Promising therapeutic potential of drugs targeting mTOR pathway

Targeting mammalian target of rapamycin (mTOR) for health and diseases

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The macrolide rapamycin is used clinically to treat graft rejection and restenosis. Mammalian target of rapamycin (mTOR) is a central controller of cellular and organism growth that integrates nutrient and hormonal signals, and regulates diverse cellular processes. New studies have linked mTOR to several human diseases including cancer, diabetes, obesity, cardiovascular diseases and neurological disorders. Recent data have also revealed that mTOR is involved in the regulation of lifespan and in age-related diseases. These findings demonstrate the importance of growth control in the pathology of major diseases and overall human health, and underscore the therapeutic potential of the mTOR pathway.

Introduction

Rapamycin is a macrolide that is produced by the bacterium *Streptomyces hygroscopicus*, which was discovered in a soil sample collected ~1970 from the Easter Island *Rapa Nui*, from where the name rapamycin is derived (Box 1). Rapamycin was developed initially by Ayerst as an antifungal agent, but was soon abandoned because of the immunosuppressive effect and rapamycin was largely forgotten during the next decade. In the 20 years following its discovery only a dozen or so papers related to rapamycin were published. However, in the early 1990s the field experienced a dramatic turn of fortune, spurred largely by studies on the mechanism of action of rapamycin and by identification of the drug target. The growth of rapamycin-related research also renewed clinical interests and, in addition to rapamycin, several rapamycin analogs have been synthesized and tested in clinical trials. In 1997 rapamycin was approved by the FDA as an anti-rejection drug for kidney transplants.

Entering the 21st century, the field has continued the explosive growth. Recent studies provide significant insights into the molecular architecture of the mammalian target of rapamycin (mTOR) pathway. More importantly, the role of the mTOR pathway as a key process that underlies many human diseases has been either discovered or confirmed. New clinical indications for rapamycin and rapamycin analogs keep arising, and the scope of these is beyond their immunosuppressive activity. Rapamycin and rapamycin analogs are in clinical trials for several conditions such as cancer and cardiovascular diseases. In 2003, the FDA approved the rapamycin-

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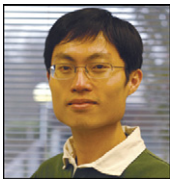

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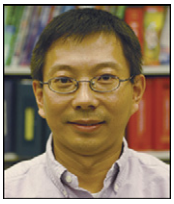

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1359-6446/06/$ - see front matter doi:10.1016/j.drudis.2006.12.008

BOX 1

A brief history of rapamycin

1970 Collected soil sample from Easter Island (Rapa nui) and isolated *S. hygroscopicus*
1975 Purified rapamycin as a natural compound from *S. hygroscopicus* and discovered its fungicidal activity
1977 Discovered immunosuppressive activity in animal models
1984 Discovered antitumor activity
1989 Proposed immunosuppressive action
1992 Identified rapamycin inhibits $p70^{S6K}$ in mitogenic pathway in mammals
1993 Isolated *TOR* gene from yeast
1994 Isolated *mTOR* gene
1995 Elucidated the mechanism of action of rapamycin
1997 FDA-approved for preventing host-rejection in kidney transplants
2003 FDA-approved for use in drug-eluting stent

eluting stent, a revolutionary coronary angioplastic procedure. The goal of this article is to comprehensively review the role of mTOR in the pathology of diverse diseases and areas that are related closely to human health. Detailed analyses of the clinical benefits and potential for the existing inhibitors of mTOR, and new therapeutic opportunities are also presented.

Inhibitors of mTOR: rapamycin and rapamycin derivatives

Rapamycin is the founding member of the family of mTOR inhibitors. Rapamycin includes two separated moieties, the TOR-binding and the FKBP12-binding regions. To be active biologically, rapamycin must form a ternary complex with mTOR and FKBP12, which is a cytosolic binding protein collectively called immunophilin. Therefore, rapamycin acts to induce the dimerization of mTOR and FKBP12. The formation of a rapamycin–FKBP12 complex results in a gain-of-function because the complex binds directly to mTOR and inhibits the function of mTOR and the mTOR-mediated signaling network. For example, rapamycin blocks interleukin 2 (IL-2)-mediated T-cell proliferation and activation, conferring an immunosuppressive effect that is useful in transplantation. In addition, rapamycin is being developed to treat autoimmune diseases such as idiopathic and lupus membranous nephropathy. Rapamycin is also a potent inhibitor of the proliferation of vascular smooth muscle (VSM) cells. Based on this property, rapamycin (Sirolimus®) was approved by the FDA in 2003 as an antirestenosis drug used in coronary-artery stents. The Development Therapeutic Branch of the National Cancer Institute (NCI) was one of the first to test rapamycin, finding that it has superior, broad antitumor activity in both *in vitro* and *in vivo* models. There are many on-going clinical trials of rapamycin against a broad range of malignancies, from childhood lymphoma (Phase I) to prostate cancer (Phase IV). Additionally, clinical trials in conditions such as autosomal-dominant polycystic kidney disease (ADPKD) are being conducted.

Because of the promising therapeutic potential of rapamycin, pharmaceutical companies started to develop rapamycin analogs in the late 1990s. Several rapamycin analogs have been synthesized to improve the pharmacokinetic properties and for patent protection. Figure 1 shows the main rapamycin analogs under development. Because rapamycin and these derivatives share the same mechanism of action, we refer to them collectively as rapamycins in this review. CCI779 (also called temsirolimus; Wyeth) was the earliest developed rapamycin analog. It is a more water-soluble ester derivative of rapamycin for intravenous and oral formulation. CCI779 has antitumor activity either alone or in combination with cytotoxic agents in several human cancer cell lines and mouse xenografts [1]. Phosphatase and tensin homolog deleted on chromosome ten (PTEN)-deficient human tumors appear to be particularly sensitive to growth inhibition by CCI779 [2]. RAD001, which is also called everolimus (Novartis), is a hydroxyethyl ether derivative of rapamycin that has been developed for oral administration. Preclinical results demonstrate that the immunosuppressive activity of RAD001 in preventing allograft rejection in experimental rat models is synergystic with cyclosporine, thereby reducing cyclosporine-associated renal toxicity [3]. Currently, this compound is undergoing Phase III and Phase IV clinical trials for transplantation in the USA, and has been approved by the European Agency for the Evaluation of Medicinal Products for renal and heart transplants. RAD001 also shows excellent activity against several human cancer cells *in vitro* and tumors in animal models, and antiangiogenic activity by inhibiting the proliferation of human vascular endothelial cells [4]. AP23573 (Ariad) is synthesized by substituting the C-43 secondary alcohol moiety of the cyclohexyl group of rapamycin with phosphonate and phosphinate groups. It retains high-affinity binding to FKBP12 and mTOR-inhibitory activity, is stable in organic solvents, aqueous solutions at various pHs, plasma and whole blood [5], and is being developed for either oral or intravenous administration. AP23573 inhibits the proliferation of diverse human tumor cell lines *in vitro* and of tumor xenografts in conjunction with cytotoxic agents [6], and clinical trials are evaluating its antitumor activity. In general, rapamycin analogs have similar therapeutic effects to rapamycin but with improved hydrophilicity and are suitable for both oral and intravenous administration.

