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

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Abstract

Despite advances in the treatment of acute lymphoblastic leukemia (ALL), the majority of children who relapse still die from 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 (mTOR) inhibitor, RAD001 (Everolimus), in a NOD/SCID 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<0.02). RAD001 together with vincristine significantly increased survival compared to either treatment alone (p<0.02). RAD001 induced a cell cycle arrest in the G0/G1 phase with associated dephosphorylation of the retinoblastoma protein, and reduced cyclin dependent kinase 4 and 6 levels. Ultrastructure analysis demonstrated the presence of autophagy and limited apoptosis in cells of RAD001 treated animals. In contrast cleaved PARP 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 mTOR inhibitors for the treatment of patients with ALL.
Introduction

Acute lymphoblastic leukemia (ALL) is the most common malignancy diagnosed in children.\(^1\) Despite intense research, a majority of children who relapse still die from ALL.\(^2\) The development of new treatments for ALL that utilize 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, that are arrested at the progenitor cell stage.\(^3\) We and others have demonstrated that the survival and proliferation of ALL cells is 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 hematological malignancies.\(^7\)-\(^{12}\) mTOR is crucial for the transmission of proliferative and anti-apoptotic signals through the PI-3K/AKT transduction pathway.\(^13\) Following activation by various growth factors and nutrients, mTOR regulates the G\(_1\) to S phase traverse by phosphorylating two proteins important in translational control: the S6 ribosomal protein kinase 1 (p70S6K) and the initiation factor 4E-binding protein 1 (4E-BP1).\(^{14,15}\) There is increasing clinical data showing promising activity of mTOR inhibitors against solid tumors\(^{14,15}\) and RAD001 has been tested in phase I/II trials for hematological malignancies, although ALL was not included in these studies.\(^11\)
In mouse experimental models, mTOR inhibitors acting as single agents have significant anti-tumor activity.\textsuperscript{10,16,18} Furthermore, data is emerging that the combination of mTOR inhibitors and other therapeutic agents is more effective.\textsuperscript{8,18} Indeed, synergistic interactions have been demonstrated between mTOR inhibitors and standard chemotherapeutics like doxorubicin,\textsuperscript{19} as well as with anti-cancer agents targeting growth factor pathways.\textsuperscript{8,20} To date, pre-clinical studies examining the potential of mTOR inhibitors in combination with standard chemotherapy for the treatment of ALL have not been reported.

In this study, we examined the efficacy of the orally bio-available mTOR inhibitor, RAD001, on ALL. The effects of RAD001 were analyzed on xenografts established from 5 patients with childhood ALL in a NOD/SCID mouse model. RAD001 strikingly reduced tumor burden via cell cycle inhibition and induction of cell death, conferring prolonged survival of engrafted animals. These effects were enhanced by the addition of vincristine, a principal drug incorporated in all current chemotherapy protocols for ALL,\textsuperscript{21} thereby proposing RAD001 as an attractive new compound in the treatment of ALL.
**Materials and methods**

