Phase I/II Study of the Mammalian Target of Rapamycin Inhibitor Everolimus (RAD001) in Patients with Relapsed or Refractory Hematologic Malignancies

Karen W.L. Yee,1 Zhihong Zeng,2 Marina Konopleva,2 Srdan Verstovsek,1 Farhad Ravandi,1 Alessandra Ferrajoli,1 Deborah Thomas,1 William Wierda,1 Efrosyni Apostolidou,1 Maher Albitar,1 Susan O’Brien,1 Michael Andreeff,1,2 and Francis J. Giles1

Abstract Purpose: Everolimus (RAD001, Novartis), an oral derivative of rapamycin, inhibits the mammalian target of rapamycin (mTOR), which regulates many aspects of cell growth and division. A phase I/II study was done to determine safety and efficacy of everolimus in patients with relapsed or refractory hematologic malignancies.

Experimental Design: Two dose levels (5 and 10 mg orally once daily continuously) were evaluated in the phase I portion of this study to determine the maximum tolerated dose of everolimus to be used in the phase II study.

Results: Twenty-seven patients (9 acute myelogenous leukemia, 5 myelodysplastic syndrome, 6 B-chronic lymphocytic leukemia, 4 mantle cell lymphoma, 1 myelofibrosis, 1 natural killer cell/T-cell leukemia, and 1 T-cell prolymphocytic leukemia) received everolimus. No dose-limiting toxicities were observed. Grade 3 potentially drug-related toxicities included hyperglycemia (22%), hypophosphatemia (7%), fatigue (7%), anorexia (4%), and diarrhea (4%). One patient developed a cutaneous leukocytoclastic vasculitis requiring a skin graft. One patient with refractory anemia with excess blasts achieved a major platelet response of over 3-month duration. A second patient with refractory anemia with excess blasts showed a minor platelet response of 25-day duration. Phosphorylation of downstream targets of mTOR, eukaryotic initiation factor 4E-binding protein 1, and/or, p70S6 kinase, was inhibited in six of nine patient samples, including those from the patient with a major platelet response.

Conclusions: Everolimus is well tolerated at a daily dose of 10 mg daily and may have activity in patients with myelodysplastic syndrome. Studies of everolimus in combination with therapeutic agents directed against other components of the phosphatidylinositol 3-kinase/Akt/mTOR pathway are warranted.

The phosphatidylinositol 3-kinase/Akt signaling pathway is important for cell growth and survival (1, 2). Akt is activated downstream of phosphatidylinositol 3-kinase and can activate several effector proteins, including the serine/threonine kinase mammalian target of rapamycin (mTOR; Fig. 1). Activated Akt can phosphorylate tuberin (TSC2), causing disruption of the inhibitory hamartin (TSC1)/TSC2 complex, and activate the GTPase Rheb, which in turn promotes the formation of the mTOR-raptor complex (TORC1). TORC1 regulates cell cycle progression (i.e., G1 to S phase transition) in part by controlling the mammalian translation machinery via activation of the p70 S6 kinase (p70S6K) protein kinase and via inhibition of the elongation initiation factor 4E inhibitor 4E-binding protein 1 (4E-BP1 refs. 1, 2). Hence, TORC1 activation results in up-regulation of effectors required for protein translation and cell cycle progression and proliferation, such as hypoxia-inducible factor-1α and cyclin D1. Up-regulation of hypoxia-inducible factor-1α leads to increased expression of angiogenic factors, such as vascular endothelial growth factor, platelet-derived growth factor, and growth-stimulatory molecules [e.g., glucose transporter 1 (Glut1); ref. 3]. mTOR has been implicated in both transformation and therapeutic resistance (1, 2).

The phosphatidylinositol 3-kinase/Akt/mTOR pathway is heavily dysregulated in hematologic malignancies and is activated by several upstream proteins, such as ras, TCL1, and bcr-abl, and membrane receptor tyrosine kinases, including vascular endothelial growth factor receptor, platelet-derived growth factor receptor, c-kit, and Flt3 (1). Increased expression and constitutive activation of the catalytic subunit...
of phosphatidylinositol 3-kinase and Akt and/or decreased or absent PTEN protein expression have been reported in primary samples from patients with acute myelogenous leukemia (AML) and/or myelodysplastic syndrome (MDS; ref. 1). Furthermore, p70S6K and 4E-BP1 are constitutively phosphorylated in primary AML cells. Tumors, such as mantle cell lymphoma, may express high levels of mTOR-regulated mRNAs (e.g., cyclin D1; ref. 1).

Everolimus [RAD001; 40-O-[2-hydroxyethyl]-rapamycin], an orally available ester derivative of the macrolide antifungal antibiotic sirolimus (rapamycin), is currently approved in Europe as an immunosuppressive agent to prevent rejection in adult cardiac and renal transplant recipients (4, 5). Everolimus has better oral bioavailability compared with the parent compound sirolimus, with steady state levels achieved within 7 days (6). Maintenance immunosuppression with everolimus or sirolimus has been associated with a significantly reduced risk of developing de novo malignancies after renal transplant (7).

Everolimus forms a complex with the immunophilin FKBP-12 (i.e., 12 kDa FK506 binding protein), which then binds to and disrupts TORC1, leading to mTOR inhibition and preventing the activation of p70S6K and inactivation of 4E-BP1. Everolimus causes G1 phase cell cycle arrest; the p21/cyclin D/cyclin-dependent kinases 2 and 4/proliferating cell nuclear antigen complexes are disrupted with down-regulation of p21, cyclin-dependent kinases 2 and 4, and subsequent inhibition of tumor growth (8–11). It may prevent nuclear factor-κB activation possibly by stabilization of its inhibitor IκBα (12). Everolimus may also exert its antitumor effect by inducing apoptosis (9, 10, 13) and suppressing angiogenesis (9).

