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Targeting the mTOR Signaling Network for Cancer Therapy

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A B S T R A C T

The serine-threonine kinase mammalian target of rapamycin (mTOR) plays a major role in the regulation of protein translation, cell growth, and metabolism. Alterations of the mTOR signaling pathway are common in cancer, and thus mTOR is being actively pursued as a therapeutic target. Rapamycin and its analogs (rapalogs) have proven effective as anticancer agents in a broad range of preclinical models. Clinical trials using rapalogs have demonstrated important clinical benefits in several cancer types; however, objective response rates achieved with single-agent therapy have been modest. Rapalogs may be more effective in combination with other anticancer agents, including chemotherapy and targeted therapies. It is increasingly apparent that the mTOR signaling network is quite complex, and rapamycin treatment leads to different signaling responses in different cell types. A better understanding of mTOR signaling, the mechanism of action of rapamycin, and the identification of biomarkers of response will lead to more optimal targeting of this pathway for cancer therapy.

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INTRODUCTION

mTOR Signaling and Cancer

mTOR signaling plays a key role in cell growth, protein translation, autophagy, and metabolism. Activation of mTOR contributes to the pathogenesis of many tumor types. Upstream, phosphatidylinositol 3′-kinase (PI3K)/Akt signaling is deregulated through a variety of mechanisms, including overexpression or activation of growth factor receptors such as human epidermal growth factor receptor 2 (HER-2) and insulin-like growth factor receptor (IGFR), mutations in PI3K and mutations/amplifications of Akt.[1-4] Tumor suppressor phosphatase and tensin homolog deleted from chromosome 10 (PTEN) is a negative regulator of PI3K signaling. PTEN expression is decreased in many cancers, including breast, endometrial, thyroid, and prostate cancers; melanoma; and glioblastoma. PTEN may be downregulated through several mechanisms, including mutations, loss of heterozygosity, methylation, aberrant expression of regulatory microRNA, and protein instability. Activated mTOR signaling is also associated with tumor-predisposition syndromes: Cowden's syndrome (*PTEN* mutations), Peutz-Jeghers syndrome (*LKB1* mutations), tuberous sclerosis (*TSC1/2* mutations), and neurofibromatosis (*NF1* mutations).[5-8] Thus mTOR signaling is activated in conditions of proliferative dysregulation and in many cancer types.

Activation of mTOR results in phosphorylation of its effectors, the best studied of which are eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) and S6 kinase 1 (S6K1). 4E-BP1 hyperphosphorylation leads to inhibition of 4E-BP binding to eukaryotic initiation factor 4E (eIF4E), activating translation. eIF4E is rate-limiting for cap-dependent translation. The translational efficiency of mRNA with highly complex 5′ untranslated regions is especially dependent on eIF4E.[9] eIF4E enhances cell proliferation, survival, and angiogenesis by leading to selective translation of mRNA such as cyclin D1, Bcl-2, Bcl-xL and vascular endothelial growth factor (VEGF)[9,10] as well as the nucleocytoplasmic transport of selected mRNA such as cyclin D1.[11] S6K1 is a key regulator of cell growth. It phosphorylates ribosomal protein S6 and, in some models, enhances the translation of mRNAs possessing a 5′ terminal oligopyrimidine tract. S6K1 also phosphorylates other important targets, including insulin receptor substrate 1 (IRS-1), eukaryotic initiation factor 4B, programmed cell death 4, eukaryotic elongation factor-2 kinase, mTOR, glycogen synthase kinase 3, and S6K1 Aly/REF-like target.[12] Both eIF4E and S6K1 are implicated in cellular transformation, and their overexpression has been linked to poor cancer prognosis.[9,13,14] Rapamycin and its analogs bind FK506 binding protein, and this complex binds to mTOR, inhibiting downstream signaling. Rapamycin causes cell cycle arrest in a broad spectrum of tumor types. In addition to direct antitumor effects, rapamycin also inhibits

endothelial cell proliferation, hypoxia inducible factor 1 and VEGF expression, angiogenesis, and vascular permeability.[15,16] Taken together, these data demonstrate the importance of mTOR signaling in cancer and support a role for mTOR as an antitumor target.

mTOR Signaling Network

The intricate mTOR signaling network (Fig 1) needs to be better understood to effectively target the pathway. mTOR exists in two multiprotein complexes: mTOR complexes 1 and 2 (mTORC1 and mTORC2). mTORC1 consists of mTOR, mammalian LST8 (mLST8), proline-rich Akt substrate 40 (PRAS40), and raptor.[17] PRAS40 has been proposed to be a negative regulator when bound to mTORC1.[18] PRAS40 itself may be a substrate of mTOR that is phosphorylated on activation by upstream regulators and released from mTORC1.[18] mTORC1 activation results in phosphorylation of 4E-BP1 and S6K1. mTORC2 consists of mTOR, mLST8 (GßL), mSIN1, PRR5 (protor), and rictor.[19-23] mTORC2 phosphorylates Akt at Ser473 and has been proposed to regulate the ability of integrin-linked kinase to promote Akt phosphorylation.[24-26] Akt Ser473 phosphorylation leads to full Akt activation and may affect its substrate specificity, with activation of Akt toward the Forkhead transcription factor FOXO and the apoptosis regulator BAD.[19] mTORC2 has been proposed to regulate phosphorylation of PKCα, control actin cytoskeleton and is linked to cell migration.[19,24,27]

