Pharmacodynamic Evaluation of CCI-779, an Inhibitor of mTOR, in Cancer Patients

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Abstract
CCI-779 is an ester of rapamycin and inhibitor of mammalian target of rapamycin (mTOR) currently in Phase II clinical development for the treatment of patients with cancer. CCI-779 interacts with mTOR and inhibits its kinase activity, resulting in inhibition of the mTOR-regulated translational controllers p70s6 kinase and 4E-BP1. Ultimately, CCI-779 decreases the translation of mRNAs involved in the control of the cell cycle, resulting in cell cycle arrest. The objective of this study was to develop a method to determine the pharmacodynamic effects of CCI-779 suitable for use in clinical trials. Exposure of Raji lymphoblastoid cells to increasing concentrations of rapamycin resulted in a linear concentration-dependent inhibition of p70s6 kinase activity, suggesting that p70s6 kinase activity could be an appropriate marker for mTOR-interacting agents. In subsequent experiments, treatment of nude mice bearing the CCI-779 susceptible breast cancer cell line MDA-468 with a single dose of 10 mg/kg CCI-779 resulted in a >80% inhibition of p70s6 kinase activity in peripheral blood mononuclear cells (PBMCs) 72 h after treatment. Importantly, the degree of p70s6 kinase inhibition was identical in PBMCs and simultaneously collected tumor tissue, suggesting that the PBMCs are an adequate surrogate tissue for p70s6 kinase activity in vivo. The intra-subject coefficient of variation of p70s6 kinase activity measured in PBMCs collected from five healthy volunteers on days 1, 4, and 8 was 14%, indicating that p70s6 kinase activity in PBMCs remains relatively stable over time. Finally, p70s6 kinase activity was measured in PBMCs from nine patients with renal cell cancer treated with a single dose of 25, 75, or 250 mg of CCI-779 i.v. (three patients each). PBMCs were collected on days 2, 4, and 8 after CCI-779 treatment. In this small data set, eight of the nine patients had evidence of p70s6 kinase activity inhibition after treatment that was independent of the administered dose. There was a significant linear association between time to disease progression and inhibition of p70s6 kinase activity 24 h after treatment. In conclusion, these results indicate that the pharmacodynamic effects of CCI-779 can be determined using a p70s6 kinase assay in PBMCs. This assay is currently being incorporated in Phase I and II studies with CCI-779 to determine its relationship with dose and plasma concentration of the agent and its value as a predictor of treatment efficacy.

Introduction
CCI-779 is an ester of rapamycin, a natural macrolide antibiotic with antifungal, immunosuppressive, and antitumor properties (1), which is currently in Phase II clinical development for the treatment of cancer. CCI-779 forms a complex with FKBP-12, a member of the immunophilin family of FK506-binding proteins, and inhibits the activity of mTOR (2–5), a kinase that functions as a checkpoint for nutritional status (6) and a downstream mediator in the phosphatidylinositol 3'kinase/Akt signaling pathway (7–9). Consequently, CCI-779 inhibits the transcriptional and translational functions regulated by mTOR. At the transcriptional level, mTOR positively controls RNA polymerase activity either by inactivation of the retinoblastoma protein, which inhibits the RNA polymerase I and III (10), or by stimulating the transcriptional activator STAT3 (11). Rapamycin treatment effectively blocks retinoblastoma protein phosphorylation and inactivation by mTOR (12). Likewise, the translational regulators p70s6 kinase and initiation factor 4E-BP1 are phosphorylated by mTOR (13–17). Ribosomal p70s6 kinase, when phosphorylated, attaches to the S6 subunit of the ribosome, and 4E-BP1, after being phosphorylated, is dissociated from the eukaryotic initiation factor eIF4E, allowing the formation of the 4F initiation complex. CCI-779, by inhibiting those translational regulators, leads to a decrease in overall protein synthesis of −15% but strongly inhibits translation of specific mRNA that encodes ribosomal proteins and other components that promote cell growth, such as insulin-like growth factor-II, c-Myc, and cyclin D1 (18–19).

CCI-779 has been evaluated in Phase II studies in patients with solid tumors (20–22). The preliminary results from Phase I clinical studies indicated that CCI-779 is generally well toler-
ated. Interestingly, the toxicity of the agent was not clearly dose related when administered on a weekly schedule of administration, complicating the selection of an appropriate dose for subsequent studies. In addition, encouraging evidence of antitumor activity was observed in Phase I clinical studies over a relatively broad range of doses and at different schedules of administration, further complicating the dose selection process. As a result, and because the maximum tolerated dose may not be the optimum dose for nontoxic agents, a range of dose levels was evaluated in Phase II trials.

