# Biochemical Correlates of mTOR Inhibition by the Rapamycin Ester CCI-779 and Tumor Growth Inhibition[1]

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## ABSTRACT

**The rapamycin ester, CCI-779, potently inhibits cell growth *in vitro*, inhibits tumor growth *in vivo*, and is currently in Phase I clinical trials. To further understand the relationship between plasma systemic exposure and inhibition of the target Ser/Thr kinase, mTOR/FRAP, two assays have been developed. The first assay involves determination of the 4E suppressor protein (4E-BP1) bound to eukaryotic initiation factor 4E (eIF4E), and the second is direct Western analysis of phosphorylation of residue $Thr^{70}$ of 4E-BP1. Under normal growth conditions *in vitro*, rapamycin caused rapid association of 4E-BP1 with eIF4E within 1 h in Rh30 and $GC_3$ human tumor cells. Association was persistent up to 16 h. In mice, administration of rapamycin (5 or 20 mg/kg) caused rapid association of 4E-BP1 with eIF4E within 4 h in both human colon adenocarcinoma $GC_3$ and rhabdomyosarcoma Rh30 xenografts. Using phospho-specific antibody against $Thr^{70}$ of 4E-BP1, rapid and persistent dephosphorylation within 30 min of exposure to rapamycin was detected in Rh18 rhabdomyosarcoma cells. Evaluation of CCI-779 against Rh18 xenografts showed this tumor to be growth inhibited at daily dose levels of ≥8.7 mg/kg. Because immunoblotting may be more suitable for assaying tumor biopsy tissue, a "blinded" comparison between the effect of CCI-779 on $Thr^{70}$ phosphorylation and growth inhibition of human tumor xenografts was undertaken. Mice were treated daily for 5 days with CCI-779 (20 mg/kg/day) or with drug vehicle, and tumor diameters were measured. Tumors were excised 1 h after the final administration and frozen, and phospho $Thr^{70}$ was determined by Western blot analysis. The correlation coefficient for decreases in $Thr^{70}$ phosphorylation and growth inhibition was high ($r^2$, 0.99). The results indicate that an assay of decreases in phosphorylation of $Thr^{70}$ of 4E-BP1 may be a useful surrogate for determining the inhibition of mTOR activity in tumor specimens.**

## INTRODUCTION

Rapamycin is a macrocyclic lactone that potently inhibits activation of T cells (Refs. 1–3; reviewed in Refs. 4–6), and causes $G_1$ arrest in many normal and tumor cells *in vitro* (7–13). Rapamycin binds to a $M_r$ 12,000 cytosolic receptor, FK506-binding protein (FKBP-12), and this complex inhibits the kinase activity of a Ser/Thr kinase mTOR/FRAP that has homology to other lipid kinases (14–17). Inhibition of mTOR kinase by rapamycin leads to a rapid inactivation of ribosomal p70 S6 kinase (18–22) possibly through disinhibition of phosphatase 2A (PP2A) associated with S6 (23). mTOR directly phosphorylates at least two residues ($Th^{37}$ and $Thr^{46}$) of 4E-BP1, the suppressor of eIF4E (24–27). In the presence of rapamycin, 4E-BP1 becomes dephosphorylated and associates with eIF4E, which prevents formation of the 4F initiation complex. Rapamycin leads to a decrease in overall protein synthesis of ~15% but strongly inhibits translation of mRNA species characterized by polypyrimidine tracts just distal to the $^{m7}$GTP cap, or by complex secondary structure of the 5′-untranslated region (28, 29). This results in suppression of translation of specific mRNA species including ribosomal proteins, and IGF[3]-II, that are dependent on the p70 S6 kinase pathway, and c-MYC, and possibly cyclin D1 in which initiation is dependent on eIF4E function (30, 31). Accumulation of rapamycin treated cells in $G_1$ is independent of p53-mediated checkpoint, and may result from stabilization of $p27^{kip1}$ (32, 33). More recently, evidence that mTOR associates with, and activates, protein kinase C isoforms δ and ϵ has been reported (34, 35).

