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Synergistic Drug Combinations with a CDK4/6 Inhibitor Targeting T-Cell Acute Lymphoblastic Leukemia

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Synergistic Drug Combinations with a CDK4/6 Inhibitor Targeting T-Cell Acute Lymphoblastic
Leukemia

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ABSTRACT

Leukemia is the most common cancer in children and teens, accounting for about 25% of pediatric cancer. The most common type of pediatric leukemia is acute lymphoblastic leukemia (ALL), with T-cell acute lymphoblastic leukemia (T-ALL) accounting for 15% to 20% of all ALL. While significant progress has been made in the treatment of T-ALL, patients with relapsed or refractory disease are unlikely to be cured. Cyclin D3 and CDK6 are two proteins that are highly expressed in T-ALL and contribute to this disease mechanism. The development of CDK4/6 inhibitors offers a promising treatment for T-ALL. However, combination therapy will be needed for the successful integration of CDK4/6 inhibitors in the treatment of human T-ALL.

In our study, we evaluated LEE011, a specific CDK4/6 inhibitor, in combination with standard chemotherapy or everolimus. We first determined that both NOTCH1 wildtype and mutant T-ALL are highly sensitive to LEE011 inhibition. Next, we determined that LEE011 is antagonistic with many of the standard chemotherapy that are currently used to treat T-ALL, including methotrexate, 6-mercaptopurine, doxorubicin, L-asparaginase, and vincristine. We also determined that LEE011 is synergistic with glucocorticoids and everolimus. The combination of LEE011 with dexamethasone prolonged survival in an orthotopic mouse model of T-ALL. We conclude that LEE011 is active against T-ALL and that combination therapy with LEE011 and glucocorticoids or mTOR inhibitors could be effective in treating T-ALL.

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Thanks also to my family and my dear friends. I am incredibly lucky to have you all by my side through this process!

INTRODUCTION

The Process of Hematopoiesis

Hematopoiesis is the process by which all new blood cells form. Hematopoietic stem cells (HSCs) reside in the bone marrow and can give rise to all types of blood cells (Orkin and Zon, 2008). HSCs give rise to myeloid and lymphoid progenitor cells, which can further differentiate to form mature blood cells (Figure 1). The myeloid progenitor leads to the production of red blood cells (erythrocytes), megakaryocytes, monocytes, neutrophils, basophils, and eosinophils, while the lymphoid progenitor contributes to the production of lymphocytes, including B and T cells.

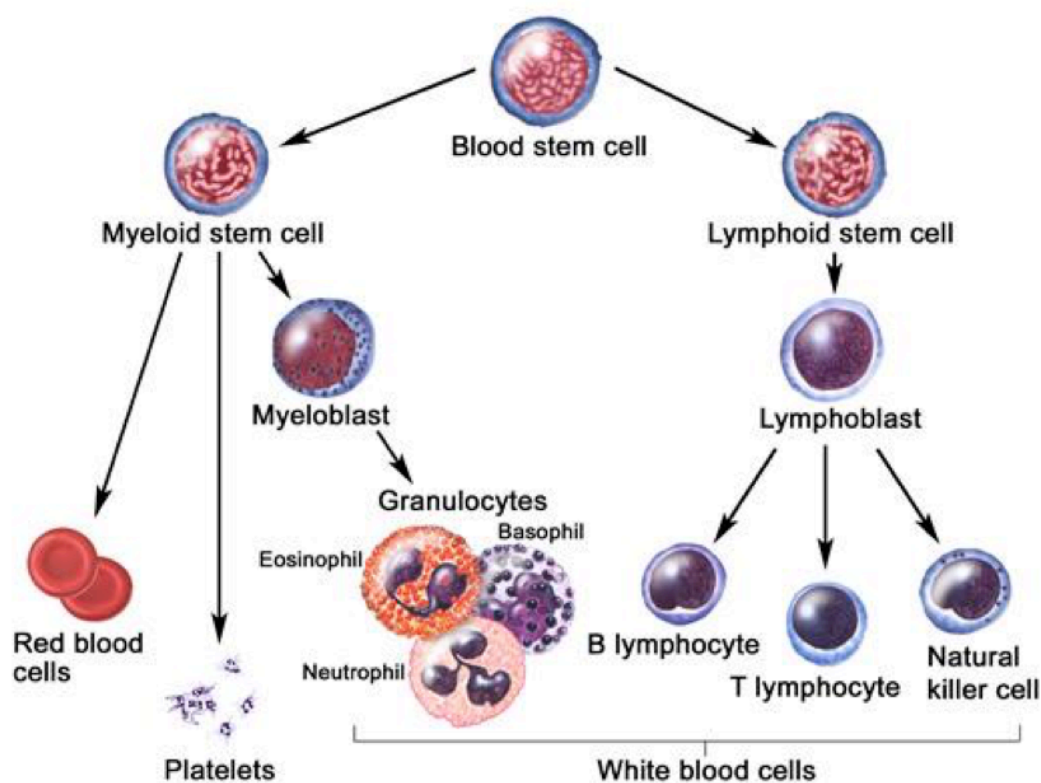


Figure 1: Schematic representation of the different stages in hematopoiesis (Cancer.gov). In normal hematopoiesis, blood stem cells can develop either along the myeloid or lymphoid lineage.

Cell Cycle and Its Controls

HSCs divide and differentiate (mature) to produce the myeloid and lymphoid progenitor cells. The cell cycle, a critically controlled process in hematopoietic cell maturation, is the series of events that take place in a cell, leading to the cell duplicating its DNA and dividing to produce two daughter cells (Cho et al., 1998; Nasmyth, 1996). In eukaryotes, the cell cycle is divided into three periods: interphase, mitosis (M), and cytokinesis. The interphase stage can be further divided into the G1 phase, during which the cell grows; synthesis (S) phase, when it duplicates its DNA; and G2 phase, when it grows and prepares for division in mitosis (Nasmyth, 1996). The cell can also exit the cell cycle (G0 phase) and stop dividing.

Cell cycle checkpoints ensure that each phase is completed accurately before the cell is able to proceed to the next phase (Blagosklonny and Pardee, 2002; Nigg, 1995). The checkpoints occur at junctions between cycles, at the G1/S transition, the G2/M transition, and the Metaphase/Anaphase transition within the M phase (Figure 2). Cyclins are proteins that are key components of this checkpoint machinery. They bind, activate, and provide substrate specificity to cyclin-dependent kinases (CDKs), proteins that are critical to this transition (Blagosklonny and Pardee, 2002; Nigg, 1995). Different cyclin-CDK complexes mediate the checkpoints by phosphorylating appropriate downstream substrates to enable transition through each phase of the cell cycle (Figure 3). For example, the CDK4/6-Cyclin D complex is critical for the G1/S transition.

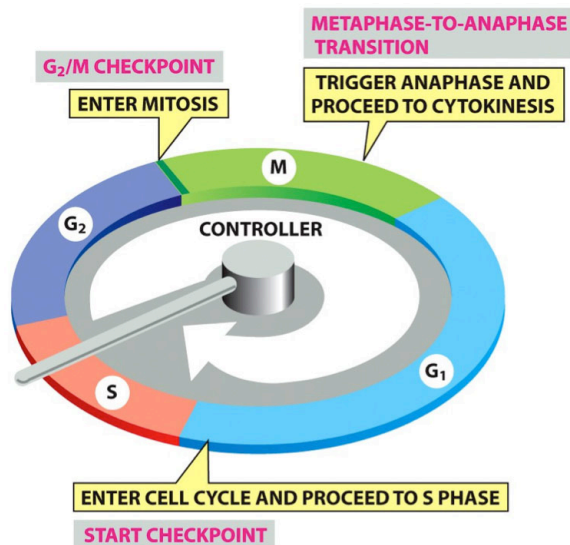


Figure 2: Three major checkpoints (G₁/S, G₂/M, and Metaphase/Anaphase) regulate the normal cell cycle (Alberts et al., 2007).

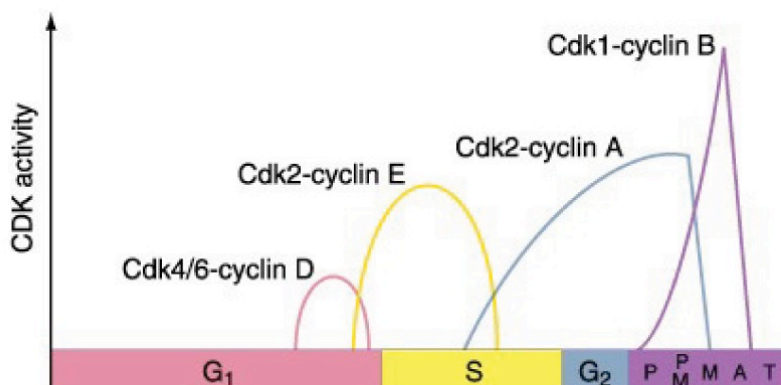


Figure 3: Cell cycle checkpoints are mediated by various CDK-cyclin complexes (Alberts et al., 2007).

D-type cyclins are the ultimate recipients of mitogenic and oncogenic signals from the environment and other cellular proteins (Sicinska et al., 2003). There are three different D-type cyclins (D1, D2, and D3) in mammalian cells with substantial amino acid similarity. These cyclins are expressed in a highly overlapping fashion in all proliferating cells (Sherr and Roberts, 1999). When D-type cyclins are induced, they bind and activate their associated CDKs, namely CDK4 and CDK6 (Dowdy et al., 1993; Kato et al., 1993). The CDK4/6-Cyclin D complexes

then phosphorylate the retinoblastoma (Rb) tumor suppressor protein (Blagosklonny and Pardee, 2002; Dowdy et al., 1993; Kato et al., 1993). This phosphorylation cancels growth inhibitory functions of Rb, prompting the release of the E2F transcription factors, which allows for the induction of E2F target genes that are required for the S phase to proceed (Figure 4; Weinberg, 1995). Therefore, D-type cyclins and CDK4/6 are critical to proper, controlled cell cycle progression.

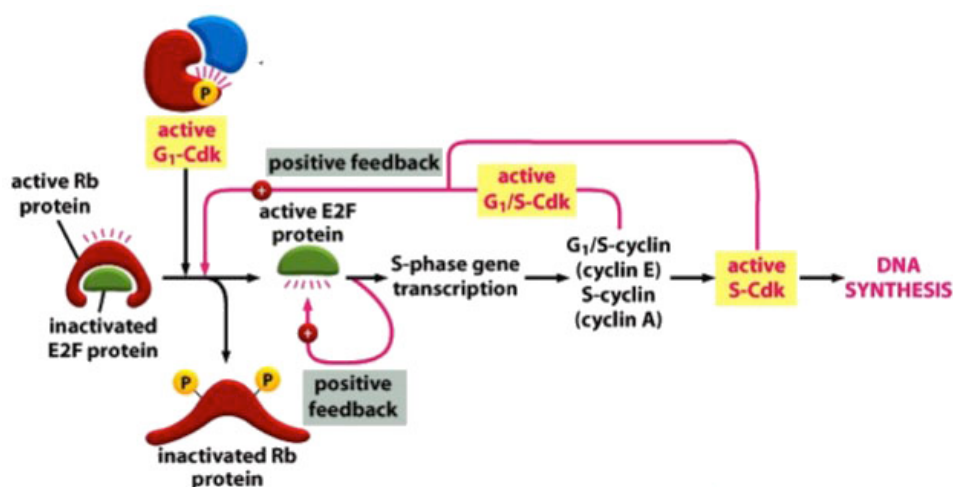


Figure 4: CDK4/6-Cyclin D complex initiates the phosphorylation of the Rb protein (Alberts et al., 2007)

Dysregulation of the Cell Cycle Leads to Cancer

Consistent with its growth promoting functions, abnormal expression of D-type cyclins is believed to be a driving force in several human cancers. Chromosomal abnormalities involving Cyclin D loci, resulting in overexpression of Cyclin D proteins, are observed in many malignancies. For example, the *cyclin D1* gene is rearranged or amplified and the protein is overexpressed in breast carcinomas, squamous cell carcinomas of the head and neck, and astrocytomas (Bartoka et al., 1995; Dickinson et al., 1995; Lammie et al., 1991; Weinstatt-Saslow et al., 1995). The *cyclin D2* gene is amplified in human testicular tumors, while the

protein is overexpressed in a wide range of B cell lymphomas and chronic lymphocytic leukemias (Delmer et al., 1995; Houldsworth et al., 1997; Motokura and Arnold, 1993; Sicinski et al., 1996). Cyclin D3 is overexpressed in B cell lymphomas, multiple myeloma, and human T-cell leukemias (Filipits et al., 2002; Shaughnessy et al., 2001; Sicinska et al., 2003). Overexpression of Cyclin D leads to the dysregulation of the G1/S transition, causing uncontrolled cellular proliferation and contributing to cancer formation.

