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Rapamycin and rapalogs

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Abstract

Inhibition of mTORC1 (mechanistic Target Of Rapamycin Complex 1) signaling promotes health and longevity in diverse model organisms. Over the past decade, excitement has built over the possibility that treatment with the mTORC1 inhibitor rapamycin can be utilized to treat or prevent age-related disease in humans. However, concerns over the side effects of rapamycin on immunity and metabolism have precluded the routine use of rapamycin as a geroprotective therapy. Here, we discuss the evidence that these negative side effects of rapamycin are largely mediated by off-target inhibition of a second mTOR Complex (mTORC2). Further, we discuss how intermittent treatment with rapamycin, specific dietary regimens, and new molecules may provide routes to the safer and more selective inhibition of mTORC1. We conclude that the time is ripe for the development of therapies based on the safe and selective inhibition of mTORC1 for the treatment or prevention of diseases of aging.

Keywords: rapamycin, mTOR, mTORC1, mTORC2, healthspan, lifespan, aging

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Inhibition of mTOR signaling extends lifespan and promotes healthspan

The mechanistic Target of Rapamycin (mTOR) is an evolutionarily conserved phosphatidylinositol-3-kinase (PI3K)-like serine/threonine protein kinase found in species ranging from yeast to worms, flies, mice and humans. mTOR signaling is acutely inhibited by rapamycin, a macrolide produced by the bacterium *Streptomyces hygroscopicus* and first found in the soil of Easter Island (1). Following many years of development, rapamycin and two rapamycin analogs (rapalogs, (2)) everolimus and temsirolimus, are now FDA-approved immunosuppressants, and are also used for the treatment of specific cancers as well as for complications of specific genetic disorders that result in hyperactive mTOR signaling.

Research over the last two decades has revealed mTOR to act as a central hub for nutrient signaling, integrating information about numerous nutrients and hormonal signals to determine if conditions favor growth and proliferation, or call for maintenance and recycling. As such, it was appreciated early on by aging researchers that mTOR signaling might be a key pathway by which calorie restriction (CR), the gold standard for anti-aging interventions in many species, promoted longevity and healthspan. Genetic inhibition of mTOR pathway signaling extends the lifespan of yeast, worms, and flies (3-6). mTOR pathway signaling was also shown to be downregulated in long-lived Ames dwarf mice (7), and it was thus hypothesized that pharmaceutical or genetic inhibition of mTOR signaling might also promote longevity in mammals (8, 9). It was first shown in 2009 that rapamycin can extend the lifespan of genetically heterogeneous mice UM-HET3 mice (10). Numerous published studies since that time (see **Table 1**) by multiple independent laboratories and groups have confirmed the ability of rapamycin to extend lifespan in multiple strains of wild-type mice as well as mouse models of a number of different diseases; importantly, the beneficial effects of rapamycin can be seen even when treatment is intermittent or conducted for only a short period of time (11-16).

Strain	Sex	Starting age	Rapa dose	Route	Control (days)	Δ lifespan (%)	Reference
Wild-type mice							
UM-HET3	Male	20 months	14 ppm	Diet	--	9	Harrison *et al.* 2009 (10)
UM-HET3	Female	20 months	14 ppm	Diet	881-895	14	
C57BL/6J.Nia	*MF*	22-24 months	4 mg/kg	IP 1x/2d	~795	>14[a]	Chen *et al.* 2009 (14)
UM-HET3	Female	9 months	14 ppm	Diet	843-891	18	Miller *et al.* 2011 (11)
UM-HET3	Male	9 months	14 ppm	Diet	780-851	10	
129/Sv	Female	2 months	1.5 mg/kg	SC 3x/week 2 weeks per 4	759	10	Anisimov *et al.* 2011 (16)
C57BL/6J.Rj	Male	4, 13, 20 months	14 ppm	Diet	~900	~10[a]	Neff *et al.* 2013 (17)
UM-HET3	Male	9 months	4.7 ppm	Diet	807	3[NS]	Miller *et al.* 2014 (18)
UM-HET3	Male	9 months	14 ppm	Diet	807	13	
UM-HET3	Male	9 months	42 ppm	Diet	807	23	
UM-HET3	Female	9 months	4.7 ppm	Diet	896	16	
UM-HET3	Female	9 months	14 ppm	Diet	896	21	
UM-HET3	Female	9 months	14 ppm	Diet	896	26	
C57BL/6J.Nia	Male	4 months	14 ppm	Diet	806*	11*	Fok *et al.* 2014 (19)
C57BL/6J.Nia	Female	4 months	14 ppm	Diet	826*	16*	
C57BL/6J.Nia	Female	20 months	2 mg/kg	IP 1x/5 days	897	7	Arriola Apelo *et al.* 2016 (12)
C57BL/6J.Nia	Male	20-21 months	8 mg/kg for 90 d	IP 1x/d	925	14	Bitto *et al*., 2016 (13)
C57BL/6J.Nia	Female	20-21 months	8 mg/kg for 90 d	IP 1x/d	847	0[NS]	
C57BL/6J.Nia	Male	20-21 months	126 ppm for 90 d	Diet	914	14	
C57BL/6J.Nia	Female	20-21 months	126 ppm for 90 d	Diet	960	9	