The objectives of these translational studies were to develop a pharmacodynamic biomarker of CCI-779 for use in clinical research. The developmental efforts focused on measurement of p70s6 kinase activity because it is a direct downstream substrate of mTOR and can be quantitated using well-developed kinase assay methods. In addition, the PBMCs were selected to measure the pharmacodynamic effects of CCI-779 because p70s6 kinase is constitutively activated in these cells, PBMCs are easily collectable from patients enrolled in clinical trials, and previous studies had used PBMCs to evaluate the pharmacodynamics of rapamycin in studies evaluating the effects of rapamycin to prevent graft rejection in transplant patients (23). This article summarizes the development, validation, and feasibility application of this method.

Materials and Methods

Drugs. Rapamycin was obtained as a gift from the Drug Synthesis & Chemistry Branch, Developmental Therapeutics Program at the Division of Cancer Treatment and Diagnosis of the National Cancer Institute (Bethesda, MD). CCI-779 was provided by Wyeth Laboratories (Pearl River, NY).

Cell Lines. Human MDA-468 breast cancer and Raji (Burkitt’s lymphoma) cells were obtained from the American Type Culture Collection. MDA-468 cells were cultured and grown until confluent in minimum essential medium containing 10% fetal bovine serum. Before injection into animals, cells were harvested in serum-free minimum essential medium. All cell culture media and reagents were purchased from Life Technologies, Inc. (Grand Island, NY). Raji cells were grown in RPMI 1640 with 2 mM glutamine modified by American Type Culture Collection to contain 10 mM HEPES, 1 mM sodium pyruvate, 4.5 grams/liter glucose, and 1.5 grams/liter sodium bicarbonate, supplemented with 10% fetal bovine serum.

In Vitro Studies. Raji cells were plated in a six-well plate at 3 × 10^5 cells/ml and treated for 30 min at the following rapamycin concentrations: 0, 0.1, 0.3, 0.5, 0.7, and 1 mM. All cell lines were incubated in a 37°C incubator containing 5% CO₂. After treatment, cells were harvested and rinsed with ice-cold PBS, and lysates were prepared for p70s6 kinase assay and immunoblotting as described below.

Animal Studies. Four- to 6-week-old nude female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were housed at the University of Texas Health Science Center at San Antonio and treated under a protocol approved by the Institutional Animal Care Committee according to local and federal regulations. After a period of 5–6 days, mice were implanted s.c. with 2 × 10^6 growing MDA-468 human breast cancer cells. After 10 days, tumor diameters were determined, and the animals were randomized to the different treatment groups. Treatment was given by a single i.p. injection of 10 mg/kg CCI-779 or vehicle. Samples of PBMCs and tumor were collected from groups of five mice each at the following time points: (a) baseline; (b) 24 h; and (c) 72 h after treatment. Blood from each animal group of five mice was collected by cardiac puncture and pooled. PBMCs were extracted by a gradient centrifugation in lymphocyte separation media (Fisher Scientific, Suwanee, GA) at 1500 × g for 20 min at room temperature. The isolated cells were snap frozen until analysis. Tumors were excised and snap frozen at −80°C until analysis. For the kinase activity analysis, lysis buffer was added to PBMCs and tumor tissues, and the subsequent protein extract was processed as described above.

Collection of PBMCs from Human Subjects. PBMCs from healthy human volunteers and cancer patients were extracted from whole blood in a CPT Vacutainer tube as recommended by the manufacturer. Briefly, 8 ml of whole blood were collected from a peripheral vein and centrifuged at 1500 × g for 20 min at room temperature to isolate the PBMC fraction. The preparation was next transferred into a 15-ml conical tube, with PBS to fill the tube, and centrifuged at 600 × g for 10 min at room temperature. Next, PBS was aspirated and disposed, and the PBMC pellet was snap frozen and stored at −80°C until use.

