A Biomarker Validation Study to Establish Whether Serial Flow Cytometric Measurements Predict Clinical Response to Sirolimus and MEC (Mitoxantrone Etoposide Cytarabine) Treatment in Patients with High-Risk Acute Myelogenous Leukemia

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List of Abbreviations

AE	Adverse Event
ANC	absolute neutrophil count
AKT	Protein Kinase B
ALL	Acute Lymphoblastic Leukemia
ALT	Alanine transaminase
AML	Acute Myeloid Leukemia
AST	Aspartate Transaminase
AUC	Area under the curve
BCR-ABL	Break point cluster-Abelson tyrosine kinase gene
BUN	blood urea nitrogen
CALGB	Cancer and Leukemia Group B
CBC	complete blood count
CCRRC	Clinical Cancer Research Review Committee
CD45	Protein tyrosine phosphatase, receptor type C
CML	Chronic Myeloid Leukemia
CNS	central nervous system
CR	complete response
CRi	Complete Response without incomplete recovery
CRp	Complete Response in the absence of total platelet recovery
CRF	case report form
CRp	Complete response in absence of total platelet recovery
CTC	common toxicity criteria
DNA	Deoxyribonucleic Acid
D5W	5% dextrose in water
DLT	dose-limiting toxicity
DSMB/DSMC	Data and Safety Monitoring Board/Data and Safety Monitoring Committee
EC	Ethics Committee
ECOG	Eastern Cooperative Oncology Group
ECHO	Echocardiogram
ERK	Extracellular signal-related kinases
FDA	Food and Drug Administration
FKBP12	FK Binding Protein 12
FLT3-ITD	F*ms*-like tyrosine kinase 3 internal tandem duplication gene
G-CSF	filgrastim (granulocyte-colony stimulating factor)
GM-CSF	sargramostim
HIDAC	High dose Cytarabine Arabinoside (ARA-C)
HPLC	High Performance Liguid Chromatography
HSCT	Hematopoeitic Stem Cell Transplant
HSV	Herpes Simplex Virus
IEC	Independent Ethics Committee
IRB	Institutional Review Board
IV	Intravenous
IWG	International Working Group
LDH	Lactate dehydrogenase
LVEF	Left Ventricular Ejection Fraction
MAPK	Mitogen-activated protein kinases

MEC	Mitoxantrone + Etoposide + Cytarabine
mTOR	Mammalian Target of Rapamycin
MTD	Maximally Tolerated Dose
MTI	mTOR Inhibitor
MUGA	Mutli Gated Acquisition Scan
NCI	National Cancer Institute
NED	No Evidence of Disease
NIH	National Institute of Health
NR	No Response
NSS	Normal Saline Solution
OPRR	US Dept of Health & Human Serv./Office for Protection from Research Risk
ORR	Objective Response Rate
OS	Overall Survival
PBMCs	Peripheral Blood Mononuclear Cells
PD	progressive disease
PD	Pharmacodynamic
PHI	Protected Health Information
PI3K	Phosphotidyl Inositol-3'-kinase
PK	Pharmacokinetic
PLT	platelets
PR	Partial Remission
PR	Partial response
pS6	Ribosomal protein S6 kinase
PS	performance status
PTEN	Phosphatidylinositol-3,4,5-trisphosphate
RFS	Relapse Free Survival
ROC	Receiver operating characteristics
RNA	Ribonucleic Acid
RR	response rate
RT	radiotherapy
RTK	Receptortyrosine Kinases
SAE	Serious Adverse Event
SD	stable disease
SKCC	Sidney Kimmel Cancer Center
SGOT	serum glutamate-oxaloacetate transaminase
SGPT	serum glutamate-pyruvate transaminase
STATs	Signal Transducer and Activator of Transcription proteins
TJUH	Thomas Jefferson University Hospital
UK-MRC	United Kingdom- Medical Research Council
ULN	upper limit of normal
US	United States
WBC	White Blood Cell

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1 Study Summary

Title	A Biomarker Validation Study to Establish Whether Serial Flow Cytometric Measurements Predict Clinical Response to Sirolimus and MEC (Mitoxantrone Etoposide Cytarabine) Treatment in Patients with High-Risk Acute Myelogenous Leukemia
Short Title	Validation of phospho-flow to predict response to sirolimus+MEC in AML
Protocol Number	15D.377
Phase	Phase 2
Methodology	Single arm, open label trial
Study Duration	7 years
Study Center(s)	Thomas Jefferson University
Objectives	**Primary objective:** The primary objective is to test the association between biochemical response as measured by flow cytometry for S6 phosphorylation and clinical response as measured by IWG. **Secondary objectives:** To estimate the efficacy of sirolimus MEC in high risk AML patients (RFS,OS). To further characterize treatment-related toxicities
Number of Subjects	Approximately 65 to enroll 49 biomarker-evaluable subjects
Diagnosis and Main Inclusion Criteria	High risk non-M3 AML: relapsed, refractory, secondary, age >60
Study Product, Dose, Route, Regimen	Sirolimus loading dose of 12 mg orally on day 1 followed by 4 mg/ day orally every 24 hours on days 2-9. MEC is Mitoxantrone 8mg/m^2/day IV, Etoposide 100mg/m^2/day IV and Cytarabine 1000mg/ m^2/day IV every 24 hours on days 4-8 days. (Starts after 4th dose of sirolimus).
Duration of administration	9 days
Reference therapy	The standard dose of MEC is Mitoxantrone 8mg/m^2/day, Etoposide 100mg/m^2/day and Cytarabine 1000mg/ m^2/day and is a common salvage regimen for AML.
Statistical Methodology	Baseline pS6 and % reduction in pS6 will be described by mean, median, standard deviation, range and coefficient of variation. Biochemical response will be scored in patients with measureable baseline pS6. Clinical response will be scored in all patients. The objective response rate (ORR) and 95% exact confidence interval will be computed for all patients and for sensitive and resistant subgroups. The association between biochemical response and clinical response will be tested by Fisher's exact test. RFS and OS will be estimated by the method of Kaplan and Meier. A landmark analysis of RFS by clinical response (CR+CRp, CRi, PR or NR) will be computed from day 45 marrow assessment. Median values and 95% confidence intervals will be calculated. Toxicities will be graded and tabled.

2 Introduction

This document is a protocol for a human research study. This study is to be conducted according to U.S. and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

2.1 Background

Therapy for relapsed or refractory acute myeloid leukemia (AML) has not changed significantly in the last 20 years and depends on high dose chemotherapy. The chemotherapeutic regimen of choice for induction of newly diagnosed patients is a combination of cytosine arabinoside with either daunorubicin or idarubicin. With improvements in supportive care, this regimen has increased the overall cure rate for newly diagnosed patients with myeloid leukemias to approximately 30%. Patients who are able to receive bone marrow transplantation may have even a better survival but still a majority of patients relapse. Therapy for relapsed myeloid leukemias and advanced CML (chronic myeloid leukemia) refractory to Gleevec (imatinib) is suboptimal. Several chemotherapeutic regimens, including high dose cytosine arabinoside (HIDAC) or mitoxantrone, etoposide and cytarabine (MEC), have up to a 30% response rate but cure for relapsed and refractory disease is less than 10%. This poor outcome demonstrates the need for improved therapeutics. Given the significant toxicity of these regimens, increased doses of chemotherapeutic drugs are unlikely to be tolerated. Therefore, we have worked to develop a better understanding of the intrinsic survival mechanisms of leukemic blasts in order to develop improved therapeutics.

Chemotherapy trials in AML have reached an unsatisfactory efficacy plateau
Acute myeloid leukemia (AML) is an aggressive hematopoietic cancer characterized by the expansion of clonal immature myeloid precursors that crowd out normal hematopoiesis and rapidly cause marrow failure.[1] Untreated, AML is universally fatal within weeks to months, but intensive combinations of myelosuppressive chemotherapy can achieve remission in 50-70% of patients.[2,3] Response rate is balanced against a significant risk of therapy-related toxicity and death, particularly in elderly patients. Despite advances in post-remission AML therapies and supportive care, disease control is typically transient and long term survival is seen in only 30% of patients below age 60 and in fewer than 10% of older patients.[4-6] Because the median age at AML diagnosis is 68, it follows that the vast majority are not cured by current approaches. Recent data suggest only a minority of patients with AML over the age of 65 receive *any* anti-leukemic therapy, [7] likely due to widespread belief among oncologists and patients that such treatments are both ineffective and unnecessarily toxic. Survival of patients with AML has not changed appreciably in 30 years and therapeutic advances to improve efficacy and reduce toxicity are eagerly sought.[8] Seventy to ninety percent of patients with AML either fail to respond to upfront therapy or relapse after successful induction, regardless of post-remission therapy intensity.[9,10] Survival in this group with relapsed or refractory leukemia is extremely poor and typically measured in the range of 3-6 months.[11] Long-term survival is generally limited to those who undergo stem cell transplantation, a risky procedure that can be offered to a small number of patients, typically after they achieve complete remission to a salvage chemotherapy regimen.

Salvage regimens have been tested with a number of traditional cytotoxic and novel agents. To summarize the large literature of clinical trials for relapsed and refractory AML, response rates to salvage chemotherapy are very poor. Prior remission durability and leukemic karyotype are better predictors of salvage response than the age of patients or the particular regimen delivered as salvage. Patients who never achieved a first remission or whose first remission was shorter than 6-12 months are unlikely to respond to a first attempt at salvage chemotherapy. Typical response rates for this group are in the range of 10-20%.[12] This group is enriched for high risk leukemic karyotype or *Fms*-like tyrosine kinase 3 internal tandem duplication (FLT3-ITD) gene mutations, both of which are biologic features that independently predict a low likelihood of salvage chemotherapy response.[13,14] Taken together, these data underscore the great demand for improved salvage regimens as well as more effective frontline treatments to

enhance response and prevent relapse. Novel agents traditionally have been tested in the context of salvage chemotherapy before successful agents are examined as initial therapy.

The optimal salvage regimen for relapsed or refractory AML is undefined but several approaches are commonly employed. High dose cytarabine has activity as a single agent and additional agents such as anthracyclines, anthracenediones, topoisomerase inhibitors, or purine analogues are postulated to further improve response rates.[15-18] Various schedules and doses of mitoxantrone, etoposide, and intermediate to high dose cytarabine have been employed in this context. The Eastern Cooperative Oncology Group (ECOG) has employed the MEC regimen in several studies.[19,20] MEC features simultaneous bolus infusion of all three drugs every 24 hours for five days. The regimen is widely used and has an established and tolerable safety record. Published large studies of MEC show a complete remission (CR) rate of 21% among relapsed and refractory AML, which is a respectable benchmark for chemotherapy activity in this patient population.[19]

The optimal approach to target oncogenic signaling in AML is undefined
Recent improvements in our understanding of leukemia biology have led to the introduction of highly effective, molecularly targeted therapies. This is exemplified by the development of BCR-ABL tyrosine kinase inhibitors such as imatinib as monotherapy for chronic myeloid leukemia (CML) and in combination with chemotherapy for BCR-ABL+ acute lymphoblastic leukemia (ALL).[21,22] Like BCR-ABL in Philadelphia chromosome positive leukemias, growth-stimulating mutations (e.g. FLT3, c-kit, and ras) occur commonly in AML**.** Unfortunately, the dependency with which AML cell growth and survival depends upon oncogenic signaling varies from patient to patient even carrying the same mutations.[23] The common recurrent mutations in signaling proteins such as FLT3, Kit, or ras are likely <u>not</u> initiating mutations for AML and often are present in a subset of tumor cells.[24] This heterogeneity in mutation representation and function within a heterogeneous tumor suggests that targeted therapy likely will be most effective using combinations of such drugs and/or integration of targeted agents within chemotherapy regimens.

