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REVIEW

Functional consequences of mTOR inhibition

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TOR (target of rapamycin) is a serine-threonine protein kinase that is conserved across a diverse range of species from fungi to mammals. The signaling pathway that is anchored by TOR is also conserved across species. In mammals, mTOR integrates growth factor, amino acid, nutrient and energy sensing signals, and thus plays a major role in cell growth and proliferation, protein synthesis and autophagy. As a result of the pivotal role of mTOR in signaling, the aberrant regulation of mTOR has been implicated in several disease processes, including cancer, diabetes, ocular diseases and neurodegenerative disorders, as well as in lifespan extension. More recently, rapamycin (sirolimus) analogs that antagonize the mTOR signaling pathway have been approved for the treatment of several cancers. This review describes some recent advances in the understanding of mTOR signaling, with an emphasis on the functional consequences of mTOR inhibition and therapeutic intervention strategies.

Keywords Cancer, diabetes, lifespan extension, mTOR, ocular disease, rapamycin, sirolimus

Abbreviations

4EBP1 Eukaryotic translation initiation factor 4E binding protein, **IRS** insulin receptor substrate protein, **PIKK** phosphotidylinsoitol-3-kinase-related kinase, **PTEN** phosphatase and tensin homolog, **TOR** target of rapamycin, **TSC** tuberous sclerosis complex

Introduction

Rapamycin was initially discovered as an antifungal agent produced by the soil bacteria *Streptomyces hygroscopicus* [1]. Yeast genetic screens were used to identify the target of rapamycin (TOR) as a serine-threonine kinase. The availability of complete genomic sequences from various species has established that TOR is conserved across species ranging from fungi to mammals. mTOR belongs to the atypical kinase family of phosphotidylinsoitol-3-kinase-related kinases (PIKK) [2]. Sequence analysis has revealed that the kinase domain of TOR is more similar to lipid kinases than to canonical protein kinases. However, despite the high degree of sequence, and perhaps also structural, homology to lipid kinases, TOR has not been demonstrated to have lipid kinase activity. The homology of mTOR to lipid kinases suggests that ATP-competitive inhibitors of mTOR may also inhibit lipid kinases but are, in general, not expected to inhibit other protein kinases. The functional consequences of the homology of mTOR to lipid kinases have not yet been explained.

Regarding the pivotal role of mTOR in several diseases, an increasing quantity of knowledge has been derived from clinical trials, animal disease models and cellular studies. The diseases in which mTOR has been implicated include cancer [3], obesity and diabetes [4], diseases of the eye [5], neurodegenerative disorders [6] and cognitive dysfunction [7]. More recently, rapamycin (sirolimus) analogs (termed rapalogs; eg, everolimus [RAD-001] and temsirolimus) have been approved for clinical use for the treatment of certain cancers. This review focuses on the functional consequences of mTOR inhibition and outlines therapeutic intervention strategies. The mTOR signaling pathway is described, with an emphasis on the complexity of the pathway and the resultant multiple potential therapeutic intervention strategies. Several recent discoveries highlighting the role of mTOR in lifespan extension and diabetes are summarized, and recent clinical developments involving mTOR inhibitors in cancer and ocular diseases are highlighted. Finally, various classes of mTOR inhibitors and therapeutic intervention strategies are described.