mTOR signaling is regulated by growth factor signaling as well as nutrient (amino acid) and energy status. PI3K/Akt signaling regulates mTOR through phosphorylation/inactivation of mTOR's negative regulator TSC2.[28-30] TSC2 contains a GTPase activating domain that inactivates Rheb GTPase, which associates with and directly activates mTORC1. Ras/MAPK signaling also inhibits TSC2.[31] Furthermore, TSC2 is regulated by cellular energy sensor AMP kinase.[7] When cellular energy stores are reduced or AMP levels increase, AMPK is activated, phosphorylating and activating TSC2 to inhibit mTOR signaling, reducing protein synthesis. Although the exact mechanism of nutrient signaling remains unclear, amino acids are thought to mediate mTORC1 signaling through class III PI3K hVps34.[32]

mTORC1 is rapamycin-sensitive; rapamycin results in dephosphorylation of 4E-BP1 and S6K1. In contrast, mTORC2 was originally thought to be rapamycin-insensitive.[19,24] However, rapamycin regulates rictor phosphorylation, suggesting that components of mTORC2 may be regulated by rapamycin.[33] Further, prolonged rapamycin treatment reduces mTORC2 levels and inhibits Akt activation in some cell lines.[24,34]

Rapamycin induces Akt activation in some models.[35,36] Insulin-like growth factor I (IGF-I) and insulin-dependent induction of the PI3K/Akt pathway leads to feedback inhibition of signaling due to mTOR/S6K-mediated phosphorylation and degradation of IRS-1. Rapamycin-induced Akt activation has been attributed to loss of this negative-feedback loop.[35,36] The effect of rapamycin on Akt may vary with drug dose, with lower doses leading to an increase in Akt activation and higher doses diminishing Akt activity.[16,37] The effect on Akt also varies with cell type, with rapamycin leading to an