p70s6 Kinase Assay. The p70s6 kinase was immunoprecipitated from protein extracts and then assayed for kinase activity. Protein extracts from Raji cells, PBMCs, or tumor tissues were obtained after homogenization in lysis buffer [50 mM Tris (pH 7.5), 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 40 mM 2-glycerophosphate, 0.1 mM sodium orthovanadate, 1 mM benzamidine, 0.5 mM phenylmethylsulfonyl fluoride, containing 1% NP40, and 10 μg/ml aprotinin, pepstatin, leupeptin, and antipain]. Protein concentration of cell lysates was determined using a Bradford assay. Equal amounts of lysate protein were incubated with antibody against p70s6 kinase (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C, and then with 25 μl of protein G-agarose for 1 h. The immune complex beads were washed twice with lysis buffer and twice with kinase buffer [50 mM Tris (pH 7.5), 10 mM MgCl₂, 0.2 mM EGTA, 1 mM DTT, 1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride]. The washed immunocomplexes were resuspended in 30 μl of kinase assay buffer containing 40 μM ATP, 2.5 μCi [γ³²P] ATP, 1 μM protein kinase A inhibitor peptide, and 250 μM p70s6 Rsk peptide substrate (Santa Cruz Biotechnology), and the reaction was incubated for 30 min at 30°C. Two different methods for the measurement of kinase activity were used to test the reliability of the assay; no significant changes were observed between results obtained by each method. In the first one, samples were subjected to SDS-PAGE, and the gel was stained with Coomassie Blue and vacuum dried. The dried gel was exposed with X-ray film at −80°C, and the kinase activity was measured by densitometry. In the second method, an aliquot of each sample was spotted onto P81 phosphocellulose paper. After extensive washing in 75 mM phosphoric acid, radioactivity on the papers was quantified by scintillation counting.

Immunoblotting. Equal amounts of Raji cell extract protein were subjected to SDS-PAGE, and proteins were transferred onto nitrocellulose membranes (Osmonics Inc., Minnetonka, MN). Membranes were blocked in 5% nonfat milk
for 1 h and then incubated overnight at 4°C with either antibody against the phospho-p70S6 kinase (Thr389) or antibody against total p70S6 kinase as primary antibodies (Cell Signaling Technology, Beverly, MA). Then, the membranes were washed and incubated with antirabbit IgG conjugated to horseradish peroxidase for 1 h. After washing the membranes again, the immunoreactive proteins were detected using the enhanced chemiluminescence method (Amersham Pharmacia Biotech, Piscataway, NJ).

**Healthy Volunteers Study.** PBMCs from five healthy volunteers who were not taking CCI-779 were isolated from whole blood using the methodology described above. Blood samples were collected on days 0 (the day of first collection), 4 (3 days after day 0 collection), and 8 (7 days after day 0 collection).

**Clinical Trial.** Nine patients with advanced renal cell cancer and treated with different doses (25, 75, or 250 mg) of CCI-779 on a weekly schedule in a randomized Phase II study were studied (21). The details of this clinical trial will be reported separately. PBMCs were obtained from whole blood samples from each patient using CPT Vacutainer tubes, at the baseline visit, at 24- and 72-h post-treatment, and on day 8 before the second weekly dose. PBMCs were processed and analyzed as detailed above.

**Statistical Analysis.** In the measurement of p70S6 kinase activity, the values are expressed as the relative variation of the 32P phosphorylation levels of the p70S6 Rsk peptide substrate between samples, according to the formula: relative p70S6 kinase activity = ([32P substrate counts − background counts]/32P Total counts]. The in vitro experiments were repeated three times, and the data presented represent the average of three experiments. The relationship between rapamycin concentration and p70S6 kinase inhibition in the Raji cells was determined by a linear regression analysis. The intersubject and insubject CV of p70S6 activity in PBMCs obtained from healthy volunteers was determined as the mean/SD. The decrement in p70S6 kinase activity in specimens collected from patients treated with CCI-779 was computed with the equation: proportional change = [(post-treatment − pretreatment)/pretreatment] × 100. The variation between the p70S6 kinase activity at the different time points was compared using a nonparametric test for multiple samples comparison. The relationship between the administered dose of CCI-779 and pharmacodynamic effects was explored using the non Kruskal-Wallis nonparametric test. The relationship between time to tumor progression and pharmacodynamic effects was explored using a linear regression analysis.

