Pharmacokinetics of long-term low-dose oral rapamycin in four healthy middle-aged companion dogs

Short title: Long-term rapamycin pharmacokinetics in dogs

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Abstract

Objective

To determine the blood concentration and pharmacokinetic parameters of rapamycin in companion dogs following long-term, low-dose oral administration of rapamycin.

Animals

Four healthy, middle-aged, medium-to-large breed privately owned dogs participated.

Procedures

All dogs had been receiving oral rapamycin at a dose of 0.025 mg/kg on Monday, Wednesday, and Friday mornings for at least one month. An initial blood sample was collected prior to morning rapamycin administration, and samples were collected at 1, 2, 6, and 24 hours after rapamycin was given. Blood samples were transferred to blood spot collection cards, air-dried and stored at -80°C. Rapamycin concentrations were determined via HPLC/MS. All blood collections occurred on Wednesdays, so that the previous dose of rapamycin had taken place 48 hours prior to blood collection.

Results

For all dogs, rapamycin T_{max} was 2 hours. Median C_{max} was 1.47 ng/ml (0.912 – 2.13), and the median $AUC_{0\text{-last}}$ was 15.7 ng*hr/mL (1.30 – 36.3). Due to sample size and timing, the only estimates related to elimination rate reported are for mean residence time with a median of 4.70 hrs (0.90 – 7.30).

Conclusions and Clinical Relevance

A 0.025 mg/kg oral dose of rapamycin, administered three times a week, resulted in concentrations of rapamycin in the blood capable of being measured in ng/ml.

Abbreviations

HPLC/MS/MS – High performance liquid chromatography – mass spectroscopy

Introduction

Rapamycin (sirolimus), an FDA-approved natural macrolide that is produced by *Streptomyces hygroscopicus*, is an inhibitor of the mechanistic target of rapamycin (mTOR). As part of the phosphatidylinositol kinase-related kinase (PIKK) family, mTOR is a serine/threonine kinase that is present within the mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). [1-3] The mTORC1 pathway is crucial for cell survival and drives cell growth and protein synthesis based in part on nutrient availability. In this regard, the activity of the nutrient/stress-sensing mTORC1 is increased during periods of nutrient abundance, and is conversely inhibited when under nutrient stress, such as reduced intracellular ATP or reduced amino acid availability. [1, 4-7] A similar inhibition of mTORC1 occurs under conditions of dietary restriction or intermittent fasting. [8] With rapamycin, inhibition of mTOR is due to the binding of rapamycin to the FK506 binding protein (FKBP12), which allosterically inhibits mTORC1. [9-11] This results in several downstream consequences including reduced translation of proteins that play a key role in the G1 to S transition of the cell cycle and activation of the autophagic degradation pathway. [1, 12, 13]

In humans, oral rapamycin is often used in immunosuppressive protocols in transplant recipients to prevent organ rejection.[14-16] In some cases, due to the mTOR pathway's close association with insulin signaling, rapamycin treatment has resulted in derangements in glucose homeostasis.[17, 18] However, multiple studies have documented potential benefits of rapamycin treatment in the context of normative aging. For instance, rapamycin-mediated

inhibition of mTORC1 has been shown to reduce age-related cancers [19, 20] and age-related declines in cognitive function [21, 22], and to improve heart function [23-25], immune function [26], kidney function [27], oral health [28, 29], intestinal function and gut dysbiosis [30, 31], and ovarian function [32] in aging mice. In both dogs and mice, rapamycin therapy improves cardiac function and can potentially treat hepatic glycogen storage disease.[4, 24, 25, 33-35] Inhibition of mTOR also results in decreased smooth-muscle cell migration and proliferation within coronary arteries, with rapamycin-coated coronary stents significantly reducing arterial stenosis following stent placement.[36] Still, one of the most striking findings is that rapamycin administration has been associated with a significant increase in subject lifespan, a result observed in multiple model organisms, including yeast [37], fruit flies [38], nematodes [39], and mice.[40-46]

In humans, oral rapamycin is absorbed rapidly with peak concentrations occurring within one to three hours depending on dosing protocol.[47, 48] In the bloodstream, the vast majority of rapamycin is distributed within red blood cells, and co-administration with a high fat meal can increase the oral bioavailability and AUC by up to 35% while decreasing the maximum blood concentration.[49, 50] In dogs, Larson *et al*. investigated rapamycin pharmacokinetics following a single oral dose of 0.1 mg/kg or a five-day course of this treatment.[51] In the single-dose protocol, rapamycin concentration peaked twice, at two- and six-hours post-administration, but this pattern was not noted when the dogs were treated on five consecutive days. These low-dose rapamycin treatments resulted in peak blood concentrations similar to those that displayed anti-tumorigenic properties in mice, with 8.39 ng/ml and 5.49 ng/ml measured in the single and consecutive dose protocols, respectively. In both instances, the time to reach maximum blood concentration was approximately three to four hours.[51] A different study found that

intramuscular injections of rapamycin doses ranging from 0.01-0.08 mg/kg, either given once or daily over a seven-day treatment schedule, also resulted in measurable and clinically relevant blood concentrations of rapamycin. However, the time to maximum blood concentration in this study ranged from 2 to 48 hours, implying significantly greater variability with this route.[52] The goal of the study reported here was to determine the pharmacokinetics of oral low-dose rapamycin after long-term administration (two to six months) to healthy middle-aged dogs.