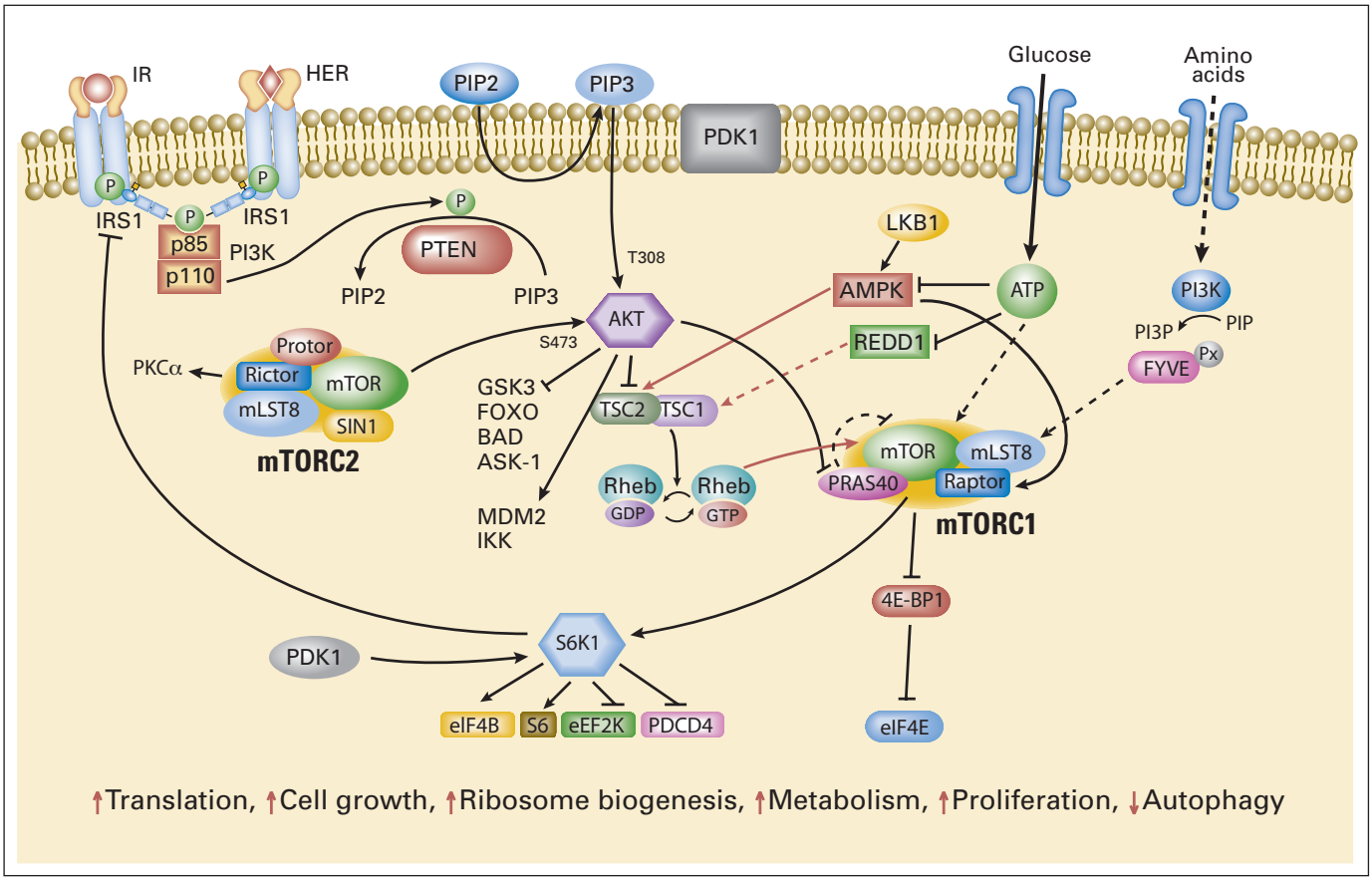

Fig 1. The mammalian target of rapamycin (mTOR) signaling network. Arrows represent activation, bars represent inhibition. mTOR signaling regulates multiple critical cellular processes by integrating energy and nutrient stutus and PI3K/Akt signaling induced by growth factors and insulin.

increase in Akt phosphorylation in some cell lines, and no change or a decrease in others.[38] The response of Akt may depend on the activity of upstream-signaling pathways and whether the mTORC2 complex is maintained.

INCORPORATION OF mTOR-TARGETED THERAPY INTO CLINICAL PRACTICE

Single-Agent Rapamycin Analogs in Clinical Trials

Cinical trials are ongoing with rapamycin and its analogs temsirolimus (Torisel, CCI-779, Wyeth Pharmaceuticals, Madison, NJ), everolimus (RAD001, Novartis, Basel, Switzerland). and AP23573 (Ariad Pharmaceuticals, Cambridge, MA) in various tumor types. Although mTOR plays a central role in many biologic processes, rapalogs have been generally well tolerated. Toxicities have included asthenia, mucositis, nausea, cutaneous toxicity, diarrhea, hypertriglyceridemia, thrombocyopenia, hypercholesterolemia, elevated transaminases, hyperglycemia, and pneumonitis.[39-41] Toxicity was more common with higher doses in some studies.[42]

mTOR is now a validated therapeutic target for renal cell carcinoma (RCC). In a multicenter phase III trial, patients with previously untreated, poor-prognosis metastatic RCC were randomized to receive 25 mg of temsirolimus intravenously weekly, interferon alfa, or combination therapy.[43] Patients who received temsirolimus alone had a significantly longer overall survival (OS) and progression-free survival (PFS) than patients who received interferon alone (Table 1). The OS in the combination group did not differ significantly from that of the interferon group. The median OS with temsirolimus, interferon, or the combination was 10.9, 7.3 and 8.4 months, respectively. The US Food and Drug Administration approved temsirolimus for the treatment of poor prognosis metastatic RCC in 2007. Recently, a randomized, double-blind, placebo-controlled phase III trial of everolimus was performed in patients with RCC whose disease progressed on VEGFR-targeted therapy.[44] At the second interim analysis, the trial showed a significant difference in efficacy and was halted early. The hazard ratio was 0.3 (95% CI, 0.22 to0.4; $P < .0001$) and the median PFS was 4 months for the everolimus arm versus 1.8 months for the control arm. The probability of being progression-free at 6 months was 26% for everolimus and 2% for placebo.

Rapalogs have been evaluated in several other cancer types (Table 1). They have shown clear evidence of single-agent activity in lymphoma. Phase II studies have shown objective response rates (ORR) of 38% to 41% in mantle-cell lymphoma[45,46] and 35% in non–mantle-cell non-Hodgkin's lymphoma.[47] A phase III trial in refractory mantle-cell lymphoma demonstrated a 22% ORR with temsirolimus given at 175 mg weekly for 3 weeks followed by 75 mg weekly, compared with 2% for the investigator's choice of therapy ($P = .0019$).[48] PFS rates were 4.8 months with the 75-mg weekly temsirolimus and 1.9 months with investigators' choice treatment ($P = .009$).[48] Rapamycin has led to regression of Kaposi's sarcoma in renal transplant recipients.[49] In preliminary analysis of phase II trials, rapalogs have also shown promise in patients with sarcoma and endometrial cancer.[50,51]

Rapamycin has also been evaluated in syndromes of proliferative dysregulation. Clinical benefit has been reported with facial angiofibroma, renal angiolypomas, and lymphangiomyomatosis.[52-54] Clinical trials are ongoing for patients with neurofibromatosis, Cowden's Syndrome, and tuberous sclerosis, as well as for sporadic lymphangiomyomatosis—a condition associated with somatic mutations in the tuberous sclerosis genes.

Overall rapalogs have achieved modest ORRs. For example, in metastatic poor-prognosis RCC, temsirolimus treatment was associated with an improvement in PFS and OS, but it was only associated with a 8.6% ORR.[43] Though everolimus improved the PFS for RCC that progressed on VEGFR-targeted therapy, the ORR was 1%.[44] Thus for rapalogs, high ORRs may not be needed to achieve clinical benefit. As demonstrated by preclinical studies,[55] rapalogs used alone are cytostatic in most tumor types and clinically may primarily stabilize disease.

