Inhibitors of mammalian target of rapamycin as novel antitumor agents: From bench to clinic

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Rapamycin and its derivatives, CCI-779 and RAD-001, inhibit the mammalian target of rapamycin (mTOR), downregulating translation of specific mRNAs required for cell cycle progression from G1 to S phase. Preclinically, mTOR inhibitors potently suppress growth and proliferation of numerous tumor cell lines in culture or when grown in mice as xenografts. CCI-779 and RAD-001 are being developed as antitumor drugs and are undergoing clinical trials. Clinically, CCI-779 has shown evidence of antitumor activity but induced relatively mild side effects in patients. Here we discuss potential antitumor mechanisms and resistance mechanisms of mTOR inhibitors, and summarize the current status of these compounds as novel antitumor agents.

Keywords Antitumor, cell cycle, mammalian target of rapamycin (mTOR), rapamycin

Introduction

Malignant disease is characterized by genetic mutations or compensatory changes in cells that result in unregulated population growth due to increased proliferation or decreased cell death. Since the early 1950s, extensive chemical synthesis and screening programs have resulted in clinical trials of many potential anticancer agents. In some cases, cytotoxic agents have significantly increased survival rates in adult diseases and notably for children with hematologic as well as solid tumors. However, relatively few cytotoxic agents have proven to be useful against a wide spectrum of cancers and most cause significant toxicity. The reasons for the relatively poor activity of cytotoxic agents are numerous. Most do not target the transforming event, rather they induce forms of damage that lead to necrosis or activation of cell-suicide, apoptosis. As a consequence, agents are cytotoxic to both malignant tumor cells and normal healthy cells, often causing severe side effects. Understanding pathways known to be critical to the growth and survival of tumors is essential to developing potentially selective treatments. Validation of this concept has been met with compounds such as imatinib (Gleevec, STI-571; Novartis AG). This compound inhibits tyrosine kinases such as BCR/ABL in chronic myeloid leukemia and c-KIT in gastrointestinal stromal tumors. Less success has been met by targeting activated Ras with inhibitors of farnesyltransferase.

Rapamycin (sirolimus, Rapamune; Wyeth-Ayerst Laboratories; Figure 1), an immunosuppressant, has emerged as a potent inhibitor of a signaling pathway that may be deregulated in some forms of cancer leading to both increased growth and malignant characteristics of cells. It is a lipophilic macrolide, that selectively inhibits a serine/theronine kinase, specifically, the mammalian target of rapamycin (mTOR). mTOR lies downstream of phosphatidylinositol 3-kinase (PI3K) in the PI3K signaling pathway. Rapamycin was originally isolated as a fungicide from the soil bacteria *Streptomyces hygroscopicus*, collected from Easter Island (known as Rapa Nui to the natives) in the South Pacific in 1975 [1,2]. Structurally similar to the immunosuppresive reagent FK-506 (tacrolimus; Fujisawa Pharmaceutical Co Ltd), rapamycin was initially developed for transplant rejection [3,4] and was approved by the US Food and Drug Administration in September 1999 and the European Commission in March 2000. While rapamycin was being developed as an immunosuppressant, it was also found to exert potent antitumor activity *in vitro* and *in vivo* [5-7]. However, perhaps because its mechanism of action was unknown at that time, rapamycin was not developed as a cancer therapeutic.

The potential for rapamycin as a cancer therapeutic was refocused, in part, by studies of Dilling *et al* [8]. These were the first studies to demonstrate potent and selective inhibition of growth by rapamycin. Rapamycin potently inhibited the growth of rhabdomyosarcoma cells at concentrations of approximately 1 ng/ml, whereas human colon cancer cells were inhibited only at micromolar concentrations in culture (Table 1). Proliferation of many rhabdomyosarcoma cells is regulated by an autocrine loop involving secretion of type II insulin-like growth factor (IGF-II) and signaling through the type I IGF receptor [9,10]. Indeed, the cell lines most sensitive to rapamycin were dependent on this autocrine pathway.