Materials and Methods

Study Participants

Seven healthy, middle-aged to senior dogs (three spayed females, four castrated males) between the ages of six to ten years old and weighing between 18.2-36.4 kg (40-80 lbs) were enrolled. All participating dogs had previously been recruited based on specified criteria into a 12-month prospective, double-blinded, placebo-controlled randomized clinical trial in which dogs received rapamycin or placebo three times per week for six months. Therefore, dogs in this ancillary study had been receiving treatment (either placebo or rapamycin) for durations ranging from one to six months based upon their date of enrollment into the clinical trial. At the time of this study, investigators remained masked to treatment assignment and collected blood samples from a total of seven dogs. Three of these dogs were later determined to have been in the placebo group, such that results from four rapamycin-treated dogs are reported here.

Drug and placebo

Both 0.5 mg tablets (Cadlia Health Care, Zydus, Ahmedabad, India) and 1.0 mg tablets (Dr. Reddy's Laboratories, Princeton, New Jersey; Greenstone LLC, Pfizer Inc.) of rapamycin were used. Rapamycin was administered to dogs at a dose of approximately 0.025 mg/kg, using 0.25 mg increments. As such, the dose of rapamycin was 0.50 mg for dogs between 18.2 and 23.0 kg (40-50 lbs), 0.75 mg for dogs between 23.1 and 30.0 kg (51-66 lbs), and 1.0 mg for dogs between 30.1 and 36.4 kg (67-80 lbs). To mask the treatment, a combination of tablets comprising the total daily dose was placed into gelatin capsules for dogs in the treatment group. Placebo capsules (Professional Compounding Centers of America; PCCA) contained lactose.

Experimental design

All dogs were fasted overnight and underwent an initial blood draw at hour 0. Approximately four ml of blood were obtained via jugular venipuncture at every time point. After the initial blood draw at 0 hour, dogs were offered their normal breakfast and given either placebo or rapamycin. Additional blood samples were obtained via the same method from each dog at 1, 2, 6, and 24 hours after treatment administration. All blood samples were promptly transferred to K-EDTA blood storage tubes. Within an hour of each time point, up to 50 μL of whole blood were pipetted from the storage tube onto two spots of a blood spot card (Whatman 903 DBS (dried blood spot) collection cards; MilliporeSigma, St. Louis, MO). Two spot cards with two spots each were prepared for each dog. The spot cards were allowed to dry at room temperature for one hour and stored at -80°C in a sealed plastic bag with a desiccant packet (0.5 gram silica gel packets, Intertek Packaging, Orchard Park NY 14127) before being shipped for analysis.

All procedures for this study were reviewed and approved by the TAMU Institutional Animal Care and Use Committee (IACUC 2018-0299 CA). Because these were client-owned animals, an IRB determination was requested, and this study was found not to be Human Subjects Research (HSR).

Analysis of Rapamycin Concentration

Measurement of Rapamycin Using HPLC/MS/MS

Rapamycin concentrations were determined via HPLC/MS/MS (Biological Psychiatry Analytical Lab [BPAL], University of Texas Health Science, San Antonio, TX). Rapamycin, ascomycin and all 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 Shimadzu SIL 20A HT autosampler, LC-20AD pumps (2), and an AB Sciex API 3200 tandem mass spectrometer with turbo ion spray. The LC analytical column was a Grace Alltima C18 (4.6 x 150 mm, 5 µm) purchased from Alltech (Deerfield, IL) and was maintained at 25^{o}C during the chromatographic runs using a Shimadzu CT-20A column oven. Mobile phase A contained 10 mM ammonium formate and 0.1% formic acid dissolved in 100% HPLC grade methanol. Mobile phase B contained 10 mM ammonium formate and 0.1% formic acid dissolved in 90% HPLC grade methanol. The flow rate of the mobile phase was 0.5 ml/min. Rapamycin was eluted with a gradient. Mobile phase gradient: 0-0.1 min, 100% B; 0.1-4 min, linear gradient to 0% B; 4.0-5.0, 0% B; 5-10 min, 100% B. The rapamycin transition was 931.6 m/z to 864.5 m/z, which was used for quantification. The internal standard (ascomycin) transition was 809.6 m/z to 756.6 m/z.

Rapamycin and ascomycin super stock solutions were prepared 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 super stock solution at a concentration of 10 µg/ml and used to spike the calibrators. Calibration samples were pipetted on spot cards, dried, and then used to quantify rapamycin in the unknown samples.