Patient Selection for Treatment With Rapamycin Analogs

Although mTOR signaling is commonly deregulated in cancer, rapalogs have failed to show any appreciable single agent activity in many tumor types. The clinical benefit seen in different tumor histologies have been attributed to rapamycin's effects on different oncogenic drivers: angiogenesis in renal cell carcinoma and Kaposi's sarcoma, t(11;14)(q13;q32) translocation with cyclin D1 overexpression in mantle-cell lymphoma, *PTEN* loss for endometrial cancer, and activation of IGF-1R signaling in sarcomas. However, these attributions have remained controversial. Further, the low ORRs seen with unselected patient cohorts demonstrate that histology-based patient selection is insufficient. Based on preclinical data, a variety of predictors of response have been proposed, but most have not yet been clinically validated. Correlative studies in many ongoing and completed clinical trials have been limited due to availability of evaluable samples and the small numbers of patients achieving objective responses. Thus there remains an urgent need to better understand rapamycin's mechanism of action and to identify predictive markers of response that can be used to prospectively select patients who will derive the greatest benefit from rapalogs.

Patients with decreased PTEN may especially benefit from rapalogs. mTOR inhibition reduces neoplastic proliferation and tumor size in *PTEN*± mice, demonstrating that mTOR is the major effector of oncogenic PI3K signaling.[56] Studies with isogenic *PTEN*+/+ and *PTEN*−/− mouse cells and with human cell lines with defined *PTEN* status have shown that PTEN-deficient tumors are preferentially inhibited by mTOR inhibition.[57-60] However, PTEN loss was not able to predict sensitivity to everolimus in glioblastoma orthotopic xenografts. The predictive role of PTEN in clinical trials remains controversial.[61,62]

Activation of PI3K signaling, regardless of mechanism (PTEN loss or activated receptor-tyrosine-kinase signaling), may sensitize tumors to mTOR inhibition.[63] Tumor growth conferred by Akt activation is also reversed by mTOR inhibitors.[57] Rapalogs also block tumor growth induced by oncogenic PIK3CA mutations,[64] suggesting that activating PI3K mutations may also have predictive value.

Predictive markers have been assessed in few clinical trials to date (Table 2). In a phase II trial of temsirolimus in RCC, paraffin-embedded tissue was available from 20 patients, five with a response (one partial and four minor).[65] A positive association of *p*-S6 (Ser235) expression and a trend toward positive expression of *p*-Akt (Ser473) was found. Patients without high *p*-Akt or *p*-S6 expression did not achieve a response. No correlation was seen between

Table 1. Efficacy of Rapamycin Analogs in Selected Clinical Trials

Study Treatment	Phase	Disease	No. of Patients	Objective Response (PR or CR, %)
Hudes, 2007[43]	III	RCC		
Temsirolimus (25 mg IV qwk)			209*	8.6
Interferon			207*	4.8
Temsirolimus + interferon			210*	8.1
Motzer, 2008[44]	III	RCC		
Everolimus (10 mg po qd)			272*	1
Placebo			138*	0
Hess, 2008[48]	III	Mantle-cell lymphoma		
Temsirolimus (175 mg × 3 doses, followed by 75 mg qwk)			54*	22
Temsirolimus (mg × 3 doses, followed by 25 mg qwk)			54*	6
Investigator's choice			54*	2
Galanis, 2005[105]	II	GBM		
Temsirolimus (250 mg IV qwk)			64†	0‡
Atkins, 2004[41]	II	RCC		
Temsirolimus (250 mg IV qwk)			37*	8.1
Temsirolimus (75 mg IV qwk)			38*	7.9
Temsirolimus (25 mg IV qwk)			36*	5.6
Witzig, 2005[46]	II	Mantle-cell lymphoma		
Temsirolimus (250 mg IV qwk)			34†	38
Chan, 2005[42]	II	Breast cancer		
Temsirolimus (250 mg IV qwk)			54*	7.4
Temsirolimus (75 mg IV qwk)			55*	10.9
Margolin, 2005[106]	II	Melanoma		
Temsirolimus (250 mg IV qwk)			33†	3
Duran, 2006[67]	II	Neuroendocrine		
Temsirolimus (25 mg IV qwk)			36*	5.6
Chawla, 2006[50]	II	Sarcoma		
Deforolimus (12.5 mg IV qd × 5, every 2 wks)			193†	3¶
Colombo, 2007[51]	II	Endometrial cancer		
Deforolimus (12.5 mg IV qd × 5, every 2 wks)			27†	7‖
Pandya, 2007[86]	II	SCLC		
Temsirolimus (250 mg IV qwk)			41†	0
Temsirolimus (25 mg IV qwk)			44†	2.3
Smith, 2008[47]	II	Lymphoma (non–mantle-cell, non-Hodgkins')		
Temsirolimus (25 mg IV qwk)			74*	35
Rizzieri, 2008[107]	II	Hematologic malignancies		
Deforolimus (12.5 mg IV qd × 5, every 2 wks)			52†	10
Slomovitz, 2008[62]	II	Endometrial		
Everolimus (10 mg po qd)			25†	0¶
Ansell, 2008[45]	II	Mantle-cell lymphoma		
Temsirolimus (25 mg IV qwk)			27†	41

