Impact of rapamycin on longevity: updated insights

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Abstract

The gradual accumulation of varying detrimental alterations during the aging process within cells and tissues contributes to a progressive decline in their functionality, which may ultimately result in death. The licensed mammalian target of rapamycin (mTOR) inhibitor rapamycin, also known as sirolimus, has recently become a promising option for anti-aging applications. Through *in vitro* and *in vivo* assessments, numerous scientific reports have illustrated diverse biochemical and clinical aspects of rapamycin's pharmacological effects in ameliorating aging-related changes and expanding longevity. Nevertheless, its clinical application has been impeded by severe adverse effects, which might be addressed by implementing an appropriate therapeutic regimen. In this regard, integrating updated insights and uncovering essential benefits and drawbacks of rapamycin as a geroprotective drug are critical for conducting further preclinical research and well-organized clinical trials, and facilitating translation to clinical practice. The present review highlights the recent findings on the role of rapamycin in improving organ health and postponing aging-related processes.

Key words: rapamycin, aging, longevity, mTOR signaling.

Introduction

Aging is a biological phenomenon marked by a gradual decline in cellular and functional capabilities over time, eventually leading to a dimin-

ished quality of life. Aging is also the paramount predisposing factor for the emergence of hard-to-treat pathologies, e.g., cardiovascular diseases, malignancies, and neurodegenerative disorders; thus, it poses a substantial worldwide socioeconomic burden and a noteworthy healthcare obstacle [1, 2]. Accordingly, it is of utmost importance to identify therapeutic interventions that facilitate "healthy aging" (i.e., maintaining functionality during old age, allowing elderly people to perform their daily activities) while restricting the promotion of various age-related pathological conditions [3].

The mammalian target of rapamycin (mTOR) is an important regulator of cellular metabolism, integrating nutrition status with cellular mechanisms that fuel cell growth and multiplication. Its dysregulation, therefore, contributes to various cellular senescence and aging-associated mechanisms. Studies have demonstrated that inhibiting mTOR signaling can expand the lifespan of model organisms and provide defense against a range of age-related ailments [4, 5]. In this regard, rapamycin, an mTOR complex1 (mTORC1) inhibitor, was demonstrated to prolong the lifespan in yeast, nematodes, fruit flies, and mice [6]. Rapamycin is licensed by the US Food and Drug Administration (FDA) for treating post-renal transplantation and lymphangioleiomyomatosis; nevertheless, due to compelling evidence regarding its anti-aging effects, it is now being considered a feasible approach to enhance lifespan [7, 8]. Multiple mechanisms have been suggested for the pro-longevity impact of rapamycin through affecting mTOR signaling, e.g., tuning protein expression, regulating mitochondrial function, rescuing stem cell activity, ameliorating inflammaging and immunosenescence, and improving autophagic flux [9–12]. However, the limitations posed by rapamycin-mediated unfavorable adverse effects necessitate thorough scientific investigation to overcome these challenges for successful clinical repositioning [13]. The current investigation endeavors to offer a contemporary and comprehensive perspective on the anti-aging properties of rapamycin, thereby illuminating forthcoming research objectives in this field.

The impact of mTOR on cell longevity and growth

As a threonine kinase belonging to the phosphoinositide 3-kinase (PI3K)-related kinase family, mTOR is situated at the intersection of multiple essential signaling pathways and performs a vital function in organizing cellular growth and longevity. To that end, it incorporates data regarding energy and food resources to regulate the production or degradation of cellular components [14]. Induction of mTOR following stresses or growth signals regulates a wide range of cellular processes, e.g., growth and multiplication, protein synthesis, mitochondria biogenesis, cytoskeleton establishment, immune reactions, and autophagy [15]. The mTOR renders two multiprotein complexes, mTORC1 and mTORC2, composed of distinct protein binding partners. mTORC1 is responsive to nutrition, while mTORC2 is controlled by PI3K and growth factor signaling [16]. The upstream regulator and downstream effectors of mTOR complexes concerning cellular growth and longevity are discussed below.

mTORC1

In order to respond to nutritional and energy fluctuations, growth factors, and cellular stresses, cells must switch on/off mTORC1 signaling. Owing to its participation in setting up anabolic regimens, mTORC1 should only be activated when growth elements are abundant [17]. Rag and Rheb GTPases are two types of small G proteins that transduce the anabolic impulses to activate mTORC1. In nutrient-replete conditions, Rag brings the mTORC1 from the cytoplasm to the lysosome, where Rheb activates the mTORC1 kinase to support sustained growth [18]. Rag and Rheb GTPases are fine-tuned by several mechanisms. Importantly, amino acids, mainly leucine and arginine, are indispensable for mTORC1 stimulation in mammalian cells via modulating Rag-GTPase action. Under deprivation and a significant fall in amino acid contents, GTPase-activating protein (GAP) activity towards Rags 1 (GATOR1) disables Rag and blocks the mTORC1 cascade [19]. Mechanistically, sestrin2, a conserved protein implicated in the cellular response to stress, detects the acute leucine shortage and inhibits GATOR2, eliminating its blockage on GATOR1, thereby suppressing mTORC1 signaling [20, 21]. Similarly, the cellular arginine sensor for mTORC1 (CASTOR1) represses GATOR2 under arginine deprivation and impedes mTORC1 activity [22]. SLC38A9 is another identified arginine sensor that collaborates with regulator in Rag activation following attachment to lysosomal arginine [23]. Furthermore, the folliculin-folliculin interacting protein 2 (FLCN-FNIP2) complex was demonstrated to activate Rag and maintain mTORC1 activity in amino acid-replete conditions [24].

The function of Rheb in triggering mTORC1 signaling is tuned by several upstream mechanisms. For example, the tuberous sclerosis complex (TSC), acting as a GAP, suppresses Rheb's function in stimulating mTORC1. Induction of PI3K/Akt signaling by insulin-like growth factor-1 (IGF-1) was demonstrated to phosphorylate and detach TSC from the lysosome surface, permitting Rheb and mTORC1 activation [25]. Insulin receptor substrate 1 (IRS-1) is then phosphorylated by mTORC1-ac-

tivated p70 S6 kinase 1 (S6K1), initiating a negative feedback loop and inhibiting additional insulin-mediated PI3K/Akt pathway induction [26]. IGF-1, tumor necrosis factor (TNF), and Wnt signaling, as well as extracellular signal-regulated kinase (ERK) and p90 ribosomal S6 kinase (RSK), have been shown to inhibit TSC and provoke mTORC1 signaling in nutrient-enriched conditions [27–30]. It is also noteworthy that growth factors may influence mTORC1 function independently of TSC via proline-rich Akt substrate 40 kDa (PRAS40), which is linked to the regulatory-associated protein of mTOR (RAPTOR) and impedes Rheb-induced mTORC1 induction. Akt-mediated phosphorylation of PRAS40 in response to insulin signaling was shown to enhance mTORC1 kinase activity [31].

As expected, stress signals inhibit mTORC1 activation and limit the anabolic pathways. Once ATP is depleted, the energy homeostasis enzyme AMP-activated protein kinase (AMPK) phosphorylates Raptor or triggers TSC2 to hinder mTORC1 and reconfigure the cell metabolism [32]. Thereby, AMPK decreases the stress imposed on mitochondrial respiration and mitigates the potential for cellular injury induced by reactive oxygen species (ROS) generation. Additionally, oxidative stress may directly inhibit mTORC1 by inducing the regulated in development and DNA damage responses 1 (REDD1) protein, which triggers TSC [33]. In response to DNA damage, mTORC1 function is also subdued by p53 target genes (e.g., phosphatase and tensin homolog (PTEN) and AMPKβ), which help to diminish the proliferation pace and conserve genome stability [34]. Another scenario is the engagement of unfolded protein response (UPR) by the endoplasmic reticulum (ER) under starvation to upregulate sestrin proteins, dampening mTORC1 activity and maintaining cell viability [35]. As explained, diverse upstream mechanisms mainly converge on Rag and Rheb GTPases to regulate mTORC1 function and cell metabolism regarding the nutritional status.

mTORC1 supports the provision of substances (e.g., proteins, lipids, and oligonucleotides) and energy necessary for cell growth [14]. Evidence shows that mTORC1 regulates protein synthesis in response to cellular demands by influencing the function of the initiation factor 4Ebinding protein (4EBP), a critical regulator of the mRNA translation process. While inactivated, 4EBP excludes the eukaryotic translation initiation factor 4E (eIF4E) from the translation process; however, after being activated by mTORC1, it allows eIF4E to enter the mRNA translation process and enables protein synthesis [36]. Besides 4EBP, mTORC1 phosphorylates S6K1, activating the S6 protein as an essential element of the 40S ribosomal subunit. S6K1 also incites eIF4B activity directly or indirectly by eliminating the eIF4A inhibitor programmed cell death 4 (PDCD4) [37]. Furthermore, SKAR, a translation regulatory factor deposited at the exon junction complex, was demonstrated to recruit S6K1 to improve the translation of spliced mRNAs [38]. Despite improving translation, S6K1 involves the biogenesis of new ribosomes through phosphorylating upstream binding factor (UBF), MAF1, and transcription initiation factor 1A (TIF1A) and resultant activation of RNA polymerases [39, 40].

