A Phase 2 Study of MLN0128 (TAK-228) in Relapsed and/or Refractory Acute Lymphoblastic Leukemia (ALL)

Summary of Changes

MCCC Addendum 3

Investigator Initiated Revisions:

#	Section	Revision
1.	Global	Updated protocol headers to the new NCI version date.
2.	Title	**EDDOP /** Early Drug Development Opportunity Program

A Phase 2 Study of MLN0128 (TAK-228) in Relapsed and/or Refractory Acute Lymphoblastic Leukemia (ALL)

Corresponding Organization: LAO-MN026 / Mayo Clinic Cancer Center LAO

Principal Investigator: Aref Al-Kali, M.D.
Mayo Clinic
200 First Street SW
Rochester, MN 55905
Telephone: (507) 284-2511
Telefax: (507) 284-5280
Email: alkali.aref@mayo.edu

Participating "Rostered" Organizations

LAO-11030 / University Health Network Princess Margaret Cancer Center LAO
LAO-CA043 / City of Hope Comprehensive Cancer Center LAO
LAO-CT018 / Yale University Cancer Center LAO
LAO-MA036 / Dana-Farber - Harvard Cancer Center LAO
LAO-MD017 / JHU Sidney Kimmel Comprehensive Cancer Center LAO
LAO-NC010 / Duke University - Duke Cancer Institute LAO
LAO-NJ066 / Rutgers University - Cancer Institute of New Jersey LAO
LAO-OH007 / Ohio State University Comprehensive Cancer Center LAO
LAO-PA015 / University of Pittsburgh Cancer Institute LAO
LAO-TX035 / University of Texas MD Anderson Cancer Center LAO
LAO-NCI / National Cancer Institute LAO
EDDOP / Early Drug Development Opportunity Program

Co-Investigators: Cristina Correia, Ph.D.*
Scott H. Kaufmann, M.D., Ph.D.
Mark Litzow, M.D.
Joel M. Reid, Ph.D. *
Andrea Wahner Hendrickson, M.D.*

*Responsible for correlative studies only

Project Manager
Sanna McKinzie, M.S.
Mayo Clinic
Telephone: (507) 538-6646
Email: mckinzie.sanna@mayo.edu

Statistician
Pamela Atherton, M.S.
Mayo Clinic
Telephone: (507) 284-0660
Email: atherton.pamela@mayo.edu

Clinical Trials.gov identifier: NCT02484430

NCI-Supplied Agent: MLN0128 (TAK-228) (NSC# 768435)

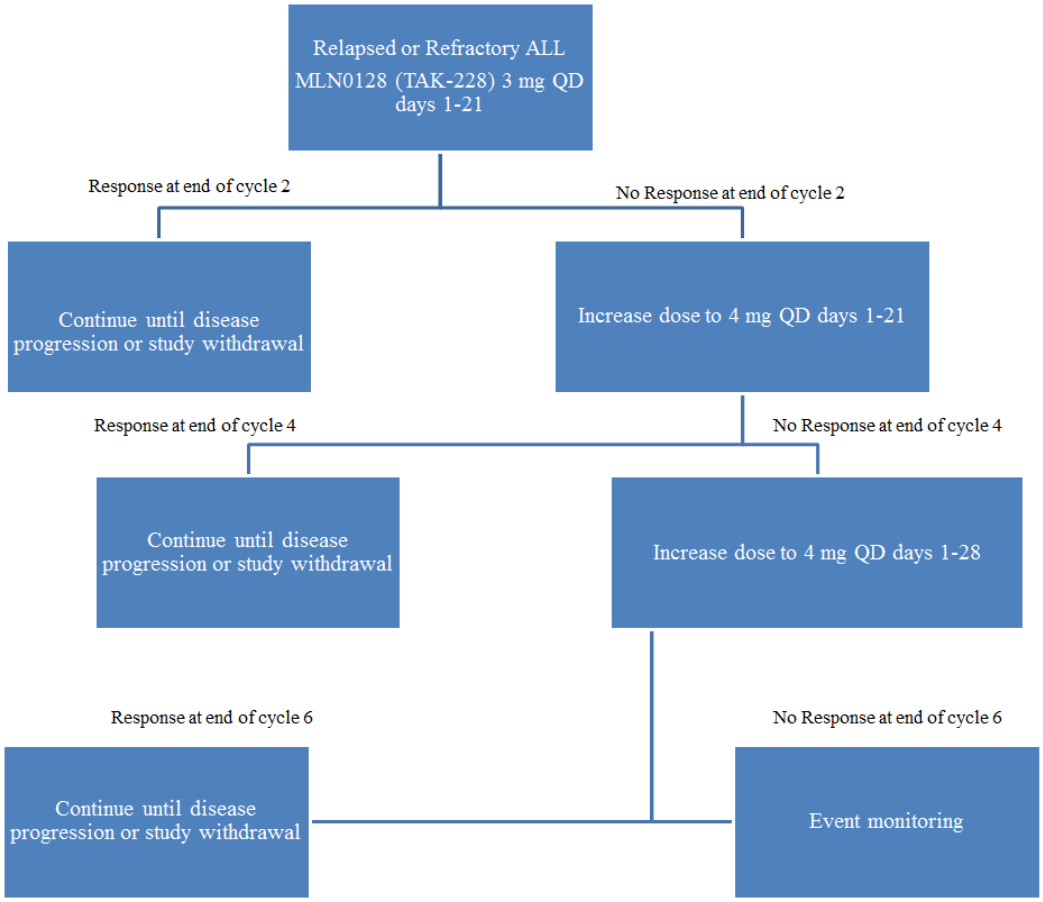
IND Sponsor: DCTD, NCI

Protocol Type / Version # / Version Date:
Original / Version 1 / May 4, 2015
Revision / Version 2 / July 1, 2015
Revision / Version 3 / July 14, 2015
Revision / Version 4 / August 5, 2015
Revision / Version 5 / September 10, 2015
Revision / Version 6 / September 29, 2015
Revision / Version 7 / May 26, 2016
Revision / Version 8 / June 21, 2016
Revision / Version 9 / July 12, 2016
Revision / Version 10 / September 9, 2016

Activation on CTSU October 20, 2016 used Version 10 dated September 9, 2016

Revision / MCCC Addendum 1 / May 9, 2017
Revision / MCCC Addendum 2 / June 29, 2017
Revision / MCCC Addendum 3 / October 2, 2017

SCHEMA



Cycle length = 28 days

Responders = CR/CRi/PR/MLFS

TABLE OF CONTENTS

1. OBJECTIVES

1.1 Primary Objectives

1.1.1 To determine the CR/CRi rate when MLN0128 (TAK-228) is administered to adult patients with relapsed/refractory ALL.

1.2 Secondary Objectives

1.2.1 To determine the overall response rate (CR, CRi/PR/morphologic leukemia free state [MLFS]).

1.2.2 To determine the CR/CRi duration when MLN0128 (TAK-228) is administered to adult patients with relapsed/refractory ALL

1.2.3 To determine the frequency of proceeding to allogeneic SCT for patients with relapsed/refractory ALL who achieve a response on MLN0128 (TAK-228)

1.2.4 To determine the overall survival for relapsed/refractory ALL patients on MLN0128 (TAK-228)

1.3 Tertiary Objectives

1.3.1 To examine the pharmacokinetics of MLN0128 (TAK-228) in ALL patients.

1.3.2 To assess whether phosphorylation of the mTOR substrate 4EBP1 decreases in leukemic blasts harvested from the bone marrow on day 8 compared to baseline.

1.3.3 To assess in an exploratory fashion whether MLN0128 (TAK-228) enhances expression of the pro-apoptotic proteins BCL2L11 (Bim)* and BBC3 (Puma) in marrow ALL cells.

1.3.4 To assess in an exploratory fashion whether MCL1 levels decrease in blast cells during MLN0128 (TAK-228) treatment.

1.3.5 To assess in an exploratory fashion whether a) the phospho-protein pattern at baseline or b) MLN0128 (TAK-228)-associated changes in the phospho-protein pattern differs between ALL samples that respond to therapy and those that do not.

*Please note that Human Gene Organization (HUGO) names have been provided throughout this protocol at the request of CTEP reviewers to allow them to track the protocol. To aid scientific and clinical reviewers who might be unfamiliar with the HUGO names, we have provided in parentheses throughout the protocol the conventional names of the proteins that are being described or analyzed.

2. BACKGROUND

2.1 Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is one of the most aggressive adult leukemias. Each year more than six thousand are diagnosed with ALL in the US (1). One of the most commonly used regimens for ALL typically is hyper-CVAD, a regimen consisting of cycles of high dose cyclophosphamide, vincristine, doxorubicin and dexamethasone alternating with methotrexate and cytarabine, with or without the addition of a Bcr/abl kinase inhibitor depending upon the presence or absence of a t(9;22) chromosomal translocation (2, 3). Despite this aggressive treatment, more than half of these patients will not survive past four years from diagnosis. Accordingly, there has been substantial interest in testing new therapeutic agents for ALL, including various monoclonal antibodies, NOTCH1 inhibitors, hypomethylating agents such as decitabine, folic acid antagonists, flavopiridol, bortezomib, and mTOR inhibitors (4, 5).

Despite advances in management, relapsed ALL is very resistant to available therapies. In a retrospective study, O'Brien *et al* reported an 18% complete remission (CR) rate and a 3-month median survival rate for relapsed ALL treated at MD Anderson with various regimens (6). Moreover, because the median age at onset for adult ALL is roughly age 65 (7), a substantial proportion of patients are not candidates for rigorous therapy such as hyper-CVAD. Therefore, novel and targeted therapies are needed for this group of patients. Finally, patients who relapse after allogeneic bone marrow transplant pose a difficult therapeutic dilemma, coupled with graft versus host disease and/or difficulty to find a second donor, making them suitable candidates for clinical trials of novel agents.

2.2 MLN0128 (TAK-228)

2.2.1 Mechanism of Action

MLN0128 (TAK-228) is an inhibitor of the mechanistic target of rapamycin (mTOR). This serine/threonine kinase is a major integrator of various types of signals within cells (8). Two distinct complexes exist, mTOR complex 1 and 2 (TORC1 and TORC2) (9). TORC1 phosphorylates p70 S6 kinase and eukaryotic translation initiation factor 4E (eIF4E) binding protein 1 (4EBP1) to regulate protein synthesis and, indirectly, progression from G1 into S phase, while TORC2 phosphorylates AKT on Ser^{473}, contributing to activation of this kinase and affecting survival pathways. MLN0128 (TAK-228) (previously called MLN0128; INK-128) has been shown to target both mTORC1 and mTORC2 (10-14), which is thought to provide improved inhibition of mTOR and enhanced cytotoxicity in lymphoid malignancies as described below.

MLN0128 (TAK-228) inhibited mTOR kinase in vitro with an IC_{50} of 1.1 nM. It was 100- to 1000-fold less potent against class I, class II and class III phosphatidylinositol-3 (PI3) kinase activities as well as PI4 kinase isoforms (15). Among a panel of 222 protein kinases, TAK-228 inhibited only 4 kinases (DNA-PK, PDGFRα, Flt3 and CK1ε) in addition to mTOR by 80% at 1 μM.

2.2.2 Nonclinical Studies

Preclinical efficacy studies (15): MLN0128 (TAK-228), like other dual mTOR inhibitors, inhibits proliferation in a wide range of human tumor cell lines *in vitro*. In particular, when examined in 105 cell lines, MLN0128 (TAK-228) inhibited growth with IC_{50} values ranging from 1 nM (SW620 colorectal cancer cells) to 2700 nM (HepG2 hepatoma cells).

Immunoblotting studies in PC-3 prostate cancer, T47D breast cancer, 786-O renal cell carcinoma lines and multiple myeloma cell lines demonstrated that MLN0128 (TAK-228) inhibits phosphorylation of the translation inhibitor 4EBP1 (an mTORC1 substrate) and the serine/threonine kinase AKT (Ser^{473}, an mTORC2 substrate) in intact cells, with almost complete inhibition at 100 nM (10, 16). In contrast, the allosteric mTORC1 inhibitor rapamycin inhibited phosphorylation of ribosomal S6 (downstream of another mTORC1 substrate p70 S6 kinase) but had little impact on either 4EBP1 or AKT Ser^{473} phosphorylation. Likewise, when administered to mice bearing U87G glioblastoma xenografts, MLN0128 (TAK-228) inhibited phosphorylation of 4EBP1, ribosomal protein S6 and AKT Ser^{473} at 1, 2, 4 and 8 hours, although some recovery of signal was observed at 24 h.

In vivo efficacy studies in mice bearing of human tumor xenografts, including U87MG (*PTEN*-mutant glioblastoma), A549 (*KRAS/LKB1*-mutant NSCLC), ZR-75-1 (*PTEN*-mutant breast cancer), MDA-MB361 (*PIK3CA/HER2*-mutant breast cancer), 786-O (*PTEN/VHL*-mutant renal cell carcinoma) and HEC-1 (*PIK3CA/KRAS*-mutant endometrial cancer) MLN0128 (TAK-228) demonstrated strong tumor growth inhibition at tolerable oral doses ranging from 0.15 mg/kg/d to 3 mg/kg every other day or 3 mg/kg every week.

Preclinical toxicology studies: Preclinical toxicology studies were performed in rats and monkeys. The dose-limiting toxicities in single-agent toxicology studies in both species were GI distress, decreased activity, hypothermia, decreased food intake, hyperglycemia, hyperinsulinemia, and lymphoid depletion.

In 28-day GLP-compliant toxicology studies, rats treated with 3.0 mg/kg MLN0128 (TAK-228) exhibited reduced body weights compared to controls; increases in aspartate aminotransferase (AST) that was attributed to nonspecific muscle injury (creatine kinase was also higher in affected animals); increases in glucose and insulin; decreases in leukocytes, lymphocytes, and eosinophils; and microscopic changes in the bone marrow (hypocellularity), thymus (lymphoid necrosis), testes (degeneration/atrophy), epididymides (oligospermia), nonglandular stomach (epithelial degeneration/ulceration/ hyperplasia; males only), and lungs (alveolar histiocytosis).

In 28-day GLP-compliant toxicology studies in cynomolgus monkeys, MLN0128 (TAK-228)-related effects observed at 0.30 mg/kg included decreased body weight, appetite, activity, and body temperature, abnormal posture, and skin cold to touch. Two of 10

monkeys treated at this dose level had to be euthanized in day 18 and 19. Clinical chemistry changes included increased serum AST, creatine kinase MM isoform, cholesterol, triglycerides, and insulin, and decreased serum sodium, potassium, and phosphorous. Hematology parameter alterations consisted of decreased lymphocytes (females only) and increased leukocytes and neutrophils, with sporadic increases in monocytes and eosinophils. Microscopic changes at 0.15 and 0.3 mg/kg were seen in the thymus, spleen, lymph nodes, gut-associated lymphoid tissue (0.30 mg/kg only), bone marrow, adrenal glands, salivary glands, pancreas, GI tract, and skin (0.3 mg/kg only). Specific MLN0128 (TAK-228)-related microscopic findings in the lymphoid organs, including gut-associated lymphoid tissue, were multicentric lymphoid depletion and splenic red pulp depletion. At 0.30 mg/kg, the spleen had moderate to severe depletion of RBCs and hematopoietic cells. These changes correlated with decreased spleen weights in males at 0.30 mg/kg and decreased thymus weights in females at 0.15 and 0.30 mg/kg. Other microscopic changes included mixed cell depletion in bone marrow; minimal to moderate bilateral cortical hyperplasia in adrenals (thought to be stress induced); acinar cell secretory depletion in the pancreas, parotid and mandibular salivary glands (thought possibly due to decreased food consumption); erosion/ulceration, edema, hemorrhage, and acute inflammation of the cardia and pylorus of the stomach; and mild and sporadic changes in the duodenum, jejunum, colon, and rectum. At 0.3 mg/kg, some monkeys exhibited mild to moderate erosion/ulceration and minimal to moderate epidermal hyperplasia of the skin on the face, neck, and forelimbs. Electrocardiography demonstrated no MLN0128 (TAK-228)-related effects, consistent with lack of significant findings in a GLP-compliant cardiovascular safety pharmacology study conducted in conscious monkeys.

2.2.3 Clinical Studies

MLN0128 (TAK-228) has been evaluated in two single-agent oral dose-finding studies, one in patients with advanced solid malignancies and one in patients with relapsed and/or refractory multiple myeloma, non-Hodgkin lymphoma, and Waldenstrom's macroglobulinemia.

In INK128-001, single-agent MLN0128 (TAK-228) has been administered to patients with advanced solid tumors in 28-day cycles according to four schedules: q day, q week, 3 consecutive days q week, and 5 consecutive days q week. The maximum tolerated doses were 6 mg q day, 40 mg q week, 16 mg q day x 3 each week, and 10 mg q day x 5 each week (15).

Treatment related adverse events, serious adverse events, and events causing withdrawal from study INK128-001 (15): In this study, ≥ 1 treatment-emergent adverse event (TEAE) was reported for 139 (98%) of 142 treated patients. Across the dosing groups, the most frequent TEAEs were hyperglycemia (65% of patients), nausea (63% of patients), and vomiting (54%). In general, TEAEs were mostly Grade 1 or 2 and manageable with supportive care and/or interruption or dose reduction of study drug. Severity ≥ Grade 3 TEAEs (SAEs) that were reported in 6% or more of patients, regardless of causality, were hyperglycemia (13% of patients), asthenia (8%), anemia

(7%), and hypophosphatemia (6%) and lymphopenia (6%). As of the clinical database cutoff date, SAEs had been reported for 58 patients (41%), with the most common terms being mucosal inflammation (4%), asthenia (3%), pneumonia (3%), abdominal pain (2%), stomatitis (2%), and renal failure (2%). Of the 142 patients treated in Study INK128-001 as of the clinical data cutoff, 29 (20%) discontinued because of 1 or more AEs, which included rash (6 patients), mucosal inflammation (4 patients), asthenia (3 patients), hyperglycemia (2 patients), nausea (2 patients), performance status decreased (2 patients), pruritus (2 patients), and acute renal failure (2 patients). Of the total 40 AEs present at study withdrawal, 21 events (53%, including 11 nonserious AEs) were reported as severity Grade 3, and 4 SAEs were Grade 5 (fatal).

A death on INK128-001 due to ventricular arrhythmia and cardiac arrest was assessed as possibly related to MLN0128 (TAK-228). After discussion with the FDA, study C31002, a phase 1 single-arm study to evaluate the effect of a single dose of 40 mg MLN0128 (TAK-228) on the QT/QTc interval was initiated in patients with advanced solid tumors. After completing the per-protocol PK/ECG/cardiac contractility monitoring, the patients continued MLN0128 (TAK-228) 30 mg QW with continued cardiac monitoring. The study results showed that treatment with MLN0128 (TAK-228) was not associated with clinically meaningful effects on the overall electrocardiographic safety profile, and that ECHO/MUGA at screening is not required on MLN0128 (TAK-228) trials. Takeda and CTEP have agreed that ECG monitoring should be performed on patients prior to entering MLN0128 (TAK-228) trials, at the time of completing treatment with MLN0128 (TAK-228), and periodically during the trial when clinically appropriate.

In INK128-002, which was reported by Ghobrial *et al.* (17) MLN0128 (TAK-228) was administered orally to 37 patients in two different schedules, either daily or three days on/four days off every week schedule with 28-day cycles. The maximum tolerated dose (MTD) was 6 mg for the daily schedule and was not defined in the 3 days on/four days off schedule. Common side effects included nausea (56%), fatigue (49%), hyperglycemia (38%), thrombocytopenia (36%), diarrhea (28%), anorexia (23%), emesis (23%), stomatitis (21%), anemia (21%), and constipation (18%). 27% discontinued drug due to an adverse event. Among 27 patients evaluable for confirmed responses, 1 multiple myeloma patient (7 mg QD) achieved a major response lasting 2 months, and 13 myeloma patients (2 at 2 mg QD, 4 each at 4 mg QD and 7 mg QD, 2 at 9 mg QDx3d QW, and 1 at 12 mg QDx3d QW) and 2 Waldenstrom's macroglobulinemia patients (at 6 mg QD and 9 mg QDx3d QW) had stable disease.

Subsequent experience with the daily schedule has indicated that MLN0128 (TAK-228) at 6 mg/day is intolerable for prolonged administration in some patients (L. A. Doyle, personal communication). Accordingly, the recommended phase 2 dose is 5 mg/day for daily administration.

2.2.4 Nonclinical Pharmacokinetics

Preclinical pharmacokinetic studies were examined in mice, rats, dogs, and monkeys. After intravenous administration, MLN0128 (TAK-228) displayed a volume of

distribution of 1.4 (dogs) to 14.3 liters/kg (rats) and a terminal half-life of 1.1 (mice) to 18.9 hours (dogs). These studies also demonstrated that MLN0128 (TAK-228) is rapidly absorbed from the GI tract and displays high bioavailability (64-73%) in all species tested except rats (15). MLN0128 (TAK-228) crossed the blood-brain barrier poorly, with plasma:brain concentrations of 5:1 in mice receiving a single oral dose (15).

MLN0128 (TAK-228) binding to plasma proteins was examined across species. Plasma protein binding ranged from 47% (dogs) to 70.4% (humans). MLN0128 (TAK-228) inhibited both drug exporters and drug importers, but only at concentrations that far exceed the anticipated clinical concentrations. In particular, MLN0128 (TAK-228) inhibited the exporter ABCG2 (BCRP)with an IC_{50} of 52 micromolar and the drug importers OCT1 and OCT2 with IC_{50}s of 19-28 micromolar and 1.9 micromolar, respectively (15).

2.2.5 Clinical Pharmacokinetics

Pharmacokinetic analysis performed in conjunction with the INK128-001 study demonstrated rapid oral absorption, with a C_{max} at approximately 2 h, and a mean terminal half-life in the plasma of 6-8 hours across dose levels (15). After the initial dose, exposures (measured as the area under the plasma concentration vs. time curve) were dose-proportional over the range of 2 to 40 mg. MLN0128 (TAK-228) did not accumulate to any appreciable extent with repeated oral dosing.

Pharmacokinetic analysis performed in conjunction with the INK128-002 study in hematological malignancies yielded similar results (15). After oral administration, C_{max} was achieved within 1-2 hours. Once again the mean plasma half-life was 8 hours and a dose-dependent increase in exposure was observed. The mean plasma C_{max} at the MTD of 6 mg/d was 46 ng/ml on cycle 1/day 1 and 31 ng/ml on cycle 2/day 1, corresponding to plasma levels of 150 and 100 nM, respectively. These results again indicate that MLN0128 (TAK-228) did not accumulate to any appreciable extent during daily oral dosing.

Metabolism in humans
Incubation of MLN0128 with human liver microsomes results in a single metabolite (M1) that is equipotent with the parent agent in inhibiting mTOR kinase. While M1 represents as much as 30% of circulating drug in rats, it represents only 4% of circulating drug in humans and is not felt to contribute significantly to MLN0128 (TAK-228) pharmacodynamic effects (15).

The capacity for MLN0128 (TAK-228) to interact with the cytochrome P450 drug metabolizing machinery has been examined (15). In a study using recombinant human isozymes, CYP2C9, 2C19, and 3A4 metabolized MLN0128 *in vitro*, whereas CYP1A2, 2B6, and 2C8 did not. MLN0128 (TAK-228) (up to 10 μ M) did not induce CYP1A2, 2B6, and 3A4 activity and expression in human hepatocytes. MLN0128 (TAK-228) did not inhibit CYP1A2, 2B6, 2C8, 2C9, 2C19, 2D6, or 3A4/5 in human liver microsomal incubations.

Potential for drug interactions
Based on the high concentrations required to inhibitor drug transporters, as well as the lack of induction or inhibition of commonly studied cytochrome P450 isoenzymes, MLN0128 (TAK-228) is felt to have limited potential for drug-drug interactions at the anticipated therapeutic concentrations (15).

2.2.6 Rationale for Proposed Starting Dose

Preclinical studies have demonstrated that prolonged mTOR inhibition (at least 72 hours) is required to see signs of apoptosis induction in ALL cell lines by dual mTORC1/mTORC2 inhibitors *in vitro* (18). Upon administration of these inhibitors to mice bearing human lymphoid xenografts, xenograft regression is not evident until days 6-8 but continues throughout the course of treatment (18). These observations, along with studies in murine Ph+ ALL showing efficacy of daily dosing (12), prompt this trial of MLN0128 (TAK-228) dosed on a daily schedule.

Preclinical studies in the Kaufmann laboratory have demonstrated that uptake of the TORC1/TORC2 dual inhibitor OSI-027 is diminished in three of four lymphoid lines selected for resistance by continuous exposure. These lines are cross-resistant to MLN0128 (TAK-228). The uptake defect is reversed by drug removal for 3 days. These observations lead to the proposal to administer MLN0128 (TAK-228) for 21 of every 28-day cycle.

2.2.7 Milled MLN0128 (TAK-228) Drug Substance (DS, process B)

In order to allow more predictable absorption of MLN0128 (TAK-228) after oral administration and to allow scale-up manufacturing of MLN0128 (TAK-228) capsules, Millennium/Takeda developed a new milled formulation of the agent. The physical milling step during the granulation process controls particle size distribution of MLN0128 (TAK-228). In order to observe whether this milling step altered the safety and PK profile of MLN0128 (TAK-228), the company performed in vivo studies with PK analysis of milled MLN0128 (TAK-228). These studies indicated that the milled formulation may result in faster absorption with possibly higher maximum concentration (Cmax), which could result in a different safety profile, compared to the previous unmilled API capsules. In rats, the mean AUC milled/unmilled was 2.97 ± 0.8, while in monkeys, it was 1.68 ± 0.86. The C_{max} ratios were 1.68 ± 0.86.

Takeda developed new MLN0128 (TAK-228) capsules containing milled active pharmaceutical ingredient (API) for clinical studies in 1 mg, 3 mg, and 5 mg strengths. Patients receiving the milled formulation were added onto ongoing studies C31001 and C31002, as well as a new study MLN0128-1004, to determine the PK parameters of milled and unmilled MLN0128 in patients administered single doses under both fasted and fed conditions, with various treatment cohorts including daily and weekly administration of milled MLN0128 (TAK-228)

The recommended dose of milled MLN0128 (TAK-228) was evaluated in 17 patients of

MLN0128-1004, with PK, safety, and tolerability assessed. Six patients were given a 4 mg QD dose of milled MLN0128 (TAK-228) and 3 patients had observed DLT (rash, appetite loss and fatigue). A dose of 3 mg QD was given to 11 patients with only 1 DLT (decreased platelets) observed. The 3 mg QD dose of MLN0128 (TAK-228) was declared the RP2D, and was generally well tolerated and demonstrating objective responses in patients.

A significant and meaningful difference in tolerability was observed in the comparison of the MTDs between unmilled and milled MLN0128 (TAK-228) when administered QD. This difference in tolerability may be possibly explained due to the effect of food on the safety/tolerability of unmilled MLN0128 (TAK-228) in study IND128-001. The GastroPlus™ simulation performed on study suggests that under fasting conditions, unmilled and milled MLN0128 (TAK-228) administrations result in comparable exposures to MLN0128 (TAK-228); whereas in the fed state, milled MLN0128 (TAK-228) resulted in higher C_{max} (1.5- to 2-fold higher) and earlier T_{max} than unmilled MLN0128 (TAK-228) with comparable AUCs. Consequently, a dose of 3 mg QD was chosen as the RP2D of milled MLN0128 (TAK-228) dose in empty stomach conditions.

The RP2D for milled MLN0128 (TAK-228) on a weekly schedule was determined to be 30 mg, the same weekly RP2D as seen for the older unmilled formulation. Six patients treated at 30 mg weekly with the milled formulation did not demonstrate any DLT, but the agent was not escalated further. No DLT had been demonstrated for milled MLN0128 (TAK-228) at the prior 20 mg QW dose as well.

2.3 Rationale

A number of studies (19-24) have demonstrated that the rapalogs temsirolimus and everolimus, which inhibit TORC1, have clinical activity in lymphoid malignancies. Unfortunately, fewer than 5 patients with ALL were treated with rapamycin analogs in phase I clinical trials (25), so the single-agent activity of rapalogs in ALL is unknown.

The rationale for the development of TORC1/TORC2 dual inhibitors has been extensively reviewed (26-33). In brief, after binding to FKBP12, rapalogs inhibit TORC1 but do not directly inhibit TORC2. As a result, there has been concern that rapalogs can activate survival pathways, either through increased TORC2-mediated phosphorylation of AKT or through derepression of insulin receptor substrate- (IRS-) mediated signaling to the PI3 kinase/AKT pathway in some cell types (34-36). To overcome this potential limitation of rapalogs, agents that simultaneously target TORC1 and TORC2 have been developed. OSI-027 and MLN0128 (TAK-228) are two such inhibitors. Both are ATP-competitive inhibitors of TORC1 and TORC2, but MLN0128 (TAK-228) is approximately 20-fold more potent.

Preclinical studies performed *in vitro* and *in vivo* have demonstrated that dual mTOR inhibitors such as OSI-027 and MLN0128 (TAK-228) appear to be particularly active in

certain lymphoid neoplasms (18, 37). When Gupta *et al*. compared the effects of dual mTOR inhibitor OSI-027 in cell lines derived from various lymphoid malignancies, including T-cell ALL (Jurkat, CEM, Molt3), B-cell ALL (Nalm6), diffuse large B cell lymphoma (HT, RL) and mantle cell lymphoma (Jeko, Mino), the ALL lines were consistently more sensitive than the other lymphoid cell lines in 5-day MTS assays (18). Subsequent experiments demonstrated that OSI-027, but not rapamycin, induced apoptosis in the ALL lines that was mediated by the upregulation of the pro-apoptotic BCL2 family members BCL2L11 (Bim) and BBC3 (Puma) (18). Further investigation has shown that BCL2L11 (Bim) upregulation reflects transactivation of the *BCL2L11* (Bim) promoter downstream of mTORC2 inhibition, whereas BBC3 (Puma) upregulation reflects inhibition of mTORC1-mediated 4EBP1 phosphorylation and consequent inhibition of translation of a BBC3 (Puma) suppressor (14).

Two studies performed in nude mice bearing Jeko (human mantle cell lymphoma) xenografts demonstrated marked tumor regressions, associated with induction of BCL2L11 (Bim) and BBC3 (Puma), after treatment with OSI-027 but not rapamycin (18). In contrast, the RL diffuse large B-cell lymphoma cell line, which contains a t(14;18) translocation that results in marked upregulation of a BCL2 protein with a gain-of-function mutation (38), did not respond to OSI-027 *in vivo* (18).

In further preclinical studies, clinical samples from patients with various lymphoid neoplasms were exposed to varying OSI-027 concentrations *ex vivo* and examined for viability at the end of 5 days. OSI-027 diminished MTS reduction in a variety of samples, including ALL, diffuse large B cell lymphoma, mantle cell lymphoma, marginal zone lymphoma, and Sezary syndrome, with ALL samples being among the most sensitive (18). In contrast, CLL and follicular lymphoma were highly resistant.

In a more detailed study in primary ALL samples using the same assay (14), IC_{50} values ranged from 80-2000 nM for OSI-027 and 2-100 nM for MLN0128 (TAK-228). Clinical ALL isolates were generally as sensitive as Jurkat cells (14), an intermediate sensitivity T-cell ALL line (18). Direct comparison of the effects of 250 nM MLN0128 (TAK-228) and 10 nM rapamycin (the highest sustainable concentrations in humans) revealed that MLN0128 (TAK-228) exhibited greater cytotoxic effects in all six clinical ALL isolates treated with both agents.

Further analysis indicated that similar biochemical effects were observed in ALL cell lines and primary ALL samples after treatment with mTORC1/mTORC2 dual inhibitors *in vitro* (14). In particular, MLN0128 (TAK-228) abolished 4EBP1 phosphorylation in Jurkat T-cell ALL cells at 200 nM and in primary ALL cells at 62.5 nM *in vitro* (N. Vincelette, K. Peterson and S. Kaufmann, unpublished observations). These observations provide part of the rationale for the correlative studies described in Sections 2.4 and 9 of this protocol

In a separate study, MLN0128 (TAK-228) suppressed proliferation of B-ALL cell lines and reduced colony formation by primary human leukemia cells from adult and pediatric B-ALL patients *in vitro* (39). In a syngeneic mouse model of lymphoid BCR-ABL

positive disease, MLN0128 (TAK-228) cleared leukemic growth. In this and additional *in vivo* models, MLN0128 (TAK-228) was well tolerated and did not suppress endogenous bone marrow proliferation.

Given the sensitivity of ALL cells and clinical ALL samples to TORC1/TORC2 dual inhibitors described above, as well as the limited treatment options for patients with relapsed ALL, the present study is a phase II trial designed to assess the activity of single-agent MLN0128 (TAK-228) in relapsed and refractory ALL.

2.4 Correlative Studies Background

2.4.1 Plasma and Marrow Pharmacokinetics

Rationale: These studies are necessary to characterize the pharmacokinetics of MLN0128 (TAK-228) in patients with acute lymphoblastic leukemia, to provide a basis for understanding differences in toxicity and responses to MLN0128 (TAK-228) and to assess pharmacodynamic relationships with phosphorylated mTOR substrates and expression levels of BCL2L11 (Bim), BBC3 (Puma), MCL1 and BCL2.

Assay description: Plasma and marrow concentrations of MLN0128 (TAK-228) will be measured by lc/ms/ms methods developed in the Mayo Clinic Cancer Center Pharmacology Shared Resource.

2.4.2 Phosphorylation of the mTOR Substrate 4EBP1 (Integrated Biomarker)

Rationale: By comparing the phosphorylation status of 4EBP1 in marrows harvested prior to treatment and again on day 8, we will assess whether levels of MLN0128 (TAK-228) sufficient to inhibit mTOR have been achieved in leukemic blasts *in situ*. Conversely, we have observed that Nalm6 B-cell ALL cells selected for resistance to mTORC1/mTORC2 dual inhibitors fail to exhibit inhibition of 4EBP1 phosphorylation upon treatment with OSI-027 or MLN0128 (TAK-228), suggesting that failure to inhibit the mTORC1/4EBP1 axis might represent a mechanism of resistance to this class of agent. Thus, this assay is being performed to confirm mechanism of action of MLN0128 (TAK-228) as well as assess a potential readout for resistance to this class of agent.

Assay description: Immunoblotting will be performed for phospho-Thr^{37,46}-4EBP1 using techniques previously described by the Kaufmann laboratory (40, 41). Whole cell lysates (42, 43) will be prepared from Ficoll/Hypaque-purified marrow mononuclear cells (assayed for blast percentage by morphological criteria). Aliquots containing equal amounts of protein from cells harvested prior to treatment and on day 8 will be subjected to SDS-PAGE in adjacent wells of gels and probed for phospho-Thr^{37,46}-4EBP1 and total 4EBP1. Further details related to the assay are found in Section 9.1.3 of this protocol.

