

mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer

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Abstract

The mammalian target of rapamycin (mTOR) is a central regulator of G1 cell cycle protein synthesis that precedes commitment to normal cellular replication. We have studied the effect of cell cycle inhibitor-779 (CCI-779), a rapamycin ester that inhibits mTOR function, on the proliferation of a panel of breast cancer cell lines. Six of eight lines studied were sensitive ($IC_{50} \leq 50$ nM) and two lines were resistant ($IC_{50} > 1.0$ μ M) to CCI-779. Sensitive lines were estrogen dependent (MCF-7, BT-474, T-47D), or lacked expression of the tumor suppressor PTEN (MDA-MB-468, BT-549), and/or overexpressed the Her-2/neu oncogene (SKBR-3, BT-474). Resistant lines (MDA-MB-435, MDA-MB-231) shared none of these properties. CCI-779 (50 nM) inhibited mTOR function in both a sensitive and a resistant line. In nu/nu mouse xenografts, CCI-779 inhibited growth of MDA-MB-468 (sensitive) but not MDA-MB-435 resistant tumors. Treatment of sensitive lines with CCI-779 resulted in a decrease in D-type cyclin and c-myc levels and an increase in p27^{kip-1} levels. There was good correlation between activation of the Akt pathway and sensitivity to CCI-779. Amplification of mTOR-regulated p70S6 kinase, which is downstream of Akt, may also have conferred CCI-779 sensitivity to MCF-7 cells. Taken together, the data suggest that mTOR may be a good target for breast cancer therapy, especially in tumors with Akt activation resulting from either growth factor dependency or loss of PTEN function.

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Introduction

Cell cycle inhibitor-779 (CCI-779) is an ester derivative of the natural product rapamycin that was developed for intravenous use for cancer chemotherapy. Rapamycin is a macrolide antibiotic with anti-fungal, immunosuppressive, and anti-tumor properties (Sehgal *et al.* 1994). Genetic studies in yeast showed that rapamycin inhibited cell growth by blocking the function of the proteins TOR1 and TOR2 (targets of rapamycin 1 and 2) (Heitman *et al.* 1991). The TOR proteins are members of the phosphatidylinositol 3-kinase (PI3-K)-related family of kinases and regulate several cellular functions (Schmelzle & Hall 2000). In order to inhibit TOR function, rapamycin initially binds to the cytoplasmic immunophilin FKBP-12 and the complex then inhibits TOR (Brown *et al.* 1994).

A mammalian homolog of the yeast TOR proteins has been cloned independently by several groups and will be referred to in this report as mTOR (Sabers *et al.* 1995), but

it is also known as FRAP (Brown *et al.* 1994), RAFT (Sabatini *et al.* 1994), and RAPT (Chiu *et al.* 1994). The mTOR protein regulates cell cycle progression, in part, by enhancing translation initiation and/or the stability of cell cycle regulatory proteins such as D-type cyclins (Hashemolhosseini *et al.* 1998, Muise-Helmericks *et al.* 1998), c-myc (West *et al.* 1998), and p27^{kip-1} (Nourse *et al.* 1994) among others. At least two direct targets of mTOR, p70 S6 kinase and 4E-BP1/PHAS-1, have been suggested to mediate the effect of mTOR on protein translation (Brunn *et al.* 1997, Thomas & Hall 1997, Burnett *et al.* 1998). 4E-BP1 (eIF-4E binding protein-1) binds to the mRNA cap recognition element of the translation initiation complex protein eIF-4E (eukaryotic initiation factor 4E) and thereby inhibits translation initiation (Beretta *et al.* 1996). mTOR phosphorylation of 4E-BP1 causes it to dissociate from eIF-4E, thus enhancing the translation initiation complex interactions with the mRNA 5' cap. The kinase p70 S6K is phosphorylated and activated by mTOR, and then

phosphorylates the S6 protein of the 40S ribosomal complex. Phosphorylation of S6 results in enhanced translation of proteins that contain a polypyrimidine tract in the 5' untranslated region (Jeffries *et al.* 1997, Volarevic & Thomas 2000). In addition to regulating protein translation, mTOR can also regulate the stability of some cell cycle regulatory proteins such as D-type cyclins and p27^{kip-1}. Activation of mTOR appears to stabilize D-type cyclins (Hashemolhosseini *et al.* 1998) and to destabilize the cyclin-dependent kinase inhibitor p27^{kip-1} (Nourse *et al.* 1994).

Several of the cell cycle targets that are regulated by mTOR have been reported to be dysregulated in human breast cancer, including eIF-4E (Kerekatte *et al.* 1995), D-type cyclins (Weinstat-Saslow *et al.* 1995), p27^{kip-1} (Fredersdorf *et al.* 1997), and c-myc (Liao & Dickson 2000). Therefore, we have begun to study the effect of the mTOR inhibitor CCI-779 in models of human breast cancer. Cell growth in culture revealed that 6 of 8 breast cancer lines studied were inhibited by CCI-779 with IC₅₀s in the low nM range. Two lines, however, were found to be markedly resistant (IC₅₀>1 μM). Cell lines sensitive to CCI-779 were estrogen receptor positive, or overexpressed Her-2/neu, or had lost the tumor suppressor gene product PTEN (phosphatase related to tensin and deleted on chromosome 10) (Li *et al.* 1997). Sensitive lines contained higher levels of the activated form of Akt, suggesting that this PI3-K downstream target may be a common link between growth factor-dependent lines (estrogen, Her-2/neu) and PTEN-deleted lines that are sensitive to mTOR inhibition. The potential for therapeutic use of an mTOR inhibitor is discussed in terms of the rationale provided by known genetic alterations in human breast cancer cells.

