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Rapamycin-Sensitive Signal-Transduction Pathways: Protein Translation Control of Cell Proliferation

By Janet E. Dancey, MD

Abstract: Rapamycin is a natural product with antimicrobial, immunosuppressant, and antitumor activities owing to its modulation of signal-transduction pathways linking mitogenic stimuli to the synthesis of specific proteins needed for cell-cycle progression from the G1 to the S phase. Rapamycin and its soluble analog CCI-779 have in vitro and in vivo antiproliferative activity against a broad range of human tumor-cell lines, and CCI-779 is just entering clinical trials to determine its value in cancer therapy. The pharmacologic action of rapamycin is mediated through its binding to the intracellular protein FK506 binding protein 12 and subsequent inhibition of the protein kinase mammalian target of rapamycin (mTOR). mTOR signals to two separate pathways, each of which controls the translation of specific mRNAs. One rapamycin-sensitive pathway affects the activity of the 40S ribosomal protein S6 kinase $p70^{s6k}$, and the other affects the function of eukaryotic initiation factor 4E-binding protein-1 (4E-BP1), also known as phosphorylated heat- and acid-stable protein. The observations by several groups that the inhibition of mTOR- mediated $p70^{s6k}$ and 4E-BP1 phosphorylation by rapamycin were coupled to growth arrest in the G1 phase led to the hypothesis that the antiproliferative properties of rapamycin result from its effects on the regulation of protein translation affected by these targets. However, the precise mechanisms of cell-cycle arrest are as yet unknown. This review will focus on recent advances in the understanding of the mechanisms by which rapamycin inhibits cell growth and the issues surrounding the development of this type of agent as a potential treatment for cancer.

CELL PROLIFERATION requires the synthesis of proteins necessary for entry into and transit through the cell cycle.[1] Thus, regulatory mechanisms to increase translation of these proteins are necessary to promoting cell proliferation. As an anticancer treatment strategy, targeting translational regulation has been a relatively unexploited area of therapeutic development. However, the identification of the antiproliferative effects of the agent rapamycin (sirolimus) and the identification of its novel mechanism of action have renewed interest in targeting the translational regulatory apparatus as a therapeutic strategy.

Rapamycin, a natural product, has antimicrobial, immunosuppressant, and antitumor activities that result from the modulation of signal transduction pathways that link mitogenic stimuli to the synthesis of specific proteins needed for cell-cycle progression from the G1 to the S phase. Rapamycin is in late phase III clinical trials as an immunosuppressive drug for organ and bone marrow transplant recipients, and its ester analog, CCI-779, is being evaluated in early clinical trials as a therapeutic agent against cancer. The immunosuppressant effects of rapamycin result from its inhibition of the biochemical events required for the progression of interleukin-2–stimulated T cells from the G1 to the S phase of the cell cycle.[2] However, the growth-inhibitory actions of rapamycin and its analog are not restricted to lymphoid cells, as these agents also have cytostatic or cytotoxic activities against solid tumor cell lines. In addition to its possible clinical utility, rapamycin is a useful pharmacologic probe for studies of the signal-transduction pathways that govern translation. This review will focus on recent advances in the understanding of the mechanisms of cell-growth inhibition by rapamycin and the issues surrounding the development of this class of agent as a potential mode of therapy in the treatment of cancer.

THE DISCOVERY OF RAPAMYCIN AND ITS ANTIPROLIFERATIVE ACTIVITY

Rapamycin, a macrolide, was first identified as a fungicide produced by the bacteria *Streptomyces hygroscopicus*, which had been isolated from soil samples from Easter Island.[3,4] Although it was

Address correspondence to Janet Dancey, MD, Investigational Drug Branch, Cancer Treatment Evaluation Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute, EPN 715, 6130 Executive Blvd, Rockville, MD 20854; email danceyj@ctep.nci. nih.gov.

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originally identified as an antifungal agent, subsequent studies demonstrated impressive antitumor and immunosuppressant activities. Rapamycin was originally evaluated by the National Cancer Institute (NCI) in the late 1970s. At that time, it was found to have antiproliferative activity in a variety of murine tumor systems, including B16 melanoma and P388 leukemia models.[5,6] Rapamycin has since been shown to inhibit the growth of B-cell lymphoma cell lines,[7] and small-cell lung cancer cell lines.[8] In addition to its growth-inhibiting effects, rapamycin induced *p53* independent apoptosis of childhood rhabdomyosarcoma cell lines.[9] Treatment with rapamycin inhibited cyclin D1 expression and proliferation of MiaPaCa-2 and Panc-1 human pancreatic cancer cell lines.[10] Rapamycin also augmented cisplatin-induced apoptosis in murine T-cell lines, the human promyelocytic cell line HL-60, and the human ovarian cancer cell line SKOV3.[11] These data suggest that the antiproliferative action of rapamycin may be an important component of the pathway that prevents cell death and that rapamycin may enhance the efficacy of selected cytotoxic agents.

