The mTOR Pathway: A New Target in Cancer Therapy

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Abstract: Mammalian target of rapamycin (mTOR) is a key protein kinase controlling signal transduction from various growth factors and upstream proteins to the level of mRNA translation and ribosome biogenesis, with pivotal regulatory effects on cell cycle progression, cellular proliferation and growth, autophagy and angiogenesis. The mTOR pathway, and its upstream regulators in the PI3K/PTEN/AKT cascade, are altered in a variety of experimental and human malignancies.This has led to the prediction that mTOR inhibitors may be used as anticancer agents. With the recent approval of two mTOR-targeted drugs (temsirolimus and everolimus) for the treatment of renal cell carcinoma and mantle cell lymphoma, this paradigm has been effectively translated into the clinical setting. In this review, we discuss mTOR biology and regulation, the mode of action of mTOR inhibitors as anti-cancer agents, and current clinical evidence supporting the use of rapamycin-like mTOR inhibitors in cancer treatment.

Keyword: mTOR, growth inhibition, angiogenesis, cancer therapy, rapamycin-like agents, renal cell carcinoma.

INTRODUCTION

The target of rapamycin (TOR) genes were originally identified in 1991 in yeast as the targets of the antifungal bacterial product rapamycin. TOR is a member of the phosphoinositide kinase−related kinase (PIKK) family and both yeast and mammalian TORs phosphorylate proteins on serine or threonine residues [1]. Genetic studies have shown that TOR is essential for cell growth and development in fruit flies, nematodes, and mammals and disruption of the gene(s) encoding TOR results in lethality in all species [2-4].

The mammalian target of rapamycin (mTOR) is a protein kinase that functions as a central element in a signaling pathway involved in the control of many processes, including protein synthesis and autophagy [5, 6]. TOR kinases are highly conserved and up to 60% identical in human and other mammalian organism. mTOR is found in the cellular cytoplasm, where it forms complexes with other molecules (Fig. **1**); there, it exists in at least two distinct complexes: a rapamycin-sensitive complex (mTORC1) defined by its interaction with the accessory protein Raptor (rapamycin-associated protein of mTOR) and a rapamycin-insensitive complex (mTORC2) defined by its interaction with Rictor (rapamycin-insensitive companion of mTOR) [7-10]. The mTORC1 complex contains the proteins mTOR, Raptor, PRAS40 (proline-rich AKT substrate 40 kDa) and mLST8 (also known as GβL) [7, 8, 10, 11]. The mTORC2 complex is composed of mTOR, Rictor, mSIN1 (mammalian stress-activated protein kinase interacting protein 1), Protor-1 (protein observed with Rictor-1) and mLST8/GbL [8, 9, 12]. A recent study reported the identification of a novel element in the mTORC2 complex, named PRR5 (PRoline-Rich protein 5), which interacts with Rictor, but not Raptor in an mTOR-independent fashion [13]. Phosphorylation of downstream target proteins by mTORC1 leads to the initiation of cap-dependent translation, whereas very little is known regarding the regulation and function of mTORC2 [10, 14]. Although mTORC2 has been shown to regulate actin cytoskeleton dynamics, the only direct target of the mTORC2 identified to date is AKT (also known as protein kinase B, PKB). Indeed, mTORC2 phosphorylates AKT on a residue of serine (Ser^{473}) that contributes to its activation [9, 15, 16].

Studies during the past decade have shown that mTOR integrates two of the most important extracellular and intracellular signaling pathways involved in the regulation of cell growth: growth factors and nutrients availability. Growth factors, such as insulin or insulin-like growth factors, and nutrients, such as amino acids or glucose, enhance mTOR function, as evidenced by increased phosphorylation of its effectors, the best studied of which are ribosomal S6 kinase 1 (S6K, formerly known as $p70^{S6K}$) and eukaryotic initiation factor 4E-binding protein 1 (4EBP-1) [1, 17, 18] (Fig. **1**).

High levels of dysregulated mTOR activity are associated with several human deseases including hamartoma syndromes and cancers (see also Table **1**). Many studies, have shown that the mTOR-regulated growth pathway is constitutively activated in numerous malignancies, suggesting mTOR as an attractive target for cancer therapy [19-21]. In this review, we discuss recent progresses in the undestranding of mTOR biology and potential therapeutic opportunities for using mTOR inhibitors in cancer therapy, either as single agents or in combination.

THE mTOR SIGNALING PATHWAY

mTOR is a critical protein that integrates signals that link the ability of cells to progress through cell cycle to the availability nutrients in their extracellular and intracellular environment [17] (Fig. **1**). The phosphoinositide 3-kinase (PI3K) signaling cascade is a key pathway that regulates mTOR signalling in response to growth factor stimuli and AKT, a downstream effector kinase of PI3K, has emerged as a critical mediator of mTOR activity [22]. Recent results provide strong evidence that AKT might stimulate mTORC1 activity through phosphorylation/inactivation of the negative mTOR regulator TSC2 (tuberous sclerosis 2, also known as tuberin) and phosphorylation of PRAS40. TSC2 and TSC1 (also known as harmartin) form a complex that acts directly

