Application for NIA Intervention Testing Program

Zelton Dave Sharp

1. Title: Inhibitors of mammalian TOR to increase life span.

2. Name: Zelton Dave Sharp

Mailing Address: Associate Professor and Interim Director/Chair

Institute of Biotechnology/Department of Molecular Medicine

University of Texas Health Science Center at San Antonio

15355 Lambda Drive

San Antonio, Texas 78245

Phone: 210-567-7226

FAX: 210-567-7277 FAX

E-mail: sharp@uthscsa.edu

3. Background Information:

Mammalian TOR. TOR (target of rapamycin) was originally identified in yeast genetic screens as two genes (*Tor1* and *Tor2*). Mutations in *Tor1/2* conferred resistance to the toxic effect of a fungicide, rapamycin, produced by a soil bacterium, *Streptomyces hygroscopicus* (Heitman et al., 1991). Subsequent cloning and characterization of ScTor1p and ScTor2p revealed a C-terminal region with homology to phosphatidylinositol 3-kinases, although no lipid kinase activity has been attributed to either protein (Kunz et al., 1993). TOR is also known in humans as FKBP12-rapamycin complex-associated protein 1 (FRAP1); in rats as RAFT1; and as sirolimus effector protein, SEP and also RAPT. Purification and cloning of mammalian TOR identified a single 290 kDa protein, which is highly related to ScTor1/2p. To date, no other mTOR orthologues have been identified.

The basic structure of TOR is conserved from fungi to humans. Studies in yeast, flies, and mammals identified a TOR-related family of proteins including MEC1, TEL1, RAD3, MEI-41, DNA-PK, ATM, ATR and TRRAP (Dennis et al., 1999; Gingras et al., 2001; Schmelzle and Hall, 2000). The characteristic C-terminal phosphatidylinositol (PI) kinase homology domain led to the nomenclature, PI-kinase (PIK)-related kinases. PIK-related kinases are involved in diverse cellular functions including cell growth, cell cycle, DNA damage checkpoints, recombination, and telomere maintenance. This family of kinases is often described as stress-response transducers.

Figure 1 below schematically illustrates a summary of the TOR signaling pathways in mammalian cells under nutrient replete and restricted conditions, and under rapamycin treatment. A developing consensus is that mTOR, dTOR and ScTor1p/2p have key roles in nutrient response systems (Blume-Jensen and Hunter, 2001; Dennis et al., 1999; Gingras et al., 2001; Manning and Cantley, 2003; Rohde et al., 2001; Schmelzle and Hall, 2000), perhaps acting a nutrient-dependent "gate keeper" (Gingras et al., 2001). Mammalian TOR-related responses to nutrients are mediated by: **1)** Regulation of translation through repressor 4EBP1 and S6 kinase (see below); **2)** Regulation RNA polymerase I, and III transcription of ribosomal RNAs; **3)** Regulation RNAPII transcription of genes important in cellular response to changes in nutrient conditions, such as nitrogen levels (Rohde et al., 2001); **4)** Regulation of autophagy. The responses in **2-4** are not shown below in the Figure for simplicity and because it is not known how rapamycin affects them. Importantly, note in Fig. 1 that current models posit that nutrient signaling through mTOR kinase is integrated with insulin/growth factor signaling through a Rheb (Ras homolog enriched in brain) (Manning and Cantley, 2003) and a raptor/GβL complex (Kim et al., 2003). Thus, mammalian TOR kinase coordinates nutritional, mitogenic and insulin metabolic signaling.

