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Sirolimus Enhances Remission Induction in Patients with High Risk Acute Myeloid Leukemia and mTORC1 Target Inhibition

Margaret T Kasner¹, Rosemarie Mick², Grace R Jeschke^{3,4}, Matthew Carabasi¹, Joanne Filicko-O'Hara¹, Neal Flomenberg¹, Noelle V Frey^{3,4}, Elizabeth O Hexner^{3,4}, Selina M Luger^{3,4}, Alison W Loren^{3,4}, James K Mangan^{3,4}, John L Wagner¹, Mark Weiss¹, Martin Carroll^{3,4}, and Alexander E Perl^{3,4}

¹Department of Medical Oncology, Sidney Kimmel Medical College and Cancer Center, Thomas Jefferson University, Philadelphia PA

²Department of Biostatistics and Epidemiology, Perelman School of Medicine at the University of Pennsylvania

³Division of Hematology-Oncology, Department of Medicine, Perelman School of Medicine at the University of Pennsylvania

⁴Abramson Cancer Center of the University of Pennsylvania, Philadelphia, PA

Abstract

Background—Mammalian Target of Rapamycin Complex 1 (mTORC1) inhibitors enhance chemotherapy response in acute myelogenous leukemia (AML) cells *in vitro*. However whether inhibiting mTORC1 enhances clinical response to AML chemotherapy remains controversial. We previously optimized measurement of mTORC1's kinase activity in AML blasts during clinical trials using serial phospho-specific flow cytometry of formaldehyde-fixed whole blood or marrow specimens. To validate mTORC1 as a therapeutic target in AML, we performed two clinical trials combining an mTORC1 inhibitor (sirolimus) and MEC (mitoxantrone, etoposide, cytarabine) in patients with relapsed, refractory, or untreated high-risk AML.

Methods—Flow cytometric measurements of ribosomal protein S6 phosphorylation (pS6) were performed before and during sirolimus treatment to determine whether mTORC1 inhibition enriched for chemotherapy response.

Results—In 51 evaluable subjects, the overall response rate (ORR) to the combination regimen was 47% (95% confidence interval 33 – 61%, 33% CR, 2% CRi, 12% PR) and similar toxicity to historic experience with MEC alone. 37 subjects had baseline pS6 measured pre-sirolimus, of

Corresponding author: alexander.perl@uphs.upenn.edu 215-615-3138.

Compliance with Ethical Standards

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Conflict of Interest

All authors declare no conflicts of interest.

whom 27 (73%) exhibited mTORC1 activity. ORR was not significantly different between subjects with and without baseline mTORC1 activity (52% vs 40%, respectively, $p=0.20$). The ORR among subjects with baseline target activation and mTORC1 inhibition during therapy was 71% (12/17) compared to 20% (2/10) in subjects without target inhibition.

Conclusions—Fixed, whole blood pS6 by flow cytometry may be a predictive biomarker for clinical response to mTORC1 inhibitor-based regimens. These data provide clinical confirmation that mTORC1 activation mediates chemotherapy resistance in patients with AML.

Keywords

Acute myeloid leukemia; mTOR; mTORC1; biomarker; phospho-flow cytometry

Introduction

Therapeutic outcomes in acute myeloid leukemia (AML) remain unsatisfactory, particularly in patients with initial presentation of high-risk disease (defined by age, adverse karyotype, prior chemotherapy, or prior clonal myeloid neoplasm), those who relapse, or those refractory to previous therapy. In these groups, the inability to achieve remission to intensive chemotherapy is a major limitation to long-term survival, which itself is largely predicted by subsequent receipt of allogeneic transplant [1]. Large studies of diagnostic AML genomes show mutations associated with activation of signal transduction pathways occurs in the majority of patient samples in AML [2,3], and development of signal transduction inhibitors to improve chemotherapy response and survival has been a longstanding goal of many research groups. The mammalian target of rapamycin complex 1 (mTORC1)'s frequent activation in AML blasts, as well as the availability of rapamycins such as sirolimus—validated, selective, potent mTORC1 inhibitors with established low toxicity and widely available therapeutic drug monitoring capability—make it an ideal target for therapeutic development of targeted agents [4]. Preclinical data suggest mTORC1 inhibition with rapamycins enhance chemotherapy response in bulk and AML stem populations [4,5]. Overall, available data support a rationale to target mTOR in AML using rapamycins.