2.4.3 Phosphorylation of the mTOR Substrate AKT Ser^{473} (Exploratory Biomarker)

Rationale: the assay for phosphorylated 4EBP1 examines inhibition of mTORC1. As indicated in Section 2.3, mTORC1/mTORC2 dual inhibitors have been developed based on the rationale that inhibiting mTORC2 will prevent feedback-mediated upregulation of pro-survival pathways. In addition, recent results have demonstrated that mTORC2 inhibition plays a major role in the MLN0128 (TAK-228)-induced upregulation of the proapoptotic BCL2 family member BCL2L11 (Bim) (14). By comparing the phosphorylation of AKT (Ser^{473}) in marrows harvested prior to treatment and again on day 8, we will assess whether TORC2 has also been inhibited in leukemic blasts *in situ*. Thus, this assay is being performed to provide further proof of the mechanism of action of this agent in clinical ALL *in situ*.

Assay description: Whole cell lysates prepared from Ficoll/Hypaque-purified marrow mononuclear cells prior to treatment and on day 8 will subjected to immunoblotting for phospho-Ser^{473}-AKT and total AKT. Further details regarding the proposed assay are contained in Section 9.1.4.

2.4.4 Expression of BCL2L11 (Bim), BBC3 (Puma), MCL1 and BCL2 (Exploratory Biomarkers)

Rationale: Previous studies have demonstrated that the mTORC1/mTORC2 dual inhibitors induce apoptosis *in vitro* and in xenograft models by upregulating the pro-apoptotic BCL2 family members BCL2L11 (Bim) and BBC3 (Puma) as well as, in some lymphoid lines, down regulating MCL1 (14, 18). Importantly, blunting BCL2L11 (Bim) or BBC3 (Puma) upregulation diminishes mTORC1/mTORC2 dual inhibitor-induced apoptosis (14, 18). By comparing expression of BCL2L11 (Bim), BBC3 (Puma) and MCL1 at the protein level by immunoblotting in marrows harvested prior to treatment and again on day 8, we will begin to assess whether the mechanisms of induction of apoptosis observed in preclinical studies are also observed in ALL in situ.

Conversely, prior results have shown that BCL2 overexpression is a mechanism of resistance to this class of agent (14, 18). Thus, assessment of pretreatment BCL2 as well as the degree of upregulation of BCL2L11 (Bim) and BBC3 (Puma) will begin to examine potential mechanisms of resistance as well.

Assay description: Immunoblotting will be performed for BCL2L11 (Bim), BBC3 (Puma), MCL1 and BCL2 (40, 41). As described in Section 2.4.2, whole cell lysates (42, 43) will be prepared from Ficoll/Hypaque-purified marrow mononuclear cells (assayed for blast percentage by morphological criteria). Aliquots containing equal amounts of protein from cells harvested prior to treatment and on day 8 will be subjected to SDS-PAGE in adjacent wells of gels and probed with commercial antibodies to BCL2L11 (Bim), BBC3 (Puma), MCL1, and BCL2. Blots will be reprobed for histone H1, a protein that should be present in similar amounts in different diploid cells, to provide a loading control. Further details regarding this assay can be found in Section 9.1.5 of this protocol. If sufficient material remains after this assay, additional BCL2 family members not currently implicated in mTOR inhibitor action in ALL samples, such as BCL2L1

(Bcl-x_L) and BCL2L2 (Bcl-w) that could in principle affect the outcome of treatment, will be considered for assessment in a similar fashion.

2.4.5 Phosphoproteomic Analysis (Exploratory Biomarker)

Rationale: Prior publications have identified differences in the pattern of protein phosphorylation between cell lines that are sensitive to kinase inhibitors and similar cell lines that are resistant (e.g., 44, 45). Preliminary studies in the Kaufmann laboratory have likewise demonstrated differences in protein phosphorylation at key nodes between parental Nalm6 B-cell ALL cells and Nalm6 cells selected for the ability to grow continuously in 8 μM OSI-027 (C. Correia, K. L. B. Knorr, S. Yun, A. E. Wahner Hendrickson, B. Madden, D. McCormick, S. Dasari, and S. H. Kaufmann, unpublished observations). Thus, we hypothesize that pretreatment patterns of phosphorylation might differ between ALLs that respond to MLN0128 and those that do not.

Assay description and analysis: Bone marrow aspirates harvested prior to therapy and again on day 8 of treatment will be enriched for leukemic blasts on Ficoll-Hypaque gradients. Whole cell lysates prepared as described in Sections 2.2 and 2.3 will be analyzed for a set of more than 60 key phosphorylations and 200 key regulatory proteins by reverse phase protein array (RPPA) in the M.D. Anderson RPPA Core Facility. Further information regarding this assay is found in Section 9.1.6.

2.4.6 Transcriptional Profiling (Exploratory Biomarker)

Rationale: Recent publications have identified additional changes that correlate with resistance to mTOR dual inhibitors, including overexpression of eIF4E (46). To search for changes such as this and provide an opportunity for further unbiased analysis of potential markers of MLN0128 sensitivity vs. resistance, samples (when cell number is sufficient) will be saved for transcriptional profiling to assess whether there is a particular gene expression profile that correlates with sensitivity vs. resistance to MLN0128 (TAK-228).

Assay description and analysis: Bone marrow aspirates harvested prior to therapy and again on day 8 of treatment will be enriched for leukemic blasts on Ficoll-Hypaque gradients. Samples will be lysed in RNAlater (Qiagen) and stored at -80 °C until analyzed. Further analysis will be performed only if the studies above do not yield robust testable hypotheses regarding responders vs. nonresponders. A further description of this assay is provided in Section 9.1.7.

2.4.7 Prioritization of Cell-Based Correlative Studies

The cell-based correlative studies are designed to provide a test of mechanism. According to current understanding, the killing of ALL cells by mTORC1/mTORC2 dual inhibitors depends upon the activation of two pathways, one involving drug-induced inhibition of mTORC1-mediated 4EBP1 phosphorylation, leading sequentially to decreased 4EBP1 phosphorylation, decreased eIF4E-facilitated translation of cMYC

mRNA, decreased c-MYC protein, increased BBC3 (Puma) mRNA stability and increased BBC3 (Puma) protein, and another pathway involving decreased mTORC2-mediated phosphorylation of a transcription factor cascade leading to increased activation of the *BCL2L11* promoter, increased BCL2L11 (Bim) mRNA, and increased BCL2L11 (Bim) protein. One or both pathways are engaged in all clinical ALL samples exposed to MLN0128 (TAK-228) *ex vivo* to date. To assure that the mechanism of action is thoroughly investigated in paired clinical samples, the order of priority will be to assay first those antigens that have been directly implicated in mTORC1/mTORC2 dual inhibitor induced killing in human ALL cells, i.e., phospho-4EBP1 (integrated biomarker, a marker of mTORC1 inhibition), phospho-Ser^{473}-AKT (exploratory biomarker, a marker of mTORC2 inhibition), BBC3 (Puma) protein levels (exploratory biomarker, responsible for killing downstream of mTORC1 inhibition) and BCL2L11 (Bim) protein levels (exploratory biomarker, responsible for killing downstream of mTORC2 inhibition). Because some of the antibodies used in the RPPAs (notably anti-BBC3) are particularly problematic (recognize a band at the expected molecular weight in cells with targeted interruption of the *BBC3* gene), we have elected to perform the analysis by immunoblotting (the technique approved by the NCI Biomarker Review Committee for the integrated biomarker) and will utilize the RPPA for confirmatory analysis of those antigens that are in common. Addition analytes recommended by the CTEP Consensus Review, including phospho-p70S6 kinase (another biomarker of mTORC1 inhibition, but one that does not correlate with mTOR inhibitor killing[18]) and the anti-apoptotic proteins BCL2L1 (Bcl-x_L) and BCL2L2 (Bcl-w), which might correlate with response but are not directly implicated in the mechanism of action of this class of compounds, have been prioritized lower. Likewise, because mTOR inhibitors affect translation of mRNA particularly dramatically, protein studies have been prioritized above mRNA studies. The proposed cell-based studies are presented above and in Section 9.1 in the order in which they are prioritized.

3. PATIENT SELECTION

3.1 Eligibility Criteria

3.1.1 WHO-defined acute lymphoblastic leukemia and **either**:

- Relapsed after achieving remission
- Refractory to therapy;
- Newly diagnosed and ineligible for intensive chemotherapy induction

Note: Patients with T lineage and B lineage ALL are eligible for this trial. Likewise, patients with Ph+ (as long as they are not candidate for other therapies for Ph+) and Ph- ALL are eligible.

3.1.2 Bone marrow blasts of at least 10%.

3.1.3 At least 4 weeks away from any previous antineoplastic or investigational agent. Patients may receive hydroxyurea or glucocorticoids for suppression of leukocytosis, but these must be stopped at least 24 h prior to initiation of therapy.

3.1.4 Age ≥18 years.

3.1.5 ECOG performance status ≤ 2

3.1.6 Life expectancy of >2 months

3.1.7 Patients must have normal organ function and metabolism as defined below (abnormal labs may be retested to determine if they normalize and meet this critieria):

- Total bilirubin ≤ 1.5 × institutional upper limit of normal
- AST(SGOT)/ALT(SGPT) ≤ 2.5 × institutional upper limit of normal
- Creatinine ≤ 1.5 × institutional upper limit of normal
- Fasting blood glucose (FBG) < 130 mg/dL
- HbA1C < 7.0%

3.1.8 Relapse after stem cell transplantation (SCT) is allowed but no active GVHD as per treating physician. Also must not exceed the number of prior induction regimens listed above. SCT does not count as line of therapy.

3.1.9 Negative serum pregnancy test result

Note: The effects of MLN0128 (TAK-228) on the developing human fetus are unknown. For this reason and because mTOR inhibitors are known to be teratogenic, women of child-bearing potential and men must agree to use 1 highly effective method of contraception and 1 additional effective (barrier) method, at the same time, from the time of signing the informed consent through 90 days (or longer, as mandated by local labeling [e.g. USPI, SmPC, etc]) after the last does of study drug. Should a woman become pregnant or suspect she is pregnant while she or her partner is participating in this study, she should inform her treating physician immediately. Men treated or enrolled on this protocol must also agree to use highly effective barrier contraception prior to the study, for the duration of study participation, and 4 months after completion of MLN0128 (TAK-228) administration.

3.1.10 Ability to understand and the willingness to sign a written informed consent document.

3.1.11 No prior therapy with mTOR inhibitors except for rapalog treatment as part of GVH prophylaxis or treatment.

3.1.12 HIV infected patients (**if HIV positive)**

HIV infected individuals are eligible provided they meet all the protocol eligibility criteria in addition to the following:

- No history of AIDS defining illness other than an historic CD4+ T-cell nadir < 200/mm^3.

- Prior to leukemia diagnosis, the HIV disease was uncomplicated as evidenced by:
  - the CD4+ T-cell counts were generally in excess of 300/mm^3
  - the HIV viral loads were less than 200 copies/ml if on anti-HIV therapy.
  - If the HIV is newly diagnosed or there is no history of using anti-HIV therapy, there are no AIDS defining conditions or other HIV-related symptoms.
  - Zidovudine is not allowed as part of the anti-HIV therapy.

3.1.13 Patients with diabetes controlled by diet or medication are allowed on trial. Controlled diabetes is defined as FBG < 130 mg/kL in the context of this study.

3.2 Exclusion Criteria

3.2.1 Patients who have had chemotherapy or radiotherapy ≤ 4 weeks prior to entering the study or those who have not recovered from adverse events due to agents administered more than 4 weeks earlier. Treatment with glucocorticoids, hydroxyurea, and tyrosine kinase inhibitors is allowed up to 24 hour prior to initiation of therapy.

3.2.2 Patients with WBC > 30,000 are not eligible to start therapy. However, it is permissible to use glucocorticoids and/or hydroxyurea to diminish peripheral WBC to less than 30,000 provided these agents are stopped at least 24 hours prior to the first dose of MLN0128 (TAK-228).

3.2.3 Patients who are receiving any other investigational agents.

3.2.4 Patients with known other active cancers. Skin cancers (basal or squamous) are exempted.

3.2.5 History of allergic reactions attributed to compounds of similar chemical or biologic composition to MLN0128 (TAK-228).

3.2.6 Because of the reportedly low potential for interaction with agents that induce or inhibit cytochrome P450 enzymes (summarized in Section 2.2.5), there are no prohibitions of specific medications on the basis of anticipated drug-drug interactions.

3.2.7 Uncontrolled intercurrent illness including, but not limited to, ongoing or active infection, symptomatic congestive heart failure, hypertension, unstable angina pectoris, cardiac arrhythmia, or psychiatric illness/social situations that would limit compliance with study requirements. No ischemic myocardial or cerebrovascular event, placement of pacemaker, or pulmonary embolism within six months of receiving first dose of MLN0128 (TAK-228).

3.2.8 Any patient receiving chronic corticosteroid administration prior to study enrollment is ineligible.

3.2.9 Baseline prolongation of the rate-corrected QT interval (QTc) > 480 milliseconds or history of congenital long QT syndrome or Torsades de pointes.

3.2.10 Concomitant administration of any proton pump inhibitor (PPI) is not permitted during the study. Patients receiving PPI therapy before enrollment must stop using the PPI for 7 days before their first dose of study drugs.

3.3 Inclusion of Women and Minorities

Both men and women of all races and ethnic groups are eligible for this trial.

4. REGISTRATION PROCEDURES

4.1 Investigator and Research Associate Registration with CTEP

4.1.1 CTEP Registration Procedures

Food and Drug Administration (FDA) regulations and National Cancer Institute (NCI) policy require all investigators participating in any NCI-sponsored clinical trial to register and to renew their registration annually.

Registration requires the submission of:

- a completed ***Statement of Investigator Form*** (FDA Form 1572) with an original signature
- a current Curriculum Vitae (CV)
- a completed and signed ***Supplemental Investigator Data Form*** (IDF)
- a completed ***Financial Disclosure Form*** (FDF) with an original signature

Fillable PDF forms and additional information can be found on the CTEP website at http://ctep.cancer.gov/investigatorResources/investigator_registration.htm.

For questions about Investigator Registration, please contact the ***CTEP Investigator Registration Help Desk*** by email at pmbregpend@ctep.nci.nih.gov.

4.1.2 CTEP Associate Registration Procedures / CTEP-IAM Account

The Cancer Therapy Evaluation Program (CTEP) Identity and Access Management (IAM) application is a web-based application intended for use by both Investigators (i.e., all physicians involved in the conduct of NCI-sponsored clinical trials) and Associates (i.e., all staff involved in the conduct of NCI-sponsored clinical trials).

Associates will use the CTEP-IAM application to register (both initial registration and annual re-registration) with CTEP and to obtain a user account.

Investigators will use the CTEP-IAM application to obtain a user account only. (See CTEP Investigator Registration Procedures above for information on registering with CTEP as an Investigator, which must be completed before a CTEP-IAM account can be requested.)

An active CTEP-IAM user account is needed to access all CTEP and CTSU (Cancer Trials Support Unit) websites and applications, and is critical to the conduct of this study, including document access, patient enrollment, and clinical data submission.

Additional information can be found on the CTEP website at http://ctep.cancer.gov/branches/pmb/associate_registration.htm.

For questions about Associate Registration or CTEP-IAM Account Creation, please contact the CTEP Registration Help Desk: ctepreghelp@ctep.nci.nih.gov

4.2 Site Registration

This study is supported by the NCI Cancer Trials Support Unit (CTSU).

Each investigator or group of investigators at a clinical site must obtain IRB approval for this protocol and submit IRB approval and supporting documentation to the CTSU Regulatory Office before they can be approved to enroll patients. Assignment of site registration status in the CTSU Regulatory Support System (RSS) uses extensive data to make a determination of whether a site has fulfilled all regulatory criteria including but not limited to: an active Federal Wide Assurance (FWA) number, an active roster affiliation with the Lead Network or a participating organization, a valid IRB approval, and compliance with all protocol specific requirements.

Sites participating on the NCI CIRB initiative that are approved by the CIRB for this studyare not required to submit IRB approval documentation to the CTSU Regulatory Office. For sites using the CIRB, IRB approval information is received from the CIRB and applied to the RSS in an automated process. Signatory Institutions must submit a

Study Specific Worksheet for Local Context (SSW) to the CIRB (via IRB Manager) to indicate their intention to open the study locally. The CIRB's approval of the SSW is then communicated to the CTSU Regulatory Office for compliance in the RSS. In order for the SSW approval to be processed, the Signatory Institution must inform the CTSU which CIRB-approved institutions aligned with the Signatory Institution are participating in the study.

4.2.1 Downloading Regulatory Documents

Site registration forms may be downloaded from the 9775 protocol page located on the CTSU Web site. Permission to view and download this protocol is restricted and is based on person and site roster data housed in the CTSU RSS. To participate, Investigators and Associates must be associated with the lead or participating protocol organization in the RSS.

- Go to https://www.ctsu.org and log in using your CTEP IAM username and password
- Click on the Protocols tab in the upper left of your screen
- Either enter the protocol number in the search field at the top of the protocol tree or
- Click on the By Lead Organization folder to expand, then select LAO-MN026 and protocol # 9775
- Click on LPO Documents, select the Site Registration documents link, and download and complete the forms provided. (Note: For sites under the CIRB initiative, IRB data will automatically load to RSS.)

4.2.2 Requirements for Protocol 9775 Site Registration

- CTSU Transmittal Sheet (optional)
- IRB approval (For sites not participating via the NCI CIRB; local IRB documentation, an IRB-signed CTSU IRB Certification Form, Protocol of Human Subjects Assurance Identification/IRB Certification/Declaration of Exemption Form, or combination is accepted)

4.2.3 Submitting Regulatory Documents

Submit required forms and documentsto the CTSU Regulatory Office, where they will be entered and tracked in the CTSU RSS. Regulatory Submission Portal: www.ctsu.org (members' area) → Regulatory Tab →Regulatory Submission

When applicable, original documents should be mailed to:
CTSU Regulatory Office
1818 Market Street, Suite 1100
Philadelphia, PA 19103

Institutions with patients waiting that are unable to use the Portal should alert the CTSU Regulatory Office immediately at 1-866-651-2878 in order to receive further instruction and support.

4.2.3 Checking **Site** Registration Status

Sites can check the status of their registration packets by querying the Site Registration subtab of the members' section of the CTSU Web site. (Note: Sites will not receive formal notification of regulatory approval from the CTSU Regulatory Office.)

- Go to https://www.ctsu.org and log in using your CTEP IAM username and password
- Click on the Regulatory tab at the top of your screen
- Click on the Site Registration sub tab
- Enter your 5-character CTEP Institution Code and click on Go

Note: If possible, please allow three working days for site registration approval before attempting to enroll your first patient.

4.3 Patient Registration

4.3.1 OPEN / IWRS

Patient enrollment will be facilitated using the Oncology Patient Enrollment Network (OPEN). OPEN is a web-based registration system available to users on a 24/7 basis. It is integrated with the CTSU Enterprise System for regulatory and roster data interchange and with the Theradex Interactive Web Response System (IWRS) for retrieval of patient registration/randomization assignment. Patient enrollment data entered by Registrars in OPEN / IWRS will automatically transfer to the NCI's clinical data management system, Medidata Rave.

The OPEN system will provide the site with a printable confirmation of registration and treatment information. Please print this confirmation for your records.

4.3.2 OPEN/IWRS User Requirements

OPEN/IWRS users must meet the following requirements:

- Have a valid CTEP-IAM account (i.e., CTEP username and password).
- To enroll patients or request slot reservations: Be on an ETCTN Lead or Participating Organization roster with the role of Registrar.
- To access cohort management: Be identified to Theradex as the "Client Admin" for the study.
- Have regulatory approval for the conduct of the study at their site.

Prior to accessing OPEN/IWRS, site staff should verify the following:

- All eligibility criteria have been met within the protocol stated timeframes. Site staff should use the registration forms provided on the CTSU web site as a tool to verify eligibility.
- If applicable, all patients have signed an appropriate consent form and HIPAA authorization form.

4.3.3 OPEN/IWRS Questions?

Further instructional information on OPEN is provided on the OPEN tab of the CTSU website at https://www.ctsu.org or at https://open.ctsu.org. For any additional questions contact the CTSU Help Desk at 1-888-823-5923 or ctsucontact@westat.com.

4.4 General Guidelines

Following registration, patients should begin protocol treatment within 7 days. Issues that would cause treatment delays should be discussed with the Principal Investigator. If a patient does not receive protocol therapy following registration, the patient's registration on the study may be canceled.

5. TREATMENT PLAN

Patients will be screened for eligibility within 7 days of day 1. Starting dose will be 3 mg daily for days 1-21 of every 28-day cycle. Concomitant corticosteroid administration is not allowed for treatment of underlying malignancy (ALL) but is allowed for treatment emergent medical conditions that would ordinarily require corticosteroids, e.g., diffuse alveolar hemorrhage or severe treatment emergent dermatological conditions.

Timing of treatment should be such that pretreatment and day 8 marrows can be shipped on Monday-Thursday as described in Section 9.2.

All patients will receive one dose of clinically indicated intrathecal (IT) chemotherapy prophylactically within one week of starting study treatment. For patients with malignant CSF and due to poor penetration of CSF by MLN0128 (TAK-228), patients can be treated with IT methotrexate or cytarabine per standard practice of participating institution. At the time of ITl chemotherapy performed prior to study treatment and any clinically indicated subsequent IT chemotherapy performed during the study, up to a 2 mLsample of CSF will be collected for MLN0128 (TAK-228) pharmacokinetics if possible.

Responders (CR/CRi/PR/MLFS) to therapy at any time can proceed to consolidation therapy at 3 mg orally daily for 21-days every 28-day cycle until disease progression and as long as they are tolerating MLN0128 (TAK-228). Responders may go off study and proceed to stem cell transplantation if candidate and donor are available at any time.

Patients who achieve a PR with MLN0128 (TAK-228) will, by definition, have normalization of

platelet and neutrophil counts despite persistent identifiable blasts in the bone marrow. It is unclear how long they might respond to MLN0128 (TAK-228) and whether they are at risk of rapid progression while on this agent. Accordingly, all patients in PR after two cycles will be discussed with the study PI and CTEP monitor in a timely fashion to determine on a case by case basis whether continued treatment at same dose, escalation to a higher dose (per Section 6.0) or removal from study for an alternative regimen with a well described response rate in relapsed/refractory ALL is the best course of action

Non-responders and PR after 2 cycles of therapy (with no evidence of significant drug-related toxicity) could proceed to dose level +1 (4 mg orally daily for 21 days every 28-day cycle). Non-responders and PR after 2 additional cycles (with no evidence of significant drug-related toxicity) of dose escalation (end of cycle 4) could proceed to dose level +2 (4 mg orally daily for 28 days every 28-day cycle). Non-responders after 2 cycles (end of cycle 6), will be taken off study.

5.1 Agent Administration

5.1.1 MLN0128 (TAK-228)

Patients will receive MLN0128 (TAK-228) orally daily on days 1-21 every 28-day cycle. Patients will complete a pill diary and return at each visit. Leftover study medication will also be returned.

Treatment will be administered on an outpatient basis, but inpatient status is allowed too. Reported adverse events and potential risks are described in Section 7. Appropriate dose modifications are described in Section 6. No investigational or commercial agents or therapies other than those described below may be administered with the intent to treat the patient's malignancy.

Antiemetics and antidiarrheal agents are not required but may be administered as needed for symptom control.

If severe emesis or mucositis prevents the patient from taking scheduled doses, that dose will be skipped. If emesis occurs after study medication ingestion, the dose will not be readministered, and patients should resume dosing at the next scheduled time with the prescribed dosage. Patients should record the occurrence of the emesis in their dosing diaries. Under no circumstance should a patient repeat a dose or double-up doses.

5.1.2 Intrathecal chemotherapy:

5.1.2.1 For patients without known CNS involvement:

Within one week (at most ten days) prior to start of therapy, patients will receive mandatory CSF prophylaxis consisting of an intrathecal regimen according to institutional or national guidelines, e.g., methotrexate 12 to 15 mg and/or cytosine arabinoside 40 mg or dexamethasone 4 mg or equivalent steroid dose. In case of

anticipated safety risks caused by lumbar puncture, e.g., in case of thrombocytopenia, CSF prophylaxis may be omitted.

5.1.2.2 For patients with CNS involvement:

Intrathecal (IT) chemotherapy (methotrexate 12 to 15 mg and/or cytarabine 100 mg) can be given for patient with malignant CSF (involved with ALL by morphology or flow cytometry) due to poor penetration of MLN0128 (TAK-228) to CSF. Treatment will be as per standard practice of each participating institution.

5.2 General Concomitant Medication and Supportive Care Guidelines

As indicated in Section 2.2.5, MLN0128 (TAK-228) has low potential for interaction with other drugs based on its limited ability to induce or inhibit cytochrome P450 isoenzymes. Therefore, there are no proscriptions against particular concomitant medications.

Supportive care: transfusions with packed RBC or platelets will be done as per each local participating institution guidelines.

G-CSF: Treatment with filgrastim (5 mcg/kg SC daily until ANC > 5000) or Pegfilgrastim (6 mg SC x1) will be allowed if severe neutropenia exists or is suspected to occur. The start date will be left to the judgment of the treating physician, but dates of administration are to be recorded in the case report forms.

Management of Hyperglycemia

On the basis of the clinical experience in TAK-228 trials, most episodes of hyperglycemia observed occurred within the first 60 days after initiation of treatment with TAK-228 and have been either Grade 1 or Grade 2, and have responded quickly to oral metformin. Hyperglycemia has not been dose-limiting since the institution of a standard regimen for early treatment of hyperglycemia.

All patients developing hyperglycemia during the study should have their glucose closely monitored by study staff. The investigator may choose to continue close monitoring of patients who develop Grade 1 hyperglycemia (fasting glucose >ULN ≤160 mg/dL) or, alternatively, consider initiating treatment with an oral hypoglycemic agent, such as metformin. All patients with ≥ Grade 2 hyperglycemia (fasting glucose >160 mg/dL) must be treated aggressively with oral hypoglycemic agents and/or insulin as clinically indicated. The investigator should consult an endocrinologist, if needed, to aid in optimizing the patient's hyperglycemia treatment plan.

It is recommended that patients with elevated fasting blood glucose be initially treated with a fast acting insulin sensitizer such as metformin at 500 mg orally QD and titrate up to a maximum of 1000 mg orally BID as needed. Concurrent addition to metformin of DPP-4 inhibitors (e.g., sitagliptin or vildagliptin) and/or insulin should also be

considered. Oral sulfonylureas (e.g., glipizide or glyburide) should be used with caution, due to the higher risk of inducing hypoglycemia in patients. The dose of oral hypoglycemic agents should be adjusted in patients with renal insufficiency. In addition, patients should be encouraged to follow a low carbohydrate diet once hyperglycemia is first observed.

If any fasting serum glucose reading performed at the site indicates hyperglycemia (> ULN or ≥ 110 mg/dL), the study staff should first confirm that the patient was fasting at the time of blood specimen collection (ie, nothing by mouth for at least 8 hours before collection).

In-Home Daily Fasting Glucose Monitoring

In addition to obtaining fasting glucose levels at the clinic visits as outlined in the Schedule of Events, all patients receiving TAK-228 will be given a glucometer to monitor their daily fasting blood glucose levels at home. The level should be collected daily, predose on dosing days, and at approximately the same time each day.

On Cycle 1 Day 1, the patient will be provided an in-home glucometer. Patients should be trained on proper use of the glucometer and instructed to collect a daily fasting blood glucose level every morning (predose on dosing days), starting on Cycle 1 Day 2. Patients will be instructed to bring the glucometer with them to each study visit so that the data collected can be reviewed and recorded in the source documents. Investigators will be responsible for reviewing the home glucose monitoring logs for hyperglycemia.

The patient will be instructed to contact the site immediately if the value is abnormal (ie, ≥150 mg/dL) for further instructions on the management of their hyperglycemia. Hyperglycemia observed during home glucose monitoring should be confirmed in the clinic.

If no irregularities in the fasting blood glucose level are observed during a minimum of 2 consecutive months, then the frequency of in-home fasting blood glucose testing can be reduced to a minimum frequency of once weekly, depending on the investigator's judgment and approval. Patients will continue to notify the investigator of fasting blood glucose levels that exceed 150 mg/dL and, if blood glucose levels are not well controlled or if the patient requires either oral hypoglycemic agents or insulin to control blood glucose levels, then the frequency of in-home testing of fasting blood glucose levels will be reinstated to daily.

5.3 Duration of Therapy

In the absence of treatment delays due to adverse event(s), treatment may continue after response until one of the following criteria applies:

- Disease progression at any time,

- Intercurrent illness that prevents further administration of treatment,
- Unacceptable adverse event(s),
- Patient decides to withdraw from the study,
- General or specific changes in the patient's condition render the patient unacceptable for further treatment in the judgment of the investigator.
- No response after 6 cycles of MLN0128 (TAK-228). Response includes CR/CRi/PR or MLFS on bone marrow
- Dose delay of more than 1 cycle due to related toxicity or more than 2 cycles to unrelated toxicity
- If responding, patient proceeds with allogeneic stem cell transplant.

5.4 Duration of Follow Up

Patients will be followed for toxicity for 1 month after removal from study or until death, whichever occurs first. Patients removed from study for unacceptable adverse event(s) will be followed until resolution or stabilization of the adverse event. Patients will be followed for survival status by a telephone call every 3 months unless patient refuses. Patients will be followed periodically until death by using public health records, for example, a local newspaper.

5.5 Criteria for Removal from Study

Patients will be removed from active treatment when any of the criteria listed in Section 5.3 applies. The reason for removal from active treatment and the date the patient was removed must be documented in the Case Report Form.

6. DOSING DELAYS/DOSE MODIFICATIONS

Dose de-escalation will be pursued if patients develop a significant related AE as below.

Dose escalation could be chosen if patients fail to show a response (CR/CRi/ MLFS) and show no evidence of drug-related toxicity. Dose escalation or re-escalation will be done after a dose has been tolerated for 2 cycles.

Dose delays are allowed for a maximum of 1 cycle if patient develops a related toxicity and 2 months if unrelated toxicity (disease related for example).

Dose Level	MLN0128 (TAK-228) Dose
-2	2 mg orally Monday-Friday, weeks 1 - 3 of each 28-day cycle
-1	2 mg orally days 1 - 21
0	3 mg orally days 1 - 21
+1	4 mg orally days 1 - 21
+2	4 mg orally days 1 - 28

Note: All treatment modifications must be expressed as a specific dose or amount rather than as a percentage of the starting or previous dose.

Nausea	**Management/Next Dose for MLN0128 (TAK-228)**
≤ Grade 1	No change in dose
Grade 2	Hold until ≤ Grade 1. Resume at same dose level.
Grade 3	If experienced for ≤ 72 hours, hold MLN0128 (TAK-228) until ≤ Grade 1, then resume MLN0128 (TAK-228) without dose modification. If experienced for > 72 hours despite optimal therapy, hold MLN0128 (TAK-228) until ≤ Grade 1, then resume treatment with the dose of MLN0128 (TAK-228) reduced by 1 level.
Grade 4	Off protocol therapy
*Patients requiring a delay of > 4 weeks if related to MLN0128 (TAK-228) should go off protocol therapy.	

Vomiting	Management/Next Dose for MLN0128 (TAK-228)
≤ Grade 1	No change in dose
Grade 2	Hold until ≤ Grade 1. Resume at same dose level.
Grade 3	If experienced for ≤ 72 hours, hold MLN0128 (TAK-228) until ≤ Grade 1, then resume MLN0128 (TAK-228) without dose modification. If experienced for > 72 hours despite optimal therapy, hold MLN0128 (TAK-228) until ≤ Grade 1, then resume treatment with the dose of MLN0128 (TAK-228) reduced by 1 level.
Grade 4	Off protocol therapy
*Patients requiring a delay of > 4 weeks if related to MLN0128 (TAK-228) should go off protocol therapy.	

Diarrhea	**Management/Next Dose for MLN0128 (TAK-228)**
≤ Grade 1	No change in dose
Grade 2	Hold until ≤ Grade 1. Resume at same dose level.
Grade 3	Hold* until < Grade 2. Resume at one dose level lower
Grade 4	Off protocol therapy
*Patients requiring a delay of > 4 weeks if related to MLN0128 (TAK-228) should go off protocol therapy. **Patients requiring > two dose reductions should go off protocol therapy.	
Recommended management: Loperamide antidiarrheal therapy Dosage schedule: 4 mg at first onset, followed by 2 mg with each loose motion until diarrhea-free for 12 hours (maximum dosage: 16 mg/24 hours) Adjunct anti-diarrheal therapy is permitted and should be recorded when used.	

Hyperglycemia	**Management/Next Dose for MLN0128 (TAK-228)**
≤ Grade 1	No change in dose. Optimize blood sugar control. *
Grade 2	No change in dose. Optimize blood sugar control.
Grade 3 Asymptomatic	No change in dose. Optimize blood sugar control.
Grade 3 Symptomatic or >7 days on therapy	Hold** until < Grade 2. Resume at one dose level lower.
Grade 4	Off protocol therapy
* If medical therapy is needed, metformin is preferred unless contraindicated. Sulfonylurea can be added if metformin does not control blood glucose level. If this fails to bring it to ≤ G1 within 1 month, then oral hypoglycemics can be stopped and insulin used. **Patients requiring > two dose reductions should go off protocol therapy.	
Hyperglycemia will be based on fasting glucose level.	

Thrombocytopenia and neutropenia	**Management/Next Dose for MLN0128 (TAK-228)**
≤ Grade 1	No change in dose
Grade 2	Hold until ≤ Grade 1. Resume at same dose level.
Grade 3	Hold* until ≤ Grade 1. Resume at one dose level lower
Grade 4	Off protocol therapy
This dose modification for thrombocytopenia and neutropenia only pertains to	

Thrombocytopenia and neutropenia	**Management/Next Dose for MLN0128 (TAK-228)**
patients in remission. Thrombocytopenia and neutropenia will not be considered dose limiting during remission induction. *Patients requiring a delay of >4 weeks if related to MLN0128 (TAK-228) should go off protocol therapy. This only will be counted if patients are in remission (CR). **Patients requiring > two dose reductions should go off protocol therapy.	

Non-hematological toxicity:

Non-hematological toxicities of fatigue, electrolyte abnormalities and abnormal liver function tests, including bilirubin, transaminases and alkaline phosphatase will only require dose modification if they are likely or definitely related to MLN0128 (TAK-228) and persist more than 7 days. Neutropenic fever and infectious toxicities are only counted for if patients achieve remission and then develop these adverse events.

Non-Hematological Toxicity

Event	**Management/Next Dose for MLN0128 (TAK-228)**
≤ Grade 1	No change in dose
Grade 2	Hold until ≤ Grade 1. Resume at same dose level.
Grade 3	Hold* until Grade 1. Resume at one dose level lower
Grade 4	Patients who develop Grade 4 nonhematologic toxicities (with the exception of isolated non-clinically significant laboratory values) should permanently discontinue study treatment, unless they derive clinical benefit, in which case they may be retreated at a reduced dose level after recovery to ≤ Grade 1 severity.
**Patients requiring a delay of >4 weeks if related to MLN0128 (TAK-228) should go off protocol therapy. **Patients requiring > two dose reductions should go off protocol therapy.	