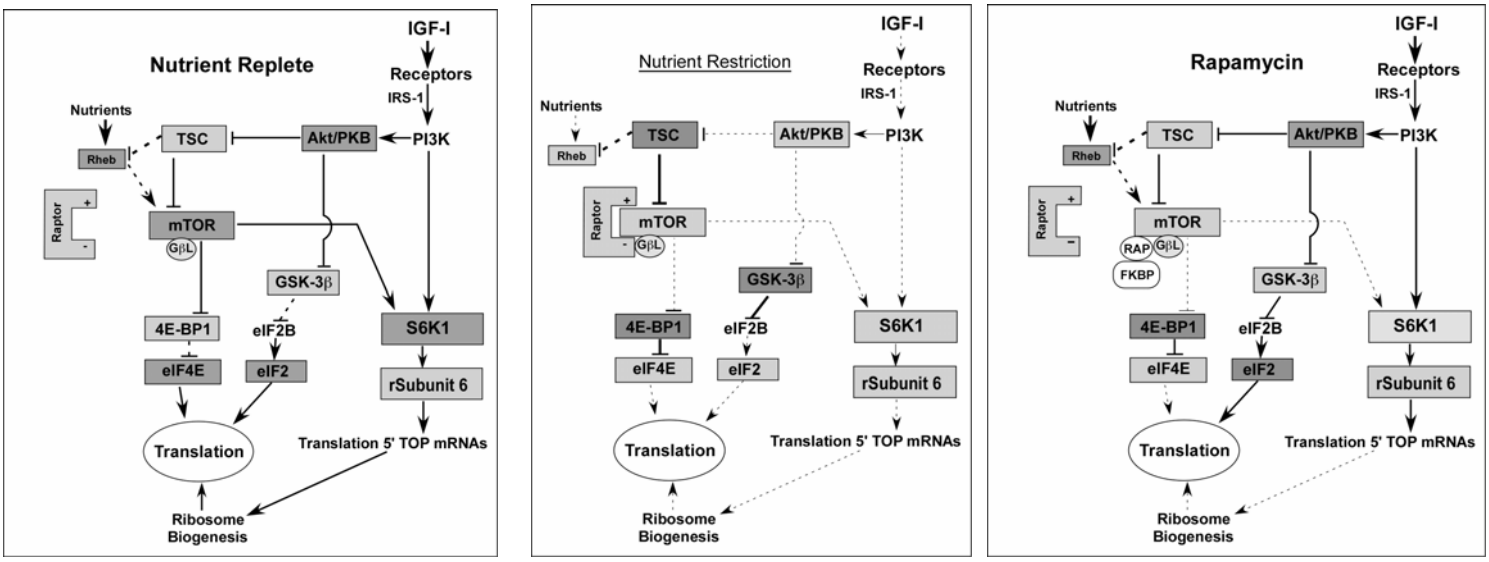


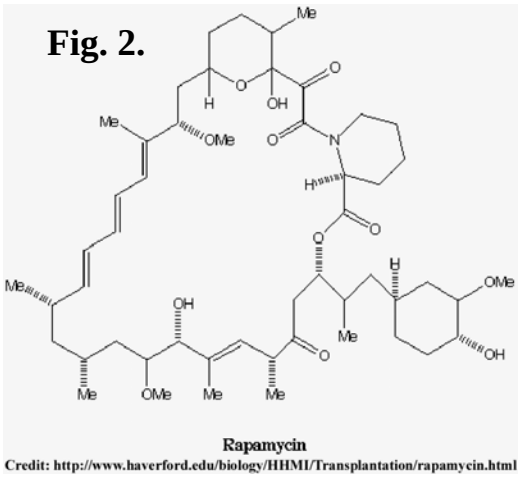
Figure 1. The pathways and components under the various conditions depicted in these figures were deduced from papers cited in the above text. Darker shaded boxes indicate active mTOR effectors. Important players in the nutrient pathways are Rheb (see above text and (Manning and Cantley, 2003)) and a nutrient-sensitive complex (consisting of mTOR, raptor and GβL (Kim et al., 2003)), which appears to be most relevant to the action of rapamycin. Under nutrient replete conditions (left panel), raptor is destabilized from interactions with mTOR, thereby activating the GβL-dependent kinase activity of mTOR. In nutrient-restricted animals (middle panel), these models (developed in cell culture systems) predict that the raptor/mTOR/GβL complex is stabilized, whereupon raptor blocks the GβL-dependent

kinase activity of mTOR (Kim et al., 2003). Interestingly, rapamycin (right panel) destabilizes the raptor/mTOR complex, which should allow activate GβL-dependent kinase activity, but instead represses GβL-dependent mTOR kinase activity (Kim et al., 2003). Note that under conditions of diet restriction, endocrine levels of IGF-I are reduced (Kari et al., 1999), which also impinges upon the mTOR kinase (middle panel). Therefore, it is predicted that rapamycin will not be a complete mimetic of diet restriction since the GSK-3β-inhibitor pathway to eIF2 (Wang et al., 2001) would remain intact. However, the restrictions that it places on the translation machinery would probably be sufficient since rapamycin severely restricted the growth of *C. elegans* (Yu and Larsen, 2001).

