The mammalian target of rapamycin (mTOR) signaling pathway is a master regulator of cell growth and metabolism. Deregulation of the mTOR pathway has been implicated in a number of human diseases such as cancer, diabetes, obesity, neurological diseases, and genetic disorders. Rapamycin, a specific inhibitor of mTOR, has been shown to be useful in the treatment of certain diseases. Here we discuss its mechanism of action and highlight recent findings regarding the effects and limitations of rapamycin monotherapy and the potential utility of combination therapy with rapamycin.

An Introduction to Rapamycin: History and Mechanism of Action
Rapamycin was initially discovered as an antifungal metabolite produced by Streptomyces hygroscopicus from a soil sample of Easter Island (also known as Rapa Nui). Subsequently, rapamycin was found to possess immunosuppressive and antiproliferative properties in mammalian cells, spurring an interest in identifying the mode of action of rapamycin. Rapamycin was shown to be a potent inhibitor of S6K1 activation, a serine/threonine kinase activated by a variety of agonists (Chung et al., 1992; Kuo et al., 1992; Price et al., 1992) and an important mediator of PI3 kinase signaling (Chung et al., 1994). Concurrently, the target of rapamycin (TOR) was identified in yeast and animal cells (Laplante and Sabatini, 2012; Loewith and Hall, 2011). Rapamycin forms a gain-of-function complex with the 12 kDa FK506-binding protein (FKBP12), and this complex binds and specifically acts as an allosteric inhibitor of mammalian TOR (mTOR, also known as mechanistic TOR) complex 1 (mTORC1).

Biochemical and genetic analysis of mTOR has demonstrated that it is present in two functionally distinct complexes. The core components of mTORC1 consist of mTOR, mammalian lethal with sec-13 protein 8 (mLST8), and regulatory-associated protein of TOR (raptor). Additional components include DEP-domain-containing mTOR-interacting protein (DEPTOR) and proline-rich Akt substrate 40 kDa (PRAS40). The mTOR complex 2 (mTORC2) core is composed of mTOR, rapamycin insensitive companion of mTOR (rictor), stress-activated protein kinase-interacting protein 1 (mSin1), and mLST8. Protein observed with rictor 1/2 (protor 1/2) and DEPTOR are additional regulatory components (Cornu et al., 2013; Laplante and Sabatini, 2012). S6 kinase 1 (S6K1) and eukaryotic inhibition factor elf4E binding protein 1 (4E-BP1) are two well-characterized substrates of mTORC1 (Ma and Blenis, 2009).

Only mTORC1 is acutely sensitive to inhibition by rapamycin. However, long-term exposure to rapamycin inhibits mTORC2 in some cell types by sequestering newly synthesized mTOR molecules (Laplante and Sabatini, 2012).

Effects of Rapamycin in Cancer
Increased activation of mTORC1 is observed in numerous human cancers due to gain-of-function mutations in oncogenes (i.e., PI3K, AKT, or Ras) and/or loss-of-function mutations in tumor suppressors (i.e., PTEN, LKB1, or TSC1/2), upstream regulators of mTORC1. These mutations provide cancer cells with a selective growth advantage in comparison to normal cells (Menon and Manning, 2008). In order to meet the high demands of proliferation, cancer cells often have fundamental alterations in nutrient uptake and energy metabolism, processes that are directly controlled by the mTORC1 pathway. Accordingly, in addition to driving protein synthesis, oncogenic activation of mTORC1 promotes a gene expression program that is involved in cancer cell metabolic reprogramming. Activation of mTORC1 promotes glycogenolysis via upregulation of hypoxia-inducible factor alpha (HIF1α) and c-Myc, stimulates lipid biosynthesis and the pentose phosphate pathway through sterol regulatory element binding protein 1 (SREBP-1) (Yecies and Manning, 2011), and positively controls glutamine metabolism by SIRT4 repression (Csibi et al., 2013). Thus, drugs that selectively target mTORC1, like rapamycin, are expected to impair cancer metabolism and are considered promising anticancer therapies.

