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Journal

Clinical Cancer Research, 21(12)

ISSN

1078-0432

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Publication Date

2015-06-15

DOI

10.1158/1078-0432.ccr-14-2888

Peer reviewed

HHS Public Access

Author manuscript

Clin Cancer Res. Author manuscript; available in PMC 2016 June 15.

Published in final edited form as:

Clin Cancer Res. 2015 June 15; 21(12): 2704–2714. doi:10.1158/1078-0432.CCR-14-2888.

A Phase I/II Study of the mTOR Inhibitor Everolimus in Combination with HyperCVAD Chemotherapy in Patients with Relapsed / Refractory Acute Lymphoblastic Leukemia

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Abstract

Purpose—Previous studies suggest a potential therapeutic role for mTOR inhibition in lymphoid malignancies. This single-center phase I/II study was designed to test the safety and efficacy of the mTOR inhibitor everolimus in combination with HyperCVAD chemotherapy in relapsed/ refractory acute lymphoblastic leukemia.

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AUTHOR CONTRIBUTIONS

MK, DT and ND developed the concept for this article and wrote the first draft. All authors reviewed and commented on the drafts and approved the final manuscript.

CONFLICT OF INTEREST

This study was supported by a grant from Novartis. ND, HK, FR, JC, and MK have received research funding from Novartis. JC, EJ, and HK have received honoraria from Novartis.

Supplemental information is attached.

Experimental Design—Twenty-four patients were treated; 15 received everolimus 5 mg/day and 9 received 10 mg/day with HyperCVAD.

Results—The median age of patients was 25 years (range, 11-64) and median number of prior treatments was 2 (range, 1-7). Grade 3 mucositis was the dose-limiting toxicity and the maximum tolerated everolimus dose was 5 mg/day. Responses included complete remission (CR) in 6 patients (25%), CR without platelet recovery (CRp) in 1 (4%), and CR without recovery of counts (CRi) in 1 (4%), for an overall response rate of 33%. Additionally, partial response (PR) was noted in 2 patients (8%). Seven of 11 patients treated in first salvage achieved CR/CRp (64%). The median OS was 29 weeks for patients in first salvage versus 15 weeks for patients in second salvage and beyond ($P \leq 0.001$). A response was noted in 5 of 10 (50%) heavily pretreated T-ALL patients (median of 4 prior salvage regimens). Everolimus significantly inhibited phosphorylation of S6RP, but this did not correlate with response. No significant decreases in p4EBP1 and pAkt levels were noted. Responders had higher everolimus dose-adjusted area under the curve ($P=0.025$) and lower clearance ($P=0.025$) than non-responders.

Conclusions—The combination of HyperCVAD and everolimus is well tolerated and moderately effective in relapsed ALL, specifically T-ALL.

Keywords

acute lymphoblastic leukemia; everolimus; HyperCVAD

INTRODUCTION

The prognosis of patients with relapsed or refractory ALL is poor (1). The median survival after salvage chemotherapy is less than 6 months for patients who are not able to undergo allogeneic stem cell transplantation (ASCT). Novel therapeutic strategies are needed.

The serine/threonine kinase protein Akt (also known as protein kinase B), a central downstream phosphatidylinositide 3-kinase (PI3K) target, is activated by phosphorylation (2-5). Activation of the PI3K/Akt–protein kinase B survival pathway promotes cell growth and metabolism (6, 7). Mammalian target of rapamycin (mTOR) is a downstream target of Akt (4, 8-10). Suppression of the PI3K/Akt prosurvival pathway explains the antileukemic activity demonstrated by mTOR inhibitors in human cell lines and ALL mouse models (11-16). In addition to single-agent activity, mTOR inhibitors may overcome drug resistance when administered in combination with cytotoxic chemotherapeutic agents, including vincristine, doxorubicin, and methotrexate (17-19).

mTOR exists in 2 complexes: mTORC1, which also contains raptor and PRAS40, and mTORC2, which also contains rictor and Sin1. These complexes have different spectra of substrates (20, 21). mTORC1 phosphorylates eukaryotic initiation factor 4E-binding protein 1 (4EBP1) and p70-kDa S6 ribosomal protein kinase (p70S6K), promoting cap-dependent mRNA translation, ribosome biogenesis, and polysome assembly (5, 22). p70S6K also acts in a feedback pathway to attenuate PI3K/Akt activation. The substrates of mTORC2 include Akt and several other members of the AGC kinase superfamily. Rapamycin analogs (termed rapalogs) such as everolimus and temsirolimus are allosteric noncompetitive inhibitors of

mTORC1 that do not acutely inhibit mTORC2 in most cells. The loss of the feedback inhibitory circuit mediated by p70S6K induced by these agents produces increased Akt phosphorylation on both T308 and S473. We reported previously that prolonged exposure to temsirolimus not only inhibited mTORC1 but also, surprisingly, blocked Akt activation via inhibition of mTORC2 formation (22). This inhibition of Akt signaling resulted in restoration of the activity of forkhead transcription factors (FKHR). FKHR mediates inhibition of cell cycle progression and transformation by transcriptional repression of D-type cyclins (23, 24). Our recent *in vitro* studies demonstrated that the culture of ALL cells under conditions mimicking a hypoxic bone marrow microenvironment promotes acquisition of a glycolytic phenotype, facilitating further glucose uptake and induction of the glycolytic enzyme HK-2 and of the anti-apoptotic protein Mcl-1, which may confer chemoresistance to standard chemotherapeutic agents. These effects were reversed by the blockade of mTOR signaling with everolimus (25). These pre-clinical findings prompted us to evaluate the combined efficacy of chemotherapy and mTOR inhibitors in ALL.

The results of a phase I study conducted at The University of Texas MD Anderson Cancer Center (UT/MDACC) to determine the safety and efficacy of everolimus in patients with relapsed or refractory hematologic malignancies suggested that everolimus is well tolerated at a dose of 10 mg daily and may have activity in patients with hematologic malignancies (26). The HyperCVAD regimen is an established chemotherapy program with clinical efficacy in *de novo* and relapsed/refractory ALL (27, 28). Because of 1) the encouraging single-agent antileukemic activity of everolimus, 2) its potential to reverse resistance to anthracyclines, methotrexate, and vincristine, and 3) its ability to enhance steroid sensitivity, we investigated the combination of everolimus with HyperCVAD in relapsed/refractory ALL. The study included pharmacokinetic and biomarker analysis to evaluate the therapeutic and molecular effects of this combination regimen.

