Inhibition of Tumor Growth and Metastasis in Non–Small Cell Lung Cancer by LY2801653, an Inhibitor of Several Oncokinases, Including MET

Wenjuan Wu, Chen Bi, Kelly M. Credille, Jason R. Manro, Victoria L. Peek, Gregory P. Donoho, Lei Yan, John A. Wijsman, S. Betty Yan, and Richard A. Walgren

Abstract

Purpose: Lung cancer is the leading cause of cancer-related death worldwide. Sustained activation, overexpression, or mutation of the MET pathway is associated with a poor prognosis in a variety of tumors, including non–small cell lung cancer (NSCLC), implicating the MET pathway as a potential therapeutic target for lung cancer. Previously, we reported on the development of LY2801653: a novel, orally bioavailable oncokinase inhibitor with MET as one of its targets. Here, we discuss the evaluation of LY2801653 in both preclinical in vitro and in vivo NSCLC models.

Experimental Design/Results: Treatment with LY2801653 showed tumor growth inhibition in tumor cell lines and patient-derived tumor xenograft models as a single agent (37.4%–90.0% inhibition) or when used in combination with cisplatin, gemcitabine, or erlotinib (66.5%–86.3% inhibition). Mechanistic studies showed that treatment with LY2801653 inhibited the constitutive activation of MET pathway signaling and resulted in inhibition of NCI-H441 cell proliferation, anchorage-independent growth, migration, and invasion. These in vitro findings were confirmed in the H441 orthotopic model where LY2801653 treatment significantly inhibited both primary tumor growth (87.9% inhibition) and metastasis (64.5% inhibition of lymph node and 67.7% inhibition of chest wall). Tumor-bearing animals treated with LY2801653 had a significantly greater survival time (87% increase compared with the vehicle-treated mice). In the MET-independent NCI-H1299 orthotopic model, treatment with LY2801653 showed a significant inhibition of primary tumor growth but not metastasis.

Conclusions: Collectively, these results support clinical evaluation of LY2801653 in NSCLCs and suggest that differences in the MET activation of tumors may be predictive of response.

Introduction

Lung cancer is the leading cause of cancer-related death in many countries, including the United States (1). Non-small cell lung cancer (NSCLC) accounts for up to 80% of all lung cancer cases; patients typically present with advanced disease at the time of diagnosis. The prognosis of patients with advanced lung cancer remains poor, and recent studies show that conventional therapies have reached a therapeutic plateau as evidenced by the 5-year survival rate for NSCLCs, which remains at 15% (2, 3). In the era of rational drug development, oncology has seen a shift from the development of cytotoxic agents to strategies that target receptor tyrosine kinase (RTK) signaling pathways. RTKs are key regulators of tumor angiogenesis, growth, survival, and metastasis. In lung cancer, the potential promise of this strategy has been illustrated by the clinical benefits associated with EGF receptor (EGFR) inhibitors gefitinib and erlotinib in a subset of patients with NSCLCs harboring EGFR mutations (4). However, the response rate to EGFR inhibitors is only 10% for patients without EGFR mutation, and even in responders, treatment response may be of limited durability due to the acquisition of resistance to gefitinib or erlotinib. Common mechanisms for acquired resistance include emergence of an EGFR gatekeeper mutation (T790M) and MET gene amplification (5–7). The limited therapeutic options currently available for patients with advanced lung cancer create a pressing need to identify new therapeutic targets.

One attractive target is the MET receptor pathway. Physiologic signaling of this pathway is initiated via ligand-dependent activation of MET at the cell surface by its sole known ligand, hepatocyte growth factor (HGF; ref. 8). Aberrant MET activation can occur through HGF-dependent

Authors' Affiliation: Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana

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Corresponding Author: Wenjuan Wu, Lilly Research Laboratories, DC0546, 307 E. McCarty Street, Indianapolis, IN 46285. Phone: 317-276-6690; Fax: 317-277-3652; E-mail: wu_wenjuan@lilly.com
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Translational Relevance

MET pathway is one of the most frequently activated receptor tyrosine kinase pathways in human cancer and is also being implicated as a resistance mechanism in other targeted therapeutics. In this study, we showed that treatment with LY2801653, a novel oncokine inhibitor with MET as one of its targets, inhibited tumor growth in non–small cell lung cancer (NSCLC) cell line–derived and patient tumor–derived xenograft mouse models as a single agent or in combination with cisplatin, gemcitabine, or erlotinib. In NSCLC orthotopic models, LY2801653 treatment inhibited both primary tumor growth and metastasis and prolonged survival time of tumor-bearing animals in MET-dependent NCI-H441 model but inhibited only primary tumor growth and not metastasis in the MET-independent NCI-H1299 model. Mechanistic studies showed that LY2801653 inhibited MET pathway signaling, anchorage-independent growth, migration, and invasion in H441 but not in H1299 cells. Collectively, these results support the ongoing early-phase clinical development of LY2801653 and suggest that differences in the MET activation status of tumors may be predictive of response.