Overall, rapamycins are tolerated well by humans. They do not exhibit the significant renal toxicity that occurs with the most common immunosuppressive drugs FK506 (tacrolimus) and cyclosporine. The immunosuppressive effects can be reduced significantly by intermittent administration of these mTOR inhibitors for the treatment of human cancer [7]. The primary side-effect of mTOR inhibitors in adult humans is a dose-dependent increase in serum cholesterol and triglycerides [8]. Other adverse effects are relatively mild, including skin reactions, muscositis, minimal myelosuppression and diarrhoea. Additionally, initial clinical studies show no significant changes in blood pressure, kidney function, such as glomerular filtration, and liver function.

mTOR and the mTOR signaling network

mTOR is a well-conserved 289 kDa phosphatidylinositol 3-kinase (PI 3-kinase)-related kinase (PIKK) that occurs in all eukaryotic organisms sequenced so far. The structural organization of mTOR is shown in Figure 2a. The C-terminal PIKK domain is conserved most highly and exhibits serine and threonine kinase activity but no lipid kinase activity as seen with other members of the PIKK family [9]. An intact PIKK domain is required for all known functions of TOR [10,11]. The FKBP12-rapamycin-binding (FRB) domain is an 11-kDa region near the PIKK domain [11,12]. FRB

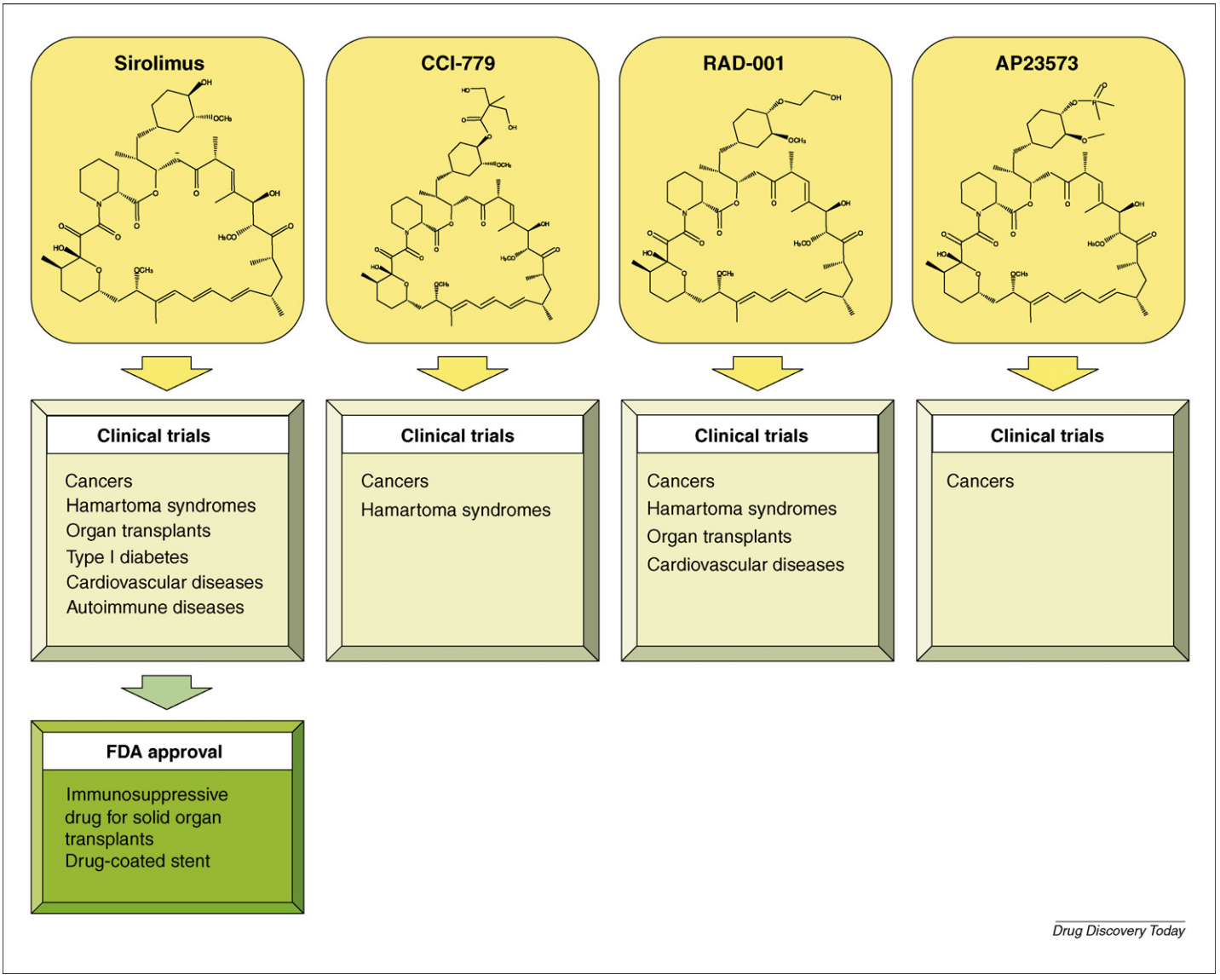

FIGURE 1

Clinical uses and trials of rapamycin and rapamycin analogs. Chemical structures, current clinical trials and FDA-approved clinical uses of rapamycin and its analogs.

forms a four-helical-bundle hydrophobic pocket that interacts with the hydrophobic rapamycin of FKBP12–rapamycin (Figure 2b) [13]. In effect, rapamycin binds FRB and FKBP12 to form a sandwich-like structure (Figure 2c). The FRB domain occurs only in the TOR protein, but not in other members of the PIKK family, which explains the unusually high specificity of rapamycin. Although the FRB domain is close to the PIKK domain, FKBP12–rapamycin does not appear to inhibit the catalytic activity of the kinase directly [11]. Therefore, how FKBP12–rapamycin interferes with the functions of mTOR remains elusive. One possibility is that FKBP12–rapamycin creates a spatial hindrance that prevents interaction between the PIKK domain and substrates that bind to the N terminus. The N terminus of TOR consists of ~20 tandem repeats of 37–43 amino acids, which are termed HEAT repeats [14]. Each HEAT unit contains two interacting α-helices and a flexible inter-helical spacer. Different HEAT units occur in tandem repeats and form super helices that are involved in interaction with other proteins. Indeed, TOR HEAT repeats interact with cofactors, such as mKog1/Raptor [15], and kinase substrates, such as Gln3 [16]. Recent studies have revealed that mTOR forms at least two distinct complexes. The mTOR complex 1 (mTORC1) is composed of mTOR, mLST8/GβL and Raptor, whereas mTORC2 consists of mTOR, mLST8/GßL, Rictor/mAvo3 and Sin1/mAvo1 [17]. Although mTORC1 is responsible for sensing nutrient signals, mTORC2 is involved in the organization of actin. In addition, mTORC2 is the Ser473 kinase for Akt/protein kinase B (PKB). FKBP12–rapamycin interacts with and inhibits mTORC1, but not mTORC2. Therefore, unless stated, our discussion relates to mTORC1, and the term mTOR refers to mTORC1.