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Mixed	Male	600-700 d	4 mg/kg	IP 1x/2d	911	13	Fang *et al.*, 2018 (20)
Mixed	Female	600-700 d	4 mg/kg	IP 1x/2d	896	22	
UM-HET3	Male	20 months	42 ppm	Diet	772	11	Strong *et al.*, 2020 (21)
UM-HET3	Male	20 months	42 ppm for 3 mo	Diet	772	11	
UM-HET3	Male	20 months	42 ppm	Diet every other mo	772	9	
UM-HET3	Female	20 months	42 ppm	Diet	905	15	
UM-HET3	Female	20 months	42 ppm for 3 mo	Diet	905	4^{NS}	
UM-HET3	Female	20 months	42 ppm	Diet every other mo	905	8	
Disease models							
Pten$^{-/-}$	*MF*	1 month	10 mg/kg (Everolimus)	Oral	66*	>292*[a]	Hernando *et al.* 2007 (22)
FVB/N HER-2/neu	Female	2 months	1.5 mg/kg	SC 3x/week 2 weeks per 4	288	13.6	Anisimov *et al.* 2010 (15)
SOD1$^{H46R/HR8Q}$	*MF*	1.5 months	14 ppm	Diet	232	*NS*	Bhattacharya *et al.* 2012 (23)
p53$^{+/-}$	Male	<5 months	1.5 mg/kg	Water	373*	28*	Komarova *et al.* 2012 (24)
p53$^{+/-}$	Male	>5 months	1.5 mg/kg	Water	373*	10*	
p53$^{-/-}$	Male	2 months	0.5 mg/kg	Oral 1x/day 5 d on/9 d off	161	35	Comas *et al.* 2012 (25)
Lmna$^{-/-}$	*MF*	1 month	14 ppm	Diet	46	35	Ramos *et al.* 2012 (26)
Lmna$^{-/-}$	*MF*	1 month	8 mg/kg	IP 1x/2 days	55	56	
Rb1$^{+/-}$	Male	2 months	14 ppm	Diet	369	13.8	Livi *et al.* 2013 (27)
Rb1$^{+/-}$	Female	2 months	14 ppm	Diet	378	8.9	
Bmal1$^{-/-}$	*MF*	16 weeks	0.5 mg/kg	Water	~240	47	Khapre *et al.* 2014 (28)
HER-2/neu	Female	2, 4, or 5 months	0.45 mg/kg	SC 3x/week 2 weeks per 4	282, 278, 289	5.7^{NS}, 6.1, 5.5	Popovich *et al.* 2014 (29)
C57BL/6NCr HFD	Male	12 months	1.5 mg/kg	IP 1x/week	684	*b*	Leontieva *et al.* 2014 (30)
Ndufs4$^{-/-}$	*MF*	weaning	8 mg/kg	IP 1x/day	52	119	Johnson *et al.* 2015 (31)
Ndufs4$^{-/-}$	*MF*	weaning	42 ppm	Diet	52	29^{NS}	
Ndufs4$^{-/-}$	*MF*	weaning	378 ppm	Diet	52	92	
Rag2$^{-/-}$	*MF*	3 months	14 ppm	Diet	310	121	Hurez *et al.* 2015 (32)
IFN-γ$^{-/-}$	*MF*	5 months	14 ppm	Diet	398	34	
C57BLKS/J *lepr*$^{db/db}$	Male	4 months	14 ppm	Diet	349	-16	Sataranatarajan *et al.* 2015 (33)
C57BLKS/J *lepr*$^{db/db}$	Female	4 months	14 ppm	Diet	487	-18	
Tk2$^{KI/KI}$	*MF*	To dams from conception	0.8 mg/kg prior to birth, 4.0 mg/kg post-birth	Water to dam	~15	~60	Siegmund *et al.*, 2017 (34)

Table 1. The effect of rapamycin on mouse lifespan. The impact of rapamycin on median lifespan in mouse studies since 2009 where longevity or mortality rate was determined. Sex is listed separately for males and females where sex-specific data exists. The rapamycin dose listed for dietary administration indicates the drug concentration in the *ad libitum* fed diet; the dose listed for administration in water, or administered intraperitoneally (IP) or subcutaneous (SC) indicates the dose in mg per kg of body weight. Control indicates median lifespan of control group in days; Δ lifespan is the change in median lifespan (* indicates that mean is reported instead). *MF* indicates that the lifespan results were not broken down by sex or that sex was not reported; *a*: Lifespan study % increase was not determined; *b*: 100% of rapamycin-treated mice survived to 2 years of age vs. 40% of control mice; *NS*: Not Statistically Significant. Control lifespan and percentage change are estimated when precise information is not listed in the referenced study. Table is adapted from Arriola Apelo et al., 2016, *JGBS* (35) and used with permission.

