Dramatic Antitumor Effects of the Dual MET/RON Small-Molecule Inhibitor LY2801653 in Non–Small Cell Lung Cancer

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Abstract

Lung cancer is a heterogeneous disease encompassing a wide array of genetic abnormalities. The MET receptor tyrosine kinase is altered in many lung cancers, especially non–small cell lung cancer (NSCLC), and clinical trials of MET inhibitors that are under way are documenting cases of acquired resistance. On the basis of the evidence that the RON receptor tyrosine kinase can also be overexpressed in NSCLC, we evaluated the potent MET/RON dual kinase inhibitor LY2801653 in this setting. LY2801653 was more efficacious than the MET/ALK/RON/ROS inhibitor crizotinib with a distinct pattern of downstream signaling effects. Using the PamGene platform, we found that inhibition of MET and RON was associated with decreased phosphorylation of CBL, PI3K, and STAT3. In classic and orthotopic mouse xenograft models of lung cancer, LY2801653 decreased tumor growth, dramatically inhibiting mitotic events and angiogenesis. Taken together, our results argued that specific targeting of the MET/RON kinases could provide robust inhibition of cell proliferation and tumor outgrowth in multiple in vitro and in vivo models of NSCLC. These findings offer a robust preclinical proof of concept for MET/RON targeting by LY2801653 as a promising small-molecule modality to treat NSCLC. Cancer Res; 74(3); 884–95. ©2013 AACR.

Introduction

Lung cancer is a global health problem, and can be divided into non–small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC can be further subdivided into various histologies such as adenocarcinoma, squamous cell carcinoma (SCC), and large cell carcinoma. As has been shown recently, there are various molecular subtypes of NSCLC, such as EGF receptor (EGFR) mutation (1, 2), ALK translocation (3), and ROS1 translocation (4). We have previously shown that MET receptor tyrosine kinase can be a good target for NSCLC (5, 6).

MET receptor tyrosine kinase (RTK) can be activated through a number of mechanisms, especially with ligand hepatocyte growth factor (HGF) stimulation. In lung cancer, MET can be overexpressed along with HGF. There also can be gain-of-function mutations within the semaphorin and juxta-membrane domain (5, 7, 8). In a subset of NSCLC, MET is amplified (9–11). RON is a RTK similar to MET; however, its activation is dependent on a separate ligand, macrophage-stimulating protein. RON has been shown to be overexpressed in NSCLC, and there are a number of isoforms in lung cancer (12). Particularly, δ 165 and 155 isoforms are known to be potent oncogenes (13, 14). It seems that there is a strong interaction of MET with its family member RON (15, 16). Recent data indicate an increasingly important role for receptor cross-talk in the activation of MET and RON. MET/RON complexes are present on the cell surface before ligand-induced dimerization, and ligand-stimulated MET activation results in direct transphosphorylation of RON (15). Catenacci and colleagues have shown that RON and MET costimulation led to enhanced malignant phenotypes over stimulation of either receptor alone using gastroesophageal adenocarcinoma cell lines. They have shown that growth inhibition, as evidenced by viability and apoptosis assays, was optimal using novel blocking monoclonal antibodies to both RON and MET, versus either alone. They have also shown that combined siRNA knockdown of RON and MET inhibited signaling feedback loops and led to optimal antitumorigenic effect (16). Because only a subset of MET-expressing tumors respond to anti-MET therapeutics, we hypothesized that MET and RON could be better therapeutic targets in NSCLC. There are various strategies to inhibit MET and RON—such as specific antibodies or small-molecule inhibitors (SMI; ref 17). The compound LY2801653 is an SMI that has shown in vitro activity against both MET and RON receptors at nanomolar concentrations.

In this study, we sought to determine the specific inhibition of NSCLC cell lines with LY2801653. Compared with crizotinib, we found LY2801653 to be more efficacious in A549, H1703, and H1993 cell lines. Concurrently, we were able to show dramatic effects on mouse xenograft models. Interestingly, we show that there are specific signal transduction pathways that are affected, which help in understanding the systems biology for MET/RON pathways. These data support potential clinical development of LY2801653 for the treatment of NSCLC.

