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eRapa Restores A Normal Life Span in a FAP Mouse Model

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Abstract

Mutation of a single copy of the *adenomatous polyposis coli* (*APC*) gene results in familial adenomatous polyposis (FAP), which confers an extremely high risk for colon cancer. $Apc^{Min/+}$ mice exhibit multiple intestinal neoplasia (MIN) that causes anemia and death from bleeding by 6 months. Mechanistic target of rapamycin complex 1 (mTORC1) inhibitors were shown to improve

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Apc$^{Min/+}$ mouse survival when administered by oral gavage or added directly to the chow, but these mice still died from neoplasia well short of a natural life span. The National Institute of Aging Intervention Testing Program showed that enterically targeted rapamycin (eRapa) extended life span for wild type genetically heterogeneous mice in part by inhibiting age-associated cancer. We hypothesized that eRapa would be effective in preventing neoplasia and extend survival of *Apc*$^{Min/+}$ mice. We show that eRapa improved survival for *Apc*$^{Min/+}$ mice in a dose-dependent manner. Remarkably, and in contrast to previous reports, most of the *Apc*$^{Min/+}$ mice fed 42 ppm eRapa lived beyond the median life span reported for wild type syngeneic mice. Furthermore, chronic eRapa did not cause detrimental immune effects in mouse models of cancer, infection or autoimmunity; thus, assuaging concerns that chronic rapamycin treatment suppresses immunity. Our studies suggest that a novel formulation (enteric targeting) of a well-known and widely used drug (rapamycin) can dramatically improve its efficacy in targeted settings. eRapa or other mTORC1 inhibitors could serve as effective cancer preventatives for people with FAP without suppressing the immune system, thus reducing the dependency on surgery as standard therapy.

INTRODUCTION

Familial adenomatous polyposis (FAP) is an autosomal dominant disease caused by mutation of the *adenomatous polyposis coli* (*APC*) gene, located on chromosome 5. APC inhibits the pro-growth WNT signaling pathway by regulating β-catenin nuclear localization and its mutation results in the development of numerous adenomatous colorectal polyps at a young age. Polyposis inevitably progresses to colorectal cancer if left untreated. Given the predictable development of colorectal cancer in patients with FAP, the safest preventative strategy is surgical resection of the colon before cancer develops. Genetic screening and endoscopy in concert with prophylactic total colectomy significantly improve the overall survival of FAP patients. Unfortunately, colonoscopies identify only 56% and 77% of tumors on the right and left side of the colon, respectively (1), and total colectomy is morbid and life altering. Further, the second leading cause of death in FAP is duodenal adenocarcinoma. Ninety percent of FAP patients develop duodenal polyps, the precursor lesions of duodenal adenocarcinoma and of these 5% will develop duodenal adenocarcinoma in their lifetime (2). Duodenal surgery is currently indicated for FAP patients with severe duodenal polyposis or duodenal carcinoma at the expense of significant morbidity. Thus, this patient population has a strong need for non-surgical treatments to prevent or reduce polyp formation and carcinogenesis in the gastrointestinal track with the possibility of reducing the need for life altering surgery.

Hence, the goal of our study was to test enteric-targeted rapamycin (eRapa) in the *Apc*$^{Min/+}$ mouse model and assess whether this intervention delayed or prevented hemorrhaging intestinal neoplasia that lead to anemia and mortality. Notably, the etiology for intestinal tumors in *Apc*$^{Min/+}$ mice is the same as most FAP lesions, so interventions that prevent neoplasia in the *Apc*$^{Min/+}$ mouse model are also likely to work for FAP.

Rapamycin has been proposed to be a cancer preventative agent. It allosterically inhibits mTORC1 when bound to FKBP12. mTORC1 promotes cell growth (mass) by coordinating numerous cellular processes including macromolecule biosynthesis in response to nutrient,

energy and growth factor stimuli (3). Upregulation of mTOR contributes to the development and growth of cancer, including intestinal tumors, making the mTOR pathway an attractive candidate for anti-cancer therapy (4). Recent evidence suggests that mTORC1 inhibition delays or prevents cancer in human kidney transplant patients treated with rapamycin (5) and mouse cancer models (6, 7). Thus, rapamycin could be an effective anti-cancer prophylactic agent.

Two previous studies support the use of rapamycin in delaying intestinal neoplasias in *Apc*–mutated mouse models. In one study, a rapamycin derivative, RAD001 (everolimus) was administered by oral gavage (3 or 10 mg/kg body weight/day, 5 times a week) (8) and the other study fed non-encapsulated rapamycin (sirolimus) in high fat food pellets (40 mg/kg) (9). Both studies showed that these agents improved survival in *Apc*-mutant mice although they died from intestinal neoplasias before the median life span for wild type syngenic C57BL/6J mice (median life spans for $Apc^{Min/+}$ and $Apc^{\Delta 716}$ mice between 420 – 550 days as compared to a range of 692–866 days for wild type mice (10, 11)).

We tested if enteric rapamycin delivery could improve efficacy to prevent intestinal polyposis in $Apc^{Min/+}$ mice. eRapa prevented or significantly delayed intestinal neoplasia in $Apc^{Min/+}$ mice and improved survival and other health span indicators without eliciting undesirable side-effects like immune suppression. Survival was prolonged beyond that of the median life span for wild type mice supporting the possibility that enteric targeting of mTORC1 inhibitors could serve as safe and effective preventatives to complement cancer surveillance procedures indicated for patients with a high risk for intestinal cancers.

MATERIALS and METHODS

Rapamycin diets

Preparation of rapamycin diets was described previously (6). The concentration of rapamycin in food was expressed as ng/mg food (parts per million).

