Potentiating effects of RAD001 (Everolimus) on vincristine therapy in childhood acute lymphoblastic leukemia

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Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in children. Despite intense research, a majority of children who relapse still die of ALL.2 The development of new treatments for ALL that use more potent but less toxic drugs will therefore be imperative to save more lives.

B-cell progenitor ALL is the most common type of ALL and arises from acquired mutations in early B-cell progenitors.3 Mutations in ALL result in highly cycling clonogenic cells, which are arrested at the progenitor-cell stage.7 We and others have demonstrated that the survival and proliferation of ALL cells are dependent on signaling through the p38MAPK, MEK, and PI-3K/AKT pathways.4-6 Although this represents a simplified chain of events, it points to crucial pathways that could be used as therapeutic targets.

Recently, the mammalian target of rapamycin (mTOR) has received much attention as a potential target in many cancers, including hematologic malignancies.7-12 mTOR is crucial for the transmission of proliferative and antiapoptotic signals through the PI-3K/AKT transduction pathway.13 After activation by various growth factors and nutrients, mTOR regulates the G1 to S phase traverse by phosphorylating 2 proteins important in translational control: the S6 ribosomal protein kinase 1 (p70S6K) and the 4E-BP1.14,15 There are increasing clinical data showing promising activity of mTOR inhibitors against solid tumors,14,15 and RAD001 has been tested in an in vivo leukemia model supporting further clinical development of target of rapamycin inhibitors for the treatment of patients with ALL. (Blood. 2009;113:3297-3306)

Despite advances in the treatment of acute lymphoblastic leukemia (ALL), the majority of children who relapse still die of ALL. Therefore, the development of more potent but less toxic drugs for the treatment of ALL is imperative. We investigated the effects of the mammalian target of rapamycin inhibitor, RAD001 (Everolimus), in a nonobese diabetic/severe combined immunodeficiency model of human childhood B-cell progenitor ALL. RAD001 treatment of established disease increased the median survival of mice from 21.3 days to 42.3 days (P < .02). RAD001 together with vincristine significantly increased survival compared with either treatment alone (P < .02). RAD001 induced a cell-cycle arrest in the G0/G1 phase with associated dephosphorylation of the retinoblastoma protein, and reduced levels of cyclin-dependent kinases 4 and 6. Ultrastructure analysis demonstrated the presence of autophagy and limited apoptosis in cells of RAD001-treated animals. In contrast, cleaved poly(ADP-ribose) polymerase suggested apoptosis in cells from animals treated with vincristine or the combination of RAD001 and vincristine, but not in those receiving RAD001 alone. In conclusion, we have demonstrated activity of RAD001 in an in vivo leukemia model supporting further clinical development of target of rapamycin inhibitors for the treatment of patients with ALL.

Methods

Leukemic cells

Leukemic blasts were obtained from 5 patients (Table 1) with ALL after informed consent was obtained in accordance with the Declaration of Helsinki and institutional ethics committee approval from the Sydney West Area Health Service Human Ethics Committee. Mononuclear cells were

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prepared and cryopreserved as described previously.22 Xenografts were established in NOD/SCID mice as previously described.23 Cells recovered from the spleens of these animals were used in experiments described here.

**Antibodies and reagents**

RAD001 was supplied by Novartis Institutes for BioMedical Research, Oncology (Basel, Switzerland). This study used the following antibodies: antihuman CD19-phycocerythrin (4G7), CD19-allophycocyanin (SJ25C1), CD10-fluorescein isothiocyanate (FITC; SS2/36), CD34-peridinin chlorophyll protein (8G12; BD Biosciences, Sydney, Australia); antimurine CD45-FITC (30-F11; Caltag, Mountain Waverley, Australia); antihuman-4E-BP1, anti-4E-BP1, anti-AKT, antiphospho-AKT, anti-poly(ADP-ribose) polymerase (PARP), anti-PTEN, anti-cdk4, anti-cdk6, anti-S6 ribosomal protein (S6RP) antiphospho-S6RP (pS6RP); Cell Signaling Technology, Danvers, MA); antiphospho-retinoblastoma protein (Rb; Abcam, Cambridge, United Kingdom); rabbit antibodies to human LC3, phospho-p38MAPK (T180/Y182), and p38MAPK (Genesearch, Arundel, Australia); horseradish peroxidase (HRP)-conjugated swine anti–rabbit immunoglobulins, FITC-conjugated swine anti–rabbit immunoglobulins (Dako Denmark, Glostrup, Denmark), and mouse anti–β-actin and HRP-conjugated goat anti–mouse immunoglobulins (Sigma-Aldrich, St Louis, MO). Antibodies were used as recommended by the manufacturers.