Abbreviations: PR, partial response; CR, complete response; RCC, renal cell carcinoma; IV, intravenously; po, by mouth; qd, every day; qwk, every week; GBM, glioblastoma multiforme; SCLC, small-cell lung cancer.
*No. of intent-to-treat patients.
†No. of assessable patients.
‡Thirty-six percent of patients had evidence of improvement on neuroimaging.
§Stable disease in 25% of patients.
‖Stable disease in 26% of patients.
¶Stable disease in 44% of patients.

response and carbonic anhydrase IX, *PTEN* or Von-Hippel Lindau mutation status. Iwenofu et al assessed the predictive value of *p*-S6 (Ser235/236) in patients with sarcoma who received deforolimus (with or without chemotherapy).[66] Among *p*-S6 high expressors there were eight patients (73%) with stable disease and three patients (27%) with progression; among low expressors there were three patients (33%) with stable disease and six (67%) experienced progression ($P = .05$). Biomarkers were assessed in a phase II trial of temsirolimus in neuroendocrine tumors using archival samples and pretreatment biopsies. Although high *p*-mTOR (S2448) and *p*-S6 (Ser235/236) in archival samples were not predictive of response, high *p*-mTOR on freshly procured pretreatment biopsies was predictive ($P = .01$), with a trend towards response with high *p*-S6 on the pretreatment samples.[67]

The assessment of markers of response remains an obstacle to predictive marker development. Immunohistochemistry (IHC) with

Table 2. Potential Predictors and Pharmacodynamic Markers of Response in Clinical Trials

Marker	Disease	Treatment	End Point
Cho, 2007[65]	Renal cell carcinoma	Temsirolimus	
High *p*-S6 (Ser235)			Response (PR or MR)
High *p*-Akt (Ser473; trend)*			
Duran, 2006[67]	Neuroendocrine	Temsirolimus	
High *p*-mTOR (Ser2448)			Response (not defined)
High *p*-S6 (Ser235/236; trend)			Response (not defined)
Increase in *p*-Akt (Ser473)			Increased TTP
Decrease in *p*-mTOR (Ser 2448)			Increased TTP
Slomovitz, 2008[62]	Endometrial	Everolimus	
Low PTEN (trend)			SD (*v* PD)
Iwenofu, 2008[66]	Sarcoma	Deforolimus with or without adriamycin	
High *p*-S6 (Ser235/236)			SD (*v* PD)
Cloughesy, 2008[80]	Glioblastoma	Rapamycin	
Increase in *p*-PRAS40 (Thr246)			Decreased TTP

Abbreviations: PR, partial response; MR, minor response; mTOR, serine-threonine kinase mammalian target of rapamycin; TTP, time to progression; PTEN, phosphatase and tensin homologue deleted from chromosome 10; SD, stable disease; PD, progressive disease.
*Trend, or difference in marker expression between responders and nonresponders, did not reach statistical significance.

PTEN and with phospho-specific antibodies such as *p*-Akt, *p*-S6K, and *p*-S6 is challenging. Their staining and quantification have not been standardized. Concerns exist about the stability of phosphoproteins.[68] The results of phospho-marker testing may vary based on specimen acquisition and processing, and may be influenced by tumor heterogeneity. Further, clinicians often assess markers in the primary tumor to make therapeutic decisions for metastatic disease; however, concordance of *p*-Akt and *p*-4E-BP1 levels by IHC in primary breast tumors and matched distant metastases was found to be poor.[69] This may reflect true biologic heterogeneity or may simply be a reflection of the poor reproducibility and process sensitivity of IHC with phospho-specific antibodies. An alternate approach may be more quantitative assays such as enzyme-linked immunosorbent array with fresh samples. Streamlining higher throughput strategies (eg, reverse-phase proteomic arrays) to quantitate the activity of several pathways simultaneously may be considered. Evaluating multiple markers may demonstrate a more robust evaluation of the oncogenic signaling drivers of each tumor. As transcriptional profiling becomes more commonplace, there is also a need to identify transcriptional profiles that correlate with mTOR activation and profiles predictive of response. Identification of genomic alterations that confer rapamycin sensitivity is also highly desirable, since genomic aberrations may be more reliably tested in paraffin.

Pharmacodynamic Markers of Target Inhibition

For mTOR, the two best studied targets are S6K1 and 4E-BP1; thus, most studies have concentrated on these proteins. Preclinically rapamycin and its analogs inhibit phosphorylation of 4E-BP1 and S6K1 in tumor, skin and peripheral blood mononuclear cells (PBMCs).[70,71] 4E-BP1 has been reported to be hypophosphorylated in PBMCs[71] while S6K1 activity has little intrasubject variation (14%)[70]; thus, PBMC S6K1 activity has been pursued in most pharmacodynamic (PD) studies. Time and dose-dependent inhibition of S6K1 was demonstrated in PBMCs. In preclinical models, a correlation with antitumor effect and prolonged ($\geq$ 7 days) PBMC-derived S6K1 activity has been observed.[71] For everolimus, preclinical simulations suggest that the administration regimen has a greater influence on S6K1 activity in the tumor than PBMCs, with daily dosing exerting greater activity than weekly doses,[72] sustained S6K inhibition occurring with $\geq$ 20-mg everolimus weekly and $\geq$ 5 mg daily.[73] These findings highlight that although PBMC S6K1 activity is often measured as a PD marker, it is not a perfect readout of target inhibition in the tumor.