Mice, eRapa chow and rapamycin blood levels

We treated and housed mice according to IACUC standards. Cohorts of $Apc^{Min/+}$ mice (Jackson Laboratories, C57BL/6-*ApcMin/J*) or C57BL/6 wild type mice were fed microencapsulated rapamycin-containing diets either containing a concentration of 14 mg/kg food, (14 ppm), which provided a dose of ~2.24 mg of rapamycin/kg body weight/day (12, 13) or 42 mg/kg food (42 ppm) (14) which provided a dose of ~6.72 mg of rapamycin/kg body weight/day. Control diet was the same but with empty capsules. We housed mice in accordance with the NIH guide for the care and use of lab animals.

In our longevity studies, mice were allowed to live out their life span, *i.e*., there was no censoring due to morbidity in the groups of mice used to measure lifespan. Mice were euthanized only if they were either (1) unable to eat or drink, (2) bleeding from a tumor or other condition, or (3) when they were laterally recumbent, i.e., they failed to move when prodded or were unable to right themselves. Rapamycin blood levels were done as previously described (6).

Measurement of Rapamycin in Intestine Tissue—Reagents and our HPLC/MS system were previously described (6). Rapamycin was quantified in mouse small intestine tissue according to the following protocol. Briefly, 100 mg of calibrator, control, and unknown tissue samples were mixed by sonication (three 5 sec bursts) with 10 μL of 0.5 μg/mL ASCO (internal standard) and 300 μL of a solution containing 0.1% formic acid and 10 mM ammonium formate dissolved in 95% HPLC grade methanol. After sonication, the samples were vortexed vigorously for 2 min, and then centrifuged at 15,000 *g* for 5 min at 23°C (subsequent centrifugations were performed under the same conditions). Supernatants were transferred to 1.5 mL microfilterfuge tubes and spun at 15,000 *g* for 1 minute and then 40 μL of the final extracts were injected into the LC/MS/MS. The ratio of the peak area of rapamycin to that of the internal standard ASCO (response ratio) for each unknown sample was compared against a linear regression of calibrator response ratios at 0, 1.78, 3.13, 6.25, 12.5, 50, and 100 μg/g to quantify rapamycin. The concentration of rapamycin was expressed as μg/g of tissue (parts per million).

Pathology

The severity of neoplastic lesions was assessed using the grading system previously described (15). Two pathologists separately examined all of the samples without knowledge of thegenotype or diet.

Immunoblots

Because Western blotting of intestinal tissue is difficult, we optimized a procedure as follows. Mouse small intestine (~15–35 mg) was ground to a powder with a mortar and pestle after cryofracture. Powdered tissue was lysed by homogenization in 5 X dry weight RIPA (modified) buffer (150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM phenylmethylsulfonyl flouride, 1% Triton X-100, 1% sodium deoxycholic acid, 0.1% sodium dodecyl sulfate, and one Pierce protease and phosphatase inhibitor mini tablet/10 ml). Debris was cleared by centrifugation. Bio-Rad Protein Assay was used to determine protein concentrations and ~40–50 μg of each sample was mixed with SDS loading dye, boiled, and separated on a gel before transfer to nitrocellulose (35 min at 20 V, Bio-Rad Semi Dry transfer) using Towbin buffer containing 0.0375% SDS. Blots were dried, cut into strips and blocked for 1 hour at room temperature in Odyssey blocking buffer. Individual strips were incubated overnight at 4° C in appropriate primary antibodies and then for 1 hour at room temperature in corresponding IRDye secondary antibodies. Blots were washed 4 times (5 minutes each) in TBS-T after each antibody incubation with a final wash in TBS before scanning and quantification on an Odyssey Imager. We quantified fluorescent signals as integrated intensities (I.I. K counts) using the Odyssey Infrared Imaging System, Application version 3.0 software. We used a local background subtraction method to subtract independent background values from each box: the median background function with a 3 pixel width border above and below each box was subtracted from individual counts. We calculated ratios for each antibody against the pan-actin loading control using I.I. K counts. The respective antibody to pan-actin ratio was then used to calculate phosphorylated protein to total protein ratio. Prism 5 (GraphPad Software, Inc.) was used to analyze and graph the data. We used an unpaired two-tailed *t*-test or Mann Whitney test (nonparametric for rpS6) to obtain p values. P values below 0.05

were considered significant. All Odyssey products and IRDye secondary antibodies were obtained from LI-COR Biosciences. All antibodies were diluted into Odyssey Blocking Buffer + 0.2% Tween 20. Actin, pan Ab-5 (clone ACTN05) mouse monoclonal primary antibody was obtained from Thermo Fisher and all other primary antibodies were obtained from Cell Signaling Technologies:phospho rpS6 (Ser240/244) rabbit polyclonal and rpS6 (5G10) mouse monoclonal, phospho AKT (Ser473) rabbit polyclonal, and AKT rabbit polyclonal.

Activity assay

Mice were individually housed in clear, Plexiglas cages (38 × 20 × 11.5 cm) for 36 hours (two light and one dark phase) to assess spontaneous activity as described (16).

Hematocrit

Red blood cells were packed using centrifugation in the presence of anti-coagulant and percentage of pellet (% packed cell volume) to supernatant was reported. Blood was either collected at time of sacrifice or from the tail vein.

Cell lines

We obtained authenticated mycoplasma free cell lines from the ATCC for use in our studies. We tested cells periodically for mycoplasma according to ATTC recommendations.

B16 tumor model

B16F10 melanoma cells were cultivated and injected subcutaneously (125,000 cells/flank), growth was measured with Vernier calipers and immunity was studied as we previously described (17).

Toxoplasma gondii challenge

Tachyzoites of *T. gondii* (Me49 strain expressing OVA) were cultivated and used to challenge mice, and immunity was studied all as we described (18–20). Mice were fed eRapa 14 ppm or Eudragit control for 30 days before, and during the infection with 10,000 tachyzoites by intraperitoneal inoculation. Animals were sacrificed 11 days after inoculation for immune studies.

Bm12 autoimmunity model

Autoimmune kidney disease was induced by transfer of bm12 splenocytes into BL6 mice, and kidney disease was monitored by albuminuria as described (21). Mice were fed eRapa 14 ppm or Eudragit control for 30 days before, and during the post-splenocyte transfer monitoring period of 12 weeks. Immune events were analyzed as for tumor and *T. gondii*-challenged mice.

