**Effects of mTOR Inhibitor Everolimus (RAD001) on Bladder Cancer Cells**

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**Abstract**

**Purpose:** We investigated the effect of the mTOR inhibitor RAD001 (everolimus) on human bladder cancer (BC) cells in vitro and in vivo.

**Experimental Design:** The effect of RAD001 on the growth of UM-UC-3, UM-UC-6, UM-UC-9, and UM-UC-14 BC cells were assessed by crystal violet and [3H]thymidine incorporation assays. Flow cytometric cell-cycle analyses were done to measure the apoptotic cell fraction. Protein synthesis was measured using tritium-labeled leucine incorporation assays. The effects of RAD001 on the mTOR pathway were analyzed by Western blotting. To test the effects of RAD001 in vivo, UM-UC-3, UM-UC-6, and UM-UC-9 cells were subcutaneously implanted into nude mice. Tumor-bearing mice were treated orally with RAD001 or placebo. Tumors were harvested for immunohistochemical analysis.

**Results:** In vitro, RAD001 transiently inhibited BC cell growth in a dose-dependent manner. This effect was augmented by re-treatment of cells after 3 days. UM-UC-14 cells were the most sensitive to RAD001, whereas UM-UC-9 cells were the least sensitive. After re-treatment with RAD001, only sensitive cell lines showed G1-phase arrest, with no evidence of apoptosis. RAD001 significantly inhibited the growth of tumors that were subcutaneously implanted in mice. Inhibition of protein synthesis through the S6K and 4EBP1 pathways seems to be the main mechanism for the RAD001-induced growth inhibition. However, inhibition of angiogenesis was the predominant mechanism of the effect of RAD001 on UM-UC-9 cells.

**Conclusions:** The mTOR inhibitor RAD001 inhibits growth of BC cells in vitro. RAD001 is effective in treating BC tumors in an in vivo nude mouse model despite the heterogeneity of in vitro responses. Clin Cancer Res; 17(9); 1–11. ©2011 AACR.

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**Introduction**

Bladder cancer is the fourth most common noncutaneous malignancy and the eighth leading cause of cancer death among men in the United States, with an estimated 13,750 deaths annually (1). Approximately 30% to 40% of patients with high-risk non–muscle-invasive bladder cancer will have progression to more advanced disease within 5 years, and up to 34% of these patients will ultimately die of bladder cancer (2, 3). Radical cystectomy, which is the mainstay of treatment of muscle-invasive bladder cancer, has a failure rate of 30% to 45%, largely because of recurrences in the form of distant metastases (4). Overall, only 20% to 40% of patients with advanced bladder cancer have a 5-year survival rate despite aggressive multimodal therapy (5, 6). To improve survival rates, there is an urgent need not only to improve current chemotherapeutic regimens but also to develop novel chemotherapeutic strategies to prevent or delay disease progression.

In recent years, mTOR, a key downstream protein kinase of the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, has been recognized to play a central role in controlling cancer cell growth (7). AKT and mTOR function as “master switch” proteins in cancer cells to modulate metabolism, the cell cycle, and apoptosis (8). Inhibition of mTOR results in a wide variety of effects on normal and malignant cells, including induction of apoptosis and inhibition of cell-cycle progression, cell growth, angiogenesis, endothelial cell proliferation, and protein translation (7, 9). mTOR inhibitors such as RAD001 (everolimus; Novartis) and AP23573 (Ariad Pharmaceuticals) have been shown to exhibit potent preclinical activities against a wide variety of cancers, including rhabdosarcoma, neuroblastoma, glioblastoma, small cell lung cancer, renal cancer, osteosarcoma, pancreatic cancer, leukemias, B-cell lymphoma, and breast and colon cancer (9). RAD001 has been clinically approved for the treatment of renal cancer.

Given the breadth of its downstream effects on cancer cells, the PI3K/AKT/mTOR signaling pathway may...
represent a promising molecular target for bladder cancer.

