Synergistic Drug Combinations with a CDK4/6 Inhibitor in T-cell Acute Lymphoblastic Leukemia

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Abstract

Purpose: Although significant progress has been made in the treatment of T-cell acute lymphoblastic leukemia (T-ALL), many patients will require additional therapy for relapsed/refractory disease. Cyclin D3 (CCND3) and CDK6 are highly expressed in T-ALL and have been effectively targeted in mutant NOTCH1-driven mouse models of this disease with a CDK4/6 small-molecule inhibitor. Combination therapy, however, will be needed for the successful treatment of human disease.

Experimental Design: We performed preclinical drug testing using a panel of T-ALL cell lines first with LEE011, a CDK4/6 inhibitor, and next with the combination of LEE011 with a panel of drugs relevant to T-ALL treatment. We then tested the combination of LEE011 with dexamethasone or everolimus in three orthotopic mouse models and measured on-target drug activity.

Results: We first determined that both NOTCH1-mutant and wild-type T-ALL are highly sensitive to pharmacologic inhibition of CDK4/6 when wild-type RB is expressed. Next, we determined that CDK4/6 inhibitors are antagonistic when used either concurrently or in sequence with many of the drugs used to treat relapsed T-ALL (methotrexate, mercaptopurine, asparaginase, and doxorubicin) but are synergistic with glucocorticoids, an mTOR inhibitor, and gamma secretase inhibitor. The combinations of LEE011 with the glucocorticoid dexamethasone or the mTOR inhibitor everolimus were tested in vivo and prolonged survival in three orthotopic mouse models of T-ALL. On-target activity was measured in peripheral blood and tissue of treated mice.

Conclusions: We conclude that LEE011 is active in T-ALL and that combination therapy with corticosteroids and/or mTOR inhibitors warrants further investigation. Clin Cancer Res; 23(4); 1012–24. © 2016 AACR.

See related commentary by Carroll et al., p. 873

Introduction

Although significant progress has been made in the treatment of T-cell acute lymphoblastic leukemia (T-ALL), approximately 20% of newly diagnosed pediatric and 50% of adult patients will experience either induction failure or relapse (1). In addition, fewer than 50% of patients with T-ALL who experience a relapse are long-term survivors despite intensive chemotherapy regimens, including stem cell transplantation. New targeted therapies are needed for the treatment of this disease.

Multiple lines of evidence point to cyclin D3 (CCND3) and CDK4/6 as potential therapeutic targets in T-ALL. A common feature of T-ALL is the activation of Notch pathway signaling by mutations in NOTCH1 and/or FBXW7, with Notch pathway activation present in about 60% of T-ALL (2). Notch is a transmembrane receptor that is cleaved on activation and translocates into the nucleus, where it alters the transcriptional programs associated with cellular proliferation and differentiation. In T-ALL, mutations in NOTCH1 typically cause ligand-independent receptor translocation and transcriptional activation (3). CCND3, a direct target of activated NOTCH1, is upregulated in T-ALL, and CCND3-null animals are refractory to NOTCH1-driven T-ALL. CCND3 binds and activates CDK4/6, and the CCND3–CDK3 complex phosphorylates the tumor suppressor RB, leading to cell-cycle progression. CDKN2A is a negative regulator of CCND3, and loss of CDKN2A, a common feature of ALL, is also predicted to activate this pathway (4–6). CDK6 is also highly expressed in human T-cell lymphoblastic lymphoma/leukemia samples (7–9). Previous studies have demonstrated that the CDK4/6 small-molecule inhibitor, PD0332991, caused cycle arrest and apoptosis in NOTCH1-driven T-ALL in vitro and delayed disease progression in NOTCH1-driven mouse models of T-ALL (10, 11). Thus, targeting of CDK4/6 and CCND3 may be particularly...
Drug Combinations with a CDK4/6 Inhibitor in T-ALL

Translational Relevance
Despite significant progress in the treatment of T-cell acute lymphoblastic leukemia (T-ALL), up to 20% of pediatric and 50% of adult patients with T-ALL will experience either induction failure or relapse of their disease. Cyclin D3 and CDK6 are both upregulated in T-ALL, and CDK4/6 inhibition may be a viable therapeutic option for these patients. However, single-agent therapy is unlikely to be effective in treating acute leukemia. Therefore, we identified synergistic combination therapies with LEE011, a CDK4/6 inhibitor, which could be readily translated to patients with T-ALL.

Effective for the treatment of T-ALL. Several CDK4/6 inhibitors have been developed [PD0332991 (Pfizer), LEE011 (Novartis), and LY2835219 (Lilly)] and are currently being tested in clinical trials for patients with solid tumors and lymphomas. This drug class, however, has not been tested in patients with T-ALL.