Regulatory T cell and myeloid derived suppressor cell suppression assays

These assays were performed as described (17). Briefly, $CD4^{+}CD25^{-}$ naïve T cells from naïve C57Bl/6 mice were incubated with carboxyfluoroscein succinimidyl ester, a fluorescent dye. Anti-CD3/anti-CD28 beads were added to induce T cell proliferation, and

flow cytometry sorted $CD4^{+}CD25^{hi}$ regulatory T cells or $CD11b^{+}Gr\text{-}1^{hi}$ myeloid derived suppressor cells were added at indicated ratios. Suppression was measured by dye dilution in indicator T cells by flow cytometry 4 days later.

Results and Discussion

We tested the ability of eRapa to inhibit mTORC1 signaling in the small intestine where polyps primarily develop in $Apc^{Min/+}$ mice. Eudragit S-100, the excipient in our eRapa microencapsulated formulation, dissolves at pH greater than 7 and is designed for colonic drug delivery in humans (22). Eudragit S-100 also provides rapamycin stability in mouse chow and improves rapamycin blood levels (12–14), which is ideal for long-term disease prevention studies. The mTORC1 substrate S6 kinase 1 phosphorylates the small ribosomal subunit protein S6 (rpS6) in response to nutrient and growth factor stimuli (23). Therefore, we measured rpS6 phosphorylation in 607–627 day old female C57BL/6 mice fed eRapa for 42 days using the same doses as the NIA Intervention Testing Program (14 and 42 ppm) (12–14). Chronic eRapa treatment inhibited rpS6 Ser240/244 phosphorylation in the proximal and distal segments of the small intestine demonstrating mTORC1 inhibition (Figure 1A). These results were reproduced in the distal intestine from multiple mice (Figure 1A, right panel, Figure 1B). Because rpS6 is encoded by a 5′ TOP-containing mRNA (24) and since mTORC1 regulates translation of 5′ TOP-containing mRNAs (25), we also assayed rpS6 content in lysates. There were no differences in the levels of rpS6 relative to actin, encoded by a non-5′ TOP mRNA (Figure 1C). We found blood levels from treated mice averaged 37 ng/ml and 170 ng/ml for the 14 and 42 ppm group, respectively (a 4.6 fold increase in response to 3 fold higher concentration in the food), Figure 1D. These blood concentrations are higher than the therapeutic range used for organ transplant recipients (26). We also observed a dose response in proximal and distal small intestine tissue levels of rapamycin (Figure 1E). Note the increase in rapamycin levels in distal intestine compared to proximal, which is consistent with the pH gradient approaching neutrality thereby resulting in an increase drug release by Eudragit. Since phosphorylation of rpS6 by S6 kinase plays a role in ribosome biogenesis (27), eRapa-mediated depression of rpS6 phosphorylation indicates decrease ribosome biogenesis, which would inhibit cell growth needed for polyp formation. Thus, eRapa is an effective and convenient method to deliver rapamycin to both the small intestine and blood indicating eRapa could have both local and systemic affects.

We also examined the status of the other major arm of mTORC1 effectors, the Cap-binding translation initiation factor, eIF4E and its binding repressor, 4E-BP1, which are implicated in cancer growth (28). We detected no significant effects on this arm of the pathway including no alterations in the ratio eIF4E to its repressor 4E-BP1 (data not shown). Thus, the major effect of chronic rapamycin treatment directed to the small intestine appears to be a reduction of S6K1 activity manifested by decreased rpS6 phosphorylation.

Chronic rapamycin improved survival for $Apc^{Min/+}$ mice (n=5 females/group) in a dose dependent manner (Figures. 2A, $p<0.005$, logrank test, and 2B for rapamycin blood levels). The $Apc^{Min/+}$ cohort fed control chow lived 164–182 days (median 174) versus the 14 ppm eRapa cohort that lived 570–685 days (median 668). The 42 ppm eRapa fed $Apc^{Min/+}$ mice lived 559–1093 days (median 937, approximately a 5.4-fold increase in life span over our

control cohort). Our Supplementary video 1 at 902 days of age shows that three surviving mice were active and appeared healthy. Previous studies have shown the median life span for C567BL/6J mice is in the range of 692–866 days (*e.g.*, see (10, 11))(shaded area in the graph in Figure 2A). The shaded area of the graph also includes female C57BL/6 survival results reported by Zhang et al., (16), and is representative of the colony in Nathan Shock Aging Animal and Longevity Assessment Core in the Barshop Center for Longevity and Aging Studies, the facility used for housing our *Apc*$^{Min/+}$ mice. Thus, the 14 ppm eRapa-fed mice live close to this range, while four out of five of the 42 ppm eRapa fed mice lived within this range and three surpassed it. In addition, the life span of eRapa fed *Apc*$^{Min/+}$ mice mimics the longevity of wild type C57BL/6Nia mice fed a 14 ppm eRapa diet beginning at 19 months of age (16). The blood levels of rapamycin achieved by 14 ppm and 42 ppm diets in the *Apc*$^{Min/+}$ mice (Figure 2B) were lower than those observed for wild type C57BL/6 mice (Figure 1D). Although the exact reason for this difference is unknown, possibilities include variation in lots of eRapa-containing foods, differential drug absorption in wild type mice compared to *Apc*$^{Min/+}$, and/or slower drug clearance in older mice (607–627 days for wild type, compare to 217 and 308 days *Apc*$^{Min/+}$).

