A Pharmacodynamic Study of Rapamycin in Men with Intermediate- to High-Risk Localized Prostate Cancer

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Abstract

Purpose: Given discrepancies between preclinical and clinical observations of mammalian target of rapamycin (mTOR) inhibition in prostate cancer, we sought to determine the pharmacodynamic effects of the mTOR/TORC1 inhibitor rapamycin in men with intermediate- to high-risk prostate cancer undergoing radical prostatectomy.

Experimental Design: Rapamycin was given at 3 or 6 mg orally for 14 days before radical prostatectomy in men with multifocal Gleason sum ≥7 prostate cancer; 10 untreated control subjects were included. The primary outcome was inhibition of phosphorylation of ribosomal S6 in posttreatment radical prostatectomy versus pretreatment biopsy tumor tissue, evaluated using a Simon two-stage design for pharmacodynamic efficacy.

Results: Thirty-two subjects were accrued: 20 at 3 mg, 2 at 6 mg, and 10 controls. No dose-limiting toxicities were observed at 3 mg; however, two of two men enrolled at 6 mg experienced dose-limiting toxicities including thrombocytopenia and fever with grade 3 stomatitis. Adverse events observed at 3 mg included stomatitis, rash, ileus, and neutropenia. Pharmacodynamic studies showed tumor S6 phosphorylation inhibition in 50% of 10 evaluable rapamycin-treated men with sufficient paired tissue [median 58% inhibition (P = 0.049) versus 2% inhibition in controls (P = 0.75)] with no significant effect on AKT activity. We observed no change in Ki-67 or caspase-3 cleavage but noted a reduction in cytoplasmic p27 staining with increased nuclear localization with rapamycin treatment. Prostate tissue rapamycin concentrations were 3- to 4-fold higher than blood.

Conclusions: At 3 mg daily, rapamycin successfully and safely inhibited prostate cancer S6 phosphorylation and achieved relatively high prostate tissue concentrations. No effect on AKT phosphorylation, tumor proliferation, or apoptosis was observed. Clin Cancer Res; 16(11): 3057–66. ©2010 AACR.

Although rapamycin analogues have provided benefit to patients with advanced renal cell carcinoma, the effect of mammalian target of rapamycin (mTOR) inhibition in other solid tumors, including prostate cancer, remains unclear (1–3). The rationale for mTOR inhibition is strong in advanced prostate cancer, given the high prevalence of activation of the phosphoinositide 3-kinase (PI3K)/AKT pathway due largely to the loss of expression/function of the tumor suppressor PTEN and the association of this pathway with adverse pathologic features, recurrence after radical prostatectomy, and systemic treatment resistance (4–8). Preclinical studies have shown an ability of TORC1 inhibitors to revert prostatic intraepithelial neoplasia and reduce tumor volume and growth/proliferation particularly in tumors with activated AKT or that lack PTEN (9–12). Thus, preclinical studies support the development of mTOR inhibitors in prostate cancer (11, 13–15).

The clinical experience of TORC1 inhibition with rapamycin analogues in men with castrate-resistant prostate cancer, however, has been disappointing with few responses and a short time to progression (16, 17), despite the common loss of PTEN observed in prostate cancer metastases (5, 18, 19). These observations mirror the modest activity of rapamycin analogues in other selected cancers (1, 20–23). Thus, studies that investigate the mechanisms...
of primary resistance to TORC1 inhibition are needed. Recent findings suggest that rapamycin inhibits one form of mTOR, TORC1, but a second mTOR complex, TORC2, may remain uninhibited in a cell lineage-specific manner (24–26). Although rapamycin may inhibit endothelial AKT activity and growth/survival (13–15), AKT activity and growth is often potentiated with rapamycin in part through the inactivation of S6 kinase with resultant feedback derepression of IRS-1 activity (27, 28). In addition, increased MYC activity and autophagy has recently been linked to rapamycin resistance through 4EBP1 modulation (29). Thus, to achieve a significant clinical effect, TORC1 inhibition with rapamycin may require the addition of upstream inhibitors, such as insulin-like growth factor signaling or PI3K signaling (27, 28, 30, 31) or, alternatively, more effective inhibition of both TORC1 and TORC2 activity.

