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*Review*

# mTORC inhibitors as broad-spectrum therapeutics for age-related diseases

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**Abstract:** Chronological age represents the greatest risk factor for many life-threatening diseases including neurodegeneration, cancer and cardiovascular disease; ageing also increases susceptibility to infectious disease. Current therapies that effectively tackle individual diseases may have little impact on the overall healthspan of older individuals, who would still be vulnerable to other age-related pathologies. However, recent progress in ageing research has highlighted the accumulation of senescent cells with chronological age as a probable underlying cause of pathological ageing. Cellular senescence is an essentially irreversible proliferation arrest mechanism that has important roles in development, wound healing and preventing cancer, but it may limit tissue function and cause widespread inflammation with age. The serine/threonine kinase mTOR is a regulatory nexus heavily implicated in both ageing and senescence. Excitingly, a growing body of research has highlighted rapamycin and other mTOR inhibitors as promising treatments for a broad spectrum of age-related pathologies, including neurodegeneration, cancer, immunosenescence, osteoporosis, rheumatoid arthritis, age-related blindness, diabetic nephropathy, muscular dystrophy, and cardiovascular disease. In this review, we assess the use of mTOR inhibitors to treat age-related pathologies, discuss possible molecular mechanisms of action where evidence is available, and consider strategies to minimize undesirable side effects. We also emphasize the urgent need for reliable, non-invasive biomarkers of senescence and biological ageing to better monitor the efficacy of any healthy ageing therapy.

**Keywords:** mTOR; mTORC1; mTORC2; rapamycin; rapalogues; rapalogs; mTOR inhibitors; senescence; ageing; aging; cancer; neurodegeneration; immunosenescence; senolytics; biomarkers

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## 1. Introduction

The greatest risk factor for all major life-threatening diseases including cancer, neurodegeneration and cardiovascular disease is age. Current therapies that target each of these age-related diseases (ARD) individually have had limited success, and a cure for one specific ARD may not greatly extend healthy lifespan, as elderly patients would still be vulnerable to other ARDs. However, mounting evidence suggests that it may be possible to develop broad-spectrum treatments for the diseases of old age by targeting the underlying biological mechanisms driving ageing and its associated pathologies. Indeed, several consistent hallmarks of ageing have been identified, including telomere attrition, epigenetic dysregulation, altered proteostasis, decreased autophagy, mitochondrial dysfunction and increased DNA damage [1]. All these processes contribute to the onset of cell senescence, a core driver of ageing, as demonstrated by improved health and extended lifespan of middle aged mice upon removal of senescent cells [2]. Furthermore, it is also possible that other hallmarks of ageing, including stem cell depletion and remodelling of the extracellular matrix [1] are in fact consequences of cell senescence.

### *1.1. Senescence*

Cellular senescence is a programme of essentially permanent proliferative arrest, induced by stresses including replicative exhaustion, DNA damage, oncogene signalling, ER stress and imbalances in ribosome biogenesis [3]. At least *in vitro*, senescent cells show greatly enlarged cell size, altered morphology, accumulation of lipid droplets and lipofuscin-type pigments [4], and prominent actin stress fibres. Mitochondrial load increases in senescence, possibly to compensate for chronically damaged mitochondria, and lysosomal stress is evident with dyes such as senescence-associated β-galactosidase [5]. Chronically elevated levels of DNA damage response proteins including 53BP1 and γH2AX in senescent cells are indicative of poor DNA repair capacity, while there is also marked restructuring of the epigenome, such that CpG methylation patterns can be used as an epigenetic ‘clock’ to determine biological age [6].

While the original evolutionary role of senescence may lie in development [7], wound healing [8], or as a barrier to viral infection [9], it also provides a failsafe mechanism against proliferation of tumorigenic or ‘aged’ cells [10]. However, this can be detrimental to tissue integrity as such cells can no longer contribute to wound healing or the cell turnover necessary for tissue maintenance. Moreover, senescent cells do not simply exist as passive but ineffective components of a tissue: instead they actively alter their microenvironment through a secretory programme termed the SASP (senescence-associated secretory phenotype) [11]. This pro-inflammatory programme comprising cytokines, chemokines, growth factors and matrix-remodelling enzymes alerts immune cells to the presence of senescent cells, which in younger organisms is thought to promote prompt immune clearance [12]. However, with increasing age comes both an increasingly unbalanced and dysfunctional immune system, and an increased rate of senescence onset via chronic exposure to extrinsic and intrinsic damaging agents, gradual loss of homeostasis and progressive telomere erosion. Together, these cause the accumulation of senescent cells, observed in various tissues with chronological age [5, 13, 14]. Pleiotropic SASP signalling also induces paracrine senescence in neighbouring cells, amplifying the senescent cell burden and possibly driving the chronic

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and sterile inflammation observed in old age – a contributing factor to the development of many ARDs. Components of the SASP also participate in paracrine pro-tumorigenic signalling (e.g. IL-6, IL-8, MMP3), promoting tumour formation and progression [11]. Several notable experiments have provided evidence for the causative role of cellular senescence in organismal ageing and age-related pathology; most convincingly, clearance of p16-expressing senescent cells *in vivo* rejuvenates naturally aged mice, improving health and extending lifespan [2].

At the biochemical level, activation of tumour suppressor proteins p53 and/or $p16^{CDKN2}$, together with cyclin-dependent kinase inhibitor $p21^{CDKN1}$, leads to cell cycle arrest and the cessation of proliferation that is characteristic of senescent cells, together with resistance to apoptosis. Notably, however, this arrest is not accompanied by a down-regulation of growth signalling, and in fact hyperactive mTOR signalling has been described as a driver of geroconversion [15] i.e. the shift from proliferation to senescence without inhibition of growth.

### *1.2. mTOR and ageing*

The serine/threonine mTOR kinase is a major regulatory nexus that integrates signals including levels of glucose, amino acids, oxygen, growth factors and hormones to direct cell growth and proliferation under suitable conditions. mTOR is the functional enzyme within two distinct complexes – mTORC1 and mTORC2 – where it associates with several other proteins which are either distinct to each complex (e.g. Raptor/Rictor) or present in both (e.g. Deptor, mLST8, see Table 1).

| | **mTORC1** | **mTORC2** |
|---|---|---|
| core subunits | mTOR<br>mLST8/ Gβ3<br>Deptor<br>Tti1/Tel2 | mTOR<br>mLST8/Gβ3<br>Deptor<br>Tti1/Tel2 |
| complex-specific subunits | Raptor<br>PRAS40 | Rictor<br><br>mSIN1<br>Protor1/2 |

Table 1: mTOR complex subunits

mTORC1 regulates pathways central to cell growth, proliferation, survival, motility, autophagy and protein synthesis, whilst mTORC2 has a role in regulating actin organization as well as metabolic control [16]. mTORC1 is activated by recruitment to the lysosome through the action of Rag GTPases and regulators such as LAMTOR/Ragulator, whereas mTORC2 is ribosomally-associated on activation by insulin-signalling, mediated through IGFR and IRS1/2 [16], though localisation at mitochondria, the plasma membrane, ER and lysosomes has also been reported [17] (Table 2). There is significant cross-talk between the two complexes through various positive and negative feedback loops (particularly through the kinase Akt/PKB) [16], and possibly also through competition for FKBP subunits [18]. Examples of key regulators, phosphorylation targets, and biochemical and biological outcomes for each complex are summarized in Table 2.