**Mouse models**

NOD/SCID mice were housed in sterile micro-isolator cages in ventilated racks. Protocols were approved by the Westmead Animal Ethics Committee. RAD001 was formulated at 2% (wt/vol) in a microemulsion vehicle (Novartis, Basel, Switzerland). RAD001 and vehicle solution were diluted to 1 mg/mL in dH2O and stored at −20°C. 100 μL of freshly thawed RAD001 (5 mg/kg) or vehicle was given 3 times weekly by gavage. Vincristine (Pharmacia, Sydney, Australia) was diluted to 30 μg/mL in 0.9% saline just before weekly intraperitoneal administration. Mice were engrafted with xenografts 1999 and 1345 received 0.15 mg/kg, whereas those engrafted with xenografts 0398 and 1196 received vehicle only, vincristine, RAD001, or both vincristine and RAD001 3 times weekly treatments with RAD001. PB was collected by cardiac puncture into ethylenediaminetetraacetic acid for the analysis of RAD001 concentrations or heparin for determination of bilirubin, liver enzymes, and creatinine levels.

**Flow cytometry**

Flow cytometric analysis of cells labeled with directly conjugated monoclonal antibodies was done as previously described and analyzed on a FACSCanto flow cytometer (BD Biosciences).23 For cell-cycle analysis, cells were fixed in 45% ethanol after surface labeling for human CD19 and washed with BD Perm/Wash Solution (BD Biosciences Pharmingen, San Diego, CA). Fixed cells were resuspended in 100 μL of BD Perm/Wash Solution containing 0.25 μg of 7-amino-actinomycin D and incubated on ice for 60 minutes. The cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences), and data were fitted using ModFit LT cell-cycle analysis software (Verity Software, Topsham, ME).

**Immunofluorescence microscopy**

Cells were treated as described and labeled with 10 μM LysoSensor Blue DND-167 (Invitrogen, Carlsbad, CA) in RPMI containing 10% fetal calf serum for 30 minutes at 37°C. Cells were resuspended in fresh medium before examination using an Olympus FV1000 confocal laser scanning microscope system, based on an Olympus IX-81 ZDC microscope (Tokyo, Japan), with BP 330- to 385-nm excitation and BA 420-nm emission filters. Images were captured using FV10-ASW 1.7 software (Olympus), and the number of acidic vacuoles in cells were quantitated using ImageJ software (National Institutes of Health [NIH], Bethesda, MD).

**Western blotting**

A single-cell suspension was obtained from spleens and red cells lysed with 0.155 M NH4Cl, 10 mM KHCO3, and 0.1 mM ethylenediaminetetraacetic acid (pH 7.5). Cell lysates were prepared, and equal amounts of protein were loaded in each lane of 7.5% or 15% sodium dodecyl sulfate–polyacrylamide gels and transferred onto nitrocellulose membranes as previously described.24 Phosphorylated and total proteins were detected sequentially on the same membrane using specific primary

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**Table 1. Clinical information**