The preprostatectomy setting (32–35) represents a reasonable venue in which paired tumor tissue can be evaluated before and after exposure to a drug to address target specificity, drug delivery, physiologic effects on tumor growth and apoptosis, and correlation of biomarkers with clinical activity. Here, we sought to characterize the pharmacodynamic (PD) effects of TORC1 inhibition in men with localized prostate cancer to elucidate the anticancer mechanisms, if any, of rapamycin in men with primary untreated prostate cancer.

Materials and Methods

Subject eligibility criteria

We conducted a two-arm open label multidose multicenter prospective clinical trial of rapamycin in men with localized intermediate-/high-risk prostate cancer undergoing radical prostatectomy. This study was conducted through the Department of Defense Prostate Cancer Clinical Trial Consortium at Duke University, Johns Hopkins University, and the University of Michigan. Eligible treated and control men had prostate cancer clinical stages T1c to T3, no metastases, Gleason sum of 7 to 10, multiple positive diagnostic cores, Eastern Cooperative Oncology Group performance status of 0 to 1, and were a candidate for radical prostatectomy. Men were sequentially enrolled to the 3-mg dosing cohort initially, followed by the 6-mg cohort, followed by the control cohort. Subjects were required to have adequate hepatic, renal, and bone marrow function; no allergy to rapamycins; avoid medications interferring with rapamycin metabolism; no active infection; no prior therapies for prostate cancer; and should be age >18 years. This trial was approved by the institutional review boards at each site and all men signed an informed consent. This trial was registered at http://clinicaltrials.gov as NCT00311623.

Treatment and safety analysis

Men were treated with oral once-daily rapamycin at either 3 or 6 mg (Wyeth Pharmaceuticals, 1- and 2-mg tablets) or no treatment on days 1 to 14. Men were instructed to take rapamycin 1 hour before or 2 hours after food. Modified retropubic open radical prostatectomy was performed on day 15; the last rapamycin dose was given the morning before surgery. Toxicity was evaluated using the National Cancer Institute Common Toxicity Criteria v3.0. Lipids, prostate-specific antigen (PSA), complete blood count with differential, and hepatic/renal function were checked at baseline, day 14, and day 90 postoperatively. Dose-limiting toxicity (DLT) was defined as grade 3/4 neutropenia with fever or lasting >7 days, platelets of <100,000/mm³ or associated with bleeding, grade ≥3 nonhematologic toxicity, or irreversible grade 2 toxicity related to rapamycin.

Pathologic and pharmacodynamic analysis

Pretreatment diagnostic transrectal paraffin-embedded core needle biopsies and posttreatment radical prostatectomy specimens were obtained on all subjects for PD analysis. Biopsies were reviewed for pathologic diagnoses at each participating institution. Representative H&E sections and unstained tissue sections were then sent for central pathology review and immunohistochemistry studies by one of two pathologists at the lead institution (GIN, AMD).

Immunohistochemistry

The primary end point was inhibition of S6 phosphorylation in radical prostatectomy tumor tissue compared with pretreatment biopsies. S6 phosphorylation at serine 240/244 was assessed by immunohistochemistry (IHC) as an indirect measure of S6 kinase activity using a validated anti-phospho S6 antibody. Scoring for S6 and phospho-AKT (serine 473) was done using the H-score, a semiquantitative measure of the percentage of cells scoring positive (0-100) multiplied by the intensity of staining (0-3). Additional secondary PD markers included Ki-67, nuclear cleaved caspase-3, p27, and PTEN. All IHC interpretations were done and scored by two urologic pathologists blinded to treatment group and sample pairing. Discrepancies were resolved...
through consensus. See Supplementary Methods for further details of tissue processing and antibodies/controls.

**Peripheral blood mononuclear cell PD analysis**
Peripheral blood mononuclear cells (PBMC) were collected at baseline and on day 15 in the operating room within 1 hour of prostate removal in rapamycin-treated subjects. Each sample was collected and processed as previously published (2); see Supplementary Methods for further details.

**Pharmacokinetic analysis**
Whole blood was collected at baseline and day 15 within 1 hour of prostatectomy in all rapamycin-treated subjects. Snap-frozen prostate tissue was evaluated for tissue rapamycin levels at the Analytical Pharmacology Core Laboratory at Johns Hopkins (2). See Supplementary Methods for details.

