Enhancing Mammalian Target of Rapamycin (mTOR)–Targeted Cancer Therapy by Preventing mTOR/Raptor Inhibition-Initiated, mTOR/Rictor-Independent Akt Activation

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Abstract
It has been shown that mammalian target of rapamycin (mTOR) inhibitors activate Akt while inhibiting mTOR signaling. However, the underlying mechanisms and the effect of the Akt activation on mTOR-targeted cancer therapy are unclear. The present work focused on addressing the role of mTOR/rictor in mTOR inhibitor-induced Akt activation and the effect of sustained Akt activation on mTOR-targeted cancer therapy. Thus, we have shown that mTOR inhibitors increase Akt phosphorylation through a mechanism independent of mTOR/rictor because the assembly of mTOR/rictor was inhibited by mTOR inhibitors and the silencing of rictor did not abrogate mTOR inhibitor-induced Akt activation. Moreover, Akt activation during mTOR inhibition is tightly associated with development of cell resistance to mTOR inhibitors. Accordingly, cotargeting mTOR and phosphatidylinositol 3-kinase/Akt signaling prevents mTOR inhibition-initiated Akt activation and enhances antitumor effects both in cell cultures and in animal xenograft models, suggesting an effective cancer therapeutic strategy. Collectively, we conclude that inhibition of the mTOR/raptor complex initiates Akt activation independent of mTOR/rictor. Consequently, the sustained Akt activation during mTOR inhibition will counteract the anticancer efficacy of the mTOR inhibitors. [Cancer Res 2008;68(18):7409–18]

Introduction
The mammalian target of rapamycin (mTOR), a phosphatidylinositol 3-kinase (PI3K)-related serine/threonine kinase, plays a central role in regulating cell growth, proliferation, and survival, in part by regulation of translation initiation, through interactions with other proteins such as raptor [forming mTOR complex 1 (mTORC1)] and rictor [forming mTOR complex 2 (mTORC2); refs. 1–3]. The best-characterized downstream effectors of mTORC1 are the 70-kDa ribosomal S6 kinase (p70S6K) and the eukaryotic translation initiation factor 4E–binding protein 1 (4E-BP1; ref. 1). In response to mitogenic stimuli or nutrient availability, mTORC1 is activated (4), leading to phosphorylation of p70S6K and 4E-BP1, and the subsequent enhanced translation of mRNAs that are critical for cell cycle progression and proliferation (1).

PI3K/Akt signaling represents a major cell survival pathway. Its activation has long been associated with malignant transformation and apoptotic resistance (5, 6). It is generally thought that mTOR (i.e., mTORC1) functions downstream of the PI3K/Akt pathway and is phosphorylated (or activated) in response to stimuli that activate the PI3K/Akt pathway (1, 7). However, the recent discovery of mTORC2 as an Akt Ser473 kinase also places mTOR upstream of Akt. Although mTORC2 is thought to be insensitive to rapamycin, it has been shown that prolonged rapamycin exposure inhibits mTORC2 assembly and Akt activity in certain types of cancer cells (8). We and others have shown that mTOR inhibitors activate Akt while suppressing mTORC1 signaling in different types of cancer cell lines and clinical human tumor samples (9–11). Currently, it is unclear how mTOR inhibitors activate Akt survival signaling.

mTOR signaling has recently emerged as an attractive therapeutic target for cancer therapy (1, 12). The potential applications of mTOR inhibitors for treating various types of cancer have been actively studied both preclinically and clinically. In the United States, several phase II or III trials are ongoing that test the effects of mTOR inhibitors on various cancers (1, 13, 14). A recent study has shown encouraging results that the mTOR inhibitor CCI-779 improved overall survival among patients with metastatic renal cell carcinoma (15).

In addition to the intrinsic resistance of cancer cells to mTOR inhibition by rapamycin, cancer cells can acquire resistance to rapamycin (16). Therefore, understanding the mechanisms by which cells become resistant to mTOR inhibitors such as rapamycin has long been an interesting subject and may eventually guide the development of successful mTOR-targeted cancer therapy by avoiding or overcoming cell resistance to mTOR inhibition.

The current study aimed at showing the relationship between mTORC2 and mTORC1 inhibition-induced Akt activation, and particularly the biological significance of Akt activation in mTOR-targeted cancer therapy.

