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

Edmund Chiong¹, I-Ling Lee², Ali Dadbin², Anita L Sabichi³, Loleta Harris², Diana Urbauer⁴, David McConkey², Rian Jason Dickstein², Tiewei Cheng², H. Barton Grossman²

¹ Department of Urology, National University Health System, Singapore
² Department of Urology, The University of Texas M. D. Anderson Cancer Center, USA
³ Department of Clinical Cancer Prevention, The University of Texas M. D. Anderson Cancer Center, USA
⁴ Division of Quantitative Sciences, The University of Texas M. D. Anderson Cancer Center, USA

1515 Holcombe Boulevard
Houston, Texas 77030

Please address all correspondence to:

H. Barton Grossman, Department of Urology
Unit 1373
The University of Texas M. D. Anderson Cancer Center
1515 Holcombe Boulevard Houston, TX 77030
Phone: 713-792-3250
Facsimile: 713-794-4824
Email: hbgrossman@mdanderson.org
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Conflict of interest

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Statement of Translational Relevance

mTOR, a key downstream protein kinase in the 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.

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Abstract

**Purpose:** We investigated the effect of the mTOR inhibitor everolimus (RAD001) 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 cytometry cell cycle analyses were performed 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 retreatment of cells after 3 days. UM-UC-14 cells were the most sensitive to RAD001, while UM-UC-9 cells were the least sensitive. After retreatment 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 4E-BP1 pathways appears to be the main mechanism for the RAD001-induced growth inhibition. However, inhibition of angiogenesis was the predominant mechanism of RAD001’s effect 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.
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 for 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 survive 5 years, 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, mammalian target of rapamycin (mTOR), a key downstream protein kinase of the phosphatidylinositol 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 et al. recently showed that 55% of human bladder tumors analyzed had increased expression of phosphorylated AKT (pAKT) 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, and that the PTEN/AKT pathway could be an important therapeutic target for bladder cancer (11). AKT, a serine-threonine kinase that is dependent on PI3K signaling for activation, is known to be involved in cell proliferation and a variety of anti-apoptotic pathways (12). Inactivation of the tumor suppressor gene PTEN, which is mapped to chromosome 10q23, has also been suggested to be involved in bladder cancer progression (13-16). Recently, a synergistic relationship between deletion of p53 and PTEN, which deregulates of mTOR signaling was shown to promote tumorigenesis in a murine model of bladder cancer (17), and rapamycin has been shown to inhibit the proliferation of bladder cancer cells in vitro (18). Thus we hypothesized that RAD001, by inhibiting the mTOR pathway, may inhibit the growth of bladder cancer cells. A recent pre-clinical
study had shown that mTOR inhibition by RAD001 has anti-proliferative activity in bladder cancer, supporting this hypothesis (19).

Here, we report the effects of RAD001 on bladder cancer 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. Barton Grossman, MD) 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; Manassas, VA). The cell lines were authenticated within 6 months of performing the experiments (20). These cell lines were maintained in Eagle’s minimum essential medium (Mediatech Inc.) supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal bovine serum, 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 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 six-well plates at a density of
1.25 × 10^4 cells/well. After 24 h, the cells were treated with one of six concentrations of
RAD001 (0.1, 0.5, 1, 10, 20, or 100 nM). 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 min 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 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 retreated 3 days after the initial treatment with the IC_{50} dose of RAD001. Each
assay was performed 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/well. After 24 h, the cells were treated with one of five
concentrations of RAD001 (0.1, 0.5, 1, 10, or 100 nM). After 48 hours of exposure to
either RAD001 or the control or DMSO, the medium was removed and replaced with
fresh cell culture medium containing 1% fetal bovine serum and 10 μCi/mL
[^3]H]thymidine (MP Biomedicals). The cells were pulsed with [^3]H]thymidine for 2 h and
the media was subsequently removed. Cells were then lysed by the addition of 0.1 mol/L
KOH and harvested onto fiberglass filters. The incorporated tritium was quantified in a
scintillation counter (1450 MICROBETA Trilux liquid scintillation and luminescence counter; PerkinElmer™ life sciences). Each assay was performed in duplicate and the experiments were repeated once.

**Flow cytometry.** Cells were grown in six-well plates and after reaching 70% confluence were exposed to various concentrations of RAD001 for 24, 48, and 72 h. Cells were harvested by trypsinization and pelleted by centrifugation. The pellets were then resuspended in phosphate-buffered saline 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 performed in a similar fashion 24 and 48 h after RAD001 exposure, without the addition of 0.1% Triton X-100. Annexin V-fluorescein isothiocyanate and PI flow cytometry were performed following the manufacturer’s instructions (TACS Annexin V-FITC Apoptosis Detection Kit from Trevigen).