MATERIAL AND METHODS

Patients

Patients aged 10 years or older with refractory or relapsed ALL were eligible for enrollment. Inclusion criteria included adequate organ function, with creatinine ≤1.5 × upper limit of normal (ULN), bilirubin ≤1.5 × ULN, alanine transaminase (ALT) and aspartate transaminase (AST) ≤2.5 × ULN; fasting serum cholesterol ≤300 mg/dL (or ≤7.75 mmol/L); fasting triglycerides ≤2.5 × ULN; and a performance status (Eastern Cooperative Oncology Group criteria) of ≤3. Exclusion criteria included active and uncontrolled disease or infection, symptomatic New York Heart Association class III or IV congestive heart failure or symptomatic pulmonary disease, prior treatment with an mTOR inhibitor, a fungal infection requiring azole antifungal therapy, and infection with human immunodeficiency virus. Pregnant and lactating mothers were not eligible for participation. Concurrent therapy for central nervous system (CNS) prophylaxis or for CNS relapse was permitted. All patients signed an informed consent form approved by the Institutional Review Board of UT/MDACC (clinicaltrials.gov identifier: NCT00968253)

Study design and objectives

This open-label, single-institution study recruited patients between 4/7/2010 and 2/9/2014. A total of 24 patients were enrolled. The latest follow-up date was 4/25/2014. The primary trial endpoint was to establish the safety and maximum tolerated dose (MTD) of everolimus in combination with HyperCVAD, as well as the efficacy (complete and overall response rates) of the combination. Secondary endpoints included analysis of the effects of everolimus on mTOR/Akt signaling pathways in leukemic blasts, and the overall survival (OS), event-free survival (EFS), and toxicities with this combination.

Treatment schema

Patients enrolled on this trial received HyperCVAD, a dose-intensive chemotherapy regimen used at our institution for adult ALL since 1992 (27, 28) (see supplemental material A for details of HyperCVAD). All patients received continuous therapy with oral everolimus, starting on Day 0 of Cycle 1, at a dose of either 5 mg/day or 10 mg/day. The central nervous system (CNS) prophylaxis comprised alternating intrathecal therapy with methotrexate and cytarabine on Days 2 and 7 of each cycle of HyperCVAD for a total of 6 or 8 doses, depending on risk for CNS relapse (29). Patients with active CNS leukemia at presentation received additional intrathecal chemotherapy with or without therapeutic cranial irradiation, as per institutional standards of care.

Pretreatment evaluations included complete history and physical examination, complete blood count with differential, comprehensive biochemistry panel, pregnancy test and counseling, and bone marrow aspiration for histologic, multiparametric flow-cytometric, and cytogenetic analyses. Multiparametric flow-cytometry and cytogenetics were performed at our institution by methods detailed previously (30).

Response definitions

CR was defined as the presence of 5% or less blasts in the bone marrow, with a granulocyte count $\geq 1.0 \times 10^9$/L, a platelet count $\geq 100 \times 10^9$/L, and no extramedullary disease. CRp was defined as CR with platelet count $<100 \times 10^9$/L. CRi was characterized as having all of the above criteria for CR but with platelet count $<100 \times 10^9$/L and/or absolute neutrophil count $<1.0 \times 10^9$/L. PR was defined as a bone marrow with >5% and <25% lymphoblasts with a granulocyte count $\geq 1.0 \times 10^9$/L and a platelet count $\geq 100 \times 10^9$/L. Relapse was defined by the recurrence of more than 5% lymphoblasts in the bone marrow aspirate or by the presence of extramedullary disease after achieving CR. OS was measured from the date of randomization to death from any cause. EFS was defined as the time from randomization to the date of relapse or death from any cause, whichever occurred first. Toxicity evaluation was based on the National Cancer Institute Common Toxicity Criteria (CTCAE) Version 3.0.

Toxicity assessment

In the phase I portion of the study, the safety of the 2 dosing regimens was assessed. A dose-limiting toxic effect (DLT) was defined as a clinically significant adverse event or abnormal laboratory value directly attributable to everolimus and assessed as unrelated to disease progression, intercurrent illness, or concomitant medications, occurring during the first or

second cycle of therapy, that met any of the following criteria: Common Terminology Criteria for Adverse Events (CTCAE version 3.0) grade 3 increased AST or ALT for ≥ 7 days, CTCAE grade 4 increased AST or ALT of any duration, or any other clinically significant CTCAE grade 3 or 4 toxic effect. Electrolyte abnormalities (changes in glucose, chemistries, liver enzymes, pancreatic enzymes) correctable by optimal therapy and without clinical impact were not considered DLTs.

A 3+3 design was used for dose escalation in the phase I portion of the study. The MTD was the highest dose level at which fewer than 2 of 6 patients developed a DLT in the first 2 cycles of therapy. Once the MTD was established, thereby defining a safe schedule, the study opened broadly for phase II at this dose.

In the phase II portion of the study, patients were not evaluated for DLT but were monitored continuously for toxicity. We denoted the probability of toxicity by θ_E, where toxicity was defined as any clinically significant CTCAE (version 3.0) grade 4 non-hematological toxic effects or death attributable to the study drug (everolimus). We assumed $\theta_E \sim$ beta (0.3, 1.7). The stopping rule was given by the following probability statement: Pr ($\theta_E > 0.15 \mid$ data) >0.90. That is, we would stop the trial if, at any time during the study, we determined that there was more than 90% chance that the toxicity rate would be greater than 15%.

Correlative studies

Peripheral blood mononuclear cells were isolated by Ficoll-Hypaque density gradient centrifugation (Sigma-Aldrich, St. Louis, MO), before (Cycle 1 Day 0) and 24 hours after (Cycle 1 Day 1) the first dose of everolimus during the first cycle of therapy. The methodology of western blot and RPPA are detailed in supplemental material B. The following biomarkers were evaluated by western blot: pAkt (Ser473), pS6-ribosomal protein (S6RP), p4EBP1 (Thr37/46), and total protein levels of Akt, S6RP, and 4EBP1.

Reverse phase protein array

Proteins were isolated from peripheral blood samples collected before (Cycle 1 Day 0) and 24 hours (Cycle 1 Day 1) after the first dose of everolimus and subjected to lysis as described for western blots. The method for analysis of correlation between treatment and change in mTOR target proteins by RPPA is detailed in supplemental material C.

Using these tools, we examined the effects on (1) the target itself (mTOR pS2448, p70S6K-pT389, S6RP pS235-S236, S6RP pS240-S244, 4EBP1 pS65, 4EBP1 pT37-T46), and (2) Akt (Akt pS473, Akt pT308).

Microarray RNA analysis

RNA was extracted from peripheral blood samples collected before (Cycle 1 Day 0) and 24 hours (Cycle 1 Day 1) after the first dose of everolimus using TRIzol reagent (Life Technologies, Carlsband, CA). RNA was precipitated with isopropyl alcohol and purified with 70% ethanol. RNA was reconstituted in RNase/DNase-free water; its integrity was determined by the Agilent Bioanalyzer 2100 (Agilent, Santa Clara, CA), and its

concentration by UV nanophotometer (Implen, München, Germany). RNA with an integrity number greater than 7 was deemed satisfactory for amplification.