Successful implementation of CDK4/6 inhibitors in the clinical setting will likely need to be done in combination with other agents because CDK4/6 inhibitors generally do not induce cell death. Often, new targeted drugs have been introduced to patients with leukemia in combination with standard-of-care cytotoxic chemotherapy. The combinations of CDK4/6 inhibitors with cytotoxic chemotherapy are predicted to be antagonistic, however, because most of these cytotoxic drugs rely on rapidly proliferating cells, and CDK4/6 inhibition induces cell-cycle arrest. Indeed, CDK4/6 inhibition protects breast cancer cells from doxorubicin- and paclitaxel-mediated cell death (12, 13). Combination studies in leukemia have not been reported.

We thus sought to identify highly effective drug combinations with CDK4/6 inhibitors in T-ALL that could be rapidly translated to the clinic. First, we confirmed the sensitivity of T-ALL cell lines to CDK4/6 inhibitors using the compound LEE011. We found that both NOTCH1-mutant and NOTCH1 wild-type T-ALL were sensitive to these inhibitors with a concentration-dependent cell-cycle arrest and modest induction of cell death. RB-null cells were highly resistant. We then performed combination studies of LEE011 with agents typically used for T-ALL treatment, including glucocorticoids, methotrexate, mercaptopurine, asparaginase, and doxorubicin, and newer targeted agents of interest in T-ALL. We conclude that LEE011 is active in T-ALL, antagonistic with many of the cytotoxic agents used to treat T-ALL but synergistic with mTOR inhibitors and glucocorticoids. Combination therapy with CDK4/6 inhibitors with glucocorticoids and/or mTOR inhibitors warrants further investigation in the clinical setting.

Materials and Methods

Cell culture, cell viability, and apoptosis assays
The human cell line MOLT16 was purchased from Leibniz-Institut DSMZ-German collection of microorganisms and cell cultures. Identity was confirmed using multiplex PCR of minisatellite markers performed by DSMZ. The LOUCY cell line was purchased from ATCC. Identity was confirmed by short tandem repeat (STR) loci profiling performed by ATCC. Human cell lines DND41, KOP1T1, MOLT4, and PB382 were kindly provided by Jon Aster (Brigham and Women’s Hospital). Identity was confirmed by STR loci profiling performed in 2012 upon receipt of the lines. SKW3, SUPT11, and HSB2 were kindly provided by James Bradner (formerly Dana-Farber Cancer Institute, now Novartis Pharmaceuticals), and Jurkat cells were kindly provided by Nicholas Haining (Dana-Farber Cancer Institute). NOTCH1 activation for cell lines HSB2, SUPT11, and Jurkat was confirmed by Western blotting. SKW3 identity was confirmed by FISH for the known MYC-TCR translocation. At the time of confirmation, all cell lines were frozen down and low passage cells were used for all current experiments.

All cell lines were maintained in RPMI1640 (CellGro) supplemented with 1% penicillin/streptomycin (CellGro) and 10% FBS (Sigma-Aldrich) at 37°C with 5% CO2. Viability was evaluated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega) after the indicated days of exposure to the specific drug or combination of drugs. Luminescence was measured using FLUOstar Omega from BMG Labtech. The IC50 values were determined using GraphPad Prism Version 6.03 software. Cell death was analyzed using Annexin V-PI staining (eBioscience).

Compounds
LEE011 and everolimus were provided by Novartis. Methotrexate, mercaptopurine, dexamethasone, and prednisolone were purchased from Sigma. Doxorubicin was purchased from Cell Signaling Technology. Bortezomib was purchased from Selleckchem, and Compound E was purchased from Enzo Pharmaceuticals. L-Asparaginase was manufactured by Lundbeck Inc. and purchased from the Dana-Farber Cancer Institute pharmacy.

Flow cytometry analysis
For flow cytometry analysis of peripheral blood samples for on-target activity measurement, fresh blood was collected after 5 days of drug treatment. Samples were transported to the laboratory within 1 hour of collection and processed immediately. Whole blood was passed through a 40-μm mesh filter to remove any clotted material, and 100 μl aliquots were prepared in 15 × 75 mm2 flow tubes. Blood samples were fixed, red blood cells lysed, and the remaining nucleated cells permeabilized as described previously (14). Antibodies used included anti-human CD45-V450 (BD Biosciences), phospho-RB5770-PE (BD Biosciences), and phospho-4EBP1-A488 (Cell Signaling Technology). For peripheral blood CD45 monitoring in the patient-derived xenograft (PDX) model, peripheral blood was obtained at the indicated time points and red blood cells lysed. Cells were then stained with anti-human CD45 antibody.