Materials and Methods

Reagents. Rapamycin and LY294002 were purchased from LC Laboratories. RAD001 powder, formulated RAD001, and matched placebo control agents were dissolved in DMSO at a concentration of 20 mmol/L, and aliquots were stored at −80°C. Stock solutions were diluted to the desired final concentrations with growth medium just before use. Rabbit polyclonal antibodies against Akt, mTOR, raptor, phosphorylated Akt (p-Akt; S473), p-Akt (T308), phosphorylated p70S6K (p-p70S6K; T389), and phosphorylated S6 (p-S6; S235/S236), respectively, were purchased from Cell Signaling Technology, Inc. Rabbit polyclonal anti-actin and mouse monoclonal anti-tubulin antibodies were purchased from Sigma Chemical Co. Rabbit polyclonal anti–glyceraldehyde-3-phosphate dehydrogenase antibody was purchased from Trevigen, Inc. Goat polyclonal mTOR (FRAP; N-19) and...
rabbit polyclonal rictor (BL2178) antibodies were purchased from Santa Cruz Biotechnology, Inc. and Bethyl Laboratories, Inc., respectively.

Cell lines and cell treatment. Human lung cancer cell lines and PC-3 cells were purchased from the American Type Culture Collection. The U937 cell line was provided by Dr. Yongkui Jing (Mount Sinai School of Medicine, New York, NY). These cell lines were grown as described previously (9). The rapamycin-resistant A549 cell line (A549-RR) was established by exposing the rapamycin-sensitive A549 parental cells (A549-P) to gradually increased concentrations of rapamycin from the initial 1 nmol/L to the final 20 nmol/L over a 6-mo period. A549-RR cells were routinely cultured in complete medium containing 1 nmol/L rapamycin. All treatments with mTOR inhibitors were done in the respective media containing 5% fetal bovine serum.

Western blot analysis. The procedures for preparation of whole-cell protein lysates and for Western blotting were described previously (9, 17).

Immunoprecipitation. mTOR complexes were immunoprecipitated with goat polyclonal mTOR (FRAP; N-19) antibody according to the same procedure described previously (18, 19). At the end, the samples containing an equal amount (20–50 μg) of whole-cell protein lysates and immunoprecipitates from 0.5 to 1 mg cell lysates captured with protein A-Sepharose were analyzed by Western blotting.

Gene knockdown by small interfering RNA. Control (nonsilencing) small interfering RNA (siRNA), raptor siRNA that targets 5′-AAGGCTTAGTCTGTTCGAAAT-3′, and rictor siRNAs that target 5′-AAGCAGCCTTGAACTGTTTAA-3′ (rictor-1) and 5′-AAACTTGTGAACTGTTATC-3′ (rictor-2), respectively, were described previously (19) and synthesized from Qiagen. The transfection of siRNA was conducted in a 12- or 24-well plate using Lipofectamine 2000 (Invitrogen) following the manufacturer’s instructions. Forty-eight hours after transfection, the cells were treated with DMSO and rapamycin for the given times and then subjected to detection of the given proteins by Western blot analysis. In addition, we also used lentiviral raptor, rictor, and scramble short hairpin RNAs (shRNA) described previously (19), which were ordered from Addgene, Inc., and used for stably knocking down raptor and rictor as described previously (19).

Growth inhibition assay. Cell number was estimated by the sulforhodamine B (SRB) assay and the growth inhibition was calculated as previously described (20).

Colony formation assay. Cells (single-cell suspension) were plated in 12-well plates at a density of 250 per well. On the second day, cells were treated with the given agents. Every 3 d, the medium was replaced with fresh medium containing the corresponding concentrations of the agents. After a 10-d treatment, the medium was removed and cell colonies were stained with crystal violet (0.1% in 20% methanol) and counted. Pictures were also taken using a digital camera to record the result.

Cell cycle analysis. The procedure for analysis of cell cycle by flow cytometry was described previously (21).

Lung cancer xenografts and treatments. Animal experiments were approved by the Institutional Animal Care and Use Committee of Emory University. Four- to 6-wk-old (~20 g of body weight) female athymic (nu/nu) mice were ordered from Taconic and housed under pathogen-free conditions.