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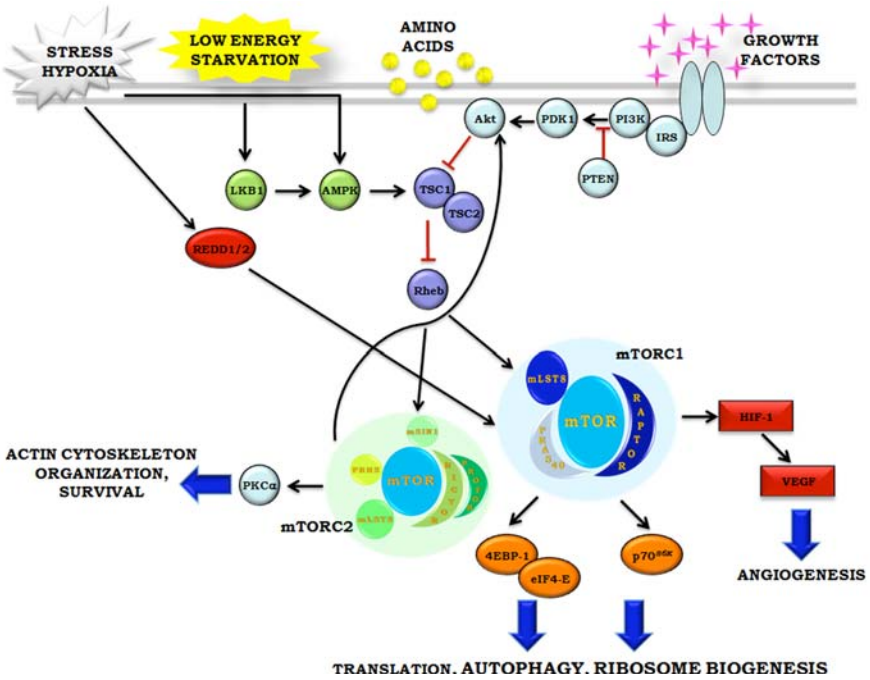

Fig. (1). Organization of the mTOR signaling pathway.

Table 1. Inherited Syndromes Involving Clear Cell and Non-Clear Cell RCC

Syndrome	Gene (Chromosome)	Protein	Normal protein function	Renal involvement	Other clinical manifestations
VHL	VHL (3p25)	pVHL	Tumor suppressor; destabilizes HIF transcription factors, prevents pseudohypoxia	Clear cell RCC	Pheocromocytoma, pancreatic endocrine tumors, CNS and retinal hemangioblastomas
TSC	TSC1 (9q34) TSC2 (16p13)	Hamartin Tuberin	Tumor suppressors, inhibit downstream mTOR activation by inhibiting Rheb activity	Angiomyolipomas, renal cysts, multiple RCC forms (clear cell, papillary, chromophobe)	Skin and brain tumors, cardiac rabdhomyomas, neurological disorders, seizures
HPRC	MET (7q31)	MET	Proto-oncogene, RTK, binds HGF	Type I papillary RCC	-
HLRCC	FH (1q42.3-q43)	Fumarate hydratase	Krebs cycle enzyme, converts fumarate to malate, prevents pseudohypoxia	Type II papillary RCC	Skin and uterine leiomyomas, uterine leiomyosarcomas
BHD	BHD (17p11.2)	Folliculin	Putative tumor suppressor, interacts with AMPK to inhibit mTOR signalling	Multiple RCC forms, including hybrid oncocytic tumors	Fibrofolliculomas, skin tags, pulmonary cysts, pneumothorax
HPT-JT	HRPT2 (1q21-32)	Parafibromin	Interacts with PAF1 and RNA polymerase II to mediate transcription elongation	Multiple RCC forms, Wilms tumors	Hyperparathyroidism, parathyroid carcinoma, fibro-osseous jaw lesions

VHL: von Hippel-Lindau; TS: Tuberous sclerosis complex; HLRCC: Hereditary leiomyomatosis and renal cell cancer; BHD: Birt-Hogg-Dubè; HPRC: Hereditary papillary renal cell carcinoma; HPT-JT: Hyperparathyroidism-jaw tumor; RCC: renal cell carcinoma; RTK: receptor tyrosine kinase; mTOR: mammalian target of rapamycin.

downstream of the serine/threonine kinase AKT and senses signal transduction from a large number of distinct signaling pathways to modulate mTORC1 activity. Within the TSC1-TSC2 complex, TSC1 stabilizes TSC2 and prevent its ubiquitin-mediated degradation [23, 24], while TSC2 acts as a GTPase actativing protein (GAP) for the Ras-related small G protein Rheb (Ras homolog enriched in brain), which associates with and directly activates mTORC1 when in its GTP-bound active form [25-27]. Genetic and biochemical studies suggest two possible mechanisms by which Rheb regulates the kinase activity of mTORC1. In the first model, the TSC1/TSC2 complex acts as a GAP for Rheb, promoting the conversion of Rheb-GTP to Rheb-GDP under poor growth conditions, while under optimal growth conditions Rheb-GTP accumulates and binds directly to mTOR within the mTORC1 complex, thereby activating it. Rheb-inhibitory effects of the TSC complex are attenuated by AKT-catalyzed TSC2 phosphorylation [27, 28]. According to the second