In an elegant phase I study of everolimus in solid tumors, pretreatment and on-treatment (day 22) tumor and skin biopsies were evaluated.[74] mTOR signaling was inhibited at all dose and schedules tested (5 and 10 mg daily, and 20, 50, and 70 mg weekly). Dose- and schedule-dependent inhibition of mTOR was observed with near-complete inhibition of *p*-S6 and *p*-eIF4G at 10 mg/d and $\geq$ 50 mg/wk. The relative inhibition of these markers differed with different dose levels. With daily dosing, *p*-S6 was inhibited with both dose levels, while *p*-eIF4G inhibition was partial with 5 mg but complete with 10 mg. With weekly dosing, *p*-S6 inhibition was almost complete at all dose levels. Inhibition was sustained in biopsies obtained 24 hours before the next weekly dose. In contrast, *p*-eIF4G inhibition was complete at 24 hours for all dose levels, but was sustained only for $\geq$ 50 mg/wk. *p*-4E-BP1 inhibition was not observed in all patients. Although there was good concordance of pathway inhibition in tumor and skin, *p*-4E-BP1 reduction was more profound in skin than tumors. This study clearly demonstrates that inhibition of mTOR signaling may be dependent on dose and schedule, and downstream targets may not always be inhibited concordantly.

Pharmacodynamic Markers of Response

To identify the potential determinants of response to rapamycin, one needs to better understand the downstream effects of mTOR inhibition in rapamycin-sensitive versus -resistant tumors and better elucidate rapamycin's mechanism of action. These molecular changes can then be followed to determine whether a patient is responding early in the treatment course, either through serial biopsies of the tumor or through molecular imaging. These markers may not only assist in better prospective patient selection, but would also allow therapy to be modified early if there is no molecular response.

Differential intrinsic sensitivity to rapamycin and analogs is not explained by differences in blockade of mTOR signaling pathway, at least not by inhibition of S6K1 or S6 phosphorylation.[57,58,63,75] A correlation was found between rapamycin-mediated decline in *p*-4E-BP1 T70 with growth inhibition in some xenograft models,[76] but in other preclinical studies, inhibition of *p*-4E-BP1 did not correlate with sensitivity.[57] Taken together, decrease of downstream signaling appears to be useful for determining whether a biologically relevant drug dose is achieved; however, this finding does not necessarily correlate with growth inhibition and thus is not a good PD marker of response.

Pathway inhibition may not be a useful marker of response because different components of downstream signaling have differing thresholds for inhibition and the critical mediators of rapamycin's growth inhibitory effect may not be measured. Thus, if one focused on *p*-S6 alone, mTOR signaling may appear to be inhibited, while different mTOR effectors have different sensitivity to mTOR inhibitors.[74] The mTOR/4E-BP1 axis which regulates eIF4E availability and cap-dependent translation may be the major driver of rapamycin-mediated growth inhibition, especially since eIF4E is a growth-regulatory target itself[10,77] and in some models confers rapamycin resistance.[78] Alternately, the pathway may be active but may not be the oncogenic driver; thus, inhibition of the pathway may be insufficient to achieve a growth-inhibitory effect.

Additionally, mTOR inhibition may in turn activate compensatory pathways such as Akt and MAPK signaling,[79] which may theoretically limit antitumor activity. However, Akt activation has been observed even in rapamycin-sensitive preclinical models; thus, the value in assessing Akt activation as a marker of rapamycin resistance remains unclear.

In a phase I trial for recurrent PTEN-deficient glioblastoma, Cloughesy et al[80] evaluated *p*-PRAS40 (Thr246) as a biomarker of Akt activity in surgical specimens obtained after 1 week of rapamycin treatment. This study differed from the usual pretreatment and on-treatment biopsy design as untreated primary tumor surgical specimens (S1) were compared with recurrent tumors treated with rapamycin for 1 week before surgery (S2). S1 and S2 samples from nine patients who did not receive rapamycin, were used as control and did not show a change in *p*-Akt. Of 14 patients in the rapamycin study, seven had an increase in *p*-PRAS40 in their S2 sample ($P = .0047$). Patients were maintained on rapamycin postoperatively. An increase in S2 *p*-PRAS40 was associated with a shorter time to progression ($P < .05$). Although it can not be determined whether *p*-PRAS40 was prognostic or whether induction of *p*-PRAS40 was predictive of poor response, these findings highlight the importance of assessing the *p*-Akt and its phosphorylation targets as potential PD markers.

Identification of the major mediators of drug response will be critical to identify ideal PD markers of response. Preclinical studies have identified a variety of alterations that occur on rapamycin treatment that may reflect direct or indirect drug effects (Table 3). These changes, alone or in combination, may be pursued as PD markers of response. Potential PD markers of response may be prioritized by concentrating on alterations critical to rapamycin's growth inhibitory effect. For example, rapamycin decreases cyclin D1 levels in several models.[63,81-83] Further, a decrease in cyclin D1 plays an important role in rapamycin-mediated growth inhibition.[82,83] However, although rapamycin decreases cyclin D1 in rapamycin-sensitive but not rapamycin-resistant cells in some studies, others report no change in cyclin D1 expression in either sensitive or resistant cells.[58,63] It is unlikely that any single marker will sufficiently separate responders from nonresponders. Evaluating a panel of rapamycin effectors may be preferable for PD monitoring. Molecular imaging with tracers that assess metabolic and proliferative function ([^{18}F]fluorodeoxyglucose and [^{18}F]fluorothymidine uptake) has also shown promise in preclinical models.[15,84] Molecular imaging with novel tracers of pathway activity is also being pursued.