Figure 1. Effects of prolonged treatment with mTOR inhibitors on Akt phosphorylation. A, the indicated cell lines were treated with DMSO and 10 nmol/L rapamycin (Rap) or RAD001 for 24 h. B, the indicated cell lines were treated with DMSO (D), 1 nmol/L rapamycin (R), or RAD001 (R1) for the given times. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. C, PC-3 cells were treated with the given concentrations of rapamycin or RAD001 for the indicated times. D, U937 or Jurkat cells were treated with the given concentrations of rapamycin or RAD001 for 24 h. The cells were then harvested from the aforementioned treatments for preparation of whole-cell protein lysates and subsequent Western blot analysis.
conditions in microisolator cages with laboratory chow and water ad libitum. A549 cells at 5 \times 10^6 in serum-free medium were injected s.c. into the flank region of nude mice. When tumors reached certain size ranges (100–500 mm^3), the mice were randomized into four groups (n = 6/group) according to tumor volumes and body weights for the following treatments: vehicle control, formulated RAD001 (4 mg/kg/d, oral gavage) and LY294002 in DMSO (25 mg/kg/d, i.p.), and the combination of RAD001 and LY294002. Tumor volumes were measured using caliper measurements once every 2 d and calculated with the formula \[ V = \frac{k}{2} \times \text{length} \times \text{width}^2. \] After a 14-d treatment, the mice were sacrificed with CO2. The tumors were then removed, weighed, and frozen in liquid nitrogen or fixed with formalin. Certain portions of tumor tissues from each tumor were homogenized in protein lysis buffer for preparation of whole-cell protein lysates as described previously (20). Western blotting results were quantitated using Kodak Image Station 2000R (Eastman Kodak Co.).

**Results**

**Effects of prolonged treatment with mTOR inhibitors on Akt phosphorylation are dose dependent.** We and others previously showed that rapamycin induces a rapid and sustained increase in Akt phosphorylation in several types of cancer cells, including lung, breast, and prostate cancer cells (9, 10). However, two recent studies have shown that prolonged treatment with mTOR inhibitors decreases Akt phosphorylation in certain cancer cell lines (e.g., PC-3 and U937; refs. 8, 22). In this study, we further examined the effects of RAD001 in comparison with rapamycin on Akt phosphorylation in a group of lung cancer cell lines after a prolonged treatment. Both RAD001 and rapamycin at 10 nmol/L increased p-Akt levels while inhibiting p70S6K phosphorylation in all of the cell lines after a 24-h treatment (Fig. 1A). We also treated H157 and A549 lung cancer cells with 1 nmol/L RAD001 or rapamycin for 24 h and then harvested them for preparation of whole-cell protein lysates. The indicated proteins were detected by Western blot analysis.
harvested the cells for analysis of Akt phosphorylation. As shown in Fig. 1B, p-Akt levels remained elevated at all the tested times over the prolonged period of time even when decreased p-p70S6K levels returned at 96 h (i.e., RAD001 at 96 h). This result clearly shows that mTOR inhibitors induce a sustained Akt activation in the tested cell lines. We noted that p-p70S6K levels recovered at 96 h after treatment with RAD001 but not with rapamycin (Fig. 1B). Because we treated cells only once, it is likely that rapamycin may have a longer half-life in cell culture than RAD001, resulting in better efficacy than RAD001 in inhibiting mTOR signaling.

Moreover, we examined the effects of prolonged treatment with rapamycin or RAD001 on Akt phosphorylation in two cell lines (i.e., PC-3 and U937), in which Akt phosphorylation was decreased by prolonged treatment with rapamycin (8), in a more detailed way. Previous studies used 100 nmol/L rapamycin (8) or >1,000 nmol/L CCI-779 (22), which decreased p-Akt levels after a 24-h treatment. In our study, we could repeat this result after both 24- and 48-h treatments with 100 nmol/L rapamycin in PC-3 cells. However, when the concentration of rapamycin was reduced to 1 nmol/L, we consistently observed an increase in Akt phosphorylation at both 24- and 48-h treatments. Similar results were also obtained from cells treated with RAD001 (Fig. 1C). In U937 cells, prolonged treatment with either 1 nmol/L rapamycin or RAD001 clearly increased the levels of p-Akt, although at 10 or 100 nmol/L they decreased p-Akt levels (Fig. 1D). Similar results with RAD001 were also observed in Jurkat cells (Fig. 1D). We noted that both rapamycin and RAD001 at 1 nmol/L sufficiently inhibited mTORC1 signaling evidenced by reduction of p-S6 or p-p70S6K.

