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RESEARCH ARTICLE

The comparison between dual inhibition of mTOR with MAPK and PI3K signaling pathways in KRAS mutant NSCLC cell lines

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Abstract KRAS mutations are found in 15–25 % of patients with lung adenocarcinoma, and they lead to constitutive activation of KRAS signaling pathway that results in sustained cell proliferation. Currently, there are no direct anti-KRAS therapies available. Therefore, it is rational to target the downstream molecules of KRAS signaling pathway, which are mitogen-activated protein kinase (MAPK) signaling pathway (RAF-MEK-ERK) and PI3K pathway (PI3K-AKT-mTOR). Here, we examined the inhibition of both these pathways alone and in combination and analyzed the anti-proliferative and apoptotic events in KRAS mutant NSCLC cell lines, A549 and Calu-1. Cytotoxicity was determined by MTT assay after the cells were treated with LY294002 (PI3K inhibitor), U0126 (MEK inhibitor), and RAD001 (mTOR inhibitor) for 24 and 48 h. The expression levels of p-ERK, ERK, AKT, p-AKT, p53, cyclinD1, c-myc, $p27^{kip1}$, BAX, BIM, and GAPDH were detected by western blot after 6 and 24 h treatment. Although PI3K/mTOR inhibition is more effective in cytotoxicity in A549 and Calu-1 cells, MEK/mTOR inhibition markedly decreases cell proliferation protein marker expressions. Our data show that combined targeting of MEK and PI3K-AKT with mTOR is a better option than single agents alone for KRAS mutant NSCLC, thus opening the possibility of a beneficial treatment strategy in the future.

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Abbreviations

NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer
PIP3	Phosphatidylinositol 3, 4, 5-triphosphate
PIP2	Phosphatidylinositol 4, 5-diphosphate
mTORC1	mTOR1 complex
TSC2	Tuberous sclerosis protein 2
MAPK	Mitogen-activated protein kinase
MEK1/2	MAP-ERK kinases 1 and 2
BEZ235	Dual PI3K and mTOR inhibitor

Introduction

According to the estimated cancer statistics for 2014, respiratory system cancers cause more than one quarter of all cancer-related deaths in males and females [1]. Lung cancer is classified into three main groups: non-small cell lung cancer (NSCLC), small cell lung cancer (SCLC), and carcinoids. NSCLC is further partitioned into adenocarcinoma, squamous cell carcinoma, and large-cell carcinoma [2]. Characterization of genetic mutations in tumors is essential for understanding cancer biology and also for designing chemotherapy. There have been several somatic mutations associated with NSCLC subtypes, which contributed to lung tumor development. In NSCLC, while EGFR and KRAS mutations and EML4-ALK rearrangements are associated with adenocarcinoma, FGFR2 and DDR2 mutations and SOX2 and FGFR1 amplifications are associated with squamous cell carcinoma [3].

RAS signaling (KRAS, HRAS, and NRAS) is frequently deregulated in one third of human cancers. KRAS protein

contains activating point mutations in codons 12, 13, and 61 those that prevent the switch between GTP and GDP and lead to constitutive activation, which no longer requires ligand binding [4, 5]. The KRAS proto-oncogene encodes a 21-kDa small GTPase, which cycles between on (GTP-bound active) and off (GDP-bound inactive) conformations [6]. KRAS mutations are found nearly in 25 % of patients with lung adenocarcinoma and associated with a history of tobacco use, 85 % of which affect codon 12. These mutations lead to constitutive activation of KRAS signaling pathway that results in sustained cell proliferation and is associated with poor progression of NSCLC [7, 8]. Currently, there are no direct anti-KRAS therapies available. Therefore, it is rational to target the downstream molecules of KRAS pathway in order to inhibit intracellular RAS signaling.

The downstream pathways of KRAS activation, mitogen-activated protein kinase (MAPK) signaling pathway (RAF-MEK-ERK), and PI3K pathway (PI3K-AKT-mTOR), are crucial for cell survival and proliferation in NSCLC. PI3K-AKT-mTOR signaling is a well-characterized and notable pathway for the proliferation and transmission of anti-apoptotic signals in cell survival. When signals reach PI3K from growth factor receptors, PI3K generates phosphatidylinositol 3, 4, 5-triphosphate (PIP3) from phosphatidylinositol 4, 5-diphosphate (PIP2). PIP3 can activate PDK1 and also binds to the PH domain of AKT and translocates it to the plasma membrane [9]. AKT is phosphorylated at T308 by PDK1 in kinase domain and at S473 by mTORC2 in regulatory domain. The phosphorylation of AKT transmits signals to its downstream molecules, which regulate cell cycle, cell death, adhesion, and migration and inhibit pro-apoptotic molecules [10, 11]. LY294002 is an inhibitor of PI3K which has been demonstrated to lead inactivation of AKT by blocking phosphorylation at T308 and S473, thereby inducing apoptotic cell death [12, 13].