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Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Materials and Methods

Cell lines
We used one human bronchial epithelial cell line (BEAS-2B) and 10 NSCLC cell lines (A549, H358, H520, H222, H661, H1703, H1993, H2170, H2228, and SKMES-1) that were obtained from the American Type Culture Collection and cultured accordingly. A TPR-MET-expressing murine pre-B cell line, BaF3 TPR-MET, was generated by transfection of an expression vector containing TPR-MET cDNA as previously described (18). BaF3 was grown in RPMI-1640 containing 10% FBS and 10% WEHI-conditioned medium as a source of murine interleukin-3. A luciferase-tagged A549 cell line, A549-luc-C8, was purchased from Caliper Life Sciences and grown in RPMI-1640 with 10% FBS.

MET/RON SMI LY2801653
The MET/RON SMI was provided by Eli Lilly and Company. The drug was received in powdered form and dissolved in dimethyl sulfoxide (DMSO) according to the company's instructions and used at the indicated concentration.

Specific knockdown of MET/RON by siRNA
Targeted siRNA to MET and RON were used on A549 cells individually and in combination for dual knockdown (Santa Cruz Biototechnology: Met siRNA, sc-29197, Ron siRNA, sc-36434, Control siRNA-D, sc-44232) using the Lipofectamine RNAiMAX Transfection Reagent kit (Invitrogen: #13778-075). Lysates from each siRNA treatment were immunoblotted to verify gene knockdown and cell viability was assessed to determine the effect of gene knockdown relative to siRNA control cells. Detailed methods for this assay are described in Supplementary Materials and Methods.

Cell viability assay
Cell lines' sensitivity to MET/RON inhibitor LY2801653 or siRNA was determined by performing cell proliferation assay using standard MTT. Detailed methods for this assay are described in Supplementary Materials and Methods. The IC₅₀ was generated for all cell lines. Similarly, the efficacy of crizotinib was determined. Crizotinib was purchased from LC Laboratories.

Immunoblot analysis and antibodies
NSCLC cells treated with drug or siRNA were immunoblotted to evaluate the biochemical effects of MET/RON inhibition. Untreated cells or control siRNA were used as controls. Immunoblotting on whole cell lysates was performed following routine protocols (19, 20). All antibodies used for immunoblot analysis are listed in Supplementary Materials and Methods.

PamGene tyrosine kinase array and run analyses
PamGene technology using the PamChip Tyrosine Kinase Array allows the kinetic detection of the phosphorylation of peptides spotted onto a three-dimensional porous well of a four-array chip, produced and commercialized by PamGene. This technology can be used to measure the activity of purified kinases and the effects of kinase inhibitors on them. Phosphorylation on these arrays is measured as described elsewhere (21). Detailed methods for PamGene analyses are provided in Supplementary Materials and Methods. The lysates of H1993 cells treated with LY2801653 (1.3, and 10 nmol/L) for 4 hours similar to the assay conducted for immunoblot analysis were hybridized in four technical replicates. The data generated were analyzed with BioNavigator (Version 0.4.2.82: PamGene).

Pathway and network analyses
Peptides found to be significantly down- or upregulated in the statistical analysis (P < 0.05) in PamGene were used for pathway and network analyses using the bioinformatics analysis software MetaCore (GeneGo, Thomson Reuters) as described in refs. 22 and 23. Several MetaCore tools were used to analyze data and are listed in Supplementary Materials and Methods. MetaCore's enrichment analysis tool uses hypergeometric modeling to determine the statistical significance of enrichment. "GeneGo Map Folders" (MetaCore tool) are organized to collect all GeneGo maps that describe different canonical pathways involved in the same process. The significant process networks were noted, and relevant signaling networks were assembled on the basis of the statistically significant data generated by PamGene fold-change calculation.

Xenograft tumor model for LY2801653
Female homozygous athymic nude mice ages 5 to 6 weeks were purchased from Harlan Laboratories and allowed to acclimate for 1 week. Animal care was in accordance with institutional animal care guidelines. Approximately 2.5 × 10⁶ A549 or 5 × 10⁶ H1993 NSCLC cells were inoculated subcutaneously in the right flank of each mouse. Tumor growth was measured with calipers and volume (mm³) was calculated as (L × W × H) / 2. When the volume reached a mean of 200 to 400 mm³, mice were randomized into two groups of 10 and 11 and treatment was initiated. LY2801653 was administered twice a day for 4 weeks by oral gavage at 20 mg/kg to one group, whereas the vehicle 10% Acacia was delivered to the second group at same duration, route, and dose. Body weight and tumor volume were recorded every 3 days until the study was terminated and animals were euthanized. Tumor tissues were excised and fixed in 10% buffered formalin and embedded in paraffin.