The poor solubility and pharmacokinetics of rapamycin triggered the development of several rapamycin analogs (rapalogs). Two water-soluble derivatives of rapamycin, temsirolimus and everolimus, were approved by the Food and Drug Administration (FDA) in 2007 and 2009, respectively, for the treatment of advanced renal cancer carcinoma (RCC). In 2011, the FDA approved the use of everolimus for patients with progressive neuroendocrine tumors of pancreatic origin (PNET). Additionally, temsirolimus was evaluated in several clinical trials for the treatment of advanced neuroendocrine carcinoma (NEC), advanced or recurrent endometrial cancer, and relapsed or refractory
mantle cell lymphoma (MCL, approved in the European Union in 2009). Moreover, a few trials of everolimus were conducted in patients with advanced gastric cancer, advanced non-small-cell lung cancer (NSCLC), and advanced hepatocellular carcinoma. Ridaforolimus, a rapamycin analog, was also examined in clinical trials for advanced bone and soft-tissue sarcomas as well as a variety of advanced solid tumors (Wander et al., 2011). Overall, however, rapalogs have only achieved modest effects in major solid tumors in the clinic. The reasons for the limited clinical success of rapalogs have not been established but are likely related to the large number of mTORC1-regulated negative feedback loops that suppress upstream signaling systems such as activation of receptor tyrosine kinases (RTKs), PI3K-Akt signaling, and Ras-ERK pathway and which can be reactivated with rapamycin (discussed more extensively below). In order to overcome these limitations, alternative strategies have been explored in the past few years. For instance, a number of ATP-competitive mTOR inhibitors have been developed, blocking both mTORC1 and mTORC2 activity. Due to high-sequence homology shared between mTOR and PI3K, some compounds that were originally identified as PI3K inhibitors were later shown to inhibit mTOR. Unlike rapamycin, which is a specific allosteric inhibitor of mTORC1, these ATP-competitive inhibitors target the catalytic site and prevent the feedback-mediated PI3K/Akt activation (described below), and therefore can potentially offer broader, more potent, and sustained mTOR inhibition (Benjamin et al., 2011) (Figure 2).

Effects of Rapamycin in Lymphangioleiomyomatosis, Tuberous Sclerosis Complex, and Neurological Diseases
Lymphangioleiomyomatosis (LAM) is a rare lung disease characterized by abnormal proliferation of smooth muscle-like cells. Infiltration of the lung with these cells by a mysterious mechanism referred to as benign metastasis can result in an anti-inflammatory response, cyst formation, and ultimately respiratory failure (Yu and Henske, 2010). LAM arises almost exclusively in women during their childbearing age and occurs either as an isolated disorder (sporadic LAM) or in association with tuberous sclerosis complex (TSC). TSC is a genetic disorder characterized by the development of benign hamartomatous tumors involving multiple organ systems. Both LAM and TSC are caused by loss-of-function mutations in the \( TSC1 \) or \( TSC2 \) genes, whose protein products function as a complex to inhibit the activity of mTORC1. Hence, the use of mTORC1 inhibitors could potentially benefit LAM and TSC patients. In the past few years, several clinical trials were conducted to examine the efficacy and safety of rapamycin-based therapy. For example, in 2011, McCormack et al. (2011) reported a double-blind, randomized, placebo-controlled trial in which 89 patients with LAM who had moderate lung impairment were assigned to either sirolimus (also known as rapamycin) or placebo for 1 year, followed by a 1-year observation period. Their study demonstrated that treatment with sirolimus for 1 year...
stabilized lung function and improved quality of life in patients with LAM. However, once patients stopped taking sirolimus, their lung function deteriorated again. The authors concluded that additional trials are required to investigate the duration of treatment and long-term safety of sirolimus (McCormack et al., 2011). Approximately 80% of all TSC patients and 50% of women with LAM also develop angiomyolipomas (AMLs), which are benign tumors of the kidney. Bisler et al. conducted two separate trials to evaluate the use of sirolimus or everolimus for AML in TSC or LAM. Their results showed that sirolimus or everolimus decreased AML volume (Bisler et al., 2008, 2013). However, the tumors returned to their original states when sirolimus was discontinued, underscoring the cytostatic and not cytotoxic effects of rapamycin and rapalogs. Since rapamycin-based therapy suppresses immune function and may cause serious side effects such as thrombocytopenia and hyperlipidemia, impaired wound healing, nephrotoxicity, and altered insulin sensitivity, the safety of long-term use still remains uncertain.

