AKT phosphorylation as a predictive biomarker for PI3K/mTOR dual inhibition-induced proteolytic cleavage of mTOR companion proteins in small cell lung cancer

Ming-Chun Hung††, Wan-Ping Wang†† and Ya-Hui Chi†,2*

Abstract

Background: Constitutive activation of PI3K signaling has been well recognized in a subset of small cell lung cancer (SCLC), the cancer type which has the most aggressive clinical course amongst pulmonary tumors. Whereas cancers that acquire a mutation/copy gain in PIK3CA or loss of PTEN have been implicated in enhanced sensitivity to inhibitors targeting the PI3K/AKT/mTOR pathway, the complexities of the pathway and corresponding feedback loops hamper clear predictions as to the response of tumors presenting these genomic features.

Methods: The correlation between the expression profile of proteins involved in the PI3K/AKT/mTOR signaling and cell viability in response to treatment with small molecule inhibitors targeting isoform-specific PI3Ks, AKT, and mTOR was assessed in 13 SCLC cancer cell lines. Athymic nude mice were used to determine the effect of PI3K/mTOR dual inhibition on the growth of xenograft SCLC tumors in vivo. The activation of caspase signaling and proteolytic cleavages of mTOR companion proteins were assessed using recombinant caspases assays and Western blot analyses.

Results: Our results indicate that the sensitivity of these SCLC cell lines to GSK2126458, a dual PI3K/mTOR inhibitor, is positively correlated with the expression levels of phosphorylated AKT (p-AKT) at Thr308 and Ser473. Inhibition of pan-class I PI3Ks or PI3K/mTOR dual inhibition was shown to induce proteolytic cleavage of RICTOR and RPTOR, which were respectively dependent on Caspase-6 and Caspase-3. A combination of a clinically approved PI3Kα-selective inhibitor and an mTORC1 inhibitor was shown to have synergistic effects in inducing the death of SCLC cells with high p-AKT. We observed no clear correlation between PTEN levels and the survival of SCLCs in response to PI3K/mTOR dual inhibition; however, PTEN depletion was shown to increase the susceptibility of low p-AKT SCLC cells to dual PI3K/mTOR inhibitor-induced cell death as well as the proteolytic cleavage of RICTOR.

Conclusions: These results suggest the level of p-AKT can be a companion diagnostic biomarker for the treatment of SCLC involving the combinational use of clinically approved isoform-specific PI3K and mTOR inhibitors.

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Keywords: PI3K inhibitor, mTOR, Small cell lung cancer

Background
Small cell lung cancer (SCLC) accounts for approximately 15% of all lung cancers, resulting in ~30,000 deaths each year in the United States. Without treatment, SCLC has the most aggressive clinical course of any type of pulmonary tumor, with median survival from diagnosis of only 2 to 4 months [1]. SCLC patients often present with metastasis at the time of diagnosis, thereby precluding surgery as a treatment option [2]. Since the early 1980s, the standard of care for limited stage (LS-SCLC) and extensive stage (ES-SCLC) disease has involved a drug combination of etoposide with cisplatin or carboplatin [3]. ES-SCLC patients are sensitive to standard chemotherapy and radiation therapy; however, tumors typically recur rapidly after primary treatment, with only 6% of patients surviving 5 years from the time of diagnosis [4]. Since 2019, the FDA has approved chemotherapy in combination with anti-PD-L1 antibodies (atezolizumab or durvalumab) for SCLC. This treatment was shown to provide a slight improvement in median overall survival (12.3 to 13.0 months) compared to chemotherapy alone (10.3 months for ES-SCLC) [5, 6]. Nonetheless, the therapeutic benefits of immune oncology-combined therapy are modest, and no targeted therapy is currently available.

SCLC is most commonly encountered in heavy smokers. The tumor cells express neuroendocrine markers and are believed to derive from cells residing in the epithelial lining of the bronchi [7]. Comprehensive genomic analyses of SCLCs have reported loss-of-function alterations in tumor suppressor genes RB1 and TP53 in ~90% of cases [8–12], in addition to a high prevalence of mutually exclusive amplification of MYCL, MYC, or MYCN (~20%), or mutations in PTEN (~8%) and NOTCH (~25%) family genes [8–12]. The cell types leading to SCLC have been more functionally characterized in terms of genetic derivation and origin than have the cells of many other tumors [13]. Conditional inactivation of Rb1 and Trp53 in lung epithelial cells (in an RP mouse model) led to the development of dysplastic precancerous lesions, which morphologically and genetically mimic human SCLC [14]. During the long latency (10–15 months) in the development of SCLC, the RP tumors were shown to acquire Mycl amplifications, Nf1b amplifications, or Pten loss [15]. Inactivation of one allele of Pten in the RP background significantly accelerated tumorigenesis, after which RP mice became moribund at an average of ~13 months, compared with ~6 months in Ptenlox/+RP and ~4 months in Ptenlox/loxRP mice [16].

In addition to Pten, the conditional knockout of Crebbp, the constitutive expression of Mycl, or a stabilized Myc mutant (MycT58A) was shown to accelerate SCLC tumorigenesis in mice [15, 17].