Histology and immunohistochemistry
Paraffin-embedded blocks of all tumors were sectioned at 5 μm. Each sample was stained with hematoxylin and eosin (H&E) for histopathologic analysis. Immunostaining was done using antibodies against cleaved caspase-3 for apoptosis, Ki-67 for cell proliferation, CD31 for angiogenesis, and total MET, phospho-MET, RON-β, phospho-RON with antibodies described in detail in Supplementary Materials and Methods. All samples from both treatment and vehicle groups (n = 10 or 11) were analyzed. In addition to scoring staining intensity, three random fields in each slide were evaluated for mitotic figures and blood vessels (n = 30 or 33). Positively and
negatively stained cell counts were divided by the total number of cells counted to generate the percentage of positive cells in a field: the area of necrosis was determined and excluded from evaluation of other factors.

Orthotopic lung cancer tumor model and imaging using Xenogen IVIS

Six-week-old female severe combined immunodeficient (SCID) mice (Harlan Laboratories) were used to establish orthotopic tumors in lung. Tumors were initiated by intrathoracic injection of 5 x 10^5 A549-luc-C8 cells in 25 μL of PBS into the right lung percutaneously as described in ref. 24. Mice were randomly divided into control and treatment groups (n = 9 per group), observed daily, and body weight measured twice weekly. One week after inoculation, mice were given LY2801653 or 10% Acacia as vehicle control by oral gavage at 20 mg/kg daily. To detect tumor, mice were imaged with Luciferin-D using the Xenogen IVIS imaging system 200 series (Xenogen Corporation) on Days 0, 7, 14, and 21 as described in refs. 24 and 25. Upon termination of study, mice were euthanized and imaged with open chest. Lungs and other organs were formalin fixed and paraffin embedded. Sections were cut at several depths to evaluate presence or absence of tumor in chest cavity. Bioluminescence data collected from IVIS were used to generate graphs comparing relative radiance in each mouse.

Statistical analysis

For cell viability assay, data were expressed as mean ± SEM and analyzed using GraphPad Prism version 5.0d. For PamGene analyses, log intensity values were analyzed using a paired two-tailed Student t test comparing untreated controls with treated. The statistically “significantly modulated” genes were defined as those that had a P value of less than 0.05. This statistical analysis was performed using BioNavigator (PamGene). For the xenograft tumor model, the P value between mean tumor volumes of vehicle and LY2801653 treated was calculated by the two-tailed Student t test using GraphPad Prism.

Results

Robust MET and RON expression in NSCLC cell lines

We first examined the expression of MET and RON in a panel of NSCLC cell lines of various histologies—adenocarcinoma (A549, H522, H1993, H2228, H358), SCC (H520, H1703, H2170, SKMES-1), and large cell carcinoma (H661). Seven of the 10 NSCLC cell lines (except H522, H2170, and H661) coexpressed both MET and RON, with the highest level of MET being expressed by H1993 (Fig. 1A). MET expression was detected at 145 kDa and RON-β was detected at 150 kDa. BaF3 TPR-MET, serving as the positive control, showed high level of MET expression, recognized at 65 kDa.

Inhibitory effect of LY2801653 on growth of NSCLC cell lines

To determine the effect of LY2801653 on cell proliferation, we treated NSCLC cell lines with LY2801653 for 72 hours in a dose range of 1 nmol/L to 10 μmol/L. BaF3 TPR-MET was used as a positive control. Of the 10 NSCLC lines tested, A549, H1703, and H1993 were found to be sensitive to LY2801653 at IC_{50} 627.6, 72.9, and 9.28 nmol/L, respectively (Fig. 1B). Similarly, cells were treated with crizotinib. A549, H1703, and H1993 were found to be sensitive to crizotinib at IC_{50} 2.478, 3.848, and 45.4 nmol/L, respectively (Fig. 1C). BaF3 TPR-MET cells were also found to be sensitive to crizotinib at IC_{50} 40.1 nmol/L. For these cells, the IC_{50} of crizotinib was higher than the IC_{50} of LY2801653 (A549; 3.9-fold IC_{50}, H1703; 53-fold IC_{50}, H1993; 4.9-fold IC_{50} and BaF3 TPR-MET; 3.1-fold IC_{50}). BEAS-2B normal bronchial epithelial cells, which do express low levels of MET (26), did not respond to MET/RON inhibition.