or -independent mechanisms. Because of the contribution of this pathway to the regulation of a large network of signaling cascades, it has been implicated to play important roles in tumor cell proliferation, survival, motility, invasion, angiogenesis, and metastasis (9). The constitutive activation of MET may occur in cancer cells via a number of mechanisms including overexpression (with or without gene amplification), activating mutation, HGF autocrine/paracrine stimulation, or through crosstalk with other RTKs (10, 11). Sustained MET activation is associated with poor outcomes for several cancer types including NSCLCs (9, 12). LY2801653 is a novel orally bioavailable type II kinase inhibitor with MET as one of its targets and is in early clinical trials (Study I3O-MC-JSBA, NCT01285037). In a previously published report (13), we described the in vitro kinase selectivity profile as well as effects on cell scattering, cell proliferation, and in vivo antitumor activity in a variety of xenograft models. Given the potential importance of MET signaling in tumor biology, we sought disease-relevant models to evaluate the potential use of MET inhibitors in NSCLCs. Orthotopic lung cancer models were developed to recapitulate the local and metastatic patterns seen in patients with lung cancer in addition to NSCLC patient–derived tumor (PDT) xenograft models (14, 15). In orthotopic models, single solid primary lung tumors progress to a widespread and fatal state characterized by the dissemination of the primary tumor to regional lymph nodes and the ipsilateral chest wall. These models offer an opportunity to study the influence of the lung microenvironment on treatment response.

In this study, we focused on evaluating the antitumor effects of LY2801653 treatment on NSCLCs with in vitro models (MET signaling, cell proliferation, migration, invasion, and anchorage-independent cell growth), in vivo xenograft (3 human NSCLC lines and 5 lung PDTs), and orthotopic models (NCI-H441, a constitutively activated MET human NSCLC line; and NCI-H1299, an MET-independent human NSCLC line). Providing further support for ongoing early-phase clinical development, the results indicated that LY2801653 is a potent MET inhibitor with antitumor growth and anti-metastasis activities.

Materials and Methods

Cell culture

Human NSCLC cell lines H441, H460, A549, H1975, and H1299 were obtained from the American Type Culture Collection. Cells were maintained in RPMI-1640 medium (H441, H460, H1975, and H1299) or F-12 Kairn’s medium (A549) supplemented with 10% FBS, sodium pyruvate, nonessential amino acids, l-glutamine, and penicillin/streptomycin (Invitrogen). Human lung cancer–associated fibroblast (CAF) cells (60093A) from Asterand were grown in fibroblast cell culture medium (#CC-3132, Lonza) with 10% FBS. All cultures were maintained in a humidified incubator at 37°C under 5% CO₂/95% air free of Mycoplasma and pathogenic murine viruses. All NSCLC cells used for in vitro experiments were at passages <7; CAF cells were used for experiments at passages 3 to 5 after recovery from the frozen stocks.

Compound and conditioned media of CAF

LY2801653 was prepared as 10 mmol/L stock solutions in dimethyl sulfoxide. CAF cells at 80% confluence, medium was changed to serum/growth factors free and collected after 48 hours, centrifuged, and the supernatants were identified as conditioned medium and stored at −20°C.

Western blotting

Cultured cells treated with recombinant human HGF (#294-HGN, R&D) and/or LY2801653 were harvested and lysed with a buffer (#9803, Cell Signaling) containing Halt protease and phosphatase inhibitor cocktail (#78441, Thermo Scientific). Total cell proteins (50 μg) were resolved by 4% to 15% SDS-PAGE, transferred to polyvinylidene difluoride membranes (90 V for 2 hours), and probed with target monoclonal antibodies. The antibodies against MET (L41G3, #3148), p-MAPK (T202/T204, #9190), MAPK (#4696), p-AKT (Ser473, #4058), and AKT (#2920) were from Cell Signaling. The p-MET antibody (pYpYpY1230/1234, #44888G) and β-actin antibody (A#2228) were from Invitrogen and Sigma, respectively. Protein bands were captured by a densitometer or luminescent image analyzer LAS-4000 (Fujifilm).