The PI3K/AKT/mTOR pathway is closely associated with growth, survival, and the invasion of cancers. Phosphatidylinositol 3-kinases (PI3Ks) belong to the lipid kinase family, which can be divided into three main classes (Class I, II, and III), in accordance with their structural and substrate specificities [18, 19]. Class IA PI3Ks are composed of a heterodimer between a p110 catalytic subunit (PIK3CA, PIK3CB, or PIK3CD) and a p85 regulatory subunit [20]. Upon receiving extracellular activation signals via tyrosine kinase receptors, PI3Ks phosphorylate PIP2 to PIP3, the process of which is counteracted by PTEN. PIP3 recruits AKT to the plasma membrane for activation by PDK1, which initiates a subsequent signaling cascade for cell growth, enhanced metabolic activity, and cell survival [21]. Mammalian target of rapamycin (mTOR) is considered a class IV PI3K-related serine/threonine kinase composed of ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3 related (ATR), DNA-dependent protein kinase (DNA-PK), and mTOR [19]. mTOR exhibits two functionally distinct complexes: mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) [22]. mTORC1 is defined by the adaptor protein RICTOR (regulatory-associated protein of mTOR) and is rapamycin-sensitive, whereas mTORC2 has been observed associating with RICTOR (rapamycin-insensitive companion of mTOR) [23]. mTORC1 is well characterized as a major driver of cell growth by promoting cap-dependent translation through the phosphorylation of the ribosomal protein S6 kinases (P70S6K1 and P70S6K2) and the eukaryotic initiation factor 4E-binding proteins (4EBP1 and 4EBP2) [21, 24]. The function of mTORC2 is less characterized; however, it is known to activate AKT, which in turn phosphorylates and inhibits mTORC1 repressor TSC2 [21, 24].

Cancers that acquire a mutation/copy gain in PIK3CA or loss of PTEN have been implicated in enhanced sensitivity to inhibitors targeting the PI3K/AKT/mTOR pathway. However, the complexities of the pathway and corresponding feedback loops hamper clear predictions as to the response of tumors presenting these genomic features [25–28]. In the current study, we evaluated the sensitivity of 13 SCLC cell lines to inhibitors targeting PI3K, AKT, and mTOR. We determined that the degree of cell growth inhibition by dual PI3K and mTOR inhibitors in SCLCs was positively correlated with AKT
phosphorylation levels at Thr308 and Ser473, but not correlated with PTEN levels or PI3KCA mutation/copy gain. These results suggest a potential strategy for the treatment of SCLC involving the combinational use of clinically approved PI3K and mTOR inhibitors.

**Methods**

**Test compounds**

MK-2206 (A10003), RAD001 (A10374), CAL-101 (A10172), LY294002 (A10547), and z-VAD-FMK (A12373) were purchased from AdooQ Biosciences. BAY 80-6946 (MedChemExpress, HY-15,346), BYL719 (Cayman Chemical, 22142), Staurosporine (Cayman Chemical, 81590), and GSK2126458 (Cayman Chemical, 17377) were purchased from vendors.

**Culturing of cell lines**

All SCLC cell lines, including NCI-H82, NCI-H446, NCI-H69, NCI-H211, NCI-H524, NCI-H526, NCI-H146, NCI-H187, NCI-H345, NCI-H841, NCI-H1930, NCI-H2171, and NCI-H2081, were obtained from American Type Culture Collection (ATCC). NCI-H345, NCI-H841, NCI-H2171, and NCI-H2081 cell lines were maintained in HITES medium [Dulbecco’s medium: Ham's F12, 50:50 mix (Gibco), insulin 0.005 mg/ml, transferrin 0.01 mg/ml, sodium selenite 30 nM, hydrocortisone 10 nM, and β-estradiol 10 nM] containing 5% fetal bovine serum (FBS, Hyclone) and penicillin–streptomycin antibiotics (Gibco). The remainder of the cell lines were cultured in RPMI1640 medium (Gibco) supplemented with 10% FBS and antibiotics. Cells were cultured for up to 8 weeks and used at passages of less than 16 from the initial source vial.

**Cell viability**

Cells were seeded in 96-well plates (Corning) at densities optimized for each line (5000–10,000 cells per well). After 24 h, cells were treated with the designated compounds for 72 h. Cell viability was determined using PrestoBlue Cell Viability Reagent (ThermoFisher Scientific). Fluorescence was measured using a Perkin Elmer Wallac 1420 Victor2 plate reader. Cell viability was calculated by normalizing each blank-corrected fluorescence value to untreated cells. The computation of IC_{50} values was based on an eight-point titration performed in triplicate. Synergy between any two compounds was determined using SynergyFinder (https://synergyfinder.fimm.fi) based on the zero interaction potency (ZIP) model [29].

**RNAi**

Small interfering RNAs (siRNAs) targeting PTEN (ThermoFisher Scientific, 4390771/Assay ID: n276787 and 4392420/Assay ID: s327), CASP3 (Qiagen, FlexiTube siRNAs SI02661939 and SI02662450), or CASP6 (Qiagen, FlexiTube siRNAs SI02654603 and SI03100041) were purchased from vendors. Two sets of siRNAs for one gene were mixed at a ratio of 1:1 for expression depletion. Cells were transfected with siRNAs using Lipofectamine™ RNAiMAX (ThermoFisher Scientific) in accordance with the manufacturer’s protocol.