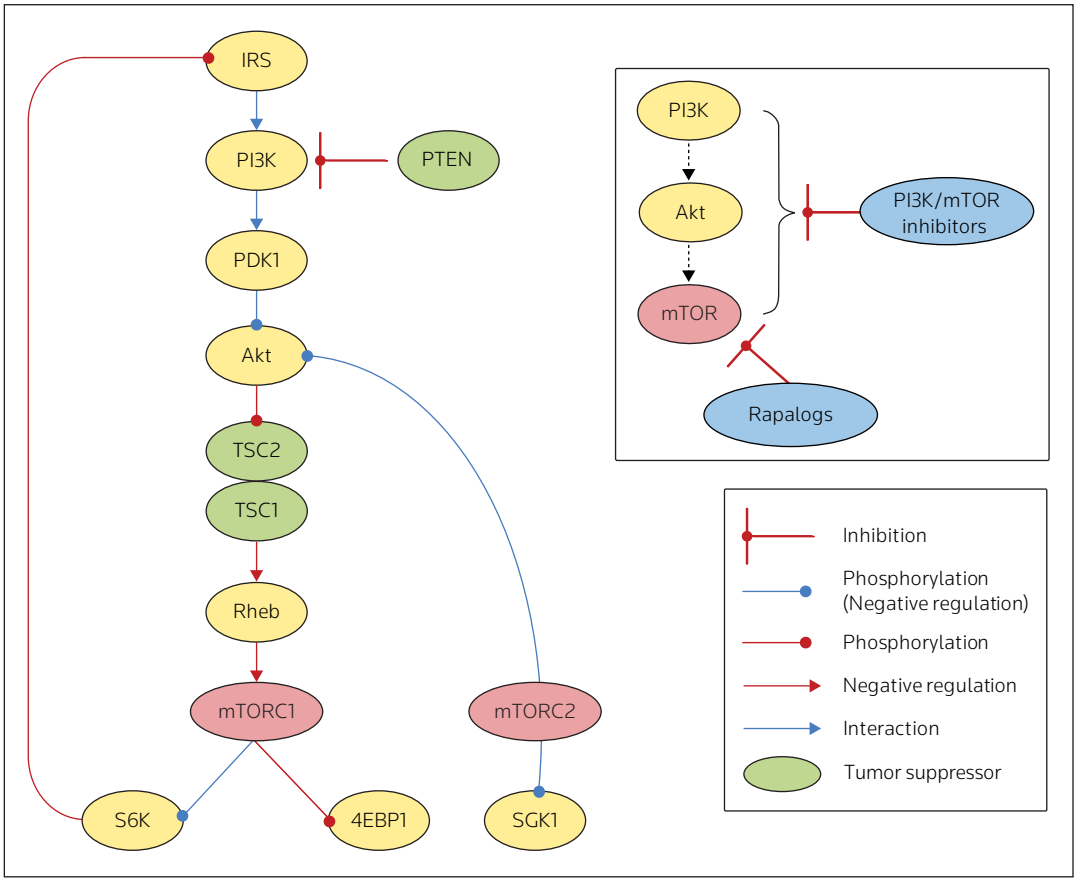
mTOR signaling components

mTOR is a highly conserved serine-threonine protein kinase that belongs to the 6-member PIKK family of proteins and has a high degree of homology to the lipid kinase PI3K. Yeast have two TOR genes, *TOR1* and *TOR2*, both of which participate in two functionally distinct complexes that are known as TORC1 and TORC2, respectively. FPR1, which is the yeast homolog of human FK506 binding protein 12 (FKBP12) and is a peptidyl-prolyl cis-trans isomerase, binds to rapamycin, and the FPR1-rapamycin complex subsequently binds upstream of the kinase domain of the TORC1 complex and inhibits its activity. The TORC2 complex in yeast is rapamycin-insensitive. In mammalian

species, TOR exists as a single gene that forms two functionally distinct complexes, mTORC1 and mTORC2, with mTOR being present in both complexes. Similar to yeast, rapamycin binds to FKBP12, and the FKBP12-rapamycin complex then binds to the FRB domain upstream of the catalytic domain of mTORC1 and inhibits its activity [8]. Prolonged exposure to rapamycin has been demonstrated to disrupt the assembly of mTORC2 complex in several cell types [9], and therefore rapamycin could also be considered to be an incomplete inhibitor of mTORC2. The two mTOR complexes have distinct interactions with their respective substrates and regulators, leading to the diverse functional roles of mTOR in processes such as nutrient, energy and amino acid sensing, the initiation of transcription, the regulation of protein translation, cell proliferation and autophagy. mTORC1 has been implicated in translation initiation, protein biosynthesis, autophagy, and amino acid and nutrient sensing; mTORC2 has been implicated in actin organization. The main components of the mTOR pathway and their interactions are presented in Figure 1 (additional details can be obtained from the references [10-12]).

The activation of mTOR by the PI3K-Akt axis has been thoroughly investigated. In response to binding insulin, IGFR activates an intracellular signaling cascade mediated by PI3K. The lipid phosphatase PTEN (phosphatase and tensin homolog) negatively regulates PI3K signaling and is frequently deleted in tumors [13]. An important target of PI3K signaling is the protein kinase Akt, which is involved in a variety of biological functions, including survival, growth and apoptosis. Akt is also overactive in various tumors and can be activated by phosphorylation at two key residues: 3-phosphoinositide-dependent kinase 1 (PDK1) phosphorylates Akt at the Thr^{308} residue; and the 'PDK2' kinase, the identity of which remained unknown until recently, phosphorylates Akt at the Ser^{473} residue. Investigators have since demonstrated that PDK2 is mTORC2 [14-16]. The discovery that Akt can be phosphorylated by mTORC2 places Akt and mTOR both

Figure 1. The mTOR pathway and therapeutic intervention points.