The PI3K signaling is critical to leukemia cell survival and can be targeted
Growth and survival stimulating signal transduction pathways are abnormally and ubiquitously activated in AML. Three signaling pathways are constitutively activated in the vast majority of primary samples: ras/raf/MEK/ERK (MAPK), STATs, and PI3K.[1] Our group chose to focus upon the phosphotidyl inositol-3'-kinase (PI3K) pathway as a therapeutic target in AML. This signal cascade is thought to contribute to survival and growth in tumor cells via downstream effects upon target proteins AKT/Protein kinase B and mammalian target of rapamycin (mTOR).[25]

In AML, we and others showed that PI3K signaling is constitutively activated in over 85% of primary samples and that the small molecule PI3K inhibitor LY294002 is cytotoxic *in vitro* to virtually all samples tested.[26-28] As LY294002 is poorly suited for drug development, we have concentrated upon other ways to inhibit signal transduction through this pathway. mTOR emerged as a logical target due to the availability of clinically available, highly specific inhibitors with favorable safety profiles. mTOR plays a central but complex role in cancer cells' metabolic regulation and survival. This serine/threonine kinase coordinates several important cellular functions and its activity is modulated in response to amino acid, glucose, oxygen, and ATP availability as well as extracellular growth factor ligation.[29] mTOR activity regulates protein translation, nutrient and amino acid uptake, mitochondrial respiration, glycolysis, cell size regulation, cell cycle entry and progression, ribosome biogenesis, and autophagy.[30] Constitutive mTOR activation is commonly seen in cancer cells and is thought to promote survival in the setting of a wide variety of cellular insults.[31] Importantly, constitutive mTOR activation may underlie chemotherapy resistance.[32-35] Although regulation of mTOR signaling in leukemia occurs through by several inputs, mTOR activity in AML (figure 1)[36] is thought to be primarily regulated by PI3K signaling through AKT via the intermediary tumor suppressor tuberous sclerosis complex (TSC1& 2) and its target rheb GTPase. Immediate downstream mTOR targets include p70S6 kinase (p70), which regulates S6 ribosomal protein (S6) to promote translational initiation and the eukaryotic initiating factor 4E binding protein 1 (4EBP1), which promotes cap-dependent protein translation.[37,38] We and others have found p70 and S6 ribosomal

protein phosphorylation to be a more robust assay to define mTOR kinase activity than 4EBP1 phosphorylation.[39]

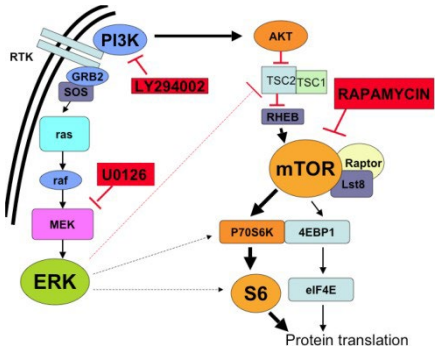

FIGURE 1: *Schematic of PI3K/AKT/mTOR (right) and ras/MAPK (left) signaling in AML. Pharmacologic inhibitors are shown in red. Black arrows show stimulation, red bars inhibition. In AML, mutations or autocrine stimulation of transmembrane receptortyrosine kinases (RTK), such as FLT3or c-KIT, initiates signaling through downstream pathways, and activate translational machinery through S6ribosomal protein (S6). Although the primary input to S6 is mTOR, note that ERK (alone or via its downstream targets RSK and/or MNK, not shown) potentially can fine tune the function of S6 cross-talk with PI3/K/mTOR at several levels (dotted lines).*

We demonstrated that p70 or S6 is constitutively activated in the vast majority of AML samples at initial diagnosis and virtually all samples at relapse.[36] In several cases where paired diagnosis and relapse samples were available, p70 or S6 phosphorylation increased at relapse (unpublished data). We also demonstrated that mTOR is necessary for cell survival in the setting of genotoxic stress (as described below). Taken together, mTOR is an attractive target for molecularly targeted therapy in AML due to its widespread activation, its necessity for AML cell survival in certain contexts, and its probable role in chemotherapy resistance and relapse.

We recently completed a pilot study of the combination of sirolimus and MEC and presented this data in combination with the data from our prior study. These studies demonstrated that patients who had baseline mTOR activation and were sensitive to therapy with sirolimus, as defined by a >40% reduction in pS6 positive blasts, had a 71% response rate while those who were “nonresponders” had a 20% objective response rate (ORR). The goal of the proposed clinical trial is to validate this finding in an adequately powered prospective clinical trial and to establish early change in pS6 positive blasts as a predictive biomarker.

2.2 Investigational Agent

Rapamycin, or sirolimus (Rapamune®, Wyeth), is a naturally occurring compound originally isolated from a soil saprophyte (*Streptomyces hygroscopicus)* found uniquely on Easter Island (Rapa Nui). In addition to its immunosuppressive properties (on which the clinical development of the drug has focused), rapamycin has antifungal, antiviral and antineoplastic properties. It is Food and Drug Administration (FDA) approved for immunosuppression following solid organ transplant. In this context, the drug is generally well tolerated and can be administered chronically. In addition to infections from chronic immuno-suppression, the drug’s side effect profile includes hypercholesterolemia and hyperglycemia but myelosuppression is uncommon and generally mild.

Rapamycin analogs such as RAD001 have shown anticancer activity in clinical trials of solid and hematologic malignancies and are undergoing FDA review for registration. It is not felt that the antitumor mechanism of these derivatives differs from that of rapamycin.

Although structurally similar to calcineurin inhibitors, rapamycin binds uniquely to FK binding protein 12 (FKBP12) and then complexes with mTOR (mammalian Target of Rapamycin). Rapamycin does not interact with calcineurin or its downstream effectors. The rapamycin-FKBP12-mTOR complex inhibits several distinct biochemical pathways, resulting in a reduction in DNA transcription, DNA translation,

protein synthesis and cell cycling. Ultimately this leads to the inhibition of the induction of activation and proliferation of mature T and B cells.[40,41]

Upstream pathways that interact with mTOR include the PTEN/PI3 kinase/AKT pathway which regulates cell growth, protein synthesis, and progression through the cell cycle. By inhibiting mTOR, rapamycin mimics growth-factor withdrawal.[42] This is a novel site of blockade not currently targeted by conventional cytotoxic agents.

2.3 Preclinical Data

There is extensive evidence that MTI (mTOR inhibitors) may inhibit the growth of and/or induce apoptosis in a wide variety of tumor types.[43-46] Additionally there is evidence of preclinical activity of MTI in leukemia as well as data supporting the potentiation of the activity of cytotoxic agents by MTI.

Researchers at the University of Pennsylvania campus have pioneered the study of mTOR inhibitors in the therapy of hematologic malignancies. The Wasik laboratory in the Department of Pathology originally demonstrated that the mTOR inhibitor, RAD001, was efficacious in a xenotransplantation model of post-transplant lymphoproliferative disease[45]. RAD increased the apoptotic rate in such cells and the drug had a profound inhibitory effect on the growth of PTLD-like Epstein-Barr virus+ B cells xenotransplanted into SCID mice. RAD001 is a derivative of rapamycin.

The Carroll laboratory has been studying the mTOR signaling pathway in primary cells from patients with acute myeloid leukemia. Dr. Carroll's group has shown that the signaling pathway is activated in the great majority (at least 80%) of patient's leukemic cells.[27] Furthermore, incubation of the cells with rapamycin or RAD001 leads to a modest decrease in survival of the leukemic cells. Other groups have published data on the ability of MTI to induce differentiation of human myeloid leukemia cells.[47] More importantly, combining RAD001 with chemotherapy leads to a dramatic enhancement in the efficacy of the chemotherapy.[36] Studies suggest that prolonged administration of RAD001 inhibits AKT. This novel effect of mTOR inhibitors may prove critical for synergy with chemotherapy.[48,49]

mTOR inhibitors inhibit cancer cell growth in vitro and in humans
Early studies of rapamycin also showed that the compound inhibited the growth of numerous cancer cell lines.[50] Although sirolimus's manufacturer chose not to develop this oral agent as an anticancer agent, the parenteral rapamycin prodrug temsirolimus (CCI-779) shows effectively an identical mechanism of action and has been tested in oncology clinical trials. In this context, temsirolimus demonstrates clinical efficacy in renal cell carcinoma and was FDA approved for this indication in 2007.[51] Temsirolimus also shows significant activity against hematopoietic tumors such as mantle cell and other lymphomas and is undergoing phase III studies in this setting.[52] Other rapamycin analogs ("rapalogs") such as everolimus (RAD-001) and the non-prodrug deforolimus (AP23573) are also undergoing development and show similar responses and toxicity profiles in early phase clinical trials of several tumor types.[48,53]

Pharmacodynamic analysis is necessary for proper use of signaling inhibitors
Pharmacodynamic study refers to the functional analysis of drugs' cellular effects. Studies of imatinib for chronic myeloid leukemia (CML) demonstrated the vital importance of this approach. Early studies confirmed imatinib's inhibition of BCR-ABL kinase in patients' leukemia cells as well as abrogation of downstream signaling during imatinib therapy.[21,54] This provided important insights into clinical response and dosing as higher imatinib doses were not clearly more efficacious at either signaling inhibition or clinical response. Based upon these data, the current strategy of using an optimal biologic dose, rather than the maximally tolerated dose has been adopted to guide initial therapy. Detailed analysis of patients' pharmacodynamic responses to mTOR inhibitors has only rarely been completed in primary tumor samples during clinical trials.[55,56] Although examination of mTOR inhibition has been widely reported from clinical trials samples, in almost all cases signaling was analyzed in non-malignant tissue, such as peripheral blood mononuclear cells (PBMCs). Such studies provide indirect pharmacokinetic information and NOT true pharmacodynamic analysis of tumor response. Such information can be useful to aid dose finding studies of novel agents, but provides no insight into tumor biology and does not define optimal

biologic doses of novel agents. Furthermore, even if PBMCs biochemical responses were known to correlate with initial tumor signal disruption, PBMC's responsiveness to signal inhibition by rapalogs has not predicted tumors' clinical responses to mTOR inhibitors. As such, these studies provide no way to evaluate for mechanisms of therapeutic resistance. Rapamycin's growth inhibitory concentrations in primary AML blast samples *in vitro* have been reported to span three orders of magnitude, which differ significantly from the narrow concentration range that inhibits lymphocyte proliferation.[57] Therefore pharmacodynamic studies of non-malignant tissue, although widely performed, provide largely uninterpretable and uninformative data to guide clinical development of these agents.

Despite the apparent ease of tumor sampling through blood draws or marrow aspirates, mTOR inhibitor trials in leukemia have generally not been subjected to detailed, systematic pharmacodynamic analysis. Recher's group, as part of their original description of rapamycin's cytotoxic *in vitro* effects upon acute myeloid leukemia (AML) blasts, treated 9 patients with refractory or relapsed AML with doses of rapamycin typically used in renal transplant. They saw reduction in peripheral or marrow blasts in 3 subjects as well as stabilization of previously rising leukocytosis in a fourth. Only one patient's pharmacodynamic response was reported, confirming p70 inhibition by Western blot.[57] The MD Anderson Cancer Center has enrolled over 50 patients in phase 1/2 clinical trials of CCI-779 and RAD-001 in leukemia and myelodysplastic syndromes. Of these patients, only 9 samples were reported for their pharmacodynamic effects, including 4 AML patients.[48,49] Another study attempted to use the quantitative method of phosphorylation-specific flow cytometry to analyze leukemia cells' biochemical response to deforolimus.[53] However, interpretation of their findings is still challenging. The investigators chose phospho-4EBP1 as a readout, which we and others have not found to be a robust predictor of mTOR inhibition by rapamycins (Carroll M unpublished, Grupp S, personal communication). Furthermore, available phospho-4EBP1 antibodies lack sufficient signal to noise ratios to produce meaningful results when analyzed by flow cytometry. The absence of positive and negative controls to the data provided from clinical samples in this report makes these results largely uninterpretable. A recent study utilized 3-color phospho-flow of gated blasts for pS6.[58] However, all samples were cryopreserved prior to flow analysis and no viability markers were employed to determine if lack of signaling changes observed reflected cells that did not survive freeze-thaw.

Taken together, it is unknown if mTOR inhibitors' modest clinical efficacy as single agents reflects marginal inhibition of tumor target, development of therapeutic resistance or both. To answer these questions, a detailed pharmacodynamic and pharmacokinetic analysis is necessary. Without such data, it is difficult to know how best to integrate mTOR inhibitors into combination chemotherapy regimens, which would be hoped to show enhanced response rates over single agent mTOR inhibitors alone.