model, the FKBP38 protein binds mTORC1 complex inactiving mTOR under poor growth conditions, while inhibition of the GAP function of TSC2 shifts the balance of its substrate Rheb-GDP to the Rheb-GTP which, in turn, binds to FKBP38 and triggers its release from mTORC1, thereby stimulating mTORC1 activation [29-31]. Recently, PRAS40 was identifed as an AKT substrate that appears to negatively regulate mTORC1. Phosphorylation of PRAS40 by AKT disrupts the binding of PRAS40 to the mTORC1 complex, thereby relieving PRAS40-mediated inhibitory constraint on mTORC1 activity [11, 32-35]. Availability of nutrients represents an important regulator of mTOR activity [36]. Recent studies reveal that the AMP-activated protein kinase (AMPK; also known as PRKAB1) serves as the 'energy sensor' for mTORC1. Energy depletion results in an increase in the intracellular levels of AMP, which in turn binds to AMPK triggering its subsequent activation by upstream kinases. The key upstream activator of AMPK is the serine-threonine kinase liver kinase Bl (LKB1; also known as STK11), a known tumor suppressor [37]. Once active, AMPK phoshorylates TSC2 to increase energy-generating catabolic processes and decrease energy-depleting anabolic pocesses such protein syntesis [28]. However, in TSC2-null cells, mTORC1 activity can still be inhibited by cellular energy-depletion, suggesting that additional components in this pathway can signal energy stress to mTORC1 [38, 39]. A recent study proposes a TSC2-independent mechanism by which AMPK can signal poor energy condition to mTORC1 [39]. This study shows that AMPK directiy phosphorylate the mTOR-binding partner Raptor on two well-conserved serine residues, resulting in the inhibition of mTORC1 activity. These new findings suggest that, similar to TSC2, Raptor is a major signal integrator that interprets cell growth cues as well as energy sufficiency.

In addition to growth factors and cellular energy levels, other envirionmental stressor also regulate mTOR signaling. For example, availability of oxygen is also essential for cellular metabolism and long-term hypoxic stress results in energy deprivation and contributes to LKB1- or AMPK-mediated mTORCl inhibition [36, 40]. Hypoxia conditions and cellular stress quickly induce cells to limit energy expenditure by inhibiting energy-consuming processes, such as protein synthesis [40]. This rapid response is mediated by two mechanisms, both of which involve regulation of the TSC1-TSC2 complex. Hypoxia cusase stress and activates AMPK thereby inhibing mTORC1 through AMPK-mediated phosphorylation and activation of the TSC1/TSC2 complex [40]. However, AMPK-independent effects of hypoxia on mTORC1 have also been described. This mechanism appears to involve two novel genes, called REDD1 and REDD2 (regulated in develompment and DNA damage responses), which suppress mTORC1 activity by direct downregulation of ribosomal $p70^{S6K}$ phosphorylation, upregulation of the hypoxia inducible factor 1 (HIF-1), and indirect activation of the TSC1/TSC2 complex [41]. While many studies have clarified the role of the mTORC1 complex, less evidence and understanding is currently available regarding the role of the mTORC2 complex. Similar to mTORC1, mTORC2 is also stimulated by growth factors and nutrients and functions as a kinase that phosphorylates AKT, thus indicating that mTORC2 is an up-stream regulator of mTORC1 [9, 16, 42]. This finding makes mTORC2 a key part of the pathway that activates AKT and, like PDK1 (3-phosphoinositide-dependent protein kinase 1) and PI3K, a potential drug target for cancers in which there is AKT deregulation. The AKT-activating function of mTORC2 sets up the intriguing situation in which mTOR, as part of two distinct complexes, is potentially both "upstream" and "downstream" of itself.

mTOR regulates cell growth through its downstream effectors such as the translation regulators 4EBP-1 and $p70^{S6K}$, which contain a TOR signaling motif, mediating their interaction with Raptor and thus facilitating their recruitment to the mTOR kinase [1, 17, 18]. 4E-BP1 acts as a translational repressor, inhibiting 5'-cap-dependent mRNA translation (which encompasses the majority of cellular translation) by binding and inactivating eIF4E. 4E-BP1 hyperphosphorylation leads to the release of eIF4E, allowing initiation of translation [43, 44]. eIF4E enhances cell proliferation, survival, and angiogenesis by leading to selective translation of mRNA coding for proteins such as cyclin D1, Bcl-2, Bcl-xL and vascular endothelial growth factor (VEGF) [45, 46]. $p70^{S6K}$ activation is initiated by mTORC1-mediated phosphorilation of Thr389. Although a full $p70^{S6K}$ activation requires multiple growth factor-induced phosphorylation events, the phosphorylation at Thr389 by mTOR is required for its activation since the substitution of this residue with other amino acids blocks its activity [47]. By acting on S6K, mTOR facilitates ribosome biogenesis and translation elongation [43, 48] (Fig. **1**).

mTOR INHIBITORS AS ANTI-CANCER AGENTS

mTOR activation is mediated by upstream signals, which are commonly deregulated in human cancer [49]. To date, no cancer-associated mutations in the TOR genes have been identified; however, many genetic aberrations located either upstream or downstream of mTOR in the PI3K/AKT/mTOR signaling pathway have been implicated in cancer development. PI3K/AKT/mTOR 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 in the PTEN gene [50, 51]. Besides, amplification and overexpression of eIF4E and $p70^{S6K}$ genes and proteins are observed in many tumor types [52, 53]. These data indicate that the mTOR pathway plays an essential role in maintaining the trasformed phenotype, supporting an important role of this pathway in the biology of human cancers [1, 14, 51, 54].