7. ADVERSE EVENTS: LIST AND REPORTING REQUIREMENTS

Adverse event (AE) monitoring and reporting is a routine part of every clinical trial. The following list of AEs (Section 7.1) and the characteristics of an observed AE (Section 7.2) will determine whether the event requires expedited reporting (via CTEP-AERS) **in addition** to routine reporting.

7.1 Comprehensive Adverse Events and Potential Risks List (CAEPR)

The Comprehensive Adverse Event and Potential Risks list (CAEPR) provides a single list of reported and/or potential adverse events (AE) associated with an agent using a uniform presentation of events by body system. In addition to the comprehensive list, a subset of AEs, the Specific Protocol Exceptions to Expedited Reporting (SPEER), appears in a separate column and is identified with ***bold*** and ***italicized*** text. The SPEER is a list of events that are protocol-specific exceptions to expedited reporting to NCI via CTEP-AERS (except as noted below). Refer to the 'CTEP, NCI Guidelines: Adverse Event Reporting Requirements' http://ctep.cancer.gov/protocolDevelopment/adverse_effects.htm for further clarification.

The CAEPR may not provide frequency data; if not, refer to the Investigator's Brochure for this information.

NOTE: The highest grade currently reported is noted in parentheses next to the AE in the SPEER. Report **ONLY** AEs higher than this grade expeditiously via CTEP-AERS. If this CAEPR is part of a combination protocol using multiple investigational agents and has an AE listed on different SPEERs, use the lower of the grades to determine if expedited reporting is required.

7.1.1 CAEPR for MLN0128 (TAK-228)

Comprehensive Adverse Events and Potential Risks list (CAEPR)
for
MLN0128 (TAK-228) (INK128, NSC 768435)

The Comprehensive Adverse Events and Potential Risks list (CAEPR) provides a single list of reported and/or potential adverse events (AE) associated with an agent using a uniform presentation of events by body system. In addition to the comprehensive list, a subset, the Specific Protocol Exceptions to Expedited Reporting (SPEER), appears in a separate column and is identified with bold and italicized text. This subset of AEs (SPEER) is a list of events that are protocol specific exceptions to expedited reporting to NCI (except as noted below). Refer to the 'CTEP, NCI Guidelines: Adverse Event Reporting Requirements' http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/aeguidelines.pdf for further clarification. *Frequency is provided based on 252 patients.* Below is the CAEPR for MLN0128 (TAK-228)(INK128).

NOTE: Report AEs on the SPEER **ONLY IF** they exceed the grade noted in parentheses next to the AE in the SPEER. If this CAEPR is part of a combination protocol using multiple investigational agents and has an AE listed on different SPEERs, use the lower of the grades to determine if expedited reporting is required.

Version 2.2, April 26, 2016

Adverse Events with Possible Relationship to MLN0128 (TAK228) (CTCAE 4.0 Term) [n= 311]			**Specific Protocol Exceptions to Expedited Reporting (SPEER)**
Likely (>20%)	**Less Likely (<=20%)**	**Rare but Serious (<3%)**	
BLOOD AND LYMPHATIC SYSTEM DISORDERS			
	Anemia		***Anemia (Gr 2)***
CARDIAC DISORDERS			
		Cardiac arrest	
		Ventricular fibrillation	
GASTROINTESTINAL DISORDERS			
	Abdominal pain		***Abdominal pain (Gr 2)***
	Constipation		***Constipation (Gr 2)***
Diarrhea			***Diarrhea (Gr 2)***
	Dry mouth		***Dry mouth (Gr 2)***
Mucositis oral			***Mucositis oral (Gr 2)***
Nausea			***Nausea (Gr 2)***
Vomiting			***Vomiting (Gr 2)***
GENERAL DISORDERS AND ADMINISTRATION SITE CONDITIONS			
	Edema limbs		
Fatigue			***Fatigue (Gr 2)***
	Fever		***Fever (Gr 2)***
	General disorders and administration site conditions - Other (mucosal inflammation)		***General disorders and administration site conditions - Other (mucosal inflammation) (Gr 2)***
INFECTIONS AND INFESTATIONS			
	Urinary tract infection		***Urinary tract infection (Gr 2)***
INVESTIGATIONS			
	Creatinine increased		***Creatinine increased (Gr 2)***
		Electrocardiogram QT corrected interval prolonged	
	Platelet count decreased		***Platelet count decreased (Gr 2)***
	Weight loss		***Weight loss (Gr 2)***
METABOLISM AND NUTRITION DISORDERS			

Anorexia			***Anorexia (Gr 2)***
	Dehydration		***Dehydration (Gr 2)***
Hyperglycemia			***Hyperglycemia (Gr 2)***
	Hypokalemia		***Hypokalemia (Gr 2)***
	Hypomagnesemia		***Hypomagnesemia (Gr 2)***
	Hypophosphatemia		***Hypophosphatemia (Gr 2)***
MUSCULOSKELETAL AND CONNECTIVE TISSUE DISORDERS			
	Arthralgia		
	Back pain		***Back pain (Gr 2)***
	Pain in extremity		
NERVOUS SYSTEM DISORDERS			
	Dizziness		***Dizziness (Gr 2)***
	Dysgeusia		***Dysgeusia (Gr 2)***
	Headache		***Headache (Gr 2)***
PSYCHIATRIC DISORDERS			
	Insomnia		
RESPIRATORY, THORACIC AND MEDIASTINAL DISORDERS			
	Cough		***Cough (Gr 2)***
	Dyspnea		***Dyspnea (Gr 2)***
		Pneumonitis	
	Respiratory, thoracic and mediastinal disorders - Other (oropharyngeal pain)		
SKIN AND SUBCUTANEOUS TISSUE DISORDERS			
Pruritus			***Pruritus (Gr 2)***
Rash maculo-papular			***Rash maculo-papular (Gr 2)***

[1]This table will be updated as the toxicity profile of the agent is revised. Updates will be distributed to all Principal Investigators at the time of revision. The current version can be obtained by contacting PIO@CTEP.NCI.NIH.GOV. Your name, the name of the investigator, the protocol and the agent should be included in the e-mail.

Adverse events reported on MLN0128 (TAK228) trials, but for which there is insufficient evidence to suggest that there was a reasonable possibility that MLN0128 (TAK228) caused the adverse event:

Cardiac disorders - Heart failure; Pericardial effusion; Sinus tachycardia
Eye disorders - Blurred vision; Eye disorders - Other (visual acuity reduced); Eye pain; Photophobia
Gastrointestinal disorders - Dyspepsia; Dysphagia; Esophagitis; Gastroesophageal reflux disease; Gastrointestinal disorders - Other (intestinal obstruction); Oral pain; Pancreatitis; Small intestinal obstruction; Small intestinal perforation; Toothache

General disorders and administration site conditions - Chills; Gait disturbance; Non-cardiac chest pain; Pain
Hepatobiliary disorders - Gallbladder obstruction
IMMUNE SYSTEM DISORDERS - Allergic reaction
Infections and infestations - Kidney infection; Lung infection; Sepsis; Skin infection; Upper respiratory infection
Injury, poisoning and procedural complications - Fall; Tracheal obstruction
Investigations - Alanine aminotransferase increased; Aspartate aminotransferase increased; Blood bilirubin increased; Cholesterol high; Lymphocyte count decreased; Neutrophil count decreased; White blood cell decreased
Metabolism and nutrition disorders - Hypercalcemia; Hypertriglyceridemia; Hypoalbuminemia; Hypocalcemia; Hyponatremia; Metabolism and nutrition disorders - Other (vitamin D deficiency)
Musculoskeletal and connective tissue disorders - Flank pain; Generalized muscle weakness; Musculoskeletal and connective tissue disorder - Other (muscle spasms); Myalgia
Neoplasms benign, malignant and unspecified (incl cysts and polyps) - Treatment related secondary malignancy
Nervous system disorders - Intracranial hemorrhage; Nervous system disorders - Other (carotid artery occlusion); Tremor
Psychiatric disorders - Anxiety; Confusion; Depression; Personality change
Renal and urinary disorders - Acute kidney injury; Hematuria; Urinary tract pain
Respiratory, thoracic and mediastinal disorders - Bronchopulmonary hemorrhage; Epistaxis; Hypoxia; Nasal congestion; Pleural effusion; Pneumothorax; Postnasal drip; Productive cough
Skin and subcutaneous tissue disorders - Dry skin; Hyperhidrosis; Rash acneiform; Urticaria
Vascular disorders - Flushing; Hypotension; Thromboembolic event

Note: MLN0128 (TAK228) in combination with other agents could cause an exacerbation of any adverse event currently known to be caused by the other agent, or the combination may result in events never previously associated with either agent.

7.2 Adverse Event Characteristics

- **CTCAE term (AE description) and grade:** The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 4.0. A copy of the CTCAE version 4.0 can be downloaded from the CTEP web site http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm.

- **For expedited reporting purposes only:**
  - AEs for the agent that are ***bold and italicized*** in the CAEPR (*i.e.*, those listed in the SPEER column, Section 7.1.1) should be reported through CTEP-AERS only if the grade is above the grade provided in the SPEER.

- Other AEs for the protocol that do not require expedited reporting are outlined in section 7.3.4.

- **Attribution** of the AE:
  - Definite – The AE *is clearly related* to the study treatment.
  - Probable – The AE *is likely related* to the study treatment.
  - Possible – The AE *may be related* to the study treatment.
  - Unlikely – The AE *is doubtfully related* to the study treatment.
  - Unrelated – The AE *is clearly NOT related* to the study treatment.

7.3 Expedited Adverse Event Reporting

7.3.1 Expedited AE reporting for this study must use CTEP-AERS (CTEP Adverse Event Reporting System), accessed via the CTEP Web site (https://eapps-ctep.nci.nih.gov/ctepaers). The reporting procedures to be followed are presented in the "NCI Guidelines for Investigators: Adverse Event Reporting Requirements for DCTD (CTEP and CIP) and DCP INDs and IDEs" which can be downloaded from the CTEP Web site (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/adverse_events.htm). These requirements are briefly outlined in the tables below (Section 7.3.3).

In the rare occurrence when Internet connectivity is lost, a 24-hour notification is to be made to CTEP by telephone at 301-897-7497. Once Internet connectivity is restored, the 24-hour notification phoned in must be entered electronically into CTEP-AERS by the original submitter at the site.

7.3.2 CTEP-AERS is programmed for automatic electronic distribution of reports to the following individuals: Study Coordinator of the Lead Organization, Principal Investigator, and the local treating physician. CTEP-AERS provides a copy feature for other e-mail recipients.

The Coordinating Center of the Lead Organization is responsible for submitting to the CTSU documentation of AEs that they deem reportable for posting on the CTSU protocol web page and inclusion on the CTSU bi-monthly broadcast.

7.3.3 Expedited Reporting Guidelines

Use the NCI protocol number and the protocol-specific patient ID assigned during trial registration on all reports.

Note: A death on study requires both routine and expedited reporting regardless of causality, unless as noted below. Attribution to treatment or other cause must be provided.

Death due to progressive disease should be reported as **Grade 5 "Neoplasms benign, malignant and unspecified (incl cysts and polyps) - Other (Progressive Disease)"**

under the system organ class (SOC) of the same name. Evidence that the death was a manifestation of underlying disease (*e.g.*, radiological changes suggesting tumor growth or progression: clinical deterioration associated with a disease process) should be submitted.

Late Phase 2 and Phase 3 Studies: Expedited Reporting Requirements for Adverse Events that Occur on Studies under an IND/IDE within 30 Days of the Last Administration of the Investigational Agent/Intervention[1, 2]

FDA REPORTING REQUIREMENTS FOR SERIOUS ADVERSE EVENTS (21 CFR Part 312)
NOTE: Investigators **MUST** immediately report to the sponsor (NCI) **ANY** Serious Adverse Events, whether or not they are considered related to the investigational agent(s)/intervention (21 CFR 312.64)
An adverse event is considered serious if it results in **ANY** of the following outcomes:

1) Death
2) A life-threatening adverse event
3) An adverse event that results in inpatient hospitalization or prolongation of existing hospitalization for ≥ 24 hours
4) A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
5) A congenital anomaly/birth defect.
6) Important Medical Events (IME) that may not result in death, be life threatening, or require hospitalization may be considered serious when, based upon medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. (FDA, 21 CFR 312.32; ICH E2A and ICH E6).

ALL SERIOUS adverse events that meet the above criteria **MUST** be immediately reported to the NCI via AdEERS within the timeframes detailed in the table below.

<table>
<tr><th>Hospitalization</th><th>Grade 1 Timeframes</th><th>Grade 2 Timeframes</th><th>Grade 3 Timeframes</th><th>Grade 4 & 5 Timeframes</th></tr>
<tr><td>Resulting in Hospitalization ≥ 24 hrs</td><td colspan="3">10 Calendar Days</td><td rowspan="2">24-Hour 5 Calendar Days</td></tr>
<tr><td>Not resulting in Hospitalization ≥ 24 hrs</td><td colspan="2">Not required</td><td>10 Calendar Days</td></tr>
</table>

NOTE: Protocol specific exceptions to expedited reporting of serious adverse events are found in the Specific Protocol Exceptions to Expedited Reporting (SPEER) portion of the CAEPR

Expedited AE reporting timelines are defined as:

- "24-Hour; 5 Calendar Days" - The AE must initially be reported via AdEERS within 24 hours of learning of the AE, followed by a complete expedited report within 5 calendar days of the initial 24-hour report.
- "10 Calendar Days" - A complete expedited report on the AE must be submitted within 10 calendar days of learning of the AE.

[1]Serious adverse events that occur more than 30 days after the last administration of investigational agent/intervention and have an attribution of possible, probable, or definite require reporting as follows:

Expedited 24-hour notification followed by complete report within 5 calendar days for:

- All Grade 4, and Grade 5 AEs

Expedited 10 calendar day reports for:

- Grade 2 adverse events resulting in hospitalization or prolongation of hospitalization

1. Grade 3 adverse events

[2]For studies using PET or SPECT IND agents, the AE reporting period is limited to 10 radioactive half-lives, rounded UP to the nearest whole day, after the agent/intervention was last administered. Footnote "1" above applies after this reporting period.

Effective Date: May 5, 2011

7.3.4 Additional Protocol-Specific Expedited Adverse Event Reporting Exclusions

For this protocol only, the AEs/grades listed below do not require expedited reporting via CTEP-AERS. However, they still must be reported through the routine reporting mechanism (Section 7.4):

CTCAE SOC	Adverse Event	Grade
Blood and lymphatic system disorders	Anemia	Grade 3 or 4
Investigations	White blood cell decreased	Grade 3 or 4
	Lymphocyte count decreased	Grade 3 or 4
	Neutrophil count decreased	Grade 3 or 4
	Platelet count decreased	Grade 3 or 4

7.4 Routine Adverse Event Reporting

All Adverse Events **must** be reported in routine study data submissions. **AEs reported through CTEP-AERS must also be reported in routine study data submissions.**

Adverse event data collection and reporting, which are required as part of every clinical trial, are done to ensure the safety of patients enrolled in the studies as well as those who will enroll in future studies using similar agents. AEs are reported in a routine manner at scheduled times during the trial using Medidata Rave. For this trial the Adverse Event is used for routine AE reporting in Rave.

7.5 Secondary Malignancy

A *secondary malignancy* is a cancer caused by treatment for a previous malignancy (*e.g.*, treatment with investigational agent/intervention, radiation or chemotherapy). A secondary malignancy is not considered a metastasis of the initial neoplasm.

CTEP requires all secondary malignancies that occur following treatment with an agent under an NCI IND/IDE be reported via CTEP-AERS. Three options are available to describe the event:

- Leukemia secondary to oncology chemotherapy (*e.g.*, acute myelocytic leukemia [AML])
- Myelodysplastic syndrome (MDS)
- Treatment-related secondary malignancy

Any malignancy possibly related to cancer treatment (including AML/MDS) should also be reported via the routine reporting mechanisms outlined in each protocol.

7.6 Second Malignancy

A second malignancy is one unrelated to the treatment of a prior malignancy (and is **NOT** a metastasis from the initial malignancy). Second malignancies require **ONLY** routine adverse event reporting unless otherwise specified.

8. PHARMACEUTICAL INFORMATION

A list of the adverse events and potential risks associated with the investigational administered in this study can be found in Section 7.1.

8.1 MLN0128 (TAK-228) (NSC # 768435)

Other Names: MLN0128, INK128, NSC# 768435

Classification: mTOR inhibitor, TORC1/2

CAS Registry Number: 1224844

Molecular Formula: $C_{15}H_{15}N_7O$ **M.W.:** 309.3

Approximate Solubility: MLN0128 (TAK-228) exhibits a pH-dependent aqueous solubility: at physiological pH the solubility is approximately 0.1 mg/mL and at or below pH 3 the solubility is greater than 15 mg/mL.

Mode of Action: MLN0128 (TAK-228) is a non-rapamycin analog mTOR (mechanistic target of rapamycin) kinase inhibitor. The mTOR kinase regulates cell growth, translational control, angiogenesis, and cell survival by integrating nutrient and hormonal signals. The mTOR complex (TORC) is an intracellular point of convergence for a number of cellular signaling pathways. MLN0128 (TAK-228) is a potent and selective adenosine tri-phosphate (ATP)-competitive inhibitor of mTOR complex 1 and 2 (TORC1/2).

Description: MLN0128 (TAK-228) drug substance is a white to off-white, crystalline powder.

How Supplied: MLN0128 (TAK-228) is supplied by Millennium Pharmaceuticals, Inc. and distributed by the Pharmaceutical Management Branch, CTEP/DCTD/NCI as size 2 hard gelatin capsules in the following strengths: 1 mg (white opaque color) and 3 mg (orange opaque color). The composition of the drug product consists of a blend of MLN0128 (TAK-228) drug substance, microcrystalline cellulose, and magnesium stearate. **Milled** formulations will have a white label with a large watermark of the strength on the label.

MLN0128 (TAK-228) capsules are packaged in 30-count, 60-cc high-density polyethylene (HDPE), white, opaque, round, tamper- and child-resistant bottles.

Storage: Capsules are to be stored in the original package between 15°C to 30°C, with allowed short-term excursions between 2°C and 40°C.

Route of Administration: Orally, given 2 hours before or 1 hour after a meal. Do not chew, open or manipulate the capsule in any way prior to swallowing. Each dose should be taken with 8 ounces (240 mL) of water.

Potential Drug Interactions: Multiple human metabolizing enzymes are involved in the Phase I metabolism of MLN0128 (TAK-228). When normalized for human liver content, the CYP isoforms CYP3A4, CYP2C9, and CYP2C19 appear to contribute to MLN0128 (TAK-228) metabolism. MLN0128 (TAK-228) displayed low potential (IC_{50} > 25 μM) for inhibition of the major human CYP isoforms.

Patient Care Implications: Women of childbearing potential should use effective methods of contraception during and through 90 days after the last dose of MLN0128 (TAK-228). Men should use effective methods of contraception and not donate sperm during and through 120 days after the last dose of MLN0128 (TAK-228).

Availability

MLN0128 (TAK-228) is an investigational agent supplied to investigators by the Division of Cancer Treatment and Diagnosis (DCTD), NCI.

MLN0128 (TAK-228) is provided to the NCI under a Collaborative Agreement between the Pharmaceutical Collaborator and the DCTD, NCI (see Section 12.3).

8.2 Agent Ordering and Agent Accountability

8.2.1 NCI-supplied agents may be requested by the Principal Investigator (or their authorized designee) at each participating institution. Pharmaceutical Management Branch (PMB) policy requires that agent be shipped directly to the institution where the patient is to be treated. PMB does not permit the transfer of agents between institutions (unless prior approval from PMB is obtained). The CTEP-assigned protocol number must be used for ordering all CTEP-supplied investigational agents. The responsible investigator at each participating institution must be registered with CTEP, DCTD through an annual submission of FDA Form 1572 (Statement of Investigator), Curriculum Vitae, Supplemental Investigator Data Form (IDF), and Financial Disclosure Form (FDF). If there are several participating investigators at one institution, CTEP-supplied investigational agents for the study should be ordered under the name of one lead investigator at that institution.

In general, sites may order initial agent supplies when a subject is being screened for enrollment onto the study.

Active CTEP-registered investigators and investigator-designated shipping designees and ordering designees can submit agent requests through the PMB Online Agent Order Processing (OAOP) application (https://eapps-ctep.nci.nih.gov/OAOP/pages/login.jspx). Access to OAOP requires the establishment of a CTEP Identity and Access Management (IAM) account (https://eapps-ctep.nci.nih.gov/iam/) and the maintenance of an "active" account status and a "current" password. For questions about drug orders, transfers, returns, or accountability, call or email PMB anytime.

8.2.2 Agent Inventory Records – The investigator, or a responsible party designated by the investigator, must maintain a careful record of the receipt, dispensing and final disposition of all agents received from PMB using the appropriate NCI Investigational Agent (Drug) Accountability Record Form (DARF) available on the CTEP forms page. Store and maintain separate NCI Investigational Agent Accountability Records for each agent, strength, formulation and ordering investigator on this protocol.

8.2.3 Useful Links and Contacts

- CTEP Forms, Templates, Documents: http://ctep.cancer.gov/forms/
- NCI CTEP Investigator Registration: PMBRegPend@ctep.nci.nih.gov
- PMB policies and guidelines: http://ctep.cancer.gov/branches/pmb/agent_management.htm
- PMB Online Agent Order Processing (OAOP) application: https://eapps-ctep.nci.nih.gov/OAOP/pages/login.jspx
- CTEP Identity and Access Management (IAM) account: https://eapps-ctep.nci.nih.gov/iam/
- CTEP Associate Registration and IAM account help: ctepreghelp@ctep.nci.nih.gov
- PMB email: PMBAfterHours@mail.nih.gov
- PMB phone and hours of service: (240) 276-6575 Monday through Friday between 8:30 am and 4:30 pm (ET)
- **IB Coordinator:** IBCoordinator@mail.nih.gov

9. BIOMARKER, CORRELATIVE, AND SPECIAL STUDIES

9.1 Biomarker Studies

9.1.1 Plasma Pharmacokinetics

9.1.1.1 Hypothesis and Rationale

Hypothesis: In those patients with inadequate drug exposure, inhibition of 4EBP1 phosphorylation and other cellular effects of mTOR dual inhibitors assayed as described in Sections 9.1.2-9.1.6 will be limited.

Rationale: These studies are necessary to characterize the pharmacokinetics of MLN0128 (TAK-228) in patients with acute lymphoblastic leukemia and to assess pharmacodynamic relationships with phosphorylated mTOR substrates and expression levels of BCL2L11 (Bim), BBC3 (Puma), MCL1 and BCL2.

9.1.1.2 Assay Validity and Appropriateness for Study

Plasma and marrow concentrations of MLN0128 (TAK-228) will be measured by lc/ms/ms methods developed in the Mayo Clinic Cancer Center Pharmacology Shared Resource. The Shared Resource, directed by Dr. Reid, is fully equipped to carry out pharmacologic investigations, including two exquisitely sensitive Xevo TQ-S mass spectrometers with Accuity UPLC systems to carry out the bioanalytical measurements, and licenses for Phoenix WINNONLIN, NONMEM and PsN Pirana to carry out standard and population PK/PD analyses. The laboratory has developed or modified assays for more than 75 antitumor agents, served as a central analytical and pharmacokinetics resource laboratory for 9 published NCI-sponsored Phase I and Phase II trials with vorinostat (for example, 47, 48, 49), and serves as one of the pharmacology resource laboratories for the Children's Oncology Group. The approach to assay development and implementation of criteria for system suitability, quality assurance and ongoing evaluation of specific assay data are consistent with recommendations from two FDA-sponsored conferences on analytical methods validation (50, 51) that are described in a recent FDA guidance document "Guidance for Industry: Bioanalytical Method Validation" (52). In particular, as we develop a specific HPLC or other method for measurement of drug and relevant metabolites, we incorporate standard assessments of drug extraction efficiency, sensitivity, linearity, precision, accuracy, storage stability and other parameters relevant to the particular method. Assay performance is thoroughly validated before implementation in the clinical trial and monitored on a routine basis during the course of the clinical trial by inclusion of appropriate quality assurance samples. The plasma concentration-time data will be will be analyzed by standard noncompartmental analysis using the program Phoenix WinNonlin (Pharsight Corp., St. Louis, MO) to determine Cmax, Tmax, half-life and accumulation. If sufficient data are available, the data will also be analyzed by non-linear mixed effects modeling using the program NONMEM 7.2.0 (ICON Development Solutions, Ellicot City, MD) to determine oral clearance, volume of distribution, sources of inter- and intra-subject variability, and pharmacodynamic relationships with phosphorylated mTOR substrates and expression levels of BCL2L11 (Bim), BBC3 (Puma), MCL1 and BCL2.

9.1.1.3 Performance Site

The assay will be performed in the Pharmacology Shared Resource of the Mayo Clinic Cancer Center. This lab has extensive experience with developing, validating and implementing assays for a variety of anticancer drugs, including mTOR dual inhibitors, as well as performing pharmacokinetic analysis after performance of the assays in preclinical and clinical studies.

9.1.1.4 Justification of the Number of Patients and Specimens

The purpose of the assay is to provide information about drug exposure that can be utilized for interpretation of pharmacodynamic and clinical response endpoints. Meaningful conclusions regarding the impact of drug exposure can only be drawn for those patients in whom the assays are performed. Accordingly, the plan is to measure serum pharmacokinetics in all patients. The number of samples (15 per patient) is required to assure sufficient data to allow calculation of standard pharmacokinetic parameters.

9.1.1.5 Description of Biological Samples Required for Assay

This assay of serum MLN0128 (TAK-228) levels requires procurement of 6 cc of blood at 15 time points as described in detail in Section 9.2.1.2. Risks of blood sample procurement include pain, infection and bleeding at the site of venous heparin lock placement as well as development of heparin-induced thrombocytopenia. Sterile technique is utilized to minimize risk of infection. With an adequate platelet count (10,000 or more) and local pressure, the risk of bleeding is low. Heparin-induced thrombocytopenia is rare; and patients' platelet counts will be monitored daily for several days after the venipuncture, with platelet transfusions mandated for thrombocytopenia as indicated in Section 5.2 (generally for bleeding or platelet count less than 10,000 unless a higher threshold is deemed appropriate by the treating physician because of fever, infection or invasive procedure). It is conceivable that infections or bleeding could be life threatening, but the risk of death after venipuncture is considered extremely rare (estimated to be 1 in 50 million).

9.1.2 Overview of Cell-Based Studies

Cell-based studies in this section are presented in the order in which they are prioritized. The rationale for this prioritization, which is that proof-of-mechanism studies (including the integrated biomarker) take higher priority than studies outside the direct mechanism of action of this class of agents, is described in detail in Section 2.4.7. **Additional details regarding these assays, including sample processing at the assay site and performance characteristics of the assays, are found in Appendix D.**

9.1.3 Phospho-Thr^{37,46}-4EBP1 analysis of pretreatment and day 8 bone marrow samples by immunoblotting (integrated biomarker)

9.1.3.1 Hypothesis and Rationale

Hypothesis: 4EBP1 phosphorylation will be inhibited by MLN0128 (TAK-228) *in situ*.

Rationale: Previous studies have shown that mTOR dual inhibitors such as MLN0128 (TAK-228) and OSI-027 diminish the mTORC1-mediated

phosphorylation of the translation inhibitor 4EBP1, causing it to bind to and inhibit the translation factor eIF4E (14, 18). In ALL cell lines the down regulation of 4EBP1 produces resistance to mTOR dual inhibitors (14), demonstrating the critical role of this protein in killing by mTOR dual inhibitors. We now propose to assess whether 4EBP1 has been dephosphorylated in leukemic blasts by therapeutically achievable MLN0128 (TAK-228) concentrations *in situ* as a proof of mechanism.

9.1.3.2 Assay Validity and Appropriateness for Study

Immunoblotting will be performed to assess 4EBP1 phosphorylation at Thr^{37} and Thr^{46} in Ficoll-Hypaque enriched cells from pretreatment and day 8 bone marrow aspirates. Alternative approaches to assessing 4EBP1 phosphorylation include ELISA, immunohistochemistry and 2-dimensional gel electrophoresis. The major advantage of immunoblotting is the ability to assess the signal at the molecular weight of interest rather than assuming that a signal observed in ELISA is due to the antigen of interest. While it is somewhat less quantitative than ELISA, the Kaufmann laboratory has demonstrated that blotting with a rabbit monoclonal antibody can detect differences in phospho-4EBP1 over a 64-fold range that encompasses the extent of phosphorylation observed in baseline ALL samples (Appendix C). Preliminary studies presented to the Biomarker Review Committee indicated a strong correlation ($R^2 > 0.94$) between results observed when samples with a wide range of 4EBP1 phosphorylation were run on separate SDS-polyacrylamide gels and probed.

9.1.3.3 Performance site

The assay will be performed in the Kaufmann laboratory of the Mayo Clinic Cancer Center. This lab has extensive experience with detecting changes in antigen content by immunoblotting (53, 54).

9.1.3.4 Data Supporting the Degree of Biomarker “Fit for Purpose”:

Credentialing of the proposed immunoblotting assay is described in Appendix C. A Standard Operating Procedure for the assay is also included in the Appendix per the instructions in the protocol template.

9.1.3.5 Justification of the Number of Patients and Specimens

The purpose of the assay is to determine whether MLN0128 (TAK-228) induces dephosphorylation of an mTORC1 target in leukemia cells *in situ* at clinically achievable concentrations. After the signal for phospho-4EBP1 is quantitated, results will be examined graphically to assess the extent and frequency of down regulation as described in Section 13.4.6. This will be the first opportunity to assess 4EBP1 dephosphorylation in ALL after treatment with an mTOR dual inhibitor, so the extent and frequency of down regulation cannot be estimated from precedent data. As a point of reference, however, complete dephosphorylation of 4EBP1 was observed in Jeko mantle cell lymphoma xenografts after treatment of mice with the mTOR dual inhibitor OSI-027, which induced regression of the xenografts (18).

9.1.3.6 Description of Biological Samples Required for Assay

This assay of paired samples depends upon procurement of bone marrow aspirates prior to treatment and again on day 8 of therapy. A pretreatment bone marrow aspirate (and biopsy) is considered standard of care for acute leukemia, so the pretreatment aspirate will simply involve the acquisition of an extra 3-5 cc of bone marrow at the time an aspirate is ordinarily obtained. The day 8 marrow sample will require an extra bone marrow for research purposes only (to determine whether MLN0128 (TAK-228) is having its intended effect on mTOR-mediated signaling and downstream apoptotic pathways). Risks of bone marrow aspiration include pain, infection and bleeding. Local anesthetic with or without conscious sedation is provided to minimize pain. Sterile technique is utilized to minimize risk of infection. With an adequate platelet count (10,000 or more) and local pressure, the risk of bleeding is low. It is conceivable that bleeding, infection or complications of the anesthesia could be life threatening, but the risk of death with the day 8 bone marrow aspirate is estimated to be on the order of one in a million.

For Patients at Mayo Clinic Rochester only:
To assess whether this alteration in signaling can also conceivably be detected at earlier timepoints and in more accessible cells, for patients treated at Mayo Clinic Rochester with a circulating blast count greater than 5000/um^3, peripheral blood drawn prior to therapy and again at 24 h after the first dose of MLN0128 (TAK-228) will be assayed as described above. To facilitate this analysis, two 7 ml EDTA tubes will be drawn prior to the first dose of MLN0128 (TAK-228) and at the time of the Cycle 1 Day 2 pharmacokinetics draw.

9.1.3.7 Comment on Feasibility of Detecting Multiple Antigens by Immunoblotting

Techniques previously reported from the Kaufmann lab (55-57) allow the sequential detection of multiple antigens on the same filter without interference. Once phospho-4EBP1 has been successfully detected, we propose to use the same filters for detection of additional antigens, which are presented below in the order in which they will be examined. Because we routinely detect 10-14 antigens on a duplicate pair of

filters (including 8 BCL2 family members, three phospho-proteins and the three corresponding total protein controls) if the molecular weights do not over lap and suitable secondary antibodies are available, we do not anticipate difficulty in completing the proposed immunoblotting with the available samples.

9.1.4 Phospho-Ser^{473} Analysis of Pretreatment and Day 8 Bone Marrow Samples by Immunoblotting (Exploratory Biomarker)

9.1.4.1 Hypothesis and Rationale

Hypothesis: AKT Ser^{473} phosphorylation will be inhibited by MLN0128 (TAK-228) *in situ*.

Rationale: Previous studies have shown that mTOR dual inhibitors such as MLN0128 (TAK-228) and OSI-027 diminish the mTORC2-mediated phosphorylation of the pro-survival kinase AKT on Ser^{473}, leading to upregulation of the pro-apoptotic protein BCL2L11 (Bim) and induction of apoptosis (14, 18). In ALL cell lines the down regulation of the mTORC2 component Rictor reproduces these effects and down regulation of BCL2L11 (Bim) produces resistance to mTOR dual inhibitors (14), demonstrating the critical role of signaling cascade in killing by mTOR dual inhibitors. We now propose to assess whether AKT Ser^{473} has been dephosphorylated in leukemic blasts by therapeutically achievable MLN0128 (TAK-228) concentration *in situ* as a proof of mechanism for this class of agent.

9.1.4.2 Assay Validity and Appropriateness for Study

Immunoblotting will be performed to assess AKT phosphorylation on Ser^{473} in mononuclear cells isolated from pretreatment and day 8 bone marrow aspirates using Ficoll-Hypaque gradients. Alternative approaches to assessing AKT Ser^{473} phosphorylation include ELISA, immunohistochemistry and 2-dimensional gel electrophoresis. The major advantage of immunoblotting is the ability to assess the signal at the molecular weight of interest rather than assuming that a signal observed in ELISA is due to the antigen of interest. While it is somewhat less quantitative than ELISA, the Kaufmann laboratory has demonstrated that blotting with a rabbit monoclonal antibody can detect differences in phospho-Akt over a 64-fold range that encompasses the extent of phosphorylation observed in baseline ALL samples (unpublished data similar to that in Appendix D for phospho-4EBP1).

9.1.4.3 Performance Site

The assay will be performed in the Kaufmann laboratory of the Mayo Clinic Cancer Center. This lab has extensive experience with detecting changes in antigen content by immunoblotting (53, 54).

9.1.4.4 Data Supporting the Degree of Biomarker "Fit for Purpose"

As indicated above, unpublished data using a commercially available anti-phospho-Ser^{473}-AKT antibody demonstrate a 32-fold dynamic range for detection of this antigen that encompasses the baseline antigen phosphorylation in clinical ALL specimens.

