A novel mTORC1/2 inhibitor (MTI-31) inhibits tumor growth, epithelial-mesenchymal transition, metastases and improves antitumor immunity in preclinical models of lung cancer

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Translational Relevance

Despite recent success in tyrosine kinase inhibitor (TKI) therapy, lung cancer patients often develop resistance and can progress on even the newest generation TKI. The mTOR signaling pathway is vital to the action of various receptor tyrosine kinase (RTK) pathways and contributes to both intrinsic and acquired TKI-resistance. With the newly developed mTORC1/mTORC2 dual inhibitors, it is important to examine their utility in non-small cell lung (NSCLC) tumors with prevalent cancer-driver mutations such as EGFR/T790M, EML4-ALK, amplified c-MET and KRAS. Our results implicate mTOR as a multifaceted regulator of NSCLC tumor growth, metastasis, EMT and immune-escape, which are critical traits directly linked to disease progression and death. The newly characterized mechanism mediated by the rapamycin-resistant mTORC2 warrants clinical investigation of mTORC1/mTORC2 inhibitors for treatment of TKI non-responders and acquired resistance in EGFR/ALK-mutant and PI3K/mTOR-dysregulated lung cancer patients.
Abstract

**Purpose:** We aimed to investigate efficacy and mechanism of MTI-31 (LXI-15029), a novel mTORC1/mTORC2 inhibitor currently in human trial (NCT03125746), in non-small cell lung cancer (NSCLC) models of multiple driver mutations and tyrosine kinase inhibitor (TKI)-resistance.

**Experimental design:** Gene depletion, inhibitor treatment, immunological, flow cytometry, cellular and animal studies were performed to determine in vitro and in vivo efficacy in NSCLC models of driver mutations and elucidate roles by mTOR-complexes in regulating migration, epithelial-mesenchymal transition (EMT), metastasis, intracranial tumor growth and immune-escape.

**Results:** MTI-31 potently inhibited cell proliferation ($IC_{50} < 1 \mu mol/L$) and in vivo tumor growth in multiple NSCLC models of EGFR/T790M, EML4-ALK, c-Met or KRAS (MED <10 mg/kg). In EGFR-mutant and/or EML4-ALK-driven NSCLC, MTI-31 or disruption of mTORC2 reduced cell migration, hematogenous metastasis to the lung, and abrogated morphological and functional traits of EMT. Disruption of mTORC2 inhibited EGFR/T790M-positive tumor growth in mouse brain and prolonged animal survival correlating a diminished tumor angiogenesis and recruitment of IBA1+ microglia/macrophages in tumor microenvironment. MTI-31 also suppressed programmed death ligand 1 (PD-L1) in EGFR- and ALK-driven NSCLC, mediated in part by mTORC2/AKT/GSK3β-dependent proteasomal degradation. Depletion of mTOR protein or disruption of mTOR-complexes profoundly downregulated PD-L1 and alleviated apoptosis in Jurkat T and primary human T cells in a tumor-T cell co-culture system.

**Conclusions:** Our results highlight mTOR as a multifaceted regulator of tumor growth, metastasis and immune-escape in EGFR/ALK-mutant and TKI-resistant NSCLC cells. The newly characterized mechanisms mediated by the rapamycin-resistant mTORC2 warrant clinical investigation of mTORC1/mTORC2 inhibitors in lung cancer patients.
Introduction

Despite significant progress in diagnosis and clinical management, lung cancer remains the leading cause of cancer-related deaths worldwide (1). Common driver mutations in Non-small cell lung cancer (NSCLC) are more prevalent in lung adenocarcinoma, in which receptor tyrosine kinases (RTKs) including EGFR, EML4-ALK and c-Met are well-known driver mutations. These mutations promote tumor growth and metastasis by activating downstream pathways (2). Approximately 15% of U.S. and 40% of Asian NSCLC cases harbor mutations in the EGFR kinase domain (3). Although majority of such patients initially benefited from treatment with EGFR tyrosine kinase inhibitor (EGFR-TKI) such as gefitinib and erlotinib (4), there is a significant heterogeneity in the clinical course of patients with EGFR-mutant lung cancers. Acquisition of EGFR-T790M is the most frequently identified resistance mechanism, and is detected in tumor cells from more than 50% of patients after disease progression (5). Despite a new generation of EGFR-TKIs are approved to treat patients with EGFR-T790M, cancer cells can still develop resistance (6, 7). Furthermore, greater than 50% of NSCLC cases are diagnosed at an advanced stage with up to 50% of patients developing brain metastases throughout their disease course (8), which presents another challenge to successful treatment.

The mechanistic target of rapamycin (mTOR) is a serine/threonine kinase related to the lipid kinases of the phosphoinositide 3-kinase (PI3K) family. The PI3K/AKT/mTOR pathway is one of the most important signaling network downstream of the oncogenic RTKs. Evidence suggests that mTOR contributes to the resistance of EGFR-TKIs in lung cancer cells (9, 10). In EGFR-mutant NSCLC that have subsequently acquired T790M mutation, mTOR signaling biomarkers are activated (11). A recent report indicates that mTOR E2419K mutation may be a novel resistance mechanism to EGFR-TKI therapy (12).
mTOR acts through two multiprotein complexes, mTORC1 and mTORC2. While the classical mTORC1 promotes cell growth via control of anabolic macromolecule synthesis, the more recently identified mTORC2 phosphorylates the kinase AKT and regulates cytoskeleton network with important implications in cancer survival, metabolism and tumor metastasis (13-16). Although the clinical use of the mTORC1-selective rapalog therapy (e.g. temsirolimus, everolimus) validated mTOR as a cancer target, the importance of mTORC2 in cancer therapy remains elusive. Epithelial-mesenchymal transition (EMT) is known to be important in tumor metastasis and resistance to therapy (17, 18) and may involve mTORC2 (19-21). Additionally, overexpression of the mTORC2 component Rictor was observed in 66% lung adenocarcinoma brain metastases (22) and Rictor overexpression was sufficient to induce glioma formation in mice (23). Thus, targeting of mTORC1 and mTORC2 with mTOR kinase inhibitor (mTOR-KI) might be a promising treatment option for RTK-driven advanced NSCLC, especially those EGFR-TKI-resistant tumors with brain metastasis potential. To date, several mTOR-KIs have entered patient trials with clinical results eagerly awaited (24-26).