Quantitative real-time PCR

Total RNA was prepared using a mirVana miRNA Isolation kit (Qiagen). First-strand cDNA was synthesized from 2 μg of total RNA using a high-capacity cDNA Reverse
Transcription kit (#4368813, Applied Biosystems). Quantitative real-time PCR (qRT-PCR) was done using first-strand cDNA with TaqMan probes and TaqMan Universal PCR Master Mix (#4304437, Roche). TaqMan probes for HGF and MET were from Applied Biosystems, and the amplification was done using an ABI PRISM 7900HT Sequence Detection System according to the manufacturer’s instructions. Quantification was done in triplicate, and the expression levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH; #4310884E, Applied Biosystems) were used as an external control.

ELISA assay
The levels of p-MET (Y1349) in lysate from cells or tumor tissues were measured with the Meso Scale Discovery (MSD) ELISA assay (#K151DDL-1, MSD), as described previously (13). For HGF (#DHG00, R&D), VEGF (#DEG00, R&D), and FGFR ELISA (#DFB50, R&D), 20 μg of protein of each sample was loaded per well in duplicate. The assay was done following the manufacturer’s instructions.

\[^{3}H\text{-thymidine incorporation cell proliferation assay}\]
H441 cells (1.5 × 10^5/well) seeded in 96-well white isoplates were cultured overnight. Cells were then treated with LY2801653 in medium with 0.5% FBS for 48 hours. One-half of a μCi of \[^{3}H\text{-thymidine}\] (Amersham) was added per well. After 6 hours, plates were washed once with PBS, and \[^{3}H\text{-thymidine\ incorporation was measured on a Wallac Microbeta JET liquid scintillation counter (Perkin Elmer).}\]

Migration and invasion assay
For the migration assay, H441 or H1299 cells were seeded into upper Transwell chambers (Costar) at 2 × 10^5 cells/well. For the invasion assay, H441 or H1299 cells were seeded into upper Transwell chambers coated with Matrigel (BD Biosciences) at 4 × 10^5 cells/well. 600-μL CAF-conditioned medium or regular medium was added in the lower chambers. LY2801653 was added in both upper and lower chambers. After overnight incubation at 37°C, the migrated or invaded cells were fixed and stained with a solution of 0.5% crystal violet/20% ethanol. Cells were counted in 5 fields under an inverted microscope at ×200 magnifications.

\[^{3}H\text{-thymidine incorporation cell proliferation assay}\]

\[^{3}H\text{-thymidine incorporation cell proliferation assay}\]

Soft agar cell growth assay
H441 or H1299 cells (10^6 cells/well) mixed with soft agar (1:1, final 0.6% agarose) were seeded in 96-well plates over a base agar layer (0.4% agarose). The plates were incubated in complete culture medium with or without LY2801653 at 37°C, with 5% CO\textsubscript{2}/95% air. After 10 days, the number and sizes of colonies were evaluated by Acumen Microplate Cytometer following 1-hour incubation with calcein solution (#C3099, Invitrogen) at room temperature. Data were analyzed using the Acumen Explorer Software.

Reverse-phase protein microarray of phosphoproteins for signaling pathway analysis
A tumor lysate from each mouse in the vehicle (n = 5) and 12 mg/kg LY2801653 (n = 3) groups was prepared in duplicate and then spotted twice per sample in the protein microarray. The reverse-phase protein microarray (RPMA) was conducted at Theranostics Health Inc. as previously described (16). Approximately 10 nl of each tumor tissue lysate sample was arrayed in duplicate.

Evaluation of LY2801653 antitumor effect in human lung cancer PDT xenograft, cell line–derived xenograft, and orthotopic tumor models
All animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Eli Lilly and Company or Oncotest GmbH. Eli Lilly and Company is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International.

For xenograft models of NSCLC cell lines (H441, A549, and H1975) and PDT (LXFL-529, LXFL-430, LXFL-1176, LXFA-1647 and LXFA-526, derived by Oncotest GmbH; refs. 13, 15, 17), detailed methods are described in the Supplementary Methods.

For the orthotopic model of NSCLC cells, logarithmically growing H441, H460, A549, and H1299 tumor cells (>95% viability) at 1 × 10^6 or 2 × 10^6 cells in 55 μL containing 50 μg Matrigel (BD Biosciences) were injected into the left lung lobe of mice as previously described (14). Seven days postimplantation, 2 mice were sacrificed to confirm the growth of the primary lung tumor (2 × 2 mm\textsuperscript{2}) and the remaining mice were randomized into various groups (n = 8–10) and treated with LY2801653. Treatment continued until vehicle control mice displayed signs of morbidity, at which point the mice were sacrificed and autopsied 2 to 4 hours following the last treatment. The growth of primary lung tumor (weight) and metastasis to lymph node (weight) and chest wall (colony counts) were measured; and the left lung lobes (including primary lung tumors and adjacent lung tissues) were collected. For survival experiments, the treatment continued until the individual mouse displayed signs of morbidity, which was indicated as the survival time. In all in vivo studies, LY2801653 was formulated in 10% acacia daily and dosed orally. LY2801653 doses used in this study were based on the pharmacokinetics and pharmacodynamic effect on p-MET reduction published previously (13).