9.1.4.5 Justification of the Number of Patients and Specimens

The purpose of the assay is to determine whether MLN0128 (TAK-228) induces dephosphorylation of an mTORC2 target in leukemia cells *in situ* at clinically achievable concentrations. After the signal for phospho-Ser^{473}-AKT is quantitated, results will be examined graphically to assess the extent and frequency of down regulation as described in Section 13.4.6. This will be the first opportunity to assess AKT phosphorylation in ALL after treatment with an mTOR dual inhibitor, so the extent and frequency of down regulation cannot be estimated from precedent data. As a point of reference, however, complete dephosphorylation of AKT Ser^{473} was observed in Jeko mantle cell lymphoma xenografts after treatment of mice with the mTOR dual inhibitor OSI-027, which induced regression of the xenografts (18).

9.1.5 Expression of BCL2L11 (Bim), BBC3 (Puma), MCL1 and BCL2 Proteins in Pretreatment and Day 8 Bone Marrow Samples by Immunoblotting (Exploratory Biomarkers)

9.1.5.1 Hypothesis and Rationale

Hypothesis: Levels of BCL2L11 (Bim) and BBC3 (Puma) in ALL will increase during MLN0128 (TAK-228) treatment, whereas levels of MCL1 and possibly BCL2 will decrease.

Rationale: Previous studies have shown that mTOR dual inhibitors such as MLN0128 (TAK-228) and OSI-027 cause upregulation of the pro-apoptotic BCL2 family members BBC3 (Puma) and BCL2L11 (Bim) in malignant lymphoid cells (14, 18). In ALL cell lines the down regulation of BBC3 (Puma) or BCL2L11 (Bim) produces resistance to mTOR dual inhibitors (14, 18), demonstrating the critical role of these proteins in killing by mTOR dual inhibitors. Conversely, the anti-apoptotic BCL2 family member MCL1 has been shown to decrease after treatment with mTOR dual inhibitors in certain malignant human lymphoid cell lines (18). Overexpression of eponymous BCL2 has also been shown to convey resistance to mTOR dual inhibitors (18). Accordingly, we propose to assess whether BCL2L11 (Bim) and BBC3 (Puma) have been upregulated or MCL1 down regulated in leukemic blasts by therapeutically achievable MLN0128 (TAK-228) concentration *in situ* as a further proof of mechanism for this class of agent.

9.1.5.2 Assay Validity and Appropriateness for Study

Immunoblotting will be performed to assess BCL2L11 (Bim), BBC3 (Puma), MCL1 and BCL2 in mononuclear cells isolated from pretreatment and day 8 bone marrow aspirates using Ficoll-Hypaque gradients. Alternative approaches to assessing expression of these proteins include ELISA, immunohistochemistry and qRT-PCR. The major advantage of immunoblotting is the ability to assess the signal at the molecular weight of interest rather than assuming that a signal observed in ELISA or immunohistochemistry is due to the antigen of interest. The advantages of immunoblotting over qRT-PCR are i) the proteins that are regulating apoptosis are actually being measured rather than mRNA levels, which might not correlate with protein levels; and ii) all of these proteins persist in mitochondria as cells undergo apoptosis, whereas mRNA is rapidly degraded at the outset of apoptosis. The Kaufmann laboratory has previously used this immunoblotting approach to examine differences in BCL2 family members among different cell lines (41) and pretreatment acute leukemia samples (58) as well as changes in BCL2, MCL1 and BCL2L11 (Bim) during the course of acute leukemia (40) and lymphoma treatment (59).

9.1.5.3 Performance Site

The assay will be performed in the Kaufmann laboratory of the Mayo Clinic Cancer Center. This lab has extensive experience with detecting changes in antigen content by immunoblotting (53, 54).

9.1.5.4 Data Supporting the Degree of Biomarker "Fit for Purpose"

Unpublished data using commercially available anti-MCL1 and anti-BCL2 antibodies demonstrate a 32-fold dynamic range for detection of these antigens.

9.1.5.5 Justification of the Number of Patients and Specimens

The purpose of the assay is to determine whether MLN0128 (TAK-228) induces upregulation of BCL2L11 (Bim) and BBC3 (Puma) or down regulation of MCL1 and BCL2 in ALL cells *in situ* at clinically achievable concentrations. After the signal for BCL2L11 (Bim), BBC3 (Puma), MCL1 and BCL2 is quantitated, results will be examined graphically to assess the extent and frequency of down regulation as described in Section 13.4.6. This will be the first opportunity to assess these BCL2 family members in clinical ALL after treatment with an mTOR dual inhibitor *in vivo*, so the extent and frequency of altered expression cannot be estimated from precedent data. As a point of reference, however, induction of BBC3 (Puma) was observed in Jeko mantle cell lymphoma xenografts after treatment of mice with the mTOR dual inhibitor OSI-027, which induced regression of the xenografts (18).

9.1.5.6 Detection of Additional Antigens

In the consensus review of this Protocol, CTEP reviewers suggested that additional BCL2 family members also be examined, specifically noting that BCL2L1 (Bcl-x_L) and BCL2L2 (Bcl-w) would be of interest. These analytes are prioritized lower than the four BCL2 family members listed above because i) changes in BCL2L1 and BCL2L2 have not been directly implicated in the mechanism of cytotoxicity of MLN0128 (TAK-228) in ALL cells and ii) we accordingly have not characterized assays for these proteins to the same extent as we have the proof-of-mechanism studies and cannot comment on the dynamic range, fitness of purpose, or appropriate standards that will encompass the expression levels likely found in clinical ALL samples.

9.1.6 Reverse Phase Protein Array for Protein Phosphorylations and Expression (Exploratory Biomarkers)

9.1.6.1 Hypothesis and Rationale

Hypothesis: Changes in phosphorylation of mTOR substrates and complementary pathway components will change during the course of mTOR dual inhibitor treatment.

Rationale: Prior publications have identified differences in the pattern of protein phosphorylation between cell lines that are sensitive to kinase inhibitors and similar cell lines that are resistant (e.g., 44, 45). Preliminary studies in the Kaufmann laboratory have likewise demonstrated differences in protein phosphorylation at key nodes between parental Nalm6 ALL cells and Nalm6 cells selected for the ability to grow continuously in mTOR dual inhibitor (C. Correia, K. L. B. Knorr, S. Yun, A. E. Wahner Hendrickson, B. Madden, D. McCormick, S. Dasari, and S. H. Kaufmann, unpublished observations). Thus, we hypothesize that pretreatment patterns of phosphorylation might differ between ALLs that respond to MLN0128 (TAK-228) and those that do not.

9.1.6.2 Assay Validity and Appropriateness for Study

Reverse phase protein arrays (RPPAs) will be utilized to assess levels of phosphorylation of 60 key proteins, including 4EBP1, ribosomal protein S6, and AKT, as well as expression levels of 200 proteins, including BCL2, BCL2L1 (Bcl-x_L), MCL1, and BCL2L11 (Bim). Alternative approaches to assessing expression of these proteins include ELISA, immunohistochemistry, immunoblotting and qRT-PCR. The major advantage of reverse phase protein array is the ability to assess the signals related to multiple antigens in a quantitative fashion. Like ELISA or immunohistochemistry, this method assumes that the signal is due to the antigen of interest; and there is no opportunity to rule out cross reactivity with an antigen of a different molecular mass. On the RPPAs, for example, the BBC3 (Puma) signal will be unreliable because the commercial antibody used recognizes a band that is present

even in BBC3$^{-/-}$ human cells (K.L. Peterson and S.H. Kaufmann, unpublished observations). The advantages of RPPA over qRT-PCR are that in RPPA the proteins and posttranslational modifications that are regulating apoptosis are actually being measured rather than mRNA levels, which might not correlate with protein levels. This analysis of key signaling pathways will be utilized to supplement the results of the more targeted immunoblotting assays.

9.1.6.3 Performance site

After mononuclear cells are isolated on Ficoll-Hypaque gradients, whole cell lysates will be prepared in the Kaufmann laboratory of the Mayo Clinic Cancer Center. Based on preliminary results, the same lysates prepared for immunoblotting (see Section 9.1.2) are suitable for RPPAs. The RPPAs will be probed and initial bioinformatic analysis performed in the RPPA Core Facility of the M.D. Anderson Cancer Center, which is a fee-for-service facility.

9.1.6.4 Data Supporting the Degree of Biomarker "Fit for Purpose"

The reproducibility and reliability of this method, as well as comparison to the gold standard of Western blotting, has been published by the founders of the RPPA Core Facility at M.D. Anderson Cancer Center (60).

9.1.6.5 Justification of the Number of Patients and Specimens

The purposes of this assay are: i) to confirm the results obtained in the assays described in Sections 9.1.2-9.1.4 using different antibodies and a different analytical platform and ii) to assess the status of other signaling pathways before and after exposure to MLN0128 (TAK-228) in situ. This will be the first opportunity to assess the performance of this assay in ALL after treatment with an mTOR dual inhibitor, so the extent and frequency of altered phosphorylation or expression of various proteins cannot be estimated from precedent data.

9.1.6.6 Plans for comparison of immunoblotting and RPPA results

Immunoblotting and blot quantification will be performed as described in Appendix C. Comparison of the relative signal in day 1 and day 8 samples from individual patients (run on adjacent lanes of the same gel and compared to a standard curve) will allow a calculation of % change in signal between the two samples. Likewise, comparison of the two samples (day 1 and day 8) subjected to RPPA will allow a calculation of a % change between day 1 and day 8. To compare the assays for any particular analyte (e.g., the integrated biomarker phospho-Thr^{37,46}-4EBP1), we will construct a scatter plot with the % change in the immunoblotting assay on the x axis and the % change in the RPPA assay on the y axis, inspect the distribution of the values, and calculate a Spearman correlation coefficient. Because these assays have not been previously performed in this manner, we cannot estimate the value of that

Spearman correlation coefficient for any of the assays that will be in common, although we suspect it will be close 0 for BBC3, where the signal for bona fide BBC3 protein (identified by comparing wild type and $BBC3^{-/-}$ cells) is detected by immunoblotting and a spurious cross-reactive antigen is detected in the RPPA assays. If correlation coefficients closer to 1.0 are observed in assays of other analytes in common between the two platforms, we will take this as a sign that the RPPA is a reasonable approximation of the immunoblotting assay and examine RPPA results for other antigens (not included in the immunoblotting) for potentially interesting patterns, e.g., differences in BCL2L2 (Bcl-w) expression that might not be feasible with the limited material available for immunoblotting.

9.1.7 Transcriptional Profiling (Exploratory Biomarker)

9.1.7.1 Hypothesis and Rationale

Hypothesis: Treatment with MLN0128 (TAK-228) will activate two transcriptional pathways related to apoptosis.

Rationale: Previous studies have shown that the mTOR dual inhibitor OSI-027 activates two distinct transcriptional pathways in ALL cell lines during the course of upregulating BCL2L11 (Bim) and BBC3 (Puma) to induce apoptosis (14). If sufficient material is available to allow isolation of RNA (after samples for immunoblotting and RPPAs have been harvested), samples will be subjected to RNA sequencing (RNAseq) to determine whether MLN0128 (TAK-228) concentrations achievable *in vivo* activate these same transcriptional pathways as a proof of mechanism study.

9.1.7.2 Assay Validity and Appropriateness for Study

RNAseq will be performed using RNA isolated from Ficoll-Hypaque purified pretreatment and day 8 bone marrow cells to assess whether transcriptional changes observed in clinical ALL leading to BCL2L11 (Bim) and BBC3 (Puma) upregulation are also observed in clinical ALL treated in vivo. Alternative approaches to assessing transcript levels include quantitative RT-PCR. The major advantages of RNAseq are: i) message can be quantitated relative to total number of mRNA molecules rather than relative to an arbitrary standard, which might or might not change during the course of treatment; ii) the entire transcriptome is interrogated in a quantitative fashion one assay; and iii) the assay also provides sequence information that can be examined to search for alterations in splicing and mutations in various messages. This is the same assay that was utilized to identify the transcriptional pathways that are altered in mTOR dual inhibitor-treated ALL cell lines, with subsequent confirmation of results by qRT-PCR (14).

9.1.7.3 Performance Site

RNA will be isolated in the Kaufmann laboratory of the Mayo Clinic Cancer Center. Library construction will take place in the Gene Expression Core of the Mayo Clinic Genome Research Facility; and sequencing of the library will take place in the Sequencing Core of the Mayo Clinic Genome Research Facility.

9.1.7.4 Data Supporting the Degree of Biomarker "Fit for Purpose"

Results currently submitted for publication (14) document the reproducibility of the assay as well as its ability to identify changes in the transcriptome that result from alterations in transcription factors as a consequence of treatment of human ALL cell lines with mTOR dual inhibitors.

9.1.7.5 Justification of the Number of Patients and Specimens

The purpose of the assay is to determine whether MLN0128 (TAK-228) induces transcriptional changes in ALL cells *in situ* that reflect its mechanism of cytotoxicity at clinically achievable concentrations. This will be the first opportunity to assess changes in the transcriptome in clinical ALL after treatment with an mTOR dual inhibitor, so the extent and frequency of transcriptional changes cannot be estimated from precedent data.

9.2 Laboratory Correlative Studies

9.2.1 MLN0128 (TAK-228) Pharmacokinetics – CSF

9.2.1.1 Patient Considerations
Patients participating in this study will have intrathecal (IT) chemotherapy as clinically indicated care within 7 to 10 days of starting study treatment. If possible, collect up to a 2 mL sample of CSF prior to the infusion of the IT chemotherapy. If a patient has another clinically indicated IT chemotherapy performed at any subsequent time in the study, collect an additional 2 mL sample of CSF prior to the infusion of the IT chemotherapy, if possible.

9.2.1.2 CSF Collection Schedule
Collect up to 2 mL of CSF prior to the infusion of the IT chemotherapy withun 7 to 10 days of starting study treatment and prior to any subsequent clinically indicated IT chemotherapy performed at any time during the study if possible.

9.2.1.3 Handling of Specimens
At Mayo Clinic Rochester only: Transport CSF sample on ice to Dr Reid's lab.

For all other sites: Transfer CSF sample to cryovial and freeze at -80º C. Ship with PK serum samples.

9.2.1.4 Shipment of Samples

Ship CSF and PK samples on dry ice Monday – Thursday by FedEx First Overnight to the following address. Please be certain to use the First Overnight service; should there be a concern about this, please contact the study principal investigator or the project manager.

Dr Joel Reid, PhD
Mayo Clinic
Guggenheim 17-37
200 First Street SW
Rochester, MN 55905

9.2.2 MLN0128 (TAK-228) Pharmacokinetics – Plasma

9.2.2.1 Patient Considerations

Patients participating in this study will be asked to provide blood samples during Cycle 1 of treatment. The morning dose of MLN0128 (TAK-228) must not be given until after pretreatment blood draw.

9.2.2.2 Blood Collection Schedule

Blood samples (6 ml) will be drawn in green top sodium heparin tubes 30 minutes before drug administration on Days 1, 2 and 8, and at the following times after administering the oral dose on Day 1 and 8: 30 min, 1 hr, 2 hrs, 3 hrs, 4 hrs, and 8 hrs. Thus, 15 samples will be collected for a total of 90 ml whole blood. Note that the windows for the pk samples are ± 10 min for the 30 minutes pre-drug sample; ± 5 minutes for the 30 minute and 1 hour post-drug samples; ± 10 min for 2 hours post-drug sample and ± 15 min for the 3, 4 and 8 hour post-drug samples. It is more important to record time accurately than to perform the blood drawn exactly at the scheduled time.

Note: The physicial exam and labs performed on Cycle 1 Day 1 may be performed the day prior in order to begin the pharmacokinetic sample collection early enough on Cycle 1 day 1 that an 8 hour pk sample is possible. If an 8 hour pk draw is not possible, please collect a 7 hour or a 7 ½ hour sample and note the timepoint on the label of the tube and a collection draw worksheet.

Please complete the pk sample blood draw list in Appendix D and include a copy with the samples at shipment.

9.2.2.3 Handling of Specimens

Collect blood samples in sodium heparin (green-top) tubes and immediately cool in an ice water bath. Isolate plasma within 20 minutes of collection.

Blood will be subjected to centrifugation (2,000 rpm for 10 minutes) in a refrigerated centrifuge kept at 4°C. Following centrifugation, plasma aliquots of at least 2 ml each should be transferred to 4 mL plastic cryovials, capped and immediately frozen

at -70°C.

Tube labels must contain the following data: patient identification code, date, draw timepoint and actual time of draw. Please complete the pk sample blood draw list in Appendix D and include a copy with the samples at shipment. with the samples.

9.2.2.4 Shipment of specimens
Ship CSF and serum pk samples on dry ice ***only Monday – Thursday*** by FedEx First Overnight to the following address. Please be certain to use the First Overnight service; should there be a concern about this, please contact the study principal investigator or the project manager.

Dr. Joel Reid, PhD
Mayo Clinic
Guggenheim 17-37
200 First Street SW
Rochester, MN 55905

9.2.2.5 Site(s) Performing the Pharmacokinetic Study

Samples will be analyzed in the Pharmacology Core of the Mayo Clinic Cancer Center, which has developed validated assays for over 85 separate anticancer drugs during the course of its existence.

9.2.3 Immunoblotting for Phosphorylation of 4EBP1 and Other Markers

9.2.3.1 Collection of Specimen(s): Bone marrow aspirate collected before treatment and on day 8 ± 2 of treatment with MLN0128 (TAK-228)

9.2.3.2 Handling of Specimens(s): Place 2-5 cc of marrow aspirate in a 5 or 7 cc heparin (green top) tube. Place the tube in a Styrofoam container with adequate padding. Between May and October add a 4 ºC cold pack to the container to prevent overheating during shipping.

9.2.3.3 Shipping of Specimen(s): Ship specimen ***only Monday-Thursday*** by FedEx First Overnight to:

Ms. Karen Flatten
c/o Scott Kaufmann, M.D., Ph.D.
Gonda 19-205B
Mayo Clinic
200 First Street S.W.
Rochester, MN 55905

Please call (507) 284-4304 or fax (507) 293-0107 Ms. Flatten on the day of shipment so that she will know a sample is coming for the next day.

9.2.3.4 Site(s) Performing Correlative Study: Kaufmann laboratory, Mayo Clinic, Rochester, Minnesota

9.2.3.5 Other marrow correlative studies: Please note that phosphorylation of AKT (Section 9.1.4), expression of BCL2 family members (Section 9.1.5), reverse phase protein array (Section 9.1.6) and transcriptional profiling (Section 9.1.7) will be performed on the same marrow aspirate specimens harvested for 4EBP1 phosphorylation. Separate specimens for these assays are not required.

9.2.4 Immunoblotting for Phosphorylation of 4EBP1 and Other Markers

9.2.4.1 Collection of Specimen(s): Bone marrow aspirate collected before treatment and on day 8 ± 2 of treatment with MLN0128 (TAK-228)

9.2.4.2 Handling of Specimens(s): Place 2-5 cc of marrow aspirate in a 5 or 7 cc heparin (green top) tube. Place the tube in a Styrofoam container with adequate padding. Between May and October add a 4 ºC cold pack to the container to prevent overheating during shipping.

9.2.4.3 Shipping of Specimen(s): Ship specimen ***only Monday-Thursday*** by FedEx First Overnight to the following address. Please be certain to use the First Overnight service; should there be a concern about this, please contact the study principal investigator or the project manager.

Ms. Karen Flatten
c/o Scott Kaufmann, M.D., Ph.D.
Gonda 19-205B
Mayo Clinic
200 First Street S.W.
Rochester, MN 55905

Please call (507) 284-4304 or fax (507) 293-0107 Ms. Flatten on the day of shipment so that she will know a sample is coming for the next day.

9.2.4.4 Site(s) Performing Correlative Study: Kaufmann laboratory, Mayo Clinic, Rochester, Minnesota

9.2.4.5 Other marrow correlative studies: Please note that phosphorylation of AKT (Section 9.1.4), expression of BCL2 family members (Section 9.1.5), reverse phase protein array (Section 9.1.6) and transcriptional profiling (Section 9.1.7) will be performed on the same marrow aspirate specimens harvested for 4EBP1 phosphorylation. Separate specimens for these assays are not required.

9.2.5 Assessment of 4EBP1 phosphorylation inhibition at earlier time points ***(Mayo Clinic Rochester only)***

9.2.5.1 Collection of Specimen(s): If circulating blast count is greater than 5000, draw two 7 mL samples in purple topped EDTA tubes prior to first dose of MLN0182 (TAK-228) and at time of Cycle 1 Day 2 MLN0128 (TAK-228) PK draw. Please call Karen Flatten to let her know that a sample will be arriving.

9.2.5.2 Handling of Specimen(s): Hand carry specimen to Kaufmann lab at Mayo Clinic Rochester at Gonda 19-205B.

9.2.5.3 Site(s) Performing Correlative Study: Kaufmann laboratory, Mayo Clinic, Rochester, Minnesota

10. STUDY CALENDAR

Baseline evaluations are to be conducted within 1 week prior to start of protocol therapy. Scans and x-rays must be done ≤ 4 weeks prior to the start of therapy. In the event that the patient's condition is deteriorating, laboratory evaluations should be repeated within 48 hours prior to initiation of the next cycle of therapy. After C1D15, a window of + 7 days for study procedures is permitted.

	Pre-Study	C 1 D 1	C 1 D2	C 1 D8	C1 D15	C1 D 22	C2 D1	C3 D1	C4 D1	C5 D1	C6 D1	C6 D28	C7 D1 and above	Off Study[d]
Informed consent	X													
Demographics	X													
Medical history	X													
Concurrent meds	X	X			X		X	X	X	X	X		X	X
Physical exam	X	X			X		X	X	X	X	X		X	X
Vital signs	X	X			X		X	X	X	X	X		X	X
Height	X	X												
Weight	X	X					X	X	X	X	X		X	X
Performance status	X	X			X		X	X	X	X	X		X	X
CBC w/diff, plts	X	X	X	X	X	X	X	X	X	X	X	X	X	X
HbA1c	X													
Serum chemistry	X	X		X			X	X	X	X	X		X	X
Serum pregnancy test	X[b]													
EKG (as indicated)	X													X
Adverse event evaluation	X	X			X		X	X	X	X	X		X	X
Bone marrow aspirate	X			X[c]				X		X		X		X[c]
CSF[R]	X[e]													
Research blood for PK[R]		X	X	X										
Bone Marrow aspirate pharmacodynamic assays[R]	X			X										X

Mayo Clinic Rochester only: Research blood for pharmacodynamic assays[h, R]	X		X											

a: Albumin, alkaline phosphatase, total bilirubin, bicarbonate, BUN, calcium, chloride, creatinine, fasting glucose, LDH, phosphorus, potassium, total protein, SGOT [AST], SGPT [ALT], sodium, uric acid.
b: Serum pregnancy test (women of childbearing potential).
c: Aspirate only for research assay is collected.
d: 1 month after end of treatment
e: CSF sample collected during clinically indicated intrathecal chemotherapy for pk analysis if possible. If subsequent clinically indicated intrathecal chemotherapy is performed, a CSF sample is requested if possible. Collect up to 2 mL if possible, transfer to a cryovial and freeze at -80º C. Ship to Dr Reid's lab with the blood pk samples.
f: Blood draws for pks are collected 30 minutes (± 10 minutes) before study drug administration on Days 1, 2 and 8, and at the following times after study drug on Day 1 and 8: 30 minutes (± 5 minutes), 1 hour (± 5 minutes), 2 hours (± 10 minutes), 3 hrs, 4 hrs, and 8 hours (all ± 15 minutes). It is more important to record time accurately than to perform the blood draw exactly on time.
g: Cycle 1 Day 1 physical exam and labs may be performed the day prior to Cycle 1 Day 1 in order to start the pharmacokinetic samples collection early on Cycle 1 Day 1.
h: Blood draws for pharmacodynamics are collected anytime prior to the first dose of MLN0128 (TAK-228) and at Cycle 1 Day 2 with the PK draw. R: Research funded

11. MEASUREMENT OF EFFECT

11.1 Antitumor Effect

11.1.1 Definitions

Evaluable for toxicity. All patients will be evaluable for toxicity from the time of their first treatment with MLN0128 (TAK-228).

Evaluable for objective response. All patients who have measurable disease present at baseline, have begun treatment, and have had their disease re-evaluated will be considered evaluable for response. These patients will have their response classified according to the definitions stated below. (Note: Patients who exhibit objective disease progression prior to the end of cycle 1 will also be considered evaluable.)

11.1.2 Response Criteria

11.1.2.1 Evaluation

Complete hematologic response (CR)

Less than 5% blasts in a non-hypocellular marrow with a granulocyte count of 1 $x10^9$/L (or above), and a platelet count of 100 $x10^9$/L (or higher) and absence of peripheral blood blasts with complete resolution of any extra medullary disease. The patient is in sustained CR if they have previously achieved a CR and continue to meet the CR criteria (at least 28 days).

CR incomplete (CRi)
is called if patient meets all CR criteria except for residual neutropenia (ANC<1 $x10^9$/L) or thrombocytopenia (platelets<100 $x10^9$/L)

Partial Response (PR):
The presence of trilineage hematopoiesis in the bone marrow with recovery of ANC and platelet count to above levels, but with 5-25% bone marrow blasts and ≥50% decrease in bone marrow blast percentage from baseline.

Morphologic leukemia-free state (MLFS)
If bone marrow blasts <5%, absence of Auer rods blasts, absence of extra medullary disease without hematological recovery.

No response (NR):
Failure to achieve CR/CRi/PR/MLFS after 2 cycles of MLN0128 (TAK-228).

Disease Progression:
At least a 25% increase in the absolute number of circulating blasts in peripheral blood, or at least 25% increase in bone marrow blasts, or development of extramedullary disease.

Relapse
Disease recurrence after achieving CR. Disease recurrence is defined by blast ≥ 5% in the bone marrow, or recurrence of peripheral blood > 5% or extramedullary involvement.

11.1.3 Duration of Response

Duration of response is the duration of CR or CRi is measured from the time measurement criteria are first met for CR until the first date that recurrence is objectively documented.

12. DATA REPORTING / REGULATORY REQUIREMENTS

Adverse event lists, guidelines, and instructions for AE reporting can be found in Section 7.0 (Adverse Events: List and Reporting Requirements).

12.1 Study Oversight

This protocol is monitored at several levels, as described in this section. The Protocol Principal Investigator is responsible for monitoring the conduct and progress of the clinical trial, including the ongoing review of accrual, patient-specific clinical and laboratory data, and routine and serious adverse events; reporting of expedited adverse events; and accumulation of reported adverse events from other trials testing the same drug(s). The Protocol Principal Investigator and statistician have access to the data at all times through the CTMS web-based reporting portal.

The Protocol Principal Investigator will have conference calls with the Study Investigators to review accrual, progress, and pharmacovigilance every two months for the first year that the study is accruing patients and then every three months after the first year. Decisions to proceed to the second stage of a Phase 2 trial will require sign-off by the Protocol Principal Investigator and the Protocol Statistician through IWRS and Medidata Rave.

All Study Investigators at participating sites who register/enroll patients on a given protocol are responsible for timely submission of data via Medidata Rave and timely reporting of adverse events for that particular study. This includes timely review of data collected on the electronic CRFs submitted via Medidata Rave.

All studies are also reviewed in accordance with the enrolling institution's data safety monitoring plan.

12.2 Data Reporting

Data collection for this study will be done exclusively through Medidata Rave. Access to the trial in Rave is granted through the iMedidata application to all persons with the appropriate roles assigned in the Regulatory Support System (RSS). To access Rave via iMedidata, the site user must have an active CTEP IAM account (https://eapps-ctep.nci.nih.gov/iam) and the appropriate Rave role (Rave CRA, Read-Only, or Site Investigator) on either the lead protocol organization or participating organization roster at the enrolling site.

Upon initial site registration approval for the study in RSS, all persons with Rave roles assigned on the appropriate roster will be sent a study invitation e-mail from iMedidata. To accept the invitation, site users must log into the Select Login (https://login.imedidata.com/selectlogin) using their CTEP-IAM user name and password, and click on the "accept" link in the upper right-corner of the iMedidata page. Please note, site users will not be able to access the study in Rave until all required Medidata and study specific trainings are completed. Trainings will be in the form of electronic learnings (eLearnings), and can be accessed by clicking on the link in the upper right pane of the iMedidata screen.

Users that have not previously activated their iMedidata/Rave account at the time of initial site registration approval for the study in RSS will also receive a separate invitation from iMedidata to activate their account. Account activation instructions are located on the CTSU website, Rave tab under the Rave resource materials (Medidata Account Activation and Study Invitation Acceptance). Additional information on iMedidata/Rave is available on the CTSU website under the Rave tab at www.ctsu.org/RAVE/ or by contacting the CTSU Help Desk at 1-888-823-

5923 or by e-mail at ctsucontact@westat.com.

12.2.1 Method

This study will be monitored by the Clinical Trials Monitoring Service (CTMS). Data will be submitted to CTMS at least once every two weeks via Medidata Rave (or other modality if approved by CTEP). Information on CTMS reporting is available at: http://www.theradex.com/CTMS. On-site audits will be conducted on an 18-36 month basis as part of routine cancer center site visits. More frequent audits may be conducted if warranted by accrual or due to concerns regarding data quality or timely submission. For CTMS monitored studies, after users have activated their accounts, please contact the Theradex Help Desk at (609) 799-7580 or by email at ctms@theradex.com for additional support with Rave and completion of CRFs.

12.2.2 Responsibility for Data Submission

For ETCTN trials, it is the responsibility of the PI(s) at the site to ensure that all investigators at the ETCTN Sites understand the procedures for data submission for each ETCTN protocol and that protocol specified data are submitted accurately and in a timely manner to the CTMS via the electronic data capture system, Medidata Rave.

Data are to be submitted via Medidata Rave to CTMS on a real-time basis, but no less than once every 2 weeks. The timeliness of data submissions and timeliness in resolving data queries will be tracked by CTMS. Metrics for timeliness will be followed and assessed on a quarterly basis. For the purpose of Institutional Performance Monitoring, data will be considered delinquent if it is greater than 4 weeks past due.

Data from Medidata Rave and CTEP-AERS is reviewed by the CTMS on an ongoing basis as data is received. Queries will be issued by CTMS directly within Rave. The queries will appear on the Task Summary Tab within Rave for the CRA at the ETCTN to resolve. Monthly web-based reports are posted for review by the Drug Monitors in the IDB, CTEP. Onsite audits will be conducted by the CTMS to ensure compliance with regulatory requirements, GCP, and NCI policies and procedures with the overarching goal of ensuring the integrity of data generated from NCI-sponsored clinical trials, as described in the ETCTN Program Guidelines, which may be found on the CTEP (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/adverse_events.htm) and CTSU websites.

An End of Study CRF is to be completed by the PI, and is to include the recommended phase 2 dose (RP2D), and a description of any dose-limiting toxicities (DLTs). CTMS will utilize a core set of eCRFs that are Cancer Data Standards Registry and Repository (caDSR) compliant (http://cbiit.nci.nih.gov/ncip/biomedical-informatics-resources/interoperability-and-semantics/metadata-and-models). Customized eCRFs will be included when appropriate to meet unique study requirements. The PI is encouraged to review the eCRFs, working closely with CTMS to ensure prospectively that all required items are appropriately captured in the eCRFs prior to study activation. CTMS

will prepare the eCRFs with built-in edit checks to the extent possible to promote data integrity.

CDUS data submissions for ETCTN trials activated after March 1, 2014, will be carried out by the CTMS contractor, Theradex. CDUS submissions are performed by Theradex on a monthly basis. The trial's lead institution is responsible for timely submission to CTMS via Rave, as above.

Further information on data submission procedures can be found in the ETCTN Program Guidelines (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/adverse_events.htm).

12.3 Collaborative Agreements Language

The agent(s) supplied by CTEP, DCTD, NCI used in this protocol is/are provided to the NCI under a Collaborative Agreement (CRADA, CTA, CSA) between the Pharmaceutical Company(ies) (hereinafter referred to as "Collaborator(s)") and the NCI Division of Cancer Treatment and Diagnosis. Therefore, the following obligations/guidelines, in addition to the provisions in the "Intellectual Property Option to Collaborator" (http://ctep.cancer.gov/industryCollaborations2/intellectual_property.htm) contained within the terms of award, apply to the use of the Agent(s) in this study:

1. Agent(s) may not be used for any purpose outside the scope of this protocol, nor can Agent(s) be transferred or licensed to any party not participating in the clinical study. Collaborator(s) data for Agent(s) are confidential and proprietary to Collaborator(s) and shall be maintained as such by the investigators. The protocol documents for studies utilizing Agents contain confidential information and should not be shared or distributed without the permission of the NCI. If a copy of this protocol is requested by a patient or patient's family member participating on the study, the individual should sign a confidentiality agreement. A suitable model agreement can be downloaded from: http://ctep.cancer.gov.

2. For a clinical protocol where there is an investigational Agent used in combination with (an)other Agent(s), each the subject of different Collaborative Agreements, the access to and use of data by each Collaborator shall be as follows (data pertaining to such combination use shall hereinafter be referred to as "Multi-Party Data"):

    a. NCI will provide all Collaborators with prior written notice regarding the existence and nature of any agreements governing their collaboration with NCI, the design of the proposed combination protocol, and the existence of any obligations that would tend to restrict NCI's participation in the proposed combination protocol.

    b. Each Collaborator shall agree to permit use of the Multi-Party Data from the clinical trial by any other Collaborator solely to the extent necessary to allow said other Collaborator to develop, obtain regulatory approval or commercialize its own Agent.

c. Any Collaborator having the right to use the Multi-Party Data from these trials must agree in writing prior to the commencement of the trials that it will use the Multi-Party Data solely for development, regulatory approval, and commercialization of its own Agent.

3. Clinical Trial Data and Results and Raw Data developed under a Collaborative Agreement will be made available to Collaborator(s), the NCI, and the FDA, as appropriate and unless additional disclosure is required by law or court order as described in the IP Option to Collaborator (http://ctep.cancer.gov/industryCollaborations2/intellectual_property.htm). Additionally, all Clinical Data and Results and Raw Data will be collected, used and disclosed consistent with all applicable federal statutes and regulations for the protection of human subjects, including, if applicable, the *Standards for Privacy of Individually Identifiable Health Information* set forth in 45 C.F.R. Part 164.

4. When a Collaborator wishes to initiate a data request, the request should first be sent to the NCI, who will then notify the appropriate investigators (Group Chair for Cooperative Group studies, or PI for other studies) of Collaborator's wish to contact them.

5. Any data provided to Collaborator(s) for Phase 3 studies must be in accordance with the guidelines and policies of the responsible Data Monitoring Committee (DMC), if there is a DMC for this clinical trial.

6. Any manuscripts reporting the results of this clinical trial must be provided to CTEP by the Group office for Cooperative Group studies or by the principal investigator for non-Cooperative Group studies for immediate delivery to Collaborator(s) for advisory review and comment prior to submission for publication. Collaborator(s) will have 30 days from the date of receipt for review. Collaborator shall have the right to request that publication be delayed for up to an additional 30 days in order to ensure that Collaborator's confidential and proprietary data, in addition to Collaborator(s)'s intellectual property rights, are protected. Copies of abstracts must be provided to CTEP for forwarding to Collaborator(s) for courtesy review as soon as possible and preferably at least three (3) days prior to submission, but in any case, prior to presentation at the meeting or publication in the proceedings. Press releases and other media presentations must also be forwarded to CTEP prior to release. Copies of any manuscript, abstract and/or press release/ media presentation should be sent to email: ncicteppubs@mail.nih.gov

The Regulatory Affairs Branch will then distribute them to Collaborator(s). No publication, manuscript or other form of public disclosure shall contain any of Collaborator's confidential/ proprietary information.