mTORC1 inhibitors are standard therapy for renal cell carcinoma, neuroendocrine tumors, and breast cancer, but there is not currently an established role of this class of agents in AML. Although *in vitro* studies suggest that mTORC1 inhibition decreases AML cell survival, limited prior phase 1/2 testing of mTORC1 inhibitors did not demonstrate substantial single-agent clinical anti-leukemic effects [5–7]. We originally proposed that mTORC1 inhibitors would act to chemosensitize AML cells [4]. We therefore performed a phase I study that confirmed the feasibility of combining rapamycins and induction chemotherapy in AML [8]. Subsequently, two clinical trials provided preliminary evidence that rapamycins might enhance chemosensitivity in relapsed AML patients [9,10]. The GOELAMS cooperative group in France showed a remarkably high rate of second remissions (19/28=68%) among 28 subjects with an initial first remission duration of over 12 months who received salvage induction containing everolimus and anthracycline/infusional cytarabine (7+3) for first relapse [9]. Using an *ex vivo* surrogate assay for mTORC1 inhibition, higher free drug levels were associated with improved response rates. Additionally, the GIMEMA observed a somewhat lower rate of remission on a study of the

lower intensity chemotherapeutic clofarabine and temsirolimus in patients over age 60 in first relapse [10]. Correlative analysis on a subset of treated subjects suggested remissions were exclusively seen among subjects with evidence of mTORC1 target inhibition. Overall, these data suggest the addition of an mTORC1 inhibitor could improve chemotherapy response rates for high-risk patients at relapse.

A challenge of extending these results to larger groups of patients is the observation that the degree of mTORC1 activation in AML is variable and is not uniformly seen in AML blasts [11]. While mutations in genes regulating signal transduction occur commonly in AML, *mTOR* or its direct regulators are only rarely mutated and a discrete genotype that consistently predicts constitutive mTORC1 activation is not established. As well, clinical responses in AML are heterogeneous and thus restricted to a subset of patients. Importantly, even in tumor types where clinical activity of rapamycins is established, there is no validated clinical predictor of response, either from genetic screens or biomarker studies. Even pharmacodynamic evidence of mTORC1 signal transduction inhibition from tumor biopsies after therapy has not consistently predicted clinical responsiveness to these agents [12]. Given these concerns, we focused previously on development of a robust biomarker for mTORC1 activation in leukemic cells that would allow for identification of patients with activation of mTORC1 at baseline and dynamic monitoring of mTORC1 activation in tumor cells during therapy.

To optimize measurement of mTORC1 activity from correlative studies, we modified our phase I regimen for subsequent trials to include sirolimus monotherapy for 3 days prior to initiation of combined sirolimus and chemotherapy (i.e., “rapamycin run-in”). This allowed for achievement of steady state levels and pharmacodynamic monitoring prior to initiation of cytotoxic chemotherapy. Real time, serial monitoring of ribosomal protein S6 phosphorylation (pS6) in immunophenotypically-identified circulating blasts was then performed in formaldehyde-fixed, unfractionated whole blood [11]. From a technical standpoint, direct blood fixation provides a close approximation of direct signaling measurements from patients’ cells during therapy and avoids biases that can occur from cell separation/blast purification methods [13].

We completed a pilot study that established our ability to detect basal mTORC1 activity by phospho-flow and then serially monitored mTORC1 activity to quantify the degree of *in vivo* inhibition in participants’ blasts from sirolimus therapy [11]. We then performed a phase II study using this regimen to further refine our biomarker evaluation. Here we present a combined analysis of the pilot and subsequent phase II study to demonstrate the association between mTORC1 target inhibition in leukemic blasts and clinical response to a regimen combining sirolimus and MEC chemotherapy. Subjects on each study had relapsed, refractory, or untreated AML with high-risk features and were treated by an identical regimen. Overall, the combined trials dataset allows for evaluation of the regimen’s clinical activity and safety as well as assessment of a pharmacodynamic bioassay to define the association of biochemical target inhibition with clinical response. We show that demonstration of *in vivo* mTORC1 inhibition in leukemic blasts by phospho-flow strongly enriches for chemotherapy response.