The combination of sirolimus and chemotherapy is feasible and shows promising activity in AML
Although sirolimus, temsirolimus, everolimus, and deforolimus have all been tested as single agents in phase 1/2 clinical trials for relapsed and refractory AML, the studies to date have generally not shown significant anti-tumor responses. Detailed studies to validate inhibition of mTOR signaling either were performed in very few samples or yielded uninterpretable results. As such, it is not possible to judge why rapalogs are not active agents in these tumors as single agents at the doses and schedules explored.
Our group chose a different approach to develop mTOR inhibitors as anti-leukemic agents.
We and others reported that mTOR inhibitors dramatically enhance response to etoposide and other chemotherapeutics in *in vitro* and murine preclinical acute leukemia models. Based upon these results, our group hypothesized that mTOR inhibitors might function best as anti-leukemic agents when combined with established AML chemotherapy.

To determine the clinical efficacy of combined mTOR inhibition and etoposide-based cytotoxic chemotherapy, we performed a phase I clinical trial of sirolimus and the regimen MEC (mitoxantrone, etoposide, and high dose cytarabine) in relapsed, refractory, and secondary AML. Our phase I study is shown in Figure 2 (UPCC 07403, top). This study enrolled 29 subjects over 32 months and demonstrated the feasibility of the combination of sirolimus and intensive chemotherapy for AML. Dose limiting toxicities included prolonged marrow aplasia and multi-system organ dysfunction in one patient apiece at the highest explored dose. Other toxicities included transient hepatic transaminase elevation and diarrhea as

is common with MEC and grade 3-4 mucositis was uncommon. 12 subjects were treated at the maximally tolerated dose (MTD), without an obvious difference in toxicity from published data and institutional MEC experience.[19]

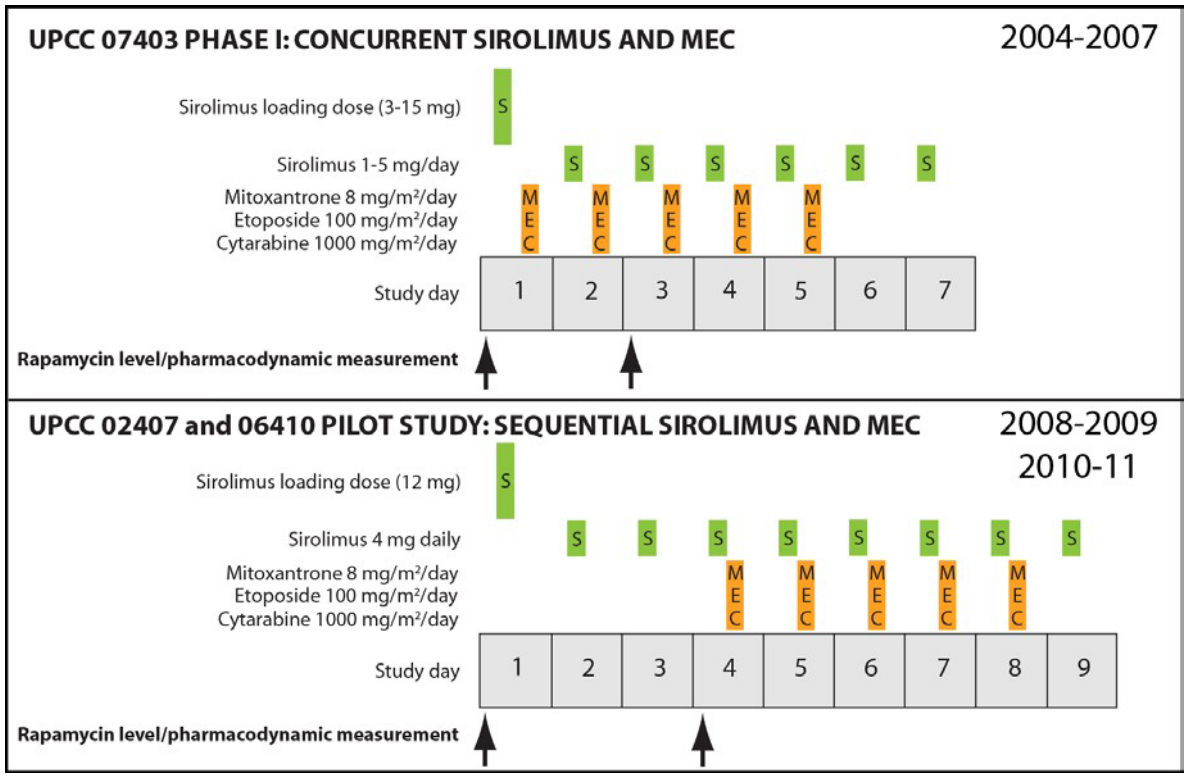

FIGURE 2: *Schema of drug administration for phase I (UPCC 07403, above) and pilot (UPCC 02407 and 06410, below) studies of sirolimus MEC. Arrows indicate pharmacodynamic sampling. Rapamycin trough levels were measured concurrent with the second pharmacodynamic measurement in each trial. In both studies, a loading dose of sirolimus was given on day 1 followed by a daily sirolimus dose that overlapped with MEC chemotherapy. UPCC 02407 and 06410 featured a "run-in" period of sirolimus monotherapy followed by combined sirolimus and MEC. Note: pharmacodynamic sampling occurred after MEC chemotherapy initiation on UPCC 07403 Phase I, but is prior to MEC on UPCC 02407 Pilot study.*

In summary, targeting the PI3 kinase is an attractive therapeutic strategy to induce cytotoxicity or enhance chemotherapy efficacy. mTOR inhibitors, such as sirolimus, can be given concurrent with induction chemotherapy such that the clinical and biochemical efficacy of this approach may be measured. Successful estimation of response rate and validation of target inhibition will require a phase 2 study with detailed pharmacodynamic analysis of leukemia cells during therapy.

2.4 Clinical Data to Date

Clinical experience with sirolimus and MEC

Since 2004, Thomas Jefferson University Hospital and the University of Pennsylvania have enrolled 81 subjects with AML to three studies of sirolimus and etoposide-based induction chemotherapy. The schema for administration of sirolimus and MEC chemotherapy (mitoxantrone, etoposide, cytarabine) are shown in Figure 2. All subjects received a standard dose of MEC and 64 of these subjects were treated at the recommended phase II dose (RP2D) of sirolimus (12 mg loading dose followed by 4 mg daily dose) in one of two sequences. The inclusion criteria for these two trials were largely similar and baseline

characteristics for age, prior therapy, and karyotype did not differ notably among subjects enrolled in these two studies.

Considering all three studies, 63 subjects treated at the RP2D are currently evaluable for response. The overall response rate (ORR) in these patients was 27/63 (43%) with 20/63 (32%) achieving complete response (CR) or complete response in the absence of total platelet recovery (CRp). Considering only patients treated with sequential sirolimus and MEC regimen, the ORR was 24/51 (47%) and CR/CRp rate was 18/51 (35%). This response rate appears improved compared to the historic CR rate of 21% in a very similar population from the ECOG 2995 study. Importantly, sirolimus MEC did not obviously increase the toxicity of MEC.

Pharmacodynamic analysis of patients treated on both sequential sirolimus MEC studies showed consistent and biologically interesting data. These studies demonstrated that patients who had baseline mTOR activation and were biochemically sensitive to therapy with sirolimus as defined by a >40% reduction in pS6 positive blasts had a 71% response rate while those who were “biochemically resistant” had a 20% ORR. The goal of the current study is to validate this finding in an adequately powered prospective clinical trial and to establish early change in pS6 positive blasts as a predictive biomarker.

Flow cytometric analysis of signal transduction in leukemic populations

Interpreting signaling data obtained from patients with relapsed/refractory AML is challenging. We suspect this largely reflects the low tumor burden at the time of enrollment to trials. At a first diagnostic sample of AML, marrow and blood samples are typically highly enriched for leukemia cells and it is not uncommon for >90% of the blood and marrow to be involved by blasts. However, at relapse, blast percentages are often low and patients may have hypocellular marrows from prior chemotherapy and pancytopenia. As such, the very population that enrolls on studies of novel agents introduces major challenges to the interpretation of resultant signaling data. This is particularly true when Western blot is employed. In this context, very few malignant cells are sampled and often a substantial amount of non-malignant tissue is collected. Because cell lysis prior to gel electrophoresis and western blot eliminates all cell-based information, it is generally not possible to determine whether signaling data obtained by Western blot is leukemia-specific or represents contaminating non-malignant cells. It is therefore not surprising that many researchers have shifted from primary tumor analysis to surrogate markers in order to validate and determine efficacy of signaling inhibitors. We sought instead to use leukemia-specific analytic tools.

Flow cytometry is a powerful technique for discriminating cells in suspension based upon their immunophenotype.[59,60] It is extraordinarily well suited to both clinical/diagnostic and research fields in leukemia, but has seen little use as a pharmacodynamic readout for clinical trials. Recently, advanced techniques have allowed for permeabilization of fixed cells such that the intracellular contents can be examined by this methodology.[61-63] While phospho-specific antibodies have demonstrated the feasibility of extensive intracellular flow cytometric studies on cell lines, marrow and blood samples from patients present a substantial challenge to the methodology due to the admixture of malignant and non-malignant populations. The harsh fixative and detergents or alcohols used to permeabilize cells traditionally create significant artifact, making interpretation challenging.[64] Careful protocols must therefore be developed that allow for preservation of cell surface epitopes throughout the various fixation, permeabilization, and red cell lysis steps. This has been a major obstacle to widespread use of this technology.

Recently, a whole blood technique for fixation and subsequent permeabilization has been developed and optimized by Chow and colleagues.[65] This methodology has been explored thoroughly in clinical leukemia samples including clinical trials of signal transduction inhibitors by David Hedley’s group in Toronto.[66-68] The method is simple and rapid and allows for examination of cytokine stimulation as well as signaling inhibition by various drugs. Importantly, the technique efficiently fixes cell processes, lyses red blood cells, and permeabilizes cells, yet preserves light scatter and cell surface epitopes. This allows for examination of signaling within cells based upon their immunophenotype and light scatter properties. Because simple dot-plots of orthogonal light scatter and hematopoietic markers such as CD45 allow for

segregation of hematopoietic cells into lymphocyte, monocyte, neutrophils, granulocytic progenitors, and blast populations, each can be gated and individually explored for intracellular signaling.[60]

After optimizing our approach for clinical samples, we explored its potential using peripheral blood samples collected from five subjects from the UPCC 02407 sequential sirolimus MEC pilot study. Of note, 3 of these 5 subjects had pancytopenia with too few circulating blasts to perform standard Western blot with confidence. All five subjects' baseline samples showed subset S6 positivity in leukemic blasts but not lymphocytes or granulocytes. Four of the five subjects showed definite inhibition of S6 on the sample acquired after 72 hours of sirolimus therapy. Interestingly, in none of these samples obtained at trough sampling was ex vivo treatment of their post-sirolimus sample by 1000 nM rapamcyin able to further inhibit S6 phosphorylation. This suggests that *in vivo* mTOR inhibition is maximal and that the optimal biologic dose has been achieved with our dose and schedule.

There are several important conclusions that we draw from these data. First, we demonstrate the feasibility of whole blood processing and flow cytometric data acquisition to provide leukemia specific signaling information devoid of contamination from non-malignant populations. Second, we demonstrate that S6 inhibition is not uniform among patient's AML blasts, confirming Western blot data suggesting rapamycin resistance in AML. The incidence and clinical significance of this finding is unknown. Third, consistent with published data from Chow and Hedley[66] we demonstrate that S6 activation only occurs in a subset of leukemic blasts. It is unknown if S6 activation represents a time dependent phenomenon from which we have only examined a snapshot. Alternatively, it is possible that subsets of blasts, or even subclones could rely upon different signaling networks, some of which may not require mTOR activation. This novel finding will require exploration outside of the context of the proposed project but is being actively pursued by members of the Carroll lab. Finally, we demonstrate that ex vivo stimulation and pharmacologic inhibition is necessary both to define positive and negative gates to compare response. It also provides useful data at the pharmacokinetic/pharmacodynamic interface to clarify dosing of mTOR inhibitors. Overall, intracellular flow cytometry is a powerful technique for the application of interpreting the results of clinical trials of signal transduction inhibitors targeting PI3K/AKT/mTOR pathway in AML.