**Immunoblotting**

Cancer cells were collected from culture plates, washed using 1× phosphate-buffered saline (PBS), and lysed using RIPA buffer (50 mM HEPES, pH 7.3, 150 mM NaCl, 2 mM EDTA, 20 mM β-glycerophosphate, 0.1 mM Na3VO4, 1mM NaF, and 0.5 mM DTT) containing 0.5% NP-40 (Sigma-Aldrich), protease inhibitor cocktail (Roche Applied Science), and phosphatase inhibitor (Roche Applied Science). Cell lysates were then prepared in 1× Laemmli protein sample buffer, and boiled at 100 °C for 10 min. For xenograft tumors, tissues were dissected into small pieces, weighed, lysed in 1× Laemmli protein sample buffer under rigorous sonication, and boiled at 100 °C for 10 min. Lysates were separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF, Millipore) membranes. Membranes were blotted with primary antibodies which were diluted in 0.2% casein, 1% bovine serum albumin (BSA), or 5% nonfat milk. After overnight blotting at 4 °C, the membranes were washed using blotting buffer [0.2% casein in 1× PBS with 0.1% Tween® 20 detergent (PBST) or 1× Tris-buffered saline with 0.1% Tween® 20 detergent (TBST)]. Corresponding horse radish peroxidase (HRP)- or alkaline phosphatase (AP)-conjugated secondary antibodies (Sigma-Aldrich) were added. The blots were developed with chemiluminescence in accordance with the manufacturer’s protocols. Primary antibodies obtained from Cell Signaling were PTEN (#9188), AKT (#04-796), p-AKT(Thr308) (#13038), p-AKT(Ser473) (#2708), P70S6K (#9234), 4EBP1 (#9644), p-4EBP1(Thr37/Thr46) (#2855), PIK3CA (#4249), RICTOR (#9476), CASP6 (#9762), cleaved CASP3 (c-CASP3, #9661), cleaved CASP6 (c-CASP6, #9761), CASP7 (#12,827), and CASP8 (#4790). The other primary antibodies were RPTOR (abcam, ab40768), PARP-1 (abcam, ab32378), CASP3 (Genetex, GTX123678), GAPDH (Genetex, GTX100118), and β-ACTIN (Sigma-Aldrich, A1978).
In vivo xenograft studies
NCI-H446 cells were prepared in cold 1× PBS, mixed with an equal volume of Matrigel® Basement Membrane Matrix (Corning), and transplanted subcutaneously (at 1 × 10^6 cells/mouse) into the right flank of 6- to 8-week-old male athymic nu/nu mice (BIOLASCO). The mice were housed in individually ventilated cages, which were maintained under 12 h light/dark cycles with controlled temperature and humidity. The size of the xenografted tumors was measured using a digital caliper (Goldsun Electronics Co.) and calculated in accordance with the following formula: tumor volume (mm^3) = length × (width)^2. Body weight and tumor size were measured at least twice a week. Mice were randomly assigned to control and treatment groups when the tumors reached 150–250 mm^3 in size. The mice were orally administered with a vehicle (0.5% methylcellulose and 0.2% Tween-80 in ddH_2O) or 1.5 mg/kg GSK2126458 [30] on a 5-on-2-off dosing regimen for 2 weeks. The animal protocol had been reviewed and approved by Institutional Animal Care and Use Committees (IACUC) of the National Health Research Institutes. The care and use of animals was conducted in accordance with regulations outlined by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

In vitro caspase reaction
NCI-H446 cells lysed in RIPA buffer were incubated with various quantities of recombinant human active Caspase-3 (CASP3, abcam, ab52101) or Caspase-6 (CASP6, abcam, ab52157) in 40 μL of 1× Caspase reaction buffer (50 mM HEPES, 50 mM NaCl, 0.1% CHAPS, 10 mM EDTA, 5% Glycerol, and 10 mM DTT) [31]. The reaction was performed at 37 °C for various durations ranging from 15 min to 3 h. Reactions were stopped by the addition of Laemmli protein sample buffer and boiling at 100 °C for 10 min, before undergoing SDS-PAGE and Western blot analyses.

Statistical analysis
Statistical differences were analyzed using two-tailed Student's t-test or two-way ANOVA. The criterion for significance (p-value) was set as described in the figures.

Results
AKT phosphorylation levels were positively correlated with the sensitivity of SCLC to PI3K and mTOR dual inhibition
The PI3K/AKT pathway is centrally involved in the transduction of mitogenic signals to mTOR. In examining the differential activation signaling of PI3K/AKT/mTOR in SCLCs, we first determined the phosphorylation levels of AKT(T308), AKT(S473), P70S6K(T389), and 4EBP1(T37/T46) as well as PTEN and PIK3CA in 13 SCLC cell lines (Fig. 1A). We then quantified protein phosphorylation inhibition and cell survival (i.e., IC_{50}) in response to treatment with GSK2126458 (a highly potent inhibitor of PI3Ka/b/δ/γ and mTORC1/2) [32], MK-2206 (a highly selective inhibitor of AKT1/2/3), RAD001 (an mTOR inhibitor of FKBP12), or LY294002 (a broad-range PI3K inhibitor) (Fig. 1B and Additional file 1: Figure S1). Genomic features of the 13 SCLC cell lines, including mutation/copy number in PIK3CA and PTEN, are listed in Table 1.

Treatment with MK-2206 or LY294002 was shown to reduce the phosphorylation of AKT(T308) and AKT(S473), as previously reported (Additional file 1: Figure S1A); however, the survival of the 13 SCLC cell lines was unaffected at the concentration of 1.0 μM (Additional file 1: Figure S1B, C). Treatment with 1.0 μM RAD001 reduced survival by less than 20% in all SCLCs except for NCI-H446 (Additional file 1: Figure S1D). Most of the SCLC cell lines that expressed lower AKT phosphorylation [i.e., p-AKT(S473) level < 0.2, Table 1] remained > 50% viable after treatment with 1.0 μM GSK2126458 (IC_{50} > 1.0 μM, including NCI-H211, NCI-H524, NCI-H526, NCI-H146, and NCI-H345). Other SCLC cell lines expressing higher levels of p-AKT [i.e., p-AKT(S473) level > 0.7, Table 1], including NCI-H187, NCI-H446, and NCI-H69, were highly sensitive (IC_{50} < 100 nM) to treatment with GSK2126458 (Fig. 1B; Table 1). Note that the sensitivity of the SCLCs to GSK2126458 was strongly correlated with the level of AKT phosphorylation at T308 (R^2 = 0.581, p = 0.0025) and S473 (R^2 = 0.723, p = 0.0002) (Fig. 1C). In these SCLC cell lines, we did not observe correlations between the IC_{50} values of GSK2126458 and PIK3CA and PTEN levels or P70S6K and 4EBP1 phosphorylation levels (Fig. 1C).

