Single-Cell Pharmacodynamic Monitoring of S6 Ribosomal Protein Phosphorylation in AML Blasts during a Clinical Trial Combining the mTOR Inhibitor Sirolimus and Intensive Chemotherapy

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Abstract

**Purpose:** Integration of signal transduction inhibitors into chemotherapy regimens generally has generally not led to anticipated increases in response and survival. However, it remains unclear whether this is because of inadequate or inconsistent inhibition of target or other complex biology. The mTOR signaling pathway is frequently activated in acute myelogenous leukemia (AML) and we previously showed the safety of combining the mTOR inhibitor, sirolimus, with mitoxantrone, etoposide, and cytarabine (MEC) chemotherapy. However, we did not reliably determine the extent of mTOR inhibition on that study. Here, we sought to develop an assay that allowed us to serially quantify the activation state of mTOR kinase during therapy.

**Experimental Design:** To provide evidence of mTOR kinase activation and inhibition, we applied a validated whole blood fixation/permeabilization technique for flow cytometry to serially monitor S6 ribosomal protein (S6) phosphorylation in immunophenotypically identified AML blasts.

**Results:** With this approach, we show activation of mTOR signaling in 8 of 10 subjects’ samples (80%) and conclusively show inhibition of mTOR in the majority of subjects’ tumor cell during therapy. Of note, S6 phosphorylation in AML blasts is heterogeneous and, in some cases, intrinsically resistant to rapamycin at clinically achieved concentrations.

**Conclusions:** The methodology described is rapid and reproducible. We show the feasibility of real-time, direct pharmacodynamic monitoring by flow cytometry during clinical trials combining intensive chemotherapy and signal transduction inhibitors. This approach greatly clarifies pharmacokinetic/pharmacodynamic relationships and has broad application to preclinical and clinical testing of drugs whose direct or downstream effects disrupt PI3K/AKT/mTOR signaling. *Clin Cancer Res; 18(6); 1716–25. ©2011 AACR.*

Introduction

It has been proposed that molecularly targeted cancer therapeutics would herald an era of improved clinical response and reduced need for more highly toxic, traditional cytotoxic chemotherapy. However, with few exceptions, neither goal has been met in many, if not most tumor types. There are several possible reasons for this dilemma. Signal transduction inhibitors (STI) may not adequately inhibit target proteins *in vivo* (1, 2). Alternatively, functional redundancy within cancer cells may allow several signaling pathways to share roles in maintaining growth or survival (3). Finally, it is possible that there is heterogeneity of signaling between different populations of tumor cells so that STI therapy may simply select for a subpopulation of cells that do not require the targeted pathway to survive (4). As none of these effects can be predicted by drug levels alone, direct pharmacodynamic measurements of signal transduction inhibitors during early-phase clinical trials may facilitate rational clinical development of molecularly targeted agents. In developmental therapeutics for most epithelial and soft tissue tumors, repeated tumor biopsy sampling is logistically challenging. However, hematopoietic cancers such as leukemia provide an excellent opportunity to serially sample circulating tumor cells to validate novel agents targeting signal transduction.

Phospho-specific flow cytometry is an emerging technique for single-cell evaluation of signal transduction. In hematologic malignancies that are well characterized immunophenotypically, it holds the promise to provide tumor-specific analysis of signal transduction in...
heterogeneous tissue (5–7). This includes peripheral blood and/or bone marrow samples containing admixtures of normal and malignant cells. Using antibodies that are highly specific for the phosphorylation state of key residues on signaling proteins, this technology allows the researcher to interpolate a signal transduction pathway’s activation state in immunophenotypically identified cells. However, the necessary reagents for sample processing potentially alter protein structure and/or antibody binding in ways that can compromise data quality for cell surface epitopes, light scatter, and intracellular phospho-proteins alike (8). These reagents typically include formaldehyde or paraformaldehyde to fix cells followed by alcohols or detergents to permeabilize their cellular membranes. Although relatively easily applied to highly uniform cell samples, such as cell lines, phospho-specific flow cytometry has thus far resisted routine application to clinical specimens.

Recent advances have identified methodologies that simplify processing and improve data quality from clinical specimens, particularly for evaluation of ras/MAPK and PI3K/AKT/mTOR signaling (9, 10). Current whole blood and marrow fixation techniques provide excellent preservation of light scatter properties and surface immunophenotype, allowing for evaluation of single or multiple phospho-proteins in specific cell populations (11, 12). However, few if any clinical trials in hematologic malignancies have used these techniques systematically to evaluate pharmacodynamic effects of drugs with concurrent therapeutic drug monitoring.