Table 3. Selected Downstream Effects of Rapamycin

Target	Rapamycin Effect	Reference No.
S6K1 (T389, T421/S424, T229)	Decrease	12
S6 (Ser235/236, Ser240/244)	Decrease	74
4E-BP1 (Thr 37*, Thr 46*; T70. Ser 65)	Decrease	74,108,109
eIF-4G (Ser1108; Ser1148; Ser1192)	Decrease	74,110,111
eIF-4B (Ser 422)	Decrease	112
FOXO1 (Ser256)	Decrease	19
PRAS40 (Ser221, Ser183)	Decrease	18
SGK1 (Ser422, Thr2560)	Decrease	113
Cyclin D1	Decrease	34,63,81,83,115
Cyclin D3	Decrease	75
c-Myc	Decrease	75,81
GLUT-1	Decrease	34
VEGF	Decrease	90
HIF-1α	Decrease	90
Ki-67	Decrease	80,91
Dusp6	Decrease	116
eEF2 (Thr56)	Increase†	85
eIF2α (Ser51)	Increase†	85
c-Jun (S63)	Increase‡	114
p27	Increase	75

Abbreviations: S6K1, S6 kinase 1; 4E-BP1, 4E-binding protein 1; eIF, eukaryotic initiation factor; PRAS40, proline-rich Akt substrate 40; SGK1, Serum/glucocorticoid-regulated kinase; GLUT-1, glucose transporter protein; VEGF, vascular endothelial growth factor; HIF-1α, hypoxia inducible factor 1α; eEF2, eukaryotic elongation factor 2 kinase.

*Although Thr 37/46 is phosphorylated in vitro by serine-threonine kinase mammalian target of rapamycin (mTOR), these residues are proposed to relatively resistant to rapamycin in the presence of serum, but they are sensitive to rapamycin under serum starvation.

†At high micromolar concentrations.

‡In cells lacking functional p53.

Effect of Dose and Schedule Selection on Efficacy

The clinical development of rapalogs has focused on the effect of dose and schedule on target inhibition. However, the ideal dose and schedule for rapamycin and analogs to achieve antitumor effect remains controversial. Rapamycin and everolimus have both shown dose-dependent antitumor efficacy in xenograft models.[71] Further, lower doses of rapamycin leads to Akt activation, whereas higher doses diminish *p*-Akt in some models.[16,37] In addition, although lower concentrations of temsirolimus and rapamycin have a selective growth inhibitory effect, at higher micromolar concentrations they have a profound antiproliferative effect in all tested cell lines with a decline in global protein synthesis and an increase in phosphorylation of eukaryotic elongation factor-2 kinase and eIF2α.[85] This highlights another means through which dose and schedule selection may affect clinical outcome.

Dosing regimens have been compared in a few randomized trials. In the phase II temsirolimus trial in RCC, 25-, 75-, and 250-mg intravenous doses were compared (Table 1): the ORR were 5.6%,

7.9%, and 8.1%, respectively.[41] The time to progression was 6.3, 6.7, and 5.2 months, respectively, and median survival was 13.8, 11.0, and 17.5 months, respectively. These were not statistically different and the authors concluded that efficacy was not significantly influenced by dose level. Thus, the 25-mg dose was pursued for the RCC trials that led to US Food and Drug Administration approval. However, some clinical data suggest that dose may be relevant to efficacy.[86] In the phase III trial of temsirolimus in mantle-cell lymphoma,[48] the 75-mg weekly regimen had a significantly higher ORR compared with investigator's choice treatment, while the 25-mg weekly regimen did not (Table 1). Furthermore, the 75-mg regimen significantly prolonged PFS (4.8 *v* 1.9 months; $P = .0009$), while the improvement in PFS with the 25-mg regimen (3.4 months) did not reach statistical significance ($P = .0618$). Thus higher doses may be more effective in some tumor types. The ideal dose and schedule needs to be further studied.

COMBINATION OF mTOR-TARGETED THERAPIES AND OTHER ANTICANCER AGENTS

In clinical trials, rapalogs have predominantly led to disease stabilization rather than tumor regression. Thus, for most tumor types, mTOR-targeted therapies will likely be used in combination therapy, with the expectation that this may induce a cytotoxic rather than cytostatic response and subsequent tumor regression.

Combination With Chemotherapy

mTOR inhibitors have been found to be additive or synergistic with paclitaxel, carboplatin, cisplatin, vinorelbine, doxorubicin, and campthotecin.[55,59,87,88] Compared with single agent therapy, the combination of rapamycin with chemotherapy enhances apoptosis in vitro and enhances antitumor efficacy in vivo.[55,87-89] Ongoing clinical trials are currently evaluating the efficacy of rapamycin and its analogs in combination with a broad spectrum of chemotherapeutic agents.