In our pilot study of sequential sirolimus and MEC, we found flow cytometry yielded consistent and interpretable data in marginal samples with so few blasts that they were not amenable to Western blot analysis at all. Furthermore, even using these samples, we were still able to look specifically at signaling *within* blasts. This yielded leukemia-specific data with confidence and allowed for improved paired comparisons from baseline to post-treatment samples. Western blot can be quite robust among clinical samples where blasts are the predominant cell type among mononuclear cells and the percentage of malignant to non-malignant cells does not vary significantly from collection time point to time point. Given that effective anti-leukemic agents make these conditions highly unlikely during AML clinical trials, these necessary stipulations for quality control decrease confidence in Western blot results from leukemia clinical trials.

Thus, we have to date demonstrated the safety of the combination of sirolimus and MEC chemotherapy and developed a reproducible assay for target inhibition. On the initial pilot/feasibility study for our novel pharmacodynamic assay, we treated 10 patients after successful development of our flow cytometric assay. Those 10 patients provide preliminary data about the presence of mTOR target activation and the ability to inhibit target. Target activation was seen in 8/10 samples within the blast population (similar to data from western blot). Six of these 8 patients inhibited target after treatment with rapamycin.

We performed a biomarker development pilot/phase 2 study, in which 36 subjects were treated at the University of Pennsylvania (UPenn) and Thomas Jefferson University Hospital (TJUH). This study largely confirmed the feasibility of larger scale trials to provide robust pharmacodynamics using flow. In summary, we established that the vast majority of high risk AML patients (relapsed, refractory, secondary

and untreated elderly patients) show detectable S6 phosphorylation in leukemic blasts by flow and that serial measurement of S6 phosphorylation were feasible on our multicenter trial.

The clinical and pharmacodynamic results of our trial provided enticing data. Combining data from the two pilot studies, we generated paired pharmacodynamic data from 37 subjects who were evaluable for clinical response. This represents informative PD data in >80% of subjects for which the correlative studies were attempted. We noted that not only was activation of signaling through S6 heterogeneous, but only a subset of patients showed in vivo downregulation of pS6 during sirolimus therapy. Using ROC analysis, we established that a 40% reduction from basal pS6 activity could be used to discriminate patients with downregulation of pS6 from those without inhibition or with increased pS6 during therapy. Considering the response rates of patients with baseline pS6 who were biochemically sensitive to or resistant to sirolimus as part of the regimen, we observed that the ORR of biochemically sensitive patients was 12/17 (71%), while those with biochemical resistance showed a response rate of 2/10 (20%). This striking difference suggests that, while the ORR for all treated patients was 47% and that of patients with basal pS6 phosphorylation was 52%, the presence of in vivo target *inhibition*, rather than target activation was the best predictor of clinical outcome in our patients.

Our group is not alone in studying the integration of mTOR inhibitors into chemotherapy regimens for AML. Various other groups have used alternative drugs and schedules of mTOR inhibitors, with recently reported results summarized here:

> The United Kingdom- Medical Research Council (UK-MRC) closed the arm of its AML-17 study that asiggned newly diagnosed patients everolimus as a single agent as chronic maintenance therapy following multiagent chemotherapy as the drug failed to improve RFS or overall survival (Alan Burnett, ASH 2012 oral presentation and Steven Knapper, personal communication). Of note, everolimus did not have prior single agent in AML,[48] and, on the UK-MRC trial, was only used in patients with *chemotherapy-responsive* leukemia. Therefore, it is unclear that this trial's results can be extrapolated to studies where mTOR inhibitors are used for short periods in combinations in an attempt to enhance concurrent chemotherapy-response in high risk patients.
>
> The French GOELAMS cooperative group recently completed a phase 1b study evaluating everolimus in relatively favorable risk relapsed patients (those with first remissions lasting >12 months).[69] This study showed a very high response rate, particularly among patients treated at the highest dose levels. While pharmacokinetics of everolimus were modeled in a surrogate marker assay (plasma inhibition assay in cell lines), and no patient tumor pharmacodynamics were performed. Interestingly, using this *ex vivo* cell line assay the group similarly found a cutoff that enriched for response among patients whose drug levels reduced p70S6K activity on western blot by 30-40%. Patients whose free drug levels exceeded this degree of inhibition showed substantially higher responses than those with lower drug concentrations.
>
> The Italian GIMEMA cooperative group recently published a study of low dose clofarabine and temsirolimus in relapsed patients.[58] Importantly, this study used phospho-flow performed on blasts obtained at baseline and day 2 of therapy. Of note, by performing these studies on ficolled, cryopreserved samples, and with only 3-color flow panels, less than half of enrolled subjects yielded interpretable correlative data. The flow data on this study used limited controls for S6 phosphorylation and viability markers for the previously cryopreserved cells were not employed. Although the low intensity salvage regimen had limited activity in this population, similar to our observations, all responses occurred among patients who downregulated pS6 during therapy (9/12, 75% CR rate if biochemically sensitive) with no activity of the regimen among patients with biochemical resistance (0/13 CRs).

Taken together our data, combined with those from French and Italian Cooperative groups show that the combining mTOR inhibitors with chemotherapy can be associated with in tumor downregulation of mTOR signaling in a subset of patients. This finding appears to strongly enrich for responding patients and thus

flow cytometric analysis for pS6 may prove to be a useful biomarker to guide patient selection for the approach.

2.5 Dose Rationale and Risk/Benefits

The pharmacokinetics of sirolimus have been extensively studied in healthy subjects, pediatric dialysis patients, hepatically-impaired adult patients, and adult renal transplant patients.[70,71] Oral doses of both liquid and solid sirolimus are rapidly, though variably, absorbed. Mean time-to-peak concentrations range from 1 hour in healthy subjects to 2 hours in renal transplant recipients. Half-life is upwards of 2 days. Metabolism is by the intestinal and hepatic CYP3A4 enzyme family and 91% of the elimination of the drug is via the GI tract. The AUC correlates well with trough and peak concentrations. Patients who ingested the drug after a high fat breakfast did have delayed C_{max} and it is recommended to consistently take sirolimus with or without food. Known toxicities are listed in 7.1.1.

In a Phase I pharmacokinetic study conducted in renal transplant patients doses ranging from 0.5 to 6.5 mg/m^2 were administered every 12 hours.[71] Phase III studies to date have had concomitant use of cyclosporine, steroid, or both. At a dose of 2mg/day the rapamycin trough concentration was 8.58 ±4.0 ng/ml and at 5 mg/day the trough was 17.3 ± 7.4 ng/ml. Rapamycin concentrations in stable renal transplant patients are dose proportional between 3 and 12 mg/m^2. Also, in this population a loading dose of 3 times the maintenance dose provided near steady-state concentrations within 1 day in most patients. Stable renal transplant recipients have received single doses of up to 21 mg/m^2. No toxicity has been observed in any of several single dosing studies with sirolimus doses ranging from 3-21 mg/m^2.

In this study, we will give a loading dose on Day 1 of 12 mg followed by daily doses of 4 mg for 2 days prior to beginning MEC and continuing until 1 day following completion of MEC. This dose of sirolimus was discovered to be the MTD in the Phase 1 trial and was verified as safe in two subsequent pilot studies.[72]

The total administered dose of chemotherapy may be rounded up or down within a range of 5% of the actual calculated dose.

Prospective subjects will be informed of all anticipated adverse effects of drug treatments. All of the drugs studied in this proposal have been used widely in humans at the doses described. Anticipated treatment-related adverse events are listed in section 7.1.2-7.1.4.. Treatment-related mortality can occur from intensive antileukemic therapy and is estimated at 5-10%. Alternative therapies, including standard chemotherapy, investigational agents, and supportive care-only, will be discussed with potential subjects prior to informed consent.

Because sirolimus is an immunosuppressant, the infectious toxicity of this regimen may be increased compared to MEC chemotherapy alone. A total of 4 infectious deaths have occurred in the 45 subjects treated on both sirolimus + MEC studies (9%), including 2/27 (7%) at the doses planned for this trial. This rate of toxic deaths does not differ from published data, but this important safety signal will be monitored closely. A formal stopping rule with respect to safety is not included, but consideration for early termination will be made in consultation with the medical monitor and biostatistician if excess toxicity is suspected.

2.6 Potential Benefits

Should sirolimus MEC improve response rate--a surrogate for survival in AML-- participation would provide substantial benefit for study participants. An additional and more certain benefit is the scientific knowledge gained from this clinical trial's correlative studies. This may improve understanding of

chemotherapy response in AML. Although study participants cannot be guaranteed benefit, the information gained may benefit cancer patients in the future.

3 Study Objectives

Primary Objective:

- The primary objective is to test the association between biochemical response and clinical response.

Secondary objectives:

- To estimate complete response rate of sirolimus MEC inpatients with high risk AML.
- To estimate progression free survival in this patient population.
- To collect further information on the safety, tolerability, and efficacy of sirolimus in combination with MEC in patients with relapsed or refractory myeloid malignancies.

4 Study Design

4.1 General Design

This is a single arm, phase 2 biomarker validation study. Subject enrollment will continue until sufficient numbers of biomarker evaluable (have sufficient blast count) patients have completed study therapy and undergone disease response assessment. Pharmacodynamic analysis will proceed in real time to ensure proper enrollment of biomarker evaluable subjects. Subjects will be followed for two years after the last enrolled subject prior to final data analysis to examine effects upon relapse-free and overall survival.

Subjects will undergo a marrow aspirate/biopsy for response evaluation within one week of peripheral count recovery but no later than Study Day 45 (i.e. 42 days after initiation of MEC) for response assessment.

We will be measuring the ability of oral sirolimus to inhibit mTOR in leukemic blasts. This will be measured by intracellular flow cytometry for phosphorylation of the downstream signaling target S6 ribosomal protein as a surrogate for mTOR activity. Inhibition of S6 phosphorylation by flow will be correlated with clinical outcome. Additionally, the *in vivo* effects of sirolimus will be compared to a whole blood *ex vivo* rapamycin dose response curve for each sample as well as to rapamycin levels obtained at

the time of pharmacodynamic measurements. Flow results will be confirmed by Western blot as appropriate.

4.2 Primary Study Endpoints

The primary study endpoint is to determine whether there is an association between biochemical response to mTOR inhibitor therapy and clinical response.

Early change in mTOR kinase activity is defined by % reduction in pS6 positive blasts = [(baseline pS6 - day 4 trough pS6)/baseline pS6].

Biochemical response is defined by >40% reduction in pS6 positive blasts is termed "rampamycin sensitive". Either ≤40% reduction or an increase in pS6 positive blasts is termed "resistant".

4.3 Secondary Study Endpoints

Response will be judged based upon International Working Group (IWG) criteria[73]
Response Criteria

Complete Remission (CR) -

- Peripheral Blood Counts -Neutrophil count ≥1 x 10^9/L.
- Platelet count ≥ 100 x 10^9/L.
- Reduced hemoglobin concentration or hematocrit has no bearing on remission status, but patients must be transfusion independent for at least one week.
- Leukemic blasts must not be present in the peripheral blood.
- Cellularity of bone marrow biopsy must trilineage with maturation of all cell lines, < 5% blasts, and no Auer rods.
- Extramedullary leukemia, such as central nervous system (CNS) or soft tissue involvement, must not be present.

Complete Response in the absence of total platelet recovery (CRp) –

- Bone marrow (<5% blasts) with trilineage bone marrow cellularity, no evidence of circulating blasts or extramedullary disease and normalization of peripheral blood counts except for platelets (neutrophil count ≥1,000/μL).
- Patients must be transfusion independent for at least one week.

Complete Response with incomplete recovery (CRi)

- Bone marrow (<5% blasts) with trilineage bone marrow cellularity, no evidence of circulating blasts or extramedullary disease and normalization of peripheral blood counts except for platelets <100 x 10^9/L or neutrophil count <1,000/μL).
- Patients must be transfusion independent for at least one week.

Partial Remission (PR)

- Requires that all of the criteria for complete remission (CR) be satisfied except that the bone marrow may contain ≥ 5% blasts but < 25% blasts.
- A marrow with <5% blasts that contain Auer rods will also be considered a PR

Progressive Disease (PD)

- An increase of at least 25% in the absolute number of leukemic cells in peripheral blood or bone marrow/aspirate, the development of extramedullary disease, or other evidence of increased tumor burden.

Relapse following complete remission

- Reappearance of leukemic blasts in the blood.

- Presence of 5% blasts, not attributable to another cause (e.g., bone marrow regeneration).
- If there are no circulating blasts and the bone marrow contains 5% to 20% blasts, then a repeat bone marrow performed 1 week later documenting more than 5% blasts is necessary to meet the criteria for relapse.

