Published in final edited form as:

Cancer Prev Res (Phila). 2015 May ; 8(5): 400–409. doi:10.1158/1940-6207.CAPR-14-0313-T.

Prevention of carcinogen and inflammation-induced dermal cancer by oral rapamycin includes reducing genetic damage

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Abstract

Cancer prevention is a cost-effective alternative to treatment. In mice, the mechanistic target of rapamycin (mTOR) inhibitor rapamycin prevents distinct spontaneous, non-inflammatory cancers, making it a candidate broad-spectrum cancer prevention agent. We now show that oral microencapsulated rapamycin (eRapa) prevents skin cancer in dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) carcinogen-induced, inflammation-driven carcinogenesis. eRapa given before DMBA/TPA exposure significantly increased tumor latency, reduced papilloma prevalence and numbers, and completely inhibited malignant degeneration into squamous cell carcinoma. Rapamycin is primarily an mTORC1-specific inhibitor, but eRapa did not reduce mTORC1 signaling in skin or papillomas, and did not reduce important pro-inflammatory factors in this model, including p-Stat3, IL-17A, IL-23, IL-12, IL-1 β , IL-6, or TNF- α . In support of lack of mTORC1 inhibition, eRapa did not reduce numbers or proliferation of CD45⁻CD34⁺CD49f^{mid} skin cancer initiating stem cells *in vivo* and marginally reduced epidermal hyperplasia. Interestingly, eRapa reduced DMBA/TPA-induced skin DNA damage and the *hras* codon 61 mutation that specifically drives carcinogenesis in this model, suggesting reduction of DNA damage as a cancer prevention mechanism. In support, cancer prevention and DNA damage reduction effects were lost when eRapa was given after DMBA-induced DNA damage *in vivo*. eRapa afforded picomolar concentrations of rapamycin in skin of DMBA/TPA-exposed mice, concentrations that also reduced DMBA-induced DNA damage in mouse and human fibroblasts *in vitro*. Thus, we have identified DNA damage reduction as a novel mechanism by which rapamycin

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The authors have no relevant financial conflicts of interest to declare.

can prevent cancer, which could lay the foundation for its use as a cancer prevention agent in selected human populations.

Keywords

cancer prevention; rapamycin; carcinogen; mTOR; DNA repair

Introduction

The US economic impact of cancer is hundreds of billions of dollars annually (1). Advances in treating cancer generally remain slow, and treatments are often toxic and expensive. Thus, a widely-applicable cancer prevention strategy would be a significant step towards improving quality of life and reducing cancer-associated health care costs. Rapamycin or related agents could be part of such a strategy, as rapamycin extends life of genetically heterogeneous mice, even when started in late life (2). The mechanism for rapamycin-mediated lifespan extension appears to include prevention of many cancer types (3–6), but whether rapamycin-mediated lifespan extension is from cancer prevention versus healthspan improvements is unclear (7, 8). Rapamycin inhibits existing tumor growth in distinct mouse cancer models (9, 10), is currently being tested in clinical trials for multiple cancers (11, 12), and a rapamycin analogue (everolimus) is FDA-approved for renal cell carcinoma, further supporting rapamycin as a potential broad spectrum cancer prevention agent.

Rapamycin inhibits mechanistic (or mammalian) target of rapamycin (mTOR), a serine/threonine kinase that is an important cellular growth and metabolism regulator whose signaling is elevated in many cancers. Thus, mTOR inhibition in tumors could be a significant mechanism for rapamycin-mediated cancer treatment and prevention (13, 14). However, other potential mechanisms for rapamycin-mediated cancer prevention are understudied. Mechanistic insights could identify other cancer prevention molecules or pathways, and help identify optimal populations for cancer prevention studies. To understand novel mechanisms of rapamycin-mediated cancer prevention, we fed mice a microencapsulated rapamycin formulation (eRapa) at a life-extending 14 ppm dose (2) in the well-defined DMBA (7,12-dimethylbenz(a)anthracene)/TPA (12-O-tetradecanoylphorbol-13-acetate)-induced skin carcinogenesis mouse model, in which inflammatory and carcinogenic signaling mechanisms are well-defined and replicate important aspects of human inflammation-driven cancers (15–17).