RNA was amplified and biotinylated by using a TargetAmp-Nano labelling kit purchased from Epicenter Biosciences (Madison, WI). Briefly, RNA was reverse-transcribed by using SuperScript III (Life Technologies) and random Oligo (dT) primers. Biotinylated mRNA was then transcribed from cDNA by using T7 RNA polymerase and biotin-UTP and purified with the RNeasy MinElute Cleanup kit (Qiagen, Venlo, Limburg, Netherlands). The integrity and concentration of amplified RNA were determined as described above. Biotinylated RNA was hybridized to a HumanHT-12v4 Bead expression chip (Illumina, San Diego, CA) by the Westmead Millennium Institute Genomics Facility according to the manufacturer's instructions and scanned by using an Illumina BeadArray Reader.

The array data were imported into the Genome Studio software (Illumina) and gene lists generated. Genes that were not significantly detected across all samples in the dataset were excluded from analysis. Gene set enrichment analysis (GSEA) was carried out by using the GSEA software from Broad institute (Massachusetts Institute of Technology, Cambridge, MA)(31). GSEA is a computational method made available through the Broad Institute that determines whether an a priori defined set of genes shows statistically significant, concordant differences between two biological states. Definitions and functional information on the specific gene signatures used in this analysis are included in supplemental material D.

Pharmacokinetic analysis

Everolimus pharmacokinetic analysis was performed on whole blood samples collected in EDTA-containing tubes 1, 2, 5, 8, and 24 hours after administration of the drug on Days 1 and 15 of Cycle 1 (Cycle 1 Day 1 and Cycle 1 Day 15) and Cycle 2 (Cycle 2 Day 1 and Cycle 2 Day 15). All the samples were stored at 4°C until analysis.

The concentration of everolimus (LC Labs, Woburn, MA) in whole blood was measured by using a validated high performance liquid chromatography (HPLC)-MS/MS method, with sirolimus (LC Labs) as an internal standard. For details of the assay, please refer to supplemental material E. Everolimus pharmacokinetic parameters, including maximum observed concentration (C_{max}), time to reach maximum concentration (T_{max}), terminal half-life ($t_{1/2}$), elimination rate (K_e), clearance (CL), area under the concentration versus time curve from time 0 to infinity ($AUC_{0-\infty}$), and volume of distribution (V), were estimated by non-compartmental pharmacokinetic methods by using Phoenix WinNonlin software (version 6.3, Pharsight Corporation, Mountain View, CA).

Statistical analysis

All *P* values were 2-sided except where noted. $P < 0.05$ was considered significant. Survival distributions were estimated by using the Kaplan-Meier method and compared by using the log-rank test (32). Correlations between everolimus pharmacokinetics and biomarker expression or treatment response were determined by using the Spearman rank test and the Kruskal-Wallis test, respectively. The association between biomarkers and treatment

response was determined by using the Kruskal-Wallis test. Statistical analyses were carried out by using IBM SPSS Statistics 21 software for Windows (SPSS Inc., Chicago, IL).

RESULTS

Patient characteristics

The first 3 patients in the phase I portion of the study were treated at dose level 0, receiving everolimus 5 mg/day in combination with HyperCVAD. There was no documented first or second cycle DLT. The next 3 patients were treated at dose level +1 with everolimus 10 mg/day in combination with HyperCVAD. One of these 3 patients experienced DLT in the form of grade 3 mucositis. Therefore, 3 additional patients were enrolled to receive everolimus 10 mg/day in accordance with the phase I 3+3 design. The protocol specified a DLT monitoring plan to include the monitoring of DLTs during the first and second cycle of HyperCVAD and everolimus therapy, implying that patients must complete 2 cycles to be evaluable for DLTs. Of the 6 patients who received everolimus 10 mg/day, 3 were taken off-protocol because of progressive disease after only 1 cycle of therapy, rendering them non evaluable for DLT, and requiring additional 3 patients to be treated. Thus, a total of 9 patients received everolimus 10 mg/day in combination with HyperCVAD in the phase I portion of the study, and 6 patients completed at least 2 cycles and were evaluable for DLTs. Two of the evaluable 6 patients experienced a DLT (both had grade 3 mucositis) with everolimus 10 mg/day in combination with HyperCVAD. The MTD of everolimus in combination with HyperCVAD was determined to be 5 mg/day, and the study was opened broadly for phase II at this dose. An additional 12 patients were enrolled in the phase II expansion of the study. Overall, a total of 24 patients were enrolled on the study.

The median age of patients was 25 years (range, 11-64). Their clinical characteristics are summarized in Table 1. Thirteen patients had pre-B-ALL, 10 had T-ALL, and 1 had a mixed phenotype acute leukemia. Cytogenetic were diploid in 12, unfavorable (including -7, +8, or 11q23 rearrangement) in 6, miscellaneous in 6, and yielded insufficient metaphases in 1. The median number of prior therapies was 2 (range, 1- 7); including 11 patients in the first salvage. The median duration of the first remission was 11 months (range, 0- 45 months). All patients were negative for the Philadelphia chromosome.

Toxicities

Toxicities are summarized in Table 2. Mucositis was the phase I dose-limiting toxicity. Two of six patients treated with everolimus 10mg/day experienced grade 3/4 mucositis within the DLT evaluation period. However, none of the 15 patients treated with everolimus 5 mg/day in combination with HyperCVAD experienced grade 3/4 mucositis while on therapy suggesting that everolimus 5 mg/day defined a safe dose in combination with HCVAD. Myelosuppression and neutropenia typical of conventional chemotherapy induction regimens occurred in all 24 patients. The incidence of grade 3/4 infections, including pulmonary infections was similar to HyperCVAD alone (90% vs. 93%) (27). No documented cases of pneumonitis seen in solid tumor trials were observed. Hyperglycemia is on-target expected toxicity of mTOR inhibitors. The incidence of grade 3/4 hyperglycemia was 50%. The hyperglycemia was not clinically significant, reversible by

optimal therapy and no hyperglycemia-related complications or mortality was documented. Grade 3/4 transaminitis were seen but could only rarely be ascribed to therapy with hyperCVAD and everolimus therapy because of the multitude of other chemotherapy drugs and antifungal agents co-administered concomitantly. The grade 3/4 transaminitis were reversible in all cases and did not require hospitalization in any of the patients.

Patient outcomes and survival

The median number of HyperCVAD and everolimus cycles administered was 2 (range, 1-4). Median duration of follow up monitoring was 31 months (range, 15-41).