MTI-31 (LXI-15029) is a latest novel and selective mTOR-KI currently in first-in-human cancer trial (NCT03125746). It has demonstrated single-agent oral antitumor efficacy in preclinical models of HER2+/PIK3CAmut breast cancer, PTEN/VHL-null renal cancer and others (27). Herein, we employed MTI-31, AZD8055 and rapamycin, as well as genetic disruption of mTOR complexes to study mTOR-targeted therapeutic effects in preclinical models of NSCLC. We found that MTI-31 inhibited in vivo growth of multiple NSCLC tumors with diverse RTK and KRAS cancer drivers. In EGFR-T790M positive NSCLC cells, treatment with mTOR-KI or depletion of mTORC2 reduced cellular EMT, lung metastasis and tumor growth in the brain. Targeting of mTOR pathway may also improve antitumor immunity.
Materials and Methods

Chemicals and shRNA

MTI-31 (27) and CM-118 (28) were synthesized as described. Gefitinib, erlotinib, afatinib, crizotinib, rapamycin, AZD8055, AZD9291 and docetaxel (all from BiochemPartner, Shanghai), PS341 (Med Chem Express) and recombinant human TGF-β (Peprotech) were purchased. All other chemicals were purchased from Sigma-Aldrich unless otherwise specified. Inhibitors were dissolved in DMSO as 20 mmol/L stock solution and were diluted before assays. pGIPZ- and/or pTRIPZ (inducible with doxycycline)-based lentiviral shRNA for human Raptor ShRNA#2 (V3LHS_329849), Rictor ShRNA#4 (V2THS_225915), mTOR ShRNA#2 (V3THS_312663) and non-targeting (NT, RHS4346) were obtained from Open Biosystems/GE Dhharmacon.

Cell culture, gene transfection, gene knockdown, growth assays and BV2 microglia conditioned media

Cell lines of NCI-H1975, NCI-H1993, NCI-H2228 and A549 were obtained from American Type Culture Collection (ATCC). HCC827, H1299, Calu-1 and Jurkat cells were obtained from the Cell Bank of Chinese Academy of Sciences (CAS, Shanghai). PC9 was obtained from European Collection of Cell Cultures (ECACC). BV2 is a gift from Professor Zhongming Qian (Fudan University School of Pharmacy). Cells were cultured using standard cell culture methods and reagents (Invitrogen). All the cells were periodically tested for mycoplasma contamination free. Luciferase-tagged H1975 cells were selected by G418 for stable expression population. Various pGIPZ/pTRIPZ-shRNA viruses were packaged in 293T before
infecting tumor cells. Gene-depletion via pTRIPZ was induced with 1 μg/mL doxycycline (Dox) for 5-7 days. For proliferation assay, cells were seeded in 96-well plate (5-10% confluence), incubated for 3-5 days before measurement using MTS reagent (Promega). The viability and growth of pGIPZ-ShRNA-expressing cells were determined by counting puromycin-resistant GFP-positive cells. For BV2-derived conditioned medium, cells were cultured in DMEM with 1% FBS for 24 or 48 h and the supernatants were collected for use as BV2-CM.

**Cell lysates and immunoblotting**

For standard assay of inhibitor effects, cells plated at pre-determined density in 12-well culture plates were treated with inhibitors for the indicated doses and times, lysed in NuPAGE-LDS lysis buffer (Invitrogen) and immunoblotted with various antibodies including P-EGFR(Y1086), P-Met(Y1234/5), P-ALK(Y1604), P-S6K(T389), S6, Cyclin D1, Raptor, Rictor, EGFR, P-GSK3β(S9), E-cadherin (Cell signaling technology); P-S6(S235/6), P-AKT(S473), AKT, mTOR, Vimentin (Abcam-Epitomics); PD-L1 (Sino biological); Fibronectin (Santa cruz); Actin and GAPDH (Bioworld).

**Cell migration and experimental metastasis**

H1975 (10×10^4), PC9 (8×10^4), H2228 (20×10^4) and A549 (8×10^4) cells in 200 μL serum-free medium were added to the upper chamber of a Transwell system (Corning, Cat#3422) without or with inhibitors and allowed to migrate for 8-16 h toward 10% serum medium in the lower chamber. The migrated cells were stained with crystal violet and counted under a microscope in 6 representative viewing fields at 200x magnification. For lung metastasis, the indicated luciferase-tagged H1975 cells (1.5×10^6 cells in 200 μL PBS) were injected into the
lateral tail vein of mice. 4 h later, mice were given D-luciferin potassium salt (150 mg/kg) intraperitoneally and imaged 6 min later for 1 min exposure in an IVIS Spectrum imaging system.