**Leukemic cells**

Leukemic blasts were obtained from five 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 Ethic Committee. Mononuclear cells were prepared and cryopreserved as described previously.\(^2^2\) Xenografts were established in NOD/SCID mice as previously described.\(^2^3\) 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 utilized the following antibodies: anti-human CD19-PE (4G7), CD19-APC (SJ25C1), CD10-FITC (SS2/36), CD34-PerCP (8G12) (BD, Sydney, Australia); anti-murine CD45-FITC (30-F11) (Caltag, Mount Waverley Australia); anti-phospho-4E-BP1, anti-4E-BP1, anti-AKT, anti-phospho-AKT, anti-PARP, anti-PTEN, anti-cdk4, anti-cdk6, anti-S6 ribosomal protein (S6RP) anti-phospho-S6RP (pS6RP), (Cell Signaling Technology, Beverly, MA); anti-phospho-retinoblastoma protein (Rb) (Abcam, Cambridge, UK); rabbit antibodies to human LC3, phospho-p38MAPK (T180/Y182) and p38MAPK (Genesearch, Arundel, QLD, Australia); horseradish peroxidase (HRP)-conjugated swine anti-rabbit immunoglobulins, FITC-conjugated swine anti-rabbit immunoglobulins (DAKO, Glostrup, Denmark) and mouse anti-β-actin and HRP-conjugated goat anti-mouse immunoglobulins (Sigma, 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% (w/v) in a microemulsion vehicle (Novartis Pharma AG). RAD001 and vehicle solution were diluted to 1mg/mL in dH2O and stored at -20°C. 100µl of freshly thawed RAD001 (5mg/kg) or vehicle was given thrice weekly by gavage. Vincristine (Pharmacia, Sydney, Australia) was diluted to 30µg/mL in 0.9% saline just before weekly intraperitoneal (i.p.) administration. Mice engrafted with xenografts 1999 and 1345 received 0.15mg/kg while those receiving remaining xenografts received 0.25mg/kg. Doses of vincristine were chosen with the intent of obtaining suboptimal responses to the chemotherapy alone and had been previously titrated for xenografts 1999, 1345 and 1196 to obtain a 50% reduction in BM infiltration after 3 weeks of treatment initiated immediately after injection of the cells.

Six to 8 week old female NOD/SCID mice received 3Gy of total body irradiation from an X-ray source delivered by a self contained cabinet (model X-RAY 320, Precision X-ray Inc, CMS Alphatech Pty Ltd, Sydney, Australia), equipped with a Pantak Seifert ISOVOLT 320 HS x-ray tube, 24h before administration of 3-5x10⁶ human leukemic cells via tail vein injection. Mice were bled weekly and the percentage of human cells determined by flow cytometry using antibodies to human CD19 and murine CD45.

In engraftment assays, treatment with RAD001 or vehicle in groups of 5-7 mice commenced 24h following the injection of ALL cells and continued for 3 weeks before elective sacrifice and analysis of tissues. Vincristine was not administered in these experiments.
In survival assays, treatment commenced once ≥5% leukemic cells were detected in the peripheral blood (PB). Groups of 6 mice received vehicle only, vincristine, RAD001 or both vincristine and RAD001 for 4 weeks. Mouse welfare was assessed daily using standardized score sheets to look for signs of leukemia including paralysis, loss of weight, ruffled coat, hunched posture, altered respiration and inactivity. Unless mice unexpectedly succumbed to their disease, a humane end point was applied when mice deteriorated in the welfare score. For mice engrafted with xenograft 1999 an elective end point was set at 10 weeks after the end of treatment.

In functional assays, treatment was delayed until the mice had an expected survival time of less than 2 weeks. Mice were sacrificed on days 1 and 7 after starting the treatment. PB, bone marrow (BM) and spleens were analyzed for total cell numbers and the presence of leukemic cells by flow cytometry. Spleen cells were collected for flow cytometry and Western blot analysis. Flow cytometry revealed that spleen cells recovered from mice bearing xenografts ALL-1345 and ALL-1999 consisted of 98±0 and 87±3% human cells respectively. Vertebral bodies and sternums were analyzed for ultrastructural changes affecting leukemic cells by light microscopy and transmission electron microscopy (TEM). Livers and femurs were collected for histological examination.

Pharmacokinetic and pharmacodynamic analyses were performed in mice engrafted with ALL-1345 or -0398 and treatment initiated as described for survival assays. Mice were sacrificed after 0, 2, 4, 7, 24 and 48h following a single administration of RAD001 and after 7 and 14 days of thrice weekly treatments with RAD001. PB was collected by cardiac puncture into EDTA (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). For cell cycle analysis, cells were fixed in 80% ethanol following surface labeling for human CD19 and washed with BD Perm/Wash Solution (BD Pharmingen, San Diego, CA). Fixed cells were resuspended in 100µL of BD Perm/Wash Solution containing 0.25µg of 7-AAD and incubated on ice for 60 min. The cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences) and data fitted using ModFit LT cell cycle analysis software (Verity Software, Topsham, ME).