Responders
- Subjects achieving a CR, CRp, CRi, or PR.

Non-responders
- Subjects achieving PD or relapse following complete remission

Objective Response Rate (ORR)
- Fraction of patients who achieve CR, CRp, or PR.

Relapse Free Survival
- The time from study entry to first documented progression, death, or last contact

Overall Survival
- The time from study entry to death or last contact

4.4 Primary Safety Endpoints
Trial toxicities will be recorded and graded per NCI CTCAE Version 4.0 guidelines.

5 Subject Selection

5.1 Inclusion Criteria
1. Patients must have histologic evidence of high risk acute myeloid leukemia defined as one of the following:
    a. Primary refractory non-M3 AML
        (i) Residual leukemia after a minimum of 2 prior courses of chemotherapy (Same or different)
        (ii) Evidence of leukemia recurrence after a nadir bone marrow biopsy demonstrates no evidence of residual leukemia.
        (iii) Evidence of leukemia after induction therapy which, in the opinion of the investigator, would be appropriate for reinduction with sirolimus/MEC therapy.
    b. Relapsed non-M3 AML
    c. Previously untreated non-M3 AML age >60 with no evidence of favorable karyotype defined by presence of t(8;21)(q22;q22) [AML1-ETO], inv16(p13;q22), or t(16;16)(p13;q22) [CBFβ;MYH11] by cytogenetics, FISH, or RT-PCR
    d. Previously untreated secondary AML (from antecedent hematologic malignancy or following therapy with radiation or chemotherapy for another disease) with no evidence

of favorable karyotype defined by presence of t(8;21)(q22;q22) [AML1-ETO], inv16(p13;q22), or t(16;16)(p13;q22) [CBFβ;MYH11] by cytogenetics, FISH, or RT-PCR

2. Subjects must be ≥ 18 years of age.
3. Subjects must have an ECOG performance status of 2 or less (see Appendix1).
4. Subjects must have a life expectancy of at least 4 weeks.
5. Subjects must be able to consume oral medication.
6. Subjects must have recovered from the toxic effects of any prior chemotherapy to ≤ Grade 1 (except alopecia).
7. Required initial laboratory values:
    a. Creatinine ≤ 2.0mg/dL;
    b. total or direct bilirubin ≤ 1.5mg/dL; SGPT (ALT) ≤ 3xULN;
    c. negative pregnancy test for women with child-bearing potential.
8. Patients must be able to sign consent and be willing and able to comply with scheduled visits, treatment plan and laboratory testing.
9. Subjects must have a left ventricular ejection fraction (LVEF) of ≥ 45%.

5.2 Exclusion Criteria

1. Subjects with FAB M3 (t (15; 17) (q22; q21) [PML-RARα]) are not eligible.
2. Subjects must not be receiving any chemotherapy agents (except Hydroxyurea).
    a. Intrathecal methotrexate and cytarabine are permissible.
3. Subjects must not be receiving growth factors, except for erythropoietin.
4. Subjects with a “currently active” second malignancy, other than non-melanoma skin cancers are not eligible.
5. Subjects with uncontrolled high blood pressure, unstable angina, symptomatic congestive heart failure, myocardial infarction within the past 6 months or serious uncontrolled cardiac arrhythmia are not eligible.
6. Subjects taking the following are not eligible:
    - Carbamazepine (e.g., Tegretol)
    - Rifabutin (e.g., Mycobutin) or
    - Rifampin (e.g., Rifadin)
    - Rifapentine (e.g., Priftin)
    - St. John's wort
    - Clarithromycin (e.g., Biaxin)
    - Cyclosporine (e.g. Neoral or Sandimmune)
    - Diltiazem (e.g., Cardizem)
    - Erythromycin (e.g., Akne-Mycin, Ery-Tab)
    - Itraconazole (e.g., Sporanox)
    - Ketoconazole (e.g., Nizoral)
    - Telithromycin (e.g., Ketek)
    - Verapamil (e.g., Calan SR, Isoptin, Verelan)
    - Voriconazole (e.g., VFEND)
    - Tacrolimus (e.g. Prograf)

    Subjects taking fluconozole, voriconizole, itraconazole, posaconazole, and ketokonazole within 72 hours of study drug starting are not eligible. Reinstitution of fluconozole, voriconizole, itraconazole, posaconazole, ketokonazole and diltiazem is permissible 72 hours after the last dose of sirolimus.

7. Subjects who require HIV protease inhibitors or those with AIDS-related illness
8. Subjects with other severe concurrent disease which in the judgment of the investigator would make the patient inappropriate for entry into this study are ineligible.
9. Subjects must not have evidence of cerebellar dysfunction at baseline or during prior cytarabine therapy
10. Subjects must not have received any investigational agents within 30 days of study entry.
11. Subjects must not be pregnant or breastfeeding. Pregnancy tests must be obtained for all females of child-bearing potential. Pregnant or lactating patients are ineligible for this study due to

the unknown human fetal or teratogenic toxicities of sirolimus. Males or females of reproductive age may not participate unless they have agreed to use an effective contraceptive method.

12. Subjects who have uncontrolled infection are not eligible. Patients must have any active infections under control. Fungal disease must be stable for at least 2 weeks before study entry.
13. Subjects with bacteremia must have documented negative blood cultures prior to study entry.

6 Study Procedures

6.1 Subject Recruitment and Screening

Patients will be recruited from the practices of the Medical Oncology Department of the Thomas Jefferson University Hospital. Advertisements will not be used to recruit patients for this study other than the Jefferson website. The patients are not excluded based on gender, race or economic status. Patients who meet eligibility criteria will be invited by their physician to participate in the study. All therapeutic options will be discussed with the patient and the patient's questions will answered to the patient's satisfaction. Patients will be asked to read, comment/ask questions about the study and then sign the informed consent form before study procedures are to take place.

Bone marrow biopsy/aspiration will be utilized to determine eligibility for the study. Screening laboratory work to be performed will include blood chemistries, pregnancy testing (if applicable) and a physical assessment

6.2 Subject Enrollment

At enrollment, each subject will have a medical history taken as well as a physical exam (including neurological exam), vital signs, performance status assessment, and laboratory studies including CBC with differential, liver function tests, electrolytes, uric acid, and glucose as well as an HSV titer, and pregnancy test, if applicable. Subjects who have not had a bone marrow biopsy sample banked in the Stem Cell and Xenograft Core within the 4 weeks prior to study entry will require a bone marrow biopsy and aspirate.

When the subject is admitted to the hospital, they will have daily assessments including adverse event assessments, laboratory studies, and physical exams through Day 9 and then weekly as detailed in the study procedure table found in Appendix 2.

Subjects will be followed for disease status and survival for 2 years following the enrollment of the last subject. These follow-ups will be via phone call, chart review, or in conjunction with their routine office visits every 3 months.

6.3 Laboratory Assessments

Plasma Rapamycin (PK): levels will be determined by commercially available assay. Rapamycin levels will be **just prior** to the dosing on Days 4. Three to 4 cc of peripheral blood will be collected in a purple

top (EDTA) tube to be sent to the local lab. The exact time that the sample is drawn along with the exact time that the last dose of drug was administered will be recorded.

Pharmacokinetic samples will be drawn on day 4 troughs and tested for sirolimus concentration by high performance liquid chromatography (HPLC) using the clinical laboratories of the respective treatment centers.

Pharmacokinetic assessment at Day 4 will assess levels of the drug in vivo. The level will be drawn prior to initiation of chemotherapy to allow for a PK/PD correlation study. No dose adjustment is planned on the basis of these studies.

Biologic Studies (PD)

Pretreatment bone marrow aspirate: 5-10 cc of Bone Marrow Aspirate in preservative-free heparin (green top) to be sent to the Stem Cell and Xenograft Core at the University of Pennsylvania. Aspirates should be drawn through a heparinized syringe.

Peripheral Blood: 20 ml in a green top tube to be sent to the Stem Cell and Xenograft Core (if peripheral blast are ≥5000/μl then a Bone Marrow Aspirate is not required). Both marrow and blood will be sent for any patient with a peripheral blast count ≤ 5000/μl and ≥ 200/μl.

Day 4 Bone Marrow: 5-10 cc of Bone Marrow Aspirate in preservative-free heparin (green top) to be sent to the Stem Cell and Xenograft Core only if pretreatment blasts are ≤200/μl otherwise 10cc peripheral blood may be substituted.

Pharmacodynamic samples are to be drawn into heparinized tubes containing >2 ml and delivered to Dr. Perl's laboratory (Room 720 Biomedical Research Building II/III, 421 Curie Blvd University of Pennsylvania Philadelphia PA 19104).

6.4 Early Withdrawal of Subjects

6.4.1 When and How to Withdraw Subjects

Subjects will continue on study treatment unless the following occur:

1. Non-compliance by the subject with protocol requirements
2. Changes in medical status of the subject such that the investigator believes that the treatment is no longer in the subject's best interest.
3. Subject refusal
4. Disease Progression

Subjects who are unable to provide samples that can be evaluated for response will be replaced.

The discontinuation of study treatment will not constitute study withdrawal or study completion. In the event of a decision to discontinue treatment, the treatment phase will be considered complete and the follow-up phase will begin.

If a subject discontinues treatment and actively withdraws consent, no additional data will be collected.

7 Study Drug & Chemotherapy

7.1 Descriptions

7.1.1 Sirolimus

Drug Name: Sirolimus

Other Names : rapamycin,Rapamune®

Classification : Immunosuppressant Agent

Mode of Action: Sirolimus inhibits T-lymphocyte activation and proliferation in response to antigenic and cytokine stimulation. Its mechanism differs from other immunosuppressants.

Storage and Stability: Store at room temperature, 20ºC to 25ºC (68ºF to 77ºF); protect from light.

Dose Specifics: Assigned dose level. Subjects will receive a12mg loading dose of Sirolimus. The following day, they will receive a daily dose of 4mg of Sirolimus once every 24 hours (±2 hours) for 8 days. The daily sirolimus dose should be administered in the morning around 9 am to facilitate research procedures, i.e. blood draws and/or marrow aspirates.

Preparation: 1 and 2 mg tablet and oral suspension.

Administration: Sirolimus is to be taken by mouth. The drug should be consistently given *without* food.

Incompatibilities: Diltiazem increases serum concentrations of sirolimus. Sirolimus serum concentrations may be increased with use of voriconazole, itraconazole, ketoconazole and fluconazole. Concurrent use of these drugs is contraindicated.

Availability: Available as Rapamune® 1mg tablet and 2mg tablets. It will be available through the Investigational Drug Services and purchased from Wyeth.

Side Effects
Common (21-100% Frequency) Immediate: Nausea, Vomiting Prompt: Within 2-3 weeks, prior to next course. Diarrhea, Hypertension, Increased creatinine Delayed: Any time after above. Hypercholesteremia, Hypertriglyceridemia
Occasional (5-20% Frequency) Immediate: Rash Prompt: Within 2-3 weeks, prior to next course. Infections; edema; weight gain, arthralgia; tremor; acne; myelosuppression; abdominal pain; myalgia; hypokalemia; hypophosphatemia Delayed: Any time after above. Stomatitis
Rare (< 5% Frequency) Hepatotoxicity Delayed: Any time after above. Hirsutism, pneumonitis, Secondary Lymphoma

Nursing/Patient Implications:
1. Monitor blood pressure and serum creatinine.

7.1.2 Mitoxantrone

Other Names: Mitoxantrone hydrochloride, Novantrone dihydroxyanthracenedione, DHAD, DHAQ.

Classification: Antitumor antibiotic (anthracenedione derivative).

Mode of Action: Mitoxantrone's precise mechanism of action is not fully known. The drug has been shown to intercalate between DNA base pairs. Mitoxantrone interacts with DNA and RNA through other mechanisms, including inhibitions of topoisomerase II, which may account for its cytocidal activity.

Storage and Stability: Intact vials are stored at room temperature. Storage under refrigeration may cause formation of a precipitate that redissolves upon warming to room temperature. Once mixed with normal saline or 5% dextrose, the drug is chemically stable for at least 48 hours at room temperature.

Concentrations of 0.02-0.5 mg/ml in normal saline or 5% dextrose are chemically stable for at least 1 week at room temperature.