Upstream of mTOR

mTOR is a central integrator of various extracellular and intracellular signals, including growth factors, nutrients, energy and stress (Figure 3). Among the signal inputs, growth factor- and hormone (e.g. insulin)-induced mTOR activation is the best characterized, and is mediated by the activation of PI 3-kinase. Active PI 3-kinase

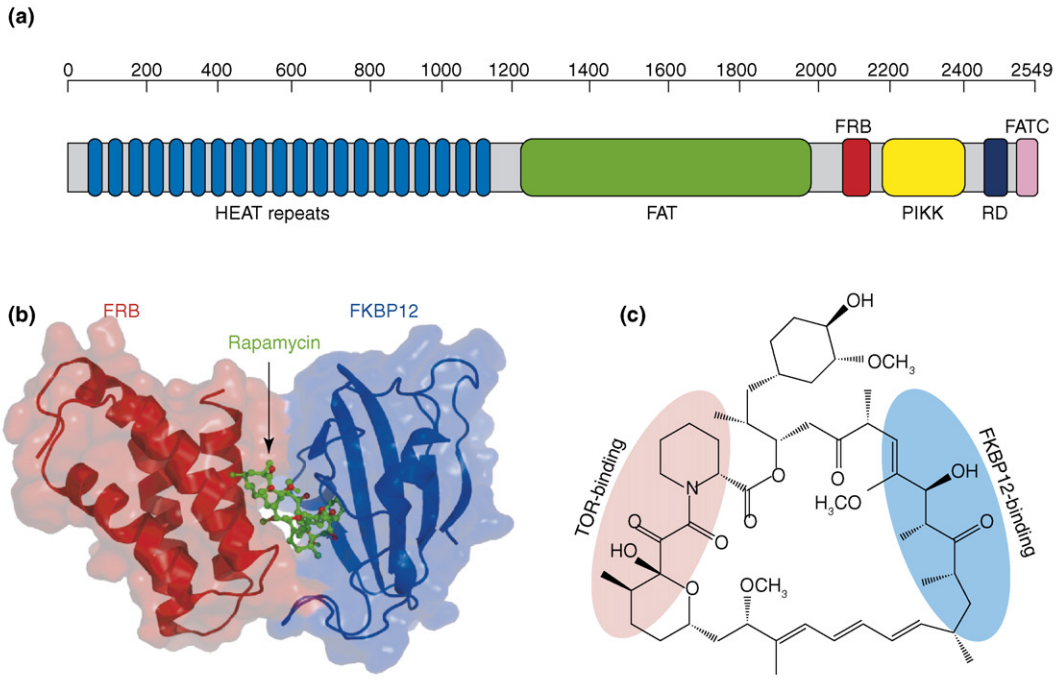

FIGURE 2

(a) Functional domains of mTOR: HEAT, huntington-elongation factor 1A-protein phosphatase 2A-A subunit-TOR; FAT, FRAP, ATM, TTRAP2; FRB, FKBP12 rapamycin binding; RD, regulatory domain; FATC, FAT, C terminal; and PIKK, PI 3-kinase-related kinase. The amino acid residue number (top) shows the relative positions of the domains. **(b)** The structure of the ternary complex of FRB (red), rapamycin (green) and FKBP12 (blue). Reprinted, with permission, from Choi *et al.* 1996, *Science* 273, 239–242 [13]. Copyright 2007 AAAS **(c)** Chemical structure of rapamycin and its binding sites for FKBP12 (blue region) and mTOR (red region).

localizes to the cell membrane and catalyzes the conversion of phosphatidylinositol (4,5)-biphosphate [PtdIns(4,5)P_2] into PtdIns(3,4,5)P_3, whose concentrations are regulated negatively by the tumor suppressor PTEN. PtdIns(3,4,5)P_3 recruits and activates Akt, which, in turn, phosphorylates and inactivates the tuberous sclerosis protein complex (TSC), which is a heterodimer composed of TSC1 and TSC2 [18]. TSC2 acts as a GTPase-activating protein (GAP) for the small GTPase Rheb, and the action of TSC is thought to be mediated by Rheb, possibly by the binding of Rheb to the kinase domain of mTOR, which activates mTOR in a GTP-dependent manner [19]. Several studies also show that GTP-loading of Rheb induces a conformational change in mTOR that results in activation of mTOR and phosphorylation of downstream effector proteins [19]. Other studies have shown that Akt might phosphorylate mTOR directly at Ser2448 [20], but the functional significance of phosphorylation of this remains to be established.

Nutrients represent another major signal input that activates mTOR. Unlike growth-factor-induced signaling, the exact mechanism of nutrient regulation of mTOR remains obscure, although the process is conserved evolutionarily from mammals to lower organisms such as *Saccharomyces cerevisiae*. One possibility is that nutrients regulate TOR signaling through energy production in the form of ATP. The cellular energy level (ATP:AMP ratio) is detected by AMP-activated protein kinase (AMPK). Under conditions of energy deprivation, the ATP:AMP ratio is low, which activates AMPK. Active AMPK phosphorylates TSC2 at multiple serine and threonine sites and activates TSC, which results in inactivation of mTOR [21]. Another study shows that the K_m of mTOR for ATP is high (mM range), which is close to the intracellular concentration of ATP. Based on this finding, it has been proposed that mTOR serves as a homeostatic sensor of ATP [22]. Various environmental stresses also lead to downregulation of mTOR signaling. For example, hypoxia inhibits mTOR and protein synthesis through two homologous proteins, REDD1 and REDD2 [23]. Upon hypoxia, both REDDs are upregulated by the transcription factor HIF1 and inhibit mTOR through TSC. DNA damage can also inhibit mTOR via p53 and the AMPK–TSC signaling pathway [24]. The regulation of TOR by stress is also conserved in lower eukaryotes. For example, inhibition of TOR by either rapamycin or disturbance of the plasma membrane activates the yeast stress-regulated mitogen-activated kinase Mpk1, which, in turn, phosphorylates silenced chromatin regulator 3 (Sir3) and leads to derepression of genes in otherwise silenced subtelomeric regions [25].

Downstream of mTOR

Growth and development require mTOR, and disruption of mTOR results in embryonic lethality and severe developmental defects [26]. At the cellular level, mTOR is central to the regulation of both catabolic and anabolic processes. In response to optimal growth stimuli, mTOR promotes the synthetic capabilities of the cell by upregulating key processes such as ribosome biogenesis and protein translation, which lead to an increase in cell mass and size, and, thus, accelerated proliferation. The best characterized downstream effectors of mTOR are the ribosomal protein S6 kinases (S6K) and 4E-BP1 (also called PHAS-I). S6K1 is phosphorylated

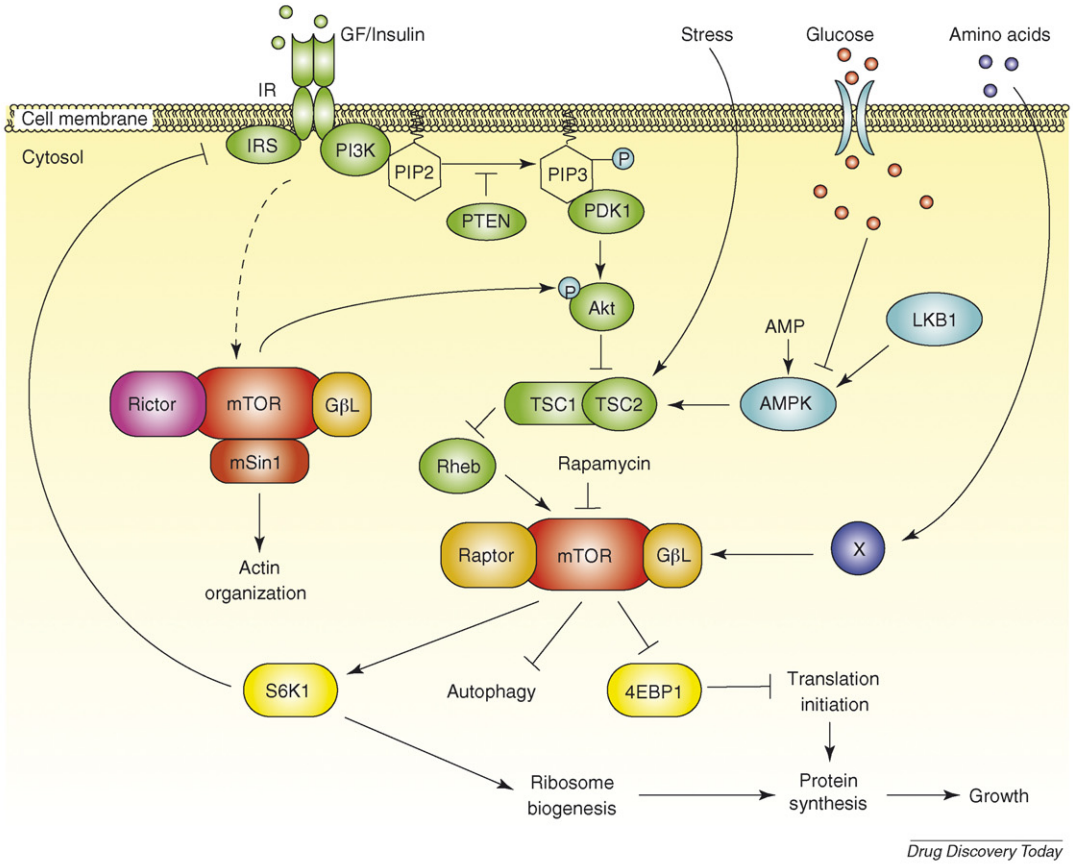

FIGURE 3

Signaling network of mTOR. mTOR integrates input signals from growth factors (GF), nutrients (glucose and amino acids) and stress to regulate cell growth via different cellular processes. Arrows and bars represent activation and inhibition, respectively. Protein X represents an unknown mediator.