In preclinical studies, everolimus inhibited proliferation and growth of a broad range of human tumor cell lines and xenograft models (8, 10, 13) and is being developed as an antitumor agent (14–16). The dose of everolimus influenced the duration of p70S6K inhibition in peripheral blood mononuclear cells in patients with solid tumors receiving escalating doses of single agent everolimus on a weekly schedule (16). At steady state, duration of p70S6K inhibition was 3 to 5 days in patients given doses of 5 to 10 mg and >7 days for those administered 20 to 30 mg (16). Pharmacokinetic and pharmacodynamic modeling has been done by combining this clinical data with preclinical data in rat pancreatic cancer xenograft models to predict intratumoral inhibition in patients (8, 16, 17). The pharmacokinetic and pharmacodynamic model predicted that the 20 mg weekly dose would be the minimal effective dose in patients and that sustained p70S6K inhibition in peripheral blood mononuclear cells should occur with daily administration as opposed to increasing the weekly dose >20 mg for the same total weekly drug administration (i.e., 10 mg daily compared with 70 mg weekly).

This was confirmed in a second phase I study of everolimus given to adults with solid tumors either weekly or daily (15). Inhibition of downstream effectors of mTOR in tumor biopsies correlated with findings in surrogate skin biopsies. Biomarker analyses showed dose-dependent and schedule-dependent inhibitions of the mTOR pathway: the daily schedule was associated with high inhibition of p70S6K and elongation initiation factor 4G at 5 mg daily, with complete inhibition occurring at 10 mg daily, whereas with the weekly schedule complete and sustained inhibition of p70S6K and elongation initiation factor 4G was observed at doses >20 mg weekly and >50 mg weekly, respectively, and nonsustained up-regulation of phosphorylated Akt was seen at doses of 50 mg. There was also a trend for decreased phosphorylation of 4E-BP1.

Although mTOR is clearly an attractive therapeutic target in leukemia, no clinical data on mTOR inhibition by rapamycin analogues have been published in this patient population.
A particular concern is whether these agents, with established immunosuppressive activity, could increase infectious agent complications.

Therefore, a phase I/II study was conducted to determine the safety, tolerability, and activity of everolimus administered daily at one of two doses (5 or 10 mg daily) in patients with a variety of relapsed or refractory or advanced hematologic malignancies. Furthermore, in consenting patients, blood samples were collected to assess surrogate markers of mTOR inhibition at baseline and at various treatment time points.

### Materials and Methods

**Patients.** Patients with histologically confirmed advanced, relapsed, or refractory AML or MDS, chronic lymphocytic leukemia (CLL), T-cell leukemia, myelofibrosis, or mantle cell lymphoma (MCL) were eligible for enrollment.

**Treatment protocol.** Patients were treated with 5 (first three patients) or 10 mg orally once daily continuously either in a fasted state or after a light fat-free meal. One treatment cycle consisted of 28 days of therapy. If emesis occurred after ingesting everolimus, the dose was not repeated. Novartis Pharmaceuticals (East Hanover, NJ) provided everolimus film-coated tablets at strengths of 5 mg. Treatment was stopped if any of the following events occurred: unacceptable toxicity, intercurrent illness or change in the patient’s condition that rendered the patient unsuitable for further therapy in the judgment of the investigator, or patient withdrawal from the study.

**Assessment of toxicity and response.** All patients were evaluable for toxicity if they received at least one dose of everolimus. Adverse events were assessed at each visit and graded according to the National Cancer Institute Common Toxicity Criteria version 3.0. Hematologic dose-limiting toxicity (DLT) was defined as myelosuppression with bone marrow hypoplasia (cellularity <5%) without evidence of leukemia for >42 days. Nonhematologic DLT was defined as any grade 4 toxicity, except for the following: grade 3nausea or vomiting, drug-related fever of any grade, alopecia, and grade 3 lipid or electrolyte abnormalities.

**Sample collection.** After obtaining written informed consent, whole-blood samples were collected into 10 mL sodium heparin tubes (Becton Dickinson, Sparks, MD) at baseline and 24 hours after administration of everolimus during the first cycle of therapy.

**Western blot analysis.** For the Western blot analyses, peripheral blood mononuclear cells were lysed in phosphoprotein lysis buffer (10 mmol/L NaF, 1 mmol/L Na3VO4, 150 mmol/L NaCl, 1 mmol/L MgCl2, 1 mmol/L CaCl2, 0.1% NaN3, 10 mmol/L iodoacetamide, 3 mmol/L phenylmethylsulfonyl fluoride, and 1% Triton X-100) supplemented with a protease inhibitor cocktail (Roche Diagnostics Corp., Indianapolis, IN). Equal amounts of proteins were then separated on a 12% polyacrylamide gel, transferred to Hybond-P membranes (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom), immunoblotted with specific antibody, and further visualized by using enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Western blots were analyzed on a Storm 860 system by using enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). Western blots were analyzed on a Storm 860 system by using enhanced chemiluminescence detection system (Amersham Pharmacia Biotech).