Spiked calibration samples were prepared at concentrations of 0, 1.56, 6.25, 25.0 and 100 ng/ml. Two blood spots, each containing 50 µl, were cut into four segments and placed in 10 X 50 mm polypropylene tubes with push caps. One ml of mobile phase A and 10 µl of a 0.5 µg/ml solution of ascomycin were added to each tube. The tubes were capped, vortexed vigorously for 30 sec and placed on a shaker Model E6010 from Eberbach Corporation (Ann Arbor, Michigan) for 30 min. After shaking, the tubes were centrifuged for 10 min at 5,000 g. The supernatants were carefully poured or pipetted into 10 X 75 mm glass tubes and dried to residue under a nitrogen stream. The residues were then redissolved in 100 µl of mobile phase A and transferred to autosampler vials. Then, 50 µl were injected into the HPLC/MS/MS system. The ratios of rapamycin peak areas to ascomycin peak areas of unknown samples were compared against the linear regression of the ratios of the calibration samples to quantify rapamycin. The concentration of rapamycin was expressed as ng/ml of blood.

Pharmacokinetic Analysis

Noncompartmental analysis using industry standard software (Certera Phoenix WinNonLin 8.2.0.4383, Princeton, NJ) was attempted using the blood concentrations from individual dogs to estimate pharmacokinetic parameters. Attempts were made to estimate or calculate the following parameters: T_{max}: time of maximum observed blood concentration; C_{max}:

maximum observed blood concentration; λz: terminal elimination rate; $t_{1/2\lambda z}$: apparent terminal half-life; $AUC_{0\text{-last}}$: area of the curve from time zero to last measurable time; $AUC_{0\text{-inf}}$: area under the curve from time zero extrapolated to infinite time; AUC % Extrap: area under the curve extrapolated from time zero to infinity as a percent of total AUC; $AUMC_{0\text{-obs}}$ = area under the moment curve from time zero to last observed concentration; $AUMC_{0\text{-inf}}$ = area under the moment curve from time zero extrapolated to infinity; $MRT_{0\text{-obs}}$ = mean resident time estimated using time zero to last observed concentrations, calculated as $AUMC_{0\text{-obs}}$ /$AUC_{0\text{-obs}}$; $MRT_{0\text{-inf}}$ = mean residence time estimated using time zero to infinity, calculated as $AUMC_{0\text{-inf}}$ /$AUC_{0\text{-inf}}$.

Results

Study Participants

The participant demographics are displayed in Table 1. A total of seven dogs were sampled. Of these, four dogs (three spayed females, one castrated male) were found to be receiving rapamycin after the clinical trial was unblinded. There were three purebred (Labrador Retriever, Australian Shepherd, Pit Bull Terrier) and one mixed breed dog represented. The median age of this group of dogs was 9.0 years (range: 8.8 – 11 years) and they were all medium to large breeds (median weight: 27.0 kg, range: 20.2 – 34.0 kg). As dogs had been previously enrolled at different dates into the clinical trial, the treatment period prior this this study varied. The range of treatment period prior to this study was one to five months; two of the four dogs had been receiving treatment for one month. Each dog had been prescribed a dose of rapamycin according to its weight, with one dog receiving 0.50 mg, two dogs receiving 0.75 mg, and one dog receiving 1.0 mg. While all dogs were offered breakfast at the time of medication administration, only one dog (#4) ate.

Table 1. Participant demographics.

Dog #	Age (years)	Breed	Sex	Weight (kg)	Treatment	Treatment Duration (months)
1	7.8	Border Collie	FS	19.0	Placebo	5.0
2	9.2	Labrador Retriever	MC	34.0	1.0 mg rapamycin	1.0
3	8.8	Australian Shepherd	FS	26.0	0.75 mg rapamycin	1.0
4[a]	9.8	Pit bull terrier	FS	28.0	0.75 mg rapamycin	5.0
5	10.8	Australian Shepherd	MC	26.2	Placebo	4.0
6	11.0	Mixed breed	FS	20.2	0.50 mg rapamycin	3.0
7	8.5	Border Collie	MC	29.0	Placebo	5.5

[a]Only participant who received rapamycin with a meal

Blood Rapamycin Concentrations

All four dogs had received rapamycin approximately 48 hours prior to baseline collection for this study. No dogs had detectable levels of rapamycin in their blood at baseline (time 0), just prior to their next scheduled administration of rapamycin. No placebo dogs had detectable levels

of rapamycin in their blood at any time. Following administration of rapamycin, all four treated dogs (#2, 3, 4, and 6) had measurable rapamycin blood concentrations that were detectable by HPLC/MS/MS (Table 2).

Table 2. Blood concentration of rapamycin (ng/ml) in four dogs following a 0.025 mg/kg oral dose of rapamycin.

