A New Pharmacologic Action of CCI-779 Involves FKBP12-Independent Inhibition of mTOR Kinase Activity and Profound Repression of Global Protein Synthesis

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Abstract

The mammalian target of rapamycin (mTOR) inhibitor CCI-779 (temsirolimus) is a recently Food and Drug Administration–approved anticancer drug with efficacy in certain solid tumors and hematologic malignancies. In cell culture studies, CCI-779 at the commonly used nanomolar concentrations generally confers a modest and selective antiproliferative activity. Here, we report that, at clinically relevant low micromolar concentrations, CCI-779 completely suppressed proliferation of a broad panel of tumor cells. This “high-dose” drug effect did not require FKBP12 and correlated with an FKBP12-independent suppression of mTOR signaling. An FKBP12-rapamycin binding domain (FRB) binding–deficient rapamycin analogue failed to elicit both the nanomolar and micromolar inhibitions of growth and mTOR signaling, implicating FRB binding in both actions. Biochemical assays indicated that CCI-779 and rapamycin directly inhibited mTOR kinase activity with IC₅₀ values of 1.76 ± 0.15 and 1.74 ± 0.34 μmol/L, respectively. Interestingly, a CCI-779–resistant mTOR mutant (mTOR-SI) displayed an 11-fold resistance to the micromolar CCI-779 in vitro (IC₅₀ 20 ± 3.4 μmol/L) and conferred a partial protection in cells exposed to micromolar CCI-779. Treatment of cancer cells with micromolar but not nanomolar concentrations of CCI-779 caused a marked decline in global protein synthesis and disassembly of polyribosomes. The profound inhibition of protein synthesis was accompanied by rapid increase in the phosphorylation of translation elongation factor eEF2 and the translation initiation factor eIF2α. These findings suggest that high-dose CCI-779 inhibits mTOR signaling through an FKBP12-independent mechanism that leads to profound translational repression. This distinctive high-dose drug effect could be directly related to the antitumor activities of CCI-779 and other rapalogs in human cancer patients. [Cancer Res 2008;68(8):2934–43]

Introduction

CCI-779 (temsirolimus) is an anticancer drug recently approved by Food and Drug Administration for the treatment of advanced renal cell carcinoma. Multiple clinical and preclinical studies have shown promising antitumor activity of CCI-779 in other tumor types, including breast cancer (1), glioma (2), endometrial cancer (3), and mantle cell lymphoma (4). The antitumor activity of CCI-779 likely involves multiple pharmacologic actions, including its antiangiogenic and antiproliferative properties (5, 6). The molecular mechanism of CCI-779 antitumor effect involves inhibition of mammalian target of rapamycin (mTOR) kinase function. mTOR is an evolutionary conserved serine/threonine kinase, the founding member of the phosphoinositide-3-kinase (PI3K)–related kinase family. In mammals, mTOR kinase acts as a key metabolic sensor that coordinates cell growth and proliferation with the availability of growth factors, nutrients, and intracellular ATP (7). Rapamycin or its analogues form tight binary complex with the small immunophilin FKBP12 (FK506 binding protein), which is highly abundant in human cells. The rapamycin-FKBP12 complex binds mTOR in a unique region called FKBP12-rapamycin binding domain (FRB; ref. 8). Accumulating evidence points to the potential regulatory role for the FRB region in ligand-mediated mTOR activity and/or mTOR signal transduction in cells. Although the exact mechanism by which rapamycin inhibits mTOR catalytic activity remains unclear, earlier findings suggest that the rapamycin-FKBP12 complex acutely destabilizes mTOR complex 1 and, in certain cell types, interferes with the assembly of the mTOR complex 2 (9–11). Rapamycin might also compete with phosphatidic acid for binding to FRB, thereby interfering with activation of mTOR by phosphatidic acid (12, 13).

In vitro, CCI-779 and rapamycin at the commonly used nanomolar dose range effectively suppress the cellular phosphorylation of the mTOR substrates S6K1 and 4E-BP1. However, the growth inhibitory effects at these same drug concentrations are generally modest and selective against certain tumor cell lines, including brain, breast, prostate, rhabdomyosarcoma cells. Recent clinical studies in patients with various solid malignancies showed that concentrations of CCI-779 in whole blood generally reach micromolar levels (1, 14–16). Although most of the in vitro studies that investigate CCI-779 and/or rapamycin action in cancer cells use nanomolar drug concentrations, they do not address the inhibitory response and underlying cellular mechanism(s) to the drug at the clinically relevant micromolar doses.