Acute myelogenous leukemia (AML) is a clinically and genetically heterogeneous hematopoietic cancer characterized by the accumulation of immature myeloid precursors in the marrow and blood, leading to rapid and inexorable exhaustion of normal hematopoiesis. Clinically, AML has a poor survival because of high relapse rates following initial chemotherapy or de novo chemotherapy resistance, which is seen in half of patients older than 65 years (13). Investigational approaches in AML have included targeting oncogenic signal transduction, either alone or in combination with chemotherapy. Preclinical data from our group and others highlight a critical role for the activation of phosphoinositide 3-kinase (PI3K) pathway signaling through its downstream effectors AKT (14, 15). These data suggest that the mTOR plays a critical role in chemotherapy resistance and inhibition of mTOR may augment chemotherapy response. We therefore designed clinical trials combining rapamycin (sirolimus, Rapamune) with cytotoxic chemotherapy for AML (16).

The cellular effects of mTOR are largely mediated by its activation of the p70S6 kinase, which itself phosphorylates ribosomal protein S6 (S6). Although p70S6 kinase antibodies are useful readouts by Western blot, they have not been optimized for flow cytometric analysis. In contrast, S6 is abundantly expressed in normal and malignant cells, and evaluation of S6 phosphorylation is commonly used as a surrogate marker for mTOR activation. Importantly, S6 phosphorylation is rapidly and thoroughly inhibited in the presence of rapamycin, whereas other functions of mTOR (e.g., phosphorylation of its other major downstream target, 4EBP1) are less clearly modulated by rapamycin treatment (17).

Here, we report the pharmacodynamic results of a pilot study of the rapamycin mTOR inhibitor sirolimus in combination with intensive AML induction chemotherapy. These data show the feasibility of serial flow cytometric monitoring of mTOR activation state in immunophenotypically identified tumor cells from fixed whole blood. To measure mTOR activation by flow cytometry, we used S6 phosphorylation as a readout. This approach provides robust data despite low circulating tumor burden, and, in combination with therapeutic drug monitoring and ex vivo dose–response modeling, highlights biologic heterogeneity in AML tumor cells that may predict clinical response or resistance to molecularly targeted agents.

Materials and Methods

Clinical trial subject selection

Subjects were recruited from the clinical practices of the University of Pennsylvania, Philadelphia, PA, between February 2008 and November 2008. Eligible subjects were older than 18 years with non-M3 AML who either had relapsed following prior chemotherapy or were refractory to induction chemotherapy. Primary refractory leukemia was defined as either persistent disease (>5% marrow blasts) following 2 induction cycles or recurrent leukemia following a single induction cycle that had resulted in complete tumor clearance from a nadir or recovering marrow biopsy. Patients older than 60 years were eligible with untreated AML provided they had no evidence of SWOG favorable risk karyotype by cytogenetics, FISH, or
multiplexed RT-PCR (for AML1-ETO or MYH11-CBFβ). Patients with untreated secondary leukemia—defined as AML arising out of an antecedent hematologic disorder or following chemotherapy or radiation therapy—were also eligible. Pathology review at the Hospital of the University of Pennsylvania was carried out to confirm diagnoses in all cases.

All subjects were required to have an Eastern Cooperative Oncology Group (ECOG) performance status of 0 to 1 and resolution of prior treatment-related toxicities. Required baseline organ function studies specified an ejection fraction above 45%, creatinine ≤ 2.0 mg/dL, total bilirubin ≤ 1.5 mg/dL, hepatic transaminases ≤ 3× upper level of normal, no uncontrolled infections, and no unstable baseline comorbidities that would jeopardize toxicity assessment. Because of significant drug–drug interactions between sirolimus and systemic imidazole or triazole antifungals, these medications were prohibited for 72 hours before study entry. Subjects whose medical regimen required drugs with marked effects upon sirolimus pharmacokinetics (e.g., rifampin, carbemazepine, verapamil, diltiazem, tacrolimus) were ineligible.

The research was approved by the Institutional Review Boards of the University of Pennsylvania and the study was monitored by the Clinical Trials Safety Review and Monitoring Committee of the Abramson Cancer Center of the University of Pennsylvania. Written informed consent was obtained from all subjects according to the Declaration of Helsinki.