Methods

Trial Eligibility

We performed two sequential clinical trials in identical patient populations ([NCT00780104](#) and [NCT01184898](#)). The pilot study was designed to optimize pharmacodynamic measurements of mTORC1 signaling in AML by phospho-flow cytometry. The subsequent phase II trial was designed to explore these same methods in a larger study group and determine whether mTORC1 inhibition was associated with clinical response. Because enrollment criteria, biomarker measurement and treatment were identical, the data from both trials were combined for analysis presented here. The trials enrolled subjects at the Hospital of the University of Pennsylvania and Thomas Jefferson University Hospital. Clinical protocols were approved by the Institutional Review Boards at each center and all subjects signed informed consent in accordance with the Declaration of Helsinki. Each trial enrolled subjects who were 18 and over with high risk non-M3 AML. High-risk included both previously treated subjects with relapsed/refractory AML as well as subjects with previously untreated AML and at least one of the following risk factors: antecedent chemotherapy or radiation for non-leukemic diagnoses, prior myelodysplastic syndrome or myeloproliferative neoplasm, or age >60 without favorable karyotype (i.e. t(8;21)(q22q22), inv16(p13q22), or t(16;16)(p13;q22)). Subjects were required to have an Eastern Cooperative Oncology Group performance score of 2 or less [14]; adequate renal, hepatic, and cardiac function; and resolution of toxicity from prior chemotherapy. Concurrent administration of medications known to strongly interact with sirolimus pharmacokinetics via the microsomal p450 CYP3A4 system was prohibited until sirolimus therapy was complete.

Treatment Plan

Subjects received a 12 mg oral sirolimus loading dose on day 1 followed by 4 mg/day every 24 hours on days 2–9. MEC chemotherapy (Mitoxantrone 8mg/m²/day IV, Etoposide 100mg/m²/day IV and Cytarabine 1000mg/ m²/day IV every 24 hours for 5 days) was administered on treatment day 4–8, following the sirolimus loading dose and 3 daily doses (c.f. Fig. 1). Actual body weights were used for chemotherapy dose calculations. Subjects had a bone marrow aspiration and biopsy on study day 17 (14 days after initiation of MEC therapy) to determine if marrow aplasia had been achieved. Subjects with evidence of persistent leukemia on a cellular nadir marrow were considered to be non-responses and were eligible for alternative therapy. Treatment response was assessed by marrow aspirate and biopsy upon hematologic recovery or day 42, whichever came first.

Clinical response, toxicities, and pharmacodynamic measurements

The primary objectives of the pilot study were to establish the safety of the regimen and the feasibility of serial phospho-flow measurements during trial therapy, which were described previously [11]. On that study, the initial 6 subjects were used primarily for optimization of methods for cell processing of pre-treatment specimens. As serial samples were not performed, target inhibition could not be assessed on these six subjects. The next 10 enrolled subjects had serial samples at baseline, day 1 and day 4 which were processed for pS6 evaluation. 15/16 subjects were evaluable for response assessment and toxicity and are included in this report, including all 10 subjects with serial pS6 measurements.

The primary objective of the subsequent phase II study was to evaluate the association between mTORC1 target inhibition during treatment and overall response, defined as complete remission (CR), complete remission with incomplete hematologic recovery (CRi), or partial response (PR). Response was defined by international working group (IWG) criteria [15]. Only subjects who received all chemotherapy were considered evaluable for response. Subjects who died prior to response assessment were considered unevaluable for biomarker assessment and were replaced. All 36 subjects were analyzed for clinical outcome and toxicity and included in this report.

Secondary endpoints for both studies included assessment of safety, overall survival and incidence of subsequent allogeneic transplantation. Toxicities were graded using the NCI Common Terminology Criteria for Adverse Events (CTCAE) Version 3.0. Adverse events were monitored until treatment response assessment or resolution of treatment associated toxicities.