In rodents, rapamycin has been shown to have numerous beneficial effects on both overall healthspan as well as specific organ systems and diseases. Rapamycin delays the onset of cancer in numerous disease models as well as wild-type mice, and it is thought that the effect of rapamycin on cancer contributes to its effects on overall longevity (17). While rapamycin does not change all age-related phenotypes (17), rapamycin has been shown to prevent or delay the onset of age-related changes in multiple rodent tissues including the heart, liver, kidney, skeletal muscle, and tendons (36-39). Rapamycin has powerful effects on the aged mouse heart, reversing pre-existing age-dependent cardiac hypertrophy and diastolic dysfunction (40, 41); the effects of rapamycin on diastolic function, hypertrophy and myocardial stiffness persist even following cessation of treatment (42). Rapamycin has been reported to rejuvenate hematopoietic stem cells of mice (14), promote the self-renewal of intestinal stem cells (43), and to reverse age-associated periodontitis, regenerating bone and reducing inflammation (44). Finally, rapamycin has been reported to have beneficial effects on cell senescence, regulating cell cycle arrest as well the senescence-associated secretory phenotype (45-48).

Thus far, there is very limited in data on the effects of rapamycin on healthspan in other organisms, but research to examine the potential benefits for rapamycin and rapalogs in companion animals, non-human primates, and even humans is now underway. A small study of the effect of rapamycin in dogs suggests that rapamycin promotes both diastolic and systolic cardiac function, and larger long-term studies will examine the effect of rapamycin on the healthspan and longevity of companion dogs (49-51). Studies of the effects of rapamycin on the healthspan and lifespan of middle-aged marmosets are already underway (52, 53), and will provide the first readout on how rapalogs effect the health and longevity on non-human primates. Finally, short-term treatment with rapamycin may rejuvenate aspects of the immune system in aged humans, boosting the subsequent response to vaccination in the aged (54).

The risks of rapamycin

The strong effects of rapamycin on mice and other model organisms has led to widespread interest in using rapamycin or rapalogs for the treatment of diseases of aging in humans. However, there is also concern about the potential side effects of chronic treatment with these compounds. The most often-expressed concern surrounds the fact that rapalogs are FDA-approved as immunosuppressants for organ transplantation; a necessary consequence of treatment with an immunosuppressant is an increased risk of infection, and indeed some rapalogs have received “black-box” warnings in part due to the risks of infection or cancer due to suppression of tumor immune surveillance (55). The risks of rapalogs when given chronically and at a high dose have been well characterized during clinical trials for the treatment of tuberous sclerosis complex (TSC), a genetic disorder resulting in hyperactivation of mTORC1, and have in a few cases led to life-threating adverse events or death (56-59).

Less well appreciated is that chronic treatment with rapamycin is associated with metabolic consequences widely perceived as deleterious, including hyperlipidemia, hypercholesterolemia, hypertriglyceridemia, insulin resistance, glucose intolerance, and an increased risk of developing new-onset diabetes (56, 57, 60-62). These side effects are observed quite often in people when high doses of rapalogs are taken chronically for the treatment of TSC; Trelinska and colleagues observed hyperlipidemia in 66% of subjects, and hyperglycemia in 22% of subjects, in a study of people taking everolimus for about 15 months for TSC (56). Much less is known about the long-term effects of lower doses, although an increased risk of new-onset diabetes has been observed in patients taking lower doses for immunosuppression (60).

Very few studies of low dose rapalogs have been done in healthy subjects, where the data is more positive. Testing several different doses (0.5 mg/d, 5 mg/wk, or 20 mg/wk) of everolimus for 6 weeks, Mannick and colleagues found very few side effects, with an adverse result—mouth ulcers—in a single subject who had received the highest dose (54). In contrast to the subjects, who tolerated everolimus relatively well, a small, 8-week long randomized clinical trial of a low dose, 1 mg/day, of rapamycin found that 5 of 11 rapamycin-treated subjects (vs. 1 of 14 control subjects) reported side effects. Laboratory results also suggested that the subjects experienced negative metabolic effects, including a small increase in glycated hemoglobin (within-group p=0.03)

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and a 40% rise in triglyceride levels (within-group p=0.05). While these effect are more rarely seen at lower doses, rapamycin treatment is associated with an increased risk of new-onset diabetes when used as a low-dose immunosuppressant (60).

Why does rapamycin have side-effects?

In order to discuss why rapamycin has side effects, we need to discuss the biology of mTOR in greater detail. The mTOR kinase is the catalytic core of two distinct protein complexes, mTOR Complex 1 (mTORC1) and mTORC2, each of which are composed of shared as well as unique protein subunits, and phosphorylate different substrates. mTORC1 is responsive to a wide range of nutrients and hormonal cues, including the availability of amino acids, glucose, oxygen, cholesterol, insulin/IGF-1, adiponectin, and FGF21 (63-65). In contrast, mTORC2 is primarily an effector of PI3K signaling (66). Both complexes associate with unique as well as shared protein components. mTORC1 is defined by interaction of the mTOR protein kinase with the scaffold protein Raptor and mLST8/GβL, which is required for complex assembly and stability, while mTORC2 is defined by the interaction of mTOR with the scaffold protein Rictor, mLST8/GβL, and mSin1 (66).