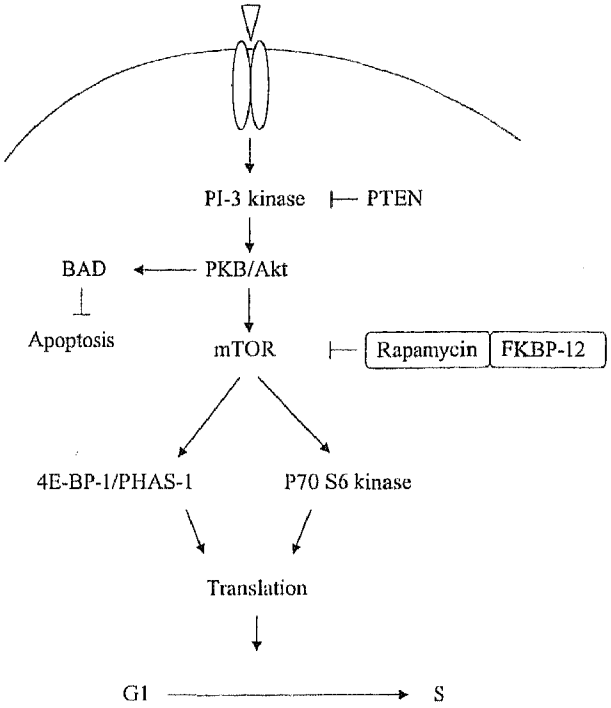

Fig 1. Rapamycin-sensitive signaling pathways. The target of rapamycin kinase (TOR) functions to regulate the activities of the translational regulators 4E-BP1/PHAS-I and p70 S6 kinase. Rapamycin binds to FKBP12 and the complex inhibits mTOR.

THE TARGET OF RAPAMYCIN

Our understanding of molecular mechanisms underlying the biologic effects of rapamycin has advanced considerably in recent years (Fig 1). Similar to other natural immunosuppressants such as cyclosporine and FK506, rapamycin binds to members of the ubiquitous immunophilin family of FK506 binding proteins (FKBPs), inhibiting their enzymatic activity as prolyl isomerases.[12,13] Although this enzymatic function is important for altering protein conformation, it is, surprisingly, not relevant to the action of rapamycin.[14] Although there are many members of the FKBP family, biochemical and genetic studies suggest that FKBP12 is the most relevant binding protein for the pharmacologic activity of rapamycin in eukaryotic cells. Yeast mutants lacking FKBP12 are viable and resistant to rapamycin toxicity, indicating that both the protein and the drug are required for rapamycin action.[14] Overexpression of FKBP12 in mammalian cells increases their sensitivity to rapamycin, and cell lines with reduced levels of FKBP12 are rapamycin-resistant, providing further evidence for a model in which the cellular effects of rapamycin result from its binding to FKBP12.[15] Thus, rapamycin may actually be considered a "prodrug" for the active agent at the cellular level, the FKBP12-rapamycin complex.

The target of the rapamycin-immunophilin complex was initially identified in yeast and confirmed in mammalian cells. In mammalian cells, the complex interacts with a large polypeptide kinase of 290 kd, termed mammalian target of rapamycin (mTOR)[16] (also known as FRAP,[17] RAFT1,[18] and RAPT1[19]), blocking its activity. The yeast TOR proteins exhibit a high degree of overall sequence identity (> 40%) to mTOR, with even greater identity (> 65%) observed in their carboxy-terminal catalytic domains (reviewed in[20]). The high level of sequence conservation, together with the strikingly similar effects of rapamycin on yeast and mammalian cell growth, suggests that TOR proteins are important to cell function because they have been highly conserved during eukaryotic evolution.

Yeast and mammalian TOR proteins are members of a recently described family of protein

kinases called phosphoinositide 3 kinase (PI3K)–related kinases (PIKKs). Members of this family are involved in a range of essential cellular functions, including cell-cycle progression, cell-cycle checkpoints, DNA repair, and DNA recombination.[21,22] Among these PIKK family members are the cell-cycle regulatory protein kinases ataxia telangiectasia mediated, ataxia telangiectasia related, and DNA-dependent protein kinase catalytic subunit. In contrast to the TOR proteins, ataxia telangiectasia mediated, ataxia telangiectasia related, and DNA-dependent protein kinases participate in cell-cycle checkpoints that govern cellular responses to DNA damage.[21] The PIKK family members share a carboxy-terminal catalytic domain that bears significant sequence homology to the lipid kinase domains of PI3Ks, although no intrinsic lipid kinase activity has been described for mTOR.