Insulin receptor substrate (**IRS**) is activated by the IGFR upon insulin and growth hormone binding. PI3K plays a key role in insulin signal transduction and activates Akt via 3-phosphoinositide-dependent protein kinase 1 (**PDK1**). The phosphatase and tensin homolog (**PTEN**) protein is a negative regulator of PI3K that is frequently deleted in tumors. Akt phosphorylates and inactivates the tuberous sclerosis complex (**TSC**). Ras homolog enriched in brain (**Rheb**) is a small GTPase that is negatively regulated by TSC and is converted to its active form upon the inactivation of TSC by Akt. Thus, the activation of Akt in cancer cells results in the inhibition of TSC, leading to the activation of mTORC1 via the activation of Rheb. The phosphorylation of the serine-threonine kinase S6K by mTORC1 eventually results in the regulation of protein translation. There are two feedback loops in this pathway: mTORC2 can phosphorylate Akt and activate downstream signaling, and S6K can directly phosphorylate IRS and inhibit IRS-mediated insulin signaling. Rapalogs are allosteric inhibitors of the mTORC1 complex, and dual PI3K-mTOR inhibitors target the catalytic domains of PI3K and mTOR. The inset highlights targets of rapalogs and dual PI3K-mTOR inhibitors.

downstream and upstream of each other in the signaling cascade, leading to a feedback loop. Tuberous sclerosis complex (TSC), which consists of TSC1 and TSC2, is an inhibitor of mTOR. Akt phosphorylates and inactivates TSC, thereby relieving the inhibition of mTOR by TSC. Ras homolog enriched in brain (Rheb) is a small GTPase that is negatively regulated by TSC and is converted to its active form upon the inactivation of TSC by Akt. Thus, the activation of Akt in cancer inhibits TSC, leading to activation of mTORC1 via activation of Rheb. A 2004 study in *Drosophila* suggested that TSC2 was not a critical substrate of Akt in normal development [17]. However, more recent research has demonstrated that Akt phosphorylates both TSC1 and TSC2 in *Drosophila*, but that Akt phosphorylation is not required for normal growth [18]. Ribosomal kinase S6K and 4EBP1 (eukaryotic translation initiation factor 4E binding protein; a negative regulator of the eukaryotic translation initiation factor EIF4E) are direct phosphorylation substrates of mTORC1. Novel substrates of mTORC1 and mTORC2 are continuing to be identified, demonstrating the complexity of the pathway. In addition to Akt, mTORC2 also phosphorylates the serum/glucocorticoid kinase SGK1 [19]. SGK1 plays an important role in the cellular stress response; however, the functional significance of the interaction between mTORC2 and SGK1 is not completely understood. Finally, S6K is also involved in a feedback loop and phosphorylates the insulin receptor substrate protein IRS-1, thus attenuating insulin-mediated PI3K-Akt-mTOR signaling [20].

Another complexity of the mTOR pathway is the crosstalk between mTOR and MAPK, as well as potentially other pathways [21]. In addition to signaling through the PI3K-Akt pathway, insulin receptor signaling can activate the MAPK cascade. ERK, a key component of the MAPK signaling cascade, phosphorylates and inhibits TSC [22]. In addition to insulin and growth factor signaling, mTOR also integrates inputs from energy, nutrient and amino acid levels [23].

The role of mTOR in oncology

The role of mTOR signaling in cancer has been the subject of numerous studies, and two rapalogs (temsirolimus and everolimus) have been approved for the treatment of renal cell carcinoma [24,25] and several dual mTOR-PI3K inhibitors are undergoing clinical trials for the treatment of cancer, including XL-765 (sanofi-aventis/Exelixis Inc; ClinicalTrials.gov Identifiers: NCT00485719, NCT00777699 and NCT00704080), and BEZ-235 (Novartis AG; NCT00620594) and BGT-226 (Novartis; NCT00700275, NCT00742105) [3,26-28]. The role of mTOR in cancer has been detailed in references mentioned in the *mTOR signaling components* section; thus, the role of mTOR signaling in cancer and the rationale for targeting mTOR is presented herein as a brief overview.

The PI3K-Akt pathway is dysregulated in cancer and can be activated by several mechanisms, including by receptor tyrosine kinases via growth factor and insulin binding [29], activating mutations in PI3K [30], the deletion of tumor suppressors such as PTEN [31] and signal amplification caused by feedback loops [32]. The activated form of mTORC1 phosphorylates substrates such as S6K and 4EBP1, both of which regulate protein translation. mTORC1 is negatively regulated by TSC, which can be inhibited in cancers via phosphorylation by Akt. Most of the early research into the components of the mTOR signaling pathway used rapamycin as a tool to inhibit mTOR. Rapamycin inhibits phosphorylation of the mTORC1 substrates S6K and 4EBP1, and thus disrupts protein translation. S6K and 4EBP1 phosphorylation levels are often used as functional measures of mTOR activity, and have been used as biomarkers in cellular assays, animal studies and clinical trials. Akt is an activator of mTORC1, with mTORC2 subsequently activating Akt [33]; therefore, Akt and mTOR can be both downstream and upstream of each other, leading to a signaling feedback loop. As a result of the complexity of mTOR signaling in cancer, several therapeutic intervention strategies have been proposed, as summarized in the *Therapeutic intervention strategies* section.

mTOR and lifespan extension

The hypothesis that calorie restriction may lead to lifespan extension has been investigated for the past 75 years [34]. Studies in yeast, worms, flies and mice support the hypothesis [35,36], and indicate a role for the insulin signaling pathway in modulating lifespan extension. Evidence suggests that the TOR signaling pathway has a pivotal role in lifespan extension in lower species as well as in mammals.