Biochemical effects of inhibition of MET and RON

To determine the biochemical consequences of MET and RON kinase inhibition by LY2801653, we first used BaF3 TPR-MET cells. Changes in tyrosine phosphorylation of cellular proteins were evaluated using phospho-specific antibodies against tyrosine phosphorylation sites in MET. We found that 50 nmol/L LY2801653 inhibited phosphorylation at Tyr361/365/366 auto-phosphorylation sites on TPR-MET (Fig. 2A). To determine whether LY2801653 also inhibited the activation of MET and RON in lung cancer cells, we used H1993 cells. Treatment of H1993 cells with LY2801653 at doses 1, 3, 10, and 30 nmol/L reduced global tyrosine phosphorylation of cellular proteins in a dose-dependent manner, as well as tyrosine phosphorylation of MET at Tyr1230/1234/1235 (homologous to Tyr361/365/366 in TPR-MET) and tyrosine phosphorylation of RON at Tyr1238/1239 (Fig. 2B).

Next, tyrosine phosphorylation of CBL (Tyr674), PI3K p85/p55 (Tyr458/199), and STAT3 (Tyr705) was examined. We have previously studied the relationship between CBL and NSCLC (27, 28), PI3K and MET in SCLC (29), and STAT3 and MET/RON in gastroesophageal adenocarcinoma (16). Because of our interest in these pathways, we picked CBL, phosphoinositide 3-kinase (PI3K), and STAT3 from the PamGene and GeneGo analyses of significant genes associated with H1993 treatment with 3 nmol/L LY2801653. Our data show that treating H1993 cells with LY2801653 reduced the phosphorylation of these proteins in a dose-dependent manner (Fig. 2B). We used Tyr674 for CBL as both Tyr674 and Tyr700 are involved in cell motility. One of the pathways LY2801653 may potentially be exerting its effect is via inhibition of MKN1/2, as reported by Yan and colleagues (30). MKN1/2 activities are measured by the phosphorylation of its substrate eukaryotic initiation factor 4e (eIF4E) at Ser209. We analyzed the phosphorylated eIF4E (ser209) level of A549 and H1993 cells treated with LY2801693 (Fig. 2C). Our data show that there was an incremental loss of p-eIF4E protein expression with increasing dose of the drug in both the cell lines, indicating that the MKN1/2 pathway may be one of the mechanisms by which LY2801653 inhibition works. This provides a potential mechanism of action for LY2801653.

Identification of 13 key peptides whose phosphorylation was modulated by treatment with LY2801653 (3 nmol/L) in H1993 cells

Using the PamGene kinase array platform, we demonstrate that treating H1993 cells with LY2801653 has a significant
effect on the phosphorylation of many peptides (Fig. 3A and B and Supplementary Fig. S1). The tyrosine kinase activity responsible for most peptides was downregulated in a concentration-dependent manner (Fig. 3A).

Analysis shows that 13 substrate sites on 12 genes were significantly modulated by LY2801653 (P < 0.05). The kinases that were inhibited are MET (Tyr1230/1234/1235), calmodulin (CALM; Tyr100), tyrosine-protein kinase SYK (KYSK; Tyr525/526), β-type platelet-derived growth factor receptor precursor (PDGFRB; Tyr771/775/778, Tyr716), RON (Tyr1353), PI3K regulatory subunit α (PI3K85A; Tyr607), erythropoietin receptor precursor (EPOR; Tyr426), linker for activation of T cells family member 1 (LAT; Tyr255), VEGF receptor 1 precursor (VEGFR1; Tyr1327/1333), STAT4 (Tyr693), and E3 ubiquitin-protein ligase CBL (Tyr700; P < 0.05). Interestingly, the T-cell surface glycoprotein CD3 ζ chain precursor (CD3ζ; Tyr153) was the only site, which was found to be significantly upregulated (P < 0.05; Fig. 3B). A network between these 12 modulated genes was created using the "Shortest Paths Algorithm, Build Network" of GeneGo (Fig. 3C).

