Lifespan extension with mTOR inhibitors rapamycin, everolimus, and temsirolimus in *Caenorhabditis elegans* (Maupas, 1899)

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Summary. A master regulator of longevity in all eukaryotes, the mechanistic target of rapamycin signalling pathway (mTOR) is thought to mediate certain effects of dietary restriction. The tumour suppressor *pRb*, whose orthologue in *Caenorhabditis elegans* is *lin-35/pRb*, is predicted to be involved in almost all human cancers. As lin-35 is linked to cancer-associated pRb function in mammals and also has a tumour suppressor effect by inhibiting mTOR signalling, the lin-35 was included in the study to investigate the effects of mTOR inhibitors. We showed that mTOR inhibitors extended the lifespan of N2 and lin-35 *C. elegans* by reducing fertilisation efficiency and resulted in reductions in the body size of worms. Additionally, *rsks-1/S6K* and *let-363/TOR* expressions increased in the presence of rapamycin, temsirolimus, or everolimus. The elucidation of molecular mechanisms of rapamycin and its analogues regulating health prolonging will expand their therapeutic applicability in treatment of human aging and age-related disorders.

Key words: ageing, *Caenorhabditis elegans*, cancer, fertilisation, *lin-35*, longevity.

The best validated antiaging interventions have been associated with two major nutrient sensing pathways, the insulin/IGF signalling (IIS) and rapamycin target (TOR) pathways. Major changes in any of these signalling pathways cause serious adverse effects, such as diabetes, cancer and embryonic death, while milder downregulation may be beneficial for health and longevity in many organisms (Wilson *et al*., 2021). Thus, the use of anti-aging IIS or TOR manipulations has an important consequence that can delay the progression of many aging-related diseases, such as diabetes and cancer (McCormick *et al*., 2011; Wilson *et al*., 2021). mTOR is a serine-threonine protein kinase that has been conserved throughout evolution and is present in many species, including humans and mice (Mannick & Lamming, 2023). mTOR kinase serves as the catalytic core of two different protein complexes (mTORC1 and mTORC2), each of which phosphorylates a different substrate and is made up of both shared and unique protein subunits. The substrates of this system, such as the well-known eukaryotic translation initiation factor 4E binding proteins (4E-BPs) and the ribosomal protein S6 kinase (S6K) β1 (S6K1), are phosphorylated as a result of amino acid, energy, oxygen, and growth signals activating TORC1. This process increases mRNA translation and inhibits autophagy. AKT (Protein kinase B), SGK1 (serum/glucocorticoid regulated kinase 1), and other related kinases are activated when ribosome contact and growth signals trigger the activation of TORC2 (Mannick & Lamming, 2023; Mir *et al*., 2023).

Rapamycin is the most specific and best studied inhibitor of TOR kinase available (McCormick *et al*., 2011). In addition, rapamycin analogues (e.g., temsirolimus and everolimus), immunosuppressive drugs approved for use in humans, are currently under clinical trials for use as anticancer agents (Ali *et al.*, 2022). Currently, there are many clinical studies on the effects of compounds that inhibit mTORC1 activity, such as sirolimus, temsirolimus, and everolimus, on various types of malignancies, alone or in combination with other compounds (Makrakis *et al*., 2023; Morken *et al*., 2023; Nelson *et al.,* 2023; Ness *et al*., 2023; Ryan *et al*., 2023; Thompson *et al*., 2023; Xiao *et al*., 2023). There is evidence that inhibition of TOR signalling extends

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lifespan in many model organisms, from yeast to mice (Vellai *et al*., 2003; Kaeberlein *et al*., 2005; Hansen *et al*., 2007; Medvedik *et al*., 2007; Syntichaki *et al*., 2007; Harrison *et al*., 2009; Selman *et al*., 2009; Bjedov *et al*., 2010; McQuary *et al*., 2016).

The genetically compatible organism, *Caenorhabditis elegans*, has been an outstanding model system for new research in aging, metabolic regulation, animal development and neuronal function. The nematode system provides unique possibilities for learning about the functioning of TOR complexes, as the relationships between these physiological functions and nutrient availability reveal many unexplained biological facts about various diseases in which TOR functions play an important role. In particular, research on *C. elegans* still continues to make important contributions to the understanding of TOR mechanisms by leading research on how to increase lifespan by reducing TOR activity (Blackwell *et al*., 2019). Consequently, understanding the involvement of the TOR pathway in aging may provide useful information for designing treatments for age-related disorders.

By generating and sustaining cellular quiescence, the DREAM (Dp/ Retinoblastoma (Rb)-like/ E2F/ MuvB) transcriptional repressor complex serves as a gatekeeper of the mammalian cell cycle (Goetsch *et al*., 2017). From invertebrates to mammals, the DREAM complex is preserved (Litovchick *et al*., 2007). This cell cycle quiescence pathway is mediated by the highly conserved DREAM transcriptional repressor complex, which stands for Dp, Retinoblastoma (Rb)-like, E2F, and MuvB (Litovchick *et al.*, 2007; Pilkinton *et al*., 2007; Sadasivam & DeCaprio, 2013). Only lin-35, a protein related to retinoblastoma protein (pRB), which is a component of the worm DREAM complex known as DRM, is a pocket protein found in C. elegans (Goetsch *et al*., 2017; Lu & Horvitz, 1998). The nematode *C. elegans* has proven helpful in learning how pocket proteins and the DRM protein complex function (van den Heuvel & Dyson, 2008; Goetsch *et al*., 2017). One of the most well-known roles of the DRM complex occurs during vulval development in *C. elegans*. The *C. elegans* vulva develops throughout larval development and is required for egg-laying and copulation with males in adult hermaphrodites (Sternberg, 2005). In addition, another important mechanism by which lin-35, the *C. elegans* orthologue of the human retinoblastoma gene (Rb), plays a role is in apoptosis (Schertel & Conradt, 2007). In *C. elegans*, apoptosis occurs in somatic tissues (developmental apoptosis) (Sulston & Horvitz, 1977; Sulston *et al*., 1983) and adult hermaphrodite gonad (physiological germ cell apoptosis) (Gumienny *et al*., 1999) during embryonic and postembryonic development, and physiological germ cell apoptosis is induced by lin-35 (Schertel & Conradt, 2007). Moreover, various conditions, including oxidative, osmotic, heat shock, and starvation stresses, can induce germ cell apoptosis (Salinas *et al*., 2006). It has also been reported that hyper-phosphorylated Rb directly binds to mTORC2, but not mTORC1, and suppresses its function (Zhang *et al*., 2016). Mechanistically, Rb, but not of Rb-related pocket proteins including p107 or p130, blocks Akt's access to mTORC2 by interacting with the mTORC2-specific component Sin1, which has been reported to lead to decreased Akt activation and increased sensitivity to chemotherapeutic drugs. Therefore, inhibition of Rb phosphorylation prevents Rb-mediated mTORC2 suppression. This leads to high Akt activation in Rb-sufficient cells, which can be attenuated by Akt inhibitors, conferring resistance to chemotherapeutic drugs (Zhang *et al*., 2016). The discovery that lin-35 inhibited the expression of epidermal growth factor (EGF) family member lin-3 showed that paracrine growth factor signalling suppression may be a mechanism through which Rb family members decrease cell development and hence operate to prevent cancer (Cui *et al*., 2006; Polley & Fay, 2012). Therefore, it has been suggested that analysis of lin-35 functions could provide valuable insights into novel mechanisms of tumour suppression by the Rb family (Bender *et al*., 2007). Finally, studies in non-mammalian systems such as *C. elegans* have identified a number of extra-cell cycle and developmental functions for the E2F-pRb network; some of these have been shown to be linked to proposed cancer-associated pRb functions in mammals (Polley & Fay, 2012). As lin-35 is linked to cancer-associated pRb function in mammals and also has a tumour suppressor effect by inhibiting mTOR signalling, the lin-35 mutant strain was included in the study to investigate the effects of mTOR inhibitors. It was aimed to elucidate the effect of mTOR inhibition on worms and the underlying mechanism by applying an inhibitor to the lin-35 strain, which is an orthologue of Rb and was among the cancer-associated *C. elegans* strains previously mentioned by Kirienko *et al*. (2010). Therefore, we aimed to reveal the effects of rapamycin and its analogues (temsirolimus and everolimus) in mutant lin-35 worms compared to wild-type N2 worms, as well as to reveal comparative differences in the expression of mTOR pathway components. Our research offers a fresh