Drugs inhibiting mTOR have been instrumental in clarifying the functionality of key mTOR components (Fig. **2**). Rapamycin (also known as sirolimus) is a macrolide antibiotic produced by *Streptomyces hygroscopicus*, a bacterial species native to the Easter Island, with immunosuppressant and anticancer properties. Rapamycin forms a complex with FK506 binding protein (FKBP12), which in turn binds to mTOR, suppressing mTORC1-mediated phosphorylation of the substrates $p70^{S6K}$ and 4EBP1 [55-58]. In contrast to mTORC1, the FKBP12–rapamycin complex cannot bind directly to mTORC2, suggesting that the effects of rapamycin on cellular signaling are due to inhibition of mTORC1 [9, 15]. A potentially important wrinkle in this seemingly

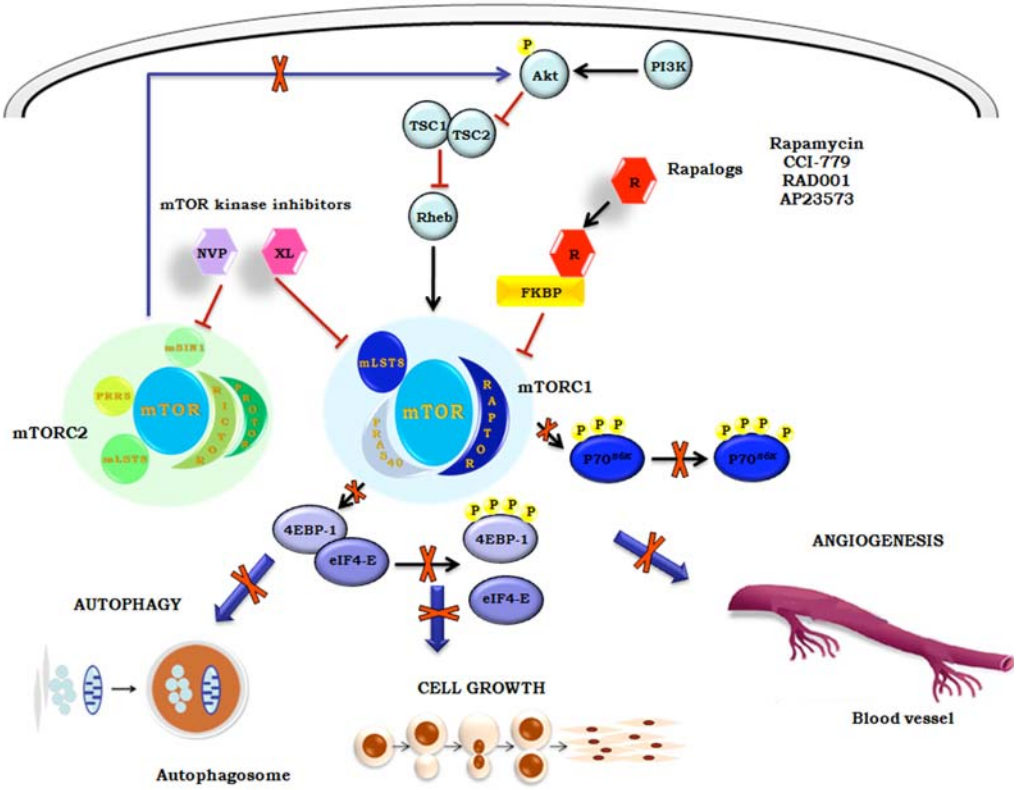

Fig. (2). Anticancer effects of clinical mTOR inhibitors.

closed story has recently emerged [59]. It turns out that prolonged treatment with rapamycin – clearly a situation that is relevant to its use in patients – perturbs mTORC2 assembly and, in about 20% of cancer cell lines, the drop in intact mTORC2 levels is sufficient to strongly inhibit AKT signaling. The binding of FKBP12-rapamycin to mTOR seems to block their subsequent binding to the mTORC2-specific components Rictor and mSin1 [59, 60] but it is unknown why in certain cell types rapamycin only partially inhibits mTORC2 assembly. No absolute correlation exists between the tissue of origin of a cell line and the sensitivity of mTORC2 formation to rapamycin, although many cell lines with this property are derived from the hematological system. Recent work provides the first evidence that mTORC2 function can be rapamycin-sensitive in patients. In more than 50% of patients with AML, rapamycin and its analogs inhibited AKT phosphorylation in primary leukemic cells and the inhibition correlated with the loss of intact mTORC2 [61]. Thus, rapamycin and its analogs are universal inhibitors of mTORC1 and S6K1, and cell-type specific inhibitors of mTORC2 and AKT. As the inhibition of mTORC2 by rapamycin is time and dose dependent, AKT activity in tumors will vary with the length of rapamycin treatment and the dosing regimen. It is important to keep in mind that, because inhibition of mTORC1 and mTORC2 will not always occur at the same time, markers of mTORC1 inhibition, such as loss of phosphorylated S6, will not necessarily reflect mTORC2 activity. The capacity to sometimes inhibit mTORC2 might help explain why the cellular effects of rapamycin vary among cancer cell lines. Moreover, in a tumor this inhibition might have the beneficial effect of preventing the activation of AKT, through inhibition of S6K1, that rapamycin would otherwise cause.

Analogues of rapamycin, such as temsirolimus (also known as CCI-779; Wyeth), everolimus (also known as RAD001; Novartis) and deforolimus (also known as AP23573; Ariad Pharmaceuticals), are the first mTOR-perturbing molecules to be approved for anticancer use in humans. These molecules inhibit mTORC1 through the same mechanism of action as rapamycin, but have different pharmacokinetic and solubility properties that increase their desirability for clinical use .