STAT3 is one of the key transcription factors involved with the genes significantly modulated by LY2801653 in H1993 cells

To determine the key transcription factors involved with genes most significantly affected by MET/RON inhibition with
LY2801653 in the PamGene kinase array, we analyzed statistically significant data generated by fold-change calculations (12 genes for 3 nmol/L; 40 genes for 10 nmol/L) by Pathway Analysis Software GeneGo’s robust and manually curated MetaCore pathway analysis solution. STAT3 and SP1 were found to be the key transcription factors affected by LY2801653 (3 nmol/L) treatment in H1993 (P = 2.15E-43). The top six transcription factors involved in the 12 genes significantly modulated are derived using the query “What are the key transcription factors and target genes in my data? Most Popular Questions” of GeneGo and detailed in Table 1. The table shows that the top six transcription factors were related to 8 to 11 of the total 12 significantly altered genes. For 10 nmol/L treatment, SP1 was the first key transcription factor (P = 9.49E-100) and STAT3 was the second key transcription factor (P = 1.86E-71) affected (data not shown).

Gene-specific dual inhibition of MET/RON
To provide evidence that dual inhibition of MET and RON by LY2801653 may be more beneficial than either MET or RON inhibition alone, we conducted gene-specific inhibition studies. siRNA was used to silence either MET, RON, or both MET and RON.
and RON in A549 cells by transient transfection. Gene silencing was confirmed by immunoblot analysis showing a marked reduction in MET and RON protein expression compared with control siRNA (Fig. 4A). Parental A549 is shown to express high levels of MET and RON, which remain unchanged with control siRNA. siMET eliminated MET protein expression almost completely with no change in RON expression. siRON eliminated RON expression almost completely. With MET/RON dual silencing, both proteins were reduced. Ninety-six hours after transfection, the viability of cells silenced for MET or RON or dual MET and RON was measured and compared against control siRNA. We show that although cell viability was reduced by 60% in either MET- or RON-silenced cells, the dual inhibition resulted in a reduced viability to 22% (Fig. 4B). These results not only show that specific inhibition of MET and RON serve to reduce cell viability in A549 cells but also that dual MET/RON inhibition has a synergistic effect, as depicted via LY2801653 treatment.

LY2801653 inhibits growth of A549 and H1993 tumor xenografts in nude mice

To further investigate the effect of MET/RON inhibition by LY2801653 in tumor proliferation, we next used the mouse flank xenograft model. Two NSCLC cell lines, A549 and H1993, were chosen on the basis of their MET/RON expression profile and in vivo tumorigenicity. Treatment with LY2801653 at 20
mg/kg/d reduced A549 and H1993 tumor growth significantly relative to vehicle control \((P < 0.05; \text{Fig. } 5A \text{ and } B)\) with unchanging body weight. Because of the rapid increase in tumor volume in untreated vehicle control mice, all mice in the vehicle groups had to be terminated on day 14 of the study.

Consistent with the in vitro experiments of GeneGo analyses, mitotic figures (H&E staining) and vessels (CD31 staining) of tumor specimens were significantly decreased in LY2801653-treated mice in both A549 and H1993 studies. Moreover, in the treated arms of H1993, necrosis was increased and cell proliferation (Ki-67) was decreased. In the treated arms of A549, apoptosis (caspase-3 staining) in tumor cells was increased (Fig. 5C). Tumor specimen from the LY2801653- and vehicle-treated mice were immunostained for phosphorylated MET and RON. Phosphorylated MET was decreased modestly in LY2801653-treated mice in both A549 and H1993 studies. Moreover, in the treated arms of H1993, phosphorylated RON was decreased modestly in LY2801653-treated mice (Supplementary Fig. S3).

LY2801653 inhibits growth of A549-luc-C8 lung orthotopic tumor xenografts in SCID mice

To examine the effects of LY2801653 in an orthotopic tumor model, we established orthotopic lung tumors using A549-luc-C8...
C8 cells in the right lung of SCID mice. Tumor progression was monitored weekly by detecting bioluminescence using IVIS imaging as described in Materials and Methods. Treatment with LY2801653 at 20 mg/kg/d reduced A549-luc-C8 tumor growth significantly relative to vehicle control on day 14 (P < 0.05; Fig. 6A and B) with unchanging body weight. Vehicle and treatment groups were euthanized and imaged with open chest and lung (Fig. 6C). Serial sections were cut of each right and left lung and stained with H&E to evaluate presence or absence of tumor in primary lung, contra-lateral lung, and chest cavity. We were able to confirm the presence of tumors in the lung of all these mice (Fig. 6D).

Discussion

In this report, we describe a novel ATP-competitive, potent, and highly selective dual SMI of MET/RON, LY2801653 and its effect on NSCLC. MET/RON are important pathways in NSCLC. In vitro and in vivo, there was considerable cell death with LY2801653 treatment.