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| | mTORC1 | mTORC2 |
|---|---|---|
| localization when active | lysosome | ribosome, plasma membrane, mitochondria, endoplasmic reticulum, lysosome |
| targets activated | $S6K^{T389}$, LIPIN1, HIF1α, GS3K, SOD1 | SGK1, PKC, paxillin, Rho GTPases, $Akt^{S473}$, IGFR, PDK1 |
| targets inhibited | 4EBP1 | FBW8 |
| activated by | insulin, growth factors, Rheb, Rag, Akt, amino acids, high $O_2$, cytokines, TFNα, IkkB | PI3K, growth factors including IGFR, Akt (on mSIN1), membrane tension, ROS, ATM/ATR |
| inhibited by | AMPK, TSC1/2 (via Rheb inactivation), low $O_2$, low ATP, low amino acids, TSC1/2 | S6K on both Rictor and mSIN1 TSC1/2 (via Rheb inactivation) |
| biochemical outcomes of activation | protein, nucleotide, lipid and mitochondrial biosynthesis; inhibition of autophagy | actin reorganization, lipid biosynthesis |
| overall outcomes of activation | cell growth (increase in volume) cell proliferation suppression of oxidative damage | cell size (surface area increase) cell shape (cytoskeletal changes) survival under oxidative stress cell cycle progression metabolic control |

Table 2. Activities and localisation of mTORC1 and mTORC2. Note that only a small subset of targets and modulators is shown.

The involvement of mTORC signalling in ageing is supported by a large body of experimental evidence. Mutations in TOR have been shown to increase the lifespan of yeast [19], *C. elegans* [20-22] and *Drosophila* [23]. Furthermore, deletion of ribosomal S6 protein kinase 1 (S6K1), a downstream target of mTOR, increases lifespan in female mice, and reduced mTOR signalling increases lifespan and reduces age-related pathologies including motor dysfunction and loss of insulin sensitivity [24]. Notably, such findings contrast with other reports that chronic mTORC inhibition induces diabetes [25]. This finding has been attributed to differential effects on mTORC1 versus mTORC2, though in some instances loss of mTORC2 signalling also increases lifespan and improves health. For instance, in the nematode worm, reduction in mTORC2 signalling by RNAi depletion of Rictor can increase lifespan under conditions of stress (high temperature) or high-quality food, whereas the opposite is seen at lower temperatures and on a less rich food source [26]. The molecular mechanism behind healthspan and lifespan extension afforded by mTOR inhibition is hence currently unclear and possibly multi-factorial, as mTOR signalling regulates a multitude of downstream signalling events (Table 2). Below, we consider major biochemical pathways important in ageing and cell senescence that are regulated by mTORC signalling, and that may therefore be amenable to modulation by mTORC inhibitors.

### *1.3 mTOR-associated pathways that contribute to senescence and ageing*

#### *1.3.1 Transcription*

mTOR signalling from both complexes can influence gene expression through interaction with a variety of transcription factors, including many involved in stress responses. For example, mTORC1 can modulate both the translational and the transcriptional activity of the hypoxia response factor HIF-1α during normoxia and hypoxia respectively [27, 28]. Furthermore, mTORC1 regulates the ROS-responsive transcription factor Nrf2 [29] as well as the heat-activated transcription factor HSF1 [30] and the osmotic

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stress transcription factor NFAT5 [31]. The effects of mTOR in modulating p53-dependent transcription are described in section 1.3.7 (DNA damage response), below.

### *1.3.2 Protein translation*

Protein translation occurs within the ribosome, a large molecular factory composed of functional RNAs and proteins. Ribosomal biogenesis (and hence subsequent protein synthesis) require the co-ordination of transcription of ribosomal RNAs (rRNA) within the nucleolus by RNA polymerase I, protein-encoding messenger RNAs (mRNA) by RNA polymerase II and transfer RNAs (tRNA) and a further 5S ribosomal RNA by RNA polymerase III. Assembly of the ribosome occurs within the nucleolus; interestingly, nucleoli are enlarged in premature ageing [32] while small nucleoli are associated with longevity [33] suggesting that enhanced ribosomal production may be associated with ageing, either as a response to imbalances in ribosomal components or as a driver through increased protein synthesis.

Protein synthesis requires not only functional ribosomes but also co-ordinated activity of a number of translation initiation and elongation factors. Two well-established phosphorylation targets of mTORC1 signalling are 4EBP1 and S6K, which act as regulators of translation initiation. Unphosphorylated 4EBP1 binds to and inhibits eIF4E, a DEAD-box helicase necessary for unwinding secondary structures at the 5' ends of transcripts, and which serves as a critical factor in recruiting 40S ribosomal subunits to mRNAs for cap-dependent translation initiation (thought to be the rate-limiting step in protein synthesis); this inhibition is removed by mTORC1-mediated phosphorylation [34]. S6K is also activated by phosphorylation by mTORC1 [16], and S6K then phosphorylates the S6 protein, a structural component of the 40S ribosomal subunit. S6K is also involved in ribosome biogenesis and in regulating translation of 5'TOP (terminal oligopyrimidine tract) mRNAs; rapamycin and similar rapalogues attenuate translation of mRNAs with complex 5' UTRs, especially those encoding HIF1α and VEGF [35]. The impact of mTORC on 4EBP1 and S6K does vary according to cell type [36], presumably allowing tailoring of translational responses to a cell's needs. Furthermore, mTOR also regulates translation elongation through activation of eEF2, which promotes translocation of the ribosome along the mRNA. While regulation of protein synthesis has largely been attributed to mTORC1, recent evidence suggests a role for mTORC2 in co-translational processing of nascent polypeptides [37, 38]. Direct activation of mTORC2 by association with the ribosome also suggests a strong link between translation and mTORC2, possibly ensuring that mTORC2 is only active in growing cells [37].

Mutations in 4EBP1, S6K and several other components of the translational machinery can confer increased longevity, and mild restriction of protein synthesis by low dose cycloheximide can prevent induction of senescence [39]. It is possible that attenuating protein translation may prevent the production of damaged proteins by enhancing quality control to prevent translational errors, co-translational misfolding, or ER- stress, and that mTORC inhibitors, by reducing rates of protein synthesis, may prevent formation of potentially toxic aggregates in the cell. mTOR is regulated by chaperone availability to link translation with quality control [40], suggesting that hyperactivated mTOR signalling with elevated levels of translation may be detrimental to cell health. Notably, dysregulation of protein synthesis and accumulation of protein aggregates are implicated in many age-related diseases, including

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neurodegenerative Alzheimer's, Parkinson's and Huntington's diseases; such dysregulation is likely to occur through a combination of high levels of translation, poor post-translational quality control and a failure of protein breakdown through autophagy.

### *1.3.3 Autophagy*

Autophagy is a selective homoeostatic degradation pathway for cellular components, which are directed via double-membrane vesicles (autophagosomes) to lysosomes for degradation. Autophagy is activated in response to nutrient limitation and suppressed by mTOR activity, through inhibitory phosphorylation of the autophagy-initiating kinase ULK1(ATG1) [41], ATG13 and lysosomally-located TFEB (reviewed in [42]). Autophagy inhibition has been linked to ageing; several proteins required for autophagy (Atg5, Atg7 and Beclin 1) are downregulated in normal human brain ageing [43] and in osteoarthritis (ULK1, Beclin 1 and LC3) [44], while knock-in of an activated form of Beclin 1 delays the onset of cardiac and renal fibrosis in normally ageing C57/BL6 mice, and even rescues the short lifespan of Klotho mutant mice [45]. Increased autophagy has been suggested to mediate the longevity effects of caloric restriction, as inhibition of autophagy prevents CR-mediated anti-ageing effects [46]. Activation of autophagy by spermidine decreases immunosenescence and improves response to influenza vaccination in mice [47]. Decreased autophagy in ageing may limit the removal of dysfunctional organelles such as mitochondria, and lead to accumulation of protein aggregates in neurodegenerative disorders. Hence reactivation of autophagy through mTORC1 inhibition is likely to be beneficial in many different diseases associated with ageing, as discussed in section 2 below.

### *1.3.4. Mitochondrial function and biogenesis*

The progressive decline of mitochondrial efficiency in senescence represents a key hallmark of ageing [1]. Mitochondrial biogenesis is a target of mTOR regulation, through several mechanisms, including selective promotion of nuclear-encoded mitochondrial-related mRNAs for translation via release of 4EBP inhibition [48], with mitochondrial oxidative function controlled through a YY1-PGC-1α transcriptional complex [49]. mTOR is thought to be a critical link between energy balance of the cell and mitochondrial biogenesis. Mitophagy is also regulated through ULK1, which is inhibited by mTORC1. Despite controversy over the mitochondrial ROS theory of ageing, mitochondria do play a critical role in the onset of cellular senescence, with senescent cells exhibiting an increased mitochondrial load and increased oxygen consumption [50]. Furthermore, DNA damage in senescence is signalled via a TGF-β-dependent retrograde pathway [51], and through mTOR to institute PGC-1-β-dependent mitochondrial biogenesis.