Time (hrs)	Dog #2	Dog #3	Dog #4	Dog #6	Median
0	0	0	0	0	0
1	1.07	1.15	0.884	0.861	0.988
2	1.08	1.86	2.13	0.912	2.03
6	0.933	1.10	1.80	0	1.02
24	0	0.875	1.14	0	0.438

By one hour post-administration, rapamycin concentrations increased for all four dogs (Figure 1), with the median rapamycin concentration one hour post-administration at 0.980 ng/ml (0.861 – 1.15). At two hours post-administration, concentrations ranged from 0.912 to 2.13 ng/ml (median = 1.47). Following this two-hour time point, blood concentrations progressively decreased in all dogs, with the median rapamycin concentrations measured at six and 24 hours being 1.02 ng/ml (0.000 – 1.80) and 0.440 ng/ml (0.000 – 1.14). By six hours post-administration, dog #6 no longer had detectable rapamycin in circulation, and by 24 hours post-administration, dog #2 also no longer had detectable rapamycin in his blood.

Figure 1: Blood concentration (ng/ml) of rapamycin in four dogs receiving long-term low-dose rapamycin therapy before (0), and 1, 2, 6, and 24 hours after oral administration.

Pharmacokinetic Parameters

Estimates of pharmacokinetic parameters are presented in Table 3. For all dogs, T_{max} was 2 hours, and the median C_{max} was 1.47 ng/ml (0.91 – 2.1). Due to the sparse sampling and lack of sufficient samples for accurate nonlinear regression of the terminal phase drug concentrations, apparent elimination half-life, AUC extrapolated to infinity, and AUMC extrapolated to infinity could not be calculated. Therefore, the only estimates related to elimination rate reported are for MRT. The median MRT was 6.8 hrs (1.3 – 10). The median $AUC_{0\text{-last}}$ was 15.7 ng*hr/mL (1.30 – 36.3).

Table 3: Pharmacokinetic parameters of rapamycin in four dogs following a 0.025 mg/kg oral dose.

Dog#	Units	2	3	4	6	Median
T_{max}	hr	2	2	2	2	2
C_{max}	ng/ml	1.1	1.9	2.1	0.9	1.47
$AUC_{0\text{-last}}$	hr*ng/ml	5.6	25.8	36.3	1.3	15.7
$AUMC_{0\text{-last}}$	hr*ng* ng/ml	17.7	272.1	376.6	1.8	144.9
MRT_{last}	hr	3.1	10.6	10.4	1.3	6.8

242 T_{max}: time of maximum observed blood concentration; C_{max}: maximum observed blood
243 concentration; $AUC_{0\text{-}last}$: area of the curve from time zero to last measurable time; $AUMC_{0\text{-}last}$:
244 area under the moment curve from time zero to last measurable time; MRT_{last}: mean residence
245 time calculated from $AUMC_{0\text{-}last}$ /$AUC_{0\text{-}last}$.
246

247 # Discussion

248 The results of this study indicate that low-dose (0.025 mg/kg, three times per week for
249 one to five months) oral rapamycin, administered to healthy, middle-aged dogs, resulted in low
250 but measurable concentrations of rapamycin in the blood. Similar concentrations have resulted in
251 reduced mTOR activity in canine tumor cells.[52] Though rapamycin has previously been
252 administered to dogs in other studies, this study is unique in the duration of treatment with a
253 lower dose than previously described. Prior to this report, the longest published study of
254 rapamycin in companion dogs was 10 weeks (2.5 months), during which Urfer *et al*. employed a
255 dosing schedule of three days a week, on Monday, Wednesday, and Friday mornings. However,
256 the doses used in that study were higher (either 0.05 mg/kg or 0.1 mg/kg). Additionally, no
257 analysis of drug concentrations was performed. [33] Larson, *et al* [51] described
258 pharmacokinetic analysis of oral rapamycin in companion dogs treated with 0.1 mg/kg
259 administered either once or on five consecutive days. By comparison, the lower-dose, three-day-
260 a-week regimen utilized in this study is markedly different. This three-times-weekly dosing
261 schedule is intended to be employed in a nationwide study, with a much larger cohort of dogs
262 who will receive rapamycin for three years. Because of this, investigation into the
263 pharmacokinetics of rapamycin administration using this novel dosing schedule was warranted.
264 Not surprisingly, some of the pharmacokinetic parameters obtained from this study
265 differed from those obtained by Larson, *et al*.[51] In the current study, the median C_{max} was 1.47
266 ng/ml, with the highest rapamycin blood concentration measured being 2.13 ng/ml. By contrast,

maximum blood concentrations following a 0.1 mg/kg oral dose of rapamycin were greater, with a single oral dose or five consecutive daily doses resulting in mean concentrations of 8.39 ng/ml and 5.49 ng/ml, respectively.[51] On the other hand, despite administering rapamycin via intramuscular injections rather than orally, Paoloni *et al*. reported a similar C_{max} to this study (median: 1.69 ng/ml, range: 1.21-1.82 ng/ml) in dogs receiving a 0.02 mg/kg dose on eight consecutive days.[52] This might indicate that in dogs, maximum blood concentrations of rapamycin are more affected by the dose utilized, rather than the frequency or route of administration. The median C_{max} in this study is significantly lower than that considered therapeutic for preventing organ transplant rejection in humans, where a blood concentration of 8-15 ng/ml is often desired.[14, 53] This is intentional, as our goal was to use a dose that results in measurable blood concentrations, but not with the objective of causing immunosuppression. The T_{max} reported in our study (two hours) is more similar to that reported by Larson *et al*. (3.3 ± 2.5 hours after one dose, and 4.5 ± 1.0 hours after five consecutive doses) compared to that observed after intramuscular (IM) injection (up to 48 hours), indicating faster absorption with oral administration.[51, 52]