Therapeutic drug monitoring

Whole blood sirolimus concentrations were determined in the individual institution laboratories by identical, commercially available assays (HPLC for the first 16 enrolled subjects and ELISA for the remainder). Sirolimus concentrations were performed from blood samples drawn 2 hours after the loading dose on day 1 and after the daily dose on day 4 (peak levels) and immediately prior to the daily doses on day 4 (trough). To determine whether MEC chemotherapy altered sirolimus steady-state concentrations, day 7 (trough) levels were also obtained.

Correlative studies

Serial monitoring of mTORC1 activity was performed in tandem with therapeutic drug level monitoring in order to investigate a pharmacokinetic/pharmacodynamic correlation. Serine 235/6 phosphorylation of the downstream mTORC1 target S6 ribosomal protein (pS6) was measured by flow cytometry using previously published methods [10]. Peripheral blood was collected to measure mTORC1 activity at baseline (day 1 prior to treatment) and prior to the sirolimus dose on day 4 in both studies. Additionally, the phase 2 study subjects had peripheral blood sampling two hours following sirolimus loading dose on day 1, and two hours after the day 4 sirolimus dose. In subjects with circulating blasts below 100 blasts per microliter, marrow aspirate at baseline and on day 4 was performed in the place of peripheral blood sampling. Whole, unfractionated peripheral blood or marrow was fixed by the direct addition of ultrapure, methanol-free formaldehyde (final concentration 4%) to the blood samples. Cells were then permeabilized by incubation in triton X-100 detergent (0.1%) at 37 degrees, washed, and stored at -20C in a glycerol-containing medium. After all time points were collected, samples were thawed, exposed to ice cold methanol (90%) to enhance the signal to noise ratio of phospho-protein antibodies, and then stained for flow cytometry at a single cytometer session.

Cytometric data analysis was performed using FlowJo (version 8, TreeStar). Leukemic blast gating was done using CD45 and right angle (side) scatter with at least two additional surface markers (e.g. CD33 and CD34) used to define this population and/or exclude other

cell populations [11]. Baseline pS6 was defined using combinations of dynamic signaling controls and staining controls (e.g. phorbol myristate acetate-treated samples, *ex vivo* sirolimus-treated samples and fluorescence minus one conditions for positive and negative controls, respectively). Baseline pS6 was defined as the percentage of gated blasts showing unequivocal phosphorylation (pS6+/all gated blast events). As AML samples generally heterogeneous and subset S6 phosphorylation, all subjects whose samples had >5% pS6+ events prior to therapy were considered to have baseline mTORC1 activation. Baseline samples in which a “tail” of phosphorylated S6 was clearly visible but constituted <5% of blast events were also considered to have baseline mTORC1 activation if exposure to *ex vivo* sirolimus clearly eliminated blasts’ S6 phosphorylation. The magnitude of mTORC1 inhibition was defined by percent change in pS6 positive blasts = $100 * [\% \text{ pS6+ at baseline} - \% \text{ pS6+ on day 4 trough}] / \% \text{ pS6+ at baseline}$.

Statistical Analysis

Descriptive statistics and scatter plots were employed to characterize baseline patient characteristics and baseline and day 4 trough pS6 levels. Treatment-related toxicities were graded by NCI CTCAE and tabled. Overall response rate, defined as the fraction of subjects who achieved CR, CRp or PR, and its 95% confidence interval were estimated. Overall survival from initiation of treatment was estimated by the method of Kaplan and Meier. Median potential follow-up was computed by the reverse Kaplan-Meier method as proposed by Schemper and Smith [16]. Overall survival was compared between sirolimus sensitive and resistant subjects by the log rank test. Cox regression with a time-varying covariate was employed to compare overall survival between patients who had allogeneic transplant after sirolimus plus MEC and patients who did not have a transplant.

