LKB1 Deficiency Renders NSCLC Cells Sensitive to ERK Inhibitors

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ABSTRACT

Introduction: Serine/threonine kinase 11 (LKB1/STK11) is one of the most mutated genes in NSCLC accounting for approximately one-third of cases and its activity is impaired in approximately half of KRAS-mutated NSCLC. At present, these patients cannot benefit from any specific therapy.

Methods: Through CRISPR/Cas9 technology, we systematically deleted LKB1 in both wild-type (WT) and KRAS-mutated human NSCLC cells. By using these isogenic systems together with genetically engineered mouse models we investigated the cell response to ERK inhibitors both in vitro and in vivo.

Results: In all the systems used here, the loss of LKB1 creates vulnerability and renders these cells particularly sensitive to ERK inhibitors both in vitro and in vivo. The same cells expressing a WT LKB1 poorly respond to these drugs. At the molecular level, in the absence of LKB1, ERK inhibitors induced a marked inhibition of p90 ribosomal S6 kinase activation, which in turn abolished S6 protein activation, promoting the cytotoxic effect.

Conclusions: This work shows that ERK inhibitors are effective in LKB1 and LKB1/KRAS-mutated tumors, thus offering a therapeutic strategy for this prognostically unfavorable subgroup of patients. Because ERK inhibitors are already in clinical development, our findings could be easily translatable to the clinic. Importantly, the lack of effect in cells expressing WT LKB1, predicts that treatment of LKB1-mutated tumors with ERK inhibitors should have a favorable toxicity profile.

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Keywords: LKB1/STK11; ERK inhibitor; SCH772984; Ulixertinib; NSCLC

Introduction

Serine/threonine kinase 11 (LKB1/STK11) is one of the most commonly mutated genes in NSCLC accounting for approximately one-third of cases. In addition, its activity is impaired in approximately 50% of KRAS-mutated NSCLC. This large number of patients has an aggressive, chemoresistant subtype of cancer and, at
present has no specific therapy. LKB1 is a serine/threonine kinase that serves as a master regulator of the cell metabolism. The best characterized LKB1 substrate is the protein kinase AMP-activated catalytic subunit alpha 2 (AMPK) and together they control cell metabolism in response to the availability of nutrients. Besides metabolism, both AMPK and LKB1 were reported to have roles in the control of cell growth. LKB1/AMPK pathway activation results in negative regulation of one of the most deregulated signaling systems in cancer, the phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha (PI3K)/AKT/mammalian target of the rapamycin (mTOR) pathway. mTOR is a pivotal protein in mTORC1 and mTORC2 complexes and acts as an integrator of nutrients and growth factor signals, resulting in the control of cell growth in all eukaryotes. These signals act on ribosome biogenesis and the translation of proteins that promote cell growth and division. The PI3K pathway is not the only regulator of mTOR. Extracellular signal-regulated kinase (ERK) and its target, p90 ribosomal S6 kinase (p90RSK), both target mTORC1 activity.

SCH772984, an analog of the orally available clinical candidate SCH900353/MK-8353, has a dual mechanism of action: allosteric inhibition of MEK1/2 binding to ERK, resulting in the lack of ERK phosphorylation and adenosine triphosphate (ATP)-competitive ERK inhibition, resulting in the absence of phosphorylation of its substrates. This compound was described as an extremely selective ERK1/2 inhibitor able to suppress the mitogen-activated protein kinase (MAPK) pathway signaling and cell proliferation in many tumor models. In particular, it has a strong activity in cell lines positive for KRAS and BRAF mutations.

Ulixertinib (BVD-523, VRT752271) is a small molecule under phase I clinical evaluation. It potently and selectively inhibits ERK1/2 kinases in a reversible, ATP-competitive way. Signal transduction, cell survival, and cell proliferation are negatively regulated particularly in cell lines harboring MAPK pathway activating mutations. Similar to SCH772984, ulixertinib inhibits cell growth in BRAF- and in KRAS-mutant tumors.