Treatment schema

The treatment regimen is shown in Fig. 1 and consisted of a loading dose of 12 mg sirolimus by mouth on day 1 followed by 8 daily doses of 4 mg sirolimus administered orally. Seventy-two hours after the sirolimus loading dose, subjects began intravenous mitoxantrone, etoposide, and cytarabine (MEC) chemotherapy (mitoxantrone 8 mg/m²/d on days 1 to 5, etoposide 100 mg/m²/d on days 1 to 5, and cytarabine 1,000 mg/m²/d on days 1 to 5) using actual body weight for dose calculations. Each intravenous chemotherapy drug was administered over 1 hour and actual body weight was used for chemotherapy calculations. Concurrent hydroxyurea was allowed on days 1 to 3 (i.e., concurrent with sirolimus monotherapy) to control leukocytosis before initiation of MEC chemotherapy. Whole blood rapamycin concentration was measured in the clinical laboratory by high-pressure liquid chromatography from samples drawn before the daily dose on treatment days 4 and 7. Sirolimus doses were not adjusted for weight or altered on the basis of measured drug concentration. Concurrent with the day 4 trough sirolimus level, peripheral blood was collected and processed as below for flow cytometric pharmacodynamic analysis.

Supportive care and toxicity assessment

Subjects care guidelines were previously described (16). Toxicity assessment used the National Cancer Institute's Common Toxicity Criteria for Adverse Events (CTCAE) version 3.0 (http://ctep.cancer.gov/protocoldevelopment/electronic_applications/docs/ctcaev3.pdf).

Response criteria

Complete remission was defined by a bone marrow aspirate showing >20% cellularity, trilineage hematopoiesis, and <5% blasts as well as the presence of an absolute neutrophil count >1,000/μL, an untransfused platelet count of >100,000/μL, and no circulating or extramedullary leukemic blasts (18). Complete remission without platelet recovery (CRp) met all criteria for CR except that the platelet count failed to exceed 100,000/μL. A partial remission (PR) met all criteria for CR, except that the marrow blast percentage was between 5% and 25%. Progressive disease was defined as 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.

Flow cytometric pharmacodynamic analysis

Peripheral blood was collected into heparinized vacutainers and was transported to the laboratory within 1 hour of collection for samples drawn after sirolimus and immediately processed upon receipt. Baseline samples were

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Treatment schema. A 12-mg loading dose of oral sirolimus was administered on day 1, followed by 8 daily doses of 4-mg sirolimus (S). Mitoxantrone (100 mg/m²/d), etoposide (100 mg/m²/d), and cytarabine (1,000 mg/m²/d) were each administered over 1 hour for 5 doses each starting after sirolimus administration on study day 4. Samples were obtained for pharmacodynamic measurement and rapamycin concentration measurement on day 4 before sirolimus dosing. A second rapamycin level was obtained before sirolimus dosing on day 7.
processed within 24 hours of collection. Prior work in our laboratory has shown that this time frame did not alter measurements of mTOR activation (data not shown) in the absence of signal transduction inhibitor therapy. Whole blood was passed through a 40 μm mesh filter to remove any possible clotted specimen. Multiple 100 μl aliquots were prepared in 15 × 75 mm² flow tubes. To these aliquots, sample conditions included unmanipulated aliquots as well as ex vivo activation of S6 phosphorylation by phorbol myristate acetate (PMA; Sigma-Aldrich) 400 nmol/L for 10 minutes, and inhibition by rapamycin (Sigma-Aldrich) 500 or 1,000 nmol/L for 30 minutes. These conditions were carried out in a dry bath (humidified incubator at 37°C, 21% O₂, 5% CO₂).

Following incubation in the dry bath, blood samples were fixed, red cells lysed, and remaining nucleated cells permeabilized as previously described (9, 10). Briefly, cells were fixed for 10 minutes by addition of room temperature methanol-free formaldehyde (Polysciences) directly to samples to achieve a final concentration of 4%. To permeabilize nucleated cells and lyse red blood cells, fixed samples were then incubated for 30 minutes at room temperature in Triton X-100 (Sigma-Aldrich) diluted in calcium and magnesium-free PBS to a final concentration 0.1%. Triton X-100 was prewarmed to 37°C before addition to the fixed blood. Samples were then centrifuged for 3 minutes at 1,000 × g at 4°C, washed with 4°C 4% bovine serum albumin (BSA) in PBS wash buffer, centrifuged again, and supernatants were discarded. The resulting cell pellets were either resuspended in medium containing 10% weight/volume glycerol (Sigma-Aldrich), 20% fetal calf serum, and 70% PBS and stored at −20°C or analyzed immediately. Frozen baseline and day 4 samples were thawed by incubation in a room temperature water bath and then were centrifuged as above, washed once as above, and the cell pellets resuspended in ice cold 50% methanol in 0.9% NaCl for 10 minutes to unmask phospho-epitopes. Samples used for ex vivo drug sensitivity studies were not frozen but were immediately placed in methanol as above and analyzed by flow cytometry on the day of collection. Following methanol incubation, samples were washed twice at 4°C and stained with antibodies (30 minutes in the dark), then washed once as above, and the cell pellets resuspended in 4% bovine serum albumin (BSA) in PBS before addition to the fixed blood.

