Rapamycin fed late in life extends lifespan in genetically heterogeneous mice

Online Supplementary Material

Methods.

Mouse production, maintenance, and estimation of lifespan. Mice were produced at each of the three test sites by mating CB6F1 females with C3D2F1 males to produce a genetically heterogeneous population. Details of the methods used for health monitoring were provided previously[15]; in brief, each of the three colonies was evaluated four times each year for infectious agents, including pinworm. All such tests were negative throughout the entire study period. Each test site enrolled approximately equal numbers of 19- to 21-day-old weanlings each month over a six-month period, housing 3 males or 4 females/cage. Each site used diets that the manufacturer stated were based on the NIH-31 standard for breeding cages and the period between weaning and the initiation of experimental diets, as follows: For breeding cages, UM used Purina 5008, UT used Teklad 7912, and TJL used Purina 5K52. For weanlings prior to 120 days of age, UM used Purina 5008, UT used Teklad 7912, and TJL used Purina 5LG6. Starting when 120 days old, mice in the Control, Enalapril, and CAPE groups received Purina 5LG6 at all three sites, without additives (control group) or with the test agent. Mice in the Rapa group remained on the weanling diet until they began to receive rapamycin, in Purina 5LG6, at 600 days of age. Separate cohorts of control and rapamycin-treated mice were established in the same way one year later, again at each test site, but with rapamycin initiated at 270 days rather than at 600 days of age. Additional husbandry details, including accounts of tests for T cell subset distribution and activity administered to a subset of each group, are provided elsewhere[16,17].

Removal of mice from the longevity population. The Cohort 2005 study population, distributed almost equally among the three test sites, consisted initially of

1960 mice, of which 674 were assigned to the Control group and 317 to 328 to each of the four treatment groups. Of these, 51 mice were removed from the study because of fighting (31 mice), accidental death (such as chip implantation or cage flooding; 13 mice), or because of technical error (error in gender assignment or diet selection; 7 mice). For survival analyses, mice were treated as alive at the date of their removal from the protocol, and lost to follow-up thereafter. These censored mice were not included in calculations of median longevity**.**

Estimation of age at death (lifespan). Mice were examined at least daily for signs of ill health, and were euthanized for humane reasons if they were so severely moribund that they were considered, by an experienced technician, unlikely to survive for more than an additional 48 hrs. A mouse was considered severely moribund if it exhibited more than one of the following clinical signs: (a) inability to eat or to drink; (b) severe lethargy, as indicated by a lack of response such as a reluctance to move when gently prodded with a forceps; (c) severe balance or gait disturbance; (d) rapid weight loss over a period of one week or more; or (e) a severely ulcerated or bleeding tumor. The age at which a moribund mouse was euthanized was taken as the best available estimate of its natural lifespan. Mice found dead were also noted at each daily inspection. Bodies were fixed for later necropsy analysis.

Control and experimental diets. TestDiet, Inc. (Richmond, IN) prepared batches of Purina 5LG6 food containing each of the test substances, as well as control diet batches, at intervals of approximately 120 days, and shipped each batch of food at the same time to each of the three test sites. Enalapril was purchased from Sigma (catalogue E6888-5G) and used at 120 mg per kg food; on the assumption that the average mouse weighs 30 gm and consumes 5 gm of food/day, this dose supplies 20 mg enalapril per kg body weight/day. CAPE, caffeic acid phenethyl ester, was purchased from Cayman (Ann Arbor, MI; Catalogue 70750), and used at either of two doses: the high dose was 300 mg/kg food (50 mg/kg body weight/day), and the low dose was 30 mg/kg food (5