Introduction to Leukemia

Leukemia, a cancer of the blood, arises from mutations in hematopoietic stem/progenitor cells, myeloid or lymphoid, found in the bone marrow. Abnormal and unregulated proliferation and impaired differentiation of cells in the lymphoid lineage, specifically lymphoblasts, cause acute lymphoblastic leukemia (ALL). The proliferating lymphoblasts displace the normal hematopoietic elements within the bone marrow. This leads to a life-threatening decrease in the normal production of red blood cells, effective white blood cells, and platelets. The absence of normal blood cells and the rapid proliferation of abnormal lymphoblasts lead to death if not treated promptly (Kampen, 2012).

Leukemia is the most common pediatric cancer, represents approximately 25% of all pediatric cancer, and is the second most common cause of cancer-related deaths in children and young adults (Lls.org). The most common type of pediatric leukemia is ALL. This disease most commonly affects children younger than five years of age, usually peaking between the ages of two and four (Cancer.net). In 2015, it was estimated that 2,670 children ages 14 and younger and 410 adolescents between the ages of 15 and 19 were diagnosed with ALL (Cancer.net).

ALL can be subdivided into B-cell ALL (B-ALL) and T-cell ALL (T-ALL), depending on the type of lymphoblast from which it is derived. B-ALL is more prevalent, accounting for 80% to 85% of cases in children with ALL; while T-ALL accounts for 15% to 20% of pediatric ALL cases (Cancer.org). In pediatrics, there are several risk stratifications for ALL, depending on age, white blood cell count at diagnosis, lineage (T-ALL versus B-ALL), and other molecular features (Pui and Evans, 2006). Treatment intensity for ALL is determined by these risk factors.

The main treatment for childhood leukemia is cytotoxic chemotherapy and sometimes cranial radiation, which for ALL is delivered over the span of at least two years. For some children with more aggressive leukemia, high-dose chemotherapy may be combined with allogeneic stem cell transplantation. Treatment of acute forms of childhood leukemia is very intensive, highly toxic, and with numerous potential acute and long-term side effects. Though the intensity of treatment is somewhat tailored to the child's specific leukemia subtype and prognostic factors, most chemotherapy affects rapidly proliferating cells and is not specific for cancer versus normal dividing cells. Thus, many children pay a steep price during leukemia-directed therapy, accumulating chemotherapy-associated toxicities if they are to survive their disease.

T-ALL and Chemotherapy

Current chemotherapy regimens to treat T-ALL are based on using a combination of therapies that target different parts of the cell cycle or cellular machinery and coordinate to effectively kill the proliferating cells. Since most traditional chemotherapy agents target rapidly proliferating cells, cellular mechanisms, such as DNA replication and cell division, are affected. Currently, a standard chemotherapy to treat T-ALL includes a combination of six drugs over the

span of a two to three year period: methotrexate, 6-mercaptopurine, doxorubicin, L-asparaginase, vincristine, and glucocorticoids. Additionally, a targeted therapy with the mTOR inhibitor, everolimus, has also shown efficacy against ALL (Baraz et al., 2014). The pathways affected in T-ALL and their respective treatments are described below (Figure 5).

Chemotherapy Impairing Nucleotide Synthesis

Folic acid is required by mammalian cells for the *de novo* synthesis of purine and thymidine nucleotides, which are required for DNA replication and repair (Jackman et al., 2008). Proteins of the folate metabolism pathway were therefore identified as anti-cancer drug targets in the 1940s, first with the use of aminopterin in the treatment of pediatric ALL (Jackman et al., 2008). Subsequently, multiple derivatives, such as methotrexate, were synthesized. Methotrexate inhibits dihydrofolate reductase (DHFR), an enzyme essential for maintaining folates in the fully reduced tetrahydrofolate form in proliferating tissues. Thus, inhibition of DHFR leads to impaired nucleotide biosynthesis.

Purine nucleotides are also critical for DNA and RNA synthesis, and thus cell division and proliferation. Phosphoribosyl pyrophosphate (PRPP) amidotransferase is the enzyme that catalyzes the rate-limiting step in purine synthesis. Similar to the function of methotrexate, 6-mercaptopurine also acts as an antimetabolite. 6-mercaptopurine inhibits purine nucleotide synthesis by interfering with PRPP amidotransferase, which catalyzes nucleotide interconversion (Dubinsky et al., 2000).

Topoisomerase Inhibitors

Topoisomerase is an essential enzyme that relaxes DNA supercoiling during replication and transcription. It controls the changes in DNA structure by catalyzing the breaking and rejoining of the phosphodiester backbone in DNA strands. In order to halt cancer cell replication, topoisomerase inhibitors block topoisomerase function, resulting in single and double stranded DNA breaks that harm the integrity of the genome and lead to cell death. Doxorubicin is one such chemotherapy agent that intercalates into DNA, interfering with the action of topoisomerase II (Bodley et al., 1989).

Chemotherapy Inhibiting Protein Synthesis

Cells need L-asparagine, a non-essential amino acid, to support protein synthesis and metabolic requirements. Because asparagine is a non-essential amino acid, normal cells synthesize it from aspartate and amine via asparagine synthetase, while leukemia cells must rely on high levels of exogenous circulating asparagine. L-asparaginase is an enzyme derived from *E. coli*, which breaks down L-asparagine into aspartic acid and ammonia (Piatkowska-Jakubas et al., 2008). Depletion of L-asparagine by L-asparaginase leads to the inhibition of protein synthesis and other metabolic alterations, resulting in subsequent cell apoptosis (Piatkowska-Jakubas et al., 2008).

Microtubule Inhibitors

During mitosis, after DNA replication, chromosomes align and then separate, so each daughter cell has one set of chromosomes. Tubulin proteins are critical to chromosome separation and form the “railroad” along which they separate. Because leukemia cells require

separation of their chromosomes as part of cell division, inhibition of this separation can be an effective therapy. Vincristine is an inhibitor that binds to the tubulin proteins, impairing chromosome separation during metaphase (Jordan, 2002). This impairs the M phase of the cell cycle, leading to cell death.

Glucocorticoid Therapy in ALL

Induction of cell cycle arrest and apoptosis affects proliferating cells. Glucocorticoids, such as dexamethasone and prednisolone, are currently part of the standard backbone of chemotherapy for T-ALL and can induce G1 cell cycle arrest in lymphoid cells via the glucocorticoid receptor. The mRNA encoding Cyclin D3, a G1 progression factor, is rapidly down-regulated when a glucocorticoid is added to T-ALL, leading to the destabilization and eventual degradation of the genetic material (Resiman and Thompson, 2013).

In addition to cell cycle arrest, glucocorticoids also induce apoptosis by intracellular mechanisms controlled by members of the Bcl-2 family and the mitochondria. In turn, this directly affects downstream caspases that play an essential role in programmed cell death (Schlossmacher et al., 2011). Ausserlechner et al. (2004) found that while Cyclin D3 was essential for glucocorticoid-induced G1 cycle arrest in human ALL cells, it was not required for apoptosis. Therefore, even though cell cycle arrest and apoptosis are both affected by glucocorticoids, these processes act independently of each other in the cell.

Targeted Therapy in T-ALL

The phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling pathway governs cellular growth, survival, and metabolism.

However, it is constitutively active in T-ALL often due to mutations (Fransecky et al., 2015). The mTOR inhibitor, everolimus, has shown pre-clinical efficacy against acute lymphoblastic leukemia cells by inducing caspase-independent cell death (Baraz et al., 2014). Induction with this targeted therapy shows T-ALL cells with morphology consistent with paraptosis, another type of programmed cell death (Baraz et al., 2014). Everolimus is currently being tested in combination with vincristine, corticosteroids, doxorubicin, and L-asparaginase in a Phase I clinical trial for patients with relapsed ALL (Dana-Farber Cancer Institute).

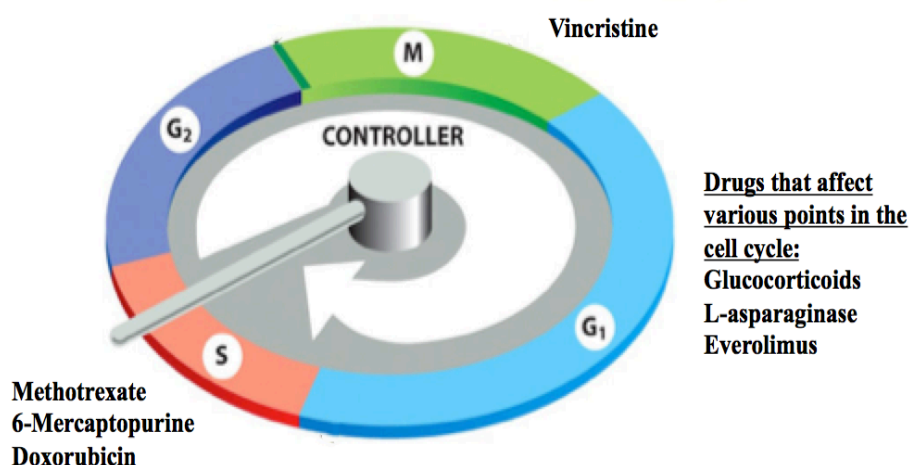


Figure 5: Chemotherapy and targeted therapy that affect the cell cycle are labeled accordingly (Adapted from Alberts et al., 2007).

Poor Outlook for Pediatric Patients with Relapsed T-ALL

While significant progress has been made in the treatment of T-ALL using a combination of chemotherapy drugs, approximately 20% of newly diagnosed pediatric and 50% of adult patients will experience either induction failure or relapse of their disease often due to drug resistance (Gökbuget, et al. 2012). Additionally, fewer than 50% of patients with T-ALL who experience a relapse are long-term survivors despite intensive chemotherapy regimens,

including stem cell transplantation. The poor outlook for pediatric patients with relapsed T-ALL calls for more effective therapy for these patients.

Notch Signaling Mediates G1/S Cell Cycle Progression in T-ALL via Cyclin D3 and CDK4/6

In the hematopoietic system, the NOTCH1 receptor functions as a ligand activated transcription factor that directly transduces extracellular signals in the cell surface into changes in gene expression in the nucleus. NOTCH1 activation and signaling play a critical role at multiple stages of T-cell development and function. A constitutively active form of NOTCH1 causes ectopic T-cell development in the bone marrow of immunodeficient mice and limited B lymphocyte production (Tanigaki and Honjo, 2007).

Activating *NOTCH1*-mutations, leading to aberrant downstream signaling, have been identified in human T-ALL. More than 50% of human T-ALLs bear mutations in *NOTCH1*, indicating a prominent role for NOTCH1 in this malignancy (Joshi et al., 2009). Gamma secretase inhibitors (GSI), which block proteolytic activation of Notch receptors, abrogate the growth of human and murine T-ALL cell lines bearing *NOTCH1* gain-of-function mutations, indicating that NOTCH1 is required in established tumors (Joshi et al., 2009; O'Neil et al., 2006).

NOTCH1 signaling is also important to the G1/S progression of the cell cycle in T-ALL. Cyclin D3 is a direct transcriptional target of activated NOTCH1, so T-ALL often up-regulates Cyclin D3. T-ALL cell lines treated with GSI show reduced Cyclin D3 expression compared to the control treatment (Joshi et al., 2009). Additionally, CDK4 and CDK6 expressions are also reduced by 50% following GSI treatment, suggesting that CDK4 and CDK6 are two putative targets of NOTCH1 regulation in T-ALL cell lines (Joshi et al., 2009). The downstream effect of

these reductions is also associated with a decrease in Rb phosphorylation (Joshi et al., 2009). Therefore, Cyclin D3 and its catalytic partners, CDK4 and CDK6, are NOTCH1-dependent targets that are important for T-ALL proliferation.

Requirement of Cyclin D3 in T-cell Development, Mouse T-cell Malignancies, and Human T-ALL Cell Lines

Since Cyclin D3 is a direct target of NOTCH1, Cyclin D3 too has a critical role in T-cell development. Sicinska et al. (2003) showed that Cyclin D3 is downstream of the pre-TCR and drives the proliferation of immature T lymphocytes. In the absence of Cyclin D3, the normally assembled pre-TCR fails to drive the expansion of immature thymocytes. Cyclin D3 is therefore required for the proliferative burst during development of immature T lymphocytes (Sicinska et al., 2003).

Cyclin D3 also plays a critical role in the maintenance of T-cell ALL and lymphomas. Sicinska et al. (2003) showed that Cyclin D3 was required for leukemias induced by the activated, intracellular form of NOTCH1. While infection of wild-type bone marrow with activated NOTCH1 led to the appearance of T-ALL leukemias in 100% of recipient mice two weeks after bone marrow transduction, mice containing NOTCH1-activated Cyclin D3^{-/-} bone marrow remained leukemia-free throughout the observation period (Sicinska et al., 2003). These results indicate that Cyclin D3 is required for the NOTCH1-mediated oncogenesis that signals through the pre-TCR (Sicinska et al., 2003).

