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 (ALL).

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%. In addition, 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 ≤ 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 nonresponders.

Conclusions: The combination of HyperCVAD and everolimus is well tolerated and moderately effective in relapsed ALL, specifically T-ALL. Clin Cancer Res; 21(12); 2704–14. ©2015 AACR.

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 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). 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 two complexes: mTORC1, which also contains raptor and PRAS40, and mTORC2, which also contains rictor and Sin1. These complexes have different spectra of substrates...
Translational Relevance

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 with 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) 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.

(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 inhibitors of mTORC1 but also, surprisingly, block 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 antiapoptotic protein Mcl-1, notype, facilitating further glucose uptake and induction of the microenvironment promotes acquisition of a glycolytic phenotype by transcriptional repression of D-type cyclins (23, 24).

Materials 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 April 7, 2010 and February 9, 2014. A total of 24 patients were enrolled. The latest follow-up date was April 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 Supplementary Material SA 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 cytogetic analyses. Multiparametric flow cytometry and cytogetic analyses 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 two 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: CTCAE version 3.0 grade 3 increased AST or ALT for $\geq 7$ 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 two 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 $\theta_t$, where toxicity was defined as any clinically significant CTCAE (version 3.0) grade 4 nonhematologic toxic effects or death attributable to the study drug (everolimus). We assumed $\theta_t \sim \beta$ (0.3, 1.7). The stopping rule was given by the following probability statement: $Pr(\theta_t > 0.15 \mid \text{data}) > 0.90$. 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), 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 analysis and RPPA are detailed in Supplementary Material SB. The following biomarkers were evaluated by Western blot analysis: pAkt (Ser473), p56-ribosomal protein (S6RP), p4EBP1 (Thr37/46), and total protein levels of Akt, S6RP, and 4EBP1.

RPPA

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 Supplementary Material SC.

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

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

RNA was amplified and biotinylated by using a TargetAmp-Nano labeling kit purchased from Epicenter Biosciences. 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). The integrity and concentration of amplified RNA were determined as described above. Biotinylated RNA was hybridized to a HumanHT-12v4 Bead expression chip (Illumina) 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. 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 biologic states. Definitions and functional information on the specific gene signatures used in this analysis are included in Supplementary Material SD.

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) in whole blood was measured by using a validated high-performance liquid chromatography (HPLC)-tandem mass spectrometry method, with sirolimus (LC Labs) as an internal standard. For details of the assay, please refer to Supplementary Material SE. 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<sub>v</sub>), clearance (CL), area under the concentration versus time curve from time 0 to infinity (AUC<sub>0-∞</sub>), and volume of distribution (V), were estimated by noncompartmental pharmacokinetic methods by using Phoenix WinNonlin software (version 6.3, Pharsight Corporation).

**Statistical analysis**

All P values were two-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.).

**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 1 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 two 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. 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

**Table 1. Clinical characteristics of study patients (N = 24)**

<table>
<thead>
<tr>
<th>No.</th>
<th>Diagnosis</th>
<th>Age (yr)</th>
<th>CG</th>
<th>No. Prior treatments/ regimen</th>
<th>CRI duration (mo)</th>
<th>Everolimus dose (mg/day)</th>
<th>Cycles, n</th>
<th>Response</th>
<th>Days on study</th>
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<td>29</td>
<td>Del 7, del 17</td>
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<td>5</td>
<td>2</td>
<td>PR</td>
<td>48</td>
</tr>
<tr>
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<td>Larson, nelarabine, other</td>
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<td>5</td>
<td>2</td>
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<td>46</td>
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<td>3</td>
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<td>t(12;21)</td>
<td>1 R-HCVAD</td>
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<td>5</td>
<td>4</td>
<td>CR</td>
<td>153</td>
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<td>4</td>
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<td>27</td>
<td>t(12;21)</td>
<td>AugBFM</td>
<td>39</td>
<td>10</td>
<td>1</td>
<td>NR</td>
<td>21</td>
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<td>del 1, inv 3</td>
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<td>10</td>
<td>1</td>
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<td>10</td>
<td>4</td>
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<td>10</td>
<td>2</td>
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<td>800</td>
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<td>AugBFM, Aug HCVAD, MOAD, PR104</td>
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<td>10</td>
<td>1</td>
<td>NR</td>
<td>81</td>
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<td>Hyper with +8</td>
<td>HCVAD + nelarabine, MOAD w/alloSCT</td>
<td>6</td>
<td>10</td>
<td>2</td>
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<td>2</td>
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<td>t(1;17), add 10, der (14;17)</td>
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<td>1</td>
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<td>5</td>
<td>1</td>
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<td>23</td>
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</table>