directly by mTOR during stimulation with either nutrients or growth factors, which results in a selective increase in the translation of mRNA transcripts that contain a 5′ tract of oligopyrimidine (5′-TOP) motif [27]. The 5′-TOP mRNAs encode components of the translation apparatus such as elongation factors and ribosomal proteins. Thus, mTOR-dependent activation of S6K1 promotes an overall increase in protein synthesis. However, S6 phosphorylation and translation of 5′-TOP mRNAs are still responsive to mitogens in a rapamycin-dependent manner in a S6K1/S6K2 double-knockout mutant, which indicates that an alternative effector is also present [28]. 4E-BP1 is an inhibitor of eukaryotic translation initiation factor 4E (eIF4E). After hyperphosphorylation by mTOR, 4E-BP1 dissociates from eIF4E, which allows eIF4E to associate with the 5′-CAP of mRNAs and initiate translation [29]. Conversely, inhibition of mTOR by growth-factor withdrawal, nutrient starvation and stress conditions lead to downregulation of ribosome biogenesis, which is a high-energy-consuming process [30]. In addition, downregulation of the mTOR pathway also stimulates catabolic processes such as autophagy. Autophagy is a membrane-trafficking process that involves the delivery of cytoplasmic contents, such as organelles and macromolecules, to lysosomes/vacuoles, where the cytoplasmic contents are broken down to provide an intracellular supply of nutrients [31]. Autophagy is essential for adaptation and survival during starvation conditions. Autophagy has also been linked to tumorigenesis. It is further involved in the removal of large protein aggregates, the underlying pathological causes for several neurological disorders [31].

mTOR in health and disease

In the past 5 years, growth has been recognized as a central process in most, if not all, aspects of cell biology. An increasing number of human diseases have been linked to the dysregulation of mTOR, including immunological disorders, cancer, metabolic diseases, cardiovascular diseases and neurological disorders. Intriguingly, most of these are due to aberrant hyperactivity of the mTOR pathway, which makes inhibitors of mTOR potentially effective therapeutics for the treatment of these diseases. One of the most exciting developments is the finding that mTOR is a key regulator of lifespan in eukaryotes and contributes significantly to age-related diseases. The fact that rapamycin can extend lifespan in model organisms indicates that pharmacological modulation of aging is, potentially, a practical approach.

Allograft rejection and autoimmune disorders

Organ transplantation often elicits a complex series of immunological responses such as inflammation, which results in allograft rejection. After organ transplantation, the graft is recognized by T

cells as carrying foreign antigens through the interaction between MHC-antigen complex and T-cell receptor (TCR). Through an autocrine mechanism, such engagement leads to the production of IL-2, and IL-2-stimulated activation and proliferation of lymphocytes. Rapamycin, FK506 and cyclosporine, the widely used immunosuppressive agents after organ transplantation, have distinct mechanisms of action [32]. Their immunosuppressive activities require that they bind to specific cytosolic binding proteins, collectively called immunophilins. Cytosporine forms a complex with cyclophilin, whereas FK506, which is structurally related to rapamycin, forms complex with FKBP12. After forming a complex with their respective immunophilin, cyclosporine and FK506 inhibit the Ca^{2+}-dependent phosphatase calcineurin, thereby blocking T-cell antigen receptor-dependent expression of IL-2, which is a key cytokine in the proliferative response of T cells [33]. By contrast, rapamycin–FKBP12 binds to and inactivates mTOR, thereby preventing IL-2-stimulated T-cell proliferation [34]. Clinical trials in the late 1990s confirmed the efficacy of mTOR inhibitors as potent immunosuppressive agents in renal transplants. Combining rapamycin with a calcineurin inhibitor achieves significant synergy, lowering the dose of each drug that is needed for immunosuppressive efficacy, and improving the rejection prevention and minimizing cyclosporine-induced nephrotoxicity [35]. Rapamycin has neither the vasomotor renal-side effects of calcineurin inhibitors nor an increased risk of malignancy [36]. As a result, calcineurin inhibitor-replacement therapy, using rapamycin plus glucocorticoids and mycophenolate mofetil, has been developed for patients at risk of renal toxicity associated with calcineurin inhibitors [37]. Moreover, a side-effect of cyclosporine and tacrolimus might be diabetes, but clinical trials reveal no increased risk of post-transplantation diabetes in rapamycin-treated patients. However, it should be noted that, in a recent study, oral glucose-tolerance tests show that rapamycin is associated with a 30% increase in the incidence in impaired glucose tolerance in kidney-transplant recipients [38]. Thus, special attention should be paid to diabetic patients who need renal transplantation. Clinical trials for transplantation of other solid organs are underway.

Rapamycins have shown efficacy in several animal models of autoimmune diseases, including allergic encephalomyelitis, insulin-dependent diabetes mellitus, lupus and adjuvant arthritis [39]. In addition, autoimmune diseases are often associated with chronic inflammatory immune responses that are perpetuated by dendritic cells. mTOR is crucial for the survival of monocyte-derived dendritic cells [40]. Furthermore, rapamycin decreases surface concentration of some MHC class II molecules on murine bone marrow-derived dendritic cells, and inhibits their maturation [41]. These observations indicate that mTOR inhibitors might be therapeutically valuable in autoimmune disorders such as rheumatoid arthritis, psoriasis and multiple sclerosis. The efficacy of mTOR inhibitors in these autoimmune disorders is being investigated in several early clinical trials.

Cancer

Soon after the isolation and characterization of rapamycin in the early 1970s, it was shown to be a potent anticancer agent by the NCI. Initially, it was thought to inhibit the cell cycle because chronic treatment with rapamycin leads to G1 cell-cycle arrest.