In addition to mouse T cell malignancies, Cyclin D3 expression is also required for the oncogenic proliferation of human T-ALL cell lines. In twelve T-ALL cell lines corresponding to immature thymocytes, knockdown of Cyclin D3 levels using siRNA impacted cell proliferation due to cell cycle arrest (Sicinska et al., 2003). Therefore, Cyclin D3 is required for proliferation

of human T-ALL derived from immature T cells, lending to the possibility of novel therapeutic approaches to treat T-ALL by inhibiting Cyclin D3.

Inhibition of CDK4/6-Cyclin D Complex Activity Suppresses Human T-ALL Cell Growth

Based on the role of Cyclin D3 and CDK6 in T-ALL, several research groups tested an inhibitor of this pathway in mouse T-ALL models. PD0332991 is a CDK4/6 specific small-molecule inhibitor that blocks Rb phosphorylation, a hallmark of a G0/G1 arrest (Sawai et al., 2012). This inhibitor also efficiently inhibited S-phase entry of all T-ALL lines carrying *NOTCH1* mutations within 15 hours, leading to an accumulation of cells in the G0/G1 phase (Sawai et al., 2012). After a 4-day exposure to PD0332991, there was a significant increase in Annexin V expression in treated cells compared to the controls, indicating progression to cell death after treatment (Sawai et al., 2012). Based on its ability to induce cell cycle arrest and apoptosis in leukemia cells, PD0332991-mediated inhibition of CDK4/6-Cyclin D3 activity was postulated to be an attractive therapy for T-ALL (Choi et al., 2012; Sawai et al., 2012).

PD0332991 versus LEE011

As with PD0332991, LEE011, developed by Novartis Pharmaceuticals, is an ATP-competitive inhibitor that interacts with cyclin-dependent kinases within their catalytic ATP-site (Mariaule and Belmont, 2014). Both molecules are selective inhibitors of CDK4 and CDK6 at clinically achievable doses. The major difference between the two compounds lies in the bicyclic core. LEE011 possesses a pyrrolo-pyrimidine, and PD0332991, a pyridopyrimidine (Figure 6; shown in black). A crucial step in the development of PD0332991's selectivity for CDK4/6 was the introduction of a 2-aminopyridyl substituent at the C2-position of pyrido[2,3-d]pyrimidin-7-

one core pharmacophore (Toogood et al., 2005). In enzymatic assays, PD0332991 displays potent inhibitory activities against the Cyclin D3 complex with CDK4 at an IC_{50} value of 11 nmol/L and with CDK6 at an IC_{50} value of 16 nmol/L (Sherr et al., 2016). Similarly, LEE011 has an IC_{50} value of 10 nmol/L for CDK4 and 39 nmol/L for CDK6 (Sherr et al., 2016). However, there are still also undesirable off-target effects associated with these compounds on other kinases, including on CDK1 and CDK2. With PD0332991, the IC_{50} values associated with CDK1 and CDK2 are both around 10 μ mol/L, while for LEE011, they are around 100 μ mol/L and 50 μ mol/L, respectively (Sherr et al., 2016). Therefore, the efficacy of LEE011 as a CDK4/6 inhibitor lies in its low nanomolar inhibition of CDK4 and CDK6 as well as its selectivity over the other CDKs.

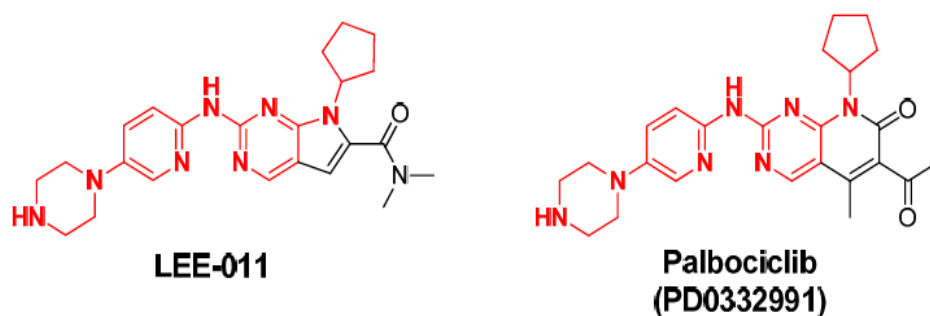


Figure 6: Comparison of the chemical structures for Novartis' LEE011 and Pfizer's PD0332991 structures (Mariaule and Belmont, 2014).

Combination Therapy with CDK4/6 Inhibitors

Currently, there are numerous clinical trials testing CDK4/6 inhibitors in patients with breast cancer, lung cancer, and other solid tumors. Despite pre-clinical evidence for CDK4/6 inhibition in leukemia, these drugs are not currently being tested in this disease. Given the rapid cellular proliferation and progression of acute leukemia, it is unlikely that a single drug that causes cell cycle arrest will be effective as a single agent. Additionally, combination therapy,

with drugs targeting multiple cellular mechanisms, has been necessary for curative leukemia treatment. CDK4/6 inhibitors induce G1 cell cycle arrest, thus combining these drugs with other molecules is critical for the successful translation of this drug class for leukemia treatment. Historically, new targeted drugs have been introduced to patients in combination on a backbone of standard-of-care cytotoxic chemotherapy. The combinations of CDK4/6 inhibitors with cytotoxic chemotherapy are predicted to be antagonistic when given concurrently, however, as most cytotoxic drugs rely on rapidly proliferating cells, and CDK4/6 inhibition induces cell cycle arrest. Combination studies of CDK4/6 inhibitors with other chemotherapy in leukemia have not been reported but are critically needed.

Thesis Aim

Despite significant progress in the treatment of T-ALL, patients who experience relapsed disease or whose disease is refractory to standard therapy, are unlikely to be cured. Since Cyclin D3 and CDK6 are both upregulated in T-ALL, CDK4/6 inhibition may be a viable therapeutic option for T-ALL treatment. However, single agent therapy is unlikely to be effective in treating acute leukemia. In this project, we aim to identify novel synergistic combinations with the CDK4/6 inhibitor, LEE011, and standard chemotherapy or everolimus that could be readily translated to effectively treat patients with T-ALL. In order to accomplish this goal, we have determined the following:

1. LEE011, Novartis's CDK4/6 inhibitor, is effective against a panel of T-ALL cell lines, regardless of *NOTCH1* mutation status.
2. *RBI* loss renders T-ALL cell lines insensitive to CDK4/6 inhibition.

3. LEE011 is antagonistic with methotrexate, 6-mercaptopurine, doxorubicin, L-asparaginase, and vincristine.
4. LEE011 is synergistic with glucocorticoids and mTOR inhibitors.

Through the use of combination drug studies, we have developed a better understanding of the synergistic and antagonistic drug combinations with LEE011 in T-ALL. Going forward, researchers at Dana-Farber Cancer Institute will be working to design a pediatric clinical trial of LEE011 in combination with glucocorticoids and mTOR inhibitors for the treatment of relapsed or refractory acute lymphoblastic leukemia.

EXPERIMENTAL METHODS

Cell Culture and Cell Viability

Human T-ALL cell lines were provided by Dr. Jon Aster from the Brigham and Women's Hospital. All the cell lines in our experiments were maintained in RPMI 1640 (Cellgro) supplemented with 1% penicillin/streptomycin (Cellgro) and 10% FBS (Sigma-Aldrich) at 37°C with 5% CO₂. Cell viability was evaluated using CellTiter-Glo Luminescent Cell Viability Assay (Promega) to quantify the effects of exposure to the specific drug or combination of drugs on day 3 and day 6 after treatment. Cell luminescence was measured using FLUOstar Omega (microplate reader) from BMG Labtech. The IC₅₀ values were determined using the Prism GraphPad software.

Compounds

LEE011 and everolimus were provided by Novartis. Methotrexate, 6-mercaptopurine, dexamethasone, and prednisolone were purchased from Sigma. Doxorubicin was purchased from Cell Signaling. L-asparaginase and vincristine were purchased from the Dana-Farber Cancer Institute pharmacy. Compound E (GSI) was purchased from Santa Cruz Biotechnology.

Drug Interaction Analysis

The expected dose-inhibitory fraction relationships for the combination therapy of LEE011 and each of the seven compounds (methotrexate, 6-mercaptopurine, doxorubicin, L-asparaginase, vincristine, dexamethasone and everolimus) were assessed based on the Bliss independence model (Bliss, 1956; Greco et al., 1995).

Bliss Independence Model

The Bliss independence model assumes that the inhibitors have independent mechanisms of action so that they can bind simultaneously and mutually non-exclusively (Bliss, 1956; Greco et al., 1995). The model predicts that if the individual drugs have the inhibitory effects of f_1 and f_2 then the *expected* combined effect of the two drugs would be given by the following equation:

$E(f_{12}) = 1 - (1 - f_1)(1 - f_2) = f_1 + f_2 - f_1f_2$. The difference between the *observed* combined effect f_{12} and the *expected* combined effect of the two drugs is called the Excess over Bliss (eob):

$$eob = f_{12} - E(f_{12}).$$

Positive *eob* values indicate synergistic interaction whereas negative *eob* values suggest antagonistic behavior. Null *eob* values mean that there is no drug interaction. For each drug pair, the *eob* scores were depicted as 3D surface plots (Excel MSOffice 10) for all the drug dose combinations.

Immunoblotting

Cells were lysed in Cell Signaling Lysis Buffer (Cell Signaling Technology), containing Complete, EDTA-free Protease Inhibitor Cocktail tablets (Roche Diagnostics), and PhosSTOP Phosphatase Inhibitor (Roche Diagnostics) (Banerji et al., 2012). Cells were then resolved by gel electrophoresis using Novex 4-12% Bis-Tris Gel (Invitrogen), transferred to a nitrocellulose membrane (Bio-Rad), and blocked for one hour in 5% Bovine Serum Albumin (BSA) (Sigma Aldrich) (Banerji et al., 2012). Blots were incubated with primary antibodies to phospho-RBS780 (Cell Signaling Technology), RB (Cell Signaling Technology), CCND3 (Santa Cruz Biotechnology), CDK4 (Neomarkers), CDK6 (Santa Cruz Biotechnology), GAPDH (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 (Perkin Elmer).

Flow Cytometry Analysis

MOLT4 (*NOTCH1*-mutated) and MOLT16 (*NOTCH1*-wildtype) cell lines were incubated with vehicle (DMSO), LEE011, dexamethasone, or the combination of LEE011 and dexamethasone and were analyzed after 24 hours (phospho-RB levels, cell cycle, and apoptosis) and 4 days (apoptosis) of treatment. For evaluation of phospho-RB levels, cells were fixed in 3.5% formaldehyde for 10 minutes, washed with PBS, and incubated in 90% ice-cold methanol for permeabilization for 24 hours at -20°C. Cells were then washed with PBS and incubated in FACS buffer (4% BSA in PBS) overnight at 4°C. Cells were subsequently stained with pRB-S780 antibody (Cell Signaling Technology) for FACS analysis (Cooper et al., 2006). For cell cycle analysis, cells were washed with PBS, and then incubated in 70% ice-cold ethanol for 24 hours at -20°C. Cells were then resuspended in a DNA staining master mix solution containing propidium iodide (1 mg/mL) and RNase (100 mg/mL) (Abcam) for analysis. The Annexin V Apoptosis Staining Kit (e-Bioscience) was used for cell death analysis. Cells were washed with PBS and then with 1X binding buffer. Cells were then resuspended with Annexin V-APC and propidium iodide staining solution and analyzed by flow cytometry.

In Vivo Studies (Reproduced from Pikman et al. (2015). Synergistic Drug Combinations with a CDK4/6 Inhibitor in T-Cell Acute Lymphoblastic Leukemia. Clin Cancer Res unpublished.)

Two million MOLT4 luciferized cells were injected via the tail vein into 8-week-old NSG (immunodeficient) mice (The Jackson Laboratory). Leukemia burden was assessed using non-invasive bioluminescence imaging by injecting mice intraperitoneally with 75 mg/kg d-Luciferin

(Promega), anesthetizing them with 2–3% 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/s/ROI (ph/s/ROI). Once detectable bioluminescence was achieved, the mice were separated into treatment cohorts and drug treatment started. Samples for pathology evaluation were collected in a subset of mice after 5 days of drug treatment. This study was conducted according to a protocol approved by the Dana-Farber Cancer Institute Animal Care and Use Committee.