CCI-779 is a blocked ester of rapamycin with improved pharmaceutical properties. *In vitro* CCI-779 appears essentially similar to rapamycin with respect to potency and inhibition of mTOR signaling. Cells engineered to express a rapamycin-resistant mTOR are highly resistant to CCI-779, further indicating the specificity for inhibiting mTOR kinase as the mechanism

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[3] The abbreviations used are: IGF, insulin-like growth factor; NCI, National Cancer Institute; T:C (ratio), treated:control (ratio of tumor volumes).

of action. Here, we report two assays to measure the activity of rapamycin and its analogues on signal transduction in drug-treated tumor cells. These assays have been applied to human tumor xenografts as a first step in evaluating whether such assays may be useful for determining the biochemical activity of these inhibitors in patient-derived tumor specimens.

## Materials and Methods

**Cell Lines.** Human cell lines Rh18 and Rh30 (rhabdomyosarcomas) and $GC_3$ colon adenocarcinoma have been described previously (36, 37). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mM L-glutamine without antibiotics.

**Tumor Growth and Measurement of Tumor Response.** Growth and chemosensitivity of Rh18 xenografts in immune-deprived CBA mice have been described previously (38). Rapamycin and CCI-779 were dissolved in ethanol and diluted with a solution of 5.2% Tween 80 and 5.2% PEG400 in sterile water and administered by i.p. injection. The final concentration of ethanol was 4%. Tumor growth was determined by weekly caliper measurements of two perpendicular diameters (38). Xenografts derived from prostate (DU-145, PC-3), glioma (SF295), and ovarian carcinoma (OVCAR5) were grown s.c. in athymic nude or severe combined immunodeficient mice as described previously (39). Tumor diameters were determined daily during administration of CCI-779. Treatment was initiated when tumors reached 400–600 mg. Tumors were collected 1 h after the last treatment and flash-frozen by immersion in liquid nitrogen.

**Assay of 4E-BP1 Associated with eIF4E.** Assay of 4E-BP1 associated with eIF4E was examined, essentially as described by Gingras *et al.* (40). Rh30 and $GC_3$ cells were plated at a density of $3.0 \times 10^6$ cells per 100-mm dish. The following day, rapamycin (100 ng/ml) was added. Cells were harvested 1–16 h later. Extracts were prepared by scraping the cells in 1 ml of ice-cold lysis buffer [50 mM Tris (pH 7.5), 150 mM KCl, 1 mM DTT, 1 mM EDTA, 50 mM β-glycerophosphate, 1 mM EGTA, 50 mM NaF, 10 mM Na-$PP_i$, 0.1 mM $Na_3VO_4$], and one Complete Mini protease inhibitor cocktail tablet for each 50 ml (Boehringer Mannheim). Lysis was accomplished by three freeze-thaw cycles. To bind eIF4E, 25 μl of $^7$methyl-GTP Sepharose (Pharmacia Biotech) was added to the lysates, which were incubated overnight on a rotator at 4°C. The complexes were pelleted and washed three times with lysis buffer. To dissociate bound eIF4E from the Sepharose, 50 μl of SDS-PAGE loading buffer was added to the samples, which were then heated to 95°C for 3 min. Samples were next analyzed by SDS-PAGE and Western blotting using standard chemiluminescent methods. Rabbit polyclonal anti-4E-BP1 antibody 11208 (generously provided by Nahum Sonenberg, McGill University, Montreal, Quebec, Canada) was used to detect 4E-BP1 associated with eIF4E. eIF4E was detected by using a commercially available monoclonal antibody (Transduction Laboratories, Lexington, KY). Tumor tissue was maintained frozen (−270°C). As needed, it was ground under liquid nitrogen with mortar and pestle into a fine powder, and suspended in extraction buffer with protease inhibitors. The suspension was sonicated three times for 10 s and centrifuged (for 30 min at 14,500 × *g* at 4°C). Supernatant was collected; total protein in the extract was measured using the bicinchoninic acid method, and aliquots of solutions were kept at −80°C.