mg/kg body weight/day). Enalapril was tested because in aged humans and in rodent models of hypertension, obesity, diabetes, and congestive heart failure, it has been reported to improve many of these conditions. CAPE was tested because this agent has been reported to possess antioxidant, anti-inflammatory, and immunomodulatory capabilities, as well as specific toxicity to transformed and tumor cells. Lifespans of mice given enalapril or CAPE are compared with controls and those given rapamycin in Supplemental Figure 1. Rapamycin was purchased from LC Labs (Woburn, MA). The rapamycin was microencapsulated by Southwest Research Institute (San Antonio, TX), using a spinning disk atomization coating process with the enteric coating material Eudragit S100 (Röhm Pharma, Germany). This thermoplastic coating material increased the fraction of rapamycin that survived the food preparation process by 3- to 4-fold[16]. Because the coating material is water soluble only in non-acidic conditions, the encapsulated rapamycin is released in the small intestine rather than in the stomach. A pilot study showed that encapsulated rapamycin led to blood concentrations approximately 10-fold higher than achieved by equivalent doses of non-encapsulated rapamycin[16]. The encapsulated rapamycin was administered at 14 mg/kg food (2.24 mg of rapamycin per kg body weight/day).

Measurement of Rapamycin. Rapamycin was obtained from LC Laboratories (Woburn, MA). 32-desmethoxyrapamycin (32-RPM) was obtained from Sigma Chemical Company (St. Louis, MO). HPLC grade methanol and acetonitrile were purchased from Fisher (Fair Lawn, NJ). All other reagents were purchased from Sigma Chemical Company (St. Louis, MO). Milli-Q water was used for preparation of all solutions. The HPLC system consisted of a Waters 510 HPLC pump, Waters 717 autosampler, Waters 2487 UV detector, and Waters Empower chromatographic software (Waters, Milford, MA). The HPLC analytical column was a Grace Alltima C18 (4.6 x 150 mm, 5 micron) purchased from Alltech (Deerfield, IL). The mobile phase was 64% (v/v) acetonitrile, and 36% water. The flow rate of the mobile phase was

1.5 ml/min and the wavelength of absorbance was 278 nm. The temperature of the HPLC analytical column was maintained at 70°C during the chromatographic runs using an Eppendorf CH-30 column heater.

Rapamycin and 32-RPM powder were dissolved in methanol at a concentration of 1 mg/ml and stored in aliquots at -80°C. A working stock solution was prepared each day from the methanol stock solutions at a concentration of 1 μg/ml and used to spike the calibrators. Calibrator samples were prepared daily by spiking either whole blood or mouse food with stock solutions to achieve final concentrations of 0, 4, 8, 12, 24, 100, and 200 ng/ml.

Rapamycin was quantified in mouse blood using HPLC with UV detection. Briefly, 0.5 mL of calibrators and unknown samples were mixed with 75 μL of 1.0 μg/mL 32-desmethoxy rapamycin (internal standard), 1.0 mL $ZnSO_4$ (50 g/L) and 1.0 mL of acetone. The samples were vortexed vigorously for 20 sec, then centrifuged at 2600 g at 23°C temperature for 5 min (subsequent centrifugations were performed under the same conditions). Supernatants were transferred to clean test tubes, then 200 μL of 100 mM NaOH was added, followed by vortexing. Then, 2 mL of 1-chlorobutane was added and the samples were capped, vortexed (1 min), and centrifuged. The supernatants were transferred to 10 mL glass tubes and dried to residue under a stream of nitrogen at ambient temperature. The dried extracts were dissolved in 750 μL of mobile phase and then 2 mL of hexane was added to each tube. The tubes were capped, vortexed for 30 sec, and centrifuged for 2 min. The hexane layers were removed and discarded. The remaining extracts were dried under nitrogen and reconstituted in 250 μL of mobile phase, and then 200 μL of the final extracts were injected into the HPLC. The ratio of the peak area of rapamycin to that of the internal standard (response ratio) for each unknown sample was compared against a linear regression of calibrator response ratios to quantify rapamycin. The concentration of rapamycin was expressed as ng/mL whole blood.