**Statistical analysis**
The minimax two-stage design of Simon was used to test a hypothesis about PD response, taken as a ≥60% inhibition in S6 kinase activity (≥60% decrease in the H-score for S6 phosphorylation in the radical prostatectomy tumor tissue compared with pretreatment biopsy tumor tissue). A reduction in S6 activity was found to correlate with potential clinical benefit in prior studies and this benchmark was chosen empirically as indicative

### Table 1. Baseline and surgical characteristics of the patients on study

<table>
<thead>
<tr>
<th>Patient characteristics</th>
<th>Rapamycin-treated men, 3 mg (n = 20)</th>
<th>Rapamycin-treated men, 3 mg (evaluable for primary end point, n = 10)</th>
<th>Rapamycin-treated men, 6 mg (n = 2)</th>
<th>Control group (n = 10)</th>
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<td>Median age, y (range)</td>
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<td>59 (50-72)</td>
<td>57.5 (51-64)</td>
<td>64 (51-68)</td>
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<td>10 (100)</td>
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<tr>
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<tr>
<td>% undetectable day 90 PSA</td>
<td>17/20 (85%)</td>
<td>8/10 (80%)</td>
<td>2/2 (100%)</td>
<td>4/5 (80%)</td>
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Abbreviations: EBL, estimated blood loss; BMI, body mass index.
of a substantial decrease (36). Assuming a two-sided type I error rate of 0.05 and null PD response rate of 10%, the trial was designed to have 95% power to reject the null hypothesis if the true response rate was 40%. In the first stage, 12 patients were to be enrolled; if <2 responses were observed, the trial was to be terminated. However, if >2 responses were observed, an additional 9 patients were to be enrolled at the second stage for a total of 21 patients. The null hypothesis is rejected if >4 patients respond at the end of the second stage. If ≥2 of 12 DLTs were observed at a given dose level, this dose was deemed unsafe for further testing in this population and accrual stopped; otherwise, dose was escalated to 6 mg and the process was repeated. Ten untreated control men were enrolled to provide estimates of variability for each PD biomarker. Descriptive graphs were developed to describe PD end points on paired tissue on an intraindividual basis and between treated and control subjects. Comparisons of the primary PD end point were conducted using the nonparametric Wilcoxon rank-sum test (between groups) or Wilcoxon signed-rank test (within groups), respectively. Pharmacokinetic (PK) data were summarized using descriptive statistics. In addition, the Spearman correlation coefficients were computed for the PK and PD end points.

**Results**

From January 2007 to November 2008, 32 men were enrolled, including 20 subjects treated at 3 mg, 2 subjects at 6 mg, and 10 control subjects. Baseline characteristics are shown in the Table 1. Most men (77% rapamycin treated, 80% controls) enrolled in each arm had intermediate-risk prostate cancer, with no observed differences across treatment groups for age, race/ethnicity, PSA, Gleason sum, or stage (37). No differences in baseline PSA values or decline in PSA over time were seen across treatment groups and >80% of men had an undetectable PSA in each arm 90 days following radical prostatectomy (Table 1).

Among control men, no adverse events were observed other than one prolonged postoperative ileus that resolved with conservative management. In the 3-mg cohort, two cases of postoperative ileus were noted, which also resolved with medical management. Known toxicities associated with rapamycin were observed at 3 mg: grade 1 maculopapular rash (1), grade 1 thrombocytopenia (1), grade 1 to 2 neutropenia without fever (2), and grade 2 stomatitis (2) without DLTs. In the 6-mg cohort, two of two subjects experienced DLTs likely related to rapamycin, consisting of thrombocytopenia requiring delay in radical prostatectomy (platelet count of 90,000/mm³), and grade 3 stomatitis, fever, and diarrhea; no further subjects were treated at this dose level per protocol. No obvious differences were noted in wound healing complications or estimated blood loss between rapamycin-treated and control subjects (Table 1). Thus, 3 mg was selected as the maximum tolerated dose in this setting.