GSK2126458 has previously demonstrated broad antitumor activity in preclinical studies across a broad panel of cancer cell lines [28]; however, the in vivo efficacy of GSK2126458 in treating SCLC has not been tested. Thus, we examined the growth inhibition activity of GSK2126458 in the NCI-H446 xenograft model, in which p-AKT was strong. Oral administration of 1.5 mg/kg GSK2126458 using a 5-on-2-off regimen for two weeks was shown to induce > 90% volume suppression of the xenograft tumors at day 12, compared to the vehicle control (Fig. 1D, p < 0.0001, t-test). We also examined the in vivo targeted inhibition of GSK2126458 in NCI-H446 xenograft tumors collected from untreated and drug-treated mice at 2, 6, and 24 h after dosing. p-AKT(T308) and p-AKT(S473) levels
were significantly lower in xenograft tumors treated with GSK2126458 at 2 h after drug administration. This treatment also led to extensive cell apoptosis, as evidenced by PARP-1 cleavage (i.e., c-PARP1, Fig. 1E). These results demonstrate the efficacy of dual PI3K and mTOR inhibition in inducing cell apoptosis in xenograft SCLC tumors presenting strong p-AKT.

**Fig. 1** SCLC cell lines with high p-AKT were sensitive to PI3K/mTOR dual inhibition. **A** Western blot analysis showing the expression of proteins involved in the PI3K/AKT/mTOR pathway in 13 SCLC cell lines. **B** Concentration-response cell survival curves of SCLC cell lines treated using GSK2126458. **C** Correlation between survival response (IC_{50}) and the levels of p-AKT(T308), p-AKT(S473), PIK3CA, PTEN, p-P70S6K, and p-4EBP1. Symbols of the cell lines are the same as those denoted in **B**. R^2, coefficient of determination. **D** Tumor growth curve of xenografted NCI-H446 cells in nude mice. Mice were orally (PO) administered with vehicle (10 ml/kg) or GSK2126458 (1.5 mg/kg) when the tumor size reached approximately 200 mm^3 using a 5-on-2-off cycle for 2 consecutive weeks. **E** Western blot analysis results showing the expression of AKT, p-AKT(T308), p-AKT(S473), cleaved PARP-1 (c-PARP1), and a GAPDH loading control in xenografted NCI-H446 tumors in mice oral administered with GSK2126458 (3.0 mg/kg). Tumors were harvested at 2, 6, and 24 h after drug treatment

**Synergistic effects of PI3Ka and mTOR in limiting the survival of SCLCs presenting high p-AKT levels**

It appears that the clinical trial of GSK2126458 may have been halted in phase I as a consequence of the high occurrence of grade ≥ 3 adverse events, including diarrhea, hyperglycemia, and skin rash [28]. Nonetheless,
our findings suggest that PI3K and mTOR dual inhibition may be an effective therapeutic strategy for the treatment of SCLCs with high p-AKT. To date, five PI3K inhibitors have obtained FDA approval. These compounds inhibit specific isoforms of PI3K, including Idelalisib (i.e., CAL-101, a PI3Kδ inhibitor), Copanlisib (i.e., BAY 80-6946, a pan-class I PI3K inhibitor with subnanomolar IC₅₀s against PI3Kα and PI3Kδ), Duvelisib (a dual inhibitor of PI3Kδ and PI3Kγ), Alpelisib (i.e., BYL719, an α-specific PI3K inhibitor), and Umbralisib (a dual inhibitor of PI3Kδ and CK1ε) [33–35]. Thus, we used these isoform-selective PI3K inhibitors to investigate the potential synergistic effects of PI3K-isoform and mTOR inhibition in SCLCs (Additional file 1: Figure S2A).

Similar to the results of GSK2126458 treatment (Fig. 1B), the survival of high p-AKT SCLCs (i.e., NCI-H187, NCI-H446, and NCI-H69) was more sensitive to BAY 80-6946 than were low p-AKT SCLCs (Fig. 2A). As expected, BAY 80-6946 rendered p-AKT(T308) and p-AKT(S473) levels undetectable and led to a decrease in p-P70S6K and p-4EBP1 levels (Fig. 2B), which is an indication of mTOR inhibition. CAL-101 and BYL719 were both shown to reduce phosphorylation of p-AKT(T308) and p-AKT(S473) by >50% at a concentration of 1.0 μM (Fig. 2B); however, SCLCs with high p-AKT were more sensitive to BYL719 than to CAL-101 (Additional file 1: Figure S2B).

We next measured the potential synergy between isoform-selective PI3K inhibitors (i.e. CAL-101 and BYL719), MK-2206, and RAD001 in SCLC survival via combination profiling. Dose matrices of increasing concentrations of any two of the four drugs were assessed in terms of their effects on cell viability. Drug synergy was evaluated using SynergyFinder (version 2.0), a web-based interactive analysis and visualization application [36]. A ZIP model was used to evaluate drug additivity and calculate a synergy score. A synergy score of greater than 10 suggests that any interaction between two drugs is likely to be synergistic [29]. Our results identified combinations of BYL719/RAD001 and MK-2206/RAD001 as synergistic with synergy scores of 12.889 and 10.688, respectively (Fig. 2C). Other combinations, including CAL-101/BYL719, CAL-101/RAD001, CAL-101/MK-2206, and MK-2206/BYL719, additively inhibited the survival of NCI-H446 (Fig. 2D and Additional file 1: Figure S2C).