Immunoblotting
Cells were lysed in Cell Signaling Lysis Buffer (Cell Signaling Technology) as previously reported (15) and resolved by gel electrophoresis using Novex 4-12% Bis-Tris Gels (Invitrogen), transferred to a nitrocellulose membrane (Bio-Rad), and blocked for 1 hour in 5% BSA (Sigma). Blots were incubated in primary antibody to phospho-RB5770 (Cell Signaling Technology), RB (Cell Signaling Technology), CCND3 (Santa Cruz Biotechnology), CDK4 (Neomarkers), CDK6 (Santa Cruz Biotechnology), GAPDH (Santa Cruz Biotechnology), phospho-P70S6K (Cell Signaling Technology), P70S6K (Cell Signaling Technology), ICN1 (Santa Cruz Biotechnology) or Vinculin (Abcam), followed by the secondary antibodies anti-rabbit HRP (Amersham) or anti-mouse HRP (Amersham). Bound...
antibody was detected using the Western Lightning Chemiluminescence Reagent (PerkinElmer).

**In vivo studies**

MOLT4 and MOLT16 luciferized cells (2 × 10⁶) were injected via the tail vein into 8-week-old, female NSG mice (The Jackson Laboratory). Leukemia burden was serially assessed using non-invasive bioluminescence imaging by injecting mice intraperitoneally with 75 mg/kg D-luciferin (Promega), anesthetizing them with 2% to 3% isoflurane, and imaging them on an IVIS Spectrum (Caliper Life Sciences). A standardized region of interest (ROI) encompassing the entire mouse was used to determine total body bioluminescence, with data expressed as photons/second/ROI (ph/s/ROI). Once detectable bioluminescence was achieved, the mice were separated into treatment cohorts and drug treatment initiated.

For the PDX study, NSG mice were injected with 0.5 × 10⁶ leukemic blasts via tail vein injection and bled weekly to determine the percentage of circulating human CD45⁺ cells in the peripheral blood. Once the leukemia burden reached more than 20% in the peripheral blood of 3 select animals, all mice were bled and assigned to one of four treatment groups to receive vehicle, LEE011 (75 mg/kg p.o. daily), dexamethasone (15 mg/kg i.p. daily), or the combination of LEE011 with dexamethasone and were treated for 21 days. Mice were monitored, bled for assessment of leukemia burden at the indicated time points, and sacrificed when determined to be moribund. All animal studies were conducted under the auspices of protocols approved by the Dana-Farber Cancer Institute Animal Care and Use Committee. Samples for pathology and flow cytometry evaluation were collected in a subset of mice after 5 days of drug treatment.

**Drug interaction analysis**
The current methodologies for estimating the drug combination are divided into two major categories: dose–effect-based approaches and effect-based approaches (16). The expected dose-inhibitory fraction relationships for the combination therapy of LEE011 and each of the 10 compounds (methotrexate, L-asparaginase, mercaptopurine, doxorubicin, dexamethasone, prednisolone, everolimus, JQ1, bortezomib, and Compound E) were assessed on the basis of the two basic methods in common use: the Chou–Talalay combination index (CI) for Loewe additivity (23–24), which employs a dose–effect strategy, and the Bliss independence model (23, 24), which uses an effect-based strategy, as detailed in the Supplementary Methods.

Loewe additivity is a basic dose–effect approach that estimates the effect of combining two drugs based on the dose of each individual drug that produces the same quantitative effect (17, 18). Chou and Talalay showed that Loewe equations are valid for enzyme inhibitors with similar mechanisms of action, either competitive or noncompetitive toward the substrate (19). They introduced the CI scores to estimate the interaction between the two drugs. If CI < 1, the drugs have a synergistic effect, and if CI > 1, the drugs have an antagonistic effect. CI = 1 means the drugs have additive effect (20).

The Bliss independence model is based on the principle that drug effects are outcomes of probabilistic processes and compares the effect resulting from the combination of two drugs directly with the effects of its individual components. Bliss independence assumes that the drugs have independent mechanisms of action and can bind simultaneously and mutually nonexclusively (23, 24). The model computes a quantitative measure called excess over Bliss (eob). Positive eob values are indicative of synergistic interaction, whereas negative eob values are indicative of antagonistic behavior. Null eob values indicate additive effect.

**Statistical analysis**
Statistical significance was determined by two-tailed Student t test for pair-wise comparison of groups and by log-rank test for survival curves. Two-way repeated-measures ANOVA was used for comparisons of two or more groups over time.