The median MRT was 6.8 hours. Because AUC could not be extrapolated to infinity using regression analysis, it is difficult to assess the sufficiency of sampling times. Typically, one would look at area under the curve extrapolated from time zero to infinity as a percent of total AUC to evaluate whether samples were collected for long enough after drug dosing, but there were not enough non-zero sampling times after C_{max} to regress appropriately. Due to this, $T_{1/2}$ was unable to be directly calculated, and MRT was reported.

In a previous study utilizing rapamycin, a mean half-life of 38.7 hours was found by Larson *et al.* following a single dose of 0.1 mg/kg orally. This was far lower than the $T_{1/2}$ of 99

hours observed following five consecutive 0.1 mg/kg oral doses in that same study.[51]. It should be noted that, similar to the variable $T_{1/2}$ calculated amongst drug recipients in both previous canine rapamycin pharmacokinetic studies (oral and IM administration), our MRT was not consistent between recipients. While two of the four dogs had an estimated MRT within 0.2 hours (10.4 & 10.6 hrs), the MRT of the other two dogs was significantly less (3.1 & 1.3 hrs).[51, 52] Whether the MRT calculated in this study is due to the low rapamycin dose utilized, the intermittent dosing schedule, the duration of administration, or a combination of these factors, is unknown. Additionally, because none of our dogs had measurable levels of rapamycin in their blood after 48 hours (that is, at time zero in our study), this may indicate that our dosing regimen was more akin to intermittent pulse-therapy as opposed to a long-term, continuous treatment. At this time, the benefit or significance of this is unknown.

This study is not without limitations. All four dogs utilized were already participating in a 12-month prospective study investigating rapamycin administration, and therefore, the duration of treatment prior to pharmacokinetic analysis was variable. Additionally, the sample size is small, attributable to the strict standards dogs must have met to be enrolled in the previously mentioned study, the duration of therapy, and the fact that three out of seven initially tested dogs were revealed to be receiving a placebo once the trial was unblinded. Blood samples were transferred to blood spot cards and then stored at -80°C for multiple months prior to analysis, which could possibly have impacted the reported rapamycin concentrations. However, blood spot cards are a highly stable form of storage, with some studies utilizing cards stored for over a decade.[54] In regard to the analysis, measurements below 1 ng/ml were obtained and reported, despite the fact that these measurements fall below the formal limit of detection for the assay, which is a statistical bound on confidence that the measured value is different from zero. We feel

comfortable including these low measurements because all of the dogs receiving rapamycin had positive measurement values, while none of the dogs receiving placebo had non-zero measurement values for any of the timepoints. We recognize, however, that the low measured values may contribute increased variance and higher error.

Finally, even though all dogs were offered food with their treatment administration, only one dog (#4) ate. Coincidentally, or perhaps consequently, this participant also had the highest blood levels of rapamycin and longest blood half-life. Rapamycin pharmacokinetics in humans have previously been shown to be impacted by the consumption of food, with a high-fat meal resulting in reductions in C_{max} but increases in AUC by 23 to 35%.[49] This, however, has not been investigated in dogs.[49] Given this, future investigation into whether food consumption does affect rapamycin absorption in dogs would be valuable, as this might influence the protocol of the upcoming long-term low-dose study, and other uses of rapamycin in this species, by recommending treatment administration at a specified time relative to a meal.

Conclusions

The results of this study indicate that low-dose (0.025 mg/kg) oral rapamycin, administered three times a week for one to five months to healthy, middle-aged large breed dogs, resulted in detectable concentrations of rapamycin in four dogs that was measurable in ng/ml. While the long-term benefits and ideal dosing schedule of rapamycin are still not known, this study demonstrates that low doses produce notable systemic exposure and that intermittent treatment may allow clearance of rapamycin from blood prior to the next dose.

Author Contributions

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Conflicts of Interest

The authors of this manuscript report no conflicts of interest.

References

1. Ballou LM, Lin RZ. Rapamycin and mTOR kinase inhibitors. J Chem Biol. 2008;1(1-4):27-36.

2. Sarbassov DD, Ali SM, Sengupta S, Sheen JH, Hsu PP, Bagley AF, et al. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell. 2006;22(2):159-68.

3. Sarbassov DD, Ali SM, Kim DH, Guertin DA, Latek RR, Erdjument-Bromage H, et al. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol. 2004;14(14):1296-302.

4. Zhang C, Liu A, Su G, Chen Y. Effect of rapamycin on the level of autophagy in rats with early heart failure. J Cell Biochem. 2019;120(3):4065-70.