In this report, we have identified a distinct pharmacologic mode of action of the rapamycin and its analogues. We show that a broad and profound suppression of tumor cell growth can be achieved by the clinically relevant low micromolar concentrations of CCI-779, a phenomenon we term as the “high-dose effect.” This enhanced antitumor activity is mechanistically linked to a previously unreported FKBP12-independent suppression of mTOR kinase activity and mTOR signaling in cancer cells. We show that the micromolar but not the nanomolar concentrations of CCI-779...
uniquely affect the function of the cellular translation machinery, leading to a marked decline in global protein synthesis, and profound inhibition of cell growth. This high-dose drug effect could be highly relevant to the treatment of human cancer patients with CCI-779 and other rapamycin analogues.

Materials and Methods

Chemicals. All general chemicals used for buffers and assays were obtained from Sigma-Aldrich unless otherwise specified. mTOR inhibitors CCI-779, rapamycin, and the rapamycin derivative WAY-179669 were provided by Wyeth Chemical and Pharmaceutical Development. FK506 was obtained from EMD Calbiochem. All inhibitors were dissolved in DMSO as concentrated stocks and diluted before use in DMSO for in vitro enzyme or binding assays or diluted in culture media for cell-based assays.

Cell culture, growth assays, and transfections. Human lung tumor lines (A549, H157, H460, H446), colon tumor lines (HCT116, HT29, SW-480, DLD1, Caco2), prostate tumor lines (LNCap, DU145), breast lines (MADB08, MAD231), and the human embryonic kidney cell line HEK293 were obtained from American Type Culture Collection. PC3-MM2 were obtained from Dr. C. Discafani of Wyeth Oncology. Cells were cultured in a 37°C incubator with 5% CO2 using standard cell culture methods. All culture media, supplements, and transfection reagents were purchased from Invitrogen.

For growth assays, cells were plated in 96-well culture plates at 1 × 10^3 cells per well. After 24 h, cells were treated with various doses of inhibitors. Three days after drug treatment, viable cell densities were determined by MTS dye conversion using CellTiter AQ assay kit (Promega). To create an mTOR-SI cell line, the empty vector ptrcHis (Invitrogen). The FRB domain of eIF2, eEF2K siRNA pool and nontargeting siRNA pool were obtained from Dharmacon. eEF2K siRNA transfections were repeated 48 h later, and the cells were cultured in DMSO as concentrated stocks and diluted before use in DMSO for in vitro enzyme or binding assays or diluted in culture media for cell-based assays.

Production and binding assays of FKBP12 and FRB. The Flag-tagged wild-type human mTOR (Flag-mTOR) and Flag-mTOR-SI (S2481/2482, created by site-directed mutagenesis) DNA constructs were transiently transfected into HEK293 cells. Protein extraction and purification of Flag-mTOR and Flag-mTOR-SI were carried out 48 h later as described previously (19). In vitro kinase assays of purified Flag-mTOR and Flag-mTOR-SI without or with FKBP12 were done and detected by dissociation-enhanced lanthanide fluorescent immunoassay (DELFIA) as described (19).

Analysis of global translation. Global protein synthesis was measured by determining incorporation of [14C]methionine into TCA-insoluble cellular components (Supplementary Materials and Methods). Analysis of polysome profiles was done as described previously (20) with minor modifications (Supplementary Materials and Methods).

Results

CCI-779 at low micromolar concentrations broadly suppresses tumor cell proliferation via an FKBP12-independent mechanism. Our studies on tumor cell proliferation in culture, we noted that CCI-779 displayed a biphasic dose response in a panel of human tumor cell lines (Fig. 1A). The most sensitive cell lines that were inhibited by >50% included LNCap, PC3MM2, MAD468, H446, and Caco2, whereas those with <25% inhibition were SW480, HT29, HCT116, and H460. Interestingly, at higher dose range of 5 to 15 μmol/L, the drug manifested a more profound antiproliferative activity in nearly all cell lines (Fig. 1A). A similar biphasic inhibition pattern was observed for rapamycin (data not shown). These observations raised the possibility that at the low micromolar doses, CCI-779 may exert a previously uncharacterized inhibition mechanism. Because rapamycin in complex with FKBP12 inhibits mTOR with high affinity (~3 nmol/L; ref. 21), we first performed FK506 competition experiments to determine whether the second phase inhibition (called micromolar inhibition) similarly requires FKBP12. CCI-779 was titrated in LNCap and MAD468 cell proliferation assay, with or without 0.3, 3, and 10 μmol/L FK506, to completely inhibit the binding of CCI-779 to FKBP12 (Fig. 1B). In both cell lines, antiproliferative effect of CCI-779 at concentrations of ≤10 μmol/L was largely reversed by FK506, indicating that the interaction with FKBP12 was essential for low-dose inhibition of cell growth by CCI-779. However, the high-dose growth inhibitory effect of the drug was completely resistant to cotreatment with 10 μmol/L FK506 in both LNCap and MAD468 cells. These data indicate that the biphasic inhibition of cell proliferation elicited by CCI-779 reflects both FKBP12-dependent and FKBP12-independent actions of this drug at low versus high concentrations respectively.