Materials and methods

Chemicals and cell culture methods

All chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA). CCI-779 was synthesized at Wyeth-Ayerst Research. Cell lines of MDA-MB-468 (MDA-468), MDA-MB-435 (MDA-435), MDA-MB-231 (MDA-231), MCF-7, T-47D, SKBR-3 and BT-474 were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). BT-474G is a sub-clone derived from BT-474. All cell lines were cultured in Minimum Essential Medium (MEM) containing 10% fetal bovine serum (FBS) and 1 mM MEM sodium pyruvate in a 37°C incubator containing 5% CO₂. All cell culture reagents were purchased from Gibco-BRL (Grand Island, NY, USA).

Proliferation assay

Cells were plated in 96-well culture plates at about 3000 cells per well. One day following plating, drugs were added to

cells. Three days after drug treatment, viable cell densities were determined by measuring metabolic conversion (by viable cells) of the dye MTS, a previously established cell proliferation assay. Stock solutions of MTS and PMS were purchased from Promega Corp. (Madison, WI, USA). For each assay, MTS and PMS stocks were freshly thawed and mixed (MTS/PMS, 20:1). The MTS/PMS mixture was then added to 96-well cell plates at 20 μl/well, and plates were incubated for 1–2 h in cell culture incubator. MTS assay results were read in a 96-well format plate reader by measuring absorbance at 490 nm. The effect of each drug treatment was calculated as a percentage of control cell growth obtained from vehicle-treated cells grown in the same culture plate.

In vivo tumor inhibition

Xenograft model athymic nu/nu female mice, 5–6 weeks of age, were obtained from Charles River Laboratories, Wilmington, MA, USA and maintained in a barrier facility in accordance with Institutional Animal Care and Use Committee (IACUC) regulations. Animals were injected s.c. with either 6 × 10⁶ MDA-MB468 cells or 6 × 10⁶ MDA-MB435 cells. When tumors reached a weight of between 80 and 120 mg, animals were randomized into treatment groups (5 mice/group). Animals were treated intraperitoneally (i.p.) for 5 consecutive days with 40, 20, or 10 mg/kg CCI-779 prepared in 2% ethanol, 8% cremophor el, (Sigma, St Louis, MO, USA), or vehicle alone. Tumor mass ($[\text{length} \times \text{width}^2]/2$) was determined on days 7, 14, 21 and 28 post staging. The data were analyzed via Student's *t*-test. A *P*-value <0.05 indicates a statistically significant reduction in relative tumor growth of the treated group compared with that of the vehicle control group.

Protein lysates and immunoblotting

For immunoblotting experiments, cells were plated in 10-cm dishes or 6-well plates. Depending on the study, after the cells had completely attached, they were either serum-starved or incubated in growth medium overnight. Treatment with various inhibitors ranged from 2 to 16 h. After drug pretreatment, the cells were rinsed once with cold PBS (phosphate buffered saline without Mg⁺⁺ and Ca⁺⁺) and then lysed in cold gentle lysis buffer (25 mM Hepes, pH 7.55, 100 mM NaCl, 20 mM β-glycerophosphate, 1.5 mM MgCl₂, 0.5 mM EGTA, 0.25 mM EDTA, 1% NP-40, 10 mM Na₃VO₄, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonylfluoride, 1 μM microcystin LR and 0.1% 2-mercaptoethanol). In some experiments, cells from 6-well plates were lysed in NuPAGE-LDS sample buffer (Invitrogen, Carlsbad, CA, USA). The crude lysates were briefly sonicated and then clarified by centrifugation for 15 min at 14 000 r.p.m. Cleared lysates (20–50 μg) were subjected to SDS-PAGE electrophoresis using the NuPAGE system (Invitrogen, Carlsbad, CA, USA) and transferred to

a nitrocellulose membrane. The sources of various primary antibodies are as follows. Phospho-AKT (Ser473), AKT, phospho-p70 S6 kinase (Thr389), p70 S6 kinase, phospho-4E-BP1 (Thr-37, Thr-46 and Ser-65) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies against cyclin D3, c-myc and 4E-BP1 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p27^{kip1} was from Transduction Laboratories (San Diego, CA, USA) and phospho-FKHRL-1 (Thr32) was from Upstate Biotechnology (Lake Placid, NY, USA). Immunoblots were blocked for 1 h with blocking buffer TBST (20 mM Tris, pH 7.5, 500 mM NaCl₂, 0.1% Tween-20) and 5% non-fat milk. After washing, they were incubated with primary antibodies overnight at 4°C according to the manufacturer's suggestions, washed and treated with appropriate secondary antibodies. Immunoreactive proteins were detected using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Results

The effect of the mTOR inhibitor CCI-779 on the growth of human breast cancer lines *in vitro*

The growth inhibitory properties of CCI-779 were studied *in vitro* on a panel of 8 human breast cancer cell lines (Table 1). The MCF-7, BT-474, and T-47D cell lines are all estradiol responsive (Yarden *et al.* 1996) and all were strongly growth inhibited by CCI-779 (IC₅₀ low nM). Similarly, growth of lines BT-549 and MDA-MB-468 which contain deletions of the PTEN tumor suppressor gene (Lu *et al.* 1999) was highly sensitive to treatment with CCI-779. The Her-2/neu overexpressing (Chen *et al.* 2000) SKBR-3 line and ER positive, Her-2 overexpressing BT-474 cells were also inhibited at low nM concentrations of CCI-779. Two lines, MDA-MB-435 and MDA-MB-231, were resistant to treatment with CCI-779 (IC₅₀s at low μM concentrations). These lines do

not respond to estradiol, do not overexpress Her-2/neu, and are wild-type for the tumor suppressor, PTEN.