**Protein synthesis.** The bladder cancer cells were plated into six-well plates at a density of $1 \times 10^5$ cells/well. After 24 h, the cells were treated with various concentrations of RAD001. After 2 and 12 h 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 mM 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 h. The radiolabeled protein–uptake assay was performed 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 performed 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 to 50 μg/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 (antirabbit 1:3,000 or antimouse 1:5,000). The antibody-probed proteins were detected using an enhanced chemiluminescence kit (Amersham Biosciences) according to the manufacturer's instructions. Anti-phosphorylated (phospho)-p70S6K (Thr 389), p70S6K, anti-phosphorylated-mTOR (Ser 2448), mTOR (Ser 2448) and anti-phosphorylated-4EBP1 (Ser 65) antibodies were purchased from Cell Signaling. The secondary antibodies were purchased from Santa Cruz Biotechnology, and the anti-actin antibody was purchased from EMD Biosciences. A cell line from another organ system was used as a positive control.

**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 x 10⁶) 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 (mm³) = length x width² x 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 two groups and either 1) fixed in 10% buffered formalin and paraffin-embedded or 2) embedded in OCT 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 min for antigen retrieval. The Lab Vision Autostainer (Lab Vision Corp., Thermo Fisher Scientific) was used for staining the samples with diaminobenzidene. Anti– phosphorylated-p70S6K (Thr 389) (Epitomics) (species reactivity: human and mouse), anti-CD31 (Lab Vision Corp., Thermo Fisher Scientific) (species reactivity: human), anti-vascular endothelial growth factor (VEGF; Lab Vision Corp., Thermo Fisher Scientific) (species reactivity: human), anti-phosphorylated-AKT (Ser 473) (Cell Signaling) (species reactivity: human, mouse, rat, hamster, monkey, D. melanogaster, bovine and zebrafish), and anti-phosphorylated-mTOR (Ser 2448) (species reactivity: human, possibly mouse and rat) antibodies (Cell Signaling) were used for immunohistochemical analyses. Negative control experiments were performed in the absence of the primary antibody. Tumor tissue sections from all
three 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 two
(Supplementary Fig. 1). The tissue specimens were evaluated by light microscopy, and
the immunostaining was scored by two independent observers who were blinded to which
samples (i.e., drug-treated or placebo) 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. 2). The
microvessel density was measured by microscopy at 100X magnification by counting the
number of blood vessels (stained with anti-CD31) in each of five fields per tumor sample
to obtain an average measurement (25). The fields with the highest density of blood
evessels on the slide were selected for analysis. The CD31 expression data were analyzed
with repeated measures analysis of variance. 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 nM to 100 nM) 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 nM, the UM-UC-3 and UM-UC-6 cell lines had moderate sensitivities, with IC₅₀s of 0.5 nM, and the UM-UC-9 cell line was the least sensitive, with an IC₅₀ of 10 nM. Retreatment of cells with RAD001 (at a dose corresponding to each cell line’s IC₅₀) 3 days after the initial therapy augmented the growth inhibition in all four 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 four 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 h (26). The [³H]Thymidine incorporation assays showed that there were 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 four cell lines were treated once with increasing doses of RAD001 (0.1 nM, 0.5 nM, 10 nM, and 100 nM), and cell cycle analysis was performed at various time points (24, 48, and 72 h). We saw no specific growth phase arrest with a single treatment of RAD001 in any of the four 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 four cell lines tested by Annexin V assays, which were performed 12 and 24 h after a single treatment of cells with RAD001, even at doses
as high as 100 nM (data not shown). We corroborated these findings with PI exclusion assays 24 and 48 h after RAD001 treatment, which showed that RAD001 induced nonapoptotic cell death at the IC_{50} 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 demonstrated that RAD001 inhibited protein synthesis in all four bladder cancer cell lines (Supplementary Fig. 3, 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, 4E-BP-1 and non-phosphorylated forms of mTOR and p70S6K, after a single 24-h exposure of UM-UC-3, UM-UC-6, UM-UC-9, and UM-UC-14 cells to various doses of RAD001. In all four cell lines, the levels of the phosphorylated forms of mTOR, p70S6K, and 4E-BP-1 were reduced with increasing doses of RAD001, but the effects of RAD001 on the non-phosphorylated 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 non-phosphorylated 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 three cell lines (Fig. 4). Immunohistochemical analysis of tumor tissue samples demonstrated reduced expression of phosphorylated p70S6K in the RAD001-treated UM-UC-3– and UM-UC-6–derived tumors compared with control-treated tumors, while the expression of phosphorylated 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 phosphorylated mTOR between the RAD001-treated and control-treated tumors. For the UM-UC-3– and UM-UC-9–derived tumors, phosphorylated AKT expression was reduced in the RAD001-treated compared with control-treated tumors, but the phosphorylated AKT levels were unchanged between the RAD001-treated and control-treated UM-UC-6–derived tumors.