AKT can also activate mTOR1 complex (mTORC1) by mediating the inhibitory phosphorylation of its negative regulators, tuberous sclerosis protein 2 (TSC2), and PRAS40. A rapamycin derivative RAD001 inhibits mTORC1 in many cancer types [14–16]. However, inhibition of mTORC1 has been shown to activate AKT by phosphorylating S473 in a negative feedback loop mechanism mediated by the mTORC1–S6K-induced phosphorylation of IRS1 [17]. On the other hand, dual mTORC kinase inhibitors such as AZD8055 block both mTOR1 and mTOR2 complexes; thus, mTORC2 cannot phosphorylate S473 and activate AKT. However, these inhibitors also relieve another feedback mechanism and cause PI3K activation, which leads to AKT T308 phosphorylation [18]. Thus, the dual targeting of PI3K/mTOR will be a better approach for blocking downstream signaling and inducing cancer cell death in KRAS mutant NSCLC cell lines.

MAPK signaling pathway is triggered by growth factors or activating mutations of its molecular members. When RAS couples are signaled from cell surface or activated by point mutations, it induces translocation of RAF (MAPK) proteins to the cell membrane. Once bound to RAS, RAF kinases will be activated by phosphorylation or dimerization [19]. Activated MAPKs induce MAP-ERK kinases 1 and 2 (MEK1/2) by phosphorylating Ser218 and Ser222 residues in the activation loop [20, 21]. MEK1/2 can be inhibited by U0126 which is a highly selective pharmacological agent for both MEK1 and MEK2 [22]. Activated MEK1/2 phosphorylates Thr202/Tyr204 residues in the activation loop of p44 and p42 MAP kinases (ERK1/2). ERKs phosphorylate several cytoplasmic and nuclear targets, including transcription factors. The targets of MAPK/ERK signaling regulate several intracellular processes such as proliferation, survival, differentiation, migration, and angiogenesis [4, 19, 23]. However, inhibition of mTORC1 with rapamycin and its analogs leads to MAPK pathway activation through a PI3K-dependent feedback mechanism in cancer cells [24].

In this study, we examined the inhibition of both PI3K-AKT and MAPK pathways alone or in combination with mTORC1 inhibitor and analyzed the anti-proliferative and apoptotic events in KRAS mutant NSCLC cell lines, A549 and Calu-1. Because both the PI3K-AKT-mTOR and MAPK signaling pathways generate anti-apoptotic signals, the combined inhibition of mTOR and either PI3K or MAPK pathways will be better for inhibiting survival and have greater promise than a single agent in the treatment of KRAS mutant NSCLC.

Materials and methods

Cell culture

The human KRAS mutant NSCLC cell lines, A549 and Calu-1, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), L-glutamine, 100 U/ml penicillin, and 50 μg/ml streptomycin in a humidified 5 % CO_2 incubator at 37 °C. The cells were plated in 75-cm^2 flasks and subcultured when they reach 70 % confluence using 0.05 % trypsin-EDTA solution. The PI3K inhibitor LY294002 and the MEK inhibitor U0126 were purchased from Cell Signaling (USA). The mTOR inhibitor RAD001 was a gift from Dr. H. Ilke Onen, which was purchased from Fluka, Hamburg, Germany. All the inhibitors were dissolved in DMSO.

Cell viability assay

Cells were plated 5000 per well in 96-well plates in DMEM+ 10 % FBS and allowed to grow for 24 h. After the attachment to the plate surface, they were incubated with LY294002, U0126, and RAD001 alone or in combination. Cell viability (cytotoxicity) was determined by MTT assay. Following the treatment with inhibitors, cells were incubated with 0.5 mg/ml MTT. After 4 h of MTT incubation, 100 μl crystal dissolving

buffer was added and cells were shaken for 5 min. The absorbance was read at a microplate reader. Equal volume of DMSO in inhibitors was used as control. Experiments were done four times for each inhibitor concentration.