A growing body of evidence suggests that the mTORC1 pathway may also be involved in a number of neurological disorders. For instance, patients with TSC are prone to a wide range of neurological diseases, such as epilepsy, neurocognitive dysfunction, and autism. Therefore, inhibition of mTORC1 is a potential therapeutic option. In a mouse model of TSC, rapamycin treatment was shown to suppress epilepsy, and shortly after, the study by Muncy et al. demonstrated that rapamycin reduced seizure frequency in a 10-year-old girl with TSC (Muncy et al., 2009). A recent study by Sato et al. reported that rapamycin reverses impaired social interactions associated with autism spectrum disorder in a mouse model of TSC. Their data suggested that the abnormal behaviors displayed by TSC-deficient mice were correlated with altered gene and protein expressions involved in the mTOR signaling (Sato et al., 2012). In 2012, the FDA approved the use of everolimus for the treatment of patients with TSC who have subependymal giant cell astrocytoma (SEGA), a neurological manifestation of TSC. Currently, there is an ongoing phase two trial assessing the possible neurocognitive benefits from treatment with everolimus (NCT01289912). Future therapies involving the use of ATP-competitive mTOR inhibitors alone or in combinations with other drugs, such as estrogen antagonists, are being explored for the treatment of LAM.

Finally, a number of studies have indicated that pharmacological inhibition of mTORC1 can provide neuroprotection in several in vivo models of neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and spinocerebellar ataxia type 3 (Bove et al., 2011). Mounting evidence suggested that accumulation of misfolded and aggregated proteins is a common feature of these diseases, possibly caused by mTORC1-driven protein synthesis and defective autophagic degradation. Thus, suppression of protein synthesis and induction of autophagy by rapamycin may prevent or diminish protein aggregation. Moreover, a recent study has shown that rapamycin improves survival and attenuates disease progression in a mouse model (Ndufs4<sup>-/-</sup>) of Leigh syndrome, which is a neurological disorder characterized by a genetic defect that disrupts mitochondrial function (Johnson et al., 2013b). For more detailed descriptions of the molecular mechanisms underlying how rapamycin exerts its neuroprotective effect, we refer the readers to a comprehensive review (Bove et al., 2011) (Figure 2).

**Effects of Rapamycin on Longevity and Aging**

mTOR signaling is an important player in longevity regulation. Genetic or pharmacological inhibition of mTOR signaling has been found to extend lifespan of invertebrates including yeast, nematodes, and fruit flies (Lamming et al., 2013). In 2009, Harrison et al. showed that rapamycin extends both median and maximal lifespan of male and female genetically heterogeneous mice when beginning treatment at 9 or 20 months of age (1.5–2 year duration) (Harrison et al., 2009), representing the first demonstration in mammals. Subsequent work by other groups confirmed the positive effect of rapamycin on lifespan in mice with different genetic backgrounds and other model organisms (Lamming et al., 2013). Two classes of explanations may account for these observations: (1) rapamycin increases lifespan by slowing aging, or (2) rapamycin inhibits detrimental metabolic diseases or lethal neoplastic diseases, independent of aging effects. To test the hypothesis that rapamycin might retard aging in mice, Wilkinson et al. used a genetically heterogeneous mouse model and analyzed a number of age-related pathologies as well as age-dependent spontaneous activity of mice upon rapamycin treatment beginning at 9 months of age (1 year duration).
Their results suggested that age-dependent changes occur more slowly in rapamycin-treated mice, including alterations in heart, liver, endometrium, adrenal gland, and tendon elasticity. Rapamycin was also shown to diminish age-related decline in spontaneous activity of mice (Wilkinson et al., 2012). As rapamycin is known to have modest antiproliferative properties in many forms of cancer, lifespan extension by rapamycin could also be caused by suppression of specific life-limiting pathologies (e.g., cancer). In a recent article by Neff et al. (2013), several concerns were raised with regard to the previous reports on the effect of rapamycin in slowing aging. For example, cancer is the main cause of death in mice, including the mice strain used in the study by Wilkinson et al. (2012). In addition, aging-independent effects by rapamycin were not examined previously. In the study by Neff et al. (2013), the authors dosed male C57BL/6j mice with rapamycin or vehicle control for 1 year at three different treatment onsets: young adulthood (i.e., at 4 months), midlife (i.e., at 13 months), and late in life (i.e., at 20–22 months). After completing the treatment, they performed a large assessment of diverse structural and functional aging phenotypes in a variety of cell types, tissues, and organ systems. Intriguingly, while rapamycin did extend lifespan in mice, age-related traits were largely unaffected. Although rapamycin was able to rescue a subset of aging-dependent phenotypes, such as spatial learning and memory impairments, as well as declined exploratory activity, similar positive effects on many of these attributes were also observed in young mice, indicating an age-independent effect. They reasoned that the discrepancy in findings could be due to different mouse models (genetic backgrounds) used in the studies or technical variations in the analysis (Neff et al., 2013), which, however, were challenged by Blagosklonny (Blagosklonny, 2013). Clearly, future studies with other mouse strains and gender are warranted (Figure 2).