During the growth phase, higher lipid synthesis is necessary for maintaining cell membrane biogenesis. Importantly, the stimulation of mTORC1 has been proven to increase the S6K1-mediated nuclear trafficking of sterol regulatory element binding protein 1/2 (SREBP1/2) transcription factor to amplify lipid and cholesterol production [41]. To further enable SREBP1/2-mediated lipid synthesis, the activated mTORC1 phosphorylates and excludes the SREBP inhibitor lipin 1 from the nucleus [42]. It is also worth noting that mTORC1 affects the action of proliferator-activated receptor γ (PPARγ) in tuning the expression of lipid homeostasis genes [43]. Besides lipids, increased nucleic acid synthesis is required to enable DNA multiplication and ribosomal RNA production during cell proliferation. Active mTORC1 promotes purine synthesis by enabling transcription factor 4 (ATF4) and its downstream target mitochondrial tetrahydrofolate cycle enzyme methylenetetrahydrofolate dehydrogenase 2 (MTHFD2) [44]. Moreover, S6K1-mediated activation of carbamoyl-phosphate synthetase 2, aspartate transcarbamoylase, and dihydroorotase (CAD) by mTORC1 leads to pyrimidine biosynthesis [45].

To further support growth, active mTORC1 considerably alters glucose metabolism and increases cell metabolic efficiency. It prioritizes glycolysis over oxidative phosphorylation via activating the transcription factor hypoxia-inducible factor-1α (HIF-1α) and the consequent upsurge in glycolytic enzyme expression [46]. Moreover, mTORC1 enables the pentose phosphate pathway via activating SREBPs to facilitate the supply of NADPH and carbon-rich molecules for lipid and nucleotide biogenesis [47]. It also enhances 4EBP1-mediated mitochondrial gene transcription and stimulates mitochondrial biogenesis by compelling the construction of the PPARγ coactivator 1α (PGC1α) transcriptional complex to expand ATP production [48, 49].

mTORC1 inhibits catabolic autophagy to preclude a fruitless process in which newly generated cellular elements are prematurely dissociated. It deactivates unc51like autophagy activating kinase 1 (ULK1) and autophagy-related 13 (ATG13) to impede autophagy initiation as well as autophagosome

generation [50]. Furthermore, mTORC1 interferes with autophagosome maturation and fusion with lysosomes by inhibiting the UV radiation resistance-associated gene (UVRAG) to prevent the degradation and recycling of proteins and organelles [51]. Conversely, starvation inhibits mTORC1 signaling and redirects resources toward autophagy. Blocking mTORC1 rescues autophagosome generation and activates genes for lysosomal biogenesis by stimulating the nuclear trafficking of transcription factor EB (TFEB) and the associated transcription factor E3 (TFE3). After a protracted deprivation period, the cytoplasmic pool of amino acids is replenished due to protein lysosomal breakdown, reactivating mTORC1 [52, 53]. Notably, the link between nutritional status and autophagy is broken during mitosis, when cyclin-dependent kinase 1 (CDK1) suppresses both mTORC1 and autophagosome formation to preserve the genome from destruction once the nuclear membrane dissolves [54]. In a nutshell, the nutrition-sensing machinery integrates with mTORC1 signaling to establish a customized growth regimen paradigm for cell longevity and growth (Figure 1).

mTORC2

It has been revealed that mTORC2 is mainly regulated by the PI3K/Akt pathway triggered by growth factors. As the unique PI3K effector and an obligate component of mTORC2, the mammalian stress-activated protein kinase-interacting protein 1 (mSin1) represses the kinase function of mTORC2. In the presence of insulin, however, the phosphatidylinositol (3,4,5)-trisphosphate (PIP3) produced owing to the induction of PI3K abolishes the inhibitory action of mSin1 on mTORC2 [55]. Furthermore, Akt was shown to directly phosphorylate mSin1 and promote mTORC2 function [56]. Of note, the function of mTORC2 is closely associated with its intracellular localization. Accordingly, PIP3 may attract mTORC2 and Akt to the cell membrane, where their mutual phosphorylation impacts their localization and function [57]. Small

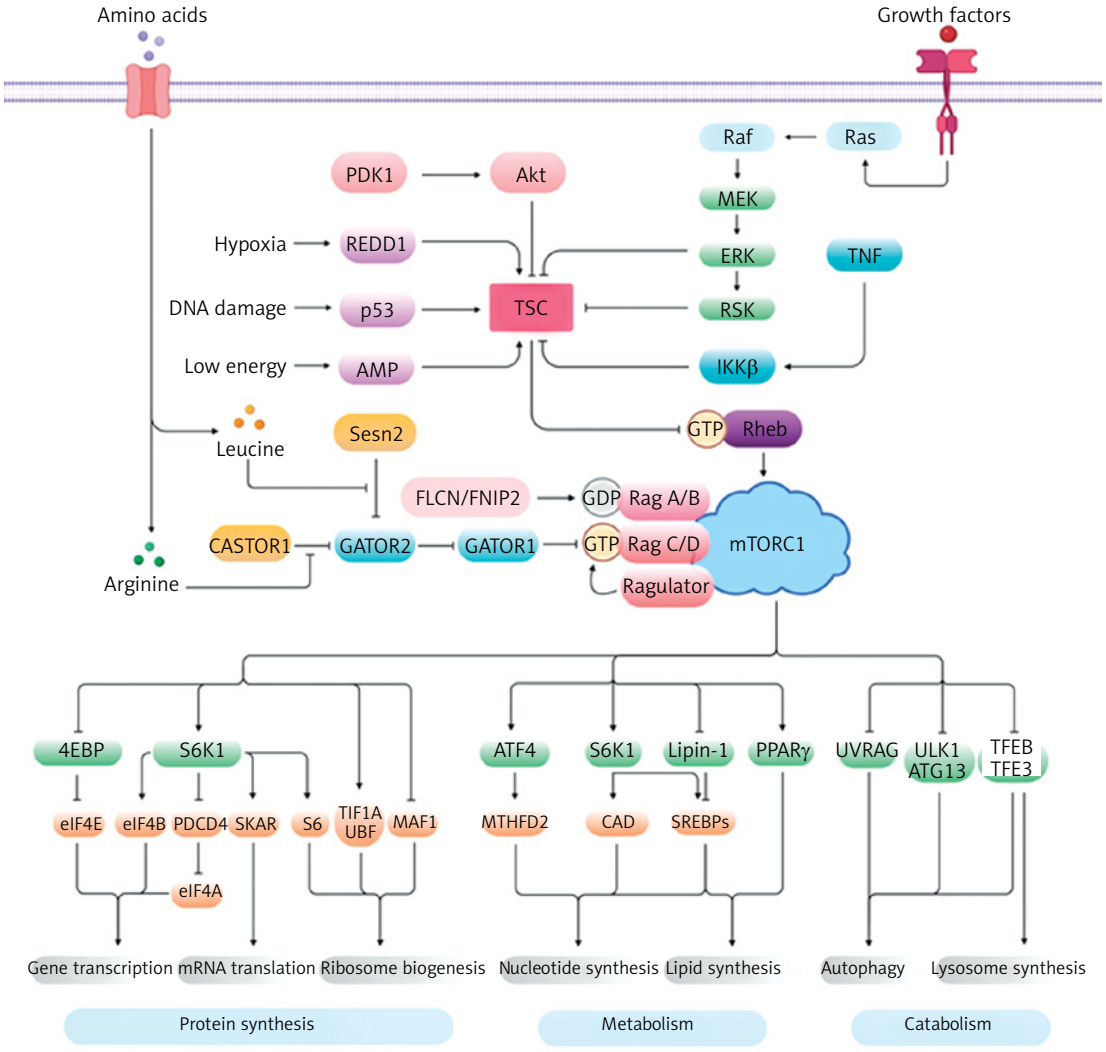

Figure 1. Upstream regulators and downstream effectors of mTORC1 signaling

GTPases (e.g., Rap1 and Ras) notably play an important regulatory role in the mentioned interaction [58, 59]. Evidence from recent studies implies that mSin1 conscripts Ras to incite the kinase function of mTORC2 at the plasma membrane [58, 60]. mTORC2 is also activated by AMPK under starvation, which may encourage cellular adaptation to oxygen/nutrient deprivation in malignant conditions [61].

It is well accepted that mTORC2 is essential for stimulating AGC kinase members such as Akt, PKC, and glucocorticoid-regulated kinase (SGK) [62]. Initial discoveries regarding the role of mTORC2 in cell biology highlighted its possible role in cell motility, since the first-identified mTORC2 substrate, protein kinase Cα (PKCα), plays critical roles in cytoskeletal regulation [63, 64]. This is consistent with the well-known function of mTORC2 in the migration and metastasis of malignant cells [65]. Furthermore, mTORC2 may work in concert with phosphoinositide-dependent kinase 1 (PDK1) to engage Akt, the major executioner in the PI3K cascade, and transduce the proliferation signals [66]. Akt likewise reshapes cell metabolism by affecting the forkhead-box O 1/3A (FOXO1/3A) transcription factor and NAD kinase and maintains the function of GSK-3β to decrease apoptosis to withstand stressful circumstances [67–69]. It is worth mentioning that the supporting feedback phosphorylation between mTORC2 and Akt regulates their localization and function; however, the phosphorylation of several substrates, such as TSC and GSK-3β, by Akt does not necessarily require mTORC2 function [57, 70]. Overall, SGK-1 seems to be the most important effector of mTORC2, since it regulates FOXO proteins, whose phosphorylation by Akt requires mTORC2 activity [70]. Importantly, mTORC1 and mTORC2 are interrelated. The activated mTORC1 potentially blocks the PI3K/Akt signaling-mediated mTORC2 activation by provoking S6K1-mediated IRS-1 degradation and activating Grb10, a negative regulator of the insulin or IGF-1 receptor [26, 71]. In return, the induction of Akt by mTORC2 inactivates TSC and promotes mTORC1 activity (Figure 2) [72].