Western blot analysis

A549 and Calu-1 cells were plated 5×10^5 per well in 6-well plates; the following day, cells were treated with inhibitors alone or in combination or equal volume of DMSO for 6 and 24 h. Cells were washed by ice-cold PBS once and lysed in 2× Laemmli sample buffer supplemented with a phosphatase and protease inhibitor cocktail. Lysates were sonicated and the protein concentration was determined using bicinchoninic acid (BCA) protein assay. An equal amount of protein was loaded, and proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h in blocking buffer (5 % milk, 1× Tris-buffered saline (TBS), 0.1 % Tween 20) and incubated with the primary antibodies those that are diluted in 5 % bovine serum albumin, 1× TBS, 0.1 % Tween 20 overnight at 4 °C. The following day, membranes were washed 3× in washing buffer (1× TBS, 0.1 % Tween 20) and placed into secondary HRP-conjugated anti-rabbit antibody. Chemiluminescent detection was done using an ECL reagent. Primary antibodies against p-AKT (#4060), AKT (#4691), p-ERK (#4370), ERK (#4695), BAX (#2772), BIM (#2819), p27 (#3686), c-myc (#5605), cyclinD1 (#2922), and GAPD H (#5174) were obtained from Cell Signaling (Danvers, MA).

Statistical analyses

Possible associations between treated groups and control groups were analyzed with Sigma Stat statistical software using *t* test. *P* values <0.05 were considered as statistically significant.

Results

In proliferation and immunoblotting assays, we chose the IC50 concentrations of all MAPK and PI3K signaling pathway inhibitors, which was determined by producers. In proliferation assays, although U0126 (10 μM), LY294002 (10 μM), and RAD001 (100 nM) decreased cell viability in Calu-1 cells, the inhibitors did not affect cell viability significantly in A549 cells at 24 h. However, the combination of RAD001 with U0126 and LY294002 resulted in higher cytotoxic activity than single agents alone at 24 h in both cell lines. Also, LY294002 and RAD001 combination was more toxic than U0126 and RAD001 combination in A549 and Calu-1 cells at 24 h (Fig. 1).

Interestingly, cell viability was higher in LY294002+ RAD001 and U0126+RAD001 combination treatments when compared to single agents in A549 cells at 48 h. In Calu-1 cells, combination treatment decreased viability more effectively than single agents but the results were not significant at 48 h.

Western blot analysis of A549 cells revealed that p-AKT was not inhibited by LY294002 alone or in combination with RAD001 at 6 and 24 h. However, p-ERK was inhibited by U0126 alone and U0126+RAD001 combination at 6 and 24 h, followed by inhibiting the activations of its downstream molecules, including cyclinD1 and c-myc. Also, c-myc expression was decreased with LY294002+RAD001 combination at 6 and 24 h, which can correlate with their cytotoxic effect. $p27^{kip1}$ expression was slightly increased with the application of all inhibitors alone or in combination at 6 h, but only increased with U0126 and RAD001 treatment at 24 h. The apoptotic protein BIM expression was generally upregulated by all inhibitors alone or in combination at 6 and 24 h. But, another apoptotic protein BAX expression was only increased with RAD001 treatment at 6 h. However, BAX expression was upregulated by all inhibitors at 24 h in A549 cells (Fig. 2).

When Calu-1 cells are treated with MAPK and PI3K signaling pathway inhibitors, immunoblots showed p-AKT signaling was only decreased with LY294002 and LY294002+RAD001 combination at 6 h but interestingly increased by all inhibitors in 24 h. On the other hand, p-ERK was downregulated by U0126 alone and U0126+RAD001 combination at 6 h, but it was also inhibited by all inhibitors at 24 h. BAX protein expression was increased at 6 h by all inhibitors but only increased by LY294002 and LY294002+RAD001 combination at 24 h. Also, $p27^{kip1}$ expression was upregulated by all inhibitors at 6 and 24 h. Interestingly c-myc expression was decreased by all inhibitors at 6 h but only reduced by LY294002 alone and LY294002+RAD001 combination which was inversely correlated with BAX expression at 24 h. CyclinD1 expression was diminished by U0126, U0126+RAD001, and LY294002+ RAD001 treatments at 6 and 24 h in Calu-1 cells (Fig. 3).

According to our results, although the cell lines used in this study was different from mutations in any other genes (Table 1), PI3K/mTOR inhibition is more effective in cytotoxicity. However, in molecular level, MEK/mTOR inhibitor combination is a better option than PI3K/mTOR inhibitor combination in A549 and Calu-1 cells. These findings suggest that the combination of MEK and mTOR inhibitors and PI3K and mTOR inhibitors compared with either drug alone can inhibit cell proliferation, induce apoptosis, and affect downstream signaling pathways.