Lessons from model systems

By using genetic and RNAi screens in *Caenorhabditis elegans*, Vellai *et al* demonstrated that animals deficient in LET-363 (the *C elegans* TOR homolog) have average lifespans that are twice as long as for wild-type animals [37]. The same study also identified a genetic interaction between *let-363* and *daf-2* (the insulin growth factor like gene in worms) that increases lifespan, thus implicating the TOR and insulin signaling pathways in lifespan extension. This role of the TOR signaling pathway in lifespan extension was confirmed in a study in *Drosophila melanogaster* by Kapahi *et al* demonstrating that the overexpression of TSC1 and TSC2 or the downregulation of TOR or S6K, which is a downstream target of TOR, also resulted in lifespan extension [38]. The same study also examined the effect of diet on longevity, and demonstrated that dietary restriction led to lifespan extension via the TOR signaling pathway. Using a genome-wide gene deletion screen in yeast, Powers *et al* demonstrated that the chronological lifespan of yeast could be extended by downregulating TOR signaling, and that the nutrient-sensing component of the TOR pathway played a significant role in the rate of aging in yeast [39]. This study also demonstrated that the inhibition of TOR by rapamycin at doses less than those required to block cell division led to a dose-dependent increase in chronological lifespan extension in yeast.

Mammalian studies

Two recent studies have confirmed the role of calorie restriction in lifespan extension in mammals [40,41]. In a 20-year longitudinal study of rhesus macaques, Colman *et al* demonstrated that monkeys receiving a moderately reduced calorie, but nutritionally complete, diet lived longer and had fewer age-related diseases than animals receiving a higher-calorie diet [40]. A more direct demonstration of the role of the downregulation of mTOR activity in lifespan extension was provided by the Harrison *et al* study, which demonstrated that rapamycin extended lifespan in mice [41]. The S6 ribosomal subunit, which is a target of the mTORC1 substrate S6K, was also demonstrated to be phosphorylated at a significantly reduced level in mice that were treated with rapamycin. A reduced level of S6K phosphorylation has also been observed in patients with glioblastoma cancer who had been treated with rapamycin; this reduction in phosphorylated S6K levels is considered to be a biomarker of mTOR inhibition [42].

Research in both lower species and mammals suggests that the TOR signaling pathway plays an important role in lifespan extension, and detailed molecular pathway components are beginning to emerge. Rapamycin is the only pharmacological agent that has been demonstrated to mimic calorie restriction and to extend lifespan. Rapamycin inhibits only the mTORC1 complex; the mTORC2 complex is rapamycin-insensitive. Future studies may use ATP-mimetic small-molecule inhibitors of mTOR to probe the functions of different components of the mTOR pathway in lifespan extension. A challenge in this field is the identification of selective inhibitors of the mTOR catalytic domain that do not antagonize other lipid kinases that are structurally similar to mTOR.

mTOR in ocular disease

Ocular diseases such as wet age-related macular degeneration and diabetic retinopathy are among the leading causes of blindness [43]. Proliferative vitreoretinopathy, diabetic retinopathy and uveitis (intraocular inflammation) have been attributed to neovascularization and abnormal cell proliferation [44]. Because of its role in angiogenesis, inflammation and cell proliferation, mTOR has been proposed as a drug target for several ocular diseases. Several preclinical and clinical investigations have reported the successful use of sirolimus, which is approved as an immunosuppressant, in treating various ocular diseases.

In mouse models of retinopathy, a study by Dejneka *et al* demonstrated that rapamycin significantly inhibited neovascularization [45]. However, the molecular mechanism of the inhibition of neovascularization by rapamycin was not evident from these data. The antitumor activity of sirolimus results in a reduction in the level of VEGF, which is believed to be a primary mediator of angiogenesis [46]. However, in the study by Dejneka *et al*, the administration of rapamycin was observed to increase the levels of VEGF while reducing neovascularization in the eye [45]. Therefore, one possible conclusion from these data is that rapamycin may act via one or more VEGF-independent pathways.