In support of this hypothesis, Wu and colleagues recently showed that 55% of human bladder tumors analyzed had increased expression of phosphorylated AKT (p-AKT) and that inhibition of the PI3K pathway could drastically reduce the invasive capacity of bladder cancer cell lines (10). PTEN mutations are also reported to be present in up to 23% of patients with bladder transitional cell carcinoma (10). In addition, we reported that inhibition of the AKT pathway by forced expression of PTEN reduced the growth of bladder cancer cells in vitro and in vivo in a subcutaneous mouse model. In vivo activity was seen despite in vitro heterogeneity. This work suggests that mTOR may be a new therapeutic target for bladder cancer.

Translational Relevance

mTOR, a key downstream protein kinase in the phosphoinositide 3-kinase (PI3K)/AKT signaling pathway, plays a central role in promoting cancer cell growth. mTOR inhibitors have been shown to exhibit potent preclinical activities against a wide variety of solid malignancies and are indicated for clinical use in some cancers. Only recently has it been reported that the PI3K/AKT pathway could be important in bladder cancer. Here, we show that the mTOR inhibitor RAD001 (everolimus) inhibits the growth of bladder cancer cells in vitro and in vivo in a subcutaneous mouse model. In vivo activity was seen despite in vitro heterogeneity. This work suggests that mTOR may be a new therapeutic target for bladder cancer.

Here, we report the effects of RAD001 on bladder cell growth both in vitro in several bladder cancer cell lines with different genetic backgrounds and in vivo in a nude mouse xenograft model.

Materials and Methods

Cell culture

The transitional cell carcinoma cell lines UM-UC-3, UM-UC-6, UM-UC-9, and UM-UC-14 (Table 1) were obtained at source (H.B.G.) from cryopreserved cells frozen over a span of more than 25 years. UM-UC-3 was also obtained from the American Type Culture Collection (ATCC). The cell lines were authenticated within 6 months of conducting the experiments (20). These cell lines were maintained in Eagle's minimum essential medium (Mediatech Inc.) supplemented with 2 mmol/L L-glutamine, 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (20). All cultures were free of bacterial, fungal, and mycoplasmal contamination.

Reagents and drug preparation

RAD001 and placebo were obtained from Novartis Pharma AG. For in vitro experiments, RAD001 was prepared in dimethyl sulfoxide (DMSO). For animal studies, RAD001 was prepared at 2% (w/w; 20 mg/g) in a microemulsion vehicle, which was diluted in 5% glucose in double-distilled water just before administration by oral gavage.

In vitro cell growth

Cell growth was measured using a crystal violet assay as previously described (21). Bladder cancer cells were plated into 6-well plates at a density of 1.25 × 10⁴ cells per well. After 24 hours, the cells were treated with 1 of 6 concentrations of RAD001 (0.1, 0.5, 1, 10, 20, or 100 nmol/L). After 4 and 6 days of exposure to either RAD001 or the control (DMSO), the medium was removed and the cells were fixed with 1% glutaraldehyde for 15 minutes and stained with 0.5% crystal violet. The dye was eluted, transferred to an ELISA 96-well plate, and the optical density was read on a microplate autoreader (Bio-Tek Instruments) at 540 nm. The optical density values of the RAD001-treated

Table 1. The characteristics of p53, PTEN, and p-AKT expression in bladder cancer cell lines are summarized (11, 25)

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>TP53</th>
<th>PTEN</th>
<th>p-AKT</th>
</tr>
</thead>
<tbody>
<tr>
<td>UM-UC-3</td>
<td>Mutated</td>
<td>Mutated</td>
<td>Increased expression</td>
</tr>
<tr>
<td>UM-UC-6</td>
<td>Wild type</td>
<td>Wild type</td>
<td>Decreased expression</td>
</tr>
<tr>
<td>UM-UC-9</td>
<td>Mutated</td>
<td>Decreased expression</td>
<td></td>
</tr>
<tr>
<td>UM-UC-14</td>
<td>Mutated</td>
<td>Wild type</td>
<td>Decreased expression</td>
</tr>
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</table>
cells were normalized to the values obtained for the DMSO-treated control cells to determine the percentage of surviving cells. For the redosing experiments, the cells were re-treated 3 days after the initial treatment with the IC_{50} dose of RAD001. Each assay was done in duplicate, and the experiments were repeated twice.