Importantly, abrogation of TOR in *C. elegans* adults extends lifespan (Vellai et al., 2003). This paper provided a critical proof-of-principle using an important metazoan experimental system. Thus, I predict that chronic treatment of mice with mTOR inhibitors (rapamycin or related drugs) will extend health span in a manner similar to diet restriction and/or CeTOR abrogation. Dwarf and diet restricted mice live long, healthy laboratory lives carrying, evidently, an acceptable tumor burden (Ikeno et al., 2003), which does not grow and progress. If the models proposed herein withstand tests, it is easy to envision technology, such as proposed here, to promote the same condition in humans. The question that this proposed intervention study could address is: is it possible to enjoy the "gains" of diet restriction without the "pains" and stigma (ie. being poor), that people associate with limiting food. If this intervention works as envisioned, another important factor is the potential reduction in overall all health cost for the US and world.

Rapamycin. The chemical structure of rapamycin is shown in Figure 2. A lipophilic macolide, it is a potent antifungal, immunosuppressive, potential anticancer drug. Its immunosuppressive actions are due to ability to inhibit proliferation of helper T cells (Lorberg and Hall, 2004).

Fig. 2.



Rapamycin

Credit: http://www.haverford.edu/biology/HHMI/Transplantation/rapamycin.html

Therapeutic interest in rapamycin as an anticancer drug (Garber, 2001) is attributed to the placement of mTOR downstream of the PI3-kinase/Akt pathway, which is up regulated in multiple cancers upon loss of the PTEN tumor suppressor gene (Mills et al., 2001; Neshat et al., 2001; Ozes et al., 2001; Podsypanina et al., 2001; Yu et al., 2001). An ester of rapamycin termed cell cycle-inhibitor 779 (CCI-779) has shown promise for certain types of cancers (Garber, 2001; Hidalgo and Rowinsky, 2000). Other analogs (e.g., RAD 001, and AP23573, Ariad Pharmaceuticals) are also highly specific inhibitors of mTOR and are under test as anticancer drugs (Mita et al., 2003b).

4. Suggested Treatment Protocol.

4a. Since rapamycin is lipophilic, it probably cannot be given in the drinking water. In its formulation as Rapamune®, Wythe distributes an oral solution and tablets for patients

(http://www.fda.gov/cder/consumerinfo/druginfo/rapamune.htm). Thus, rapamycin or Rapamune®) would probably have to be administered by injection or in the food. Based on a review of the literature (Guba et al., 2002; Lieberthal et al., 2001; Podder et al., 2001), and in consultation with Dr. Dan Riley (a colleague in the Division of Nephrology who has clinical experience with Rapamune® in kidney transplant patients), I have arrived at a daily dose of 1 mg of rapamycin or analog (CCI-779, see above) per kilogram of body weight. However, it is important to point out that the critical issue is blood concentrations of the drug. It will be imperative to monitor blood levels of rapamycin (or analogs if available) to achieve 10 ng rapamycin per ml. This benchmark was arrived at in consultation with Dr. Riley as the level at which rejection of kidney transplant are inhibited and is well tolerated in long-term treatments.

4b. Rapamycin is available from commercial sources such as Sigma-Aldrich ($198/mg) and Calbiochem ($167/mg), and KC Laboratories (see below).

However, as mentioned in 4a, Wythe markets Rapamune® and has CCI-779 is in clinical trials. The most convenient, and perhaps ideal, drug for this intervention study would be CCI-779, which is a water-soluble ester of rapamycin. In addition, CCI-779 is purported to be less immunosuppressive, and is under intensive study as an anticancer drug (Dudkin et al., 2001; Geoerger et al., 2001; Hidalgo and Rowinsky, 2000; Mita et al., 2003a; Neshat et al., 2001; Yu et al., 2001). This investigator applied to Wyeth for sufficient CCI-779 to conduct small-scale pilot experiments, the purpose of which was to compare its actions with those in a diet restriction protocol using cross sectional analyses of key signaling molecules in mice. In its denial, the company indicated that it is more interested in using their limited supply of this drug for cancer studies rather than aging, although it is well known that cancer is primarily a disease of the aged. Thus, whether or not its use for this intervention could be successfully negotiated is unknown. It is also not known at the present time if the laboratory tests for determining blood concentrations of rapamycin could be used for NCI-779. If not, this is clearly a test that would have to be developed.

Using bulk rate commercial sources (e.g., KC Laboratories, http://www.lclabs.com/PRODFILE/P-R/R-5000.php4), this would be a modestly expensive project. KC Labs advertises 200 mg of rapamycin for $1,590, or $7.95 per mg. The cost per mouse at this price would be about $58/mouse/year (20 gram mouse X 1 mg/kg X $7.95/mg X 365). KC Labs also advertises lower bulk rates upon request, so the costs per mouse could be lower.