The 3-mg cohort was expanded after greater than two PD responses and no DLTs were observed in the first 12 subjects. Among the 20 men treated at 3 mg, 10 men had adequate paired tissue for evaluation of PD response, largely due to the lack of availability or inadequacy of either biopsy
or radical prostatectomy (n = 10) specimens. There were no significant differences in baseline characteristics in men with versus those without adequate paired tissues (Table 1). Five of 10 men (50%; 95% confidence interval (CI), 19-81%; P < 0.0001 versus null hypothesis of 10%) achieved a ≥60% tumor S6 kinase inhibition (inhibition of S6 phosphorylation) with rapamycin treatment, thus meeting the prespecified primary end point of the study. One of eight (12.5%; 95% CI 0.3-53%) men in the control arm experienced a PD response (P = 0.81 versus null hypothesis). Median S6 kinase inhibition was 58% [interquartile range (IQR) = −15 to 82%] in rapamycin-treated subjects versus 2% (IQR = −6 to 30%) among eight controls with adequate available paired tissue. Posttreatment reduction in S6 kinase activity was statistically significant in rapamycin-treated men (Wilcoxon P = 0.049) but not in control men (P = 0.75; Fig. 1). The median posttreatment S6 activity H-score was 70 in 13 rapamycin-treated men and 140 in 9 control men with evaluable tissue (Wilcoxon P = 0.006). In addition, we found that a decrease in serum PSA between baseline and day 14 was observed with rapamycin treatment in none of four evaluable men who had a tumor S6 PD response (median, 23% increase), whereas three of four men without a tumor S6 PD response had a PSA decline (median, 7% decline; χ²P = 0.028).

We next examined the relationship of pretreatment PTEN and AKT status with PD efficacy. Biopsy PTEN H-scores ranged from 0 to 250 (median 80), whereas biopsy phospho-AKT H-scores ranged from 0 to 240 (median 80). We found a modest negative correlation (R² = −0.83) between biopsy PTEN expression and inhibition of S6 kinase in rapamycin-treated men (n = 10). For example, two of two (100%) treated men with PTEN-null tumors (H-Score, 0-50) on biopsy had a PD response; one of three (33%) men with PTEN-low tumors (H-Score, 50-80) had a PD response; and one of four (25%) men with tumors expressing moderate levels of PTEN (H-score, >80) had a PD response. Only one rapamycin-treated subject had no PTEN expression detectable in the biopsy specimen; likewise, only two subjects had no PTEN expression at the time of radical prostatectomy. We observed no correlation between biopsy and radical prostatectomy PTEN expression, despite efforts to score radical prostatectomy tumor samples from the same location as the pretreatment biopsy. Of the biopsies with low PTEN expression (H Score, <100), 6 of 12 (50%) had AKT phosphorylation; of the biopsies with intact PTEN (H Score, >100), 2 of 7 (29%) had AKT phosphorylation. No correlation was observed between pretreatment biopsy AKT phosphorylation, or PTEN status with biopsy S6 phosphorylation or subsequent S6 inhibition.

In men with evaluable paired tumor tissue (10 rapamycin-treated men at 3 mg and 7 controls), we detected no difference in phosphorylated AKT induction across treatment groups (Fig. 2A). Among rapamycin-treated men, any increase in AKT phosphorylation was seen in four men; any decrease was seen in four men; and no change was seen in two men. Among controls, we observed an increase in AKT phosphorylation in three men, a decrease in three men, and no change in one man. We observed no relationship between posttreatment AKT phosphorylation induction and biopsy PTEN status. The median posttreatment phospho-Akt H-score was 100 in 13 rapamycin-treated men and 20 in 8 control men with evaluable tissue.

No changes were observed in proliferation (Ki-67 expression) in paired samples in either rapamycin-treated (median, 7.5% versus 8.2%) or control subjects (17%)
versus 15.1%; Fig. 2B). We did not observe a difference in Ki-67 expression among rapamycin-treated men who had tumor S6 inhibition compared with those without S6 inhibition (2% versus 0% reduction; n = 10). No difference in proliferation was noted in posttreatment Ki-67 levels in 13 rapamycin-treated versus 10 control men with evaluable tissue (median, 5% versus 10%). No relationship between posttreatment PSA declines and changes in tumor phospho-Akt was observed. However, a PSA decline was observed in three of six men (50%) with a reduction in tumor Ki-67 after rapamycin and in one of seven (14%) men without a Ki-67 reduction.