Over the past decade, there has been considerable data generated on MET biology and therapy for lung cancer. In lung cancer, MET and phosphorylated MET (especially juxtamembrane domain site pY1003 and autocatalytic site pY1234/1235) are overexpressed in 40% of tumor tissues (31), and are prognostic biomarkers (32). Like MET, RON and phosphorylated RON also are overexpressed in lung cancer (12), but unlike MET, which can be mutated in lung cancer, RON is not mutated. However, RON can have differential transcripts in various tumors (33, 34) and it is not known which transcripts exist in lung cancer. This would be important to determine because oncogenic functionality of epithelial cells in the lung may be dependent on different RON transcripts.

As has been shown, MET can be overexpressed or amplified in tumors resistant to cytotoxic agents such as cisplatin (35) or SMIs such as gefitinib (36–38). Recently, MET resistance was observed using anti-MET antibody (MetMAb; ref. 39). The mechanism by which MET acquires resistance is not known. MET can also synergize with other targets such as EGFR in NSCLC (40). A number of MET inhibitors are being tested with EGFR inhibitors in lung cancers (41–43). However, in the most recent trial using tivantinib with erlotinib versus erlotinib alone (phase III trial; NCT01244191; ref. 44), no difference in overall survival was observed (45). Our data show that MET/RON may be important to cotarget in NSCLC. To better treat tumors, a strategy that uses MET and RON dual inhibition would be more desirable than inhibiting one pathway alone.

We found LY2801653 to be more efficacious than crizotinib in A549 (KRAS G12S), H1703 (PDGFRA amplified), and H1993 (MET amplified) NSCLC cell lines. We also showed that LY2801653 was effective in in vivo models. Because of the focused studies on LY2801653, it would be useful for the future to compare all the SMIs versus antibody inhibition of MET versus MET and RON. Interestingly, LY2801653 may also be effective against tumors via inhibition of MKNK1/2 (30). Thus, for the future, it would be interesting to determine the therapeutic inhibition of MET/RON in patients with lung cancer harboring various mutations. We have determined the sensitivities of the LY2801653 in various NSCLC cell lines harboring different "driver" mutations. Because NSCLC is a heterogeneous group of diseases, it is important to note that not all NSCLCs will respond to the same strategy of inhibition. Although the LY2801653 inhibited A549, H1703, and H1993 cells, not all others tested. It is possible that this is related to the various other abnormalities that exist within these cells. As well, these are in vitro experiments, and more in vivo experiments should be done in the future to determine if tumor–stromal interactions play important roles in the inhibition of the various cells. In the future, it will also be prudent to have isogenic cell lines with the various oncogenic and tumor suppressor abnormalities that exist or coexist in NSCLC.

Kinase assays on PamChip peptide microarrays provide rapid identification of substrates for the kinase under investigation. PamGene assay removes many limitations to assay
development because 144 peptides can be tested simultaneously. Computational pathway analysis (MetaCore; GeneGo) provides detailed knowledge of the highest-ranked common network and helps validate the implicated disease pathways. Using PamGene and MetaCore tools, we found that several downstream signal transduction molecules were affected with MET/RON inhibition, including CBL, PI3K, and STAT3 (Fig. 2B, Fig. 3, Table 1).

CBL is an E3 ubiquitin ligase that regulates RTK activity in a number of different receptors, including MET, EGFR, PDGFR, and VEGFR. In our initial study, we evaluated expression and mutational status of CBL in archival NSCLC tumor tissue collected from African Americans, Caucasians, and East Asians (27). Interestingly, we found that CBL was expressed at relatively low levels and was mutated occasionally. The overall mutation rate of CBL in NSCLC was 8.4% (10 of 119). Preliminary functional studies of these mutants revealed defects in their ability to ubiquitinate MET, and no discernable effects on the ubiquitination of EGFR. Moreover, forced expression of these mutants in an NSCLC cell line enhanced cell viability in vitro (28). From these data and our data, this pathway may play an important role in lung cancer carcinogenesis.

The PI3K pathway is fundamental for cell development, growth, and survival. Its deregulation and contribution to carcinogenesis has been well documented and reviewed in the past, including in carcinomas of the lung. We have reported that the PI3K pathway is crucially targeted via MET stimulation in SCLC (29). Now, this study finds that inhibition of the MET by LY2801653 targets the PI3K pathway. Hence, PI3K also may be an important target in NSCLC.