Temsirolimus is a synthetic, rapamycin ester available in oral and intravenous formulations. Upon injection, temsirolimus is rapidly converted to rapamycin, which is probably responsible for most of its pharmacological effects. The anticancer activity of temsirolimus likely involves multiple pharmacologic actions, including its antiangiogenic and antiproliferative properties [62, 63]. Preclinical studies indicate that temsirolimus has synergistyc effects in combination with conventional cytostatic agents [64, 65]. This drug was the first of its class to receive FDA approval, and current indications include the treatment of poor-risk, untreated, advanced renal cell carcinoma (RCC) patients (see below). Multiple clinical and preclinical studies have shown promising antitumor activity of temsirolimus in other tumor types, including breast cancer [66], glioma [67], and mantle cell lymphoma [68]. Everolimus is an oral mTOR inhibitor with antineoplastic activity similar to other rapamycin-like inhibitors. In clinical pharmacokinetic studies, it was found to have a relatively low bioavailability. Everolimus is currently approved by the FDA for the treatment of patients with ad-

vanced RCC after the failure of treatment(s) with VEGF receptor tyrosine kinase inhibitors. Deforolimus is a non-prodrug rapamycin analog, currently under clinical testing. Antitumor activity was seen in non small cell lung cancer (NSCLC), carcinosarcoma, RCC, and Ewing sarcoma [69-71].

A new generation of mTOR inhibitors is being developed (Fig. **2**). New mTOR kinase inhibitors are small molecules designed to compete with ATP at the catalytic site of mTOR. In contrast to rapamycin-like inhibitors that only target mTORC1, these molecules inhibit both mTORC1 and mTORC2. Agents such as NVP-BEZ235 (Novartis) and XL765 are currently being tested to evaluate their tolerability and anticancer activity. These drugs are dual inhibitors of PI3K and downstream mTOR, whose activity in advanced solid tumors is being evaluated in phase I/II clinical trials [72, 73].

MOLECULAR MECHANISMS UNDERLYING THE ANTI-CANCER EFFECTS OF mTOR INHIBITORS

Rapamycin and its analogues can inhibit several processes that are regulated by mTOR, including cell proliferation, survival, and angiogenesis (Fig. **2**). The main effect of mTOR inhibition in many tumor cell lines is growth retardation. Indeed, mTORC1 contributes to overall cap-dependent translation by phosphorylation of 4EBP-1; inhibition of mTOR results in a block of phosphorylation of 4EBP-1, resulting in the sequestration of eIF4E and failure to form the m^7GTP cap-dependent preinitiation complex, greatly reducing translation of m^7GTP cap containing transcripts. Many of these m^7GTP cap-containing transcripts encode proteins required for cell cycle progression, including cyclin D1. Multiple evidence indicate that inhibiting this mechanism of translational control contributes to the cytostatic effects of rapamycin-like drugs [74]. The inhibition of mTOR leads to increased levels of the cyclin-dependent kinase 2 (CDK2) inhibitor, $p27^{kip1}$, an inhibitor of G1-S cell cycle progression [75, 76]. Induction of $p27^{kip1}$ by rapalogs appears to be a significant contributor to the block in cell proliferation observed *in vivo* and in some cell lines [76, 77].

A case in point is the effect of mTOR inhibition on apoptosis, which varies depending on which cell types are tested. There are some reports of rapamycin promoting pro-apoptotic effect but there are also reports of it promoting cell survival [54, 78-80]. The effect of mTOR inhibition on apoptosis might correlate with its varying effects on AKT, an anti-apoptotic molecule that regulates cell survival. In fact, studies have shown that when rapamaycin inhibits mTORC1, but not mTORC2, AKT is activated by mTORC2 and the drug might protect against apoptosis. Conversely, in cells in which the drug inhibits mTORC2 and AKT, it may promote apoptosis [59]. Among the primary cells that undergo apoptosis are certain populations of dendritic cells, and renal tubular cells [81, 82].

mTOR is also involved in the regulation of the autophagic process and represents the major negative regulator of autophagy in human cells [83, 84]. Then, if mTOR is inactive autophagy proceeds, while when mTOR is activated the autophagic process is inhibited. The first evidence that mTOR has a role in regulating autophagy came from experiment involving rat hepatocytes that showed that rapamaycin partially reverses the inhibitory effects of amino acids on autophagy proteolysis [85]. The autophagy-stimulatory effect of rapamycin and its analogs has since been confirmed in different models [86]. This results suggest that TORC1 is directly involved in the regulation of autophagy.

A particularly interesting property of rapamycin and its analogues is their ability to suppress angiogenesis [63] . Angiogenesis is a fundamental process for both solid and hematologic malignancies, by which a tumor develops its own blood supply by hijacking the surrounding vasculature to invade the growing tumour mass. mTOR plays a key role in the formation of new blood vessels to provide oxygen and nutrients to growing and dividing cells [87, 88]. Specifically, mTOR regulates the translation and activity HIF1-α, the inducible subunit of the HIF transcription factor, a master regulator of the expression of a wide variety of genes whose products play a role in angiogenesis (such as VEGF, PDGF-β, TGF-α, and Ang-1) [87, 88]. VEGF attracts vascular endothelial cells to hypoxic areas where new blood vessels are needed and orchestrate the formation of these blood vessels. For example, VEGF production is increased in HER2/neu-positive breast cancer cells and is offset by temsirolimus treatment *in vitro,* thereby reducing angiogenesis *in vivo*. This effect correlates with temsirolimus-mediated inhibition of HIF-1α expression [62, 89]. In addition to their effects on VEGF production, rapamycin and its analogs may also directly inhibit endothelial cell proliferation and tube formation resulting in significant inhibition of angiogenesis, tumor growth and metastasis [21, 63, 90]. Clearly, not all tumor types will respond to these rapalogs, and although constitutive mTORC1 activation might be widely observed in a given cancer subtype, the genetic context in which this aberrant phenotype is expressed plays a determinant role in patient sensitivity or resistance to rapalog therapy [91, 92] (see below).