TABLE 1

Components of the mTOR pathway involved in human cancers

Mutant protein	Clinical diseases and types of cancer
PTEN	Glioblastoma, prostate cancer, endometrial cancer
PI 3-kinase	Transformation, cancer
Akt	Breast cancer, chronic myeloid leukaemia, ovarian cancer
TSC1/2	Tuberous sclerosis
eIF4E	Lymphoma
S6K1	Breast cancer
NF1	Neurofibromatosis type 1, peripheral nerve-sheath tumors
LKB1	Peutz-Jeghers syndrome, gastrointestinal hamartomas
P53	Tumors
4EBP	Transformations
Beclin-1	Breast carcinomas
HIF	Kidney cancer
Myc	Burkitt's lymphoma
Cyclin D1	Mantle cell lymphoma

However, it has been appreciated recently that growth is an important prerequisite for proliferation [19,42]; without sufficient cell mass and size, the cell cycle cannot be sustained and, eventually, cells become arrested in G1. Recent advances in cancer biology reveal that numerous human cancers, including lymphomas, melanomas, gliomas, malignancies of the CNS, and carcinomas of the lung, bladder, kidney, ovary, breast, prostate, stomach, pancreas, head and neck, contain mutations in genes that encode components of the mTOR signaling network [43]. These genetic abnormalities can cause hyperactivity of PI 3-kinase; overexpression of Akt, Rheb, eIF4E and S6Ks; either loss-of-function or deficiency of the tumor suppressors PTEN, TSC1/2 and LKB1, and underexpression of beclin-1 (Table 1), although a mutation of mTOR itself has not been reported. Consistent with these data, cancer cells in which mTOR becomes hyperactive because of either a mutation in *PTEN* or overexpression of *AKT* are particularly susceptible to rapamycins [2,44]. This provides the conceptual basis for the use of mTOR inhibitors to block the downstream pathways that are important for the growth of cancer cells, interrupting proliferation and accelerating apoptosis [45].

Rapamycins are undergoing active clinical trials to evaluate their antiproliferative action in cancer. Clinical trials have demonstrated their efficacy and mild side-effects. The most encouraging results of the anticancer effect of mTOR inhibitors have been obtained in renal cell carcinoma, mantle cell lymphoma (MCL) and endometric cancers. Compelling results have also been observed in refractory patients. Based on these promising results, CCI779 has been filed for registration in the USA and Europe for first-line treatment of patients with advanced renal cell carcinoma. More importantly, these clinical studies provided useful information on the types of cancer that might be more sensitive to mTOR inhibitors. For example, MCL and endometrial cancers have functional apoptosis pathways, and are usually accompanied by genetic mutations, such as loss of PTEN and overexpression of cyclin D1, that lead to aberrantly overactive mTOR-signal transduction. In such cases, they are likely to be more sensitive to rapamycin and apoptosis might be triggered by relatively low

doses of mTOR inhibitor. By contrast, some types of cancer, such as renal cell carcinoma and breast cancer, have either redundant signaling pathways or non-functional apoptosis pathways and so might require high doses of mTOR inhibitor to elicit a cytostatic response, and no dose-dependent apoptosis can be observed. However, as yet, it is not possible to predict accurately which cancer types are sensitive to mTOR inhibitors.

Another function of mTOR that is related to cancer development is tumor angiogenesis, the growth of blood vessels that provide tumor cells with nutrients and oxygen. The role of mTOR in tumor vascularization is interesting because preclinical studies have demonstrated that rapamycins have antiangiogenetic effects. Rapamycin lowers the concentration of vascular endothelial growth factor (VEGF), which leads to the suppression of endothelial-cell proliferation, survival and migration, and, eventually, tumor-vessel thrombosis [46]. It has been shown that the expression and function of hypoxia-induction factor (HIF) depends on mTOR [47]. Moreover, hypoxia also regulates mTOR in HIF-dependent and HIF-independent manners [48]. HIF-dependent inhibition of mTOR is mediated by upregulation of REDD1 and REDD2 [23]. Frequently, the hypoxia pathway is constitutively active in solid-tumor cells and, therefore, might be selectively reliant on mTOR for survival in hypoxic environments. Rapamycin treatment might cause apoptosis of hypoxic cancer cells that depend upon mTOR for survival, but not affect normal cells because they do not grow under these conditions. It is possible that the antiangiogenic effect of rapamycin is as important as growth inhibition in the suppression of solid tumors.

The direct and indirect effects of rapamycins on the growth of cancer cells make them excellent candidates for therapeutic agents in cancer. The hallmark of some cancers is the evasion of apoptosis. Thus, cancer cells occasionally become resistant to conventional chemotherapeutic agents, especially when the apoptotic regulator, such as Akt, is mutated. A recent finding using a murine lymphoma model shows that rapamycin reverses the chemoresistance that results from mutant Akt [44]. The important implications of this study are that induction of apoptosis by pharmacological inhibition of mTOR might restore drug sensitivity, and that combining conventional chemotherapy agents with rapamycin might be an important strategy to reverse drug resistance in human cancer.

A major challenge in rapamycin-based cancer therapy is to identify the right population of patients. Typically, $\leq$10% of patients respond to rapamycin. Some cancer cells are highly sensitive to rapamycin whereas others are significantly resistant. Most advances in research on the sensitivity of tumors to rapamycin have been made using cancers in which the PI 3-kinase–PTEN–AKT pathway is altered. However, the percentage of rapamycin-sensitive tumors that can be attributed to changes in PI 3-kinase–PTEN–AKT is unclear. Recently, some $PTEN^{-/-}$ tumors have been reported to be highly resistant to rapamycin [49]. Further investigation is needed to address these issues. A recent genomic screen of rapamycin sensitivity in yeast has identified mutations in many genes that affect rapamycin sensitivity [50], most of which are well conserved in humans. It will be interesting to determine whether either mutations or changes in expression of their human homologues have similar roles in cancer cells. The identification and treatment of molecularly defined patient cohorts, based on molecular phenotype, is a top priority for patient-orientated cancer therapy of mTOR-dependent cancers. Such studies would also provide important rationales for combinational therapy with rapamycin and other FDA-approved anticancer drugs. In addition, effective surrogate markers need to be developed to follow rapamycin-based therapy conveniently. Currently, most laboratory and clinical studies use phosphorylation of S6K1 to follow inhibition of mTOR. However, S6K1 is inhibited efficiently, even in cancer cells that are highly resistant to rapamycin. It is equally important to evaluate the combinational use of an mTOR inhibitor with other anticancer drugs to achieve maximal efficacy and minimal side-effects.

Diabetes

Diabetes is a metabolic disorder of glucose homeostasis. Insulin regulates blood-glucose levels by promoting the uptake and conversion of glucose to glycogen and its storage in the liver and muscles [51]. Insulin is produced by the β-cells in pancreatic islets. Type 1 diabetes is caused by loss of insulin production due to destruction of pancreatic β-cells. Currently, the only way to restore and sustain the normal concentration of blood glucose in patients with type 1 diabetes is either transplanting a pancreas or infusing isolated islets. Clinical studies using immunosuppressive regimens that contain rapamycin to prevent the rejection of islet transplants have shown significant efficacy in type 1 diabetic patients [52]. Thus, the use of rapamycins is crucial in the treatment of type 1 diabetes. By contrast, mTOR has a positive role in the growth of pancreatic β cells [53]. Acting downstream of PI 3-kinase–Akt, mTOR mediates insulin-induced protein translation and proliferation of β cells. In addition, glucose and branched amino acids stimulate β-cell growth in an mTOR-dependent manner [55]. This role of mTOR is supported further by the phenotype of mice deficient in S6K1, a kinase that acts downstream of mTOR. The $S6K1^{-/-}$ mice have smaller islets, are hypoinsulinaemic and glucose intolerant [54]. These mice have a sharp reduction in glucose-induced insulin secretion and in pancreatic insulin content because of a selective decrease in β-cell size. Thus, the opposing effects of mTOR inhibitors must be balanced carefully in islet transplantation.