Our Western blot results were in line with the synergy scores, indicating that RAD001 in combination with BYL719 or MK-2206 had a synergistic effect on the level of cleaved PARP-1 (c-PARP1), which is indicative of synergistically enhanced cell death. Combinations of CAL-101/BYL719, CAL-101/RAD001, CAL-101/MK-2206, RAD001/MK-2206, and MK-2206/BYL719 were also shown to increase c-PARP1 levels, compared to single drug treatments (Fig. 2E). These results indicate that the

| Cell Line   | PIK3CA mutation/copy number | PTEN mutation | Relative level of p-AKT (T308)/(S473) | GSK2126458 IC₅₀ ± SD (nM) |
|-------------|-----------------------------|---------------|--------------------------------------|---------------------------|
| NCI-H187    | WT/1.87³                     | WT            | 0.348/0.753                          | 21.2 ± 6.0                |
| NCI-H446    | WT/NA                       | Exon 4, 46 bp deletion from codon 82¹ | 0.891/0.791                          | 31.7 ± 13.8              |
| NCI-H69     | G106-R108 del/3.69¹         | WT            | 0.544/0.960                          | 47.3 ± 19.2               |
| NCI-H2081   | WT/NA                       | Missense Mutation, C124E² | 0.240/0.782                          | 158.4 ± 98.8             |
| NCI-H1930   | WT/1.90²                     | WT            | 0.064/0.231                          | 285.3 ± 74.4             |
| NCI-H841    | WT/copy number gain⁴        | WT            | 0.434/0.295                          | 329.6 ± 59.3             |
| NCI-H2171   | WT/1.28³                     | WT            | 0.043/0.051                          | 358.8 ± 239.6            |
| NCI-H82     | WT/1.69³                     | WT            | ND/ND                                | 710.6 ± 204.4            |
| NCI-H146    | WT/1.74³                     | WT            | 0.033/0.021                          | 1572.3 ± 388.4           |
| NCI-H211    | WT/1.54³                     | WT            | ND/ND                                | 2354.5 ± 973.3           |
| NCI-H526    | WT/2.19³                     | WT            | 0.019/ND                             | 2665.9 ± 1979.0          |
| NCI-H524    | WT/1.66³                     | WT            | 0.137/0.154                          | 4035.2 ± 455.9           |
| NCI-H345    | WT/3.45³                     | WT            | 0.029/0.122                          | 4622.3 ± 1183.9          |

WT wild type, NA not available, ND not detectable

1 See reference [51]
2 Information retrieved from DepMap Portal (Broad Institute) https://depmap.org/portal/
3 See reference [46]
4 See reference [52]
A combination of PI3K and mTOR inhibitors presented synergistic effects in limiting the survival of SCLC cells with high p-AKT. 

**A** Concentration-response cell survival curves of SCLC cell lines treated using BAY 80-6946.

**B** Western blot analysis showing the expression of proteins involved in the PI3K/AKT/mTOR pathway in NCI-H446 cells treated with indicated concentrations of GSK2126458 (GSK), BAY 80-6946 (BAY), CAL-101 (CAL), BYL719 (BYL), RAD001 (RAD), or MK-2206 (MK) for 24 h. Levels of p-AKT(T308) and p-AKT(S473) were quantified and listed below the profiles.

**C** Dose matrix of RAD001 in combination with BYL719 or MK-2206. Percent growth inhibition at each dose was assessed at 72 h after drug treatment (see left panel). Synergy distribution and synergy scores based on a zero interaction potency (ZIP) model (shown on the right).

**D** List of ZIP scores presenting synergy of drug combinations (any two of CAL, BYL, RAD and MK) to the survival of NCI-H446 cells.

**E** Western blot analysis showing the expression of the indicated proteins in NCI-H446 cells following single or combination treatment using GSK, BAY, CAL, BYL, RAD001, or MK-2206 at a concentration of 1.0 µM. The lower band under full-length RICTOR is cleaved RICTOR (c-RICTOR). The expression of c-PARP1 used to denote cell apoptosis. Levels of c-PARP1, c-RICTOR, c-RPTOR, p-AKT(T308) and p-AKT(S473) were quantified and listed below the profiles.

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**Fig. 2** A combination of PI3K and mTOR inhibitors presented synergistic effects in limiting the survival of SCLC cells with high p-AKT.

A Concentration-response cell survival curves of SCLC cell lines treated using BAY 80-6946. B Western blot analysis showing the expression of proteins involved in the PI3K/AKT/mTOR pathway in NCI-H446 cells treated with indicated concentrations of GSK2126458 (GSK), BAY 80-6946 (BAY), CAL-101 (CAL), BYL719 (BYL), RAD001 (RAD), or MK-2206 (MK) for 24 h. Levels of p-AKT(T308) and p-AKT(S473) were quantified and listed below the profiles. C Dose matrix of RAD001 in combination with BYL719 or MK-2206. Percent growth inhibition at each dose was assessed at 72 h after drug treatment (see left panel). Synergy distribution and synergy scores based on a zero interaction potency (ZIP) model (shown on the right). D List of ZIP scores presenting synergy of drug combinations (any two of CAL, BYL, RAD and MK) to the survival of NCI-H446 cells. E Western blot analysis showing the expression of the indicated proteins in NCI-H446 cells following single or combination treatment using GSK, BAY, CAL, BYL, RAD001, or MK-2206 at a concentration of 1.0 µM. The lower band under full-length RICTOR is cleaved RICTOR (c-RICTOR). The expression of c-PARP1 used to denote cell apoptosis. Levels of c-PARP1, c-RICTOR, c-RPTOR, p-AKT(T308) and p-AKT(S473) were quantified and listed below the profiles.
PI3K isoforms work differently with mTOR in terms of the survival of SCLCs with high p-AKT.