One of the major differences between the complexes is that mTORC1 is acutely sensitive to rapamycin, while mTORC2 is not; however, mTORC2 is inhibited both in cell culture and *in vivo* in mice by rapamycin when high levels of rapamycin are present for a prolonged period of time (67, 68). This ability of rapamycin to inhibit both mTORC1 and mTORC2 is key to understanding the molecular basis for the metabolic effects of rapamycin. Building on work in rats and mice that demonstrated that prolonged rapamycin treatment causes glucose intolerance (69, 70), we performed a hyperinsulinemic-euglycemic clamp study, and determined that rapamycin causes hepatic insulin resistance (68). Intriguingly, they found that in the liver, both mTORC1 and mTORC2 were disrupted by chronic rapamycin treatment, and using mice in which either *Raptor* (mTORC1) or *Rictor* (mTORC2) was deleted in hepatocytes, they determined that the effects of rapamycin on hepatic insulin sensitivity was mediated not by inhibition of mTORC1, but instead was due to inhibition of mTORC2 (68).

mTORC1 is well-characterized as a regulator of lipid homeostasis (reviewed in (71)), but in contrast to rapamycin which promotes hyperlipidemia, genetic inhibition of either hepatic or adipose mTORC1 was originally shown to protect from diet-induced elevation of triglycerides and cholesterol (72, 73). As chronic treatment with rapamycin disrupts mTORC2 in the majority of mouse tissues examined (68, 74), a logical question to ask is if the side-effects of rapamycin treatment on lipid metabolism may due to inhibition of mTORC2. Consistent with the hypothesis that the negative effects of rapamycin on serum lipids do not result from inhibition of mTORC1, but are a consequence of inhibition of mTORC2, genetic inhibition of mTORC2 in either adipose tissue or the hypothalamus elevates plasma triglycerides (75, 76). However, recent work suggests that inhibition of mTORC1 in adipose tissue can lead to feeding-dependent systemic hyperlipidemia (77), while inhibition of hepatic mTORC2 lowers plasma cholesterol and triglycerides (78). Ultimately these genetic models are limited in part by the complete effectiveness of the inhibition; genetic deletion of *Raptor* results in the inhibition of both rapamycin-sensitive and rapamycin-resistant functions of mTORC1 (79). To address this more clearly, we recently examined the effects of chronically treating mice with an mTORC1-selective rapamycin analog, DL001. In contrast to rapamycin, DL001 inhibits mTORC1 without negatively affecting glucose tolerance and without increasing plasma cholesterol, triglycerides, or free fatty acids in fasted mice (80). These results clearly suggest that the negative side effects of rapamycin on circulating lipids is mediated by disruption of mTORC2.

The immunoregulatory effects of rapamycin have been the subject of intensive research. Rapamycin is generally viewed as immunosuppressive due to its effects on T regulatory cells (Tregs), which requires inhibition of both mTORC1 and mTORC2 (reviewed in (81)). In contrast, other aspects of immunity, including macrophage polarization and the innate immune system, has been shown to be regulated individually when either mTORC1 or mTORC2 is inhibited genetically (82-85). We compared the effect of chronic treatment with rapamycin or DL001 on the immune cell profile of mice, and found that DL001 had a substantially smaller impact on T cells, including on Tregs (80). While further research is necessary, in combination these findings suggest that some, but not all, of the effects of rapamycin on the immune system are likely mediated by disruption of mTORC2.

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A simple model: Inhibition of mTORC1 is beneficial, inhibition of mTORC2 is detrimental

As chronic treatment with rapamycin can inhibit both mTORC1 and mTORC2 *in vivo* in mice, a logical question is if mTORC1, mTORC2, or both mediate the beneficial effects of rapamycin on longevity. Numerous studies in model organisms have shown that inhibition of mTORC1 itself or signaling pathways downstream of mTORC1, including S6K and translation initiation factors, can extend the lifespan of yeast (3, 5, 86), C. elegans (6, 87, 88), and D. melanogaster (4). Several studies in mice demonstrate that inhibition of mTORC1 is beneficial. Mice doubly heterozygous for *mTOR* and *mLST8*, which have decreased mTORC1 activity, show a significant increase in lifespan (68), while inhibition of hepatic mTORC1 rescues an age-associated defect in ketogenesis (89). Specific inhibition of S6K1, a major mTORC1 substrate, extends the lifespan of mice (90), while activation of 4E-BP1, a substrate normally repressed by mTORC1, in skeletal muscle preserves metabolic health during aging or exposure to a high fat diet (91, 92). Other genetic mutations that reduce mTORC1 or S6K1 signaling, including overexpression of the TSC complex member *Tsc1*, deficiency of mitochondrial arginase type II, or mutations in growth hormone signaling likewise increase lifespan (93-95).