**In vitro cell proliferation**

Bladder cancer cells were plated into 96-well plates at a density of 8 × 10^3 cells per well. After 24 hours, the cells were treated with 1 of 5 concentrations of RAD001 (0.1, 0.5, 1, 10, or 100 nmol/L). After 48 hours of exposure to RAD001 or the control or DMSO, the medium was removed and replaced with fresh cell culture medium containing 1% FBS and 10 μCi/ml [3H]thymidine (MP Biomedicals). The cells were pulsed with [3H]thymidine for 2 hours, and the media were subsequently removed. Cells were then analyzed by the addition of 0.1 mol/L KOH and harvested onto fiberglass filters. The incorporation of tritium was quantified in a scintillation counter (1450 MICROBETA Trilux liquid scintillation and luminescence counter; PerkinElmer life sciences). Each assay was carried out in duplicate, and the experiments were repeated once.

**Flow cytometry**

Cells were grown in 6-well plates and after reaching 70% confluence were exposed to various concentrations of RAD001 for 24, 48, and 72 hours. Cells were harvested by trypsinization and pelleted by centrifugation. The pellets were then resuspended in PBS containing 50 μg/ml propidium iodide (PI), 0.1% Triton X-100, and 0.1% sodium citrate. DNA staining with PI was measured by fluorescence-activated cell-sorting analysis, using the FL-3 channel (FACSCalibur flow cytometer; Becton Dickinson), to determine the cell-cycle distribution. Cells displaying a hypodiploid DNA content, which is indicative of DNA fragmentation, were scored as apoptotic. PI exclusion was carried out in a similar fashion 24 and 48 hours after RAD001 exposure, without the addition of 0.1% Triton X-100. Annexin V-fluorescein isothiocyanate (FITC) and PI flow cytometry were carried out following the manufacturer’s instructions (TACS Annexin V–FITC Apoptosis Detection Kit from Trevigen).

**Protein synthesis**

The bladder cancer cells were plated into 6-well plates at a density of 1 × 10^5 cells per well. After 24 hours, the cells were treated with various concentrations of RAD001. After 2 and 12 hours of exposure to either RAD001 or control (DMSO), the medium was removed, 2 μCi/ml of l-[4,5-3H]leucine (GE Healthcare) and leucine-free media (Dulbecco’s modified Eagle’s medium without l-glutamine and leucine; MP Biomedical) supplemented with 20 ml/L of 200 mmol/L l-glutamine and 110 mg/L of sodium pyruvate were added, and the cells were incubated at 37°C in a humidified chamber with 5% CO₂ for 2 hours. The radiolabeled protein–uptake assay was done as previously described (22). The radioactivity of the solution was measured as disintegrations per minute (DPM), using a liquid scintillation counter (Beckman LS 6500; Global Medical Instrumentation, Inc.). All experiments were carried out in triplicate and repeated once.

**Western blot analysis**

Cells were harvested at 70% to 80% confluence, and cell lysates were obtained as previously described (11). Protein extracts (30–50 μg per lane) were electrophoretically separated on a 4% to 20% Tris-glycine gel (Invitrogen) and transferred onto nitrocellulose membranes (11). The nitrocellulose membranes were incubated with 1:200 to 1:1,000 dilutions of primary antibodies, followed by a horseradish peroxidase–linked secondary antibody (anti-rabbit 1:3,000 or anti-mouse 1:5,000). The antibody-probed proteins were detected using an enhanced chemiluminescence kit (Amersham Biosciences) according to the manufacturer’s instructions.

**Animal studies**

All animal experiments were conducted according to institutional guidelines established for the Animal Core Facility at The University of Texas M. D. Anderson Cancer Center. UM-UC-3, UM-UC-6, and UM-UC-9 cells (1 × 10^6) were injected subcutaneously into the right flank of 4- to 6-week-old female nude mice. Tumor growth was measured with calipers twice per week. The tumor volume was calculated according to the following equation: volume \((\text{mm}^3) = \text{length} \times \text{width}^2 \times 0.5236\). When the tumors reached approximately 5 mm in diameter (1 week after injection), the animals received RAD001 by oral gavage at a dose of 5 mg/kg in 100 μl 5% glucose in water or a placebo (Novartis Pharma AG) twice weekly for 1 month or until the tumor burden (approximately 1.5 cm in size) required euthanasia (anesthetized with CO₂ and euthanized by cervical dislocation). Harvested tumor specimens were divided into 2 groups and either (i) fixed in 10% buffered formalin and paraffin-embedded or (ii) embedded in OCT (optimum cutting temperature) and frozen in liquid nitrogen. All experiments were repeated once.