<table>
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<tr>
<th>ID</th>
<th>Sex/age</th>
<th>Source</th>
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<th>Stage</th>
<th>Immunophenotype</th>
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<td>D</td>
<td>CD10+</td>
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<td>BM</td>
<td>94</td>
<td>D</td>
<td>CD10-CD34</td>
<td>46 XX</td>
</tr>
<tr>
<td>0398</td>
<td>M/15</td>
<td>BM</td>
<td>96</td>
<td>R</td>
<td>CD10-CD34</td>
<td>46 XY add(3)(q29) t(14;19)(q32p13)</td>
</tr>
<tr>
<td>1196</td>
<td>F/8</td>
<td>NA</td>
<td>86</td>
<td>D</td>
<td>CD10-CD34</td>
<td>46 XX – 19,del(19), t(11;19)(q23p13)</td>
</tr>
<tr>
<td>2032</td>
<td>M/12</td>
<td>NA</td>
<td>96</td>
<td>D</td>
<td>CD10-CD34</td>
<td>46 XY add (9)(p24), del(9)(p21), del(13)(q11q21), der(19)t(1;19)(q23;p13)</td>
</tr>
</tbody>
</table>

D indicates diagnosis; NA, not available; and R, relapse.
antibodies, appropriate secondary antibodies conjugated to HRP, and enhanced chemiluminescence (PerkinElmer Life and Analytical Sciences, Waltham, MA). Bands were quantitated by densitometry (GE Healthcare, Little Chalfont, United Kingdom) using ImageQuant software.

Electron microscopy
Vertebral bodies and sternums of mice were fixed in modified Karnofsky fixative (2.5% formaldehyde prepared freshly from paraformaldehyde; 2.5% EM grade glutaraldehyde in 0.1 M 3-[N-morpholino]propanesulfonic acid buffer, pH 7.4) and then decalcified in 0.5 M ethylenediaminetetraacetic acid for 14 days. Tissue blocks were trimmed and postfixed in osmium tetroxide, dehydrated in increasing concentrations of ethanol, and embedded in epoxy resin. Semithin (500-nm) sections were cut on a Reichert ultratcut microtome and assessed by light microscopy. Ultrathin (80-90 nm) sections were cut and grid stained with 2% ethanolic uranyl acetate and then Reynolds lead citrate. The ultrastructure was examined using a Philips CM-10 transmission electron microscope (FEI, Portland, OR) operated at 80 kV. Images were recorded using Kodak electron microscope film type 4489 (Eastman Kodak, Rochester, NY). Black and white prints were scanned using a Hewlett-Packard scanner (model number 5300C) and digitalized using Photoshop software, version 8 (Adobe Systems, San Jose, CA), and composite images were compiled using Adobe Photoshop software, version 8 (Adobe Systems, San Jose, CA).

Histology
Femurs and livers were fixed in 10% buffered formalin. Femurs were decalcified for 14 days in 0.5 M ethylenediaminetetraacetic acid. Decalcified femurs were processed into paraffin; 5-μm sections were cut on a Reichert ultratcut microtome and examined by light microscopy. Slides were examined by transmission light microscopy using an Olympus BX51 microscope fitted with an UPlanFl 20× objective at room temperature. Images were captured using a Spot RT slider camera (Diagnostic Instruments, Sterling Heights, MI) and SPOT Advanced software. Composite figures prepared using Adobe Photoshop software.

Pharmacokinetics and clinical chemistry
RAD001 concentrations were determined by high-performance liquid chromatography-electrospray tandem mass spectrometry by SydPath Laboratories (St Vincent’s Hospital, Sydney, Australia). Standard noncompartamental pharmacokinetic parameters were derived, including the highest and lowest concentration (Cmax, Cmin), and the area under the concentration-time curve (AUC) over the 48-hour dosing interval (calculated by trapezoidal summation; AUC48). Serum aspartate aminotransferase, alanine aminotransferase, bilirubin, and creatinine were determined by routine laboratory testing at Institute for Clinical Pathology and Medical Research, Westmead Hospital (Westmead, Australia).