Histology and immunohistochemistry
For the H441 lung orthotopic tumor model, lung tumors and adjacent lung tissues were collected after the last dose, fixed with 10% neutral-buffered formalin, embedded in paraffin, and sectioned by microtome. Immunolabeling for p-MET (rabbit anti-human p-MET, Y1234/Y1235, #3129, Cell Signaling) was conducted on an autostainer (Dako) using heat-induced antigen retrieval and standard techniques with 3,3’-diaminobenzidine as chromogen and hematoxylin counterstain.

For human tumor samples from patients with NSCLCs, materials and methods are described in the Supplementary Methods.
Statistical analysis
Nonlinear regression was used to fit the 4-parameter logistic model to estimate the IC_{50} value for LY2801653 in the p-MET, 3^H-thymidine incorporation, and soft agar assays. For the RPMA, the log_2 of the geometric mean of the 4 measurements per tumor was used for subsequent statistical analysis. A pooled t test for each phosphoprotein was used to test for statistical differences between the 2 groups. The false discovery rate (FDR) method (SAS v9.2, Multitest procedure) was used to provide control of the rate of false positives among the statistical tests of the 40 analytes.

For all in vivo tumor models, the analysis methods are described in detail in the Supplementary Methods.

Results
Expression of MET in human NSCLC specimens
MET expression in patient samples was initially evaluated using a tumor microarray (TMA) containing 39 tumor samples of lung adenocarcinomas in 1.5-mm cores. The MET immunolabeling of the tumor cell membranes was scored semiquantitatively by a pathologist using a conventional scoring system of 0 (negative), 1+ (weak), 2+ (medium), and 3+ (strong). Results showed that 46% (18 of 39) of the tumor samples had 2+ or 3+ membrane expression for MET. Next, a set of 13 larger sized samples of adenocarcinomas from patients was immunolabeled and scored in a similar fashion. Heterogeneity of MET expression within an individual tumor was noted in these larger samples. Overall, 39% (5 of 13) of these samples had 2+ or 3+ MET membrane expression (Supplementary Table S1 and Fig. S1).

LY2801653 treatment inhibited tumor growth in lung tumor cell line or PDT xenograft models as a single agent or in combination with cisplatin, gemcitabine, or erlotinib

We have previously reported that as a single agent LY2801653 inhibited tumor growth in multiple xenograft tumor models, including NSCLC H441 (low MET amplification) and NCI-H1993 (high MET amplification; ref. 13). As described here, treatment with LY2801653 inhibited the tumor growth in A549 (low MET expression, 50.6% inhibition) and H1975 (MET overexpression, EGFR mutations L858R/T790M, 61.8%–85.3% inhibition) xenograft models; and LY2801653 treatment inhibited more tumor growth in H441 xenograft tumors when combined with gemcitabine (80.8% inhibition), cisplatin (81.6% inhibition), or erlotinib (86.3% inhibition; Fig. 1A and Supplementary Table S2). Similarly, LY2801653 treatment alone inhibited tumor growth in all PDT xenograft models, including LXFL-529 (low MET expression with mutation T992I, 39.3% inhibition), LXFL-430 (low MET expression, 38.7% inhibition), LXFL-1176 (MET overexpression, high p-MET, 37.4%–60.3% inhibition), LXFA-526 (MET amplification, 64.7% inhibition), and LXFA-1647 (MET amplification, 78.8%–90.0% inhibition). The antitumor activity was further increased in LXFA-526 PDT xenograft model with LY2801653 treatment in combination with erlotinib (Fig. 1B and Supplementary Table S2).

Expression of HGF, MET, and phospho-MET in human NSCLC cell lines and cell line–derived lung orthotopic tumors
To further evaluate LY2801653, 4 NSCLC cell lines (A549, H460, H441, and H1299) were selected on the basis of their robust ability to establish orthotopic lung tumors in mice (Fig. 2). When grown in cell culture, all cell lines showed low expression levels of HGF mRNA or protein. In contrast, HGF levels (mRNA and protein) were significantly higher in orthotypically grown lung tumors from H1299 and A549 cells than in cultured cells and measured protein levels were increased by approximately 100-fold in H1299 and 20-fold in A549 cell tumors (Fig. 2A). MET (mRNA and protein) and p-MET were overexpressed in H441 cells and tumors but not in the other 3 NSCLC cell lines (Fig. 2A and B), even though HGF (mRNA and protein) was barely detected in H441 cells and tumors.