Immunofluorescence Microscopy

Cells were treated as described and labeled with 10µM Lysosensor Blue DND-167 (Molecular Probes, Eugene, OR) in RPMI containing 10% fetal calf serum for 30 minutes at 37°C. Cells were resuspended in fresh medium prior to examination using an Olympus FV1000 confocal laser scanning microscope system, based on an Olympus IX-81 ZDC microscope, with BP 330-385 nm excitation and BA 420nm emission filters. Images were captured using FV10-ASW 1.7 software and the number of acidic vacuoles in cells quantitated using ImageJ software.

Western blotting

A single cell suspension was obtained from spleens and red cells lysed with 0.155M NH₄Cl, 10mM KHCO₃ and 0.1mM EDTA (pH 7.5). Cell lysates were prepared and equal amounts of protein loaded in each lane of 7.5 or 15% SDS-PAGE gels and transferred onto nitrocellulose
membranes as previously described. Phosphorylated and total proteins were detected sequentially on the same membrane using specific primary antibodies, appropriate secondary antibodies conjugated to HRP and enhanced chemiluminescence (Perkin-Elmer, Boston, MA). Bands were quantitated by densitometry (Molecular Dynamics) using ImageQuant software.

**Electron microscopy**

Vertebral bodies and sternums of mice were fixed in modified Karnovsky’s fixative (2.5% formaldehyde prepared freshly from paraformaldehyde; 2.5% EM grade glutaraldehyde in 0.1M MOPS (3-[N-Morpholino]propanesulphonic acid) buffer, pH 7.4) then decalcified in 0.5M EDTA for 14 days. Tissue blocks were trimmed and post fixed in osmium tetroxide, dehydrated in increasing concentrations of ethanol and embedded in epoxy resin. Semi-thin (500nm) sections were cut on a Reichert ultracut microtome and assessed by light microscopy. Ultrathin (80-90nm) sections were cut and grid stained with 2% ethanolic uranyl acetate, then Reynolds lead citrate. The ultrastructure was examined using a Philips CM-10 transmission electron microscope operated at 80 KV. Images were recorded using Kodak electron microscope film type 4489. Black and white prints were scanned using an HP scanjet flatbed scanner and composite images compiled using Adobe Photoshop (Version 8) software.

**Histology**

Femurs and livers were fixed in 10% buffered formalin. Femurs were decalcified for 14 days in 0.5M EDTA (pH7.4). Decalcified femurs were processed into paraffin. Five micron sections were cut and stained using haematoxylin and eosin as described previously. Slides were examined by transmission light microscopy using an Olympus BX51 microscope fitted with an UPlanFl 20x/0.5 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 in duplicate by HPLC-electrospray tandem mass spectrometry by SydPath Laboratories (St Vincent's Hospital, Sydney). Standard non-compartmental pharmacokinetic parameters were derived including the highest and lowest concentration ($C_{\text{max}}, C_{\text{min}}$), and the area under the concentration-time curve (AUC) over the 48h dosing interval (calculated by trapezoidal summation; AUC48). Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), bilirubin and creatinine were determined by routine laboratory testing at Institute for Clinical Pathology and Medical Research, Westmead Hospital (Westmead, Sydney).

**Statistics**

Comparisons between 2 groups were performed using Student’s t-tests and between multiple groups using ANOVA analysis. Log transformation was made prior to analysis to stabilize for variance. Pairwise comparisons between groups were adjusted for multiple comparisons using Bonferroni’s 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. 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 analysed 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\(^{23}\) and shown that PI-3K/AKT signaling is important for spontaneous and CXCL12-induced ALL cell proliferation \textit{in vitro}.\(^{6}\) 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 two xenografts (1345 and 1196) where significant numbers of human cells were detected within the 3 week period (Figure 1A). At sacrifice on day 21, significantly fewer ALL cells were present in the BM of RAD001 treated mice for all three 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 a dramatic reduction in the degree of ALL infiltration in the livers of RAD001 treated mice bearing xenografts ALL-1345 and 1196 as compared to control treated animals (Figure 1C). Xenograft ALL-0398 did not significantly infiltrate the liver at the time point examined (data not shown). These data demonstrate that RAD001 dramatically inhibits ALL cell engraftment \textit{in vivo}.