**Determination of Phospho-Thr70 of 4E-BP1.** To detect the phosphorylated 4E-BP1 protein, samples (~30 μg of total protein) were loaded onto 12% SDS-PAGE Bio-Rad Ready gels. Electrophoresis was conducted at 4°C, 100V, for 1 h. Under these conditions, 4E-BP1 isoforms do not separate. For separation of isoforms, samples (~18 μg) were electrophoresed in 18% gels (at 4°C, 125V, for 3.5 h). The proteins were transferred to polyvinylidene difluoride membrane using a wet-transfer method. Membranes were probed with polyclonal rabbit antibody against 4E-BP1 phosphorylated on $Thr^{70}$ (New England Biolab). The blots were developed with chemiluminescent Western blotting detection reagent ECL Plus (Amersham Pharmacia Biotech), and scanned with Storm 860 Scanner Control (Molecular Dynamics) to quantify the intensity of the bands. Chemifluorescence intensity was calculated per μg of total protein.

## RESULTS

Under conditions of growth factor or serum-starvation, the activity of mTOR decreases, and 4E-BP1 becomes dephosphorylated and associates with eIF4E. Stimulation with IGF-I leads to rapid dissociation of 4E-BP1 from eIF4E, and this is inhibited in Rh30 cells by rapamycin. To determine the kinetics of rapamycin-induced association of 4E-BP1 with eIF4E, $GC_3$ and Rh30 cells were treated with rapamycin (100 ng/ml) and lysates were prepared from 1 to 16 h. As shown in Fig. 1*A*, under control conditions, little 4E-BP1 was associated with eIF4E in the colon carcinoma cells, and none was detected in lysates from the rhabdomyosarcoma cell line. Rapamycin induced an association within 1 h in Rh30 cells, which remained unchanged over 16 h. In $GC_3$ cells, maximum association was determined at 4 h and remained unchanged at 16 h. On the basis of these results, the effect of rapamycin *in vivo* was examined in control tumors and 4 h after a single administration of rapamycin. Lysates from Rh30 tumors were prepared; protein concentration was diluted to 500, 250, or 125 μg/ml; and samples were incubated with a fixed volume of $^{7m}$GTP-Sepharose. As shown in Fig. 1*B*, detection of eIF4E was proportional to the protein concentration, which indicated an excess of $^{7m}$GTP-Sepharose for binding this protein. Rh30 tumor samples that were prepared 4 h after the injection of 20 mg/kg rapamycin showed a marked increase in 4E-BP1 associated with eIF4E. Similarly, rapamycin administered at 5 mg/kg resulted in an increased association of 4E-BP1 in $GC_3$ tumors, which indicated inhibition of mTOR signaling. These results suggest that rapamycin at these dose levels causes inhibition of mTOR, and this can be detected reproducibly by this assay.

Because of the potential technical difficulties applying the "pull-down" assay to tumor biopsy material, this assay was compared with an assay measuring changes in phosphorylation of $Thr^{70}$ of 4E-BP1. Rh18 cells were grown overnight and treated with rapamycin (100 ng/ml). As shown in Fig. 2*A*, rapamycin caused a time-dependent increase in association of 4E-BP1 with eIF4E. As anticipated, rapamycin treatment caused a decrease of the slowest mobility hyperphosphorylated form