Combination With IGF-IR Inhibitors

The rapamycin-induced Akt activation observed in some cancer cell lines and in clinical trials increased interest in overcoming this feedback loop activation by using mTOR inhibitors in combination with antagonists of upstream signaling such as IGF-IR inhibitors.[35,36,90] IGF-IR inhibition prevents rapamycin-induced Akt activation and sensitizes tumor cells to mTOR inhibition in preclinical models.[35,90] The combination of rapalogs and IGF-IR inhibitors are now being studied in clinical trials.

Combination With Octreotide

In neuroendocrine tumors, although a phase II trial with temsirolimus obtained a relatively low ORR, a phase II trial of everolimus in combination with octreotide demonstrated clinical efficacy with an ORR of 20% by intent-to-treat analysis.[91] This may reflect differences between patient cohorts, differences in mTOR inhibition with different drug and dosing regimens, or may be attributable to the combination of mTOR inhibitors with octreotide in the latter trial. Somatostatin analogs such as octreotide decrease PI3K/Akt signaling in some models[92] and thus theoretically may enhance rapamycin's antitumor activity. However, preclinical work in carcinoid cells demonstrated that although rapamycin causes significant growth inhibition in vitro and in vivo, it did not enhance rapamycin's antiproliferative effects and did not inhibit rapamycin-mediated Akt activation.[93] Yet, preclinical models have clear limitations. Randomized prospective trials are being conducted to determine whether octreotide enhances the antitumor effects of mTOR inhibitors.

Combination With Trastuzumab

In HER-2–positive breast cancer cell lines, trastuzumab has been shown to inhibit feedback-loop activation of Akt.[94] This is especially notable as PTEN loss is a known mediator of trastuzumab resistance,[95,96] providing another rationale to use mTOR inhibitors to restore or enhance trastuzumab sensitivity. In vitro, low doses of everolimus significantly increased growth inhibition by trastuzumab, and in vivo everolimus enhanced the antitumor efficacy of trastuzumab by a modest amount.[94] The combination of everolimus and trastuzumab is currently in clinical trials. A recent multicenter phase I trial of everolimus in combination with paclitaxel and trastuzumab in patients with HER-2–overexpressing metastatic breast cancer with prior resistance to trastuzumab demonstrated that the combination was well tolerated, with the preliminary evidence of efficacy.[97]

Combination With Antiestrogen Therapy

Akt/mTOR signaling has been associated with resistance to endocrine therapy in breast cancer,[98] providing rationale for combining endocrine therapy with mTOR inhibitors. In preclinical models, rapalogs enhance the efficacy of selective estrogen receptor modulators tamoxifen, raloxifene, and ERA-923; estrogen receptor downregulator fulvestrant; and aromatase inhibitor letrozole.[71,99-101] However, the interim analysis of a phase III randomized placebo controlled trial of letrozole with or without temsirolimus reported no improvement in PFS[102]; final analysis has not been published. The combination of everolimus with letrozole has been pursued with more promising results. A phase I study of everolimus with letrozole demonstrated some clinical responses.[103] The combination of daily oral everolimus plus letrozole versus placebo plus letrozole was recently tested in a randomized phase II neoadjuvant trial in 270 postmenopausal women with estrogen receptor–positive breast cancer.[61] The clinical response rate with everolimus and letrozole was significantly more than letrozole alone at the preplanned alpha of 0.1 (68% *v* 59%; $P = .062$). These results were confirmed by ultrasound (objective response 58% *v* 47%; $P = .035$). Cell cycle response was also higher in the combination arm (57% *v* 30% for Ki-67 ≤ 2 at day 15; $P < .01$). Thus, mTOR inhibition may increase the efficacy of endocrine therapy. However, everolimus was associated with an increase in grade 3/4 adverse events (22.6% in the combination arm *v* 3.8% in the letrozole arm). Although, the addition of everolimus to letrozole, a drug that has excellent baseline tolerability, increases adverse effects,[61,103] this strategy may be warranted in patients with higher-risk hormone receptor–positive tumors, especially if predictors of response can be utilized to select patients most likely to benefit from this combination.

NEW mTOR-TARGETED THERAPIES

A new generation of mTOR inhibitors is being developed. In contrast to rapalogs, catalytic site inhibitors of mTOR inhibit both mTORC1 and mTORC2, and inhibition of mTORC2 will affect the activation of Akt. Agents such as BEZ235 (Novartis, East Hanover, NJ) and EX147 (Exelixis, San Francisco, CA) are dual PI3K/mTOR inhibitors and thus may bypass feedback loops, potentially increasing their efficacy

compared with rapalogs. The tolerability and efficacy of these agents are currently being tested in clinical trials. In addition, other strategies to downregulate mTOR signaling, such as the use of antidiabetic drug metformin—an activator of AMPK[104]—are being pursued in clinical trials.

SUMMARY AND CONCLUSION

mTOR is now a validated target in the treatment of some tumor types. Careful patient selection and rational selection of combination therapies will enhance the success of mTOR therapies. Used effectively, mTOR inhibitors will play an important role in delivering more effective, personalized cancer therapy.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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