### *1.3.5. Hypoxia*

The transcription factor HIF-1, active under hypoxic conditions, has been linked to ageing in *C. elegans* – with increased and reduced activity both causing lifespan extension, dependent on context. mTORC1 signalling is inhibited on HIF-1 activation, through transcription of REDD1, which activates the TSC1/TSC2 complex, resulting in mTORC1 inhibition. Conversely, high oxygen tensions lead to mTORC1 activation, while reactive oxygen species may specifically activate mTORC2 [52, 53] to promote survival under

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oxidative stress. However high Rheb activity in many cancers leads to hyperactive mTOR signalling and increased HIF1 activity, resulting in upregulation of VEGF and high vascularisation of the tumour [54]; hence inhibition of mTORC through rapalogues or second-generation mTOR inhibitor ATP mimetics may have a beneficial impact on cancer through blocking this pathway. Whether this has direct relevance to ageing remains to be determined, though it has been suggested that ageing induces an mTOR-dependent pseudo-hypoxic state with high HIF1 and lactate production under normoxic conditions [55, 56], which may be amenable to mTORC inhibition.

### *1.3.6. Immunomodulatory signaling*

A common feature of age-related pathologies is chronic sterile inflammation. The secretory phenotype (SASP) of senescent cells, through which pro-inflammatory mediators are released to stimulate clearance by immune cells, may be the source of such inflammation. The SASP has pleiotropic signalling effects, exhibiting not only paracrine immunomodulatory signalling but also autocrine and paracrine pro-senescence and paracrine pro-tumorigenic signalling. Therefore, the SASP may amplify the senescent cell burden of an elderly individual, exacerbate tissue dysfunction, and stimulate age-related tumorigenesis. The SASP is at least partially regulated by mTOR, possibly through inhibiting feedback loops of IL1A translation or MAPKAPK2 signalling, and can be suppressed using rapamycin or Torin [57, 58], or MAP kinase inhibitors [59]. These findings conflict with earlier studies showing the central importance of mTOR in innate immunity, specifically in production of anti-inflammatory IL-10 and suppression of pro-inflammatory cytokines IL-21 and IL1β; rapamycin and Torin are also reported to suppress the anti-inflammatory effects of circulating glucocorticoids [60], and transplant patients receiving mTORC inhibitors showed more than double the expected rate of non-infectious fever [61], suggesting excess inflammation. It is possible that these marked – and important – discrepancies relate to dosage, with pro-inflammatory effects of mTORC inhibition at high dose, while SASP-suppressive doses are much lower.

### *1.3.7 DNA damage response*

Following DNA damage, cell cycle progression is halted through activation of multiple checkpoints and cyclin-dependent kinase inhibitors. The damage-responsive ATM/ATR kinases phosphorylate and activate mTORC, which can then phosphorylate Chk1, leading to proliferative arrest at either S phase or G2/M; mTORC2 is specifically implicated in this arrest, at least in breast cancer cells [62]. In addition to Chk1, components of the mTOR/S6K axis are also phosphorylated by p38α MAPK following DNA damage. While mTOR activity can itself be modulated by the tumour suppressor protein p53 (e.g. through p53 transcriptional targets such as TSC2, AMPK and REDD1 [63]), p53 activity is sensitive to mTOR signalling; mTORC1 can enhance the translation rate of p53 [64, 65] or activate p53 through S6K1-dependent phosphorylation of and binding to MDM2, which releases p53 from inhibition [66] so that it can act as a transcription factor for repair factors such as Gadd45 or pro-apoptotic factors Bax and PUMA (reviewed in [67, 68]). Moreover,

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mTOR activity enhances p53-dependent transcription of $p21^{CDKN1/SDI1}$ and induction of senescence [69], a possible molecular explanation for geroconversion.

The importance of mTORC in DNA damage responses suggests that mTORC inhibitors may be beneficial in cancer by sensitising cells to genotoxic agents, though conflicting results have also been reported [70]. Very recent work suggests that the DNA damage response is defective in cells with hyper-activated mTORC1 signalling that lack the LKB1 tumour suppressor [71]. Chronic persistent DNA damage – and hyperactive mTOR – are also features of senescent cells. Hence mTOR inhibitors may alleviate the burden of DNA damage on ageing, though their impact on cell cycle control should be closely monitored.

### *1.4 Rapamycin and other mTOR inhibitors*

Rapamycin is the natural macrolide antibiotic lactone produced by *Streptomyces hygroscopius,* discovered in soil samples from Easter Island, and initially noted for inhibiting the proliferation of yeast [72]. At high doses (e.g. 5mg/day), rapamycin has immunosuppressive effects and is FDA-approved for prevention of transplant rejection [73]. It is also in clinical use or in trials for a large number of cancers where mTORC signalling appears to be a key factor in promoting and/or sustaining oncogenic transformation (see section 2.8 below). Reported side-effects of chronic administration include ulceration of mucosal tissues, haematological abnormalities, induction of insulin insensitivity, obesity and diabetes, though these adverse effects may be largely dose-dependent.

As discovered through *S. cerevisiae* genetic screens [74], rapamycin mechanistically acts by binding the protein FKBP12, producing a complex which can bind the FRB region of mTOR and partially occlude the active site of mTOR kinase in the mTORC1 complex [75]. This induces cellular effects including a decrease in protein synthesis, increase in autophagy and inhibition of cellular growth [76]. Rapamycin does not inhibit the phosphorylation of all mTORC1 substrates equally – it completely inhibits S6K1 phosphorylation while only partially blocking 4EBP1 phosphorylation [36]. A crystal structure of mTOR, rapamycin and FKBP12 [77] suggests that this may be due to differential substrate access to the kinase active site, controlled by the mTOR FRB domain, though differential substrate quality (i.e. degree of divergence from the consensus sequence of the phosphorylation site) could also be important.

Structural and functional analogues of rapamycin ('rapalogues') that also act by allosterically modulating the enzyme have been developed to improve bioavailability and pharmacokinetics, including drugs such as everolimus (RAD001). These agents also act by recruiting the immunophilin/prolyly isomerase FKBP12 to mTORC1.

By contrast to mTORC1, mTORC2 is not particularly sensitive to inhibition by rapamycin or rapalogues, though chronic administration does impact mTORC2 signalling [78], either through feedback via the insulin signalling pathway, and/or through competition for key subunits FKBP12, 51 and 52, which may set different thresholds for rapamycin sensitivity between the two complexes [18]. In human cells in culture, the 'chronic' effect on mTORC2 is observed as little as 24 hours after drug treatment, though metabolic effects in animals and human patients require more prolonged treatment (over weeks or months).

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mTORC2 inhibition is implicated in impaired glucose homeostasis, insulin insensitivity and diabetes, though studies on worms with tissue-specific RNAi have suggested that it is loss of mTORC2 activity specifically in the intestine that results in dysregulation of glucose metabolism [26]. It is important to note that such studies often rely on phosphorylation of mTORC2 target Akt on S473 as a readout of mTORC2 activity, but this site on Akt may also be targeted by kinases IKKε, TBK1 [79] and DNA-PK [80], potentially skewing the interpretation of mTORC2-specific effects.

Second-generation mTOR inhibitors have been developed, primarily as anti-cancer agents to target hyperactive mTOR observed in many cancers [81]. These drugs compete with ATP for the active site of the TOR kinase, and hence are effective in inhibiting both mTORC1 and mTORC2. Some agents have extremely high specificity and selectivity for the mTORC kinase (e.g. AZD8055 has 1000-fold greater effect on mTORC than other PI3 kinases [82], whereas others (e.g. BEZ235) have dual inhibitory effects on both mTORC and PI3K [83], with a 3-5 fold higher $K_d$ for damage response kinase ATR [84]. While these ATP-competitive inhibitors exhibit more potent apoptotic effects *in vitro* compared with rapalogues, and a number of such agents have been tested in clinical trials for safety, larger scale trials have not yet demonstrated greater efficacy than current best treatment regimens [81]. Therefore, drugs such as AZD8055, AZD2014 and WYE354 have not yet received FDA approval. The differential specificities of rapalogues and second generation mTORC inhibitors have proven useful in primary research to dissect the effect of mTORC1 inhibition (rapalogues) versus dual mTORC1/2 inhibition (competitive ATP mimetics) in senescence [85]. The major classes of mTOR inhibitors and other pathway modulators are listed in Table 3.