LY2801653 treatment inhibited phosphorylation and downstream signaling of MET, cell proliferation, anchorage-independent cell growth, migration, and invasion in H441 cells

Ligand-independent constitutive activation of MET may be induced through gene amplification, overexpression, or mutation of MET. MET gene amplification analysis of H441 cells by FISH revealed that approximately 75% of the cells harbored 3 to 5 copies of the MET gene (13). No MET mutation was observed in H441 cells based on the Broad Institute Cancer Cell Line Encyclopedia database.

MAPK1 and AKT are important downstream effectors of the MET signaling pathway, and the LY2801653 treatment effect on this signaling cascade was evaluated in H441 cells. The IC_{50} value for LY2801653 inhibition of MET autophosphorylation at the docking site Y1349 was 2.7 mmol/L (Fig. 3A). H441 cells were treated with LY2801653 in the absence or presence of HGF. Autophosphorylation at the activation loop Y1234/Y1235 was completely blocked by LY2801653 at 30 nmol/L. Downstream activation of AKT (p-AKT) and MAPK (p-MAPK) was also inhibited by LY2801653 (Fig. 3B). While exogenous HGF did not elicit a detectable elevation of p-MET in the constitutively activated H441 cells, it did increase levels of both p-AKT and p-MAPK. LY2801653 treatment inhibited this HGF-dependent enhancement in MET pathway signaling to a greater extent for p-AKT than for p-MAPK (Fig. 3C). The in vitro proliferation of H441 cells was inhibited by LY2801653 with an IC_{50} of 0.22 μmol/L (Fig. 3D). Anchorage-independent H441 colony growth in soft agar was inhibited by LY2801653 with an IC_{50} of 1.53 μmol/L (Fig. 3E). With the addition of CAF-conditioned medium, H441 cell migration and invasion were enhanced by 100- and 10-fold, respectively, when compared with those in absence of CAF-conditioned medium. LY2801653 treatment blocked these responses with an IC_{50} of approximately 0.1 μmol/L for migration and <0.01 μmol/L for invasion (Fig. 3F).
LY2801653 Inhibits Orthotopic Tumor Growth and Metastasis

LY2801653 suppressed lung tumor growth and metastasis and enhanced survival of tumor-bearing mice in H441 lung orthotopic tumor model

Orthotopic implantation of H441 cells into mouse lungs resulted in the growth of a primary tumor in the lung and the metastasis to the lymph nodes and chest wall. Tumor-bearing mice were treated with LY2801653 (0.8, 3, or 12 mg/kg) or vehicle control. Primary lung tumors were detected in all groups. Representative primary lung tumors of each dose group are shown in Fig. 4A. For all in vivo studies, administration of LY2801653 was well tolerated with no significant loss in body weight. LY2801653 treatment resulted in a dose-dependent reduction of primary tumor growth (Fig. 4B). At the time when vehicle-treated animals were becoming moribund (postimplantation day 54), the reductions of primary tumor weight, as compared with the vehicle-treated animals, were 56.0% (P = 0.005) and 87.9% (P < 0.0001), respectively, for the animals treated with 3 and 12 mg/kg of LY2801653 (Fig. 4B). Compared with the control, LY2801653 treatment at 12 mg/kg also reduced the spontaneous metastasis to the mediastinal lymph nodes by 64.5% (P = 0.0021) and to
the chest wall by 67.7% (Fig. 4B). On macroscopic inspection, 3 mice were found to be free of gross lymph node metastasis and 5 mice were free of gross thoracic wall metastasis when treated with 12 mg/kg of LY2801653.

The antitumor effects of LY2801653 were also correlated with the increase in the survival time of mice bearing H441 orthotopic tumors by 21% and 87% ($P < 0.0001$) for the 3 and 12 mg/kg groups, respectively, as compared to the vehicle group (Fig. 4C).

LY2801653 inhibited MET phosphorylation and downstream effectors in H441 orthotopic tumors

To elucidate the mechanism underlying the observed antitumor growth and anti-metastasis effects of LY2801653 in vivo, downstream effectors of MET pathway signaling and MET-targeted molecules were assessed in the tissues and/or lysates from lung tumor (Fig. 4A) by IHC, ELISA, or RPMA. LY2801653 treatment at 12 mg/kg markedly decreased p-MET (Y1234/Y1235; Fig. 4D). The IHC results were consistent with the p-MET (Y1349) level measured by MSD ELISA (Fig. 4E). VEGF and FGF2 levels are regulated, in part, by MET signaling (18, 19). The levels of VEGF, but not those of FGF2, were significantly reduced in tumor tissues from animals treated with LY2801653 (Fig. 4E).