Effects of Rapamycin on Metabolism

In response to nutrients and growth factors, mTOR signaling serves as a key regulator of cell metabolism to coordinate the balance between anabolic and catabolic processes. When fasting, muscle and liver produce glucose via glycogenolysis (glycogen breakdown) and gluconeogenesis (glucose synthesis), and adipose tissue generates fatty acids through lipolysis, whereas, upon feeding, glycogenesis (glycogen synthesis) is favored in muscle and liver, and lipid uptake is favored in adipose tissue. Dysregulation of mTOR signaling has been linked to the development of a few metabolic diseases, such as diabetes and obesity (Laplante and Sabatini, 2012). Inhibition of the mTOR pathway by rapamycin has been demonstrated to have both beneficial and detrimental effects on metabolism. For example, acute rapamycin treatment improves insulin sensitivity in vitro and in vivo by disrupting a S6K-mediated feedback loop (described below) (Krebs et al., 2007; Tremblay and Marette, 2001). Moreover, rapamycin treatment inhibits human adipocyte differentiation in vitro (Bell et al., 2000) and protects against high-fat-diet-induced obesity in C57BL/6J mice (16 weeks) (Chang et al., 2009a). As discussed above, rapamycin appears capable of extending the lifespan of mice and preventing the onset of many age-related diseases; however, deleterious metabolic effects associated with rapamycin were also reported in other studies. For instance, Fraenkel et al. found that rapamycin treatment (2 weeks) worsened hyperglycemia in a nutrition-dependent type 2 diabetes mouse model (Fraenkel et al., 2008). Similarly, the study by Chang et al. demonstrated that rapamycin administration (6 weeks) exacerbates glucose intolerance in diet-induced obesity KK/HIJ mice (Chang et al., 2009b). Moreover, the study by Houde et al. reported that rapamycin treatment (2 weeks) promoted insulin resistance and hyperlipidemia in rats (Houde et al., 2010). It is currently unclear how rapamycin can have both positive and negative effects. In 2012, Lamming et al. showed that rapamycin exerts different effects via separate mechanisms. They found that while reduced mTORC1 increases longevity and maintains normal glucose homeostasis, disruption of mTORC2 contributes to insulin resistance in vivo (Lamming et al., 2012). Additionally, Fang and colleagues suggested that these seemingly controversial findings might be explained by the duration of rapamycin treatment (Fang et al., 2013). Their study compared various metabolic effects in male mice after 2, 6, or 20 weeks of rapamycin treatment. After 2 weeks of rapamycin treatment, the mice displayed smaller pancreas and enlarged liver. However, with prolonged treatment, these features returned to normal levels, while adiposity, body weight (BW), and food consumption were significantly reduced. More strikingly, insulin sensitivity was altered with respect to different lengths of rapamycin treatment. Under normal physiological conditions, insulin suppresses hepatic gluconeogenesis while increasing lipogenesis, but in individuals with impaired insulin sensitivity, excess insulin is secreted to compensate for the insulin resistance. In agreement with previous findings by Houde et al. (2010), insulin levels were increased after 2 weeks of rapamycin treatment, and the treated mice became glucose intolerant and insulin resistant. Interestingly, after 6 weeks of treatment the mice exhibited a transition to improved insulin sensitivity and by 20 weeks of rapamycin treatment insulin levels were decreased while insulin sensitivity increased. Additional metabolic effects including lipid profile, oxygen consumption, and ketogenesis were also altered in a manner relative to treatment durations (Fang et al., 2013). Together, these remarkable findings may provide insight into how long-term treatment of rapamycin extends longevity in mice. However, how these studies will translate to human aging is unclear (Figure 2).