Type 2 diabetes arises when insulin secretion from pancreatic β cells fails to compensate for the peripheral insulin resistance (or insensitivity to insulin) in skeletal muscle, liver and fat cells [55]. Evidence indicates that insulin resistance might be caused by inhibition of insulin-receptor substrate (IRS) proteins by phosphorylation, which abolishes the signal transduction from insulin receptor to PI 3-kinase [51]. Although many distinct pathways have been implicated in downregulating IRS function, recent data indicate that sustained activation of mTOR signaling is a crucial event that renders IRS irresponsive to insulin [56]. Moreover, it has been demonstrated that rapamycin restores the sensitivity of IRS to insulin. Because mTOR is downstream of the IRS–PI3K–Akt pathway, the reported mTOR-mediated inhibition of IRS is thought to act via a negative-feedback loop [56]. Furthermore, it has been demonstrated that S6K1 phosphorylates IRS directly, which results in the inhibition of the association of IRS with the insulin receptor [57]. Another recent finding indicates that chronic hyperglycemia can lead to chronic activation of mTOR in β-cells [58]. Chronically active mTOR triggers phosphorylation

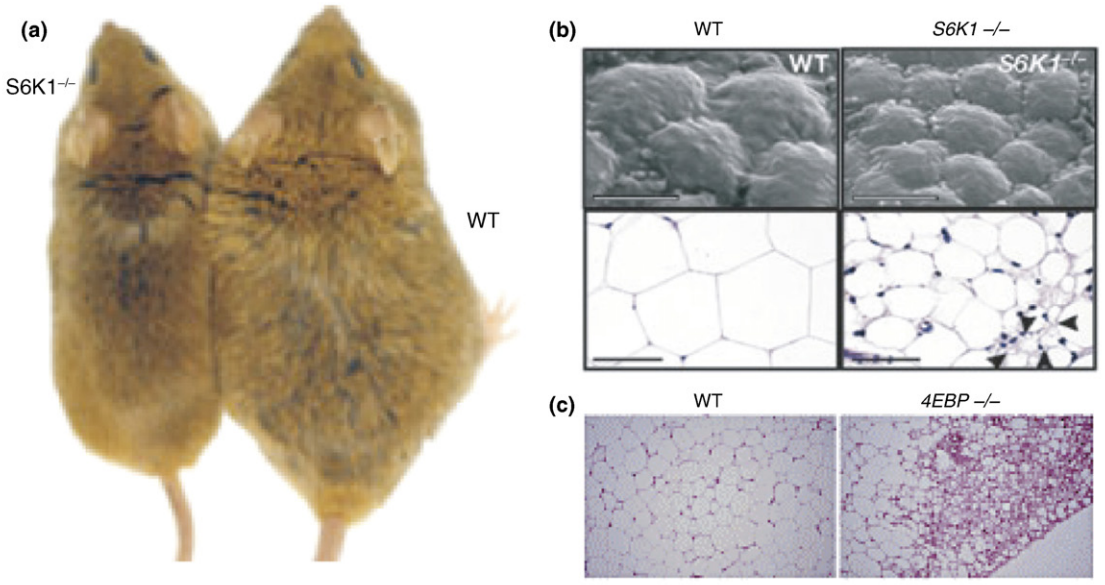

FIGURE 4

Regulation of mTOR-mediated fat accumulation and metabolism. (a) Wild-type (WT) and S6K1$^{-/-}$ mice after 6 months on a high-fat diet. **(b)** Scanning electron microscopic (upper panel) and histological (lower panel) analyses of epididymal white adipose tissue of wild-type and S6K1$^{-/-}$ mice. Reprinted, with permission, from McMillan Publishers Ltd: Um *et al.* 2004, *Nature* 431, 200–205 [65], copyright 2007. Arrowhead indicates multilocular adipocytes. Magnification for scanning microscopy and histology are 500× and 200×, respectively. **(c)** Retroperitoneal white adipose tissue of wild-type and 4EBP$^{-/-}$ mice. Sections stained with HandE. Reprinted, with permission, from McMillan Publishers Ltd: Tsukiyama–Kohara *et al.* 2001, *Nature Med.* 7, 1128–1132 [66], copyright 2007.

and subsequent proteosomal degradation of IRS2, which leads to an increase in β-cell apoptosis. Together, these studies indicate that persistent activation of mTOR in insulin-responsive cells downregulates insulin signaling and contributes to insulin resistance; whereas, persistent activation of mTOR in β-cells reduces cell mass and insulin secretion. Both situations can increase the risk of type 2 diabetes. Therefore, rapamycin is potentially useful in management of type 2 diabetes.

Obesity

According to the American Heart Association, >60% of Americans are either overweight or obese, which creates serious problems in healthcare and the overall economy. Understanding the cause of obesity and finding effective prevention and treatment methods are paramount tasks. Accumulating evidence indicates that mTOR has a role in lipid metabolism. During adipogenesis, the expression of mTOR increases dramatically, from barely detectable in pre-adipocytes to highly expressed in fully differentiated adipocytes [59,60], and rapamycin inhibits adipocyte differentiation [61]. Moreover, mTOR is required for the expression and activity of peroxisome proliferator-activated receptor-γ, a transcription factor that is crucial for adipogenesis [59,60]. Lipin, a lipodystrophy and obesity protein, is another possible target of mTOR. Phosphorylation of lipin is stimulated by insulin and amino acids in a rapamycin-sensitive manner [62]. Adipose tissue is not only for storing fat, it is also responsible for the secretion of hormones that regulate appetite. Leptin is a protein hormone that is synthesized predominantly by adipocytes and acts on receptors in the CNS and other sites to inhibit food intake and promote energy expenditure. mTOR regulates the synthesis and secretion of leptin from adipose cells [63]. Thus, by regulating leptin production in adipocytes, mTOR might have a broader role in controling whole-body energy metabolism.

Experiments in fruit flies indicate that TOR is required for fat accumulation, which further supports the notion that fat accumulation and metabolism are regulated by activity of mTOR [64]. It has been suggested that the regulation of mTOR-mediated fat metabolism involves signaling through S6K1 and 4E-BP1. More direct evidence of the role of the mTOR–S6K1 pathway in fat metabolism has been obtained from the S6K1-mutant mice. In these mice, high-fat diet- and age-dependent obesity is reduced markedly (Figure 4A,B), possibly because of the lower amounts of adipose tissue and fat accumulation that result from higher β-oxidation of fatty acids (lipolysis) in adipocytes [65]. Reduction of adipose tissue is also observed in mice that lack the translational inhibitor 4E-BP1 (Figure 4C) [66]. These observations indicate that S6K1 and 4E-BP1 might be novel therapeutic targets for the development of antiobesity drugs.

Cardiovascular diseases

Cardiac hypertrophy (heart enlargement) is caused by abnormally large cardiomyocytes. Cardiac hypertrophy accompanies many forms of heart disease, including ischemic disease, hypertension, valvular disease and heart failure. At the cellular level, cardiac hypertrophy is characterized by an increase in cell size and enhanced protein synthesis [67]. Although there are various hypertrophic stimuli, such as neurohormones and peptide growth factors, and several protein kinase cascades are involved in cardiac hypertrophy, it is likely that all forms of hypertrophic stimuli activate the general protein translational machinery in an mTOR-

dependent manner. Remarkably, inhibition of mTOR by rapamycin prevents cardiac hypertrophy in numerous transgenic mouse models. In addition, stress-induced cardiac hypertrophy is dependent on mTOR in mice [68]. These results indicate that mTOR is crucial for the abnormal cardiac overgrowth, and that mTOR inhibitors are promising agents for treatment of human cardiac hypertrophy.

Since the first surgery of percutaneous transluminal angioplasty in 1977, coronary intervention has led to the successful treatment of patients with narrowing of the coronary arteries [69]. Traditional balloon angioplasty and the stents that were developed later have been used widely to unblock and widen the affected arteries. However, after angioplasty, the artery often narrows again (a phenomenon called restenosis) because of vessel recoil or/and the overgrowth of scar tissue on the artery wall. Although insertion of a stent eliminates the vessel-recoil problem and is, therefore, a big improvement over balloon angioplasty, restenosis still occurs in many patients because of overgrowth of VSM cells. This is because the interaction between blood components and the metal surface of the stent stimulates proliferation of VSM cells. To circumvent this issue, drug-eluting stents have been developed to inhibit the growth of VSM cells [70]. Rapamycin (sirolimus)-coated stents effectively reduce restenosis and have been approved by the FDA [70]. The proposed mechanism of inhibition of proliferation of VSM cells by rapamycin involves inactivation of S6K1, impairment of Rb phosphorylation, and prevention of p27 downregulation [70]. Additionally, studies *in vitro* show that rapamycin inhibits platelet-derived growth factor-induced migration of human VSM cells without affecting their cytoskeletal components and ability to bind collagen [71].