Necropsies were performed on *Apc*$^{Min/+}$ mice that were sacrificed as they became moribund (unable to move and severely dehydrated) or recently after death. At necropsy, there was a reduction in the number of intestinal neoplasms for the 42 ppm cohort as compared to the control and 14 ppm eRapa dose cohorts (Table 1 and Figure 2C). The first mouse to die in the 42 ppm eRapa dose cohort (#15, Table 1) was found soon after death with an intact intestine. There were no gross pathological lesions including intestinal adenomas. Therefore, this mouse appeared to die from something other than intestinal neoplasms. The second mouse to die in this cohort (#13) was sacrificed when she was no longer able to move. Intestinal cancer likely contributed to her death since she had multiple grade 5 adenocarcinomas (Table 1). There was also a large skin ulcer (hyperplasia of squamous epithelium and amyloidosis) on her right lateral side that may have contributed to her death in addition to the intestinal neoplasia. Pathology on the third 42 ppm mouse to die (#11, 937 days of age) revealed one area of focal hyperplasia (mucosal epithelium) in the proximal small intestine and a necrotic liver with grade 4 (moderately severe) lymphoma (Table 1). Thus, this mouse died from a common age-related disease in mice, which is unrelated to the usual cause of death in *Apc*$^{Min/+}$ mice. The fourth mouse to die (# 12, 995 days of age) had one intestinal tumor (moderately severe adenocarcinoma, Table 1). During manuscript review and revision, one *Apc*$^{Min/+}$ mouse was still alive at 1087 days (October 3, 2013, Supplementary video 2), and she appeared to be a healthy old mouse. This mouse died 6 days later with a diagnosis of one grade 2 adenocarcinoma located in the distal segment of the small intestine and a thrombus located in the atrium of the heart (Table 1). Our results suggest that eRapa improves survival of *Apc*-mutant mice better than the delivery methods used in previous studies. eRapa's superior performance was not due to higher rapamycin doses since the 14 ppm dose (2.24 mg/kg/day) was lower than all other reported doses [3 and 10 mg/kg/day by oral gavage (8) and 40 mg/kg food pellet (9)], yet exerted a greater improvement in survival. Analyses of eRapa effects on intestines and tumors of *Apc*$^{Min/+}$ mice were not done in our survival studies since we find necropsy materials unsuitable for immunoassays of mTORC1 status. As mice approach death, they stop eating and drug levels

likely drop significantly. Thus, off-tumor target effects of higher doses of rapamycin could also explain the dose responses we observed and require additional investigation.

As observed by Fujishita et al. study of $Apc^{\Delta 716}$ mice (8), $Apc^{Min/+}$ mice treated with both low and high dose eRapa also developed adenocarcinoma in the intestine, albeit in lower number in the high doses group (Table 1). One possibility for resistance of these tumors to chronic rapamycin is due to resistance of the mTORC1-4E-BP1 axis resulting from prolonged treatment (29–31). In agreement, our cross sectional analysis of mouse organs show no significant effects of chronic rapamycin treatment of 4E-BP1 phosphorylation. However, we do see a trend toward an increase in the ratio of eIF4E to 4E-BP1 levels (data not shown), which has been reported to cause resistance of cancer cells to active-site mTOR inhibitors (28). Finally, treatments with rapamycin or rapalogs results in the activation of a negative feedback loop resulting in activation of mTORC2 that phosphorylates and activates Akt (32, 33), which is a pro-growth, pro-survival pathway suggested to cause rapamycin resistance (34). In our analysis of tissues, we observe no significant effects on Akt Ser473 phosphorylation and a small decrease in Akt levels in distal small intestine (Figure 2D & E). This suggests that rebound activation of this pro-growth signaling pathway is less likely to be a factor in resistant tumors.

We measured the activity of $Apc^{Min/+}$ mice as a surrogate for health. At 164–167 days, the eRapa-fed mice (both doses) exhibited a higher level of activity than control-fed mice during both the light (inactive) and dark (active) phases (Figure 2F, $p<0.001$). eRapa fed mice at both doses showed greater activity during the dark phaseas compared to the light phase ($p<0.001$), whereas control-fed mice show no difference between these phases ($p = 0.415$). Thus, $Apc^{Min/+}$ mice on eRapa maintained diurnal rhythm and activity levels similar to wild type C57BL/6 mice suggesting better health versus $Apc^{Min/+}$ mice on control chow.

Hematocrits were measured (Figure 2G) to determine the severity of intestinal bleeding that causes death. A normal hematocrit for wild type C57BL/6 mice is 44 ± 0.4 (35). At 164 days, two of the control mice exhibited hematocrits of 17% and 19% consistent with their early demise. The 14 ppm eRapa-fed cohort exhibited an average hematocrit of 45% at 217 days (n=5), 40% at 308 days (n=5) and 24% at 588 days (n=4), consistent with adenomas developing much later as verified by end-of-life necropsy. The 42 ppm eRapa-fed cohort exhibited average hematocrits of 46% at 217 days (n=5), 48% at 308 days (n=5), 44% at 588 days (n=4), 37% at 734 days (n=4), 43% (n=3) at 849 days and 40% (n=3) at 902 days. For the 734-day time point, one of the 42 ppm eRapa-fed cohort had an abnormally low hematocrit (27%), suggesting she had intestinal adenomas by this time. This mouse died at 750 days with adenocarcinomas (Table 1). The other mice in the 42ppm eRapa-fed cohort had normal or near normal hematocrits as late as 902 days suggesting a low adenoma burden. Thus, our study supports the possibility that eRapa could be administered prophylactically to FAP or similar patients to delay or prevent intestinal neoplasia and surgical intervention. Rapamycin targeted for release in the upper GI tract might also be an effective preventive for patients who have had colectomies but are at high risk for duodenal polyps and adenocarcinoma.

Rapamycin would need to have few harmful side effects in healthy adults if it is to be used as a prophylactic. One study found rapamycin caused glucose intolerance and insulin insensitivity in C57BLBL/6 mice (36) but these problems were not found in another study on the HET3 mice used by the Intervention Testing Program) (37) suggesting a difference in strain background (C57Bl/6 versus HET3), environment and/or delivery method (IP injections versus intestinal release by eRapa). Another study on C57BL/6 mice found that eRapa extended the life span of male C57BL/6 mice but also caused nephrotoxicity and testicular degeneration (38). However, nephrotoxicity was not found in other studies on C57BL/6 mice (16) or HET-3 mice (14). Thus, the only common toxicity associated with chronic rapamycin treatment is testicular degeneration (39).

