Inhibition of c-Met as a Therapeutic Strategy for Esophageal Adenocarcinoma

Gregory A. Watson*,1, Xinglu Zhang*,1, Michael T. Stang*, Ryan M. Levy*, Pierre E. Queiroz de Oliveira*, William E. Gooding†, James G. Christensen† and Steven J. Hughes*

Departments of *Surgery and †Biostatistics, University of Pittsburgh, Pittsburgh, PA, USA; ‡Cancer Biology, Pfizer, Inc., La Jolla, CA, USA

Abstract
The hepatocyte growth factor (HGF) receptor c-Met is a tyrosine kinase receptor with established oncogenic properties. We have previously shown that c-Met is usually overexpressed in esophageal adenocarcinoma (EA), yet the implications of c-Met inhibition in EA remain unknown. Three c-Met–overexpressing EA cell lines (Seg-1, Bic-1, and Flo-1) were used to examine the effects of a c-Met–specific small molecule inhibitor (PHA665752) on cell viability, apoptosis, motility, invasion, and downstream signaling pathways. PHA665752 demonstrated dose-dependent inhibition of constitutive and/or HGF-induced phosphorylation of c-Met, which correlated with reduced cell viability and inhibition of extracellular regulated kinase 1/2 phosphorylation in all three EA cell lines. In contrast, PHA665752 induced apoptosis and reduced motility and invasion in only one EA cell line, Flo-1. Interestingly, Flo-1 was the only cell line in which phosphatidylinositol 3-kinase (PI3K)/Akt was induced following HGF stimulation. The PI3K inhibitor LY294002 produced effects equivalent to those of PHA665752 in these cells. We conclude that inhibition of c-Met may be a useful therapeutic strategy for EA. Factors other than receptor overexpression, such as c-Met–dependent PI3K/Akt signaling, may be predictive of an individual tumor’s response to c-Met inhibition.

Neoplasia (2006) 8, 949–955

Keywords: c-Met, hepatocyte growth factor (HGF), PHA665752, phosphatidylinositol 3-kinase (PI3K), extracellular regulated kinase (ERK).

Introduction
Esophageal adenocarcinoma (EA) is a highly aggressive malignancy with propensity for early local invasion and systemic metastasis. The incidence of EA is increasing rapidly, and EA currently represents the most common histologic type of esophageal cancer in the United States [1,2]. Despite advances in diagnosis and treatment, the overall 5-year survival remains approximately 14% [3,4]. The rising incidence of EA and the dismal prognosis associated with current treatment strategies warrant a search for innovative therapies.

The hepatocyte growth factor (HGF) receptor c-Met is a tyrosine kinase receptor with established oncogenic properties. Activation of c-Met results in phosphorylation of the receptor that leads to the recruitment of adaptor proteins and to the subsequent activation of various signal transducers, including phosphatidylinositol 3-kinase (PI3K) and extracellular regulated kinase (ERK) 1/2, resulting ultimately in the stimulation of growth, survival, motility, and invasion in certain cell types [5]. c-Met is known to contribute to these properties of malignant cells in a variety of human tumors, including lung cancer [6–8], pancreatic cancer [9], ovarian cancer [10], glioma [11], and gastric cancer [12], but the role of c-Met in EA remains poorly defined.

Herrera et al. [13] and Miller et al. [14] have recently shown that c-Met is overexpressed in EA compared to normal esophageal squamous epithelium and Barrett’s esophagus columnar epithelium without dysplasia, suggesting that c-Met may be an attractive candidate for targeted therapy in EA. In the present study, we investigated the effects of PHA665752, a small molecule inhibitor specific for c-Met kinase [15], on EA cell viability, apoptosis, motility, invasion, and downstream signaling pathways. Our findings demonstrate variability in the response of EA cell lines to c-Met inhibition, suggesting that factors other than receptor overexpression may determine the response of an individual neoplasm to c-Met inhibition.

Materials and Methods

Cell Lines
Three human EA-derived cell lines (Seg-1, Bic-1, and Flo-1) have been previously described [16]. A549 is a human-derived non–small cell lung cancer (NSCLC) cell line previously shown to be c-Met–responsive [17]. Seg-1 was maintained in RPMI 1640 medium, and Bic-1, Flo-1, and A549 were maintained in
Anti–phospho-AktSer473 and anti-Akt antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and anti–phospho ERK and anti-ERK antibodies were purchased from BioSource International, Inc. (Camarillo, CA). Absorbance was normalized to unabsorbed, cells were resuspended in dimethylsulfoxide (Sigma-Aldrich, Inc.), and absorbance was recorded at 570 nm for 20 minutes at 53°C.