Figure 3. Comparison of cell sensitivities to mTOR inhibitors (A), growth rates (B), phosphorylation of p-Akt, p-p70S6K, and p-S6 (C), and the levels of mTORC1 and mTORC2 (D) between A549 parental (A549-P) and rapamycin-resistant A549 cells (A549-RR). A, the indicated cell lines were plated in 96-well plates and then treated with different concentrations of rapamycin or RAD001 as indicated on the second day. After 3 d, the cell numbers were estimated using the SRB assay. Points, mean of four replicate determinations; bars, SD. B, the similar number of A549-P and A549-RR cells was seeded in 96-well plates. At the indicated times, one plate was fixed for estimation of cell number using the SRB assay. Points, mean of four replicate determinations; bars, SD. C, the indicated cell lines were plated in 10-cm-diameter cell culture dishes and treated on the second day with DMSO (D) and the given concentrations of rapamycin (R) or RAD001 (R1) for 1 h. The cells were then harvested for preparation of whole-cell protein lysates and subsequent Western blot analysis. Rapamycin was removed from the culture medium for at least 24 h before A549-RR cells were used in the experiments. D, the whole-cell protein lysates were prepared from A549-P and A549-RR cells and then subjected to immunoprecipitation using mTOR antibody. Cell lysates and mTOR immunoprecipitates were analyzed by Western blotting.
levels (Fig. 1C and D). Thus, the effects of prolonged treatment with mTOR inhibitors on Akt phosphorylation are clearly dose dependent in these cell lines. We also noted that both rapamycin and RAD001 at 1 to 100 nmol/L increased Akt phosphorylation at Thr308 in a dose-dependent manner in PC-3 cells (see Supplementary Fig. S1), suggesting that mTOR inhibitors also activate phosphoinositide-dependent protein kinase 1 kinase. We noted that our data here on Akt phosphorylation at Thr308 by rapamycin or RAD001 in PC-3 cells are different from previous report that our data here on Akt phosphorylation at Thr308 by rapamycin or RAD001 in PC-3 cells were at least 10,000-fold more resistant to either rapamycin or RAD001 than A549-P cells by comparing their IC50s. The A549-RR cell line had a comparable growth rate to that of A549-P (Fig. 3B). To maintain the acquired resistance to rapamycin, we routinely cultured A549-RR cells in complete medium containing 1 μmol/L rapamycin. Twenty-four hours before each experiment, rapamycin was withdrawn from the medium. We observed that A549-RR cells had much higher basal levels of p-Akt than A549-P cells; these high levels of p-Akt were not increased further by either rapamycin or RAD001 (Fig. 3C). In A549-P cells, rapamycin at either 1 nmol/L or 1 μmol/L increased p-Akt levels. The total levels of Akt in both A549-P and A549-RR cell lines were not altered (Fig. 3C, bottom). Both glycogen synthase kinase 3β (GSK3β) and FOXO3a are well-known substrates of Akt. The basal levels of phosphorylated GSK3β (p-GSK3β) but not phosphorylated FOXO3a were accordingly elevated in A549-RR cells compared with those in A549-P cells (Fig. 3C). We noted that p-p70S6K levels were not decreased by rapamycin or RAD001 in A549-RR cells, although the p-S6 levels were slightly decreased by high concentration (i.e., 1 μmol/L) of rapamycin or RAD001 (Fig. 3C). These results indicate that A549-RR cells lose responses to mTOR inhibitor-mediated inhibition of mTORC1-p70S6K signaling while exhibiting increased levels of p-Akt. It has been suggested that down-regulation of 4E-BP1 is associated with rapamycin resistance (23). Therefore, we compared the levels of 4E-BP1 and its phosphorylation between A549-P and A549-RR cell lines. As presented in Fig. 3C, we did not find an obvious difference in basal levels of 4E-BP1 between A549-P and A549-RR cell lines. The expression levels of 4E-BP1 were not altered by mTOR inhibitors in both cell lines. We found that both cell lines had comparable levels of phosphorylated 4E-BP1 (p-4E-BP1), p-4E-BP1 levels were reduced by both low (1 nmol/L) and high (1 μmol/L) concentrations of rapamycin or RAD001 in A549-P cells but not in A549-RR cells, except for the high dose (1 μmol/L) of rapamycin. These results suggest that 4E-BP1 levels cannot account for cell resistance to mTOR inhibitors in our system. Following these studies, we determined whether the assembly of mTOR complexes was altered in A549-RR cells. Therefore, we compared the levels of mTORC1 and mTORC2 between A549-P and A549-RR cells. The total levels of mTOR, raptor, and rictor in cell lysates were not altered in A549-RR cells; however, the amounts of raptor and rictor in mTOR complexes precipitated by a mTOR antibody were strikingly decreased (Fig. 3D), indicating that both mTORC1 and mTORC2 were inhibited in A549-RR cells. Under such circumstances, the levels of p-Akt (S473), p-Akt (T308), and p-GSK3β (S9) were elevated in cell lysates from A549-RR cells compared with those in A549-P cells (Fig. 3C). The total levels of p-Akt were not decreased by rapamycin or RAD001 in A549-RR cells, although the p-S6 levels were slightly decreased by high concentration (i.e., 1 μmol/L) of rapamycin or RAD001 (Fig. 3C). These results indicate that A549-RR cells lose responses to mTOR inhibitor-mediated inhibition of mTORC1-p70S6K signaling while exhibiting increased levels of p-Akt.