4c. The best way to determine if the intake of the compound is having the optimal biological effects is to monitor the status of key signaling proteins downstream of mTOR signaling. This would entail sacrificing at least 10 mice/year in the treatment group and controls to harvest tissues (liver, kidney, brain, skeletal muscle, testis, heart, e.g.). From my experience on similar experiments with Ames dwarf mice, this number of mice would be required to obtain statistically significant data on the phosphorylation and binding status of these proteins. The mTOR targets that I would suggest monitoring would be 4EBP1 (for both phosphorylation status and eIF4E

binding), S6 kinase phosphorylation status and activity (Fingar et al., 2002) and S6 ribosomal protein phosphorylation status (See Fig. 3 for examples that the sponsor has done in his laboratory using dwarf (*df/df*) and normal size mouse liver). Blocking mTOR should increase the binding activity the translation repressor, 4EBP1, to the translation initiation factor 4E (eIF4E), which would decrease Cap-dependent translation. It should also decrease the activation of S6 kinase 1 (S6K1), which should decrease ribosome biogenesis. As mentioned above, it would also be important to also monitor blood concentrations of rapamycin (or analog) to achieve ~10 ng/ml.

The treatment should commence on postnatal week seven. One week after treatment, the first blood tests in one or two test animals should be conducted. If blood levels of rapamycin are optimal (10 ng/ml), the first sacrifice for organ harvest should be done. These tests should be repeated at least once during the first year, and twice/yr in succeeding years. The blood tests for rapamycin could be done in clinical labs, and the tests of the tissues could be done in the sponsor's lab. If we do not observe the expected down-regulation of mTOR effectors (Fig. 3) in the initial assays, then the dose will have to be adjusted upward. If we see too great a response in later years, then the dose and target blood levels might need to be reevaluated along with their overall health status of the mice.