Until recently, there have been very few studies which have examined the role of mTORC2 in lifespan and healthspan. While genetic inhibition of mTORC2 signaling in *C. elegans* was initially shown to extend lifespan (96), subsequent work has shown that the effects of mTORC2 inhibition on worm lifespan are dependent upon the tissue that is targeted, the temperature, and the food source (97, 98). In flies, overexpression of the mTORC2 component *Rictor* extends lifespan (99). Whole body deficiency of mTORC2 signaling, or tissue-specific inhibition of mTORC2 signaling in the brain, liver, or adipose tissue, impairs healthspan and reduces lifespan in wild-type and long-lived mice (75, 76, 100, 101). Conversely, mTORC2 activity is elevated in long-liver Snell dwarf mice and $Ghr^{-/-}$ mice (94, 102), as well as in acarbose-treated and 17-α estradiol-treated male mice, which live longer when fed diets containing these compounds (102, 103).

There is essentially no data to support a beneficial effect of mTORC2 inhibition on the health and longevity of wild type mice, but the beneficial effect of rapamycin on a mouse model of Leigh's syndrome, a mitochondrial disorder, is mediated at least in part by disruption of mTORC2 signaling, which results in the inhibition of multiple PKC isoforms (104). As PI3K/mTORC2 signaling is critical to the growth of some cancers, it is likely that mTORC2 inhibition contributes to the beneficial effects of rapamycin on the survival of at least some mouse models of cancer (105, 106). There may be also some downstream effectors of mTORC2 that, when inhibited, promote longevity; partial genetic inhibition of *Akt1* extends the lifespan of both *C. elegans* and mice (107). On balance, these data show that inhibition of mTORC2 not only results in negative changes to glucose and lipid metabolism and immunity, but impair overall health and survival.

In light of our model that inhibition of mTORC1 is beneficial, while inhibition of mTORC2 is detrimental, we and others have suggested that compounds that target mTORC1 more specifically than rapalogs will allow us to realize the beneficial effects arising from mTORC1 inhibition while reducing or eliminating the mTORC2-mediated side effects. In order to discuss strategies for specifically inhibiting mTORC1, we must first discuss how mTORC1 activity is normally regulated. Many of these mechanisms for mTORC1 regulation, as well as potential points through which mTORC1 could be specifically inhibited, are highlighted in **Figure 1**, and discussed in detail below.

Regulation of mTORC1 activity by nutrients and environment

mTORC1 integrates numerous environmental signals that indicate if conditions are favorable for a wide range of anabolic processes, including ribosomal biogenesis, protein translation, autophagy, lipogenesis and nucleotide biogenesis. The activation of mTORC1 requires the allosteric binding of mTORC1 by its co-activator Rheb-GTP, which results in the realignment and activation of kinase-site residues (108, 109). Two major pathways converge on mTORC1 to control its regulation: One pathway, regulated by amino acids as well as other stimuli, controls the recruitment of mTORC1 to the lysosome, a physical location inside the cell where the Rheb GTPase can be found. A second pathway, regulated by insulin/IGF-1 signaling as well as other hormonal and environmental cues, regulate the GTP/GDP binding status of Rheb.

The recruitment of mTORC1 to the lysosome by amino acids has been the subject of intensive research for more than a decade, and is reviewed in detail elsewhere (110). Briefly, mTORC1 is recruited to the lysosome by interacting with heterodimeric pairs of the Rag family of small GTPases (111, 112). When amino acid levels drop within a cell, RagA/B bind GDP and RagC/D bind GTP (e.g., $RagA^{GDP}$/$RagC^{GTP}$); when so configured, the Rags do not interact with mTORC1. However, when amino acids are present, the Rags flip their nucleotide-bound state (e.g., $RagA^{GTP}$/$RagC^{GDP}$) and interact with the mTORC1 component Raptor, localizing mTORC1 to the lysosome (111, 112).

The nucleotide binding status of the Rag GTPases is controlled by several different protein complexes. One of the most important of these is the Ragulator, a lysosomal protein complex with guanine nucleotide exchange factor (GEF) activity for two of the Rag proteins, RagA and RagB (113-116), and which senses the availability of amino acids in part via the lysosomal vacuolar ATPase (v-ATPase), which has extensive amino acid-dependent interactions with the Ragulator (117). A lysosomal amino acid transporter, SLC38A9, which interacts with both the Ragulator and the v-ATPase, acts as an amino acid sensor upstream of mTORC1 for several different amino acids, including asparagine, arginine, glutamine, histidine and lysine (118-120). It is not yet clear if SLC38A9 regulates Ragulator activity in response to lysosomal levels of amino acids, or if instead SLC38A9 regulates the lysosomal efflux of amino acids into the cytoplasm, which then activate mTORC1 (121). SLC38A9 is also required for mTORC1 activation by cholesterol (63).

The nucleotide binding status of the Rag GTPases is also controlled by the GATOR complexes; GATOR1 functions as a GTPase-activating protein (GAP) for RagA and RagB, while GATOR2 acts to inhibit the activity of GATOR1 (122, 123). Three different amino acid sensors have been found that regulate mTORC1 activity by controlling GATOR1 or GATOR 2 activity. When leucine levels are low, the Sestrin family of proteins binds to and inhibit the action of GATOR2 permitting GATOR1 to inhibit the recruitment of mTORC1 to the lysosome. Leucine binding to the Sestrins, particularly Sestrin 2, relieves the inhibitory action of these proteins upon GATOR2, and thereby permits mTORC1 to be recruited to the lysosome (124, 125). The CASTOR proteins function similarly, inhibiting GATOR2 activity when arginine levels are low (126, 127). Finally, SAMTOR protein acts as an indirect sensor of methionine levels, regulating GATOR1 activity in response to levels of the methionine metabolite S-adenosylmethionine (SAM) (128), which is extremely responsive to methionine levels both in cell culture and *in vivo* (129).