The growth inhibitory action of rapamycin at nanomolar concentrations is well known to be largely cytostatic. To determine whether the micromolar CCI-779 also elicits cytostasis, we measured viability of several representative cell lines after 24 and 48 h of exposure to 20 nmol/L or 20 μmol/L CCI-779. Consistent with the previous finding, no cell death was detected in 20 nmol/L vector pGE2X2 (GE Healthcare). His6-FKBP12 and glutathione S-transferase–FRB were produced and purified according to the respective vendor's instructions. FKBP12 binding assay was done in the 96-well format nickel chelate flashplate with [3H]FK506 (NEW-PerkinElmer; Supplementary Materials and Methods). FRB binding by the rapamycin analogues was assessed in an FKBP12-rapamycin-FRB ternary complex formation assay in the 96-well MaxiSorp Fluoro plates (Nunc; Supplementary Materials and Methods).

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CCI-779–treated cells. In cells treated with 20 μmol/L CCI-779, cell death for HCT116, HT29, A549, and HEK293 was minimal at both time points. Interestingly, both PTEN-negative cell lines LNCap and MDA468 exhibited a small but significant reduction in viability as early as 24 h (Supplementary Table S1). To further examine whether these cells undergo apoptosis, we performed immunoblotting for PARP cleavage as well as caspase activity assays. CCI-779 (20 μmol/L) caused PARP cleavage in LNCap and MDA468 cells but not in HT29 and A549 cells (Supplementary Fig. S1A). The caspase-3/caspase-7 activity was also increased in LNCap (4.9-fold) and MDA468 (6.6-fold) cells (Supplementary Fig. S1B). Thus, micromolar CCI-779 seems to inhibit proliferation via a largely cytostatic mechanism in most of the cells tested, whereas it induces apoptosis in the PTEN-negative tumor cells.

Micromolar inhibition of proliferation correlates with FKBP12-independent inhibition of cellular mTOR signaling.

To define the mechanism responsible for high-dose growth inhibition by CCI-779, we initially examined the effect of CCI-779 on cell proliferation and mTOR signaling at nanomolar versus micromolar drug concentrations. These assays were done in the absence or presence of FK506, which competes with CCI-779 for binding to intracellular FKBP12. At low concentrations (e.g., 20 nmol/L), CCI-779 exhibited an FK506-reversible inhibition of growth (Fig. 2A), which correlated with the reversible dephosphorylation of mTOR substrates S6K1 (Thr389) and 4E-BP1 (Thr70; Fig. 2B). In contrast, the inhibitory effects of high-dose CCI-779 (10–20 μmol/L) on growth and mTOR substrate phosphorylation were not reversed by FK506, suggesting that high-dose CCI-779...
suppresses both mTOR signaling and cell growth through an FKBP12-independent mechanism. The mTOR complex 2 is insensitive to low concentrations of rapamycin and its analogues (22, 23). However, the effects of higher drug concentrations on this second mTOR complex are unclear. Consequently, we examined the phosphorylation state of the known mTORC2 substrate AKT and its downstream target GSK-3 in CCI-779–treated cells. At concentrations of 10 and 20 μmol/L, CCI-779 dramatically inhibited phosphorylation of Ser473 of AKT and Ser21/9 of GSK-3 with maximal suppression of both responses observed after 24 h of drug exposure (Fig. 2C). Partial inhibition of AKT and GSK-3 phosphorylation was also observed after 24 h of cellular treatment with a low concentration (20 nmol/L) of CCI-779, and this CCI-779 effect was fully reversed by cotreatment with FK506. The decreases in AKT and GSK-3 phosphorylation after long-term CCI-779 exposure were consistent with previous findings, and the blockade of the CCI-779 effects by FK506 indicates that the low-dose effect of CCI-779 was fully dependent on FKBP12. In contrast, FK506 cotreatment failed to reverse the inhibitory effects of 10 or 20 μmol/L CCI-779. In fact, concomitant FK506 exposure noticeably augmented the acute (6 h) effects of CCI-779 on AKT and GSK-3 phosphorylation. Although the actual mechanism underlying this enhancing effect is unknown, we speculate that FK506 may saturate irrelevant binding sites (including those exposed by FKBP family members), thereby increasing the pool of free intracellular CCI-779.