Statistics
Comparisons between 2 groups were performed using Student t tests and between multiple groups by analysis of variance. Log transformation was made before analysis to stabilize for variance. Pairwise comparisons between groups were adjusted for multiple comparisons using the Bonferroni method. Linear regression was used to determine correlations between variables. Survival was measured from the onset of disease until death and analyzed using SPSS, version 15.0 (SPSS, Chicago, IL). The Kaplan-Meier method was used to construct survival curves, and results were compared using the log-rank test of survival distribution by treatment stratified by cell line. The number of cells containing acidic vacuoles was compared between groups using Student t tests and comparison of the number of acidic vacuoles/cell analyzed using the Kruskal-Wallis test, to determine differences between treatments, and the Jonckheere-Terpstra test to demonstrate association between increasing numbers of AV/cell with increasing concentrations of RAD001.

Results
RAD001 improves survival of NOD/SCID mice engrafted with ALL
We have previously demonstrated the inhibition of ALL engraftment in NOD/SCID mice after blockade of CXCR4/CXCL12 and shown that PI-3K/AKT signaling is important for spontaneous and CXCL12-induced ALL cell proliferation in vitro. Therefore, we investigated the effect of inhibition of mTOR, which is downstream of AKT, on ALL engraftment using our established NOD/SCID xenograft model. NOD/SCID mice were treated with the mTOR inhibitor, RAD001, starting the day after the injection of ALL-1345, -1196, or -0398 cells. RAD001 treatment prevented disease progression as determined by weekly assessment of the percentage of leukemic cells in the PB of the 2 xenografts (1345 and 1196) where significant numbers of human cells were detected within the 3-week period (Figure 1A). At death on day 21, significantly fewer ALL cells were present in the BM of RAD001-treated mice for all 3 xenografts and in the spleens and blood of xenografts 1345 and 1196 (Figure 1B). For xenograft ALL-1345, leukemic cells were below the limit of detection (< 0.01%) in the spleens of 5 and the blood of 4 of the 7 treated mice. Similarly, there was
Surprisingly long-term complete remission was persistent in ALL-1999 with a rate of 83% and 100%, for single and combined treatments, respectively, 10 weeks after the end of treatment. The impact of treatment on ALL expansion and dissemination was reflected in the improved overall survival of animals. The median survival of vehicle-treated mice was 21.3 days (range, 13.8-35.0 days) after leukemia was detected in the PB for all 5 ALL xenografts (Figure 2B). RAD001 significantly increased survival for each xenograft with a median survival of 42.0 days (range, 24.5-104 days). As anticipated from a log-rank test of survival distribution by treatment. As anticipated from the doses selected, vincristine as a single agent enhanced survival in only 2 xenografts (ALL-1345 and -0398) with no overall increase in survival (median survival, 24.1 days; range, 14.0-39.5 days, P = not significant). Despite this, the combination of RAD001 and vincristine enhanced overall survival to a greater extent than treatment with RAD001 (4 of 5) or vincristine alone (3 of 5) with a median survival of 50.0 days (range, 33.0-105 days), which was 8 days greater than the survival induced by RAD001 alone and 25.9 days longer than mice receiving vincristine alone (P < .02). Detailed statistical analysis of survival data for each xenograft is given in Table 2. In one xenograft (ALL-1999), all RAD001 and RAD001 and vincristine-treated animals survived for 10 weeks after the completion of treatment. Mice were electively culled at this time. Leukemia was detected in only one mouse receiving RAD001 as a single agent. However, the disease was below the limit of detection in all

<table>
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<tr>
<th>Xenograft</th>
<th>RAD001 vs placebo</th>
<th>Vincristine vs placebo</th>
<th>RAD001 + vincristine vs RAD001 alone</th>
<th>RAD001 + vincristine vs vincristine alone</th>
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</thead>
<tbody>
<tr>
<td>1345</td>
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<td>.001</td>
<td>.383</td>
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The significance values shown were determined using a Mantel-Cox log rank test.
hematopoietic compartments in all mice receiving vincristine and RAD001 (Figure 2C and data not shown). Femur sections revealed the return of normal hematopoiesis, and murine blood counts showed white cell counts, hemoglobin concentration, and platelet counts returning to within the normal range in animals receiving RAD001 or RAD001 and vincristine (Figure 2D and data not shown). This suggests that mice recovered well from both their disease and the treatments received. Overall, these data confirm the potent inhibitory effect of RAD001 on the expansion of ALL in vivo and show an interactive effect when combined with the chemotherapeutic agent vincristine.