Rapamycin and longevity

Regarding numerous reports, mTOR is a critical longevity regulator, and its dysregulation due to metabolic disorders, lethal neoplastic diseases, or age-related ailments disturbs cellular homeostasis and limits lifespan [73]. Pharmacological inhibition of mTOR by rapamycin has demonstrated intriguing anti-aging properties in multiple organs and extended the lifespan of diverse invertebrate (e.g., yeast, nematode, and fruit fly) and vertebrate (e.g., mouse) models [74]. This section provides an updated insight into the role of rapamycin in regulating longevity pathways, promoting healthy aging, and ameliorating age-related ailments. Furthermore, summarized preclinical evidence regarding the anti-aging mechanisms of rapamycin in several organs is presented in Table I.

The impact of rapamycin on protein expression

Protein homeostasis, often known as proteostasis, is the process by which proteins inside the cell are regulated to maintain the integrity of the cellular proteome and the viability of the organism. Recent research has demonstrated that the ability of multiple cells and organs to preserve proteostasis under a variety of situations diminishes with age, and predictably, proteostasis failure contributes to the pathogenesis of a wide range of human diseases associated with aging. Accordingly, alterations in levels or mutations in translational machinery elements have a substantial influence on longevity in many mammals [75, 76]. One of the most vital duties of mTORC1 is to govern mRNA translation under growth-promoting settings; however, it impairs translational fidelity [77]. Inhibiting processes that promote growth and proliferation, particularly mTORC1 signaling, may extend longevity in eukaryotes, since a gen-

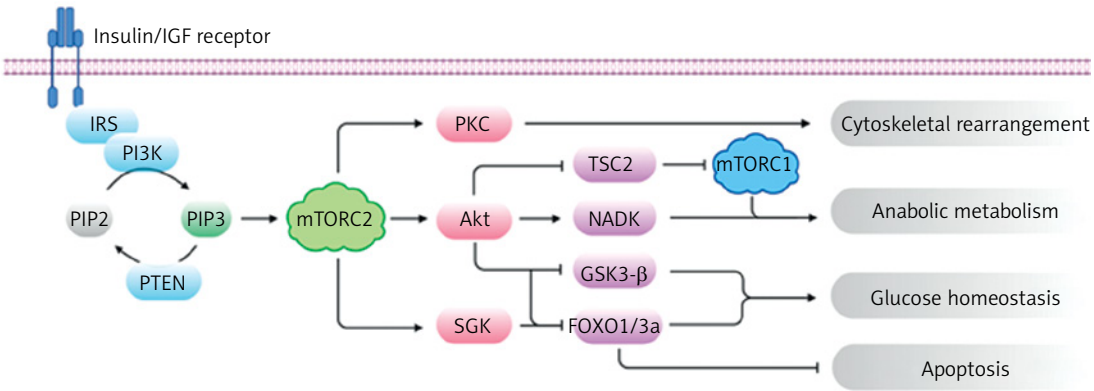

Figure 2. Upstream regulators and downstream effectors of mTORC2 signaling

Table I. Anti-aging mechanisms of rapamycin in several organs regarding preclinical findings

System	Disorder	Study model	Dose of rapamycin	Beneficial effects/mechanism of action	Ref.
Cardiovascular	*Trypanosoma cruzi*-induced acute myocarditis	Elderly mice	4 mg/kg/3 days (88 weeks)	- Improved parasitological control - Diminished heart inflammation and microstructural insults - Enhanced the balance between Th1 and Th2 effectors - Induced Th1 cytokines and the iNOS pathway - Inactivated the arginase pathway	[202]
	Cardiac aging	Aged mice	42 ppm (8 weeks)	- Enhanced diastolic function and myocardial stiffness - Modified the cardiac proteome without metabolic changes - Altered mitochondrial respiratory chain activity	[203]
	Age-related vascular dysfunction	Old mice	2.24 mg/kg/day (6 weeks)	- Improved endothelium-dependent dilation in the carotid artery - Reduced aortic pulse-wave velocity and collagen content - Normalized NADPH oxidase expression - Reduced age-related arterial senescence marker, p19 - Activated AMPK signaling - Upregulated cell cycle-related proteins PTEN and p27kip	[204]
	Atherosclerosis	ApoE$^{-/-}$ mice	5 mg/kg/day (7 days)	- Decreased atherosclerotic plaque burden - Decreased proliferating macrophage population - Reduced key proinflammatory cytokines (MCP-1 and IL-b1) - Decreased MMP activity	[163]
Nervous	Brain aging	Aged mice	mg/kg/day (3 months)	- Affected aging-associated brain functions, including brain development, neuronal apoptosis, and cell adhesion - Reduced gene expression changes associated with aging - Decreased aging-related DNA methylation alterations	[205]
	Ischemic brain damage	Diabetic rats	3 mg/kg/day (3 days)	- Mitigated diabetes-enhanced ischemic brain damage - Inhibited the mTOR pathway and reversed the imbalance in mitochondrial dynamics	[206]
	Alzheimer's disease	Zinc-treated SH-SY5Y cells and rats	20 ng/ml (1 h) 1.5 mg/kg (three times for 1 week)	- Ameliorated synaptic deficit and cognitive impairments - Repressed zinc-induced mTOR/p70S6K signaling - Inverted zinc-mediated Nrf2/HO-1 inhibition - Reduced tau phosphorylation - Suppressed oxidative stress	[207]
	Parkinson's disease	MPTP-treated rat	7.5 mg/kg (twice a day for 7 days)	- Ameliorated behavioral symptoms. - Inhibited the d mTOR/4EBP1 pathway.	[208]
		MPTP-treated astrocytes and mice	100 nM (4 h) 7.5 mg/kg (11 days)	- Showed neuroprotective effects *in vitro* and *in vivo* - Ameliorated behavioral symptoms - Diminished expression of the E3 ubiquitin ligase Nedd4-2 and decreased colocalization of glutamate transporters with ubiquitin - Enhanced IL-6 expression via the mTOR/Akt/NF-κB signaling - Decreased expression of inflammatory cytokines, e.g., TNF-α and IFN-γ	[209]

Table I. Cont.

System	Disorder	Study model	Dose of rapamycin	Beneficial effects/mechanism of action	Ref.
Gastrointestinal	Intestinal aging	Old *Drosophila*	200 μM	- Extended lifespan - Slowed the proliferation rate of intestinal stem cells in the aging guts - Provoked autophagy in the intestinal epithelium - Affected FOXO-related genes and upregulate the negative regulators of the IMD/Rel pathway, delaying the microbial expansion in the aging guts	[210]
	Periodontal disorder	Aged mice	42 ppm (8 weeks)	- Rejuvenated the aged oral cavity of the elderly mice - Attenuated RANKL expression and $TRAP^+$ cells in periodontal bone - Attenuated enhanced NF-κB expression and inflammatory cytokine profiles in the periodontium - Shifted aged oral microbiome towards young oral microbiome	[211]
Hepatic	Hepatic aging	Middle-aged mice	14 ppm (7 weeks)	- Reduced age-related hepatic insults and extended longevity - Attenuated oxidative stress and induced autophagy - Reduced mtDNA fragments inside nuclear DNA - Lowered mitochondrial protein lipoxidation - Decreased lipofuscin accumulation	[142]
	Fatty liver disease	HepG2 NAFLD model	25 ng/ml	- Reduced intracellular concentration of triacylglycerol - Hindered mTORC1 and provoked autophagy, as evidenced by downregulated P62 and increased LC3-II/I ratio	[212]
Respiratory	COPD	Transgenic mice model	2.5 mg/kg (twice a week for 3 months)	- Inhibited lung cell senescence as well as the development of lung emphysema and pulmonary hypertension - Inhibited mTOR, suppressed inflammation, and normalized cytokine levels (IL-6, IL-8, and CCL2)	[213]
		Patient-derived PBMCs	20 nM	- Increased therapeutic response to corticosteroids - Repressed mTORC1/S6K activity - Showed anti-inflammatory effect against CXCL8 release by TNF-α	[214]
Renal	Renal aging	Type-2 diabetes murine model	14 ppm (16 weeks)	- Inhibited mTOR and ameliorated diabetic nephropathy - Improved kidney function - Reduced albumin/creatinine ratio	[215]
		Homocysteine-induced MPC-5 podocyte cell senescence	1 ng/μl	- Inhibited the emergence of the senescent phenotype - Repressed mTOR and provoked autophagy - Reversed the upregulation of aging-related proteins (e.g., p16, p21, and p53)	[216]
Reproductive	Ovarian aging	Mice oocytes	10 nM	- Ameliorated the developmental competence and quality of oocytes - Reduced ROS levels - Inhibited mTOR and improved DNA repair response - Diminished chromosome aberration and reduced γ-H2AX levels	[217]
	Testicular dysfunction	Spontaneously hypertensive rats	1 mg/kg (3 weeks)	- Restored the testicle size and histological alterations and prevented apoptosis - Limited mitochondrial superoxide production - Depleted mitochondrial membrane potential via induction of Nrf2-mediated Gpx4 and SOD2 expression	[218]

Table I. Cont.