CCI-779/rapamycin micromolar action requires binding to the FRB domain of mTOR. Structural information regarding the interactions between rapamycin, FKBP12, and the FRB domain of mTOR has supported rational approaches to the generation of rapamycin derivatives with varying affinities for FKBP12 or FRB. We used select rapamycin analogues to further investigate the requirements for FKBP12 and/or mTOR binding in the dose-dependent effects of CCI-779 on cell proliferation and signaling functions. WAY-179639, a triene-perturbed derivative, retained potent FKBP12 binding (EC50, 8 nmol/L) but was inactive in an FKBP12-rapamycin-FRB ternary complex formation assay (EC50, >5,000 nmol/L; Fig. 3A). Interestingly, this compound was not growth inhibitory against LnCap, HEK293 (Fig. 3B), and other cells (data not shown) at the doses up to 30 μmol/L. The observed lack of activity in proliferation assays is presumably due to the lack of inhibition of cellular mTOR signaling as evidenced by the normal levels of S6K1 phosphorylation (Fig. 3C). To ascertain whether WAY-179639 actually penetrated cells, we

Figure 2. Micromolar CCI-779 inhibition of HEK293 cell proliferation correlates with FKBP12-independent inhibition of cellular mTOR signaling. A, HEK293 cells were assayed in growth inhibition by CCI-779 with 0, 5, and 10 μmol/L of FK506. The assays were done and analyzed as in Fig. 1. B, cells were plated in six-well plates in growth media overnight, and treated for 6 h with indicated doses of CCI-779 with or without 5 and 10 μmol/L of FK506. Total cellular lysates were prepared and subjected to immunoblotting. The blots were stained with Ponceau-S for total proteins and probed with antibodies for phosphorylated S6K1, total S6K1, phosphorylated 4E-BP1, cyclin D3, and p21/Cip1. Arrowheads, migration of phosphorylated versus dephosphorylated form of p70 S6K1. C, cells were similarly treated for 6 and 24 h with or without 10 μmol/L of FK506. Blots were probed with antibodies against phosphorylated AKT, total AKT, phosphorylated GSK-3, and total GSK-3.
used it as a competitor against CCI-779 in a growth inhibition assay. Cotreatment with WAY-179639 overcomes the growth inhibitory effect of CCI-779 at concentrations of \( \geq 10 \) \( \mu \text{mol/L} \), consistent with the ability of WAY-179639 to penetrate cells and to compete with CCI-779 for binding to FKBP12 (Fig. 3D). These results indicate that the high-dose inhibition of cell growth by CCI-779 correlates with its ability to bind the mTOR FRB domain.