Additional findings from various research groups around the world support rapamycin as a good candidate for a cancer therapeutic agent. In many malignant cells in culture, rapamycin can act as a cytostatic agent by arresting cells in G1 phase. Another potential mode of its antitumor action is via the induction of apoptosis. Rapamycin potently inhibits proliferation or growth of cells derived from rhabdomyosarcoma, neuroblastoma, glioblastoma, medulloblastoma, small cell lung cancer [8,11-16], osteoscarcoma [17], pancreatic carcinoma [18,19], breast and prostate carcinoma [20-22], murine melanoma and leukemia, and B-cell lymphoma [6,23-25] (Table 1).

Despite the antiproliferative effects of rapamycin, it has poor water-solubility and stability in solution, precluding its formulation for parenteral use as an anticancer agent. Two rapamycin ester analogs, CCI-779 (Wyeth-Ayerst Research; Figure 1) and RAD-001 (everolimus; Novartis AG; Figure 1), with improved pharmaceutical properties, but similar cellular effects to rapamycin [16,20-22,26,27,28•,29•], are currently undergoing antitumor phase III and phase I clinical trials, respectively. This review will discuss potential antitumor and resistance mechanisms of rapamycin and its derivatives, and summarize the preliminary data about these compounds, from the bench to the clinic.

Table 1. Sensitivity of different tumor cell lines to rapamycin.

Cell Line	Rapamycin (IC_{50})	Reference
Rhabdomyosarcoma cells	**(ng/ml)**	[8]
Rh1 (10% serum)	4680	
Rh1 (serum free)	3.6	
Rh18	0.1	
Rh28	8.0	
Rh30	0.37	
Colon carcinoma cells	**(ng/ml)**	[8]
GC_3/c1	9800	
VRC_8/c1	1280	
CaCo	1570	
HCT8	8400	
HCT29	> 10,000	
HCT116	> 10,000	
Small cell lung cancer cells	**(nM)**	[11,12]
H69	~ 1	
H345	~ 1	
H510	~ 1	
Neuroblastoma cells	**(ng/ml)**	[13]
NB-SD	~ 1	
NS-1643	~ 1	
NB-EB	2.9	
NB-1691	18	
NB-1382.2	639	
Glioblastoma cells	**(ng/ml)**	[13]
SJ-G2	0.5	
SJ-G3	> 10,000	
Medullablastoma cells	**(ng/ml)**	[16]
DAOY	~ 1	
Osteoblasts-like osteosarcoma cells	(nM)	[17]
ROS 17/2.8	< 100	
Pancreatic cancer cells	**(ng/ml)**	[18,19]
Panc-1	1	
MiaPaCa-2	3	
Leukemia cells	**(nM)**	[23]
RBL-2H3	~ 10	
B-cell lymphoma cells	**(ng/ml)**	[25]
BKS-2	0.31	
L1.2	0.27	
NFS1.1	0.31	
WEHI-279	0.60	
Thymoma cells	**(ng/ml)**	[25]
EL4	0.17	

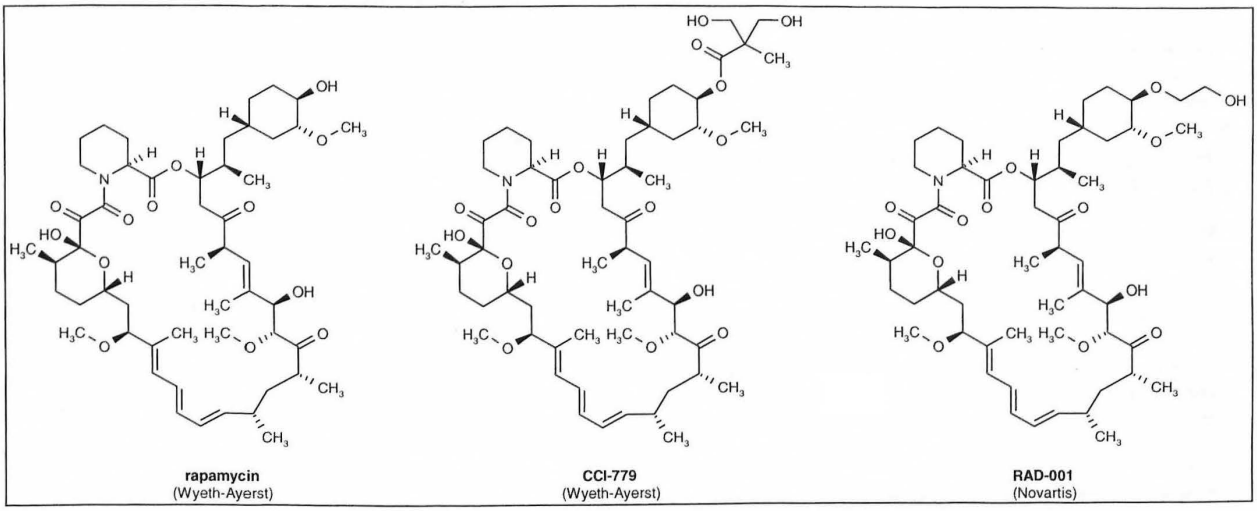
Antitumor mechanism of inhibitors of mTOR