System	Disorder	Study model	Dose of rapamycin	Beneficial effects/mechanism of action	Ref.
Skeletal muscle	Muscle aging	Aged rats	14 mg/kg (14 weeks)	- Improved muscle mass, strength, and function - Affected the expression of age-associated genes in muscle	[219]
		Zmpste24$^{-/-}$ mouse-derived muscle-derived stem/progenitor cells	10 nM	- Promoted myogenic and chondrogenic differentiation - Inhibited apoptosis and senescence	[190]
		Old mice	14 ppm (from ~270 days to ~900–1,099 days of age)	- Reduces muscle fiber loss - Reduced STAT3 and S6 protein phosphorylation - Decreased expression of GDFs - Relieved oxidative stress and protein oxidation - Downregulated cleaved caspase-3 levels and apoptosis	[220]

Th – T helper, iNOS – inducible nitric oxide synthase, NADPH – nicotinamide adenine dinucleotide phosphate, AMPK – AMP-activated protein kinase, PTEN – phosphatase and tensin homolog, MCP-1 – monocyte chemoattractant protein-1, IL – interleukin, MMP – matrix metalloproteinase, mTOR – mammalian target of rapamycin, p70S6K – 70-kDa ribosomal protein S6 kinase, Nrf2 – nuclear factor erythroid 2-related factor 2, HO-1 – heme oxygenase-1, 4EBP1 – eukaryotic translation initiation factor 4E-binding protein 1, Nedd4 – neural precursor cell-expressed developmentally downregulated gene 4, MPTP – 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, NF-κB – nuclear factor kappa B, TNF-α – tumor necrosis factor α, IFN-γ – interferon gamma, FOXO – forkhead box O, IMD/Rel – immune deficiency/relish, RANKL – receptor activator of nuclear factor kappa-Β ligand, TRAP – tartrate resistant acid phosphatase, NAFLD – nonalcoholic fatty liver disease, mtDNA – mitochondrial DNA, LC3 – light chain 3, COPD – chronic obstructive pulmonary disease, CCL2 – chemokine ligand 2, PBMCs – peripheral blood mononuclear cells, S6K – S6 kinase, CXCL8 – C-X-C motif chemokine ligand 8, ROS – reactive oxygen species, Gpx4 – glutathione peroxidase 4, SOD2 – superoxide dismutase 2, STAT3 – signal transducer and activator of transcription 3, GDFs – growth differentiation factors.

eral decline in mRNA translation is advantageous during aging by enabling natural protein repair and degrading mechanisms to properly preserve protein homeostasis while protecting against toxic protein aggregates and oxidative insults [78, 79]. Indeed, elevating the translation accuracy as well as protein synthesis fidelity is among the most critical mechanisms of rapamycin in enhancing organismal health and longevity [80].

An investigation conducted by Martinez-Miguel *et al.* demonstrated that rapamycin enhanced translation fidelity in *Drosophila* S2R^{+} cells by lowering both stop codon readthrough and misincorporation errors and extended the lifespan of wild-type flies [81]. mTORC1 signaling was shown to inhibit eukaryotic elongation factor 2 kinase (eEF2K), which phosphorylates and inactivates eEF2, resulting in the motion of ribosomes along mRNAs, and hastens the elongation stage of protein synthesis. In this regard, blocking mTORC1 with rapamycin suppresses eEF2, reducing the pace of elongation, improving protein synthesis accuracy, and lowering misreading or termination readthrough errors. In supporting the mentioned hypothesis, deletion of eEF2K and impairing the translation fidelity were demonstrated to decrease the lifespan of *Caenorhabditis elegans* [82]. Another study on *C. elegans* showed that age and other factors that reduce longevity, such as high temperature, lead to the buildup of detergent-insoluble proteins. In the *C. elegans* strain harboring a green fluorescent protein (GFP) transcriptional reporter under the control of a heat shock promoter, rapamycin treatment considerably suppressed mTOR signaling, delayed aberrant expression, slowed the buildup of these insoluble proteins, reduced proteostatic stress, and increased longevity. It has also been observed that suppressing S6K1 downstream of mTORC1 exhibited comparable effects on the reduction of protein translation and the enhancement of lifespan [83].

In addition, rapamycin has been found to modify the scope and pace of protein translation. It inhibits the function of the ribosomal protein S6 and the eukaryotic translation initiation component 4EBP1, lowering canonical cap-dependent mRNA translation and indirectly enhancing cap-independent translation [84–86]. Evidence supports cap-independent translation as a regulator of stress tolerance as well as maintaining metabolic functions and survival through preserving the synthesis of critical proteins [87]. Certain elongation and initiation elements and proteins that identify sequences or changes in the 5′ untranslated region (UTR) sequence, such as 6-methyl-adenosine residues (m6A), are essential for cap-independent translation processes. Notably, the ability of rapamycin to intercept cap-dependent translation may facilitate the translation of m6A-containing

mRNA, increasing protein levels without commensurate modifications in mRNA transcription [87, 88]. As proof of concept, a study by Shen *et al*. revealed that inhibiting mTORC1 by rapamycin therapy may contribute to lifespan expansion in UM-HET3 mice via preventing age-related decline in mitochondrial and stress proteins, e.g., O-6-methyl guanidine-DNA methyltransferase (MGMT), N-myc downstream regulated gene-1 (NDRG1), mitochondrial transcriptional factor A (TFAM), and heat shock protein 70 (Hsp70) as cap-independent translation targets in the liver and kidney tissues [89].

In line with the mentioned findings, a recent investigation revealed that rapamycin-mediated cap-dependent mRNA translation suppression in *C. elegans* evoked preferential ATF4 expression, independent of the integrated stress response (ISR), upregulating expression of the cystathionine gamma-lyase-2 (CTH2) transsulfuration enzyme as well as hydrogen sulfide (H2S) production. Such alterations promote stress resistance and longevity by ramping up protein persulfidation, a protective adjustment of redox-reactive cysteines [90]. The ATF4 ortholog in *Saccharomyces cerevisiae*, Gcn4, a key transcriptional regulator of amino acid biosynthesis genes, was demonstrated to promote longevity. As a repressor of protein synthesis, Gcn4 has a vital function in the rapamycin-mediated extension of yeast lifespan [91]. The abovementioned discoveries illustrate promising novel strategies for enhancing longevity via rapamycin-mediated enhancement of protein expression scope and fidelity.

In addition, rapamycin may be capable of alleviating late-life malignancies by selective inhibition of the pro-tumorigenic senescence-associated secretory phenotype (SASP). Despite the fact that cellular senescence inhibits cancer cell growth, the buildup of senescent cells with age develops the SASP, which can destabilize tissues and contribute to age-associated diseases such as cancer. In senescent human fibroblasts subjected to oncogenic RAS or radiation, active mTOR signaling was demonstrated to enhance the translation of several SASP factors, including interleukin (IL)-1 and mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MK2) [92, 93]. In this regard, rapamycin was shown to repress the transcriptional function of nuclear factor κB (NF-κB), critical for producing SASP proteins. Practically, the growth-stimulating action of senescent fibroblasts on prostate tumors in mice was inhibited by rapamycin [92]. Furthermore, rapamycin has been shown to reduce the expression of signal transducer and activator of transcription 3 (STAT3) in cancerous tissue, acting as a crucial downstream mediator of SASP signaling [94, 95].

The impact of rapamycin on autophagy

In eukaryotes, autophagy is a powerful breakdown mechanism for starvation-induced amino acid recycling and removing defective organelles and macromolecules from the cytoplasm [96]. It is an internal mTORC1-tuned mechanism that has been retained through evolution and is necessary to maintain cellular homeostasis in response to the stresses that trigger cellular senescence [97]. Intriguing investigations in yeasts, worms, flies, and mice have established considerable involvement of autophagy-associated genes in lifespan expansion in various longevity scenarios. Particular tissues may need or profit from autophagy engagement, since it precisely targets defective cellular elements and prevents their buildup. Even in a non-cell autonomous manner, autophagy may affect organismal health and aging; therefore, promoting autophagy in certain tissues may prolong longevity [98]. There is mounting evidence that autophagic breakdown slows with aging, contributing to the buildup of harmed proteins and dysfunctional mitochondria that underpins age-related cellular failure [99, 100]. Inhibiting mTORC1, and hence stimulating autophagy, is thought to preserve cellular activity throughout aging by expediting the breakdown of damaged or obsolete cellular components. As proof of concept, lifespan prolongation in response to food restriction or rapamycin has been shown to entail mTORC1-mediated autophagy activation in diverse species, reversing senescence and restoring regenerative functions [101–103].

Direct suppression of mTORC1 via administration of rapamycin was shown to increase lifespan by promoting autophagy and inhibiting the adverse impacts of aging on the heart. It has been revealed that the buildup of lipofuscin, i.e., pigment granules made of lipid-containing lysosomal digesting residues, as well as reduced autophagy levels, is associated with aging and the senescence of cardiomyocytes. Interestingly, increasing autophagy flux by 6 months of rapamycin feeding has been proven to decrease lipofuscinogenesis, increase lipofuscin breakdown, and improve cardiomyocyte senescence in aged rats [104]. Furthermore, rapamycin demonstrated cardioprotective effects in heart-related pathologies such as ischemic heart disease via regulating the balance between cardiomyocyte apoptosis and autophagy. Rapamycin was demonstrated to improve cardiac function, inhibit cardiac remodeling, and prevent apoptosis by regulating the crosstalk between the mTOR and ER stress pathways and promoting autophagy [105, 106].