The mTOR pathway is activated in CCI-779 sensitive MDA-MB-468 cells and minimally activated in CCI-779 resistant MDA-MB-435 cells

There was a marked difference in the ability of CCI-779 to inhibit MDA-468 (PTEN^{-/-}) cells compared with MDA-435 (PTEN^{+/+}) cells (Fig. 1A). The PTEN^{-/-}, CCI-779 sensitive MDA-468 line showed evidence of Akt activation, as has been reported by others (Lu *et al.* 1999, Weng *et al.* 1999). A comparison of Western blots using a phospho-specific antibody for the activated form of Akt shows marked activation relative to total Akt protein in the MDA-468 cells compared with the MDA-435 cells (Fig. 1B). The forkhead transcription factor (FKHRL⁻¹), a downstream target of Akt (Biggs *et al.* 1999) is also highly phosphorylated, confirming that the Akt signaling is activated in these cells. p70 S6K, a direct target of mTOR, is also highly phosphorylated in the MDA-468 cells, suggesting that the mTOR pathway is activated in the PTEN^{-/-} cells. Both the Akt and mTOR pathways were only minimally activated in the MDA-435 PTEN wild-type cells.

Inhibition of mTOR function inhibits growth in xenografts of MDA-468 (PTEN^{-/-}) cells but not MDA-435 (PTEN^{+/+}) cells

CCI-779 produced a similar differential effect on the growth of MDA-468 cells compared with MDA-435 cells *in vivo* (Fig. 2). The PTEN mutant MDA-468 cells were sensitive whereas the PTEN wild-type MDA-435 cells were not. In these experiments, nu/nu mice were injected in the flank with tumor cells and tumors were allowed to grow to a size of 100 mg. Staged mice were then randomized into treatment groups and treated with CCI-779 by i.p. injection at 10, 20, or 40 mg/kg or with vehicle for 5 consecutive days. Although the MDA-468 cells did not grow as well as the MDA-435 cells in nude mice, there was a clear regression of tumor size in MDA-468-treated tumors at doses of 40 and 20 mg/kg. Even at the low dose of 10 mg/kg, the delay in growth of MDA-468 tumors extended 10 days beyond the last dose of CCI-779 on day 5. The growth of MDA-435 tumors was not affected by CCI-779 at any dose. The effect of CCI-779 *in vivo* has not been limited to slow growing tumors as we have seen similar growth inhibitory effects in the fast growing U87 MG glioblastoma (data not shown).

Treatment with CCI-779 inhibits mTOR function in both sensitive (MDA-468) and resistant (MDA-435) cells

MDA-468 cells were sensitive to growth inhibition by the mTOR inhibitor CCI-779 *in vitro* and *in vivo* while

Table 1 Effect of CCI-779 on the growth of human breast cancer lines *in vitro*.

Cell line	IC ₅₀ (nM)	Estrogen receptor α	Her-2/Neu	PTEN ^{+/-}
MCF-7	10–50	+	–	–
BT-474	0.6	+	+	–
T-47D*	<10.0	+	–	–
BT-549*	<10.0			+
MDA-MB-468	0.7			+
SKBR-3	0.7	–	+	–
MDA-MB-435	1600	–	–	–
MDA-MB-231	5900	–	–	–

*Data from National Cancer Institute 60 cell panel screen (Monks *et al.* 1991).