The MET signaling pathway plays key roles in cell proliferation, survival, scattering, motility, invasion, and metastasis through regulating a large network of signaling cascades (20–22). Thirty-nine components of the MET signaling network were evaluated in the H441 lung tumors using RPMA (Table 1 and Supplementary Table S3). These cellular markers include the phosphoproteins that regulate transcriptional control (e.g., STAT3), cell proliferation and differentiation (e.g., MAP2K1 and EIF4E), cell survival and apoptosis (e.g., AKT and MAPK8), cell stress and inflammation response (e.g., MAPK14), cell motility (e.g., PTK2 and EZR), and transphosphorylation of potential heterodimerization partner proteins with MET (e.g., EGFR, ERBB2, and ERBB3). As compared to the controls, 12 mg/kg LY2801653 treatment led to significant reductions in phosphorylation of 14 of the 39 phosphoproteins (Table 1), including EGFR, EIF4E, MAP2K1, MAPK14, RPS6KB2, and STAT3.

LY2801653 in vitro and in vivo effects in MET-independent H1299

MET and p-MET were not detected in any significant levels in H1299 cells in vitro or as orthotopic tumors (Fig. 2). To understand LY2801653 activity on MET-independent cells and tumors, the effect of LY2801653 on tumor growth and metastasis was evaluated in H1299 cells of in vitro and in vivo orthotopic models. In contrast to the responses seen in H441 cells (constitutively activated MET), LY2801653 treatment did not inhibit H1299 anchorage-independent cell growth, migration, or invasion (Fig. 5A–C).

Orthotopic implantation of H1299 cells into the mouse lung resulted in the growth of primary tumor in the lung and metastasis to lymph node and chest wall, in a pattern similar to that of the H441 orthotopic lung tumor. Compared with the vehicle-treated animals, a significant reduction of primary tumor growth was observed by 45.7% ($P = 0.014$) and
75.4% (P = 0.0001), for the 3 and 12 mg/kg LY2801653-treated animals, respectively. However, LY2801653 treatment at either dose did not significantly inhibit spontaneous metastasis to the mediastinal lymph nodes or to the chest wall (Fig. 5D).

Discussion

The dysregulation of MET signaling has been extensively described in many types of human cancers including NSCLCs (8–12). The sustained activation, overexpression, mutation, or gene amplification of MET is generally associated with a worse prognosis and contributes to tumor growth, angiogenesis, and metastasis. In these studies, we have investigated the effect of LY2801653, a novel, orally bioavailable, type II inhibitor of several oncoproteases, including MET, on NSCLCs. Our results indicated that LY2801653 treatment inhibited tumor growth in both tumor cell line and PDT xenograft models. None of the cell lines or PDT tumors in this study harbor MET mutation except LXFL-529. T992I MET mutation in LXFL-529 may not be an activating mutation (9). Tumor growth inhibition also occurred with LY2801653 treatment in combination with cisplatin, gemcitabine, or erlotinib. Among the PDT xenograft models described here, LY2801653 showed more antitumor activity in tumors with MET amplification (LXFA-1647, LXFA-526) or MET overexpression (LXFL-1176) than it did in tumors with low MET expression.
Furthermore, LY2801653 treatment also inhibited primary tumor growth and metastasis (to lymph nodes and chest wall) and prolonged survival of tumor-bearing mice in H441 orthotopic model (constitutively activated MET). These in vivo data were consistent with the in vitro inhibition of cell proliferation, migration, invasion, and anchorage-independent growth by LY2801653 treatment in H441 cells.

For many tumor types, the development of tumor metastasis is a defining characteristic of advanced or relapsed disease and is associated with significant shifts in morbidity and mortality. The development of metastatic foci is dependent on multiple processes, including dissemination of tumor cells from the primary tumor, migration, invasion through the basement membrane and stroma, and regrowth in distal sites with angiogenesis. As such, the process of metastasis is dependent on contributions of the cellular and molecular biology of tumor and stromal cells in the tumor microenvironment. The CAFs surrounding the tumor cells have been shown to be particularly important for regulating migration and invasion of tumor cells (23). HGF was identified as a fibroblast-derived factor which is capable of causing epithelial cell scattering and stimulating the migration and invasion of cancer cells (24–26). MET is overexpressed in a substantial portion of the NSCLC tumors, ranging from 26% to 51% (27), and has also been associated with advanced cancer stage and poor patient survival (28, 29). Consistent with these reports, MET was expressed in 39% to 46% of specimens from patients with adenocarcinoma evaluated in this study. The expression and signaling of the HGF/MET pathway were evaluated here in both