Neurological functions and disorders

Recent findings show that mTOR inhibitors might treat some human neurological disorders such as Huntington's, Alzheimer's and Parkinson's diseases. Because rapamycins are hydrophobic, they pass through the blood–brain barrier, making them attractive drug candidates in treating the aforementioned diseases and brain tumors. Huntington's disease is a neurodegenerative condition caused by a mutant form of huntingtin with abnormally long glutamine repeats at the N terminus. The mutant protein accumulates as intraneuronal aggregates that are thought to cause nerve cell damage and toxicity, possibly by interacting with and disrupting the transcriptional activity of several transcription factors. Rapamycin attenuates the accumulation of huntingtin and cell death, and protects against neurodegeneration in animal models of Huntington's disease [72]. Moreover, rapamycin induces an autophagy response that has been suggested to play a role in the 'clearance' of huntingtin aggregates. Similar large-protein aggregates occur in other neurodegenerative disorders such as aggregation of the insoluble β-amyloid in the brain of Alzheimer's patients. Thus, the rapamycin-induced autophagy response might help to remove and degrade β-amyloid in Alzheimer's patients. Interestingly, several reports indicate that mTOR might also be linked to Alzheimer's disease through translation of tau mRNA and degradation of tau protein [73]. The tau protein is found frequently in the brains of Alzheimer's patients, and is thought to contribute to the formation of neurofibrillary tangles. In a fly model, it has been demonstrated that rapamycin reduces the concentration of tau and lowers the toxicity caused by tau accumulation [74]. Therefore, mTOR inhibitors might be useful in preventing the accumulation of toxic tau protein in Alzheimer's patients.

As mentioned previously, mTOR signaling has a role in the response to stress. Under stressful conditions such as oxidative insult, mTOR signaling is inhibited, which results in the induction of some stress-responsive genes. One such stress-induced gene, which is involved in oxidative response in yeast is *YDR533C* [75]. Mutation of the human homolog, *DJ-1*, is associated with autosomal recessive, early-onset Parkinson's disease [76]. It is postulated that inhibition of mTOR might induce the expression of *DJ-1* and so protect neurons in Parkinson's patients. There is also evidence that tuberous sclerosis complex (TSC) mutations cause neuropsychiatric disorders such as epilepsy, mental retardation and autism [77]. The neuropathology of TSC is probably due to developmental abnormalities of the cerebral cortex, including missing normal six-layered structure of the cortex, dysmorphic neurons, large astrocytes and a unique type of cell known as a giant cell [78]. As the major downstream effector of TSC proteins, mTOR might also be associated with TSC-mediated neuropsychiatric disorders, and the therapeutic inhibition of mTOR might benefit these diverse neurological diseases.

In addition to being involved in neurological disorders, mTOR is implicated in the normal function of the CNS. Learning and memory are achieved by long-term synaptic plasticity, often called long-term potentiation (LTP) in mammals and long-term facilitation (LTF) in the marine snail *Aplysia*. Long-term changes in synaptic function and structure are confined to the stimulated synapses and require the local synthesis of proteins from pre-existing mRNAs. mTOR and translational components are enriched at postsynaptic sites [79] and both localized protein synthesis and the induction of LTP/LTF are inhibited by rapamycin [80]. These findings demonstrate that mTOR controls synaptic protein synthesis, and indicate that mTOR has a positive role in learning and memory, and in brain function in general.

Regulation of lifespan and aging

The world is facing a rapid expansion of an aging population. Aging is characterized as a progressive accumulation of functional impairments in organs and tissues, leading to increased risk of diseases and death. Age-related diseases and lifespan are two important readouts of aging. Recently, studies using model organisms such as yeast, worms, fruit flies and mice have identified key genetic and environmental factors in aging. Many genes that affect longevity were discovered in *Caenorhabditis elegans*. Among them, genes that are involved in the insulin or insulin-like growth factor (IGF) signaling pathway have attracted much attention. In *C. elegans*, mutations in the IGF1–PI3K pathway extend lifespan: mutation in *daf-2*, the insulin/IGF receptor results in a twofold increase in lifespan and mutation in *age-1*, the PI 3-kinase that acts downstream of *daf-2*, leads to 65% extension of lifespan. In *Drosophila*, mutation in either *dTOR* or its substrate *dS6K*, results in a longevity phenotype, and overexpression of *dTSC1* and *dTSC2*, which leads to inhibition of dTOR, also extends lifespan [81]. In yeast, extension of the replicative and chronological lifespan have also been observed as a consequence of mutations in the

TOR pathway [82,83]. Recent studies indicate that the role of the TOR pathway in lifespan regulation is conserved in mammals. For example, heterozygous deletion of the IGF receptor in mice extends lifespan by 26% [84], and polymorphisms in the genes that encode the IGF1 receptor and PI 3-kinase are linked with longevity in humans [85].

Lifespan can be also modified by the simple, non-genetic manipulation of caloric restriction (CR), which is a powerful method that enhances longevity in yeast, *C. elegans*, fruit flies, mice and other nonhuman mammals. Usually, CR is achieved by reducing the food intake by 30–40% of *ad libitum*, without compromising the supply of essential nutrients. This simple manipulation extends lifespan by 15–40%, and also delays the onset of age-related diseases [86]. CR leads to several physiological changes, including lower plasma concentration of insulin, lower body temperature, and decreased oxidative damage to proteins, DNA and lipids [87]. The molecular mechanism by which CR increases longevity is unclear. However, the IGF/TOR pathway appears to have a key role. First, CR reduces the plasma IGF-1 concentration [88]. Second, the lifespan of fruit flies and yeast subjected to CR is not increased further by mutations in the IGF/TOR pathway [82]. Finally, the role of the TOR pathway as cellular-nutrient sensor is consistent with the idea that it mediates the CR effect on lifespan.

Identification of the downstream effectors of the IGF/TOR pathway in lifespan regulation is an important future task. One possible mechanism is upregulation of autophagy as a result of TOR inhibition. siRNA knockdown of BEC-1, a protein that is involved in autophagy, suppresses the longevity phenotype of *daf-2* in *C. elegans* [89]. However, the lifespan of wild-type worms is not affected by autophagy deficiency. It is possible that autophagy plays a role in the aging of long-lived organisms, where clearance of damaged proteins and organelles is necessary to maintain the normal functions of organs. Another possible mechanism is upregulation of the anti-stress response. Mutation in *daf-16*, the gene that encodes the forkhead transcription factor in *C. elegans*, suppresses the longevity phenotype of *daf-2* mutation [90]. Daf-16 has been shown to cross-talk with the TOR pathway by affecting transcription of the Raptor ortholog daf-15, a presumed TORC1 component, providing more evidence that dTOR is involved [91]. The involvement of the TOR pathway in lifespan regulation, together with the effects of rapamycin on aged-related diseases such as cancer, obesity, type II diabetes mellitus, autoimmune diseases, cardiovascular diseases and neuronal degeneration diseases, demonstrate the possibility that pharmacological approaches might extend human lifespan and improve the quality of life.