13. STATISTICAL CONSIDERATIONS

13.1 Study Design/Endpoints

This is a phase II study in relapsed or refractory ALL patients treated with MLN0128 (TAK-228). This study will use a one-stage design with an interim analysis to evaluate the efficacy of MLN0128 (TAK-228) in this patient population.

13.1.1 Primary Endpoint

The primary endpoint of this trial is complete response (CR, CRi) rate. A complete response will be considered synonymous with "success". All patients meeting the eligibility criteria who have signed a consent form and have begun treatment will be evaluable for response.

13.1.2 Primary Analyses Plan

The interim and final analyses for this trial will commence at the time the patients have become evaluable for the primary endpoint. Analyses at other time points will be a decision made by the Statistician and Study Chair, in accord with CCS Standard Operating Procedures, availability of data for secondary endpoints, and the level of data maturity. It is anticipated that the earliest date in which the results will be made available via manuscript, abstract, or presentation format is when the last patient registered has been followed for at least 6 months.

13.1.2.1 Definition of Primary Endpoint

The primary endpoint of this trial is complete response rate. A complete response is defined to be a CR or CRi noted as the objective status at any time during treatment. Response will be evaluated using all cycles of treatment. All patients meeting the eligibility criteria who have signed a consent form and have begun treatment will be evaluable for response.

13.1.2.2 Estimation

The proportion of successes will be estimated by the number of successes divided by the total number of evaluable patients. Ninety-five percent Duffy-Santner confidence intervals for the true success proportion will be calculated.

13.1.2.3 Primary Endpoint Evaluable Patient Exception

Patients not being treated at the safe dose level (see section 13.5) will be summarized separately for the primary endpoint and will not be included when evaluating the final decision rule.

13.1.2.4 Decision Rule

Previous studies in relapsed or refractory ALL have shown a CR rate ranging from 20 to 47% (61, 62). Responses can vary by factors such as first or second relapse and age. Due to the heterogeneity of this disease, a response rate of 10% with MLN0128 (TAK-228) would not be of interest. The largest success proportion where the proposed treatment regimen would be considered ineffective in this population is 10%, and the

smallest success proportion that would warrant subsequent studies with the proposed regimen in this patient population is 30%. The following one-stage design with an interim analysis uses 26 evaluable patients to test the null hypothesis that the true success proportion in a given patient population is at most 10%.

13.1.2.5 Interim Analysis

Enter 11 evaluable patients into the study. If 1 or fewer successes are observed in the first 11 evaluable patients, we will consider this regimen ineffective in this patient population and terminate this study. Otherwise, if the number of successes is at least 2, we will continue accrual.

13.1.2.6 Final Decision Rule

Enter 26 evaluable patients into the study. If 4 or fewer successes are observed in the first 26 evaluable patients, we will consider this regimen ineffective in this patient population. If 5 or more successes are observed in the first 26 evaluable patients, we may recommend further testing of this regimen in subsequent studies in this population.

13.1.2.7 Over Accrual

If more than the target number of patients are accrued at the safe dose, the additional patients will not be used to evaluate the stopping rule or used in any decision making process; however, they will be included in final endpoint estimates and confidence intervals.

NOTE: We will not suspend accrual at the interim analysis to allow the first 11 patients to become evaluable, unless undue toxicity is observed. Given the limited overall sample size and the inclusion of an adverse events stopping rule, we feel it is ethical to not halt accrual for the interim analysis. However, if accrual is extremely rapid, we may temporarily suspend accrual in order to obtain safety data on these patients before re-opening accrual to further patients.

13.1.3 Power and Significance Level

Assuming that the number of successes is binomially distributed, the significance level is .09, i.e. there is a 9% chance of finding the drug to be effective when it truly is not. The probability of declaring that this regimen warrants further study (i.e. statistical power) and the probability of stopping at the interim analysis under various success proportions can be tabulated as a function of the true success proportion as shown in the following table.

If the true success proportion is...	0.10	0.15	0.20	0.25	0.30
Then the probability of declaring that the regimen warrants further study is…	0.09	0.28	0.52	0.72	0.85
and the probability of stopping at the interim analysis is ...	0.70	0.49	0.32	0.20	0.11

13.1.4 Other Considerations

Adverse events, quality/duration of response, and patterns of treatment failure observed in this study, as well as scientific discoveries or changes in standard care will be taken into account in any decision to terminate the study.

13.2 Sample Size/Accrual Rate

13.2.1 Sample Size

The one-stage design with an interim analysis to be utilized is fully described above. A maximum of 26 evaluable patients will be accrued onto this phase II study unless undue toxicity is encountered. If there is undue toxicity in the initial safety cohort (see section 13.5), an additional 6 patients will be accrued. Further, we anticipate accruing an additional 3 patients to account for ineligibility, cancellation, major treatment violation, or other reasons. Therefore, the maximum projected accrual is 35 patients.

13.2.2 Accrual Rate and Study Duration

The anticipated accrual rate is 1 evaluable patient per month. Therefore, the accrual period for this study is expected to be approximately 3 years. The final analysis can begin approximately 3.5 years after the trial begins, i.e. as soon as the last patient has been on treatment for 6 months.

13.2.3 Inclusion of Women and Minorities

13.2.3.1 This study will be available to all eligible patients, regardless of race, gender, or ethnic origin.

13.2.3.2 There is no information currently available regarding differential effects of this regimen in subsets defined by race, gender, or ethnicity, and there is no reason to expect such differences to exist. Therefore, although the planned analysis will, as always, look for differences in treatment effect based on racial and gender groupings, the sample size is not increased in order to provide additional power for subset analyses.

13.2.1.3 The geographical region served by Mayo Clinic has a population that includes approximately 7% minorities. Based on prior Mayo phase 2 studies involving similar disease sites, we expect about 7% of patients will be classified as minorities by race and about

55% of patients will be women. Expected sizes of racial by gender subsets are shown in the following table:

DOMESTIC PLANNED ENROLLMENT REPORT					
Racial Categories	**Ethnic Categories**				**Total**
	Not Hispanic or Latino		**Hispanic or Latino**		
	Female	**Male**	**Female**	**Male**	
American Indian/ Alaska Native	0	0	0	0	0
Asian	0	0	0	0	0
Native Hawaiian or Other Pacific Islander	0	0	0	0	0
Black or African American	1	1	0	0	2
White	18	13	1	1	33
More Than One Race	0	0	0	0	0
Total	19	14	1	1	35

13.3 Stratification Factors

None.

13.4 Analysis of Secondary/Tertiary Endpoints

13.4.1 The overall response rate will be estimated by the total number of complete or partial responses (CR, CRi, PR, or MLFS) divided by the total number of evaluable patients. All evaluable patients will be used for this analysis. Exact binomial 95% confidence intervals for the true overall response rate will be calculated.

13.4.2 Duration of complete response (CR/CRi) is defined for all evaluable patients who have achieved a complete response as the time from the date at which the patient's objective status is first noted to be a CR to the earliest date progression is documented. The distribution of duration of complete response will be estimated using the method of Kaplan-Meier.

13.4.3 The frequency of proceeding to allogeneic SCT after achieving response (CR, CRi, PR, or MLFS) to MLN0128 (TAK-228) will be estimated by the number of patients who proceed to allogeneic SCT after achieving response divided by the total number of

evaluable patients who achieved a response. All evaluable patients who achieved a response will be used for this analysis. Exact binomial 95% confidence intervals for the true overall response rate will be calculated.

13.4.4 Overall survival time is defined as the time from registration to death due to any cause. The distribution of survival time will be estimated using the method of Kaplan-Meier.

13.4.5 Adverse Events
All eligible patients that have initiated treatment will be considered evaluable for assessing adverse event rate(s). The maximum grade for each type of adverse event will be recorded for each patient, and frequency tables will be reviewed to determine patterns. Additionally, the relationship of the adverse event(s) to the study treatment will be taken into consideration.

13.4.6 Correlative Analyses
All analyses with respect to the correlative component of this study (Goal 1.2.5-1.2.9) are intended to be hypothesis-generating and descriptive in nature. Due to the exploratory nature of the correlative endpoints, we will not adjust for multiple comparisons for these analyses.

Descriptive statistics and simple scatterplots will form the basis of presentation of these data and any calculated changes from baseline. Single sample t-tests, Wilcoxon signed rank tests or McNamar's tests may be employed as appropriate. Data may be compared between success and failure groups using two sample t-tests or Wilcoxon rank sum tests. Correlations between these laboratory values and other outcome measures will be carried out by standard parametric and nonparametric correlation procedures (Pearson's and Spearman's coefficients).

13.5 Safety Analysis

13.5.1 A safety analysis will be performed after the first 6 patients have been accrued to the study on dose 0 (3 mg, orally days 1-21). If 2 or more of the first 6 patients experience a dose-limiting toxicity (DLT) as defined below, then 6 patients will be enrolled on dose -1 (2 mg, orally days 1-21). If 2 or more of the second 6 patients experience DLT, then the study will be terminated.

13.5.2 Toxicity will be measured per NCI-CTCAE version 4. DLT is defined as an adverse event possibly, probably, or definitely related to study treatment that meets one of the following:

- Any grade 4 drug-related non-hematological toxicity
- Any grade 3 drug-related non-hematological toxicity that does not resolve to < grade 3 within 3 days, with the following exceptions:
    - Grade 3 Bilirubin, AST, ALT, Alkaline phosphatase will be considered DLT only if it does not recover to <grade 3 within 7 days
- Myelosuppression: hypoplasia (bone marrow cellularity <10%) >50 days

13.6 Data Monitoring

The principal investigator(s) and the study statistician will review the study at least twice a year to identify accrual, adverse event, and any endpoint problems that might be developing. The accrual and safety data provided by Theradex for this trial will be reviewed and monitored by the Mayo Clinic Cancer Center (MCCC) Data Safety Monitoring Board (DSMB) at least twice a year, or more frequently as warranted.

13.6.1 Adverse Event Stopping Rule

The stopping rules specified below are based on the knowledge available at study development. We note that the Adverse Event Stopping Rule may be adjusted in the event of either (1) the study re-opening to accrual or (2) at any time during the conduct of the trial and in consideration of newly acquired information regarding the adverse event profile of the treatment(s) under investigation. The study team may choose to suspend accrual because of unexpected adverse event profiles that have not crossed the specified rule below.

Accrual will be temporarily suspended to this study if at any time we observe events considered at least possibly related to study treatment (i.e. an adverse event with attribute specified as "possible", "probable", or "definite") that satisfy any of the following:

- If 3 or more out of the first 12 treated patients experience grade 4 non-hematological toxicities at least possibly related to treatment excluding fatigue, fever, infection, and lab abnormalities (hyper- or hypokalemia, hypocalcemia, hyperuricemia, hypophosphatemia) that resolve within 7 days without jeopardizing patient safety.

- If after the first 12 patients have been treated, 25% of patients experience grade 4 non-hematologic toxicities at least possibly related to treatment excluding fatigue, fever, infection, and lab abnormalities (hyper- or hypokalemia, hypocalcemia, hyperuricemia, hypophosphatemia) that resolve within 7 days without jeopardizing patient safety.

We note that we will review grade 4 and 5 adverse events deemed "unrelated" or "unlikely to be related", to verify their attribution and to monitor the emergence of a previously unrecognized treatment-related adverse event.

13.7 Reporting and Exclusions

13.7.1 Evaluation of Toxicity

All patients will be evaluable for toxicity from the time of their first treatment with MLN0128 (TAK-228).

13.7.2 Evaluation of Response

All patients included in the study must be assessed for response to treatment, even if there are major protocol treatment deviations or if they are ineligible. Each patient will be assigned one of the following categories: 1) complete response, 2) partial response, 3) stable disease, 4) progressive disease, 5) early death from malignant disease, 6) early death from toxicity, 7) early death because of other cause, or 9) unknown (not assessable, insufficient data). [Note: By arbitrary convention, category 9 usually designates the "unknown" status of any type of data in a clinical database.]

All of the patients who met the eligibility criteria (with the possible exception of those who received no study medication) should be included in the main analysis of the response rate. Patients in response categories 4-9 should be considered to have a treatment failure (disease progression). Thus, an incorrect treatment schedule or drug administration does not result in exclusion from the analysis of the response rate. Precise definitions for categories 4-9 will be protocol specific.

All conclusions should be based on all eligible patients. Sub analyses may then be performed on the basis of a subset of patients, excluding those for whom major protocol deviations have been identified (*e.g.*, early death due to other reasons, early discontinuation of treatment, major protocol violations, etc.). However, these sub analyses may not serve as the basis for drawing conclusions concerning treatment efficacy, and the reasons for excluding patients from the analysis should be clearly reported. The 95% confidence intervals should also be provided.

REFERENCES

1. Cancer Facts and Figures 2012. American Cancer Society. 2012.
2. Kantarjian H, Thomas D, O'Brien S, Cortes J, Giles F, Jeha S, et al. Long-term follow-up results of hyperfractionated cyclophosphamide, vincristine, doxorubicin, and dexamethasone (Hyper-CVAD), a dose-intensive regimen, in adult acute lymphocytic leukemia. Cancer. 2004;101:2788-801.
3. Ravandi F, O'Brien S, Thomas D, Faderl S, Jones D, Garris R, et al. First report of phase 2 study of dasatinib with hyper-CVAD for the frontline treatment of patients with Philadelphia chromosome-positive (Ph+) acute lymphoblastic leukemia. Blood. 2010;116:2070-7.
4. Litzow MR. Novel therapeutic approaches for acute lymphoblastic leukemia. Hematology/Oncology clinics of North America. 2011;25:1303-17.
5. Hoelzer D, Gokbuget N. Chemoimmunotherapy in acute lymphoblastic leukemia. Blood reviews. 2012;26:25-32.
6. O'Brien S, Thomas D, Ravandi F, Faderl S, Cortes J, Borthakur G, et al. Outcome of adults with acute lymphocytic leukemia after second salvage therapy. Cancer. 2008;113:3186-91.
7. Faderl S, O'Brien S, Pui CH, Stock W, Wetzler M, Hoelzer D, et al. Adult acute lymphoblastic leukemia: concepts and strategies. Cancer. 2010;116:1165-76.

8. Martelli AM, Evangelisti C, Chiarini F, McCubrey JA. The phosphatidylinositol 3-kinase/Akt/mTOR signaling network as a therapeutic target in acute myelogenous leukemia patients. Oncotarget. 2010;1:89-103.
9. Polak R, Buitenhuis M. The PI3K/PKB signaling module as key regulator of hematopoiesis: implications for therapeutic strategies in leukemia. Blood. 2012;119:911-23.
10. Maiso P, Liu Y, Morgan B, Azab AK, Ren P, Martin MB, et al. Defining the role of TORC1/2 in multiple myeloma. Blood. 2011;118:6860-70.
11. Hsieh AC, Liu Y, Edlind MP, Ingolia NT, Janes MR, Sher A, et al. The translational landscape of mTOR signalling steers cancer initiation and metastasis. Nature. 2012;485:55-61.
12. Janes MR, Vu C, Mallya S, Shieh MP, Limon JJ, Li LS, et al. Efficacy of the investigational mTOR kinase inhibitor MLN0128/INK128 in models of B-cell acute lymphoblastic leukemia. Leukemia. 2013;27:586-94.
13. Hassan B, Akcakanat A, Sangai T, Evans KW, Adkins F, Eterovic AK, et al. Catalytic mTOR inhibitors can overcome intrinsic and acquired resistance to allosteric mTOR inhibitors. Oncotarget. 2014;5:8544-57.
14. Yun S, Vincelette ND, Knorr KLB, Schneider PA, Peterson KL, Flatten KS, et al. Dual mTOR Inhibitiors Induce Bim-Dependent Apoptosis in Acute Lymphoblastic Leukmeia via Inhibition of eIF4E/eIF4G and Activation of NF-kB/Egr-1. Submitted. 2015.
15. Investigator's Brochure MLN0128. 7th edition ed, 2014.
16. ABT-767 Investigator's Brochure. 3rd ed. North Chicago, IL: AbbVie, Inc.; 2013.
17. Ghobrial IM, Siegel D, Vij R, Wolf JL, Berdeja JG, Matous JV, et al. MLN0128 (INK128), an Investigational Oral Dual TORC1/2 Inhibitor, in Patients (pts) with Relapsed or Refractory Multiple Myeloma (MM), Non-Hodgkin's Lymphoma (NHL), or Waldenstrom Macroglobulinemia (WM): Preliminary Results From a Phase 1 Dose-Escalation Study. ASH Annual Meeting Abstracts. 2012;120:4038-.
18. Gupta M, Wahner Hendrickson A, Seongseouk Y, Han JJ, Schneider PA, Stenson M, et al. Dual TORC1/TORC2 Inhibition Diminishes Akt Activation and Induces Puma-Dependent Apoptosis in Lymphoid Malignancies. Blood. 2012;119:476-87.
19. Witzig TE, Geyer SM, Ghobrial I, Inwards DJ, Fonseca R, Kurtin P, et al. Phase II trial of single-agent temsirolimus (CCI-779) for relapsed mantle cell lymphoma. Journal of Clinical Oncology. 2005;23:5347-56.
20. Ansell SM, Inwards DJ, Rowland KM, Flynn PJ, Morton RF, Moore DF, Jr., et al. Low-dose single-agent temsirolimus for relapsed mantle cell lymphoma: A phase 2 trial in the North Central Cancer Treatment Group. Cancer. 2008;113:508-14.
21. Zent CS, LaPlant BR, Johnston PB, Call TG, Habermann TM, Micallef IN, et al. The treatment of recurrent/refractory chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL) with everolimus results in clinical responses and mobilization of CLL cells into the circulation. Cancer. 2010;116:2201-7.
22. Johnston PB, Inwards DJ, Colgan JP, Laplant BR, Kabat BF, Habermann TM, et al. A Phase II trial of the oral mTOR inhibitor everolimus in relapsed Hodgkin lymphoma. Am J Hematol. 2010;85:320-4.
23. Witzig TE, Reeder CB, Laplant BR, Gupta M, Johnston PB, Micallef IN, et al. A phase II trial of the oral mTOR inhibitor everolimus in relapsed aggressive lymphoma. Leukemia. 2011;25:341-7.

24. Ansell SM, Tang H, Kurtin PJ, Koenig PA, Inwards DJ, Shah K, et al. Temsirolimus and rituximab in patients with relapsed or refractory mantle cell lymphoma: a phase 2 study. Lancet Oncol. 2011;12:361-8.
25. Yee KW, Zeng Z, Konopleva M, Verstovsek S, Ravandi F, Ferrajoli A, et al. Phase I/II study of the mammalian target of rapamycin inhibitor everolimus (RAD001) in patients with relapsed or refractory hematologic malignancies. Clinical cancer research : an official journal of the American Association for Cancer Research. 2006;12:5165-73.
26. Bjornsti MA, Houghton PJ. The TOR pathway: a target for cancer therapy. Nat Rev Cancer. 2004;4:335-48.
27. Dancey J. mTOR signaling and drug development in cancer. Nat Rev Clin Oncol. 2010;7:209-19.
28. Benjamin D, Colombi M, Moroni C, Hall MN. Rapamycin passes the torch: a new generation of mTOR inhibitors. Nat Rev Drug Discov. 2011;10:868-80.
29. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol. 2011;12:21-35.
30. Blachly JS, Baiocchi RA. Targeting PI3-kinase (PI3K), AKT and mTOR axis in lymphoma. Br J Haematol. 2014;167:19-32.
31. Roti G, Stegmaier K. New Approaches to Target T-ALL. Front Oncol. 2014;4:170.
32. Eyre TA, Collins GP, Goldstone AH, Cwynarski K. Time now to TORC the TORC? New developments in mTOR pathway inhibition in lymphoid malignancies. Br J Haematol. 2014;166:336-51.
33. Mohindra NA, Giles FJ, Platanias LC. Use of mTOR inhibitors in the treatment of malignancies. Expert Opin Pharmacother. 2014;15:979-90.
34. Sengupta S, Peterson TR, Sabatini DM. Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Molecular Cell. 2010;40:310-22.
35. Sabatini DM. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer. 2006;6:729-34.
36. Hsu PP, Kang SA, Rameseder J, Zhang Y, Ottina KA, Lim D, et al. The mTOR-regulated phosphoproteome reveals a mechanism of mTORC1-mediated inhibition of growth factor signaling. Science. 2011;332:1317-22.
37. Neri LM, Cani A, Martelli AM, Simioni C, Junghanss C, Tabellini G, et al. Targeting the PI3K/Akt/mTOR signaling pathway in B-precursor acute lymphoblastic leukemia and its therapeutic potential. Leukemia. 2013.
38. Smith AJ, Dai H, Correia C, Lee S-H, Takahashi R, Kaufmann SH. Noxa/Bcl-2 Interactions Contribute to Bortezomib Resistance in Human Lymphoid Cells. J. Biol. Chemistry. 2011;286:17682-92.
39. Janes MR, Vu C, Mallya S, Shieh MP, Limon JJ, Li LS, et al. Efficacy of the investigational mTOR kinase inhibitor MLN0128/INK128 in models of B-cell acute lymphoblastic leukemia. Leukemia. 2012.
40. Kaufmann SH, Karp JE, Svingen PA, Krajewski S, Burke PJ, Gore SD. Elevated Expression of the Apoptotic Regulator Mcl-1 at the Time of Leukemic Relapse. Blood. 1998;91:991-1000.
41. Dai H, Meng XW, Lee S-H, Schneider PA, Kaufmann SH. Context-dependent Bcl-2/Bak Interactions Regulate Lymphoid Cell Apoptosis. J. Biol. Chemistry. 2009;284:18311-22.
42. Kaufmann SH, Okret SH, Wikström A-C, Gustafsson J-Å, Shaper JH. Binding of the Glucocorticoid Receptor to the Rat Liver Nuclear Matrix: The Role of Disulfide Bond

Formation. J. Biol. Chemistry. 1986;261:11962-7.
43. Kaufmann SH, Svingen PA, Gore SD, Armstrong DK, Cheng Y-C, Rowinsky EK. Altered Formation of Topotecan-Stabilized Topoisomerase I-DNA Adducts in Human Leukemia Cells. Blood. 1997;89:2098-104.
44. Casado P, Alcolea MP, Iorio F, Rodriguez-Prados JC, Vanhaesebroeck B, Saez-Rodriguez J, et al. Phosphoproteomics data classify hematological cancer cell lines according to tumor type and sensitivity to kinase inhibitors. Genome Biol. 2013;14:R37.
45. Casado P, Rodriguez-Prados JC, Cosulich SC, Guichard S, Vanhaesebroeck B, Joel S, et al. Kinase-substrate enrichment analysis provides insights into the heterogeneity of signaling pathway activation in leukemia cells. Sci Signal. 2013;6:rs6.
46. Cope CL, Gilley R, Balmanno K, Sale MJ, Howarth KD, Hampson M, et al. Adaptation to mTOR kinase inhibitors by amplification of eIF4E to maintain cap-dependent translation. J Cell Sci. 2013.
47. Galanis E, Jaeckle KA, Maurer MJ, Reid JM, Ames MM, Hardwick JS, et al. Phase II trial of vorinostat in recurrent glioblastoma multiforme: a north central cancer treatment group study. J.Clinical Oncol. 2009;27:2052-8.
48. Fouladi M, Park JR, Stewart CF, Gilbertson RJ, Schaiquevich P, Sun J, et al. Pediatric phase I trial and pharmacokinetic study of vorinostat: a Children's Oncology Group phase I consortium report. J..Clinical Oncol. 2010;28:3623-9.
49. Lee EQ, Puduvalli VK, Reid JM, Kuhn JG, Lamborn KR, Cloughesy TF, et al. Phase I study of vorinostat in combination with temozolomide in patients with high-grade gliomas: North American Brain Tumor Consortium Study 04-03. Clinical Cancer Research. 2012;18:6032-9.
50. Shah VP, Midha KK, Dighe S, McGilveray IJ, Skelly JP, Yacobi A, et al. Analytical methods validation: bioavailability, bioequivalence and pharmacokinetic studies. Conference report. European journal of drug metabolism and pharmacokinetics. 1991;16:249-55.
51. Shah VP, Midha KK, Findlay JW, Hill HM, Hulse JD, McGilveray IJ, et al. Bioanalytical method validation--a revisit with a decade of progress. Pharm Res. 2000;17:1551-7.
52. Guidance for Industry: Bioanalytical Method Validation. U.S. Department of Health and Human Services Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM). Draft Guidance, September 2013. In: Administration FaD, editor.
53. Kaufmann SH, Svingen PA. Immunoblot Analysis and Band Depletion Assays. In: Bjornsti M-A, Osheroff N, editors. Protocols for DNA Topoisomerases: Humana Press; 1998. p. 253-68.
54. Svingen PA, Loegering DA, Rodriquez J, Meng XW, Mesner PW, Jr., Monks A, et al. Components of the Cell Death Machine and Drug Sensitivity of the NCI Cell Line Panel. Clinical Cancer Research. 2004;10:6807-20.
55. Kaufmann SH, Ewing CM, Shaper JH. The Erasable Western Blot. Analytical Biochemistry. 1987;161:89-95.
56. Kaufmann SH. Reutilization of Immunoblots After Chemiluminescent Detection. Analytical Biochemistry. 2001;296:283-6.
57. Kaufmann SH. Reutilizaton of Western Blots After Chemiluminescent or Autoradiographic Detection. In: Walker JM, editor. Protein Protocols Handbook, 3rd edition. Totowa, N.J.: Humana Press; 2009. p. 785-801.

58. Kaufmann SH, Karp JE, Letendre L, Kottke TJ, Safgren S, Greer J, et al. Phase I and Pharmacological Study of Infusional Topotecan and Carboplatin in Relapsed and Refractory Leukemia. Clinical Cancer Research. 2005;11:6641-9.
59. Witzig TE, Tang H, Micallef IM, Ansell SM, Ding H, Link BK, et al. A Multi-Institution Phase 2 Study of the Farnesyltransferase Inhibitor Tipifarnib (R115777) in Patients with Relapsed and Refractory Lymphomas. Blood. 2011;118:4882-9.
60. Tibes R, Qiu Y, Lu Y, Hennessy B, Andreeff M, Mills GB, et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. Molecular Cancer Therapeutics. 2006;5:2512-21.
61. Faderl S, Thomas DA, O'Brien S, Ravandi F, Garcia-Manero G, Borthakur G, et al. Augmented hyper-CVAD based on dose-intensified vincristine, dexamethasone, and asparaginase in adult acute lymphoblastic leukemia salvage therapy. Clin Lymphoma Myeloma Leuk. 2011;11:54-9.
62. O'Brien S, Schiller G, Lister J, Damon L, Goldberg S, Aulitzky W, et al. High-dose vincristine sulfate liposome injection for advanced, relapsed, and refractory adult Philadelphia chromosome-negative acute lymphoblastic leukemia. J.Clinical Oncol. 2013;31:676-83.

APPENDIX A: PERFORMANCE STATUS CRITERIA

ECOG Performance Status Scale	
Grade	**Descriptions**
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.
1	Symptoms, but ambulatory. Restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature (*e.g.*, light housework, office work).
2	In bed < 50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.
3	In bed > 50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.
5	Dead.

APPENDIX B: STUDY MEDICATION CALENDAR

STUDY # _____

Patient's Name _____ Cycle # _____ Day 1 of Cycle _____ / _____ / _____ PATIENT ID # _____

INSTRUCTIONS:

- The capsules are to be swallowed intact, not less than 2 hours before or 1 hour after a meal. Do not chew, open or manipulate the capsule in any way prior to swallowing. Each dose should be taken with 8 ounces (240 mL) of water. Doses are to be taken at about the same time each day. If a dose is missed, it can be taken up to 6 hours past the scheduled time. If more than 6 hours past the scheduled time, the dose should not be taken and will not be made up. Vomited doses will not be made up.
- Please record the times that you take your study medications. Return this diary and the medication bottles (even if unopened or empty) along with any unused pills at your next visit.

DAY OF WEEK: _____	**DAY OF WEEK:** _____	**DAY OF WEEK:** _____	**DAY OF WEEK:** _____	**DAY OF WEEK:** _____	**DAY OF WEEK:** _____	**DAY OF WEEK:** _____
Day 1 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 2 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 3 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 4 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 5 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 6 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 7 of Cycle **# Pills:** _____ **__ :___ AM or PM**
Day 8 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 9 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 10 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 11 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 12 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 13 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 14 of Cycle **# Pills:** _____ **__ :___ AM or PM**
Day 15 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 16 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 17 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 18 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 19 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 20 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 21 of Cycle **# Pills:** _____ **__ :___ AM or PM**
Day 22 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 23 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 24 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 25 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 26 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 27 of Cycle **# Pills:** _____ **__ :___ AM or PM**	Day 28 of Cycle **# Pills:** _____ **__ :___ AM or PM**

Patient's Signature _____ Date _____

APPENDIX C: PATIENT DRUG INFORMATION HANDOUT AND WALLET CARD

Information for Patients, Their Caregivers, and Non-Study Healthcare Team on Possible Interactions with Other Drugs and Herbal Supplements

The patient _____ is enrolled on a clinical trial using the experimental study drug, MLN0128 (TAK-228). This clinical trial is sponsored by the National Cancer Institute. This form is addressed to the patient, but includes important information for others who care for this patient.

These are the things that you as a healthcare provider need to know: Because of the low potential for interaction with agents that induce or inhibit cytochrome P450 enzymes, there are no prohibitions of specific medications on the basis of anticipated drug-drug interactions.

To the patient: Take this paper with you to your medical appointments and keep the attached information card in your wallet.

MLN0128 (TAK-228) may interact with other drugs which can cause side effects. For this reason, it is very important to tell your study doctors of any medicines you are taking before you enroll onto this clinical trial. It is also very important to tell your doctors if you stop taking any regular medicines, or if you start taking a new medicine while you take part in this study. When you talk about your current medications with your doctors, include medicine you buy without a prescription (over-the-counter remedy), or any herbal supplements such as St. John's Wort. It is helpful to bring your medication bottles or an updated medication list with you.

Many health care providers can write prescriptions. You must tell all of your health care providers (doctors, physician assistants, nurse practitioners, pharmacists) you are taking part in a clinical trial.

These are the things that you and they need to know:

- Please be very careful! Over-the-counter drugs (including herbal supplements) may contain ingredients that could interact with your study drug. Speak to your doctors or pharmacist to determine if there could be any side effects.

- Your regular health care provider should check a frequently updated medical reference or call your study doctor before prescribing any new medicine or discontinuing any medicine. Your study doctor's name is _____ and he or she can be contacted at _____.

STUDY DRUG INFORMATION WALLET CARD

You are enrolled on a clinical trial using the experimental study drug MLN0128. This clinical trial is sponsored by the NCI.

It is very important to:

- Tell your doctors if you stop taking any medicines or if you start taking any new medicines.
- Tell all of your health care providers (doctors, physician assistants, nurse practitioners, or pharmacists) that you are taking part in a clinical trial.
- Check with your doctor or pharmacist whenever you need to use an over-the-counter medicine or herbal supplement.
- Before prescribing new medicines, your regular health care providers should go to a frequently-updated medical reference for a list of drugs to avoid, or contact your study doctor.

- Your study doctor's name is _____ and can be contacted at _____.

APPENDIX D: PHARMACOKINETIC BLOOD SAMPLES LIST

9775 / MC1482: A Phase 2 Study of MLN0128 (TAK-228) in Relapsed and/or Refractory Acute Lymphoblastic Leukemia (ALL)

Pharmacokinetic Blood Samples List
Cycle 1 Day 1

Date:	Patient ID:	Site Name and CTEP ID:

Instructions: Please complete the scheduled times and the actual times the pk blood samples were collected below. Please include a copy of this document with the samples at time of shipment.

Sample Collection Times (All times hh:mm in 24 hour clock)		
Time Point	**Scheduled Time**	**Actual Time**
30 minutes pre-dose (± 10 minutes)		
Administration of MLN0128 (TAK-228)	_____	
30 minutes post-dose (± 5 minutes)		
1 hour post-dose (± 5 minutes)		
2 hours post-dose (± 10 minutes)		
3 hours post-dose (± 15 minutes)		
4 hours post-dose (± 15 minutes)		
8 hours post-dose* (± 15 minutes)		

***Note: If unable to draw an 8 hour post dose sample, please specify what timepoint blood sample was collected.**

9775 / MC1482: A Phase 2 Study of MLN0128 (TAK-228) in Relapsed and/or Refractory Acute Lymphoblastic Leukemia (ALL)

Pharmacokinetic Blood Samples List
Cycle 1 Day 2

Date:	Patient ID:	Site Name and CTEP ID:

Instructions: Please complete the scheduled times and the actual times the pk blood samples were collected below. Please include a copy of this document with the samples at time of shipment.

Sample Collection Times (All times hh:mm in 24 hour clock)		
Time Point	**Scheduled Time**	**Actual Time**
30 minutes pre-dose (± 10 minutes)		
Administration of MLN0128 (TAK-228)	_____	

9775 / MC1482: A Phase 2 Study of MLN0128 (TAK-228) in Relapsed and/or Refractory Acute Lymphoblastic Leukemia (ALL)

Pharmacokinetic Blood Samples List
Cycle 1 Day 8

Date:	Patient ID:	Site Name and CTEP ID:

Instructions: Please complete the scheduled times and the actual times the pk blood samples were collected below. Please include a copy of this document with the samples at time of shipment.

Sample Collection Times (All times hh:mm in 24 hour clock)		
Time Point	**Scheduled Time**	**Actual Time**
30 minutes pre-dose (± 10 minutes)		
Administration of MLN0128 (TAK-228)	_____	
30 minutes post-dose (± 5 minutes)		
1 hour post-dose (± 5 minutes)		
2 hours post-dose (± 10 minutes)		
3 hours post-dose (± 15 minutes)		
4 hours post-dose (± 15 minutes)		
8 hours post-dose* (± 15 minutes)		

***Note: If unable to draw an 8 hour post dose sample, please specify what timepoint blood sample was collected.**

APPENDIX E: ADDITIONAL INFORMATION REGARDING PROPOSED BIOMARKER STUDIES

Per the instructions in the CTEP Protocol Template, this appendix is constructed to provide information on endpoint validation including additional background (as needed), description of the assay(s) used, materials and methods, and assay validation. The integrated biomarker (phospho-Thr^{37,46}-4EBP1 phosphorylation by immunoblotting in blasts from pretreatment and day 8 marrows) has previously been reviewed by the Biomarker Review Committee; and that material is provided here in case that review is not provided to reviewers of this protocol. Similar information about the exploratory biomarkers is provided in more abbreviated form per our interpretation of the template instructions.

Biomarkers are provided in the order of their priority, highest to lowest. The rationale for this priority is provided in Section 2.4.7 of the main body of the protocol.