Tolerance of RAD001 in mice engrafted with ALL

To determine whether the therapeutic effect of RAD001 was dependent on mTOR inhibition in our NOD/SCID model of childhood ALL, systemic exposure to RAD001 was quantified. Peak plasma concentrations of 1.86 ± 0.38 μM (mean ± SD; n = 3) and 2.51 ± 0.69 μM (mean ± SD; n = 3) were achieved 2 hours after a single orally administered dose in NOD/SCID mice engrafted with ALL-1345 and -0398, respectively (Figure 3A). The t1/2 was approximately 10 hours. Repetitive administration (3 times weekly) of RAD001 maintained similar trough blood concentrations at days 2, 7, and 14 (Figure 3B). Despite the higher than anticipated blood concentrations, RAD001 was well tolerated in animals engrafted with ALL. There was no consistent trend or finding with respect to several biochemical assessments during the course of the study, in particular serum renal or liver function tests (Table S1, available on the Blood website; see the Supplemental Materials link at the top of the online article). Indeed, mice engrafted with ALL-1345 showed elevated alanine aminotransferase levels that were significantly attenuated by RAD001 treatment, reflecting reduced ALL cell infiltration of the liver. In addition, nonengrafted mice treated with RAD001 showed no signs of cytotoxicity or cell degeneration in the hematopoietic cell compartment, with normal reconstitution of the white blood cell counts after sublethal irradiation (data not shown).

RAD001 attenuates phosphorylation of 4E-BP1 and S6RP in ALL cells

To confirm that RAD001 is acting on target, we examined the phosphorylation status of the mTOR target proteins 4E-BP1 and S6RP as well as the nontarget proteins AKT and p38MAPK. For xenografts ALL-1999 and -1345, this was performed on human cells recovered from the spleens of mice after 24 hours of RAD001 treatment. Samples analyzed after in vitro culture revealed a similar reduction in phosphorylated 4E-BP1 and S6RP after incubation with 2 μM RAD001 (Figure 3C). In ALL-0398, the reduction in phosphorylation was less than that observed in the other samples, a finding consistent with the reduced efficacy of RAD001 in this xenograft. Consistent with the data
Mechanisms underlying improved survival of mice engrafted with ALL receiving RAD001

To determine the mechanisms involved in RAD001-mediated cytocidal effect of ALL xenografts, histologic analysis of microenvironmental niches at the endosteum of vertebral bodies of the lower spine was performed. TEM revealed that, consistent with the light microscopy, vincristine treatment resulted in patchy cellular apoptosis, the ultrastructure of which was characterized by uniformly electron dense round nuclear chromatin bodies associated with early preservation of the cytoplasmic membrane and organelles (Figure 6B). In contrast, RAD001 treatment showed only occasional apoptotic cells (Figure 6C). However, a small but significant number of leukemic cells showed double-walled peripheral cytoplasmic polyphagocytic vacuoles (Figure 6E-G, Figure S5). These vacuoles contained a mixture of complex structures consistent with mitochondria, rough endoplasmic reticulum, polyribosomes, lysosomes among other organelles in various stages of breakdown. The combination of RAD001 and vincristine (Figure 6D) produced both sets of features (ie, the presence of both apoptotic cells and cells containing autophagic vacuoles). This ultrastructure data suggest the induction of autophagy, but not apoptosis, by RAD001.

Induction of apoptosis by vincristine was confirmed by the detection of increased PARP cleavage in lysates of spleen cells recovered from mice receiving vincristine or RAD001 plus vincristine, but this was not observed in those receiving RAD001 alone (Figure 7A). Cleavage of caspases could not be detected in cells recovered from mice. However, cleaved caspase 3 was detected in the ALL cell line NALM6 after in vitro exposure to vincristine, but not in cells incubated with RAD001. These cells also demonstrated the same pattern of PARP cleavage (ie, only increased in response to vincristine) as had been observed in cells recovered from the mice, although PARP cleavage was more pronounced after in vitro culture (Figure 7B).