In the present work, we investigated the activity of SCH772984 and ulixertinib in different NSCLC models with different LKB1 and KRAS status.

Methods

Cell Cultures, Drugs, Growth Assay, Small Interfering RNA, and Plasmids

NSCLC cell lines (H1299, LU99, H460, A549, and H358) were grown in Roswell Park Memorial Institute (RPMI)-1640 supplemented with 10% fetal bovine serum. H1299-derived clones (wild-type [WT] and G12C KRAS) were grown in normal medium including 500 μg/mL of G418 (Gibco, Monza Italy). LKB1 knockout (KO) clones in H1299, LU99, and H358 were generated using LKB1 CRISPR/Cas9 KO Plasmid and HDR Plasmid (Santa Cruz Biotechnology, Santa Cruz, California) according to the manufacturer’s instructions. Cell lines and clones are routinely tested by polymerase chain reaction for mycoplasma contamination and authenticated with the PowerPlex 16 HS System (Promega, Milan, Italy) every 6 months by comparing the short tandem repeat profiles with those deposited in American Type Culture Collection and/or DSMZ-German Collection of Microorganisms and Cell Cultures GmbH databases. SCH772984 (TargetMol, Boston, MA), ulixertinib (TargetMol, Boston, MA), ARQ 751 (ArQule, Burlington, MA), and PIK-75 (Selleckchem, Munich, Germany) DMSO stock solutions were dissolved in medium just before use. In all the cytotoxicity experiments, cells were continuously treated for 72 hours. Drug effects were estimated by the CellTiter AQueous MTS (Promega, Milan, Italy) as reported. The mean and SD of at least three independent experiments are presented. Endoribonuclease-prepared small interfering RNA (EsiRNA) were purchased from Sigma Aldrich (Milan, Italy) and transfected by Lipofectamine 2000 (Invitrogen, Monza, Italy). pcDNA3-FLAG-LKB1 was a gift from Lewis Cantley (Addgene plasmid # 8590). The colony assay was performed as reported.

Western Blotting Analyses

Proteins were extracted and visualized as reported. Immunoblotting was performed with the antibodies listed in the Supplementary Material.

Xenograft Studies

The Istituto di Ricerche Farmacologiche Mario Negri (IRFMN, Milan, Italy) adheres to the principles declared in the Supplementary Material. For the xenograft experiments, 6-week-old female nude athymic mice (Envigo, Bresso, Italy) were housed at constant temperature and humidity, according to institutional guidelines. SCH772984 (ChemieTek, Indianapolis, IN) was dissolved in 50% DMSO, 20% 2-hydroxypropyl-beta-cyclodextrin (Carlo Erba Reagents S.r.l, Cornaredo, Italy) and given intraperitoneally twice a day for 14 days consecutively. ARQ 751 (ArQule, Burlington, MA) was dissolved in 0.5% methylcellulose (Carlo Erba Reagents S.r.l, Cornaredo, Italy) and given orally for 3 days a week for 3 cycles. Additional details are reported in the Supplementary Material.

Genetically Engineered Murine Models of NSCLC and Derived Cell Lines

Conditional transgenic models (KRAS<sup>16ValCAF/L</sup> and LKB1/STK11<sup>-/-;Lkb1<sup>fl/fl</sup></sup>) were purchased from Jackson
Laboratories. KRAS<sup>tm4Tyr/I</sup> (KRAS<sup>G12D/WT</sup>) and LKB1/STK11<sup>tm1Sjm/I</sup> (LKB1<sup>KO/KO</sup>) mice were crossed to generate conditional double KO mice (KRAS<sup>G12D/WT</sup>/LKB1<sup>KO/KO</sup>) which were genotyped using polymerase chain reaction protocols obtained from the Jackson Laboratories. KRAS<sup>G12D/WT</sup> and KRAS<sup>G12D/WT</sup>/LKB1<sup>KO/KO</sup> mice were treated intranasally with Adeno-Cre (SignaGen Laboratories, Rockville, MD, USA) at 6 weeks of age as previously reported. Four weeks after Adeno-Cre treatment, the animals were randomized to receive SCH772984 or vehicle as for xenograft experiments. At the end of treatment, six animals per group were sacrificed and lungs fixed in formalin for histopathologic evaluation.