Cell Viability and Repopulation Assays

Cultured cells were serum-starved for 24 hours, treated with various concentrations of PHA665752 or LY294002 for 2 hours, and stimulated with HGF (50 ng/ml) for 10 minutes. Protein was extracted using lysis buffer (Cell Signaling Technology, Inc.) containing 1 mM phenylmethylsulfonylfluoride (Sigma-Aldrich, Inc.) and quantified using the BCA protein assay kit (Pierce, Rockford, IL). Proteins were resolved using sodium dodecyl sulfate (SDS) polyacrylamide gels and subsequently transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked in 5% milk solution, incubated with primary antibody, washed, and incubated with HRP-conjugated secondary antibody. Immunoreactivity was detected using Supersignal West Pico Chemiluminescent Substrate (Pierce) and X-ray film (Eastman Kodak, Rochester, NY). Blots were stripped with 2% SDS, 100 mM β-mercaptoethanol, and 62.5 mM (pH 6.8) Tris (Sigma-Aldrich, Inc.) for 20 minutes at 53°C and reprobed with control antibody. Each presented immunoblot was selected as a reproducible representative of a minimum of three individual experiments.

Statistical Analysis

All data were checked for distributional properties by estimating Box–Cox transformation parameters. Both log and square root transformations were applied, as required, to improve symmetry and to stabilize variances. Analyses were conducted by parametric two-way and three-way analyses of variance. Individual contrasts were tested with either an F test for contrasts involving three or more groups or a t-test for two-group comparisons. Dose effects were tested with orthogonal contrasts. All tests were two-sided. Raw P values are reported without adjustment for multiple comparisons.

Results

PHA665752 Inhibits Constitutive and HGF-Induced Phosphorylation of c-Met

We have previously reported the activation status and HGF responsiveness of c-Met in three EA cell lines (Seg-1, Bic-1, and Flo-1) known to overexpress c-Met [13]. For this study, we sought to characterize the effects of PHA665752, a c-Met–specific small molecule inhibitor, on c-Met phosphorylation [15]. We have previously shown the constitutive phosphorylation of c-Met in all of these cell lines by immunoblotting with prolonged exposure and immunofluorescence [13]. Using short exposure to facilitate the observation of differences in band intensity between treatments and to make comparisons between cell lines, a detectable level of the constitutive phosphorylation of c-Met is observed in the Bic-1 cell line, and c-Met phosphorylation was induced by HGF in constitutive phosphorylation of c-Met is observed in the Bic-1 cell line, and c-Met phosphorylation was induced by HGF in
all three EA cell lines (Figure 1A). Treatment with PHA665752 inhibited either constitutive (Bic-1) or HGF-induced (all three EA cell lines) phosphorylation of c-Met in a dose-dependent manner (Figure 1A). Prolonged exposure of an anti–c-Met immunoblot using lysates from Flo-1 cells shows that abrogation of identifiable phosphorylated c-Met is technique-dependent and that larger doses of PHA665752 may be required to completely abolish c-Met phosphorylation (Figure 1B). Taken together, these observations suggest that c-Met is phosphorylated in all three EA cell lines in response to HGF and that PHA665752 is a viable strategy to inhibit c-Met activity in EA.

c-Met Inhibition Reduces EA Cell Viability and Differentially Induces Apoptosis

Because c-Met promotes growth and survival in some tumor types [5], we hypothesized that inhibition of c-Met would reduce EA cell viability and induce apoptosis. PHA665752 is appropriately applied at doses ranging from 0.1 to 2.5 μM [15]. No significant effects on cell viability were apparent within 24 hours of treatment with HGF or PHA665752 (Figure 1A). Following 48 hours of HGF stimulation, the number of viable Bic-1 cells and, to a lesser extent, Seg-1 cells increased, whereas HGF had no effect on Flo-1 cell viability (Figure 2, A and B), suggesting that c-Met induces proliferation in Bic-1 and Seg-1. Treatment with 250 nM PHA665752 decreased the number of viable Bic-1 and Flo-1 cells, whereas a similar effect was observed in Seg-1 cells at higher doses of PHA665752 (Figure 2, A and B).

