amplified and highly phosphorylated, lapatinib had little effect on phosphorylation or cell growth at 1 μmol/L. Foretinib inhibited pMET and partially inhibited pHER2 and pHER3, whereas lapatinib plus foretinib almost completely inhibited pMET, pHER2, and pHER3 with similar results in the presence and absence of HGF (Fig. 4B). These results show that foretinib synergizes with lapatinib by inhibiting activated MET in the HER2-amplified cancer cells.

**MET expression and lapatinib response in HER2-positive, advanced, or metastatic breast cancer patients**

To test whether overexpression of MET is associated with lapatinib response in the clinic, baseline MET expression was measured in 60 archived tumor samples obtained during the EGF20009 lapatinib clinical trial, in which patients with HER2-positive, advanced, or metastatic breast cancer, documented by FISH in primary or metastatic tumor tissue, were treated with first-line, single-agent lapatinib (20). Of the 60 lapatinib-treated patients for whom we had sufficient tissue to extract RNA, 9 of 60 had symptoms of progressive disease (PD), 30 of 60 had stable disease (SD), 5 of 60 are not evaluable (NE), and 16 of 60 had a partial response (PR) based on RECIST (Response Evaluation Criteria in Solid Tumors) criteria. **MET** gene expression was found to vary considerably, however, was not correlated with HER2 expression across this set of samples (Fig. 5A). A cutoff value of 0.29, log_{2} = −1.78) of MET relative expression was determined by ROC analysis from these 9 PD and 16 PR patients (data not shown). The ROC cutoff showed an assay sensitivity of 63% and specificity of 89% [area under the curve (AUC) = 0.736]. Using this cutoff, low relative MET expression (<0.29) was associated with longer PFS (median PFS = 28.14 weeks), whereas high relative MET expression (>0.29) was associated with shorter PFS (median PFS = 19.29 weeks; P = 0.0225; Fig. 5B). The difference in median survival time for patients in the low and high MET-expressing groups was 8.86 weeks (62 days). Thus, in this data set, increased MET expression is associated with reduced median PFS. These clinical data further support the idea that foretinib could be useful in combination with lapatinib for treating patients with HER2-positive and MET-overexpressing breast cancer.

**Discussion**

MET receptor tyrosine kinase stimulates cell proliferation and increases cell motility and invasion and thus represents a promising target for cancer treatment. Tumor cells with MET amplification are more susceptible to MET inhibition for cell survival (26). However, MET amplification is a relatively rare event with approximately 5% to 10% in gastric, 1% to 7% in lung, and 4% to 7% in esophageal cancers (27–29). MET amplification has been shown to confer resistance to HER1 selective inhibitors such as erlotinib and gefitinib through the activation of HER3/P13K/AKT signaling in HER1 mutant lung tumor cells (7). In fact, a subset of HER1 mutant lung cancer patients (~20%) who initially responded to erlotinib or gefitinib developed acquired resistance to these agents via MET amplification (30). On the other hand, MET is more widely overexpressed and associated with poor prognosis in various cancers such as lung, breast, and head and neck (31–35). Recently, Turke and colleagues (36) reported that MET activation by its ligand, HGF, also induced drug resistance to these HER1 inhibitors through GAB1 signaling in HER1 mutant lung tumor cells. Additional studies on cross-talk among MET, HER1, HER2, and/or HER3 RTKs further support the potential benefit of inhibiting both MET and HER RTKs in HER-activated and MET-amplified or -overexpressing cancers (37, 38).

This study shows that foretinib is a potent inhibitor of MET phosphorylation and downstream signaling of MET via MAPK and PI3K/AKT in MET-amplified and highly expressed cells (Figs. 2B, 3D, and 4). The cell growth inhibition profile of foretinib (Fig. 2A and Supplementary Table S1) is consistent with its potent MET inhibition when MET is upregulated by amplification (26). The results presented here support 4 major conclusions concerning the role of MET in susceptibility to HER-targeted oncotherapeutics: (i) amplification of MET confers insensitivity to erlotinib or lapatinib in HER1-amplified, HER1 ligand overexpressing, or HER2-amplified cells, consistent with the known mechanism of resistance to EGFR inhibitors in EGFR mutant lines (30, 39); (ii) overexpression of MET is associated with decreased sensitivity to erlotinib or lapatinib in vitro in HER1-amplified or HER2-amplified tumor cells in the presence of HGF (Tables 1 and 2); (iii) combining MET-targeted agents, foretinib, FF02341066, or PHA665752, or siRNA to MET with HER-targeted agents, erlotinib or lapatinib, or siRNA to HER1 or HER2 is beneficial in HER1-activated (by amplification or overexpression of HER1 ligands) or HER2-amplified tumor cells with MET amplification or overexpression (Tables 1 and 2, Supplementary Fig. S2 and
S3); and (iv) high relative expression of MET is associated with shorter PFS in lapatinib-treated EGF20009 patients with HER2-amplified metastatic or advanced breast cancer (Fig. 5B). In addition, we used the EOH-SATD method to quantify combination synergy on the response scale. It provides an additional measure of synergy and is applicable to combination analysis on the response scale even when the CI cannot be computed.

The results of this study strongly support the hypothesis that MET and HER1/2 cooperate in promoting cell proliferation and survival of transformed cells by engaging in cross-talk between MET and HER1/HER3 or HER2/HER3, which leads to downstream MAPK and PI3K/AKT signaling as illustrated in Fig. 5C. Cross-talk of the pathways is further suggested by the fact that in MET-amplified lines (H1993, H1648, and OE-33), foretinib alone decreased the phosphorylation of HER1 and HER3 or HER2 and HER3, even though foretinib is not a potent inhibitor of the enzymatic activity of HER1 and HER2 (IC50 values >2 μmol/L). Similarly, MET-specific siRNA decreased the phosphorylation of HER1 and HER3 in H1648 cells (data not shown). The data show that foretinib can potentially inhibit cross-talk between MET and HER1 or HER2; however, the combination of foretinib with HER-targeted agents is more effective at suppressing growth of HER1-activated or HER2-amplified and MET-amplified or -overexpressing tumors.

The finding of MET overexpression associated with decreased sensitivity to lapatinib in HER2-amplified tumor cells is novel. Clinical data from EGF20009 strengthens the hypothesis that MET status influences responsiveness of human breast cancer patients to lapatinib. As shown in Fig. 5B, patients with high MET expression progressed quicker on lapatinib than those with low MET expression ($P = 0.0225$). Furthermore, on the basis of the synergistic effects of foretinib and...
Synergism of MET- and HER-Targeted Agents

Lapatinib described in the preclinical data, it can be predicted that foretinib and lapatinib may be an effective combination therapy for MET-overexpressing HER-positive tumors that are resistant to HER-targeted agents. Therefore, this study provides a strong rationale for clinical studies of foretinib in combination with erlotinib or lapatinib in patients with HER1 activation or HER2-positive tumors with high MET expression.

Disclosure of Potential Conflicts of Interest

All authors are employees and stockholders of GlaxoSmithKline.

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Synergistic Effects of Foretinib with HER-Targeted Agents in MET and HER1- or HER2-Coactivated Tumor Cells

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