**Quantitative real-time reverse transcription-PCR.** Total RNAs were prepared using Trizol reagent as described by the manufacturer (Life Technologies, Inc., Gaithersburg, MD). Total RNA (1 µg) was reverse transcribed by avian myeloblastosis virus reverse transcriptase (Roche Diagnostics) under standard conditions. Duplicate samples of 1 µl of each cDNA were amplified by PCR in the ABI Prism 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA). The amplification reaction mixture (25 µl) contained cDNAs, forward primers, reverse primers, probes, and Taqman Universal PCR Master Mix (PE Applied Biosystems). β2-microglobulin was coamplified as an internal control to normalize for variable amounts of cDNA in each sample. The thermocycler variables were as follows: 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Taqman gene expression assay for cyclin D1 (assay Hs00277039_m1), cyclin D2 (assay Hs00277041_m1), and Glut1 (assay Hs00197884_m1) was purchased from PE Applied Biosystems and used as directed by the manufacturer. Results were collected and analyzed to determine the PCR cycle number that generated the first fluorescence signal above a threshold (threshold cycle, Ct; 10 SD above the mean fluorescence generated during the baseline cycles). The abundance of each transcript of interest relative to that of β2-microglobulin was calculated as follows: relative expression = 2^(-ΔΔCt), where ΔCt is the mean Ct of the transcript of interest less the mean Ct of the transcript for β2-microglobulin.

**Statistical analysis.** At least three patients had to be observed for at least 4 weeks during their first cycle of treatment with 5 mg daily without a DLT. With each DLT, three additional assessable patients had to be accrued, and further escalation could occur only if no more DLTs were observed. In the absence of such toxicity, the highest dose level (i.e., 10 mg daily) was to be used for phase II testing.

For the phase II part of the study, the primary objective was to estimate the overall response rate (responses were defined as per standard criteria; refs. 18–21). As everolimus has a noncytotoxic mechanism of action, a response rate of 10% in a very poor prognosis group of patients was considered of sufficient interest to warrant additional investigation. A maximum total of 25 evaluable patients were to be entered in each group of patients with advanced or refractory disease: (a) AML or MDS, (b) myelofibrosis, and (c) CLL, T-cell leukemia, or MCL. This sample size would yield an 82% posterior credibility interval for probability of response of width 0.16. Interim analyses were to be done in each group after 14 patients had been evaluated for response. Thus, in each patient cohort, the trial would be terminated after the first 14 patients if no responses were observed. As a further safeguard to prevent exposure of patients to an agent with minimal activity, the overall response rate was reviewed periodically. If overall response rate was <1 in 26 patients, 2 in 42 patients, or 3 in 55 patients, this would indicate that the probability of an overall response rate of <10% among this patient population was >90%. The study was closed to accrual after 26 evaluable patients were enrolled.

**Results**

**Study group.** A total of 27 patients with AML, MDS, CLL, MCL, myelofibrosis, natural killer cell/T-cell cell leukemia, and T-cell prolymphocytic leukemia was entered onto the study between April 2004 and May 2005. Patient characteristics are listed in Table 1. Fourteen patients had received three or more prior treatment regimens for their disease, including 2 patients who had received an allogeneic stem cell transplant. Two patients had not received prior therapy for their disease: a 75-year-old patient who developed therapy-related AML 2 years after being treated for Burkitt-like non–Hodgkin’s lymphoma and a 59-year-old man who developed MDS 9 years after being treated with uracil and tegafur plus leucovorin for metastatic colon cancer. A third patient had been diagnosed with essential
Three of four patients with MCL were in leukemic phase with leukemia had received two or more prior treatment regimens. The patients with myelofibrosis, blasts (RAEB) in transformation while receiving therapy with imatinib for myelofibrosis. The patients with myelofibrosis, thrombocythemia in 1992, which evolved to myelofibrosis in 1999; the patient progressed to refractory anemia with excess blasts (RAEB) in transformation while receiving therapy with imatinib for myelofibrosis. The patients with myelofibrosis, natural killer cell/T-cell leukemia, and T-cell prolymphocytic leukemia had received two or more prior treatment regimens. Three of four patients with MCL were in leukemic phase with circulating lymphoma cells. All six patients with CLL had received prior fludarabine-based therapy.

**Side effects.** All 27 patients were evaluable for toxicity. No DLT occurred in the first three patients treated with 5 mg daily; all subsequent patients received 10 mg daily. The frequency and grading of potentially treatment-related adverse effects are summarized in Table 2. These included grade 1 or 2 hyperlipidemia (44%), elevation of transaminases and/or alkaline phosphatase (41%), anorexia (37%), oral aphthous ulcers (37%), diarrhea (29%), hyperglycemia (26%), and hypomagnesemia (22%). Uncomplicated grade 3 hyperglycemia occurred in six patients requiring insulin therapy; five had a history of diabetes mellitus with grade 2 hyperglycemia noted at baseline in four patients. The hyperglycemia was resolved to baseline or better with cessation of everolimus. Other grade 3 toxicities included hypophosphatemia and fatigue in two patients each and anorexia and diarrhea in one patient.

One patient with refractory anemia with ringed sideroblasts developed a biopsy-proven grade 3 cutaneous leukocytoclastic vasculitis (LCV) after 120 days of therapy, which was believed to be related to everolimus. Everolimus was discontinued. She did not have any systemic manifestations of vasculitis nor malignancy. Systemic immunosuppressive therapy, including corticosteroids, was not used, and no new lesions have appeared. She required surgical debridement and skin grafting.

Thirty-eight infectious episodes were noted in 21 (78%) patients at some time during the therapy. These included fever of unknown origin in 6 (22%) patients and documented infections in 20 (74%) patients: 2 patients with sinusitis, 2 patients with bronchitis, 2 patients with bacteremia involving *Enterococcus faecalis* and *Staphylococcus aureus*, four urinary tract infections in 4 patients (one of whom developed a vaginal yeast infection and another *Clostridium difficile*–positive diarrhea.