Rapamycin and its analogs, CCI-779 and RAD-001, are the most potent and selective inhibitors of mTOR reported so far. The three agents share a common mechanism of antitumor action, ie, inhibiting mTOR, which, links mitogen stimulation to protein synthesis and cell cycle progression.

mTOR

To better understand the antitumor mechanism of rapamycin and its derivatives, we will briefly review the emerging cellular role of mTOR. mTOR is referred to by various other names, some of which are derived from its binding partner FK-506-binding protein, FKBP12 (discussed below). These names are FRAP (FKBP12 and rapamycin-associated protein), RAFT1 (rapamycin and FKBP12 target 1), RAPT1 (rapamycin target 1) and SEP (sirolimus effector protein). In the mid-1990s, mTOR was identified as a mammalian serine/threonine kinase of approximately 289 kDa in humans, mice and rats [30-33]. TOR proteins represent a class of evolutionarily conserved kinases in eukaryotes. In the yeasts, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, two TOR genes, *TOR1* and *TOR2*, have been cloned, which share 67% identity and encode proteins of approximately 280 kDa [34-36]. In the fruit fly, *Drosophila melanogaster*, a single *TOR* ortholog, termed *dTOR*, has been characterized, sharing 38% identity with *TOR2* from *Saccharomyces cerevisiae* [37,38]. *mTOR* shares approximately 45% identity with *TOR1* and *TOR2* from the yeast *Saccharomyces cerevisiae*, and 56% identity with *dTOR* in overall sequence [39,40]. Human, mouse and rat mTOR proteins share 95% identity at the amino acid level [40,41]. mTOR contains a catalytic kinase domain and a FKBP12-rapamycin binding (FRB) domain near the C-terminus, and up to 20 tandemly repeated HEAT (Huntingtin, EF3, A subunit of PP2A and TOR) motifs at the N-terminus, as well as FAT (FRAP-ATM-TRRAP) and FATC (FAT C-terminus) domains (Figure 2). Since the C-terminus of mTOR shares strong homology to the catalytic domain of PI3K, mTOR is considered a member of the PIK-related kinase family, which also includes MEC1, TEL1, RAD3, MEI-41, DNA-PK, ATM, ATR and TRRAP [42••]. Both PI3K and potentially protein kinase B (PKB; Akt) lie upstream of mTOR, whereas ribosomal p70S6 kinase (p70S6K) and eukaryotic initiation factor-4E (eIF-4E) binding protein isoforms (4E-BP1-3) are the

Figure 1. Molecular structures of rapamycin, CCI-779 and RAD-001.

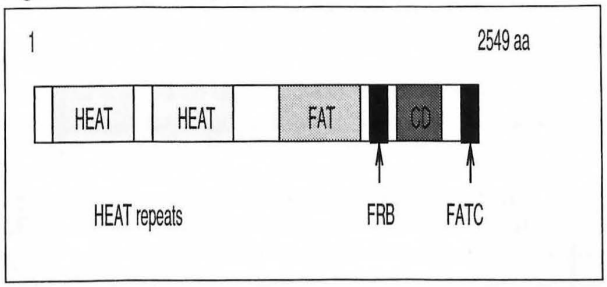


best characterized downstream mTOR effector molecules. Increasing evidence has implicated mTOR as a central controller of cell growth and proliferation. mTOR may directly or indirectly regulate translation initiation, actin organization, membrane traffic and protein degradation, protein kinase C signaling, ribosome biogenesis and tRNA synthesis, as well as transcription [42••]. Recent results also suggest that mTOR may sense cellular ATP levels, suppressing protein synthesis when ATP levels decrease [43].