**Immunohistochemical analysis**

Paraffin-embedded tissue sections were incubated in citrate buffer, pH 6.0, at 100°C for 20 minutes for antigen retrieval. The Lab Vision Autostainer (Lab Vision Corp.; Thermo Fisher Scientific) was used for staining the samples with diaminobenzidene. Anti-p-p70S6K (Thr 389; Epi-
tomics; species reactivity: human and mouse), anti-CD31 (Lab Vision Corp.; Thermo Fisher Scientific; species reactivity: human), anti-VEGF (Lab Vision Corp.; Thermo Fisher Scientific; species reactivity: human), anti-p-AKT (Ser 473; Cell Signaling; species reactivity: human, mouse, rat, hamster, monkey, Drosophila melanogaster, bovine, and zebrafish), and anti-p-mTOR (Ser 2448; species reactivity: human, possibly mouse and rat) antibodies (Cell Signaling) were used for immunohistochemical analyses. Negative control experiments were conducted in the absence of the primary antibody. Tumor tissue sections from all 3 implanted cell lines were concurrently incubated with each type of antibody and stained with the autostainer. Additional tumor tissue sections from each cell line were used for immunohistochemical staining as positive controls for the other 2 cell lines (Supplementary Fig. S1). The tissue specimens were evaluated by light microscopy, and the immunostaining was scored by 2 independent observers who were blinded to samples (i.e., drug-treated or placebo) that they were evaluating. Only cytoplasmic staining was considered for scoring. The staining intensity was assigned a score for the most dominantly stained area, and this stained area should occupy at least 20% of the specimen. The intensity of staining was scored on a scale of 0 to 3+, with 0 for no staining, 1+ for weak staining intensity, 2+ for moderate staining intensity, and 3+ for the most intense staining (examples of staining intensities are shown in Supplementary Fig. S2). The microvessel density (MVD) was measured by microscopy at 100x magnification by counting the number of blood vessels (stained with anti-CD31) in each of 5 fields per tumor sample to obtain an average measurement (23). The fields with the highest density of blood vessels on the slide were selected for analysis. The CD31 expression data were analyzed with repeated-measures ANOVA. Statistical significance was determined to be P < 0.05.

Results

Growth inhibitory effects of RAD001 in vitro

The bladder cancer cell lines UM-UC-3, UM-UC-6, UM-UC-9, and UM-UC-14 were treated with increasing doses of RAD001 (0.1–100 nmol/L) and incubated for up to 6 days. RAD001 inhibited the growth of all bladder cancer cells tested in a dose-dependent manner, with a partial loss of response seen 4 days after initial treatment (Fig. 1A). The UM-UC-14 cell line was the most sensitive to RAD001, with an IC50 of 0.1 nmol/L; the UM-UC-3 and UM-UC-6 cell lines had moderate sensitivities, with IC50s of 0.5 nmol/L; and the UM-UC-9 cell line was the least sensitive, with an IC50 of 10 nmol/L. Re-treatment of cells with RAD001 (at a dose corresponding to each cell line IC50) 3 days after the initial therapy augmented the growth inhibition in all 4 bladder cancer cell lines (Fig. 1B). The timing of redosing at day 3 was based on the observation that there was loss of response to RAD001 in all 4 cell lines from day 4 of incubation (Fig. 1A). The transient effect of a single dose of RAD001 is likely related to its half-life of 60 hours (24).

[3H]Thymidine incorporation assays showed that there was a dose-dependent decrease in the number of bladder cancer cells proliferating when treated with RAD001 (Fig. 1C). This was consistent with the growth inhibitory effects of RAD001 on bladder cancer cells seen in the crystal violet assays (Fig. 1A).