Association between baseline pS6 activity and overall response was tested by Fisher’s exact test. The day 4 trough pS6 level, which reflected an early change in mTORC1 kinase activity, was selected as a candidate predictive biomarker of clinical response based on preliminary data that maximal inhibition is achieved by Day 4. Baseline and day 4 trough pS6 levels were compared by Wilcoxon signed ranks test for paired data. Both visual inspection of scatter plots of mTORC1 inhibition by overall response and receiver operating characteristic (ROC) curve analysis guided our exploratory analysis to identify an optimal cut point in which to dichotomize subjects as being either sirolimus resistant (i.e., low inhibition) or sensitive (i.e., high inhibition). A subsequent descriptive analysis estimated the overall response rates for sirolimus resistant and sensitive subjects. Statistical significance was set at 0.05. All tests were 2-sided. Statistical analyses were performed using SPSS 20.0 software (SPSS, Inc., Chicago, IL) and Stata 10 (College Station, TX).

Results

Between April 2008 and January 2012, 52 subjects were enrolled to two consecutive trials with identical eligibility criteria. One subject was withdrawn for serious infection that precluded delivery of the planned regimen. Thus, 51 response evaluable subjects are included in this report, 15 from [NCT00780104](#) and 36 from study [NCT01184898](#). Table 1 lists baseline characteristics of all subjects. The median age at enrollment was 60, 56% had

abnormal cytogenetics, and 69% had received prior chemotherapy to which they were relapsed or refractory. Because of concurrent studies of FLT3 inhibitors, relatively few subjects with FLT3-ITD enrolled. The median follow-up was 56.4 months overall and 80.6 months for 5 living subjects.

Safety and toxicity

Sirolimus plus MEC was generally well tolerated and treatment-emergent adverse events were comparable in frequency and severity to those previously reported with MEC therapy alone (Table 2) [17]. Three subjects (6%) died prior to response assessment, all from infection. Grade 4 toxicities included one each of sepsis, mucositis, neutropenic fever, QTc prolongation/torsades de pointes, muscle weakness/gait disturbance, CHF, and LFT abnormalities. No cases of prolonged aplasia were observed.

Summary of Clinical Responses

The overall response rate was 47% (24/51, 95% confidence interval 33 – 61%). There were 17 (33%) complete responders. An additional patient had a CRi and 6 subjects had partial responses (PR). The rates of CR did not differ by age, baseline disease status, prior CR duration, or prior SCT (data not shown). Twenty subjects (39%) proceeded from sirolimus plus MEC chemotherapy to allogeneic transplant, including subjects with both CR/CRi and PR responses. Among partial responders, 2 experienced progressive leukemia and died and 3 underwent allogeneic transplant without intervening therapy. Of these, two achieved CR after transplant and remain alive; the other died of non-engraftment.

Baseline mTORC1 activity

Thirty-seven subjects had pre-sirolimus samples that were adequate for basal pS6 analysis. In 27 of these 37 (73%) subjects, baseline pS6 activity was detected. Consistent with prior publications, the degree of pS6 was heterogeneous among samples (median 9.7% pS6+ blasts, range <2% to 41%) [18]. The pS6 percent did not vary by baseline clinical characteristics (data not shown). Although initially we arbitrarily defined a cutoff of 5% pS6+ blast events as indicative of mTORC1 activation, five samples with % pS6+ between 2–5% pS6+ could be shown to have abrogation in pS6 signal after a 30 minute *ex vivo* exposure to 1000 nM sirolimus administration. These subjects' samples were additionally considered to have basal pS6 activation.

Ten (27%) subjects did not have detectable pS6 at baseline, consistent with previous reports that mTORC1 is not activated in all AML subjects [11,18]. The overall response rate among 27 subjects with baseline mTORC1 activity was not significantly different than that of the 10 subjects without baseline mTORC1 activity (52% vs. 40%, $p=0.20$, Fisher's exact test). Thus, baseline pS6 was not a predictor of response to sirolimus plus MEC chemotherapy.

Pharmacodynamic Assessment of mTORC1 inhibition

Pharmacodynamic effects to inhibit drug targets are predicated upon adequate drug concentrations. 33 subjects on the phase 2 study had pharmacokinetic samples drawn 2 hours (predicted peak) after the day 4 sirolimus dose was administered. 49 subjects across both studies had pharmacokinetic samples obtained prior to day 4 sirolimus dosing (trough).