---

**Table 1. Patient characteristics (N = 27)**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y, median (range)</td>
<td>64 (18-77)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>13 (48)</td>
</tr>
<tr>
<td>Male</td>
<td>14 (52)</td>
</tr>
<tr>
<td>Diagnosis</td>
<td></td>
</tr>
<tr>
<td>MDS (n = 5)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>RARS (n = 4)</td>
<td>3 (11)</td>
</tr>
<tr>
<td>RAEB (n = 1)</td>
<td>1 (4)</td>
</tr>
<tr>
<td>RAEB in transformation</td>
<td></td>
</tr>
<tr>
<td>AML (n = 10)</td>
<td>9 (33)</td>
</tr>
<tr>
<td>Prior MDS or MPD</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Treatment related</td>
<td>2 (8)</td>
</tr>
<tr>
<td>CLL (n = 6)</td>
<td>6 (22)</td>
</tr>
<tr>
<td>MCL (n = 4)</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Myelofibrosis</td>
<td>1 (4)</td>
</tr>
<tr>
<td>NK/T-cell leukemia</td>
<td>1 (4)</td>
</tr>
<tr>
<td>T-PLL</td>
<td>1 (4)</td>
</tr>
<tr>
<td>Duration of disease, mo, median (range)</td>
<td>33.5 (0.2-172.1)</td>
</tr>
<tr>
<td>Eastern Cooperative Oncology Group</td>
<td>1</td>
</tr>
<tr>
<td>performance status, median</td>
<td></td>
</tr>
<tr>
<td>0-1</td>
<td>23 (85)</td>
</tr>
<tr>
<td>2</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Prior therapy</td>
<td></td>
</tr>
<tr>
<td>Median treatment regimens (range)</td>
<td>3 (0-11)</td>
</tr>
<tr>
<td>AML (n = 9)</td>
<td></td>
</tr>
<tr>
<td>Prior chemotherapy</td>
<td>8 (89)</td>
</tr>
<tr>
<td>Prior targeted therapy</td>
<td>3 (33)</td>
</tr>
<tr>
<td>Prior allogeneic stem cell transplant</td>
<td>2 (22)</td>
</tr>
<tr>
<td>CLL (n = 6)</td>
<td></td>
</tr>
<tr>
<td>Prior chemotherapy</td>
<td>6 (100)</td>
</tr>
<tr>
<td>MDS (n = 5)</td>
<td></td>
</tr>
<tr>
<td>Prior cytokines</td>
<td>3 (60)</td>
</tr>
<tr>
<td>Prior targeted therapy</td>
<td>3 (60)</td>
</tr>
<tr>
<td>MCL (n = 4)</td>
<td></td>
</tr>
<tr>
<td>Prior chemotherapy</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Prior targeted therapy</td>
<td>3 (75)</td>
</tr>
<tr>
<td>Prior autologous stem cell transplant</td>
<td>1 (25)</td>
</tr>
<tr>
<td>Baseline WBC count ( × 10⁹/L), median (range)</td>
<td>8.6 (0.2-132.8)</td>
</tr>
<tr>
<td>AML</td>
<td>5.8 (0.3-20.3)</td>
</tr>
<tr>
<td>CLL</td>
<td>12.9 (1.8-132.8)</td>
</tr>
<tr>
<td>MDS</td>
<td>5.9 (2.3-16.9)</td>
</tr>
<tr>
<td>MCL</td>
<td>14.4 (3.1-28.5)</td>
</tr>
<tr>
<td>Baseline platelet count ( × 10⁹/L), median (range)</td>
<td>33 (6-349)</td>
</tr>
<tr>
<td>AML</td>
<td>33 (6-183)</td>
</tr>
<tr>
<td>CLL</td>
<td>45 (9-85)</td>
</tr>
<tr>
<td>MDS</td>
<td>32 (26-304)</td>
</tr>
<tr>
<td>MCL</td>
<td>63.5 (15-230)</td>
</tr>
<tr>
<td>Baseline peripheral blast count ( × 10⁹/L), median (range)</td>
<td>0 (0-5.9)</td>
</tr>
<tr>
<td>AML</td>
<td>0.61 (0.5-9.9)</td>
</tr>
<tr>
<td>MDS</td>
<td>0 (0-1.69)</td>
</tr>
</tbody>
</table>

Abbreviations: RARS, refractory anemia with ringed sideroblasts; MPD, myeloproliferative disease; NK/T cell leukemia, natural killer cell/T-cell leukemia; T-PLL, T-cell prolymphocytic leukemia.

---

**Table 2. Potentially everolimus-related adverse effects**

<table>
<thead>
<tr>
<th>Adverse effect</th>
<th>Grade 1/2, n (%)</th>
<th>Grade 3/4, n (%)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperglycemia</td>
<td>7 (26)</td>
<td>6 (22)</td>
<td>13 (48)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>12 (44)</td>
<td>0</td>
<td>12 (44)</td>
</tr>
<tr>
<td>Anorexia</td>
<td>10 (37)</td>
<td>1 (4)</td>
<td>11 (41)</td>
</tr>
<tr>
<td>Hepatic*</td>
<td>11 (41)</td>
<td>0</td>
<td>11 (41)</td>
</tr>
<tr>
<td>Oral aphthous ulcers</td>
<td>10 (37)</td>
<td>0</td>
<td>10 (37)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>8 (29)</td>
<td>1 (4)</td>
<td>9 (33)</td>
</tr>
<tr>
<td>Hypophosphatemia</td>
<td>5 (18)</td>
<td>2 (7)</td>
<td>7 (26)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>4 (15)</td>
<td>2 (7)</td>
<td>6 (22)</td>
</tr>
<tr>
<td>Hypomagnesemia</td>
<td>6 (22)</td>
<td>0</td>
<td>6 (22)</td>
</tr>
<tr>
<td>Dermatologic</td>
<td>5 (18)</td>
<td>0</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Hypocalcemia</td>
<td>5 (18)</td>
<td>0</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Dysgeusia</td>
<td>5 (18)</td>
<td>0</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Constipation</td>
<td>5 (18)</td>
<td>0</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Cramps</td>
<td>5 (18)</td>
<td>0</td>
<td>5 (18)</td>
</tr>
<tr>
<td>Nausea/vomiting</td>
<td>4 (15)</td>
<td>0</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Weight loss</td>
<td>4 (15)</td>
<td>0</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Xerostomia</td>
<td>4 (15)</td>
<td>0</td>
<td>4 (15)</td>
</tr>
<tr>
<td>Headache</td>
<td>3 (11)</td>
<td>0</td>
<td>3 (11)</td>
</tr>
<tr>
<td>Hypokalemia</td>
<td>2 (8)</td>
<td>0</td>
<td>2 (8)</td>
</tr>
<tr>
<td>LCV</td>
<td>0</td>
<td>1 (4)</td>
<td>1 (4)</td>
</tr>
</tbody>
</table>