**Results and Discussion**

As mentioned above, inhibition of mTOR by CCI-779 inhibits the translational regulators 4E-BP-1 and p70S6 kinase. Previous studies have explored the analysis of changes in 4E-BP-1 phosphorylation to evaluate the biological effects of CCI-779. These studies have shown treatment with the agent results in a dose- and concentration-dependent inhibition in 4E-BP1 phosphorylation in in vitro and in vivo models (24–28). A recent study (28) reported a correlation between decreases in 4E-BP1 phosphorylation at site Thr70 and tumor growth inhibition. These results suggest that an assay to measure decreases in phosphorylation for this particular site of 4E-BP1 may be a useful surrogate for determining inhibition of mTOR activity in clinical specimens. The use of 4E-BP1 to assess the pharmacodynamic effects of CCI-779, has, however, an important limitation from an analytical perspective. 4E-BP1 is not a kinase itself, and therefore, its activity cannot be measured using quantitative kinase assays as with p70S6 kinase. The availability of a quantitative assay for p70S6 kinase increases the rationale to use it rather than 4E-BP-1 for pharmacodynamics studies. For this reason, this study focuses on the development, validation, and feasibility testing of this approach.

**Linear Concentration-dependent Inhibition of p70S6 Kinase by Rapamycin in Raji Lymphoid Cells.** The first experiment determined the effects of the mTOR inhibitor rapamycin on p70S6 kinase activity in the Raji cell line. Raji cells were selected for these experiments because they can be reliably cultured in vitro and have a lymphoid origin. As shown in Fig. 1A, p70S6 kinase activity decreased in a linear fashion (R² = 0.93) in response to increasing concentrations of rapamycin ranging from 0 to 1 nM. To confirm the results obtained with the kinase assay, we also performed Western blot analysis of protein extracts from Raji cells exposed to rapamycin with antibodies against total and active (phosphorylated) p70S6 kinase. As shown in Fig. 1B, exposure to rapamycin resulted in a concentration-dependent inhibition of p70S6 kinase activity in Raji cells.

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

**Fig. 1** Dose-response effect of rapamycin on p70S6 kinase activity in Raji cells. A, Raji cells were grown in full media and treated with increasing concentrations of rapamycin from 0 to 1 nM. Cell lysates were analyzed for p70S6 kinase activity by scintillation counting, and the results are plotted as relative levels of kinase activity. B, immunoblotting analysis on phospho-p70S6 kinase expression in Raji cells and total p70S6 kinase as a loading control. Immunoprecipitates (IP) of total p70S6 kinase were analyzed by Western blot (WB) for either phospho-p70S6 or total p70S6 kinase. The panels show a representative result for at least three experiments with similar results.
with no change in the expression of total protein, confirming the observations obtained with the quantitative kinase assay. These results, therefore, indicate that the activity of p70s6 kinase can be quantitated in lymphoid cells and varies in a predictable mode after exposure to an mTOR inhibitor, reinforcing the use of this method to evaluate CCI-779 pharmacodynamic effects.

CCI-779 Inhibits p70s6 Kinase Activity in PBMCs and Tumor Tissue in Vivo. The next question was to determine whether or not p70s6 kinase could be measured in PBMCs in vivo. To address this question, groups of five mice were treated with a single dose of CCI-779 of 10 mg/kg, a dose that has been shown to inhibit tumor growth in previous in vivo studies, and sacrificed 24 and 72 h after treatment. PBMCs were isolated from pooled blood collected from treated mice. As shown in Fig. 2, p70s6 kinase activity decreased by ≈80% compared with the baseline activity. These results indicate that, indeed, p70s6 kinase activity in PBMCs is inhibited on treatment of mice with CCI-779 and can be quantitated. These results suggest that PBMCs, which are easily collected from patients in large scale clinical trials, could be an appropriate specimen to study the pharmacodynamics of CCI-779.

A major problem with the use of normal tissues to determine pharmacodynamic effects of anticancer agents is that the dose and/or concentration required to inhibit a biological target in tumor and normal tissue may not necessarily be similar. To address this problem, the p70s6 kinase activity was compared between PBMCs and simultaneously collected tumor tissues. For these experiments, we selected the PTEN-negative MDA-468 breast cancer cell line that has constitutive p70s6 kinase activity and has been shown to be susceptible to CCI-779 in in vitro and in vivo experiments. As demonstrated in Fig. 2, the activity of p70s6 kinase decreased in a parallel fashion in tumor tissues and PBMCs, indicating that, indeed, the PBMCs could be an appropriate surrogate tissue to study the effects of CCI-779 on p70s6 kinase activity.