The spreading and migration of retinal pigment epithelium cells are considered to be significant initial events in proliferative vitreoretinopathy. mTOR-specific siRNA has been observed to inhibit cell spreading and migration in human retinal pigment epithelial cell lines [47]. These *in vitro* results were confirmed in a rabbit model of proliferative vitreoretinopathy. Sirolimus has also been demonstrated to reduce VEGF levels in human retinal pigment epithelium cells and to be superior as a therapeutic agent to other established anti-VEGF therapeutics [48].

In a study by Shanmuganathan *et al*, the clinical benefits of sirolimus in treating severe non-infectious uveitis were assessed, and sirolimus therapy was observed to be effective in reducing uveitis in five out of eight patients [49]. Sirolimus is the only mTOR inhibitor used in the study of ocular diseases, and additional specific inhibitors of mTOR need to be tested to understand the conflicting data regarding VEGF levels.

mTOR in diabetes and obesity

Although a complete molecular understanding of diabetes has not been established, one of the primary symptoms of type 2 diabetes mellitus is insulin resistance. The binding of insulin to its tyrosine kinase receptor triggers the phosphorylation of various IRS proteins at tyrosine residues. One of the major signaling pathways activated by the tyrosine phosphorylation of IRS proteins is the PI3K-Akt-mTOR pathway, which leads to a normal insulin response. IRS proteins can also be phosphorylated at serine-threonine residues, resulting in the negative regulation of insulin signaling and leading to insulin resistance [50].

One of the important IRS kinases is S6K, the downstream effector and substrate of mTORC1. S6K is phosphorylated by mTORC1, and phosphorylated S6K regulates protein synthesis. In an S6K knockout mouse model, levels of serine-threonine phosphorylation of IRS-1 were decreased, leading to the restoration of insulin sensitivity, even in diet-induced obese mice [51]. More recently, S6K has been demonstrated to directly phosphorylate IRS-1 in response to TNFα and to promote insulin resistance [20]. The inhibition of mTORC1 with rapamycin resulted in reduced activation of S6K and could potentially be exploited to treat insulin resistance. Moreover, several studies reviewed in reference [52] demonstrated that, in animal models of type 1 and 2 diabetes, rapamycin (primarily acting as an inhibitor of mTORC1) blocked renal hypertrophy in the early stages of diabetic nephropathy. The blockade of diabetic nephropathy by rapamycin did not affect blood glucose levels, suggesting that mTORC1 signaling is activated by hyperglycemia [52].

Rapamycin prevented vascular senescence in a diet-induced obese mouse model, with the molecular basis of vascular senescence demonstrated to be the overactivation of Akt, which is regulated by the mTORC2 complex [53]. Although mTORC2 is considered to be rapamycin-insensitive, prolonged exposure to rapamycin can also inhibit mTORC2, which consequently inhibits Akt activation. This study assigns a central role for Akt in vascular senescence in

the diet-induced obesity model, but does not eliminate the possibility of additional roles for mTOR or S6K in vascular senescence [53]. Because rapamycin is not a known inhibitor of Akt and given that rapamycin prevents vascular senescence, it is possible that mTOR has additional, and as yet unexplored, roles in vascular senescence.

Leptin-mediated mTOR signaling

Leptin, a hormone/cytokine that is produced mainly in the adipose tissue, is a sensor of fat content that regulates food intake and basal metabolism. Leptin is structurally similar to IL-6 and other cytokines, and is common to neuroendocrine and immune systems. Leptin knockout mice are obese and exhibit a range of other abnormalities, including insulin secretion [54]. The binding of leptin to the leptin receptor results in the phosphorylation of the tyrosine kinase JAK2. JAK2 can induce the phosphorylation of IRS proteins, eventually triggering the PI3K pathway in a manner analogous to insulin signaling. Because the downstream effects of PI3K are mediated by mTOR, the potential role of mTOR in leptin signaling has been considered. Leptin has been demonstrated to induce lipid formation in macrophages by activating PI3K via an mTOR-dependent, rapamycin-sensitive mechanism [55]. Leptin-induced lipid formation was inhibited by the PI3K inhibitor LY-294002, and also in mice deficient in PI3K. Other markers of the activation of the mTOR pathway, such as the phosphorylation of S6K and 4EBP1, were also induced by leptin. The same study also demonstrated that rapamycin inhibited lipid body biogenesis both *in vivo* and *in vitro* [55]. Therefore, mTOR plays a central role in the formation of lipids in macrophages in response to leptin signaling, and mTOR inhibitors may be useful in studying lipid accumulation caused by inflammatory disorders.