The overall response rate was 33%, including 6 CR's, 1 CRp, and 1 CRi. Seven of the 11 patients in first salvage achieved CR/CRp, 1 of 3 in second salvage achieved CRi, and no responses were seen in the 10 patients beyond the second salvage. Additionally, PR was seen in 2 patients who had received 3 and 4 prior salvage regimens, respectively. Four of the patients who achieved CR underwent ASCT and came off study; 2 remain alive. Figure 1 shows the OS without and with censored at the time of ASCT, respectively. We compared response rates in recent first salvage patients treated with HyperCVAD alone (n=45) to those treated with everolimus-HyperCVAD (n=11) at our institution. A response (CR+PR) was obtained in 7 of 11 (64%) patients treated with everolimus-HyperCVAD as compared to 24 of 45 (53%) treated with HyperCVAD alone in first salvage (P=0.54).

The median OS and median EFS were 29 weeks and 22 weeks for patients in first salvage, and 15 weeks and 7 weeks, respectively, for patients in second salvage and beyond, respectively ($P \leq 0.001$ and P=0.01) (Supplemental Figure 2).

Ten T-ALL patients, 13 B-ALL patients, and one mixed phenotype patient were treated on protocol. The T-ALL patients were heavily pretreated with a median of 4 (range, 1-7) prior therapies as compared to B-ALL patients who had received a median of 1 (range, 1-4) prior therapy. In spite of this difference, a response was noted in 5 of 10 (50%) T-ALL patients (including 2 CR, 1 CRp, 1 CRi, and 1 PR) as compared to 5 of 13 (39%) B-ALL patients (all CR). The median OS was similar between the T-ALL (23 weeks) and B-ALL (23 weeks) patients.

The major reasons for discontinuation of the therapy included no response (n=12), transplant (n=4), relapse (n=3), disease progression (n=2), death on study (n=2), and one patient discontinued intensive chemotherapy and switched to maintenance after 2 courses due to severe infections.

Cytogenetic and minimal residual disease at response

Among the 8 patients who achieved a CR/CRp/CRi, cytogenetic studies showed: diploid karyotype in 5 patients, unfavorable karyotype in 2 patients (11q23 and +8), and miscellaneous (t 2:9) in 1 patient. The 3 patients with abnormal cytogenetics at diagnosis achieved a complete cytogenetic response.

Multiparametric flow cytometry for minimal residual disease (MRD) showed no detectable MRD at response in 5 of the 8 patients who achieved CR/CRp/CRi. Of the 5 patients with

no detectable MRD at response, 3 have experienced relapse and died, and 2 remain alive in CR, including 1 patient who underwent ASCT. The 3 patients who achieved CR but had residual MRD by flow cytometry at response eventually underwent ASCT: 1 patient had ALL relapse and died; 1 patient died from complications of ASCT; and the third patient eventually achieved MRD negative status and underwent ASCT with no detectable MRD, he is still alive and in CR.

Modulation of mTOR signaling by everolimus in ALL blasts

Pharmacodynamic studies in peripheral blood samples collected before (Cycle 1 Day 0) and 24 hours (Cycle 1 Day 1) after the first dose of everolimus included western blotting in 8 patient samples and RPPA in 10 samples.

Western blot showed that phosphorylation of the downstream mTOR marker S6RP was reduced by everolimus in 5 of 8 (62%) tested samples, at both the 5 mg and the 10 mg dose levels (Figure 2).

RPPA incorporated a comprehensive proteomic profile of 10 proteins and their phosphorylated forms. Of the 10 patients for whom RPPA was performed, 3 received everolimus at 5 mg/day and 7 at 10 mg/day. Inhibition of mTOR signaling (S6RP) was observed in 7 of the 10 (70%) tested patient samples. Western blot and RPPA changes of S6RP phosphorylation were concordant in 7 of the 8 patients whose samples were tested by Western blot (the exception was patient #7, who had no difference by RPPA and expected change by Western blot analysis). The degree of inhibition of any of the proteins or their phosphorylated form, including Akt targets cyclin D1, FOXO3A, PRAS40 and Mcl-1, did not differ with the 5 mg/day and the10 mg/day doses of everolimus.

In all 10 patients, everolimus significantly (1-sided paired *t*-test) inhibited phosphorylation of S6RP on both pS235-S236 and pS240-S244 sites (P=0.007 and P=0.01, respectively). When adjusted for multiple comparisons, the P-value was 0.07 (Figure 2 and Supplemental Table 1). No statistically significant differences in phosphorylation of 4EBP1, Akt, or other proteins were found (Supplemental Table 1). We next used a binomial test to analyze whether changes in phosphorylation of S6RP, 4EBP1 (both T37/46 and T70) or Akt occurred in a significant number of patients. Supplemental Table 2 shows the results of this analysis with calls of changes (“expected”, “indifferent,” or “opposite”) for each patient. This analysis confirmed significantly common decrease in phosphorylation of S6RP on pS235-S236 and pS240-S244, but not of other proteins tested.

We investigated the association between biomarkers and outcome. Lower levels of p4EBP1 at T37 and T46 at baseline Cycle 1 Day 0 (P=0.047) and Cycle 1 Day 1 (P=0.058, not shown) were associated with a greater propensity to achieve response (P=0.0253, Supplemental figure 1). However, there was no correlation between the baseline levels of any of the other mTOR pathway biomarkers (including pS6RP, pPRAS40, pAKT, pFOXO3p) and treatment response. Similarly, there was no correlation between the degree of inhibition of any of the mTOR pathway biomarkers and treatment response.

Of interest, 10 patients had serial analysis for PTEN levels by RPPA. Five of the analyzed patients demonstrated elevation of PTEN levels, including 3 T-ALL patients and 2 B-ALL patients. The 3 T-ALL patients were heavily pretreated and had received 4, 3, and 2 prior salvage therapies, respectively. Interestingly, all 3 of the T-ALL patients with a documented increase in PTEN achieved a response (partial remission in two and CRi in one). The two B-ALL patients with increased PTEN did not achieve response.

Microarray analysis for gene enrichment in patients on everolimus

The results of GSEA analysis of microarray data (*GSE60540*) from samples collected from 5 patients before (Cycle 1 Day 0) and 24 h after (Cycle 1 Day 1) the first dose of everolimus and the top 4 gene sets and alignment with the consensus miR-21 signature are illustrated in Figure 3. Expression of mTOR-sensitive genes induced by overexpression of Akt was significantly reduced in response to everolimus treatment, and the inflammation-associated genes, including those induced by the tumor necrosis factor (*TNF*), epidermal growth factor (*EGF*), and interleukin-6 (*IL-6*) genes were similarly reduced. In contrast, the target genes of miR-21 were increased following exposure to everolimus. Although not listed in these miR-21 gene signatures, *PTEN*, a previously identified target of miR-21 and a key negative regulator of PI3K signaling (33), was also increased in 4 of the 5 patient samples (P=0.016), consistent with RPPA findings.