**Results**

ALL is highly responsive to CDK4/6 inhibitors

We used an independent dataset, The Genomics of Drug Sensitivity in Cancer Project, to evaluate whether ALL is sensitive to CDK4/6 inhibition and to validate the previous finding that NOTCH1-mutated T-ALL is particularly sensitive to a CDK4/6 inhibitor. The Genomics of Drug Sensitivity in Cancer Project profiled 633 cancer cell lines representing a wide range of tumor types in a viability assay across a range of concentrations of 138 compounds (25). The cell lines screened in this study have been characterized in the Catalogue of Somatic Mutations in Cancer database, which includes information on somatic mutations in cancer genes, gene amplifications and deletions, tissue type, and transcriptional data, to allow for identification of biomarkers of drug response. Analysis of this publicly available dataset revealed that T-ALL cell lines were among the most sensitive to treatment with the CDK4/6 inhibitor PD0332991 (Fig. 1A) compared with the other 608 cell lines. B-ALL cell lines did not quite achieve statistical significance (P = 0.007; Fig. 1B). NOTCH1 mutations were a biomarker of response when all cell lines across all disease types were analyzed (Fig. 1C). When the analysis was limited exclusively to T-ALL cell lines, however, both NOTCH1-mutated and wild-type cells were equally responsive to PD0332991, suggesting that NOTCH1 mutations do not predict responsiveness within the T-cell lineage (Fig. 1D).

We next tested LEE011, a structurally distinct CDK4/6 inhibitor, in a panel of 10 T-ALL cell lines (Fig. 2A). We found sensitive cell lines to have IC₅₀s in the range of 0.7 to 3.2 μmol/L after 6 days of treatment with viability measured by the CellTiter-Glo ATP-based assay. In support of the analysis performed in The Genomics of Drug Sensitivity in Cancer Project, we found that some of the most sensitive T-ALL cell lines actually lacked activating NOTCH1 mutations, suggesting that NOTCH1 mutations do not required for a strong response to CDK4/6 inhibitors in T-ALL. RB1 expression, however, was critical to response to LEE011. Cell lines with RB1 loss by Western blot analysis did not show a significant response to the drug (Fig. 2B and C).

We next selected two cell lines, MOLT4 (NOTCH1 mutated) and MOLT16 (NOTCH1 wild type) for further testing. In these cell lines, LEE011 decreased phosphorylation of RB in a concentration-dependent manner, which corresponded to a G₁ cell-cycle arrest after 24 hours of treatment (Fig. 2D and E). There was an increase in cell death as shown by Annexin V–positive staining after 4 days of treatment. Cell death was more prominent in the NOTCH1 wild-type MOLT16 cell line compared with the NOTCH1-mutated MOLT4 cells (Fig. 2F). There was a correlation between the ratio of Annexin V to the ratio of viability as measured by the CellTiter-Glo ATP-based assay. With increased cell death,...
A CDK4/6 inhibitor is antagonistic with methotrexate, mercaptopurine, and l-asparaginase in vitro

Standard chemotherapy used in T-ALL treatment relies on rapidly proliferating cells for its activity. Because LEE011 induces cell-cycle arrest, we hypothesized that LEE011 would be antagonistic with many chemotherapy agents used to treat T-ALL. We concurrently treated the MOLT4 and MOLT6 cell lines with LEE011 in combination with methotrexate, mercaptopurine, l-asparaginase, or doxorubicin across a range of drug concentrations in a serially 2-fold dilution. Cells were treated in 384-well format in quadruplicate for each drug concentration combination, and viability was assessed after 3 and 6 days of treatment using the CellTiter-Glo ATP-based assay.

Because there are multiple approaches to measuring synergy, each with advantages and disadvantages, we evaluated a minimum of two approaches. We used an effect-based approach, the Bliss independence model, which assumes that the inhibitors have independent mechanisms of action and can bind simultaneously and mutually nonexclusively (23, 24). On the basis of this model, the combinations of LEE011 with methotrexate, mercaptopurine, and l-asparaginase were all antagonistic at the day 6 assessment while there was synergy in combination with doxorubicin over a narrow range of doses (Supplementary Fig. S1).

In addition, we used a dose–effect-based approach, the Loewe additivity model (17, 18), and the Chou–Talalay CI (19, 20, 22). Using the Chou–Talalay CI for Loewe additivity model, LEE011 treatment in combination with methotrexate, mercaptopurine, doxorubicin, or l-asparaginase was antagonistic (Fig. 3).