viewpoint on mammalian life extension, which could inspire the development of fresh methods for extending human lifespans.

MATERIAL AND METHODS

***C. elegans* and *Escherichia coli* Strains**. Strains used in this study was Wild-type (N2 Bristol) and lin-35 mutant AWR54 (kea7[*Lin-35* p::degron::GFP::*Lin-35*]) I; ieSi57 [eft-3p::TIR1::mRuby::unc-54 3'UTR + Cbr-unc-119(+)] II. *C. elegans* worms and the OP50 strain of *E. coli* as food source of *C. elegans*, which were obtained from the Caenorhabditis Genetics Centre (CGC), University of Minnesota (Minneapolis–St. Paul, MN 55455, USA.

***C. elegans* culture.** Worms were maintained according to the method of Brenner (1974). Briefly, worms were grown at 20°C on nematode growth medium (NGM) agar [3 g NaCl, 17 g agar, and 2.5 g peptone 975 ml H_2O were added, mixed and autoclaved. After autoclaving, the mixture was cooled to 55°C, 1 ml of 1 M $CaCl_2$, 1 ml of 5 mg ml^{-1} cholesterol in ethanol, 1 ml of 1 M $MgSO_4$ and 25 ml of 1 M KPO_4 (pH 6) buffer were added]. The prepared NGM solution was dispensed into Petri dishes (60 × 15 mm) seeded with *Escherichia coli* OP50 (food). Prior to seeding, bacteria were incubated overnight at 37°C. The 'chunking' method was applied to transfer worms grown on NGM plates. According to this method, a chunk of agar was transferred from an old plate to a new plate using a sterilised scalpel or spatula. Worm stocks were transferred every 1-3 generations. Worms were controlled by visualising them under a dissecting microscope.

Obtaining axenised *C. elegans* eggs and synchronous cultures of *C. elegans* were carried out according to the method described by Stiernagle (2006), with some modifications. Four *C. elegans* stock plates containing many gravid hermaphrodites were washed with sterile H_2O by pipetting several times across the plate to loosen the eggs, and the solution containing eggs was collected in a sterile 15 ml conical centrifuge tube. 0.5 ml of 5 N NaOH and 1 ml of bleach (5% sodium hypochlorite solution) were added to this solution and mixed rapidly. This process was performed for 6 min. It was then centrifuged at 1800 *g* for 1 min to pellet the released eggs. After centrifugation, up to 0.1 mL of supernatant was removed and made up to 15 ml with sterile H_2O. After continuous shaking, it was centrifuged again at 1800 *g* for 1 minute. After centrifugation, up to 0.1 ml of supernatant was removed and the eggs in 0.1 ml of H_2O were transferred to a Petri dish containing 5 ml of M9 buffer [3 g KH_2PO_4, 6 g Na_2HPO_4, 5 g NaCl, 1 ml 1 M $MgSO_4$, H_2O to 1l] and incubated overnight at 20°C to obtain fasted L1 nematodes. After overnight incubation, M9 buffer containing L1 worms was transferred to a 15 ml conical centrifuge tube and centrifuged at 1800 *g* for 1 min. The remaining solution after centrifugation was removed. Worms were transferred to a Petri dish (60 × 15 mm) containing 5 ml of S Basal [5.85 g NaCl, 1 g K_2HPO_4, 6 g KH_2PO_4, 1 ml cholesterol (5 mg ml^{-1} in ethanol), dissolved in 1l H_2O] inoculated with concentrated *E. coli* OP50. The culture was observed by checking under a dissecting microscope. At 20°C, it took approximately 8 h to obtain mid-L1 larvae, 18 h to gain mid-L2 larvae, 25 h to obtain mid-L3 larvae, and 37 h to produce mid-L4 larvae. Synchronised well-nourished young adults were used in all experiments.

Drugs and reagents. The mTOR inhibitor rapamycin (sirolimus), temsirolimus and everolimus were purchased from Biosynth (Turkey) dissolved in dimethyl sulfoxide (DMSO) as concentrated 10^{-3}M stock solution (stored at −20°C). In drug dilutions, the 0.5% DMSO was applied with little or no modifying effect on *C. elegans* survival or physiological rates (AlOkda & Van Raamsdonk, 2022).

Fertilisation assay. For the assay, the age-matched adult worm (one worm per well for each strains) were transferred to 24-well plates containing 1 ml $well^{-1}$ S Medium [1l S Basal, 10 ml 1 M potassium citrate pH 6, 10 ml trace metals solution (1.86 g disodium EDTA, 0.69 g $FeSO_4$ •7 H_2O, 0.2 g $MnCl_2$•4 H_2O, 0.29 g $ZnSO_4$•7H_2O, 0.025 g $CuSO_4$•5 H_2O, dissolved in 1l H_2O), 3 ml 1 M $CaCl_2$, 3 ml 1 M $MgSO_4$] with 3µl of *E. coli* OP50 and increasing concentrations of rapamycin, temsirolimus or everolimus (0.01 nmol l^{-1}, 0.1 nmol l^{-1}, 1 nmol l^{-1}, 5 nmol l^{-1} and 10 nmol l^{-1}) and cultured at 20°C. Only DMSO was added to the control group. The number of eggs laid in each well for 7 days was counted under microscope. The experiments were repeated twice, and each experiment was performed in quadruplicate.