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biological significance of sustained Akt activation in mTOR-targeted cancer therapy. To this end, we took advantage of the rapamycin-resistant cell line (i.e., A549-RR) that has elevated levels of p-Akt as described above. We first determined whether the acquired rapamycin resistance in A549-RR cells was reversible. To do so, we cultured A549-RR cells in rapamycin-free complete medium for up to 5 months and monitored cell responses to mTOR inhibitors and p-Akt levels at 1-month intervals. At 2 months after rapamycin withdrawal, the cell line, which was named A549-RR2W, was slightly more sensitive than A549-RR cells to either rapamycin or RAD001 (Fig. 4A). Even at 3 or 4 months after rapamycin withdrawal, the cells (i.e., A549-RR3W and A549-RR4W) were still partially resistant to mTOR inhibitors, although their sensitivities to rapamycin or RAD001 were increased compared with A549-RR cells (data not shown). After a 5-month withdrawal of rapamycin, the cell line, which was named A549-RR5W, was as sensitive as A549-P cells to the rapamycin and RAD001 (Fig. 4B), indicating a complete restoration of rapamycin sensitivity. Collectively, these results indicate that the acquired rapamycin resistance in A549 cells is reversible, although it sustains for over 5 months.

Accordingly, we examined basal p-Akt levels and their modulation by mTOR inhibitors in rapamycin-resistant cell lines during rapamycin withdrawal. After a 2-month withdrawal of rapamycin, we found that the basal levels of p-Akt in A549-RR2W cells were still much higher than that in A549-P cells and were only increased by high concentrations of rapamycin or RAD001 (i.e., 1 μmol/L; Fig. 4C). The basal levels of p-p70S6K in A549-RR2W and A549-P cells were comparable and could be effectively inhibited by both rapamycin and RAD001. Similarly, the p-S6 levels in A549-RR2W and A549-P cells were also comparable and inhibited by mTOR inhibitors (Fig. 4C). After 5-month withdrawal of rapamycin when cell sensitivity to rapamycin is fully restored, we noted that p-Akt levels in A549-RR5W cells were as low as those in A549-P cells (Fig. 4D). On treatment with rapamycin or RAD001, p-Akt levels were substantially increased in A549-RR5W cells as was observed in A549-P cells (Fig. 4D). As we already showed in A549-RR2W cells, p-p70S6K levels in A549-RR5W cells were comparable with those in A549-P cells and could be effectively decreased by rapamycin or RAD001 (Fig. 4D). Collectively, our results clearly indicate that sustained Akt activation (i.e., increase in p-Akt levels) during mTOR-targeted cancer therapy is associated with cell resistance to mTOR inhibitors.

To further show this association, we examined whether enforced reduction of p-Akt levels by Akt siRNA alter cell sensitivity to rapamycin. To this end, we decreased p-Akt levels by knocking...
down the levels of total Akt using Akt siRNA and then examined its effect on cell sensitivity to rapamycin. As presented in Supplementary Fig. S2, silencing of Akt by Akt siRNA substantially reduced the levels of p-Akt (Supplementary Fig. S2A). Accordingly, these cells were much more sensitive than control siRNA-transfected cells to rapamycin (Supplementary Fig. S2B), indicating that enforced reduction of p-Akt levels restores cell sensitivity to rapamycin. Thus, these results further support the notion that sustained increase in p-Akt levels is associated with the development of cell resistance to mTOR inhibitors.

The rapamycin-resistant cell line retains sensitivity to PI3K inhibitors. Because of the increased levels of p-Akt in A549-RR cells, we determined whether A549-RR cells were cross-resistant to PI3K inhibitors. A549-RR cells responded as well as A549-P cells to either LY294002 or wortmannin in terms of a 3-day monolayer culture assay (Supplementary Fig. S3A). By a long-term (10-day) colony formation assay, we found that LY294002 effectively inhibited the growth of both A549-P and A549-RR cells (Supplementary Fig. S3B). At the tested concentrations of up to 15 μmol/L, LY294002 failed to induce apoptosis in either A549-P or A549-RR cells.