Specificity of rapamycin action

Rapamycin inhibits proliferation and growth of many tumor cells, which is clearly a consequence of binding mTOR. Whether this action is a consequence of inhibiting mTOR kinase activity *per se* is less clear. Rapamycin cannot directly bind to mTOR. It first has to bind to the 12 kDa cytosolic immunophilin, FKBP12, found in mammalian cells, to form the FKBP12-rapamycin complex. The complex then interacts with the FRB domain in mTOR (Figure 2), and inhibits function of mTOR. High concentrations of rapamycin together with FKBP12 are required to inhibit mTOR kinase activity *in vitro* and mTOR autophosphorylation. However, the specificity of rapamycin action can be demonstrated *in vivo* as certain mutations in the FRB domain of mTOR affect FKBP12-rapamycin binding, and significantly reduce the cellular sensitivity of rapamycin. The first rapamycin-resistant alleles, *TOR1-1* and *TOR2-1*, identified in a *Saccharomyces cerevisiae* genetic screen were shown to confer dominant resistance. These mutant TOR proteins lost the ability for FKBP-rapamycin complex binding [44]. Similarly, mammalian cells also became highly resistant to rapamycin when a mutation ($Ser^{2035} \rightarrow Ile^{2035}$) occurred in the FRB domain of mTOR, which resulted in decreased affinity for binding of FKBP12-rapamycin complex [14,45,46]. In the yeast, *Saccharomyces cerevisiae*, decreased RBP1, a homolog of mammalian FKBP12, or mutation at Tyr^{89}, led to decreased binding of rapamycin and conferred a recessive resistance phenotype [47].

Figure 2. Schematic representation of mTOR domains.



HEAT Huntingtin, EF3, A subunit of PP2A and TOR, **FAT** FRAP-ATM-TRRAP, **FRB** FKBP12-rapamycin binding, **CD** catalytic domain, **FATC** FAT C-terminus.