Dose Specifics: 8 mg/m^2/day for 5 days

Preparation: Dilute in at least 50ml normal saline or 5% dextrose prior to administration

Administration: Mitoxantrone has been given by IV push (over 3 minutes or more), but it is recommended that the drug be administered by slow IV infusion, over 5-15 minutes. The drug has also been given as a

continuous IV infusion, intraperitoneally, intravesicularly, intramuscularly and intrathecally. Although it is not classified as a vesicant, tissue damage due to extravasation has been reported.

Incompatibilities: Heparin (1-10 U/ml with mitoxantrone 50-200 µg/ml) causes an immediate precipitate. Hydrocortisone sodium phosphate (2 mg/ml with mitoxantrone 50 µg/ml) causes an immediate precipitate.

Availability: Commercially available as a 2 mg/ml solution (20, 25, and 30 mg/vial).

Side Effects
1. Hematologic: Leukopenia, thrombocytopenia, and anemia.
2. Dermatologic: Alopecia (mild), pruritus, dry skin.
3. Gastrointestinal: Nausea and vomiting (usually preventable); diarrhea, mucositis, abdominal pain.
4. Cardiovascular: Cumulative cardiomyopathy (congestive heart failure); arrhythmias; tachycardia; chest pain.
5. Allergic: Hypotension, urticaria, and rash.
6. Hepatic: Transient increases in serum glutamate-oxaloacetate transaminase (SGOT); jaundice (rare); hyperbilirubinemia.
7. Neurologic: Headache; seizures (rare).
8. Pulmonary: Cough, dyspnea.
9. Other: Blue discoloration of sclerae, veins; blue discoloration of the urine and stool, may persist for 24 to 48 hours after administration; fever; conjunctivitis; phlebitis; amenorrhea; tissue ulceration and necrosis upon extravasation (rare).

Nursing Implications
1. Monitor complete blood count (CBC), platelet count, SGOT, bilirubin.
2. Administer antiemetics as indicated.
3. Advise patient that blue/green discoloration of urine and stool may occur for 24-48 hours.
4. Monitor for signs and symptoms of cardiomyopathy and calculate total cumulative dose with each administration.
5. Monitor IV site for signs of phlebitis or extravasation.
6. Monitor for GI symptoms (diarrhea, stomatitis, and abdominal pain) and treat symptomatically.
7. Advise patient that mild alopecia may occur.

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7.1.3 Etoposide

Other Names: VP-16, VePesid, VP-16-213, EPEG, and epipodophyllotoxin, NSC # 141540

Classification: Podophyllotoxin derivative.

Mode of Action: Etoposide inhibits the enzyme topoisomerase II and causes DNA breakage.

Storage and Stability: The injection should be stored at room temperature; the capsules must be refrigerated (2-8E C). Following in 0.9% sodium chloride or 5% dextrose to dilution concentrations of 0.2-

0.4 mg/ml the drug is chemically stable for 96 and 48 hours at room temperature, respectively. Bristol-Myers in- house data indicate that etoposide may be stable in 5% dextrose or normal saline for 24 hours (0.6 mg/ml), 4 hours (1mg/ml), and 2 hours (2 mg/ml), but these concentrations may lead to precipitation and are not recommended for general practice.

Dose Specifics: 100 mg/m^2/day for 5 days, as per protocol outlined by Greenberg in the ECOG 2995 study[73]

Preparation: The desired dose is usually diluted to a concentration of 0.4 mg/ml in normal saline or 5% dextrose. More concentrated solutions may be used but have shorter stability (and may precipitate).

Administration Slow IV infusion over 60 minutes.

Compatibilities: Compatible with cytarabine.

Availability: Commercially available as an injection in 50 mg and 100 mg (20 mg/ml) multiple dose vials and in 50 mg pink capsules for oral use.

Side Effects

1. Hematologic: Leukopenia, dose-related, primarily granulocytopenia; nadirs within 7-14 days and recovery within 20 days of administration; thrombocytopenia, uncommon; anemia.
2. Dermatologic: Alopecia is generally mild, reversible and is reported to occur in 20-66% of the patients, although some patients develop total baldness; rash (rare), severe pruritus (rare), radiation recall reaction (rare), phlebitis, local pain at injection site, pigmentation (rare).
3. Gastrointestinal: Nausea and vomiting, relatively uncommon, but more frequent with oral dosing; anorexia in 10-13% of patients; significant mucositis and enteritis requiring narcotic analgesia and

parenteral nutrition was seen in some patients; abdominal pain, diarrhea, aftertaste, parotitis, dysphagia, and constipation occur rarely.
4. Hypersensitivity: Anaphylaxis (rare).
5. Hepatic: Hyperbilirubinemia and increased transaminase levels, usually mild and transient.
6. Cardiovascular: Transient hypotension, associated with rapid administration; transient hypertension (rare); other cardiovascular events (*e.g.* congestive heart failure) thought to be related to large amounts of sodium chloride administered with the drug.
7. Neurologic: Peripheral neuropathy, somnolence, fatigue, headache, vertigo, transient cortical blindness (all rare); transient confusion with high doses, perhaps due to the alcohol-containing vehicle.
8. Other: Rarely, fever, muscle cramps, metabolic acidosis, and hyperuricemia.
9. Secondary leukemia: high risk with large cumulative doses, other drugs in same family add to risk (*i.e.*, teniposide).

Nursing Implications
1. Monitor CBC, platelet count.
2. Advise patient of possible alopecia. Instruct how to obtain wig, hairpiece, etc.
3. Infuse drug over at least 30 minutes. A more rapid infusion may cause hypotension.
4. Observe for possible phlebitis at injection site or burning pain with infusion.
5. Monitor for anaphylactoid reaction (rare).
6. Administer antiemetics as indicated.
7. Track cumulative doses, particularly in pediatrics, adolescents.

References
1. van Maanen JMS, *et al.* Mechanism of action of antitumor drug etoposide: A review. J Natl Cancer Instit 80:1526-1533, 1988.
2. Stewart CF, Hampton EM. Stability of cisplatin and etoposide in intravenous admixtures. Am J Hosp Pharm 46:1400-1404, 1989.
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4. Fleming RA, *et al.* Etoposide: An update. Clin Pharm 8:274-293, 1989.
5. O'Dwyer PJ, *et al.* Etoposide, current status of an active anticancer drug. N Engl J Med 312:692- 700, 1985.

7.1.4 Cytarabine

Other Names: Cytosar-U, Arabinosyl, Ara-C, cytosine arabinoside

Classification: Antimetabolite.

Mode of Action: Converted to cytarabine triphosphate (Ara-CTP), a competitive inhibitor of DNA polymerase. The drug is also incorporated into cellular DNA and RNA. Active against cells in S-phase and is considered phase specific.

Storage and Stability: Intact vials are stored at room temperature. After reconstitution, the solution is stable for 7 days at room temperature and 15 days when refrigerated. Solutions with a slight haze should be discarded.

Dose Specifics: 1000 mg/m^2/day for 5 days, as per protocol outlined by Greenberg in the ECOG 2995 study.[73]

Preparation: Preservative-free sterile water should be used for reconstitution. Add 5 ml to the 100mg vial, resulting in a concentration of 20 mg/ml. Add 10 ml of sterile water to the 500 mg vial to get 50 mg/ml.

Add 10 ml to the 1-gram and 20 ml to the 2 gram vials to get 100 mg/ml. Note: Since cytarabine is very soluble, smaller amounts of diluent may be used.

Administration: Intravenous infusion over 1 hour; longer infusions (especially if administered over >3 hours) potentially enhance neurotoxicity.

Incompatibilities: Possible interaction with fluorouracil has been reported to decrease the cellular uptake of methotrexate and thereby reduce its effectiveness.

Compatibilities: Cytarabine (0.26 mg/ml) and etoposide (0.4 mg/ml) are stable in D5/0.45% NaCl for 72 hours at room temperature. Cytarabine is also compatible with sodium chloride, potassium chloride, calcium, and magnesium sulfate.

Availability: Commercially available in 100 mg, 500 mg, 1000 mg, and 2000 mg vials.

Side Effects

1. Hematologic: Leukopenia, thrombocytopenia, anemia; recovery generally occurs in 16-42 days.
2. Dermatologic: Transient skin erythema without exfoliation, alopecia.
3. Ophthalmic: Conjunctivitis, keratitis (usually on days 1-3), and photophobia, reduced with prophylactic glucocorticoid eye drops.
4. Gastrointestinal: Nausea, vomiting, diarrhea, (potentiated with the addition of anthracycline), metallic taste, dysphagia, stomatitis; severe gastrointestinal ulceration, peritonitis, necrosis has occurred.
5. Hepatic: Transient elevations of SGOT/SGPT, lactate dehydrogenase (LDH), and bilirubin.
6. Neurologic: Cerebral and cerebellar dysfunction, ataxia, confusion and coma; more pronounced when receiving anthracycline concomitantly; transient (3-7 days), dose-related (> 3 g/day or > 36 g/m^2/course), and associated with previous CNS therapy; usually reversible. Peripheral and sensory neuropathies also occur.
7. Pulmonary: Rare syndrome of sudden respiratory distress, rapidly progressing to pulmonary edema and cardiomegaly.
8. Cardiovascular: Rare cardiomegaly, pericarditis with tamponade.
9. Other: Flu-like syndrome, fever and arthralgias; hyperuricemia, hyperphosphatemia if tumor lysis syndrome develops; phlebitis.

Nursing Implications

1. Neurological assessment prior to each dose. Hold drug and notify physician if any neurological changes have occurred (***e.g.***, nystagmus, ataxia, and dysarthria). Administration period should not be

prolonged beyond recommended 1 or 3 hours per dose. Longer administration periods at these doses are felt to increase cardiotoxicities.
2. Patient teaching related to prolonged myelosuppression.
3. Monitor CBC, platelet counts.
4. Administer antiemetics as needed.
5. Monitor for stomatitis and make recommendations for oral care.

References

1. Early AP, Preisler HD, Slocum H, Rustum YM. A pilot study of high-dose 1-B-Darabinofuranosylcytosine for acute leukemia and refractory lymphoma: Clinical response and pharmacology. Cancer Res 42:1587-1594, 1982.
2. Hines JD, Oken MM, Mazza JJ, *et al.* High dose cytosine arabinoside and M-AMSA is effective therapy in relapsed ANLL. J Clin Oncol 2:545-549, 1984.
3. Salinsky MC, Levin RL, Aubuchon JP, Schutta HS. Acute cerebellar dysfunction with high-dose Ara-C therapy. Cancer 51:426-429, 1983.
4. Lass JH, *et al*. Topical corticosteroid therapy for corneal toxicity from systemically administered cytarabine. Am J Ophthalmol 94:617-21,1982.
5. Nand S, *et al*. Neurotoxicity associated with systemic high dose cytosine arabinoside. J Clin Oncol 4:571-575, 1986.
6. Herzig R, *et al*. Central nervous system effects of high dose cytosine arabinoside. Semin Oncology 14:21-24, 1987.

7.2 Treatment Regimen

Sirolimus will be given by mouth as a 12mg loading dose on day 1, followed by 4mg/day on days 2-9. Sirolimus should be taken around 9 a.m. without food.
MEC (Mitoxantrone 8mg/m^2/day IV over 15 minutes in 150 ml D5W, Etoposide 100mg/m^2/day IV over 1 hour in 500 ml NSS, and Cytarabine 1000mg/ m^2/day IV over 1 hour in 250 cc NSS) will be administered once every 24 hours on days 4-8, starting after the sirolimus dose on day 4.

Actual body weight will be used to calculate chemotherapy dose. Institutional standards for supportive care and monitoring should be followed, such as hydration/tumor lysis prophylaxis, antiemetics, steroid eye drops (keratitis prophylaxis), and cerebellar exam monitoring during cytarabine therapy

Subjects will undergo a nadir bone marrow biopsy within one week of hematologic recovery (but not later than day 45) to document response.

7.3 Preparation and Administration of Study Drug

Sirolimus will be stored and dispensed from the Investigational Drug Service (IDS). It will be dispensed in pill bottles containing a full cycle's amount of Sirolimus. Subjects might start a cycle as an outpatient and self-administer their Days 1, 2, 3, 4 doses prior to admission. The subject will be asked to bring their pill

bottle as well as their pill diary to the hospital at the time of admission. For the rest of the cycle, each daily dose will be administered by nurses in the inpatient setting.