Although pharmacodynamic studies should ideally be conducted in tumor tissues rather than normal tissue, this is rarely possible in clinical trials in which only a minority of patients has accessible tumor tissues for sequential sampling. In addition, the collection of tumor tissues for biological studies in a sufficient number of patients to explore the relationship between pharmacodynamic effects and outcome is not possible. For this reason, validation of a surrogate tissue that is easily collectable in a large number of patients in disease-oriented clinical trials is needed. The data reported here suggest that PBMCs may indeed be an appropriate surrogate tissue to examine the activity of p70s6 kinase on the basis of in vivo data. Additional clinical studies are currently in development to explore if this correlation also exists in patients in intensive studies in which tumor tissue and PBMCs will be simultaneously collected in a limited number of patients.

p70s6 Kinase Activity in PBMCs Collected from Human Subjects Remains Stable Over Time. Subsequently, we examined the degree of spontaneous variation of p70s6 kinase activity in PBMCs collected from normal individuals. Five healthy volunteers not exposed to CCI-779 had PBMCs collected from whole blood at days 0, 4, and 8. p70s6 kinase activity assays by scintillation counting were performed in cell lysates, and the activity results were tested for the intra and intersubject CV.
p70\textsuperscript{e6} kinase activity as a function of time. In the prototypical patient, p70\textsuperscript{e6} kinase activity decreased by 24 h after treatment and reached a maximum inhibition at day 8.

No relationship was observed between the administered dose of CCI-779 and inhibition of p70\textsuperscript{e6} kinase in PBMCs in this small cohort of patients, suggesting that CCI-779 exerts pharmacodynamic effects at doses as low as 25 mg. In this regard, preliminary data from randomized Phase II clinical trials of patients treated with CCI-779 do not suggest a clear association between dose and treatment outcome (21). Importantly, as illustrated in Fig. 5, there was a linear association between inhibition of p70\textsuperscript{e6} kinase and time to tumor progression (P < 0.04). Although the limited sample size in this feasibility study does not permit one to draw firm conclusions regarding the value of this biomarker to predict the outcome of patients treated with the drug, the results support the incorporation of this test in additional larger studies.

On the basis of these results, this method has been implemented in additional Phase I and II clinical trials with CCI-779. The results of Phase I dose escalation and pharmacokinetic studies will permit one to explore the relationship between dose and pharmacokinetic parameters and pharmacodynamic effects and model the mathematical equations governing these relationships. Furthermore, the data from Phase II studies will be used to explore correlations between pharmacodynamic effects and outcome.

A key issue that needs to be considered in the development of pharmacodynamic end points, particularly when using normal tissues, is whether these end points are merely pharmacological end points whose target inhibition is not necessarily synonymous with antitumor effects. Indeed, for CCI-779, it has been demonstrated that the degree of inhibition of p70\textsuperscript{e6} kinase activity is not different in susceptible versus resistant cell lines, and other factors, such as the expression and modulation of CCI-779 of other oncogenes and cell cycle regulators, such as c-myc, cyclin D3, and p27, are also important. These studies, therefore, need to be followed by additional preclinical and clinical studies to determine the relationship between inhibition of the immediate intratumor target and other parameters of antitumor effects, such as cell cycle arrest, apoptosis, and tumor growth. Ultimately, as mentioned above, the final validation will be to prove the relationship between target inhibition and indices of patient outcome. These various aspects are currently the subjects of active investigation.

In summary, the results from these studies demonstrate that the pharmacodynamic effects of CCI-779 can be measured in PBMCs using a fully quantitative p70\textsuperscript{e6} kinase assay. The activity of p70\textsuperscript{e6} kinase decreases in a linear fashion after exposure to an mTOR inhibitor, and there is a good correlation in magnitude of p70\textsuperscript{e6} kinase inhibition between PBMCs and tumor tissues. In addition, this parameter appears to be relatively constant over time in healthy subjects. The combination of these findings reinforces the potential value of measuring p70\textsuperscript{e6} kinase activity as a pharmacodynamic marker of CCI-779. Studies in progress will help to elucidate the relationship between dose and pharmacokinetic parameters of CCI-779 and p70\textsuperscript{e6} kinase inhibition and the value of this measurement in predicting the clinical activity of this agent.

Fig. 5 Relationship between proportional decrement in p70\textsuperscript{e6} kinase activity and time to tumor progression in nine patients with renal cell cancer treated with CCI-779. P = 0.04, R\textsuperscript{2} = 0.45.

References
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