RESULTS

T-ALL is Highly Responsive to CDK4/6 Inhibitors

Using an independent data set from The Genomics of Drug Sensitivity in Cancer Project, we first aimed to validate the previous finding that *NOTCH1*-mutated T-ALL is sensitive to CDK4/6 inhibition. This study profiled 633 cancer cell lines in a viability assay against 138 compounds using a range of concentrations (Yang et al., 2013). This dataset revealed that T-ALL cell lines were very sensitive to treatment with the CDK4/6 inhibitor, PD0332991 (Figure 7A and 7B). Additionally, the T-ALL cell lines screened in this study have all been characterized in the Catalogue of Somatic Mutations in Cancer database, which includes information on somatic mutations in cancer genes, gene amplifications and deletions, and tissue type and transcriptional data, to allow for identification of biomarkers in response to drug treatment. From this database, *NOTCH1* mutations were found to be a biomarker for response to PD0332991 (Figure 7C). However, among T-ALL cell lines, both *NOTCH1* mutated and wildtype cells were equally as responsive to PD0332991, suggesting that *NOTCH1* mutations are not necessary for inhibitor response in T-ALL lines (Figure 7D).

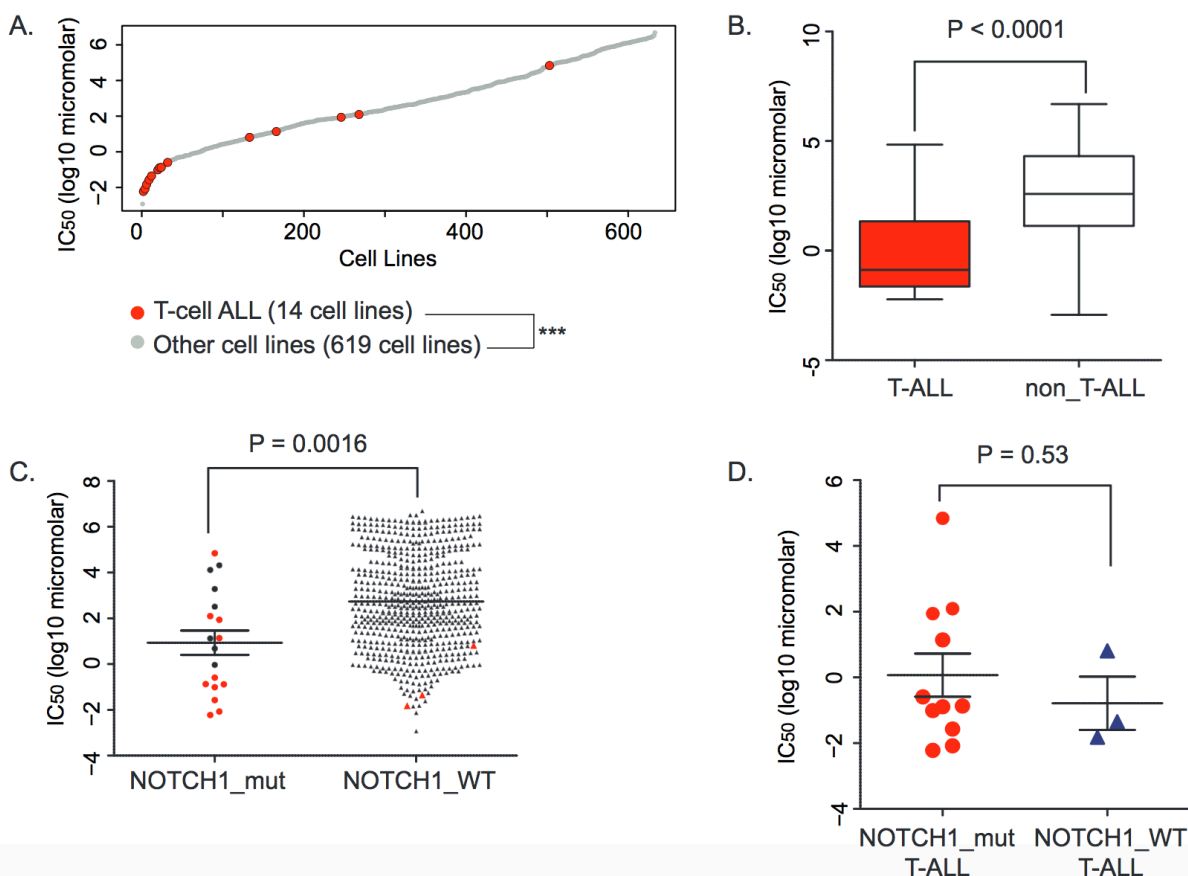


Figure 7: (A, B) The dataset from The Genomics of Drug Sensitivity in Cancer Project profiled 633 cell lines against PD0332991. T-ALL cell lines were found to be very sensitive to a CDK4/6 inhibitor. (C) *NOTCH1* mutations were found to be a biomarker of response to PD0332991 in the Catalogue of Somatic Mutations in Cancer database. (D) Among T-ALL cell lines, *NOTCH1* mutations are not a marker for responsiveness to PD0332991. (Reproduced from Pikman et al. (2015). Synergistic Drug Combinations with a CDK4/6 Inhibitor in T-Cell Acute Lymphoblastic Leukemia. Clin Cancer Res unpublished.)

Similar to PD0332991, LEE011 is also a CDK4/6 inhibitor that functions as an ATP competitive inhibitor. We tested LEE011 in a panel of nine T-ALL cell lines (Figure 8). The cells lines were found to be quite sensitive to the inhibitor since the IC₅₀s ranged between 0.7 μ M to 2.7 μ M after six days of treatment as measured by the CellTiter-Glo ATP-based assay. Confirming the analysis from The Genomics of Drug Sensitivity in Cancer Project, we also found that the T-ALL cell lines that lacked mutated *NOTCH1* were still sensitive to LEE011. This suggests that *NOTCH1* mutations are not required for a strong response to CDK4/6

inhibitors in T-ALL. However, Rb expression was critical to achieve a response to LEE011 in T-ALL since the cell lines with *RB1* loss (HSB2 and SUPT11) did not show a significant response to the drug. Overall, LEE011 seemed to be an effective CDK4/6 inhibitor against T-ALL cell lines expressing the Rb protein.

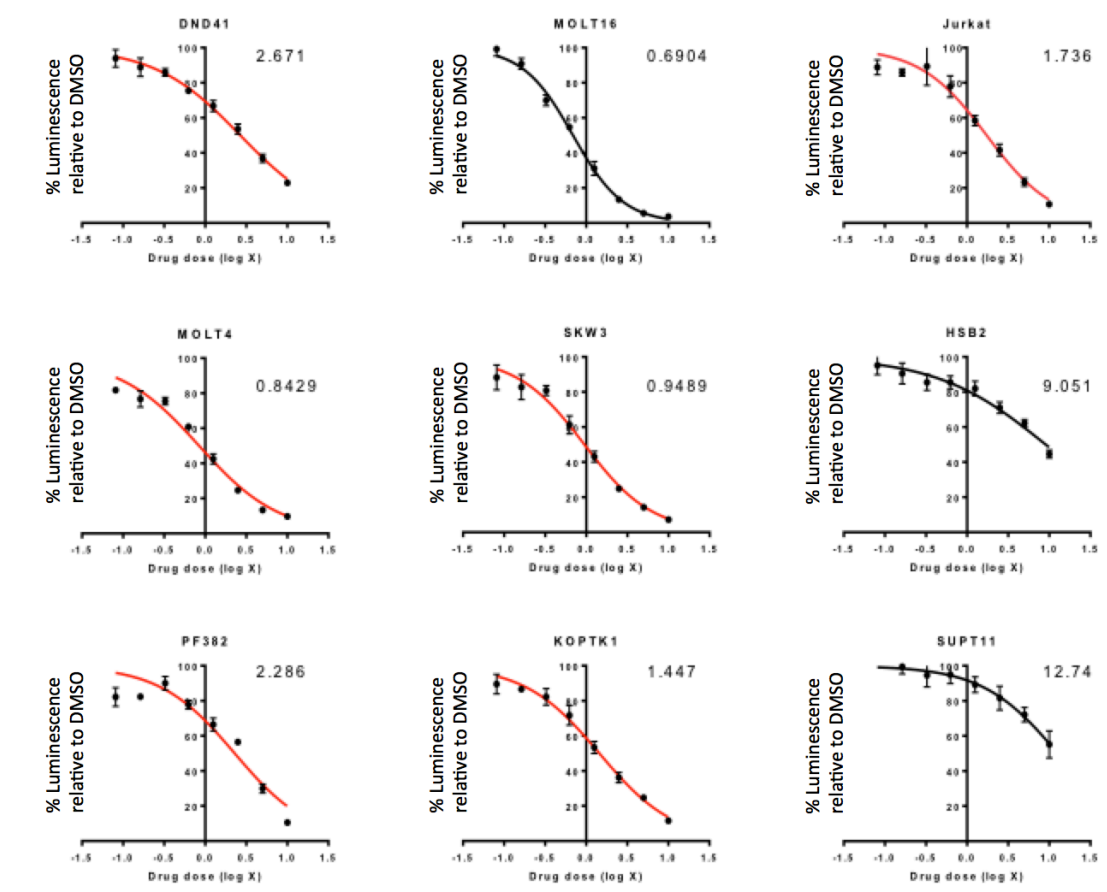


Figure 8: T-ALL cell lines are responsive to LEE011 treatment, dependent on *RB1* expression. The red curves indicate a *NOTCH1*-mutated cell line. The black curves indicate a *NOTCH1* wildtype cell line. The IC_{50} values are labeled next to each curve in micromolar concentrations. Two of the lines, HSB2 and SUPT11, have *RB1* deletion.

LEE011 Treatment of T-ALL Cell Lines Leads to a Decrease of Rb Phosphorylation, Cell Cycle Arrest, and Cell Death

Since most T-ALL cell lines were found to be sensitive to LEE011, we selected two cell lines, MOLT4 (*NOTCH1*-mutated) and MOLT16 (*NOTCH1*-wildtype), for further testing. In the

MOLT4 and MOLT16 cell lines, after 24 hours of LEE011 treatment, there was decreased phosphorylation of Rb and G1 cell cycle arrest in a concentration-dependent manner (Figure 9A and 9B). Corresponding to the increase in the percentage of the cells remaining in the G0/G1 phase, there was also increased cell death as shown by increased Annexin V positive staining after 4 days of drug treatment (Figure 9C). Cell death was more prominent in the *NOTCH1*-wildtype MOLT16 cell line than *NOTCH1*-mutated MOLT4 cells. As a single drug, LEE011, affected the phosphorylation of Rb, cell cycle, and cell death in T-ALL cell lines.

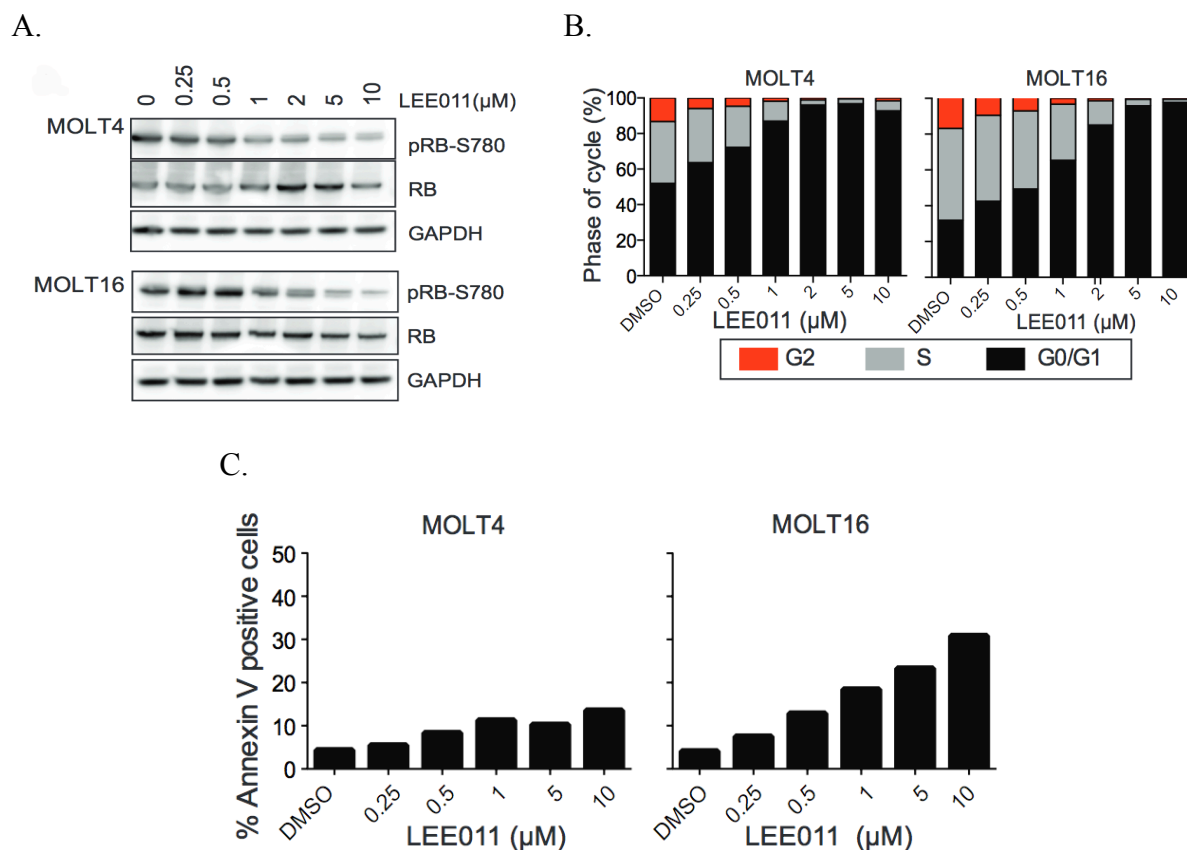


Figure 9: MOLT4 and MOLT16 cell lines are sensitive to LEE011 treatment. (A) Immunoblot showed a concentration-dependent decrease in pRb level after 24 hours. (B) Cell cycle analysis in MOLT4 and MOLT16 cells showed increased cell cycle arrest with increasing concentrations of LEE011. (C) Percent Annexin V positive cells increased with increasing concentrations of LEE011 treatment in MOLT4 and MOLT16 cells after 4 days of treatment. (Performed in collaboration with Yana Pikman and Andy Furman.)