CLINICAL APPLICATIONS OF mTOR INHIBITORS

mTOR Inhibitors in RCC

The first indication approved for mTOR inhibitors in clinical oncology is the treatment of advanced renal cell carcinoma (RCC) [93]. The rationale for targeting mTOR in RCC stems from the observation that most RCC tumors exhibit dysfunctional signaling pathways that either increase the activity of mTOR or depend on mTOR activity for their pathology. Although most of RCC cases occur in a sporadic form, both clear cell and non-clear cell RCC can occur in the context of inherited cancer syndromes, whose molecular genetics has shed light on the pathogenetic mechanisms of different RCC subtypes [94, 95] (Table **1**). This is probably best exemplified by von Hippel-Lindau disease (VHL, [96]) and tuberous sclerosis (TS, [97]), two autosomal dominant inherited syndromes with variable penetrance that carry a high lifetime risk of developing clear cell RCC. The VHL gene, which targets HIF-1α for degradation by the proteasome, is mutated or silenced in up to 75% of sporadic clear cell RCC, suggesting that genetic abnormalities involved in

inherited RCC syndromes (and subsequent alterations downstream intracellular signaling cascades) may also play a central role in sporadic RCC and maybe utilized to develop novel, target-oriented, treatment strategies. In tumors carrying a mutated VHL, the increased levels of HIF-1α play a critical oncogenic role, including stimulation of VEGF transcription. Activated mTOR, in turn, exacerbates the loss of VHL function by further elevating HIF-1α through increased translation. Indeed, in a murine xenograft model, RCC tumors with higher HIF-1α levels were more sensitive to mTOR inhibition than tumors with lower HIF-1α levels [98]. Because unregulated angiogenesis is a prominent feature of RCC, the inhibition of mTOR is relevant clinically and may inhibit angiogenesis through a mechanistic approach that differs from that of VEGFR-targeted agents [62]. TS, on the other hand, is an autosomal dominant disorder with 95% penetrance, caused by mutations in either the *TSC1* (9q34) or the *TSC2* (16p13.3) genes, encoding for the hamartin and tuberin proteins, respectively. Hamartin and tuberin physically interact to form a complex, which, through the GAP activity of tuberin, inactivates the small G protein Rheb, thereby relieving Rheb-mediated mTOR inhibition (see above). Therefore, genetic inactivation of *TSC1/2* results in the uncontrolled activation of the mTOR pathway, leading, among other effects, to increased synthesis and accumulation of HIF-1, even in the absence of hypoxia, and transcription of HIF-dependent genes. In addition to clear cell RCC, the spectrum of renal manifestations in TS also includes development of multiple angiomyolipomas, renal cysts, and non-clear cell RCC (papillary and chromophobe carcinomas). Other hereditary RCC syndromes involving non-clear cell RCC have also been identified and characterized in terms of the underlying genetic lesions (Table **1**). Interestingly, common molecular themes underlying renal carcinogenesis can be identified by combined genetic analysis of heritable RCC forms and molecular profiling of sporadic cases. For example, activation of the mTOR pathway appears to be central to the development of different renal manifestations of disease, including benign (angiomyolipomas, renal cysts, oncocytomas), borderline (hybrid oncocytic tumors), and frankly malignant (papillary and chromophobe RCC) lesions. Indeed, genetic aberrations of both *TSC1/2* and *BHD* directly impinge on the activation of the mTOR pathway, leading to the development of an array of renal lesions that can be partially reversed by rapamycin-mediated inhibition of mTOR, both in preclinical models and human patients with TSC [99-105]. In the highly aggressive papillary type 2 RCC observed in HLRCC, FH deficiency creates a pseudohypoxic intracellular environment, leading to HIF-1α accumulation; from a molecular standpoint, this situation is similar to that observed in *VHL* mutant RCC cells, where HIF-1α translation and accumulation can be prevented by temsirolimus-mediated mTOR inhibition, thereby rendering HIF-1α-overexpressing cells particularly prone to the growth inhibitory effects of temsirolimus, both *in vitro* and *in vivo* [98]. More recently, computational analysis of gene expression data derived from papillary RCC revealed that a transcriptional signature indicative of MYC pathway activation is present in high-grade type 2 papillary RCC. The MYC signature was associated with amplification of chromosome 8q and overexpression of MYC that maps to chromosome 8q24 and, reflective of the association of an active MYC signature component with papillary type 2, the presence of this pathway signature component was also associated with a highly aggressive clinical behavior and poor overall survival [106]. Recent evidence indicates the existence of an important growth-regulatory crosstalk between the MYC and the mTOR pathway, mediated by the regulation of tuberin (TSC2) expression: indeed, although overexpression of Drosophila MYC and TSC1/2 cause opposing growth and proliferation defects, transcriptional controls are potentially important regulators of tuberin expression and MYC is a direct repressor of its expression. Since tuberin loss de-represses MYC protein, the connection between these two growth regulators is positioned to act as a feed-forward loop that would amplify the oncogenic effects of decreased tuberin or increased MYC [107], again suggesting a possible therapeutic role of mTOR inhibitors in RCC subtypes caused by either TSC loss or MYC gain. Consistent with the above-highlighted central position of the mTOR cascade along different molecular pathways that lead to an array of diverse renal lesions, including both clear cell and non-clear cell RCC, other components of the mTOR have recently been foung dysregulated in sporadic RCC: loss or inactivation of PTEN occurs frequently in RCC and is a prognostic indicator of poor survival; PTEN, p27, phosphorylated AKT, and phospho-S6K1 ribosomal protein may predict prognosis and may serve as surrogate parameters for the selection of candidates for treatment with mTOR inhibitor therapy [108]. Overall, both genetic and molecular data strongly indicate that common avenues do exist in renal carcinogenesis and that mTOR activation may represent a common molecular theme across different benign renal lesions and RCC subtypes, including both clear cell and non-clear cell forms, and may therefore constitute a widespread therapeutic target in both sporadic and familial RCC.