Even though the abovementioned evidence underlines that the reduced cardiac autophagic capacity is implicated in cardiovascular aging

and deterioration, several studies have indicated that morbidities accelerating cardiovascular aging, such as glucotoxicity and lipotoxicity, actually boost autophagy in cardiac tissue and result in cardiotoxicity [98, 107]. For instance, diabetes-mediated chronic cardiomyopathy is accompanied by excessive autophagy induction in the cardiac tissue, as demonstrated by elevated autophagy indices (e.g., light chain (LC)B-II and beclin-1) [108]. It has been hypothesized that an autophagy-induced increase in amino acid availability may contribute to protein synthesis for SASP-mediated cardiomyocyte senescence [109]. Also, the literature shows the controversial effect of autophagy on ischemia-reperfusion injury (IRI). Increased autophagy during ischemia-reperfusion has been revealed to promote cardiomyocyte death and heart failure [110, 111]. During the reperfusion stage, oxidative stress leads to a rise in ROS generation, the primary cause of autophagy activation; however, the autophagosome clearance is impaired, resulting in accelerated autophagy and cardiomyocyte death [112, 113]. Likewise, suppressing autophagy was demonstrated to improve cardiac function and myocardial infarct size in a myocardial ischemia-reperfusion injury murine model [114].

Accordingly, rapamycin may exert paradoxical effects on longevity owing to various heart complications. Furthermore, as adaptive responses to rapamycin treatment, the precise progression of autophagy in cardiac tissue is extensively controlled by post-transcriptional processes. Rapamycin therapy was demonstrated to provoke autophagy in the cardiac tissue, which in turn triggers an autophagy-suppressing miRNA network either as a protective mechanism to stop aberrant autophagy that causes cardiomyopathy and heart failure or as a harmful process that halts autophagy advancement and accelerates cardiac senescence [107]. As a result, a crucial step in identifying whether the rapamycin-mediated autophagy induction is proceeding adequately to preserve the heart tissue is to invent novel non-invasive imaging methods carefully monitoring changes in cardiac function and autophagic flux.

Brain aging is a complex and natural phenomenon primarily defined by oxidative stress, the buildup of oxidatively injured macromolecules, and changes in the architecture and longevity of neurons, all of which raise the risk for neurological illnesses [115]. The mTOR signaling and autophagy have vital actions in preserving the proper functioning of the central nervous system; however, defective autophagy associated with aging has been linked to the development and frequency of neurological disorders [116]. By maintaining cellular homeostasis, as well as the structural and functional integrity of neurons, autophagy plays a principal function in postponing brain aging. In this regard, rapamycin-stimulated autophagy has been shown to confer considerable protection to the aging rat brain by lowering age-induced oxidative stress, apoptotic cell extinction, and neurodegeneration [117]. It is also believed that adult neurogenesis, i.e., the continual process of producing functioning neurons in the human brain from neural progenitor cells, slows down with aging and contributes to the deterioration of brain functionality [118]. A recent study discovered a strong correlation between deteriorating adult neurogenetic function and reduced autophagy. In subventricular/subgranular zone homogenates acquired from the brain of middle-adult rats, the expression of autophagy-related genes and autophagic function were drastically diminished. Furthermore, suppressing autophagy by small interfering RNA (siRNA)-based RNAi gene therapy repressed proliferation and differentiation of neural progenitor cells. Strikingly, rapamycin-stimulated autophagy promoted neurogenesis in the subventricular/subgranular zone and restored the survival of neural progenitor cells while enhancing the olfactory sensitivity and cognitive abilities of middle-aged rats [119].

In addition, including rapamycin in preventing and alleviating neurodegenerative alterations is a true-to-life opportunity owing to its capacity to revive the dysregulated mTOR signaling identified in the etiology of such disorders [120]. Significantly, faulty protein processing contributes to the emergence of various neurodegenerative disorders, as reflected by the buildup of misfolded and hazardous proteins within particular brain structures. Growing data suggest that faults in the autophagic breakdown process are to blame for these protein changes [121]. Consequently, rapamycin-induced autophagy may be advantageous by avoiding or attenuating harmful protein aggregation [122]. It has been discovered that the neuroprotective impact of fibroblast growth factor 21 (FGF21) gene delivery in the Aβ42-induced rat Alzheimer's disease model is improved by rapamycin treatment. FGF21 cross-talks with autophagy; thus, adding rapamycin to the treatment potentiated the impact of FGF21 in autophagic clearance of toxic protein aggregates, as revealed with increased expression of central autophagy proteins, reduced protein levels of Aβ42 and phosphorylated tau, alleviated oxidative stress, and renovated neuronal density [123]. Similarly, rapamycin has been shown to strengthen the impact of trehalose, a bioactive natural disaccharide [124–129], in promoting autophagy as well as the removal of toxic proteins and structures in the brain of a rat model of Parkinson's disease [130]. Another recent investigation demonstrated

that rapamycin diminished the loss of dopaminergic neurons and improved behavioral symptoms in a mouse model of Parkinson's disease by repressing ferroptosis through activating autophagy [131]. While autophagy initiation impairment is responsible for harmful protein aggregations, the defective autophagy-lysosome pathway (i.e., the fusion of autophagosomes with lysosomes) also has a significant role in developing protein aggregates throughout the pathologic aging process, driven by upregulation of autophagy and accumulation of autophagosomes [121]. It seems that neuronal autophagy primarily has a pro-survival role before gradually transitioning to a pro-death function. As a result, adopting rapamycin to promote autophagy and lengthen longevity may have contradictory effects given the brain's pathologic situation [132].

The impact of rapamycin on mitochondrial function

Mitochondrial activity and homeostasis are fundamental in the proper functioning of signaling pathways that govern longevity among species. Mitochondrial dysfunction is, therefore, a crucial contributor to the emergence of age-associated ailments such as neurological and cardiovascular disorders [11]. There are studies underlying the importance of tuning mitochondrial function in the mechanism of rapamycin in prolonging longevity; however, such effects are complicated and include multiple processes [133]. Aging and age-related disorders are linked to a mismatch in the energy supply and demand, which may be ameliorated by several therapies, including medications (e.g., rapamycin) [11, 134]. In this regard, preserving the nicotinamide adenine dinucleotide (NAD) redox balance was demonstrated to be an important mechanism of rapamycin to maintain energy balance as well as cellular health compromised during the aging process [135, 136]. The drop in nuclear NAD^+ and resultant impaired oxidative phosphorylation (OXPHOS) system in mitochondria occurs during the aging process and is attributed to the emergence of cellular pseudo-hypoxia under normoxic conditions represented by the accumulation of HIF-1α and increased lactic acid production [137]. In this regard, the investigation by Zhang *et al.* on myoblasts showed that rapamycin favored a more oxidized NAD^+/NADH ratio in aged muscle and probably ameliorated OXPHOS through affecting the function of NAD^+-dependent enzymes. The mentioned effect is likely conducted through the rapamycin-mediated reduction in energetic demand [138]. There is also evidence that mitochondrial dysfunction may be secondary to hyperactive mTOR-driven pseudo-hypoxia. Interestingly, rapamycin has been shown to rescue pseudo-hypoxia, demonstrated by downregulating HIF-1α and lactate production through suppressing mTOR signaling and independent of mitochondrial respiration [135].

Improving mitochondrial biogenesis is one of the most important roles of rapamycin in maintaining the functionality of critical organs and expanding longevity. It has been revealed that rapamycin enhances diastolic function in aged rats, beginning between 2 and 4 weeks of therapy and continuing throughout 10 weeks of treatment. Rapamycin prompted temporary upregulation of autophagy, as indicated by ULK phosphorylation, and mitochondrial biogenesis, as evidenced by PGC1α and TFAM upregulation, while canonical mTORC1 signaling through S6 phosphorylation was hindered throughout rapamycin treatment. These findings imply that freshly generated mitochondria replace defective ones to renew mitochondrial homeostasis. This remodeling is demonstrated to swiftly invert the age-associated decline in fatty acid oxidation to modify the myocardial metabolism and reinstate fresh substrates and suitable energetic status in elderly isolated perfused hearts [139]. In brown adipocytes, which contain many mitochondria and govern energy consumption via thermogenesis, inhibiting mTOR signaling with rapamycin has also been demonstrated to enhance the expression of mitochondrial biogenesis, dynamics, and mitophagy-relevant proteins and strengthen mitochondrial quality control [140].

In addition, rapamycin treatment was suggested to improve mitochondrial DNA (mtDNA) quality in aging mice. Cell death and tissue deterioration may originate from age-related mutations in the mtDNA. With this in mind, Bielas *et al.* showed that long-term administration of rapamycin (42 ppm) causes a remarkable decrease in mtDNA deletion frequency and electron transport chain deficient fibers in mouse quadriceps muscle [141]. The mentioned effect is probably due to the alleviation of mitochondrial ROS generation and oxidative damage, which is among the well-documented mechanisms of interventions expanding longevity. In this regard, seven-week treatment with rapamycin at doses known to improve the longevity of mice (14 mg/kg) was found to reverse mitochondrial ROS generation, oxidative damage, buildup of mtDNA fragments, and mitochondrial protein lipoxidation in middle-aged mice [142]. Of note, there are also several mechanisms for ameliorating the negative consequences of mtDNA injuries. For example, low-dose oral rapamycin was shown to enhance the longevity of the murine model of mtDNA depletion syndrome with no detectable improvement in mitochondrial dysfunction or canonical pathways. This effect is thought to be driv-

en by rapamycin-induced metabolic changes that allow mice to utilize alternative energy resources (e.g., amino acids and lipids) and induce indirect signaling that modify mortality via developmental reprogramming [143]. Besides age-related conditions, oxidative damage is associated with certain pathologies restricting lifespan. It has been established that mitochondrial oxidative stress contributes to the development of anti-phospholipid antibodies (aPL) in systemic lupus erythematosus (SLE) patients and subsequent chronic inflammation, playing a role in the emergence of liver disease progressing from cirrhosis to hepatocellular carcinoma. In this regard, rapamycin may attenuate aPL production and resultant inflammation, thereby attenuating liver disorders and prolonging patients' survival by inhibiting mTOR, known as a regulator of oxidative stress [144–146].