Lifespan assays. The L4 stage worms (~12 worms per well for each strains) were transferred to 24-well plates containing 1 ml $well^{-1}$ S Medium with 3 µl of *E. coli* OP50 and increasing concentrations of rapamycin, temsirolimus or everolimus (0.01 nmol l^{-1}, 0.1 nmol l^{-1}, 1 nmol l^{-1}, 5 nmol l^{-1} and 10 nmol l^{-1}), and worms were cultured at 20°C for lifespan assay. FUDR (5-Fluoro-2'-deoxyuridine) was used to prevent reproduction. Only DMSO was added to the control group. The

medium was changed every 7 days and worms counted under the microscope every other day. Animals that did not move after being gently nudged to move the plate were deemed dead. For body size analyses of worms, 0-, 7-, and 14-day photomicrographs were obtained using the Zeiss Axiovert A1 with Zeiss Digital Microscope Camera (AxioCam ICc 5). Body length was determined using ImageJ software. Data were analysed using GraphPad Prism. The experiments were repeated twice, and each experiment was performed in duplicate.

RNA isolation and cDNA synthesis. After synchronisation, approximately 3000 L4 stage worms from two different generations of N2 and lin-35 were transferred to 24-well plates containing 1 ml well^{-1} S Medium with 3µL of *E. coli* OP50 and 10 nM of rapamycin, temsirolimus, or everolimus and cultured at 20°C for 7 days. After 7 days of incubation with the rapamycin, temsirolimus or everolimus, 3000 worms in each well collected and transferred to a 15 mL conical centrifuge tube, 3 ml of M9 buffer was added to them and incubated again on ice for 15 min. Worms kept on ice were centrifuged at 4800 *g* for 5 min at 4°C. The supernatant was removed until 1 ml remains after centrifugation and top up to 15 ml with DNase/RNase-free water and incubated on ice again for 15 min. It was then centrifuged at 4800 *g* for 5 min. All supernatant was removed until only pellets remained. 1 ml of the homogenisation buffer (HT buffer) included in the RNA Isolation kit was added to the pellet and the worms RNA were extracted by sonication (Bhaskaran *et al*., 2011) for 2 min at amplitude 9 (BANDELIN Sonopuls HD 2070). After sonication, the homogenate was incubated on ice for 5 min and the entire homogenate was transferred to a 2 ml centrifuge tube. After these stages, total RNA was isolated using the RNA Isolation kit (HibriGen Biotechnology, Istanbul, Turkey, Total RNA Isolation kit; Cat. No: MG-RNA-01-100) according to the manufacturer's instructions. The cDNA synthesis has been performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA; Cat. No: 1708891) for obtaining cDNA product with 500 ng µl^{-1} of RNA according to manufacturer's recommendations. The cDNA that was produced was either immediately examined by qPCR or was kept at +4 °C for later use.

Quantitative real-time PCR. Expression in N2 and *lin-35* at L4 stage worm of components of the mTOR system (*rsks-1*, and *let-363*) mRNA was evaluated by RT-qPCR. Quantitative PCR was performed with the SYBR green assay and the StepOnePlus^{TM} Real Time PCR system (Applied Biosystems, USA) according to the manufacturer's instructions for real-time amplifications. The assay was performed using 10 µL of BlasTaqTM 2X qPCR Master Mix (Abm, Vancouver, Canada), 0.5 µl of each primer and 2 µl of cDNA template in a total reaction volume of 20 µL. Table 1 provides primer details; 96-well plates were used for all reactions. PCR was performed using the real-time instrument according to the manufacturer's protocol. Typically, 3 min of initial denaturation at 95°C followed by 40 cycles of denaturation at 95°C for 10 s, 30 s annealing at 60°C, and 15 s extension at 72°C were followed. A cycle of dissociation steps (58°C for 1 min followed by 0.5°C for 10 s up to 95°C) was added for melting curve analysis. A melting curve was produced at the expiration of each PCR experiment to validate the presence of a single peak and rule out the synthesis of primer dimers and non-specific products. Two biological samples were taken for each gene, and each sample was measured twice. *act-1* and *cdc-42* were used to normalise mRNA levels using the method as previously described (Vandesompele *et al*., 2002). The relative expression of genes was calculated using the comparative threshold method, $2^{-\Delta Ct}$. Primers

Table 1. Primer sequences and gene information for RT-qPCR.

Gene symbol	Gene description	Sequence name	Function	Primer sequence	Reference
act-1	Actin-1	T04C12.6	Cytoskeletal structural protein	F: 5'-CTCTTGCCCCATCAACCATG-3' R: 5'-CTTGCTTGGAGATCCACATC-3'	(Hoogewijs *et al*., 2008)
cdc-42	Cell division control protein 42 homolog	R07G3.1	RHO GTPase	F: 5'-CTGCTGGACAGGAAGATTACG-3' R: 5'-CTCGGACATTCTCGAATGAAG-3'	(Hoogewijs *et al*., 2008)
rsks-1	Ribosomal protein S6 kinase beta	Y47D3A.16	ribosomal protein S6 kinase activity	F: 5′-CCGTTTGTGGGATTCACC-3′ R: 5′-TGGCTTTCTCGGGCTCTT-3′	(Zhuang *et al.,* 2016)
let-363	Target of rapamycin homolog	B0261.2	protein serine/threonine kinase activity	F: 5'-GCCACTCTCTGATTACCCTGT-3' R: 5'-GTGAGCCGCGTGTTTCAAAT-3'	(Chalorak *et al.*, 2020)

were purchased from BMLabosis (Ankara, Turkey). Ct values >35 did not yield consistent results and were considered below the detection limit of the assay.

Statistical analysis. All experiments were repeated at least twice. The outcomes of the repeated experiments were comparable. The statistical software GraphPad Prisim 6.0 (GraphPad software, San Diego, CA) was utilised for the analysis. For survival, log-rank tests were used. A one-way ANOVA test was used to compare statistical results between groups. When significant differences were discovered, the Newman-Keuls test was used to compare groups. $P < 0.05$ was considered statistically significant in all analyses. The data are shown as mean ± SEM (Standard Error of the Mean).

RESULTS

Effects of mTOR inhibitors on *C. elegans* fertilisation. Our results showed that rapamycin, temsirolimus and everolimus at 0.01, 0.1, 1, 5 and 10 nM concentrations exerted a significant inhibitory effect on the number of eggs laid by *C. elegans* N2 (Fig. 1A, C and E) and lin-35 (Fig. 1B, D and F) worms. It was observed that the number of