## 2. Ageing and age-related pathologies amenable to treatment by mTOR inhibition

### *2.1 Ageing*

A landmark study from 2009 in which rapamycin was fed to middle aged mice provided the first evidence that any small molecule drug, taken orally, could significantly extend both mean and maximum lifespan in mammals [103]. In this multi-centre, large cohort study of genetically heterogeneous (UM-HET3) mice, rapamycin delayed the ageing of 20-month old male and female mice. Further studies have not only validated these results but have demonstrated that rapamycin improves health, in terms of lower incidence or decreased severity of age-related disease, as well as prolonging life [104]. Below, we assess the impact of mTOR inhibition on a number of age-associated diseases and pathologies, collating findings from model systems and human clinical trials.

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| Drug class | Mode of action | Drug name | Ki or IC50 | Status |
|---|---|---|---|---|
| mTORC1 inhibitor | Binds FKBP12 which then associates with mTORC1 and partially occludes kinase active site; mTORC2 inhibited on chronic treatment (possibly through feedback loops) | Rapamycin (sirolimus) | mTORC1 $IC_{50}$ 0.1 nM (in HEK293 cells) | FDA-approved for cancer and as immunosuppressant to prevent rejection in renal transplant; eluting stents in cardiovascular disease. Delays senescence in cell culture [86] Extends lifespan and health in lab animals and improves cardiovascular health in companion dogs (see text). |
| | | Everolimus (RAD001) | mTORC1 $IC_{50}$ 1.6-2.4 nM (cell-free assay) | FDA-approved for cancer (e.g. monotherapy against advanced renal cell carcinoma, neuroendocrine tumours of pancreatic, gastrointestinal or lung origin, and SEGA associated with TSC, and as combination therapy with exemestane for HER2-negative breast cancer (Novartis)). Clinical trials show immune system rejuvenation [87, 88] |
| | | Temsirolimus; (CCI-779, NSC 683864) | $IC_{50}$ 0.3-0.5 nM in cell culture | FDA approved, used at 10 mg/kg/day in acute lymphocytic leukaemia. |
| Pan-mTOR inhibitor (inhibits both mTORC1 and mTORC2) | ATP-competitive mTORC1/2 inhibitor | AZD8055 | mTOR $IC_{50}$ 0.8 nM (MDA-MB-468 cells); 1000-fold selectivity against PI3K isoforms and ATM/DNA-PK | Acceptable safety profile for treatment of advanced solid tumours and lymphoma in phase I trial. [89]; reverses phenotypes of senescence in cell culture [85] |
| | | Sapanisertib (AK-228, INK 128, MLN0128) | mTORC1 and mTORC2 1nM (PI3K isoforms ~200nM) | Phase 1 trials (cancer). |
| | | OSI-027 | 22nM mTORC1, 65nM mTORC2 (>100x selectivity over PI3K) | Phase 1 trials. In experimental colorectal xenograft, OSI-027 (65 mg/kg) more effective than rapamycin [90], reviewed [91]. |

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| | | | | |
|---|---|---|---|---|
| mTORC2-specific inhibitor | prevents interaction of Rictor with mTOR hence blocking mTORC2 | JR-AB2 | ? | Experimental, xenograft tumour models [92] |
| Dual PI3K and mTOR inhibitor | ATP-competitive dual PI3K and mTORC1/2 inhibitor | Apitolisib (GDC-0980, RG7422) | Dual PI3K/mTOR 5-14nM Ki, 17nM mTOR | Phase 2 trials (cancer). |
| | | Dactolisib (NVP-BEZ235, BEZ235) | mTOR $IC_{50}$ 6 nM, PI3K p110α/γ/δ $IC_{50}$ 4/5/7 nM respectively; $IC_{50}$ ATR 21 nM (cell-free assays) | Passed phase I initial dose discovery trial [93]; modest efficacy in advanced or metastatic carcinoma in phase II [94] but poorly tolerated in advanced pancreatic neuroendocrine tumour patient phase II study [95]; beneficial outcomes in trial with everolimus for reversal of immune senescence [88]. |
| | | PF-04691502 | PI3K(α/β/δ/γ)/mTOR dual inhibitor with $K_i$ of 1.8 nM/2.1 nM/1.6 nM/1.9 nM and 16 nM (respectively) | Phase 1 clinical trials. |
| PI3K, DNAPK and mTOR | ATP binding site competitor | PI-103 | PI3K 2-15nM, mTOR and DNAPK 30nM | Experimental [96] |
| .Other components of signaling pathway | PI3K and BRD bromodomain proteins | SF2523 | DNAPK 9nM, 34-158 nM; BRD4 241nM, mTOR 280nM | Blocks Brd4; blocks Brd2 to overcome insulin resistance – may be useful as adjunt to prevent diabetic complications of mTOR inhibitors [97] |
| | Highly selective GS3K inhibitor; ATP binding competitor | CHIR-98014 | GS3Kα 0.65 nM GS3Kβ 0.58nM | Experimental [98, 99] |
| mTOR activator | FKBP1A | 3BDO | n/a | Experimental; inhibits autophagy; provides vascular protection [100].; improves neuronal function in App and Psen1 transgenic mice [101] |

343 Table 3. Classes of mTOR pathway modulators with examples of each class. $IC_{50}$ and Ki data derived from [102].

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## 2. Ageing and age-related pathologies amenable to treatment by mTOR inhibition

### *2.1 Ageing*

A landmark study from 2009 in which rapamycin was fed to middle aged mice provided the first evidence that any small molecule drug, taken orally, could significantly extend both mean and maximum lifespan in mammals [103]. In this multi-centre, large cohort study of genetically heterogeneous (UM-HET3) mice, rapamycin delayed the ageing of 20-month old male and female mice. Further studies have not only validated these results but have demonstrated that rapamycin improves health, in terms of lower incidence or decreased severity of age-related disease, as well as prolonging life [104]. Below, we assess the impact of mTOR inhibition on a number of age-associated diseases and pathologies, collating findings from model systems and human clinical trials.

### *2.2. Immunosenescence*

The immune system undergoes a functional decline with age that both contributes to organismal ageing through decreased senescent cell clearance, and also compromises its ability to fight infection. The term 'immunosenescence' is specifically associated with a decline in the haematopoietic stem cell proliferation compartment, a higher proportion of exhausted, PD-1$^+$ lymphocytes, an inverted CD4/CD8 ratio (<1), a low number of B cells, and CMV seropositivity [105]. Age is associated with a high mortality rate from infectious disease, thought to be a direct consequence of loss of immune function. Activation of autophagy has been shown to rejuvenate the immune system in mice [47]; since mTOR activity inhibits autophagy, it follows that mild inhibition of mTOR could be beneficial for immune function with increasing age. Deriving an appropriate dose is critical, as at high doses rapamycin is immunosuppressive, as it blocks both protein synthesis and cell division; both are required in order to mount an adaptive immune response.