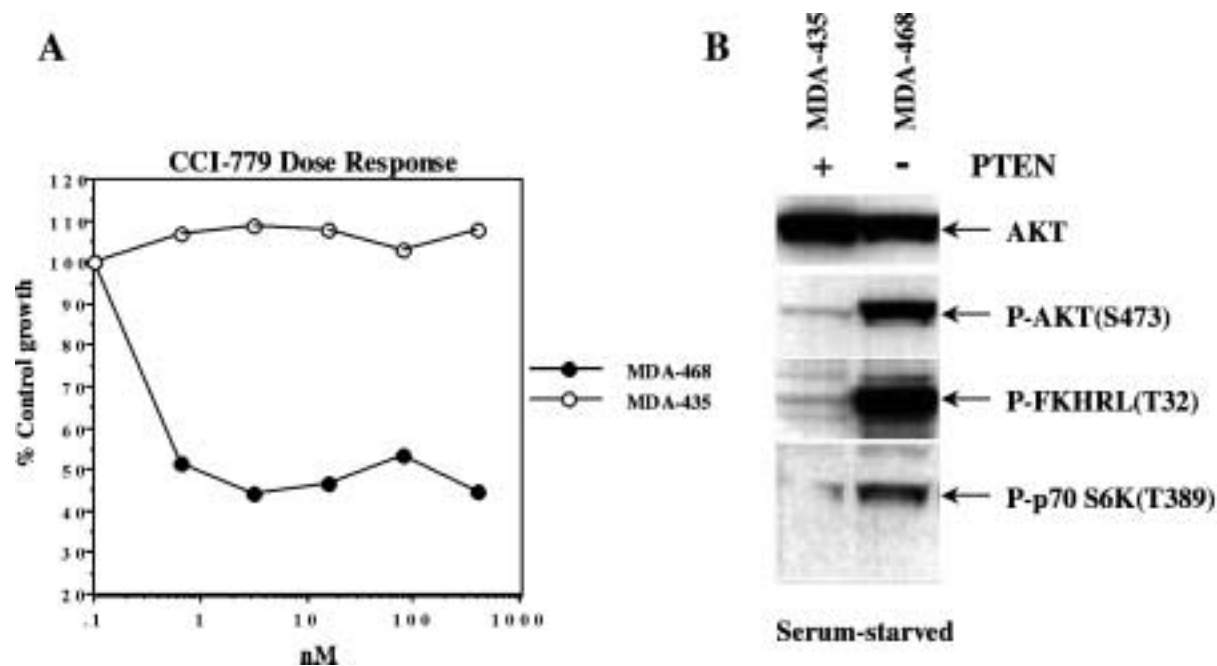


Figure 1 Loss of PTEN tumor suppressor in MDA-468 cells results in an elevated sensitivity to growth inhibition by CCI-779. (A) Growth inhibition curves. Cells were plated in 96-well cell culture plates at 3000 cells per well overnight before treatment with CCI-779 for 3 days. Cell growth assays were performed by standard MTS assay as described in Materials and methods. (B) Elevated AKT and mTOR signaling in MDA-468 cells. Cells were plated in 10-cm culture plates overnight and were then serum-starved for 24 h with culture medium containing 0.1% serum. Total cellular lysates were prepared using the gentle lysis buffer described in Materials and methods. Equal amounts (50 μ g) of total proteins were analyzed by immunoblotting with antibodies of AKT, phospho (P)-AKT (S473), phospho (P)-FKHRL-1 (T32) and phospho (P)-p70 S6K (T389) as described in Materials and methods.

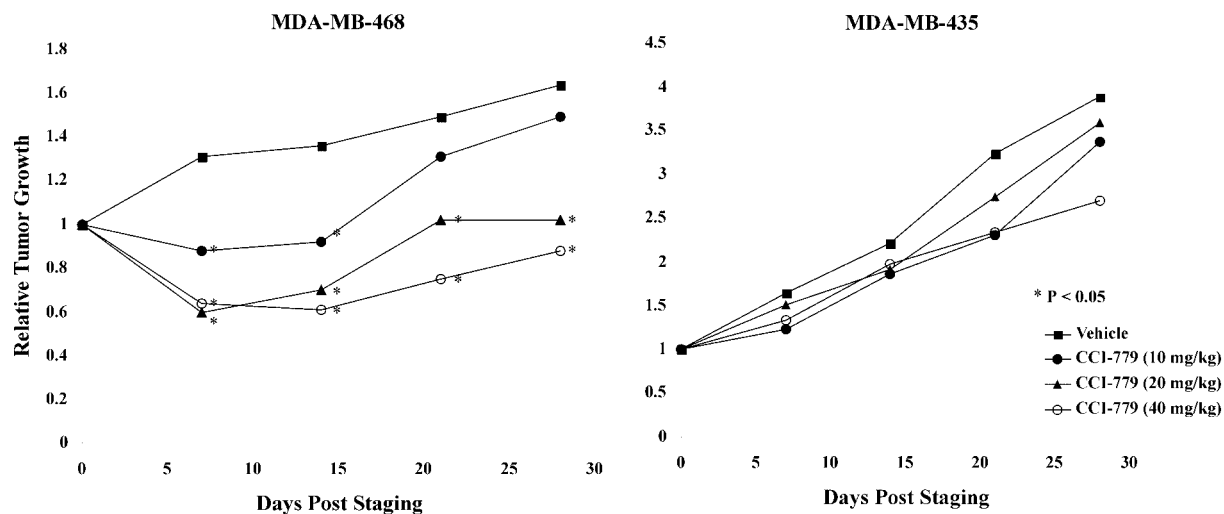


Figure 2 Nu/nu mice (5 mice/group) were injected in the flank with either MDA-MB-468 or MDA-MB-435 cells (6×10^6 /mouse). Mice were randomized into treatment groups after tumors reached a size of about 100 mg. Treatment with CCI-779 or vehicle was for days 1–5 after staging and mice were not treated thereafter.

MDA-435 cells were not. We studied by Western blot the effect of CCI-779 on mTOR function in these cells by looking at the phosphorylation status of the mTOR targets p70 S6K and 4E-BP1 (Fig. 3). Cells were treated *in vitro* for 16 h with CCI-779 at a concentration (50 nM) that inhibited growth of MDA-468 cells by about 50% and had no effect on growth of MDA-435 cells. The level of phosphorylated p70 S6K was higher in the MDA-468 cells, presumably due to loss of PTEN and activation of Akt (Fig. 1B), and CCI-779 treatment resulted in complete dephosphorylation of p70 S6K without affecting the total protein levels of p70 S6K (not shown). There was considerably less but detectable phosphorylated p70 S6K in the PTEN^{+/+} MDA-435 cells; however, treatment with CCI-779 also resulted in complete dephosphorylation of p70 S6K in these cells (Fig. 3). Similar results have been reported for rhabdomyosarcoma lines sensitive or resistant to rapamycin (Hosoi *et al.* 1998). Using an antibody specific for threonine 46 (T46) on 4E-BP1, a site directly phosphorylated by mTOR (Gingras *et al.* 1999), we