It is worth mentioning that the impact of rapamycin on mitochondrial function is dose-dependent. Rapamycin exhibits biphasic effects on cells at high and low doses, known as hormesis. Rapamycin's hormetic property enables modification of the mTOR-mitochondrial cross-talk, which supports anti-aging actions in cells. While rapamycin is lethal to cells at high doses, it can improve lifespan at low levels. This may be explained by the hypothesis that rapamycin causes partial inhibition of mTOR activity at low concentrations, as opposed to total mTOR suppression at high doses. Rapamycin at low doses was shown to alleviate mitochondrial oxidative injury, metabolic dysregulation, and membrane depolarization [147]. Moreover, rapamycin is not a feasible treatment option for every type of mitochondrial dysfunction since the primary therapeutic mechanism is unknown, the least effective dose must be identified, and whether this therapy can be employed in general is still being determined. Therefore, it is important to at least consider the unique facets of each mitochondrial condition when rapamycin treatment is introduced to extend the patient's lifespan [148, 149].

The impact of rapamycin on inflammation and immune function

Dysregulation of immune function is a phenotype associated with aging. Innate and adaptive immunity can be negatively impacted by immunosenescence owing to the malfunction of immune cells as well as higher levels of inflammation [150, 151]. Of note, dismal immune responses and inflammation in diverse organs during the aging process may be regulated by manipulating the mTOR signaling pathway [152]. A recent preprint published by Zhang *et al.* revealed that suppressing TORC1/S6K in *Drosophila* ameliorates inflammaging and immunosenescence and thereby extends longevity [153]. Immunosenescence is assumed to underlie many age-related diseases, including cancer, autoimmune conditions, infections, and the inefficient removal of senescent cells; thus, it offers a tempting explanation for aging as well as possible treatment pathways [154, 155]. Rapamycin has been demonstrated to inactivate hyperactive lymphocytes by decreasing their reaction to cytokine receptor-associated signaling; thereby, it is effective in prolonging lifespan through ameliorating autoimmune disease (e.g., systemic lupus erythematosus and rheumatoid arthritis) and preventing organ rejection after transplantation [156, 157]. For example, rapamycin has shown promising results in ameliorating orbitopathy in a preclinical model of Graves' disease via downregulating $CD4^+$ cytotoxic T lymphocytes [158]. T-cell dysfunction has also been reported in patients with SLE in association with mTOR hyperactivation and chronic inflammation. Assessments in SLE patients demonstrated that one-year rapamycin treatment suppressed pro-inflammatory T-cell lineage specification and IL-4 and IL-17 production via expanding $CD4^+CD25^+FoxP3^+$ regulatory T-cells depleted in these patients [159]. Unfortunately, inhibition of mTORC1 in fibroblasts by rapamycin was shown to inhibit the wound healing process, explaining the emergence of non-healing oral ulcers in SLE patients following prolonged rapamycin therapy [159–161]. The effect of rapamycin on the immunological response, however, appears to be dose-dependent. When looking at patterns across diverse organism models studying the immunologic impacts of rapamycin, greater dosages are often linked with immunosuppression, whereas lower doses produce excitement. The immunostimulatory properties of rapamycin, which presumably contribute to its anti-neoplastic actions, may be explained in this way [162].

Inflammation has a fundamental contribution to many age-related diseases. Chronic renal failure, atherosclerosis, and lung infections are only some of the many inflammatory conditions linked to mTOR hyperactivation, and rapamycin was demonstrated to pose anti-inflammatory actions in these conditions. Thereby, one of the most appealing mechanisms through which mTORC1 suppression might delay numerous age-associated diseases, extend longevity, and improve health span is a drop in chronic, age-related inflammation [163–165]. As proof of concept, rapamycin administration to aged mice has been shown to extend their lifespan and downregulate the acute phase response proteins incorporated in inflammation [166]. Likewise, a recent investigation by Zwaans *et al.* revealed that rapamycin repressed inflammation in aortic vascular smooth muscle cells. Hyperactive mTOR-mediated tumor necrosis factor-α (TNF-α) induction causes matrix metallo-

proteinase overstimulation in these cells, which then facilitates the destruction of collagen fibers. Rapamycin treatment rescued these vascular changes by targeting mTOR and repressing TNF-α production [167].

As noted, rapamycin increases longevity and health span by preventing inflammaging, i.e., chronic, low-grade inflammation, mainly reliant on NF-κB signaling. It has been revealed that rapamycin inhibits the NF-κB nuclear translocation by improving the interaction between p65 and the inhibitor of κBα (IκBα) [168]. Rapamycin, for instance, suppresses high glucose-induced inflammation in THP-1-derived macrophages by suppressing mTOR and reducing NF-κB phosphorylation, inhibiting activation of the NLRP3 inflammasome – an essential element of the innate immune system that mediates the secretion of proinflammatory cytokines [169]. In an experimental model of inflammatory lung injury, rapamycin consistently suppressed inflammation by repressing NF-κB, leading to decreased IL-1β and IL-18 release and diminished leukocyte infiltration into lung tissue and bronchoalveolar lavage fluid [170]. Nonetheless, there is evidence that inhibiting NF-κB-mediated aging phenotypes and promoting healthspan is not necessarily associated with lowering inflammation. In a murine model of genetically induced NF-κB activity associated with expedited aging, rapamycin mitigated indicators of cellular senescence, lowered weakness, and promoted long-term memory, neuromuscular integration, and tissue structure, despite having no positive impacts on lifespan or inflammaging [171].

The immunomodulation brought on by rapamycin medication has also been shown to help survive infectious diseases [172]. Interestingly, a connection has been established between inflammaging and the vulnerability of older individuals to community-acquired pneumonia (CAP), supported by a positive correlation between increased levels of serum TNF-α and IL-6 and a higher occurrence of CAP in otherwise healthy seniors aged 70 to 79 years [173]. In addition, age-associated cell senescence is a major contributor to the pro-inflammatory lung exacerbation associated with chronic obstructive pulmonary disease, a major risk factor for pneumococcal pneumonia [174, 175]. Regarding the preventive impact of rapamycin on inflammaging and cell senescence, it may attenuate the predisposition to pneumonia. There is likewise evidence underlying the negative impact of chronic inflammation on HIV-1 infection. Mu *et al*. reported that rapamycin therapy in HIV-1-infected humanized mice markedly attenuated persistent interferon (IFN)-I-mediated inflammation and enhanced antiviral T-cell responses. Indeed, chronic inflammation causes loss of $CD4^+$ T-cells and exhaustion of antiviral immunity. Autophagy induction by rapamycin was shown to repress IFN-I-mediated inflammation, thus improving antiviral T-cell responses [176]. These findings suggest that rapamycin may reduce the risk of emergence and progression of infectious diseases during chronic inflammatory situations; however, there are reports warning about the risk of late-onset pneumocystis pneumonia after solid organ transplantation, underscoring the importance of targeted prophylactic therapies in such conditions [177, 178].

In addition, rapamycin has been suggested to enhance the efficacy of various types of vaccines. It has been established that rapamycin stimulates the production of memory $CD8^+$ T-cells, which have a pivotal role in the adaptive immune response against pathogens, being faster and stronger than a primary immune response [179]. Intriguingly, rapamycin was shown to enhance the effect of memory $CD8^+$ T-cells induced by immunization with amastigote surface protein-2 (ASP2) of *Trypanosoma cruzi* [180]. Likewise, stimulation of dendritic cells harboring the Bacille Calmette-Guerin (BCG) vaccine using rapamycin resulted in improved efficacy of dendritic cell vaccines in inducing immunity against tuberculosis in mice [181]. Indeed, rapamycin-mediated autophagy enhances the antigen presentation by dendritic cells and subsequent activation of $CD8^+$ naïve T-cells [182]. Accordingly, combining vaccination with rapamycin may open up new possibilities for the emergence of novel vaccine design approaches against infectious diseases.

This approach might also potentially help improve the effectiveness of cancer vaccinations. Of note, a brief period of high-dose rapamycin administration was shown to inhibit the activity of mTOR in $CD8^+$ T-cells following viral vaccination, promoting the persistence of $CD8^+$ T-cells and enhancing their ability to recall antigen responses rather than promoting their maturation into type 1 effector cells. However, extended administration of high-dose rapamycin suppresses memory responses [183]. In a similar vein, short-term exposure to mTOR inhibitors in dendritic cells while they are responding to toll-like receptor (TLR) agonists was shown to enhance their ability to activate naïve $CD8^+$ T-cells, thereby enhancing the control of B16 melanoma in a therapeutic autologous vaccination mouse model and extended lifespan [184]. It is critical to consider short-term inhibition of mTORC1 to enhance cancer vaccine therapy since long-term rapamycin may lead to the development of autoimmunity due to the reliance of effector regulatory T-cells on mTORC1 signaling [185]. Short-term rapamycin's effect on cancer vaccination, however, is also debatable. It

was reported that short-term and long-term rapamycin can eliminate $CD8^+$ T-cell recruitment to the tumor site and inhibit the antitumor immune response when combined with the human papillomavirus E7 peptide vaccine in a mouse model of cervical cancer [186]. This is probably attributed to the cell- and context-dependent nature of mTOR signaling. Furthermore, regulatory T-cell expansion has the potential to suppress effector T-cells and promote immune suppressive environments, which might confer protection against different types of cancers [186]. Therefore, it is critical not only to thoroughly investigate the effect of mTOR inhibitors on antitumor immunity in both animal and human subjects but also to identify the optimal mTORC1 activity level for memory formation without jeopardizing effector T-cell function or expanding regulatory T-cells [187].