Details for isolation of cell lines from lung nodules are reported in the Supplementary Material. Two independent clones from KRAS<sup>G12D/WT</sup>/LKB1<sup>KO/KO</sup> lung tumors (KL 7 and KL 95) were isolated and their behavior compared to that of a KRAS<sup>G12D/WT</sup> cell line (KP1.9).

**Magnetic Resonance Imaging Scan**

Before treatment and after 7 days post-treatment, the group of animals for nodule count was analyzed by magnetic resonance imaging using a small rodents 7 Tesla BRUKER scanner (Bruker Biospec 70/30), equipped with a 72 volume coil in transmission and a saddle shaped 2 × 2 surface array for receiving the signal. Details are reported in the Supplementary Material.

**Histopathology**

Lungs were formalin-fixed, paraffin-embedded, and 4-μm thick sections were routinely stained with hematoxylin and eosin. Pulmonary proliferative lesions were classified according to international guidelines. The tumoral burden was assessed by counting the number of pulmonary tumors on an approximately 50 mm<sup>2</sup> of parenchymal area.

**Statistical Analyses**

Statistical analyses were performed using GraphpadPrism version 7. Differences between groups were considered significant when the p values were less than 0.05.

**Results**

While exploring drug responses in NSCLC cell lines in vitro, we found strikingly different responses to the ERK inhibitor SCH772984 in two cell lines, H727 and H1299. In H1299 the drug was completely inactive (concentration that inhibits 50% [IC<sub>50</sub>] > 7 μM), whereas in H727 there was a very good response, with an IC<sub>50</sub> of approximately 0.13 μM (confidence interval [CI]: 0.07–0.24 μM) (Supplementary Fig. 1A). Among the mutations harbored in the two cell lines, our attention was caught on KRAS and LKB1 given that the H727 cell line is mutated in both KRAS and LKB1 whereas these proteins were WT in H1299 (COSMIC database, https://cancer.sanger.ac.uk/cosmic). Because H727 cells werederived from carcinoid, we no longer used this model for subsequent experiments and concentrated our experiments on carcinomas.

**Lack of LKB1 Expression Increases Sensitivity to the ERK Inhibitor SCH772984 in KRAS WT NSCLC Cells by Impairing S6 Ribosomal Protein Activation Through p90RSK Protein**

The H1299 cell line (KRAS WT/LKB1 WT) was genetically manipulated with CRISPR/Cas9 technique to generate a deletion in the LKB1 gene (resulting in lack of protein expression), and we selected two independent LKB1 KO clones (H1299-LKB1 KO 1 and 2) (Fig. 1A). SCH772984 treatment gave a marked, statistically significant response in LKB1-deficient cells compared to the parental cell line (Fig. 1B). Whereas for the latter it was not possible to calculate an IC<sub>50</sub> with the drug concentrations tested, the IC<sub>50</sub> of the two LKB1-deficient clones were, respectively, 2.4 μM (CI: 1.9–3.0 μM) and 2.8 μM (CI: 2.3–3.5 μM). These results were confirmed by using the colony formation assay (Figs. 1C and D).

To corroborate these data with a different approach, LKB1 was silenced with specific LKB1 EsiRNA transfection, which strongly downregulated LKB1 protein expression, whereas universal negative EsiRNA had no effect (Fig. 1E). As in H1299-LKB1 KO clones, transient silencing of LKB1 in H1299 cells induced significant sensitization to SCH772984 compared to cells transfected with the negative EsiRNA (Fig. 1F). In agreement with these data, the re-introduction of LKB1 in the H1299 LKB1 KO clone (Fig. 1G) strongly reduced the activity of SCH772984 to the level of parental H1299 cells, whereas LKB1 overexpression did not alter the behavior of H1299 after drug treatment (Fig. 1H).