Discussion

The cells proliferate in an uncontrolled manner by unregulated survival signals, which could proceed to malignancy with

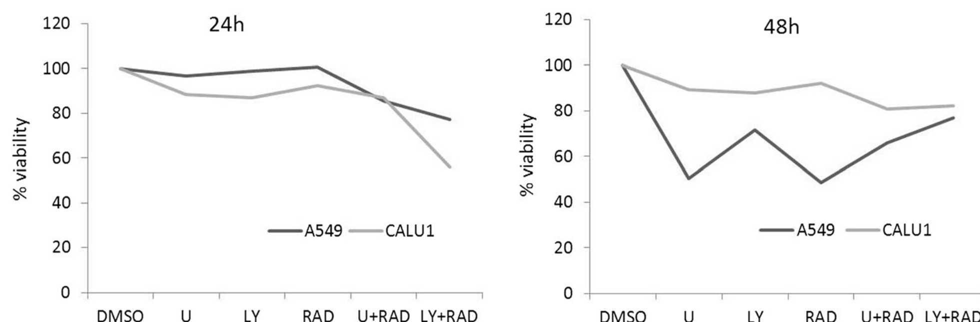

Fig. 1 The anti-proliferative effects of U0126, LY294002, and RAD001 as a single agent and combination in KRAS mutant cells for 24 and 48 h

additional mutations. Cancer cells can invade surrounding normal tissues and spread through the body, that makes cancer difficult to treat. PI3K and MAPK pathways generate survival signals and are commonly upregulated in human cancers. Therefore, the molecular members of these pathways have become popular targets for cancer therapy. Targeted therapy blocks the specific molecule of interest that is involved in the growth, progression, or metastasis of cancer. However, there is no specific inhibitor developed for KRAS, which is mutated in almost one quarter of all malignancies.

KRAS mutant cancers generally exhibit resistance to targeted therapies. This may be due to their cross-talk activation. In addition to their individual signaling programs, the MAPK and PI3K-AKT pathways can regulate each other's activity negatively or positively. While ERK phosphorylates GAB1 and inhibit PI3K signaling [25], AKT can also phosphorylate and inhibit RAF [26, 27]. On the other hand, ERK can positively regulate PI3K-AKT signaling by phosphorylating Raptor and activate mTORC1 [28]. There are several agents developed to inhibit PI3K, AKT, or mTOR alone or in combination. In our study, we used MEK inhibitor and mTOR inhibitor alone and in combination thereby trying to inhibit both MAPK and PI3K-AKT-mTOR pathways. Although they both have KRAS mutation, the cell lines used in this study behave differently to inhibitors due to their mutational status in other genes.

In our proliferation assays, combination of RAD001 (100 nM) with U0126 (10 μM), and LY294002 (10 μM) resulted in higher cytotoxic activity than single agents alone at 24 h in both cell lines. Similar to our results, Iida et al. found RAD001 and U0126 combinations have synergistic effects in cell proliferation and migration in neuroendocrine tumor cell lines [29].

It has been shown that there is a negative feedback loop between AKT and mTOR signaling. When mTORC1 is inhibited by rapamycin or its derivatives, mTORC2 phosphorylates and activates AKT on Ser473 position [17, 30]. It has been shown that although RAD001 inhibited mTOR downstream activity, it induces p-AKT (Ser473) activation in breast cancer cells. The addition of LY294002 to RAD001 improved the anti-tumor effects and decreased p-AKT (Ser473) activity [31]. The same as this feedback mechanism in our study, RAD001 treatment resulted in AKT activation at 6 h in Calu-1 cell line. On the other hand, although p-AKT was upregulated with almost all inhibitors at 24 h in both cell lines, it was inhibited by LY294002 and RAD001+LY294002 in A549 and Calu-1 cells at 6 h. The upregulation of p-AKT with the inhibitors at 24 h might be a cause of confluency difference between the control group and treated groups, suggesting that decreasing cell number due to inhibitors in the wells may influence intracellular signaling [32, 33]. Also, Ishibe et al. showed while confluent cells lose AKT activation,

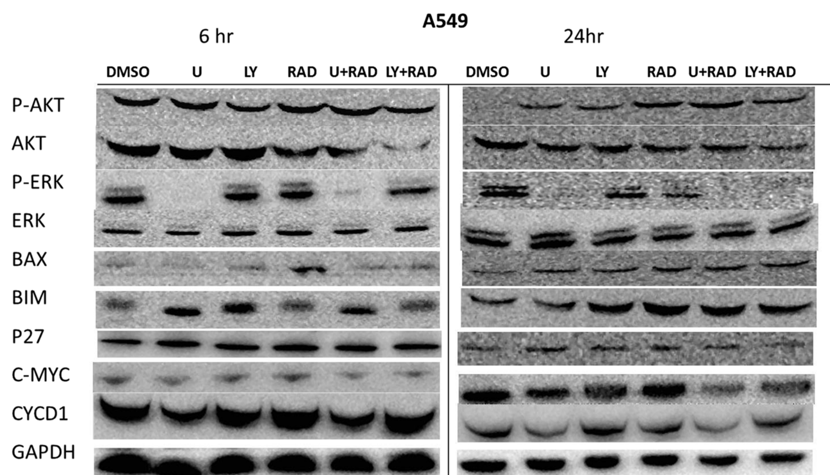

Fig. 2 A549 cells were treated with U0126, LY294002, RAD001, U0126+RAD001, and LY294002+RAD001 for 6 and 24 h. The cells were lysed and western blots were probed with indicated antibodies