In mouse models, increased immune activity against both viruses and bacterial pathogens has been observed on mild mTOR inhibition [106], suggesting that it is possible to improve at least some aspects of the ageing immune system with low dose mTOR inhibitors. Furthermore, a placebo-controlled, randomized, double-blind human clinical trial of over 200 elderly volunteers has shown similar results [87]. Volunteers were assigned to one of three regimes of the mTORC1 inhibitor RAD001 (everolimus - low: 0.5 mg daily or 5 mg weekly; high: 20 mg weekly) for a 6-week period, followed by a two week drug-free interval. These volunteers were then challenged with the seasonal influenza vaccine. Though the relatively small size of the study impeded powerful statistical analysis, the two low-dose RAD001 regimens improved immune function without causing serious side effects. Patients produced a broader and more powerful immune response, with improved HSC function and a decreased proportion of PD-1$^+$ lymphocytes. The increased breadth of the immune response was particularly promising; older individuals are more likely to die from influenza than younger people, but generally produce a narrow, weak response to vaccination. Despite the lack of a young control population in the study, the improved response is thought to correspond to a rejuvenated immune system. In a subsequent follow-up study using combined BEZ235 and RAD001 treatment, again for just six weeks, better infection control was reported in older adults for a year after treatment ended [88]. Given the important role of the

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immune system in cancer surveillance and senescent cell clearance, it would be very interesting to test whether such a rejuvenated immune system was better equipped to clear senescent or tumorigenic cells *in vivo*.

### *2. 3. Age-related neurodegeneration*

mTOR hyperactivation is associated with cognitive deficit and brain dysfunction, as seen in Tuberous Sclerosis, where loss of TSC1/2 prevents negative regulation of mTOR. Hence mTOR inhibition is being trialled for TS treatment, with beneficial results reported (reviewed in [107]). Lifelong rapamycin administration to mice prevents the usual age-related decline in cognitive function, thought to be through suppression of IL1β [108]. Neurodegenerative diseases characterized by accumulation of abnormal protein aggregates (Alzheimer's disease, Parkinson's disease, and Huntington's disease) are further candidates for treatment with mTOR inhibitors. Not only does mTORC1 exert tight control over protein synthesis and degradation (autophagy) through 4EBP1/S6K, ULK1 and SCF/FBW8, but the mTOR pathway is involved in regulating inflammatory responses known to be involved in the progression of neurodegeneration; it may also contribute to an energetic deficiency observed in such diseases. Conversely, however, the mTOR pathway has been proposed to regulate synaptic plasticity and memory consolidation, through control of actin reorganization by mTORC2 [109] and neuronal Rictor knock-out mice do indeed show cognitive effects due to alterations in actin reorganisation needed for dendritic spine growth and formation of memories [110]. However human trial data suggest that pharmacological inhibition, which is not equivalent to total loss of mTORC2, is if anything supportive of brain function: patients taking everolimus for immunosuppression after heart transplantation actually showed improvements in memory and concentration in comparison to those on calcineurin inhibitors [111].

#### *2. 3. 1. Alzheimer's disease.*

Alzheimer's disease (AD) is the most prevalent neurodegenerative disease, characterized by accumulation of aggregated extracellular amyloid β (Aβ) plaques and intracellular neurofibrillary tangles composed of tau protein. Neuronal loss and brain atrophy worsen with disease progression. mTOR signalling has been implicated in AD pathogenesis: evidence from human post-mortem exams suggests that mTOR activity is upregulated in AD brains compared to age-matched controls, as levels of phosphorylated mTOR, p70S6K and eIF4E are all increased in AD [112]. This upregulation of mTOR signalling could be mediated via Aβ accumulation, which may activate the PI3K/AKT pathway and in turn, increased mTOR signalling has been linked to the development of tau pathology [113]. Aβ upregulates mTOR and mTOR is thought to increase levels of Aβ (reviewed in [114]) potentially generating a positive feedback loop in disease progression.

Rapamycin has been shown to prevent cognitive decline in Alzheimer's (AD-Tg) mouse models [115-117], and even to reverse already established memory deficits [118], though these effects were limited to mild cognitive decline before widespread plaques and tangles were observable. Improvements in memory and cognition with rapamycin or tersolimus treatment correlated with improvements in the three major hallmarks of AD (Aβ plaques, tau tangles and microglia activation) [116-118]. A genetic mouse model lacking one mTOR gene

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copy in the brain exhibited reduced Aβ deposits, and rescued memory deficits [119], hence reduced mTOR activity associates with cognitive improvement. It is likely that treatment must happen prior to major deposition as cognitive improvements are seen in mice on whole-life but not late-life administration of rapamycin – a therapeutic window exists, though it is not yet known what constitutes the point of no return.

Though the mechanism of improvement is still unclear, it is possible that decreased protein synthesis may avoid the build-up of toxic Aβ, or that induction of autophagy through mTORC1 inhibition may result in removal of protein aggregates. Healthy neurons have highly efficient and active autophagy, but this decreases with age (reviewed in [120]). In the mouse models where rapamycin was shown to decrease levels of Aβ, autophagy induction was necessary [115]. Further, in rapamycin-treated AD-Tg mice brains, increased localization of Aβ into lysosomes was detected, suggesting a more active degradation of these peptides [115], and the decrease in Aβ levels induced by rapamycin could be prevented by blocking autophagy. Hence mTOR inhibition leading to increased autophagy may be beneficial in treating neuropathies associated with protein aggregation. Other components of the mTOR signalling cascade are also implicated in neurodegeneration, including GS3K, overactivity of which results in decreased lysosomal acidification. Hence GS3K inhibitors (eg peptide L803-mts) present a novel alternative to mTORC inhibition in AD, and appear active in the 5xFAD mouse model of AD [121].

### *2.3.2 Huntington's disease*

Huntington's disease (HD) is a neurodegenerative disorder where a genetic mutation causes an expansion of the polyglutamine tract within the Huntingtin protein (HTT), resulting in protein aggregation. As mTORC1 signalling suppresses autophagy, responsible for recycling protein aggregates, it has been implicated in HD pathology. Counter-intuitively however, mTORC1 activation may actually be beneficial: in HD mouse models with increased mTOR activity, motor performance was improved relative to controls, coincident with improved mitochondrial function, cholesterol synthesis and decreased HTT abundance. Further, phosphorylation of S6 was actually decreased in human HD patients compared to controls, further suggesting a complicated association between mTOR signalling and HD [122].

### *2.3.3. Parkinson's disease*

Parkinson's disease is a progressive age-associated neurodegenerative disorder with death of neurons in the substatia nigra. It manifests as loss of motor co-ordination, often associated with mood disturbance and in many cases followed by dementia. Current treatment is symptomatic, using L-DOPA to reinforce failing dopaminergic signalling. Though a number of genes are associated with PD, there is little overall understanding of the etiology, though lysosomal dysfunction (allowing a build-up of intracellular α-synuclein as Lewy bodies) is implicated. Failure of mitophagy, through defects in PINK1/Parkin, may also be important, and defective mitochondria are observed in PD [123].

mTORC1 has been suggested to be neuroprotective and consistent with this, suppression of mTORC1 signalling by several routes (AMPK, PTEN or REDD1 activation, or rotenone treatment) results in neuronal cell death in models of PD [124, 125]. Moreover,

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L-DOPA, the current symptomatic treatment of PD, activates mTORC1, supporting the idea that mTORC1 activity is beneficial. However, the opposite has also been reported: elevated mTORC signalling (by deletion of the gene Engrailed, or exposure to paraquat) leads to neuronal apoptosis, suggesting that a balance of mTORC activity is required for neuronal health.

To achieve this balance, mTORC inhibition is being explored as a possible treatment route for PD. Rapamycin has been shown to overcome dyskinesia in mice, a major side effect of treatment with L-DOPA, without interfering with the therapeutic effects of L-DOPA [126], while a number of other studies have also demonstrated benefits of rapamycin use in PD (reviewed in [127]). As in AD, other mTOR pathway factors such as GS3K might present therapeutic targets, particularly as lysosomal function appears important. It will be interesting to determine if mTORC inhibition promotes autophagic clearance of aggregated α-synuclein and/or dysfunctional mitochondria, and whether this is enhanced by co-treatment with GS3K-inhibiting peptides. However, it has been argued that specific pro-autophagic interventions may provide even better therapeutic outcomes than global autophagy stimulation [128].