Cell cycle and apoptosis

All 4 cell lines were treated once with increasing doses of RAD001 (0.1, 0.5, 10, and 100 nmol/L), and cell-cycle analysis was done at various time points (24, 48, and 72 hours). We saw no specific growth-phase arrest with a single treatment of RAD001 in any of the 4 cell lines tested (Fig 2A). When we re-treated UM-UC-6 and UM-UC-9 cell lines with RAD001, 3 days after the initial dose, only the UM-UC-6 cells were arrested in the G0/G1 phase (Fig 2A). We did not see any evidence of apoptosis in any of the 4 cell lines tested by Annexin V assays, which were carried out 12 and 24 hours after a single treatment of cells with RAD001, even at doses as high as 100 nmol/L (data not shown). We corroborated these findings with PI exclusion assays 24 and 48 hours after RAD001 treatment, which showed that RAD001 induced nonapoptotic cell death at the IC50 doses in all of the bladder cancer cell lines except UM-UC-9 (Fig. 2B, data not shown for cell lines UM-UC-3 and UM-UC-14).

Protein synthesis

Using a radioactively labeled amino acid (leucine), we showed that RAD001 inhibited protein synthesis in all 4 bladder cancer cell lines (Supplementary Fig. S3, data not shown for cell lines UM-UC-3 and UM-UC-14).

Downstream effects of mTOR blockade by RAD001

To investigate the effects of RAD001 treatment on mTOR signaling, we used Western blotting to analyze the levels of the phosphorylated forms of mTOR, p70S6K, 4EBP1, and nonphosphorylated forms of mTOR and p70S6K after a single 24-hour exposure of UM-UC-3, UM-UC-6, UM-UC-9, and UM-UC-14 cells to various doses of RAD001. In all 4 cell lines, the levels of the phosphorylated forms of mTOR, p70S6K, and 4EBP1 were reduced with increasing doses of RAD001, but the effects of RAD001 on the nonphosphorylated form of mTOR protein levels in each cell line were different, with only UM-UC-3 and UM-UC-6 having a slight decrease in mTOR levels with increasing RAD001 dose (Fig. 3). No downstream effect of RAD001 was seen in the nonphosphorylated form of p70S6K in all 4 cell lines (Fig 3). Overall, the effects of RAD001 on the levels of these proteins in the mTOR signaling pathway did not correlate with the relative sensitivity of each cell line to the growth inhibitory effects of RAD001.

Effects of RAD001 in vivo

UM-UC-3, UM-UC-6, and UM-UC-9 cells were implanted subcutaneously into nude mice, and when the xenograft tumors reached 5 mm in diameter, the mice...
were treated with RAD001 by oral gavage twice per week for 1 month. RAD001 significantly inhibited the growth of tumors derived from all 3 cell lines (Fig. 4). Immunohistochemical analysis of tumor tissue samples showed reduced expression of p-p70S6K in the RAD001-treated UM-UC-3- and UM-UC-6–derived tumors compared with control-treated tumors, whereas the expression of p-p70S6K in RAD001-treated UM-UC-9–derived tumors was unchanged (Table 2 and Fig. 5). There was no difference in the expression level of p-mTOR between the RAD001-treated and control-treated tumors. For the UM-UC-3- and UM-UC-9–derived tumors, p-AKT expression was reduced in the RAD001-treated compared with control-treated tumors, but the p-AKT levels were unchanged between the RAD001-treated and control-treated UM-UC-6–derived tumors.