Figure 3. A triene-perturbed rapamycin derivative deficient in FRB-binding fails to confer nanomolar and micromolar inhibitions of mTOR signaling and proliferation. A, left, chemical structure of rapamycin analogue WAY-179639. Right top, the FKBP12 binding assay was done using His6-FKBP12 and \(^{[3}H\)FK506 as described in Materials and Methods. FK506, rapamycin, and WAY-179639 were assayed at 0.3 to 5,000 \( \text{nmol/L} \) for competing with \(^{[3}H\)FK506 for binding. An \( EC_{50} \) value was determined as the dose required for 50% inhibition of bound radioactivity. Right bottom, FK506, rapamycin, and WAY-179639 were assayed at 0.1 to 5,000 \( \text{nmol/L} \) in an FKBP12-rapamycin-FRB ternary complex assay as described in Materials and Methods. Note that rapamycin, but not FK506 or WAY-179639, dose-dependently supported complex formation. B, WAY-179639 and CCI-779 were assayed for growth inhibition in LNCap and HEK293 as in Fig. 1. Note that WAY-179639 was inactive in both the nanomolar and micromolar inhibitions of growth. C, HEK293 cells were treated with the indicated doses of CCI-779 or WAY-179639 for 6 h and subjected to immunoblotting similarly as in Fig. 2. Arrowheads, migration of phosphorylated versus dephosphorylated form of p70 S6K1. D, CCI-779 was tested with 0, 1, 3, and 10 \( \mu \text{mol/L} \) WAY-179639 in the LNCap cell growth assay done as in Fig. 1. Note that WAY-179639 efficiently competed with CCI-779 for its nanomolar activity.
Direct inhibition of mTOR enzymatic activity by CCI-779 and rapamycin in the absence of FKBP12. To obtain biochemical evidence of inhibition of mTOR directly by CCI-779 and rapamycin, we performed in vitro kinase assays with purified, recombinant Flag-mTOR in the presence of various concentrations of CCI-779 without FKBP12. Assay products were first analyzed by immunoblot analysis with phosphorylation-specific antibodies. Phosphorylation of both mTOR substrates, His6-S6K1 (Thr389) and His6-4E-BP1 (Thr46S), in these assays was inhibited by CCI-779 at concentrations of ≥800 nmol/L (Fig. 4A). Direct inhibition of mTOR kinase activity was quantified with a DELFIA-based mTOR kinase assay using His6-S6K1 as the substrate (19). Pooled data from three experiments yielded mean IC50 values of 1.76 ± 0.15 and 1.74 ± 0.34 μmol/L for CCI-779 and rapamycin, respectively (Fig. 4B). Our results show that both CCI-779 and rapamycin directly inhibit mTOR catalytic activity in the absence of FKBP12. Furthermore, the IC50 values for rapamycin obtained in our DELFIA assay were well correlated with those reported in a recent study in which mTOR activity was measured in an immune complex kinase assay (24).

FRB mutant mTOR-SI confers resistance to nanomolar and micromolar CCI-779 inhibitions in vitro and in vivo. Rapamycin-resistant mTOR alleles can be created by substitution of a conserved residue Ser2035 in the FRB domain with a more bulky amino acid, such as isoleucine (21, 25). To assess whether CCI-779 shows a similar dependence on Ser2035 for binding to the FRB domain in the absence of FKBP12, we examined the sensitivity of an mTOR (Ser2035→Ile) mutant, termed mTOR-SI, to CCI-779 in the presence or absence of FKBP12. Wild-type Flag-mTOR was efficiently inhibited by the FKBP12-rapamycin complex (IC50, 11 ± 1 nmol/L); however, mTOR-SI was not inhibited by the immunophilin-drug complex at concentrations of up to 160 nmol/L (Fig. 5A, top). In the parallel assays without FKBP12, inhibition of mTOR-SI was only observed at drug concentrations considerably higher than those required for inhibition of wild-type mTOR. The mean IC50 value of 20 ± 3.2 μmol/L (Fig. 5A, bottom) for CCI-779 was ~11-fold higher than that measured with the wild-type mTOR enzyme (Fig. 4B). Thus, the Ser2035→Ile mutation in the FRB domain also confers substantial resistance to CCI-779 in the absence of FKBP12.

To determine whether these results could be extended to intact cells, we generated HEK293 cell–derived stable cell lines expressing either the control vector or mTOR-SI alleles. Phosphorylation of S6K1 at Thr389 and AKT at Ser473 and autophosphorylation of mTOR itself at Ser2481 was restored in mTOR-SI cells acutely (6 h) treated with 0.02 μmol/L CCI-779, consistent with previously published studies (Fig. 5B; refs. 26, 27). Importantly, compared with vector control, mTOR-SI cells were also protected against the inhibition of above markers by 10 μmol/L and, to a lesser extent, by 20 μmol/L CCI-779 (Fig. 5B). We further investigated whether such restoration of mTOR signaling is sufficient to rescue long-term (3 days) growth suppression induced by various concentrations of CCI-779. As expected, although the vector control cells showed 55% reduction in growth in response to 0.05 μmol/L CCI-779, growth of mTOR-SI cells was inhibited only minimally (Fig. 5C). Interestingly, although 15 and 20 μmol/L drug equally inhibited the growth of both vector and mTOR-SI cells, mTOR-SI cells remained significantly more resistant to 1 and 10 μmol/L CCI-779 (Fig. 5C). The failure of mTOR-SI cells to exhibit significant protection against higher doses of CCI-779 is likely due to the fact that the mTORC1 complexes in these cells contain both wild-type mTOR (rapamycin-sensitive) and mTOR-SI mutant (rapamycin-resistant) proteins. Collectively, our data support the hypothesis that the micromolar effects of CCI-779/rapamycin in the cells are specifically mediated through mTOR and require the binding of the drug to FRB.