Taken together, these two analytical methods are highly concordant with one another, supporting an antagonistic relationship between LEE011 and methotrexate, mercaptopurine,
Figure 2.
LEE011 inhibits the growth of T-ALL cell lines. A, Ten T-ALL cell lines were grown in a range of LEE011 concentrations and viability evaluated at day 6 by an ATP-based assay as the percentage of viable cells relative to a DMSO control. Shown are the mean ± SD of four replicates. B, Western immunoblotting showing expression of ICN1, RB, cyclin D3, CDK4, and CDK6 in a panel of T-ALL cell lines. C, Table showing calculated IC50 values for LEE011 treatment from the dose-response curves in A. Also annotated is the ICN1 and RB1 status for each cell line. D, Western immunoblotting showing a decrease in phosphorylation of RB with increasing concentrations of LEE011 in ICN1-positive (MOLT4) and -negative (MOLT16) cell lines. E, Cell-cycle analysis in MOLT4 and MOLT16 cells treated with increasing concentrations of LEE011. F, Ratio of Annexin V-positive cells to control with increasing concentrations of LEE011 treatment of MOLT4 and MOLT16 cells. Shown are the mean ± SEM of two separate experiments. G, Correlation of ratio of Annexin V-positive cells (relative to control) versus ratio of Cell Titer-Glo (CTG) luminescence (relative to control) of cells treated with increasing concentrations of LEE011 at the indicated doses. Numbers indicate LEE011 concentration in μmol/L units.
LEE011 is antagonistic with methotrexate, mercaptopurine, doxorubicin, and L-asparaginase. A–D, CI analysis for the combinations of LEE011 with methotrexate (A), mercaptopurine (B), L-asparaginase (C), and doxorubicin (D) in MOLT4 and MOLT16 cells treated for 6 days in replicates of 4.
and L-asparaginase. While the algorithms did not completely agree on the relationship with doxorubicin, the Chou-Talalay combination index may be the more relevant model in this case in light of the complexity in confirming completely independent mechanisms of action of two drugs [24].

It is possible that the antagonism seen by treating cells with two drugs at the same time can be overcome by sequential addition of the drugs. We thus tested sequential addition of drugs by adding LEE011 or the chemotherapy agent to cells in a 384-well format in a range of concentrations and subsequently added the second drug after 24 hours. Viability was assessed after 3 days of combined treatment using the CellTiter-Glo ATP-based assay. Using the Chou–Talalay CI for Loewe additivity model, this sequential addition did not significantly alter the pattern of antagonism (Supplementary Fig. S2).

CDK4/6 inhibitor is synergistic with glucocorticoids and experimental T-ALL therapies

To further characterize the combination of LEE011 with other drugs that do not rely on rapidly proliferating cells, we tested LEE011 in combination with glucocorticoids and newer targeted therapies of interest in T-ALL. We first tested the MOLT4 and MOLT16 cell lines as above in a 2-fold dilution series with LEE011 in combination with drugs that could be rapidly translated to the clinic: dexamethasone, prednisolone, and everolimus. Viability was assessed after 3 and 6 days of treatment using an ATP-based assay. The combination of either everolimus or a glucocorticoid with LEE011 showed synergy (Fig. 4A-C and Supplementary Fig. S3). We thus expanded testing to include other T-ALL cell lines, both NOTCH1-mutated (PF382, KOPTK1) and wild type (SKW3). In KOPTK1, SKW3, and PF382, the combinations of LEE011 and dexamethasone or LEE011 and everolimus were also synergistic (Fig. 4A and B). Next, we tested the combination of LEE011 with newer agents: JQ1, bortezomib, and Compound E. JQ1 and bortezomib were generally additive across multiple concentrations, whereas compound E was synergistic (Supplementary Fig. S4). In light of the strong synergy with LEE011 in combination with glucocorticoids and everolimus, and the ease of clinical translation, we focused our further study on these two combinations. In the MOLT4 and MOLT16 cell lines, we first validated the synergistic concentrations of LEE011, dexamethasone, and everolimus in a low-throughput assay and measured the effects on phosphorylation of RB. MOLT4 and MOLT16 cells were incubated with LEE011, dexamethasone, everolimus, and combinations of these drugs. Viability was assessed after 3 and 6 days of treatment with an ATP-based assay. The combination of LEE011 with either dexamethasone or everolimus was confirmed to have a greater effect on viability than any single treatment (Supplementary Fig. S5). At the selected concentrations, combinations of LEE011 with either everolimus or dexamethasone also had a greater effect on phosphorylation of RB than any single drug treatment as assessed by Western blotting at 24 hours in the MOLT4 cells (Fig. 4D). Everolimus showed on-target activity with a decrease in phosphorylation of P70S6K after 24 hours of treatment. A more modest decrease in P70S6K phosphorylation was also observed with LEE011 treatment. Moreover, treatment with LEE011 alone led to an increase in CCND3 in both MOLT4 and MOLT16 cell lines, an effect tempered by cotreatment with either everolimus or dexamethasone.