mTOR signals integrate multiple cues (*e.g.*, growth factors, energy, nutrients) to regulate important cellular processes (*e.g.*, cell cycle, autophagy, lipid/protein biosynthesis) (18). mTOR forms two distinct complexes, mTORC1 and mTORC2 with rapamycin considered primarily an mTORC1 inhibitor (19). We now show that eRapa affords only picomolar concentrations of skin rapamycin yet significantly prevents skin carcinogenesis without classical mTORC1 inhibition in target tissue, or effects on known cancer initiating cells and pro-inflammatory mediators in this model. It reduced DNA damage *in vivo* in DMBA/TPA-treated skin and reduced the *hras* gene codon 61 mutation responsible for skin carcinogenesis in this model, suggesting a potential broad-spectrum cancer prevention

mechanism. Consistent with this conclusion, eRapa's cancer prevention and DNA damage reduction properties are abrogated when eRapa is given after DMBA-induced DNA damage. We also verified that picomolar rapamycin concentrations reduce DMBA-induced DNA damage in mouse and human fibroblast cell lines *in vitro*. Since cancer treatment with rapamycin often induces nanomolar levels of rapamycin in target tissues (11), these data suggest that far less rapamycin could be needed for cancer prevention versus treatment. Chronic low doses could limit potential adverse effects of rapamycin or other mTOR inhibitors.

Materials and Methods

Mice and eRapa

Mice (wild type, $\beta\delta$ TCR KO) were 8-12-week old male C57BL/6J (BL6, Jackson Labs) maintained in specific pathogen free conditions. For pre-DMBA studies, mice were placed on eRapa or Eudragit control (Rapamycin Holdings) *ad libitum* 30 days before carcinogen challenge, and for study duration at the life-extending 14 ppm rapamycin concentration (2). For post-DMBA studies, mice were initiated with DMBA first, then fed eRapa or Eudragit one week later for one month before starting TPA.

Cell lines and rapamycin

For *in vitro* studies, 3T3 mouse fibroblasts and human foreskin fibroblasts (HFF, both from the American Type Culture Collection but not authenticated) were cultured in DMEM (Corning Life Sciences) plus 10% fetal bovine serum. Rapamycin (InvivoGen) and DMBA (Sigma) were diluted in culture media for *in vitro* studies.

DMBA/TPA two-stage skin carcinogenesis

The protocol was adapted from (20). Briefly, mice were shaved dorsally one week before application of 100 μ g DMBA (Sigma) in acetone. Mice were then placed in disposable housing (Taconic) for one week, then 12.5 μ g 12-O-tetradecanoylphorbol-13-acetate (TPA, LC Labs) in ethanol was applied twice weekly for 24 weeks. Mice were assessed weekly for body weight and tumors, which were identified as skin lesions >1 mm persisting >1 week. Tumor latency is time to 50% prevalence of tumor-bearing mice. Tumor area is length $\times$ width. At study conclusion, tumors were excised, formalin-fixed, paraffin-embedded, and H&E stained. A blinded pathologist performed tumor histopathology. Photomicrographs used an Olympus IX71 inverted scope.

Bone mineral density and content

Mouse femurs and tibias were surgically dissected and placed into 70% ethanol. Bone mineral density and content were measured by dual-energy X-ray absorptiometry (Lunar PIXImus Densitometer, GE Medical Systems) and analyzed with the manufacturer's software.

Isolation of genomic DNA and quantitative PCR for *hras* mutation

Genomic DNA (gDNA) was isolated from epidermal scrapings of mice using the Quick-gDNA MiniPrep kit (Zymo Research) per manufacture's protocol and tested for purity by NanoDrop (Thermo Scientific). To quantitate the CAA → CTA mutation in *hras* codon 61, validated primer sequences (21) were used to generate custom Taqman primers and probe (ABI Life Technologies). Quantitative PCR reaction was run on a MyIQ2 thermocycler (BioRad) using Taqman Gene Expression Master Mix (ABI Life Technologies). Obtained Ct values were subtracted against *β-actin* (ABI Life Technologies) and expression was calculated using the equation 2^{-Ct} . Normalization to naïve skin (no DMBA/TPA) yielded 2^{-Ct} .

Tissue preparation and immunoblot

Snap frozen dorsal skin, epidermal scrapings, and tumor tissues were homogenized in RIPA buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM disodium EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1% triton-X100) plus 1 mM phenylmethylsulphonyl fluoride and Halt protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford) and handled as described for the bullet blender protocol (Next Advance). Homogenization was for 5 min, speed 10 (skin) and 4 min, speed 9 (tumor) plus beads. Lysates were centrifuged at 140,000 rpm, 4° C, 10 min. Cleared samples were collected into pre-chilled Eppendorf tubes without disturbing fat. Protein was measured by Bradford method (Thermo Scientific). 50 µg protein was separated by 4–15% sodium dodecyl sulfate polyacrylamide gels (BioRad Hercules), transferred to PVDF membranes (GE water and Process Technologies), blocked in Tris-buffered saline (pH 7.4) plus 0.1% Tween-20 and 5% skim milk, and incubated overnight at 4° C with 1:1000 diluted phospho- and/or total antibodies against indicated proteins (Cell Signaling) plus anti-mouse *β-actin* (Santa Cruz Biotechnology). Membranes were incubated with HRP-conjugated antibodies, 1 h. Proteins were detected by enhanced chemiluminescence (Pierce). Band quantification was by Image J software (National Institutes of Health).