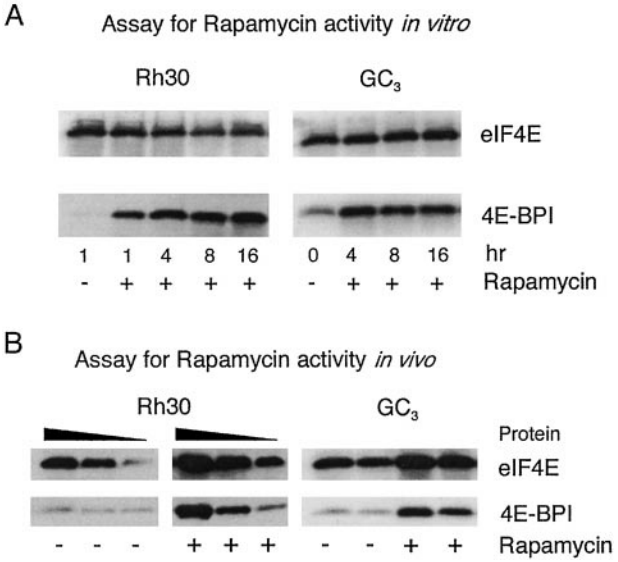


*Fig. 1* Rapamycin induces association of 4E-BP1 with eIF4E both *in vitro* and *in vivo*. *A*, $GC_3$ colon carcinoma and Rh30 rhabdomyosarcoma cells were grown in complete medium overnight. Control (no treatment) or cells treated with rapamycin (100 ng/ml) were harvested between 1 and 16 h, and association of 4E-BP1 with eIF4E was determined using the $^{7m}$GTP-Sepahrose assay. *B*, Lysates were prepared from Rh30 tumors from untreated mice (−) or 4 h after treatment with a single administration of rapamycin (+). Lysates were diluted to a final concentration of 500, 250, or 100 μg/ml, and the association of 4E-BP1 with eIF4E determined as above. For GC3 tumor, lysates (50 μg/ml) were prepared from untreated mice or 4 h after rapamycin administration (5 mg/kg), and assayed as above. Results show duplicate determinations.

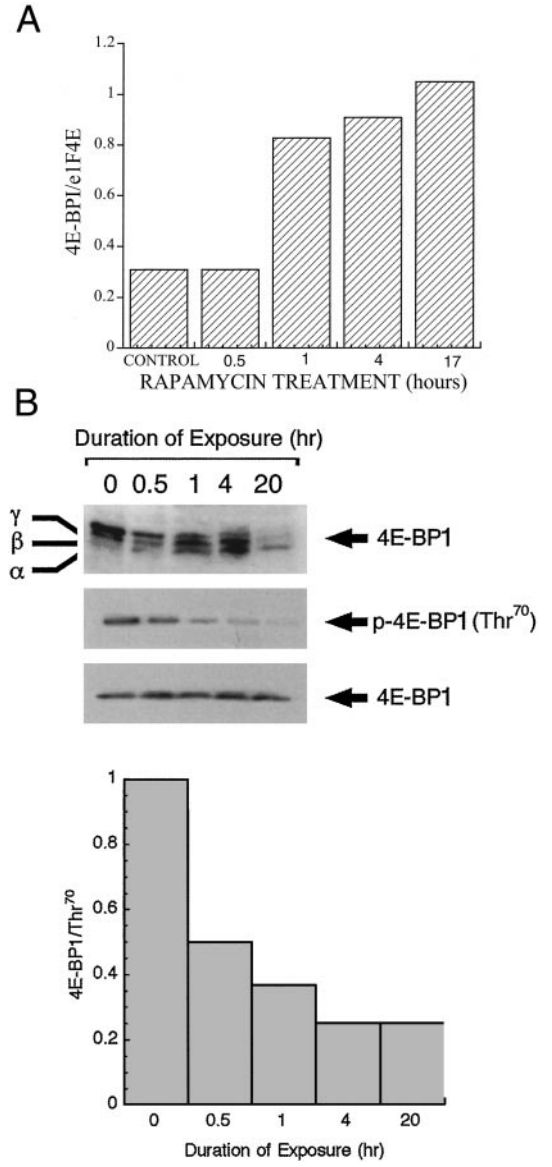


*Fig. 2* Correlation between rapamycin-induced association of 4E-BP1 with eIF4E and decreased phosphorylation of $Thr^{70}$ of 4E-BP1. *A*, Rh18 cells were in complete medium overnight. Control (0 h) or cells treated with rapamycin (100 ng/ml) were harvested from 0.5 to 17 h, and the association of 4E-BP1 with eIF4E was determined using the $^{7m}$GTP-Sepahrose assay. *B*, changes in mobility of 4E-BP1 isoforms and $Thr^{70}$ phosphorylation after rapamycin exposure. Rh18 cells were exposed to rapamycin (100 ng/ml) for 0–20 h. Cell lysates were prepared and diluted to 500 μg/ml protein. *Top panel*, 4E-BP1 isoforms using electrophoretic conditions allowing separation of hypo- and hyperphosphorylated 4E-BP1; *center panel*, phospho-$Thr^{70}$ detected under electrophoretic conditions that do not resolve isoforms; *bottom panel*, total 4E-BP1 under electrophoretic conditions that do not resolve isoforms. *Histogram*, phospho-$Thr^{70}$ signal was normalized to total 4E-BP1. Data show a representative experiment.