CDK4/6 inhibitor enhances the effects of glucocorticoids and mTOR inhibitors in vitro

We next extended testing to MOLT16 and MOLT4 orthotopic mouse models of T-ALL. MOLT16 cells were labeled with luciferase (MOLT16-Luc) and were injected into NOD/SCID IL2Rgnull (NSG) mice. Mice were treated in six groups: vehicle, LEE011 (75 mg/kg), everolimus (5 mg/kg), dexamethasone (15 mg/kg), the combination of LEE011 and dexamethasone, and LEE011 and everolimus. The LEE011 dose was selected as it corresponds to a clinically achievable dose in patients. All treatments were associated with a decrease in spleen weight after 5 days of treatment (Fig. 5A). In this model, mice treated with dexamethasone alone did not have an increase in survival. In addition, mice treated with the combination of LEE011 with dexamethasone had the same survival as mice treated with LEE011 alone (Fig. 5B). Given these unexpected results, we measured serum drug levels in mice treated with dexamethasone, LEE011, and the combination of LEE011 and dexamethasone at 1 and 4 hours after drug administration because dexamethasone is a reported inducer of the p450 system (26, 27). There was no significant difference in serum concentrations of dexamethasone when the drug was given alone or in combination with LEE011 at 1 hour with a small difference at 4 hours (Supplementary Fig. S6A). In contrast, at hour 1, there was a decrease in LEE011 levels in mice receiving the combination of LEE011 and dexamethasone, although this difference did not persist at the hour 4 measurement (Supplementary Fig. S6B). Although not conclusive, these data suggest that dexamethasone may increase LEE011 metabolism and thus decrease serum levels of LEE011 in this mouse model.

The combination of LEE011 and everolimus resulted in a significantly prolonged survival in the MOLT16-Luc mouse model compared with either drug alone (Fig. 5C). Using flow cytometry on mouse peripheral blood, we confirmed on-target activity of everolimus and LEE011. Treatment with everolimus resulted in a decrease in the phosphorylation of 4E-BP1 (Thr37/46), a downstream target of mTOR, in the peripheral blood from everolimus-treated versus vehicle-treated mice after 5 days of drug administration (Fig. 5D). Treatment with LEE011 led to a decrease in phosphorylation of RB in mouse peripheral blood after 5 days of daily treatment (Fig. 5E). Given the discordance between the in vitro and in vivo results for the combination of LEE011 with dexamethasone in the MOLT16 models, we tested this combination in a MOLT4 orthotopic xenograft model. Here, MOLT4 cells were labeled with luciferase (MOLT4-Luc) and were injected into NSG mice. Mice were treated in four groups: vehicle, LEE011, dexamethasone, and the combination of LEE011 and dexamethasone. In this model, the combination of LEE011 with dexamethasone resulted in a decrease in spleen weight, whereas single-agent treatment was ineffective (Fig. 5F). Although single-drug treatments resulted in prolonged survival in this model, the combination had a greater effect than either single treatment alone (Fig. 5G).

We measured the serum drug levels of dexamethasone and LEE011 in this model at 1 and 4 hours after drug dosing (Supplementary Fig. S6C and S6D). In this experiment, there was no difference in dexamethasone or LEE011 levels in mice treated with single drugs alone or in combination at either time point (Supplementary Fig. S6C and S6D). Histopathology evaluation showed a decrease in pRB-S807/811, a measure of on-target...
Figure 4. LEE011 is synergistic with dexamethasone, prednisolone, and everolimus. A–C, CI analysis for the combinations of LEE011 with everolimus (A), dexamethasone (B), and prednisolone (C) in the indicated cell lines treated for 6 days in replicates of 4. D, Western immunoblotting showing changes in protein levels associated with treatment with LEE011 (L), everolimus (E), dexamethasone (D), and combinations of LEE011 with everolimus (L + E) or LEE011 with dexamethasone (L + D).
activity of LEE011 in bone marrow collected after 5 days of drug treatment (Fig. 5H).

We next tested the LEE011 and dexamethasone combination in a PDX model. Leukemia cells from a pediatric patient with NOTCH-activated relapsed T-ALL were engrafted into NSG mice. We then injected these cells (P1) into NSG mice and monitored for engraftment. At an average of 25% leukemia in the peripheral blood, mice were assigned to one of four groups (vehicle, dexamethasone, LEE011, and combination of LEE011 with dexamethasone) and treatment initiated. The mice were treated daily for 21 days, and the effects on peripheral leukemia burden and overall survival were determined. The combination of LEE011 with dexamethasone significantly decreased leukemia burden and prolonged survival compared with all other groups (Fig. 6A and B). There was also a decrease in spleen size after 5 days of treatment (Fig. 6C). Histopathology showed a decrease in pRB in the spleen, (15), flow cytometry evaluation showed a decrease in pRB in peripheral blood leukemia cells, both collected after 5 days of drug treatment (Fig. 6D and E).