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 three 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, in spite of the variations in genetic backgrounds of the bladder cancer cell lines. This presents further evidence that the PI3K/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 phosphorylated AKT, and mutated TP53; UM-UC-6 cells have wild-type PTEN and TP53; UM-UC-9 cells have reduced PTEN expression and active phosphorylated AKT; and UM-UC-14 cells have wild-type PTEN and no phosphorylated AKT expression (Table 1) (11, 27). 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 (28, 29). 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 phosphorylated AKT (Tables 1A and 1B). 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, 30). mTOR comprises two multiprotein complexes: mTOR complexes 1 and 2 (mTORC1 and mTORC2) (28). It is known that mTORC1 is rapamycin-sensitive and its activation results in phosphorylation of 4EBP1 and S6K1, while mTORC2 may be regulated by rapamycin to cause either AKT activation or inactivation (28). The variable effect of RAD001 on phosphorylated 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 demonstrate that RAD001 inhibits the growth of bladder cancer cells at concentrations that are physiologically safe in humans (31). 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} of UM-UC-9 was approximately 20 times higher than 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 retreatment 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. Since mTOR inhibition was not seen to correlate with expression of upstream targets (PTEN and AKT), it is therefore likely that the variability of 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 4E-BP1 and S6 kinase 1 (S6K1), both of which are
regulators of protein translation (7, 9). Activation of mTOR leads to phosphorylation of
4E-BP1, which prevents it from binding to eukaryotic initiation factor 4E (eIF4E). EIF4E
is then free to bind to mRNA transcripts and other translation initiation complex proteins
and activate cap-dependent translation (7, 28, 32). S6K1, a key regulator of cell growth,
phosphorylates ribosomal protein S6 and enhances the translation of 5’-terminal
oligopyrimidine tract mRNAs in some models (28). S6K1 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, 28). Mansure et al 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 phosphorylated p70S6
kinase and phosphorylated 4E-BP1 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 MEK-ERK signaling pathway (33, 34). It was also shown that RAD001 can activate MAP Kinase (MAPK), which is mediated by S6K-PI3K-Ras signaling, and that this feedback could be a cause of the poor anticancer activity of mTORC1 inhibitors (35). These complex relationships between mTOR and other pathways may account for differences in various cell lines’sensitivity 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. Retreatment 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. Since 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 non-apoptotic 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 (24). 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 vitro responded to RAD001 treatment with different sensitivities, this difference was not seen in vivo. 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, that are only functional in vivo. Consistent with the in vitro findings, phosphorylated 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 where expression was measured after a single RAD001 dose, UM-UC-9–derived tumors exhibited similar levels of phosphorylated p70S6K in RAD001- and control-treated tumors but had a significantly reduced mean vascular density (Table 1B). Interestingly, VEGF expression was not different between the RAD001- and control-treated tumors in any of the three cell lines tested (Table 1B). On the basis of these findings, we hypothesize that inhibition of protein synthesis through the S6K and 4E-BP1 pathways, rather than inhibition of angiogenesis, appears to be the more important mechanism for bladder cancer cell growth inhibition in UM-UC-3 and UM-UC-6 cells (Table 1B and Fig. 5). However, in the UM-UC-9 cells, inhibition of angiogenesis, possibly through a non-VEGF–related pathway, appears 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 PTEN 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 pre-clinical studies that describe the use of mTOR inhibition in bladder cancer. Phospho-mTOR expression was recently shown to be increased in 32% of human bladder cancers compared to non-cancerous 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, 36). These findings, together with current limited but growing pre-clinical 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.

Conclusion

The mTOR inhibitor RAD001 inhibits protein synthesis and growth of bladder cancer cells in vitro at doses ranging from 0.1 nM to 100 nM. 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.

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Figure Legend

**Fig. 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 *A*, RAD001 and incubated for 6 days. *B*, Cells were treated with RAD001 initially at day 0 and then retreated at day 3 with a dose equivalent to the IC₅₀ for each cell line and incubated for 5 days. *C*, RAD001 and incubated for 48 h.