Rapamycin content of mouse chow was verified using HPLC with UV detection. Briefly, 100 mg of chow for spiked calibrators and unknown samples were crushed with a mortar and pestle, then vortexed vigorously with 20 µL of 100 µg/mL 32-RPM (internal standard) and 0.5 mL methanol. The samples were then mechanically shaken for 10 min. Next, 0.5 mL of Millipore water was added and the samples were vortexed vigorously for 20 sec. The samples were centrifuged for 10 min and then 40 µL were injected into the HPLC. The ratio of the peak area of rapamycin to that of the internal standard (response ratio) was compared against a linear regression of calibrator response ratios at rapamycin concentrations of 0, 2, 4, 8, 10, and 20 ng/mg of food to quantify rapamycin. The concentration of rapamycin in food was expressed as ng/mg food (parts per million).

Rapamycin effectiveness. To assay for the status of an mTORC1 downstream effector, we measured phosphorylation of ribosomal protein S6 (Ser240/244), a substrate of S6 kinase 1, in visceral adipose tissue lysates in mice fed an encapsulated rapamycin diet for 420 days or a control diet with empty microcapsules. Tissues were dissected and snap frozen in liquid nitrogen for storage at -80°C, ground into powder under liquid nitrogen and dissolved in 10 volumes of buffer (50mM Tris-HCl (pH 7.5), 120 mM NaCl, 1% NP-40, 1 mM EDTA, 50 mM NaF, 40 mM 2-glycerophosphate, 0.1mM Na orthovanadate (pH 10), 1 mM benzamidine, and 1X Complete protease inhibitor cocktail (Roche). After sonication and microcentrifugation, lysates were quantified[30]. 40 µg of soluble protein from each extract was loaded on a 4–12% gradient PAGE and electrophoresed overnight at 5V. Gels were then transferred to nitrocellulose membranes (dry procedure), blocked and incubated with the primary antibodies [S6 Ribosomal Protein (5G10) Rabbit mAb cat. #2217; Phospho-S6 Ribosomal Protein (Ser235/236) Antibody cat. #2215; and Cat. #4968 Pan-Actin Antibody; Cell Signaling Technologies, Danvers MA], followed by secondary antibody [Anti-rabbit IgG, (H+L), Peroxidase Conjugated Antibody, cat. #31460 Pierce, Rockford IL] for detection by

chemiluminescence. Signal intensities for each immunoblot were captured using a Kodak Image Station, which were analysed using Kodak 1D image analysis software.

Supplementary Table S1: Details of calculation for comparison of surviving proportion of mice at the 90th percentile age.

Site	Sex	Age for 90th percentile	Group	Number alive	Number dead	Total	% Live	Youngest live mouse	p-value
TJL	F	1167	Controls	4	91	95	4.2%	1192	p = 0.0006
			Rapa	11	37	48	22.9%	1192	
UM	F	1162	Controls	2	93	95	2.1%	1187	p = 0.0001
			Rapa	13	35	48	27.1%	1147	
UT	F	1123	Controls	8	91	99	8.1%	1180	p = 0.22
			Rapa	7	41	48	14.6%	1189	
Pooled	F		Controls	14	275	289	4.8%		p < 0.0001
			Rapa	31	113	144	21.5%		
TJL	M	1088	Controls	8	118	126	6.3%	1146	p = 0.008
			Rapa	11	46	57	19.3%	1243	

UM	M	1154	Controls	9	103	112	8.0%	1161	p = 0.07
			Rapa	9	42	51	17.6%	1228	
UT	M	1112	Controls	4	115	119	3.4%	1157	p = 0.0001
			Rapa	14	46	60	23.3%	1156	
Pooled	M		Controls	21	336	357	5.9%		p < 0.0001
			Rapa	34	134	168	20.2%		