Figure 5. Combination of RAD001 and LY294002 augments growth inhibition of lung cancer cells in cell culture (A and B) and in nude mice (C and D). A, the individual cell lines, as indicated, were seeded in 96-well plates. On the second day, they were treated with the indicated concentrations of RAD001 (RAD) alone, 2 μmol/L LY294002 alone, and their respective combinations. After 3 d, plates were subjected to determination of cell number using a SRB assay. Columns, mean of four replicate determinations; bars, SD. B, H460 cells at a density of ~250 per well were seeded in 12-well plates. On the second day, cells were treated with the indicated concentrations of RAD001 alone, 2.5 μmol/L LY294002 alone, and their respective combinations. The same treatments were repeated every 3 d. After 10 d, the plates were stained for the formation of cell colonies with crystal violet dye. The picture of the colonies was then taken using a digital camera.

C and D, four groups of mice with either A549 (C) or H460 (D) xenografts were treated with vehicle control, RAD001 alone, LY294002 (LY) alone, and RAD001 plus LY294002 on the same day after grouping. After 14 d, the mice were sacrificed and the tumors were removed. Tumor sizes were measured once every 2 d. Points, mean (n = 6); bars, SD. *, P < 0.05; **, P < 0.01; and ***, P < 0.001, compared with vehicle control; #, P < 0.05, compared with RAD001 treatment.
cells by examining cell morphologic changes and analysis of sub-G1 populations (data not shown). However, LY294002 induced G1 arrest in both A549-P and A549-RR cells with comparable potencies (Supplementary Fig. S3C). Moreover, we compared the effects of LY294002 on p-p70S6K and p-Akt in A549-P and A549-RR cells and found that LY294002 effectively decreased the levels of not only p-p70S6K and p-S6 but also p-Akt in both cell lines, although A549-RR cells had very high basal levels of p-Akt (Supplementary Fig. S3D). Collectively, these results indicate that A549-RR cells do not exhibit cross-resistance to PI3K inhibitors.

**Cotargeting mTOR and PI3K/Akt signaling augments inhibition of tumor growth.** Given that sustained Akt activation is associated with development of cell resistance to mTOR inhibitors, whereas mTOR inhibitor-induced Akt activation was suggested to be PI3K dependent (9), it was plausible to speculate that blockage of mTOR inhibitor-induced Akt activation by a PI3K inhibitor would enhance the anticancer efficacy of the mTOR inhibitors and prevent development of cell resistance to mTOR inhibitors. Thus, we examined the effects of RAD001 combined with LY294002 on the growth of lung cancer cells in cell culture.

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Figure 6. Detection of p-Akt and p-S6 levels in tumor tissues. A and B, tissue from each tumor generated in the experiments described in Fig. 5C and D was homogenized for preparation of whole-cell protein lysates and subsequent analyses of p-Akt (A) and p-S6 (B) by Western blot analysis. The results were quantitated using Kodak Image Station 2000R. Columns, mean (n = 6); bars, SD. C, p-Akt in H460 xenografts was detected with immunohistochemistry.
LY294002 combination exhibited growth-inhibitory effects that are greater than that caused by each single agent in a 3-day monolayer culture (Fig. 5A). In the long-term colony formation assay, we obtained similar results. This combination worked better than either single agent in decreasing colony size and number (Fig. 5B).

Furthermore, we tested the effects of the combination of RAD001 and LY294002 on the growth of lung cancer xenografts in nude mice. In agreement with the results in cell cultures, the combination of RAD001 and LY294002 exhibited a significantly greater effect than RAD001 or LY294002 alone in inhibiting the growth of A549 xenografts ($P < 0.001$; Fig. 5C). During the 2-week period of treatment, the tumor sizes in mice receiving both RAD001 and LY294002 were smaller in comparison with other groups receiving either vehicle or single-agent treatment (Fig. 5C), indicating an effective anticancer efficacy for the combination treatment. In a H460 xenograft model, we began treatments with relatively larger tumors (in average 300–400 mm$^3$). Both RAD001 and LY294002 alone failed to achieve significant effects on inhibiting the growth of tumors; however, the combination of RAD001 and LY294002 significantly inhibited the growth of H460 xenografts compared with control ($P < 0.05$ or 0.01; Fig. 5D). Collectively, these results clearly show that cotargeting mTOR and PI3K/Akt signaling exhibits enhanced anticancer efficacy.