Dual PI3K and mTOR inhibition induce proteolytic cleavage of RICTOR and RPTOR in SCLCs with elevated p-AKT expression levels

We described above that the simultaneous inhibition of PI3K and mTOR (or AKT and mTOR) reduced the viability of SCLC cells with high p-AKT levels (Figs. 1 and 2). Note that a lower band (approximately 140 kDa) appeared below the full-length RICTOR, the intensity of which coincided with c-PARP1 levels (Fig. 2E). The proteolytic cleavage of RICTOR was consistently observed in NCI-H446 treated with the PI3K/mTOR dual inhibitor GSK2126458 and the pan-class I PI3K inhibitor BAY 80-6946. Cleaved RICTOR (c-RICTOR) was observed as early as 3 h after treatment with GSK2126458 or BAY 80-6946, and the level was found to increase with time.
the molecular size of which was approximately 130 kDa.

induce the proteolytic cleavage of RPTOR (c-RPTOR), using GSK2126458 or BAY 80-6946 was also shown to
mediated AKT dephosphorylation was correlated with
(Fig. 1C), we next sought to determine whether PTEN-
viability of SCLCs under dual PI3K/mTOR inhibition
the protein level of PTEN was not associated with the
role in the proteolytic cleavage of RICTOR and RPTOR,
mediation‑induced cell death and proteolytic cleavage
PTEN depletion sensitizes SCLC to PI3K/mTOR
inhibitors (Additional file 1: Figure S3B). Note that the
observations pertaining to GSK2126458-treated cells,
the level of RICTOR and RPTOR in NCI-H446 respec-
teolytic cleavages were on-target effects, we measured
We next investigated whether p-AKT levels play a
role in the proteolytic cleavage of RICTOR and RPTOR,
which was achieved by shutting down PI3K/mTOR
mediation‑induced cell death and proteolytic cleavage
the dual depletion of CASP3 and CASP6 (Fig. 5B, C). The
deployment of CASP3 ameliorated GSK2126458-induced cleavage of RICTOR and RPTOR.
Among the caspases examined in this study (CASP3, CASP6, CASP7, and CASP8), we observed the expres-
sion of cleaved CASP3 (i.e., c-CASP3) and cleaved
The cleavage level of RICTOR was further ameliorated by
We next sought to determine whether RICTOR and
RPTOR are respective direct substrates of CASP6 and CASP3
To identify the caspase(s) that could be implicated in
the cleavage of RICTOR and RPTOR, we first examined
GSK2126458-induced caspase activation in NCI-H446.
Among the caspases examined in this study (CASP3, CASP6, CASP7, and CASP8), we observed the expression
of cleaved CASP3 (i.e., c-CASP3) and cleaved
CASP6, CASP7, and CASP8), we observed the expres-
sion of cleaved CASP3 (i.e., c-CASP3) and cleaved
On the other hand, PTEN deletion did not alter survival rates
in the two cell lines following treatment with CAL-101,
BYL719, MK-2206, or RAD001 (Fig. 4A).
Western blot results verified that the depletion of
PTEN elevated the levels of p-AKT (S473) and
p-AKT (T308) (Fig. 4D). Under PTEN depletion, treat-
ment with GSK2126458 eliminated AKT phosphoryl-
ation and promoted cell apoptosis, as evidenced by
elevated c-PARP1 levels. Furthermore, the depletion of
PTEN increased proteolytic cleavage of RICTOR, com-
pared to PTEN-intact NCI-H146 and NCI-H526 cells
(Fig. 4D). These results indicate that PTEN antagonized
dual PI3K/mTOR inhibition-induced cell apoptosis in
SCLCs with low intrinsic p-AKT levels.

PTEN depletion sensitizes SCLC to PI3K/mTOR
inhibition‑induced cell death and proteolytic cleavage
of RICTOR

PTEN is a protein phosphatase that antagonizes the
kinase activity of PI3K [37]. Having determined that
the protein level of PTEN was not associated with the
viability of SCLCs under dual PI3K/mTOR inhibition
(Fig. 1C), we next sought to determine whether PTEN-
mediated AKT dephosphorylation was correlated with
the viability of SCLCs under PI3K and/or mTOR inhibi-
tion. In NCI-H146 and NCI-H526 cells (in which p-AKT
levels were nearly undetectable), the depletion of PTEN
significantly reduced the viability of cells under treatment
with GSK2126458 or BAY 80-6946 (Fig. 4A–C). On the
other hand, PTEN depletion did not alter survival rates
in the two cell lines following treatment with CAL-101,
BYL719, MK-2206, or RAD001 (Fig. 4A).

Using RNAi, we noted the depletion of CASP3 ame-
liorated GSK2126458-induced cleavage of RICTOR and
RPTOR. On the other hand, the depletion of CASP6
ameliored the cleavage of RICTOR but not of RPTOR.
The cleavage level of RICTOR was further ameliorated by
the dual depletion of CASP3 and CASP6 (Fig. 5B, C). The
GSK2126458-elicted proteolytic cleavage of RICTOR
and RPTOR can be eliminated through the addition of
z-VAD-FMK, a general caspase inhibitor (Fig. 5D).
These results suggest that the activation of caspases, includ-
ing CASP3 and CASP6, is responsible for the proteolytic
processing of RICTOR and RPTOR.

We next sought to determine whether RICTOR and
RPTOR are direct substrates of protein caspases. This was
achieved by incubating NCI-H446 lysates with recombi-
nant CASP3 and CASP6 (Fig. 5E).

Western blot results verified that the depletion of
PTEN elevated the levels of p-AKT (S473) and
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RPTOR are direct substrates of protein caspases. This was
achieved by incubating NCI-H446 lysates with recombi-
nant CASP3 and CASP6 (Fig. 5E). We then compared the
degree of RICTOR and RPTOR cleavages in cells treated with
multiple dosages and corresponding time durations
to elucidate the specificity of the recombinant caspases to substrates. RICTOR could be efficiently processed by CASP6 into a ~140 kDa protein, whereas RPTOR was more responsive to the enzymatic activity of CASP3, resulting in a cleaved form of ~130 kDa (Fig. 5E). The molecular sizes of c-RICTOR and c-RPTOR processed by recombinant CASP6 and CASP3 were similar to those of c-RICTOR and c-RPTOR in GSK2126458-treated cells. Researchers have previously reported on caspase-mediated cleavages of RICTOR and RPTOR following treatment with stimuli, such as the death ligand or small molecules [31, 38]. Martin et al. reported that the death signal-induced proteolytic cleavage of RPTOR was primarily mediated by CASP6, whereas Zhao et al. reported that RICTOR was a direct substrate of CASP3 [31, 38]. In the current study, RICTOR and RPTOR could both