(γ) and increased the higher mobility less-phosphorylated isoforms (β,α), consistent with the increased association of 4E-BP1 with eIF4E (Fig. 2*B*). However, it has recently been shown that $Thr^{70}$ phosphorylation of the α-form converts it to β-4E-BP1 (41). Thus, α-4E-BP1 lacks phospho-$Thr^{70}$. Consequently, electrophoretic conditions that allow separation of 4E-BP1 would not be suitable for quantitating the ratio of $Thr^{70}$:total 4E-BP1 using a phospho-$Thr^{70}$-specific antibody. To more accurately quantitate phospho-$Thr^{70}$:total 4E-BP1, electrophoretic conditions that did not resolve the isoforms were used. Using phospho-specific antibody, decreased phosphorylation of $Thr^{70}$ was detected within 30 min and remained depressed for 20 h (Fig. 2*B*). The $Thr^{70}$ signal was normalized to total 4E-BP1. Rh18 cells demonstrated significant decrease of phospho-4E-BP1 protein (about 30% of control). To determine whether biochemical end points were related to tumor sensitivity to rapamycin *in vivo*, mice bearing Rh18 xenografts were treated with CCI-779. The schedule used was daily administration for 5 days in consecutive weeks. Cycles of treatment were repeated twice at 21-day intervals. As shown in Fig. 3, treatment with daily dose levels of 8.7–20 mg/kg caused significant inhibition of tumor growth, with no difference between the lowest and highest dose levels evaluated. CCI-779 treatment reduced growth during the period of administration, but tumors progressed when treatment was terminated (week 8). The ratio of T:C tumor volumes after one or two cycles of treatment are presented in Table 1.

To test the value of the phospho-$Thr^{70}$ immunoblot assay in a blinded manner, mice bearing prostate, glioma, or ovarian