7.4 Handling and Disposal of Study Drug

Qualified personnel who are familiar with procedures that minimize undue exposure to them and to the environment should undertake the preparation, handling, and safe disposal of chemotherapeutic agents in an appropriate environment.

Discard unused portions of injectable chemotherapeutic agents that do not contain a bacteriostatic agent or are prepared with unpreserved diluents (i.e., Sterile Water for Injection USP or 0.9% Sodium Chloride for Injection USP) within eight hours of vial entry to minimize the risk of bacterial contamination.

7.5 Subject Compliance Monitoring

Each subject will receive a pill bottle containing a full cycle's amount of Sirolimus. The labeling will contain explicit dosing instructions and the subject will be asked to keep a pill diary to record the time of each dose of Sirolimus taken. If subject starts a cycle as an outpatient, they will self-administer their Days 1, 2 3, and perhaps 4 doses. The subject will be asked to bring their pill bottle as well as their pill diary to the hospital at the time of admission. For the rest of the cycle, each daily dose will be administered by nurses in the inpatient setting.

7.6 Prior and Concomitant Therapy

1. Information of prior cytotoxic regimens will be collected.
2. All concomitant medical therapy will be collected as part of the history.
3. All concomitant medicines/therapies are permitted during the study except for those noted in the exclusion criteria in section 5.2.
4. All patients should receive acyclovir unless documented to be HSV negative.
5. Growth factors are permitted beginning on Day 12 of therapy at the discretion of the treating physician.

8 Statistical Plan

8.1 Statistical Considerations

This is a proof of concept trial to establish early change in mTOR kinase activity during treatment with Sirolimus and MEC chemotherapy as a predictive biomarker for clinical response in patients with high risk AML. The trial will be conducted in one stage, with no provision for early stopping. This is an enrichment study, since only patients with measureable mTOR kinase activity at baseline, and who have the potential to demonstrate change in activity, will be evaluated for biochemical response.

8.1.1 Objectives

The primary and secondary objectives are listed in section 3.

8.1.2 Endpoints

Early change in mTOR kinase activity is defined by % reduction in pS6 positive blasts = [(baseline pS6 - day 4 trough pS6)/baseline pS6]. Biochemical response defined by >40% reduction in pS6 positive blasts is termed "rampamycin sensitive". Either ≤40% reduction or an increase in pS6 positive blasts is termed "resistant". Clinical response is based on hematologic recovery/day 45 marrow using IWG criteria (CR, CRp, CRi, PR, NR). Objective response rate (ORR) is defined as the fraction of patients who achieve CR, CRp, or PR. RFS and OS are defined as the time from study entry to first documented progression, death

or last contact (RFS) or to death or last contact (OS). Toxicity is graded by NCI CTCAE Version 4.0. Worse toxicity grades observed during treatment will be described.

8.1.3 Plans for Data Analysis

Baseline pS6 and % reduction in pS6 will be described by mean, median, standard deviation, range and coefficient of variation. Biochemical response will be scored in patients with measureable baseline pS6. Clinical response will be scored in all patients. The objective response rate (ORR) and 95% exact confidence interval will be computed for all patients and for sensitive and resistant subgroups. The association between biochemical response and clinical response will be tested by Fisher's exact test. RFS and OS will be estimated by the Kaplan-Meier method. A landmark analysis of RFS by clinical response (CR+CRp, CRi, PR or NR) will be computed from day 45 marrow assessment. Median values and 95% confidence intervals will be calculated. Toxicities will be graded and tabled.

8.1.4 Preliminary Data

In our previous study of Sirolimus and MEC, the ORR was 47.1% (24/51 patients). Of 37 patients evaluable for paired pharmacodynamic assessment, 73.0% of patients (27/37) had baseline pS6 detected. Therapy induced changes in pS6 positive blasts ranged from an increase of 225% to a decrease of 98%. The ORR was 51.9% in these 27 patients. Both visual inspection and ROC statistical analysis suggested that >40% reduction in pS6 positive blasts discriminated responders from nonresponders. In rapamycin sensitive patients, the ORR was 70.6% (12/17 patients) while in resistant patients, the ORR was 20.0% (2/10 patients). The goal of this study is to validate this finding in an adequately powered prospective clinical trial and to establish early change in pS6 positive blasts as a predictive biomarker. We have chosen to test a slightly more conservative difference in ORR (65% vs. 25%) than observed in our preliminary data.

8.1.5 Sample size

A study of 49 biomarker evaluable subjects (31 sensitive; 18 resistant) is needed to detect a difference in ORR of 65% in sensitive subjects versus 25% in resistant subjects with 80% power for a Fisher's exact test at 1-sided type I error of 5%. **The total sample size** must be increased by 30% to **65 subjects** to account for subjects that will not have measureable pS6 at baseline, although such subjects will contribute to analyses of overall ORR, RFS, OS and toxicity. Accrual to rampamycin sensitive or resistant subgroups (known by analysis of day 4 blasts) will be monitored to ensure sufficient numbers of each subgroup.

8.1.6 Accrual Duration

With an estimated total accrual of 65 patients, accrual will continue for 6 year and follow-up will continue for an additional year prior to final statistical analysis.

8.1.7 Clinical outcomes

Complete remission (CR) is a well-validated surrogate for survival in AML.[74] Complete remission with incomplete recovery (Cri) is routinely reported from AML studies from the UK and continental Europe, but has not until recently been widely used in the US. Response criteria of CRp (all CR criteria met, except platelet count <100,000/uL) have been validated in the relapsed patients for certain novel agents, notably gemtuzumab ozogamicin. However, CRi and CRp responses are frequently not published in older US clinical trials, making comparison of our results to historic controls problematic. Because many relapsed patients are able to receive potentially curative allogeneic transplants following a CRi or PR, we remain convinced that these can reflect meaningful treatment responses. Of the 4 patients with PR response to sequential sirolimus and MEC, 2 were subsequently transplanted. Both patients achieved CR post hematopoietic stem cell transplant (HSCT) and remain alive at >2 years of follow up with no evidence of disease (NED). Therefore, morphologic CR will be evaluated as the key clinical outcome variable, but

CRp, CRi and PR, as defined by the International Working Group (IWG) will be collected as secondary endpoints.

The short term benefits of remission-induction are obvious for high risk AML but long-term disease control and cure generally requires HSCT. Thus, rates of HSCT will be collected as secondary endpoints.

8.2 Subject Population(s) for Analysis

All samples obtained will have correlative studies performed. All subjects who receive any study related therapy will be evaluable for toxicity. All subjects will be evaluated for response. Any subject who does not complete 9 days of treatment for reasons other than treatment related toxicity or disease progression will be replaced.

9 Safety and Adverse Events

Subjects will be followed for toxicity until count recovery or day 45 of the treatment cycle, whichever is earlier, or until resolution of serious adverse events if one is noted.

9.1 Definitions

Unanticipated Problems Involving Risk to Subjects or Others
Unanticipated problems (UAPs) include, in general, any incident, experience, or outcome that meets the following criteria:

- unexpected in terms of nature, severity, or frequency given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the participant population being studied;

UAPs are considered to pose risk to participants or others when they suggest that the research places participants or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

Adverse Event
An adverse event is any untoward or unfavorable medical occurrence in a human participant, including any abnormal sign (for example, abnormal physical exam or laboratory finding), symptom, or disease, temporally associated with the participant's participation in the research, whether or not considered related to the participant's participation in the research.
Serious Adverse Event
Adverse events are classified as serious or non-serious. A ***serious adverse event*** is any AE that is:

- fatal
- life-threatening
- requires or prolongs hospital stay
- results in persistent or significant disability or incapacity
- a congenital anomaly or birth defect
- an important medical event

Important medical events are those that may not be immediately life threatening, but are clearly of major clinical significance. They may jeopardize the subject, and may require intervention to prevent one of the other serious outcomes noted above. For example, drug overdose or abuse, a seizure that did not result

in in-patient hospitalization, or intensive treatment of bronchospasms in an emergency department would typically be considered serious.

All adverse events that do not meet any of the criteria for serious should be regarded as ***non-serious adverse events***.

Adverse Event Reporting Period
The study period during which adverse events must be reported is normally defined as the period from the initiation of any study procedures to the end of the study treatment follow-up. For this study, the study treatment follow-up is defined as 14 days following the last administration of study treatment. Toxicity is followed until Day 45 or count recovery.

Preexisting Condition
A preexisting condition is one that is present at the start of the study. A preexisting condition should be recorded as an adverse event if the frequency, intensity, or the character of the condition worsens during the study period.

General Physical Examination Findings
At screening, any clinically significant abnormality should be recorded as a preexisting condition. At the end of the study, any new clinically significant findings/abnormalities that meet the definition of an adverse event must also be recorded and documented as an adverse event.

Post-study Adverse Event
All unresolved adverse events should be followed by the investigator until the events are resolved, the subject is lost to follow-up, or the adverse event is otherwise explained. At the last scheduled visit, the investigator should instruct each subject to report any subsequent event(s) that the subject, or the subject's personal physician, believes might reasonably be related to participation in this study.

Abnormal Laboratory Values
A clinical laboratory abnormality should be documented as an adverse event if any one of the following conditions is met:

- The laboratory abnormality is not otherwise refuted by a repeat test to confirm the abnormality
- The abnormality suggests a disease and/or organ toxicity
- The abnormality is of a degree that requires active management; e.g. change of dose, discontinuation of the drug, more frequent follow-up assessments, further diagnostic investigation, etc.

Hospitalization, Prolonged Hospitalization or Surgery
Any adverse event that results in hospitalization or prolonged hospitalization should be documented and reported as a serious adverse event unless specifically instructed otherwise in this protocol. Any

condition responsible for surgery should be documented as an adverse event if the condition meets the criteria for an adverse event.

Neither the condition, hospitalization, prolonged hospitalization, nor surgery are reported as an adverse event in the following circumstances:

- Hospitalization or prolonged hospitalization for diagnostic or elective surgical procedures for a preexisting condition. Surgery should ***not*** be reported as an outcome of an adverse event if the purpose of the surgery was elective or diagnostic and the outcome was uneventful.
- Hospitalization or prolonged hospitalization required to allow efficacy measurement for the study.
- Hospitalization or prolonged hospitalization for therapy of the target disease of the study, unless it is a worsening or increase in frequency of hospital admissions as judged by the clinical investigator.

9.2 Recording and Reporting of Adverse Events

Toxicities will be assessed throughout the cycle of therapy. Toxicity will be graded according to the NCI CTCAE version 4.0 (http://ctep.cancer.gov/reporting/ctc.html).

At each contact with the subject, the investigator must seek information on adverse events by specific questioning and, as appropriate, by examination. Information on all adverse events should be recorded immediately in the source document, and also in the appropriate adverse event module of the case report form (CRF). All clearly related signs, symptoms, and abnormal diagnostic procedures results should recorded in the source document, though should be grouped under one diagnosis.

All non-hematologic adverse events occurring during the study period must be recorded. The clinical course of each event should be followed until resolution, stabilization, or until it has been determined that the study treatment or participation is not the cause. Serious adverse events that are still ongoing at the end of the study period must be followed up to determine the final outcome. Any serious adverse event that occurs after the study period and is considered to be possibly related to the study treatment or study participation should be recorded and reported immediately.

All emergent ≥ Grade 2 non-hematologic adverse events (AEs) will be reported to the DSMC. The only hematologic event which will be considered an AE/SAE for this study and will be reported is marrow aplasia (defined as <10% cellularity with <10% blast) > 4 weeks not attributable to disease. Grades 1 to 4 of neutropenia, anemia, or thrombocytopenia would not qualify as an AE/SAE as the purpose of the treatment is to create a period of complete aplasia in the marrow. However, all hematologic Grade 5 SAEs will be reported to the DSMC regardless of causality.

9.2.1 Safety Assessment and Follow-Up

The relationship to study intervention or study participation must be assessed and documented for all adverse events. Evaluation of relatedness must consider etiologies such as natural history of the underlying disease, concurrent illness, concomitant therapy, study-related procedures, accidents, and other external factors.

The following guidelines are used to assess relationship of an event to study intervention:

1. Related (Possible, Probable, Definite)

    a. The event is known to occur with the study intervention.

    b. There is a temporal relationship between the intervention and event onset.

    c. The event abates when the intervention is discontinued.

d. The event reappears upon a re-challenge with the intervention.