To check whether the absence of LKB1 increased the sensitivity to SCH772984 in vivo, both LKB1 WT and KO cells were subcutaneously implanted into nude mice. Although the LKB1 KO clone took much longer (40 days) to reach randomization weight than the LKB1 WT cell line (19 days), their growth rates from this point were comparable. SCH772984 treatment induced a tumor growth reduction in LKB1 KO clone reaching statistical significance on day 19 (p < 0.05) and 21 (p < 0.01) after treatment start, with a best treated/control ratio (T/C) of 33% on day 16 (Fig. 1J). The same treatment schedule in LKB1 WT expressing tumors had a best T/C of only 77% on day 9 and did not reach a significant reduction at any time during the experiment (Fig. 1J). SCH772984...
was well tolerated and there was no significant reduction in the animals’ body weights (Supplementary Fig. 1B). The drug reached and inhibited its target in both clones, as shown by the similar reduction of ERK phosphorylation 6 hours after drug administration (Fig. 1K).

To clarify the molecular mechanism responsible for the selective activity of SCH772984 in LKB1 deleted cells, we analyzed the MAPK pathway and mTOR downstream effector activation in cells growing in culture (Fig. 2A). ERK phosphorylation was abolished at 6 hours and reappeared 24 hours after treatment in both WT and LKB1 deleted cell lines. In both cells the drug induced phosphorylation of AKT. The phosphorylation status of p70S6K, the direct target of mTOR, was qualitatively comparable for both cells. However, the activation of the S6 ribosomal protein (S6) was strongly reduced by SCH772984 in LKB1 KO cells but only slightly perturbed in the LKB1 WT counterpart. Because the activation of p70S6K was comparable, we investigated the role of p90 ribosomal S6 kinase (p90RSK) given it is an ERK target and regulates S6. The phosphorylation at both investigated sites was almost abolished only in cells deleted in LKB1. To further address the role of p90RSK as a mediator of the SCH772984 response, we used CRISPR/Cas9 technology to generate H1299 clones lacking the p90RSK1 protein.
expression. Figure 2B shows the expression of p90RSK1 in the heterozygous (H1299-RSK K016) and the homozygous (H1299-RSK K09 and H1299-RSK K031) deleted clones. The lack of p90RSK1 resulted in cell responses to the ERK inhibitor (IC$_{50}$ H1299-RSK K09 = 5.70 µM, CI: 5.04–6.55 µM; and IC$_{50}$ H1299-RSK K031 = 6.03 µM, CI: 5.60–6.50 µM) comparable to those obtained by the loss of LKB1 (IC$_{50}$ = 4.04 µM, CI: 3.57–4.56 µM) (Fig. 2C). The p90RSK1 heterozygous clone’s response was comparable to that observed in the parental cell line (IC$_{50}$ >5 µM for both). Molecular analyses indicated that homozygous loss of p90RSK1 impaired the phosphorylation of S6 even when the WT LKB1 was expressed (Fig. 2D).

In vivo too, the inhibition of S6 was noteworthy in the LKB1 KO-derived tumor but not in the parental-derived tumor (Fig. 2E).