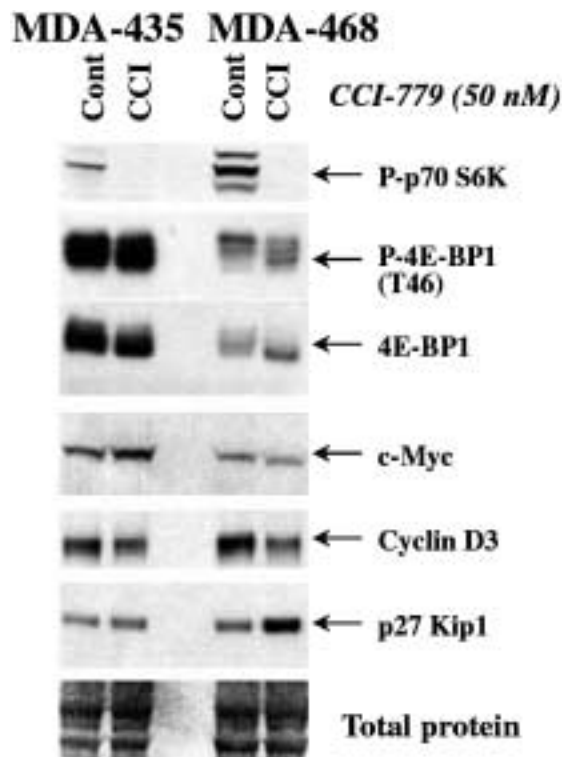


Figure 3 Differential effects of CCI-779 on cellular mTOR targets in CCI-sensitive and CCI-resistant cells. MDA-435 and MDA-468 cells were plated in complete growth medium in 6-well culture plates and treated with CCI-779 for 16 h. Total cellular proteins were prepared by NuPAGE-LDS sample buffer. Immunoblotting assays with antibodies of phospho (P)-p70 S6K (T389), phospho (P)-4E-BP1 (T46), 4E-BP1, c-Myc, cyclin D3 and p27^{Kip1} were performed using the NuPAGE system. Cont, control.

observed that CCI-779 treatment in MDA-468 cells resulted in a shift to a faster migrating species, suggesting inhibition of phosphorylation of other residues due to mTOR inhibition (Fig. 3). We were surprised to find that the resistant MDA-435 cells markedly overexpressed 4E-BP1 relative to MDA-468 cells. Nevertheless, there was also a shift to faster migrating species of 4E-BP1 after CCI-779 treatment of MDA-435 cells. This shift was more easily observed with an antibody that recognized total 4E-BP1 levels where a more condensed faster migrating band was seen after CCI-779 treatment. Thus treatment with CCI-779 resulted in mTOR inhibition in both a sensitive and a resistant line as evidenced by a decrease in phosphorylation of the direct mTOR targets p70 S6K and 4E-BP1.

We also looked at downstream cell cycle regulatory proteins that are reported to be modulated by mTOR (Fig. 3). In the sensitive MDA-468 cells, we observed decreases in total c-myc and cyclin D3 protein levels after treatment with CCI-779 but not in the resistant MDA-435 cells. Similarly, we observed an increase in p27^{Kip1} levels in the sensitive MDA-468 cells, but not in the resistant cells. These data suggest that mTOR function is inhibited in both sensitive and resistant lines but the downstream consequences are greater in PTEN^{-/-} MDA-468 cells, suggesting they are more dependent on mTOR function. Alternatively, there may be other targets of mTOR inhibition besides p70 S6K and 4E-BP1 that are operative in sensitive lines but not in resistant lines.

The effect of mTOR inhibition on the growth of estrogen-dependent MCF-7 cells

MCF-7 cells do not contain a PTEN mutation or deletion but are growth inhibited by about 50% by treatment with 50 nM CCI-779. We looked by Western blot at the effect of CCI-779 treatment on proximal (p70 S6K, 4E-BP1) and downstream (c-myc, cyclin D3, p27^{Kip1}) targets in these cells (Fig. 4A). We found that MCF-7 cells markedly overexpressed the mTOR target p70 S6K (data not shown). There were high levels of mTOR-dependent phosphorylated p70 S6K in these cells that were completely inhibited by CCI-779. Similarly, there was a shift to faster migrating species of 4E-BP1 in CCI-779-treated MCF-7 cells. These results suggest that phosphorylation of two specific targets of mTOR (p70 S6K and 4E-BP1) is inhibited by the drug. We also observed a slight decrease in c-myc and cyclin D3 levels in CCI-779-treated MCF-7 cells. The levels of p27^{Kip1} appear unchanged after CCI-779 treatment, although very high levels of p27^{Kip1} were seen in untreated MCF-7 cells making small changes difficult to detect. We have also looked at the status of other targets regulated by Akt signaling (Brunet *et al.* 1999). Phosphorylation of the forkhead transcription factor FKHRL-1 is highly elevated (Fig. 4B) compared with MDA-435 and MDA-231 cells. Similarly, we also observed