Micromolar CCI-779 profoundly inhibits cellular translation. Treatment of sensitive cell types with low concentrations of rapamycin provokes the accumulation of cells in G1 phase of cell cycle, in part due to the translational suppression of a subset of proliferation-related mRNAs, including the c-Myc and cyclin D1 transcripts (28, 29). The considerably more profound inhibition of cellular proliferation induced by high-dose CCI-779 treatment prompted more detailed analysis of the effects of micromolar CCI-779 on global protein synthesis. We first measured serum-stimulated incorporation of radiolabeled methionine into newly synthesized proteins in LNCap, HCT116, and HEK293 cells after treatment with 20 nmol/L CCI-779 (Fig. 5D). The considerable more profound inhibition of protein synthesis was observed in any of the tested populations (Fig. 6A and Supplementary Fig. S2A). In contrast, treatment with 20 μmol/L CCI-779 strongly inhibited global translational activity to a level comparable with that observed with a benchmark protein synthesis inhibitor, cycloheximide. Such profound suppression of global protein synthesis by micromolar CCI-779 is unlikely due to a secondary response of the cells to the profound suppression of translational activity.
growth delay, as an unrelated control drug Taxol, a potent cell growth inhibitor, was not active in this assay. To extend these findings, we analyzed polyisome profiles of HEK293 and HCT116 cells treated with 0.02 or 20 μmol/L CCI-779 (Fig. 6B and Supplementary Fig. S2B). These studies revealed that 0.02 μmol/L CCI-779 caused a significant decrease in the ratio of polyisomes to monosomes, an effect consistent with previously reported for rapamycin (30, 31). However, exposure to 20 μmol/L CCI-779 caused much more profound reduction in polyisomes, accompanied by a strong increase in the 80S monosomal peak. Thus, the dramatic decrease in translational activity induced by 20 μmol/L CCI-779 is likely related to the disassembly of actively translating polyisomes into monosomes.

Modulation of phosphorylation of multiple translation factors. To shed light on the mechanism underlying the translational arrest provoked by high-dose CCI-779, we investigated phosphorylation states of several translation factors in HEK293 cells treated with 0.02, 10, or 20 μmol/L CCI-779, with or without 10 μmol/L FK506, to competitively inhibit the binding of CCI-779 to FKBP12. Consistent with the profound suppression of translational activity (Fig. 6A and B), we observed striking increase in inhibitory phosphorylations of eukaryotic translation initiation factor eIF2α on Ser51 and eukaryotic translation elongation factor eEF2 on Thr56 (Fig. 6C). Both of these alterations occurred within 1-hour exposure to 10 to 20 μmol/L CCI-779 and were not reversed by concomitant exposure to FK506 (Fig. 6C). Similar results were obtained in HCT116 colon carcinoma cells exposed to 10 to 20 μmol/L CCI-779 (Supplementary Fig. S2C, left). It is also noteworthy that micromolar CCI-779 induced accumulation of the transcription factor ATF-4 (Fig. 6C, left), which might be secondary to the phosphorylation of eIF2α.

The nucleotide exchange activity of eIF2 complex is negatively regulated by phosphorylation of eIF2α subunit on Ser51 in response to numerous growth inhibitory signals (32), although a role for mTOR in regulating eIF2α in mammalian cells has not been reported. The phosphorylation of key inhibitory residue Thr56 in eEF2 is attributed to the activation of eEF2K, which itself is regulated by an inhibitory phosphorylation event on Ser366. The mTORC1-stimulated protein kinase S6K1 has been identified as one of the upstream effectors of eEF2K phosphorylation at Ser366 (33). Nevertheless, the eEF2 phosphorylation elicited by micromolar CCI-779 seemed to involve an S6K1-independent mechanism because it did not correlate with S6K1 activity or Ser366 phosphorylation of eEF2K (Fig. 6C, right). To further explore the role of eEF2K in eEF2 phosphorylation, we depleted this protein from HCT116 cells using specific siRNAs. Knockdown of eEF2K completely abolished the eEF2 (Thr56) phosphorylation induced by high-dose CCI-779 (Supplementary Fig. S2C, right), indicating that eEF2K was fully responsible for the dramatic increase in eEF2 (Thr56) phosphorylation seen in these cells. A similar result was observed in U87MG glioma cells (data not shown). The exact mechanism whereby high-dose CCI-779 activates eEF2K remains to be elucidated; regardless, the combined effects of this drug on eIF2α and eEF2 phosphorylation suggest that high-dose CCI-779 interferes with cellular protein synthesis by blocking both the initiation and elongation steps of translation.