5. Ehninger D, Neff F, Xie K. Longevity, aging and rapamycin. Cell Mol Life Sci. 2014;71(22):4325-46.

6. Kapahi P, Chen D, Rogers AN, Katewa SD, Li PW, Thomas EL, et al. With TOR, less is more: a key role for the conserved nutrient-sensing TOR pathway in aging. Cell Metab. 2010;11(6):453-65.

7. Blenis J. TOR, the Gateway to Cellular Metabolism, Cell Growth, and Disease. Cell. 2017;171(1):10-3.

8. Blagosklonny MV. Rapamycin-induced glucose intolerance: hunger or starvation diabetes. Cell Cycle. 2011;10(24):4217-24.

9. Saxton RA, Sabatini DM. mTOR Signaling in Growth, Metabolism, and Disease. Cell. 2017;168(6):960-76.

10. Martin DE, Hall MN. The expanding TOR signaling network. Current opinion in cell biology. 2005;17(2):158-66.

11. Lee MB, Carr DT, Kiflezghi MG, Zhao YT, Kim DB, Thon S, et al. A system to identify inhibitors of mTOR signaling using high-resolution growth analysis in Saccharomyces cerevisiae. Geroscience. 2017;39(4):419-28.

12. Hidalgo M, Rowinsky EK. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. Oncogene. 2000;19(56):6680-6.

13. Hashemolhosseini S, Nagamine Y, Morley SJ, Desrivieres S, Mercep L, Ferrari S. Rapamycin inhibition of the G1 to S transition is mediated by effects on cyclin D1 mRNA and protein stability. J Biol Chem. 1998;273(23):14424-9.

14. Morath C, Arns W, Schwenger V, Mehrabi A, Fonouni H, Schmidt J, et al. Sirolimus in renal transplantation. Nephrol Dial Transplant. 2007;22 Suppl 8:viii61-viii5.

15. Badve SV, Pascoe EM, Burke M, Clayton PA, Campbell SB, Hawley CM, et al. Mammalian Target of Rapamycin Inhibitors and Clinical Outcomes in Adult Kidney Transplant Recipients. Clin J Am Soc Nephrol. 2016;11(10):1845-55.

16. Johnston O, Rose CL, Webster AC, Gill JS. Sirolimus is associated with new-onset diabetes in kidney transplant recipients. J Am Soc Nephrol. 2008;19(7):1411-8.

17. Di Paolo S, Teutonico A, Leogrande D, Capobianco C, Schena PF. Chronic inhibition of mammalian target of rapamycin signaling downregulates insulin receptor substrates 1 and 2 and AKT activation: A crossroad between cancer and diabetes? J Am Soc Nephrol. 2006;17(8):2236-44.

18. Khan KH, Wong M, Rihawi K, Bodla S, Morganstein D, Banerji U, et al. Hyperglycemia and Phosphatidylinositol 3-Kinase/Protein Kinase B/Mammalian Target of Rapamycin (PI3K/AKT/mTOR) Inhibitors in Phase I Trials: Incidence, Predictive Factors, and Management. Oncologist. 2016;21(7):855-60.

19. Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk ML, et al. Rapamycin increases lifespan and inhibits spontaneous tumorigenesis in inbred female mice. Cell Cycle. 2011;10(24):4230-6.

20. Zhang Y, Bokov A, Gelfond J, Soto V, Ikeno Y, Hubbard G, et al. Rapamycin extends life and health in C57BL/6 mice. J Gerontol A Biol Sci Med Sci. 2014;69(2):119-30.

21. Halloran J, Hussong SA, Burbank R, Podlutskaya N, Fischer KE, Sloane LB, et al. Chronic inhibition of mammalian target of rapamycin by rapamycin modulates cognitive and non-cognitive components of behavior throughout lifespan in mice. Neuroscience. 2012;223:102-13.

22. Majumder S, Caccamo A, Medina DX, Benavides AD, Javors MA, Kraig E, et al. Lifelong rapamycin administration ameliorates age-dependent cognitive deficits by reducing IL-1beta and enhancing NMDA signaling. Aging Cell. 2012;11(2):326-35.

23. Chiao YA, Kolwicz SC, Basisty N, Gagnidze A, Zhang J, Gu H, et al. Rapamycin transiently induces mitochondrial remodeling to reprogram energy metabolism in old hearts. Aging (Albany NY). 2016;8(2):314-27.

24. Dai DF, Karunadharma PP, Chiao YA, Basisty N, Crispin D, Hsieh EJ, et al. Altered proteome turnover and remodeling by short-term caloric restriction or rapamycin rejuvenate the aging heart. Aging Cell. 2014;13(3):529-39.

25. Flynn JM, O'Leary MN, Zambataro CA, Academia EC, Presley MP, Garrett BJ, et al. Late-life rapamycin treatment reverses age-related heart dysfunction. Aging Cell. 2013;12(5):851-62.

426 26. Chen C, Liu Y, Zheng P. mTOR regulation and therapeutic rejuvenation of aging
427 hematopoietic stem cells. Sci Signal. 2009;2(98):ra75.