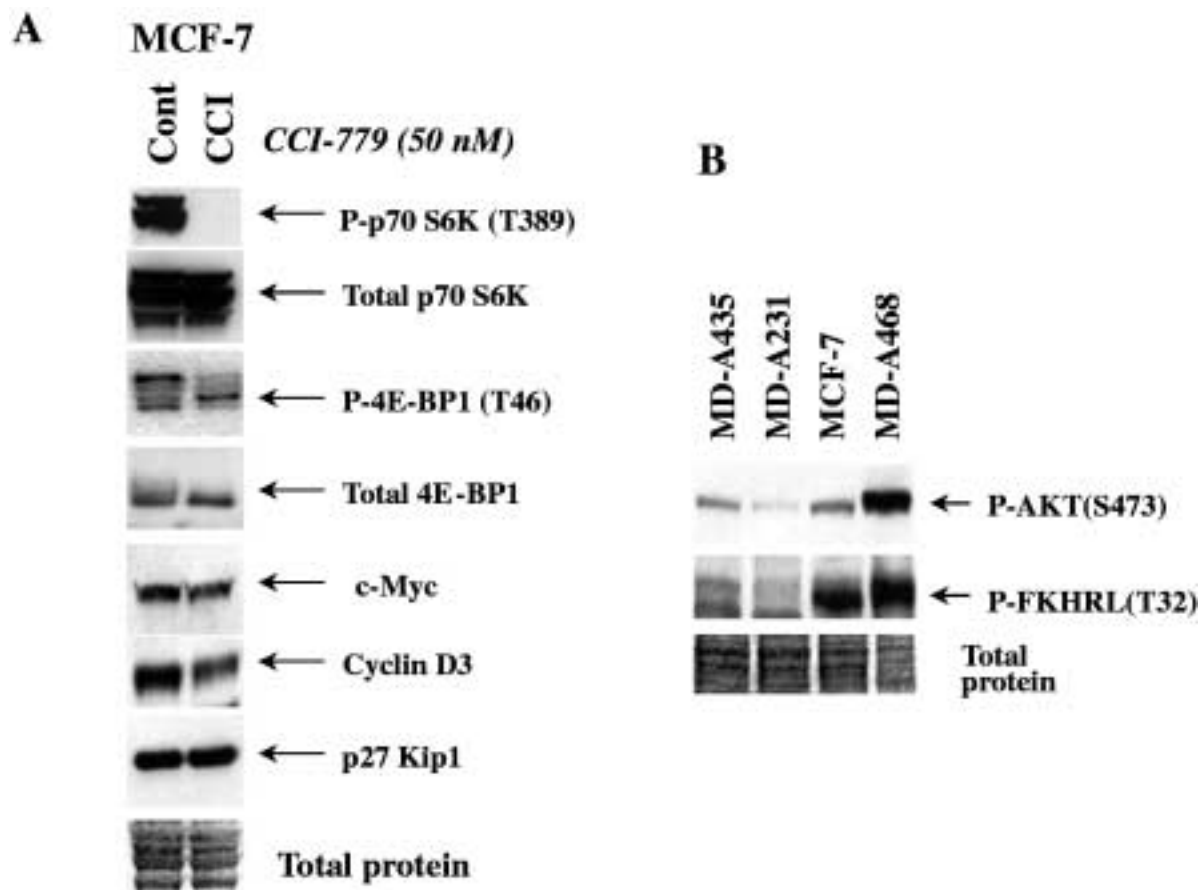


Figure 4 Biochemical characterizations of cellular mTOR and AKT targets in MCF-7 cells. (A) Effects of CCI-779 on cellular mTOR targets. Cells were plated, treated and analyzed in a similar manner as described in Fig. 3. (B) Cells of the indicated cell lines were plated in complete growth medium in 10-cm culture plates. Total cell lysates were made from exponentially growing cells in the gentle lysis buffer. Fifty micrograms total proteins per lane were immunoblotted for phospho (P)-AKT (S473) and phospho(P)-FKHRL-1 (T32) in a similar manner as described in Fig. 1B.

an increased phosphorylation of the Akt target GSK-3 β (data not shown). Since MCF-7 cells do not have a high level of active Akt-1, the mechanism for elevated phosphorylation of FKHRL-1 and GSK-3 β remains to be identified. It is also possible that deregulation of these targets may contribute to its sensitive response to inhibition of mTOR function.

Cells resistant to mTOR inhibition contain lower levels of activated Akt than sensitive lines

The phosphorylation status of 4E-BP1 is also affected by Akt (Gingras *et al.* 1999) and several laboratories have suggested that mTOR is either downstream of PI3-K \rightarrow Akt activation or activated in parallel with PI3-K/Akt to collaborate on the regulation of 4E-BP1 and p70 S6K. Therefore, we compared the levels of Akt phosphorylation in a panel of breast cancer lines containing cells sensitive or resistant to the mTOR

inhibitor CCI-779 (Fig. 5). Cells were grown in 10% serum and harvested prior to achieving confluency. The two resistant lines, MDA-231 and MDA-435, showed the least phosphorylation of Akt relative to total Akt levels. The highest levels of phospho-Akt, as expected, were seen in the PTEN^{-/-} MDA-468 cells. In the Her-2 overexpressing and ER positive BT-474 cells, Akt was also highly phosphorylated and only slightly less so in SKBR-3 cells which also overexpress Her-2/neu. The estrogen responsive cells MCF-7 and T-47D were intermediate in the level of Akt phosphorylation.