**KRAS Mutated NSCLCs Deficient in LKB1 Are Sensitive to the ERK Inhibitor SCH772984**

To investigate the activity of SCH772984 in cells harboring both a KRAS mutation and an impairment of LKB1, we generated further ad hoc isogenic cell systems. We selected the already available H1299-derived isogenic system expressing WT or G12C KRAS and applied the CRISPR/Cas9 system, targeting the LKB1 gene.¹⁸⁻²¹ We selected the KRAS(WT)/LKB1 KO and KRAS(G12C)/LKB1 KO clones.²² The presence of a mutant KRAS did not change the response to SCH772984 which remained negligible (IC$_{50}$ >3 µM). In contrast, treatment of LKB1-deleted clone resulted in a marked response (IC$_{50}$ KRAS WT/LKB1 KO = 0.31 µM, CI: 0.24–0.41 µM; and IC$_{50}$ KRAS G12C/LKB1 KO = 0.87 µM, CI: 0.71–1.07 µM) (Fig. 3A).
We then extended our findings in a cell model derived from lung tumors developed in GEMMs expressing mutated KRAS (KP1.9) or mutated KRAS and LKB1 loss (KL7 and KL95). As reported in Figure 3B, the treatment of KL cells resulted in a strong response (IC50 KL7 cells = 1.20 μM, CI: 1.14–1.26 μM; IC50 KL95 cells = 0.49 μM, CI: 0.45–0.53 μM) whereas the same treatment was ineffective in KP cells (IC50 > 10 μM). The transient re-introduction of LKB1 in the KL95 clone (Fig. 3C) reduced the activity of SCH772984 (Fig. 3D).

The same cells were implanted subcutaneously in the syngeneic C57Bl/6J mice. The KL tumors treated with SCH772984 showed a tumor weight reduction compared to the control groups reaching statistical significance on days 9, 12, and 15 (p < 0.0001) after treatment start, with a best T/C of 39% on day 9 (Fig. 3E). The KP tumors had a best T/C of only 77% on day 9 and did not reach a statistically significant reduction at any time in the experiment (Fig. 3F). SCH772984 was well tolerated and there was no significant reduction in the animals’ body weights (Supplementary Fig. 1C). Also, in this in vivo model there was a similar inhibition of ERK phosphorylation in tumor samples from both KP and KL mice, whereas the reduction in S6 phosphorylation could be appreciated only in KL tumors, thus confirming previous data (Fig. 3G).

To corroborate these data, we moved to the well-characterized Cre-inducible transgenic NSCLC model KRASG12D/WT/LKB1KO/KO. Four weeks after viral induction, mice were randomized to receive SCH772984 or vehicle. By magnetic resonance imaging scan we were able to detect a clear effect of the drug (Supplementary Fig. 2), and 7 days after treatment start six mice per group were sacrificed and lungs histologically examined to determine the tumoral burden. Histologic proliferative lesions were represented by multifocal areas of alveolar hyperplasia and multiple adenomas. The mean number of adenomas counted in SCH772984 treated group was 2.5 ± 0.34 compared to 7 ± 0.63 in the vehicle group (p < 0.0001) (Fig. 3H).

To further extend these data in physiologically KRAS-mutated cells, LKB1 was selectively knocked down in mutated KRAS NSCLC cell lines proficient in LKB1. Considering the previous data, which strongly

Figure 3. KRAS-mutated NSCLC murine models deficient in LKB1 are sensitive to the ERK inhibitor SCH772984. A, Activity of SCH772984 in KRAS (wild-type [WT]) or KRAS(G12C) H1299 and their isogenic LKB1 KO clones. B, Activity of SCH772984 in KP or KL cells. C, Expression of LKB1 in KL95 cells transiently transfected with pCDNA3/LKB1 or pCDNA3 empty vector. Ran was used as loading control. D, Activity of SCH772984 in KL95 cells transfected with pCDNA3 empty vector (upper panel) or pCDNA3/LKB1 (lower panel). E, Tumor growth inhibition on KL cells injected mice (n = 8) treated with SCH772984 25 mg/kg or vehicle. ****p < 0.0001 SCH772984 versus vehicle. F, Tumor growth inhibition on KP cells injected mice (n = 8) treated with SCH772984 25 mg/kg or vehicle. G, Activation of ERK and S6 in KP and KL tumors removed from mice 6 hours after the last treatment. Actin was used as loading control. H, Mean number of counted masses in lungs of KL mice treated with SCH772984 (n = 6) or vehicle (n = 6).
suggest that the mTOR pathway is involved in the action of SCH772984, we searched for cells with WT LKB1 and not mutated PI3K/AKT/mTOR pathway. However, all of the analyzed NSCLC cell lines harbored a mutation which leads to a constitutive activation of the PI3K/AKT/mTOR pathway which, according to our prediction, could prevent the activity of SCH772984.