Fig. 4 PTEN contributes to the survival of low p-AKT cells treated using PI3K/mTOR inhibitors. A Percent survival of NCI-H146 and NCI-H526 in response to treatment using the indicated compounds for 72 h. *p < 0.05; **p < 0.01; ***p < 0.001 (t-test). B, C Concentration-response cell survival curves of NCI-H146 and NCI-H526 cells pretreated using siRNAs targeting PTEN for 48 h, followed by treatment using GSK2126458 (B) or BAY 80-6946 (C) for an additional 72 h. ***p < 0.001 (two-way ANOVA). D NCI-H146 and NCI-H526 cells were first transfected with siRNAs targeting PTEN for 48 h. After another 12 h treatment using GSK2126458 (1.0 µM), cells were harvested and subjected to Western blot analysis using the indicated antibodies. Levels of c-RICTOR, c-PARP1, PTEN and p-AKT(S473) were quantified and listed below the profiles. The results indicated that c-RICTOR and c-PARP1 were increased in GSK2126458-treated cells depleted for PTEN.
be processed by recombinant CASP3 and CASP6; however, RICTOR was more sensitive to CASP6 and RPTOR appeared more sensitive to CASP3, under the same experimental conditions (Fig. 5E). Taken together with the RNAi data (Fig. 5B, C), these results indicate that in high p-AKT SCLC cells, the dual inhibition of PI3K and mTOR can induce cell death, accompanied by CASP6- and CASP3-mediated proteolytic cleavage of mTOR companion proteins.

Discussion
This study led us to devise a strategy by which to select SCLC patients who are more likely to benefit from isoform-specific PI3K and mTOR inhibitors already in clinical use. Inhibition of both PI3K and mTOR induced apoptosis in SCLCs with acquired high p-AKT levels, in conjunction with CASP6- and CASP3-mediated proteolytic cleavages of mTOR companion proteins RICTOR and RPTOR. The fact that the combined targeting
of mTOR and PI3K\(\alpha\) was more effective than inhibiting mTOR and PI3K\(\delta\) suggests that PI3K\(\alpha\)-mediated signaling is more important to cell survival in SCLC cell lines with high p-AKT under mTOR inhibition.

Patients with lung adenocarcinomas presenting well-defined genomic alterations benefit greatly from genotype-directed therapies; however, researchers have yet to identify actionable targeted therapy strategies for SCLC. Note that the repurposing of existing drugs, such as tyrosine kinase inhibitors, Hedgehog signaling inhibitors, anti-apoptotic inhibitors, and anti-angiogenesis agents, has not had a meaningful clinical impact in treating SCLCs [13, 39, 40]. Gene mutations (e.g., PIK3CA, PTEN, and RICTOR) and activation of the PI3K/AKT/mTOR have been reported in genomic and proteomic profiling studies of SCLC tumor samples; however, there have been notable discrepancies in the results, due perhaps to relatively small sample sizes, differences in population (e.g., race, naïve or chemo-resistant) and sequencing techniques [8, 10, 12, 41]. Nevertheless, the effectiveness of PTEN inactivation in accelerating SCLC in genetic mutant mouse models suggests that the PTEN-associated signaling pathway is a strong therapeutic pathway for SCLC, as long as predictive biomarkers can be identified [15, 16, 40]. In the current study, we observed notable differences in PTEN levels among SCLC cell lines; however, there was an absence of correlation between PTEN levels (Fig. 1A) and the degree of cellular sensitivity to dual PI3K/mTOR inhibitors (Fig. 1C). Nonetheless, PTEN knock-down increased the expression of p-AKT as well as the likelihood of cell death induced by dual PI3K/ mTOR inhibitors in SCLC cell lines with low p-AKT levels (i.e., NCI-H146 and NCI-H526) (Fig. 4D). These results supported the notion that PTEN-mediated AKT dephosphorylation is important to the survival of SCLCs when the activities of PI3Ks and mTORs are blocked.

Constitutive activation of PI3K activity has been well recognized in a subset of SCLCs, and the inhibition of PI3K/AKT signaling has been shown to block growth and promote apoptosis in SCLCs activated in AKT [42, 43]. Two pioneering works published when only an early-generation PI3K inhibitor (LY294002) was available suggested that LY294002 at a high molar concentration is required to erase AKT phosphorylation [42], and the cancer cell growth inhibition efficiency was suboptimal [43]. Moore et al. reported that the administration of LY294002 at a concentration of 10 \(\mu\)M reduced the growth of NCI-H69 cells by ~25%; however, the administration of Rapamycin (20 nM) in conjunction with LY294002 (10 \(\mu\)M) inhibited NCI-H69 cell growth by ~50%, which suggests that PI3Ks and mTORC1 may act synergistically in their effects on SCLC survival. Umemura et al. demonstrated that the survival of cisplatin-resistant SCLC cell lines could be suppressed using the new PI3K/mTOR dual inhibitor (BEZ235); however, not all SCLC cell lines harboring alterations in the PI3K/AKT/mTOR pathway exhibited the same degree of sensitivity to BEZ235 [10]. BEZ235 failed in subsequent clinical trials on advanced renal cell carcinoma, due to a high frequency of grade 3–4 adverse (~50%). In another study, BEZ235 treatment targeting advanced pancreatic neuroendocrine tumors and other solid tumors was discontinued in 39% of cases [44]. Similar adverse effects have been reported in other studies on dual PI3K/mTOR inhibitors, including GSK2126458, VS-5584, and GDC-0980. These on-target adverse effects raise doubts as to whether a full blockade of PI3Ks and mTORs is clinically applicable [28, 44]. In the current study, we examined the effects of clinically approved pan-class I and isoform-selective PI3K inhibitors on the survival of SCLCs. This led to the discovery of potential treatment strategies involving BAY 80-6946 alone or BYL719 and RAD001 in combination for SCLC (Fig. 2), which is a disease indication that has not previously been clinically investigated for these drugs. Similar to GSK2126458, the clinically approved pan-class I PI3K inhibitor BAY 80-6946 reduced viability and induced RICTOR/RPTOR cleavage in high p-AKT cells (Fig. 2A and Additional file 1: Figure S3). BYL719 and RAD001 in combination induced a feedback reactivation of PI3K/ mTOR (Additional file 1: Figure S3A) and survival signaling soon after drug treatment; however, this could be overcome by adjusting dosing frequency of the drugs in clinical design. The availability of clinical toxicology results from these FDA-approved PI3K inhibitors would facilitate the design of clinical plans pertaining to the use of PI3K inhibitors for the treatment of SCLC, particularly in terms of working dosages and adverse effects.