**Fig. 2.** Flow cytometry analysis of bladder cancer cell lines. *A*, Cell cycle analysis was performed at 48 h for control- and RAD001-treated (0.5 nM) UM-UC-6 cells, control- and RAD001-treated (10 nM) UM-UC-9 cells, UM-UC-6 cells treated with RAD001 (0.5 nM) every 3 days, and UM-UC-9 cells treated with RAD001 (10 nM) every 3 days. Bar charts comparing the differences in the percentage of G1 phase cells between controls and RAD001-treated cells are shown. *B*, Propidium iodide exclusion assays were performed 24 h after UM-UC-6 cells and UM-UC-9 cells were treated with RAD001. The flow cytometric profiles showing the percentage of cells undergoing nonapoptotic cell death (*G*) are shown.

**Fig. 3.** Western blot analysis of UM-UC-3, UM-UC-6, UM-UC-9, and UM-UC-14 cells 24 h after treatment with RAD001.
**Fig. 4.** *In vivo* growth-inhibitory effect of prolonged treatment of subcutaneous tumor-bearing nude mice. The graphs show tumor size with time after RAD001 or placebo treatment, in mice with UM-UC-3–, UM-UC-6– and UM-UC-9-derived tumors respectively.

**Fig. 5.** Immunohistochemical staining of representative tumor tissue sections harvested after prolonged treatment with either a placebo or RAD001. Phospho-mTOR, phospho-p70S6K and phospho-AKT are shown at 400X magnification, and CD31 is shown at 100X magnification.
Table 1. The characteristics of p53, PTEN, and pAKT expression in bladder cancer cell lines are summarized (11, 27)

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>TP53</th>
<th>PTEN</th>
<th>pAKT</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>increased expression</td>
</tr>
<tr>
<td>UM-UC-14</td>
<td>mutated</td>
<td>wild type</td>
<td>decreased expression</td>
</tr>
</tbody>
</table>
Table 2. Summary of the scoring results of immunohistochemical analysis for phospho-mTOR, phospho-p70S6K, phospho-AKT, CD31, and VEGF expression in UM-UC-3, UM-UC-6, and UM-UC-9 tumors from mice treated with RAD001. Microvessel density (MVD) was quantified at 100X magnification.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>pmTOR</th>
<th>pP70S6K</th>
<th>pAKT</th>
<th>VEGF</th>
<th>CD31</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Staining intensity score</td>
<td>Mean *MVD (range)</td>
<td>*P-value</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UM-UC-3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control treated</td>
<td>0 /1+</td>
<td>2+</td>
<td>2+</td>
<td>1+</td>
<td>21.8 (18.6 – 25.0)</td>
</tr>
<tr>
<td>RAD001 treated</td>
<td>0</td>
<td>1+</td>
<td>1+</td>
<td>1+</td>
<td>17.4 (14.1 – 20.7)</td>
</tr>
<tr>
<td>UM-UC-6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control treated</td>
<td>0</td>
<td>2+</td>
<td>2+</td>
<td>3+</td>
<td>16.0 (14.0 – 18.0)</td>
</tr>
<tr>
<td>RAD001 treated</td>
<td>0</td>
<td>1+</td>
<td>2+</td>
<td>3+</td>
<td>16.7 (14.7 – 18.7)</td>
</tr>
<tr>
<td>UM-UC9</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control treated</td>
<td>1+ / 2+</td>
<td>1+</td>
<td>3+</td>
<td>2+</td>
<td>31.1 (25.0 – 37.2)</td>
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<tr>
<td>RAD001 treated</td>
<td>1+</td>
<td>1+</td>
<td>2+</td>
<td>2+</td>
<td>20.9 (14.8 – 27.0)</td>
</tr>
</tbody>
</table>

*MVD: Microvessel density
References

Fig 1A

UM-UC-6

UM-UC-14

UM-UC-3

UM-UC-9
Fig 1C

![Graph showing the effect of RAD001 on ³H-Thymidine incorporation for different cancer cell lines.](image)

The graph illustrates the inhibition of ³H-Thymidine incorporation by increasing concentrations of RAD001 in various cancer cell lines. The experimental conditions include control, DMSO, and different concentrations of RAD001 (0.1nM to 100nM). The cell lines tested are UM-UC-3, UM-UCC-6, UM-UC-14, and UM-UC-9.
Fig 2B
Fig 4

**UM-UC-3 implanted mice**

- Control
- RAD001

**UM-UC-6 implanted mice**

- Control
- RAD001

**UM-UC-9 implanted mice**

- Control
- RAD001

Tumor Size (mm³) vs. Day
Clinical Cancer Research

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


Clin Cancer Res Published OnlineFirst March 17, 2011.

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