The LU99 cell line harbors an activating mutation (p.T1025A) in the PIK3CA gene encoding the PI3K catalytic isoform p110α (COSMIC database). This cell line (KRAS mut/LKB1 WT) was used to generate isogenic clones deleted in LKB1 (Fig. 4A). As predicted, SCH772984 resulted in an equal lack of response in clones and in the parental cell line (Supplementary Fig. 3A). Co-treatment of the LU99 system with the PI3K p110α-specific inhibitor PIK-75, using a concentration able to kill less than 15% of cells when used as single agent (Supplementary Fig. 3B), restored the sensitivity of LKB1-deficient cells to the ERK inhibitor (IC50 = 0.28 μM; CI: 0.16–0.42 μM) whereas no effect was observed for the parental line (IC50 > 5 μM) (Fig. 4B). To corroborate these results, the PI3K downstream effector AKT was inhibited with ARQ 751 using a concentration able to kill less than 15% of cells when used as single agent (Supplementary Fig. 3C) but able to inhibit the target as shown by the phosphorylation reduction of the AKT effector PRAS40 (Fig. 4C). Again, we restored the sensitivity of LKB1-deficient cells to SCH772984 (IC50 = 0.93 μM; CI: 0.35–1.57 μM) (Fig. 4B). From a molecular point of view, as expected, SCH772984 was able to inhibit ERK phosphorylation in both cell lines and a slight S6 inactivation in LKB1 KO cells. The combination SCH772984/ARQ 751 almost completely abolished the S6 activation in the responsive cells only (Fig. 4C).

We performed the same experiments in the H358 (KRAS mut/LKB1 WT) isogenic system, which is mutated in the PHLP2 gene (p.D724Y) (COSMIC database). This gene encodes for a phosphatase involved in the regulation of PI3K signaling, in particular acting on ser473 of AKT.23 Knocking down LKB1 in H358 (Supplementary Fig. 3D) did not significantly alter the sensitivity to SCH772984 (Supplementary Fig. 3E). As predicted, co-treatment with the AKT inhibitor ARQ 751, using a dose able to kill less than 15% of cells when used as single agent but able to inhibit the target (Supplementary Figs. 3F and G) strongly sensitized LKB1 KO (IC50 = 0.40 μM; CI: 0.23–0.71 μM) but not LKB1 WT cells (IC50 > 3 μM) (Fig. 3H) to SCH772984. The transient re-introduction of LKB1 in the H358 LKB1 KO clone (Supplementary Fig. 3I) strongly reduced the activity of SCH772984 (Supplementary Fig. 3J).

These findings were extended in vivo using LU99 model where the combination ARQ 751/SCH772984 induced a tumor growth reduction in LKB1 KO clone reaching statistical significance on days 14 (p < 0.05) and 16 (p < 0.001) after treatment start, with a best T/C of 33% on day 10 (Fig. 4D). The LKB1 WT expressing cells had a best T/C of 58% on day 5 and did not reach a significant reduction at any time in the experiment (Fig. 4E). Mice bearing WT cells were sacrificed before the end of the scheduled treatment because the maximum average tumor volume allowed (1500 mg) was reached. All treatments were well tolerated and there was no significant reduction in the animals’ body weights (Supplementary Figs. 3K and L).