Discussion

Although there has been an explosion of targeted therapies for the treatment of cancer, most cancers are still treated with cytotoxic chemotherapy. For integration of targeted therapy into clinical practice, it is essential to find appropriate combination therapies. CDK4/6 inhibitors are showing promise in clinical trials, but drugs that cause cell-cycle arrest may be particularly difficult to combine with other chemotherapy, as most cytotoxic chemotherapies rely on rapidly proliferating cells. The combination of palbociclib with lutezolone, an aromatase inhibitor, was recently approved by the FDA for treatment of breast cancer based upon promising phase II clinical trial results (28). Several combinations with CDK4/6 inhibitors utilizing a combination of a cell-cycle inhibitor with an inhibitor of an activated signaling pathway are currently in clinical trials for patients with solid tumors. These include clinical trials of palbociclib with tramtinib, a MEK inhibitor, in patients with solid tumors; LEE011 with everolimus, an aromatase inhibitor, in patients with breast cancer; and LEE011 and LGG1816, a BRAF inhibitor, in patients with BRAF-mutant solid tumors (29–31). For patients with myelofibrosis, a phase I trial is currently evaluating the safety of combining PIM447, a PIM kinase inhibitor, with ruxolitinib, a JAK inhibitor, and LEE011 (32). Combination studies of CDK4/6 inhibitors with other drugs have not been reported for models of acute leukemia.

We have tested the combination of LEE011 with standard chemotherapy agents used to treat ALL (methotrexate, mercaptopurine, 1-asparaginase, glucocorticoids, and doxorubicin) and with experimental agents (everolimus, IQ1, bortezomib, and Compound E). Many chemotherapy agents, such as mercaptopurine and methotrexate, rely on rapidly proliferating cells for activity. Combinations of these drugs with a CDK4/6 inhibitor would thus be predicted to be antagonistic. Indeed, we found LEE011 to be antagonistic when simultaneously or sequentially administered with mercaptopurine, methotrexate, doxorubicin, or 1-asparaginase in T-ALL cell lines. While we have found CDK4/6 inhibitors to be antagonistic with doxorubicin in T-ALL and others report antagonism in breast cancer (12,13), there is evidence that in some cellular contexts this combination may enhance cell death, such as in MYCN-amplified T-34P5 and wild-type neuroblastoma (33). Importantly, TPS3 is mutated in the T-ALL cell lines that we have studied (34). Similarly, while paclitaxel has been reported to be antagonistic in breast cancer, in some KRAS mutant lung adenocarcinoma cell lines, it has been reported to be synergistic (35). Moreover, there are examples of chemotherapy agents not tested in this study that have been combined with CDK4/6 inhibitors without antagonism between the two drugs, such as in the case of cytarabine in AML (36) and gemcitabine in a Calu-6 lung cancer xenograft model (37). Thus, there may be cell context, genotype, and specific cytotoxic drug determinants modifying the final outcome of drug combinations.

We focused on two promising synergistic drug combinations with LEE011 that can be rapidly translated to clinical trial, glucocorticoids, and everolimus, although further study of GSI in combination with CDK4/6 inhibitors is warranted given the synergy we observed in vitro in NOTCH1-mutant T-ALL cells. Glucocorticoids have a variety of effects on ALL cells, including cell-cycle arrest and induction of programmed cell death. One mechanism of glucocorticoid activity is downregulation of D-type cyclins, particularly cyclin D3 (33–35). Everolimus is an inhibitor of the mTOR and has shown to have clinical efficacy in patients with leukemia. Inhibition of the mTOR signaling pathway inhibits the G1-S transition, partly via suppression of CCND3 (36). In addition, mTOR inhibitors have been reported to increase glucocorticoid sensitivity in glucocorticoid-resistant ALL (37). Treatment of T-ALL cell lines with LEE011 caused an increase in CCND3 protein levels by Western blotting, a possible mechanism attenuating activity of the drug. CCND3 was decreased by both
dexamethasone and everolimus by Western blotting, with a decrease in CCND3 protein with the combination of LEE011 and dexamethasone or LEE011 and everolimus, a possible mechanism for these synergies. Consistent with these findings, in a pancreatic ductal carcinoma model, mTOR/Pi3K inhibitors enhanced the effects of palbociclib, an effect also thought to be