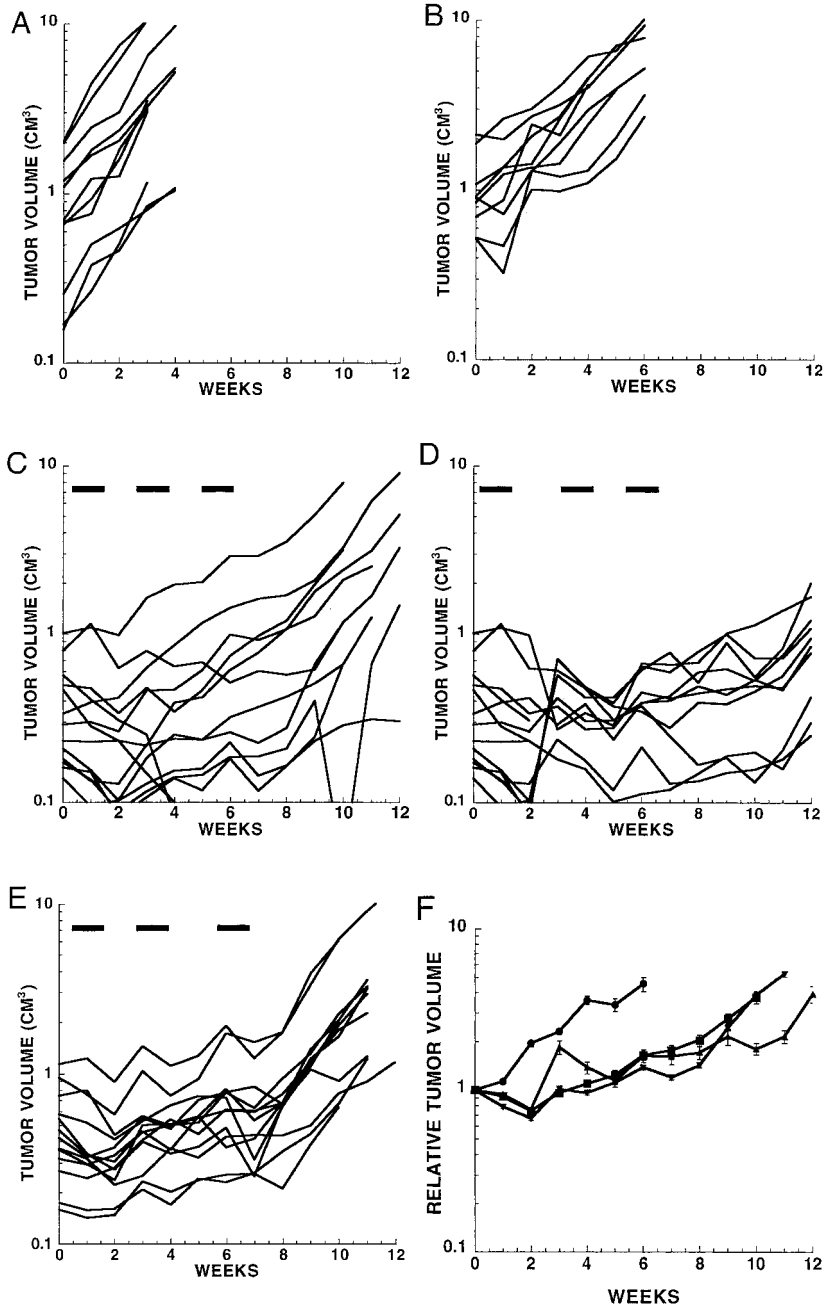


*Fig. 3* Sensitivity of Rh18 xenografts to CCI-779 treatment. Mice bearing s.c. tumors were randomized to receive: *A*, no treatment; *B*, vehicle control; *C*, CCI-779 (20 mg/kg); *D*, CCI-779 (13.2 mg/kg); *E*, CCI-779 (8.7 mg/kg). Drug was administered i.v. 5 days each week for 2 consecutive weeks. Cycles of treatment were repeated every 21 days over 8 weeks (*horizontal bars*). *F*, relative tumor volume plots for each treatment group ± SE: ●, vehicle control; ▼, CCI-779 (20 mg/kg); ▲, CCI-779 (13.2 mg/kg); ■, CCI-779 (8.7 mg/kg).

carcinoma xenografts were treated daily for 5 days with CCI-779 (20 mg/kg) or with vehicle alone. Tumor diameters were measured daily, and the ratio of T:C tumor growth over the 5-day period was calculated after the final drug administration. Tumors were excised 1 h after the final drug injection and were frozen, shipped, and assayed without the tumor response results being revealed. Rh18 xenografts were included as a positive control. Changes in $Thr^{70}$ phosphorylation and inhibition of tumor growth are presented in Fig. 4. Tumor growth inhibition studies demonstrated that CCI-779 retarded the growth of each tumor line except OVCAR5, with which the growth was not significantly retarded (Fig. 4*A*). However, it is noted that the growth of tumors within a treatment or control group over this period was quite varied as shown by the large SDs. The most comprehensive data on tumor sensitivity was the study using Rh18 xenografts (presented in Fig. 3), in which CCI-779 was administered daily for 5 days for 2 consecutive weeks, with cycles repeated every 21 days over an 8-week period. This study demonstrated a consistent and significant inhibition of growth; hence, this xenograft was used as a positive control for assessing the biochemical effects of CCI-779. Compared with control tumors, Rh18 tumors from mice treated with CCI-779 for 5 days

*Table 1* *In vivo* activity of CCI-779 against Rh18 xenografts

| Dose (mg/kg) | Cycle 1 (day 21)[a] T/C | Cycle 2 (day 42)[b] T/C |
|---|---|---|
| Vehicle control | 1.00 | 1.00 |
| 8.7 | 0.44 | 0.30 |
| 13.2 | 0.80 | 0.36 |
| 20 | 0.42 | 0.37 |

[a] Volumes of treated tumors: volumes of controls at day 21 after start of CCI-779 administration.