Samples were then centrifuged for 3 minutes at 1,000 × g at 4°C, washed with 4°C 4% bovine serum albumin (BSA) in PBS wash buffer, centrifuged again, and supernatants were discarded. The resulting cell pellets were either resuspended in medium containing 10% weight/volume glycerol (Sigma-Aldrich), 20% fetal calf serum, and 70% PBS and stored at −20°C or analyzed immediately. Frozen baseline and day 4 samples were thawed by incubation in a room temperature water bath and then were centrifuged as above, washed once as above, and the cell pellets resuspended in ice cold 50% methanol in 0.9% NaCl for 10 minutes to unmask phospho-epitopes. Samples used for ex vivo drug sensitivity studies were not frozen but were immediately placed in methanol as above and analyzed by flow cytometry on the day of collection. Following methanol incubation, samples were washed twice at 4°C and stained with antibodies (30 minutes in the dark), then washed once and subjected to flow cytometric analysis.

Antibodies used included murine anti-human CD45 PerCP, CD45 APC-Cy7, CD34 APC, CD33 PE-Texas Red (Invitrogen), CD45 PE-Cy5, CD33 PE, CD34 PE, CD14 PE, CD10 APC (all from Becton Dickinson), rabbit monoclonal phospho-S6 Alexa 488 or Alexa 647 (Cell Signaling). A CD10 APC (all from Becton Dickinson), murine anti-human CD45 PerCP, CD45 APC-Cy7, CD34 APC, CD33 PE-Texas Red (Cell Signaling). A rabbit monoclonal phospho-S6 Alexa 488 or Alexa 647 (Cell Signaling). A

**Table 1. Baseline characteristics of the 10 subjects who provided paired samples for flow cytometric pharmacodynamic analysis**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n = 10</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (range), y</td>
<td>61 (32–75)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>9</td>
</tr>
<tr>
<td>Female</td>
<td>1</td>
</tr>
<tr>
<td>Disease status</td>
<td></td>
</tr>
<tr>
<td>First relapse/primary refractory</td>
<td>6</td>
</tr>
<tr>
<td>Primary refractory/first CR ≤ 6 mo.</td>
<td>3</td>
</tr>
<tr>
<td>First CR 6–12 mo</td>
<td>2</td>
</tr>
<tr>
<td>First CR &gt; 12 mo</td>
<td>1</td>
</tr>
<tr>
<td>&gt;First relapse</td>
<td>2</td>
</tr>
<tr>
<td>Secondary AML (antecedent MDS)</td>
<td>1</td>
</tr>
<tr>
<td>Age &gt;60 (without favorable karyotype)</td>
<td>1</td>
</tr>
<tr>
<td>Karyotype</td>
<td></td>
</tr>
<tr>
<td>Favorable</td>
<td>2</td>
</tr>
<tr>
<td>Intermediate</td>
<td>8</td>
</tr>
<tr>
<td>Unfavorable</td>
<td>0</td>
</tr>
<tr>
<td>FLT3-ITD</td>
<td>3</td>
</tr>
<tr>
<td>Prior therapy has been defined as</td>
<td></td>
</tr>
<tr>
<td>Anthracycline/cytarabine (7 + 3)</td>
<td>8</td>
</tr>
<tr>
<td>High-dose cytarabine (postremission)</td>
<td>3</td>
</tr>
<tr>
<td>High-dose cytarabine (at relapse)</td>
<td>2</td>
</tr>
<tr>
<td>Myeloablative transplant</td>
<td>2</td>
</tr>
<tr>
<td>No prior AML therapy</td>
<td>2</td>
</tr>
</tbody>
</table>

Note: High-dose cytarabine regimens were defined by ara-C doses of at least 1 g/m².

Abbreviations: CR, complete remission; FLT3-ITD, Fms-like tyrosine kinase 3/internal tandem duplication; MDS, myelodysplastic syndrome.
myelomonocytic/monoblastic leukemic blast populations may extend into or exclusively occupy a CD45 bright region otherwise enriched for monocytes.