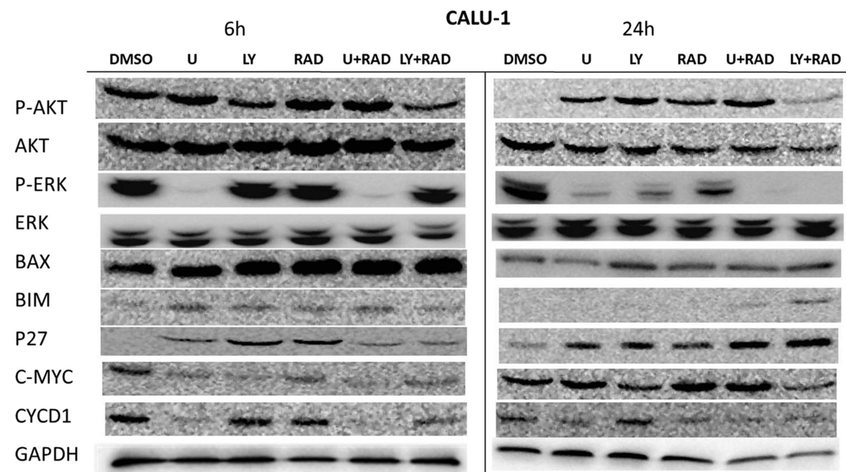

Fig. 3 Calu-1 cells were treated with U0126, LY294002, RAD001, U0126+RAD001, and LY294002+RAD001 for 6 and 24 h. The cells were lysed and western blots were probed with indicated antibodies

nonconfluent cells have high levels of AKT activation following HGF stimulation [32]. In our study, p-AKT signaling was diminished due to increased cell number in control groups in both cell lines.

LY294002 and RAD001 combination was effective on cyclinD1 and c-myc expression at 24 h which can correlate with their cytotoxic effect in our study. Besides, the same with Gao et al.'s [34] and Ko et al.'s [35] study, cyclinD1 was downregulated at 6 and 24 h with U0126 in both cell lines. Withal, we observed RAD001+U0126 combination have inhibitory effects on cyclinD1 expression in A549 and Calu-1 cell lines. Although c-myc was inhibited with LY294002 and LY294002+RAD001 combination in both cell lines at 24 h, we did not observe c-myc inhibition with RAD001 single treatment, suggesting that when mTOR inhibitor is used alone, it contributes to therapy by stabilizing the disease rather than tumor regression [36]. Also, Ji et al. showed that the combination of RAD001 and an AKT inhibitor exhibited cytotoxic effects such as G1/S cell cycle arrest, cyclinD1 downregulation, and growth inhibition in PTEN mutant gastric cancer cells. However, the combination did not cause apoptotic cell death but induced beclin-1 expression which is an indicator of autophagy [37]. Zito et al. showed LY294002 and rapamycin combination was synergistic in six NSCLC cell lines, and low rapamycin concentrations (1 nM) resulted in sensitization to PI3K inhibitors [38]. Thereby, the dual targeting of PI3K/mTOR would be highly effective inhibiting survival in cancer cell lines. When PI3K mutant tumors were treated with a dual PI3K and mTOR inhibitor (BEZ235), it reduced tumor formation. Although it was ineffective alone, the combination of BEZ235 with a MEK inhibitor was effective in KRAS mutant lung cancers [39]. It was shown that AZD6244 was not effective at inhibiting H460 injected athymic nude mice tumors. However, AZD6244 and BEZ235 combination reduced cell viability and tumor volume [40]. Hata et al. determined MEK and PI3K inhibitor combinations do not cause apoptosis in most human KRAS mutant NSCLC cell lines. They identified BIM and PUMA downregulation and BCL-XL upregulation in resistant cells. So, leading to apoptosis is essential for overcoming resistance and benefit from concomitant use of inhibitors [41]. Our western blots revealed that U0126 directly inhibited the phosphorylation of ERK at 6 and 24 h, followed by inhibiting the activations of its downstream molecules, including cyclinD1 and c-myc but increased the expression of $p27^{kip1}$, BAX, and BIM at 6 and 24 h.