Due to the potent effects of RAD001 in an engraftment setting, we investigated the efficacy of RAD001, alone and in combination with vincristine in established ALL. Five established xenografts were studied. Treatment with RAD001 and/or vincristine began after leukemia was engrafted and disseminated in the PB (>5% human ALL cells) and continued for 4 weeks, a time
consistent with induction protocols. The percentage of leukemic cells in the PB of vehicle treated animals continued to rise throughout the treatment period until sacrifice due to disease progression (Figure 2A and data not shown). RAD001 decreased leukemic burden in ALL-1999, 1345 and 2032, stabilized disease in ALL-1196 and had no effect in ALL-0398. Despite low cytotoxicity of vincristine alone, the combination with RAD001 further improved the rate of regression over those observed with RAD001 alone in all samples examined, suggesting interplay between the 2 compounds (Figure 2A and data not shown). 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 (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, p<0.02 using 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 of 24.1 days, range 14.0-39.5, p=ns). 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), which was 8 days greater than the survival induced by RAD001 alone and 25.9 days longer than mice receiving vincristine alone (p<0.02). 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 following the completion of treatment. Mice were electively culled at this time. Leukemia
was detected in only 1 mouse receiving RAD001 as a single agent. However the disease was below the limit of detection in all 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 if 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±0.38µM (mean±SD; n=3) and 2.51±0.69µM (mean±SD; n=3) were achieved 2h after a single orally administered dose in NOD/SCID mice engrafted with ALL1345 and 0398 respectively (Figure 3A). The t₁/₂ was approximately 10h. Repetitive administration (thrice 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 (Supplementary Table 1). Indeed, mice engrafted with ALL-1345 showed elevated ALT levels that were significantly attenuated by RAD001 treatment, reflecting reduced ALL cell infiltration of the liver. Also, non-engrafted 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 following sub-lethal 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 non-target proteins AKT and p38MAPK. For xenografts ALL-1999 and -1345, this was performed on human ALL cells recovered from the spleens of mice after 24h of treatment. In xenograft ALL-0398, the purity and quantity of the human cells recovered from the spleens was insufficient for analysis. The remaining xenografts (ALL-1196, -2032 and -0398) were analyzed following a 24h *in vitro* culture in the presence or absence of 2µM RAD001 based on the peak plasma levels obtained in the mice following oral administration. In the two *in vivo* cases, phosphorylated 4E-BP1 and S6RP were significantly reduced after 24h of RAD001 treatment. Samples analyzed after *in vitro* culture revealed a similar reduction in phosphorylated 4E-BP1 and S6RP after incubation with 2µM 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 observed by Western blotting, flow cytometric assessment of the phosphorylation of 4E-BP1 on ALL-1345 cells obtained from spleens confirmed similar reductions (Figure 3D & E). On day 7, ALL-1345 no longer demonstrated reduced phosphorylation of S6RP, although phosphorylation of 4E-BP1 was still suppressed. Analysis of total proteins revealed that total 4E-BP1, and in ALL-1999, S6RP protein was also reduced in response to RAD001 in the xenografts on day 7, a finding consistent with the mTOR inhibitor CCI-779. In contrast, no consistent effects on the phosphorylation of AKT or p38MAPK were observed (Supplementary Figure 3).
Mechanisms underlying improved survival of mice engrafted with ALL receiving RAD001.

To determine the mechanisms underlying the reduction of leukemia in vivo, we examined cells recovered from animals engrafted with ALL-1345, 1999, and 0398, which had high tumor burden and had been treated as above for 24h or 7 days. One-way ANOVA was used to analyze the effect of treatment separately in each ALL xenograft, since analysis of variance identified a significance of treatment by cell line interaction (p=0.009). Treatment with RAD001 and/or vincristine did not significantly reduce ALL cell number after 24h. However after 7 days of treatment, RAD001 and the RAD001/vincristine combination, reduced the extent of the disease in the PB and spleens of all three xenografts compared to placebo treated animals (Figure 4A, B & C). No significant effect on the number of ALL cells was observed in the BM at any time point. As observed in childhood ALL patients with high initial leukemic burden, day 7 might be too early to detect a reduction of ALL infiltration in the BM.26 This data demonstrates that in the 3 xenografts studied, 7 days treatment with RAD001 alone or in combination with vincristine, resulted in a reduction in the level of ALL in the animals.