1. Phospho-Thr^{37,46}-4EBP1 Analysis of Pretreatment and Day 8 Bone Marrow Samples by Immunoblotting

   1.1 Role in Trial: Integrated Biomarker. This is the top priority assay and will be performed first on every available sample from the trial.

   1.2 Hypothesis and Rationale

   Hypothesis: 4EBP1 phosphorylation will be inhibited by MLN0128 (TAK-228) *in situ*.

   Rationale: Previous studies have shown that mTOR dual inhibitors such as MLN0128 (TAK-228) and OSI-027 diminish the mTORC1-mediated phosphorylation of the translation inhibitor 4EBP1, causing it to bind to and inhibit the translation initiation factor eIF4E (1, 2). In acute lymphocytic leukemia (ALL) cell lines, the down regulation of 4EBP1 produces resistance to mTOR dual inhibitors (2), demonstrating the critical role of this protein in killing by mTOR dual inhibitors. We now propose to assess whether 4EBP1 has been dephosphorylated in leukemic blasts by therapeutically achievable MLN0128 concentrations *in situ* as a proof of mechanism.

   Results of these assays will not help individual patients on this trial but might be particularly useful to clinical investigators thinking about future development of this class of agents: If ALL fails to respond and 4EBP1 remains phosphorylated, this might imply that the 4EBP1 pathway has been inadequately targeted at therapeutically achievable concentrations, e.g., because toxicities in normal tissues preclude adequate drug levels, transport-mediated resistance diminishes ALL drug uptake, or signaling alterations restore 4EBP1 phosphorylation in the absence of mTOR. On the other

hand, if 4EBP1 is extensively dephosphorylated but ALL fails to respond, this will suggest that clinical ALL is not dependent on this target, but would not preclude combining mTOR inhibition with other therapies.

1.3 Assay Procedure

The assay procedure is described in detail in the attached Standard Operating Procedure previously provided to the Biomarker Review Committee (pp. 16- 26 of this appendix). In brief, 4EBP1 phosphorylation will be assessed by immunoblotting as previously described (1). Detailed procedures for immunoblotting have been previously published by the Kaufmann laboratory (3, 4). Sources of critical reagents are identified in the following description.

After sequential samples of bone marrow blasts (harvested prior to treatment and on day 8 of therapy) are subjected to SDS-polyacrylamide gel electrophoresis in adjacent wells using standard techniques and electrophoresis grade reagents from BioRad as previously described (3, 4), proteins will be transferred to Biotrace NT nitrocellulose (Pall #66485). After blocking according to standard techniques (4), blots will be incubated overnight with rabbit anti-phospho-Thr^{36,47}-4EBP1 monoclonal antibody 236B4 (Cell Signaling Technology product #2855) diluted 1:1000 in buffer consisting of 5% bovine serum albumin (Sigma-Aldrich #A7906) in 150 mM NaCl-10 mM Tris-HCl (pH 7.4 at 20 °C) containing 0.05% Tween 20, 1 mM sodium azide, 100 U/ml penicillin G, and 100 µg/ml streptomycin. Blots will be washed, incubated with 0.125 µg/ml horseradish peroxidase-coupled goat anti-rabbit IgG (KPL #074-1506) in 5% (w/v) powdered nonfat dry milk in 150 mM NaCl-10 mM Tris-HCl, washed again, reacted with Supersignal West Dura enhanced chemiluminescence reagents (Thermo #34075), and exposed to high sensitivity x-ray film (Phenix Research Products #F-BX810).

Because it is difficult to purify and reproducibly phosphorylate 4EBP1, aliquots of lysate from the Nalm6 human ALL cell line (authenticated by short tandem repeat profiling in the Mayo Advanced Genomics Technology Center) will be lyophilized in single-use aliquots and stored at -80 °C. Serial dilutions of these lysates on each blot will serve as a positive control and provide a standard curve for quantitation. This cell line was chosen because our studies have shown that it contains high levels of phospho-Thr^{37,46}-4EBP1 (see Fig. 2 below). Blots exposed in the linear range will be scanned on an Epson 4870 scanner and quantitated using NIH Image version 1.63. A complete description of the immunoblotting procedure, including sources of all reagents not specified above, is provided in the Standard Operating Procedure attached at the end of this appendix.

1.4 Source of Samples

Marrow mononuclear cells (typically at least 80% malignant lymphoid cells in ALL specimens—ref. 5, 6) will be harvested by Ficoll-Hypaque gradient centrifugation (7)

from bone marrow aspirates harvested prior to therapy and on day 8 ± 1 of treatment with single-agent MLN0128 (TAK-228). Day 8 was chosen for the post-treatment sample based several considerations:

- In published murine xenograft experiments (1), 4EBP1 dephosphorylation is detectable in xenografts at early time points after dual mTOR inhibitor treatment and maintained for the duration of drug exposure. This observation is presented in Fig. 4 below. On the other hand, induction of critical signaling steps downstream of 4EBP1 such as PARP cleavage, which could be assessed in the same specimens in an exploratory manner, is not consistently evident at day 3 in the xenograft experiments but is evident at day 6 (1).
- Xenograft regression was not extensive until beyond day 8 (1).
- Day 8 is a standard time for follow up appointments and toxicity assessment in patients who are being treated in the outpatient setting.
- Picking an arbitrary time such as day 5 or day 6 will harm patient accrual to the trial and sample acquisition: If samples were required on day 5, then patients could only start treatment on Monday, Thursday or Friday so that day 5 marrow procurement and processing would fall on a weekday.

1.5 Assay Performance Characteristics

The following performance characteristics of the phospho-4EBP1 assay have been evaluated:

1.51 Characterization of antibody used in these studies: A critical antibody for this procedure, a rabbit monoclonal from Cell Signaling Technology, Inc., recognizing phospho-Thr^{37,46}-4EBP1, is supplied as a pre-titered reagent. In proprietary information provided to the Biomarker Review Committee, this antibody has been shown by ELISA to be specific for the phosphorylated peptide compared to the non-phosphorylated peptide. Moreover, additional experiments have demonstrated that the antibody reacts with cells expressing 4EBP1 but not with 4EBP1 knockout cells (personal communication from Kimberly McCabe, Cell Signaling Technology, 11/19/14), further establishing specificity of the antibody. The supplier's practice of titering each lot of antibody before release helps assure that Western blots performed with the same dilution will yield similar results with different lots of antibody whenever the assay is run.

1.52 Dynamic range of the assay: To assess the dynamic range of the proposed assay, serial dilutions of the Nalm6 ALL cell line were subjected to immunoblotting. As indicated in Fig. 1, signal was detectable in undiluted sample and six 2-fold dilutions (64-fold range). Moreover, this range encompassed the baseline phosphorylation of 4EBP1 observed in four ALL samples harvested from newly diagnosed ALL patients prior to therapy and processed as described above.

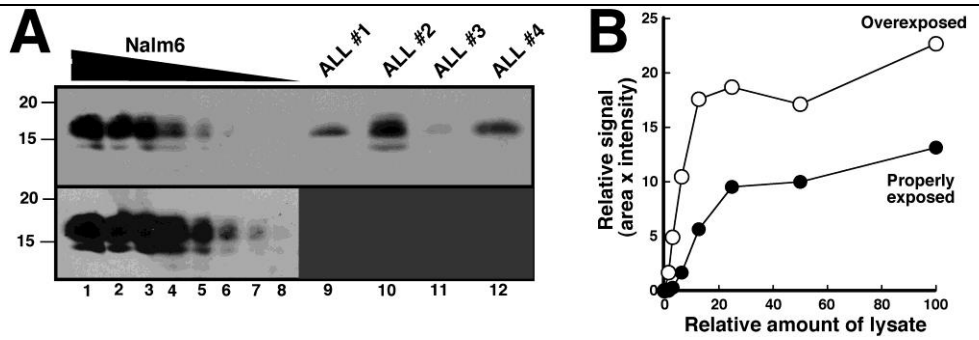


Figure 1. Assessment of dynamic and linear ranges of phospho-4EBP1 blotting. A, a sample containing 50 µg of total cellular protein from the human B-cell ALL Nalm6 line (lane 1) and serial 2-fold dilutions (lanes 2-8) were subjected to SDS-PAGE and probed with antibody that recognizes phospho-Thr^{37,46}-4EBP1. Aliquots containing 50 µg of total cellular protein from four pretreatment human ALL samples were also contained on the blot (lanes 9-12). Two different exposures of the same blot are shown. **B,** quantitation of integrated signals [area x (intensity – background)] of blots shown in panel A using NIH Image v1.63. Note that signals can be detected in a lysate over 64-fold range (lower panel of A, lanes 1-7), although the linear range is more limited (panel B).

1.53 Reproducibility of the blotting for phospho-4EBP1 and quantification: Lysates prepared from 15 different malignant human lymphoid cell lines were subjected to SDS-PAGE, transferred to nitrocellulose on three separate days, and probed with replicate aliquots of anti-phospho-4EBP1 antibody as described above. As indicated in Fig. 2, the blots were qualitatively similar. Quantification using NIH

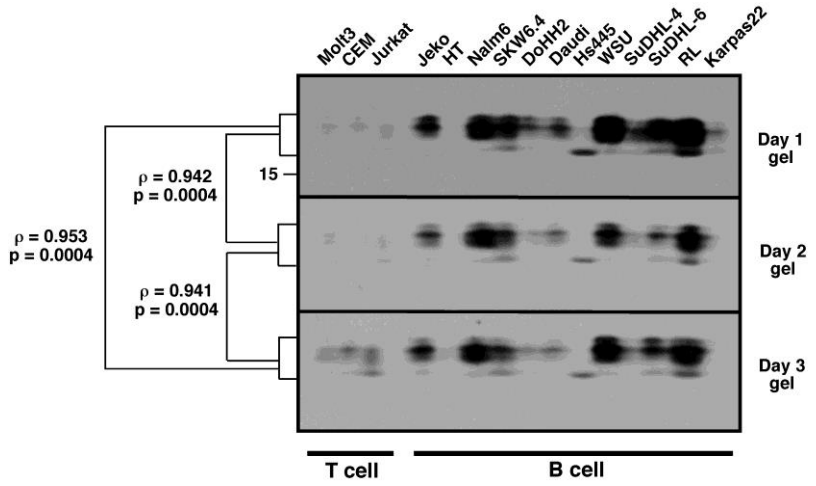


Figure 2. Assessment of reproducibility of blotting for phospho-Thr^{37,46}-4EBP1. Aliquots containing 50 µg of total cellular protein from the indicated human neoplastic lymphoid cell lines were subjected to SDS-PAGE followed by transfer to nitrocellulose on three consecutive days. Blots were probed with replicate aliquots of the same anti-phospho-4EBP1 antibody, visualized by enhanced chemiluminescence reagents, and exposed to x-ray film exactly as proposed for the clinical samples. After blots were scanned, the integrated signal in individual lanes [area x (intensity – background)] was quantitated using NIH Image. Numbers at left indicate Spearman

correlation coefficients for signals in corresponding lanes of the indicated blots.

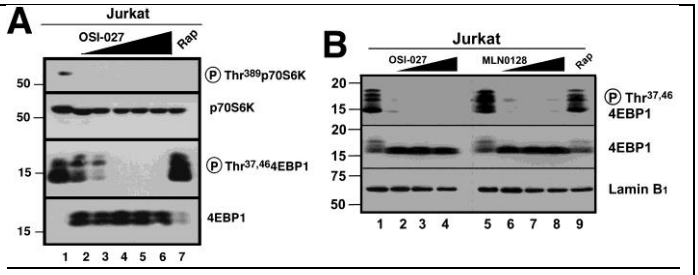


Figure 3. Inhibition of 4EBP1 Thr^{37,46} phosphorylation in Jurkat cells. A, Jurkat cells were treated for 8 h with diluent or 1.25, 2.5, 5, 10 or 20 μM OSI-027 or 10 nM rapamycin, then harvested for blotting. From ref. (1). **B,** after Jurkat cells were treated with diluent (lanes 1 and 5); OSI-027 at 5, 10 or 20 μM (lanes 2-4); MLN0128 (TAK-228) at 0.25, 0.5 or 1 μM (lanes 6-8); or 10 nM rapamycin (lane 9), whole cell lysates were subjected immunoblotting.

Image revealed a correlation between integrated signals for each cell line on pairs of blots as indicated on the figure.

1.54 Assessment of the reproducibility of 4EBP1 dephosphorylation: To assess whether 4EBP1 phosphorylation is reproducibly inhibited upon exposure to dual mTORC1/mTORC2 inhibitors, multiple investigators in the Kaufmann laboratory assessed the impact of the dual mTORC1/mTORC2 inhibitor OSI-027 on 4EBP1 phosphorylation in the ALL cell line Jurkat independently over a 5-year period using different batches of phospho-Thr^{37,46}-4EBP1 antibody from the indicated supplier. Two of these assays are shown in Figs. 3A (lanes 2-6) and 3B (lanes 2-4) below. These results show that 4EBP1 dephosphorylation is detectable by different lab personnel using different lots of antibody, nitrocellulose, x-ray film, detection reagents and buffers as long as the Standard Operating Procedure is followed.

1.55 Occurrence of the proposed 4EBP1 dephosphorylation in human neoplastic lymphoid cells after dual mTORC1/mTORC2 inhibitor treatment of xenografts *in vivo*: To assess whether 4EBP1 phosphorylation would be inhibited by therapeutically achievable concentrations *in vivo*, mice bearing xenografts of the human mantle cell lymphoma cell line Jeko were treated with the maximum tolerated dose of OSI-027 for 3, 5 or 6 days. Xenografts were snap frozen, then subjected to sample preparation and immunoblotting as described in Section 1.3 of this Appendix. Results of this analysis demonstrated inhibition of 4EBP1 Thr^{37,46} phosphorylation *in vivo* in all samples from treated mice compared to pretreatment samples (Fig. 4).

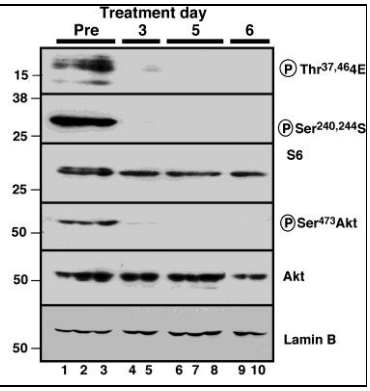


Figure 4. Dephosphorylation of 4EBP1 Thr^{37,46} and AKT Ser^{473} in Jeko xenografts after OSI-027 treatment *in vivo*. Whole cell lysates prepared (8) from separate xenografts of untreated mice (lanes 1-3) or xenografts harvested 4 h after treatment with OSI-027 on day 3 (lanes 4, 5), day 5 (lanes 6-8) or day 6 (lanes 9, 10) were subjected to SDS-PAGE and probed with antibodies to the indicated antigen.

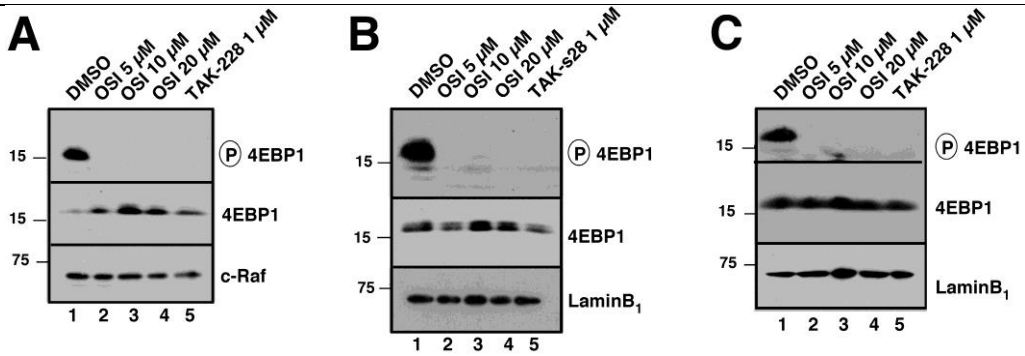


Figure 5. Effect of dual mTOR inhibitors OSI-027 (OSI) and MLN0128 (TAK-228) on 4EBP1 phosphorylation in clinical ALL samples exposed *ex vivo*. Clinical ALL specimens were subjected to Ficoll-Hypaque gradient centrifugation as described above. Aliquots containing 5 x 10^6 cells were incubated for 48 h with 0.1% DMSO (lane 1) or dual mTORC1/mTORC2 inhibitor at the indicated final concentration in the presence of the broad-spectrum caspase inhibitor Q-VD-OPh to prevent caspase-medicated changes in protein expression. At the end of the incubation, cells were sedimented, washed in serum-free medium, lysed, and subjected to immunoblotting for phospho-Thr^{37,46}-4EBP1 or total 4EBP1 as described above. Because degradation of 4EBP1 is phosphorylation dependent (explaining the increase in drug-treated cells), c-Raf and lamin B_1 were included as loading controls.

1.56 Occurrence of 4EBP1 dephosphorylation in clinical ALL samples upon treatment with dual mTORC1/mTORC2 inhibitors *ex vivo*: To assess whether the biochemical change previously shown to be critical for killing of ALL cells (2) could occur in clinical ALL, ALL specimens harvested from three newly diagnosed ALL patients were exposed to the dual mTORC1/mTORC2 inhibitors OSI-027 and MLN0128 (TAK-228) *ex vivo*. Results of this analysis indicated that 4EBP1 phosphorylation was reduced to undetectable levels in all samples upon drug exposure *ex vivo* (Fig. 5A-C; see also Fig. 6B).

1.57 Occurrence of 4EBP1 dephosphorylation in the range of MLN0128 (TAK-228) concentrations that are anticipated to be achievable *in vivo*: To assess the ability of MLN0128 (TAK-228) to inhibit 4EBP1 phosphorylation at submicromolar concentrations, Jurkat T cell ALL cells were treated for 24 h with various concentrations of MLN0128 (TAK-228) and blotted for 4EBP1. As indicated in Fig. 6A, 4EBP1 dephosphorylation was readily detectable at MLN0128 (TAK-228) concentrations of 100 nM and above. To assure that 4EBP1 dephosphorylation would occur over the same MLN0128 (TAK-228) concentration range in clinical ALL specimens, a clinical specimen from a patient with newly diagnosed ALL (distinct from those in Fig. 5) was treated with varying concentrations of MLN0128 (TAK-228) for 48 h, harvested, and subjected to immunoblotting for 4EBP1. As indicated in Fig. 6B, diminished 4EBP1 phosphorylation was detectable at the lowest concentration tested, 62.5 nM. These results compare favorably with the

peak MLN0128 (TAK-228) serum concentration of ~130 nM achieved in phase I solid tumor trials at the 6 mg dose level (9).

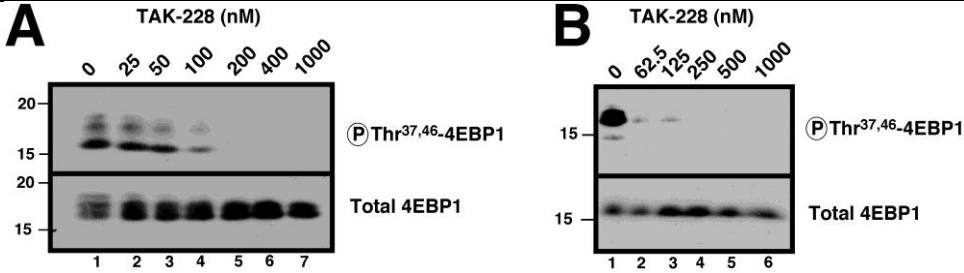


Figure 6. Effect of various MLN0128 (TAK-228) concentrations on 4EBP1 phosphorylation. A, Log phase Jurkat cells were treated for 24 h with diluent (0.1% DMSO, lane 1) or MLN0128 (TAK-228) at the indicated concentration, lysed, and subjected to immunoblotting for phospho-Thr^{37,46}-4EBP1 or total 4EBP1 as described above. **B,** replicate aliquots isolated from a pretreatment ALL specimen were treated for 48 h with 0.1% DMSO or the indicated concentration of MLN0128 (TAK-228) in the presence of 5 μM Q-VD-OPh, washed, and subjected to SDS-PAGE followed by blotting for the indicated antigen.

2. Phospho-Ser473 analysis of pretreatment and day 8 bone marrow samples by immunoblotting

2.1 Note on probing blots for multiple antigens

Techniques previously reported from the Kaufmann lab (4, 10, 11) allow the sequential detection of multiple antigens on the same filter without interference. Once phospho-4EBP1 has been successfully detected, we propose to use the same filters for detection of additional antigens, which are presented below in the order in which they will be examined. Because we routinely detect up to 14 antigens on a duplicate pair of filters from cell lines (including eight BCL2 family members, three phospho-proteins and the three corresponding total protein controls), we do not anticipate difficulty in completing the proposed immunoblotting with the available samples.

2.2 Role in trial: Exploratory biomarker

2.3 Hypothesis and rationale:

Hypothesis: AKT Ser^{473} phosphorylation will be inhibited by MLN0128 (TAK-228) *in situ*.

Rationale: Previous studies have shown that mTOR dual inhibitors such as MLN0128 (TAK-228) and OSI-027 diminish the mTORC2-mediated phosphorylation of the pro-survival kinase AKT on Ser^{473} (e.g., Fig. 10 below), leading to upregulation of the pro-apoptotic protein BCL2L11 (Bim) and induction of apoptosis (1, 2). In ALL cell lines the downregulation of the mTORC2 component Rictor reproduces these effects and downregulation of BCL2L11 (Bim) produces resistance to mTOR dual inhibitors

(2), demonstrating the critical role of signaling cascade in killing by mTOR dual inhibitors. We now propose to assess whether AKT Ser^{473} has been dephosphorylated in leukemic blasts by therapeutically achievable MLN0128 (TAK-228) concentration *in situ* as a proof of mechanism for this class of agent.

2.4 Assay procedure:

The assay procedure will be performed as described in Section 1.3 and the Standard Operating Procedure (pp. 16-26) except that immunoblotting will use Cell Signaling Technology rabbit monoclonal antibody D9E, which recognizes AKT when phosphorylated in Ser^{473} but not when unphosphorylated at this site (See p. 27 of this appendix).

2.41 Characterization of antibody used in these studies: A critical antibody for this procedure, a rabbit monoclonal from Cell Signaling Technology, Inc., recognizing phospho-Ser^{473}-AKT, is supplied as a pre-titered reagent. The supplier's practice of titering each lot of antibody before release helps assure that Western blots performed with the same dilution will yield similar results with different lots of antibody whenever the assay is run. The inability of the antibody to recognize unphosphorylated AKT is documented by the supplier in their datasheet appended to the end of this Appendix.

2.42 Reproducibility of the blotting for AKT Ser^{473} and quantification: Lysates prepared from 15 different malignant human lymphoid cell lines were subjected to SDS-PAGE, transferred to nitrocellulose on two separate days, and probed with replicate aliquots of anti-phospho-Ser^{473} antibody as described above. As indicated in Fig. 7, the blots were qualitatively quite similar. Quantification of integrated signal density [area x (intensity – background)] using NIH Image J revealed a correlation between integrated signal on blot 1 and blot 2 for the various cell lines on the two blots, with $\rho = 0.95$ ($p = 0.0004$).

2.43 Occurrence of the proposed AKT Ser^{473} dephosphorylation in human neoplastic

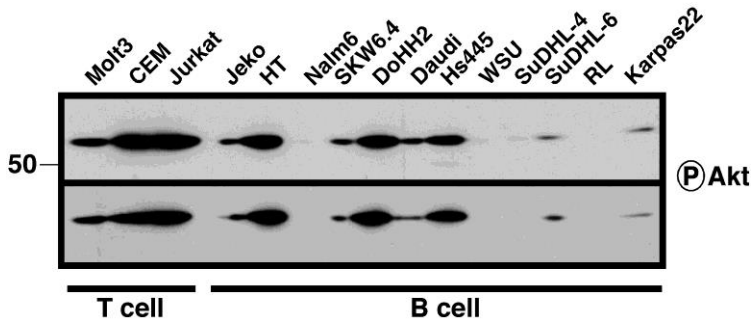


Figure 7. Reproducibility of blotting for phospho-Ser^{473}-AKT. Aliquots containing 50 µg of total cellular protein from the indicated human neoplastic lymphoid cell line were subjected to

SDS-PAGE followed by transfer to nitrocellulose on two consecutive days. Blots were probed with replicate aliquots of the same anti-phospho-Ser^{473}-AKT antibody, visualized by enhanced chemiluminescence reagents, and exposed to x-ray film exactly as proposed for the clinical samples.

lymphoid cells after dual mTORC1/mTORC2 inhibitor treatment in xenografts *in vivo*: To assess whether 4EBP1 phosphorylation would be inhibited by therapeutically achievable concentrations *in vivo*, mice bearing xenografts of the human mantle cell lymphoma cell line Jeko were treated with the maximum tolerated dose of OSI-027 for 3, 5 or 6 days. Xenografts were snap frozen, then subjected to sample preparation and immunoblotting as described in Section 1.3 of this Appendix. Results of this analysis demonstrated inhibition of AKT Ser^{473} phosphorylation *in vivo* in all drug-exposed xenografts compared to pretreatment samples (Fig. 4).

3. Expression of BCL2L11 (Bim), BBC3 (Puma), MCL1 and BCL2 proteins in pretreatment and day 8 bone marrow samples by immunoblotting

3.1 Roles in study: exploratory biomarkers

3.2 Hypothesis and rationale:

Hypothesis: Levels of BCL2L11 (Bim) and BBC3 (Puma) in ALL will increase during MLN0128 (TAK-228) treatment, whereas levels of MCL1 and possibly BCL2 will decrease.

Rationale: Previous studies have shown that mTOR dual inhibitors such as MLN0128 (TAK-228) and OSI-027 cause upregulation of the pro-apoptotic BCL2 family members BBC3 (Puma) and BCL2L11 (Bim) in malignant lymphoid cells (1, 2). In ALL cell lines, down regulation of BBC3 (Puma) or BCL2L11 (Bim) produces resistance to mTOR dual inhibitors (1, 2), demonstrating the critical role of these proteins in killing by mTOR dual inhibitors. Conversely, the anti-apoptotic BCL2 family member MCL1 has been shown to decrease after treatment with mTOR dual inhibitors in certain malignant human lymphoid cell lines (1). Overexpression of eponymous BCL2 has also been shown to convey resistance to mTOR dual inhibitors (1). Accordingly, we propose to assess whether BCL2L11 (Bim) and BBC3 (Puma) have been upregulated or MCL1 down regulated in leukemic blasts by therapeutically achievable MLN0128 (TAK-228) concentration *in situ* as a further proof of mechanism for this class of agent.

3.3 Assay procedure and sources of critical reagents:

The assay procedure will be performed as described in Section 1.3 and the Standard Operating Procedure included in this Appendix with three exceptions. First, the following antibodies will be used (data sheets included at the end of this Appendix):

BCL2L11 (Bim): C34C5 rabbit monoclonal antibody, Cell Signaling Technology

BBC3 (Puma): G-3 murine monoclonal antibody (sc-374223), Santa Cruz Biotechnology
BCL2: Clone 124 mouse anti-human BCL2 antibody (Dako)
MCL1: D35A5 rabbit monoclonal antibody, Cell Signaling Technology

Second, appropriate secondary antibody will be utilized. Third, as standards for these studies, we currently plan to purify recombinant human BCL2 (12-14), MCL1 (12, 13), BCL2L11 (Bim) (15) and BBC3 (Puma) (16) and apply known amounts in a serial dilution to serve as a standard curve.

3.4 Assay performance characteristics

The following performance characteristics of the assay have been evaluated:

3.41 Characterization of antibodies used in these studies: Critical antibodies for these procedures are the antibodies listed above. For the antibodies from Cell Signaling Technology, Inc., the supplier's practice of titering each lot of antibody before release helps assure that Western blots performed with the same dilution will yield similar results with different lots of antibody whenever the assay is run. The absence of signal in lysates from gene-deleted cells (BBC3) or diminished signal in cells with siRNA/shRNA-mediated knockdown (BCL2L11, BCL2 and MCL1) has been documented in the Kaufmann laboratory for each of these antibodies (data not shown).

3.42 Dynamic range of the assay: To assess the dynamic range of the proposed assay, serial dilutions of the Nalm6 ALL cell line were subjected to immunoblotting with antibodies to BCL2 and BCL2L11 (Bim). As indicated in Fig. 8, signals were detectable in undiluted sample and six 2-fold dilutions 64-fold range) for BCL2 and in the undiluted sample and four 2-fold dilutions (16-fold range) for BCL2L11 (Bim). MCL1 likewise had a 16- to 32-fold dynamic range. BBC3 (Puma) could not be effectively assayed in these cells prior to treatment because its baseline levels are too low. Note, however, that we plan to utilize

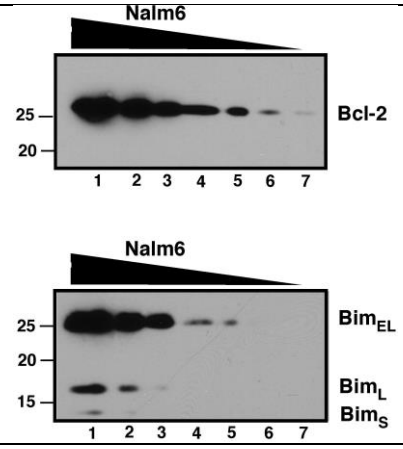


Figure 8. Assessment of dynamic ranges of BCL2 and BCL2L11 (Bim) blotting. A sample containing 50 µg of total cellular protein from the human B-cell ALL Nalm6 line (lane 1) and serial 2-fold dilutions (lanes 2-7) were subjected to SDS-PAGE and probed with antibodies that recognize BCL2 (top) or Bim (bottom). The locations of the extra-long, long and short splice variants of Bim (17) are indicated.

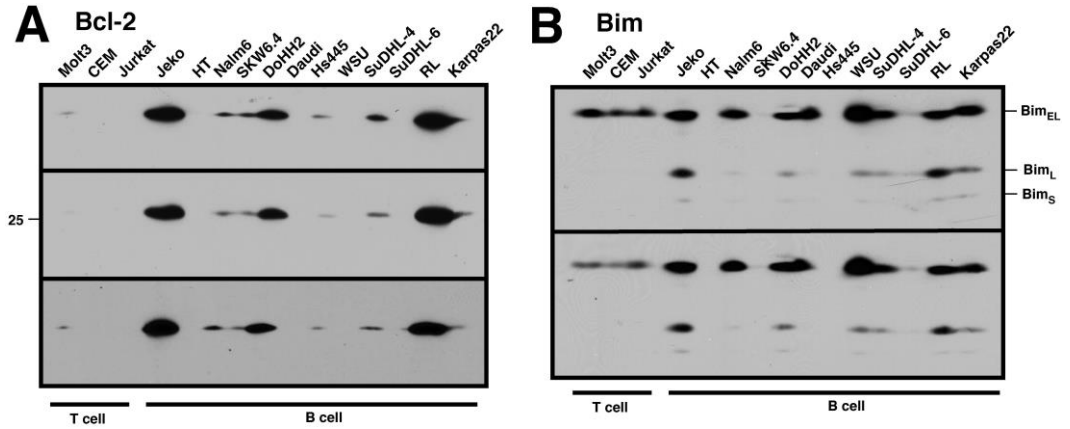


Figure 9. Assessment of reproducibility of blotting for BCL2 and BCL2L11 (Bim). Aliquots containing 50 µg of total cellular protein from the indicated human neoplastic lymphoid cell line were subjected to SDS-PAGE followed by transfer to nitrocellulose on three (A) or two (B) consecutive days. Blots were probed with replicate aliquots of the same antibodies, visualized by enhanced chemiluminescence reagents, and exposed to x-ray film exactly as proposed for the clinical samples.

serial dilutions of purified BBC3 (Puma) protein rather than a healthy cell line as a loading control for this assay (see Section 3.3 above).

3.43 Reproducibility of the blotting for BCL2 and BCL2L11 (Bim): Lysates from 15 malignant human lymphoid cell lines were subjected to SDS-PAGE, transferred to nitrocellulose on multiple separate days, and probed with replicate aliquots of anti-BCL2 and anti-BCL2L11 (Bim) antibody as described above. As indicated in Fig. 9, the blots were qualitatively similar. Quantification of integrated signals [area x (intensity – background)] using NIH Image revealed a strong correlation between the BCL2 signals obtained for each cell line on blot 1 vs. blot 2, blot 2 vs. blot 3 and blot 1 vs. blot 3 in A [ρ = 0.90 - 0.95 ($p = 0.0004 – 0.008$)] or $BCL2L11_{EL}$ (Bim_{EL}) on blot 1 vs. blot 2 in B [$\rho = 0.95$ ($p = 0.0004$) in B].

3.44 Assessment of BCL2L11 (Bim) and BBC3 (Puma) induction: To assess whether BCL2L11 (Bim) and BBC3 (Puma) are reproducibly upregulated upon exposure to dual mTORC1/mTORC2 inhibitors, multiple investigators in the Kaufmann laboratory assessed the impact of the dual mTORC1/mTORC2 inhibitor OSI-027 on levels of these proteins in the ALL cell line Jurkat independently over a 5-year period using different batches of BCL2L11 (Bim) and BBC3 (Puma) antibodies from the indicated supplier. Two of these assays are shown in Figs. 10A (lanes 2-6) and 10B (lanes 2-4) on the next page. These results show that upregulation of BCL2L11 (Bim) and BBC3 (Puma) is detectable by different lab personnel using different lots of antibody, nitrocellulose, x-ray film, detection reagents and buffers as long as the procedure described in Section 1.3 of this Appendix is followed.

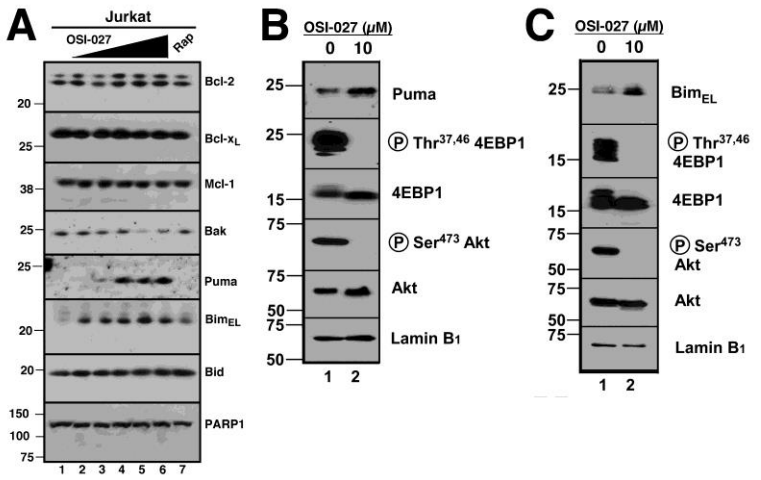


Figure 10. Induction of BCL2L11 (Bim) and BBC3 (Puma) by OSI-027. A, after Jurkat cells were treated for 48 h with diluent (lanes 1); OSI-127 at 1.25, 2.5, 5, 10 or 20 μM (lanes 2-6); or 10 nM rapamycin (lane 7) in the presence of the caspase inhibitor Q-VD-OPh (5 μM—ref. 18) to inhibit apoptosis, whole cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies that recognize the indicated antigen. PARP1 served as a loading control. **B, C,** in two separate experiments designed to assess various aspects of OSI-027-induced signaling (2), control Jurkat cells were treated with diluent or 10 μM OSI-027 for 48 h and harvested for immunoblotting for the indicated antigens.