Figure 1. PHA665752 inhibits constitutive and HGF-induced phosphorylation of c-Met. (A) Simultaneously performed representative immunoblots of phosphorylated c-Met (p-Met) in three EA cell lines (Seg-1, Bic-1, and Flo-1) following PHA665752 treatment (2 hours) in the presence or in the absence of HGF stimulation (50 ng/ml for 10 minutes). Constitutive phosphorylation of c-Met was observed in Bic-1 cells. All three EA cell lines demonstrated phosphorylation of the mature (140 kDa) form of c-Met following HGF stimulation, and phosphorylation of the precursor (170 kDa) form of c-Met was also observed in Seg-1 cells. PHA665752 inhibited the phosphorylation of c-Met in a dose-dependent fashion. (B) Prolonged exposure immunoblot demonstrating that larger doses of PHA665752 are required to completely abolish c-Met phosphorylation (Flo-1 cells).

Figure 2. Effects of c-Met inhibition on EA cell viability and apoptosis. (A) MTT assay time course (24 – 72 hours) in Bic-1 cells following treatment with HGF (50 ng/ml) or PHA665752 (0.25 μM), alone and in combination. Absorbance at 570 nm is presented as the mean ± SEM of two individual experiments. Following 48 hours of treatment, HGF resulted in a significant increase in the number of viable cells (P = .01), whereas PHA665752 resulted in a significant decrease in the number of viable cells (P < .001) relative to controls, even in the presence of HGF. These effects persisted to 72 hours. (B) MTT assay of EA cells 48 hours following treatment with HGF (50 ng/ml) or various concentrations of PHA665752. Absorbance was normalized to controls (% control viability) and is presented as the mean ± SEM of four individual experiments. The number of viable Bic-1 (P = .01) and Seg-1 (P = .05) cells, but not Flo-1 cells, increased significantly following HGF stimulation. PHA665752 (0.25 μM) reduced the number of viable Bic-1 (P < .001) and Flo-1 (P = .01) cells, and a similar effect was observed in Seg-1 cells (P < .001) at higher doses (1.25 μM). (C) FACScan analysis of Annexin V– and propidium iodide–stained cells 48 hours following treatment with HGF (50 ng/ml), alone or in combination with PHA665752 (250 nM shown). Positive staining for Annexin V suggests early apoptosis (right lower quadrant). Positive staining for propidium iodide suggests loss of membrane integrity late in apoptosis (right upper quadrant) or due to necrosis (left upper quadrant). HGF treatment reduced the number of apoptotic Flo-1 cells observed relative to controls but had no effect on Bic-1 (not shown) or Seg-1 (not shown) cells. PHA665752 induced apoptosis in Flo-1 cells, but not in Bic-1 or Seg-1 cells.
We next examined the effects of c-Met inhibition on EA cell apoptosis. HGF stimulation decreased the number of early and late apoptotic Flo-1 cells (relative to controls), whereas treatment with PHA665752 resulted in an increase in both apoptotic fractions (Figure 2C), suggesting that c-Met promotes survival in Flo-1. Although inhibition of c-Met reduced the number of viable Bic-1 and Seg-1 cells compared to controls (Figure 2, A and B), treatment with PHA665752 did not induce apoptosis at the time points assessed in the present study (Figure 2C). Cell cycle analysis indicates that arrest is not responsible for this observation (data not shown), suggesting that PHA665752 inhibited proliferation rate in these two cell lines. This is further supported by the continued growth of Bic-1 and Seg-1 cells, albeit at a slower rate, following treatment with PHA665752 (Figure 2A). Taken together, these findings show that c-Met inhibition variably affects EA cell viability and apoptosis, and suggests that differential response of EA cells to c-Met inhibition may exist.

**c-Met Differentially Stimulates EA Cell Motility and Invasion**

In addition to promoting growth and survival, c-Met–dependent signal transduction has been shown to induce motility and invasion in some tumor types [5], and we hypothesized that inhibition of c-Met would reduce EA cell motility and invasiveness. HGF-treated A549 cells (control cells that express levels of Met protein similar to those of EA cell lines) [13] and Flo-1 cells demonstrated pseudopod formation and migration within 24 hours of wounding, whereas no effect was observed in Seg-1 cells, even at later (72 hours) time points (Figure 3A). Bic-1 cells do not achieve confluence in culture and were not analyzed. PHA665752 inhibited HGF-induced pseudopod formation and migration in both A549 and Flo-1 cells (Figure 3A), suggesting that HGF induces motility through c-Met–dependent signaling in these two cell lines.