*Elevated aspartate aminotransferase, alanine aminotransferase, and/or alkaline phosphatase.*
while on antibiotics), 14 pneumonias in 12 patients (one of whom developed oral thrush while on antibiotics), and 1 patient each with cellulitis, folliculitis, paronychia, and presumed herpetic stomatitis/mucositis. Fourteen (37%) of the 38 infectious episodes that occurred in 9 patients required either hospitalization or prolonged hospitalization.

One patient with RAEB-1 developed a prolonged Escherichia coli urinary tract infection during the eleventh cycle of therapy, which was complicated by recurrent hematuria, requiring platelet transfusions, antibiotics, and hospitalization. Therefore, everolimus was held during the twelfth cycle of therapy. Cytoscopy with bladder biopsy revealed a marked chronic inflammatory infiltrate, vascular congestion, and hemorrhage with no leukemic cells present. The hematuria was resolved 37 days after discontinuation of everolimus. This has been observed with prolonged symptomatic urinary tract infections and was not believed to be associated with administration of everolimus.

Of the 12 patients with pneumonia, only 2 had positive cultures (Aspergillus species and coagulase-negative Staphylococcus and Aspergillus niger, Klebsiella species, and methicillin-resistant Staphylococcus aureus). Two episodes of pneumonia occurred in one patient with myelofibrosis who was subsequently diagnosed with biopsy-proven bronchiolitis obliterans with organizing pneumonia, which was treated successfully with steroids. Of the remaining 11 patients with pneumonia, 1 patient had a chronic esophageal stricture, requiring dilations every 2 months, due to prior radiotherapy for Burkitt-like lymphoma and was believed to have an aspiration pneumonia involving the right lower lobe. 4 had grade 3 or 4 neutropenia, and 2 had CLL with hypogammaglobulinemia. Two patients died while on study: one patient with natural killer cell/T-cell lymphoma and was believed to have an aspiration pneumonia, which was complicated by recurrent hematuria, requiring hospitalization. While off treatment and after resolution of the hematuria, this patient has had a sustained increase in platelet counts ranging from 29 to 42 \( \times 10^9/L \) of \( >2 \)-month duration. The patient with refractory anemia with ringed sideroblasts (patient 7), who developed a cutaneous LCVC, had decreased red cell transfusion requirements, with only three transfusions administered over a 9-month period since discontinuing everolimus. The maximum interval between transfusions was 116 days compared with a baseline of every 21 to 26 days before treatment with everolimus.

Although no patients with CLL patients achieved a complete or partial response, three patients had a 27% to 34% reduction in adenopathy, documented radiographically, after two to three cycles of therapy. A fourth patient had a 65% reduction in peripheral lymphadenopathy, documented clinically, after two cycles of therapy; this patient subsequently died from uncontrollable gastrointestinal bleeding due to arteriovenous malformations.

Fifteen patients subsequently received other therapies for their underlying disease (median, 1; range, 1-4), including hydroxyurea (n = 2), induction chemotherapy with cytarabine and an anthracycline or purine analogue (n = 3), fludarabine-based regimen (n = 2), azacytidine (n = 1), vincristine and decadron (n = 1), hyperCVAD and alemtuzumab (n = 1), allogeneic stem cell transplant (n = 1), and other investigational agents (n = 8). After a median follow-up of 18 weeks (range, 3 to 68+) from the time of study entry, 16 patients have died, including the 2 patients who died while on study.

Effects of everolimus on mTOR signaling pathways. The ability of everolimus to modulate mTOR signaling was examined in samples from nine patients by comparing profiles of phosphorylated mTOR target proteins before and 24 hours after everolimus administration. To investigate everolimus-specific effects on mTOR signaling in vivo, phosphorylation of the 70-kDa 40S ribosomal protein kinase (p70S6K) and 4E-BP1 was examined. Both phosphorylated p70S6K and 4E-BP1 in Thr70 were simultaneously inhibited after 24 hours of everolimus administration in samples from four patients (patients 2, 3, 7, and 9; Table 3). 4E-BP1 exhibited a decrease in Thr70 phosphorylation in two additional samples (patients 5 and 6). Interestingly, the phosphorylation of another rapamycin-sensitive residue (Thr37/Thr46) was affected only in the sample from patient 3, indicating that mTOR-insensitive phosphorylation of this site can occur as previously suggested (22). No significant changes in mTOR signaling were observed in samples from four other patients. Notably, patients 3 (CLL), 5 (RAEB-1), 6 (RAEB-1), and 7 (refractory anemia with ringed sideroblasts) displayed some evidence of clinical responses to everolimus as detailed above, and patient 5 had objective response (hematologic improvement, see above). Examples of Western blot analyses for samples 5 and 3 are represented in Fig. 2.