**Detection of cell surface PD-L1 or PD-1**

H1975 and H2228 cells grown on a chamber slide were washed with PBS, fixed with 4% paraformaldehyde and blocked with 1% BSA. The slides were incubated with anti-PD-L1 (CST, Cat#13684) overnight, subsequently rinsed and detected with Alexa Fluor 647- or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (Invitrogen, Cat#A32733 or Jackson, Cat#711-545-152, respectively). The images were acquired using a confocal microscope (Zeiss, LSM710).

Jurkat T cells and CD3+ primary human T cells sorted from human peripheral blood mononuclear cells (PBMCs) (obtained from Xidier Biotech, Jiangsu) using T cell isolation kit (Stemcell, Cat#17951) were activated with pre-immobilized anti-CD3 (1 μg/mL, 0.2 mL/well) plus soluble anti-CD28 (500 ng/mL) (eBioscience, Cat#16-0037, Cat#16-0289) for 24 h or 48 h. The cells were stained with 10 μg/mL nivolumab (Selleck, Cat#A2002) or hIgG control for 60 min followed by secondary rPE-Goat anti-human IgG-Fc (Abcam, Cat#ab98596) for 30 min. The stained cells were detected in a CytoFlex S Flow Cytometer (Beckman Coulter) and analyzed using FlowJo 7.6.1 software.

**Apoptosis assay of Jurkat T and CD3+ primary human T cells**

The indicated H1975 and A549 cells (8×10^4/well) were seeded in 12-well culture plates overnight, then co-cultured with the pre-activated Jurkat T or CD3+ primary human T cells at the T cell to attached tumor cell ratio of 1:2 or 1:1, respectively. After 72 h (Jurkat) or 48 h (T) were harvested from the co-culture system and analyzed using the Annexin V-FITC/PI detection kit.
(KeyGEN Biotech, Cat#KGA108) in a CytoFlex S Flow Cytometer. The CD3+ and Annexin V-FITC/PI-positive cells were gated as apoptotic T cells.

**In vivo tumor growth inhibition**

Animal studies were performed under protocols approved by Institutional Animal Care and Use Committee (IACUC) of Fudan University. Balbc female nude mice bearing H1975, H1993, H2228 or A549 tumors were staged at initial tumor volume of 150-200 mm\(^3\) and randomized into treatment groups (n=8). MTI-31 (salt-form) was formulated in citrate buffer (pH 4.5). Erlotinib and crizotinib were formulated in 0.5% hydroxypropyl methyl cellulose (HPMC)-0.2% tween-80 as homogeneous suspension. All test agents were prepared twice weekly. Mice were dosed orally once daily (qd), except for docetaxel once weekly (qw). Tumor growth was calculated using the formula \(V=\frac{LW^2}{2}\) (where \(V=volume, L=length\) and \(W=width\)).

**Intracranial tumor model and immunohistochemistry (IHC)**

Balbc female nude mice (6-8 weeks old) were anesthetized and fixed in a stereotactic frame. H1975-luc \((5\times10^5\) cells\) was injected into the right striatum using a 5 \(\mu\)L microsyringe to deliver tumor cells to a 3-mm intraparenchymal depth. Tumor growth was monitored using an IVIS Spectrum imaging system. For IHC, tumor slides were deparaffinized, rehydrated and permeablized with 1% Triton X-100. Antigens were retrieved with Tris/EDTA (pH 9.0) under microwave heating for 20 min, then blocked in TBS with 5% BSA-0.3 M glycine and probed with anti-Rictor (CST, Cat#2114), P-AKT(S473) (Abcam, Cat#81283), CD31 (Servicebio, Cat#GB13036) or IBA1 (Servicebio, Cat#GB13105) and detected with peroxidase-conjugated
secondary antibody (Jackson, Cat#111-035-003). Images were acquired using Leica microscope (model DMI4000D), analyzed by Image pro plus 6.0 software.

**Statistical analysis**

Numerical data processing and statistical analysis were performed with Microsoft Excel and GraphPad Prism 5 software; values were expressed as mean±SE. P values were calculated using unpaired two-tailed Student-t test.

**Results**

**Lung cancer cells harboring diverse cancer-driver mutations are sensitive to mTOR inhibition**

We first assessed anti-proliferative activity of MTI-31 in a panel of 6 NSCLC cell lines, which included representative lines with oncogenic EGFR (HCC827, PC9 and H1975), c-Met (H1993), EML4-ALK (H2228) or KRAS (A549). While as expected, the EGFR-KI gefitinib potently inhibited proliferation in HCC827 and PC9 cells, it was poorly effective against the EGFR-T790M positive line H1975, c-Met-driven H1993, EML4-ALK-driven H2228 or KRAS-mutant A549 (Fig. 1A). Interestingly, MTI-31 inhibited proliferation of all 6 NSCLC cell lines with IC$_{50}$ values 0.247±0.03, 0.31±0.004, 0.477±0.073, 0.424±0.041, 0.505±0.032, 0.707±0.024 μmol/L, respectively (Fig. 1A). Importantly, treatment of H1975 cells with a second-generation EGFR inhibitor afatinib or treatment of H1993 and H2228 cells with a c-Met/ALK dual inhibitor CM-118 (28) each reduced P-S6(S235/6) and P-AKT(S473) indicating an involvement of mTORC1 and mTORC2 in EGFR/c-Met/ALK-targeted therapy (Fig. 1B). Consistently, when applied as a single agent, both mTOR-KIs MTI-31 and AZD8055 elicited a stronger inhibition of
cyclin D1 compared with that of the mTORC1 inhibitor rapamycin correlating well with mTOR-KI’s deeper suppression of mTOR signaling function (Fig. 1C). To clarify the relative contribution of mTOR complexes in cell growth, we transfected ShRNA for depleting Raptor (disrupting mTORC1) or Rictor (disrupting mTORC2) in H1975, H1993 and H2228 cells (Fig. 1D), which resulted in a significant growth retardation in both settings in all three cell lines (Fig. 1E). Collectively these results demonstrated that NSCLC cells with diverse oncogenic-RTK backgrounds are susceptible to mTOR-targeted growth suppression.