We next examined the effects of c-Met inhibition on the property of cell invasion [18]. In the absence of HGF, substantial invasion was observed only in A549 and Flo-1 cells, whereas HGF treatment induced invasion in A549, Flo-1, and, to a lesser extent, Seg-1 cells (Figure 3B). Interestingly, Bic-1 cells, which demonstrate strong constitutive phosphorylation of c-Met (Figure 1), did not invade either in the absence or in the presence of exogenous HGF (Figure 3B). PHA665752 inhibited HGF-induced invasion in A549, Flo-1, and Seg-1 cells (Figure 3B), suggesting that c-Met is involved in the regulation of invasion in these three cell lines. Collectively, these observations show that HGF differentially induces EA cell motility and invasion through c-Met signaling and further supports the notion that cell line–specific differences exist in response to c-Met inhibition.

**c-Met Variably Modulates ERK and AKT Signaling in EA**

Pleiotropic response to c-Met activation may be explained, in part, by diverse intracellular mediators that convey c-Met signaling [19–21]. Because ERK and Akt are involved in c-Met signal transduction and contribute to cell growth, survival, motility, and invasion [22,23], we hypothesized that c-Met differentially modulates ERK and Akt signaling in EA. All three EA cell lines demonstrated constitutive ERK phosphorylation, which was further augmented following HGF stimulation (Figure 4A). PHA665752 modestly attenuated constitutive ERK phosphorylation in Bic-1 and Seg-1 cells and inhibited HGF-induced ERK phosphorylation in all three EA cell lines (Figure 4A). Although the effects of PHA665752 on constitutive ERK phosphorylation in Seg-1 cells raise the possibility of inhibitor nonspecificity, Seg-1 cells express HGF, and we have reported the constitutive phosphorylation of c-Met in these cells [13]. Constitutive phosphorylation of Akt was not observed in any of the EA cell lines, and treatment with HGF induced Akt phosphorylation only in Flo-1 cells (Figure 4B). Consistent with induction of activity by HGF, Akt phosphorylation was inhibited in a dose-dependent fashion by PHA665752 only in Flo-1 cells (Figure 4B). Taken together, these findings demonstrate that c-Met differentially modulates ERK and Akt signaling in EA cell lines and suggest that the response of EA cells to c-Met inhibition
may be dependent, at least in part, on intracellular mediators that participate in c-Met signal transduction.

The Effects of PI3K Inhibition on Cell Survival, Motility, and Invasion Are Similar to Those of c-Met Inhibition in Flo-1 Cells

Because stimulation of c-Met promoted the greatest effects on survival, motility, and invasion in Flo-1 cells, we hypothesized that PI3K/Akt signaling mediated these HGF-induced effects. Inhibition of PI3K with LY294002 (at doses of ≥ 10 μM) abolished HGF-induced phosphorylation of Akt (Figure 5A) and resulted in an increased number of both early and late apoptotic Flo-1 cells (Figure 5B). Compared to c-Met inhibition, PI3K blockade by LY294002 was associated with a larger fraction of early apoptotic cells (Figures 2C and 5B) and a greater inhibition of invasion (Figure 5D), suggesting that some PI3K activity in these cells is not c-Met-dependent. HGF-induced motility of Flo-1 cells was similarly abrogated following both c-Met and PI3K inhibition (Figures 3A and 5C). Collectively, these findings support the current opinion that PI3K/Akt signaling is critical in the regulation of c-Met-induced survival, motility, and invasion [24,25], and suggest that the effects of c-Met inhibition on EA may be dependent, at least in part, on the involvement and/or the dependence of the PI3K/Akt pathway on c-Met signal transduction.

Discussion

Our earlier observation that c-Met was not expressed in normal squamous esophagus or nondysplastic Barrett’s esophagus but was usually overexpressed in EA [13] supports the potential for therapies that inhibit c-Met in the treatment of EA. We have shown that HGF/c-Met-dependent signaling differentially induces proliferation, survival, motility, and invasion, as well as ERK and Akt signaling, in a panel of EA cell lines. Although all three EA cell lines overexpress c-Met [13], PHA665752 induced apoptosis and inhibited motility and invasion only in cells (Flo-1) in which PI3K/Akt signaling was stimulated by HGF. Our findings support the use of strategies to inhibit c-Met as a viable therapeutic option for EA and suggest that factors other...
than overexpression of c-Met, such as involvement of PI3K/Akt in c-Met signal transduction, may determine the response of an individual neoplasm to c-Met inhibition.