4. Reverse phase protein array for protein phosphorylations and expression (exploratory biomarkers)

4.1 Hypothesis and rationale:

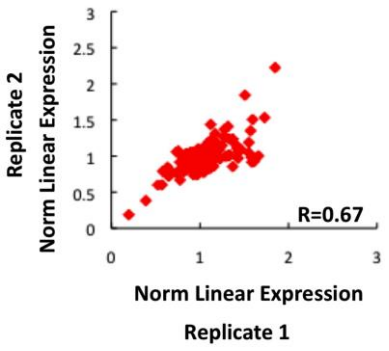
Hypothesis: Phosphorylation of mTOR substrates and complementary pathway components will change during the course of mTOR dual inhibitor treatment.

Rationale: Prior publications have identified differences in the pattern of protein phosphorylation between cell lines that are sensitive to kinase inhibitors and similar cell lines that are resistant (e.g., 20, 21). Preliminary studies in the Kaufmann laboratory have likewise demonstrated differences in protein phosphorylation at key nodes between parental Nalm6 ALL cells and Nalm6 cells selected for the ability to grow continuously in mTOR dual inhibitor (C. Correia, K. L. B. Knorr, S. Yun, A. E. Wahner Hendrickson, B. Madden, D. McCormick, S. Dasari, and S. H. Kaufmann, unpublished observations). Thus, we hypothesize that pretreatment patterns of phosphorylation might differ between ALLs that respond to MLN0128 (TAK-228) and those that do not.

4.2 Assay validity and appropriateness for study:

Reverse phase protein arrays (RPPAs) will be utilized to assess levels of phosphorylation of 60 key proteins, including 4EBP1, ribosomal protein S6, and AKT, as well as expression levels of roughly 200 proteins, including BCL2, BCL2L1 (Bcl-x_L),

Figure 11. Biological replicates in RPPA samples run at M. D. Anderson Cancer Center Reverse Phase Protein Array facility. Samples of control Jurkat cells harvested on separate days were prepared for reverse phase protein arrays. Shown are normalized linear expression values across 260 antigens. The QC score for each antibody was >0.8. Between the two samples, variation averaged 10%.



MCL1, and BCL2L11 (Bim), using lysates from pretreatment and day 8 bone marrow blasts. This analysis of key signaling pathways will be utilized to supplement the results of the more targeted immunoblotting assays described above.

4.3 Performance site:

Whole cell lysates will be prepared in the Kaufmann laboratory of the Mayo Clinic Cancer Center. Based on preliminary results, the same lysates prepared for immunoblotting are suitable for RPPAs. The RPPAs will be probed and initial bioinformatic analysis performed in the RPPA Core Facility of the M.D. Anderson Cancer Center, which is a fee-for-service facility. Secondary bioinformatic analysis will be performed at Mayo.

4.4 Performance characteristics of the assay:

The reproducibility and reliability of this method, as well as comparison to the gold standard of Western blotting, has been published by the founders of the RPPA Core Facility at M.D. Anderson Cancer Center (22).

To further assess the reproducibility of the assay in our hands, samples of control Jurkat cells harvested on sequential days were submitted to the M.D. Anderson Cancer Center RPPA Core Facility. Quantitative results across 260 antigens were scored in each sample. Information returned from the Core Facility (Fig. 11) indicated a quality score of >0.8 for each antibody. Variation between the replicates averaged 9.9%; and a correlation coefficient of 0.67 was observed between samples on day 1 and day 2.

5. Transcriptional profiling (exploratory biomarker)

5.1 Role in trial: Exploratory biomarker

5.2 Hypothesis and rationale:

Hypothesis: Treatment with MLN0128 (TAK-228) will activate two transcriptional

pathways related to apoptosis.

Rationale: Previous studies have shown that the mTOR dual inhibitor OSI-027 activates two distinct transcriptional pathways in ALL cell lines during the course of upregulating BCL2L11 (Bim) and BBC3 (Puma) to induce apoptosis (2). If sufficient material is available to allow isolation of RNA (after samples for immunoblotting and RPPAs have been harvested), samples will be subjected to RNA sequencing (RNAseq) to determine whether MLN0128 (TAK-228) concentrations achievable *in vivo* activate these same transcriptional pathways as a proof of mechanism study.

5.3 Assay methodology:

RNAseq will be performed on pretreatment and day 8 bone marrow aspirates to assess whether transcriptional changes observed in clinical ALL leading to BCL2L11 (Bim) and BBC3 (Puma) upregulation are also observed in clinical ALL treated *in vivo*. In brief, after Ficoll purification, total RNA will be extracted from marrow mononuclear cells using Qiagen RNA extraction kit. After RNA sample quality is assessed in the Gene Expression Core of the Mayo Clinic Cancer Center by measuring RNA Integration Number (RIN), an Illumina TruSeq mRNA kit will be used to generate cDNA for next generation sequencing. RNAs will be poly-A selected and fragmented, then subjected to reverse transcription with random primers and second-strand synthesis to generate double-stranded cDNA. Ends will be repaired and poly(adenyl)ated, followed by adaptor and index ligation. The cDNAs will then denatured and PCR-enriched to generate the final genomic library, which will be analyzed on an Illumina HiSeq 2000. Data will analyzed according to a Mayo Clinic developed protocol for analyzing RNA-Sequencing data, (http://www.biomedcentral.com/content/pdf/1471-2105-15-224.pdf). Each mRNA count number will be normalized to counts per million.

5.4 Assessment of assay performance:

To assess assay performance, on two successive days Jurkat T cell ALL cells were treated with diluent vs. 10 μM OSI-027 for 48 h in the presence of the broad spectrum caspase inhibitor Q-VD-OPh (to prevent apoptosis-associated RNA degradation), RNA was extracted and subjected to the RNA sequencing procedure described in the preceding paragraph. As indicated in Fig. 12, results obtained with the duplicate samples under each condition were highly reproducible, with R values of 0.99 in diluent-treated cells and 0.97 in drug-treated cells, respectively.

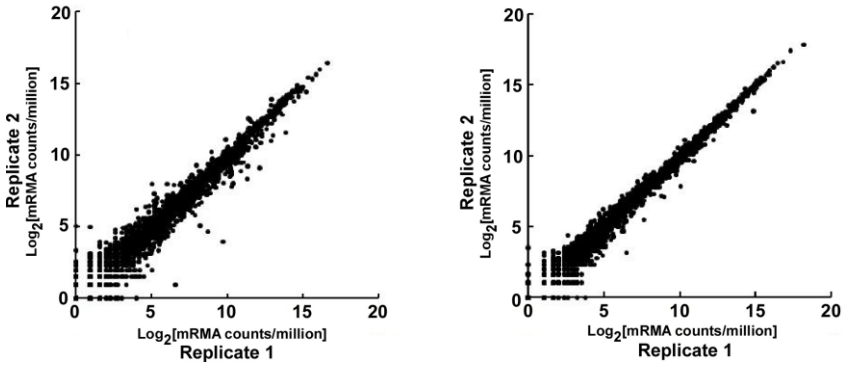


Figure 12. Reproducibility of RNAseq data. Jurkat cells treated with diluent + 5 µM Q-VD-OPh (left) or 10 µM OSI-027 + 5 µM Q-VD-OPh for 48 h (right) were harvested on two successive days and subjected to RNAseq analysis as described in Section 5.3. Plotted are relative reads for each mRNA in the first vs. second sample.

REFERENCES

1. Gupta M, Wahner Hendrickson A, Seongseouk Y, Han JJ, Schneider PA, Stenson M, et al. Dual TORC1/TORC2 Inhibition Diminishes Akt Activation and Induces Puma-Dependent Apoptosis in Lymphoid Malignancies. Blood. 2012;119:476-87.
2. Yun S, Vincelette ND, Knorr KLB, Schneider PA, Peterson KL, Flatten KS, et al. Dual mTOR Inhibitiors Induce Bim-Dependent Apoptosis in Acute Lymphoblastic Leukmeia via Inhibition of eIF4E/eIF4G and Activation of NF-kB/Egr-1. Under revision. 2015.
3. Kaufmann SH, Svingen PA. Immunoblot Analysis and Band Depletion Assays. In: Bjornsti M-A, Osheroff N, editors. Protocols for DNA Topoisomerases: Humana Press; 1998. p. 253-68.
4. Kaufmann SH. Reutilizaton of Western Blots After Chemiluminescent or Autoradiographic Detection. In: Walker JM, editor. Protein Protocols Handbook, 3rd edition. Totowa, N.J.: Humana Press; 2009. p. 785-801.
5. Kaufmann SH, Karp JE, Burke PJ, Gore SD. Addition of Etoposide to Initial Therapy of Adult ALL: A Combined Clinical and Laboratory Study. Leukemia and Lymphoma. 1996;23:71-83.
6. Gore SD, Rowinsky EK, Miller CB, Griffin C, Chen T-L, Borowitz M, et al. A Phase II "Window" Study of Topotecan in Untreated Patients with High Risk Adult Acute Lymphocytic Leukemia. Clinical Cancer Research. 1998;4:2677-89.
7. English D, Andersen BR. Single-Step Separation of Red Blood Cells. Granulocytes and Mononuclear Leukocytes on Discontinuous Density Gradients of Ficoll-Hypaque. Journal of Immunological Methods. 1974;5:249-52.
8. Friedman HS, Dolan ME, Kaufmann SH, Colvin OM, Griffith OW, Moschel RC, et al. Elevated DNA Polymerasea, DNA Polymeraseb, and DNA Topoisomerase II in a Melphalan-Resistant Rhabdomyosarcoma Xenograft that is Cross-Resistant to Nitrosoureas and Topotecan. Cancer Research. 1994;54:3487-93.
9. Investigator's Brochure MLN0128. 7th edition ed2014.
10. Kaufmann SH, Ewing CM, Shaper JH. The Erasable Western Blot. Analytical

Biochemistry. 1987;161:89-95.
11. Kaufmann SH. Reutilization of Immunoblots After Chemiluminescent Detection. Analytical Biochemistry. 2001;296:283-6.
12. Dai H, Meng XW, Lee S-H, Schneider PA, Kaufmann SH. Context-dependent Bcl-2/Bak Interactions Regulate Lymphoid Cell Apoptosis. Journal of Biological Chemistry. 2009;284:18311-22.
13. Smith AJ, Dai H, Correia C, Lee S-H, Takahashi R, Kaufmann SH. Noxa/Bcl-2 Interactions Contribute to Bortezomib Resistance in Human Lymphoid Cells. Journal of Biological Chemistry. 2011;286:17682-92.
14. Dai H, Ding H, Meng XW, Lee S-H, Kaufmann SH. Contribution of Bcl-2 Phosphorylation to Bak Binding and Drug Resistance. Cancer Research. 2013;73:6998-7008.
15. Dai H, Smith A, Meng XW, Schneider PA, Pang Y-P, Kaufmann SH. Transient Binding of an Activator BH3 Domain to the Bak BH3-Binding Groove Initiates Bak Oligomerization. Journal of Cell Biology. 2011;194:39-48.
16. Dai H, Pang Y-P, Kaufmann SH. Evaluation of the BH3-Only Protein Puma as a Direct Bak Activator. Journal of Biological Chemistry. 2014;289:89-99.
17. Puthalakath H, Huang DC, O'Reilly LA, King SM, Strasser A. The proapoptotic activity of the Bcl-2 family member Bim is regulated by interaction with the dynein motor complex. Mol Cell. 1999;3:287-96.
18. Caserta TM, Smith AN, Gultice AD, Reedy MA, Brown TL. Q-VD-OPh, a broad spectrum caspase inhibitor with potent antiapoptotic properties. Apoptosis. 2003;8:345-52.
19. Kaufmann SH, Svingen PA, Gore SD, Armstrong DK, Cheng Y-C, Rowinsky EK. Altered Formation of Topotecan-Stabilized Topoisomerase I-DNA Adducts in Human Leukemia Cells. Blood. 1997;89:2098-104.
20. Casado P, Alcolea MP, Iorio F, Rodriguez-Prados JC, Vanhaesebroeck B, Saez-Rodriguez J, et al. Phosphoproteomics data classify hematological cancer cell lines according to tumor type and sensitivity to kinase inhibitors. Genome Biol. 2013;14:R37.
21. Casado P, Rodriguez-Prados JC, Cosulich SC, Guichard S, Vanhaesebroeck B, Joel S, et al. Kinase-substrate enrichment analysis provides insights into the heterogeneity of signaling pathway activation in leukemia cells. Sci Signal. 2013;6:rs6.
22. Tibes R, Qiu Y, Lu Y, Hennessy B, Andreeff M, Mills GB, et al. Reverse phase protein array: validation of a novel proteomic technology and utility for analysis of primary leukemia specimens and hematopoietic stem cells. Mol Cancer Ther. 2006;5:2512-21.

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 1
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

Division of Oncology Research

Mayo Clinic – Rochester

Technical Reviewer: Scott H. Kaufmann Date: _____

Secondary Reviewer: Charles Erlichman Date: _____

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 2
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

TABLE OF CONTENTS

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 3
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

1. PURPOSE

To standardize methods of probing bone marrow mononuclear cells (BMMCs) from bone marrow samples with anti-phospho-Thr^{37,46}-4EBP1 antibody to determine whether anticancer agents are inhibiting phosphorylation of this protein.

2. SCOPE

This procedure is used by all lab personnel responsible for probing immunoblots for phospho-Thr^{37,46}-4EBP1 antibody to assess this phosphorylated protein in BMMC from patients participating in clinical trials of chemotherapeutic agents to ensure consistency in the handling of these samples.

3. ABBREVIATIONS

4-EBP1 – Eukaryotic initiation factor 4E binding protein 1
BMMC – Bone marrow mononuclear cells
ECL – Enhanced chemiluminescence
PBS – Dulbecco's calcium- and magnesium-free phosphate buffered saline
RT – Room temperature (20-22 °C)
SOP – Standard Operating Procedure
TS – Tris-Saline

4. INTRODUCTION

Consistent processing of immunoblots is very important for reliably assessing posttranslational modifications in clinical leukemia samples. This SOP describes in detail the procedures to probing immunoblots to assess the phosphorylation status of the translation inhibitor 4E-BP1.

5. ROLE AND RESPONSIBILITIES

Principal Investigator/Supervisor

The Principal Investigator/Supervisor oversees laboratory operations and supervises research personnel. S/he is responsible for probing of immunoblots. S/he is also responsible for ensuring proper training and certification of research personnel who probe blots according to this SOP.

Certified blot processor

A certified blot processor may be a research technologist, post-doctoral fellow,

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 4
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

graduate student or research scientist.

5.1 The Principal Investigator/Supervisor is responsible for ensuring proper training of certified blot processors in processing of immunoblots according to this SOP.

5.2 The certified blot processor is responsible for proper handling and documentation of blot processing according to this SOP.

6. MATERIALS AND EQUIPMENT REQUIRED

6.1 Pipettors (20 µl, 200 µl, 1000 µl) and tips

6.2 Orbital shakers (2, e.g., Lab-Line Model #3520)

6.3 5 ml (Falcon #357543) and 10 ml (Falcon #357551) sterile pipettes, individually wrapped.

6.4 50 ml (Falcon #352098) sterile polypropylene conical tubes

6.5 Microfuge tubes, 500 µl (e.g., Sarstedt #72.699)

6.6 Magnetic stir platform (e.g., Cole Parmer 9” x 9” stirrer)

6.7 Magnetic stir bars

6.8 Beakers (100 ml, 250 ml and 1000 ml, plastic or glass)

6.9 Graduated cylinders (25 ml, 100 ml and 250 ml, plastic or glass)

6.10 Automatic pipettor (e.g., Drummond Scientific 4-000-040)

6.11 Ziploc bags (quart size, Ziploc #868624)

6.12 10X PBS (Invitrogen 14200-075)

6.13 Deionized water (e.g., produced by properly maintained Barnsted Nanopure II)

6.14 Powdered nonfat dry milk (HyVee #14616)

6.15 Tween 20 (Sigma-Aldrich P1379-1L)

6.16 NaCl (ACS grade, Fisher 5271-3)

6.17 Sodium azide (Sigma-Aldrich S2002-100G)

6.18 100X Penicillin/streptomycin (American Type Culture Collection 30-2300)

6.19 Bovine serum albumin (Sigma-Aldrich A7906-100G)

6.20 Primary antibody: Rabbit anti-phosphospho-Thr^{37,46}-4EBP1 (monoclonal 236B4, Cell Signaling Technology product #2855S)

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 5
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

6.21 Horseradish peroxidase-coupled anti-rabbit IgG (KPL #074-1506, 1 mg)

Preparation of stock solution (per supplier instructions):

6.21.1 Add 0.5 ml sterile water to 1 mg powdered antibody
6.21.2 Gently invert vial until antibody is dissolved
6.21.3 Slowly add 0.5 ml glycerol, mixing antibody up and down in pipettor without introducing bubbles
6.21.4 Aliquot antibody into 25 µl aliquots in 500 µl microfuge tubes
6.21.5 Store tubes at -20 °C in freezer. (Do not use a frost-free freezer because the temperature is not stable.)
6.21.6 Transfer working tube to 4 °C refrigerator, where it is stable for 4 weeks.

6.22 1X PBS

Preparation: 100 ml 10X PBS + 900 ml deionized water
Storage: Stable for 30 days at room temp.

6.23 TS buffer

Composition: 150 mM NaCl, 10 mM Tris-HCl (pH 7.4)

Preparation: Weigh 8.72 gm NaCl into 1 L beaker
Add 800 ml RT deionized water
Add 1.21 gm Tris base
Adjust pH to 7.4 with concentrated HCl
Bring final volume to 1000 ml
Sterile filter into sterile bottle using bottle top filter.

Storage: 22 °C (stable for 1 year)

6.24 Blot wash buffer

Composition: PBS-0.05% Tween 20

Preparation: Weigh out 0.5 gm Tween 20 in 1 L beaker
Add 100 ml 10X PBS
Add 900 ml deionized water
Stir on magnetic stirrer until Tween 20 is dissolved

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 6
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

Storage: Stable for 7 days at RT.

6.25 Primary antibody dilution buffer

Composition: 5% bovine serum albumin in TS buffer containing 0.05% Tween 20, 100 U/ml penicillin G, 100 µg/ml streptomycin and 1 mM sodium azide.

Preparation:
Weigh out 0.10 gm Tween 20
Place in 250 ml beaker
Add 150 ml TS buffer
Stir until Tween 20 is dissolved
Weigh out 10 gm bovine serum albumin
Add to TS-Tween 20 and stir until dissolved
Add 2 ml Penicillin/streptomycin solution
Weigh out 13 mg of sodium azide
Add to TS-Tween 20-Penicillin/streptomycin solution
Stir until dissolved
Bring to 200 ml in a graduated

cylinder. Storage: Stable for 30 days at 4 °C.

6.26 Secondary antibody dilution buffer

Composition: 3% (w/v) milk in 1X PBS

Preparation: Weigh 0.75 gm powdered nonfat dry milk into 50 ml conical tube
Add 2.5 ml 10X PBS
Add 15 ml deionized water
Dissolve milk by inverting tube 6-8 times gently
Bring to final volume of 25 ml

Storage: Prepare fresh for each blot.

6.27 SuperSignal West Dura extended duration ECL substrate (Thermo #34075)

6.28 ECL working solution

Preparation: Combine 2 ml of solution 1 + 2 ml of solution 2 from West Dura

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 7
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

ECL kit for each blot

Storage: Use within 10 min of combining these solutions

6.29 X-ray film processor (e.g., Kodak X-omat 2000)

6.30 X-ray cassettes (e.g., Fisher Biotech FBXC 810)

6.31 X-ray film (e.g., Denville Scientific E3018 8 x 10 in HyBlot CL or Phenix Research Products #F-BX810)

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 8
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

7. OPERATING PROCEDURES

7.1 Record blot ID number and data on Blot Information Sheet (Appendix 1).

7.2 Each blot will be recorded separately.

7.21 This will ensure that deviations from procedure will be documented.

7.3 Blot rehydration

7.3.1 Blots are stored dry.

7.3.1 Place each blot in a separate 1 quart Ziploc bag.

7.3.2 Using a sterile 10 ml pipet, add 15 ml TS buffer to bag. Zip the top.

7.3.3 Make certain that the blot is completely submerged in the buffer.

7.3.4 Incubate 5 min at room temperature.

7.3.5 Pour TS buffer down sink.

7.3.6 Using a 10 ml pipet and an automatic pipetter, remove any remaining buffer from the bag.

7.4 Add primary antibody to blot

7.4.1 Prepare an aliquot of antibody by adding 10 µl of undiluted anti-phospho-Thr^{37,46}-4EBP1 antibody to 10 ml of primary antibody dilution solution in a 15 ml conical tube. Invert tube slowly 5 times to mix antibody.

7.4.2 Pour contents of antibody tube into bag containing blot.

7.4.3 Placing the bag with blot on a flat surface, gently work as much air as possible from the bag, then seal it.

7.4.4 Fold top of bag over and affix with tape to prevent leakage while rocking.

7.4.5 Incubate on orbital shaker at 200 rpm for 12-16 hours at 4 °C.

7.5 Washing after primary antibody

7.5.1 Remove antibody solution from bag with a 10 ml pipet.

7.5.2 Because of the presence of sodium azide, which can react with copper in pipes to cause explosions if present in concentrated solutions, the removed antibody solution is to be discarded by flushing down a sink containing non-copper pipe along with at least 25 volumes of cold water (250 ml per

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 9
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

10 ml aliquot of antibody solution) per Mayo Clinic Department of Laboratory Medicine Procedure [PROC 032915.013] dated 04/07/2014.

7.5.3 Add 100 ml of RT blot wash buffer to bag.

7.5.4 Incubate on orbital shaker at RT for 15 min.

7.5.5 Pour off blot wash buffer.

7.5.6 Repeat step 7.5.3-7.5.5 for a total of 3 washes

7.5.7 Add 100 ml 1X PBS.

7.5.8 Incubate on orbital shaker at RT for 5 min.

7.5.9 Pour off 1X PBS.

7.5.10 Repeat steps 7.5.7-7.5.9 for a total of two washes.

7.6 Preparation of secondary antibody solution (during last three washes in Section 7.5)

7.6.1 Prepare fresh aliquot of RT secondary antibody dilution buffer.

7.6.2 Transfer to 50 ml conical tube.

7.6.3 Add 3 µl of stock horseradish peroxidase-coupled anti-rabbit IgG to secondary blotting buffer.

7.6.4 Invert tube gently 8 times to mix solution

7.7 Incubation of blot with secondary antibody

7.7.1 Pour secondary antibody solution into Ziploc bag containing blot.

7.7.2 Incubate on orbital shaker for 60 min at RT.

7.8 Washing after secondary antibody

7.8.1 Remove antibody solution from bag with a 10 ml pipet.

7.8.2 Add 100 ml of RT blot wash buffer to bag.

7.8.3 Incubate on orbital shaker at RT for 5 min.

7.8.4 Pour off blot wash buffer.

7.8.5 Repeat step 7.8.2-7.8.4 for a total of 2 washes

7.8.6 Add 100 ml of RT blot wash buffer to bag.

7.8.7 Incubate on orbital shaker at RT for 15 min.

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 10
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

7.8.8 Pour off blot wash buffer.

7.8.9 Repeat step 7.8.6-7.8.8 for a total of 2 washes

7.8.10 Add 100 ml RT wash buffer.

7.8.11 Incubate on orbital shaker at RT for 5 min.

7.8.12 Pour off blot wash buffer.

7.8.13 Repeat steps 7.8.10-7.8.12 for a total of 2 washes.

7.9. Signal detection (per instructions that accompany the SuperSignal ECL kit)

7.91 Remove blot wash buffer from blot.

7.92 Add ECL working solution.

7.93 Incubate for 60 sec. at room temp. with manual gentle agitation.

7.94 Pour off solution. Do not remove the small amount of liquid adhering to the blot.

7.95 Seal bag, taking care to remove as much air as possible.

7.96 Immediately place in x-ray cassette and start timer counting for 3 minutes.

7.97 When timer gets to 3 minutes, remove film from x-ray cassette and insert into X-ray film
developer.

7.98 Examining the 3 minute exposure, determine whether increased or decreased exposure of x-ray film is required and insert a new piece of film in the cassette.

7.99 Reset timer for desired length of exposure.

7.910 Continue until you acquire a series of exposures, some of which show various gradations of signal in the first (most concentrated) few samples on the standard curve and some of which show gradations of signal in the last (least concentrated) samples on the standard curve.

Title:	Immunoblotting for phospho-Thr^{37,46}-4EBP1				Page 11
Doc. #	SOP00002	Revision	A	Effective Date:	01 Dec 2014

APPENDIX 1: BLOT INFORMATION SHEET **PAGE 1**

Each blot must be accompanied by a completed Blot Information Sheet with each page ***dated and initialed***. A **separate** Blot Information Sheet should be started for each blot.

NOTE: ***Record times using military time (24-h designation), for example specify 16:15 to indicate 4:15 PM.***

Lab Technician (Certification number): _____
Date: _____
Reviewer/Supervisor: _____
Date: _____
Clinical Protocol Number: _____

Blot ID (date and clinical samples contained): _____

Processing Records:

1. Consumables and Equipment

Primary antibody (Lot # and receipt date) _____

Secondary antibody (Lot # and preparation date) _____

Serial numbers:

P-20 Pipetman: _____ P-200: _____ P-1000: _____

°C shaker (Manufacturer and Serial #): _____

RT shaker (Manufacturer and Serial #): _____

2. Blot processing

Time primary antibody added to blot (date and time):

Time primary antibody removed from blot (date and time):

Time primary secondary antibody added to blot (date and time):

Time first x-ray film applied to blot (date and time):

BLOT INFORMATION SHEET: INITIALS _____
DATE _____

#4060 Store at –20°C

Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb

☐ Small 100 µl (20 western blots)
☐ Large 300 µl (60 western blots)

rev. 06/15/11

Cell Signaling TECHNOLOGY®

Orders ■ 877-616-CELL (2355) orders@cellsignal.com
Support ■ 877-678-TECH (8324) info@cellsignal.com
Web ■ www.cellsignal.com

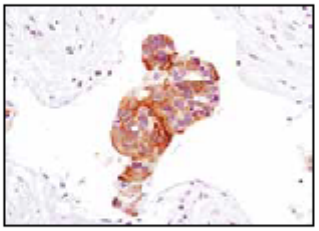
Entrez-Gene ID #207
Swiss-Prot Acc. #P31749

Applications	Species Cross-Reactivity*	Molecular Wt.	Isotype
W, IP, IHC-P, IHC-F, IF-IC, F Endogenous	H, M, R, Mk, Hm, B, Dm, Z, (Pg, C, X, Dg)	60 kDa	Rabbit IgG**

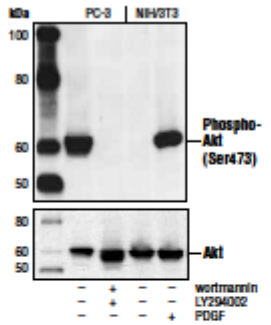
Background: Akt, also referred to as PKB or Rac, plays a critical role in controlling survival and apoptosis (1-3). This protein kinase is activated by insulin and various growth and survival factors to function in a wortmannin-sensitive pathway involving PI3 kinase (2,3). Akt is activated by phospholipid binding and activation loop phosphorylation at Thr308 by PDK1 (4) and by phosphorylation within the carboxy terminus at Ser473. The previously elusive PDK2 responsible for phosphorylation of Akt at Ser473 has been identified as mammalian target of rapamycin (mTor) in a rapamycin-insensitive complex with rictor and Sin1 (5,6). Akt promotes cell survival by inhibiting apoptosis by phosphorylating and inactivating several targets, including Bad (7), forkhead transcription factors (8), c-Raf (9) and caspase-9. PTEN phosphatase is a major negative regulator of the PI3 kinase/Akt signaling pathway (10). LY294002 is a specific PI3 kinase inhibitor (11).

Another essential Akt function is the regulation of glycogen synthesis through phosphorylation and inactivation of GSK-3α and β (12,13). Akt may also play a role in insulin stimulation of glucose transport (12).

In addition to its role in survival and glycogen synthesis, Akt is involved in cell cycle regulation by preventing GSK-3β mediated phosphorylation and degradation of cyclin D1 (14) and by negatively regulating the cyclin dependent kinase inhibitors p27 Kip (15) and p21 Waf1/CIP1 (16). Akt also plays a critical role in cell growth by directly phosphorylating mTOR in a rapamycin-sensitive complex containing raptor (17). More importantly, Akt phosphorylates and inactivates tuberin (TSC2), an inhibitor of mTOR within the mTOR-raptor complex (18). Inhibition of mTOR stops the protein synthesis machinery by inactivating p70 S6 kinase and activating the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), an inhibitor of translation (18,19).



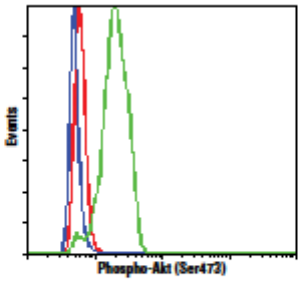
Immunohistochemical analysis of paraffin-embedded human lung carcinoma using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb.



Western blot analysis of extracts from PC-3 cells, untreated or LY294002/wortmannin-treated, and NIH/3T3 cells, serum-starved or PDGF-treated, using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (upper) or Akt (pan) (C67E7) Rabbit mAb #4691 (lower).

Specificity/Sensitivity: Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb detects endogenous levels of Akt only when phosphorylated at Ser473.

Source/Purification: Monoclonal antibody is produced by immunizing animals with a synthetic phosphopeptide corresponding to residues around Ser473 of human Akt.



Flow cytometric analysis of Jurkat cells, untreated (green) or treated with LY294002, wortmannin and U0126 (blue), using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb compared to a nonspecific negative control antibody (red).

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

***Species cross-reactivity is determined by western blot.**

****Anti-rabbit secondary antibodies must be used to detect this antibody.**

Recommended Antibody Dilutions:

Western blotting	1:2000
Immunoprecipitation	1:50
Immunohistochemistry (Paraffin)	1:50†
Unmasking buffer:	Citrate
Antibody diluent:	SignalStain® Antibody Diluent #8112
Detection reagent:	SignalStain® Boost (HRP, Rabbit) #8114
†Optimal IHC dilutions determined using SignalStain® Boost IHC Detection Reagent.	
Immunohistochemistry (Frozen)	1:50†
Fixative:	3% Formaldehyde/MeOH
Detection reagent:	SignalStain® Boost (HRP, Rabbit) #8114
†Optimal IHC dilutions determined using SignalStain® Boost IHC Detection Reagent.	
Immunofluorescence (IF-IC)	1:200
Flow Cytometry	1:100

For application specific protocols please see the web page for this product at www.cellsignal.com.

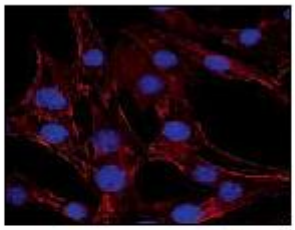
Please visit www.cellsignal.com for a complete listing of recommended companion products.

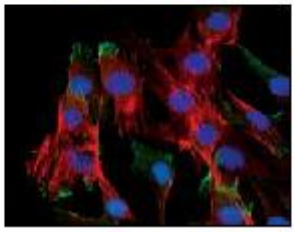
DRAQ5® is a registered trademark of Biostatus Limited.

Alexa Fluor® is a registered trademark of Molecular Probes, Inc.

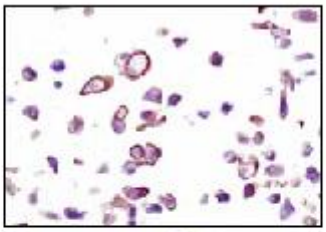
IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

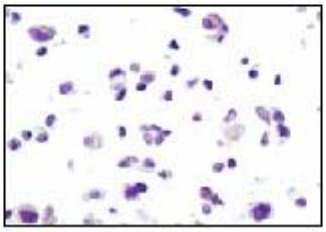
Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—Horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.



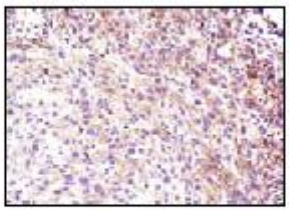


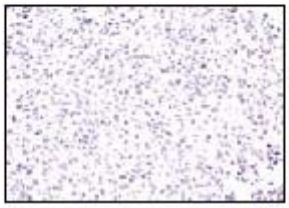
Confocal immunofluorescent analysis of C2C12 cells, LY294002-treated (upper) or insulin-treated (lower), using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb (green). Actin filaments have been labeled with Alexa Fluor® 555 phalloidin (red). Blue pseudocolor = DRAQ5® #4084 (fluorescent DNA dye).



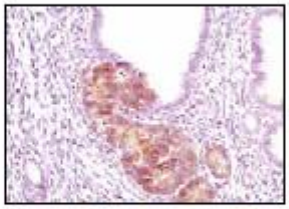


Immunohistochemical analysis using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb on SignalSlide® Phospho-Akt (Ser473) IHC Controls #8101 (paraffin-embedded LNCaP cells, untreated (upper) or LY294002-treated (lower).

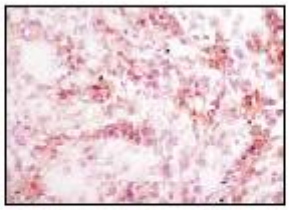




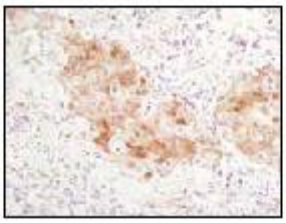
Immunohistochemical analysis of paraffin-embedded U-87 MG xenograft, untreated (upper) or λ phosphatase-treated (lower), using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb.

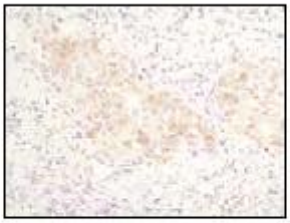


Immunohistochemical analysis of paraffin-embedded PTEN heterozygous mutant mouse endometrium using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb. (Tissue section courtesy of Dr. Sabina Signoretti, Brigham and Women's Hospital, Harvard Medical School, Boston, MA.)



Immunohistochemical analysis of frozen SKOV3 xenograft using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb.





Immunohistochemical analysis of paraffin-embedded human breast carcinoma comparing SignalStain® Antibody Diluent #8112 (upper) to TBST/5% normal goat serum (lower) using Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060.