The impact of rapamycin on stem cells

Defective tissue regeneration is partly a consequence of the decline in adult stem cell activity. In this regard, stem cell dysfunction may participate in developing age-related diseases in mammals. There is growing evidence that mTORC1 is a critical player in this process and that blocking the mTORC1 pathway can protect and even restore stem-cell activity in different organs [188, 189]. For example, upregulated mTORC1 signaling is associated with faulty multiplication and differentiation of muscle-derived stem cells, obtained from mice deficient in zinc metalloproteinase STE2, in culture and during tissue regeneration. These mice exhibit early age-related-like musculoskeletal pathologies. Intriguingly, rapamycin repression of mTORC1 increased myogenic and chondrogenic differentiation while decreasing apoptosis and senescence of these stem cells [190]. Bone marrow-derived mesenchymal stem cells were the subject of another recent study in Klotho-deficient mice, a murine model of human aging with multiple bone defects. The results revealed that the stem cells have hyperactive proliferation but diminished functionality owing to enhanced mTORC1 signaling. Intraperitoneal rapamycin administration restored stem cell quiescence, improved bone phenotype, and prolonged the longevity of model mice [189]. Rapamycin has also been demonstrated to reestablish the proangiogenic function of senescent mesenchymal stem cells, which is important in the management of ischemic conditions. Cao *et al.* observed that rapamycin reversed the senescent phenotype and considerably improved the proangiogenic function of human umbilical cord mesenchymal stem cells *in vitro*. Furthermore, intramuscular administration of rapamycin-primed senescent stem cells into the ischemic limb dramatically improved neovascularization and ischemic limb salvage in a murine model of hindlimb ischemia [191].

Evidence shows that autophagy activation may be among the mechanisms by which rapamycin improves the viability and differentiation of stem cells. Autophagy modulation is an intriguing strategy that may change mesenchymal stem cells' characteristics and affect their regenerative therapeutic effects. Furthermore, it was postulated that the capacity of mesenchymal stem cells to influence the autophagy of cells in damaged tissues/organs plays a role in their regeneration [192–194]. Specifically, mesenchymal stem cells can influence autophagy in immune cells involved in injury-induced inflammation, regulating immune cell viability, multiplication, and activity and promoting the resolution of inflammation [195, 196]. Moreover, mesenchymal stem cells can influence autophagy in native adult or progenitor cells, boosting their survival, multiplication, and differentiation to assist in tissue repair and functional reconstitution [194, 197].

In light of these findings, rapamycin-mediated elevated autophagic activities and lysosome production in rat bone marrow-derived stem cells significantly improved survival and resistance to apoptosis under hypoxia and serum deprivation. Furthermore, intracardial transplantation of rapamycin-pretreated stem cells dramatically enhanced cardiomyogenesis and angiogenesis in the infarcted myocardium due to augmented expression of growth factors (e.g., IGF-1 and VEGF), decreased expression of inflammation mediators (e.g., IL-1β and TNF-α), and differentiation of stem cells into cardiomyocytes or endothelial cells [198]. It has also been demonstrated that rapamycin induces the proliferation of myeloid cells by provoking the expression of G-CSF in mesenchymal stem cells. G-CSF is the primary driver of stem cell hematopoiesis, reinforcing their differentiation into common myeloid or granulocyte/macrophage progenitor cells [199, 200]. Another recent investigation by Xing *et al.* demonstrated that baseline autophagy in bone marrow mesenchymal stem cells declines gradually throughout osteogenic differentiation, and rapamycin promotes their osteogenic differentiation by activating autophagy [201]. Furthermore, rapamycin-triggered autophagy has been shown to enhance Nrf2/Keap1 signaling in cartilage endplate stem cells, enabling the expression of antioxidant proteins, thereby eradicating ROS, ameliorating cell senescence, lowering osteogenic differentiation of stem cells, and eventually rescuing cartilage endplate from chronic inflammation-mediated degeneration [9]. These findings support rapamycin-mediated stem cell revitalization and preservation of the homeostasis of the adult stem cell pool as a therapeutic approach for healthy aging [202–220].

Major challenges facing the use of rapamycin in anti-aging therapies

The emergence of diverse and serious adverse effects precluded the clinical utilization of rapamycin as a longevity drug. Even though rapamycin does not directly impact mTORC2, prolonged therapy may sequestrate mTOR from mTORC2, thereby impeding mTORC2 assembly, which is believed to be a contributing factor to the metabolic issues with rapamycin therapy, such as abnormal lipid profiles, glucose intolerance, and insulin resistance [13]. Despite the extensive research conducted on the advantageous impacts of rapamycin in age-associated ailments and geroprotection, limited studies have been conducted to determine the optimal dose and duration of administration and prevent the negative adverse effects of rapamycin. It has been suggested that intermittent administration could serve as a prospective approach to mitigate certain adverse effects associated with rapamycin. Administrating rapamycin at a dosage of 2 mg/kg on a single occasion every 5 days was the most commonly employed therapeutic regimen that did not adversely affect glucose homeostasis in mice. Additionally, this particular dosing schedule exhibited a diminished effect on the immune system while still effectively suppressing mTORC1 in numerous tissues and increasing lifespan [221]. Moreover, several studies have demonstrated the beneficial impacts of rapamycin on longevity following short-term administration at different periods of life. An investigation by Bitto *et al.* suggested that administering rapamycin for a brief period during the later stages of life can yield long-lasting outcomes that effectively postpone the aging process, impact the incidence of cancer, and regulate the microbiome. Three-month rapamycin (8 mg/kg) administration caused a significant increase in the lifespan of middle-aged mice by up to 60% and an enhancement in health span indicators [222]. There is, however, mounting evidence that rapamycin administered during development or early adulthood results in durable impacts on longevity. A recent study revealed interesting findings indicating that the geroprotective benefits of prolonged rapamycin treatment can be acquired through short-term administration of the drug during the early stages of adulthood in female *Drosophila* and mice. The administration of rapamycin to adult *Drosophila*, either briefly or throughout their lifespan, was found to extend their longevity and mitigate age-associated deterioration in the intestine to a comparable extent. The enduring recollection of prior therapy was mediated by heightened autophagy in enterocytes of the intestinal tract. Indeed, transient upregulation of autophagy during early adulthood resulted in a sustained enhancement of autophagic activity. The early administration of rapamycin to mice for a duration of 3 months likewise produced a memory effect and gastrointestinal geroprotection, even 6 months after rapamycin withdrawal [10]. Another recent investigation by Shindyapina *et al.* revealed that administering rapamycin (42 ppm) to genetically diverse UMHET3 mice during the initial 45 days of their life resulted in slower growth and delayed reproductive age whilst not affecting offspring numbers. Such a treatment protocol increased the median lifespan of the mice while also contributing to preserving their health as determined by assessments of frailty index scores, gait speed, and glucose and insulin tolerance tests [223]. The abovementioned findings provide novel insights into the importance of the treatment protocol in achieving optimum pro-longevity impacts with minimum adverse effects. However, further preclinical investigations, as well as clinical trials, are required to provide satisfactory data for approving rapamycin as a suitable anti-aging drug in humans.

An additional obstacle in the integration of rapamycin into anti-aging interventions pertains to the unclear differences in its impact on different genders. Numerous investigations demonstrate the sex-specific variations in the pro-longevity impact of rapamycin. For example, the sexual identity of enterocytes has been shown to control autophagy and determine the impact of rapamycin on intestinal health and lifespan; accordingly, rapamycin was shown to prolong the longevity of female *Drosophila* but not males. Evaluation in mice also revealed the sex differences in autophagy and response to rapamycin (42 mg/kg, 6 months) in the intestine, brown adipose tissue, and muscle tissue. Accordingly, sex is a significant determinant in regulation of metabolic processes by mTOR and the effectiveness of mTOR-targeted pharmacological interventions for anti-aging purposes [224]. There are several explanations for such sex-specific differences. It has been hypothesized that Rictor, a crucial constituent of mTORC2, has a vital contribution to the viability of male mice; however, the absence of Rictor does not seem to affect the lifespan of female mice. Therefore, suppressing mTORC2 signaling via rapamycin and its adverse impact on males could potentially account for the sexually dimorphic advantage [225]. Furthermore, there is a natural elevation in mTORC1 signaling in several organs of juvenile female mice in comparison to male mice of the same age; hence, females may experience more benefits from the administration of rapamycin [226]. Moreover, it has been proposed that the fitness cost of prolonging lifespan is sex-specific, and the smaller sex likely incurs a lower cost while experiencing a comparatively greater lifespan extension. Given that males

have larger body sizes than females across many mammalian species, interventions targeting the mTOR signaling pathway may have a more significant positive impact on females than males [227]. In contrast to the previously mentioned data, an investigation by Strong *et al.* revealed that administrating three-month rapamycin (42 ppm) through diet had a greater prolongevity effect on middle-aged male mice compared to female mice [228]. Furthermore, short-term rapamycin treatment (42 ppm) during the developmental phase of UMHET3 mouse growth had a greater effect on the lifespan of males compared to females [223]. In summary, the existing data are not sufficient to provide an in-depth definition of the gender-specific effects of rapamycin, necessitating further investigation. Incorporating findings on the rapamycin treatment protocol (dose and duration) and sex-specific effects into comprehensive studies will help move faster toward clinical application.