**MTI-31 inhibits in vivo tumor growth in oncogenic-EGFR, EML4-ALK, c-Met and KRAS lung cancer models**

Because the EGFR-mutant H1975 cells possess a secondary EGFR-T790M mutation rendering resistance to gefitinib and erlotinib, we conducted an efficacy study with MTI-31 in this model. As expected, the tumors did not respond to 60 mg/kg erlotinib but were inhibited by a second-generation EGFR-KI afatinib. Daily treatment with 10, 20 and 40 mg/kg of MTI-31 produced a dose-dependent tumor inhibition with comparable efficacy by 20 mg/kg MTI-31 or 20 mg/kg afatinib (Fig. 2A). MTI-31 was also efficacious in the c-Met-driven H1993 model (Fig. 2B) and the EML4-ALK-driven H2228 model (Fig. 2C), where 20 mg/kg MTI-31 or 20 mg/kg crizotinib (c-Met/ALK dual inhibitor) achieved comparable efficacy (Fig. 2B, 2C). Additionally, MTI-31 inhibited growth of KRAS-mutant A549 tumors, in which 20 mg/kg MTI-31 was similarly effective as the clinical regimen of docetaxel (Fig. 2D). Taken together, orally administrated MTI-31 strongly inhibited mTOR biomarker in vivo (Fig. S1) and demonstrated a dose proportional antitumor efficacy in NSCLC tumors with diverse cancer-driver mutations.
**MTI-31 mediates suppression of cell migration and experimental metastasis**

Lung adenocarcinoma is well known for high incidence of metastasis and death. We examined the effect of MTI-31 and mTORC2-disruption in tumor cell migration and hematogenous metastasis. Treatment with 5 or 1 μmol/L MTI-31 resulted in a dose-dependent inhibition of cell migration in the EGFR-mutant H1975 and PC9, ALK-mutant H2228 and KRAS-mutant A549 cells (Fig. 3A) but not in the unrelated H1299 cells (Fig. S2). Rapamycin had little effect in cell migration, implying a more prominent involvement for mTORC2 in this setting (Fig. 3A). Indeed, disruption of mTORC2 but not mTORC1 in H1975 or PC9 cells resulted in a profound inhibition of cell migration (Fig. 3B). Next, pretreatment of H1975 cells with 5 μmol/L MTI-31 significantly reduced lung colonization in mice as measured by bioluminescence imaging, while similar pretreatment with rapamycin had little effect (Fig. 3C, 3D). Additionally, mTORC2-disrupted H1975 cells also showed a significant reduction in lung colonization compared to the control cells (Fig. 3E, 3F). These results strongly indicate that mTORC2 plays an important role in tumor cell early metastasis. Blockade of mTORC1 and mTORC2 function by mTOR-KIs can attenuate this process.

**Antagonism in TGF-β-induced EMT and drug resistance**

Comparative analysis of 230 lung adenocarcinoma dataset from The Cancer Genome Atlas (TCGA) revealed a higher mRNA expression of TGF-β1 in the EGFR-mutant tumors (n=40) compared to that of EGFR-wild type tumors (n=190) (Fig. 4A). We then investigated the role of mTOR in TGF-β-induced EMT. After incubation with TGF-β for 3 days, H1975 and Calu-1 cells both converted to a more mesenchymal-like morphology. Interestingly, only the conversion in H1975 cells was blocked by MTI-31 or AZD8055, indicating a specific involvement of mTOR
in these cells (Fig. 4B). In H1975, H2228 and A549 cells, the TGF-β-induced fibronectin (FN1) and vimentin (VIM) as well as the decrease in E-cadherin (E-cad) correlated well with the mTORC2 biomarker P-AKT and were completely blocked by MTI-31/AZD8055 and partially attenuated by rapamycin (Fig. 4C). However, the biomarker inhibitions were not observed in Calu-1 cells (Fig. S3), which may explain the lack of morphological reversion (Fig. 4B). To further clarify the role of mTOR complexes in EMT response, we conducted similar TGF-β treatments using H1975 stable clones expressing the doxycycline (Dox)-inducible Raptor-ShRNA or Rictor-ShRNA. The Dox-induced disruption of mTORC1 or mTORC2 each blocked TGF-β-stimulated upregulation of FN1 (Fig. 4D). Moreover, disruption of mTORC2 enhanced basal level of E-cadherin and partially attenuated its loss after TGF-β treatment (Fig. 4D). We then examined effects of mTOR-inhibition in TGF-β-induced cell migration and chemotherapy resistance. In H1975 and H2228 cells, TGF-β promoted cell migration, which was consistently blocked by MTI-31 while the inhibition by rapamycin was variable (Fig. 4E). Disruption of mTORC2 also reduced both the basal level and TGF-β-induced cell migration (Fig. 4F). In the presence of TGF-β, H1975 cells were more resistant to chemotherapy docetaxel (DTX) but a low dose MTI-31 (0.5 μmol/L) effectively restored DTX sensitivity in TGF-β-treated cells (Fig. 4G, 4H). Taken together, these results suggest that MTI-31 can effectively antagonize TGF-β-mediated invasive phenotype and DTX resistance in H1975 cells.