Discussion

CCI-779 blocks growth of different types of tumors, in part, by inducing G1 arrest and inhibiting proliferation of tumor cells.
Although some malignancies respond well to CCI-779 treatment, others exhibit intrinsic or acquired resistance to the drug both in preclinical or clinical settings. In this report, we showed for the first time that increasing concentrations of CCI-779 to 10 to 20 μmol/L not only led to a sharp decrease in proliferation of sensitive cells, but also overcame the resistance of many tumor cell lines to the drug, such as colon cancer cells HCT116 or HT29. Similar doses of rapamycin were used in several reports; however, this phenomenon has been neither noted nor studied previously (34–36). We showed that the profound antiproliferative effect of micromolar CCI-779 is largely attributed to its cytostatic action on most of the tested cells; however, it also elicits apoptotic response in some PTEN-negative tumor cells.

Whereas the mechanisms of intrinsic resistance of tumor cells to the nanomolar CCI-779 and rapamycin analogues in general are not fully defined, deregulation of several signaling pathways is

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**Figure 6.** Micromolar CCI-779 elicits global repression of cellular translation. A, [3H]methionine incorporation. Serum-starved LNCap and HCT116 cells were treated with DMSO (Veh), 20 μmol/L cycloheximide (CHX), 20 nmol/L CCI-779 (CCI-L), 20 μmol/L CCI-779 (CCI-H), 100 nmol/L Taxol in methionine-free medium for 1 h. [3H]Methionine labeling and protein synthesis were done and analyzed as described in Materials and Methods. Mean values for micromolar CCI-779 were equal to 38 ± 7% of control in LNCap and 37 ± 13% of control in HCT116 cells. The presented graphs are based on mean values of three experiments. B, polysome profiles of HEK293 cells treated for 6 h with DMSO, 20 nmol/L and 20 μmol/L CCI-779. The absorbance profiles at 254 nm (A_{254}) recorded during gradient fractionation of the samples. The direction of sedimentation is from left to right. Polysomes-to-monomeres ratio was calculated as described in Supplementary Materials and Methods. The profiles shown are representative of the results from three independent experiments. C, HEK293 cells were treated for 1 h with the indicated doses of CCI-779 without or with 10 μmol/L of FK506, processed for immunoblotting similarly as in Fig. 2. Blots were probed with antibodies against phosphorylated S6K1, phosphorylated 4E-BP1, phosphorylated eIF2α, total eIF2α, ATF-4 (left) and against phosphorylated eEF2, total eEF2, and phosphotyliated eEF2K (right). The blot was stained with Ponceau-S for total proteins.
known to contribute to the sensitivity of cancer cells to rapamycin and its analogues in vitro and in vivo. For example, breast, prostate, or renal cancer cell lines that display a loss of PTEN or VHL tumor suppressors or an activation of AKT signaling are more sensitive to CCI-779 inhibition (37–39). Response of tumor cells to the commonly used nanomolar concentrations of rapamycin may be limited by the feedback loop, whereas suppression of S6K1 signaling by rapamycin stimulates IRS-PI3K pathway to promote AKT activity. Herein, we found that micromolar CCI-779 dramatically reduced mTORC1 and mTORC2 signaling, as evidenced by the decrease in the phosphorylation of Thr389 of S6K1, Thr70 of 4E-BP1, as well as phosphorylation of Ser472 of AKT and Ser9/21 of GSK-3, which may in part contribute to the uniform inhibition of proliferation in our cell line panel by micromolar CCI-779. It is also interesting to speculate that micromolar CCI-779 may disrupt the function of both mTORC1 and mTORC2 complexes in cancer cells in a more efficient manner than nanomolar compound. Further studies will be needed to address this hypothesis.