MAPK pathway promotes survival by inhibiting the pro-apoptotic BIM and BAD protein expressions and caspase 9 activity or by activating anti-apoptotic BCL-2 and BCL-XL protein expressions [42, 43]. ERK activation also interferes extrinsic apoptotic pathway by inhibiting the death receptors FAS, TRAIL, or TNF [44]. Besides, activated ERK signaling can enhance the expression of CDK inhibitor proteins, p16, p21, and p27, thereby leading to cell cycle arrest at the G1 phase [45, 46]. However, MAPK pathway activation can lead to cross-talk through PI3K-AKT pathway. MEK inhibition alone usually exhibit resistance in KRAS mutant NSCLC by upregulating p-AKT signaling. So, combination treatment with MEK and PI3K-AKT pathway inhibitors may be a beneficial approach to overcome resistance. Selumetinib (AZD6244), a MEK inhibitor, and BYL719, a PI3K inhibitor, combination resulted in higher toxicity compared with single agents alone in KRAS mutant NSCLC cells. Although selumetinib alone upregulated AKT and BAD phosphorylation, the combination with BYL719 was more effective for p-ERK and p-AKT inhibition in A549 xenografts [47]. Also,

Table 1 Mutation status of NSCLC cell lines used in this study

NSCLC cell line	KRAS	P53	LKB1	EGFR
A549	G12S	WT	Q37[a]	WT
Calu-1	G12C	HD	WT	WT

WT wild type, *HD* homozygous deletion

[a] Reflects nonsense mutation

Chen et al. found the inhibition of AKT and the activation of ERK-MAPK pathway are associated with neuroendocrine differentiation in NSCLC H157 cell line, which is a marker of SCLC and carcinoid tumors [48].

There have been some clinical trials going on for targeted therapies, but the toxicity and efficacy of these inhibitors remain in question. This work provides further knowledge on molecular signaling for biomarker-driven translational therapies and for future drug combinations. As shown in our study, dual inhibition of mTOR and ERK-MAPK or PI3K-AKT pathways may overcome the disadvantage of single-agent therapies and enhance the efficacy of mTOR-targeted therapies. Also, targeting PI3K, MEK, and mTOR all together is more effective than dual inhibitors [49]. However, considering the side effects, it would be difficult to treat patients with all these inhibitors at the same time. Our findings suggest that the combination of MEK and mTOR inhibitors and PI3K and mTOR inhibitors compared with either drug alone can inhibit cell proliferation, induce apoptosis, and affect downstream signaling pathways. Although PI3K/mTOR inhibition is more effective in cytotoxicity, MEK/mTOR inhibition markedly decreases cell proliferation protein marker expressions. In the molecular level, U0126/RAD001 combination is a better option than LY294002/RAD001 combination in A549 and Calu-1 cells. Once again, the molecular expression differences within cell lines may due to the mutational status of cells used in this study. The results of this study were only observed in cell lines and need to be clarified in mice and human primary tumor samples.