Other regulators of the Rags include the FLCN complex, which acts as a GAP for Rag C and Rag D (130); the FLCN complex is itself recruited to the lysosome by RagA/RagB when amino acids are depleted (131, 132). Phosphorylation of FLCN by CDK4 allows FLCN to depart the lysosome and permits the Rags to recruit mTORC1 if amino acids are abundant (133). The leucyl-tRNA synthetase (LRS) has also been reported to function as a leucine sensor for mTORC1; it has been proposed that LRS functions as a GAP for RagD (134). Similarly, the mitochondrial threonyl-tRNA synthetase TARS2 is reported to function as a threonine sensor, interacting with GTP-RagC to promote the GTP loading of RagA, likely via the recruitment of an unidentified RagA GEF (135). Finally, aminoacyl-tRNA synthetases, including LRS, can catalyze the aminoacylation of specific lysine residues, and LRS-mediated leucylation of RagA and RagB may be important in the sensing of leucine by mTORC1 (136).

The regulation of mTORC1 activity by glucose is not yet fully understood. Glucose was originally thought to stimulate mTORC1 via suppression of AMPK, which phosphorylates components of both mTORC1 and the Tuberous Sclerosis Complex (TSC) (137, 138). However, the Rag proteins also play a role in glucose sensing (139, 140). Finally, it was recently shown that while glucose itself is not sensed by mTORC1, the glycolytic intermediate dihydroxyacetone phosphate (DHAP) is sensed via a mechanism that is dependent upon both GATOR complexes (141).

Once mTORC1 is localized to the lysosome, its activation depends upon interaction with Rheb-GTP; however, in the presence of the Tuberous Sclerosis Complex (TSC), which acts as a GAP for Rheb, Rheb is found bound GDP. The activity of TSC is controlled by many different kinases, including AKT, AMPK, CDK4/6, ERK, GSK3 and IKKβ, which phosphorylate different residues and proteins within the TSC complex (138, 142-148). In the absence of insulin/PI3K/AKT signaling, TSC localizes to the lysosome; when AKT is activated, it

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phosphorylates TSC on multiple residues, and TSC departs from the lysosome, which allows Rheb to be loaded with GTP (149). Other kinases may also regulate TSC by controlling its localization.

As much as we know about the regulation of mTORC1, there remains much we do not know. The specific molecular sensors by which mTORC1 senses amino acids other than leucine, arginine, and methionine have not yet been identified. The RagGTPases were recently reported to recruit TSC to lysosomes in response to amino acid or growth factor restriction (150); although details surrounding this process are unclear, GATOR2 and Sestrin2 may regulate TSC2 phosphorylation (151). Perhaps most importantly, the details of mTORC1 activation by amino acids were investigated in cell culture using exogenous amino acids, and a new study suggests that that lysosomal-derived amino acids activate mTORC1 via a RagGTPase-independent mechanism (152). Indeed, the Rag-GATOR pathway acts as a negative regulator of mTORC1 activity with respect to lysosomal-derived amino acids.

Rapamycin acts to inhibit mTORC1 by first forming a complex with FK506-binding protein 12 (FKBP12) which then binds to the FKBP12-rapamycin binding (FRB) domain of mTOR (153-155). In contrast, mTORC2 is not acutely sensitive to rapamycin, as components of mTORC2, specifically Rictor and mSin1, hinder the binding of FKBP12-rapamycin to mTOR (156-158). mTORC2 activity is decreased in many cell lines as well as the majority of mouse tissues when rapamycin treatment is continued for a prolonged time (67, 68, 74); mTORC2 inhibition is believed to occur indirectly, with rapamycin sequestering free mTOR and hindering the formation of new mTORC2 (67).

Specifically targeting mTORC1 signaling to promote healthy aging

The incredible detail that has been discovered about the regulation of mTORC1 by nutrient signaling as well as rapamycin has suggested that there may be multiple potential ways to selectively inhibit mTORC1 signaling. Here, we will discuss intermittent dosing regimens, dietary manipulations, novel rapamycin analogs, and other novel compounds that selectively target mTORC1.

As we have previously discussed at greater length (55), genetic mouse models in which mTORC1 is moderately inhibited (*S6K1*$^{-/-}$, *mTOR*$^{+/-}$ *mLST8*$^{+/-}$, *mTOR*$^{\Delta/\Delta}$, *TSC1*^{tg}) have demonstrated that a moderate reduction in mTORC1 activity can extend lifespan and healthspan in mice, particularly in females (68, 90, 95, 159). This suggests that strong inhibition of mTORC1 signaling may not be required to extend lifespan, and in combination with the fact that mTORC2 is only inhibited when rapamycin treatment is chronic, suggests that intermittent dosing might reduce mTORC2-dependent phenotypes while still being able to extend lifespan.