Cotargeting mTOR and PI3K/Akt signaling enhances inhibition of mTORC1 signaling while preventing Akt phosphorylation in vivo. We also determined whether continuous RAD001 treatment in cancer xenograft models led to an increase in Akt phosphorylation as we observed in cell cultures. By Western blot analysis, we detected p-Akt levels in tumors exposed to RAD001 for 14 days and found that p-Akt levels were significantly increased ($P < 0.05$) in the RAD001-treated group compared with the vehicle control group in both A549 and H460 xenografts (Fig. 6A). As expected, p-Akt levels in tumors exposed to the combination of RAD001 and LY294002 were not increased (Fig. 6A). Immunohistochemical analysis of p-Akt in H460 xenografts also showed that p-Akt levels were increased in RAD001-treated tumors but not in tumors exposed to the combination treatment of RAD001 and LY294002 (Fig. 6C). Thus, these results clearly indicate that continuous treatment of lung tumors with a mTOR inhibitor in nude mice leads to an increase in Akt phosphorylation and this increase can be abrogated by inclusion of a PI3K inhibitor.

Moreover, we determined whether the presence of LY294002 affected the inhibitory effect of RAD001 on mTORC1 signaling in tumor tissues. As presented in Fig. 6B, RAD001 alone significantly decreased the levels of p-S6 ($P < 0.001$), indicating that RAD001 indeed inhibits mTORC1 signaling; however, the presence of LY294002 further reduced the levels of p-S6, which were significantly lower than those in tumors exposed to RAD001 alone ($P < 0.05$ or 0.01). Thus, these results indicate that cotreatment of tumors with a mTOR inhibitor (e.g., RAD001) and a PI3K inhibitor (e.g., LY294002) not only blocks RAD001-induced Akt phosphorylation but also exhibits an enhanced effect on inhibiting mTORC1 signaling.

Discussion

In the current study, we further showed that prolonged treatment with either rapamycin or RAD001 increased p-Akt levels in several human lung cancer cell lines (Fig. 1). A549-RR cells, which were routinely cultured in the presence of 1 μmol/L rapamycin, still exhibited increased levels of p-Akt compared with the parental A549 cells (Fig. 3). Moreover, we detected significantly increased levels of p-Akt in lung cancer xenografts exposed to RAD001 for 14 days (Fig. 6). In current studies, we used 1 or 10 nmol/L of rapamycin or RAD001, which is lower than concentrations (100 or >1,000 nmol/L) used in other studies, showing that prolonged treatment with a mTOR inhibitor decreases p-Akt levels (8, 22). At 100 nmol/L (or even at 10 nmol/L), both rapamycin and RAD001 indeed decreased p-Akt levels after a 24- or 48-h treatment in PC-3, U937, and Jurkat cells as reported (8, 22). However, both rapamycin and RAD001 at 1 nmol/L consistently increased p-Akt levels even after a 48-h exposure in these cell lines (Fig. 1). Thus, it seems that there are two types of cancer cells: one type exhibits increased levels of p-Akt after a prolonged treatment with a mTOR inhibitor regardless of concentrations (e.g., A549 and H157 cells), whereas another type shows dose-dependent alterations in p-Akt levels after prolonged treatment with a mTOR inhibitor (e.g., PC-3 and U937 cells). In the latter cell type, low doses (e.g., 1 nmol/L) of mTOR inhibitors, which sufficiently block mTORC1 signaling (9), clearly increase p-Akt levels.

It has been suggested that mTORC2 is rapamycin insensitive (18), although it can be inhibited by prolonged rapamycin treatment (8). It has been suggested that an equilibrium may exist between mTORC1 and mTORC2 complexes (7). Therefore, it is possible that inhibition of mTORC1 by a mTOR inhibitor somehow shifts the equilibrium to favor or facilitate formation and activation of mTORC2, leading to increase in Akt phosphorylation. In our study, we found that a prolonged treatment with rapamycin (i.e., 24 h) inhibited not only mTORC1 but also mTORC2 (e.g., H157 and A549) with increased Akt phosphorylation in all three lung cancer cell lines (Fig. 2A). In rapamycin-resistant A549-RR cells where p-Akt levels were increased, the assembly of both mTORC1 and mTORC2 was also clearly inhibited (Fig. 3D). Thus, our results clearly indicate that p-Akt levels can be increased under the condition that mTORC2 activity is inhibited.

Although mTORC2 has been recently shown to be an Akt Ser$^{273}$ kinase (19), our results indicate that mTOR inhibitor-induced Akt phosphorylation is unlikely to be mediated by mTORC2 because it is inhibited during mTOR inhibitor treatment. This notion is further supported by our findings that disruption of mTORC2 by knocking down rictor did not block rapamycin-induced Akt phosphorylation (Fig. 2). In agreement with previous findings that raptor knockdown increases Akt phosphorylation (19), we also observed that inhibition of mTORC1 by silencing raptor was sufficient to increase Akt levels in our cell lines tested. These results indicate that mTOR inhibitor-induced Akt activation is the consequence of mTORC1 inhibition. Collectively, we conclude that mTOR inhibitors induce Akt activation through a mTORC1-dependent mechanism independent of mTORC2.