Although our cell processing reduced the brightness of cell surface antigen staining to some degree, it nonetheless provided excellent discrimination of light scatter and CD45 expression. This was sufficient to define discrete cell population gates in all subjects (Fig. 2, top left). Of note, 4 of 10 subjects on our study had a total white blood cell (WBC) count below $5 \times 10^3/\mu L$ and an absolute blast counts (ABC, defined as blast percentage $\times$ total WBC count) below $1 \times 10^3/\mu L$ on at least one pharmacodynamic peripheral blood draw. An additional subject had no visible circulating blasts by peripheral smear and underwent paired marrow aspiration at baseline and day 4 for pharmacodynamic sampling. Morphologic hematopathology review of the marrow aspirate in this subject showed only 15% blasts. Depending upon the subjects’ clinical immunophenotypes, antibodies directed against myeloid and/or immaturity surface antigens (e.g., CD33 and CD34) were used to confirm the blast gate and were informative in all cases. These findings show the feasibility of obtaining consistent tumor-specific data on peripheral blood samples even on patients with a low WBC.

S6 phosphorylation was evaluable in all subjects samples, even in cases where the WBC was as low as $1.1 \times 10^3$ with 10% blasts (i.e., $ABC = 110/\mu L$). In several subjects with acute monocytic or myelomonocytic leukemias, expansion of both a CD45 dim, SSC low blast gate and a CD45, SSC-intermediate monocytic gate was noted. In these cases, each population was evaluated for S6 phosphorylation. Discrimination between monocyte and lymphocyte populations was made on the basis of CD33 staining and both blast and monocyte population analysis were carried out in these samples helped confirm myeloid lineage. CD34 was not expressed on this subject’s granulocytes, but a small population has variable, but meaningful data evaluation in this population. PMA-stimulated cells (right column) define a positive region for S6 phosphorylated cell events. The majority of blasts at baseline show no constitutive phosphorylation, but a small population has variable, but constitutive activation. Ex vivo rapamycin treatment of the baseline blood sample (middle column) inhibits mTOR and markedly reduces the percentage of blasts with constitutive S6 phosphorylation.
cases (Fig. 2, top right). Thus, we were able to analyze S6 phosphorylation in separate populations of normal and malignant cells in all samples.

Comparing samples drawn at baseline to samples treated ex vivo with PMA or rapamycin, we saw dynamic changes in S6 phosphorylation (Fig. 2, bottom). In all subjects’ samples, PMA led to marked upregulation of S6 phosphorylation in all CD45+ cell populations, regardless of staining pattern in the unmanipulated sample. This serves as an internal control for cell viability and served as a positive control for the purpose of estimating the percentage of internal control for cell viability and served as a positive control in the unmanipulated sample. This serves as an internal control for cell viability and served as a positive control for the purpose of estimating the percentage of blasts with mTOR activation at baseline. It further shows that the phospho-S6 epitope is fully unmasked by our cell processing method and capable of resolution if phosphorylated. Ex vivo treatment with ≥500 nmol/L rapamycin markedly reduced or abrogated S6 phosphorylation in all samples that showed baseline constitutive S6 phosphorylation. Comparing PMA as a positive control and rapamycin and/or FMO treatment as positive and negative controls, respectively, we created discrimination lines for positive and negative phospho-S6 populations and scored percentage of positive cells for each group of cells.

For baseline samples, the most consistent finding was marked heterogeneity of S6 phosphorylation among leukemic blasts. The majority of samples (8 of 10) showed both a dominant discrete population that lacked S6 phosphorylation (similar phospho-S6 brightness to either the FMO control or lymphocytes) and a smaller subset of cells with heterogeneous, but constitutive S6 phosphorylation, comparable in brightness to the range of PMA-stimulated blasts. In 2 subjects’ baseline samples, phospho-S6 staining fell uniformly in the negative gate and no obvious positive phospho-S6 events were seen. From these gates, we defined baseline percentages of positive cell events. In cases where there was marked separation between narrowly clustered positive and FMO populations, a midpoint between the 2 populations was chosen for discrimination, as previously described (20).