References

1. Siegel R, Ma J, Zou Z, Jemal A. Cancer statistics, 2014. CA Cancer J Clin. 2014;64:9–29.
2. Beasley MB, Brambilla E, Travis WD. The 2004 world health organization classification of lung tumors. Semin Roentgenol. 2005;40:90–7.
3. A genomics-based classification of human lung tumors. Sci Transl Med 5: 209ra153. 2013.
4. Steelman LS, Pohnert SC, Shelton JG, Franklin RA, Bertrand FE, et al. JAK/STAT, Raf/MEK/ERK, PI3K/Akt and BCR-ABL in cell cycle progression and leukemogenesis. Leukemia. 2004;18:189–218.
5. Diaz-Flores E, Shannon K. Targeting oncogenic Ras. Genes Dev. 2007;21:1989–92.
6. Pylayeva-Gupta Y, Grabocka E, Bar-Sagi D. RAS oncogenes: weaving a tumorigenic web. Nat Rev Cancer. 2011;11:761–74.
7. Mascaux C, Iannino N, Martin B, Paesmans M, Berghmans T, et al. The role of RAS oncogene in survival of patients with lung cancer: a systematic review of the literature with meta-analysis. Br J Cancer. 2005;92:131–9.
8. Sekido Y, Fong KM, Minna JD. Molecular genetics of lung cancer. Annu Rev Med. 2003;54:73–87.
9. Sable CL, Filippa N, Filloux C, Hemmings BA, Van Obberghen E. Involvement of the pleckstrin homology domain in the insulin-stimulated activation of protein kinase B. J Biol Chem. 1998;273: 29600–6.
10. Liang J, Slingerland JM. Multiple roles of the PI3K/PKB (Akt) pathway in cell cycle progression. Cell Cycle. 2003;2:339–45.
11. Testa JR, Bellacosa A. AKT plays a central role in tumorigenesis. Proc Natl Acad Sci U S A. 2001;98:10983–5.
12. Bondar VM, Sweeney-Gotsch B, Andreeff M, Mills GB, Mcconkey DJ. Inhibition of the phosphatidylinositol 3'-kinase-AKT pathway induces apoptosis in pancreatic carcinoma cells in vitro and in vivo. Mol Cancer Ther. 2002;1:989–97.
13. Tsurutani J, Fukuoka J, Tsurutani H, Shih JH, Hewitt SM, et al. Evaluation of two phosphorylation sites improves the prognostic significance of Akt activation in non-small-cell lung cancer tumors. J Clin Oncol. 2006;24:306–14.
14. Boulay A, Lane HA. The mammalian target of rapamycin kinase and tumor growth inhibition. Recent Results Cancer Res Fortschritte der Krebsforschung Progres dans les Recherches sur le Cancer. 2007;172:99–124.
15. O'donnell A, Faivre S, Burris 3rd HA, Rea D, Papadimitrakopoulou V, et al. Phase I pharmacokinetic and pharmacodynamic study of the oral mammalian target of rapamycin inhibitor everolimus in patients with advanced solid tumors. J Clin Oncol. 2008;26:1588–95.
16. Tabernero J, Rojo F, Calvo E, Burris H, Judson I, et al. Dose- and schedule-dependent inhibition of the mammalian target of rapamycin pathway with everolimus: a phase I tumor pharmacodynamic study in patients with advanced solid tumors. J Clin Oncol. 2008;26:1603–10.
17. O'reilly KE, Rojo F, She QB, Solit D, Mills GB, et al. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res. 2006;66:1500–8.
18. Rodrik-Outmezguine VS, Chandarlapaty S, Pagano NC, Poulikakos PI, Scaltriti M, et al. mTOR kinase inhibition causes feedback-dependent biphasic regulation of AKT signaling. Cancer Discov. 2011;1:248–59.
19. Mccubrey JA, Steelman LS, Chappell WH, Abrams SL, Wong EW, et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. Biochim Biophys Acta. 2007;1773:1263–84.
20. Papin C, Eychene A, Brunet A, Pages G, Pouyssegur J, et al. B-Raf protein isoforms interact with and phosphorylate Mek-1 on serine residues 218 and 222. Oncogene. 1995;10:1647–51.
21. Adjei AA. Signal transduction pathway targets for anticancer drug discovery. Curr Pharm Des. 2000;6:361–78.
22. Favata MF, Horiuchi KY, Manos EJ, Daulerio AJ, Stradley DA, et al. Identification of a novel inhibitor of mitogen-activated protein kinase kinase. J Biol Chem. 1998;273:18623–32.
23. Shaul YD, Seger R. The MEK/ERK cascade: from signaling specificity to diverse functions. Biochim Biophys Acta. 2007;1773: 1213–26.
24. Carracedo A, Ma L, Teruya-Feldstein J, Rojo F, Salmena L, et al. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest. 2008;118:3065–74.
25. Yu CF, Liu ZX, Cantley LG. ERK negatively regulates the epidermal growth factor-mediated interaction of Gab1 and the phosphatidylinositol 3-kinase. J Biol Chem. 2002;277:19382–8.
26. Zimmermann S, Moelling K. Phosphorylation and regulation of Raf by Akt (protein kinase B). Science. 1999;286:1741–4.
27. Guan KL, Figueroa C, Brtva TR, Zhu T, Taylor J, et al. Negative regulation of the serine/threonine kinase B-Raf by Akt. J Biol Chem. 2000;275:27354–9.
28. Carriere A, Romeo Y, Acosta-Jaquez HA, Moreau J, Bonneil E, et al. ERK1/2 phosphorylate raptor to promote Ras-dependent activation of mTOR complex 1 (mTORC1). J Biol Chem. 2011;286: 567–77.