Inhibition of mTOR is known to induce cell cycle arrest in tumor cells making it possible that RAD001 could be inhibiting ALL expansion in the mice by inhibiting proliferation.27 Cell cycle analysis performed on cells recovered from the spleens of engrafted mice 24h after drug administration revealed that RAD001 induced a G0/G1 arrest in all 3 xenografts examined (Figure 5A & B). The increase of cells in G0/G1 was accompanied by a loss of cells in S phase. Vincristine induced a significant G2/M arrest in ALL-0398 only. The changes in quantitative DNA staining by flow cytometry could be confirmed in the loss of mitotic figures in femoral sections of RAD001 treated animals (Figure 5C). Similar effects on cell cycle status were apparent in cells
Mechanisms of RAD001 induced cell death

To determine the mechanisms involved in RAD001 mediated cytoreduction of ALL-xenografts, histologic analysis of micro-environmental 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 characterised 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 polyphagic vacuoles (Figure 6E-G and Supplementary Figure 5). These vacuoles contained a mixture of complex structures consistent with mitochondria, rough ER, polyribosomes, lysosomes amongst other organelles in various stages of breakdown. The combination of RAD001 and vincristine (Figure 6D) produced both sets of features, i.e. the presence of both apoptotic cells and cells containing autophagic vacuoles. This ultra-structure data suggests 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 (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 following \textit{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 following \textit{in vitro} culture (Figure 7B).

The induction of autophagy by RAD001 was further supported by the detection of increased proportion of cells containing acidic vacuoles, and an increase in the number of acid vacuoles detected in each cell (p<0.001 in all 3 experiments), in response to RAD001 treatment in the ALL cell lines NALM6 and REH (Figure 7D, E & F). A positive association between the number of acidic vacuoles and the dose of RAD001 applied to the cells was apparent (p<0.001). 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).\textsuperscript{28} Overall, the data demonstrates 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 re-induction protocols can be as high as 40% making recruitment into trials of new agents, that often have similar predicted outcomes and increased potential toxicities, difficult. One strategy to improve access onto phase I trials in pediatric ALL is to identify new drugs likely to enhance leukemia killing by standard multi-drug re-induction 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 pre-clinical 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 nano-molar 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. The clearance of RAD001 was approximately four times faster in ALL engrafted mice as compared to healthy human volunteers but the overall bioavailability was comparable based on dose-adjusted AUC following a single dose. This was surprising since a considerably lower bioavailability of RAD001 has been previously reported in rats. 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 transplant trials but the drug concentrations were well tolerated, with no toxicity observed with respect to several biochemical and hematological assessments, and were comparable to previous in vitro IC₅₀s of survival inhibition and cell death induction of different tumor types to RAD001.²⁷,³⁴

Applying our treatment regime of 3x/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 out 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. Since responses of human ALL xenografts to chemotherapeutic agents, such as vincristine, correlate significantly with patient outcome,³⁶ the enhanced anti-leukemic activity of RAD001 in a multi-agent 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.\textsuperscript{38,39,40} In addition, RAD001 can paradoxically increase AKT phosphorylation in some malignant cells resulting in resistance to mTOR inhibitors.\textsuperscript{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 following 24h of \textit{in vitro} culture with 2µM RAD001 (Supplementary Figure 3). 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.\textsuperscript{8} RAD001-induced inhibition of cell cycle progression \textit{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.\textsuperscript{42,43,44} Surprisingly, we did not detect increased levels of apoptosis in response to RAD001 treatment, although this was evident following exposure to vincristine. This contrasts with previous reports that mTOR inhibition with rapamycin induces apoptosis in ALL cells.\textsuperscript{7,45} The most likely explanation for this discrepancy is the examination of patient ALL cells cultured \textit{in vitro} in these studies. Under these conditions, ALL cells undergo significant spontaneous apoptosis, which mTOR inhibition may have enhanced, while 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, the induction of Beclin-1 and lipidation of LC3 following RAD001 exposure raises the possibility that ALL cells are dying as a result of excessive autophagy. While
mTOR inhibition is well known to induce autophagic cell death in other cell types, \(^4^6\) our current data falls 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, since resistance to current chemotherapies has been linked to defects in their apoptotic machinery. \(^4^7\) Therefore, targeting the non-apoptotic pathway may yield better clinical outcomes for patients undergoing cytotoxic cancer therapies. \(^4^8 \ 4^9\)