Our observation that chronic eRapa exposure extends life span and ameliorates many age-dependent changes supports the notion that eRapa is safe, since toxic compounds would predictably shorten life span, not extend it. Furthermore, eRapa-fed $Apc^{Min/+}$ mice with blood levels of 10–28 ng/ml (Figure 1) (often exceeding human therapeutic levels) live longer than wild type controls is inconsistent with clinically significant immunosuppression or other toxicities. Yet, a criticism of mouse studies is that mice are housed in a pathogen free environment that could mask potential problems with long-term rapamycin treatments.

To test for the possibility that eRapa is immunosuppressive in our setting, we evaluated its effects in several additional, relevant models. In a mouse melanoma model, four month-old mice were fed 14 ppm eRapa for 3 months and then challenged with B16 tumor cells. Tumor growth was similar between the eRapa and control mice (Figure 3A) as previously reported (40). Regulatory T cells and myeloid derived suppressor cells inhibit anti-tumor immunity generally and specifically in the B16 tumor model (17). However, 14 ppm eRapa did not increase regulatory T cell or myeloid derived suppressor cell numbers or suppression in the B16 tumor model (Figure 3B, C). Thus, in this tumor model we found no detrimental immune effects of chronic eRapa and specifically no increase in tumor-mediated immune suppression. In a *T. gondii* challenge model in which we and others have shown that survival following infection with this parasite depends on antigen-specific immunity and interferon-γ production (18, 20), eRapa at 14 ppm did not alter the numbers of antigen-specific $CD8^+$ T cells or their interferon-γ production (Figure 4). In the well-established bm12 transfer model of autoimmunity, eRapa at 14 ppm did not accelerate autoimmune kidney disease, affect Treg numbers or function or alter host T cell numbers or activation versus Eudragit controls (not shown). Similarly, recent publications specifically examining rapamycin immune effects demonstrate that rapamycin boosts immunity to infection (41) including in a transplant model without worsening graft-versus-host disease (42), all underscoring the fact that rapamycin at clinically relevant concentrations that prolong life are not apparently immunosuppressive. When the optimal dose for increasing FAP survival is established, additional work will be required to assess specific immune effects at that dose.

Non-steroidal anti-inflammatory drugs (NSAIDs) and various dietary supplements have been studied as potential chemoprevention agents. A large aspirin study showed daily consumption for five years or longer reduced cancer death in humans including deaths from gastrointestinal cancers (43). Aspirin inhibits mTOR and activates AMPK. However, in contrast to eRapa, aspirin extended median lifespan only in males (44). Aspirin can also

cause significant side effects including gastrointestinal bleeding. Sulindac can be given to delay the progression of polyposis in the retained rectum among patients after colectomy with ileorectal anastomosis in conjunction with a strict endoscopic surveillance regimen (45) while the cyclooxygenase inhibitor, celecoxib reduced colorectal adenomas in a 6-month trial in FAP adults (46). Despite apparent effectiveness, neither sulindac nor celecoxib are recommended as primary prevention agents since reports suggest potential cardiovascular toxicity with COX-2 inhibitors in FAP. Furthermore, non-selective (COX-1 and COX-2) NSAIDs, including sulindac and naproxen, were suggested to increase cardiovascular thrombotic events. Thus, these agents are not ideal for cancer prevention.

Chemoprevention should ideally be well-tolerated, non-toxic, effective and inexpensive, for long-term use. As shown in Figure 2, enteric targeting of mTORC1 inhibitors demonstrates significant potential in meeting these first criteria and cost will decrease significantly when the rapamycin US patent (#5,100,899) expires in 2013.