**Disruption of mTORC2 inhibits EGFR/T790M-positive H1975 tumor growth in mouse brain**

The rate of brain metastasis (BM) is higher in EGFR-mutant tumors (29) including those with the secondary T790M mutation (30). We therefore examined role of mTORC2 in
intracranial growth of the T790M-positive H1975 cells. To do this, luciferase-tagged H1975 stable clones expressing an inducible non-targeting (NT)- or Rictor ShRNA were implanted in mouse brain parenchyma. Tumor growth was monitored with a bioluminescence imaging system. Depletion of Rictor resulted in a significant reduction in overall tumor burden (Fig. 5A) and a prolonged animal survival (Fig. 5B). Median survival for the Sh-NT group was 25.5 days compared to the 50.5 days for the Sh-Ric group (p<0.001) (Fig. 5B). Immunohistochemistry (IHC) analysis confirmed depletion of Rictor and reduced expression of the mTORC2 substrate P-AKT (S473) in Sh-Ric tumors (Fig. 5C). We then analyzed blood vessel distribution by CD31 staining. Depletion of Rictor reduced level of CD31 positive staining (p<0.01) implicating a compromised tumor angiogenesis (Fig. 5D). Because tumor-associated microglia/macrophages could promote growth of brain tumor or metastasis (31), we examined tumor recruitment of microglia cells by IBA1 staining of tumor tissue sections. There were significantly less amounts of IBA1+ microglia cells in the Rictor-depleted tumors compared to that of control tumors (p<0.01) (Fig. 5E) suggesting an altered tumor-microglia interaction in mTORC2-disrupted tumors. We next employed BV2 microglial cells as an in vitro model to mimic tumor-microglia interaction in brain microenvironment (31). BV2-derived conditioned medium (BV2-CM) markedly activated P-AKT and P-EGFR in the H1975-Sh-NT cells and this action was abrogated in the Rictor-depleted cells (Fig. 5F). Treatment with MTI-31 also blocked the BV2-CM-stimulated activation of AKT while rapamycin had negligible effect (Fig. 5G). Although a direct inhibition of H1975 brain xenograft tumor growth was not attainable with MTI-31 due to its insufficient brain penetration (Fig. S4), these results overall highlight a requirement for mTORC2 in H1975 tumor growth in the brain, which involves angiogenesis and tumor-microglia interaction in tumor microenvironment.
MTI-31 induces proteasomal degradation of PD-L1 in EGFR/ALK-mutant NSCLC cells

Dysregulation of the programmed death ligand-1 (PD-L1) plays an important role in immune escape of numerous cancer types (32, 33). In EGFR-mutant H1975 and ALK-mutant H2228 cells, treatment with MTI-31, rapamycin or AZD9291/PF02341066 each reduced cell surface PD-L1 as measured by immunofluorescence (Fig. 6A). In A549 cells, MTI-31/AZD8055 or rapamycin blocked the EGF-stimulated PD-L1 (Fig. 6B). Treatment of EGFR-mutant H1975, PC9 or ALK-mutant H2228 cells for 24 h caused nearly complete (MTI-31) or partial (rapamycin) suppression of the steady state total PD-L1 protein levels (Fig. 6C), which was not due to a reduction in PD-L1 mRNA (Fig. S5). Next, H1975 cells were tested for MTI-31 without or with pre-incubation with a proteasome inhibitor PS-341 or a lysosome acidification inhibitor chloroquine (CQ). Immunoblotting demonstrated that while CQ had little effect, PS-341 significantly rescued the MTI-31-induced loss of PD-L1 thus implicating proteasome degradation in this process (Fig. 6D). Because MTI-31 targets mTORC2 leading to activation of GSK3β, a known inducer of proteasome pathway (34), we evaluated whether the GSK3β inhibitor LiCl could overcome PD-L1 degradation induced by MTI-31. Pretreatment of H1975 cells with LiCl (20 mmol/L) largely blocked the MTI-31-invoked degradation of PD-L1 (Fig. 6E). Collectively, these results suggest that MTI-31 induces proteasomal degradation of PD-L1 in part through mTORC2/AKT-mediated activation of GSK3β.

Disruption of mTOR-complexes profoundly downregulate PD-L1 and alleviate apoptosis in Jurkat T and primary T cells
Given our results and previous report implicating mTOR in regulating PD-L1, we assessed PD-L1 levels in cells disrupted for mTOR complexes. Steady-state level of PD-L1 protein was reduced in mTORC1- or mTORC2-disrupted H1975 (Fig. 7A) and PC9 cells (Fig. S6). To examine whether the reduced mTOR signaling in these cells would alleviate T cell apoptosis, we pre-activated Jurkat T cells with CD3/CD28 antibodies (35, 36) to induce surface PD-1 (Fig. 7B). The activated Jurkat cells were co-cultured with the parent H1975 (high PD-L1) or A549 (low PD-L1) cells for 3 days (Fig. 7C). There was an increased Jurkat cell apoptosis when co-cultured with H1975 compared to that with A549 or Jurkat alone, and this increase was blocked by an anti-PD-1 nivolumab (Fig. 7D). We then co-cultured Jurkat cells with mTORC1- or mTORC2-disrupted H1975 cells and observed a significant protection against apoptosis in both groups (Fig. 7E). Results pooled from three independent experiments are shown (Fig. 7F). To extend these results, CD3+ primary human T cells were re-stimulated with CD3/CD28 antibodies (PD-1+ staining 10-26%) and subjected to a similar co-culture system (Fig. 7G, 7H). There were significant rescues of T cell apoptosis in both mTORC1- and mTORC2-disrupted and in nivolumab-treated H1975 co-cultures (Fig. 7I). Results pooled from three independent experiments are shown (Fig. 7J). Collectively, these results suggest that mTOR-targeted inhibition of PD-L1 may functionally improve antitumor immune response.