STAT3, one of the seven members of the STAT family, has been the most strongly implicated member in tumorigenesis...
We have previously shown in a gastric cancer cell line that STAT3 is activated upon RON and/or MET stimulation (16). As shown here in the MetaCore analysis data, STAT3 is the key transcription factor affected by LY2801653 treatment of H1993 cells. Furthermore, tyrosine phosphorylation of STAT3 was also reduced by LY2801653. The HGF/MET signaling pathway is responsible for stimulation of cell motility and invasion (20, 48), and STAT3 is responsible for at least part of the MET signal.

In this study, the antitumor efficacy of LY2801653 was evaluated in tumor models that (i) express MET/RON, (ii)

Figure 6. LY2801653 inhibits growth of orthotopic tumor xenografts in SCID mice. A, quantitative analysis of bioluminescence photon counts as a measure of A549-luc-C8 cell line tumor growth in SCID. Oral gavage treatment with LY2801653 at 20 mg/kg/d reduced A549-luc-C8 tumor growth significantly relative to vehicle control (P < 0.05). B, representative images of the whole body. C, representative fluorescence images of whole body with open chest and lung. D, H&E histologic staining to evaluate presence or absence of tumor in chest cavity. Error bars, SEM. P value between mean radiance of vehicle control and LY2801653 by the two-tailed Student t test; *, P < 0.05.
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We have confirmed the effect of LY2801653 against NSCLC cell lines using both heterotopic and orthotopic human xenograft mouse models. In the heterotopic mouse model, LY2801653 inhibited the proliferation of tumors and decreased mitotic cells and vessels significantly. Modest but not notable differences in the expression of phosphorylated MET and RON was detected in the treated versus untreated tumor tissues, although, we had seen considerable effects on tumor growth. It is unclear if there is a potential feedback loop that is cross-activating the phosphorylation and needs further exploration. In the orthotopic mouse model, LY2801653 also inhibited tumor proliferation.

The tumor microenvironment plays a major role in promoting tumor growth (49). In xenografts models, tumor location is important in relation to sensitivity to chemotherapy (50) and is a critical factor in targeted therapies. Taking these points into consideration, orthotopic models have advantages over heterotopic models. These advantages include use of the relevant site for tumor-host interaction, the ability to study site-specific dependence of therapy, organ-specific expression of genes, and replication of clinical scenarios. For these reasons, we used the heterotopic models with two NSCLC cell lines, and only the luciferase-tagged A549-luc-C8 for the orthotopic modeling. A luciferase-tagged H1993 cell was not obtainable.

Finally, others and we have previously shown gain-of-function mutations of MET in lung cancers (5, 8, 20). There also seems to be a differential response to the MET SMI SU1274 in E160D versus N755S MET-mutant lung cancer cells (8). Although MET and RON synergize in function, in terms of mutation mutations of MET in lung cancers (5, 8, 20). There also seems to be a differential response to the MET SMI SU1274 in E160D versus N755S MET-mutant lung cancer cells (8). Although MET and RON synergize in function, in terms of being investigated in clinical trials (17, 43). LY2801653 is also being investigated in patients with advanced cancer (adenocarcinoma of the colon or rectum, head and neck squamous cell carcinoma, and cholangiocarcinoma; phase I trial D0-MC-JSBA, NCT01285037). Because of its potent efficacy at lower concentration and against multi-targeted kinase especially MET/RON, we believe that LY2801653 has a promising therapeutic potential in treating NSCLC.

In summary, LY2801653 is a novel, orally available multi-targeted kinase inhibitor, which inhibits MET and RON signaling. It has significant in vitro and in vivo antitumor activities against MET- and RON-driven tumors. The results described in this report provide justification for advancing LY2801653 to clinical development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors’ Contributions

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Development of methodology: I. Kawada, R. Hasina, R. Salgia

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Kawada, R. Hasina, E. Smithberger, A.N. Husain, E.E. Vokes, R. Salgia

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Kawada, R. Hasina, E. Smithberger, A.N. Husain, R. Salgia

Writing, review, and/or revision of the manuscript: I. Kawada, R. Hasina, E.E. Vokes, R. Salgia

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): I. Kawada, R. Hasina, E.E. Vokes, R. Salgia


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References


Novel Inhibitor LY2801653 with Effects on MET and RON in NSCLC


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