[b] T/C values after cycle 2 of therapy (day 42).

demonstrated a 27% decrease in phospho-Thr$^{70}$ reactivity. Similarly, DU-145, PC-3, and SF295 xenografts demonstrated reduced levels of phospho-Thr$^{70}$ (>20%; Fig. 4*B*). In contrast, levels were elevated in OVCAR5 tumors relative to vehicle-treated controls. The correlation between these two data sets was high ($r^2$, 0.992; Fig. 4*C*).

## DISCUSSION

Rapamycin and many analogues, including the rapamycin ester CCI-779, are relatively novel agents in that they inhibit the initiation of translation and induce growth arrest or apoptosis (42). Rapamycin and CCI-779 specifically inhibit a Ser/Thr kinase, mTOR, that is downstream of phosphatidylinositol 3′ kinase. mTOR controls two pathways involved in the initiation of translation involving subsets of mRNA, and inhibition results in an ~15% decrease in overall protein synthesis. The best characterized pathways downstream of mTOR are ribosomal p70 S6 kinase and 4E-BP1. Activity of mTOR is necessary for the growth factor stimulation of p70 S6 kinase activity and for the suppression of 4E-BP1 binding to eIF4E. Certain data suggests that rapamycin exerts its growth inhibitory effects by preventing eIF4E-dependent translation. Thus, to inhibit T-cell activation, rapamycin has to be present within 6 h of stimulation, however, even after this time, rapamycin still inhibits p70 S6 kinase. In tumor cells intrinsically resistant to rapamycin, p70 S6 activity is inhibited, but rapamycin is unable to suppress translation of c-MYC after IGF-I stimulation (36). More recent data indicates that tumor cells that are selected for acquired resistance to rapamycin down-regulate levels of 4E-BP1, thus deregulating 4E-dependent translation, without significant changes in p70 S6 kinase levels or activity (43). Indeed, very preliminary data suggest that cells that are intrinsically resistant to rapamycin may have low levels of 4E-BP1 relative to eIF4E.[4] On the basis of these data, we have focused on assays that determine alterations in the 4E-pathway downstream of mTOR. The first assay functionally determines the level of 4E-BP1 associated with eIF4E. Under normal conditions of growth, 4E-BP1 is phosphorylated and is not associated with eIF4E. Rapamycin treatment of cells *in vitro* resulted in a rapid and prolonged association of 4E-BP1 with eIF4E. Similarly, within 4 h of treatment of tumor-bearing mice with either 5 or 20 mg/kg rapamycin, levels of 4E-BP1 associated with eIF4E in tumor tissue increased significantly. Because of the complexity