LEE011 is Antagonistic with Methotrexate, 6-mercaptopurine, Doxorubicin, L-Asparaginase, and Vincristine In Vitro

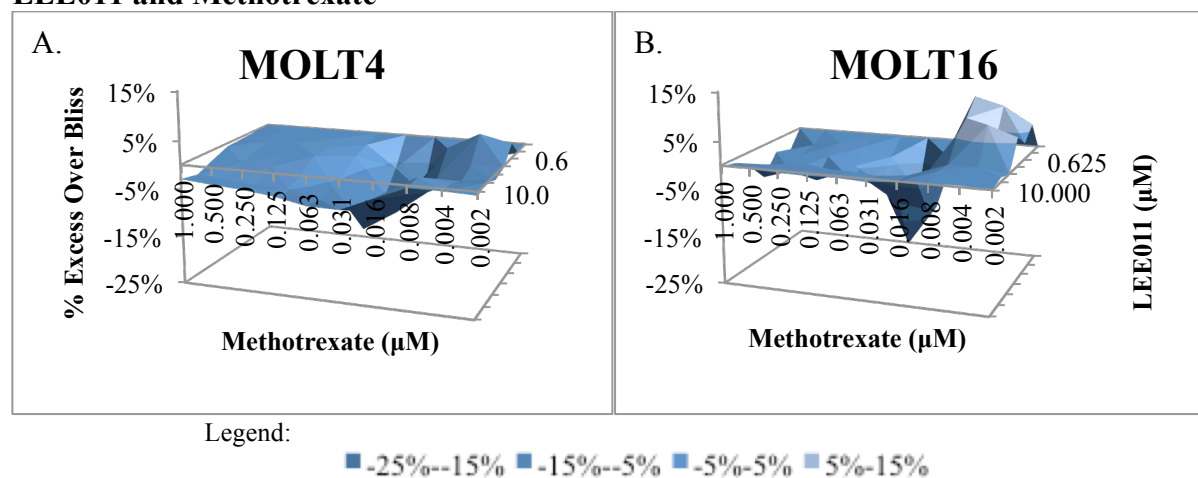
Since LEE011 was effective against T-ALL cell lines as a single agent, we next tried to introduce this CDK4/6 inhibitor in combination with cytotoxic chemotherapy. As shown in Figure 9B, LEE011 induces G1 cell cycle arrest, and thus combining this drug with chemotherapy agents that induce cell death is critical for the successful translation to leukemia treatment. However, standard chemotherapy used in T-ALL treatment relies on rapidly proliferating cells for its activity. Therefore, we hypothesized that LEE011 would be antagonistic with many chemotherapy agents used to treat T-ALL.

In order to determine the effects of combination therapy on T-ALL, we first treated T-ALL cell lines with each chemotherapy drug individually to determine the dose range for response. Next, we treated two T-ALL cell lines, MOLT4 and MOLT16, with LEE011 and methotrexate, 6-mercaptopurine, doxorubicin, L-asparaginase, or vincristine across a range of drug concentrations in a serially 2-fold dilution. For both the individual and combination drug treatments, cells were treated in a 384-well format in quadruplicates for each drug combination. Viability was assessed after 3 and 6 days of treatment using the CellTiter-Glo ATP-based assay.

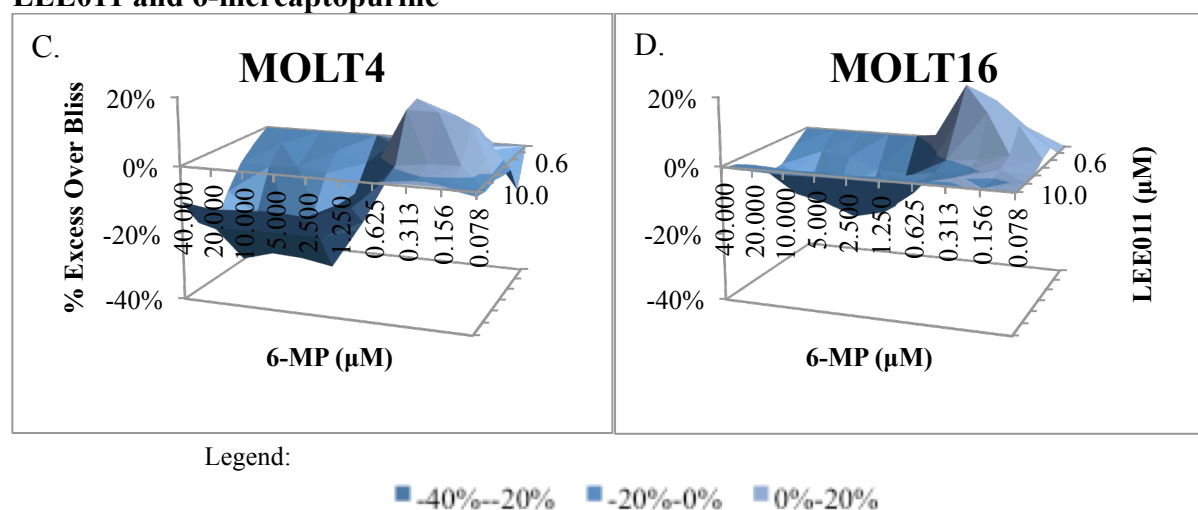
To evaluate synergy between the two drugs, we used the Bliss independence model, which assumes that the inhibitors have independent mechanisms of action and can bind simultaneously and mutually non-exclusively (Bliss, 1956; Greco et al., 1995). Positive Excess above Bliss values are indicative of a synergistic interaction whereas negative values are indicative of antagonism. Using this model, the combinations of LEE011 with methotrexate, 6-mercaptopurine, L-asparaginase, and vincristine were all found to be antagonistic at Day 6 of the assessment (Figure 10). Although LEE011 and doxorubicin showed positive Excess over Bliss values over a narrow range of concentrations, further analysis using Combination Index showed

this combination to be antagonistic (Figure 11). The latter analysis does not assume independence between the two drugs tested in combination. Thus Combination Index may be the more relevant model for synergy assessment, supporting an antagonistic relationship between LEE011 and doxorubicin. In summary, the combinations of LEE011 with methotrexate, 6-mercaptopurine, L-asparaginase, doxorubicin, or vincristine were antagonistic when used simultaneously.

LEE011 and Methotrexate



LEE011 and 6-mercaptopurine



LEE011 and L-asparaginase

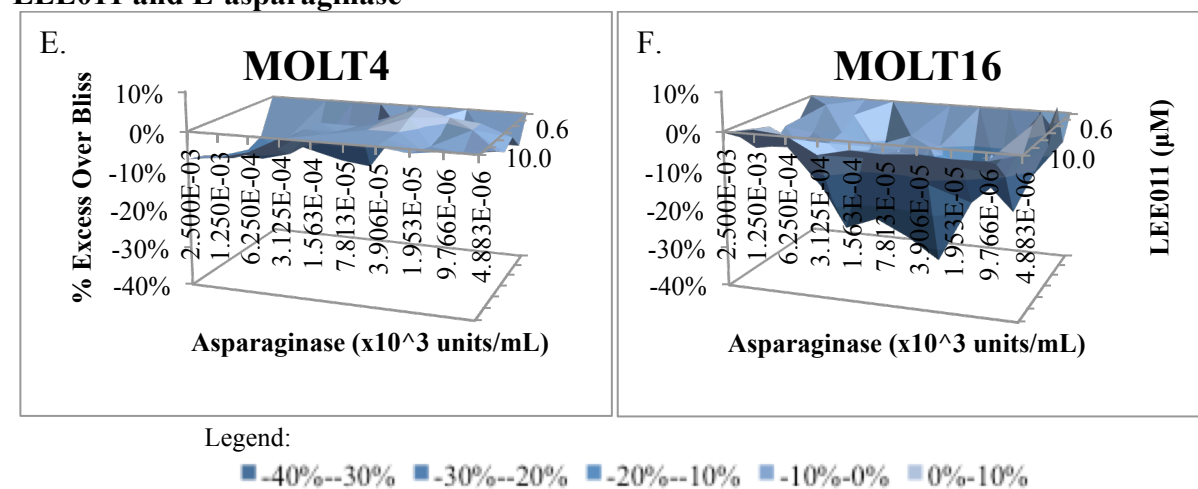
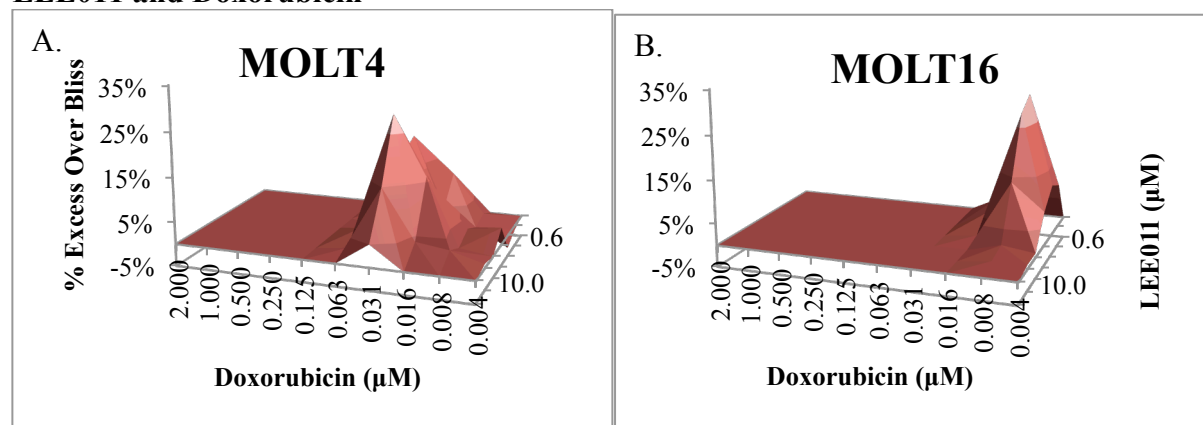


Figure 10: LEE011 is antagonistic with methotrexate, 6-mercaptopurine, and L-asparaginase. The Bliss independence model was used to evaluate the effect of the drug combinations in MOLT4 and MOLT16 cell lines.