These data show that, consistent with previous results from our laboratory and others, mTOR is constitutively activated in AML blasts in the majority of primary samples. However, this activation is actually only present in a subset of blasts at any one time. The described intracellular phospho-specific flow cytometry of fixed whole blood allowed for several applications. For example, we used the methodology to determine the sensitivity of AML cells to rapamycin. To do this, we evaluated the effect of a 30-minute ex vivo incubation of the baseline sample in increasing concentrations of rapamycin to estimate a dose–response curve for mTOR inhibition in leukemic blasts. A representative sample showing low nanomolar sensitivity to rapamycin is shown in Fig. 3 (top). Note that concentrations below 10 nmol/L (corresponding to clinically measured concentrations of 9.1 ng/mL) fail to produce substantial reduction in S6 phosphorylation, but concentrations at or above 20 nmol/L show similar reduction to that of 1,000 nmol/L rapamycin. This suggests that near-maximal inhibition occurs between 10 and 20 nmol/L in this sample, which is similar to clinically achieved concentrations. These studies show that we can assay in vitro drug sensitivity on AML cells and compare the effects of in vitro inhibition of target with in vivo results. This allows us to discriminate between patients who received inadequate concentrations of drug in vivo compared with samples with endogenous rapamycin insensitivity.

Flow cytometry also allowed for analysis of signaling in different subpopulations of blast cells. In one subject with AML arising from underlying chronic myelomonocytic leukemia, the baseline sample showed abnormal expansion of both an immature monoblast population and a more differentiated monoblast population by CD45 and SSC (Supplementary Fig. S1). These 2 populations were confirmed as discrete populations by CD34, CD33, and CD14 staining (CD34+, CD33 dim+, CD14− myeloblasts vs. CD34−, CD33 bright+, CD14+ monoblasts). Interestingly, the myeloblast population showed no baseline phosphorylation of S6, although constitutive phosphorylation was readily seen in monoblasts. Thirty-minute ex vivo treatment with rapamycin failed to potently inhibit S6 phosphorylation in monocytes until concentrations above 50 nmol/L were evaluated. Notably, 50 nmol/L exceeds measured blood rapamycin concentrations from the trial. Thus, methodologically, we are able to show variations of signaling within subpopulations of leukemic cells, which may be important for understanding the diverse response of leukemia to signal transduction inhibitors.

Finally, we focused on clinical evaluation of the effect of oral sirolimus therapy upon S6 phosphorylation (Fig. 3, bottom). Because the majority of the blast events showed phospho-S6 staining in a range comparable to FMO, shifts in the mean fluorescence intensity were less dramatic than changes in the percentage of cells in the positive phospho-S6 gate. To quantify the degree of mTOR inhibition, we calculated an inhibition percentage, defined by 1 minus the fraction of positive phospho-S6 blasts on day 4 divided by the percentage at baseline. Thus, if a subject had 20% phospho-S6–positive blasts at baseline and 5% on day 4, the inhibition percentage was 1 − (5/20) = 75% inhibition. We therefore could use changes in S6 phosphorylation during therapy to group subjects based upon their blasts’ biochemical sensitivity to sirolimus.

Three patterns of baseline mTOR activation and response to sirolimus emerged from our analysis. Of the 8 subjects that showed baseline constitutive phosphorylation of S6, 6 showed marked reduction in the percentage of phospho-S6–positive events (Fig. 4) Such subjects blasts were considered biochemically “sirolimus-sensitive” and showed a mean inhibition percentage of 75% reduction in the percentage of cells in the positive gate for S6 phosphorylation (range, 53%–98%). The 2 subjects without constitutive phosphorylation of S6 showed no appreciable change in their blasts’ S6 phosphorylation on day 4 and were considered uninformative for sirolimus sensitivity. Two subjects showed persistent phosphorylation in their blasts on day 4 and were considered sirolimus-resistant. In one subject, baseline and day 4 percentage positive phospho-S6 events were similar, and in the other, a marked

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increase in phospho-S6 events was seen. Of note, this subject's baseline sample was noted to require relatively higher concentrations of rapamycin in \textit{ex vivo} testing to show inhibition of S6 phosphorylation. Interestingly, measured sirolimus troughs were similar in biochemically sirolimus-sensitive and -resistant leukemia. Overall, 8 of 10 (80\%) patients showed activation of S6 at baseline. Of these 8 subjects, 6 of 8 (75\%) were inhibited \textit{in vivo}. One of these 2 patients showed sirolimus resistance both \textit{in vivo} and \textit{in vitro}. The other subject's samples were not evaluated for \textit{in vitro} sensitivity. Four subjects had \textit{ex vivo} testing carried out on their baseline samples. Of note, 3 of 3 subjects whose \textit{ex vivo} testing of their leukemia sample for rapamycin sensitivity showed sensitivity at \( \leq 20 \text{ nmol/L} \) had biochemical sensitivity to sirolimus during therapy. One subject baseline showed no inhibition of S6 phosphorylation \( \leq 20 \text{ nmol/L} \), modest reduction at \( 50 \text{ nmol/L} \), and marked inhibition at \( 1,000 \text{ nmol/L} \). This subject's day 4 sample showed biochemical sirolimus resistance.