REGULATION OF PROTEIN SYNTHESIS BY mTOR

Stimulation of quiescent cells with growth factors leads to a dramatic increase in the translation of a subset of mRNAs whose protein products are required for progression through the G1 phase of the cell cycle.[23] mTOR regulates key pathways affecting the efficiency of protein translation. The upstream signaling pathway that couples growth-factor–receptor occupancy to mTOR protein activation is only partially understood. mTOR is a phosphoprotein, and its phosphorylation state, as well as its catalytic activity, have been reported to be modulated by the mitogen-activated phosphatidylinosol (PI) 3 kinase-Protein Kinase B (PKB)/Akt.[24,25] PI3 kinase and Akt are considered to be proto-oncogenes, and the pathway is inhibited by the tumor suppressor gene *PTEN*.[26] Although other signaling pathways are activated downstream of PI3K, the Akt pathway is of particular interest because of its role in inhibiting apoptotic pathways and promoting cell proliferation (reviewed in[26,27]). In mammalian cells, activated mTOR signals to two separate pathways that control translation of specific subsets of mRNAs. These are the 40S ribosomal protein S6 kinase, $p70^{s6k}$,[8] and the eukaryotic initiation factor (eIF)-4E-binding protein-1 (4E-BP1), also known as PHAS-I (phosphorylated heat- and acid-stable protein).[28,29] Among subset mRNAs regulated by these pathways are those encoding components of the protein synthesis machinery itself.

Several recent reports indicated that activation of either PI3K or Akt is sufficient to induce the phosphorylation of both $p70^{s6k}$ and 4E-BP1/PHAS-1 through mTOR.[30,31] Treatment of activated PI3K or Akt expressing cells with rapamycin blocks the $p70^{s6k}$ and 4E-BP1/PHAS-1 phosphorylation, indicating that mTOR is required for these responses.[29,32] In addition, there is evidence that Akt phosphorylates the carboxyl terminus of mTOR, contributing to its activation.[24,25] These results clearly link the PI3K-Akt pathway to $p70^{s6k}$ and 4E-BP1/PHAS-1 translational control pathways through mTOR.

Compared with its upstream effectors, the downstream actions of mTOR on translation are more fully characterized. For the subset of mRNAs that contain regulatory elements located in the 5′-untranslated regions, the binding of the mRNA to the ribosomal subunit and the efficient initiation of translation is mediated by the multisubunit eukaryotic initiation factor-4 (eIF-4) complex.[33] 4E-BP1/PHAS-I is a low-molecular-weight protein that inhibits the initiation of translation through its association with eIF-4E, the mRNA cap-binding subunit of the eIF-4F complex.[23] Binding of 4E-BPs to eIF-4E is dependent on the phosphorylation status of 4E-BP. In quiescent cells, 4E-BP1/PHAS-1 is relatively underphosphorylated and binds tightly to eIF-4E (reviewed in[34]). Stimulation of cells by hormones, mitogens, growth factors, cytokines, and G-protein–coupled agonists results in 4E-BP1/PHAS-1 phosphorylation through the action of mTOR and, possibly, other kinases, which promotes the dissociation of the 4E-BP1/PHAS-I–eIF-4E complex (Fig 2). eIF-4E can then bind to the eIF-4F complex, and this interaction will then lead to an increase in translation rates. Conversely, growth-factor deprivation or treatment with rapamycin results in 4E-BP1/PHAS-1 dephosphorylation, an increase in eIF-4E binding, and a concomitant decrease in translation.[34]

The second downstream target of mTOR is $p70^{s6k}$, the kinase that phosphorylates the 40S ribosomal protein S6. In response to mitogenic stimuli, $p70^{s6k}$ phosphorylates S6 on multiple sites, and these modifications favor the recruitment of the 40S subunit into actively translating polysomes[35] and enhance the translation of mR-