### *2. 4. Age-related blindness: AMD*

Age-related macular degeneration (AMD) is the most common cause of blindness in the Western world, whereby retinal damage leads to loss of vision in the centre of the visual field (macula). In senescence-accelerated OXYS rats, rapamycin administration in food decreased the incidence and severity of AMD-like retinopathy and prevented destruction of ganglionar neurons in the retina [129]. These promising results accelerated rapamycin as an AMD therapeutic through to clinical trials, however conflicting results have since been produced, potentially because of dosing issues. For example, one small phase II clinical trial administered 440 µg rapamycin to one eye every three months for 24 months to eleven patients with an advanced form of dry AMD, but was terminated early after finding that treatment may be detrimental to visual acuity [130]. High dose rapamycin is known to elicit unwanted side effects, so it is unfortunate that such high dosage trials are likely to reinforce clinical prejudice against use of mTOR inhibitors for non-life-threatening illness. Full dose-response trials to obtain maximal benefit with minimal side effects are still needed, particularly as AMD treatment options are limited and pharmacological therapies should provide a cheaper and more accessible option to the successful stem cell treatments recently reported [131].

### *2.5 Musculoskeletal disorders*

#### *2.5.1 Sarcopenia and muscle wasting*

Structural and functional remodelling of skeletal muscle throughout ageing causes sarcopenia, a muscle-wasting syndrome that results in frailty. Muscle loss is consistently observed in premature ageing syndromes and associated with mTOR signalling. For example, muscle-derived stem/progenitor cells (MDSPCs) from the premature ageing Ercc1$^{-/\Delta}$ mouse show upregulated mTOR signalling and are defective in differentiation. Treatment with rapamycin improved myogenic differentiation with increased levels of

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autophagy detected in the isolated cells [132]. Hutchinson-Gilford progeria syndrome (HGPS), a human early onset premature ageing syndrome, is also associated with musculoskeletal abnormalities. HGPS results from a splice site mutation in the lamin A (LMNA) gene leading to production of an aberrant lamin protein termed progerin, though even in normal individuals, progerin accumulates during ageing and is associated with vascular pathology. Rapamycin treatment can induce autophagy and reduce phenotypes of senescence induced by progerin in cell culture models of HGPS [133]. Based on such studies, everolimus is now included in a clinical trial for 17 children with HGPS { [134]}.

The muscle loss in premature ageing HGPS is highly similar to that seen in various other laminopathies including Emery-Dreifuss muscular dystrophy, Limb-girdle muscular dystrophy and dilated cardiomyopathy. mTORC1 is implicated in these LMNA-related dystrophies: both $Lmna^{H222P/H222P}$ and $Lmna^{-/-}$ mice show aberrant mTORC1 signalling [135]; $Lmna^{-/-}$ mice specifically showed increased mTORC1 signalling in cardiac and skeletal muscle, with impaired cardiac autophagy, while rapamycin treatment enhanced cardiac and skeletal muscle function and survival in the mutant mice [136].

Targeting mTORC1 signalling is the only therapeutic avenue yet explored for laminopathies that has promise against both dystrophic and progeroid laminopathies [137], but it has yet to be tested in sarcopenia. However, as a note of caution, patients taking rapamycin for more than 6 months for treatment of renal cell carcinoma or paracrine neuroendocrine tumours demonstrated an increase in sarcopenia [138], a worrying finding as sarcopenia is predictive of outcomes in cancer patients. Longitudinal rapamycin studies in healthy subjects, such as those ongoing in companion dogs [139] are needed to inform on whether low dose mTOR inhibition may be able to delay or even prevent the onset of sarcopenia.

### *2.5.2 Osteoporosis*

Osteoporosis is a common ARD characterized by loss of bone density, causing fragility. Falls, as a consequence of co-morbid sarcopenia and age-associated changes to vision and balance perception, often result in hip fractures, and a high number of elderly fracture patients die within 6 months of pneumonia (exacerbated by co-morbid immunosenescence) [140, 141]. Increased activity of osteoclasts, which mediate bone resorption, together with decreased osteoblast activity is frequently seen in multiple forms of bone loss (osteoporosis, rheumatoid arthritis and cancer-induced bone loss). mTOR signalling regulates osteoclast differentiation by altering ratios of the LIP/LAP isoforms of transcription factor C/EBPβ [142] which enhances osteoclastogenesis. In mouse models and human cells, inhibition of mTORC1 signalling lowers activity of the translation initiation factor eIF4E, in turn diminishing expression of the LIP isoform by inhibiting translation re-initiation. This increased the LAP to LIP ratio and inhibited osteoclastogenesis, hence rapamycin can limit bone resorption [143, 144]. Furthermore, mTORC1 inhibitor everolimus inhibits bone loss in an experimental rat model of osteoporosis induced by ovariectomy [145].

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### *2.5.3 Rheumatoid Arthritis*

Rheumatoid arthritis (RA) is a chronic and progressive age-related disease. Highly effective treatments include methotrexate and infliximab, but have limited utility in elderly patients because of underlying renal insufficiency; factors such as transport/mobility difficulties also limit attendance at treatment centres for regular antibody infusion. Hence a safer therapy is required in this patient cohort, which may be provided by mTOR inhibitors. Active mTOR signalling has been detected in synovial tissue from RA patients, and is crucial for joint destruction in experimental arthritis [146]; senescent cells have been detected in OA joints (Clinicaltrials.gov identifier NCT03100799), and SASP secretion of collagenase and other metalloproteases is likely to impact significantly on joint integrity. Hence mTOR inhibition could be beneficial in OA, by targeting mTOR hyperactivity in senescent cells. Intraperitoneal administration of rapamycin reduced cartilage destruction and synovitis in experimentally-induced osteoarthritis in mice [147]; this may occur at least in part through increased ULK1-mediated autophagy and through suppression of MMP secretion by chondrocytes (reviewed in [148]). Such results appear to be relevant to human joints: in a recent proof-of-concept study (a multi-centre, randomized, double-blind study of 121 patients with RA), 6 mg everolimus daily for 6 months, in combination with methotrexate, showed improved clinical efficacy compared with methotrexate alone, as well as causing few side effects [149]. OA presents an ideal opportunity for intervention as intra-articular administration should avoid side-effects associated with systemic mTORC inhibitor treatment.

## *2. 6. Cardiovascular disease*

Cardiovascular disease is the leading cause of death in developed nations and its incidence increases with age. A number of studies have shown beneficial effects of rapamycin on cardiovascular disease in mice: for example, rapamycin has been shown to attenuate pressure overload-induced cardiac hypertrophy [150], to regress established cardiac hypertrophy and improve cardiac function [151], and to suppress experimental aortic aneurysm growth [152]. Recent studies have elaborated on this research. In female 24 month-old C57BL/6J mice fed rapamycin for 3 months, the greatest benefit measured was in cardiac health, with reversal or attenuation of age-related cardiac decline. Specifically, rapamycin appeared to slow or reverse progression of age-related hypertrophy; ventricular function of the ageing heart was also improved [153]. Through RNA-seq analysis, validated at the protein level and with bioinformatics analysis, it appeared that rapamycin reduced age-related sterile inflammation in the heart, while promoting expression of RAD (Ras associated with diabetes), which promotes anti-hypertrophic signalling and enhances cardiomyocyte excitation-contraction coupling [154]. Caloric restriction and rapamycin treatment (both for 10 weeks) were also shown to rejuvenate the aging mouse heart [155]. Improvements in mitochondrial function were implicated in the mechanism, as the mitochondrial proteome was rejuvenated [155], consistent with the known action of mTORC1 in mitochondrial biogenesis, and the contribution of mitochondrial accumulation to senescence. Hence, rapamycin could act both to suppress excessive mitochondrial biogenesis and to activate mitophagy. It is of note that improved cardiovascular function was also the most marked outcome of the first year of a trial feeding rapamycin to companion dogs [139], reinforcing

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595 the potential for rapamycin to treat cardiovascular disease. It is possible that the mechanism
596 here is through induction of autophagy by ULK1 upregulation on mTORC inhibition, as
597 cardiac fibrosis is also decreased in older mice on activation of autophagy by disrupting the
598 Beclin 1-Bcl2 interaction [45] – decreased inflammation by suppression of the SASP is also
599 a potential mechanism.
600 Furthermore, rapamycin-eluting stents are now in widespread clinical use in coronary
601 angioplasty to treat cardiovascular disease, after being approved in Europe in 2002 as the
602 RAVEL trial produced very promising results [156]. In this context, rapamycin may benefit
603 coronary function by restricting cell proliferation and thus preventing fibrosis that could
604 block the artery; everolimus is now also in clinical trials for this use. To date, therefore,
605 mTOR inhibition appears to be a safe and effective intervention to improve cardiovascular
606 function during ageing.