Limitations of Monotherapy

The beneficial effects of rapamycin have largely been shown in preclinical animal models. However, the clinical success of rapamycin has been associated with only a few benign and malignant cancers. Several factors may contribute to the modest outcome in the clinic. (1) Rapamycin and rapalogs are generally cytostatic instead of cytotoxic. Clinical trials have demonstrated that while rapamycin treatment can induce tumor regression, tumors regrow upon the cessation of treatment (Bissler et al., 2008; Marsh et al., 2008). (2) Rapamycin fails to completely inhibit a subset of mTORC1-mediated processes, such as protein synthesis and autophagy. Choo et al. reported that rapamycin differentially regulates S6Ks versus 4E-BP1. In several cell types, short-term treatment of rapamycin dephosphorylates 4E-BP1; however, phosphorylation of 4E-BP1 reemerges after 12+ hr treatment, resulting in a recovery in cap-dependent translation despite continued inhibition of S6K (Choo et al., 2008). In addition, although it was known that rapamycin effectively activates...
autophagy in yeast, the induction of autophagy is only mild in mammalian cells (Thoreen and Sabatini, 2009). These partial inhibitory effects may offer a potential explanation for the poor efficacy of rapamycin in clinical trials. However, it is puzzling why mTORC1 substrates have differential sensitivities to rapamycin treatment in cells. In a recent study, Kang and colleagues found that mTORC1 phosphorylation sites are one determinant for the differential responses to rapamycin. It was shown that the ability of mTORC1 to phosphorylate a specific site in vitro has a strong correlation with the respective rapamycin-resistant site in cells. For example, mTORC1 potently phosphorylated the synthetic peptides encompassing 4E-BP1 (T37) and (T46), which are rapamycin resistant. In contrast, mTORC1 weakly phosphorylated S6K1 (T389), which is rapamycin sensitive. Furthermore, their findings indicated that the sequence composition of mTORC1 phosphorylation sites is one essential factor in determining substrate quality within cells. Lastly, Kang et al. were able to control mTORC1 activity toward a substrate phosphorylation site in vitro, and subsequently alter substrate sensitivity to rapamycin, nutrient, and growth factor levels in cells as well (Kang et al., 2013). (3) Another reason for the limited clinical success of rapalogs is the existence of several feedback loops involved in cell survival responses. For instance, under normal circumstances, mTORC1-mediated activation of S6K1 promotes degradation of insulin receptor substrate (IRS) and subsequent downregulation of PI3K signaling. Inhibition of mTORC1 by rapamycin relieves this negative feedback loop, thereby increasing PI3K signaling. Likewise, mTORC1-mediated signaling via S6K1 also inhibits mTORC2 by phosphorylation of Rictor. Rapamycin blocks this feedback loop, resulting in mTORC2-mediated AKT activation. Additionally, mTORC1 negatively regulates growth factor receptor-bound protein 10 (Grb10), an inhibitor of PI3K signaling. Thus, rapamycin released mTORC1-mediated inhibition of Grb10, leading to PI3K pathway activation in certain cell types (Laplante and Sabatini, 2012). (Figure 3). (4) Recent studies also demonstrate that inhibition of PI3K/AKT/mTOR pathway can lead to reactivation of many RTKs and their downstream signaling systems (Chandarlapaty et al., 2011). (5) Compensatory pathways exist that are often activated in cancer cells or upregulated in response to chronic rapamycin treatment (discussed above). For example, other AGC kinase family members can compensate for S6K1 inhibition such as RSK and Akt (Mendoza et al., 2011). (6) Finally, elf4E expression can be upregulated with chronic inhibition of mTOR (Alain et al., 2012). One of the major unanswered questions in the field is why these various compensatory mechanisms and negative feedback loops are differentially regulated in different organisms or in different cancer cell types. Fully addressing this question will lead to an improved understanding as to why rapalog work better in some cases than in others, and that should lead to improved therapeutic responses with mTOR inhibitors in monotherapy or combination therapy.