Following this logic, we identified an intermittent dosing regimen (2 mg/kg I.P. rapamycin once every 5 days) that inhibited mTORC1 moderately while substantially reducing the effects on glucose metabolism and immunity (160). Importantly, this dosing regimen was still able to extend lifespan of aged female C57BL/6J mice (12). Similarly, cancer prone 129/Sv mice treated with 1.5 mg/kg rapamycin three times per week for 2 weeks of every month had a significant extension of lifespan (15). However, a limitation of these studies was the lack of a continuous treatment control group. In part to address the question of comparative efficacy, the National Institute on Aging (NIA) Interventions Testing Program (ITP) examined the effect of treating HET3 mice with diet containing 42-ppm rapamycin every other month or continuously starting at 20 months of age. They determined that every-other-month rapamycin treatment was almost as effective at extending lifespan as continuous rapamycin treatment (21).

An alternative to intermittent treatment with rapamycin may be treatment for only a short period of life, as many of the metabolic and immunological effects of rapamycin are reversible (161). Relatively short-term treatment with rapamycin functionally rejuvenates many aged tissues, including the heart, where treatment for 8 weeks reverses age-associated declines in diastolic function as well as cardiac hypertrophy (40, 41). Importantly, these effects persist after the cessation of treatment (42). In agreement with the idea that a short-term therapy with rapamycin has persistent benefits, two different studies observed that treatment of aged C57BL/6J mice with rapamycin for 2–3 months extends lifespan (13, 14). The NIA ITP program recently examined this idea in HET3 mice, and found that treatment of 20-month-old mice for 3 months with 42-ppm rapamycin extended the

lifespan of males, but not females; male lifespan was extended to the same extent by this short treatment as male mice that continuously received rapamycin (21).

Related to the idea of intermittent or transient treatment with rapamycin is the idea of using very low doses of rapamycin. A few recent studies suggest that very low doses of the rapamycin analog everolimus given for just a few weeks can help to rejuvenate the immune system (54, 162). In this short-term intermittent low-dose context, mTORC2-mediated side effects are expected to be extremely low. A related possibility is exemplified by the new "third-generation mTOR inhibitor" RapaLink-1, which tethers a mTOR kinase inhibitor to rapamycin in order to more selectively target a mTOR kinase inhibitor to mTORC1 (163). At a low dose, this approach achieves stronger and more completely mTORC1 inhibition than a low dose of rapamycin alone can achieve (164).

mTORC1 activity, but not the activity of mTORC2, is regulated by the levels of amino acids, glucose, and insulin as described in detail above. As such, dietary interventions to decrease mTORC1 signaling have been of significant interest, particularly protein restriction. Several studies have shown that protein restriction, which extends lifespan, reduces activity of mTORC1 (165, 166). Methionine restriction, which extends the lifespan of mice and rats (167, 168), likewise reduces mTORC1 signaling (169). Restriction of branched-chain amino acids (BCAAs; leucine, isoleucine, and valine) has also recently been shown to inhibit mTORC1 but not mTORC2 and extend the lifespan of mice when begun early in life (170). Finally, low-glucose ketogenic diets also promote longevity and reduce mTORC1 signaling (171).

In addition to the possibility of eating diets reduced in protein or one or more specific amino acids, or consuming ketogenic diets, we have previously discussed the possibility that drugs could be developed that partially block intestinal uptake of specific dietary amino acids from the intestine (172). The neutral amino acid transporter B^0AT1 (SLC6A19) is the major amino acid transporter for methionine and BCAAs in the intestine, and although the effect on mTOR signaling has not been examined, mice lacking this transporter have improvements in metabolic health similar to that observed in BCAA and methionine restricted mice (173). Research to identify chemical inhibitors of this transporter are underway (174, 175). SLC7A5, also known as LAT1, is the major transporter for many essential amino acids including the BCAAs, and treatment of cancer cells with a LAT1 inhibitor, JPH203 reduced mTORC1 activity (176-178). However, as LAT1 function is also required for many normal cells and tissues, it is unlikely that LAT1 inhibitors could be used as geroprotectors.

The past few years has seen a dramatic increase in our knowledge of the structure of mTORC1 itself as well as its regulators. Building in part on an initial 26 Å resolution of mTORC1 (154), new high-resolution Cryo-EM structures (down to 3 Å) of human mTORC1 (179-181) have been developed, which have provided new insight into how mTORC1 is activated, recruits substrates, and is inhibited by rapamycin. An expanding number of structures have also been developed for specific regulators of mTORC1, including the TSC complex (182), the Rag GTPases in complex with mTORC1 and Ragulator (183-185), as well as the binding of arginine by CASTOR1 (127) and the binding of leucine by Sestrin2 (124). This knowledge is already being utilized to generate new mTORC1-selective drugs; one such compound is NR1, which binds the mTORC1 activator Rheb and blocks the activation of mTORC1 (109). A mTORC1-selective activator, NV-5138, was recently designed based in part on structural information about the binding of Sestrin2 to leucine (186); potentially, a mTORC1-selective inhibitor could be designed based on this structural information as well. Compounds that block sensing of leucine or threonine by LRS or TARS2, respectively, may also hold potential as selective mTORC1 inhibitors as well, and several recent compounds including (S)-4-isobutyloxazolidin-2-one and BC-LI-0186 have been identified that inhibit mTORC1 by interfering with LRS sensing or activity (187-190).