The median peak and trough sirolimus concentrations on day 4 were 22 and 8.9 ng/mL (range 10.6–49.4 and 3.5–27.9, respectively). By phospho-flow, we previously demonstrated that 30 minute ex vivo exposures to 20 ng/ml sirolimus is sufficient to achieve potent mTORC1 inhibition in primary AML samples. This suggests mTORC1 inhibitory sirolimus concentrations were likely achieved during the trials in response-evaluable subjects [11]. Sirolimus concentrations did not vary by presence or absence of clinical response (data not shown). Next we evaluated change in pS6 in the 27 subjects with activated pS6 at baseline (Fig. 2a). The day 4 post-sirolimus pS6 levels were significantly reduced ($p=0.004$, Wilcoxon signed rank test, Fig. 2a). Of these 27 subjects, 15 (55%) had decreased pS6 levels on Day 4, while 9 of the remaining 12 subjects showed no change or modest decreases. Interestingly, 3 subjects showed dramatically increased pS6 levels during sirolimus therapy.

As baseline pS6 level did not correlate with response and adequate drug concentrations were achieved, we next studied the relationship between degree of mTORC1 inhibition and clinical response. We first stratified subjects by presence or absence of clinical response and then examined the distribution of day 4, sirolimus-induced pS6 changes (Fig. 2b). Although the mean degree of reduction in pS6 was greater in patients with as compared to those without clinical response, this did not reach statistical significance ($p=0.14$, Wilcoxon rank sum test). More notable, however, was the distribution of these changes as a function of clinical response. Day 4 pS6 changes were heterogeneous in the 14 non-responding subjects (range 98% reduction to 225% increase), while all 13 responding subjects showed >30% reductions in pS6 (range 31.5–87%).

Although all responders had >30% reduction in pS6 and the receiver operating characteristic (ROC) analysis indicated the optimal cut point was 32% reduction, we noted a mixed population near this value (Fig. 2b) which included 2 responders (pS6 levels were 31.5%, 32.5%) and 1 non-responder (pS6 level was 31.9%). Thus, we chose a more conservative cut point of 40% reduction, to discriminate between sirolimus sensitive and resistant subjects. Since this cut point was data-driven (i.e., based on clinical response), the overall response rates were vastly different in the sensitive (ORR= 71%) and resistant (ORR = 20%) groups, as expected. This analysis identified a possible predictive biomarker and due to the small sample of patients and exploratory nature, these results must be validated in an independent study.

Fig. 3 shows response rates and changes in pS6 during therapy for representative subjects with evidence of biochemical sensitivity and resistance to sirolimus, as well as subjects without basal pS6. In addition to the low response rates for sirolimus resistant subjects, similarly low response rates were observed with no basal mTORC1 activation (ORR 40%).

In this high-risk group of subjects studied on these trials, a single round of even highly intensive chemotherapy is not anticipated to achieve cure nor do chemotherapy-only strategies reliably protect against relapse. For this reason, subjects who are eligible for allogeneic transplant proceed to this treatment following induction therapy. In order to determine if the sirolimus plus MEC regimen can be incorporated into an overall approach to definitive AML therapy for high-risk subjects, analysis was done of event free and overall survival in this cohort. Note that survival was not a primary endpoint of our trial design and

all survival analyses are performed only for hypothesis generation. Median overall survival (OS) and event free survival (EFS) were 6.6 and 7.9 months, respectively (Fig. 4a/b) with one and two year survivals (+/- standard error) of 30.4% (+/- 9.6%) and 21.7% (+/-8.6%), respectively. Cox regression modeling with a time varying covariate demonstrated that subjects who proceeded to allogeneic transplant after sirolimus plus MEC did not have a significantly longer survival compared to subjects who did not have a transplant ($p=0.093$). Regardless of post-remission treatment--including HSCT--the number of long-term survivors was low, confirming the high-risk nature of this population. Finally, although the survival of subjects with basal pS6 and mTORC1 inhibition on day 4 was better than that of subjects with basal pS6 but no inhibition, as well as that of all other subjects, these improvements did not reach statistical significance (log rank $p=0.347$ and $p=0.23$, respectively; Supplemental fig S1A, S1B).