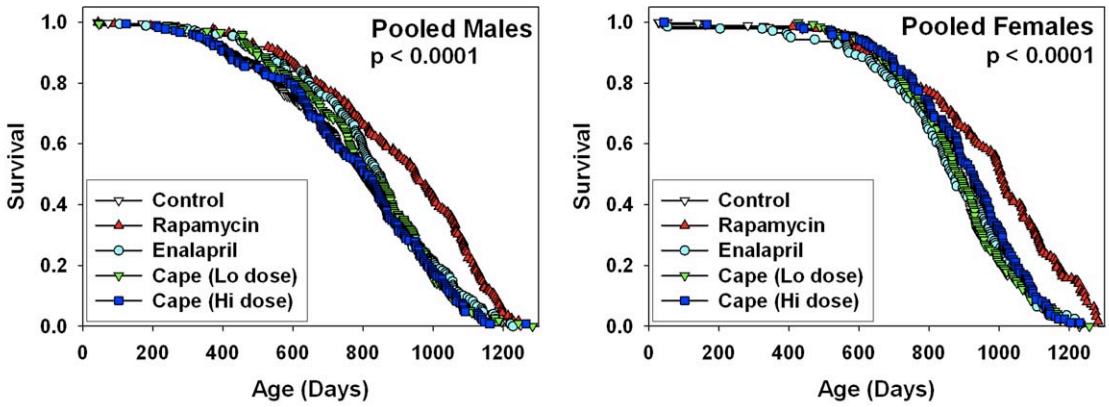
The table lists, for each combination of site, gender, and treatment group, the number of mice that were alive (and number dead) at the age (column 3) at which 90% of the joint distribution (control plus rapamycin for the site/gender combination) had died. For example, for females at TJL, 4.2% of the controls (4/95) and 22.9% of the rapamycin-treated mice (11/48) were still alive at the age of 1167 days. At the time of analysis (Feb 1, 2009), there were no live control mice at ages below the 90th percentile age in any of the groups. The was one live female, at UM, at an age below the 90th percentile threshold, but this mouse was in the rapamycin group, and its age at death would therefore not have a major effect on the statistics and p-values listed in the table.

Supplementary Table S2: Lesions in rapamycin-treated mice and in controls at the time of death.

Cause of death	Controls	Rapamycin
Abscesses	1	1
Adrenal tumor	1	
Carcinoma (GI)	1	
Carcinoma (renal)	1	
Cardiac degeneration		1
Cardiomyopathy		1
Fibrosarcoma		2
Gastric ulcer	1	
Heart failure	2	1
Heart fibrosis		1
Hemangiosarcoma	3	5
Hepatocarcinoma	3	3
Leiomyosarcoma		1
Lymphoma	10	15
Mammary adenocarcinoma		1
Myocardial infarct		1
Pleuritis	1	
Prostatitis	2	
Pulmonary tumor	4	7

Septicemia	1	
Diagnosable cases	31	40
Autolysis	17	12
Unknown	2	1
Grand Total	50	53

The mean age at death was 977 for controls (N = 31) and 1005 days for rapamycin-treated (N = 40) mice, among those animals for which a presumptive cause of death could be determined. Cause of death was inferred, where possible, based on gross evaluation, followed by histopathologic examination of a standard set of tissues from each mouse by an experienced veterinary pathologist. Tumors were deemed the cause of death based on tumor type, size, number, and distribution. Cause of death for mice with inflammatory or degenerative lesions was based on the location and severity of the lesions and the likelihood that such lesions were severe enough to cause morbidity and mortality. Many animals had small, localized tumors and various degenerative lesions, which were deemed unlikely to have contributed to their death. Autolysis precluded diagnosis in 29 cases, and the cause of death could not be determined in three other cases as indicated.



Supplementary Figure 1. Survival plots for male (left) and female (right) mice, comparing control mice to those fed enalapril, CAPE or rapamycin pooling across the three test sites. Enalapril and CAPE were added to the diet at 4 months of age, and rapamycin at 20 months. P-values were calculated by the log-rank test.