Rapamycin in clinical trials: optimal doses and safety concerns

Given the multifaceted involvement of mTOR in various vital biological processes, e.g., nucleotide, protein, and lipid synthesis, legitimate concerns arise regarding the prudent utilization of rapamycin within anti-aging programs. Oral mucositis, gastrointestinal illnesses, metabolic disorders, arthralgia, thrombocytopenia, anemia, renal toxicity, rash/eczema, and delayed wound healing have been associated with rapamycin administration as an immunosuppressor in organ transplantation [229]. However, these adverse effects may be prevented or alleviated by incorporating proper doses and courses of rapamycin administration [230]. Ten years of extensive research on the use of rapamycin in cardiac transplant recipients has indicated that there is an association between rapamycin blood levels and unfavorable events, implying that maintaining rapamycin concentrations within the lower range of its therapeutic window may enhance tolerability; however, it should be borne in mind that any improvement in tolerability must be weighed against the potential for reduced efficacy [231].

Taking the safety data from rapamycin clinical trials in healthy or diseased subjects into account would greatly aid in assessing potential adverse effects, both in terms of their nature and severity, regarding the rapamycin dose and course of treatment. Notably, prolonged periods of rapamycin administration at higher doses have been associated with greater adverse effects in various clinical trials. For instance, 12-month rapamycin therapy (2 mg/day) in individuals with lymphangioleiomyomatosis was associated with significant adverse effects, including mucositis, diarrhea, nausea, hypercholesterolemia, acneiform rash, and swelling in the lower extremities [232]. Another clinical trial in patients with TSC demonstrated oral aphthous ulcers, hypertriglyceridemia, microcytosis, and hypochromia as frequently observed adverse events following one-year rapamycin (1 mg/day) treatment [233]. In addition, prolonged administration of rapamycin (1 mg/kg) was attributed to the emergence of respiratory tract infections and stomatitis in TSC patients [234]. Long-term rapamycin therapy (1.6 mg/m^2/day) in patients with complicated vascular anomalies also caused blood/bone marrow toxicity in 27% of patients, as well as gastrointestinal and metabolic toxicity in 3% [235]. However, administrating the mentioned dose even for 2 weeks was likewise reported to cause mouth sores in these patients [236]. Interestingly, a modest dosage (0.5 mg/day) of rapamycin for 24-week therapy in individuals with active rheumatoid arthritis was proven to be well tolerated and exhibited no evaluable side effects [237]. Accordingly, most adverse events of rapamycin are likely dose- and time-dependent; therefore, it is essential to carefully monitor therapeutic effects, lower the rapamycin trough concentrations as much as possible, and determine a rational course of treatment [238]. It is also important to consider that certain risk factors may contribute to the occurrence of adverse effects associated with rapamycin. Therefore, it is advisable to avoid or delay rapamycin administration in patients at high risk while also addressing any modifiable risk factors. This approach should be taken as the initial step in mitigating the undesired effects of rapamycin [238].

Strikingly, multiple clinical trials are currently assessing the safety and effectiveness of rapamycin in diminishing clinical aging indicators, as well as biochemical and physiological endpoints linked to deteriorating health and aging in healthy or diseased adults/older adults of both genders (Table II). Consistent with the abovementioned data, these trials used low doses of rapamycin (< 1 mg/day) for long-term and higher doses of rapamycin (up to 2 mg/day) for short-term treatments. A phase II randomized controlled trial was executed by Kraig *et al.* to establish the feasibility and safety of 8-week rapamycin therapy (1 mg/kg) in an older human cohort. The participants were administered 1 mg of rapamycin over eight weeks. Five of the 11 subjects who completed the trial reported experiencing facial rash, stomatitis, and gastrointestinal issues. Blood indicators, e.g., hemoglobin, hematocrit, and red blood cell count, were decreased considerably; however, none of these alterations displayed clinically relevant consequences during the short-term rapamycin administration. Furthermore, no significant cognitive, immune, insulin-related, physical performance, or

Table II. Clinical trials assessing the anti-aging impacts of rapamycin in adults and older adults

Rapamycin dose	Phase	Subjects	Assessment	Year	Status	Trial number
0.5 mg/day, 1 year	II	Adults and older adult	Functional biomarkers of aging	2023	Not yet recruiting	NCT05237687
5 mg/week, 1 year	II	Adults and older adult	Long-term safety and efficacy	2022	Active, not recruiting	NCT04488601
1 mg/day, 8 weeks	II	Older adults	Cardiac function	2022	Recruiting	NCT04742777
2 mg/day for 5–7 days	II	Adults and older adults with breast cancer	Malignant markers and aged mammary stem/progenitor cell number	2022	Active, not recruiting	NCT02642094
Topical (8%, daily)	I	Older adults	Epigenetic and inflammatory markers in the skin	2022	Active, not recruiting	NCT04608448
1 mg/day, 8 weeks	II	Older adults	Immune, cognitive, and physical function	2018	Completed	NCT02874924
0.5, 1, or 2 mg/day, 12 weeks	I	Adults and older adults with coronary artery disease	Safety, feasibility, SASP, and frailty	2016	Completed	NCT01649960

SASP – senescence-associated secretory phenotype.

self-perceived health alterations were observed in healthy older individuals, confirming the safety of short-term rapamycin usage [239]. In terms of efficacy, administrating a low dose of rapamycin (0.5 mg/day) for 12 weeks in a phase I clinical trial was observed to reduce SASP generation in elderly patients undergoing cardiac rehabilitation but failed to alleviate frailty [240]. Furthermore, a phase II clinical trial is going to evaluate the impact of short-term rapamycin (1 mg/kg) treatment on the cardiac function of healthy older adults (NCT04742777). Another phase II clinical trial is also underway to investigate the short-term impact of 2 mg/day rapamycin on the number of aged mammary stem/progenitor cells as well as malignant markers in adults and older adults with breast cancer (NCT02642094). In addition, a few clinical investigations are assessing the long-term

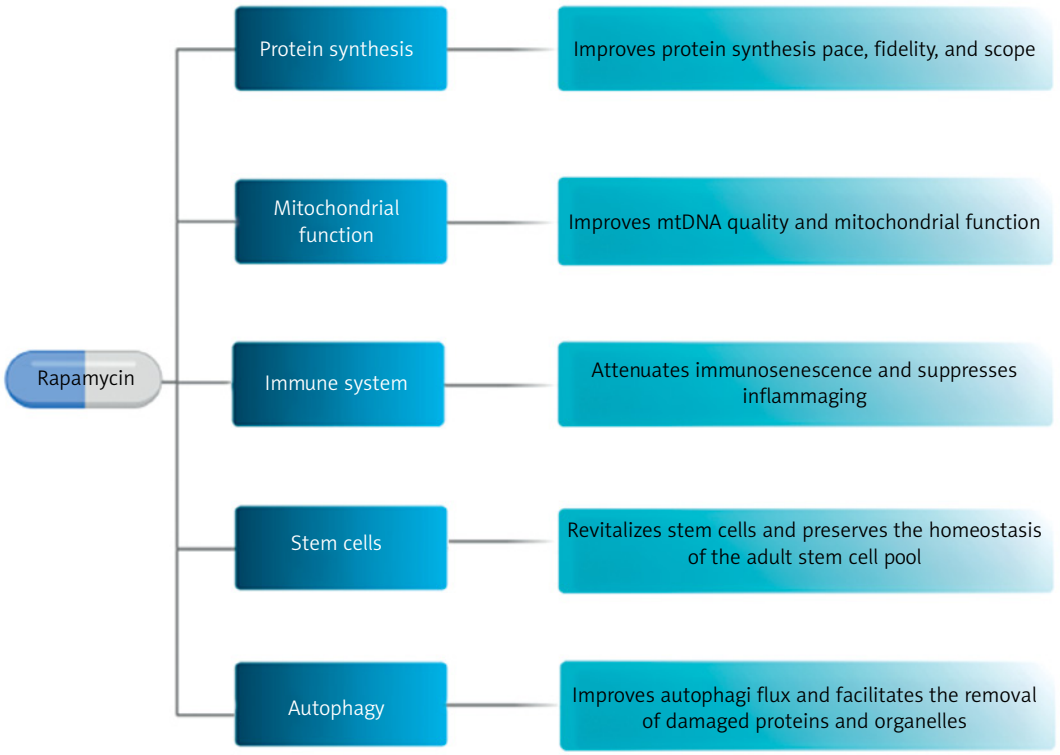

Figure 3. Rapamycin's major actions in promoting healthy aging

safety and efficacy of low-dose rapamycin (e.g., 0.5 mg/day or 5 mg/week) in fighting aging phenomena (NCT05237687 and NCT04488601). The results of these trials will pave the way to provide more accurate insights into the therapeutic usefulness of rapamycin as a pro-longevity medicine.

Conclusions

Pursuing an approach to prolong the human lifespan has been a long and challenging endeavor [241–244]. Despite the lack of certainty, there is hope that the mTOR inhibitor rapamycin might achieve this objective. The rapid and intriguing advancements in this field suggest that the future of rapamycin anti-aging therapy holds promise [245]. A substantial amount of preclinical research supports the potential pro-longevity effects of rapamycin in diverse species, which is mediated through mTORC1 inhibition (Figure 3). In this light, several clinical trials are underway, translating these effects. The findings of these clinical assessments, along with further preclinical studies, will aid in addressing issues related to the adverse effects and gender-specific impacts, as well as identifying appropriate doses and therapeutic regimens. Hopefully, this will finally facilitate the clinical application and repositioning of rapamycin as an anti-aging medication.

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Ethical approval

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Conflict of interest

The authors declare no conflict of interest.

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