Discussion

We have performed a clinical evaluation of the sirolimus plus MEC regimen in patients with high-risk AML and developed a method to select patients most likely to respond to the combination regimen. Overall, we confirm our earlier observation of substantial clinical activity of the combination of sirolimus and MEC in high-risk AML. The response rate to sirolimus plus MEC (ORR=47%, CR/CRp= 35%) was higher than published studies of MEC in similar populations (CR=21%) [17], despite no obvious increase in observed toxicity. We conclude that sirolimus plus MEC is an effective, well-tolerated regimen for salvage or initial therapy of high-risk AML. The regimen is particularly well suited as a bridge to allogeneic transplantation as definitive therapy. Although we were unable to confirm a survival benefit to allogeneic transplantation among responding patients, this likely relates to the small numbers studied.

This study represents the largest study of ribosomal S6 phosphorylation in AML blasts performed on fresh tissue and the results have implications both for AML biology and the use of rapamycins for AML therapy. We find that circulating blasts from high-risk AML patients have variable degrees of constitutive pathway activation as measured by baseline ribosomal S6 phosphorylation. Of note, 27% of the subjects on this study, had samples with no detectable S6 activation and in no subjects was S6 activation seen in a majority of AML blasts. Thus single cell analysis resolves a heterogeneity of signal transduction in AML cells that cannot be appreciated by Western blotting or other methods analyzing aggregated populations of cells.

Clinically, we demonstrate that subjects with baseline activation of the mTORC1 signal transduction and *in vivo* pathway inhibition by sirolimus show a high clinical response rate to the combination of sirolimus and MEC chemotherapy. However, roughly half of enrolled subjects either lacked pathway activation or did not achieve *in vivo* biochemical inhibition. These latter two groups of subjects showed substantially lower rates of clinical response to sirolimus plus MEC. Although we observed a trend for improved survival among subjects with demonstrable mTORC1 inhibition during therapy, this did not reach statistical significance and requires confirmation in an appropriately powered study. Taken together, our data suggest that chemotherapy response in high-risk AML is modulated by PI3K/

mTOR signaling and that effective mTORC1 inhibition enhances chemotherapy response and possibly survival in a subset of AML patients that can be identified by phospho-flow.

Although we observed evidence of mTORC1 activation in the majority of enrolled subjects at baseline, sirolimus nonetheless demonstrated a heterogeneous ability to de-phosphorylate S6. This was true despite steady state trough levels in the range targeted for transplant immunosuppression for essentially all treated subjects. The mechanism through which S6 phosphorylation was maintained in the presence of sirolimus therapy requires further study, but potentially represents incomplete mTORC1 kinase inhibition, oncogenic re-wiring of signaling networks downstream of mTORC1, or marked upregulation of parallel signaling inputs to S6 that bypass mTORC1 (e.g. via ras/MAPK and/or RSK). Additionally, whether alternative inhibitors would similarly enhance chemotherapy response is worthy of future study. Such approaches could include more potent or alternative methods to inhibit mTORC1 kinase, including PI3 kinase inhibitors, mTOR catalytic site inhibitors, or parenteral rapamycins. Alternatively agents targeting parallel S6 inputs, such as MEK inhibitors might be integrated with chemotherapy or rapamycins. Such agents may similarly increase chemotherapy response in patients not benefited from sirolimus, but this approach clearly requires methods that rationally allocate patients to one approach or another.

Our data have implications for the interpretation of other clinical trials testing mTOR inhibitors in AML. At present, the ECOG, GIMEMA, UK-MRC, and EORTC have each embarked upon randomized clinical trials utilizing sirolimus and/or everolimus in combination with chemotherapy for AML. These studies may ultimately show a benefit for the approach overall, though early results have been disappointing [19]. Importantly, our data suggest caution in the interpretation of these and other trials that lack enrichment strategies or correlative assays to identify mTORC1 activation prior to therapy and/or monitor sensitivity of leukemic blasts to tested mTORC1 inhibitors. Indeed, the magnitude of clinical benefit observed could be substantially diluted by subjects without target activation and/or sensitivity who may be unlikely to benefit from the approach.