Discussion

We have studied the effect of the mTOR inhibitor CCI-779 on cell growth and cell signal transduction in a panel of human breast cancer cell lines. We found that most breast cancer lines were responsive to CCI-779. While the

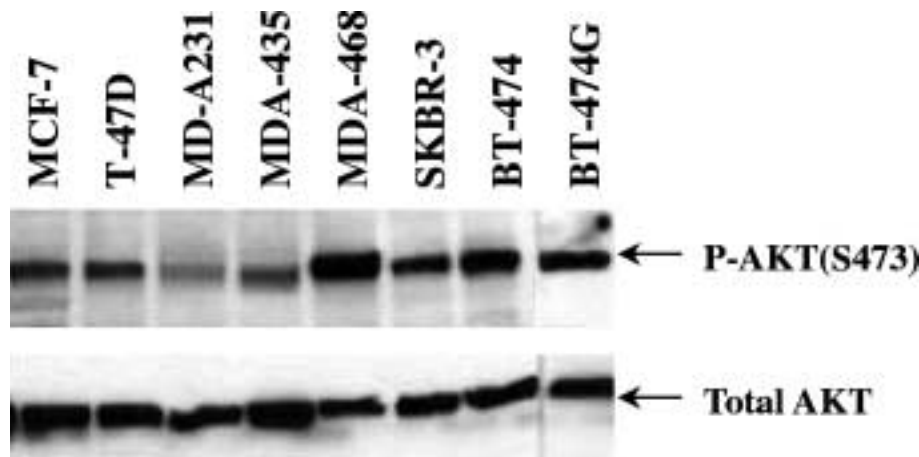


Figure 5 Survey of the phosphorylated AKT proteins in breast tumor lines. Indicated cell lines were plated in 10-cm dishes in complete growth medium. Lysates were prepared in the gentle lysis buffer and immunoblotted for phospho (P)-AKT (S473) and AKT in a similar manner as described in Fig. 1B.

molecular basis of responsiveness to mTOR inhibition is not known, some observations from the literature regarding the responsive cells may be pertinent. Cells with known dependence on the hormonal growth factor estradiol or with aberrant expression of a growth factor receptor (Her-2/neu) were sensitive to *in vitro* growth inhibition by the mTOR inhibitor. Similarly, cells that had lost PTEN, a negative regulator of growth factor signaling through PI3-K, were also sensitive to CCI-779. These observations are consistent with the known effect of mTOR regulation on growth factor-induced proliferation (Sehgal *et al.* 1994, Wiederrecht *et al.* 1995). Cell lines in which growth was not inhibited by the mTOR inhibitor did not respond to estrogen, did not overexpress Her-2/neu, and were wild-type for PTEN.

We chose one sensitive (MDA-MB-468) and one resistant (MDA-MB-435) line to study *in vivo* in nu/nu mouse xenografts and for further biochemical characterization of mTOR inhibition. *In vivo*, MDA-468 tumors were inhibited by treatment with CCI-779 (10, 20, or 40 mg/kg). At the higher doses there was regression of the staged tumors and even at the low dose of 10 mg/kg, the delay in growth extended 10 days beyond the last dose given. The MDA-435 line was not inhibited in the xenograft model at any of the doses tested, similar to the refractory phenotype observed *in vitro*.

By Western blot we showed that the serine threonine kinase Akt was highly activated in exponentially growing MDA-468 cells but only minimally in MDA-435 cells. Activation of Akt in these cells results from loss of function of the PTEN tumor suppressor gene (Lu *et al.* 1999, Weng *et al.* 1999) which negatively regulates PI3-K activation of Akt. In addition to Akt activation, p70 S6K (a downstream target of mTOR) was also highly phosphorylated on Thr-389 in MDA-468 cells but only marginally in MDA-435 cells,

suggesting activation of the mTOR pathway in the sensitive but not in the resistant line. Treatment of both cell lines with CCI-779 resulted in complete dephosphorylation of p70 S6K at the mTOR-dependent site Thr-389. With respect to the other mTOR target 4E-BP1, treatment with CCI-779 resulted in a shift to faster migrating species in both sensitive and resistant lines, suggesting that mTOR-dependent phosphorylation of 4E-BP1 was inhibited in both lines. The level of expression of 4E-BP1 was markedly higher in the resistant MDA-435 cells and although there was a shift to the more dephosphorylated form after CCI-779 treatment, constitutive levels of the unphosphorylated form were high, suggesting a possible mechanism for MDA-435 cell resistance to mTOR regulation. This could occur if mTOR was able to phosphorylate only a portion of the 4E-BP1, leaving high residual levels of unphosphorylated 4E-BP1 bound to eIF-4E. Additional studies to determine if the unphosphorylated 4E-BP1 caused a difference in free 4E levels in growing MDA-435 versus MDA-468 cells will be necessary to address this possibility. Nevertheless, we have shown that the difference in responsiveness to CCI-779 in these two breast cancer lines is not due to the failure of CCI-779 to inhibit mTOR function in the resistant cells. Hosoi *et al.* (1998) have reported the same observation in rapamycin sensitive and resistant rhabdomyosarcoma cell lines.

Although CCI-779 inhibited mTOR function in both sensitive and resistant cells, downstream of mTOR the response to CCI-779 was different in MDA-468 compared with MDA-435 cells (Fig. 3). Inhibition of mTOR by CCI-779 in MDA-468 cells resulted in decreased cyclin D3 and c-myc levels and an increase in p27^{kip-1} levels. These effects were not seen in the resistant MDA-435 cells. Lu *et al.* (1999) reported that transfection of PTEN into the PTEN^{-/-} MDA-468 cells decreased phosphorylation of p70

S6K on the same residues as the mTOR inhibitor rapamycin. PTEN transfection also resulted in increased p27^{kip-1} levels in the MDA-468 cells. Thus, PTEN, like the mTOR inhibitor CCI-779, inhibited p70 S6K and increased p27^{kip-1}. These data suggest that the growth advantage of cells that have lost PTEN may, at least in part, be mediated by mTOR. Our data showing an increase in p27^{kip-1} after CCI-779 treatment in MDA-468 cells support this contention and suggest that p27^{kip-1} is regulated by PTEN in an mTOR-dependent manner. The effect of mTOR inhibition on p27^{kip-1} levels in normal T cells has long been known (Nourse *et al.* 1994). Taken together, these data suggest that loss of PTEN results in activation of mTOR and that mTOR inhibition may be therapeutically effective in breast tumors lacking PTEN.