The induction of autophagy by RAD001 was further supported by the detection of increased proportion of cells containing acidic vesicles among other organelles in various stages of breakdown. The combination of RAD001 and vincristine (Figure 6D) produced both sets of features (ie, the presence of both apoptotic cells and cells containing autophagic vacuoles). This ultrastructure data suggest the induction of autophagy, but not apoptosis, by RAD001.

Mechanisms of RAD001-induced cell death

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vacuoles, and an increase in the number of acid vacuoles detected in each cell \((P < .001\) in all 3 experiments), in response to RAD001 treatment in the ALL cell lines NALM6 and REH (Figure 7D-F). A positive association between the number of acidic vacuoles and the dose of RAD001 applied to the cells was apparent \((P < .001)\). Western blotting also revealed an increase in the autophagy-associated protein Beclin-1 and the processing of LC3 to the lipidated form (LC3-II), which associates with autophagosomes (Figure 7C). Overall, the data demonstrate that apoptosis is not the major mechanism of cell death induced by RAD001 alone and is consistent with the picture observed by electron microscopy implicating autophagy as the predominant form of RAD001-induced cell death.

**Discussion**

Despite significant improvements in primary therapy, long-term outcomes after relapse remain poor, with a 15-year overall survival of only 37% among patients enrolled in the ALL-BFM-87 series of studies. Patients who have a second BM relapse have a 5-year survival of only 8%. Considering the poor outcome, there is a critical need for new drugs with novel mechanisms of action to improve or even prevent relapse of ALL. Complete response rates using familiar reinduction protocols can be as high as 40%, making recruitment into trials of new agents, which often have similar predicted outcomes and increased potential toxicities, difficult. One strategy to improve access onto phase 1 trials in pediatric ALL is to identify new drugs that probably enhance leukemia killing by standard multidrug reinduction regimens, but with minimal added systemic toxicity. With the latter philosophy in mind, we have examined the potential of RAD001, a small orally bioavailable mTOR inhibitor, to treat leukemia as a single agent and as a combinational agent with vincristine, a widely used agent of multimodal chemotherapy in several study groups.

The primary goal of this study was to provide preclinical data to form a rational basis for combining mTOR inhibitor therapy with conventional cytotoxic agents. Thus, we adapted RAD001 treatment regimens of animal experiments in other tumor types to our model of ALL. RAD001 was rapidly absorbed, and consistent
steady-state concentrations in the low to mid nanomolar range were achieved. This is consistent with animal data but contrasts with human studies where increasing blood concentrations are observed over the first days of treatment.33 The clearance of RAD001 was approximately 4 times faster in ALL-engrafted mice compared with healthy human volunteers,32 but the overall bioavailability was comparable based on dose-adjusted AUC after a single dose.32 This was surprising because a considerably lower bioavailability of RAD001 has been previously reported in rats.33 The high blood trough concentration achieved may be explained by the very high binding (> 99%, Novartis) of RAD001 to plasma proteins in mice, limiting the amount of drug available to enter cells. As a result, mice in this study had a higher RAD001 exposure than pediatric patients in transplantation trials, but the drug concentrations were well tolerated, with no toxicity observed with respect to several biochemical and hematologic assessments, and were comparable with previous in vitro IC_{50} values of survival inhibition and cell death induction of different tumor types to RAD001.27,24