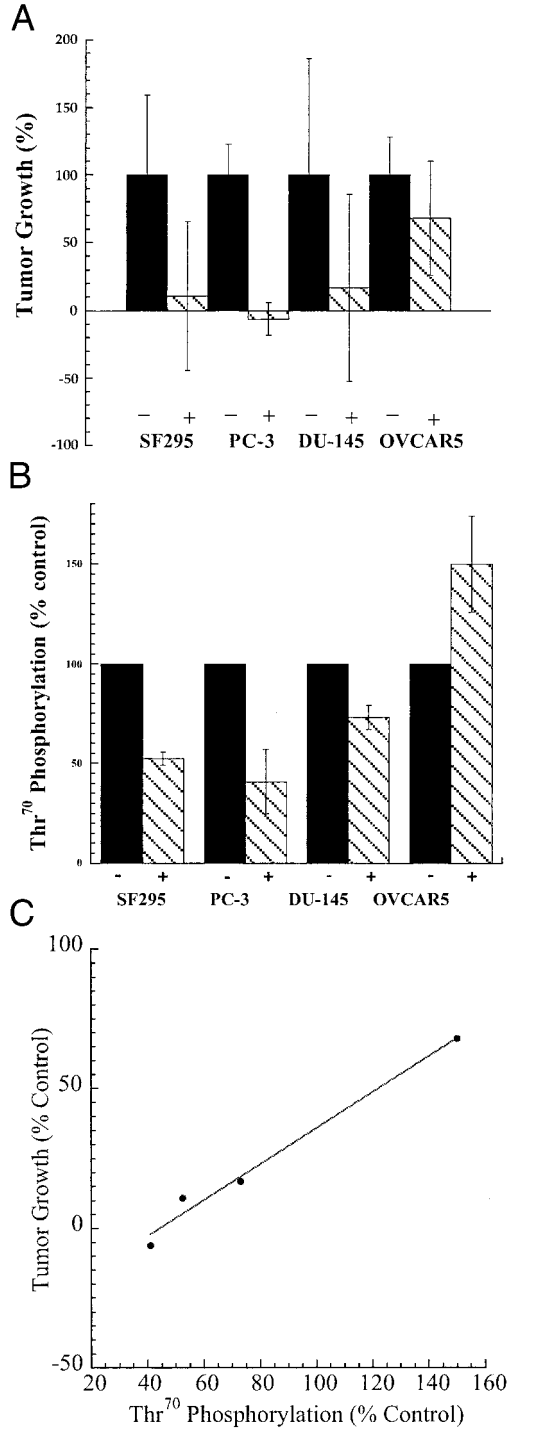


*Fig. 4* Correlation between decreases in Thr70 phosphorylation and tumor growth inhibition. Tumor bearing mice were treated daily with CCI-779 (20 mg/kg). Tumor diameters were recorded daily. One h after the last administration, tumors were excised, and 4E-BP1 phosphorylation was determined using phospho-specific antibody against Thr70. *A*, tumor growth (%) in control (−, ■) and CCI-779 treated (+, ▨) groups. Tumor growth was determined by the change in tumor volume during treatment (volume day 5/volume day 0 × 100), and growth of treated tumors expressed as percentage control growth. Data are mean ± SD ($n$ = 5). *B*, Thr$^{70}$ phosphorylation relative to controls (percentage control ± SD; $n$ = 3). *C*, correlation between inhibition of Thr$^{70}$ phosphorylation and growth inhibition during the period of treatment ($r^2$, 0.992).

[4] Unpublished data.

of this assay, when applied to potential surgical biopsies, a second method, the determination of phosphorylation of $Thr^{70}$ of 4E-BP1, was evaluated. This residue becomes hypophosphorylated relatively rapidly when cells are treated with rapamycin. Similarly, in Rh18 xenografts, decreased $Thr^{70}$ phosphorylation was detected within 4 h of treatment with rapamycin. The growth of Rh18 xenografts was significantly inhibited by CCI-779 treatment; hence, changes in $Thr^{70}$ phosphorylation of 4E-BP1 in this sensitive tumor could be used as a positive control for assessing the biochemical effects of CCI-779 in tumors in which drug responsiveness was less well characterized. We next tested whether this assay was correlated with tumor-growth inhibition in additional tumor models. Mice bearing s.c. xenografts were treated with vehicle control or with CCI-779 for 5 consecutive days at NCI, and tumor diameters were measured. Tumor tissue was assayed for changes in phospho-$Thr^{70}$ without knowledge of the antitumor testing results. Although the response of individual tumors in a treatment group was quite variable, comparison of these results with the inhibition of $Thr^{70}$ phosphorylation shows a high correlation between decreased phosphorylation $Thr^{70}$ of 4E-BP1 and tumor growth inhibition. These results suggest that this assay may be valuable for determining the relationship between systemic exposure to CCI-779 and inhibition of mTOR signaling in tumor or surrogate tissues. The data suggest, also, that relatively small changes in $Thr^{70}$ phosphorylation (~20–30% decrease) may be associated with significant retardation of tumor growth. A secondary question, whether inhibition of mTOR results in tumor response, will also be more readily answered by having assays that determine target inhibition in tumor tissue.

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