Other diseases

Increasingly, mTOR is implicated in other, less well-known diseases. An interesting example is ADPKD, which is an inherited condition that frequently leads to renal failure. Most cases of ADPKD are caused by mutations in polycystin-1 (PC1). It was shown recently that the cytoplasmic tail of PC1 interacts with tuberin, which results in inappropriate activation of the mTOR pathway in the epithelial cells that line the cysts in the kidneys of humans with ADPKD and in mouse models [92]. Rapamycin is effective in reducing renal cystogenesis in mouse models and the size of polycystic kidneys in human ADPKD patients after receiving a transplant. Phase I/II clinical trials are ongoing for rapamycin-treatment of ADPDK patients.

Discovery of new inhibitors of mTOR

A major challenge is to identify and understand the long-term negative effects of mTOR inhibitors. Although rapamycin and the currently available rapamycin analogs are well tolerated, their side-effects are not documented fully. Nevertheless, because mTOR is involved in many cellular processes and disease pathways, selective inhibitors that target a subset of mTOR-regulated functions are likely to reduce the undesirable side-effects. By contrast, many tumors are not very responsive to rapamycin therapy, the mechanism of which is still unclear. Conceivably, an agent that disrupts both mTOR complexes is likely to be a more potent anticancer drug. In addition, new rapamycin derivatives might have improved pharmacological properties and/or reduced production costs.

Three rapamycin analogs, namely CCI779, RAD001 and AP23573, have been designed and manufactured for development as anticancer and immunosuppressive drugs. There is an on-going effort to identify additional rapamycin analogs with pharmacological improvements over rapamycin, reduced production costs, minimized side-effects, and to study SARs. Usually, drug analogs are generated by synthetic methods, semi-synthetic methods, precursor feeding and biosynthetic engineering. Recent advances in the area of rapamycin biosynthesis have shown promise for novel rapamycin analogs. For example, precursor-directed biosynthesis has been evaluated by feeding alternative rapamycin-precursor molecules to *Streptomyces hygroscopticus*. During rapamycin biosynthesis, L-lysine is converted to L-pipecolate, which is then incorporated into the molecule just before the final closure of the macrocyclic ring. It has been demonstrated that (±)-nipecotic acid enhances the incorporation of pipecolate analogs into rapamycin, leading to the production of two new sulfur-containing rapamycin analogs, 20-thiarapamycin and 15-deoxo-19-sulfoxyl-rapamycin [93]. Because rapamycin is synthesized via a mixed polyketide synthase–nonribosomal peptide synthetase enzymatic complex, genetic engineering of these polyketide synthases of *S. hygroscopticus* is a potential route for generating novel rapamycin analogs. Such a combinatorial biosynthesis method has produced a library of rapamycin analogs with altered oxidation and alkylation patterns. Moreover, exogenous carboxylic acids can be used to increase the diversity through mutasynthesis of the engineered rapamycin-producing strains for novel rapamycin analogs [94,95]. This approach has the advantage of rapid generation of many rapamycin analogs for SAR analysis and drug discovery.

Another approach, SAR by nuclear magnetic resonance spectroscopy, has been used to identify structurally simplified analogs of FK506 (the immunosuppressive compound that is structurally related to rapamycin) that bind to FKBP12 with high affinity [96]. This technology seems to be particularly useful in target-directed drug research and might be applied in the high-throughput screening of natural-product libraries for therapeutic analogs of rapamycin. Computational database screening such as Ludi has also been used as a tool to assist the *de novo* design of FKBP12 ligands and SAR studies, resulting in the identification of the simplified analog of FK506 [97]. This might represent an alternative approach for finding rapamycin analogs with tractable synthetic routes that block mTOR

function. With respect to modification of rapamycin itself, there is little room to improve the potency because rapamycin is already a potent inhibitor of mTOR. Nevertheless, rapamycin can be optimized further: for example, a hydroxyl group on carbon 43 of rapamycin is unstable because of its intrinsic metabolic site, and a carbonyl group on carbon 15 is important for FKBP12 binding [98]. Optimization of these sites might improve the stability and pharmacokinetic properties of the drug.

Of the two distinct mTOR complexes, mTORC1 and mTORC2, only mTORC1 is sensitive to inhibition by rapamycin [17]. A recent study reported that chronic rapamycin treatment also affects mTORC2, and it suggested that inhibition of mTORC2 is more important for the anticancer effect of rapamycin in some tumors [99]. The PIKK kinase domain of mTOR appears to be crucial for the function of both mTORC1 and mTORC2 [100]. Therefore, targeting the PIKK domain is likely to lead to more potent inhibitors of mTOR for cancer therapy. Recently, Shokat and colleagues synthesized a series of isoform-selective inhibitors of the PI 3-kinase family and defined the structural basis for their specificity [101]. They also assayed systematically the activity of these compounds on different members of the PI 3-kinase family. One of the compounds, PtdIns-103 has dual specificity for mTOR and PI 3-kinase α [102]. When used in a mouse model of malignant glioma, PtdIns-103 has excellent antitumor activity. However, one major concern is that inhibition of both mTOR complexes might have considerable toxicity. However, PtdIns-103 has no detectable side-effects, which indicates that the kinase-inhibitor approach is feasible.

Recent advances in the understanding of the molecular interactions between the components of the mTOR pathway provide excellent opportunities for developing novel inhibitors of this pathway. For example, stable association of mTOR with several proteins, including GβL/mLST8 and raptor/mKog1, is important for the function of mTOR [17]. It is conceivable that targeting these proteins might provide effective, alternative approaches for the development of new drugs. More recently, a study has demonstrated that farnesylthiosalicyclic acid promotes the dissociation of raptor and mTOR, and decreases phosphorylation of S6K1 in breast cancer cells [103], which demonstrates that this approach is feasible. It is estimated that, on average, each human kinase phosphorylates ~20 substrates. Although mTOR is known to control diverse cellular processes, only two proteins (4E-BP1 and S6K1) have been shown convincingly to be direct substrates of mTOR. More efforts are needed to identify substrates of mTOR, which are instrumental to understanding the overall growth regulatory functions of mTOR. Each substrate tends to be involved in a subset of mTOR-regulated processes and, thus, either one or a subset of diseases, so targeting mTOR substrates is likely to generate more-specific, small-molecule compounds. For example, mice that lack either S6K1 or 4E-BP1 prevent high fat diet- and age-dependent obesity (Figure 4) [65,66]. Conceivably, these proteins might be used to screen for small molecules that might prevent and/or treat obesity.

Future perspective and conclusions

We have experienced amazing advances in the understanding of mTOR signaling during the past 15 years. This basic research has inspired clinical studies that reveal crucial roles of mTOR in a range of human disorders. Further research on mTOR will generate new understanding of how cells control their functions, with growth as the centerpiece. They will also reveal molecular details of how malfunctions at various steps lead to disease states, through genetic and/or environmental changes. Inhibitors of mTOR have already been shown to be well-tolerated, effective therapeutics in several disease areas (organ transplantation and drug-eluting stent). The future looks even brighter for mTOR inhibitors, many of which are either already in clinical trials (e.g. cancer) or have been implicated as useful therapeutic agents. We anticipate that the existing inhibitors of mTOR will have a much greater role in managing many major human diseases. Understanding the precise role of mTOR in regulating human aging and neurodegeneration are of paramount importance in helping with the health issues associated with an aging society. Although mTOR inhibitors are undergoing clinical trials to evaluate their effects in organ transplantation and inhibiting cancer cell growth, their potential to treat obesity, diabetes and neurological disorders are far behind on the clinical-development track. Evaluation of the clinical efficacy of mTOR inhibitors in these age-related diseases will have a long-lasting impact on society.

Acknowledgement

We apologize for being unable to cite all the relevant publications due to space constraint. Research in the authors' laboratories was supported by grants from the National Institutes of Health (L.F.L. and X.F.S.Z).

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