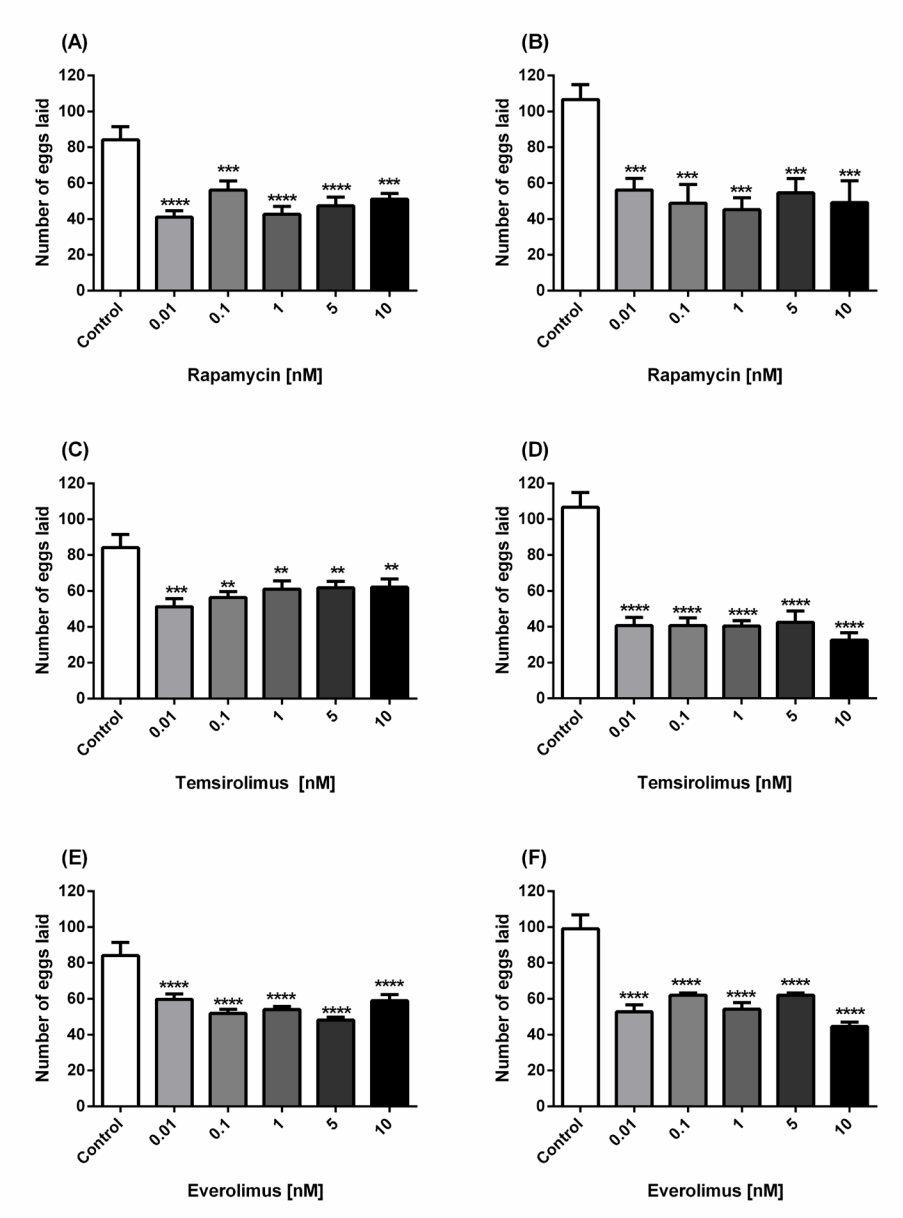

Fig. 1. The effect of mTOR inhibitors (rapamycin, temsirolimus and everolimus) on egg-laying in N2 (Panel A, C and E) and lin-35 (Panel B, D and F) *Caenorhabditis elegans*.

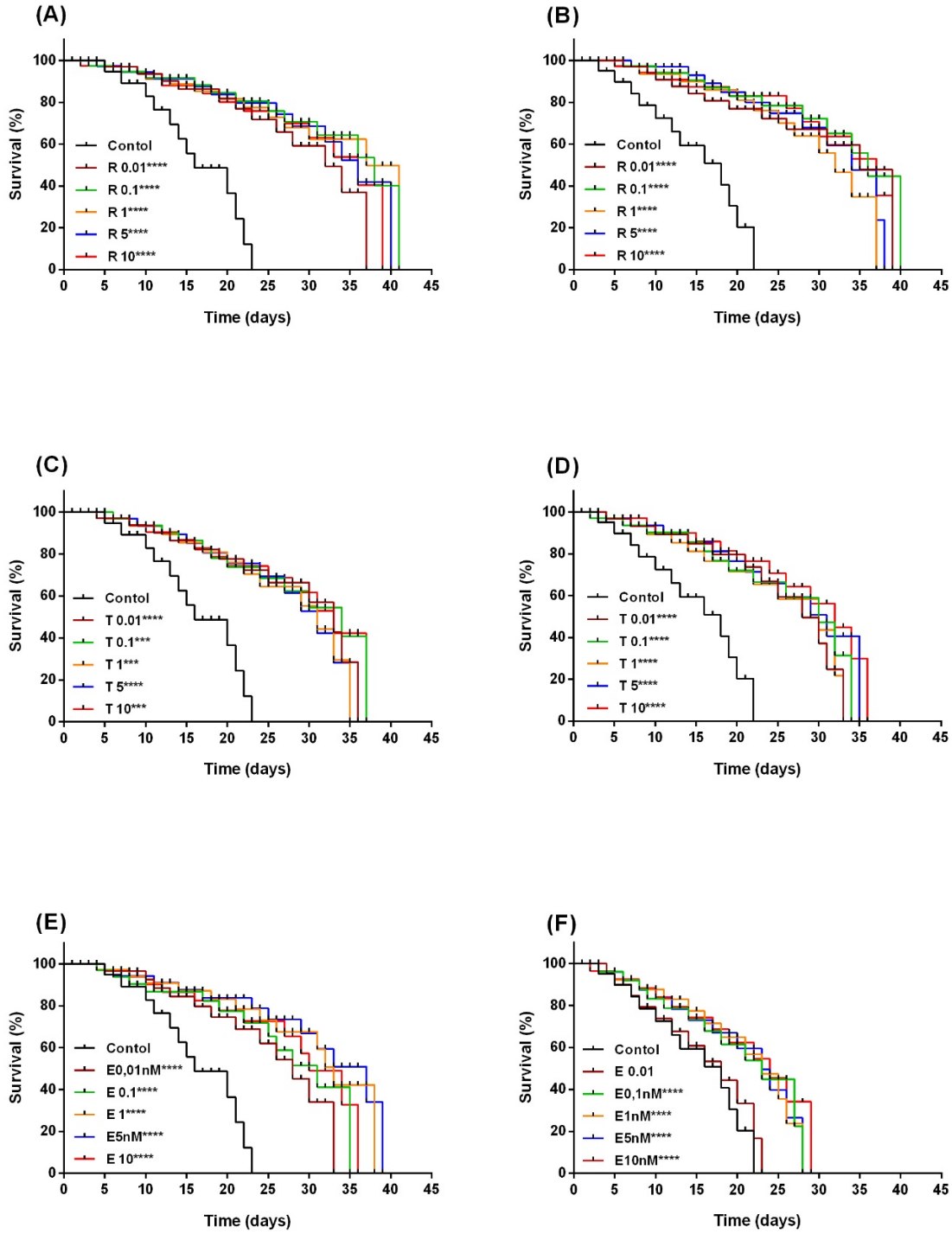

Fig. 2. Lifespan survival curves of N2 (Panel A, C and E) and lin-35 (Panel B, D and F) *Caenorhabditis elegans* in the presence of mTOR inhibitors. The experiments were carried out on synchronised populations. The synchronised populations of wild-type and mutant *C. elegans* were transplanted to 24-well plates containing liquid media with mTOR inhibitors (0.01-10 nM). Significance relates to a comparison of lifespan in *C. elegans* exposed to mTOR inhibitors and control results (worms exposed to DMSO vehicle). Labels denote the drug name, followed by the concentration of drug used (R: Rapamycin, T: Temsirolimus, E: Everolimus). Values represent the mean ± SEM of two measurements from two independent experiments. ***$P < 0.001$, ****$P < 0.0001$.