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

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References

Table 1. Clinical information

<table>
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<tr>
<th>Patient ID</th>
<th>Sex/Age</th>
<th>Source</th>
<th>% Blasts</th>
<th>Stage</th>
<th>Immuno-phenotype</th>
<th>Cytogenetics</th>
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<tr>
<td>1345</td>
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<td>BM</td>
<td>95</td>
<td>D</td>
<td>CD10+</td>
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<td>F/14</td>
<td>BM</td>
<td>94</td>
<td>D</td>
<td>CD10+CD34-</td>
<td>46 XX</td>
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<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>
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<td>BM</td>
<td>-</td>
<td>D</td>
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<td>46 XX -19,del(19), t(1;19)(q23p13)</td>
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<td>D</td>
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<td>46 XY add (9)(p24), del(9)(p21), del(13)(q11q21), der(19) t(1;19)(q23;p13)</td>
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Abbreviations: -, not available; R, relapse; D, diagnosis.
### Table 2 Statistical Analysis of Survival in Response to Individual Treatments and Treatment Combinations

<table>
<thead>
<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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<tbody>
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<td>0.325</td>
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<tr>
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<td>0.001</td>
<td>0.068</td>
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<tr>
<td>0398</td>
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<td>0.002</td>
<td>0.004</td>
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<td>1999</td>
<td>0.001</td>
<td>0.393</td>
<td>0.317</td>
<td>0.001</td>
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</table>

The significance values shown were determined using a Mantel-Cox Log Rank Test.
Figure legends

Figure 1. RAD001 inhibits ALL cell engraftment in NOD/SCID mice.

NOD/SCID mice, injected with 3-5x10^6 cells from indicated xenografts, were treated with RAD001 starting 1 day after the injection of cells. (A) The percentage of human CD19^+ cells (mean±SD) in the PB at weekly bleeds of animals for each group is shown. (B) The total number of human CD19^+ cells in the PB, BM and spleen for each animal at sacrifice as determined by flow cytometry. The bar indicates the mean of the group. (C) Infiltration of livers with human ALL. H&E staining was used. Arrows indicate ALL cells. Original magnification 200x. Larger images available in Supplementary Figure 1.

Figure 2. RAD001 improves survival of NOD/SCID mice engrafted with ALL.

(A) Percentage of human ALL cells in the blood of mice engrafted with the indicated samples over time. Mice were treated with vehicle, vincristine (Vin), RAD001 or RAD001 and vincristine (RAD+Vin). The time indicated is from the commencement of treatment and the arrow indicates the completion of treatment. Mean±SD of surviving animals is shown. (B) Kaplan-Meier plots of the survival of mice engrafted with xenografts from 5 patients with childhood ALL. The time indicated is from the commencement of treatment and the arrow indicates the completion of treatment. (C) Femur (a-c) or vertebral body H&E stained sections (d) from mice engrafted with ALL-1999 10 weeks following the completion of treatment with RAD001 (a, c and d) or RAD001 + vincristine (b). Sections a and b show normal hematopoiesis while c and d are from the single animal showing disease relapse at the time of sacrifice. Regions of leukemic infiltration are indicated by arrows. Original magnification 200x. Larger images available in Supplementary Figure 2. (D) Murine white blood cell counts (mean±SD) of surviving NOD/SCID mice engrafted with ALL-1999 during and following the completion of treatment. Time is from the
initiation of treatment and the end of treatment indicated by the arrow. The white cell counts were very low at the start of treatment because the mice were still recovering from the sublethal radiation administered to facilitate ALL engraftment. The shaded area represents the normal range, and the line the mean value for white blood cell counts observed in our NOD/SCID mouse colony. Note that the vehicle and vincristine treated mice had all died of disease by day 20.