Finally, the molecular basis of the cell and tissue specificity of mTORC2 inhibition by chronic rapamycin treatment has been a long-standing mystery. PC3 cells, have mTORC2 activity that is extremely sensitive to rapamycin, while mTORC2 activity in other cell lines is completely resistant (67), and mTORC2 in vivo likewise has tissue-specific differences in the response to chronic rapamycin treatment (67, 74). Rapamycin inhibits mTOR in a stepwise manner, first binding to the protein FKBP12; this FKBP12-rapamycin complex than binds to and mTORC1. Recently it was found that other FK506-binding proteins (FKBPs) can also complex with rapamycin and inhibit mTORC1 (191). Intriguingly, while rapamycin-FKBP12 can inhibit both mTORC1 and mTORC2 when treated chronically, rapamycin in complex with another FKBP, FKBP51, does not inhibit

mTORC2 (74). The reason for this difference is unknown; recent detailed structural data on mTORC2 shows that Rictor blocks the site on mTOR that interacts with rapamycin and FKBPs (157).

These results suggested that rapamycin analogs may exist that, through preferential interaction with FKBP51 or through other mechanisms, could more specifically target mTORC1. Screening of a library of rapalogs identified DL001, a compound with significantly increased selectivity for mTORC1 (80). In agreement with our model in which inhibition of mTORC2 mediates the negative metabolic effects of rapamycin, treatment with DL001 does not cause glucose intolerance or dyslipidemia, while mice treated in parallel with rapamycin experienced these effects. The effect of DL001 on the immune system was also significantly less than that of rapamycin (80). However, contrary to our initial hypothesis that DL001 may function through FKBP51, we determined that knockdown of FKBP12, but not FKBP51, inhibited the ability of both rapamycin and DL001 to inhibit mTORC1 as well as mTORC2 (80). The exact molecular mechanisms responsible for the reduced impact of DL001 on mTORC2 therefore remains to be determined.

Conclusion

Building on the strong evidence that rapamycin can extend the lifespan and healthspan of model organisms, there has been tremendous excitement in developing therapies for age-related diseases based on mTOR inhibition. Concerns over the risks of infection and metabolic disruption have slowed the testing of rapalog-based therapies for diseases of aging in humans, and will likely limit their adoption. Over the next decade, work in canines, non-human primates, and human clinical trials will begin to answer some of the outstanding questions regarding the efficacy and safety of such regimens.

Many of the side effects of rapalogs are mediated not by the inhibition of mTORC1, which is believed to be therapeutic, but due to “off-target” inhibition of mTORC2. The balance of evidence from model organism and mouse studies in which mTORC2 is specifically inhibited strongly indicates that this inhibition of mTORC2 has deleterious effects on metabolic health as well as survival. Further, genetic evidence from a range of organisms suggests that inhibition of mTORC1 signaling is sufficient to promote healthspan and lifespan.

Here we have discussed a range of options to more selectively inhibit mTORC1. These include the use of rapalogs in intermittent or time-limited periods, which is sufficient to promote healthspan and lifespan, while minimizing side effects; the use of dietary regimens with altered levels of dietary protein or specific amino acids; and new mTORC1-specific small molecules. Each of these strategies, as well as treatment with standard rapalogs, may come with a unique set of side effects or limitations, and thus the best strategies to reduce mTORC1 signaling may be different for different diseases of aging or for different people. Collectively, this research in ways to sustainably and safely reduce mTORC1 activity may soon provide new options to treat or prevent age-related diseases, and improve healthspan and lifespan for everyone.

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Conflict of Interest

D.W.L. has received funding from, and is a scientific advisory board member of, Aeovian Pharmaceuticals, which seeks to develop novel, selective mTOR inhibitors for the treatment of various diseases.

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Figure Legends

Figure 1. An overview of the mechanistic Target Of Rapamycin Complex 1 (mTORC1) signaling pathway with areas of potential pharmaceutical inhibition highlighted. Negative regulators (CASTOR1, GATOR1, SAMTOR, Sestrin2, tuberous sclerosis complex [TSC]) and positive regulators (FLCNFNIP2, GATOR2, KICKSTOR, LRS, RAG GTPases, RAGULATOR, Rheb, SLC38A9, v-ATPase) are shown. Potential mechanisms for the development of mTORC1 specific inhibitors include: A, B, C, D. Identifying small molecules that block the ability of amino acid sensors upstream of mTORC1 to sense the availability of leucine, arginine, or SAM; (E, F) developing compounds such as BC-LI-0186 that inhibit the GAP or GEF activities of FLCN-FNIP2, LRS, or RAGULATOR; (G) Inhibiting the interaction of mTORC1 and Rheb, the mechanism of action of NR1; and (H) Identifying rapamycin derivatives that specifically inhibit mTORC1. Figure is from Dumas and Lamming, *JGBS*, 2019 (55) and used with permission.