Discussion

MTI-31 demonstrated efficacy in several NSCLC models representing oncogenic EGFR/T790M, EML4-ALK, c-Met or KRAS both in vitro and in vivo. While these results confirmed a critical role for mTOR in three distinct RTK-driven lung tumor cells, they also showed mTOR-dependence in KRAS-mutated A549 tumors. Interestingly, A549 contains not only the mutated-KRAS but also deletion of LKB1, a tumor suppressor and negative regulator of
mTOR (37). LKB1-inactivation occurs in ~30% of KRAS-mutated NSCLC (38) and warrants expanded studies to explore mTOR-targeted efficacy in preclinical models of this NSCLC subpopulation.

EMT plays an increasingly recognized role in cancer progression and resistance to therapy (17, 18). We found that the TGF-β-induced EMT in EGFR/ALK-mutant and KRAS-mutant/LKB1-deficient cells requires both mTORC1 and mTORC2, indicated by the activation of P-AKT and P-S6 and by the blockade of mesenchymal morphology and EMT signature proteins upon MTI-31/AZD8055 treatment. Disruption of mTORC1 and especially mTORC2 interfered with TGF-β-induced EMT. The functional involvement of mTOR in EMT appears to be more prominent in subsets of NSCLC cells with EGFR/ALK mutations and/or dysregulated mTOR signaling. Tumor metastasis and chemoresistance are the two foremost challenges in cancer treatment. MTI-31 treatment and/or mTORC2-disruption strongly inhibited cell migration in H1975, H2228 and A549 cells, effectively restored docetaxel sensitivity in the presence of TGF-β and attenuated lung invasion in mice. Similar treatment with rapamycin or mTORC1-disruption exerted weaker and more variable inhibition. These results highlighted a critical role for mTORC2 in regulating cellular invasive properties, drug resistance and metastasis.

Disruption of mTORC2 inhibited the TKI-resistant H1975 tumor growth in the mouse brain leading to prolonged mice survival. To our knowledge, these results suggest for the first time that mTORC2 is involved in the EGFR-mutant NSCLC tumor growth in the brain. We observed less amounts of CD31+ and IBA1+ staining in the Sh-Ric brain xenografts compared to that of Sh-NT xenografts, suggesting that mTORC2 plays a role in tumor angiogenesis and recruitment of microglia in the brain environment. The role of mTORC2 was further supported by the BV2 microglia in vitro model where the BV2-CM can directly promote P-EGFR and P-
AKT via a tumor cell mTORC2-dependent manner. While these observations are consistent with the recent reports that microglia cells could enhance outgrowth of brain metastatic breast tumor cells (31) and glioblastoma cells (39), future studies are required to elucidate the mechanistic details on mTORC2 mechanism of action.

Tumor cell PD-L1, through binding to its receptor PD-1 on T lymphocytes, plays an important role in immune escape in certain cancer types (40, 41). Several PD-1/PD-L1 blocking antibodies are recently approved antitumor agents (42). In another approach, we speculated that downregulation of PD-L1 in tumor cells may also improve antitumor immunity. Given the critical role of mTOR in RTK signaling in NSCLC, we focused on whether and how mTOR controls PD-L1 expression. mTOR-KIs MTI-31/AZD8055 significantly inhibited PD-L1 expression in the EGFR/ALK-mutant NSCLC. We further showed that the mTOR-targeted loss of PD-L1 was mediated through the suppression of mTORC1 and especially mTORC2 activity, where the diminished mTORC2-AKT induces proteasomal degradation of PD-L1 in part through the activating dephosphorylation of GSK3β. Previously, PD-L1 was shown to be upregulated by the loss of tumor suppressor PTEN in glioma (32), mutant-EGFR (33, 43) and EML4-ALK (44) in NSCLC cells. It thus appears that mTOR pathway can mediate PD-L1 expression in diverse cancer settings.

mTOR represents a multifaceted regulator of immune responses. In solid organ transplantation, rapalogs promote Tregs induction and create an immunosuppressive environment required to prevent from graft rejection (45, 46). Interestingly, it has been recently reported that organ transplant recipients treated with rapalogs have a lower risk of developing cancer, suggesting an impact of mTOR inhibition on antitumor immune responses (47). Notably, recent clinical studies have associated rapalogs with active antitumor immune responses via
promoting CD8+ memory T cells (48, 49). However, the impact of mTOR inhibition in the context of particular tumor environment remains unknown. Considering the immune-suppressive role of PD-1/PD-L1 and the fact that mTOR-KI exerts a stronger inhibition of PD-L1 in tumor cells, one may envision a further improved antitumor immune response by mTOR-KI therapy. In the current study, we found that depletion of tumor cell mTORC1 or mTORC2 each elicited a significantly decreased apoptosis in Jurkat T cell or primary human T cells in a tumor /T cell co-culture system. The efficacy is comparable with that by the anti-PD-1 nivolumab treatment. Although our result is preliminary, it supports the idea that mTOR-targeted inhibition of PD-L1 may functionally improve the antitumor immune response and might be a novel mechanistic aspect relevant for therapeutic response.