Figure 3. RAD001 concentrations and inhibition of phosphorylation of S6RP and 4E-BP1 after drug administration.

Mice engrafted with ALL-1345 or ALL-0398 cells were treated with RAD001 or vehicle and blood harvested after the indicated time points of continuing treatment and evaluated for RAD001 concentrations. (A) RAD001 (mean±SD, n=3) concentration-time profiles in mice engrafted with ALL-1345 (solid line) and ALL-0398 (dotted line) up to 48h after administration of a single dose of RAD001. (B) RAD001 (mean±SD, of duplicate determinations from 3 experiments) trough concentrations in mice treated for up to 14 days with RAD001. (C) Ex vivo analysis of the phosphorylation of S6RP and 4E-BP1 in ALL cells from spleens recovered from mice engrafted with ALL-1345 or ALL-1999, 1 or 7 days into the treatment protocol (left and centre panels). Cells from xenografts ALL-1196, -2032 and -0398 recovered from untreated mice were treated in vitro for 24h with or without 2µM RAD001 (right panel). The numbers below the blots indicate the intensity of phosphorylation specific bands for 4E-BP1 and S6RP relative to total 4E-BP1 and S6RP. (D) Representative plots showing the levels of phosphorylated 4E-BP1 assessed by flow cytometry in ALL cells recovered from spleens of mice engrafted with ALL-1345. (E) Time course of the reduction of phosphorylated 4E-BP1 following administration of RAD001 measured by flow cytometry in ALL cells recovered from mice engrafted with ALL-1345.
Figure 4. Reduction of ALL cells in the blood and spleens of mice engrafted with human ALL and treated with RAD001, vincristine or the combination of both for 7 days.

Mice engrafted with ALL-1999, -1345 or 0398 were treated with vehicle, RAD001, vincristine or RAD001 + vincristine for 7 days. (A) Representative flow cytometric analysis showing the percentage of human ALL cells in the PB of mice engrafted with ALL-1345. The percentage human ALL cells is shown. (B) Photographs of spleens recovered from mice engrafted with ALL-1345 following the indicated days of treatment with RAD001 or vehicle. (C) The absolute number of human ALL cells in the PB and spleens of mice treated as indicated are shown. Bars indicated the mean±SD of groups of between 2 and 4 mice. *p<0.05 compared to vehicle treated animals.

Figure 5. RAD001 arrests ALL cells in G0/G1 phase of the cell cycle.

Mice were treated with vehicle, RAD001, vincristine or RAD001 + vincristine for 1 or 7 days. (A) ALL cells isolated from spleens were labeled with 7-AAD and analyzed by flow cytometry. Cell-cycle profiles of ALL-1345 are shown as representative examples. (B) The percentage of cells in each stage of the cell cycle after 1 day of treatment as indicated is shown. The mean±SD of the percentage of cells in each cell phase from replicate mice is shown (n≥4). (C) H&E stained sections from femurs of mice engrafted with ALL-0398 are shown with examples of mitotic (arrow heads) and apoptotic (thin arrows) figures. Original magnification 200x. Higher power examples of mitotic and apoptotic figures produced by a further 20x digital enlargement are shown below. Larger images available in Supplementary Figure 3. (D) Effect of RAD001 and/or vincristine treatment on the phosphorylation status of Rb and the levels of cdk5 and 6 after 1
day of treatment of NOD/SCID mice engrafted with ALL-1345 or ALL-1999. The numbers below the plots indicate the ratio of cdk4 and 6 to \( \beta \)-actin.