Applying our treatment regimen of 3 times weekly administration of RAD001 in 5 ALL xenograft cases, we present convincing evidence of significant inhibition of leukemic growth. The NOD/SCID mouse model more closely reflects disease progression in pediatric patients because leukemic cells disseminate in extramedullary organs, such as spleen and liver, once the BM is engrafted.35 Treatment with RAD001 inhibited engraftment of ALL in BM and extramedullary organs. RAD001 produced significant reductions in ALL once leukemia was established, resulting in prolonged survival. Notably, in xenografts of ALL-1999, sustained remission with recovery of normal hematopoiesis was demonstrated in 5 of 6 mice up to 10 weeks after finishing RAD001 treatment. Strikingly, we observed a positive interaction between RAD001 and vincristine in the majority of xenografts tested. Because responses of human ALL xenografts to chemotherapeutic agents, such as vincristine, correlate significantly with patient outcome,36 the enhanced antileukemic activity of RAD001 in a multagent combination regimen observed in xenografts may predict similar activity in patients. Indeed, another mTOR inhibitor, CCI-779, was similarly shown to enhance the efficacy of methotrexate using a similar model system.37

Despite clear and potent effects of RAD001, considerable variation was observed between the responses of the 5 xenografts studied. Loss of PTEN and subsequent constitutive phosphorylation of AKT results in resistance to chemotherapy in ALL, which can be overcome by inhibition of mTOR.38-40 In addition, RAD001 can paradoxically increase AKT phosphorylation in some malignant cells resulting in resistance to mTOR inhibitors.41 However, the PTEN protein was present at similar levels in all 5 xenografts (data not shown), and there was no evidence of increased AKT phosphorylation in the ALL cells recovered from either RAD001-treated mice or after 24 hours of in vitro culture with 2 μM RAD001 (Figure S3). Therefore, the variation in responses cannot be explained by alterations in PTEN expression or AKT phosphorylation.

We report here that levels of phosphorylated 4E-BP1 and S6RP were decreased after RAD001 treatment, consistent with current studies on mTOR signaling.3 RAD001-induced inhibition of cell cycle progression in vivo was associated with reduced levels of phosphorylated Rb, and cdk4 and 6 levels, consistent with previous reports of mTOR inhibition in other cell types.42-44 Surprisingly, we did not detect increased levels of apoptosis in response to RAD001 treatment, although this was evident after exposure to vincristine. This contrasts with previous reports that mTOR inhibition with rapamycin induces apoptosis in ALL cells.45 The most probable explanation for this discrepancy is the examination of patient ALL cells cultured in vitro in these studies. Under these conditions, ALL cells undergo significant spontaneous apoptosis, which mTOR inhibition may have enhanced, whereas in our study, cells were either recovered ex vivo or were continuous cell lines where spontaneous apoptosis is minimal. However, ultrastructural analysis demonstrated the presence of autophagic vacuoles as well as limited apoptosis in vertebral bodies infiltrated with ALL cells after RAD001 treatment. The clear specific induction of autophagic vacuoles as well as limited apoptosis in vertebral bodies infiltrated with ALL cells after RAD001 treatment. The clear specific induction of autophagic vacuoles, the induction of Beclin-1, and lipidation of LC3 after RAD001 exposure raise the possibility that ALL cells are dying as a result of excessive autophagy. Although mTOR inhibition is well known to induce autophagic cell death in other cell types,46 our current data fall short of providing a causal link between the increased autophagy and reduced viability in our model of ALL. However, the successful induction of prolonged...
remissions in animals with extensive disease suggests that, in the absence of apoptosis, autophagy is a plausible alternative death mechanism. Enhancement of autophagy through mTOR inhibition might be of particular interest for the treatment of ALL because resistance to current chemotherapies has been linked to defects in their apoptotic machinery. Therefore, targeting the nonapoptotic pathway may yield better clinical outcomes for patients undergoing cytotoxic cancer therapies.

In conclusion, we have shown that RAD001 can effectively inhibit the growth of childhood ALL, providing prolonged survival of mice engrafted with ALL. We conclude that RAD001 warrants clinical investigation as a combination therapy for relapsed ALL and potentially as front-line therapy for relatively chemotherapy-resistant ALL subgroups.

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Authorship

Contribution: R.C. and J.H. designed and performed experiments, analyzed data, and contributed to writing the manuscript; A.C., R.B., and M.T. performed experiments and analyzed data; K.F.B. supported research and contributed to writing the manuscript; and L.J.B. supported
research, designed experiments, analyzed data, and contributed to writing the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

References