Figure 1. The inhibitory effects of RAD001 on bladder cancer cell growth and proliferation determined by crystal violet assays (A and B) and [3H]thymidine incorporation assays (C), respectively. UM-UC-3, UM-UC-6, UM-UC-9, and UM-UC-14 cells were treated with RAD001 and incubated for 6 days (A), with RAD001 initially at day 0 and then re-treated at day 3 with a dose equivalent to the IC50 value for each cell line and incubated for 5 days (B), and with RAD001 and incubated for 48 hours (C).
Figure 2. Flow cytometric analysis of bladder cancer cell lines. A, cell-cycle analysis was done at 48 hours for control- and RAD001-treated (0.5 nmol/L) UM-UC-6 cells, control- and RAD001-treated (10 nmol/L) UM-UC-9 cells, UM-UC-6 cells treated with RAD001 (0.5 nmol/L) every 3 days, and UM-UC-9 cells treated with RAD001 (10 nmol/L) every 3 days. Bar charts comparing the differences in the percentage of G\(_1\)-phase cells between controls and RAD001-treated cells are shown. B, PI exclusion assays were conducted 24 hours after UM-UC-6 cells and UM-UC-9 cells were treated with RAD001. The flow cytometric profiles with the percentage of cells undergoing nonapoptotic cell death (G) are shown.
The vascular density in the tumors was measured by counting the number of blood vessels that expressed CD31. RAD001 treatment did not affect the vascular density of UM-UC-6–derived tumors ($P = 0.5503$; Table 2 and Fig. 5). However, RAD001 treatment modestly reduced the vascular density of the UM-UC-3–derived tumors ($P = 0.0597$) and significantly reduced the vascular density of the UM-UC-9–derived tumors ($P = 0.036$; Table 2 and Fig. 5).

Expression levels of VEGF were similar in the RAD001- and control-treated tumors derived from all 3 cell lines (Table 2), suggesting that the changes in vascular density were not due to changes in the VEGF pathway.

Discussion

In this study, we show that the inhibition of the mTOR pathway effectively reduces the growth of bladder cancer cells despite the variations in genetic backgrounds of the bladder cancer cell lines. This presents further evidence that the PTEN/AKT/mTOR signaling pathway is important in bladder cancer and that mTOR may be used as a molecular target for bladder cancer therapy.

Like wild-type bladder cancer cells, the cell lines we tested have different alterations in the PTEN/AKT and p53 pathways. The UM-UC-3 cells have a PTEN deletion, constitutively active p-AKT, and mutated TP53; UM-UC-6 cells have wild-type PTEN and TP53; UM-UC-9 cells have reduced PTEN expression and active p-AKT; and UM-UC-14 cells have wild-type PTEN and no p-AKT expression (Table 1; refs. 11, 25). The effect of mTOR inhibition on AKT activity has been reported to be variable, depending on the cell type, and inhibition of mTOR has led to increased activation, no change, or reduced phosphorylation of AKT after prolonged treatment (26, 27). We show that RAD001 reduces the expression of AKT in the UM-UC-3– and UM-UC-9–derived tumors, both cell lines constitutionally express p-AKT (Tables 1 and 2). This reduction in AKT expression was not seen in the RAD001-treated UM-UC-6–derived tumors, a cell line that has a normal PTEN/AKT pathway (Tables 1 and 2). Although it has been reported that in other tumors, PTEN and AKT status may correlate with the sensitivity of cell lines to mTOR inhibitors, we did not find this relationship to be present in our panel of bladder cancer cells despite having chosen cell lines with known variable PTEN/AKT status for our study (19, 28). mTOR comprises 2 multiprotein complexes: mTOR complexes 1 and 2 (mTORC1 and mTORC2; ref. 26). It is known that mTORC1 is rapamycin-sensitive and its activation results in phosphorylation of 4EBP1 and S6K1, whereas mTORC2 may be regulated by rapamycin to cause either AKT activation or inactivation (26). The variable effect of RAD001 on p-AKT levels in different bladder cancer cell types could be due in part to the differential effects of mTOR inhibition on mTORC1 and mTORC2 in different cell lines. The complexity of the multiple pathways involved may be one of the reasons for not seeing a direct relationship between the PTEN/AKT status and effects of mTOR inhibition.

Our in vitro results show that RAD001 inhibits the growth of bladder cancer cells at concentrations that are physiologically safe in humans (29). However, the response of bladder cancer cells to RAD001 in vitro was heterogeneous, with UM-UC-14 being the most sensitive cell line and UM-UC-9 being the least sensitive. The IC$_{50}$ value of UM-UC-9 was approximately 20 times higher than that in the UM-UC-3 and UM-UC-6 cell lines, and 100 times higher than in the UM-UC-14 cell line, respectively. The loss of the inhibitory effect of a single dose of RAD001 4 days after treatment was overcome by re-treatment of cells on the third day following the first dose. This transient effect of RAD001 provided the basis for the dosing schedule in our in vivo murine experiments. Because mTOR inhibition was not seen to correlate with expression of upstream targets (PTEN and AKT), it is therefore likely that the variability in sensitivity and the transient nature of response of different cell types to single doses of RAD001 was mainly due to the differential effects of mTOR.
inhibition on downstream effectors. It is known that the downstream effectors of mTOR are 4EBP1 and S6 kinase 1 (S6K1), both of which are regulators of protein translation (7, 9). Activation of mTOR leads to phosphorylation of 4EBP1, which prevents it from binding to eukaryotic initiation factor 4E (eIF4E), which, in turn, is then free to bind to mRNA transcripts and other translation initiation complex proteins and activate cap-dependent translation (7, 26, 30). S6K1, a key regulator of cell growth, phosphorylates ribosomal protein S6 and enhances the translation of 5′-term-