LEE011 and Doxorubicin



Legend:

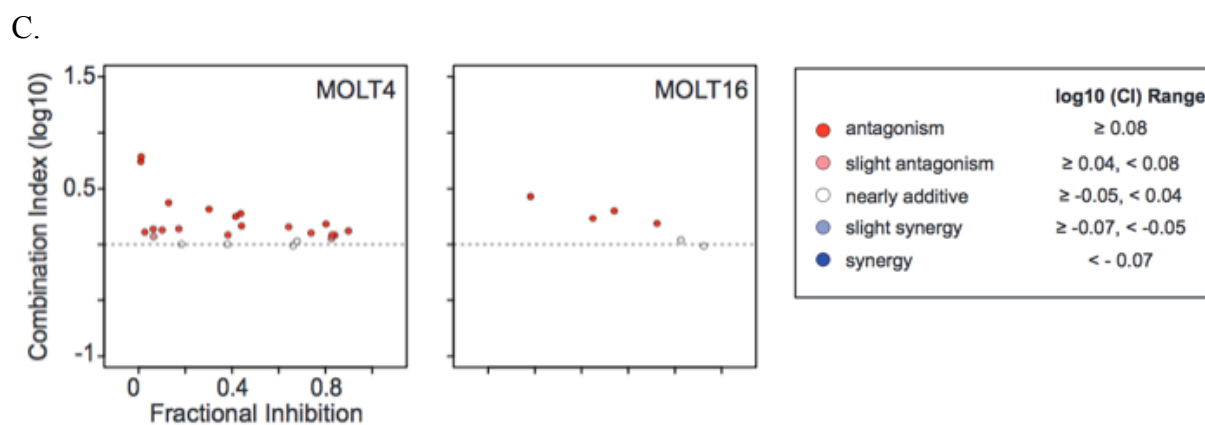
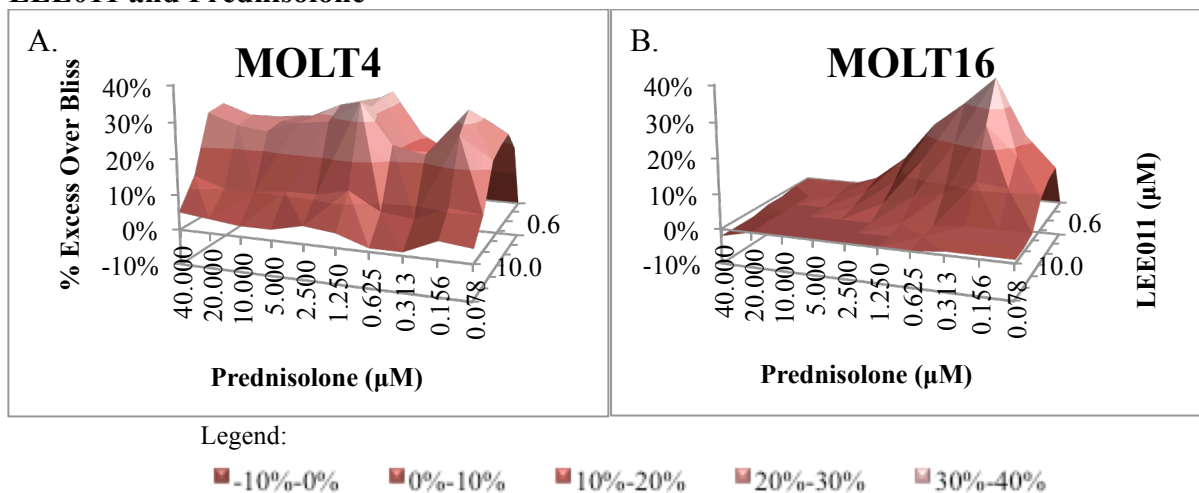


Figure 11: LEE011 is antagonistic with doxorubicin as shown by Combination Index. The Bliss model (A, B) assumes independence between LEE011 and doxorubicin, while Combination Index (C) does not. (Combination Index analysis reproduced from Pikman et al. (2015). Synergistic Drug Combinations with a CDK4/6 Inhibitor in T-Cell Acute Lymphoblastic Leukemia. Clin Cancer Res unpublished.)

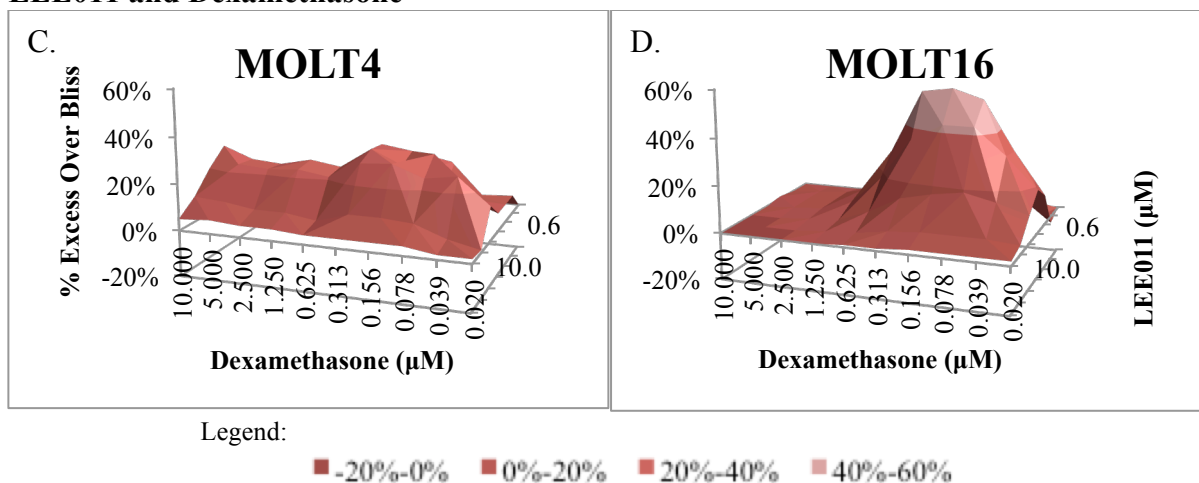
CDK4/6 Inhibitor is Synergistic with Glucocorticoids and mTOR Inhibitors In Vitro

In addition to the five cytotoxic drugs tested above, LEE011 was also further characterized in combination with other chemotherapy agents that do not rely on rapidly proliferating cells, including glucocorticoids (prednisolone and dexamethasone) and the targeted therapy, everolimus, an mTOR inhibitor. MOLT4 and MOLT16 cell lines were treated in 2-fold dilution series in quadruplicate with LEE011 in combination with prednisolone, dexamethasone, or everolimus. Viability was assessed after 3 and 6 days of treatment using the CellTiter-Glo ATP-based assay. The combination of LEE011 with glucocorticoids or everolimus showed synergy after 6 days of drug treatment, as demonstrated by a positive Excess over Bliss analysis over a large range of doses (Figure 12). Since glucocorticoids are a backbone of standard chemotherapy regimens for the treatment of T-ALL, we thus focused on the combination of LEE011 with dexamethasone for further study as this combination can be rapidly translated to the clinic.

LEE011 and Prednisolone



LEE011 and Dexamethasone



LEE011 and Everolimus

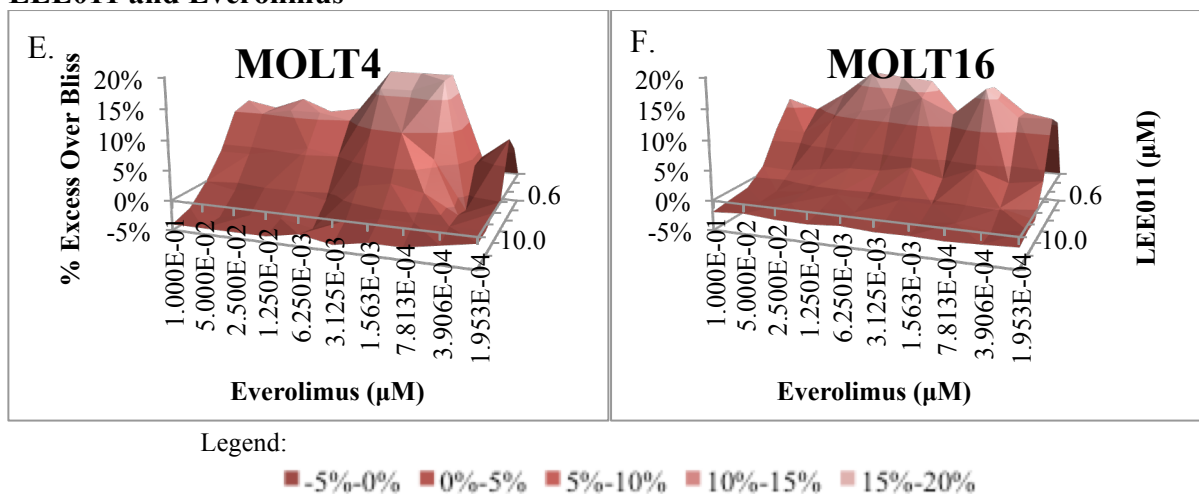


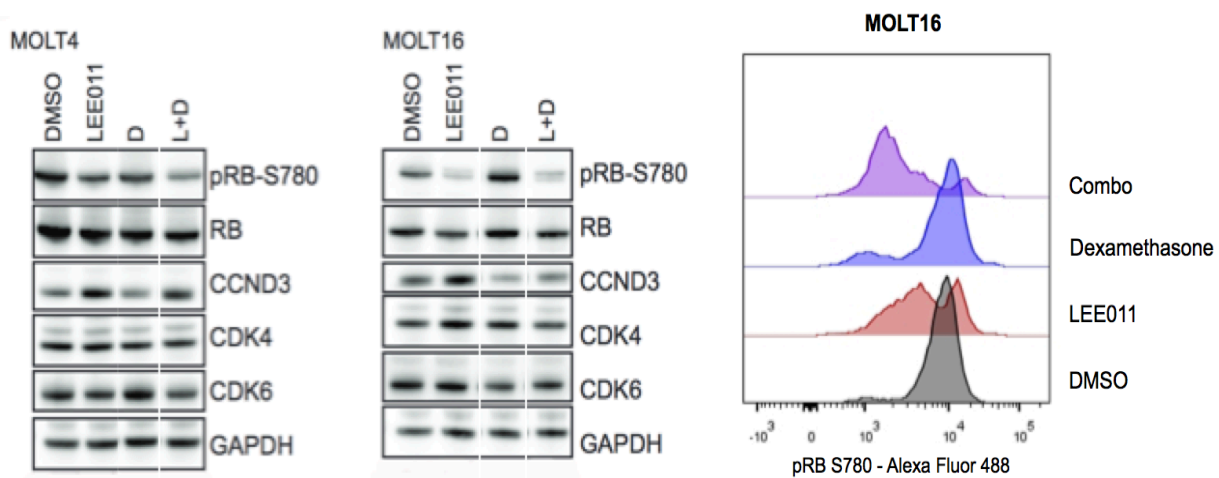
Figure 12: LEE011 is synergistic with prednisolone, dexamethasone, and everolimus. The Bliss independence model was used to evaluate the effect of the drug combinations in MOLT4 and MOLT16 cell lines.

The Combination of LEE011 and Dexamethasone Promotes Cell Cycle Arrest and Cell Death More than either Drug Alone

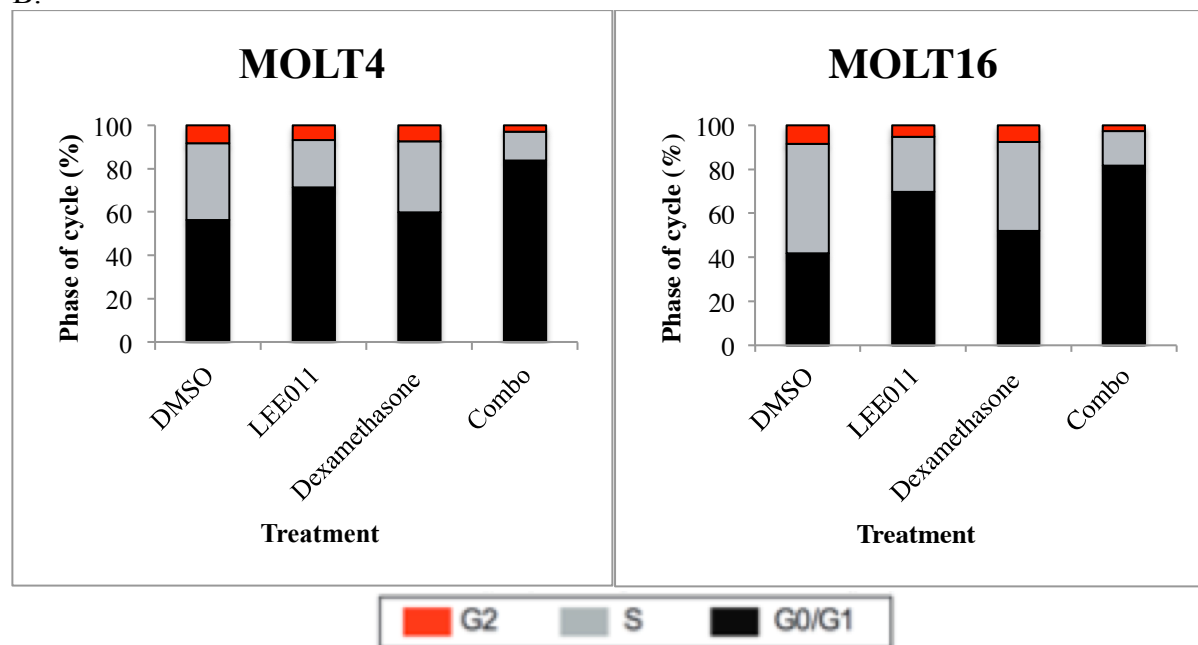
Based on the synergy results using the combination of LEE011 and dexamethasone, we next tested its effects on Rb phosphorylation, cell cycle, and cell death. MOLT4 and MOLT16 cell lines were incubated with LEE011, dexamethasone, and the combination of both drugs. At the selected concentrations, combinations of LEE011 with dexamethasone had a greater effect on the phosphorylation of Rb than any single drug treatment as assessed by immunoblot and flow cytometry after 24 hours (Figure 13A). Additionally, treatment with LEE011 alone led to an increase in Cyclin D3 levels, as seen by immunoblot, in both MOLT4 and MOLT16 cell lines, an effect tempered by the co-treatment with dexamethasone in the MOLT16 cell line. The levels of Rb, CDK4, and CDK6 were consistent across the various treatments with single agents or combinations of the drugs (Figure 13A).