D-type cyclins have been reported to be overexpressed in human breast cancer (Weinstat-Saslow *et al.* 1995). The mTOR pathway can regulate both the stability and translation of D-type cyclins (Hashemolhosseini *et al.* 1998, Muise-Helmericks *et al.* 1998). Reduction of cyclin D3 in the MDA-468 cells by CCI-779, coupled with the increase of p27^{kip-1} may result in redistribution of p27^{kip-1} towards cyclin E-CDK-2 complexes, thereby preventing entry into the S phase of the cell cycle.

Breast lines that are wild-type for PTEN but dependent on estrogen were also sensitive to CCI-779. Western analysis of MCF-7 cells showed that these cells markedly overexpressed p70 S6K and had high levels of the mTOR-dependent phosphorylated form of p70 S6K when grown in 10% serum. The increased p70 S6K expression is a function of gene amplification (Barlund *et al.* 2000, Wu *et al.* 2000) and has been reported to occur in as many as 9% of primary breast cancers. Treatment of MCF-7 cells with CCI-779 completely inhibited phosphorylation of p70 S6K. Similarly, CCI-779 caused a nearly complete shift of 4E-BP1 to the under-phosphorylated fast migrating species. Cyclin D3 levels in MCF-7 cells were reduced by CCI-779 by about 50% and similar results were seen for cyclin D1 (data not shown). It has recently been shown that as little as 30–40% reduction in cyclin D1 levels by anti-estrogens in MCF-7 cells is sufficient to induce a shift in p21^{cip1} molecules to cyclin E-Cdk2 complexes, causing inhibition of progression from G1→S phase of the cell cycle (Carroll *et al.* 2000). Inasmuch as anti-estrogens inhibit D-type cyclin production at the transcriptional level and mTOR inhibition decreases D-type cyclins at the translational and/or protein stability level, there is a strong rationale for combining CCI-779 with anti-estrogen therapy. In experiments to be reported elsewhere, one of us (P Frost) has found that CCI-779 acted synergistically in combination with an anti-estrogen to inhibit proliferation of MCF-7 cells *in vitro* and also potentiated the effect of anti-estrogens *in vivo* in a MCF-7 mouse-xenograft model.

mTOR inhibition also effectively inhibited proliferation of Her-2/neu-expressing cells BT-474 and SKBR-3 (Table

1). Lee *et al.* (2000) recently showed that neu-dependent transformation requires cyclin D1 and is induced through an E2F-dependent signaling pathway. Although we did not directly study the effects of CCI-779 on D-type cyclins in the Her-2/neu overexpressing lines, the amply demonstrated effect of mTOR inhibition on D-type cyclin levels suggests a plausible mechanism for sensitivity of Her-2/neu positive tumors to mTOR inhibition.

The upstream activator(s) of mTOR is not well characterized. Studies in yeast (Cardenas *et al.* 1999) and more recently in mammalian cells suggest that mTOR may act as a sensor to ensure appropriate nutritional status before the cells commit to division (Schmelzle & Hall 2000). Activation of Akt appears to be upstream of mTOR activation in that Akt has been shown to phosphorylate mTOR (Sekulic *et al.* 2000). However, mutation of the site on mTOR phosphorylated by Akt did not inhibit downstream signaling to p70 S6K or 4E-BP1 (Sekulic *et al.* 2000). This has led to the hypothesis that Akt and mTOR are activated by parallel pathways and converge to activate downstream targets (Gingras *et al.* 1999). Nevertheless, it appears that Akt and mTOR are activated by growth factors in a coordinated if not linear fashion and suggests that Akt activation may be a marker for enhanced mTOR dependency in tumor cells. We found good correlation between Akt activation and responsiveness to CCI-779 in the breast cancer lines studied. The two resistant lines, MDA-MB-231 and MDA-MB-435, showed the least activation of Akt as evidenced by phosphorylation of Akt and its downstream targets FKHRL-1 (Fig. 4B) and GSK-3 β (data not shown). One exception was the MCF-7 line, which was sensitive to CCI-779 but did not show evidence of strong activation of Akt. MCF-7 cells did overexpress highly activated mTOR-dependent p70 S6K, suggesting that mTOR may be activated in an Akt-independent manner in these cells. The resistant line MDA-MB-231 has been shown to overexpress Akt-3 (Nakatani *et al.* 1999). There are three Akt isozymes, Akt 1–3, that are reported to be regulated similarly. Our data for MDA-MB-231 show no evidence of phosphorylation of downstream targets of Akt such as FKHRL-1 and GSK-3 β , suggesting that even though Akt-3 is overexpressed, it does not appear to be constitutively active in these cells. This differs from PTEN-deficient cells where it has been shown the Akt is constitutively active, suggesting a greater dependency of these cells on the Akt and mTOR pathways. In support of this, Aoki *et al.* (2001) have recently reported that the mTOR inhibitor, rapamycin, selectively blocked Akt-induced transformation of chicken fibroblasts when compared with 11 other oncogenes. Taken together, the data suggest that Akt activation confers sensitivity to mTOR inhibitors but is not a requirement for responsiveness to mTOR inhibitors. Nevertheless, cells with activated Akt whether through PTEN deletion, growth factor stimulation,

or aberrant growth factor receptor signaling are growth inhibited by the mTOR inhibitor CCI-779.

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