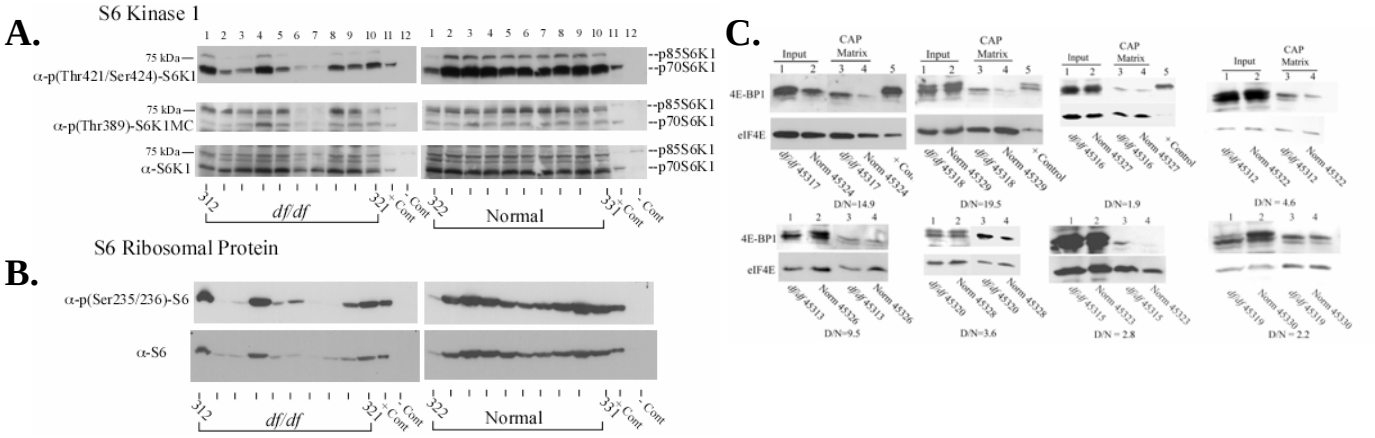


Figure 3. *Assays for the status of downstream mTOR effectors.* All the experiments shown here used protein lysates prepared from mouse livers from Ames dwarf (*df/df*) and normal size littermates, which are indicated above the lanes (1-10) in A & B and individually below the lanes in C. The tissues were provided by A. Bartke, and the data are from a paper being prepared for submission. **A.** An analysis of S6 kinase 1 of which there are two isoforms, p70 and p85. Phosphorylation of Thr421/Ser424 and Thr389 was detected using antibodies specific for these epitopes (Cell Signaling Tech). Total S6K1 was detected in stripped blots using antibodies from Santa Cruz. There is about a 3 fold increase in the phosphorylation of these residues in normal liver compare to dwarf, reflecting, we postulate, growth hormone stimulation. **B.** These data are an analysis of Ser235/236 phosphorylation in the sixth ribosomal subunit, a target of S6 kinase, which also shows similar increases in normal livers. **C.** Assays for 4E-BP1 binding to eIF4E (eukaryotic initiation factor 4E). The binding of the translation inhibitor, 4E-BP1 (aka,

PHAS-1), to eIF4E is inhibited by hyper-phosphorylation of 4E-BP1. To directly assay 4E-BP1/eIF4E association in tissue extracts, a co-precipitation procedure was used. The assay, previously described by Gingras, et al., (Gingras et al., 1998), makes use of ^{7}methyl-GTP-Sepharose (Amersham/Pharmacia). Varying concentrations of cell lysates are incubated with a fixed volume of the resin, washed and the bound proteins assayed using immunoblotting with 4E-BP1 and eIF4E antibodies (Santa Cruz and Cell Signaling, respectively). The data presented here shows a comparison of *df/df* and normal size littermates. The D/N is a ratio of the co-precipitating 4EBP1 in the lysates from dwarf and normal livers. Note that the ratio indicates a substantial increase in co-precipitating 4EBP1, leading to the postulate that liver translation is inhibited in dwarf mice, perhaps due to less stimulation by growth hormone. A similar approach would be used in the intervention to document the biological effects of rapamycin.

4d. As indicated above, the test group should be started at postnatal week seven, and continued throughout the life span. Assuming rapamycin (or analog) mimics caloric restriction, and testing a cohort of 100 mice, a 10% difference in mean survival could be detected with a power of 0.92 (Liang et al., 2003).

5. Animal Safety Information. I am not aware of any acute side effects of rapamycin. Rapamycin is an immunosuppressant, taken by humans (Rapamune®, Wyeth) for prevention of transplant rejection. For some reason, lymphocytes are particularly sensitive to this drug. Humans take the drug long term, with some side effects (per Dr. Dan Riley in Nephrology), but little could be found about rapamycin in mice long term. As mention previously, CCI-779, is in clinical trials for cancer treatment. It is purported that this drug has reduced immunosuppressant activity. Most of the publications (Jiang et al., 2001) use rapamycin short term. If the mice are kept in microisolator cages, and monitored carefully for this caveat, I would not expect unscheduled euthanizing of mice due to infections. If it is obvious that a mouse is sick, the treatment should, of course, be stopped.

Although it has never been noted in patients, it is possible that cognitive function could be negatively affected by this treatment since *ex vivo* experiments show that long-term hippocampal synaptic plasticity is sensitive to rapamycin (Takei et al., 2001; Tang et al., 2002). Thus, the tests for cognitive abilities of the mice described in the application materials would be important.

6. Costs. As discussed above, the costs using commercial sources would be modest -- $58/mouse/year, or about $6,000/year for a study involving 100 mice. Using commercial sources of rapamycin, it would also be necessary to contract with a company to formulate a special chow, which would add more to the cost.

However, as discussed above, enlisting a pharmaceutical company (e.g., Wyeth) to supply the drug would be ideal, especially one like CCI-779, which would be much easier to administer. The issue for Wyeth would probably be the supply of the drug needed for a mean survival study.

7. Statement of understanding.

In submitting this proposal I agree to the following:

7a. I understand that all information presented in the proposal can be freely shared with members of the ITP Steering Committee and Access Panel during their evaluation of proposals, but will otherwise be considered confidential in the same sense that NIH grant applications are treated as confidential throughout the review process.

7b. If my proposal, or a modification of it (such as altered dosage or frequency of administration), is accepted for inclusion in a research protocol, I will be asked to help evaluate the data and to prepare the data for written and oral publications, on each of which I will be offered co-authorship. I understand that the ITP intends to submit for publication the results of all ITP-supported studies whether or not they produce data showing positive or negative effects on health status in mice.

7c. I understand that data generated by ITP-supported experiments using the compound/diet proposed will be made publicly available and can be used in applications for further research support by anyone. I will also be free to use ITP-generated data in the context of applications for research support or for any other purpose.

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