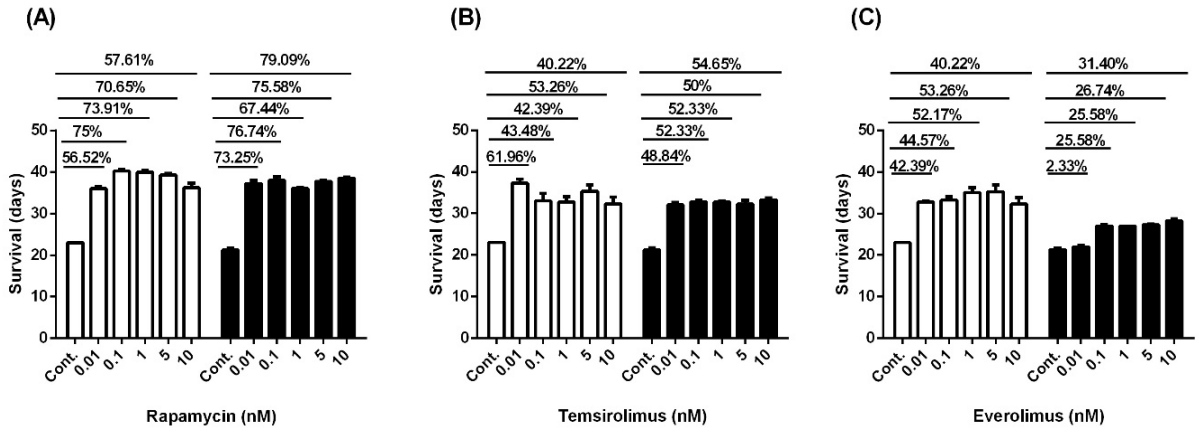

Fig. 3. Graph showing the % increase in lifespan of N2 (white bars) and lin-35 (black bars) *Caenorhabditis elegans* in the presence of rapamycin (A), temsirolimus (B) and everolimus (C) compared to control.

eggs laid was significantly reduced compared to the control group at all concentrations of mTOR inhibitors applied to wild type N2 and mutant lin-35 worms. In addition, when the effects of rapamycin and temsirolimus on the number of eggs were compared between N2 and lin-35 worms, it was seen that rapamycin and temsirolimus had more inhibitory effects on lin-35 worms. In the presence of the highest applied concentration of temsirolimus and everolimus (10 nM), the lowest egg count was obtained in lin-35 worms (Fig. 1D, F). When the control groups of N2 and lin-35 worms were compared among themselves, the number of lin-35 worms was higher than that of N2 worms. Moreover, both strains of *C. elegans* exposed to rapamycin, temsirolimus, and everolimus caused a delay in the onset of fertility behaviour. While it normally takes 4-5 days for L4 worms to reach fertile adulthood, it took 7-10 days in the presence of these inhibitors.

The experiments were carried out on synchronised populations. Worms were assessed to be exposed to mTOR inhibitors throughout their development from egg to young adulthood for the experiment, in which exposure to mTOR inhibitors was shown at 7 days (the method by which L4 individuals attain fertile adulthood) according to the worm's developmental stage. During the egg stage, synchronised populations of wild-type and mutant *C. elegans* were transplanted to 24-well plates containing S-medium with mTOR inhibitors (0.01-10 nM). Significance relates to a comparison of egg-laying behaviour in *C. elegans* exposed to mTOR inhibitors and control results (worms exposed to DMSO vehicle). The number of eggs laid by each worm was counted every day for 7 days. Values represent the mean ±SEM of two independent experiments in quadruplicate. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; n = 1.

Screening of mTOR inhibitors for lifespan effects of *C. elegans.* We verified the effects on worm lifespan of rapamycin, temsirolimus, and everolimus. Rapamycin at 0.01, 0.1, 1, 5 and 10 nM increased mean lifespan of N2 worms by 56.52%, 75%, 73.91%, 70.65% and 57.61% (Figs 2A, 3A; white bars), whereas in lin-35 worms' lifespan was increased by 73.25%, 76.74%, 67.44%, 75.58% and 79.09%, respectively (Figs 2B, 3A; black bars). Temsirolimus at 0.01, 0.1, 1, 5 and 10 nM also increased lifespan, by 61.96%, 43.48%, 42.39%, 53.26% and 54.65% in N2 worms, respectively (Figs 2C, 3B, white bars). On the other hand, in lin-35 worms temsirolimus at 0.01, 0.1, 1, 5 and 10 nM increased lifespan by 48.84%, 52.33%, 52.33%, 50% and 54.65%, respectively (Fig. 2D, 3B; black bars). Everolimus at 0.01, 0.1, 1, 5, and 10 nM increased survival in N2 worms by 42.39%, 44.57%, 52.17%, 53.26%, and 40.22% (Figs 3E, 4C; white bars), while everolimus at 0.01, 0.1, 1.5, and 10 nM increased the lifespan of lin-35 worms by 2.33%, 25.58%, 25.58%, 26.74%, and 31.40% (Figs 2F, 3C, black bars), respectively. As expected, it was observed that the extension of the lifespan of the worms was concentration-independent. In other words, as the concentration of the inhibitors increased, the lifespan did not increase in parallel (Figs 2, 3).

Body size measurement. To investigate the effect of the mTOR inhibitors, rapamycin, temsirolimus and everolimus at 0.01, 0.1, 1, 5 and 10 nM after 14 days, on the body size of *C. elegans,* we measured the body sizes of the mutant lin-35 and wild-type N2 worms (Fig. 4). Body length of wild-type N2 worms were significantly shortened in length