We next analyzed the percent of MOLT4 and MOLT16 cells in each phase of the cell cycle for cells treated with LEE011, dexamethasone, or the combination of LEE011 with dexamethasone. The combination drug treatment resulted in increased G1 cycle arrest compared to the individual drug treatments (Figure 13B). Additionally, the combination treatment resulted in increased cell death after 4 days as indicated by increased Annexin V positive staining (Figure 13C). Cell death was more prominent in the MOLT16 cell line than in the MOLT4 cells. Overall, LEE011 and dexamethasone in combination showed greater effects on cell viability than the single agents alone.

A.



B.



C.

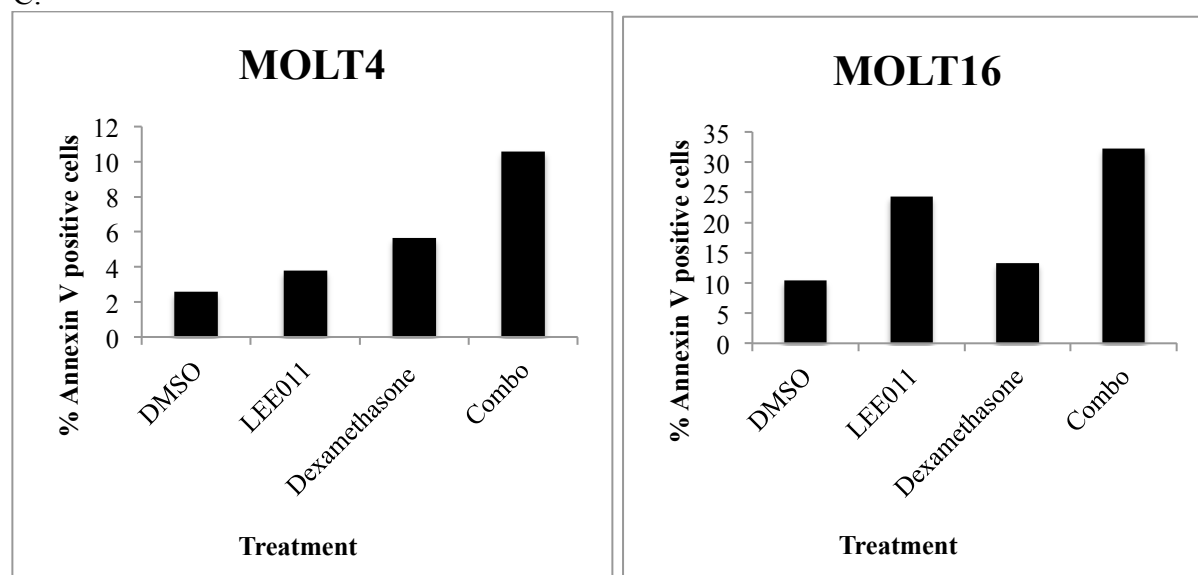


Figure 13: Treatments with LEE011 and dexamethasone in MOLT4 and MOLT16 cell lines leads to decreased phosphorylation of Rb (24 hours), increased G1 cycle arrest (24 hours), and increased cell death (4 days). MOLT4 lines were treated with 3 μ M LEE011 and 5 μ M dexamethasone; MOLT16 lines were treated with 1 μ M LEE011 and 0.02 μ M dexamethasone. (A) Immunoblot and flow cytometry showed a decrease in the levels of phosphorylated Rb. The numbers indicated next to the histogram represent the median. (B) G1 cycle arrest increased with the combination drug treatments compared to the single agents. (C) Annexin V staining revealed greater cell death with the combination drug treatment than with either single drug alone.

CDK4/6 Inhibitor is Synergistic with Gamma Secretase Inhibitor (GSI)

Previously we had mentioned that more than 50% of T-ALL has activating mutations in *NOTCH1*. This would render T-ALL cells sensitive to treatment with a gamma secretase inhibitor (GSI), such as Compound E, which blocks the proteolytic cleavage of NOTCH1. The MOLT4 cell line was tested as above in 2-fold dilution series in quadruplicate with the combination of LEE011 with Compound E. Viability was assessed after 3 and 6 days of treatment using the CellTiter-Glo ATP-based assay. Treatment with LEE011 and Compound E synergistically impaired cell viability (Figure 14). The combination of LEE011 with Compound E will be studied in more detail in future experiments.

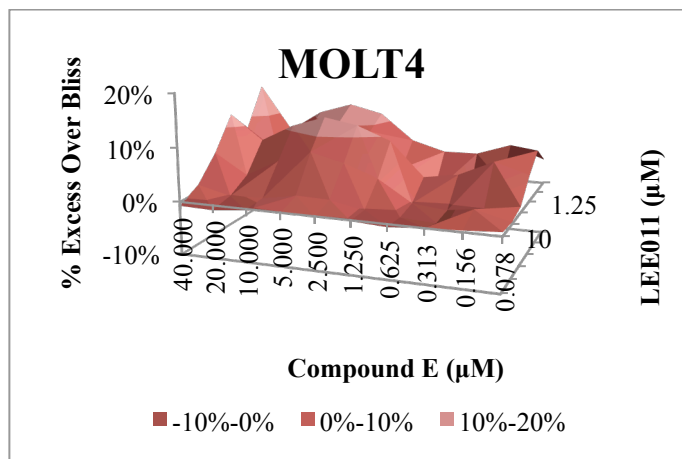


Figure 14: LEE011 is synergistic with Compound E (GSI) in the MOLT4 cell line. The Bliss independence model was used to evaluate the effect of the drug combination.

CDK4/6 Inhibitors Enhance the Effects of Glucocorticoids In Vivo

With all the combination drug studies performed in cell lines, we next extended our testing from the laboratory to a MOLT4 orthotopic xenograft model of T-ALL. MOLT4 cells were labeled with luciferase (MOLT4-Luc), an oxidative enzyme that can produce

bioluminescence, and were injected into NOD-SCID IL2R γ^{null} (NSG) mice. Mice were treated in four separate groups: vehicle, LEE011, dexamethasone, and the combination of LEE011 and dexamethasone. In this study, the combination treatment with LEE011 with dexamethasone resulted in a decrease in spleen weight while little change was observed from the single agent treatment (Figure 15A) after five days. The combination drug treatment also had the greatest effect on survival compared to both the single agent treatments alone (Figure 15B) following 21 days of treatment. Histopathology evaluation showed a decrease in the levels of phosphorylated Rb, a measure of on-target activity of LEE011 in bone marrow collected after 5 days of drug treatment (Figure 15C). Therefore, the *in vivo* response to the combination of LEE011 with dexamethasone seemed most effective. These encouraging results warrant further testing of the combination of LEE011 with dexamethasone in other mouse models and possible integration into a clinical trial for patients with T-ALL.

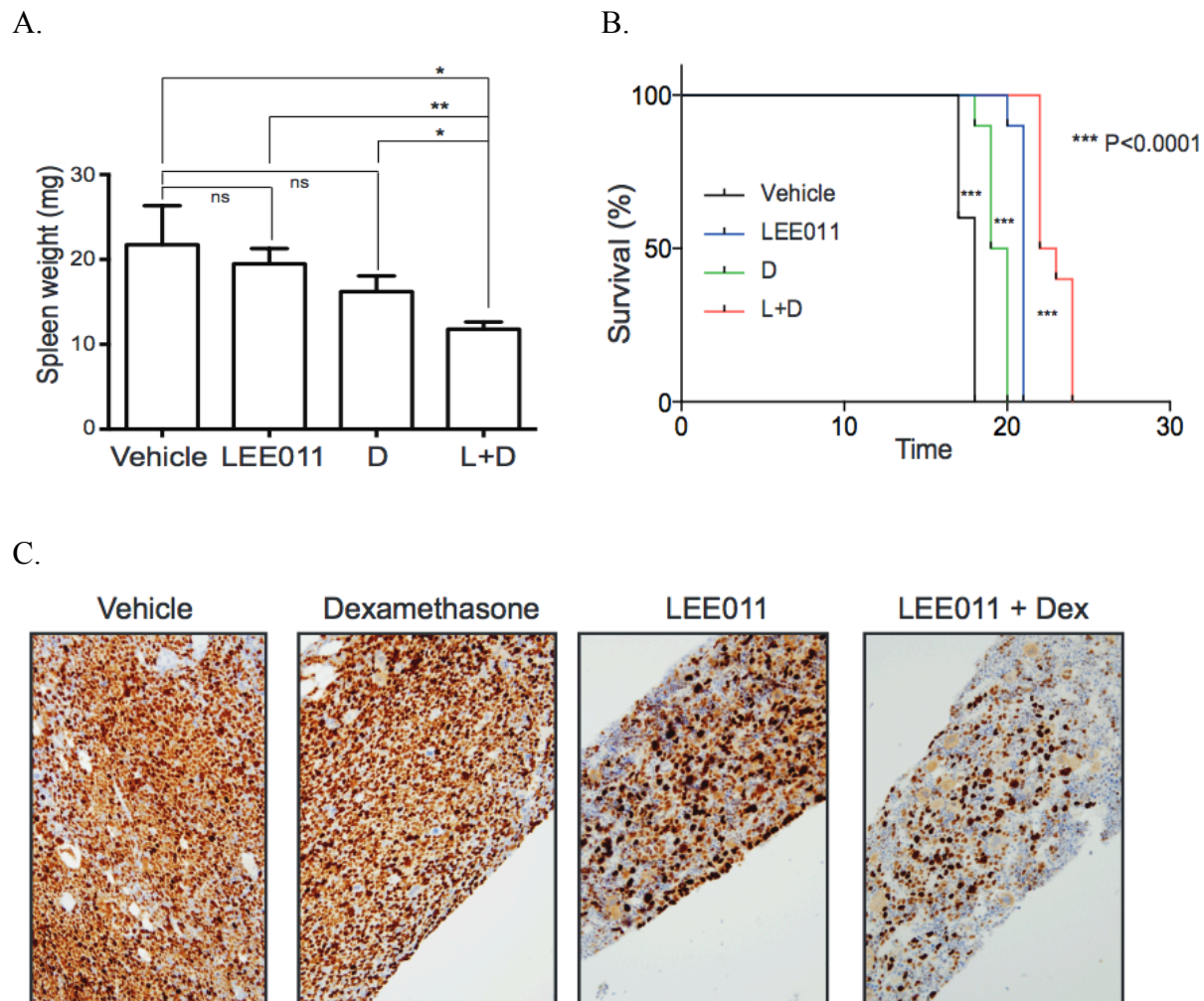


Figure 15: LEE011 enhances the effect of dexamethasone in vivo. (A) Weight of spleen significantly decreased with the LEE011 and dexamethasone combination treatment in three mice after five days of drug treatment. (B) In a group of ten mice following 21 days of treatment, those receiving the LEE011 and dexamethasone combination showed the longest survival rate. (C) pRb levels decrease with the combination drug treatment after five days. (Reproduced from Pikman et al. (2015). Synergistic Drug Combinations with a CDK4/6 Inhibitor in T-Cell Acute Lymphoblastic Leukemia. Clin Cancer Res unpublished.)

DISCUSSION

Combination Studies with CDK4/6 Inhibitors

Although T-ALL only accounts for a small subset of pediatric ALL cases, patients with T-ALL often face induction failure or relapse due to drug resistance. Therefore, there is a need for more effective therapies. Currently, T-ALL is treated with a combination of six standard chemotherapy agents: methotrexate, 6-mercaptopurine, doxorubicin, L-asparaginase, vincristine, and glucocorticoids. Most standard chemotherapy drugs work by directly interfering with cell division, often at the DNA level, and killing cells in the body that grow and divide quickly. Tumors are especially sensitive to these drug treatments since cancer cells are also fast-growing. Targeted therapy is a newer type of cancer treatment that can more precisely identify and attack cancer cells, sparing normal tissues. Targeted therapy has higher specificity for cancer, targeting specific proteins or pathways that are differentially altered in the cancer cells versus normal tissues, and thus promises the potential for tumor eradication with decreased toxicity (Masui et al., 2013). Because the majority of cancer is still treated with standard chemotherapy, specifically with cytotoxic drugs, one approach to integrate targeted therapy into cancer treatment is to identify additive or synergistic combinations with cytotoxic chemotherapies.