From a clinical standpoint, two mTOR-targeted agents have recently been approved for the treatment of advanced, metastatic 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 [109]. Patients who received temsirolimus alone had a significantly longer overall survival (OS) and progression-free survival (PFS) than patients who received interferon alone. 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 [110]. 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 to 0.4; $P<0.0001$) 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. The benefits of mTOR inhibition in the second-line setting are being explored further in a nation-wide phase 2 trial conducted in Italy, open to patients progressing after any first-line treatment, as well as

in an international randomized phase 3 trial of temsirolimus compared with sorafenib in sunitinib-refractory patients. In that trial, sorafenib is an active comparator rather than the placebo comparator that was used in the everolimus phase 3 trial. Various combination regimens that include mTOR inhibitors also are being explored in patients with solid tumors, including RCC. For example, results have been reported for phase 1 dosing studies of temsirolimus or everolimus in combination with sorafenib, bevacizumab, erlotinib, gefitinib, or imatinib. Overall, acceptable tolerability has been observed with mTOR inhibitors combined with other agents. One exception was reported in a phase 1 dose-escalation study of sunitinib plus temsirolimus, which indicated that this combination was poorly tolerated in patients with advanced RCC, even at the lowest dose level of temsirolimus (reviewed in [93, 111]). A large randomized phase 3 trial (Investigation of Torisel and Avastin Combination Therapy; INTORACT) is evaluating the efficacy and safety of the combination of temsirolimus plus bevacizumab versus bevacizumab plus IFN as first-line treatment in patients with advanced RCC and a similarly designed randomized phase 2 study is evaluating the combination of everolimus plus bevacizumab.

mTOR Inhibitors in Other Tumor Types

Rapalogs have been evaluated in several other cancer types. 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 and 35% in non–mantle-cell non-Hodgkin's lymphoma with temsirolimus [68, 112]. Temsirolimus activity was also demonstrated in multiple myeloma [113]. 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=0.0019). PFS rates were 4.8 months with the 75-mg weekly temsirolimus and 1.9 months with investigators' choice treatment (P=0.009) [114]. Rapamycin has led to regression of Kaposi's sarcoma in renal transplant recipients [115]. Everolimus and deforolimus have also shown antitumor activity in various hematologic malignancies [93]. In preliminary analysis of phase II trials, rapalogs have also shown promise in patients with sarcoma and endometrial cancer [93, 111]. Rapamycin has also been evaluated in syndromes of proliferative dysregulation. Clinical benefit has been reported with facial angiofibroma, renal angiolypomas, and lymphangiomyomatosis [101-104]. 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.

Combinations with Other Agents

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. Though everolimus improved the PFS for RCC that progressed on VEGFR-targeted therapy, the ORR was 1%. This is consistent with preclinical studies demonstrating that rapalogs, when used alone, are cytostatic in most tumor types (see above) and clinically may primarily stabilize disease. However, although mTOR plays a central role in many biologic processes, rapalogs have been generally well tolerated, making them attractive candidates for the development of combination strategies. Toxicities include asthenia, mucositis, nausea, cutaneous toxicity, diarrhea, hypertriglyceridemia, thrombocyopenia, hypercholesterolemia, elevated transaminases, hyperglycemia, and pneumonitis [116-118].

mTOR inhibitors have indeed been found to be additive or synergistic with several chemotherapeutic drugs (such as paclitaxel, carboplatin, cisplatin, vinorelbine, doxorubicin, and campthotecin) and combinations of rapamycin and its analogs in with a broad spectrum of chemotherapeutic agents are currently being evaluated in ongoing clinical trials (reviewed in [111]).

Rapamycin-induced AKT activation has increased interest in overcoming this feedback loop activation by using mTOR inhibitors in combination with antagonists of upstream signaling such as HER-2 or IGF-IR inhibitors. In HER-2–positive breast cancer cell lines, trastuzumab has been shown to inhibit feedback-loop activation of AKT [119]. This is especially notable as PTEN loss is a known mediator of trastuzumab resistance 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 [119]. The combination of everolimus and trastuzumab is currently being tested 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. IGF-IR inhibition prevents rapamycin-induced AKT activation and sensitizes tumor cells to mTOR inhibition in preclinical models [120]. Combinations of rapalogs and IGF-IR inhibitors are now being studied in clinical trials. In breast cancer, AKT/mTOR signaling has been associated with resistance to endocrine therapy in breast cancer [121], 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 (reviewed in [111]). However, the interim analysis of a phase III randomized placebo controlled trial of letrozole with or without temsirolimus reported no improvement in PFS, although the final analysis of such trial has not been published yet. 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. 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. The clinical and ultrasound-assessed response rate with everolimus and letrozole was significantly higher than letrozole alone, as it was cell cycle response. Thus, mTOR inhibition may in-

crease the efficacy of endocrine therapy, although at the expense of increased toxicity.