Furthermore, the activity of mTOR in the rat hypothalamus was increased by leptin; fasting rats had significantly lower levels of phosphorylated S6K in hypothalamic regions, but not in extra-hypothalamic regions [56]. Similarly, the number of cells with activated phosphorylated mTOR was significantly decreased in hypothalamic areas as a result of fasting. To investigate whether the manipulation of mTOR activity plays a role in food intake, the effect of L-leucine was also examined, because the activity of mTOR is sensitive to levels of this amino acid. The administration of L-leucine was observed to cause anorexia and weight loss in rats. The effect of mTOR inhibition was tested directly by the intracerebroventricular administration of rapamycin, resulting in the reduced phosphorylation of S6K; in addition, the administration of rapamycin followed by L-leucine inhibited anorexia. Leptin-induced anorexia was also inhibited by rapamycin [56]. Thus, mTOR plays a central role in food and nutrient sensing, and in the related leptin-mediated signaling in the hypothalamus.

Therapeutic intervention strategies

Given the complexity of the mTOR signaling pathway, which contains multiple feedback loops and crosstalk with other pathways, compounds that inhibit multiple targets may be required to effectively modulate the pathway. Inhibitors of mTOR that are used in therapeutic intervention strategies can be broadly divided into five classes: (i) rapamycin and rapalogs that are allosteric inhibitors of mTORC1; (ii) dual PI3K-mTOR catalytic domain inhibitors that modulate both mTORC1 and mTORC2 negative feedback loop signals; (iii) selective mTOR inhibitors that exhibit activity against PI3K that is an order of magnitude less than their mTOR activity; (iv) the combination of an mTOR inhibitor with other compounds that target additional components of a crosstalk pathway; and (v) compounds that exhibit properties associated with mTOR pathway inhibition in addition to desirable mTOR-independent mechanisms of action.

Rapalogs

Sirolimus was first approved for clinical use in 1999 as an immunosuppressant, and in 2003 for restenosis in stent coating. However, a detailed mechanism of action of sirolimus, which does not bind to the ATP-binding pocket of mTOR but antagonizes mTOR signaling, has not been elucidated. Sirolimus can be described as an allosteric inhibitor of mTOR, or more generally as an mTOR pathway inhibitor. Rapalogs for the potential treatment of cancer have been designed to exhibit improved pharmacological properties as well as to gain IP protection [57]. For example, temsirolimus, which can be administered either intravenously or orally, was the first rapalog to be approved for treating advanced renal cell carcinoma [24]. In addition, in 2009, everolimus, another rapalog with an oral formulation, was approved for the treatment of advanced renal cell carcinoma [25], and the rapalog ridaforolimus (deferolimus; ARIAD Pharmaceuticals Inc/Merck & Co Inc) has been investigated in clinical trials for various cancer indications [58]. All of the rapalogs that have been evaluated appear to have a similar mechanism of action when characterized in terms of biomarkers of mTOR inhibition. In addition, although sirolimus has been used extensively as an immunosuppressant in transplant patients, no significant immune suppression has been observed in patients with cancer treated with rapalogs [59].

Rapamycin resistance

Resistance to rapamycin therapy may occur because of several reasons, including undiscovered mutations of key genes in the mTOR pathway or in drug transporting genes. The negative feedback loop from mTORC2 that activates Akt appears to be a key contributor to rapamycin resistance [60,61]. A 2008 proof-of-concept phase I clinical trial reported the occurrence of rapamycin resistance in patients with glioblastoma and a PTEN deficiency [42]. In this trial, Akt phosphorylation levels increased in 7 out of 14 patients, indicating that the negative feedback loop from mTORC2 was active. However, tumor cells harvested from non-responders were sensitive to sirolimus *ex vivo*, suggesting that the resistance mechanism was not intrinsic to the cell. To overcome sirolimus resistance caused by mTORC2 signaling, several compounds that inhibit the catalytic domains of PI3K as well as mTOR complexes have been developed.

Dual mTOR-PI3K inhibitors

Several compounds that inhibit the signaling outputs from both mTORC1 and mTORC2 have been reported. These compounds disrupt mTORC1 output in a similar manner to rapamycin and also inhibit the negative feedback loop from mTORC2 that phosphorylates and activates Akt. Because of the high level of sequence homology between mTOR and the lipid kinase PI3K, these compounds are not selective inhibitors of mTOR, but also inhibit PI3K and, in some cases, other PIKK members as well. Dual PI3K-mTOR inhibitors include XL-765 (sanofi-aventis/ Exelixis Inc) [26], which is undergoing phase I clinical trials for the treatment of solid tumors and gliomas (NCT00485719, NCT00704080 and NCT00777699), PI-103 [27] and NVP-BEZ235 (Novartis AG) [62]. All three of these compounds exhibit IC_{50} values in the range of 10 to 99 nm against PI3K and mTOR, and inhibit the activity of PI3K-mTOR axis biomarkers in a superior manner compared with sirolimus. As a class, the dual PI3K-mTOR inhibitors are still in early-phase trials, and a detailed understanding of the mechanisms of action of these drugs is yet to emerge.