428 27. Shavlakadze T, Zhu J, Wang S, Zhou W, Morin B, Egerman MA, et al. Short-term Low-
429 Dose mTORC1 Inhibition in Aged Rats Counter-Regulates Age-Related Gene Changes and
430 Blocks Age-Related Kidney Pathology. J Gerontol A Biol Sci Med Sci. 2018;73(7):845-52.

431 28. An JY, Kerns KA, Ouellette A, Robinson L, Morris HD, Kaczorowski C, et al.
432 Rapamycin rejuvenates oral health in aging mice. eLife. 2020;9.

433 29. An JY, Quarles EK, Mekvanich S, Kang A, Liu A, Santos D, et al. Rapamycin treatment
434 attenuates age-associated periodontitis in mice. Geroscience. 2017;39(4):457-63.

435 30. Bitto A, Ito TK, Pineda VV, LeTexier NJ, Huang HZ, Sutlief E, et al. Transient
436 rapamycin treatment can increase lifespan and healthspan in middle-aged mice. eLife.
437 2016;5:e16351.

438 31. Yilmaz OH, Katajisto P, Lamming DW, Gultekin Y, Bauer-Rowe KE, Sengupta S, et al.
439 mTORC1 in the Paneth cell niche couples intestinal stem-cell function to calorie intake.
440 Nature. 2012;486(7404):490-5.

441 32. Garcia DN, Saccon TD, Pradiee J, Rincon JAA, Andrade KRS, Rovani MT, et al. Effect
442 of caloric restriction and rapamycin on ovarian aging in mice. Geroscience. 2019;41(4):395-
443 408.

444 33. Urfer SR, Kaeberlein TL, Mailheau S, Bergman PJ, Creevy KE, Promislow DEL, et al. A
445 randomized controlled trial to establish effects of short-term rapamycin treatment in 24
446 middle-aged companion dogs. Geroscience. 2017;39(2):117-27.

447 34. Ramos FJ, Chen SC, Garelick MG, Dai DF, Liao CY, Schreiber KH, et al. Rapamycin
448 reverses elevated mTORC1 signaling in lamin A/C-deficient mice, rescues cardiac and
449 skeletal muscle function, and extends survival. Sci Transl Med. 2012;4(144):144ra03.

450 35. Yi H, Brooks ED, Thurberg BL, Fyfe JC, Kishnani PS, Sun B. Correction of glycogen
451 storage disease type III with rapamycin in a canine model. J Mol Med (Berl).
452 2014;92(6):641-50.

453 36. Ruygrok PN, Muller DW, Serruys PW. Rapamycin in cardiovascular medicine. Internal
454 Medicine Journal. 2003;33(3):103-9.

455 37. Powers RW, 3rd, Kaeberlein M, Caldwell SD, Kennedy BK, Fields S. Extension of
456 chronological life span in yeast by decreased TOR pathway signaling. Genes Dev.
457 2006;20(2):174-84.

458 38. Bjedov I, Toivonen JM, Kerr F, Slack C, Jacobson J, Foley A, et al. Mechanisms of life
459 span extension by rapamycin in the fruit fly Drosophila melanogaster. Cell Metab.
460 2010;11(1):35-46.

461 39. Robida-Stubbs S, Glover-Cutter K, Lamming DW, Mizunuma M, Narasimhan SD,
462 Neumann-Haefelin E, et al. TOR signaling and rapamycin influence longevity by regulating
463 SKN-1/Nrf and DAF-16/FoxO. Cell Metab. 2012;15(5):713-24.

464 40. Anisimov VN, Zabezhinski MA, Popovich IG, Piskunova TS, Semenchenko AV, Tyndyk
465 ML, et al. Rapamycin extends maximal lifespan in cancer-prone mice. Am J Pathol.
466 2010;176(5):2092-7.

467 41. Bitto A, Ito TK, Pineda VV, LeTexier NJ, Huang HZ, Sutlief E, et al. Transient
468 rapamycin treatment can increase lifespan and healthspan in middle-aged mice. Elife. 2016;5.

469 42. Harrison DE, Strong R, Sharp ZD, Nelson JF, Astle CM, Flurkey K, et al. Rapamycin fed
470 late in life extends lifespan in genetically heterogeneous mice. Nature. 2009;460(7253):392-
471 5.

472 43. Wilkinson JE, Burmeister L, Brooks SV, Chan CC, Friedline S, Harrison DE, et al.
473 Rapamycin slows aging in mice. Aging Cell. 2012;11(4):675-82.

474 44. Miller RA, Harrison DE, Astle CM, Baur JA, Boyd AR, de Cabo R, et al. Rapamycin, but
475 not resveratrol or simvastatin, extends life span of genetically heterogeneous mice. J
476 Gerontol A Biol Sci Med Sci. 2011;66(2):191-201.

477 45. Miller RA, Harrison DE, Astle CM, Fernandez E, Flurkey K, Han M, et al. Rapamycin-
478 mediated lifespan increase in mice is dose and sex dependent and metabolically distinct from
479 dietary restriction. Aging Cell. 2014;13(3):468-77.