607 *2. 7. Kidney disease*

608 *2.7.1 Adult polycystic kidney disease*

609 Age-related incontinence is a common cause of depression and isolation in the elderly.
610 A possible heritable disease model for this condition, adult polycystic kidney disease, also
611 known as autosomal-dominant polycystic kidney disease (ADPKD) is the most common
612 heritable kidney disorder, with a prevalence of between 1/400 and 1/1000. Mutations in two
613 genes are responsible for the condition: PKD1 (85% of cases – severe, early onset) and
614 PKD2. PKD1 codes for polycystin-1, a membrane receptor protein, while PKD2 codes for
615 polycystin-2, a $Ca^{2+}$-permeable channel that binds PKD1. Polycystins are involved in
616 maintaining a differentiated epithelium in the kidney, liver and pancreas, but when mutated,
617 excessive epithelial proliferation results in renal cysts. Mechanistically, they play a role in
618 signalling – there are direct physical interactions between the cytoplasmic tail of polycystin-
619 1 and tuberin, the product of the TSC2 gene, which regulates mTOR [157]. As mTOR
620 signalling is therefore regulated by polycystin-1, and mTOR signalling is increased in murine
621 models and in human ADPKD, mTOR activation may contribute to renal cyst expansion
622 through excessive tubular epithelial cell proliferation. Hence mTOR inhibition may be
623 beneficial, and rapamycin has been shown to decrease proliferation in cystic and non-cystic
624 tubules, to inhibit renal enlargement and prevent loss of kidney function in the Han:SPRD
625 rat model of ADPKD [158-160]. While this model results from mutations in genes other than
626 PKD1 and PKD2, rapamycin treatment was also effective in a more human-orthologous
627 mouse model of conditional inactivation of PKD1 [161]. Still, both models exhibit early-
628 onset, rapidly progressive disease, whereas human ADPKD is characterized by complex,
629 slow and heterogeneous progression. Therefore, retrospective analyses of human ADPKD
630 patients after renal transplantations have been very informative. Using MRI-determined
631 increases in kidney volume as a marker of disease progression, rapamycin-based regimens
632 showed significantly reduced cystic kidney volumes compared to alternative treatments [157,
633 162, 163]. Clinical trials using rapamycin to treat ADPKD have however produced varied
634 results [164-166], though may have been impeded by small sample size, reliance on poor
635 markers of clinical progression, short follow up time for such a slow-progressing disease,
636 and insufficient rapamycin doses [167].

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637 *2. 7.2. Diabetic neuropathy*

638 High doses of rapamycin used for immunosuppression in renal transplantation and
639 cancer are associated with type II diabetes [25]. However, there is some evidence that low
640 doses of rapamycin may have therapeutic benefit in treatment of diabetic nephropathy (DN),
641 one of the major complications of both type I and II diabetes [168] that currently has very
642 limited treatment options.
643 In diabetes, hyperglycaemia increases mTOR activity through activation of Akt and
644 inhibition of AMPK, which has consequences for development of podocytes, critical in
645 production of the renal filtration barrier. Experimentally increasing mTORC1 activity in
646 mouse podocytes induced DN phenotypes, podocyte loss and mis-localization of Nephrin, a
647 cell surface protein important in production of the renal filtration barrier [169], while reduced
648 mTORC1 activity prevented DN progression [169]. Rapamycin and everolimus treatment
649 has also shown therapeutic benefit for DN in other models including rats with STZ-induced
650 diabetes [170] [171] [172] [173] [174]. Some caution is required, however, as mTORC1
651 activity appears to protect diabetic livers from steatosis [175], though active mTORC2
652 promotes steatosis through induction of fatty acid and lipid synthesis [176], hence any
653 treatment with mTORC inhibitors in diabetic patients must include close monitoring of a
654 number of biomarkers for liver and kidney function as well as glucose homeostasis.

655 *2.8. Age-related cancer*

656 Consistent with its role as a central regulator of cell growth, proliferation and
657 angiogenesis, many oncogenic mutations activate mTOR signalling [177], meaning the
658 pathway is a key target in anti-cancer therapy. Elderly patients are particularly vulnerable to
659 tumorigenesis; their inflamed tissue microenvironment and the paracrine pro-tumorigenic
660 signalling in the SASP of accumulating senescent cells can drive progression of age-related
661 cancer. In parallel, DNA-damaging chemotherapies given to cancer patients of any age can
662 induce senescence (and the resulting SASP) in both tumorigenic and healthy collateral
663 cells. This is thought to underlie the increased occurrence of secondary tumours as a side
664 effect of chemotherapy [11, 178, 179]. Since the SASP is under the control of the mTOR
665 pathway, treating senescent cells with mTOR inhibitors can suppress the secretion of
666 inflammatory cytokines [57, 58]. Notably, rapamycin treatment can prevent stimulation of
667 prostate tumour growth by senescent fibroblasts in mice [57]. Thus, rapamycin may be
668 useful not only as an anti-cancer treatment but also as a preventative therapeutic against
669 age-related cancers or those arising after genotoxic chemo- or radio-therapy.
670 Despite promising early findings, mTOR inhibitors have not fulfilled their potential as
671 monotherapies against cancer. However, combination regimens of mTOR inhibitors
672 together with current best-in class chemotherapeutics show efficacy against a range of
673 cancers. For example, combination treatment with rapamycin and resveratrol may be
674 effective in inducing cell death in bladder cancer cells [180], with resveratrol blocking the
675 Akt activation induced by rapamycin. Similarly, rapamycin has been shown to enhance
676 mitomycin C-induced apoptosis in peritoneal carcinomatosis [181]. In combination with
677 anti-cancer agents such as trastuzumab or exemestane, mTOR inhibitors exhibit promising
678 anti-tumour activity even against aromatase inhibitor-resistant breast tumours [182].

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Rapamycin may also be beneficial in combination with radiotherapy treatment, for example inducing a significant decrease in tumour metabolic activity (assessed by PET-scan) of rectal cancers before surgical resection [183].

Currently, 461 clinical trials are listed on Clinicaltrials.gov involving use of mTOR inhibitors in cancer, in a range of tissue types including breast, cervix, prostate, ovary, pancreas, lung and colon carcinomas, various sarcomas and lymphomas, while PubMed lists 601 publications for the search terms "mTOR inhibitor cancer clinical trial". The reported outcomes are highly variable, with some suggesting markedly better outcomes (e.g. Hodgkin's lymphoma on mTOR inhibition [184, 185]), while others showed no improvement or even faster disease progression. It is likely that the variability represents both the stage and grade of cancer, and mTOR status, which should be assessed by 'personalised medicine' prior to use of mTOR inhibitors in cancer treatment, as not all will be driven by hyperactive mTOR, and even those that are may not be sensitive to rapalogue inhibition (e.g. if mutated in the FKBP12 binding site). For those tumours with activated drug-sensitive mTOR, however, mTOR inhibition can give remarkably good outcomes, with complete response to therapy reported in one patient during a Phase I trial of everolimus in combination with pazopanib [186]. Use of specific mTORC2 inhibitors has been suggested as route to overcoming the pro-survival effect of PI3K/PDK1/Akt feedback loops [187], though pan-mTOR inhibitors may be equally valuable in this context. The choice to test mTOR inhibitors in aggressive and treatment-refractory or relapsing tumours would present significant challenges to any drug therapy, as the cancers by this stage will be genetically heterogeneous and hard to treat. It is possible that earlier intervention with mTOR inhibitors, and in combination therapies, may provide more reliable anti-cancer activity. However, a major goal would instead be prevention. In this context, it is possible that use of mTOR inhibitors to intervene in other age-related disease may in fact serve a preventative role in cancer, possibly by blocking the deleterious SASP.