2. Not Related (Unlikely, Not Related)

a. There is no temporal relationship between the intervention and event onset.

b. An alternate etiology has been established.

9.2.2 Expectedness

The PI is responsible for determining whether an AE is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the intervention. Risk information to assess expectedness can be obtained from preclinical studies, the investigator's brochure, published medical literature, the protocol, or the informed consent document.

9.2.3 Intervention

Any intervention implemented to treat the adverse event must be documented for all adverse events.

9.3 Safety Reporting

9.3.1 Reporting to IRB

9.3.1.1 Unanticipated Problems

All incidents or events that meet criteria for unanticipated problems (UAPs) as defined in Section 9.1 require the creation and completion of an unanticipated problem report form (OHR-20).

UAPs that pose risk to participants or others, and that are not AEs, will be submitted to the IRB on an OHR-20 form via the eazUP system within 5 working days of the investigator becoming aware of the event.

UAPs that do not pose risk to participants or others will be submitted to the IRB at the next continuing review.

9.3.1.2 Adverse Events

Grade 1 AEs will be reported to the IRB at continuing review.

Grade 2 AEs will be reported to the IRB at the time of continuing review.

9.3.1.3 Serious Adverse Events

SAEs will be reported to the IRB on OHR-10 forms via the electronic reporting system (eSAEy) according to the required time frames described below.

Grade 3-4 AEs that are unexpected and deemed to be at least possibly related to the study will be reported to the IRB within 2 working days of knowledge of the event.

Grade 3-4 AEs that are deemed unrelated to the study will be reported to the IRB within 5 working days.

Grade 5 AEs will be reported to the IRB within one working day of knowledge of the event.

All SAEs will be submitted to the IRB at continuing review, including those that were reported previously.

9.3.2 Reporting to SKCC DSMC

All AEs and SAEs, safety and toxicity data, and any corrective actions will be submitted to the DSMC per the frequency described in the SKCC DSMP. The report to the SKCC DSMC will also include any unanticipated problems that in the opinion of the PI should be reported to the DSMC.

All emergent ≥ Grade 2 non-hematologic adverse events (AEs) will be reported to the DSMC. The only hematologic event which will be considered an AE/SAE for this study and will be reported is marrow aplasia (defined as <10% cellularity with <10% blast) > 4 weeks not attributable to disease. Grades 1 to 4 of neutropenia, anemia, or thrombocytopenia would not qualify as an AE/SAE as the purpose of the treatment is to create a period of complete aplasia in the marrow. However, all hematologic Grade 5 SAEs will be reported to the DSMC regardless of causality.

For expedited reporting requirements, see table below:

DSMC AE/SAE Reporting Requirements

	Grade 1	Grade 2		Grade 3				Grades 4 and 5
				Unexpected		Expected		
	Unexpected and Expected	Unexpected	Expected	With Hospitalization	Without Hospitalization	With Hospitalization	Without Hospitalization	Unexpected and Expected
Unrelated Unlikely	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	5 Working Days	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	5 Working Days	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Phase I - 48 Hours (Death: 24 Hours) Phase II - 5 working days
Possible Probably Definite	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	48 Hours (Death: 24 Hours)	Phase I - 48 Hours Phase II - 5 working days	48 Hours (Death: 24 Hours)	Reviewed at Quarterly DSMC Meeting and IRB Annual Review	Phase I and Phase II - 48 Hours (Death: 24 Hours)

9.3.3 Protocol Deviations/Exceptions

Deviations/exceptions from eligibility will not be allowed under any circumstances. An accidental or unintentional deviation from the approved protocol, that in the opinion of the investigator placed one or more participants at increased risk, or affects the rights or welfare of subjects, will be reported to the IRB and DSMC within 10 working days of notification of investigator. Deviations to protect subjects from immediate harm/danger should be reported immediately following the event to both the IRB and DSMC.

There may be times, when a planned deviation from the protocol seems warranted. Such deviations should only occur with the **prior** assessment of the DSMC. All entities should be given sufficient time to evaluate the request.

9.3.4 Study Oversight

In addition to the PI's responsibility for oversight, study oversight will be under the direction of the SKCC's Data and Safety Monitoring Committee (DSMC). The SKCC DSMC operates in compliance with a Data and Safety Monitoring Plan (DSMP) that is approved by the NCI.

9.4 Clinical Site Monitoring and Auditing

Clinical site monitoring and auditing is conducted to ensure that the rights of human participants are protected, that the study is implemented in accordance with the protocol and/or other operating procedures, and that the quality and integrity of study data and data collection methods are maintained. Monitoring and auditing for this study will be performed in accordance with the SKCC's Data and Safety Monitoring Plan (DSMP) developed by the SKCC Data and Safety Monitoring Committee (DSMC). The DSMP specifies the frequency of monitoring, monitoring procedures, the level of clinical site monitoring activities (e.g., the percentage of participant data to be reviewed), and the distribution of monitoring reports. Some monitoring activities may be performed remotely, while others will take place at the study site(s). Appropriate staff will conduct monitoring activities and provide reports of the findings and associated action items in accordance with the details described in the SKCC DSMP.

10 Data Handling and Record Keeping

10.1 Confidentiality

Information about study subjects will be kept confidential and managed according to the requirements of the Health Insurance Portability and Accountability Act of 1996 (HIPAA). Those regulations require a signed subject authorization informing the subject of the following:

- What protected health information (PHI) will be collected from subjects in this study
- Who will have access to that information and why
- Who will use or disclose that information
- The rights of a research subject to revoke their authorization for use of their PHI.

In the event that a subject revokes authorization to collect or use PHI, the investigator, by regulation, retains the ability to use all information collected prior to the revocation of subject authorization. For subjects that have revoked authorization to collect or use PHI, attempts should be made to obtain permission to collect at least vital status (i.e. that the subject is alive) at the end of their scheduled study period.

10.2 Source Documents

Source data is all information, original records of clinical findings, observations, or other activities in a clinical trial necessary for the reconstruction and evaluation of the trial. Source data are contained in

source documents. Examples of these original documents, and data records include: hospital records, clinical and office charts, laboratory notes, memoranda, subjects' diaries or evaluation checklists, pharmacy dispensing records, recorded data from automated instruments, copies or transcriptions certified after verification as being accurate and complete, microfiches, photographic negatives, microfilm or magnetic media, x-rays, subject files, and records kept at the pharmacy, at the laboratories, and at medico-technical departments involved in the clinical trial.

10.3 Case Report Forms

The study case report form (CRF) is the primary data collection instrument for the study. All data requested on the CRF must be recorded. All missing data must be explained. If a space on the CRF is left blank because the procedure was not done or the question was not asked, write "N/D". If the item is not applicable to the individual case, write "N/A". All entries should be printed legibly in black ink. If any entry error has been made, to correct such an error, draw a single straight line through the incorrect entry and enter the correct data above it. All such changes must be initialed and dated. DO NOT ERASE OR WHITE OUT ERRORS. For clarification of illegible or uncertain entries, print the clarification above the item, then initial and date it.

10.4 Records Retention

Upon completion and termination of protocol, records will be kept for a period of 2 years.

11 Ethical Considerations

This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Institutional research policies and procedures.

This protocol and any amendments will be submitted to a properly constituted independent Ethics Committee (EC) or Institutional Review Board (IRB), in agreement with local legal prescriptions, for formal approval of the study conduct. The decision of the EC/IRB concerning the conduct of the study will be made in writing to the investigator and a copy of this decision will be provided to the sponsor before commencement of this study. The investigator should provide a list of EC/IRB members and their affiliate to the sponsor.

All subjects for this study will be provided a consent form describing this study and providing sufficient information for subjects to make an informed decision about their participation in this study. This consent form will be submitted with the protocol for review and approval by the EC/IRB for the study. The formal consent of a subject, using the EC/IRB-approved consent form, must be obtained before that subject undergoes any study procedure. The consent form must be signed by the subject, and the investigator-designated research professional obtaining the consent.

12 Study Finances

12.1 Funding Source

Clinical research costs and investigational compound will be paid for by departmental funds and pharmacodynamics (PD) studies will be paid for by Hematologic Malignancies Translational Center of Excellence of the Abramson Cancer Center. The work will be performed in Dr. Perl's laboratory at the University of Pennsylvania.

12.2 Conflict of Interest

Any investigator who has a conflict of interest with this study (patent ownership, royalties, or financial gain greater than the minimum allowable by their institution, etc.) must have the conflict reviewed by a properly

constituted Conflict of Interest Committee with a Committee-sanctioned conflict management plan that has been reviewed and approved by the study sponsor prior to participation in this study.

13 Publication Plan

We intend to publish our results in a peer-reviewed journal. The primary responsibility for publication lies with the PI, co-investigators, and statistician.

14 References

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15 Attachments

Appendix 1: ECOG Performance Status
Appendix 2: Study Schedule

Appendix 1:

ECOG PERFORMANCE STATUS

Grade	ECOG
0	Fully active, able to carry on all pre disease performance without restriction
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light house work, office work
2	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	Capable of only limited self-care, confined to bed or chair more than 50% of waking hours
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair
5	Dead

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Appendix 2

Study Procedures/Study Table

		Base Line[1]	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Q Wk[11]	Follow -up[10]
Informed Consent		X[4]											
Medical/Oncologic History		X[4]											X[14]
Physical Examination/Vital Signs		X	X[2]	X[2]	X[2]	X	X	X	X	X	X	X	
Performance Status		X											
Weight/Height/Body Surface Area		X											
Adverse Event Assessment		X	X[2]	X[2]	X[2]	X	X	X	X	X	X	X	X[10]
Laboratory Studies													
CBC, Diff, Platelets		X	X[2]	X[2]	X[2]	X	X	X	X	X	X	X	
Serum Chemistry, Electrolytes[12]		X[12]			X[12]			X[12]			X[12]		
Liver Function Tests[12]		X[12]			X[12]			X[12]					
Herpes Simplex Titer		X[13]											
Pregnancy test[3]		X											
Pharmacokinetics[5]						X[5]							
Pharmacodynamics[6]			X[6]			X[6,8]							
Tests & Studies													
MUGA scan or ECHO *cardiogram*		X[4]											
Bone Marrow Biopsy/Aspirate		X[6,7]				X[6,7]							X[9]
Investigational Agent & Chemotherapy													
Sirolimus Administration			X	X	X	X	X	X	X	X	X		
Pill Diary Given to Patient			X										
Mitoxantrone/ Etoposide/ Cytarabine (MEC)						X	X	X	X	X			

1. To be done within ten days prior to study entry unless otherwise indicated.
2. On inpatient days only Day 1 through Day 9. Vital signs Q8 hours and physical exam daily. Physical exam includes a neurological exam.
3. Only perform for females of child bearing potential
4. To be done within 4 weeks of study entry.
5. Blood to be drawn prior to sirolimus administration for trough level on day 4
6. 5-10cc of bone marrow aspirate will be collected and sent to Stem Cell and Xenograft Core. The bone marrow aspirate may be replaced by 20 mLs of peripheral blood if the subject has a peripheral leukemic blast count ≥5000/μl.
7. The on-study bone marrow study may be done up to 28 days prior to enrollment. If a diagnostic bone marrow biopsy has been done within 28 days of study enrollment, and a sample is available in the stem cell core registry at the University of Pennsylvania, a repeat bone marrow biopsy and aspirate is not necessary.
8. 20 mL of peripheral blood to be collected prior to first dose of Sirolimus. Samples will also be collected within 4 hours prior to Sirolimus dose on Day 4. This will be a bone marrow aspirate if there are insufficient circulating blasts (see section 6.3 for details)
9. Bone marrow studies to be done within 1 week of count recovery but no later than day 45
10. All AE's will be followed until resolution/stabilization or new baseline. All subjects are followed until death or end of the study.
11. To be done weekly while subject on study (until hematologic recovery or 45 days, whichever happens first)
12. Serum chemistry and electrolytes labs should include: creatinine, BUN, glucose, magnesium, phosphate, and urate. Liver function labs should include: bilirubin, SGOT (AST), SGPT (ALT), alk phos.
13. Herpes simplex titer is recommended within 4 weeks of starting.
14. Subjects will be followed for disease status until relapse/progression and for survival until death or end of the study *(2 years after enrollment of last subject)*