It is well documented that PI3K/Akt represents a major survival pathway that is often associated with resistance to cancer therapy (24–26). The biological significance of mTOR inhibitor-induced Akt activation in mTOR-targeted cancer therapy is unclear. In our study, we observed that p-Akt levels were drastically increased in the rapamycin-resistant cell line (A549-RR). Moreover, when the selective pressure (i.e., rapamycin) was removed, the acquired high levels of p-Akt remained for a long period of time and were tightly associated with cell resistance to mTOR inhibitors. When the sensitivity of rapamycin-resistant (A549-RR) cells to mTOR inhibitors was fully restored after a 5-month removal of rapamycin, p-Akt levels dropped to normal levels comparable with those in rapamycin-sensitive parental cells (A549-P; Fig. 4). Additionally,
enforced reduced p-Akt levels by silencing total Akt levels with Akt siRNA increase cell sensitivity to rapamycin (see Supplementary Fig. S2). Thus, our results suggest a critical role of Akt activation in the development of cell resistance to mTOR inhibitors. Although we suggest the association between sustained Akt activation and development of acquired resistance to mTOR inhibitors, the mechanistic insights into how sustained Akt activation negatively regulates the efficacies of the mTOR inhibitors are still unclear and need further investigation.

PI3K/Akt works upstream of mTORC1 and regulates mTORC1 activity. Therefore, inhibition of PI3K/Akt signaling using PI3K inhibitors should affect mTORC1 activity as well. Moreover, mTOR is a PI3K-related serine/threonine kinase, and its activity can be directly inhibited by the PI3K inhibitors LY294002 and wortmannin (27, 28). Thus, it has been proposed that PI3K inhibitors may share similar signaling pathways with rapamycin, such as mTOR/p70S6K, to exert their biological function (27). If PI3K inhibitors suppress cell growth solely through inhibition of mTOR signaling, cells resistant to rapamycin should be cross-resistant to PI3K inhibitors as was seen with RAD001. In our study, LY294002 or wortmannin was equally effective in inhibiting the growth of A549-P and A549-RR cells. Moreover, LY294002 induced G1 arrest in both A549-P and A549-RR cells with comparable potencies. We also found that LY294002 effectively decreased the levels of p-p70S6K, p-S6, and p-Akt in both A549-P and A549-RR cells (see Supplementary Fig. S3). Together, these results indicate that rapamycin resistance does not interfere with the action of PI3K inhibitors, suggesting that mTOR and PI3K inhibitors exert their biological functions through different mechanisms or PI3K inhibitors suppress cell growth through other mechanisms in addition to inhibition of mTOR signaling. Rapamycin resistance is an important subject of mTOR-targeted cancer therapy in the clinic. Our finding that rapamycin-resistant cells retain sensitivity to PI3K inhibitors has important clinical implications. To overcome or avoid cell resistance to mTOR inhibitors during mTOR-targeted cancer therapy, combination of a mTOR inhibitor with a PI3K inhibitor or intermittent use of a PI3K inhibitor and a mTOR inhibitor may be good approaches. Indeed, our results clearly show that RAD001 combined with LY294002 exhibited enhanced inhibitory effects on the growth of human lung cancer cells in cell cultures (Fig. 5). Importantly, the RAD001 and LY294002 combination worked better than each single agent alone in inhibiting the growth of human lung cancer xenografts in nude mice (Fig. 5), indicating an enhanced anticaner activity in vivo. As expected, treatment of xenografts with RAD001 increased p-Akt levels, which could be abrogated by cotreatment with LY294002. Besides, we found that RAD001 plus LY294002 also exerted an enhanced effect on reduction of p-S6 levels, indicating that inhibition of PI3K/Akt enhances the effect of the mTOR inhibitor on inhibition of mTORC1 signaling (Fig. 6). Collectively, our results validate the strategy for cancer therapy by cotargeting mTOR and PI3K/Akt signaling and warrant clinical evaluation of this strategy for cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Acknowledgments

References

Enhancing Mammalian Target of Rapamycin (mTOR)—Targeted Cancer Therapy by Preventing mTOR/Raptor Inhibition-Initiated, mTOR/Rictor-Independent Akt Activation

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