In summary, our studies have established MTI-31 as an antitumor agent in preclinical lung cancer models harboring diverse cancer-driver mutations and explored several new mechanistic aspects relevant for therapeutic response. Given that mTORC2 plays an important role in metastasis, EMT and microenvironment, and mTORC2 is also activated via genetic mutations in the mTORC2-components Rictor or Sin1 in lung cancer patients (50), the new generation mTORC1/mTORC2 inhibitors might be a novel strategy for treating lung cancer.

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Authors' Contributions
Conception and design: Q. Zhang, K. Yu
Development of methodology: Q. Zhang, Y. Zhang
Acquisition of data: Q. Zhang, Y. Zhang, Y. Chen, J. Qian, X. Zhang
Analysis and interpretation of data: Q. Zhang, Y. Zhang, K. Yu
Writing, review, and/or revision of the manuscript: Q. Zhang, K. Yu
Administrative, technical, or material support: Q. Zhang, K. Yu
Study supervision: Q. Zhang, K. Yu

Reference


**Figure legends**

**Figure 1.** Lung cancer cells with diverse cancer-driver mutations are sensitive to mTOR inhibition. **A,** The indicated cell lines were treated with various doses of MTI-31 and Gefitinib for 3 days, IC\textsubscript{50} are plotted. **B,** Cells were treated with 3, 1 μmol/L afatinib or CM118 for 6 h and immunoblotted. **C,** Cells were treated as indicated for 48 h then immunoblotted. **D** and **E,** The indicated cell pools expressing pGIPZ-ShRNA were cultured and analyzed by immunoblotting (D) or growth measurement via cell counting (E). ****, P<0.001.
Figure 2. MTI-31 inhibits in vivo tumor growth in oncogenic-EGFR, EML4-ALK, c-Met and KRAS lung cancer models. A-D, Female nude mice bearing tumors of H1975 (A), H1993 (B), H2228 (C) or A549 (D) were treated orally with MTI-31, erlotinib or crizotinib via a once daily (qd) regimen, or docetaxel once weekly (qw). Tumor growth curves are shown. **, P<0.01; ***, P<0.001.

Figure 3. MTI-31 inhibits cell migration and experimental metastasis. A and B, H1975, PC9, H2228 and A549 cells were assayed for migration (8-16 h) with inhibitor treatment (A) or mTOR-complex disruption (B). Migrated cells were quantified and plotted. C and D, Luciferase-tagged H1975 cells were pretreated with inhibitors for 24 h and injected into the tail vein of nude mice (n=5). Bioluminescence was measured 4 h later (C) and results quantified based on total photon flux are plotted (D). E and F, H1975-luc cells as in B were injected into the tail vein of Balb/c nude mice (n=5). Bioluminescence was similarly measured and analyzed. **, P<0.01; ***, P<0.001.

Figure 4. mTOR inhibition blocks TGF-β-induced EMT and drug resistance. A, RNAseq data of TGF-β1 mRNA levels in EGFRmut versus EGFRwt lung adenocarcinoma were plotted (TCGA datasets from www.cbioportal.org, accessed 5/2018). B and C, H1975, H2228, A549 and Calu-1 cells were treated with TGF-β1 (10 ng/mL) without or with inhibitors for 72 h. Cells were imaged for morphology at 100x magnification (B) or immunoblotted (C). D, ShRNA-expressing H1975 cells were treated with TGF-β1 for 72 h, then immunoblotted. E, H1975 and H2228 cells were pre-induced with TGF-β1 for 72 h followed by cell migration (8-16 h) with or without
inhibitors. F, Cells as in D were assayed for cell migration. G and H, H1975 cells as in E were treated with docetaxel (DTX), MTI-31 or combination, without or with TGF-β1 for 3 days. Cell viabilities were analyzed and plotted. *, P<0.05; ***, P<0.001.

**Figure 5.** Disruption of mTORC2 inhibits H1975 tumor growth in the mouse brain. A, H1975-luc cells expressing pTRIPZ-Sh-NT or Sh-Ric were pre-induced with Dox for 5 days and injected intracranially to the bregma and maintained with 2 mg/mL Dox in drinking water. Bioluminescence was measured and results quantified based on total photon flux are plotted. B, Kaplan-Meier survival curves from Sh-NT group (n=8) and Sh-Ric group (n=6). This experiment was performed twice with same results. C, Tumor sections were analyzed by IHC using anti-Rictor and anti-P-AK (200x magnification). D and E, Tumor sections were analyzed by IHC with anti-CD31 (D, 200x magnification) and IBA1 (E, 100x magnification then enlarged to 400x). Five view fields per tumor were assessed for quantification. Representative views (left) and quantification plot (right) are shown. F, Cells as in A were treated for 24 h with BV2-conditioned medium (BV2-CM; 0, 24, 48 h after BV2-cell incubation), then immunoblotted. G, H1975 cells were similarly treated with BV2-CM without or with inhibitors, then immunoblotted. *, P<0.05; **, P<0.01.