Akt and mTOR are linked to each other via positive and negative regulatory circuits, and in model systems, tumors
exhibiting activation of Akt are hypersensitive to mTOR inhibitors (23). We therefore analyzed effects of everolimus in the context of Akt signaling. At baseline, Akt was phosphorylated on Thr\(^{308}\) in all seven of seven samples tested and on Ser\(^{373}\) in eight of nine samples. Unexpectedly, we found inhibition of phosphorylation of Akt at Thr\(^{308}\) in four of six (patients 2, 3, 5, and 7) and at Ser\(^{373}\) in five of eight (patients 2, 3, 5, 6, and 9) patient samples (see examples on Fig. 2). Interestingly, Akt was down-regulated only in samples where inhibition of mTOR signaling was observed, suggesting that mTOR inhibition in vivo may inhibit Akt signaling. Activation (phosphorylation) of ERK was documented in samples from eight of nine patients, and unanticipated decrease in phosphorylated ERK levels was observed in samples from two patients (patients 3 and 8).

mTOR signaling has been reported to affect the expression of the target genes by different mechanisms (transcription, translation, or protein degradation; refs. 24–26). We examined the effects of mTOR inhibition on the transcription of the glucose transporter Glut1 and D-type cyclins that control the G\(_1\)/S transition in the cell cycle. In samples from six patients, in glucose transporter Glut1 and D-type cyclins that control the effects of mTOR inhibition on the transcription of the translation, or protein degradation; refs.24–26). We examined the target genes by different mechanisms (transcription, translation, or protein degradation; refs.24–26).

### Discussion

Because of accumulating evidence documenting the importance of the phosphatidylinositol 3-kinase/Akt/mTOR pathway in the pathogenesis of a variety of hematologic malignancies, several therapeutic strategies are being developed to modulate this signaling pathway. The importance of mTOR is underscored by its key regulatory role in protein translation (1, 2). The current phase I/II study is the first to evaluate everolimus in patients with advanced hematologic malignancies. In addition, the effect of everolimus on multiple mTOR-regulated proteins was evaluated.

Therapy with everolimus at a dose of 10 mg orally once daily was relatively well tolerated. Toxicities observed with everolimus were consistent with those reported in previous studies using mTOR inhibitors and consisted of hyperglycemia, hyperlipidemia, anorexia, elevated liver enzymes, oral aphthous ulcers, diarrhea, hypophosphatemia, fatigue, and hypomagnesemia (27–30). The frequency of infectious episodes did not seem to be increased in the study patients, an important consideration for future studies in the hematologic malignancies. There were no documented episodes of cytomegalovirus infections or varicella zoster infections.

One patient with advanced myelofibrosis had recurrent pulmonary infiltrates while receiving therapy with everolimus. The first episode occurred 57 days after initiating therapy and resolved with antibiotics and ongoing therapy with everolimus. This was subsequently biopsy proven to be bronchiolitis obliterans with organizing pneumonia and treated successfully with steroids. Everolimus was discontinued due to the intercurrent illness. She was not rechallenged. Bronchiolitis obliterans with organizing pneumonia is usually idiopathic, although it has been associated with hematologic malignancies and cytotoxic drugs (31, 32) Nonspecific pneumonitis has been described in patients treated with temsirolimus and sirolimus (27, 33). Six of 42 patients were diagnosed with bronchiolitis obliterans with organizing pneumonia, all of whom were recipients of solid organ transplants. Approximately one third of patients experienced improvement of the infiltrates after discontinuation of the drug, and two of four patients with renal cell cancer experienced recurrent pneumonitis on rechallenge (27). Preclinical studies indicated that everolimus could prevent the development of bronchiolitis obliterans in a porcine bronchial model (34). Similarly, the combination of mycophenolate and sirolimus could attenuate the progression of bronchiolitis obliterans syndrome in lung and heart-lung transplant recipients (35). Therefore, the relationship of
bronchiolitis obliterans with organizing pneumonia to everolimus is unclear.

A patient with MDS developed cutaneous LCV while receiving therapy with everolimus on this study. Causes of LCV include malignancy, including MDS, and drugs (36). LCV has been reported with sirolimus in four patients. This occurred 0.75 to 4 months after initiation of sirolimus to prevent solid organ rejection. The involved sites were skin in three cases and gastrointestinal in the fourth. Symptoms were resolved within 0.2 to 4 months after discontinuation of sirolimus. Rechallenge with sirolimus resulted in recurrence of the LCV in one case.

Only one patient with MDS achieved an objective response (i.e., major hematologic improvement in platelet count), although one other patient achieved a minor response in platelet count of 25-day duration. Although only five patients with MDS were treated with everolimus, the response rate (20%) was comparable with what has been observed with sirolimus in this patient population (16%; ref. 28). None of the nine patients with AML treated with everolimus had an objective response, in contrast to an overall response rate of 44% (44% partial response) reported with sirolimus in patients with AML (37). It is unclear whether this is due to differences in patient characteristics, duration of therapy (only 28 days with sirolimus), or significant differences in the agents (1). We observed no responses in 10 patients with AML treated with temsirolimus (38). On interim analysis, we observed that only 2 of 26 (8%) of patients with AML or MDS treated with AP23573 had a hematologic improvement (39). Response rates of 38% (3% complete response; 35% partial response) were observed in patients with relapsed or refractory MCL receiving single agent temsirolimus (38). On interim analysis, we observed that only 2 of 26 (8%) of patients with AML or MDS treated with AP23573 had a hematologic improvement (39). Response rates of 38% (3% complete response; 35% partial response) were observed in patients with relapsed or refractory MCL receiving single agent temsirolimus; median time to response was 1 month (range, 1-8 months; ref. 29). All four patients with MCL treated with everolimus had progressive disease; three of whom did not complete a course of therapy. Furthermore, three patients had circulating lymphoma cells at baseline and all four patients in the current study had refractory disease compared with 54% of those given temsirolimus.