Everolimus pharmacokinetics and association with outcome and biomarkers

The pharmacokinetic parameters of everolimus were calculated on day 1 and day 15 of cycle 1 (Cycle 1 Day 1, Cycle 1 Day 15) and day 1 and day 15 of cycle 2 (Cycle 2 Day 1, Cycle 2 Day 15) (Supplemental table 3). Pharmacokinetic sampling was performed on a total of 8 patients on Cycle 1 Day 1 and Cycle 1 Day 15, of which 3 patients received everolimus 5 mg/day and 5 received 10 mg/day. Pharmacokinetic sampling was performed on 5 patients on Cycle 2 Day 1 (3 patients on everolimus 5 mg/day and 2 on 10 mg/day) and 2 patients on Cycle 2 Day 15 (both patients on everolimus 5 mg/day). As in previous studies, AUC and C_{max} were dose-proportional between everolimus 5 mg/day and 10 mg/day (34, 35). At the 10-mg/day dose, the AUC and C_{max} at steady state (C1D15 and C2D0) were higher than in previous published studies. However, it must be noted that the significant variability and small number of patients in the10 mg/day group preclude truly meaningful comparison (34-36).

When all the patients with available pharmacokinetic data were combined (N = 8), those who achieved treatment response had a significantly higher everolimus AUC and lower clearance at steady state (Cycle 1 Day 15) than patients who achieved PR or no response (P=0.025) (Figure 4). The trough levels of everolimus were not associated with response or toxicity. The pharmacokinetic parameters were not associated with toxicity to everolimus. Similarly, we were unable to identify an association between the pharmacokinetic parameters and the biomarker expression.

DISCUSSION

The study described here is the first to evaluate the safety and efficacy of an mTOR inhibitor in combination with HyperCVAD in patients with relapsed and refractory ALL. The study demonstrates the feasibility of combining everolimus, a specific mTOR inhibitor, with an intensive chemotherapy regimen, HyperCVAD.

The PI3K/Akt/mTOR signaling pathway is essential for cell growth, survival and suppression of apoptosis (37, 38). Our previous findings (39, 40) and reports from other groups (41, 42) demonstrated constitutive activation of PI3K/Akt signaling in acute leukemia. However, the mechanisms that up-regulate PI3K/Akt signaling in ALL cells remain unclear, with conflicting reports suggesting that Akt activation in acute myeloid leukemia blasts may be dependent on, or independent from, PI3K (43). A number of different approaches have been pioneered to inhibit the PI3K/Akt/mTOR pathway, predominantly focusing on small molecules designed to selectively target key components of this signal transduction cascade, thereby inducing apoptosis and/or increasing sensitivity of the leukemic blasts to conventional drugs (37). mTOR inhibitors are the most developed class of compounds that target the PI3K/Akt pathway. The mTOR kinases are especially attractive targets as they are located downstream of Akt.

Of the 24 patients, 11 were in first salvage, 3 in second salvage, and 10 were beyond second salvage. The T-ALL patients were heavily pretreated with a median of 4 prior therapies. A response was noted in 5 of 10 (50%) T-ALL patients and the median OS among these heavily pretreated T-ALL patients was 23 weeks. It is difficult to definitively compare these results as there is little published data specific to T-ALL outcomes beyond first salvage. However, the outcomes in T-ALL patients with everolimus-HyperCVAD seemed to compare favorably to previously reported outcomes in heavily pretreated T-ALL patients treated at our institution(44) wherein response rate (CR+PR) was 20% and median OS was 12 weeks.

Among the toxic effects observed, hematologic effects were the most common, as would be expected with an intensive combination chemotherapy regimen. Grade 3 infections and grade 4 sepsis were also prominent and were managed successfully with broad-spectrum antibiotic and antifungal therapy. The infection rates were similar to those observed with HyperCVAD alone (28). Mucositis was the DLT attributed to everolimus, and the maximum tolerated everolimus dose was determined to be 5 mg/day.

Pharmacokinetic analysis showed that higher plasma exposure of everolimus resulted in better treatment response (Figure 4). As in previously reports, inhibition of S6RP was observed in the majority of patients for whom samples were analyzed (45). Our pharmacodynamic study results suggest that everolimus at a dose of 5 mg/day sufficiently blocked phosphorylation of S6RP at pS235-S236 and pS240-S244 sites. Furthermore, our results showed no significant decrease in p4EBP1 levels (on both T37/46 and T70). This is in contrast with the findings by Tabernero *et al*., who noted a correlation between increased everolimus plasma trough concentrations and reduced p4EBP1 levels in skin and tumor tissue (45). Interestingly, lower baseline p4EBP-T37/46 levels were associated with a better

response to therapy in our study (Supplemental Figure 1). This was not true for baseline p4EBP1-T70 levels. It may be postulated that, since these patients had low p4EBP1-T37/46 at baseline, they may not have required significant further suppression of this phosphoprotein to block eukaryotic initiation factor 4E (elF4E)–mediated protein translation and achieve a response. Furthermore, in leukemia cells with low baseline 4EBP1-T37/46 phosphorylation, eIF4E would be less active, so the S6 kinase arm may be the primary driver of tumor growth.

In concordance with previous reports, there was no direct correlation between everolimus exposure and phosphorylation of 4EBP1 (T37/46 or T70) (51) or Akt in our study (45). These findings confirm preclinical reports showing that rapalogs fail to reduce the ability of mTORC1 to phosphorylate 4EBP1 (46). In contrast, second generation active-site mTOR inhibitors more effectively prevent 4EBP1 binding to eIF4E, reduce protein synthesis, and inhibit p-AKT. Recent studies have demonstrated the superior efficacy of active-site mTOR inhibitors in preclinical ALL models, arguing for the incorporation of these agents into salvage ALL regimens (47). Similarly, Wunderle et al noted an encouraging response rate of 30%, including sustained molecular remission in one patient among relapsed B-cell ALL patients treated with a dual PI3K/mTOR inhibitor(48).