The observed activation of PI3K/AKT/mTOR signaling and corresponding increase in AKT phosphorylation in non-small cell lung cancer (NSCLC) tumor specimens (50–73%) was associated with poor disease prognosis [45]. The constitutive stimulation of this pathway could be attributed to genetic mutations/amplifications of PIK3CA (14–25%), mutations/loss of PTEN (24–44%), and/or genetic mutations in their upstream regulators EGFR (10–20%) and KRAS (8–21%) [45–48]. Agents that target this pathway could potentially shut down survival signaling in NSCLC; however, early phase clinical trials using specific PI3K and/or mTOR inhibitors have yielded disappointing results [47]. The non-exclusive genetic alterations in PIK3CA and EGFR or KRAS have amplified complications associated with the clinical use of PI3K/mTOR inhibitors to NSCLC. Unlike NSCLC, it appears that EGFR and KRAS mutations are extremely rare in SCLC [9, 49]. Differences between NSCLC and SCLC in terms of genetic background could affect the clinical benefits of using PI3K/mTOR pathway inhibitors.
Our results revealed that inhibiting PI3K, AKT, or mTOR signaling induced caspase-mediated proteolytic cleavage of RICTOR and/or RPTOR, albeit to varying degrees (Figs. 2, 3, 4 and 5). The caspase-mediated cleavage of proteins basically ensures the irreversible commitment of cells to apoptosis; therefore, it appears that the proteolytic cleavage of RICTOR and RPTOR could ensure the blockade of the survival signals mediated via mTORC1 and mTORC2. This would prevent intrinsic and acquired resistance mechanisms, which would otherwise limit the therapeutic efficacy of small-molecule inhibitors targeting this pathway. Many of the drugs targeting the PI3K pathway are currently under clinical evaluation; however, only one phase I clinical trial (NCT02069158) is currently investigating the potential use of the dual PI3K/mTOR inhibitor PF-05212384 in combination with paclitaxel and carboplatin for the treatment of SCLC [50]. Our findings in this study using preclinical cell and animal models provide possible avenues by which to accelerate the development of SCLC therapeutic strategies using PI3K inhibitors, which are already in clinical use.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13578-022-00862-y.

Additional file 1: Figure S1. Cell survival of SCLC cell lines treated using PI3K, AKT, or mTOR inhibitors. A, Western blot analysis showing the expression of proteins involved in the PI3K/AKT/mTOR pathway in NCH-446 cells treated using RAD001, Wortmannin, MK-2206, LY294002, and GSK2126458 at indicated concentrations for 24 h. B–D, Percent survival of the thirteen SCLC cell lines in response to treatment using MK-2206 (B), LY294002 (C), and RAD001 (D). Figure S2. Cell survival of SCLC cell lines treated using isoform-selective PI3K inhibitors. A, Compound name and major molecular target of the PI3K inhibitors. B, Percent survival of SCLC cell lines in response to treatment using CAL-101 or BYL719. C, Dose matrix of drug combinations between any two of the following compounds: MK-2206, BYL719, RAD001, and CAL-101. Percent growth inhibition at each dose assessed after drug treatment for 72 h was shown on left. Synergy distribution and synergy scores based on a ZIP model were shown on right. Figure S3. Proteolytic cleavages of RICTOR and RPTOR in NCH-446 cells treated using PI3K/mTOR inhibitors. A, Western blot analysis showing the time-dependent cleavages of RICTOR and RPTOR in NCH-446 cells treated using BY 80-6946 (1 μM) or BYL719 (10 μM) and RAD001 (1 μM) in combination. B, Western blot analysis showing the expression of AKT, p-AKT (S473), RICTOR, and RPTOR in NCH-446 cells treated using BY 80-6946 or NVP-BG722 at indicated concentrations for 24 h. C, Western blot analysis showing the expression of AKT, p-AKT (S473), RICTOR, and RPTOR in high p-AKT (NCH-446 and NCH-187), medium p-AKT (NCH-841), and low p-AKT (NCH-211) cells treated using BY 80-6946 at indicated concentrations for 24 h. The expression levels of c-RICTOR and c-RPTOR were quantified and listed below the profiles in (A), (B) and (C). Figure S4. Activation of caspases in high p-AKT cells via dual PI3K/mTOR inhibition. Western blot analysis showing the cleavage of RICTOR and RPTOR and the expression of caspases in NCH-446 cells treated using Staurosporine (STS, 2 μM) or GSK2126458 (200 and 1000 nm) for 7 h.

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Author contributions

MCH performed Western blot analysis, cell viability assays, in vitro caspase cleavage assays, and prepared the figures. WPW performed Western blot analysis and the experiments on animal xenografts and drug treatment. YHC designed the study, performed cell cultures, analyzed the results, and wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

All data generated in this study have been included in the article and additional files.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

All the listed authors have participated in the study, and have read and approved the submitted manuscript.

Competing interests

The authors declare no potential conflicts of interest.

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