Finally, we verified the activity of the ERK inhibitor in two non-manipulated cell lines, A549 and H460, where the expression of LKB1 is naturally lost. Both cell lines, in addition to the LKB1 nonsense mutation (p.Q37*), harbor activating mutations in the KRAS gene (A549: p.G12S; H460: p.Q61H) and mutations activating the PI3K pathway (A549: CPPED1 p.Q188H involved in the regulation of AKT, in particular acting on ser47324; H460: PIK3CA p.E545K; all mutations are described in COSMIC database). As expected, SCH772984 alone was not effective in either cell lines (IC50 > 7.5 μM) but the combination of the ERK inhibitor with the AKT inhibitor ARQ 751 induced a cell response (IC50 A549 = 1.20 μM, CI: 0.97–1.47 μM; and IC50 H460 = 1.85 μM, CI: 1.38–2.52 μM) (Fig. 4F). SCH772984 alone was able to inhibit ERK phosphorylation in both cell lines but had no effect on S6 regulation. The combination SCH772984/ARQ 751 almost completely abolished the activation of S6 (Fig. 4G).

**Ablation of LKB1 Sensitizes NSCLC Cells to the ERK Inhibitor Ulixertinib**

To corroborate and generalize our findings, avoiding a peculiar mechanism of SCH772984, we treated cells with ulixertinib, a different ERK inhibitor. All the isogenic systems described above were treated with the second ERK inhibitor alone or with the drug combinations that were previously shown to be active.

H1299 and the LKB1 KO-derived clone were treated with ulixertinib and the lack of LKB1 resulted in a marked response to the drug whereas cells harboring WT LKB1 were resistant to the treatment (Supplementary Fig. 4A). Again, ulixertinib treatment of the H1299 system expressing different mutant isoforms of KRAS and the derived LKB1 KO clones resulted in responses of cells deficient for LKB1 expression, independently from KRAS status, whereas WT LKB1 cells did not respond to the ERK inhibitor (Supplementary Fig. 4B).
Similarly, only the KRAS-mutated LU99-derived clone deleted in LKB1, but harboring a PIK3CA gene mutation responded to ulixertinib when combined with PIK-75, whereas ulixertinib alone was not active (Supplementary Fig. 4C), in accordance with what was found for SCH772984. The results were the same when AKT was inhibited with ARQ 751 (Supplementary Fig. 4D). In the H358 cell line, the combination of ulixertinib with the AKT inhibitor ARQ 751 resulted in a response of the LKB1 KO clone, whereas the parental cell
It has been reported that the tumor phenotype with high prevalence in mouse models, in combination with mutant KRAS leads to an aggressive invasion, and metastasis. Our data suggest that ERK inhibition in murine lung tumors promotes tumor burden, concentration is low. One of the main activities of AMPK is the inactivation of mTOR when ATP levels are too low to sustain protein synthesis and cell growth. In this scenario, the lack of LKB1 leads to dysregulation of cellular processes such as cell growth and metabolism under energy stress conditions, resulting in particular sensitivity to treatments that target energy pathways. Our idea, therefore, was to target the MAPK pathway, another signaling pathway involved in cell proliferation, metastatization and metabolism, closely connected to the LKB1/AMPK/mTOR pathway. Consistent with our hypothesis, LKB1-deficient cells were particularly sensitive to the inhibition of ERK, a protein belonging to the MAPK pathway.

Approximately 25% to 30% of NSCLC patients present with KRAS activating mutations. Approximately 50% of the NSCLC tumors with activating KRAS mutations also harbor LKB1 inactivating mutations. Current estimates suggest that at least 10% of all NSCLCs are comutated for both KRAS and LKB1. LKB1 deficiency in combination with mutant KRAS leads to an aggressive tumor phenotype with high prevalence in mouse models. It has been reported that the KRAS/LKB1 double mutation in murine lung tumors promotes tumor burden, invasion, and metastasis. Our data suggest that ERK inhibition could also be effective in tumors harboring KRAS/LKB1 double mutation, thus increasing the therapeutic strategies for this prognostically unfavorable subgroup of patients for whom no targeted therapy is currently available.