Changes in gene expression in response to everolimus were consistent with decreased miR-21, the expression of which is frequently increased in cancers, including leukemia and lymphoma, with enforced overexpression in pre-B cells resulting in lymphoid malignancies (49, 50). miR-21 is commonly associated with aggressive disease, reduced patient survival duration, and *in vitro* resistance to chemotherapy (51, 52). Furthermore, *PTEN*, a major negative regulator of PI3K/Akt/mTOR signaling and a known target of miR-21, was increased by everolimus in our study, a finding consistent with decreased miR-21 expression (33). By RPPA, 5 patients (3 T-ALL, 2-B-ALL) had elevations in PTEN on serial evaluation. The 3 T-ALL patients with elevated PTEN achieved a response whereas neither of the B-ALL patients achieved response. Although these are small numbers these data suggest that the mTOR/PTEN network may play a more central role in T-ALL leukemogenesis and that adequate suppression of this pathway may produce a response even in heavily pretreated T-ALL patients. miR-21 is also a known regulator of inflammatory responses, and it is possible that changed miR-21 expression could explain the inflammatory gene signatures detected in the array data (49).

In conclusion, the combination HyperCVAD and everolimus regimen in patients with ALL did not have significantly increased toxicity as compared to HyperCVAD alone. Of interest, the regimen produced a response in 50% of heavily pretreated T-ALL patients (median of 4 prior therapies). This study provides a first proof of concept that targeting ALL with the combination of everolimus and HyperCVAD is feasible. These data in addition to recently published reports (53-55) suggest that evaluation of next-generation mTOR inhibitors and/or dual PI3K/mTOR for ALL is warranted, with specific emphasis on T-ALL.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Kathryn L Hale, Scientific Editor, Scientific Publications, MDACC for editing the manuscript.

Trial Registration ID: NCT00968253

Funding source: Novartis Pharmaceuticals Corporation and The University of Texas MD Anderson Cancer MD Anderson Cancer Center Leukemia Support Grant (CCSG) CA016672 supported the study. The UT/MDACC RPPA Core facility is supported by the Cancer Center Core Support Grant P30CA016672. The biomarker studies were funded by the Faculty Start-up Award (to MK). LB is supported by an NHMRC Senior Research Fellowship (1042305) and a Cancer Institute NSW Fellowship.

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STATEMENT OF TRANSLATIONAL RELEVENCE

A total of 24 patients with relapsed/refractory ALL were enrolled. No unexpected toxicities were encountered with the combination. A response was noted in 41% of the patients. Responses were significantly higher in first salvage patients as compared to second salvage or beyond. Reverse phase protein array (RPPA) and western blot assays demonstrated that everolimus at a dose of 5 mg/day sufficiently blocked phosphorylation of S6RP at pS235-S236 and pS240-S244 sites, but this did not correlate with response. As expected, the type 1 rapalog everolimus did not significantly inhibit the phosphorylation of 4EBP1 and Akt suggesting a potential benefit of type 2 rapalogs in lymphoid malignancies. Gene set enrichment analysis (GSEA) analysis of microarray data demonstrated that expression of mTOR-sensitive genes induced by overexpression of Akt was reduced in response to everolimus treatment. This manuscript provides rationale for further exploration of rapalogs in lymphoid malignancies.

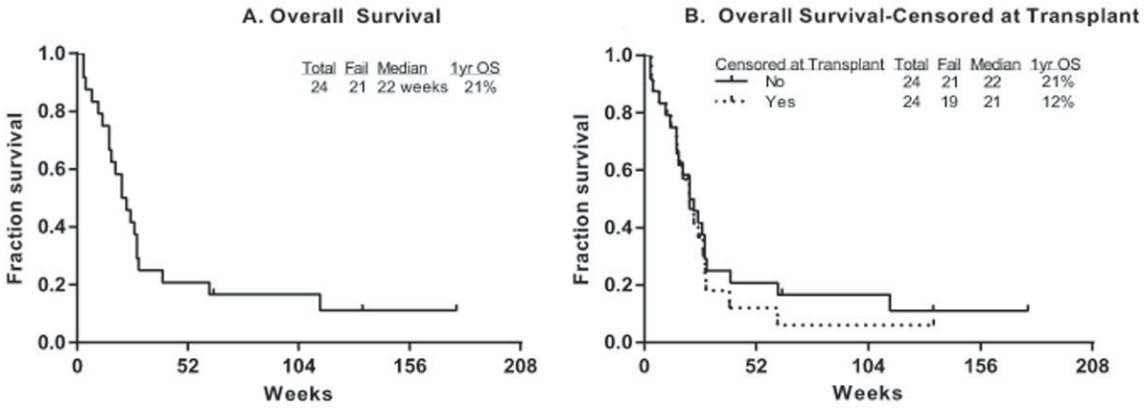

Figure 1.

Overall survival: (A) Overall survival for all 24 patients treated with HyperCVAD and everolimus (B) Overall survival censored at allogeneic stem cell transplant for all patients treated with HyperCVAD and everolimus.