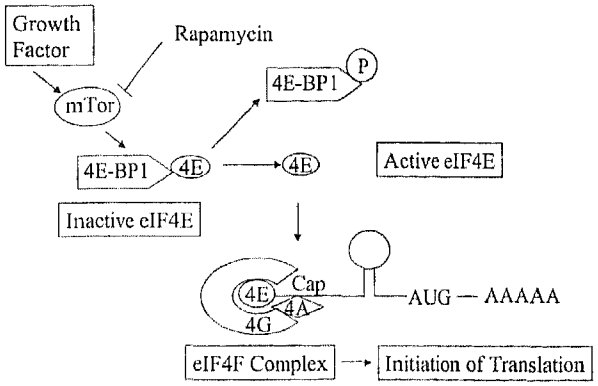

Fig 2. Activation of mTOR leads to the phosphorylation of 4E-BP1/PHAS-1. eIF-4E is released and then binds to the eIF-4G scaffolding protein of the eIF-4F complex at the 5′ cap site of the mRNA template. The eIF-4F complex contains a 7-methylguanosine cab-binding subunit called eIF-4E, an RNA helicase called eIF-4A, and a multifunctional scaffolding protein eIF-4G that bridges the 40S ribosome and the mRNA, while also binding eIF-4E, eIF-4A, eIF-3, and the poly(A)-binding protein.

NAs bearing 5′ terminal oligopolypyrimidine tracts. Although these transcripts represent only 100 to 200 genes, they can encode up to 20% of the cell's mRNA.[36]

Rapamycin treatment triggers the rapid dephosphorylation and inactivation of $p70^{s6k}$ in mitogen-stimulated cells. Although $p70^{s6k}$ activation involves a complex series of phosphorylation events catalyzed by multiple protein kinases, the prompt inhibitory effect of rapamycin suggests that persistent stimulatory input from mTOR leads to the activated state of $p70^{s6k}$.[36] The exact nature of the input supplied by mTOR is unclear; however, a recent study suggested that the mTOR phosphorylates and suppresses the activity of a type 2A protein phosphatase bound directly to $p70^{s6k}$.[37]

The observations by several groups that the inhibition of mTOR-mediated $p70^{s6k}$ and 4E-BP1 phosphorylation by rapamycin were coupled to growth arrest led to the hypothesis that the antiproliferative properties of rapamycin are a result of its effects on translational control.[38-40] Inhibition of these key signaling pathways results in the inefficient translation of the mRNAs of proteins such as cyclin D1[41] and ornithine decarboxylase,[42] which are important for cell-cycle progression through the G1 phase. However, in addition to its actions on $p70^{s6k}$ and 4E-BP1/PHAS-1, rapamycin prevents cyclin-dependent kinase activation and retinoblastoma protein (pRb) phosphorylation.[43-46] Rapamycin also seems to accelerate the turnover of cyclin D1, both at the mRNA and protein levels, resulting in a deficiency of active cdk4/cyclin D1 complexes required for pRB phosphorylation and the release of E2F transcription factor and increased association of $p27^{kip1}$ with cyclin E/cdk2. These two events, along with the inhibition of translation of other mRNAs, can certainly explain the observed inhibition at the G1/S phase transition.[40,47] However, cells derived from mice in which the *p27* gene has been disrupted by homologous recombination are only partially rapamycin-resistant, indicating that rapamycin can inhibit cell-cycle progression by *p27*-independent mechanisms.[48] Whether the effects of rapamycin on cyclin cdks and cdk inhibitor *p27* are mediated through its inhibition of translation remains to be defined with precision in different cell types. Thus, although the direct target of rapamycin has been identified, the downstream pathway from the target to the inhibition of cell-cycle progression requires further study.

CLINICAL DEVELOPMENT

Unfortunately, poor aqueous solubility and instability compromised the development of rapamycin as an anticancer agent. However, the NCI, in collaboration with Wyeth-Ayerst, examined several derivatives of rapamycin and selected one agent, CCI-779, for further development, on the basis on its mechanism of action and favorable in vitro and in vivo efficacy and toxicity data.

CCI-779 is a soluble ester analog of rapamycin with impressive in vitro and in vivo cytostatic activity. Results from the NCI human tumor cell line screen showed that CCI-779 and its parent compound, rapamycin, share a mechanism of action that is distinct from those of other cancer therapeutic agents. The two agents are similar: the Pearson correlation coefficient of the in vitro antiproliferative activities and potencies of the two agents across the 60-cell line screen is .86. In vitro, human prostate and breast cancer lines as well as CNS, melanoma, small-cell lung carcinoma, and T-cell leukemia human tumor lines were among the most sensitive to CCI-779 with a 50% inhibitory concentration of less than 10^{-8} M.[49] Platelet-derived growth factor stimulation of the human glioblastoma line T98G was markedly

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