Selective mTOR inhibitors

In order to investigate signaling outputs from mTORC1 and mTORC2 that are independent of PI3K inhibition, compounds that inhibit the catalytic site of mTOR potently and inhibit PI3K with a potency by at least one less order of magnitude have been developed [14,15]. These inhibitors block the phosphorylation of the Akt Ser^{473} residue, which is the target of the negative feedback loop emanating from mTORC2, as well as blocking the phosphorylation of S6K that is caused by mTORC1. The inhibition of Akt phosphorylation by these mTOR-selective inhibitors confirms that mTOR, and indirectly mTORC2, contribute significantly to the negative feedback loop signaling. These selective inhibitors are generally superior to rapamycin in blocking cell proliferation, 4EBP1 phosphorylation and protein translation [14,15]. One surprising discovery from this research has been that the superiority of the selective inhibitors compared with rapamycin was not a result of mTORC2 inhibition, but was caused by the enhanced inhibition of mTORC1. Several selective mTOR inhibitors are in the discovery stage of development, and the recent advances regarding this class of compounds may improve the understanding of the complexity of mTOR pathway and potential future therapeutic interventions.

mTOR pathway inhibitors

An additional class of mTOR inhibitors has molecular targets that are yet to be identified. For example, EM-101 (LY-303511; Emiliem Inc), which was initially discovered as a negative control for the pan-PI3K inhibitor LY-294002, inhibits mTOR autophosphorylation and S6K phosphorylation, and blocks the proliferation of A549 cells [63]. EM-101 also demonstrated efficacy as an antitumor agent in a mouse prostate cancer xenograft model. The precise molecular target of EM-101 has not been characterized, but functional analyses of EM-101 action suggest mTOR pathway modulation [63]. Palomid-529 (Paloma Pharmaceuticals Inc) also inhibits both of the mTOR complexes, as indicated by reduced levels of Akt and S6K phosphorylation [64]. Tumor growth in a mouse xenograft model was also inhibited by Palomid-529, but the precise target of the drug has not been characterized.

Changes in Akt and S6K phosphorylation have been used to identify several compounds derived from natural products as inhibitors of the mTOR pathway. However, the molecular targets of most of these natural products have not been identified. For example, NV-128 (Marshall Edwards Inc), a novel isoflavone derivative, decreased levels of phosphorylated Akt and S6K, and induced cell death in paclitaxel- and carboplatin-resistant cells [65]. Curcumin has been reported to have anti-inflammatory and antiproliferative properties, and has been demonstrated to disrupt the association of RICTOR and mTOR, thereby inhibiting mTORC1 signaling [66]. Curcumin [67] and isoflavone derivatives [68] are undergoing clinical testing for various cancer indications.

Combination therapies

Crosstalk between mTOR and other pathways has been observed in specimens obtained from patients in clinical trials as well as from animal models of cancer. For example, solid tumor biopsies from patients treated with the rapalog everolimus exhibited administration schedule-dependent activation of the MAPK pathway [69]. The activation of the MAPK pathway by everolimus was confirmed in a mouse model of prostate cancer and in both cancerous and normal cell lines. Moreover, S6K was identified as the key mediator of MAP activation in this study. In addition, the coadministration of sirolimus and PD-0325901, a MEK1/2 inhibitor (MEK1/2 is upstream of ERK, which is a key MAPK pathway kinase), synergistically enhanced the action of everolimus [69]. Another study in a mouse model of androgen-independent prostate cancer confirmed that the MAPK pathway was activated by rapalogs, and that the combination of everolimus and PD-0325901 acted synergistically in this model [70].

Several combinations of protein tyrosine kinase (PTK) inhibitors and sirolimus have been demonstrated to increase the efficacy of PTK inhibitors that target leukemias [71]. For example, the combination of the MAPK inhibitors sirolimus and imatinib was superior in action to imatinib alone. Of particular importance was the observation that sirolimus enhanced imatinib action even in cancer cell lines bearing imatinib-resistant mutations. Thus, synergistic actions of sirolimus and PTK inhibitors may help to overcome drug resistance in certain cancers.

Given that the combination of PTK and mTOR inhibitors demonstrates synergistic activity, the possibility of designing single compounds that inhibit pathways anchored by mTOR and PTKs may be considered. Structurally divergent kinases such as PTKs and PI3K have been demonstrated to be amenable to inhibition by single compounds [72]. Computational techniques, biochemical

screening and X-ray crystallographic studies have been used to identify several compounds that exhibit PI3K, mTOR and PTK inhibitory activities. Of particular importance is the compound PP-121, which was active against PI3K, mTOR and non-receptor tyrosine kinases such as Abl and Src, as well as the receptor tyrosine kinase PDGFR [72].