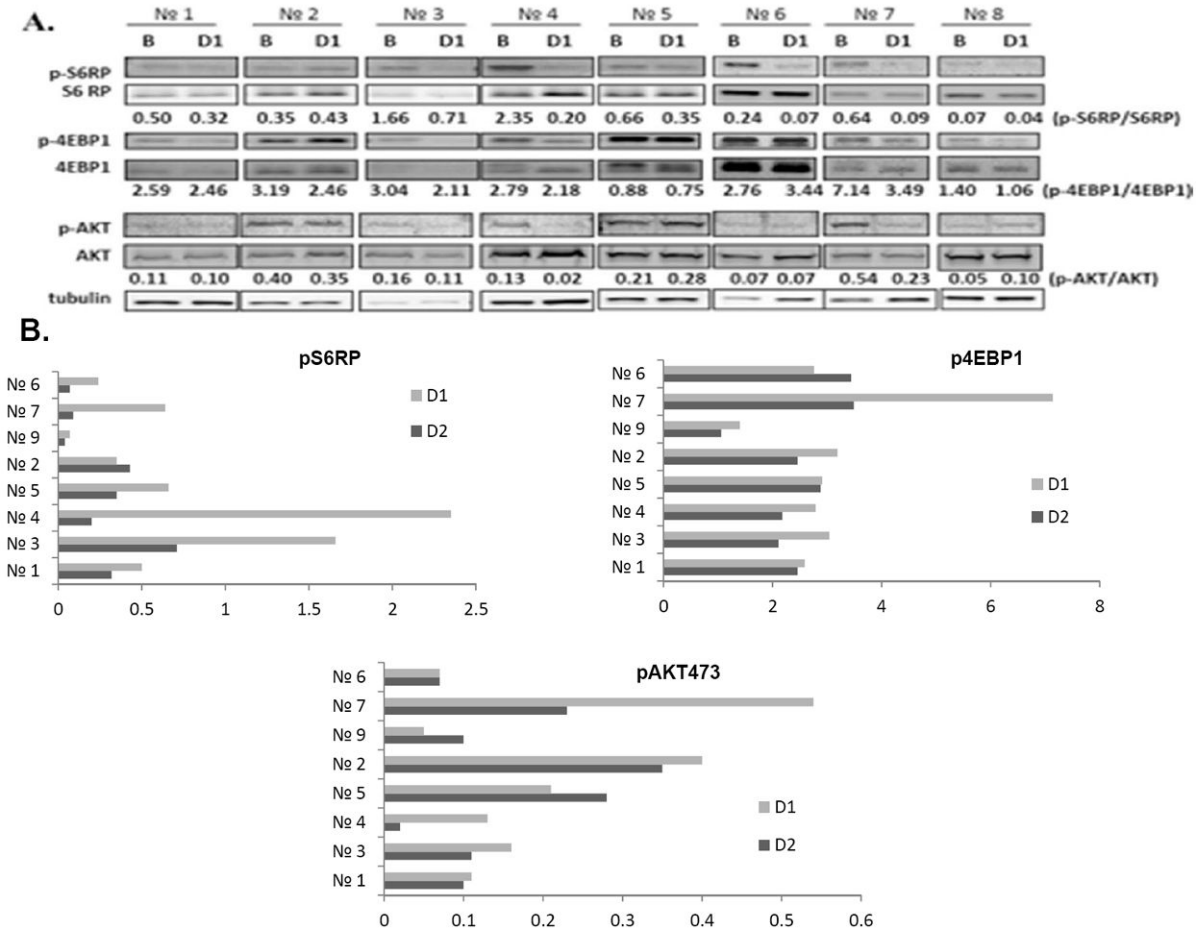

Figure 2.

Modulation of mTOR signaling by everolimus in patient-derived ALL blasts. Patients 1, 2, and 3 received everolimus at a dose of 5 mg/day; patients 4, 5, 6, 7, 9, 10, and 11 received everolimus at a dose of 10 mg/day. (A) Western blot analysis of representative patients. Primary patient samples were analyzed by applying pS6RP, S6RP, p4EBP1, 4EBP1, pAkt, Akt, and tubulin antibodies. Ratios of phosphorylated over total proteins for S6RP, 4EBP1, and Akt (as determined by densitometry) are indicated (B) Ratios of pS6RP, p4EBP1 and pAkt473 on days 0 and 1 as determined by western blot are shown. Response status is indicated for each patient (CR, complete remission; CRi, complete remission with incomplete blood cell count recovery; PR, partial remission; NR, no response). (C) Ratios of pS6RP, pEBP1 and pAkt473 on days 0 and 1 as determined by RPPA are shown.

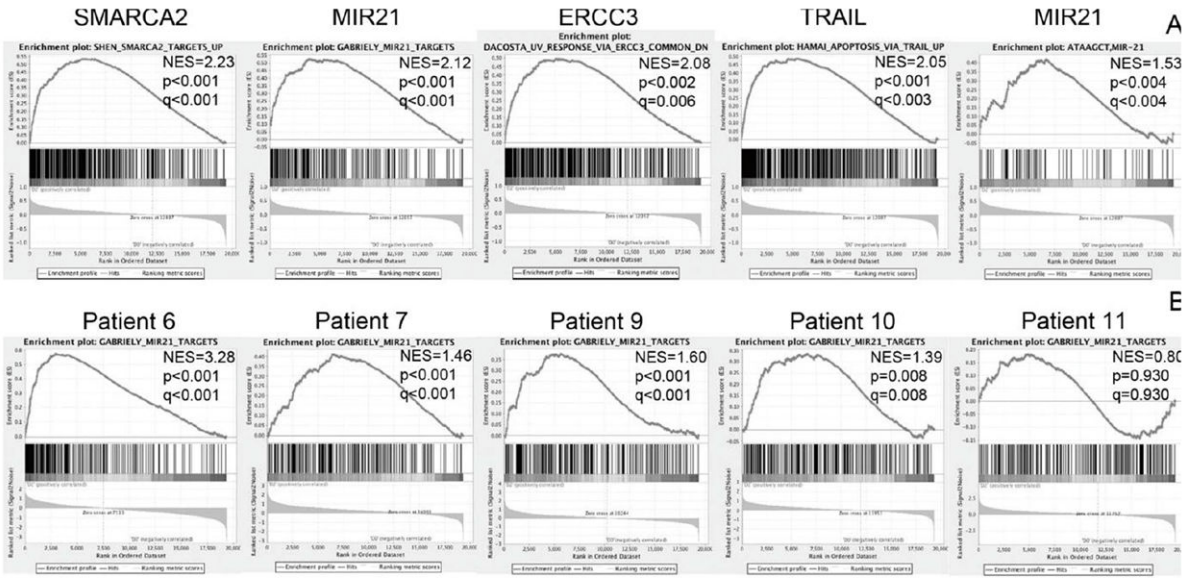

Figure 3. GSEA analysis

GSEA analysis of microarray data of samples collected before (C1D0) and 24 hours after (C1D1) the first dose of everolimus. In the upper panel, columns 1-4 show the top 4 gene signatures (SMARCA2, MIR21, ERCC3, TRAIL) and column 5 the consensus miR-21 signature (GABRIELY_MIR21_TARGETS) when all available samples were analyzed. The lower panel shows the results for the miR-21 targets for each patient pre- and post-treatment. Definitions and functional information regarding these gene signatures is included in supplemental material C.

Abbreviations: SMARCA2, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 2; MIR21, MicroRNA 21; ERCC3, excision repair cross-complementation group 3; TRAIL, TNF-related apoptosis-inducing ligand (TRAIL).