We now need to determine the optimal eRapa dose to extend life span without toxic side effects and unravel its mechanism(s) of action. In this regard, it is interesting that mTORC1 mediates caloric restriction and rapamycin effects on intestinal crypt stem cell function and renewal (47), and identified as the cells-of-origin of intestinal cancer in *Apc*$^{Min/+}$ mice (48). In sum, this report shows that targeted delivery of rapamycin (which is demonstrated here and elsewhere to be safe and to extend maximum lifespan in preclinical models) could be an effective prevention strategy for FAP patients and others predisposed to intestinal cancer. Further work is required to assess potential benefits of chronic eRapa treatment versus possible risks associated with chronic mTOR inhibition, which experience in transplant and cancers patients clearly demonstrate are manageable (49, 50), before moving into cancer prevention clinical trials.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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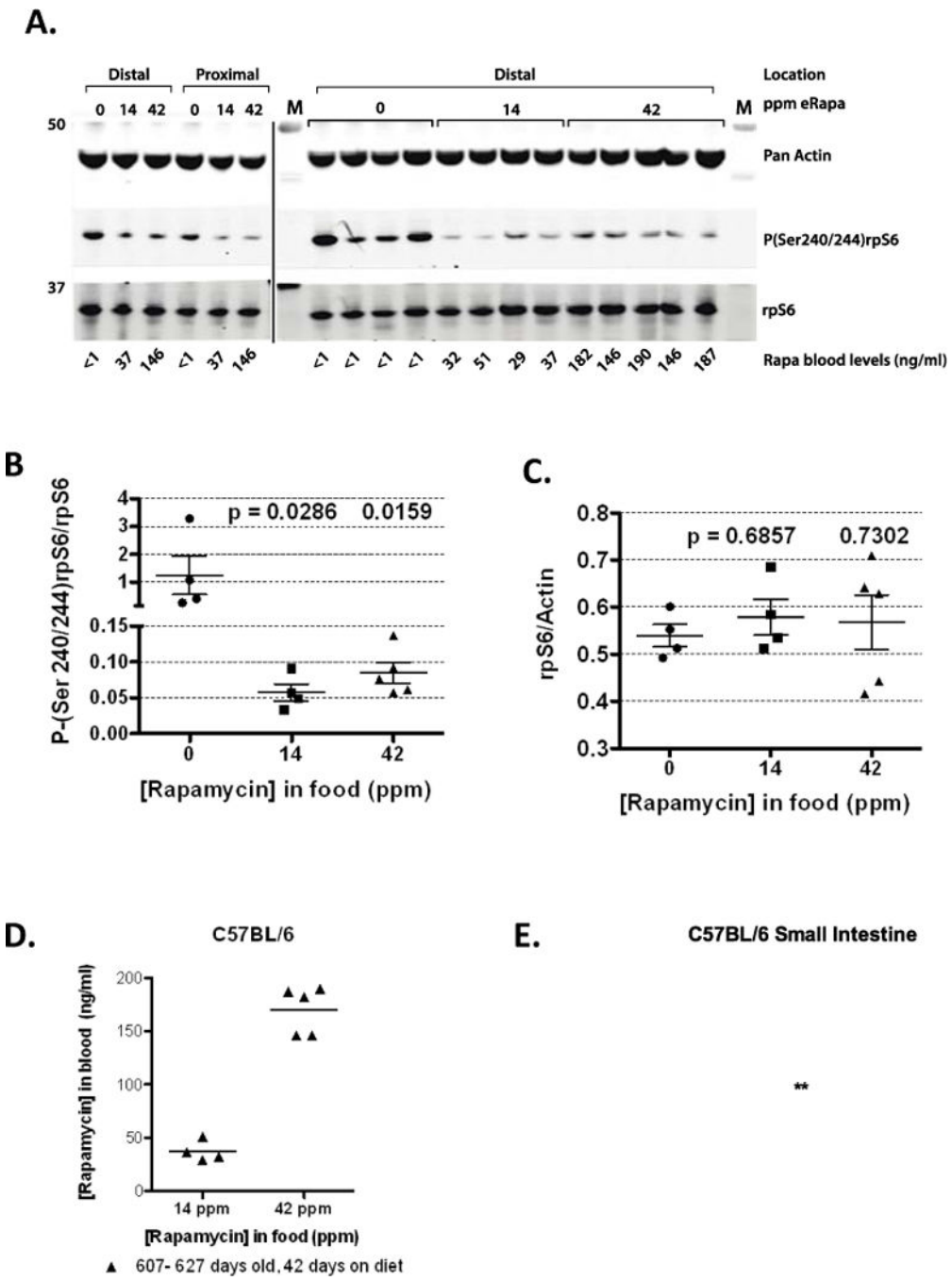

Figure 1.

Pharmacodynamic data for C57BL/6 mice fed eRapa. (A) Western blots developed with indicated antibodies. C57Bl/6 mice (607–627 days old) were fed eRapa for 42 days, intestine dissected and immunoassayed as described in methods. Segments of the small intestine (proximal and distal) were assayed as indicated. Graphs showing the quantitation of the immunoblot data as measured by the ratio of intensity values for the phosphorylation state-dependent signal (P(240/244)rpS6) to phosphorylation state-independent (rpS6) signal (B)and rpS6 to actin antibody signal (C). (D) Rapamycin blood levels of C57BL/6 mice at

607–627 days of age (42 days on eRapa diets, which averaged 37 ng/ml and 170 ng/ml for the 14 and 42 ppm group, respectively). (E) Rapamycin tissue levels in proximal and distal small intestine of C57BL/6 mice (607–627 days of age and 42 days on diet). Mean levels (μg/g) are: proximal 14 ppm 91.62 ± 14.91, 42 ppm 266.7 ± 26.35; distal 14 ppm 627.3 ± 135.5, 42 ppm 1488 ± 141.6. P values are: 0.001 (proximal 14 ppm compared to 42 ppm); 0.035 (distal 14 ppm compared to 42 ppm); 0.0077 (proximal 14 ppm compared to distal 14 ppm); <0.0001 (proximal 42 ppm compared to distal 42 ppm).