29. Iida S, Miki Y, Ono K, Akahira J, Nakamura Y, et al. Synergistic anti-tumor effects of RAD001 with MEK inhibitors in neuroendocrine tumors: a potential mechanism of therapeutic limitation of mTOR inhibitor. Mol Cell Endocrinol. 2012;350:99–106.
30. Wan X, Harkavy B, Shen N, Grohar P, Helman LJ. Rapamycin induces feedback activation of Akt signaling through an IGF-1R-dependent mechanism. Oncogene. 2007;26:1932–40.
31. Chen X, Zhao M, Hao M, Sun X, Wang J, et al. Dual inhibition of PI3K and mTOR mitigates compensatory AKT activation and improves tamoxifen response in breast cancer. Mol Cancer Res. 2013;11:1269–78.
32. Ishibe S, Haydu JE, Togawa A, Marlier A, Cantley LG. Cell confluence regulates hepatocyte growth factor-stimulated cell morphogenesis in a beta-catenin-dependent manner. Mol Cell Biol. 2006;26:9232–43.
33. Swat A, Dolado I, Rojas JM, Nebreda AR. Cell density-dependent inhibition of epidermal growth factor receptor signaling by p38alpha mitogen-activated protein kinase via Sprouty2 downregulation. Mol Cell Biol. 2009;29:3332–43.
34. Gao J, Zhao Y, Lv Y, Chen Y, Wei B, et al. Mirk/Dyrk1B mediates G0/G1 to S phase cell cycle progression and cell survival involving MAPK/ERK signaling in human cancer cells. Cancer Cell Int. 2013;13:2.
35. Ko JC, Wang YT, Yang JL. Dual and opposing roles of ERK in regulating G(1) and S-G(2)/M delays in A549 cells caused by hyperoxia. Exp Cell Res. 2004;297:472–83.
36. Meric-Bernstam F, Gonzalez-Angulo AM. Targeting the mTOR signaling network for cancer therapy. J Clin Oncol. 2009;27:2278–87.
37. Ji D, Zhang Z, Cheng L, Chang J, Wang S, et al. The combination of RAD001 and MK-2206 exerts synergistic cytotoxic effects against PTEN mutant gastric cancer cells: involvement of MAPK-dependent autophagic, but not apoptotic cell death pathway. PLoS One. 2014;9, e85116.
38. Zito CR, Jilaveanu LB, Anagnostou V, Rimm D, Bepler G, et al. Multi-level targeting of the phosphatidylinositol-3-kinase pathway in non-small cell lung cancer cells. PLoS One. 2012;7, e31331.
39. Engelman JA, Chen L, Tan X, Crosby K, Guimaraes AR, et al. Effective use of PI3K and MEK inhibitors to treat mutant Kras G12D and PIK3CA H1047R murine lung cancers. Nat Med. 2008;14:1351–6.
40. Qu Y, Wu X, Yin Y, Yang Y, Ma D, et al. Antitumor activity of selective MEK1/2 inhibitor AZD6244 in combination with PI3K/mTOR inhibitor BEZ235 in gefitinib-resistant NSCLC xenograft models. J Exp Clin Cancer Res CR. 2014;33:52.
41. Hata AN, Yeo A, Faber AC, Lifshits E, Chen Z, et al. Failure to induce apoptosis via BCL-2 family proteins underlies lack of efficacy of combined MEK and PI3K inhibitors for KRAS-mutant lung cancers. Cancer Res. 2014;74:3146–56.
42. Ballif BA, Blenis J. Molecular mechanisms mediating mammalian mitogen-activated protein kinase (MAPK) kinase (MEK)-MAPK cell survival signals. Cell Growth Differ Mol Biol J Am Assoc Cancer Res. 2001;12:397–408.
43. Allan LA, Morrice N, Brady S, Magee G, Pathak S, et al. Inhibition of caspase-9 through phosphorylation at Thr 125 by ERK MAPK. Nat Cell Biol. 2003;5:647–54.
44. Sahu RP, Batra S, Kandala PK, Brown TL, Srivastava SK. The role of K-ras gene mutation in TRAIL-induced apoptosis in pancreatic and lung cancer cell lines. Cancer Chemother Pharmacol. 2011;67:481–7.
45. Mirza AM, Gysin S, Malek N, Nakayama K, Roberts JM, et al. Cooperative regulation of the cell division cycle by the protein kinases RAF and AKT. Mol Cell Biol. 2004;24:10868–81.
46. Gysin S, Lee SH, Dean NM, Mcmahon M. Pharmacologic inhibition of RAF→MEK→ERK signaling elicits pancreatic cancer cell cycle arrest through induced expression of p27Kip1. Cancer Res. 2005;65:4870–80.
47. Ku BM, Jho EH, Bae YH, Sun JM, Ahn JS, et al. BYL719, a selective inhibitor of phosphoinositide 3-kinase alpha, enhances the effect of selumetinib (AZD6244, ARRY-142886) in KRAS-mutant non-small cell lung cancer. Investig New Drugs. 2014.
48. Chen Y, Nowak I, Huang J, Keng PC, Sun H, et al. Erk/MAP kinase signaling pathway and neuroendocrine differentiation of non-small-cell lung cancer. J Thorac Oncol Off Publ Int Assoc Study Lung Cancer. 2014;9:50–8.
49. Haagensen EJ, Kyle S, Beale GS, Maxwell RJ, Newell DR. The synergistic interaction of MEK and PI3K inhibitors is modulated by mTOR inhibition. Br J Cancer. 2012;106:1386–94.