inal oligopyrimidine tract mRNAs in some models (26). It has also been reported to play a role in glucose homeostasis by phosphorylating insulin receptor substrate 1 and glycogen synthase kinase 3 and by regulating other factors such as eukaryotic initiation factor 4B, programmed cell death 4, eukaryotic elongation factor-2 kinase, and S6K1 Aly/REF-like target (7, 26). Mansure and colleagues recently suggested that the reduction of phosphorylation levels of S6 activation correlated with the sensitivity of bladder cancer cells to RAD001 and that S6 inhibition may reflect the sensitivity of these cells to mTOR inhibition (19). In our study, RAD001 reduced the expression of p-p70S6K and p-4EBP1 in vitro in all cell lines tested. However, we did not observe a correlation between S6 phosphorylation levels and sensitivity to mTOR inhibition by RAD001. One reason for the difference may be due to the fact that the panel of bladder cancer cell lines tested was different in both studies. The differential sensitivity of bladder cancer cells to mTOR inhibition may be due to a variety of other factors, such as variability of mTORC1 and mTORC2 response or activation/inhibition of other pathways in response to mTOR inhibition. It has been shown that mTORC1 and S6K may be activated independent of mTOR, possibly through the mitogen-activated protein (MAP)/extracellular signal-regulated kinase (ERK)/ERK signaling pathway (31, 32). It was also shown that RAD001 can activate MAP kinase (MAPK), which is mediated by S6K/P13K/Ras signaling, and that this feedback could be a cause of the poor anticancer activity of mTORC1 inhibitors (33). These complex relationships between mTOR and other pathways may account for differences in sensitivity of various cell lines to RAD001 and the inability to reliably correlate expression of effectors such as S6K and response to mTOR inhibition. Such relationships deserve further investigation.

Contrary to other reports (7, 19), we observed that a single treatment of RAD001 did not cause a specific growth-phase arrest in any of the cell lines we tested. Re-treatment with RAD001 induced G0/G1 arrest in UM-UC-3 and UM-UC-6 but not in UM-UC-9 cells, which are resistant to RAD001 in vitro. Because MAPK is known to regulate transcription of genes that are important for cell cycle, effects of the MAPK feedback loop with mTOR may contribute to the differential effects on cell-cycle arrest seen in various cell lines. In our study, we show that RAD001 induced nonapoptotic cell death in all the cell lines tested. As autophagic activation by mTOR inhibition is known to occur, this may be another possible mechanism of bladder cancer cell growth inhibition by RAD001 (34). Further evaluation of the relationship between mTOR inhibition and autophagy is currently underway.

Our in vivo data confirm that mTOR inhibition by RAD001 is effective in inhibiting the growth of UM-UC-3, UM-UC-6, and UM-UC-9 bladder cancer cells subcutaneously implanted into nude mice. Although the bladder cancer cell lines we tested in vivo responded to RAD001 treatment with different sensitivities, this difference was not seen in vitro. We hypothesize that the differences in
sensitivity to RAD001 between the cell lines in vitro and in vivo reflect the results of mTOR inhibition of alternate pathways such as the angiogenesis pathway, which are only functional in vivo. Consistent with the in vitro findings, p-p70S6K expression in RAD001-treated tumors was reduced in tumors derived from UM-UC-3 and UM-UC-6 cell lines. However, contrary to our in vitro findings wherein expression was measured after a single RAD001 dose, UM-UC-9–derived tumors exhibited similar levels of p-p70S6K in RAD001- and control-treated tumors but had a significantly reduced mean vascular density (Table 2). Interestingly, VEGF expression was not different between