## 3. Perspectives

### *3.1. Balancing efficacy against side effects*

Treating otherwise healthy aging individuals with mTOR inhibitors to treat or prevent progression of age-related disease is only viable if the treatment does not induce unacceptable and undesirable side effects. The studies of immunosenescence from Mannick et al ([87] and [88] may provide critical insights into side effect profiles of low-dose mTOR inhibition in aging humans. These studies showed that everolimus was generally well tolerated, although with increased incidence of mouth ulceration. Particularly promising is the finding that the lowest dose regimens (0.5 mg daily and 5 mg weekly) proved both the most effective and the best tolerated with fewest overall adverse events per cohort. Hence using as low dose as possible whilst retaining efficacy is critical in minimising side effects.

High dose rapamycin (~20 ng/ml blood) used for immunosuppression after transplant or cancer treatment is associated with deleterious side effects, such as development of type II diabetes [25], though evidence from experimental models produces conflicting results. For example, two short-term studies in mice found that chronic rapamycin treatment induced deleterious metabolic side effects such as weight gain, glucose intolerance [188], and

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progression of type II diabetes [189], while a longer study showed that these effects could be transient [153]. The dose of rapamycin used may be of critical importance in determining the side effect profile; far lower doses are required for anti-ageing effects than for cancer treatment or immunosuppression and as doses decrease, so do serious adverse events. Disruption of mTORC2 may be behind the metabolic side effects of rapamycin treatment, since it is widely considered that mTORC2 primarily drives the response to insulin signalling and causes lipid biosynthesis (though note the caveats above concerning single $Akt^{S473}$ phosphorylation as a sole readout of mTORC2 activity). An alternative strategy to circumvent high dose rapalogue-induced glucose intolerance is to use in combination with anti-diabetes medicines such as metformin – another promising longevity therapeutic in its own right. Indeed, this strategy has been shown highly effective in HET3 female mice treated with both rapamycin and metformin, where glucose tolerance readings were indistinguishable from control mice, though the protective effect was not seen in males [190]. Hence complex-specific mTORC inhibitors, with additional agents to counteract adverse side effects, could retain treatment efficacy over the long-term, a necessary requirement for anti-ageing medicines.

An alternative approach to minimising side effects would be to use topical application of mTOR inhibitors. This is possible in age-related diseases that occur in discrete compartments, such as OA and AMD, where injection into the affected site is possible. However, as ageing affects the entire body, systemic therapies should be more effective at treating aging *per se* and hence in minimising the onset of multiple age-related diseases. mTOR inhibitors currently provide a really promising avenue for further research and development, and may promote healthy ageing by modulating the harmful aspects of senescent cells, but they should be considered in combination with other treatment approaches.

In this context, alternative anti-ageing therapies are also being developed – notably the growing field of senolytic drugs that are designed to selectively target and kill senescent cells. These agents exploit the reliance of senescent cells on survival pathways, and can induce apoptosis specifically in senescent cells, for example by inducing p53 or disrupting Bcl2. Treatment of aged mice with such treatments has been shown to rejuvenate mice and reverse several age-related pathologies (e.g. [191, 192]) and a human clinical trial for OA is currently recruiting (Clinicaltrials.gov identifier NCT03513016). However, while senolytics are indisputably exciting, it is well established that senescent cells are beneficial in various instances, such as in wound healing and regeneration. Furthermore, a recent study investigating the senescent cell burden of several tissues of old mice found that up to 14% of cells were senescent [13]. It is therefore important to investigate whether killing a significant proportion of cells in the tissues of elderly patients is safe, whether stem cells are able to refill this empty niche to restore structural and functional tissue integrity, and to assess whether wound healing and regeneration are compromised by senolytic agents. Furthermore, senescent cells from different tissues and in different contexts rely on different survival pathways to avoid apoptosis and are therefore only vulnerable to specific agents, meaning that a range of senolytics will be required to treat different ARDs. Modulation of the antagonistically pleiotropic cell senescence undoubtedly requires careful and context-dependent consideration.

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765 *3.2. Monitoring therapeutic outcomes: the need for ageing biomarkers*

766 There is an urgent need for reliable, non-invasive and quantitative biomarkers of
767 senescence and ageing to both measure disease susceptibility or progression, and promptly
768 monitor the outcome of any intervention. It is highly likely that single factors will not be able
769 to adequately reflect the panoply of changes associated with ageing and that instead a panel
770 of biomarkers will be required to account for the multi-factorial and complex nature of
771 pathological ageing. Molecular markers currently in use include telomere length analysis,
772 DNA methylation patterns and SAβGAL staining, while functional and morphological
773 markers are also available. The choice of marker may depend on the trial to be conducted –
774 for example, PET scanning for amyloid deposition may be necessary in AD trials, though a
775 recently described blood test for amyloid could substitute [193]. Notably, a number of simple
776 biochemical biomarkers selected for inclusion in UK Biobank appear to be valid for
777 assessment of age-related changes, including glycated haemoglobin, while functional
778 readouts including hand grip strength appear good measure of frailty. Clinical trials and any
779 licensed treatments may thus require the development and validation of a panel of biomarkers
780 that could be analysed in a low cost, straightforward and quick in-house procedure from
781 readily available patient material e.g. urine or blood.

782 In conclusion, ageing and age-related diseases that arise from hyperactive mTORC
783 signalling may benefit from use of mTORC inhibitors. However, any such treatment strategy
784 must consider both beneficial effects, such as those afforded by activation of autophagy and
785 improved protein synthesis quality control, as well as potential detrimental effects from
786 modifying cellular or organismal metabolism. We believe that mTORC inhibitors hold much
787 promise in the field of anti-ageing medicine, and that clinical prejudice against their use needs
788 to be overcome by careful dosage trials. To obtain maximal therapeutic benefit whilst
789 minimising side-effects, combinatorial therapies may prove useful. Overall outcomes on
790 ageing and age-related diseases require the use of a panel of robust biomarkers which should
791 provide rapid readouts of age-associated factors in a minimally invasive and cost-effective
792 format. Biochemical pathways that intersect with mTORC signalling may also provide
793 fruitful avenues for anti-ageing drug discovery.
794

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801

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802 **Abbreviations**

| | |
|---|---|
| AMD | age-related macular degeneration |
| ADPKD | adult polycystic kidney disease |
| ARD | age-related disease |
| AD | Alzheimer's disease |
| Aβ | amyloid beta |
| ATM | ataxia telangiectasia mutated |
| ATR | ATM-related |
| CR | caloric restriction |
| DN | diabetic neuropathy |
| 4EBP1 | eIF4E binding protein |
| eIF4E | eukaryotic translation initiation factor 4E |
| FKBP12 | FK506 binding protein |
| FRB | FKBP12-Rapamycin Binding (FRB) domain of mTOR |
| FDA | Food and Drug Administration |
| GS3K | glycogen synthase kinase 3 |
| HTT | huntingtin protein |
| HD | Huntington's disease |
| HIF1 | hypoxia inducible factor 1 |
| HGPS | Hutchinson Gilford progeroid syndrome |
| IKK | IkB kinase |
| IGFR | insulin-like growth factor receptor |
| IL | interleukin |
| LMNA | lamin A |
| L-DOPA | L-dopamine |
| mTOR | Mechanistic target of rapamycin |
| mTORC1/2 | mTOR complex 1 or 2 |
| OA | osteoarthritis |
| PD | Parkinson's disease |
| PI3K | **Phosphatidylinositol-4,5-bisphosphate 3-kinase** |
| PD-1 | programmed death 1 |
| Akt/PKB | protein kinase B |
| S6K | protein kinase that phosphorylates S6 ribosomal protein |
| REDD1 | regulated in development and DNA damage 1 |
| RA | rheumatoid arthritis |
| SAβGAL | senescence associated beta galactosidase |
| SASP | senescence-associated secretory phenotype |
| FK506 | tacrolimus |
| TSC1/2 | tuberous sclerosis complex 1 or 2 |
| ULK1 | Unc-51 like autophagy activating kinase |
| VEGF | vascular endothelial growth factor |

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