We next evaluated tumors for evidence of nuclear caspase-3 cleavage as a biomarker of apoptosis in paired tissues (Fig. 2C). Nuclear expression of caspase-3 cleavage was overall low in most biopsy and radical prostatectomy specimens (median, 3.0%; IQR = 2.0-6.0 in 20 biopsies; median, 2.5%; IQR = 0-4.0 in 20 radical prostatectomy specimens) with no significant differences in caspase-3 induction observed in rapamycin-treated (n = 10 pairs) or control men (n = 5 pairs), nor in PD responders versus nonresponders. Nuclear caspase-3 cleavage was higher in radical prostatectomy specimens in control men (5.5%, n = 6) compared with rapamycin-treated men (1%, n = 14). Although pre-treatment biopsy caspase-3 cleavage was also higher in control men (4% versus 3%), this difference was not significant.

Among rapamycin-treated subjects (n = 9 with adequate paired tissue), p27 cytoplasmic staining frequency was reduced (median pretreatment p27 expression, 70% versus 27.5% posttreatment), whereas no difference was noted in control subjects (n = 4, 82.5% versus 85%; Fig. 3A). An increase in p27 nuclear localization was noted in rapamycin-treated subjects (6/11 pretreatment versus 12/13 posttreatment) but not in control subjects (6/8 pretreatment versus 1/6 posttreatment), indicating no difference in baseline (biopsy) nuclear p27 localization but an increase in nuclear localization in posttreatment (radical prostatectomy) samples with rapamycin but not control subjects (Fig. 3B).

PBMC S6 kinase was inhibited on day 15 by a median of 32% in rapamycin-treated subjects (3 mg) and 9 of 19 rapamycin-treated subjects with evaluable paired PBMCs had ≥60% S6 inhibition posttreatment. PBMC S6 kinase inhibition did not correlate with tumor S6 kinase inhibition and PD response in tumor versus PBMCs did not correlate in nine subjects with evaluable paired tissues, with six of nine subjects having discordant results. Only four of nine paired PBMC-tumor results showed a similar directional trend (Fig. 4). Induction of PBMC total S6 expression by rapamycin was observed in 44% of men. There was a modest correlation of baseline PBMC S6 kinase activity with day 15 S6 kinase inhibition in rapamycin-treated subjects (R² = 0.68).

Mean day 15 blood rapamycin level was 9.9 ng/mL (range, 2.7-17.8) in the 3-mg cohort and mean day 15 prostate tissue rapamycin level was 28.7 ng/g (range, 7.0-69.2; Fig. 5). Mean relative systemic exposure (tumor/whole blood ratio) was 3.54 (range, 0.9-8.5; median, 2.09), indicating a 3- to 4-fold increase in rapamycin tissue levels over whole-blood concentrations. We found no correlation between whole-blood or tissue rapamycin levels and any PD effects in rapamycin-treated men.

**Discussion**

In this study, we found that rapamycin entered the prostate at physiologically relevant concentrations 3- to 4-fold higher than whole blood, despite the use of a 3 mg oral daily dose that is half the maximum tolerated dose in other settings (2). We found that rapamycin inhibited the activity of a downstream target of TORC1, S6 kinase, in over
half of evaluable patients without DLT, thus demonstrating the intended target inhibition and meeting the study's primary end point. However, we found no physiologically relevant effects of rapamycin on tumor cellular proliferation, posttreatment tumor grade or stage, PSA, or apoptosis over a 2-week exposure period. Rapamycin administration was safe at 3 mg and this dose can be considered the maximum tolerated dose in the preoperative setting. In addition, we found no correlation between PBMC and tumor PD efficacy, which suggests a limited role for PBMCs as surrogate measures of efficacy and the need for tumor-based PD assessments of drug mechanism.

This study has several limitations. This was a multicenter study and some tissues were nonevaluable due to the absence of tumor or limited residual biopsy material for analyses. These limitations significantly reduced the overall sample size for PD analysis for all end points and emphasizes the need for standard operating procedures for collection, prioritization, tracking, and shipping of tumor tissue to maintain integrity and statistical power in these studies. Thus, many of our secondary PD analyses are underpowered to detect moderate biomarker effect sizes and should be considered exploratory in nature. In addition, many assays had wide variability despite attempts to isolate identical tumor foci in the radical prostatectomy specimen compared with the original biopsy. Despite this, we were able to collect sufficient paired samples to meet our primary end point and investigate several secondary PD and PK end points of rapamycin effect and mechanism in vivo, which have not previously been reported in the literature.