Figure 4. Effect of LY2801653 treatment on tumor growth, metastasis, survival of mice bearing H441 lung tumors, and levels of p-MET, VEGF, and FGF2 in lung tumor tissues in H441 orthotopic model. A and B, H441 cells were implanted into the left lungs of mice and LY2801653 treatment (0.8, 3, and 12 mg/kg, orally dosed twice daily) began on day 7 following implantation. Mice were sacrificed when significant morbidity was observed (day 54 after cell implantation) and the primary lung tumor (A, pictures were taken from the representative primary tumors in each group) and metastasis to lymph nodes and chest wall were evaluated. Data were expressed as mean ± SE of 8 to 10 mice. * statistical significance of LY2801653 group compared with vehicle group (B). C, H441 cells were implanted into the left lungs of mice and LY2801653 treatment (3 and 12 mg/kg, orally dosed twice daily) began on day 7 following implantation. Mice were monitored daily and sacrificed when they became moribund. Data are expressed as mean ± SE of 10 mice. * statistical significance of LY2801653 group compared with vehicle group. D, representative sections obtained from primary lung tumors were immunolabeled with antibody directed against p-MET (scale bar = 50 μm). E, p-MET (top) in lysate of lung tumor tissues were measured by MSD ELISA. VEGF level (middle) and FGF2 level (bottom) in lysate of lung tumor tissues were detected by ELISA.
LY2801653 Inhibits Orthotopic Tumor Growth and Metastasis

Table 1. Inhibition of phosphoproteins in H441 lung orthotopic tumor tissues treated by LY2801653

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<th>Target protein</th>
<th>Phosphorylation site(s)</th>
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<th>FDR q value</th>
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</tbody>
</table>

NOTE: Tumor lysates from vehicle group (n = 5) or 12 mg/kg LY2801653 (n = 3) were assessed with RPMA for phosphorylation status of various signaling pathway proteins. Thirty-nine phosphoprotein analytes were evaluated. The log2 of the geometric mean of the 4 measurements per tumor (duplicate lysate per tumor and each lysate evaluated twice) was used for subsequent analysis. Treatment group was compared to the vehicle control group as Inhibition%. The statistical analysis (FDR) is described in Materials and Methods. The 14 analytes with significant FDR q value are listed here. The data for other 25 analytes are in the Supplementary Table S3.

NSCLC cell lines and tumor tissues, providing data and insights on the impact of the tumor microenvironment on the MET pathway in tumor cells. HGF expression was barely detected in four NSCLC cell lines tested (H441, A549, H1299, H460). It is of particular interest to note that HGF expression was highly increased in the orthotopic tumors from A549 and H1299 but not from H441 and H460. These data suggest the contextual importance of the interaction between tumor and stromal cells in the tumor microenvironment on HGF/MET pathway signaling. The higher observed HGF levels in the A549 and H1299 lung tumors, however, did not result in increased MET activation as indicated by the p-MET levels (Fig. 2A). It is possible that the observed levels of HGF (100–450 pg/mL) may be too low to activate MET. Activation of MET in A549 in vitro has been previously reported to require addition of 40 ng/mL of exogenous HGF (30, 31). Constitutive activation of MET was detected only in H441 cells and H441-derived tumors, which overexpressed MET. On the basis of the lack of high HGF expression in H441 cells and due to the fact that mouse HGF is not recognized by human MET, the MET signaling in H441 cells would appear to principally function in a ligand-independent manner. This constitutive activation is most likely the result of MET gene amplification (13).

The HGF/MET pathway has been hypothesized to play critical roles in promoting epithelial-to-mesenchymal transition of tumor cells, tumor cell migration, invasion, and metastasis (10, 32–34). An increase in MET activity has been shown to potently induce migration and invasion, bolstering the ability of cells to degrade the matrix and undergo chemotaxis (8). The constitutive activation of MET in NSCLC cells has also been found to correlate with anchorage-independent growth and the ability to avoid anoikis (35). Consistent with these findings, the migration and invasion of H441 cells were stimulated by lung CAF conditioned medium (data not shown), which were totally blocked by MET inhibitor LY2801653 treatment. In vivo studies were conducted in an orthotopic model of lung cancer to further explore the mechanistic effects of MET pathway inhibition with LY2801653 treatment.