We found that ERK inhibition was likely to act through inhibition of the mTOR downstream pathway, as S6 phosphorylation was selectively reduced in cells sensitive to the ERK inhibitors. This is not surprising because there is evidence of connections between the MAPK, PI3K/mTOR, and LKB1 pathways. From this, we hypothesized that constitutive activation of the parallel PI3K/mTOR pathway would abolish the selective activity of ERK inhibitors observed in LKB1 KO cells. The experiments in cell lines with activated PI3K or AKT clearly showed that this was the case. Using specific inhibitors targeting PI3K (PIK-75) and AKT (ARQ 751) at concentrations able to inhibit the targets without inducing cytotoxicity when used as single agent, we showed that LKB1 KO cells, but not LKB1 WT cells, were selectively sensitized to ERK inhibitors. A recently published paper shows that the dual inhibition of mTOR and PI3K can be a promising therapeutic strategy for LKB1-deficient tumors. These data are in line with our results where we inhibited the PI3K and mTOR target S6 through the inhibition of ERK/p90RSK/S6 axis.

These results are potentially and easily translatable to the clinic where the combination of PI3K inhibitors and ERK inhibitors would selectively kill LKB1-deficient (tumor) cells but not LKB1 WT (normal) cells. On the other hand, we were not able to find a panel of PI3K pathway WT NSCLC cell lines to further proof that loss of LKB1 is sufficient to sensitize cells with different mutational background to ERK inhibitors.

Although SCH772984 is not currently under clinical evaluation, we showed that the results obtained with this inhibitor were also true for another molecule, ulixertinib, an ERK inhibitor already in clinical development together with other ERK inhibitors.

In conclusion, our data suggest that the lack of a functional LKB1, now associated with a poor prognosis for patients with NSCLC, could offer a strong therapeutic advantage. The lack of effect in cells expressing WT LKB1 suggests that treatment of LKB1-mutated tumors with ERK inhibitors should have a low toxicity profile. In this context, we must always consider the status of PI3K/AKT/mTOR pathway, given that the activation of the latter renders unsuccessful the ERK inhibitor treatment. This limitation can be easily overcome considering a combination treatment with, at least, PI3K and AKT inhibitors. Although PI3K inhibitors were described to have several limitations due to the emergence of dose-limiting for the presence of adverse effects, our data open the possibility to reconsider these drugs for the use in combinations with ERK inhibitors, given that these inhibitors should be used at low doses (sufficient to inhibit the target) and hence likely not to induce severe toxicity. On the other hand, the analysis of data available in the cBioPortal revealed that, although the NSCLC LKB1 loss cell lines are 100% mutated in the PI3K/AKT/mTOR pathway, the 60% to 85% of NSCLC patients mutated in LKB1 do not present additional mutations in genes belonging to the PI3K/AKT/mTOR pathway, rendering the majority of LKB1-mutated patients suitable for a monotherapy treatment.

LKB1 mutation in NSCLC is associated with loss of protein. WT LKB1 expression is regulated both at transcriptional and post-transcriptional level. Any treatment that downregulates LKB1 expression (as we
showed using siRNAs in cell cultures) should result in sensitization to ERK inhibitors. Transient and specific (in tumor) \( \text{LKB1} \) downregulation in NSCLC is potentially feasible, for example by targeted delivery of small interfering RNAs or microRNAs in the lung, thus increasing the number of the patients who could potentially benefit from this treatment. The selective advantage for NSCLC patients with \( \text{LKB1} \)-mutated tumors might therefore possibly be extended to other NSCLC patients with WT \( \text{LKB1} \).

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**Supplementary Data**

Note: To access the supplementary material accompanying this article, visit the online version of the *Journal of Thoracic Oncology* at [www.jto.org](http://www.jto.org) and at [https://doi.org/10.1016/j.jtho.2019.10.009](https://doi.org/10.1016/j.jtho.2019.10.009).

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