We also showed that robust and strong PD effects (S6 inhibition) after drug exposure in prostate cancer can be observed with relatively small sample sizes, given reduced statistical variability with paired tissues. We did not find a correlation between PTEN expression in biopsy and radical prostatectomy specimens, despite using a validated PTEN assay with genetic controls, indicating that different heterogeneous tumors were sampled and analyzed before and after rapamycin, that PTEN expression may have been altered by rapamycin, or a limited power to detect these changes. We additionally found that many of the PD biomarkers such as Akt, p27, caspase-3, Ki-67, and PTEN have wide variability in this setting during specimen collection and processing across multiple centers, highlighting the challenges inherent in these PD studies and the need for standardized collection, processing, and ascertainment of these markers, with consideration of final sample size based on evaluable tissues.

We found that rapamycin reduced the cytoplasmic expression and increased the nuclear localization of the cyclin-dependent kinase inhibitor p27 in radical prostatectomy specimens compared with nontreated men. The finding of increased p27 nuclear localization is intriguing but not clearly correlated with a change in cellular proliferation as might be expected. A role for p27 as a prognostic marker in prostate cancer is relatively strong, with multiple (38–41) but not all (42) studies showing an adverse relationship between low nuclear p27 expression and recurrence after surgery. In addition, knockout of p27 combined with constitutive Akt activation in model systems leads to an aggressive, invasive prostate cancer phenotype (43). Variability in fixation techniques or localization of paired tumor foci may explain some of this heterogeneity; thus, a reduction in p27 nuclear localization is currently of unclear physiologic relevance (44). However, defective p27 localization has been linked to rapamycin.

![Fig. 4. Paired bar plot showing PBMC and prostate tumor phospho-S6 inhibition among those rapamycin-treated subjects (n = 9) who had available PBMC and tumor tissue for biomarker assessment. Phospho-S6 percent change (before and after rapamycin) is calculated using the equation depicted in the figure.](image-url)
resistance and further genomic studies are planned to characterize these molecular alterations (45). Longer periods of rapamycin exposure or higher doses may also be required to observe an antiproliferative cytostatic effect; however, higher doses were not well tolerated in this treatment-naïve preoperative setting.

These PD findings, along with clinical studies in the metastatic setting, call into question the clinical relevance of single agent TORC1 inhibition in unselected men with advanced prostate cancer and point to strategies that dissect mechanisms of resistance (16, 17). Given the preclinical efficacy of TORC1 inhibitors, it is essential to improve upon model systems while investigating the PI3K pathway, and which components, including TORC1 or TORC2, are most relevant to prostate cancer progression.

The mechanistic evaluation of novel agents and biomarkers in prostate cancer requires careful consideration of many factors including disease state, tumor heterogeneity, reliability and validation of biomarker assays, and the physiologic and clinical significance of biomarkers in determining the relevance of trial outcomes (46). Biomarkers should be associated with clinical outcomes and should be assessed using appropriate intrapatient and interpatient controls, which account for assay variability. Consideration of duration of exposure is crucial, given that early changes may be missed if assessment is deferred beyond the point of maximal effect on a biomarker and inadequate exposure windows may not allow for sufficient time to observe PD effects. Our choice of S6 activity as a stable and validated marker of TORC1 activity reflected the predominant thinking when this study was conceived; however, recent studies have questioned its clinical relevance and suggest that other markers, such as measures of proliferation or apoptosis, 4EBP-1, TORC2, and AKT activity, may be more relevant in assessing a favorable effect on the PI3K/AKT pathway (8, 24, 47, 48). Our investigation of several of these markers strengthen the impact of our findings and the absence of effects on these parameters supports the concept that target inhibition (as measured by reduced S6 phosphorylation) does not correlate necessarily with physiologic or clinical benefit. Further analyses across different disease states will further define an optimal biomarker and the cellular mechanisms of benefit from or resistance to mTOR inhibition to guide in the development of agents targeting this important survival pathway.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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