Although the intent of this pilot study was not to evaluate clinical efficacy, clinical responses to sirolimus and MEC were evaluable in 9 of 10 subjects who provided paired samples for flow analysis. One subject died of bacterial infection before hematopoietic recovery from sirolimus MEC and was not evaluable for treatment response. Among the 5 evaluable sirolimus-sensitive subjects, 1 CR and 2 PRs were seen. Neither of the 2 evaluable sirolimus-resistant patients showed a clinical response. One of the 2 evaluable subjects without baseline S6 phosphorylation had a PR.

\section*{Discussion}

These data provide the first evidence that serial intracellular flow cytometric monitoring of fixed peripheral blood can be used to evaluate the biochemical efficacy of drugs targeting the PI3K/AKT/mTOR signal transduction pathway in leukemia clinical trials. In addition, they show heterogeneity in rapamycin sensitivity and suggest that evaluation for \textit{ex vivo} drug sensitivity prior to or early in therapy may have a role in predicting chemotherapy response to regimens combining mTOR inhibitors and chemotherapy. This approach may promote more rational enrollment of subjects to clinical trials of novel therapeutics and eventually is hoped to help develop personalized approaches in the future.

\section*{Figure 3. \textit{Ex vivo} inhibition of mTOR predicts \textit{in vivo} effects of oral sirolimus therapy. Top, exposing aliquots of a representative baseline sample (Fig. 2) to an increasing concentration of rapamycin \textit{ex vivo} suggest that target inhibition occurs at a whole blood concentration between 10 and 20 nmol/L (8.1–18.2 ng/mL). Of note, higher concentrations do not further impair S6 phosphorylation, predicting that mTOR inhibition in this sample will occur at clinically achievable concentrations. Bottom, serially obtained samples show abrogation of S6 phosphorylation \textit{in vivo} during oral sirolimus therapy. Measured rapamycin concentration on day 4 was 4.7 ng/mL (5.1 nmol/L). Exposure of the day 4 sample to \textit{ex vivo} rapamycin in excess shows no significant additional effect, suggesting that near maximal target inhibition occurred \textit{in vivo}. Note: Although all samples shown in this figure were obtained from the same patient's sample, the staining and cytometer data acquisition for the 2 experiments were not carried out simultaneously. This accounts for minor variation in phospho-S6 staining across the \textit{ex vivo} and \textit{in vivo} experiments. Within experiments, cell samples were exposed to a single staining antibody cocktail, such that dynamic changes in phospho-signal can be directly compared.
Using serial flow cytometric analysis of clinical trial samples, we confirm widespread constitutive activation of mTOR in circulating peripheral blood AML cells. Consistent with prior data, however, we show marked heterogeneity in the degree of this activation among patients and within particular patients’ blast populations. We further show that oral administration of the mTOR inhibitor sirolimus during an intensive combination chemotherapy regimen achieves successful disruption of mTOR enzymatic function in the malignant cells of the majority of treated patients. Finally, we show that, in a small number of patient samples, constitutive phosphorylation is present but resistant to mTOR inhibition by oral sirolimus. This finding was not predicted by therapeutic drug monitoring, suggesting cell-intrinsic resistance, as confirmed in one case by ex vivo bioassays of rapamycin sensitivity. These results show that flow cytometric assays highlight biologic heterogeneity that is invisible to techniques such as Western blot.

Drug development of rapamycin analogues for cancer therapy has emphasized parenteral formulations and/or drugs with improved bioavailability in comparison to rapamycin. The goal of these second generation drugs is to produce comparably high rapamycin concentrations. Interestingly, our data show relatively low doses of rapamycin lead to effectively maximal mTOR inhibition in tumor cells. This is particularly true in samples that show sensitivity by ex vivo testing. Thus in many, if not most patients, second-generation rapalogs may unnecessarily add expense and/or dose-dependent toxicity compared with sirolimus.