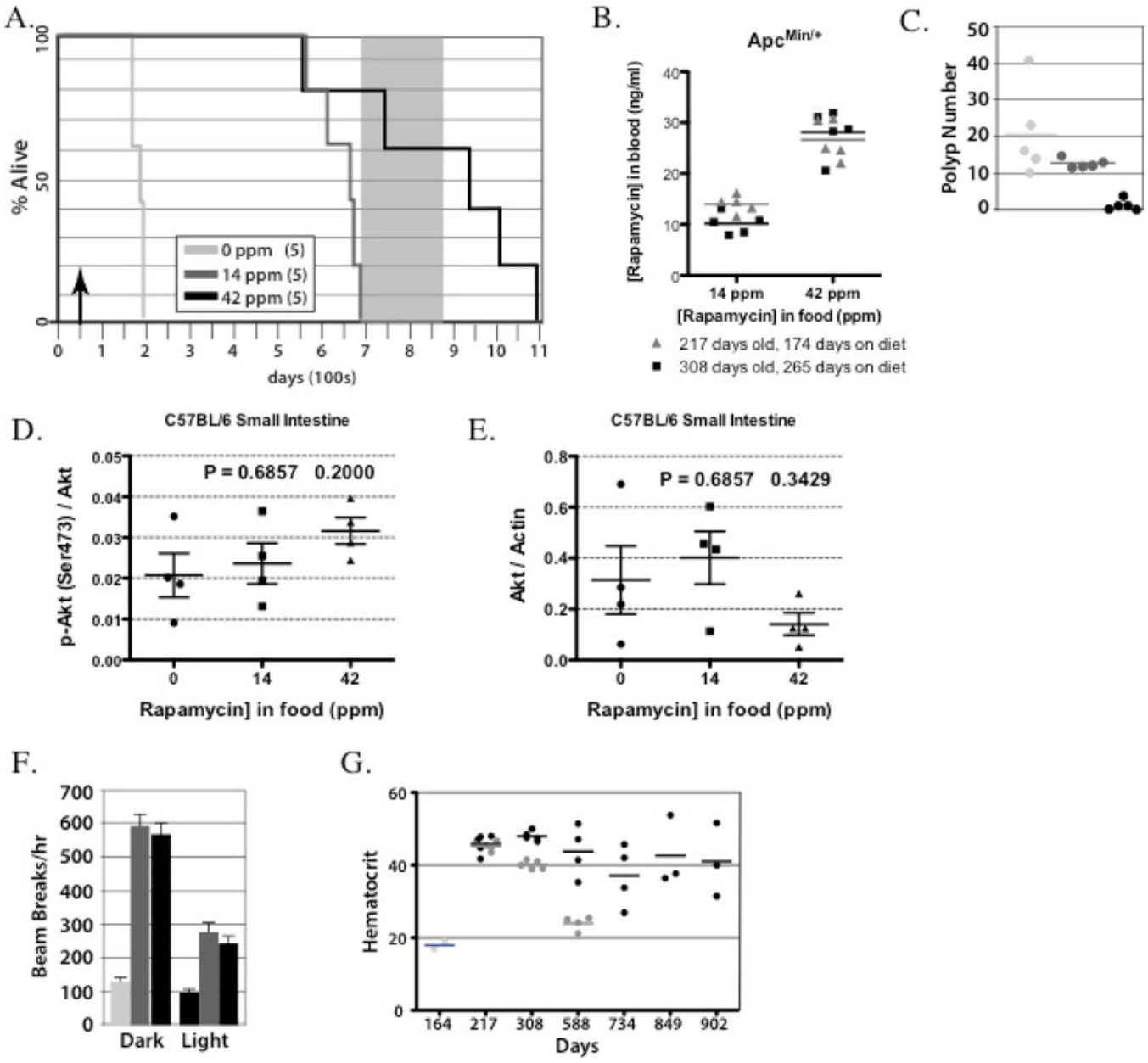

Figure 2.

eRapa increases life and health span for *Apc*$^{Min/+}$ mice. (A) eRapa increases life span for *Apc*$^{Min/+}$ mice. The grey box represents a range of median life spans for C57BL/6J female mice taken from previous studies {Wijnhoven et al., 2005, #5122; Yuan et al., 2009, #78681; Zhang et al., 2013, #44400}, which is also representative of syngeneic colonies at the Barshop Institute animal facility used in our study. Note that 60% of the 42 ppm eRapa fed *Apc*$^{Min/+}$ mice have lived beyond this range. (B) Rapamycin blood levels in *Apc*$^{Min/+}$ mice at 217 days (174 days on eRapa diets, which averaged 14 ng/ml or 26.6 ng/ml on 14 ppm or 42 ppm diets, respectively) or at 308 days of age (265 days on eRapa diet, which averaged 10.2 ng/ml or 28.1 ng/ml on 14 ppm or 42 ppm diets, respectively) (**C**) Polyp count for *Apc*$^{Min/+}$ mice at the time of death. Note the first and third mouse that died from

the 42 ppm cohort had no polyps. (**D&E**) Graph showing quantification of phosphorylation state-dependent signal P(Ser473)Akt to phosphorylation state-independent signal (D) and Akt to actin antibody signal (E). (**F**) eRapa improves physical activity in $Apc^{Min/+}$ mice. Graphed is the mean number of beam breaks (activity) for light (=inactive) and dark (=active) phases of the day. The food area of the cage is excluded. (**G**) eRapa maintains normal hematocrits in $Apc^{Min/+}$ mice. Note the dose response. Also note the normal hematocrit as compared to wild type C57Bl/6 mice (~40%) in the high dose group even at late time points when many wild type C57Bl/6 mice would die from natural causes.

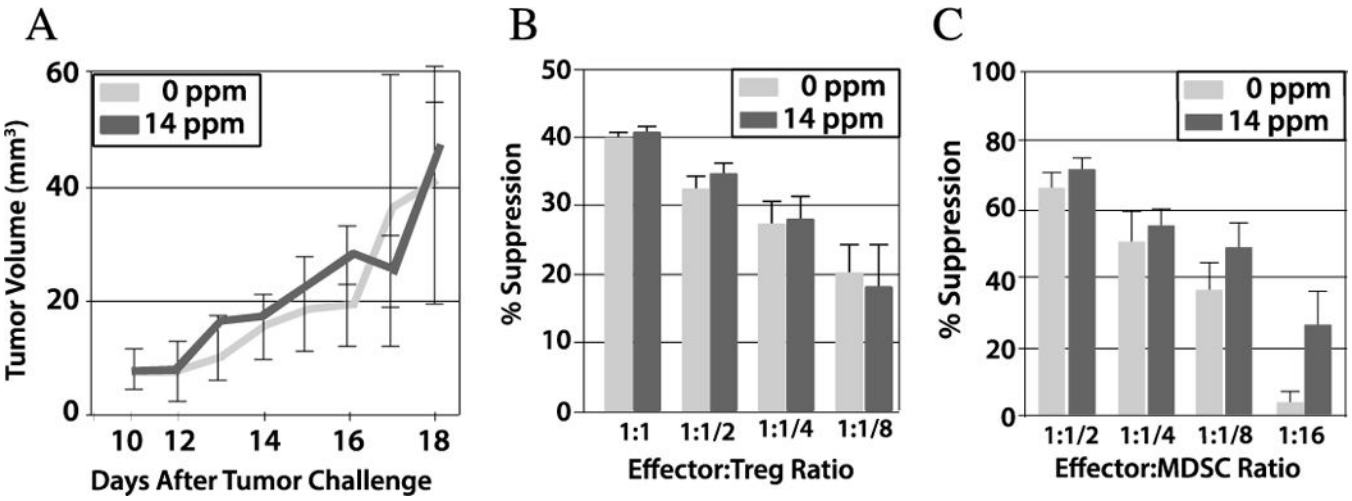

Figure 3.

eRapa does not boost tumor growth or tumor-mediated immune suppression. C57BL/6J mice (n=6) were given either Eudragit or 14 ppm eRapa for 3 months and challenged with B16 melanoma. **A.** Tumor volume. **B.** Spleen Treg suppression assessed at various $CD4^+$responder: Treg ratios. **C.** Spleen myloid derived suppressor cell (MDSC) suppression assessed at various $CD4^+$responder:MDSC ratios. P>0.05 for all in **B** and **C**. Treg, regulatory T cell; MDSC, myeloid derived suppressor cell.