Potential models for rapamycin inhibition of mTOR

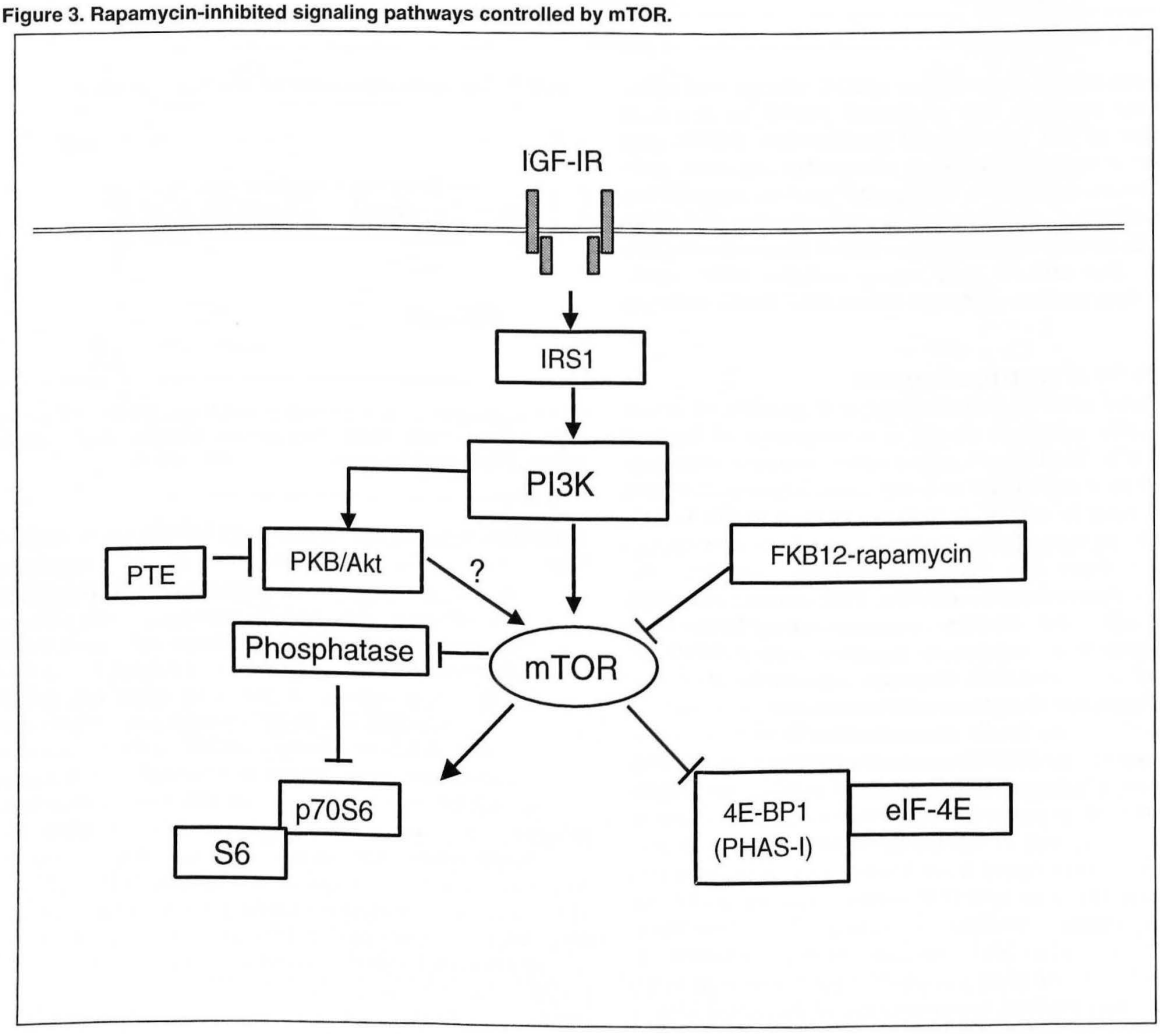
Small molecule kinase inhibitors act directly, regulating kinase activity generally by competition for ATP binding. However, whether the FKBP12-rapamycin complex or rapamycin alone directly inhibits the kinase activity of mTOR is still controversial. *In vitro*, rapamycin inhibited the modest increase in kinase activity of immunoprecipitated mTOR induced by insulin [48]. The FKBP12-rapamycin complex also inhibited the autokinase activity of mTOR, although a much higher concentration of rapamycin was needed *in vitro* than *in vivo* to inhibit the activity of mTOR [49]. Conversely, treatment of cells with rapamycin did not alter the autophosphorylation level of Ser^{2481}, and had little or no effect on the kinase activity of immunoprecipitated mTOR [37,49]. More recently, an alternative model for mTOR function has been proposed. Specifically, mTOR may repress phosphatase activity associated with downstream targets. The inhibition of mTOR induced by bound FKBP12-rapamycin complex, may result in activation of this phosphatase, which then dephosphorylates downstream effector molecules such as p70S6K [50••,51•]. Consistent with this model, we [52, Houghton & Huang, unpublished data] have suggested that mTOR regulates the catalytic subunit of PP2A associated with p44/42 mitogen-activated protein (MAP) kinases in some

cells. In these cells, rapamycin inhibits p44/42 phosphorylation on Thr^{202} following IGF-I stimulation. However, more studies are necessary to confirm the generality of this phosphatase model. An alternative model is for mTOR to act as a scaffold and for the FKBP12-rapamycin complex to disrupt higher order mTOR-protein complexes.

Rapamycin inhibition of mTOR-controlled signaling pathways

Although specific details of how rapamycin inhibits function of mTOR remain to be resolved, it has been widely accepted that inhibition of mTOR by rapamycin blocks growth factor stimulation of 40S ribosomal p70S6 kinase and phosphorylation of 4E-BP1 (also designated PHAS-I). This results in a 15 to 20% inhibition of overall protein translation and arrests cell cycle progression in G1. Consistent with this observation, mTOR controls the synthesis of essential proteins involved in cell cycle progression (cyclin D1 and ornithinine decarboxylase) [53,54] and survival (c-Myc) [55]. A scheme of mTOR-controlled signaling pathways based on rapamycin effects is shown in Figure 3. 4E-BP1, the suppressor of eIF-4E, has been reported to be a direct substrate for mTOR in cells [56,57]. *In vitro*, mTOR selectively phosphorylates 4E-BP1 at least at two and possibly four Ser/Thr residues (Thr^{37}, Thr^{46}, Thr^{70} and Ser^{65}) in the N-terminal region [58••,59]. Phosphorylation of 4E-BP1 appears to be an ordered process [58••,59,60]. Phosphorylation of Ser^{65} depends on phosphorylation of all three Ser/Thr phosphorylation sites [59,60], whereas mutations of Thr^{37} and/or Thr^{46} to Ala(s) prevents phosphorylation of Ser^{65} and Thr^{70}, suggesting that phosphorylation of Thr^{37} and Thr^{46} serves as a requisite 'priming' event [51•]. It appears that mTOR also plays a critical role in regulating the phosphorylation of Ser^{65} and Thr^{70}. In the presence of rapamycin, 4E-BP1 becomes hypophosphorylated and associates with eIF-4E. This prevents formation of the eIF-4F initiation complex and cap-dependent translation of mRNA.