We found that the profound inhibition of mTOR signaling and cell proliferation elicited by micromolar CCI-779 did not require FKBP12, suggesting that FKBP12 independent binding of CCI-779 to the FRB domain of mTOR is required for the observed phenotypes. This view is further supported by the observation that an FRB-binding defective rapamycin analogue failed to display any growth inhibitory properties, even at micromolar concentrations. These results implicate the rapamycin triene interaction with FRB as necessary for both the nanomolar and micromolar dose activity of the drug. Additional findings helped to explain the mechanism of micromolar CCI-779 action at the biochemical level. First, CCI-779 was able to inhibit mTOR kinase activity at micromolar doses directly in the absence of FKBP12, with an IC50 value of 1.76 μmol/L. Second, the kinase activity of the FRB mutant mTOR-SI was 11-fold more resistant to the micromolar CCI-779 compared with the wild-type mTOR. mTOR-SI also conferred a partial protection to mTOR signaling and cell growth in response to micromolar CCI-779. The lack of significant resistance of mTOR-SI cells to the growth inhibitory effect of the drug at the highest concentrations (≥15 μmol/L) can be explained by the fact that (a) endogenously expressed wild-type mTOR allele renders at least a subset of mTORC1 complexes sensitive to CCI-779, (b) mTOR-SI allele seems to be hypomorphic with respect to reconstitution of mTORC1 signaling functions (25), and (c) the mTOR-SI mutant enzyme is partially inhibited by 10 to 20 μmol/L CCI-779 in vitro (Fig. 5A). Together, our current findings are in accord with recent reports that rapamycin is able to bind FRB domain of mTOR directly, without FKBP12, and our IC50 value for CCI-779 agrees well with Kd value reported in these studies (12, 24, 40). These reports, however, do not address biological effects of direct interactions between rapamycin and FRB on mTOR signaling in the cells.

mTOR kinase coordinates protein synthesis and abundance of the various components of the translational machinery with the continuous changes in nutrient, mitogen, or energy status of the cell. Despite numerous studies implicating mTOR in control of general translational factors, the effects of nanomolar rapamycin on global protein synthesis rates are subtle and cell selective. A marked reduction in general protein synthesis in response to micromolar CCI-779, as indicated by both methionine incorporation and polysome profiles data, suggests a potential mechanism for the suppression of cell proliferation at elevated concentrations of the drug. In our studies, micromolar CCI-779 dramatically increased the phosphorylation of the translation initiation factor eIF2α on Ser51 and elongation factor eEF2 on Thr56 in an FKBP12-independent manner. It is likely that modulation of the above factors, as well as reduction in phosphorylation of S6K1 and 4E-BP1, may collectively contribute to the observed decrease in global protein synthesis. Phosphorylation of eIF2α on Ser51 by four kinases (PERK, GCN2, PKR, HRI) inhibits eIF2α-GTP-Met-tRNA ternary complex formation and results in attenuation of global protein synthesis under diverse stress conditions (32). The kinase(s) that phosphorylates eIF2α following the exposure to micromolar CCI-779 remains to be determined. It is well established that phosphorylation of Thr56 of eEF2 by micromolar CCI-779 is not mediated by dephosphorylation of eEF2α at Ser51 or by eEF2 hyperphosphorylation. Whereas AMPK pathway also impinges on eEF2 in certain cellular contexts, preincubation of cancer cells with compound C, a direct and specific inhibitor of AMPK, did not eliminate the effect of micromolar CCI-779 on eEF2 phosphorylation (data not shown).

Finally, the silencing of eEF2K using siRNA prevented the effects of micromolar CCI-779 on eEF2 phosphorylation, thus substantiating the role of eEF2K in regulating eEF2 activity under above conditions. Alternative mode of regulation independent on eEF2K cannot be ruled out where the exposure to micromolar dose of CCI-779 may suppress the activity of PP2A phosphatase, thereby delaying the rate of Thr56 dephosphorylation. Similar mechanism was recently proposed for the ethanol-induced eEF2 phosphorylation in C2C12 myocytes (44).

Whereas it remains an open question whether this newly described mechanism of CCI-779 action contributes to antitumor efficacy in the clinic, a number of recent clinical studies suggest that micromolar drug levels are present in blood of CCI-779-treated patients with breast cancer (1), advanced renal cancer (15), and malignant glioma (16). Therefore, the high-dose drug concentrations that are used in our work are well within the clinically relevant drug concentrations. Interestingly, in a randomized phase II renal cancer trial, patients who received the highest weekly dose of CCI-779 of 250 mg (whole blood exposure of 16 μg/mL) had a higher median survival and higher probability of survival than those in the lower dosage groups (15). Therefore, we speculate that the newly described pharmacologic effects exerted by micromolar CCI-779/rapamycin action in cancer cells contribute to its therapeutic efficacy in vivo.
References


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