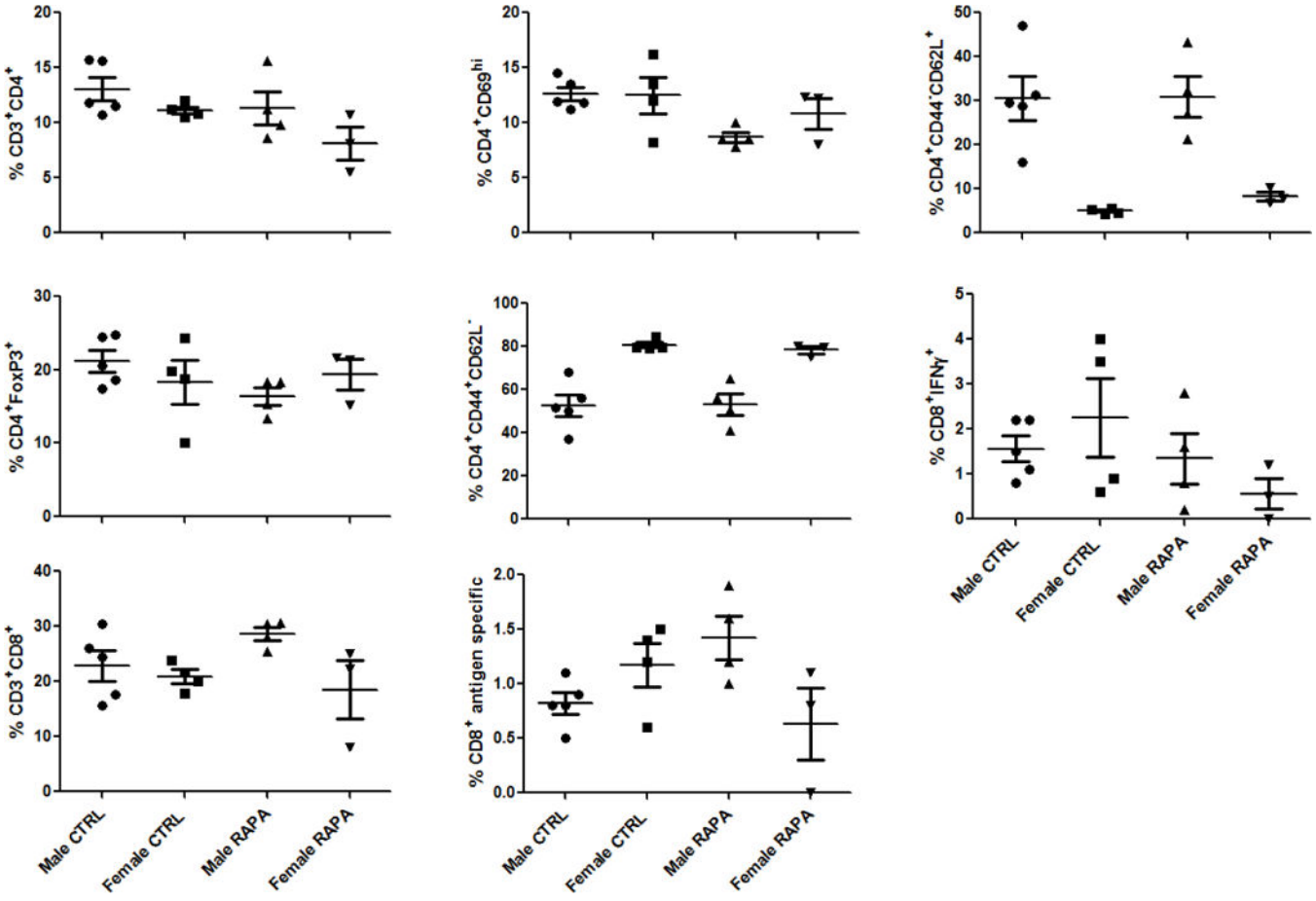

Figure 4.

eRapa does not reduce mediators of *T. gondii* immunity during active infection. BL6 mice were infected with 10,000 Me49 strain *T. gondii* tachyzoites expressing an OVA reporter antigen by intraperitoneal challenge and mice were sacrificed 11 days later. Immune events were acquired from single-cell spleen suspensions on a Becton-Dickinson LSRII. Individual symbols represent individual mice and the bar is the median value. CD69 is an activation marker. Foxp3 is a regulatory T cell marker. $CD44^{+}CD62L^{-}$ cells are memory T cells. $CD44^{-}CD62L^{+}$ cells are naive T cells. CTRL, mice fed Eudragit control chow. RAPA, mice fed chow with rapamycin 14 ppm. IFNγ, interferon-γ. "Antigen specific" refers to $CD8^{+}$ T cells recognizing the SIINFEKL sequence of the OVA antigen engineered into *T. gondii* tachyzoites. No differences were statistically significant.

Table 1

Pathology 42 ppm *Apc*$^{Min/+}$

Mouse	Age at Death (days)	Diagnosis	Diagnosis Grade	Location
#11	937	focal hyperplasia lymphoma necrosis	1 4 3	proximal small intestine Liver
#12	995	1 adenocarcinoma	4	distal small intestine
#13	750	3 adenocarcinomas 1 adenocarcinoma 1adenoma	5 5 5	distal small intestine proximal small intestine middle small intestine
#14	1093	1 adenocarcinoma thrombus	2 2	distal small intestine atrium
#15	559	not determined	NA	no intestinal polyps

Grades: 0 = normal; 1 = minimal; 2 = mild; 3 = moderate; 4 = moderately severe; 5 = severe

NA: not applicable