Figure 6.
LEE011 enhances response to dexamethasone in a PDX model of relapsed T-ALL. A primary pediatric patient T-ALL sample was transplanted into NSG mice and disease established. Spleen cells from this PDX were reinfused into NSG mice and leukemia burden monitored by peripheral blood hCD45 staining. Once leukemia burden was greater than 20% in peripheral blood as measured by human CD45 staining, mice were assigned to four treatment groups: vehicle, LEE011, dexamethasone, or the combination of LEE011 with dexamethasone. A, Leukemia burden was monitored by CD45 staining at indicated time points in 3 mice per group. Shown is the average percent of CD45+ cells; error bars represent SD for 3 mice. Mice were treated for 21 days. *, P < 0.05; **, P < 0.0001 calculated using multiple t tests with Holm–Sidak correction. B, Kaplan–Meier curves showing overall survival of mice (n = 10/group). P value calculated using log-rank test. P < 0.0001 between the group treated with combination of LEE011 and dexamethasone and any individual treatment groups. C, Spleens were collected from 1 mouse per group after 5 days of treatment. D, Histology of mice treated with vehicle, LEE011, dexamethasone, and the combination of LEE011 and dexamethasone. Mice were treated for 5 days and tissue collected 1 hour after dosing. Shown are representative sections of spleen stained with p-RB S807/811. Images were taken at ×400 magnification. Scale bar, 100 μm. E, pRB levels from peripheral blood cells measured by flow cytometry from mice treated for 5 days with drugs as indicated. Each bar represents a single mouse.
mediated via the inhibitory effect of mTOR/P13K inhibitors on a palbociclib-mediated increase in cyclin E1 and cyclin D1 (38). In addition, LEE011 treatment caused a mild decrease in phosphorylation of P70-S6 kinase. Cyclin D1/CDK4 complex has previously been shown to interact with TSC2, upstream of mTOR, and more recently, suppression of CDK4 using the CDK4/6 inhibitor abemaciclib was shown to reduce TSC2 phosphorylation, leading to a reduction in P70-S6K activity in breast cancer (39, 40). Regulation of the mTOR pathway by the CDK–cyclin D complex may thus be another mechanism contributing to the synergy between LEE011 and everolimus.

For effective use of targeted therapy, it will be important to select patients who are likely to respond to treatment based on molecular characteristics. We tested a panel of 10 T-ALL cell lines with LEE011. Cell lines harboring deleterious mutations in RB1, HSB2, and S UPI T1 did not respond to LEE011. Deleterious events in RB1 can be identified in 8% to 12% of patients with T-ALL (41, 42), and effective implementation of this drug in clinical trials will need to exclude patients with RB1 mutations. Activated NOTCH1 has previously been reported to be a biomarker of response to CDK4/6 inhibitors. Analysis of data from the Genomics of Drug Sensitivity in Cancer Project database showed T-ALL cell lines as a group to be differentially sensitive to PD-0332991. NOTCH1 mutations did not appear to be a biomarker of response within the T-ALL subset of cell lines, although this analysis is limited by having only three NOTCH1 wild-type cell lines. A clinical trial in T-ALL of a CDK4/6 inhibitor will be needed to definitively determine whether clinical response is based on NOTCH1 mutational status, but our data suggest that NOTCH1 mutation should not be a requirement for entry onto initial trials testing these inhibitors.

Measuring drug levels and on-target activity of targeted therapies in combination is important for interpretation of results of early-phase clinical trials. In this study, we used flow cytometry to evaluate the effect of LEE011 and everolimus on its respective targets, RB and 4EBP1, in peripheral blood from mice receiving the drugs. Future preclinical and clinical studies may use these assays for ensuring on-target activity of the drug in its recipients. Our efforts to measure serum levels in two models showed no alteration in LEE011 levels in combination with dexamethasone in one of the models but a slight decrease in LEE011 levels in a second study but only at the earliest time point. Therefore, it will be important to carefully assess for drug–drug interactions in the context of any clinical trials testing CDK4/6 inhibitors in combination with glucocorticoids.

Given prior studies reporting CDK4/6 inhibitor activity in mouse models of T-ALL, we have focused this investigation on T-ALL, although the utility of these drug combinations will likely extend to the treatment of other acute leukemias. For example, recent studies have shown CDK6 to be a direct target of MLL-AF9 in AML and MLL-AF4 in infant ALL (43). Although CDK4/6 inhibitors may be active in these types of acute leukemia, and particularly in infant ALL, the drug combinations that we have identified should be tested in preclinical studies. Thus, discovery of combination therapies with CDK4/6 inhibitors has impact for the treatment of numerous hematologic malignancies and our findings may be relevant beyond T-ALL.

In summary, this work supports the testing of CDK4/6 inhibitors in the treatment of T-ALL. Successful implementation of new drugs for the treatment of leukemia requires effective combination therapies. In this study, we have discovered novel synergistic combinations between LEE011 and corticosteroids and LEE011 and everolimus that could be readily translated to a clinical trial for patients with T-ALL.

Disclosure of Potential Conflicts of Interest
K. Stegmaier is a consultant for and reports receiving commercial research grants from Novartis. No potential conflicts of interest were disclosed by the other authors.

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Grant Support
This research was supported by Team Ari and the Boston Children’s Hospital Translational Research Program Pilot grant. K. Stegmaier is a Leukemia and Lymphoma Society Scholar. Y. Pikman is the recipient of an Alex’s Lemonade Stand Young Investigator Award and NIH National Institute of Child Health & Human Development (SK12HD052896-09).

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Received November 25, 2015; revised September 20, 2016; accepted October 13, 2016; published OnlineFirst November 9, 2016.

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Clin Cancer Res 2017;23:1012-1024. Published OnlineFirst November 9, 2016.

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