Abbreviations: No., number; CG, cytogenetics; CRI, first complete remission; PR, partial response; NR, nonresponder; CR, complete response; Pseudo, pseudodiploid; Hyper, hyperdiploid; IM, insufficient metaphases; Unk, unknown; mrt, maintenance.
Table 2. Toxicities by everolimus dose levels

<table>
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<th>Toxicity</th>
<th>5 mg, N (%)</th>
<th>10 mg, N (%)</th>
<th>Overall, N (%)</th>
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<td>G1/2</td>
<td>G3/4</td>
<td>G1/2</td>
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<td>Infection (one or more)</td>
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<td>Mucositis/stomatitis</td>
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<td>4 (40)</td>
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<tr>
<td>Metabolic</td>
<td>8 (57)</td>
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<td>6 (60)</td>
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<td>7 (50)</td>
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<td>Bilirubin</td>
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<td>Urinary retention</td>
<td>2 (14)</td>
<td>0</td>
<td>1 (10)</td>
</tr>
<tr>
<td>Tachycardia</td>
<td>0</td>
<td>1 (7)</td>
<td>0</td>
</tr>
<tr>
<td>Pericardial effusion</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: N, number; G, grade.

 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 1 dose-limiting toxicity. Two of 6 patients treated with everolimus 10 mg/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%; ref. 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 coadministered 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 CRs, 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. In addition, 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 with 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 ≤ 0.001 and P = 0.01; Supplementary Fig. S2).

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 with 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 with 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 two 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 analysis 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 (Fig. 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 analysis and RPPA changes of S6RP phosphorylation were concordant in 7 of the 8 patients whose samples were tested by Western blot analysis. 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 the 10 mg/day doses of everolimus.

In all 10 patients, everolimus significantly (one-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 (Fig. 2 and Supplementary Table S1). No statistically significant differences in phosphorylation of 4EBP1, Akt, or other proteins were found (Supplementary Table S1). We next used a binomial test to analyze whether changes in phosphorylation of S6RP, 4EBP1, Akt, or other proteins were found (Supplementary Table S1). When adjusted for multiple comparisons, the $P$ value was 0.07.
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 analysis are shown. Response status is indicated for each patient. C, ratios of pS6RP, pEBP1 and pAkt473 on days 0 and 1 as determined by RPPA are shown.
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; Supplementary Table S3). 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 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 the 10 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 C_{max} at steady state (cycle 1 day 15) than patients who achieved PR or no response (Fig. 4; P = 0.025). 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 upregulate 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 are 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 with 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 (45). Interestingly, lower baseline p4EBP1-T37/46 levels were associated with a better response to therapy in our study (Supplementary Fig. S1). This was not true for baseline p4EBP1-T70 levels. It may be postulated that as 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 (eIF4E)–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; ref. 46) or Akt in our study (45). These findings confirm preclinical reports showing that rapalogs fail to reduce the ability of mTORC1 to phosphorylate 4EBP1 (47). 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 (48). Similarly, Wunderle and colleagues 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 (49).

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 (50, 51). miR-21 is commonly associated with aggressive disease, reduced patient survival duration, and in vitro resistance to chemotherapy (52, 53). 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 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 with 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.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N. Daver, H. Kantarjian, D. Thomas, M. Konopleva
Development of methodology: N. Daver, D. Thomas
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): N. Daver, H. Kantarjian, J. Cortes, M.E. Ryting, J. Banzerti, M.A. Richer, R. Garris, E. Iabbour, J. Burger, D. Thomas, M. Konopleva
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): N. Daver, H. Kantarjian, J. Cortes, J.D. Kawedia,

![Figure 4](image-url)

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.
HyperCVAD plus Everolimus in Patients with ALL

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