480 46. Kaeberlein M, Kennedy BK. Ageing: A midlife longevity drug? Nature.
481 2009;460(7253):331-2.

482 47. Zimmerman JJ, Kahan BD. Pharmacokinetics of sirolimus in stable renal transplant
483 patients after multiple oral dose administration. J Clin Pharmacol. 1997;37(5):405-15.

484 48. MacDonald A, Scarola J, Burke JT, Zimmerman JJ. Clinical pharmacokinetics and
485 therapeutic drug monitoring of sirolimus. Clin Ther. 2000;22 Suppl B:B101-21.

486 49. Zimmerman JJ, Ferron GM, Lim HK, Parker V. The effect of a high-fat meal on the oral
487 bioavailability of the immunosuppressant sirolimus (rapamycin). J Clin Pharmacol.
488 1999;39(11):1155-61.

489 50. Trepanier DJ, Gallant H, Legatt DF, Yatscoff RW. Rapamycin: distribution,
490 pharmacokinetics and therapeutic range investigations: an update. Clin Biochem.
491 1998;31(5):345-51.

492 51. Larson JC, Allstadt SD, Fan TM, Khanna C, Lunghofer PJ, Hansen RJ, et al.
493 Pharmacokinetics of orally administered low-dose rapamycin in healthy dogs. Am J Vet Res.
494 2016;77(1):65-71.

52. Paoloni MC, Mazcko C, Fox E, Fan T, Lana S, Kisseberth W, et al. Rapamycin pharmacokinetic and pharmacodynamic relationships in osteosarcoma: a comparative oncology study in dogs. PLoS One. 2010;5(6):e11013.

53. Kahan BD. Two-year results of multicenter phase III trials on the effect of the addition of sirolimus to Cyclosporine-based immunosuppressive regimens in renal transplantation. Transplantation Proceedings. 2003;35(3):S37-S51.

54. Torok D, Muhl A, Votava F, Heinze G, Solyom J, Crone J, et al. Stability of 17a-hydroxyprogesterone in dried blood spots after autoclaving and prolonged storage. Clin Chem. 2002;48:370-2.

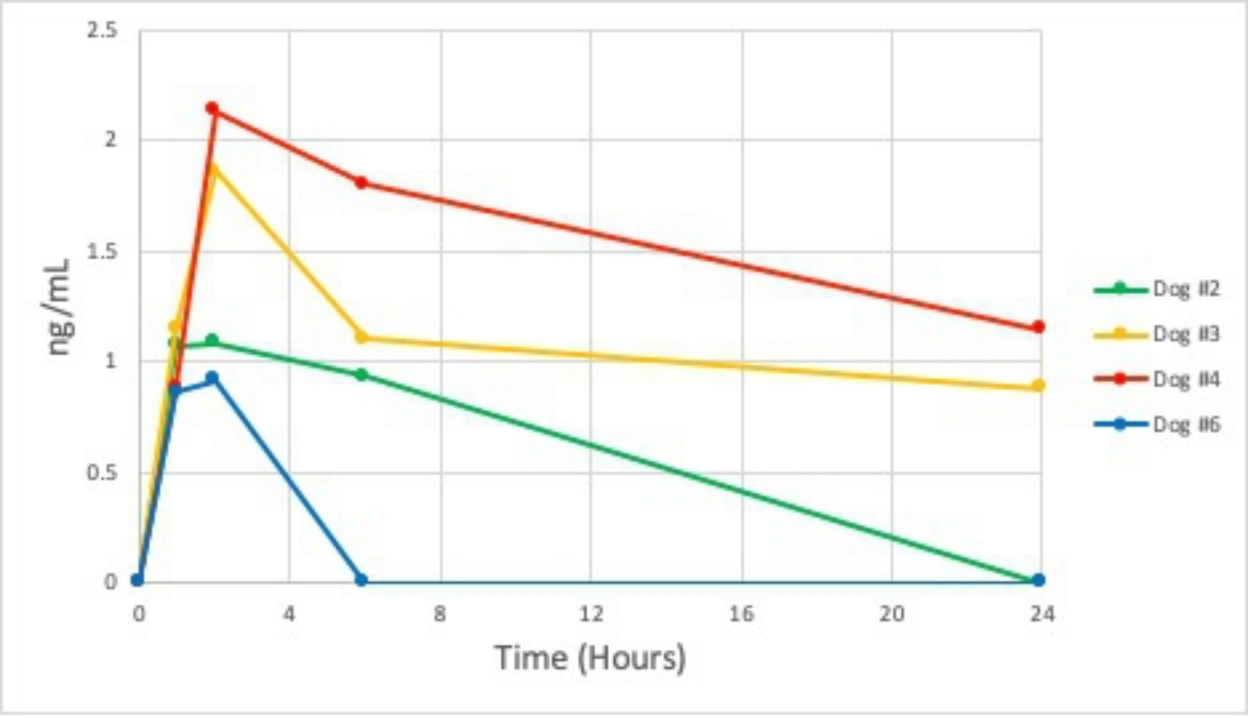

Figure 1