In summary, we show sirolimus plus MEC chemotherapy to be a tolerable, clinically active regimen in subjects with high risk AML with a promising rate of allogeneic transplantation and survival for responding subjects. From our phospho-flow analysis, we conclude that the benefits of sirolimus' addition to MEC chemotherapy appear to be limited to subjects with evidence of baseline mTORC1 activation in leukemia cells and mTORC1 inhibition during therapy. These correlative data support our original hypothesis that mTORC1 regulates chemotherapy sensitivity in a substantial subset of patients with AML. Future studies are needed to prospectively validate the use of S6 phosphorylation measurements by flow as a predictive biomarker of response to sirolimus plus MEC among high-risk AML patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Treatment schema showing the sirolimus and MEC treatment regimen. Arrows indicate pharmacokinetic/pharmacodynamic sampling time points used on final analysis. To estimate peak concentration effects of sirolimus, drug concentration (and associated phospho-flow analysis) was also measured on a subset of subjects 2 hours following sirolimus dosing on study days 1 and 4, not shown

Fig. 2.

(a) Paired analysis of pS6 positivity at baseline and trough level on day 4 in gated blasts. Median percent pS6 positivity was 9.7% (range 2.2 – 41.4%) at baseline and 3.0 (0.43 – 78.0%) at day 4 (Wilcoxon signed ranks test, $p=0.004$). (b) Percent change in pS6 positivity from baseline to day 4, for 27 patients with baseline pS6 activation. Patients are grouped by overall response. Responders include CR, CRi and PR. The solid line indicates no change from baseline. The dotted line indicates the cut point of >40% reduction used to distinguish sirolimus sensitive patients from sirolimus resistant patients.

Fig. 3.

Frequency of clinical responses to sirolimus plus MEC among subjects grouped for baseline mTORC1 activation and/or target inhibition as measured by percentage pS6 change in gated blasts by phospho-flow. Dotplots show gated blasts pS6 on vertical axis. Horizontal line to define pS6+ events was drawn for individual samples using dynamic controls. Biochemical sensitivity is defined by >40% reduction in pS6 on day 4, compared to baseline. CR= complete remission, CRi= complete remission with incomplete hematologic recovery, PR= partial remission, ORR= overall response rate

Fig. 4.

Survival of patients treated with sirolimus plus MEC

(a) Kaplan-Meier estimate of overall survival from first day of treatment. (b) Event free survival for 23 patients who responded to sirolimus plus MEC.

Table 1

Baseline characteristics (n=51). Percentages were rounded to nearest integer.

Age, median (range)		60	23–77
Sex		#	%
	Male	32	62
	Female	19	37
Disease status			
	Untreated, age >60	7	14
	Untreated secondary AML	8	16
	Primary refractory	12	24
	First relapse	18	35
	First CR <6 months	8	16
	First CR 6–12 months	6	12
	First CR >12 months	4	8
	>First or refractory relapse	6	12
Cytogenetic Risk Group			
	Poor risk	18	35
	Intermediate	27	53
	Favorable	3	6
	monosomal	8	16
	No growth	3	6
	FLT3-ITD+	7	14

Table 2
Treatment-emergent adverse events Grade 3 or greater, regardless of attribution to sirolimus

Toxicity	Grade 3	Grade 4	Grade 5	Total	%
Neutropenic fever	22	1		23	43
Shortness of breath/hypoxia	4			4	8
Sepsis/Infection *	14	1	3 [†]	18	35
Diarrhea	2			2	4
Electrolyte abnormalities	5			5	10
LFT abnormalities	1	1		2	4
Dehydration	2			2	4
Bleeding	2			2	4
Anorexia	1			1	2
Mucositis		1		1	2
QTc prolongation/Torsades de pointes		1		1	2
Muscle weakness/Gait disturbance		1		1	2
Congestive heart failure		1		1	2

* includes bacteremia and pneumonia

[†] all deaths occurred prior to treatment response assessment