Variability in everolimus concentrations has been observed in different ethnic groups, with lower bioavailability in African-Americans compared with Caucasians or non-African-Americans (40). In the current study, 3 of the 26 evaluable patients were African-Americans (1 CLL, 1 natural killer cell/T-cell leukemia, and 1 AML); none of whom achieved an objective response, although the patient with CLL did have a 33% reduction in lymphadenopathy after three cycles of therapy with everolimus.

Akt was activated as evidenced by phosphorylation of Ser473 and Thr308 in all but one sample tested, confirming the high frequency of Akt activation in primary leukemia cells. (1) Analysis of phosphorylation of 4E-BP1 and p70S6K, established biomarkers of mTOR blockade by rapamycin analogues, showed inhibition of phosphorylation of both proteins in four of nine samples and inhibition of phosphorylated Thr70 in 4E-BP1 in two additional samples (i.e., inhibition in ~66% of patients). Notably, evidence of inhibition of mTOR signaling by everolimus was documented in samples from all patients with clinical responses, with the exception of samples from three patients with CLL, where the patients did not consent to provide samples. mTOR inhibition was associated with transcriptional down-regulation of D-type cyclins (either cyclin D1 or cyclin D2) and a decrease in Glut1 mRNA levels in a subset of patients, suggesting that, in leukemic cells, everolimus attenuates transcription of these genes. However, inhibition of mTOR downstream targets did not translate into clinical responses in AML patients 2 and 9, indicating that the ability to inhibit the mTOR pathway is not always sufficient to elicit clinical responses.

Modest antitumor activity of mTOR inhibitors may also be associated with activation of alternative prosurvival signaling pathways, such as mitogen-activated protein kinase/ERK. Furthermore, ERK1/2 has been shown to phosphorylate and inactivate TSC2 and 4E-BP1 and activate p70S6K in vitro (41–43), and preclinical data indicate that inhibition of both mTOR and ERK signaling pathways is required to prevent de novo protein translation. We observed high levels of phosphorylation of ERK in the majority of samples studied, confirming our previously reported data in AML that showed phosphorylated ERK in >80% of AML patients and providing a rationale for future combination studies with Raf or mitogen-activated protein kinase/ERK kinase inhibitors (44, 45).

Recent work has shown that selective inhibition of mTOR downstream signals paradoxically increases phosphorylated Akt (46, 47), likely attributable to lack of feedback inhibition by p70S6K on the insulin-like growth factor-I receptor. We unexpectedly observed inhibition of Akt phosphorylation in five of six samples, in which everolimus modulated mTOR signaling. mTOR partitions between two scaffold proteins, the rapamycin-sensitive raptor and the rapamycin-insensitive rictor (23). Recent data show that the rictor-mTOR complex (TORC2) directly phosphorylates Akt on Ser473 and facilitates Thr308 phosphorylation by PDK1 (48). Although rapamycin does not bind to a preformed rictor-mTOR complex (49), it has been proposed that, during prolonged rapamycin treatment, the drug

### Table 3. Effects of everolimus on intracellular signaling pathways and transcription of target genes (Cont’d)

<table>
<thead>
<tr>
<th>Phosphorylated Akt Thr308</th>
<th>Phosphorylated Akt Ser473</th>
<th>Phosphorylated ERK p42/44 (Thr202/Tyr204)</th>
<th>Glut1</th>
<th>Cyclin D1</th>
<th>Cyclin D2</th>
</tr>
</thead>
<tbody>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.8</td>
<td>0.5</td>
<td>0.6</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.6</td>
<td>0.9</td>
<td>0.5</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.2</td>
<td>0.02</td>
<td>0.7</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.6</td>
<td>1.5</td>
<td>1.3</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.8</td>
<td>0.02</td>
<td>0.4</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.4</td>
<td>6.5</td>
<td>0.2</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>2.8</td>
<td>0.06</td>
<td>3.1</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>1.0</td>
<td>1.0</td>
<td>1.2</td>
</tr>
<tr>
<td>ND</td>
<td>ND</td>
<td>No phosphorylated ERK</td>
<td>1.3</td>
<td>0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>
will sequester the newly synthesized mTOR molecules, interfere with reassembly of rictor-mTOR complex, and thereby inhibit rictor-mTOR activity. In fact, prolonged, but not acute, treatment of some human cells with rapamycin has been reported to partially inhibit Akt phosphorylation (50).

The data reported here provide the first *in vivo* evidence that rapamycin analogues are capable of suppressing Akt activity in hematopoietic cells, perhaps in contrast to solid tumor cell types. Although the antitumor activity of single agent mTOR inhibitors is modest in patients with hematologic malignancies, combination therapy of these agents with modulators of upstream stimulants of the pathway (e.g., vascular endothelial growth factor, Flt3, bcr-abl) is warranted. Based on data from the current study, concerns about the immunosuppressive effects of mTOR inhibitors in patients with advanced hematologic malignancies should not pose a barrier to the conduct of further studies, although this issue will require careful monitoring.