Background References:

(1) Franke, T.F. et al. (1997) *Cell* 88, 435–7.
(2) Burgering, B.M. and Coffer, P.J. (1995) *Nature* 376, 599–602.
(3) Franke, T.F. et al. (1995) *Cell* 81, 727–36.
(4) Alessi, D.R. et al. (1996) *EMBO J* 15, 6541–51.
(5) Sarbassov, D.D. et al. (2005) *Science* 307, 1098–101.
(6) Jacinto, E. et al. (2006) *Cell* 127, 125–37.
(7) Cardone, M.H. et al. (1998) *Science* 282, 1318–21.
(8) Brunet, A. et al. (1999) *Cell* 96, 857–68.
(9) Zimmermann, S. and Moelling, K. (1999) *Science* 286, 1741–4.
(10) Cantley, L.C. and Neel, B.G. (1999) *Proc Natl Acad Sci USA* 96, 4240–5.
(11) Vlahos, C.J. et al. (1994) *J Biol Chem* 269, 5241–8.
(12) Hajduch, E. et al. (2001) *FEBS Lett* 492, 199–203.
(13) Cross, D.A. et al. (1995) *Nature* 378, 785–9.
(14) Diehl, J.A. et al. (1998) *Genes Dev* 12, 3499–511.
(15) Gesbert, F. et al. (2000) *J Biol Chem* 275, 39223–30.
(16) Zhou, B.P. et al. (2001) *Nat Cell Biol* 3, 245–52.
(17) Navé, B.T. et al. (1999) *Biochem J* 344 Pt 2, 427–31.
(18) Inoki, K. et al. (2002) *Nat Cell Biol* 4, 648–57.
(19) Manning, B.D. et al. (2002) *Mol Cell* 10, 151–62.

Store at -20°C

Bim (C34C5) Rabbit mAb

www.cellsignal.com

Support: 877-678-TECH (8324)
www.cellsignal.com/support

#2933

Small 100 µl (10 western blots)
Petite 40 µl (4 western blots)

Orders: 877-616-CELL (2355)
orders@cellsignal.com

rev. 07/30/14

Entrez-Gene ID #10018
UniProt ID #043521

For Research Use Only. Not For Use In Diagnostic Procedures.

Applications	Species Cross-Reactivity*	Molecular Wt.	Isotype
W, IP, IHC-P, IF-IC, F Endogenous	H, M, R, (Mk, B, Dg, Pg)	12, 15, 23 kDa	Rabbit IgG**

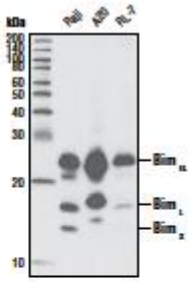
Background: Bim/Bod is a pro-apoptotic protein belonging to the BH3-only group of Bcl-2 family members including Bad, Bid, Bik, Hrk, and Noxa that contain a BH3 domain but lack other conserved BH1 or BH2 domains (1,2). Bim induces apoptosis by binding to and antagonizing anti-apoptotic members of the Bcl-2 family. Interactions have been observed with Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bfl-1, and BHRF-1 (1,2). Bim functions in regulating apoptosis associated with thymocyte negative selection and following growth factor withdrawal, during which Bim expression is elevated (3-6). Three major isoforms of Bim are generated by alternative splicing: Bim_{EL}, Bim_L, and Bim_S (1). The shortest form, Bim_S, is the most cytotoxic and is generally only transiently expressed during apoptosis. The Bim_{EL} and Bim_L isoforms may be sequestered to the dynein motor complex through an interaction with the dynein light chain and released from this complex during apoptosis (7). Apoptotic activity of these longer isoforms may be regulated by phosphorylation (8,9). Environmental stress triggers Bim phosphorylation by JNK and results in its dissociation from the dynein complex and increased apoptotic activity.

Specificity/Sensitivity: Bim (C34C5) Rabbit mAb detects endogenous levels of total Bim (EL, L and S isoforms) protein.

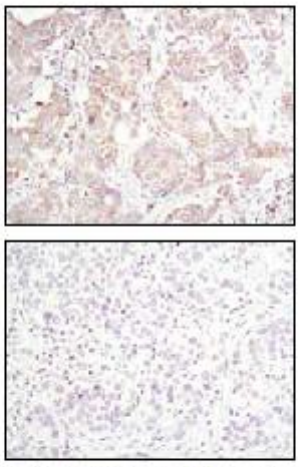
Source/Purification: Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Pro25 of Bim.

Background References:

(1) O'Connor, L. et al. (1998) *EMBO J* 17, 384-95.
(2) Hsu, S.Y. et al. (1998) *Mol Endocrinol* 12, 1432-40.
(3) Bouillet, P. et al. (2002) *Nature* 415, 922-6.
(4) Whitfield, J. et al. (2001) *Neuron* 29, 629-43.
(5) Dijkers, P.F. et al. (2000) *Curr Biol* 10, 1201-4.
(6) Ley, R. et al. (2003) *J Biol Chem* 278, 18811-6.
(7) Puthalakath, H. et al. (1999) *Mol Cell* 3, 287-96.
(8) Lei, K. and Davis, R.J. (2003) *Proc Natl Acad Sci USA* 100, 2432-7.
(9) Putcha, G.V. et al. (2003) *Neuron* 38, 899-914.



Western blot analysis of extracts from Raji, A20 and RL-7 cells using Bim (C34C5) Rabbit mAb.



Immunohistochemical analysis of paraffin-embedded human breast carcinoma using Bim (C34C5) Rabbit mAb in the presence of control peptide (upper) or antigen-specific peptide (lower).

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

*Species cross-reactivity is determined by western blot.

**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:

Western blotting	1:1000
Immunoprecipitation	1:100
Immunohistochemistry (Paraffin)	1:100†
Unmasking buffer:	Citrate
Antibody diluent:	SignalStain® Antibody Diluent #8112
Detection reagent:	SignalStain® Boost (HRP, Rabbit) #8114
†*Optimal IHC dilutions determined using SignalStain® Boost IHC Detection Reagent.*	
Immunofluorescence (IF-IC)	1:100
Flow Cytometry	1:400

For product specific protocols and a complete listing of recommended companion products please see the product web page at www.cellsignal.com

DRAQ5® is a registered trademark of Biostatus Limited.
MitoTracker® is a registered trademark of Molecular Probes, Inc.
U. S. Patent No. 5,675,063
Tween® is a registered trademark of ICI Americas, Inc.

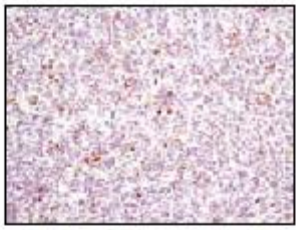
IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween®20 at 4°C with gentle shaking, overnight.

Thank you for your recent purchase. If you would like to provide a review visit www.cellsignal.com/comments.

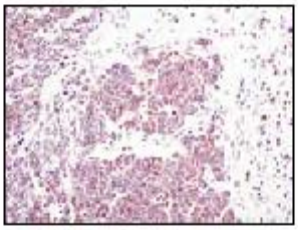
Cell Signaling TECHNOLOGY®

Applications: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide Species Cross-Reactivity: H—human M—mouse R—rat Hm—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—Horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.

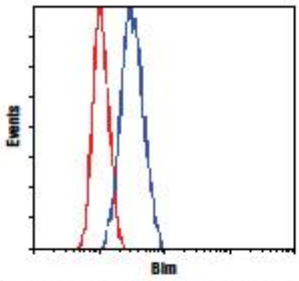
Confocal immunofluorescent analysis of MCF-7 cells using Bim Antibody (green) showing colocalization with mitochondria that have been labeled with MitoTracker® Red CMXRos (red). Blue pseudocolor – DRAQ5® #4084 (fluorescent DNA dye).



Immunohistochemical analysis of paraffin-embedded human lymphoma using Bim (C34C5) Rabbit mAb.



Immunohistochemical analysis of paraffin-embedded human lung carcinoma using Bim (C34C5) Rabbit mAb.



Flow cytometric analysis of Raji cells using Bim (C34C5) Rabbit mAb (blue) compared to a nonspecific negative control antibody (red).

SANTA CRUZ BIOTECHNOLOGY, INC.

PUMAα/β (G-3): sc-374223

BACKGROUND

PUMA (Bcl-2 binding component 3, JFY1, PUMA/JFY1) is a mitochondrial pro-apoptotic Bcl-2 homology domain (BH3)-only protein that induces rapid apoptosis through a Bax- and mitochondria-dependent pathway. The PUMA gene encodes four proteins originating from different splice variants of the same transcript: PUMAα, β, γ and δ. Both PUMAα and PUMAβ contain a BH3 domain, while PUMAγ and PUMAδ lack this domain. The BH3 domain is essential for binding of PUMAα and PUMAβ to Bcl-2 or Bcl-x_L. PUMA is an initiator of γ-radiation apoptosis and glucocorticoid-induced apoptosis in lymphoid cells *in vivo*. Bcl-2 family members generally regulate apoptosis and transmit death signals to mitochondria. Members of this family include both pro- and anti-apoptotic proteins that share homologous sequences known as Bcl-2 homology domains (BH1-4). The BH3 proteins, BID, NOXA, PUMA, NBK, Bim and Bad, are all pro-apoptotic and share sequence homology within the amphipathic α-helical BH3 region.

REFERENCES

1. Han, J., et al. 2001. Expression of BBC3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc. Natl. Acad. Sci. USA 98: 11318-11323.
2. Yu, J., et al. 2001. PUMA induces the rapid apoptosis of colorectal cancer cells. Mol. Cell 7: 673-682.
3. Nakano, K., et al. 2001. PUMA, a novel pro-apoptotic gene, is induced by p53. Mol. Cell 7: 683-694.
4. Bouillet, P., et al. 2002. BH3-only proteins-evolutionarily conserved pro-apoptotic Bcl-2 family members essential for initiating programmed cell death. J. Cell Sci. 115: 1567-1574.
5. Jeffers, J.R., et al. 2003. PUMA is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell 4: 321-328.
6. Hemann, M.T., et al. 2004. Suppression of tumorigenesis by the p53 target PUMA. Proc. Natl. Acad. Sci. USA 101: 9333-9338.
7. Cregan, S.P., et al. 2004. p53 activation domain 1 is essential for PUMA upregulation and p53-mediated neuronal cell death. J. Neurosci. 24: 10003-10012.

CHROMOSOMAL LOCATION

Genetic locus: BBC3 (human) mapping to 19q13.32.

SOURCE

PUMAα/β (G-3) is a mouse monoclonal antibody raised against amino acids 57-193 mapping at the C-terminus of PUMAα of human origin.

PRODUCT

Each vial contains 200 µg IgG_{2b} in 1.0 ml of PBS with < 0.1% sodium azide and 0.1% gelatin.

STORAGE

Store at 4° C, **DO NOT FREEZE**. Stable for one year from the date of shipment. Non-hazardous. No MSDS required.

APPLICATIONS

PUMAα/β (G-3) is recommended for detection of PUMAα and PUMAβ of human origin by Western Blotting (starting dilution 1:100, dilution range 1:100-1:1000), immunoprecipitation [1-2 µg per 100-500 µg of total protein (1 ml of cell lysate)], immunofluorescence (starting dilution 1:50, dilution range 1:50-1:500) and solid phase ELISA (starting dilution 1:30, dilution range 1:30-1:3000).

Suitable for use as control antibody for PUMA siRNA (h): sc-37153, PUMA shRNA Plasmid (h): sc-37153-SH and PUMA shRNA (h) Lentiviral Particles: sc-37153-V.

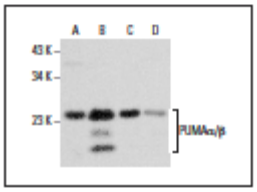
Molecular Weight of PUMAα/β: 18-24 kDa.

Positive Controls: HeLa whole cell lysate: sc-2200, K-562 whole cell lysate: sc-2203 or Hep G2 cell lysate: sc-2227.

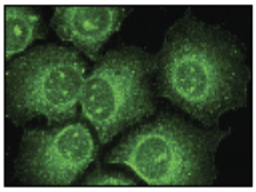
RECOMMENDED SECONDARY REAGENTS

To ensure optimal results, the following support (secondary) reagents are recommended: 1) Western Blotting: use goat anti-mouse IgG-HRP: sc-2005 (dilution range: 1:2000-1:32,000) or Cruz Marker™ compatible goat anti-mouse IgG-HRP: sc-2031 (dilution range: 1:2000-1:5000), Cruz Marker™ Molecular Weight Standards: sc-2035, TBS Blotto A Blocking Reagent: sc-2333 and Western Blotting Luminol Reagent: sc-2048. 2) Immunoprecipitation: use Protein A/G PLUS-Agarose: sc-2003 (0.5 ml agarose/2.0 ml). 3) Immunofluorescence: use goat anti-mouse IgG-FITC: sc-2010 (dilution range: 1:100-1:400) or goat anti-mouse IgG-TR: sc-2781 (dilution range: 1:100-1:400) with UltraCruz™ Mounting Medium: sc-24941.

DATA



PUMAα/β (G-3): sc-374223. Western blot analysis of PUMAα,β expression in A549 (A), Hep G2 (B), HeLa (C) and K-562 (D) whole cell lysates.



PUMAα,β (G-3): sc-374223. Immunofluorescence staining of methanol-fixed HeLa cells showing cytoplasmic localization.

SELECT PRODUCT CITATIONS

1. Sistrunk, C., et al. 2013. Skp2 deficiency inhibits chemical skin tumorigenesis independent of $p27^{Kip1}$ accumulation. Am. J. Pathol. 182: 1854-1864.
2. Quast, S.A., et al. 2013. ROS-dependent phosphorylation of Bax by wortmannin sensitizes melanoma cells for TRAIL-induced apoptosis. Cell Death Dis. 4: e839.

RESEARCH USE

For research use only, not for use in diagnostic procedures.

Dako

CE

Monoclonal Mouse Anti-Human BCL2 Oncoprotein
Clone 124
Code M0887

ENGLISH

Intended use

For in vitro diagnostic use.

Monoclonal Mouse Anti-Human BCL2 Oncoprotein, Clone 124, is intended for use in immunohistochemistry. The antibody labels cells expressing BCL2 oncoprotein. Positive results aid in the classification of follicular lymphomas and various diffuse lymphoproliferative diseases. The clinical interpretation of any staining or its absence should be complemented by morphological studies using proper controls and should be evaluated within the context of the patient's clinical history and other diagnostic tests by a qualified pathologist.

Summary and explanation

BCL2 oncoprotein is a blocker of apoptotic cell death. Gene transfer experiments have shown that elevated levels of this protein can protect a wide variety of cells from diverse cell death stimuli ranging from growth factor withdrawal and cytotoxic lymphokines to virus infection and DNA-damaging, anticancer drugs and radiation (2, 3). BCL2 oncoprotein resides on the cytoplasmic side of the mitochondrial outer membrane, endoplasmic reticulum and nuclear envelope (2, 4), and has a molecular mass of 26 kDa (3).

The *BCL2* gene is involved in the t(14;18) chromosomal translocation found in 85% of human follicular lymphomas and 20% of diffuse B-cell lymphomas (4). In this translocation, the *BCL2* gene at chromosome segment 18q21 is juxtaposed with the Ig heavy chain locus at 14q32, resulting in deregulated expression of BCL2 oncoprotein (4).

Reagent provided

Monoclonal mouse antibody provided in liquid form as cell culture supernatant dialysed against 0.05 mol/L Tris/HCl, pH 7.2, and containing 15 mmol/L NaN_3.

Clone: 124 (1). Isotype: IgG1, kappa.

Mouse IgG concentration: see label on vial.

The protein concentration between lots may vary without influencing the optimal dilution. The titer of each individual lot is adjusted to ascertain comparable lot-to-lot immunohistochemical staining performance with a reference lot.

Immunogen

Synthetic peptide comprising amino acids 41-54 of human BCL2 oncoprotein (1).

Specificity

In Western blotting of extracts of normal human spleen (1, 5), t(14;18)-positive follicular lymphoma (1), and myloid leukaemic cell lines (5) the antibody labels solely a band of 26 kDa, corresponding to BCL2 oncoprotein under both nonreducing (1) and reducing conditions (1, 5).

The antibody labels the myeloid leukaemic cell lines, HL-60 (promyelocytic), KG1 (myeloblastic), GM-1 (monoblastic) and K562 (erythromyeloid) (5).

Precautions

1. For professional users.
2. This product contains sodium azide (NaN_3), a chemical highly toxic in pure form. At product concentrations, though not classified as hazardous, sodium azide may react with lead and copper plumbing to form highly explosive build-ups of metal azides. Upon disposal, flush with large volumes of water to prevent metal azide build-up in plumbing.
3. As with any product derived from biological sources, proper handling procedures should be used.
4. Wear appropriate Personal Protective Equipment to avoid contact with eyes and skin.
5. Unused solution should be disposed of according to local, State and Federal regulations.

Storage

Store at 2-8 °C. Do not use after expiration date stamped on vial. If reagents are stored under any conditions other than those specified, the conditions must be verified by the user. There are no obvious signs to indicate instability of this product. Therefore, positive and negative controls should be run simultaneously with patient specimens. If unexpected staining is observed which cannot be explained by variations in laboratory procedures and a problem with the antibody is suspected, contact Dako Technical Services.

Specimen preparation

Paraffin sections: The antibody can be used for labeling paraffin-embedded tissue sections fixed in formalin or Bouin's (5). Pre-treatment of tissues with heat-induced epitope retrieval is required. For tissues fixed in formalin, optimal results are obtained with 10 mmol/L Tris buffer, 1 mmol/L EDTA, pH 9.0. Less optimal results are obtained with Dako Target Retrieval Solution, High pH, code S3308, Dako Target Retrieval Solution, code S1700, or 10 mmol/L citrate buffer, pH 6.0. Pre-treatment of tissues with proteinase K was found inefficient. The tissue sections should not dry out during the treatment or during the following immunohistochemical staining procedure.

Frozen sections and cell preparations: The antibody can be used for labelling acetone-fixed, frozen sections and cell smears (1).

Staining procedure

Dilution: Monoclonal Mouse Anti-Human BCL2 Oncoprotein, code M0887, may be used at a dilution range of 1:50-1:100 when applied on formalin-fixed, paraffin-embedded sections of tonsil tissue and using 20 minutes heat-induced epitope retrieval in 10 mmol/L Tris buffer, 1 mmol/L EDTA, pH 9.0, and 30 minutes incubation at room temperature with the primary antibody. Optimal conditions may vary depending on specimen and preparation method, and should be determined by each individual laboratory. The recommended negative control is Dako Mouse IgG1, code X0931, diluted to the same mouse IgG concentration as the primary antibody. Unless the stability of the diluted antibody and negative control has been established in the actual staining procedure, it is recommended to dilute these reagents immediately before use, or dilute in Dako Antibody Diluent, code S0809. Positive and negative controls should be run simultaneously with patient specimen.

Visualization: Dako LSAB™+/HRP kit, code K0679, and Dako EnVision™+/HRP kits, codes K4004 and K4006 are recommended. For frozen sections and cell preparations, the Dako APAAP kit, code K0670, is a good alternative if endogenous peroxidase staining is a concern. Follow the procedure enclosed with the selected visualization kit.

Automation: The antibody is well suited for immunocytochemical staining using automated platforms, such as the Dako Autostainer.

Performance characteristics

Cells labelled by the antibody display a cytoplasmic staining pattern.

Normal tissues: The antibody labels almost all peripheral blood lymphocytes. In lymphoid tissue, small lymphocytes in the mantle zones and T-cell areas are positive whereas very few cells in germinal centres are labelled. In the spleen, many cells in both T- and B-cell areas and the red pulp are labelled by the antibody. In the thymus, many cells in the medulla are labelled, while most cells in the cortex show weak or negative staining (1).

Dako Denmark A/S | Produktionsvej 42 | DK-2600 Glostrup | Denmark | Tel. +45 44 85 95 00 | Fax +45 44 85 95 95 | CVR No. 33 21 13 17

FRANÇAIS

Intérêt

Résumé et explication

Réactif fourni

Immunogène

Spécificité

Précautions d'emploi

Conservation

Préparation de l'échantillon

Procédure d'immunomarquage

Performances

Dako Denmark A/S | Pro

Dans le thymus, de nombreuses cellules de la medulla ont été marquées, alors que presque toutes les cellules du cortex présentaient un marquage faible ou négatif (1).

Tissus anormaux : L'anticorps a marqué de nombreuses cellules néoplasiques, notamment 31 cas sur 38 de lymphomes diffus et maladies lymphoprolifératives de grades inférieur et élevé, incluant des leucémies lymphocytaires chroniques, des leucémies tricholeucocytaires, des lymphomes à cellules T, des lymphomes anaplasiques à grandes cellules B et T et des lymphomes anaplasiques à grandes cellules Ki-1, et 37 cas sur 43 de follicules néoplasiques dans des cas de lymphomes folliculaires (1). L'expression de l'oncoprotéine BCL2 a également été détectée dans 15 cas sur 19 de sarcome synovial (6) et dans des tumeurs d'origine musculaire (7).

DEUTSCH

Zweckbestimmung

Zur Verwendung für in-vitro-Untersuchungen.

Monoclonal Mouse Anti-Human BCL2 Oncoprotein, Clone 124, ist für die immunhistochemische Anwendung bestimmt. Der Antikörper markiert BCL2-Onkoprotein exprimierende Zellen. Positive Ergebnisse helfen bei der Klassifizierung follikulärer Lymphome und verschiedener diffuser lymphoproliferativer Krankheiten. Differentialdiagnose wird durch die Ergebnisse von einem Antikörperpanel unterstützt. Die klinische Auswertung einer eventuell eintretenden Färbung sollte durch morphologische Studien mit geeigneten Kontrollen ergänzt werden und von einem qualifizierten Pathologen unter Berücksichtigung der Krankengeschichte und anderer diagnostischer Tests des Patienten vorgenommen werden..

Zusammenfassung und Erklärung

Das BCL2-Onkoprotein ist ein Hemmer der Zellapoptose. Durch Experimente zum Gentransfer wurde nachgewiesen, dass erhöhte Spiegel dieses Proteins eine große Vielzahl von Zellen vor unterschiedlichen, zu Zelltod führenden Stimuli schützen können. Diese reichen von Wachtumsfaktor-Entzug und zytotoxischen Lymphokinen bis hin zu Virusinfektion und DNA-Schäden, Zytostatika und Strahlentherapie (2, 3). Das BCL2-Onkoprotein ist auf der zytoplasmatischen Seite der äußeren Mitochondrienmembran, auf dem endoplasmatischen Retikulum und der Kernhülle angesiedelt (2, 4), und seine relative Molekülmasse beträgt 26 kDa (3).

Das *BCL2*-Gen ist an der t(14;18) chromosomalen Translokation beteiligt, die bei 85 % der follikulären Lymphome und bei 20 % der diffusen B-Zell-Lymphome des Menschen festgestellt wird (4). Bei dieser Translokation wird das *BCL2*-Gen am Chromosomensegment 18q21 mit dem IgH-Schwerkettenlocus bei 14q32 in Juxtaposition gebracht, was in deregulierter Expression des BCL2-Onkoproteins resultiert (4).

Geliefertes Reagenz

Der monoklonale Mausantikörper wird in flüssiger Form als Zellkulturüberstand geliefert, wurde gegen 0,05 mol/L Tris/HCl, pH-Wert 7,2, dialysiert und enthält 15 mmol/L NaN_3.

Klon: 124 (1). Isotyp: IgG1, Kappa.

Maus-IgG-Konzentration: Siehe Produktetikett.

Die Proteinkonzentration kann bei den Chargen verschieden ausfallen, ohne die optimale Verdünnung zu beeinflussen. Der Titer wird bei jeder einzelnen Charge mit einer Referenzcharge verglichen und dieser angeglichen, um konstante immunhistochemische Färbeergebnisse zwischen den Chargen zu gewährleisten.

Immunogen

Synthetisches Peptid mit den Aminosäuren 41-54 des humanen BCL2-Onkoproteins (1).

Spezifität

Beim Westernblotting von Extrakten der gesunden menschlichen Milz (1, 5), des t(14;18)-positiven follikulären Lymphoms (1) und myeloider Leukämie-Zellinien (5) markiert der Antikörper ausschließlich eine Bande von 26 kD, die dem BCL2-Onkoprotein unter Bedingungen der Nicht-Reduktion (1) und Reduktion (1, 5) entsprechen.

Der Antikörper markiert die myeloiden Leukämie-Zellinien HL-60 (promyelozytisch), KG1 (myeloblastisch), GM-1 (monoblastisch) und K562 (erythromyeloid) (5).

Hinweise und Vorsichtsmaßnahmen

1. Für geschultes Fachpersonal.
2. Dieses Produkt enthält Natrium-Azid (NaN_3), eine in reiner Form hochtoxische chemische Verbindung. Bei den in diesem Produkt verwendeten Konzentrationen kann Natrium-Azid, obwohl nicht als gefährlich klassifiziert, mit in Abflussrohren enthaltenem Blei oder Kupfer reagieren und zur Bildung von hochexplosiven Metall-Azid-Anreicherungen in den Abflussrohren führen. Nach der Entsorgung muss mit reichlich Wasser nachgespült werden, um Metall-Azid-Anreicherung in den Abflussrohren zu vermeiden.
3. Wie bei allen aus biologischen Materialien gewonnenen Produkten müssen die ordnungsgemäßen Handhabungsverfahren eingehalten werden.
4. Geeignete Schutzkleidung tragen, um Augen- und Hautkontakt zu vermeiden.
5. Nicht verwendete Lösung ist entsprechend örtlichen, bundesstaatlichen und staatlichen Richtlinien zu entsorgen.

Lagerung

Bei 2 – 8 °C lagern. Nicht nach dem auf dem Fläschchen angegebenen Verfallsdatum verwenden. Falls die Reagenzien unter anderen Bedingungen als den beschriebenen aufbewahrt werden, so müssen diese vom Anwender verifiziert werden. Es gibt keine offensichtlichen Anhaltspunkte für die mögliche Instabilität dieses Produktes. Es sollten daher die Positiv- und Negativkontrollen gleichzeitig mit den Patientenproben mitgeführt werden. Wenn unerwartete Verfärbung beobachtet wird, welche durch Änderungen in den Labormethoden nicht erklärt werden kann, und falls Verdacht auf ein Problem mit dem Antikörper besteht, ist bitte Kontakt mit unserem technischen Kundendienst aufzunehmen.

Probenvorbereitung

Paraffinschnitte: Der Antikörper kann für die Färbung von paraffineingebetteten, in Formalin oder Bouin-Lösung fixierten Gewebeschnitten genutzt werden (5). Eine Vorbehandlung der Gewebe mit hitzeinduzierter Epitopdemaskierung ist erforderlich. Für formalinfixierte Gewebeproben werden optimale Ergebnisse mit 10 mmol/L Tris-Puffer, 1 mmol/L EDTA, pH 9,0, erzielt. Weniger gute Ergebnisse werden mit Dako Target Retrieval Solution, pH 9,9, Code Nr. S3308, Dako Target Retrieval Solution, Code-Nr. S1700, bzw. 10 mmol/L Citratpuffer, pH 6,0, erzielt. Die Vorbehandlung der Gewebe mit Proteinase K hat sich als ineffizient erwiesen. Während der Gewebevorbehandlung oder während der sich anschließenden immunhistochemischen Färbeprozedur dürfen die Gewebeschnitte nicht austrocknen.

Gefrierschnitte und zytologische Präparate: Der Antikörper kann für die Markierung von azetonfixierten Gefrierschnitten und Zellausstrichen verwendet werden (1).

Färbeprozedur

Verdünnung: Monoclonal Mouse Anti-Human BCL2 Oncoprotein, Code-Nr. M0887, kann bei einem Verdünnungsbereich von 1:50-1:100 eingesetzt werden, wenn es für formalinfixierte, paraffineingebettete Schnitte aus Tonsillengewebe genutzt wird und wenn 20 Minuten lang die hitzeinduzierte Epitopdemaskierung in 10 mmol/L Tris-Puffer, 1 mmol/L EDTA, pH 9,0, durchgeführt wird, gefolgt von 30minütiger Inkubation mit dem primären Antikörper bei Raumtemperatur. Die optimalen Bedingungen schwanken je nach Probe und Methode der Probenvorbereitung und sollten von jedem einzelnen Labor bestimmt werden. Als Negativkontrolle wird Dako Mouse IgG1, Code-Nr. X0931 empfohlen, das auf dieselbe Maus-IgG-Konzentration wie der primäre Antikörper verdünnt worden ist. Solange mit dem eigentlichen Testsystem die Stabilität des verdünnten Antikörpers und der Negativkontrolle nicht sichergestellt ist, wird empfohlen, diese Reagenzien unmittelbar vor Gebrauch zu verdünnen oder die Verdünnung mit Dako Antibody Diluent, Code-Nr. S0809, vorzunehmen. Die Positiv- und Negativkontrollen sollten gleichzeitig mit den Patientenproben mitgeführt werden.

Visualisierung: Folgende Kits werden empfohlen: Dako LSAB™+/HRP-Kit, Code-Nr. K0679, und Dako EnVision™+/HRP-Kits, Code-Nr. K4004 und K4006. Falls bei Gefrierschnitten und Zellpräparaten Probleme mit endogener Peroxidasefärbung auftreten, bietet der Dako

Dako Denmark A/S | Produktionsvej 42 | DK-2600 Glostrup | Denmark | Tel. +45 44 85 95 00 | Fax +45 44 85 95 95 | CVR No. 33 21 13 17

Leistungseigenschaften

Referenoes/ Références/ Lite

Explanation of symbols/ Lég

Catalogue number
Référence du catalo
Bestellnummer

In vitro diagnostic m
Dispositif médical d
in vitro
In-Vitro-Diagnostiku

Consult instructions
Consulter les instruc
d'utilisation
Gebrauchsanweisu

(104935-005)

Dako Denmark A/S | Pro

#5453 Store at -20°C

Mcl-1 (D35A5) Rabbit mAb

☑ 100 µl
(10 western blots)

Cell Signaling TECHNOLOGY

Orders ■ 877-616-CELL (2355)
orders@cellsignal.com
Support ■ 877-678-TECH (8324)
info@cellsignal.com
Web ■ www.cellsignal.com

rev. 02/06/12

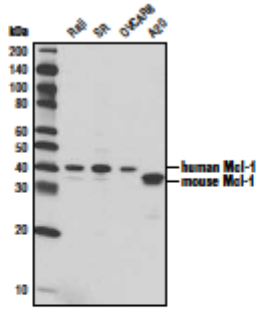
Entrez-Gene ID #4170
Swiss-Prot Acc. #Q07820

Applications	Species Cross-Reactivity*	Molecular Wt.	Isotype
W Endogenous	H, M, Mk, (B)	40 kDa (human), 35 kDa (mouse)	Rabbit IgG**

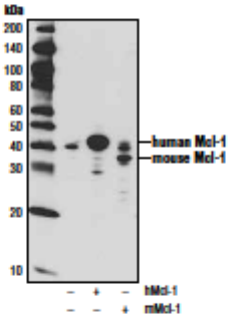
Background: Mcl-1 is an anti-apoptotic member of the Bcl-2 family originally isolated from the ML-1 human myeloid leukemia cell line during phorbol ester-induced differentiation along the monocyte/macrophage pathway (1). Similar to other Bcl-2 family members, Mcl-1 localizes to the mitochondria (2), interacts with and antagonizes pro-apoptotic Bcl-2 family members (3), and inhibits apoptosis induced by a number of cytotoxic stimuli (4). Mcl-1 differs from its other family members in its regulation at both the transcriptional and post-translational level. First, Mcl-1 has an extended amino-terminal PEST region, which is responsible for its relatively short half-life (1,2). Second, unlike other family members, Mcl-1 is rapidly transcribed via a PI3K/Akt dependent pathway, resulting in its increased expression during myeloid differentiation and cytokine stimulation (1,5-7). Mcl-1 is phosphorylated in response to treatment with phorbol ester, microtubule-damaging agents, oxidative stress, and cytokine withdrawal (8-11). Phosphorylation at Thr163, the conserved MAP kinase/ERK site located within the PEST region, slows Mcl-1 protein turnover (10) but may prime the GSK-3 mediated phosphorylation at Ser159 that leads to Mcl-1 destabilization (11). Mcl-1 deficiency in mice results in peri-implantation lethality (12). In addition, conditional disruption of the corresponding mcl-1 gene shows that Mcl-1 plays an important role in early lymphoid development and in the maintenance of mature lymphocytes (13).

Specificity/Sensitivity: Mcl-1 (D35A5) Rabbit mAb detects endogenous levels of total Mcl-1 protein.

Source/Purification: Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to residues surrounding Leu210 of human Mcl-1.



Western blot analysis of extracts from various cell lines using Mcl-1 (D35A5) Rabbit mAb.



Western blot analysis of extracts from 293T cells, mock transfected or transfected with human or mouse Mcl-1 constructs, using Mcl-1 (D35A5) Rabbit mAb.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA, 50% glycerol and less than 0.02% sodium azide. Store at –20°C. *Do not aliquot the antibody.*

*Species cross-reactivity is determined by western blot.

**Anti-rabbit secondary antibodies must be used to detect this antibody.

Recommended Antibody Dilutions:
Western blotting 1:1000

For application specific protocols please see the web page for this product at www.cellsignal.com.

Please visit www.cellsignal.com for a complete listing of recommended companion products.

Background References:

(1) Kozopas, K.M. et al. (1993) *Proc Natl Acad Sci USA* 90, 3516-20.
(2) Yang, T. et al. (1995) *J Cell Biol* 128, 1173-84.
(3) Sato, T. et al. (1994) *Proc Natl Acad Sci USA* 91, 9238-42.
(4) Zhou, P. et al. (1997) *Blood* 89, 630-43.
(5) Wang, J.M. et al. (1999) *Mol Cell Biol* 19, 6195-206.
(6) Jourdan, M. et al. (2003) *Oncogene* 22, 2950-9.
(7) Chao, J.R. et al. (1998) *Mol Cell Biol* 18, 4883-98.
(8) Domina, A.M. et al. (2000) *J Biol Chem* 275, 21688-94.
(9) Inoshita, S. et al. (2002) *J Biol Chem* 277, 43730-4.
(10) Domina, A.M. et al. (2004) *Oncogene* 23, 5301-15.
(11) Maurer, U. et al. (2006) *Mol Cell* 21, 749-60.
(12) Rinkenberger, J.L. et al. (2000) *Genes Dev* 14, 23-7.
(13) Opferman, J.T. et al. (2003) *Nature* 426, 671-6.

IMPORTANT: For western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

Applications Key: W—Western IP—Immunoprecipitation IHC—Immunohistochemistry ChIP—Chromatin Immunoprecipitation IF—Immunofluorescence F—Flow cytometry E-P—ELISA-Peptide
Species Cross-Reactivity Key: H—human M—mouse R—rat Ha—hamster Mk—monkey Mi—mink C—chicken Dm—D. melanogaster X—Xenopus Z—zebrafish B—bovine
Dg—dog Pg—pig Sc—S. cerevisiae Ce—C. elegans Hr—horse All—all species expected Species enclosed in parentheses are predicted to react based on 100% homology.