**Figure 6.** mTOR inhibitors downregulate PD-L1 expression in EGFR/ALK-mutant NSCLC cells. A, H1975 and H2228 cells were treated as indicated for 24 h, subjected to anti-PD-L1 immunofluorescence (200x magnification) and quantification. B, Serum-starved A549 cells were stimulated with EGF (100 ng/mL) without or with inhibitors before immunoblotted. C, H1975, PC9 and H2228 cells were treated as indicated for 24 h followed by immunoblotting. D and E,
H1975 cells treated with 5 μmol/L MTI-31 alone or in combination with 10 μmol/L CQ or 0.01 μmol/L PS-341 (D) or 20 mmol/L LiCl (E), then immunoblotted. Quantified results are plotted. *, P<0.05; ***, P<0.001.

**Figure 7.** Disruption of mTOR complexes suppress PD-L1 and alleviate apoptosis in T cells. A, ShRNA-expressing H1975 cells were induced with Dox for 5 days then immunoblotted. B, Expression of surface PD-1 in activated Jurkat cells was confirmed by flow cytometry and immunoblotting. C, Levels for surface PD-L1 in H1975 and A549 cells were compared by immunofluorescence. D, Apoptosis rates of Jurkat cells co-cultured for 72 h with H1975, A549 cells or H1975 with 10 μg/mL nivolumab were analyzed by Annexin V-FITC/PI assay (Left) and quantified (Right). E, Apoptosis rates of Jurkat cells co-cultured with H1975 Sh-NT, Sh-Rap, Sh-Ric or Sh-NT with nivolumab were analyzed. F, Normalized results pooled from three independent Jurkat co-culture experiments are plotted. G, Expression of surface PD-1 in primary human CD3+ T cells was confirmed by flow cytometry. H, Depletion of surface PD-L1 in ShRNA-expressing H1975 cells is shown. I, Apoptosis rates of CD3+ T cells co-cultured (48 h) with H1975 Sh-NT, Sh-Rap, Sh-Ric or Sh-NT with nivolumab were analyzed by CD3 and Annexin V-FITC/PI double staining. J, Normalized results pooled from three independent T cell co-culture experiments are plotted **, P<0.01; ***, P<0.001.
Figure 1

A

IC_{50} (μM)

HCC827 PC9 H1975 H1993 H2228 A549

<0.027 <0.027

B

H1975 (EGFRT790M)

Afatinib

H1993 (C-MET)

CM-118

H2228 (EML4-ALK)

CM-118

Veh Veh Veh

Phosphor-EGFR/MET/ALK

P-S6 S6 AKT mTORC1 mTORC2

C

PC9 H1975 H1993 H2228

DMSO Rapa_1 MTI-31-1.5 8051-0.1 Rapa_1 MTI-31-1.5 8051-0.1 Rapa_1 MTI-31-1.5 8051-0.1 Rapa_1 MTI-31-1.5 8051-0.1

μmol/L

P-EGFR P-S6 P-AKT Cyclin D1 Actin

D

NT Rap Ric

NT Rap Ric

NT Rap Ric

ShRNA

Rictor Raptor P-S6K1 P-AKT GAPDH

E

Relative growth

H1975 H1993 H2228

Sh-NT Sh-Rap Sh-Ric Sh-NT Sh-Rap Sh-Ric Sh-NT Sh-Rap Sh-Ric

Day 1 Day 8 Day 1 Day 8 Day 1 Day 8

** ** ** ** ** **
Figure 3

A

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Figure 4

**A**

Lung Adenocarcinoma (n=230)

**B**

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Relative migrated cells

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**F**

Migrated cells (% control)

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**G**

% Cell growth

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**H**

% Cell growth

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*Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.*
Figure 5

A) [Images showing fluorescence at D5, D18, and D25 for Sh-NT and Sh-Ric samples with a graph showing total flux (×10^4) ± S.E. from Day 5 to Day 52.]

B) [Graph showing median survival: NT: 25.5 days, Ric: 50.5 days. P<0.001.]

C) [Images of Rictor and P-AKT staining for Sh-NT and Sh-Ric samples.]

D) [Immunohistochemistry for CD31 showing relative positive area for Sh-NT and Sh-Ric samples. **]

E) [Images showing IBA1 cell number and normalized P-EGFR for Sh-NT and Sh-Ric samples. **]

F) [Western blots for P-AKT, AKT, P-EGFR, Rictor, and Actin normalized to Sh-NT and Sh-Ric samples at 0, 24, and 48 hours. * and ** indicates significance.]

G) [Table showing DMSO, MTI-31, Rapa_1 concentrations (μmol/L) and BV2-CM (h) for P-AKT and AKT with corresponding Western blots.]
Figure 6

A. PD-L1/DAPI staining in H1975 and H2228 cells treated with DMSO, MTI-31, Rapa, and AZD9291/PF.

B. Western blot analysis of A549 cells treated with DMSO, MTI-31, Rapa, and 8055-1 at 0.5 and 12 hours.

C. Western blot analysis of H1975, PC9, and H2228 cells treated with DMSO, MTI-31, Rapa, and MTI+CQ at 5 and 1 hour.

D. Western blot analysis of H1975 cells treated with DMSO, MTI-31, CQ, MTI+CQ, PS341, and MTI+PS341.

E. Western blot analysis of DMSO, MTI-31, LiCl, and MTI+LiCl treated cells.
A novel mTORC1/2 inhibitor (MTI-31) inhibits tumor growth, epithelial-mesenchymal transition, metastases and improves cancer immunity in preclinical lung cancer models

Qianwen Zhang, Yan Zhang, Yaqing Chen, et al.

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