Observations in various tumor models [5] suggest that c-Met signaling induces pleiotropic effects, yet few studies have examined this phenomenon in a panel of cell lines derived from the same tumor type. Similar to our findings, Coltella et al. [26] observed differential responses to c-Met stimulation in five osteosarcoma cell lines that overexpress c-Met. Treatment with HGF induced proliferation and ERK phosphorylation in four of the cell lines, stimulated motility/invading and Akt phosphorylation in two of the cell lines, and had no effect in one cell line. Additionally, differential effects of c-Met inhibition on anchorage-independent growth have been reported in panels of cell lines derived from lung and gastric cancers, as well as in gliomas [15]. In contrast, Miller et al. [14] recently demonstrated global induction of apoptosis following treatment with the heat shock protein 90 (Hsp90) inhibitor geldanamycin in the same three EA cell lines used in our study; however, the specificity of this response for c-Met is unclear as Hsp90 is involved in signal transduction from a variety of tyrosine kinase receptors [3,27]. Similar to our observations in EA, these studies suggest that the response of other neoplasms to c-Met inhibition therapy may also be dependent on factors other than receptor overexpression.

Although our findings suggest that optimal response to c-Met inhibition will be observed in cells that signal through PI3K/Akt, other possibilities should be considered. Similar to other receptor tyrosine kinase–targeted therapies, such as Herceptin, Gleevec, and Iressa, the most robust clinical response to c-Met inhibition on anchorage-independent growth have been reported in panels of cell lines derived from lung and gastric cancers, as well as in gliomas [15]. In contrast, Miller et al. [14] recently demonstrated global induction of apoptosis following treatment with the heat shock protein 90 (Hsp90) inhibitor geldanamycin in the same three EA cell lines used in our study; however, the specificity of this response for c-Met is unclear as Hsp90 is involved in signal transduction from a variety of tyrosine kinase receptors [3,27]. Similar to our observations in EA, these studies suggest that the response of other neoplasms to c-Met inhibition therapy may also be dependent on factors other than receptor overexpression.

Constitutive activation of c-Met has been correlated with PI3K-dependent cell survival in NSCLC cell lines [31], suggesting that the most robust response to c-Met inhibition may be expected in cells with constitutive c-Met activity. We did not observe constitutive or HGF-induced activation of PI3K/Akt (Figure 4B) in the EA cell line with basal activation of c-Met (Bic-1; Figure 1), and inhibition of c-Met did not induce apoptosis in this cell line (Figure 2C). Bic-1 cells express HGF [13], suggesting that autocrine activation is likely, whereas an HGF-independent mechanism is responsible for c-Met activation in NSCLC cell lines [31] and may account for these differences.

The mechanism(s) responsible for the differential involvement of PI3K/Akt signaling in c-Met signal transduction requires further investigation. Our findings are most consistent with differential recruitment of adaptor proteins, such as Gab1, to the carboxy-terminal docking site of c-Met, and we intend to perform further experiments to test this hypothesis. Alternatively, the PTEN (phosphatase and tensin homologue deleted in chromosome 10) tumor-suppressor protein is one of the most widely studied inhibitors of PI3K [32], and PTEN loss has been associated with resistance to other forms of tyrosine kinase inhibition therapy [33–35]. However, loss of PTEN function is generally associated with constitutive PI3K activity [36], and PTEN mutation has not been identified in over 80 samples of EA [37], suggesting that loss of PTEN is unlikely to be responsible for our observations.

Two limitations of this study are the lack of a molecular method of blocking c-Met function and the lack of an in vivo model. The specificity of PHA665752 for c-Met has been previously established [15], and off-target effects are generally not seen at doses less than 2 μM (J. G. Christensen, personal communication), suggesting that effects are c-Met–specific. Furthermore, PHA665752 has been compared with other techniques of c-Met inhibition (anti-HGF antibody and c-Met RNA inhibition), and its effects have been shown to be c-Met–dependent [38]. Molecular HGF/c-Met inhibition strategies [8,39–41] and other strategies including HGF antagonists or neutralizers [42–45], c-Met dimerization blockers [46–49], and inhibitors of the c-Met intracellular pathway [20] have been reported. Phosphorylation of a catalytic domain (Tyr1230/1234/1235) is believed to be required for c-Met signaling [21]. Thus, unlike these other inhibition strategies, one advantage of our approach is that PHA665752 should inhibit the HGF/c-Met pathway irrespective of the mechanism of activation. Unfortunately, PHA665752 causes vein sclerosis and peritonitis in mice precluding in vivo experimentation.

In summary, our study is the first to investigate the effects of a c-Met–specific inhibitor on EA. Using a panel of c-Met–overexpressing EA cell lines, we have demonstrated variability in the response of EA to c-Met inhibition that correlated with downstream pathway activation. Our data support c-Met inhibition as a potential therapy for EA.

References