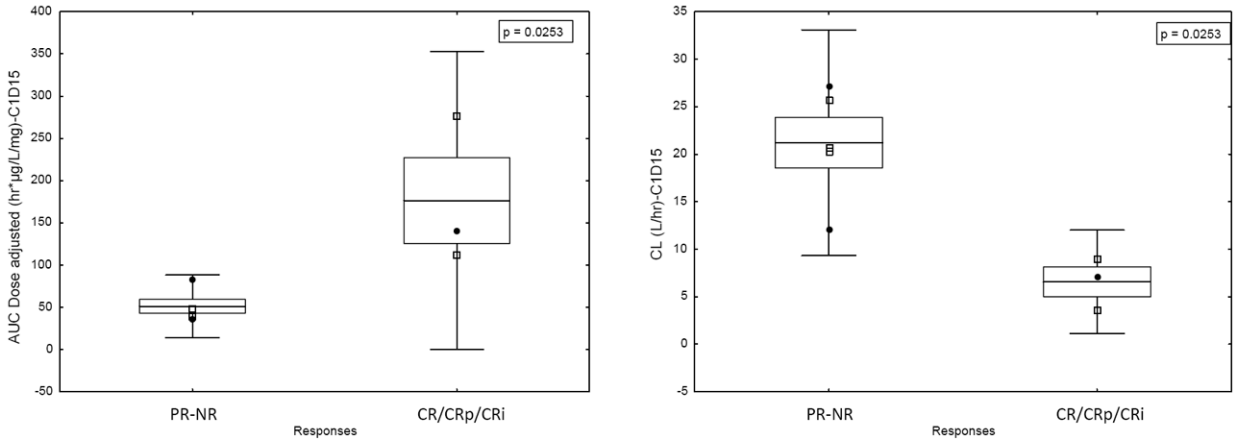

Figure 4. Association of Everolimus PK parameters at C1D15 with outcome

The association of everolimus pharmacokinetics at C1D15 with outcome. Patients who achieved response (CR/CRp/CRi) had significantly higher dose-adjusted AUC (A) and significantly lower clearance (CL) (B) than patients who achieved partial response (PR) or no response. Open squares are patients in the 10 mg/day dose group and solid circles are patients in the 5 mg/day dose group. (CR, complete remission; CRi, complete remission with incomplete blood cell count recovery; PR, partial remission; NR, no response).

Table 1

Clinical characteristics of study patients (N = 24)

No.	Diagnosis	Age	CG		No. Prior treatments/Regimen	CR1 Duration (months)	Everolimus Dose (mg/day)	No. Cycles	Response	Days on Study
1	Pre-T-ALL	29	Del 7, del 17	4	ESHAP, CHOP, AugHCVAD, alloSCT	7	5	2	PR	48
2	Pre-T-ALL	24	Diploid	4	Larson, Nelarabine, other	11	5	2	NR	46
3	Pre-B-ALL	52	11q23	1	R-HCVAD	26	5	4	CR	153
4	Pre-B-ALL	27	t(12;16)	1	AugBFM	39	10	1	NR	21
5	Pre-T-ALL	11	del 1, inv 3	1	CCG-1961 w/XRT	12	10	1	NR	28
6	Pre-T-ALL	24	Hyper with 11q23	3	HCVAD, Nelarabine, alloSCT	30	10	4	PR	145
7	Pre-B-ALL	59	Diploid	1	R-CHOP	12	10	2 +mnt	CR	800
8	Pre-B-ALL	24	Diploid	4	AugBFM, Aug HCVAD, MOAD, PR104	38	10	1	NR	81
9	Pre-T /ALL	26	Hyper with +8	2	HCVAD+Nelarabine, MOAD w/alloSCT	6	10	2	CRi	148
10	Pre-B-ALL	24	Del 7, del 17	3	ALL0232, other	12	10	2	NR	49
11	Pre-B-ALL	23	t(2;9)	1	AugBFM	21	10	2	CR	73
12	Pre-B-ALL	22	Diploid	2	AugBFM, HCVAD+Decitabine w/alloSCT	27	10	2	NR	73
13	Pre-B-ALL	64	Diploid	1	R-CHOP	0	5	2	CR	153
14	Pre-B ALL	43	Diploid	4	HCVAD w/FLAG-IDA, Clofarabine, BiTE	9	5	2	NR	64
15	Pre-T-ALL	40	t(9;17), add 10, der (14;17)	7	HCVAD, Aug HCVAD, Nelarabine, Clofarabine+CTX+ Etoposide, BID FA	Unk	5	1	NR	23
16	Pre-T-ALL	22	Diploid	1	AugBFM	15	5	4	CR	102
17	Pre-B-ALL	14	IM	3	AALL0232, Bortezomib	11	5	1	NR	29
18	Pre-T-ALL	22	Diploid	5	HCVAD, Nelarabine, MOAD, Clofarabine + ara-c, Alemtuzumab	3	5	2	NR	29
19	Pre-B-ALL	24	Diploid	1	AugBFM	45	5	5	CR	150
20	Pre-B-ALL	31	Hyper with +21,+5,+6	1	HCVAD	8	5	2	NR	65
21	Pre-T-ALL	19	Diploid	1	AugBFM	9	5	2	CRp	68
22	Pre-T-ALL	29	Del 5, del 12, del 6	5	HCVAD+Nelarabine, MOAD, IPI-145, CIA+VCR+DXM, Nelarabine	6	5	1	NR	12
23	Mixed phenotype	44	Diploid	2	HCVAD w/allo SCT, MorphoSys	7	5	1	NR	26
24	Pre-B-ALL	39	Hyper with +8	1	HCVAD+Ofa w/alloSCT	11	5	1	NR	23

Abbreviations: No., number; CG, cytogenetics; CR1, first complete remission; PR, partial response; NR, non-responder; CR, complete response; Pseudo, pseudodiploid; Hyper, hyperdiploid; IM, insufficient metaphases; Unk, unknown; mnt, maintenance; CRi, complete remission with incomplete blood cell count recovery; CRp, complete remission with incomplete recovery of platelets.

Table 2

Toxicities by everolimus dose levels

Toxicity	Everolimus dose				Overall; N (%)	
	5 mg; N (%)		10 mg; N (%)			
	G1/2	G3/4	G1/2	G3/4	G1/2	G3/4
Infection (one or more)	0	13 (93)	0	10 (100)	0	23 (96)
Mucositis/stomatitis	6 (43)	0	4 (40)	3 (30)	10 (42)	3 (13)
Metabolic						
ALT/AST	8 (57)	4 (29)	6 (60)	3 (30)	14 (58)	7 (29)
Bilirubin	9 (64)	1 (7)	5 (50)	1 (10)	14 (58)	2 (8)
Hyperglycemia	3 (21)	7 (50)	4 (40)	5 (50)	7 (29)	12 (50)
Gastrointestinal						
Diarrhea	4 (29)	0	4 (40)	2 (20)	8 (33)	2 (8)
Nausea	3 (21)	0	6 (60)	0	9 (38)	0
Constitutional						
Fatigue	9 (64)	0	4 (40)	0	13 (54)	0
Headache	5 (36)	0	1 (10)	4 (40)	6 (25)	4 (17)
Pulmonary						
Pleural effusions	0	1 (7)	1 (10)	1 (10)	1 (10)	2 (8)
Dyspnea	1 (7)	0	0	1 (10)	1 (4)	1 (4)
Renal						
Renal insufficiency	0	2 (14)	0	2 (20)	0	4 (17)
Urinary retention	1 (7)	0	0	0	1 (4)	0
Cardiac						
Tachycardia	2 (14)	0	1 (10)	1 (10)	3 (13)	1 (4)
Pericardial effusion	0	1 (7)	0	1 (10)	0	2 (8)

Abbreviations: N, number; G, grade; ALT, alanine aminotransferase; AST, aspartate transferase