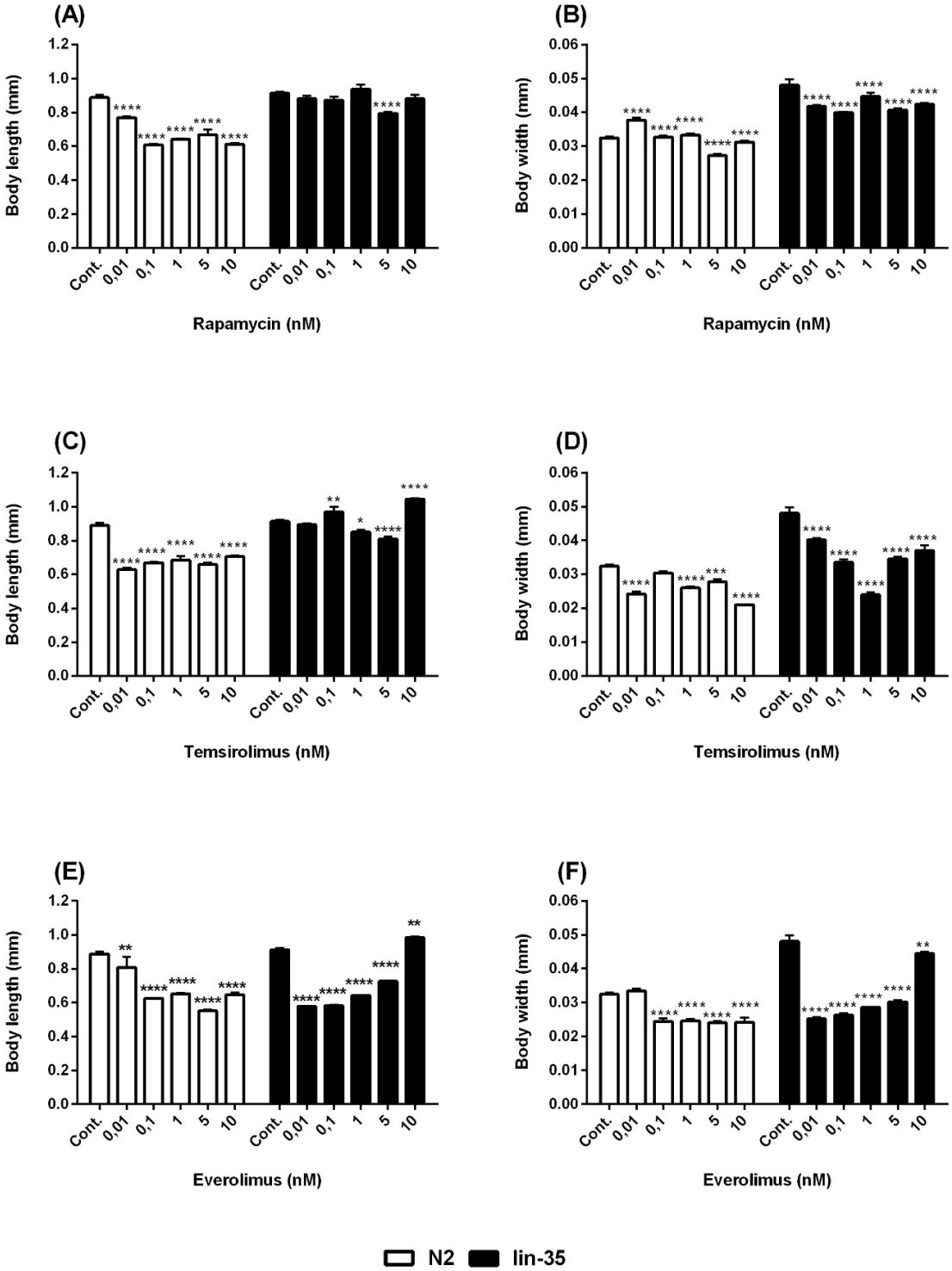

Fig. 4. Body size of wild-type N2 and mutant lin-35 strains of *Caenorhabditis elegans* grown in S-medium including different concentrations of rapamycin, temsirolimus or everolimus (A, C and E). Body length of N2 (white bars) and lin-35 worms (black bars) grown in S-medium including rapamycin, temsirolimus and everolimus compared with control (B, D and F). Body width of N2 (white bars) and lin-35 worms (black bars) grown in S-medium including rapamycin, temsirolimus and everolimus compared with control. Body length and width size derived N2 and lin-35 worms after 14 days is in mm using ImageJ software. Values represent the mean ± SEM of two independent experiments in duplicate. ****$P < 0.0001$, ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$.

as a result of rapamycin (Fig. 4A; black bars), temsirolimus (Fig. 4C; black bars) or everolimus (Fig. 4E; black bars) treatment at all concentrations (0.01, 0.1, 1, 5, and 10 nM) compared to control. When the body lengths of mutant type lin-35 worms were examined, it was observed that the body lengths of the animals exposed to everolimus at all concentrations (0.01, 0.1, 1, 5, and 10 nM) were significantly shortened (Fig. 4E; grey bars). On the other hand, significant shortening was observed in lin-35 worms exposed to 5 nM and 0.1 nM rapamycin and temsirolimus, respectively (Fig. 4A, C; grey bars).

Body width of wild-type N2 worms decreased after treatment with 5 nM and 10nM rapamycin (Fig. 4B; black bars), 0.01 nM, 1 nM, 5 nM and 10 nM temsirolimus (Fig. 4D; black bars), and 0.1nM, 1nM, 5nM, and 10 nM everolimus (Fig. 4F, black bars). On the other hand, a width reduction in lin-35 worms was observed at all rapamycin (Fig. 4B; grey bars), temsirolimus (Fig. 4D; grey bars), and everolimus (Fig. 4F; grey bars) concentrations.

mRNA expression of components of the mTOR system in *C. elegans.* Compared with control, *let-363* mRNA expression was significantly increased in lin-35 worms cultured with rapamycin, temsirolimus, or everolimus, whereas there was a significant increase in N2 worms when cultured with everolimus alone (Fig. 5). In the presence of these three inhibitors, no significant difference was observed in *rsks-1* mRNA expression in N2 worms compared to control groups, while a significant increase in *rsks-1* mRNA expression was observed in lin-35 worms (Fig. 5). When we compared the mRNA expression differences between N2 and lin-35 worms, a significant difference in *let-363* mRNA expression was observed between N2 and lin-35 worms cultured with rapamycin or temsirolimus, while a significant difference in *rsks-1* mRNA expression was observed between N2 and lin-35 worms only in the presence of everolimus (Fig. 5).

DISCUSSION

The purpose of this study was to investigate the antiaging potential of a pharmaceutical intervention to inhibit TOR signalling in *C. elegans* and to pinpoint the underlying mechanisms. To our knowledge, there are no previous studies demonstrating the antiaging effects of a pharmacological intervention, specifically rapamycin analogues (temsirolimus and everolimus) to reduce TOR signalling, in *C. elegans* and subsequently evaluating the expression of mTOR pathway components. For this purpose, we investigated the effects of rapamycin and rapamycin analogues on fertilisation, lifespan, and body size in N2 and lin-35 worms *in vivo*. We also evaluated differential mRNA expression of mTOR system components in these worms. Furthermore, in light of the fact that lin-35 in *C. elegans* is associated with cancer-related pRb function in mammals and has a tumour suppressor effect by inhibiting TOR signalling, the results of this study will provide a new perspective on mammalian life extension through the *C. elegans* model organism.

For this purpose, we first examined the effects of mTOR inhibitors on fertilisation. The self-fertile hermaphrodites of *C. elegans* produce oocytes after storing sperm in the spematheca. Hermaphrodites store fertilised eggs in their uterus during the first day of the L4/adult moult; a young adult hermaphrodite will typically have 10–15 eggs stored there at any given time (Schafer, 2005). Every day, eggs were counted while the hermaphrodite was transferred to a new plate to make counting the totals easier the following day. Usually, animals are moved to fresh plates until no more eggs are laid. According to the result of our fertilisation analysis, mTOR inhibitors (rapamycin, temsirolimus and everolimus), at concentrations of 0.01, 0.1, 1, 5 and 10 nM, was shown to have a significant inhibitory effect on the number of eggs laid by both N2 and lin-35 worms. We also observed the lowest egg count in lin-35 worms treated with 10 nM temsirolimus or everolimus.