Because of the abundance of tumor cells that are readily accessible during clinical care, leukemia is perhaps an ideal disease in which direct pharmacodynamic monitoring technology can be developed. Despite this apparent ease, pharmacodynamic monitoring in leukemia in the past has not been entirely straightforward. Studies of signal transduction traditionally used density centrifugation (Ficoll separation) and Western blot of isolated mononuclear fractions. Although marrow aspirates often contain highly purified populations of leukemic cells, the quality of tumor sampling is invariably compromised by peripheral blood “hemodilution” of the specimen during aspiration. In addition, density centrifugation enriches for but does not exclusively isolate tumor cells. Because both leukemic and non-malignant cells express target signaling proteins, it is notable that the concentrations necessary to abrogate enzymatic function of several kinases may differ from that of normal tissue and can vary substantially among tumor samples. Finally, cell lysis for Western blot eliminates all ability to resolve the heterogeneity of these samples. Taken together, obtaining pharmacodynamic signaling data in leukemia trials is procedurally simple, but interpretation of these data can prove challenging.

Phospho-specific intracellular flow cytometry thus provides an attractive method to directly measure changes in key signaling proteins in response to clinical therapeutics and has appropriate sensitivity to provide robust readout despite low circulating tumor burden. Despite these potential advantages, we note several limitations of this report. We recognize that our data were obtained in real-time and our expertise in flow cytometry to immunophenotype samples developed during this research effort. Although the majority of samples in this study were evaluated using 3 or 4 color cytometry, we currently are using a more sophisticated 6 to 10 fluorophore panel to provide further
specificity for leukemic populations and to evaluate subpopulations among leukemia samples because of observed heterogeneity in signaling activation and inhibition. As only 10 subjects were evaluated by these methods, we are currently completing a larger study to better characterize mTOR activation and inhibition to this regimen, as well as the predictive ability for flow cytometric studies for chemotherapy response.

We also recognize that S6 phosphorylation can be upregulated by cytokine stimulation as well as stromal and/or microenvironment marrow factors that are absent or present in low concentrations in peripheral blood. Therefore, another potential limitation is our choice of peripheral blood samples. Direct comparisons of our methods in marrow and blood are ongoing to estimate the magnitude, if any, of these differences. Thus far, we have not seen a consistent pattern to suggest that peripheral blood sampling provides inferior estimation of basal S6 phosphorylation or signal transduction inhibition. However, our measurements of mTOR activation state among circulating blasts should be considered only estimates of the effects of sirolimus therapy upon marrow blasts. Flow cytometric analysis in the context of an in vitro model of the marrow microenvironment (e.g., stroma-supported culture of pretreatment blast samples) might further enhance our ability to predict clinical response to mTOR or other signaling inhibitors.

We hypothesize that mTOR inhibition and chemotherapy are synergistic in their anti-leukemic effect based upon preclinical assays; whether this translates into improved clinical response remains to be determined. Furthermore, if these agents are indeed synergistic, it is unknown whether partial inhibition or abrogation of S6 phosphorylation predicts this interaction. We expect our current studies to clarify whether the degree of S6 signal disruption predicts chemotherapy response to sirolimus MEC and as well whether mTOR inhibition itself is sufficient to reverse chemotherapy resistance. Such larger studies may require multiple treatment sites, which potentially jeopardizes the data quality of our techniques. This is in part because transit time from samples obtained at remote centers might alter the robustness of our data. We therefore are exploring local cell processing such that data quality on multicenter trials is maintained.

In summary, we show that real-time pharmacodynamic evaluation of leukemia cells by serial flow cytometric analysis of S6 phosphorylation is a powerful tool to evaluate in vivo biochemical efficacy of signal transduction inhibitors. Beyond the monitoring of mTOR inhibitors in AML, we are also currently testing whether monitoring peripheral blood S6 phosphorylation by flow can be used to directly confirm the pharmacodynamic effects of other agents that secondarily inhibit PI3K/AKT/mTOR signaling. This includes clinical trials of AKT and FLT3 inhibitors in AML and JAK2 inhibitors in myeloproliferative disorders. We also seek to document whether compensatory changes in upstream or parallel signaling proteins occur during mTOR inhibition with these agents through the use of multiple phospho-specific antibodies. Such data may help elucidate resistance mechanisms to these agents. Finally, we hope that our flow cytometric approach can ultimately be used as screening tools to predict patients likely to benefit from specific clinical therapeutics, thus promoting personalized approaches to molecularly targeted agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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In Memoriam
The manuscript is dedicated in memory of Dr. Alan M. Gewirtz, whose intellect, wit, and compassion continue to inspire all who knew him.

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