Toxicity related to mTOR inhibitors

The safety profile of sirolimus has been well-documented because of the widespread use of this drug in organ transplantation and cancer. Overall, adverse events associated with sirolimus are considered to be minor to moderate, and are usually managed adequately by supportive care and/or dose reduction [59]. Rapalogs, including temsirolimus, everolimus and ridaforolimus, generally share the same safety profiles as sirolimus. The most common side effects of these drugs are mucositis, stomatitis and oral sores, all of which occur at a fairly high incidence (> 50%). The mechanism by which these symptoms occur is unknown; the lesions appear to be distinct from similar radio- or chemotherapy-induced effects, but can be exacerbated with combination therapy [59]. Pulmonitis is also a well-documented adverse effect of sirolimus and the rapalogs, and may be related to a cell-mediated autoimmune response [73] or a T-cell-mediated delayed hypersensitivity reaction [74]. Skin lesions such as maculopapular or acneform rashes have been reported at a high incidence, particularly with the rapalogs. Other toxicities observed with all rapalogs in clinical trials include hyperglycemia caused by the disruption of insulin signaling and bone marrow suppression [59].

Conclusion

Research on mTOR-mediated signaling has been intense, and has highlighted the myriad functions of mTOR, which integrates several signaling pathways. As a consequence of this research, mTOR has been implicated to play a key role in several normal biological processes as well as disease pathologies. Some of these processes include protein translation, cell growth and regulation, cell survival, cytoskeleton organization, autophagy, energy and amino acid sensing, and glycogen metabolism. The dysregulation of mTOR signaling has been implicated in several disease processes, including cancer, obesity and diabetes. Recently, mTOR has also been demonstrated to regulate pathologies associated with aging. Sirolimus, the prototypical mTOR inhibitor, has been approved for clinical use as an immunosuppressant and in stent coatings for the prevention of restenosis. In addition, two rapalogs have been approved for treating renal cell carcinoma, and several other compounds that target the mTOR active site are undergoing clinical evaluation. Reported toxicities associated with compounds that target mTOR have been mild to moderate and can be controlled by general supportive therapy. Hyperglycemia, which has been observed in several patients, appears to be related to the disruption of insulin signaling.

Most of the research on the mTOR signaling pathway has focused on using rapamycin to block mTOR activity. The discovery that mTOR exists in the two complexes mTORC1 and mTORC2 and the demonstration that rapamycin is an incomplete inhibitor of mTOR suggests that some complexities of mTOR signaling have not been identified by using rapamycin. Novel compounds that inhibit mTORC1 and mTORC2 are likely to reveal as yet undiscovered components and complexities of the mTOR pathway. The crosstalk between various signaling pathways (eg, the inhibition of one pathway leads to the activation of another) may result in the identification of compounds that inhibit multiple targets in these interacting pathways, yielding more effective agents for the treatment of certain diseases. Although compounds that inhibit structurally divergent kinases are now being described in the literature, dissecting the polypharmacology of these multifunctional compounds is likely to be challenging.

Similar to canonical protein kinase inhibitors, compounds that inhibit mTOR in combination with other kinases could be hypothesized to be effective, and perhaps required, for the successful treatment of cancer. One important challenge for the development of anti-mTOR therapy for cancer is the identification of predictive biomarkers of efficacy. There are no known predictive biomarkers of rapamycin efficacy or resistance in cancer, and there is an urgent need to discover biomarkers that would predict the efficacy of anti-mTOR therapies. In addition, as new therapeutic approaches are translated into clinical trials, the safety profile established by the rapalogs are expected to change, and may resemble that of other multikinase inhibitors.

Given the initial success of various rapalogs in the treatment of cancer, most of the clinical studies of mTOR inhibitors have focused on cancer indications. As research on mTOR continues to identify the role of this protein in other diseases, an evaluation of the chronic administration of mTOR inhibitors may be required. It is likely that chronic mTOR inhibition for the treatment of diseases other than cancer may require highly selective mTOR inhibitors. Identifying selective inhibitors of mTOR may be difficult because of the structural similarities between mTOR and lipid kinase, and perhaps also canonical protein kinases. In addition, compounds that inhibit specific mTOR complexes may also be required. One possible approach to identifying selective mTOR inhibitors is to screen for allosteric inhibitors that disrupt complex formation. In this context, rapamycin is an allosteric inhibitor of the mTORC1 complex, and the search for an allosteric inhibitor of the mTORC2 complex is worth pursuing. The functional consequences of inhibiting the evolutionarily conserved protein mTOR may prove to be substantially more complex than has yet been established, and is likely to produce unexpected results.

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References

•• of outstanding interest
• of special interest

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