Based on the longevity analysis results, both wild-type N2 and mutant lin-35 worms extended their lifespan in the presence of all concentrations of these three mTOR inhibitors. Moreover, we found that the lin-35 worms had a greater prolongation of lifespan compared to N2 worms after treatment of rapamycin, temsirolimus, or everolimus. As a result, mTOR inhibitors extended the lifespan of N2 and lin-35 worms by reducing fertilisation efficiency. A study by Robida-Stubbs *et al.* (2012) showed that exposure to 100 μM (100000 nM) rapamycin, a much higher concentration than used in cultured cells, extended the lifespan of worms. However, as in many studies in the literature, drug effectiveness was investigated by culturing worms on NGM agar in this study. The reason for this is the high bioavailability of drugs for worms. Since the high effectiveness of bioavailability in drug applications on NGM agar has always been questionable, it has been recommended to apply drugs at high concentrations to ensure effectiveness of bioavailability (Kokel *et al*., 2006). In this study, in order not to raise any doubts about bioavailability, worms were exposed to mTOR inhibitors in liquid culture medium because the drugs spread more

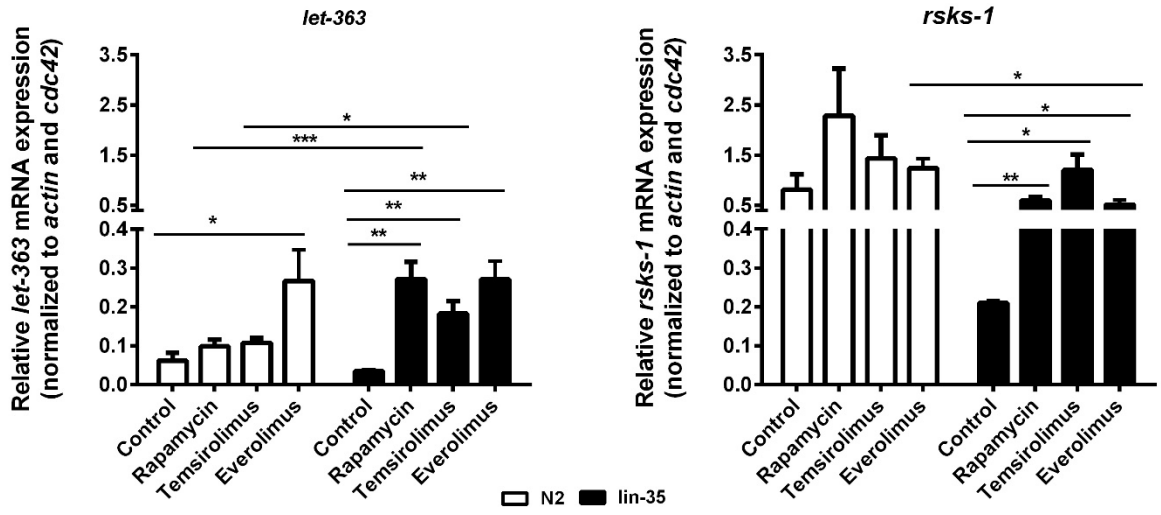

Fig. 5. *let-363* and *rsks-1* mRNA expression in N2 (white bars) and lin-35 (black bars) of *Caenorhabditis elegans*. *let-363/TOR* and rsks-1/S6K expression was measured relative to the levels of the house-keeping genes *cdc-42* and *act-1*. Values represent the mean ± SEM of two measurements from two independent experiments. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

homogeneously in liquid culture medium and bioavailability by worms is high in liquid conditions. Therefore, in many cancer studies related mTOR signalling in the literature (De Martino *et al*., 2012; Fagone *et al*., 2013), optimum drug concentration ranges applied under cell culture conditions have been tested. Our findings showed that the lifespan of worms was also extended at mTOR inhibitor concentrations applied in cell culture. In another study by Schinaman *et al.* (2019), 10 µg ml^{-1} (11000 nM) rapamycin was applied to *Drosophila* flies and it was shown that drugs extended *Drosophila* lifespan. Bjedov *et al.* (2010) applied 1×10^6, 50×10^6, 200×10^6, and 400×10^6 nM concentrations of rapamycin to *Drosophila* flies and the lifespan of the flies was prolonged in the three highest concentrations. Additionally, rapamycin treatment in yeast has previously been shown to prolong chronological (Powers *et al*., 2006) and replicative (Medvedik *et al.*, 2007) survival. A study with mice showed that when rapamycin was administered late in life, it also prolonged the lifespan in mice (Harrison *et al*., 2009). In another recent study by Shindyapina and colleagues (2022), rapamycin treatment was applied to rats for the first 45 days of their lives and it was found that the rats grew slower and remained smaller compared with control group. In the same study, similar to the findings on rats, it was also observed that the lifespan of *Daphnia magna* exposed to rapamycin during development was significantly prolonged and their body size was reduced (Shindyapina *et al*., 2022). These findings show that the antiaging properties of rapamycin are evolutionarily conserved; together with our finding that rapamycin treatment increases lifespan in *C. elegans*. Additionally, a growing amount of evidence indicates that shortened lifespan and decreased fecundity can be uncoupled (Partridge *et al*., 2005; Grandison *et al*., 2009). According to our findings, egg production decreased and lifespan increased in both worm strains when exposed to mTOR inhibitors. According to the results of the body size analysis of the worms, significant reductions in length and width were observed in wild type N2 and mutant type lin-35 worms exposed to rapamycin, temsirolimus, or everolimus.

The first major nutrient sensing pathways closely related to the most effective antiaging treatments that have been best characterised and approved to date are the insulin/IGF signalling (IIS) and target of rapamycin (TOR) pathways (Stein & Murphy, 2012). The IIS pathway includes among the main components the gene expression regulatory factors FoxOs, whose localisation in the cytoplasm or nucleus depends on nutrient conditions (Picca *et al*., 2017). In nematodes, insulin-like peptides bind to daf-2 and activate intracellular signalling. Daf-2 signalling results in phosphorylation of age-1, pdk-1, and akt-1/2 and inactivates DAF-16/FOXOs by retaining the protein in the cytosol. As with nutrient deficiency, in the absence of daf-2, age-1, and akt-

1/2, daf-16 migrates to the nucleus and induces expression of longevity-associated genes. On the other hand, the IGF-1 signalling pathway is activated by nutrient abundance and induces sequential protein synthesis and activation of TOR and ribosomal protein 6 kinase (S6K) while inhibiting FoxO-mediated transcription (Murphy & Hu, 2013). The TOR orthologue in *C. elegans*, let-363, is a serine/threonine kinase, but no protein has been identified as a direct phosphorylation target of TORC1 or TORC2 for nearly all of the TOR-regulated processes in *C. elegans* (Blackwell *et al*., 2019). S6K is a downstream target of mTOR and rapamycin, and *in vitro* studies in mammalian cell culture and other model organisms have revealed that S6K is a direct phosphorylation target of mTORC1. Decreased translation of S6K protein is the result of mTOR signalling inhibition. Since reducing the expression of the S6K homologue in *C. elegans* (*rsks-1*) alone is sufficient to extend lifespan, this mechanism likely contributes to lifespan extension through mTOR inhibition. Reduced *S6K/rsks-1* transcription and deletion mutations have been shown to extend the lifespan of *C. elegans* (Blackwell *et al*., 2019).