Figure 3. Rapamycin-inhibited signaling pathways controlled by mTOR.



Arrows represent activation, whereas bars represent inhibition. **IGF-IR** type I insulin-like growth factor, **IRS1** insulin receptor substrate 1, **PI3K** phosphatidylinositol 3-kinase, **PTEN** phosphatase and tensin homolog deleted on chromosome ten, **PKB/Akt** protein kinase B, **FKB12-rapamycin** FK-506-binding protein 12-rapamycin complex, **mTOR** mammalian target of rapamycin, **eIF-4E** eukaryotic initiation factor-4E, **4E-BP1 (PHAS-I)** eIF-4E-binding protein 1, **S6** 40S ribosomal protein, **p70S6K** p70S6 kinase.

Ribosomal p70S6K represents the other well characterized downstream target of mTOR. Two p70S6 kinases have been characterized, namely, p70S6K1 and p70S6K2. The activation of both these kinases can be inhibited by rapamycin [61,62]. mTOR may directly or indirectly phosphorylate p70S6K1 at Thr^{229} or Thr^{389} [50••,63,64,65••,66•,67]. Phosphorylation of these two residues is blocked by rapamycin. Furthermore, mutation of either of these residues can abrogate the ability of rapamycin to inhibit p70S6K activation. p70S6K functions to increase translation of 5' terminal oligopyrimidine (5'TOP) tract mRNAs, primarily coding for elements of the translational machinery, such as ribosomal proteins, elongation factors, the poly(A) binding protein [61] and IGF-II [68]. Inhibition of mTOR by rapamycin thus selectively causes decreased translation of 5'TOP-containing mRNAs.

In addition to pathways controlling translation initiation, mTOR has been implicated in regulating the retinoblastoma protein (pRb), RNA polymerase (Pol) I/II/III-transcription and translation of rRNA and tRNA, and phosphatases (PP2A, PP4, PP6) [69]. It seems that these pathways are cell type-dependent. For example, in vascular smooth muscle cells, rapamycin may act upstream of pRb to slow or arrest cell cycle transit [70]. In this model, rapamycin inhibits activation of cyclin-dependent kinases (CDKs), which results in hypophosphorylation of pRb protein, and inhibits cells progressing from G1 to S-phase [70]. In T-lymphocytes, rapamycin induces G1 arrest, in part through inhibition of activation of CDK1 ($p34^{cdc2}$) and the formation of the cyclin E-$p33^{cdk2}$ complex [71,72]. G1 arrest by rapamycin may also be due to prevention of the degradation of CDK inhibitory protein $p27^{Kip1}$ that occurs when cells are stimulated by growth factors [73,74]. This is further supported by the observation that $p27^{Kip1}$ deficient fibroblasts are somewhat resistant to rapamycin as determined by assaying for DNA synthesis [75]. In NIH3T3 cells, rapamycin inhibits the G1 to S transition in part through decrease of cyclin D1 mRNA level and protein stability [76], or delay of the expression of cyclin A [77].

Antitumor activity of rapamycin

As previously mentioned, rapamycin has been approved as an immunosuppressive drug for organ transplantation by the FDA. So far, rapamycin has been used clinically in organ transplantation with great success, particularly in kidney transplantation [78,79]. This is because rapamycin can inhibit T-cell activation and proliferation. Increasing evidence indicates that rapamycin is not only a potent immunosuppressant, but also a promising antitumor agent. As reviewed above (Table 1), rapamycin potently inhibits the growth of many tumor cell lines *in vitro*, and has demonstrated antitumor activity in both xenograft and syngeneic murine tumor models.