| Table 2. Summary of the scoring results of immunohistochemical analysis for p-mTOR, p-p70S6K, p-AKT, CD31, and VEGF expression in UM-UC-3, UM-UC-6, and UM-UC-9 tumors from mice treated with RAD001 |
|---|---|---|---|---|
| Cell lines | Immunohistochemical marker |
| | p-mTOR | p-p70S6K | p-AKT | VEGF | CD31 |
| | Staining intensity score | | | Mean MVD (range) | |
| UM-UC-3 | | | | |
| Control treated | 0 | 2+ | 2+ | 1+ | 21.8 (18.6–25.0) | 0.0597 |
| RAD001 treated | 0/1+ | 1+ | 1+ | 1+ | 17.4 (14.1–20.7) | |
| UM-UC-6 | | | | |
| Control treated | 0 | 2+ | 2+ | 3+ | 16.0 (14.0–18.0) | 0.5503 |
| RAD001 treated | 0 | 1+ | 2+ | 3+ | 16.7 (14.7–18.7) | |
| UM-UC9 | | | | |
| Control treated | 1+/2+ | 1+ | 3+ | 2+ | 31.1 (25.0–37.2) | 0.0365 |
| RAD001 treated | 1+ | 1+ | 2+ | 2+ | 20.9 (14.8–27.0) | |

NOTE: MVD was quantified at 100× magnification.

Figure 5. Immunohistochemical staining of representative tumor tissue sections harvested after prolonged treatment with either a placebo or RAD001. p-mTOR, p-p70S6K, and p-AKT are shown at 400× magnification, and CD31 is shown at 100× magnification.
the RAD001- and control-treated tumors in any of the 3 cell lines tested (Table 2). On the basis of these findings, we hypothesize that inhibition of protein synthesis through the S6K and 4EBP1 pathways, rather than inhibition of angiogenesis, seems to be the more important mechanism for bladder cancer cell growth inhibition in UM-UC-3 and UM-UC-6 cells (Table 2 and Fig. 5). However, in the UM-UC-9 cells, inhibition of angiogenesis, possibly through a non–VEGF-related pathway, seems to play a dominant role.

Although the cell lines we tested had different genetic backgrounds, RAD001 was active against all cell lines tested both in vitro and in vivo, but the sensitivities of the different cell lines to RAD001 in vitro did not correlate with the in vivo results. Thus, our results suggest that the growth inhibitory action of RAD001 on bladder cancer cell lines is independent of genomic genomic alterations in the AKT pathway or the AKT phosphorylation status.

Limitations of our study include the small number of cell lines used, as responses of other cell lines to RAD001 may yield additional insights in the differential response to mTOR inhibition. Furthermore, there are potentially multiple mechanisms for differences in sensitivity to mTOR inhibition that have yet to be tested. Despite these limitations, this study is one of very few preclinical studies that describe the use of mTOR inhibition in bladder cancer. p-mTOR expression was recently shown to be increased in 32% of human bladder cancers compared with noncancerous bladder tissue, and activation of p-S6K was also seen in 25% of invasive human bladder cancers, which displayed altered p53 and PTEN expression (17, 35). These findings, together with current limited but growing preclinical evidence of the efficacy of mTOR inhibition in bladder cancer cells, suggest mTOR inhibition may have a future role in the treatment of selected human bladder urothelial cancers.

Conclusions

The mTOR inhibitor RAD001 inhibits protein synthesis and growth of bladder cancer cells in vitro at doses ranging from 0.1 to 100 nmol/L. Despite the heterogeneity of responses seen in vitro, RAD001 is effective in vivo, in part, because of changes in angiogenesis. In conclusion, RAD001 affects tumor growth through different mechanisms, depending on the genotype of the bladder cancer cells. This work suggests that mTOR is potentially a new therapeutic target for bladder cancer. Future studies are required to determine its role in bladder cancer therapy.

Disclosure of Potential Conflicts of Interest

H.B. Grossman is on Novartis Advisory Board 2007. All other authors have no conflict of interest.

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