According to our mRNA expression analysis results, an increase in *let-363/TOR* and *rsks-1/S6K* mRNA expression was observed in wild-type and mutant worms exposed to all three inhibitors compared to the control groups. On the other hand, when wild-type and mutant worms exposed to all three inhibitors were compared among themselves, an increase in *let-363/TOR* mRNA expression was observed in lin-35 worms compared to N2 worms in the presence of rapamycin or temsirolimus, whereas a decrease in *rsks-1/S6K* mRNA expression was observed in lin-35 worms exposed only to everolimus compared to N2 worms. The longer survival of everolimus-treated mutant individuals compared to wild-type animals may be due to the decrease in *rsks-1/S6K* mRNA expression.

In *C. elegans*, downregulation of the TOR, ribosomal proteins, *S6K*, or *elF* genes also decreases fertility, and reduced TOR signalling can shorten lifespan of mutants (Hansen *et al*., 2007). TOR inhibition, rapamycin, and dietary restriction all appear to prolong longevity through lowering mRNA translation (Stanfel *et al.*, 2009; Zid *et al*., 2009; Bjedov *et al*., 2010; Kapahi *et al*., 2010; Kenyon, 2010). A lower level of translation might be advantageous since it eases the burden of protein synthesis, but current research indicates that when translation is decreased, protective mechanisms are engaged, maintaining or even enhancing the translation of some genes (Stanfel *et al*., 2009; Zid *et al*., 2009; Kapahi *et al*., 2010). Genetically modifying translation initiation lengthens longevity in *C. elegans*, and some research contends that this impact depends on conserved transcription factors like *DAF-16/FoxO* and *SKN-11/Nrf* (Henderson *et al*., 2006; Hansen *et al*., 2007; Tohyama *et al*., 2008; Wang *et al*., 2010; Rogers *et al*., 2011). This implies that the advantages of decreased protein synthesis might potentially include transcriptional regulation. DAF-16 and SKN-1 controls genes that keep different species alive longer and protect them from environmental, metabolic, and proteotoxic stress (Tullet *et al*., 2008; Oliveira *et al*., 2009; Kenyon, 2010; Kwon *et al*., 2010; Sykiotis & Bohmann, 2010; Li *et al*., 2011). According to our results, overall, we observed increased expression of all genes in wild-type and mutant worms compared to control groups. This situation may indicate that when worms are exposed to inhibitors, protective mechanisms come into play, causing the expression of *let-363/TOR* and *rsks-1/S6K* genes to increase. Therefore, the existence of this protective mechanism will need to be investigated by evaluating the expression of DAF-16 and SKN-1 genes.

Furthermore, in mammalian cell culture, *sgk* inhibits FoxO3 activity (Brunet *et al*., 2001), and *sgk-1* is thought to shorten the lifespan of *C. elegans* by inhibiting DAF-16/FoxO activity (Hertweck *et al*., 2004). However, some studies have shown that *sgk-1* mutations reduce the lifespan of *C. elegans*, which conflicts with the lifespan extension phenotype of *sgk-1* following RNAi knockdown. Additionally, they suggested that the mechanisms by which Akt/PKB and *sgk-1* affect lifespan, stress resistance, and FoxO transcription factor activity in *C. elegans* are different (Hertweck *et al*., 2004; Soukas *et al*., 2009; Alam *et al*., 2010; Kwon *et al*., 2010; Chen *et al*., 2013). Based on all these findings, studies on *C. elegans* aging and longevity remain complex, especially in relating these two processes to signalling pathways. In summary, reduced transcription and deletion mutation of *let-363/TOR* and *S6K/rsks-1* are known to contribute to lifespan extension through mTOR inhibition. However, as mentioned above, the fact that the increase in *sgk-1* expression extends lifespan is contrary to the generally accepted situation, and the increase in *let-363/TOR* and *S6K/rsks-1* expression may be associated with longevity, contrary to this lifespan extension phenotype observed in our results. However, we only measured mRNA and not the actual protein levels of mTOR signalling pathway components. More research is needed in this regard, as mRNA levels do not necessarily

reflect protein levels. Therefore, it will be useful to elucidate these mechanisms with molecular and biochemical experiments in the future. It would also be useful to examine the gene and protein expression levels of insulin/IGF-1-like signalling (IIS) components, which are closely associated with longevity and fertilisation.

In conclusion, we showed that mTOR inhibitors administered to worms at all concentrations reduced fertilisation capacity. Mutant worms exposed to the highest doses of temsirolimus and everolimus had a lower fertilisation capacity than wild-type animals. We also observed that in the presence of all inhibitors, both strains prolong the fertility period in their animals. N2 and lin-35 worms exposed to rapamycin, temsirolimus, and everolimus inhibitors had a prolonged lifespan, and mutant worms treated with all three mTOR inhibitors had a longer lifespan than wild-type animals. In addition, reductions in length and width were observed in all three inhibitor applications. There was an increase in *rsks-1/S6K* and *let-363/TOR* expressions in the presence of applied concentrations. However, additional studies to reveal protein expression levels may reveal potential co-treatments for a wide variety of age-related conditions to investigate the effects of rapamycin and its analogues in disease model systems, particularly in lin-35 worms with the cancer-related gene.

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Demirel Tuğba, Ülkü Özdemir Ö. and Ş. Berk. Увеличение продолжительности жизни *Caenorhabditis elegans* (Maupas, 1899) при действии mTOR ингибиторов: рапамицина, эверолимуса и темсиролимуса.

Резюме. Считается, что главный регулятор долголетия у всех эукариот, механическая мишень сигнального пути рапамицина (mTOR), опосредует определенные эффекты ограничения в питании. Супрессор опухоли pRb, ортологом которого у *Caenorhabditis elegans* является lin-35/pRb, по прогнозам, участвует почти во всех видах рака у человека. Поскольку lin-35 связан с функцией pRb, связанной с раком у млекопитающих, а также оказывает супрессорное действие на опухоль путем ингибирования передачи сигналов mTOR, lin-35 был включен в исследование для изучения эффектов ингибиторов mTOR. Мы показали, что ингибиторы mTOR продлевают продолжительность жизни N2 и lin-35 *C. elegans* за счет снижения эффективности оплодотворения и приводят к уменьшению размеров тела червей. Кроме того, экспрессия rsks-1/S6K и let-363/TOR увеличивалась в присутствии рапамицина, темсиролимуса или эверолимуса. Выяснение молекулярных механизмов действия рапамицина и его аналогов, регулирующих продление здоровья, расширит их терапевтическое применение в лечении старения человека и возрастных заболеваний.