Finally 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 [122]. 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 [123] and thus theoretically may enhance rapamycin's antitumor activity. Randomized prospective trials are now being conducted to determine whether octreotide enhances the antitumor effects of mTOR inhibitors.

CONCLUSIONS AND FUTURE PROSPECTS

Signal transduction inhibitors, including inhibitors of the mTOR pathway, have successfully entered the clinical arena. Meanwhile, our knowledge of signal transduction pathways has evolved, over the past 20 years, from the classical notion of "linear" signaling pathways, whereby a single receptor would transduce signals through specific "intermediates" to a limited number of final "effectors", to the much more complex vision of "signaling networks", in which every single component is closely intertwined with an array of different players. This creates an extremely complex scheme of vertical and parallel signaling pathways regulated by positive and negative feedback loops. In this context, even the most specific interference with a single signaling component, as it is the case for rapalogs, may actually lead to unexpected, and sometimes "undesired" from a therapeutic perspective, functional outputs. Such a new level of complexity obviously requires completely novel strategies to approach both pathway investigation (for example, the use of high-throughput technologies and "omics" approaches) and interpretation of the results (the thriving science of "systems biology" applied to cancer biology and anticancer drug discovery). This may help explain why, in addition to a handful of success stories (such as the development of temsirolimus and everolimus for the treatment of RCC and mantle cell lymphoma), the clinical development of these and other mTOR inhibitors in other clinical settings (such as breast cancer) may be more troublesome. In addition to the inherent complexity of cancer signaling as a therapeutic target, these setbacks reflect a variety of other factors specifically related to the inadequacy of classical drug development paradigms when applied to "targeted" therapies, including a rush to get compounds into the clinic, a lack of validated biomarkers, insufficient characterization of patient populations appropriate for treatment, and oversight of pharmacodynamic and scheduling issues. Indeed, the major limit of mTOR-targeted therapeutic approaches currently lies in the lack of validated biomarkers that would enable the identification of patients at the highest likelihood of deriving a benefit from such a therapeutic approach.

Deeper understanding of the intricate signaling networks regulating mTOR activity and extensive preclinical and early clinical modeling, also taking into account indirect mechanisms of action that counteract paraphysiological processes which turn normal tissue surrounding the tumor into a powerful cancer ally (such as neo-angiogenesis), are expected to rapidly lead to the effective translation of exciting preclinical findings into new therapeutic strategies for our patients suffering from cancer.

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ABBREVIATIONS

mTOR	=	mammalian Target Of Rapamycin
PIKK	=	phosphoinositide kinase–related kinase
Raptor	=	rapamycin-associated protein of mTOR
mTORC1	=	Rapamycin-sensitive complex
mTORC2	=	rapamycin-insensitive complex
Rictor	=	rapamycin-insensitive companion of mTOR
PRAS40	=	proline-rich AKT substrate 40 kDa
mLST8	=	mammalian lethal with Sec13 protein 8
mSIN1	=	mammalian stress-activated protein kinase interacting protein 1
Protor-1	=	protein observed with Rictor-1
PRR5	=	PRoline-Rich protein 5
AKT	=	protein kinase B
Ser	=	serine
4EBP-1	=	eukaryotic initiation factor 4E-binding protein 1
$p70^{S6K}$	=	p70 ribosomal S6 kinase
PI3K	=	the phosphoinositide 3-kinase
TSC2	=	tuberous sclerosis 2
TSC1	=	tuberous sclerosis 1
PRAS40	=	proline rich AKT substrate 40 KDa
GAP	=	GTPase actativing protein
Rheb	=	Ras homolog enriched in brain
GDP	=	guanosine diphosphate
GTP	=	guanosine 5' triphosphate
FKBP12	=	FK506 binding protein 12
FKBP38	=	FK506-binding protein 38
AMPK	=	AMP-activated protein kinase
LKB1	=	liver kinase B1
HIF-1	=	hypoxia inducible factor 1
PDK1	=	3-phosphoinositide-dependent protein kinase 1

eIF4E = eukaryotic translation initiation factor 4E

Bcl-2 = B cell lymphoma gene-2

Bcl-xL = B-cell leukemia XL

VEGF = vascular endothelial growth factor

HER-2 = human epidermal growth factor receptor 2

IGFR = insulin-like growth factor receptor

AML = acute myeloic leukemia

RCC = renal cell carcinoma

FDA = food and Drug Amministration

NSCLC = non small cell lung cancer

ATP = adenosine 5'-triphosphate

PDGF-β = platelet derived growth factor β

TGF-α = tumor growth factor α

Ang-1 = angiopoetin 1

VHL = von Hippel-Lindau disease

VEGFR = vascular endothelial growth factor receptor

BHD = Birt Hogg Dubè syndrome

HLRCC = hereditary leiomyomatosis and renal cell cancer

FH = fumarate hydratase

OS = overall survival

PFS = progression-free survival

Myc = myelocytomatosis viral oncogene homolog

INTORACT = Investigation of Torisel and Avastin Combination Therapy

IFN = interferon

ORR = objective response rate

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