However, rather than acting as a cytostatic, rapamycin induces cell death under some conditions. Early data show that rapamycin induces programmed cell death or apoptosis of B-cells [24,25]. Consistent with these findings, recent studies indicate that rapamycin alone can also induce apoptosis of certain rhabdomyosarcoma cells [14,15], and monocyte- and CD34-derived dendritic cells [80]. When combined with other chemotherapeutic agents *in vitro*, rapamycin enhanced cisplatin-induced apoptosis in human small cell lung cancer cell lines [11], potentiated apoptosis of the murine T-lymphoblastoid cell line S49, induced by dexamethasone [81] and augmented cisplatin- or camptothecin-induced cytotoxicity in DAOY human medulloblastoma cell lines [16]. Rapamycin produced additive cytotoxicity with 5-fluorouracil and cyclophosphamide in a Colon 38 tumor model [7]. At present, little is known about the molecular mechanism by which rapamycin induces apoptosis of tumor cells. However, Huang *et al* [15] have observed that the responses of malignant and normal cells to rapamycin are qualitatively different. When treated with rapamycin, cells with wild-type p53 arrest in G1 phase and maintain viability. In contrast, when grown under autocrine conditions (ie, serum-free) in the presence of rapamycin, p53 mutant cells accumulate in G1 phase, but progress to S-phase and undergo apoptosis. More than 90% of apoptotic Rh30 cells (mutant p53 alleles, $Arg^{273}\rightarrow Cys^{273}$) were BrdU-labeled, suggesting that the cells died after initiating replication. Thus, rapamycin-induced death appears to be a consequence of continued cell cycle progression, suggesting that p53 senses inhibition of mTOR and co-operates to reinforce a G1 arrest. This model has been further tested using Rh30 infected with adenovirus expressing wild-type p53 (Ad-p53) and p53 +/+ or p53 -/- murine embryo fibroblasts (MEFs) [15]. Restoring the p53-mediated G1 checkpoint by Ad-p53 infection causes Rh30 rhabdomyosarcoma cells to arrest in G1 and prevents rapamycin-induced apoptosis (Figure 4). Similarly, when exposed to rapamycin, p53 -/- MEF cells continue cell cycle progression, and become apoptotic, whereas p53 +/+ MEF cells arrest in G1 and remain viable. Exactly why cells that fail to arrest in G1 in the presence of rapamycin die is currently unknown.

Mechanisms of resistance to rapamycin

As shown by Dilling *et al* [8], under similar conditions of growth, various cell lines demonstrated several thousand-fold differences in sensitivity to rapamycin. The mechanism for this intrinsic resistance is under investigation. Cells may also acquire resistance either with or without mutagenesis.

Mutations in FKBP12, mTOR and p70S6K

Budding yeast *Saccharomyces cerevisiae* treated with rapamycin irreversibly arrested in the G1 phase. However, when yeast TOR1 and TOR2 were genetically mutated to TOR1-1 and TOR2-1, these strains were completely resistant to the growth-inhibitory effect of rapamycin. These resistant alleles encode proteins that have reduced affinity for binding the FKBP12-rapamycin complex [44]. Also in yeast, a recessive resistance phenotype was associated with decreased RBP1, a homolog of mammalian FKBP12, or a mutation altering Tyr^{89}, leading to decreased binding of rapamycin [47]. In mammalian cells, resistance to rapamycin selected after mutagenesis is related to a dominant phenotype consistent with mutation in mTOR [45]. Similar to results in yeast, mTOR mutants are associated with decreased affinity for binding of the FKBP12-rapamycin complex. High-level resistance to rapamycin is obtained when a mutant mTOR ($Ser^{2035}\rightarrow Ile^{2035}$), having reduced affinity for binding the FKBP12-rapamycin complex, is expressed [14,46]. mTOR is essential for activation of ribosomal p70S6K1 through phosphorylation of the rapamycin-sensitive sites at Thr^{229} or Thr^{389} [63,64]. Substitution of either of these residues can also abrogate the ability of rapamycin to inhibit p70S6K activation. Whether this results in resistance to the growth inhibitory effect of rapamycin is less clear, and may be cell context-specific.

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