**Figure 6. RAD001 promotes autophagy and induces apoptosis.**

Ultrastructural analysis by means of TEM of ultrathin BM sections in vehicle (A), vincristine (B), RAD001 (C) and vincristine + RAD001 (D) treated mice. Apoptotic cells (black arrow heads), extravasated erythrocytes (black asterix), filipodia (white arrows), phagolysosomes (white arrow heads) are indicated. Double walled polyphagic vacuoles in the cytoplasm of ALL cells from ALL-1345 (E) and ALL-1999 (F and G) recovered from mice treated with RAD001. Bars indicate 1\( \mu \). Larger images are available in Supplementary Figure 4.

**Figure 7. Mechanisms of cell death induced by RAD001 and vincristine alone and in combination.**

(A) Mice engrafted with ALL-1345 or ALL-1999 cells were treated with vehicle, vincristine, RAD001 or RAD001 + vincristine for 24h and lysates prepared from recovered spleen cells. Lysates were subjected to Western blotting with anti-PARP, or anti–\( \beta \)-actin antibodies. (B) NALM6 cells were cultured with 2\( \mu \)M RAD001, 1nM vincristine or the combination of both for 24h and cell lysates prepared. Lysates were subjected to Western blotting with anti-PARP, anti-caspase 3 or anti–\( \beta \)-actin antibodies. (C) NALM6 cells were treated with the indicated doses of RAD001 for 24h and cell lysates analysed for Beclin-1 and LC3 expression by Western blotting. \( \beta \)-actin is shown as a loading control for Beclin-1 and the ratio of LC3-II/LC3-I is indicated below the blots for LC-3. NALM6 or REH cells were treated with vehicle alone (Control), or 2 or 16\( \mu \)M RAD001 for 24h. Cells were stained for acidic vacuoles using Lysosensor Blue. Representative fields of view are shown in (D). The proportion of cells containing acidic
vacuoles (AV) (E) and the number of AV in each cell (F) are shown. Quantitation was obtained from the analysis of over 120 cells for each condition in each of two separate experiments. The mean±SD of the independent experiments is shown for NALM6 cells.
Figure 1
Figure 2
A

Blood Concentrations of RAD001

<table>
<thead>
<tr>
<th></th>
<th>C_{min} (μM) (ALL-1345)</th>
<th>C_{min} (μM) (ALL-0398)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 days</td>
<td>0.099 ± 0.037</td>
<td>0.090 ± 0.021</td>
</tr>
<tr>
<td>7 days</td>
<td>0.089 ± 0.019</td>
<td>0.065 ± 0.012</td>
</tr>
<tr>
<td>14 days</td>
<td>0.106 ± 0.009</td>
<td>0.090 ± 0.017</td>
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Indicated concentrations are the mean ± SD of duplicate determinations.

C

[Images of western blots for ALL-1345 on Day 1 and Day 7, ALL-1999 on Day 1 and Day 7, and in vitro culture with RAD001, pS6RP, S6RP, β-actin, p4E-BP1, and 4E-BP1 on Day 1 and Day 7]

D

[Graph showing flow cytometry data for p4E-BP1 FITC with counts on the y-axis and p4E-BP1 FITC on the x-axis, comparing Isotype Control, Vehicle, and RAD001]

E

[Graph showing p4E-BP1 Relative (MFI) over time (h) with error bars]

Figure 3
Figure 4
Figure 6
Figure 7

A: Western blots showing the expression of PARP and Cleaved PARP in ALL-1345 and ALL-1999 cell lines treated with RAD001 and Vincristine.

B: Western blots showing the expression of Cleaved Caspase 3 in NALM6 cell lines treated with RAD001.

C: Western blots showing the expression of Beclin 1 and Cleaved PARP in NALM6 cell lines treated with increasing concentrations of RAD001.

D: Fluorescence images of NALM6 and REH cell lines treated with control, 2μM RAD001, and 16μM RAD001.

E: Bar graph showing the proportion of cells with AVs in NALM6 cell lines treated with increasing concentrations of RAD001. * indicates significant difference compared to control.

F: Bar graph showing the proportion of cells with AVs in NALM6 cell lines treated with 2μM and 16μM RAD001.
Potentiating effects of RAD001 (Everolimus) on vincristine therapy in childhood acute lymphoblastic leukemia

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