**References**

9. Majumder PK, Fedbo PG, Bikoff R, et al. mTOR inhi-
bitors reverses Akt-dependent prostate intrapitheli-
al neoplasia through regulation of apoptotic and HIF-1-
10. Aguirre D, Boya P, Bellet D, et al. Bcl-2 and CD34/ CD4 expression levels predict the cellular effects of
mTOR inhibitors in human ovarian carcinoma. Apopto-
11. Law M, Forrester E, Chytli A, et al. Rapamycin dis-
rupts cyclin/cyclin-dependent kinase/p21/prolifer-
ating cell nuclear antigen complexes and cyclin D1
reverses rapamycin action by stabilizing these com-
12. Jurdi F, Raettel N, Muller C, et al. A rapamycin de-
rivative (everolimus) controls proliferation through
down-regulation of truncated CAAAT enhancer bind-
ing protein 1 and NF-κB activity in Hodgkin and
anaplastic large cell lymphomas. Blood 2005;106:
1801–7.
13. Boehm A, Aichberger KJ, Mayenhofe Mea. Target-
ing of mTOR in AML is associated with decreased
growth or leukemic cells and downregulation of VEGF
pharmacodynamic evaluation of dose and schedule of
RAD001 (everolimus) in patients with operable
with tumor molecular pharmacodynamic evaluation of
dose and schedule of the oral mTOR-inhibitor Evero-
limus (RAD001) in patients with advanced solid
study of the oral mTOR inhibitor RAD001 as a mono-
therapy to identify the optimal biologically effective
dose using toxicity, pharmacokinetic, and pharmaco-
dynamic endpoints in patients with solid tumours
17. Lane H, Tanaka C, Kovarik J, et al. Preclinical and
clinical pharmacokinetic/pharmacodynamic modeling to
help define an optimal biological dose for the oral
mTOR inhibitor, RAD001, in oncology [abstract 951].
Working Group (IWG) consensus criteria for treat-
ment response in myelofibrosis with myeloid meta-
plasia: on behalf of the IWG for myelofibrosis research
of an international working group to standardize
response criteria for myelodysplastic syndromes.
the National Cancer Institute-sponsored workshop on
definitions of diagnosis and response in acute myeloid
regulation of 4e-BP1 in regenerating rat liver. J Biol
22. Hay N. The Akt-mTOR tango and its relevance to
23. Raval RR, Lau KW, Tran MG, et al. Contrasting prop-
terties of hypoxia-inducible factor 1 (HIF-1) and HIF-2
24. Gera JF, Mellinghoff IK, Shi Y, et al. AKT activity
determines sensitivity to mammalian target of rapamy-
cin (mTOR) inhibitors by regulating cyclin D1 and
25. Hashemolhosseini S, Nagamine Y, Morley SJ, Des-
vieres S, Mercel F, Ferrari S. Rapamycin inhibi-
tion of the G to S transition is mediated by effects on
cyclin D1 mRNA and protein stability. J Biol Chem
ized phase II study of multiple dose levels of CCI-779,
a novel mammalian target of rapamycin kinase inhibi-
tor, in patients with advanced refractory renal cell
of sirolimus in patients with myeloproliferative syn-
of single-agent temsirolimus (CCI-779) for relapsed
mantle cell lymphoma. J Clin Oncol 2005;23:
5347–66.
29. Raymond E, Alexandre J, Faivre S, et al. Safety and
pharmacokinetics of escalated doses of weekly intra-
venous infusion of CCI-779, a novel mTOR inhibitor,
30. Oymak FS, Demirbas HM, Mavili E, et al. Bronchi-
olitis obliterans organizing pneumonia. Clinical and
histopathologic features in 26 cases. Respiration 2002;
single pathway inhibition: MEK inhibitors as a platform
for the development of pharmacological combinations
with synergistic anti-leukemic effects. Curr Pharm Des
32. O'Reilly KE, Rojo F, She QB, et al. mTOR inhibition
induces upstream receptor tyrosine kinase signaling and
33. Sun SY, Rosenberg LM, Wang X, et al. Activation of
Akt and eIF4E survival pathways by rapamycin-
mediated mammalian target of rapamycin inhibition.
34. Barbassov DD, Guertin DA, Ali SM, Sabatini DM.
Phosphorylation and regulation of ERK phosphorylation
and regulation in relapsed and refractory primary
single pathway inhibition: MEK inhibitors as a platform
for the development of pharmacological combinations
with synergistic anti-leukemic effects. Curr Pharm Des
36. Yee KW, Hymes SR, Heller L, Prieto VG, Welch MA,
Giles FJ. Cutaneous leukocytoclastic vasculitis in a
patient with myelodysplastic syndrome after therapy
with the rapamycin analogue everolimus: case report
and review of the literature. Leuk Lymphoma 2006;47:
926–9.
37. Recher C, Bayne-Rauzy O, Demur C, et al. Antileu-
kemic activity of rapamycin in acute myeloid leukemia.
phase II study of temsirolimus (CCI-779) in patients
with advanced leukemias [abstract 4523]. Blood
2004;104:214a.
clinical trial of AP23573, an mTOR inhibitor, in patients
with relapsed or refractory hematologic malignancies
40. Dirks NL, Huth B, Yates CR, Meibohm B. Pharmacoco-
netics of immunosuppressants: a perspective on
ethnic differences. Int J Pharmacol Ther 2004;
42:701–18.
41. Naegele S, Morley SJ. Molecular cross-talk be-
 tween MEK1/2 and mTOR signaling during recovery
of 293 cells from hypertonic stress. J Biol Chem
2004;279:46023–43.
Pharmacogenomic profiling of the PI3K/PTEN-AKT-
mTOR pathway in common human tumors. Int J Oncol
43. Hammerman PS, Fox CJ, Bimbaur MJ, Thompson
CB. Pim and Akt oncogenes are independent regula-
tors of hematopoietic cell growth and survival. Blood
Phase I/II Study of the Mammalian Target of Rapamycin Inhibitor Everolimus (RAD001) in Patients with Relapsed or Refractory Hematologic Malignancies

Karen W.L. Yee, Zhihong Zeng, Marina Konopleva, et al.


Updated version Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/12/17/5165

Cited articles This article cites 48 articles, 20 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/12/17/5165.full#ref-list-1

Citing articles This article has been cited by 39 HighWire-hosted articles. Access the articles at:
http://clincancerres.aacrjournals.org/content/12/17/5165.full#related-urls

E-mail alerts Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.