When used in combination with other chemotherapy that does not rely on rapid cell proliferation, CDK4/6 inhibitors, a type of targeted therapy, have shown promising results in the treatment of various cancers. For example, the combination of PD0332991 with letrozole, an aromatase inhibitor, was recently approved by the FDA for the treatment of breast cancer after significantly improving progression-free survival in women with advanced estrogen receptor-positive and HER2-negative breast cancer in Phase 2 clinical trial (Finn et al., 2014). Additionally, the inhibition of both the anaplastic lymphoma kinase (ALK) and CDK4/6 in

neuroblastoma models with LDK378, an ALK inhibitor, and LEE011, demonstrates potent on-target in vitro synergy and in vivo activity with the inhibition of respective molecular targets, resulting in decreased cell proliferation and induction of cell death (Wood et al., 2014). Currently, there are also several combination therapies with LEE011 in clinical trials: a Phase 2 study of LEE011 in combination with LGX818, a BRAF (serine/threonine kinase) inhibitor, and MEK162, a MEK (mitogen-activated protein kinase) kinase inhibitor, in adult patients with BRAF-dependent advanced solid tumors (Novartis Pharmaceuticals); and a Phase 1b trial of LEE011 with everolimus and exemestane in the treatment of ER+ HER2- advanced breast cancer (Novartis Pharmaceuticals). However, combination studies of CDK4/6 inhibitors with other drugs have yet to be reported for acute leukemia.

Previously, PD0332991 (palbociclib) has shown promising results in the treatment of T-ALL cells with decreased phosphorylation of Rb, leading to G1 cell cycle arrest (Choi et al., 2012; Sawai et al., 2012). In our studies, we tested LEE011, which is structurally and effectively similar to PD0332991, against T-ALL cell lines. As a single agent, LEE011 showed promising results with decreased Rb phosphorylation, caused G1 cell cycle arrest, and increased cell death in T-ALL cell lines. Since LEE011 causes cell cycle arrest and many of the standard chemotherapy agents rely on rapidly proliferating cells for activity, combinations of these drugs would thus be predicted to be antagonistic and need to be tested in the laboratory first. In our combination studies, we found that LEE011 was antagonistic with methotrexate, 6-mercaptopurine, doxorubicin, L-asparaginase, and vincristine in T-ALL cell lines and synergistic with glucocorticoids (dexamethasone and prednisolone) and everolimus.

Mechanism of Synergy

Once the synergistic combinations with LEE011 were determined, we were interested in evaluating why there was synergy between certain drug combinations. Based on our studies, the combination of LEE011 with dexamethasone was synergistic in T-ALL cell lines. This combination treatment led to decreased levels of pRb and increased cell cycle arrest and cell death, which are all cellular events that were altered with treatment of LEE011 alone. We therefore focused on this synergistic drug combination to characterize the mechanism of synergy. Glucocorticoids, such as dexamethasone, have a variety of effects on ALL cells, including cell cycle arrest and induction of programmed cell death. One mechanism of glucocorticoid activity is through the down-regulation of D-type cyclins, particularly Cyclin D3 (Garcia-Gras et al., 2000; Rhee et al., 1995; Rogatsky et al., 1997). Treatment of T-ALL cell lines with LEE011 caused an increase in Cyclin D3 protein levels as seen on western blot analysis, a possibly attenuating response to the drug. However, the combination of LEE011 and dexamethasone decreased Cyclin D3 levels, especially in the MOLT16 cells, suggesting this as a possible mechanism for synergy (Figure 16). This will need to be evaluated more definitely in future studies. We plan to use recombinant DNA technology to overexpress Cyclin D3 in MOLT16 cells and then determine whether Cyclin D3 overexpression attenuates response to LEE011 treatment. I hypothesize that increasing the levels of Cyclin D3 will make cells more resistant to LEE011, increasing the effective IC_{50} .

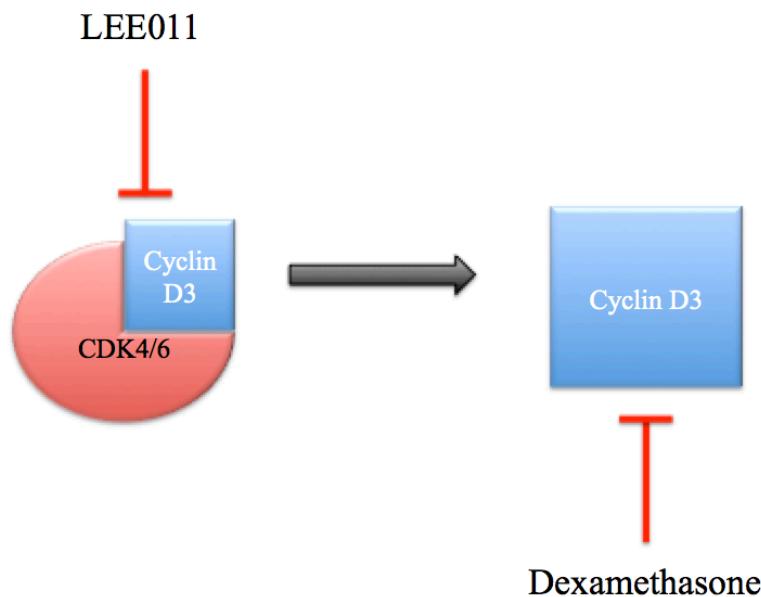


Figure 16: Schematic showing the potential mechanism of synergy for the combination treatment of LEE011 and dexamethasone in T-ALL cell lines.

Importance of Chemotherapy Sequence

When we tested LEE011 in combination with either standard chemotherapy or everolimus, the drugs were added concurrently. Adding the drugs simultaneously was the most efficient way to evaluate for synergy and antagonism in the combinations. However, we realized that the sequence of drug treatment could affect combination outcomes as it had been shown in other acute leukemias. For example, in the treatment of acute myeloid leukemia (AML), the sequence with which standard cytotoxic chemotherapy and CEP-701, a FLT3 inhibitor that induces apoptosis in FLT3/ITD-expressing cell lines, are combined appears to be important. Treatment of AML cells with CEP-701 prior to the addition of chemotherapy seemed to antagonize the cytotoxic effects of chemotherapy agents such as cytarabine and etoposide (Levis et al., 2004). In contrast, when CEP-701 was added simultaneously with or immediately following exposure of cells to chemotherapy, synergistic cytotoxicity was observed (Levis et al.,

2004). Similarly, there is also a sequence-dependent synergy between CEP-701 and chemotherapy agents on HB-1119 and SEM-K2 cells lines (Brown et al., 2006). These lines are derived from patients with *MLL*-rearranged ALL, which express activated FLT3 and demonstrate pronounced dependence on FLT3 signaling for survival. In this case, treatment with chemotherapy first, followed by exposure to CEP-701, showed synergy (Brown et al., 2006). Simultaneous exposure to both agents was additive (Brown et al., 2006). However, when cells were exposed to CEP-701 first and then chemotherapy, the interaction was antagonistic (Brown et al., 2006). For our future experiments, we plan to test two additional sequences of exposure for the combination studies of LEE011 and standard chemotherapy or everolimus in T-ALL: LEE011 followed by chemotherapy and chemotherapy followed by LEE011 (Figure 17).

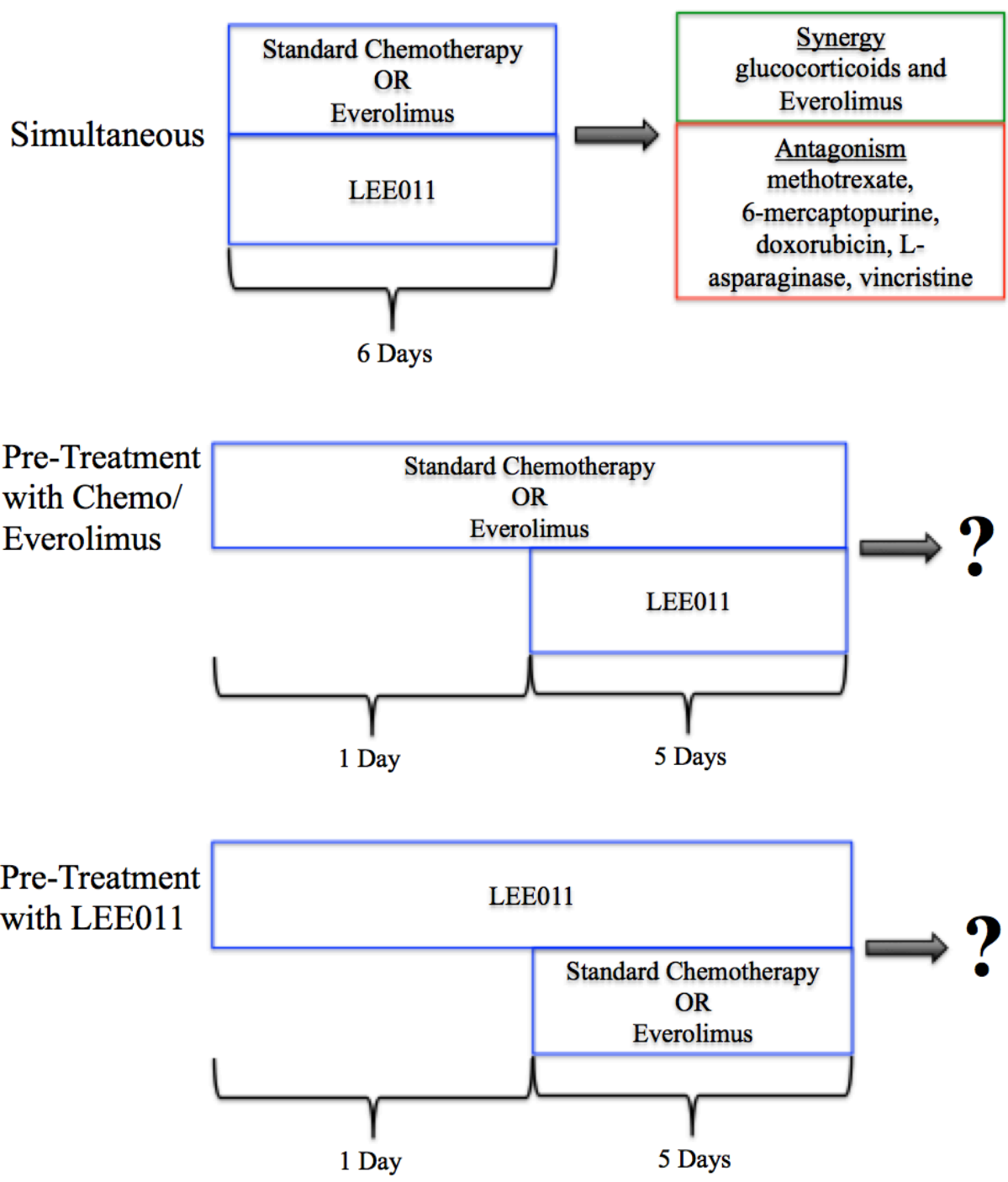


Figure 17: Schematic illustrating the three sequences of drug addition that should be utilized in characterizing the effects of the combination between LEE011 and standard chemotherapy or everolimus.

Clinical Relevance for Targeted Therapies

Based on our study, we have identified a synergistic drug combination of LEE011 and glucocorticoid or everolimus when added concurrently. This finding could be translated to the clinic to help patients with T-ALL, especially those with the relapsed and refractory disease and are in need of new therapies. For effective use of targeted therapy, it will be important to select patients who are likely to respond to treatment based on molecular characteristics. When we had tested a panel of nine T-ALL cell lines with LEE011, we found that cell lines with *RBI* deletion, HSB2 and SUPT11, did not respond to LEE011. While *RBI* deletion is found in 8-12% of patients with T-ALL, effective implementation of this drug in clinical trials will need to exclude patients with *RBI* mutations (Mansur et al., 2015; Mullighan et al., 2007). Activated *NOTCH1* has previously been reported to be a biomarker of response to CDK4/6 inhibitors. However, *NOTCH1* mutations did not appear to be a marker of response within the T-ALL subset of cell lines we tested, even though this analysis was limited by only three *NOTCH1*-wildtype cell lines. A clinical trial in T-ALL patients with LEE011 will be needed to determine whether clinical response is based on *NOTCH1* mutational status. Based on our data, *NOTCH1* mutation should not be a requirement for patients entering the initial clinical trials testing combination drug treatments with LEE011.

Given prior studies reporting CDK4/6 inhibitor activity in mouse models of T-ALL, we have focused our investigation on T-ALL. However, these drug combinations will likely extend to the treatment of other acute leukemias. We are currently studying these drug combinations in B-ALL. Our initial testing of LEE011 and standard chemotherapy drugs in B-ALL also show a similar pattern of synergies as in T-ALL. Recent studies have shown CDK6 to be a direct target of fusion gene MLL-AF9 in AML and MLL-AF4 in infant ALL (Placke et al., 2014). This suggests

that CDK4/6 inhibitors may also be active in these types of acute leukemia. Therefore, the discovery of combination therapies with CDK4/6 inhibitors could impact the treatment of acute leukemias beyond T-ALL.

Overall, our work supports the use of combination therapy with CDK4/6 inhibitors in treating T-ALL. Successful implementation of a new treatment for leukemia requires effective combination therapies. In this study, we have discovered novel synergistic combinations between LEE011 with glucocorticoids and LEE011 with mTOR inhibitors that could be readily translated to a clinical trial for patients with T-ALL and potentially other leukemias.

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