**Rapamycin in Combination Therapy**

The modest effect of rapamycin-based therapy has prompted investigators to consider other therapeutic options. Combination therapy with rapamycin or rapalogs has become a promising approach, as various strategies have the potential of improving drug efficacy by inhibiting multiple targets including those activated by removal of feedback loops or those involved in parallel pathways. Additionally, combination approaches may delay the emergence of drug resistance.

In 2012, everolimus was approved by the FDA in combination with exemestane (aromatase inhibitor) for the treatment of postmenopausal women with advanced hormone receptor-positive HER2-negative breast cancer who experience recurrence or progression after treatment with letrozole or anastrozole (aromatase inhibitors) alone. In addition, multiple clinical trials are currently evaluating the efficacy of rapalog with several chemotherapeutic agents, including paclitaxel, doxorubicin,
and capcitabine. Furthermore, preclinical and clinical data suggested that rapalogs work effectively with targeted therapies to enhance antitumor activity, such as RTK-targeted therapy, hormone-based therapy, angiogenesis inhibitors, and IGF-IR inhibitor. For example, the combination of rapamycin and erlotinib (epidermal growth factor receptor [EGFR] inhibitor) resulted in a synergistic effect in vitro and significantly reduced tumor growth in vivo compared to single-agent treatment. This may be linked to the activation of RTKs by inhibition of the PI3K/Akt/mTOR pathway (Chandarlapaty et al., 2011). In addition, a phase one trial examined the combination of everolimus with pasireotide, a somatostatin analog, in patients with advanced neuroendocrine tumors. The preliminary data showed that the combination induced antitumor activity, and further studies are underway (Zaytseva et al., 2012). Furthermore, everolimus was shown to potentiate inhibition in growth rates in many tumors with activated mTOR in combination with lower doses of the catalytic mTOR inhibitor BEZ235, used to reduce survival via feedback loops activating Akt (Nyfeler et al., 2012). Many more clinical trials are currently investigating different combination approaches. Exciting outcomes from these studies are anticipated in the near future.

**Conclusion and Future Perspective**

Since the serendipitous discovery of rapamycin, considerable achievements have been made in understanding the mechanism of action and unraveling the intricate signaling network of mTOR. Uncontrolled mTORC1-mediated signaling is often observed in human diseases. Therefore, it was thought that pharmacological inhibition of mTOR by rapamycin would have a substantial and wide range of clinical effects. Although rapamycin-based therapy has shown benefits for patients with RCC-, TSC-, and LAM-related tumors, the use of rapamycin monotherapy in a broad spectrum of metabolic diseases, especially in treating cancers, is limited due to its modest efficacy. This may be explained by the inability of rapamycin to completely block mTORC1-mediated signaling events, the presence of several feedback loops, and the upregulation of compensatory pathways that promote cell survival and growth. Thus, there is a critical need to further define these signaling processes and to develop new strategies that can overcome these drawbacks. The recent emergence of combination therapy with rapamycin may further increase efficacy and bypass feedback activation of survival pathways. Efforts that focus on exploring novel drug combinations with optimal doses will have great potential to yield an improvement of efficacy and safety profiles. Additionally, significant promise remains for the discovery of new pathway inhibitors as well as the possibility that existing bioactive may directly or indirectly reduce mTORC1 and/or mTORC2 activity in monotherapy or in combination.

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