Typically, preclinical in vivo evaluation of MET inhibitors for antitumor activity has been conducted in subcutaneous xenograft models (36, 37). This approach may not adequately address the interaction between tumor and stromal cells in the specific organ environment and the metastatic potential of the MET signaling (38, 39). Consistent with the in vitro results, LY2801653 treatment led to the inhibition of both primary tumor growth and spontaneous metastasis to the lymph nodes and to the chest wall in the H441 orthotopic model. In contrast, in the MET-independent H1299 cells, migration and invasion were not affected by LY2801653 in the presence of the lung CAF conditioned medium. The in vivo spontaneous metastasis in H1299 lung orthotopic tumors was also not inhibited by LY2801653 treatment. Thus, the data in this study further support the hypothesis that the MET pathway plays a critical role in tumor migration, invasion, and metastasis; and the treatment with the MET inhibitor may be able to inhibit metastasis in cancer driven by the activation of MET pathway signaling.

The activation of MET signaling has also been reported to induce angiogenesis of lung cancer via the KDR pathway (19). LY2801653 has little or no inhibitory activity against KDR, and treatment was shown to induce vessel normalization in the U-87MG xenograft tumor (13). In this H441 orthotopic model, LY2801653 treatment resulted in significant reduction of VEGF level in the tumors. Whether VEGF reduction by LY2801653 treatment in this model contributed to the inhibition in tumor growth and metastasis requires further study.

MET signaling is important in modulating cancer cell proliferation, apoptosis, survival, scattering, motility, invasion, and metastasis through several signaling axes, including the PI3K/AKT/mTOR, RAS/RAF/MAP2K/MAPK, and PTK2/ERZ/STAT3 (20–22). In the H441 tumors treated with LY2801653, downregulation of multiple downstream signaling steps in the MET pathway was observed, including STAT3, MAP2K1/2, MAPK14, RPS6KB2, and

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EIF4E. These data are consistent with the inhibition of MET-mediated biological effects, such as cell migration and tumor metastasis. The anchorage-independent growth in soft agar, one in vitro feature of cancer cells, has been linked to STAT3 activation through MET signaling (20). Therefore, inhibition of anchorage-independent growth in vitro and metastasis in vivo by LY2801653 may result from the inhibition of MET-dependent STAT3 signaling. It is also of note that the phosphorylation of EGFR, a potential heterodimerization partner of MET, was also reduced with LY2801653 treatment.

LY2801653 treatment showed in vivo inhibition of primary tumor growth and anti-metastatic activity in a spontaneous metastatic lung orthotopic tumor model. These in vivo activities of LY2801653 may not be entirely due to the inhibition of MET kinase activity as the kinase selectivity profile of LY2801653 includes other kinases such as MST1R, FLT3, AXL, MER, TEK, ROS1, DDR1/2, and MKNK1/2 (13). The reduction of p-EIF4E (a substrate of MKNK1/2) in the H441 tumor tissues treated by LY2801653 may be a contribution from inhibition of both MET and MKNK1/2 (40–42). On the other hand, H1299 cells express high level of AXL, a receptor tyrosine oncokine, and LY2801653 was shown to inhibit AXL with an IC50 of 2 nmol/L (13). The observed inhibition of primary lung tumor growth in the H1299 lung tumors (Fig. 5D) may result, at least in part, from LY2801653 inhibition of AXL.

In conclusion, data from this study showed that LY2801653 treatment of H441 cells in vitro inhibited the constitutive activation of the MET signaling pathway, cell proliferation, anchorage-independent growth, migration, and invasion. Consistent with the in vitro findings, LY2801653 treatment showed significant in vivo inhibition of both primary tumor growth and metastasis which was associated with a prolonged survival of mice bearing H441 lung tumors. These data support the ongoing early-phase clinical evaluation of LY2801653 in patients with advanced cancer (trial I3O-MC-JSBA, NCT01285037).
Disclosure of Potential Conflicts of Interest

G.P. Donoho has ownership interest (including patents) in Eli Lilly and Company and J.A. Wijsman is employed as scientist in Eli Lilly and Company. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: W. Wu, V.L. Peek, G.P. Donoho, S.-C. B. Yan, R.A. Walgren

Development of methodology: W. Wu, C. Bi, L. Yan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): W. Wu, K. Credille, J.A. Wijsman

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): W. Wu, C. Bi, K. Credille, J.R. Manro, V.L. Peek, J.A. Wijsman, S.-C. B. Yan, R.A. Walgren

Writing and/or revision of the manuscript: W. Wu, C. Bi, K. Credille, J.R. Manro, V.L. Peek, G.P. Donoho, J.A. Wijsman, S.-C. B. Yan, R.A. Walgren

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Wu, J.R. Manro

Study supervision: W. Wu, G.P. Donoho

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Wenjuan Wu, Chen Bi, Kelly M. Credille, et al.

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