Cytokine detection

100 mg epidermal scrapings were placed in Eppendorf tubes with 300 µl PBS plus protease and phosphatase inhibitor (Thermo Scientific), then mechanically disrupted with a bullet blender (Next Advance) and homogenized for 5 min, speed 10 with beads. Lysates were centrifuged at 14,000×g, 4° C, 10 min. Supernatants were collected as for immunoblots. A mouse 26-plex kit (Ebioscience) tested skin lysates per the manufacturer's protocol. Data was acquired and analyzed on a Luminex 200 (Millipore).

Tissue rapamycin

Whole blood by cardiac puncture was placed into EDTA tubes. Blood, dorsal skin, and epidermal scrapings from the same mice were stored at −80° C until rapamycin concentration were determined as described (4).

Flow cytometry

Epidermal digests for general single cell suspensions and specifically for keratinocyte stem cells were done as described (22, 23). We used validated commercial reagents: LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies), anti-mouse CD3, CD34, Langerin, Ki-67 (Ebioscience), anti-mouse TCR $\gamma\delta$, TCR β chain, CD4, CXCR3 (Biolegend), anti-mouse CD45, CD49f, p-H2AX pS139, CD8, FITC BrdU (BD Biosciences), on Becton-Dickinson hardware and software as described (24).

Immunofluorescence and microscopy

Dorsal skin sections (5 μ m) were deparaffinized 2 $\times$ 10 min then hydrated 1 $\times$ 5 min each through graded ethanol (100%, 95%, 70%, 0%). Antigen retrieval was by incubation in 10 mM sodium citrate, pH 6.0 for 15 min at 95° C, then ambient temperature (in solution) for 20 min before water rinse. Endogenous peroxidases were quenched in 3% hydrogen peroxide, 5 min. Slides were then blocked, 20 min in TBS-T (containing 0.02% Tween 20) + 4% BSA. Primary antibody incubations were done at 4° C using anti-pH2AX (Millipore) diluted 1:200. Secondary antibody incubations used anti-mouse IgG Alexa Fluor 488 (Life Technologies) diluted 1:1000. Both antibodies were diluted into blocking buffer. Then slides were rinsed and washed 2 $\times$ 5 min in TBS-T, followed by 10 min incubation in 0.5 μ g/ml DAPI. Slides were then dehydrated 1 $\times$ 5 min each through graded ethanol, then coverslip mounted (Fluoroshield, Sigma). Slides were imaged on a Carl Zeiss Axioplan2 microscope with AxioVision Release 4.5 software.

Statistics

Statistical tests were by GraphPad Prism 5.00 using appropriate analyses as indicated.

Results

eRapa prevents DMBA/TPA-induced dermal neoplasia

To determine if eRapa prevents carcinogen (DMBA)-induced and inflammation (TPA)-driven dermal neoplasia, we fed WT C57BL/6J (BL6) male mice eRapa or Eudragit control for one month, then initiated tumors with 100 μ g DMBA and promoted with TPA, 25 μ g/week for 24 weeks (21). eRapa significantly increased tumor latency (11 versus 15 weeks for Eudragit or eRapa, respectively, Fig. 1A), and reduced tumor prevalence (Fig. 1A), tumor multiplicity (Fig. 1B) and total tumor burden (Fig. 1C). There was no difference in average size of individual tumors (Fig. 1D) indicating that eRapa's primary effect was reduction of tumor numbers (representative photographs, supplementary Fig. S1). Thus, eRapa prevents dermal neoplasia in DMBA/TPA skin carcinogenesis. eRapa-mediated lifespan extension is greater in females versus males (2) but both sexes exhibited similar eRapa-mediated protection in our DMBA/TPA studies (data not shown).

eRapa prevents malignant papilloma degeneration

Benign DMBA/TPA-induced papillomas can degenerate into cancers (squamous cell carcinoma or spindle cell carcinoma) (15). eRapa completely protects against malignant degeneration (17.9% of mice on Eudragit with cancers versus 0% on eRapa) in WT BL6

mice (Fig. 1E). Thus, in addition to preventing benign neoplasia, eRapa also prevents DMBA/TPA-induced carcinomas that can be fatal. Tumors in Eudragit controls included papillomas, keratoacanthomas and well differentiated squamous cell carcinomas as reported (15) (representative photomicrographs, Fig. 1F).

eRapa protection occurs without local mTORC1 suppression or high skin rapamycin concentrations

Rapamycin can suppress mTOR signaling that drives cell growth and contributes to DMBA/TPA carcinogenesis (25). We assessed mTOR signals in skin from DMBA/TPA-treated mice. Contrary to expectation and published results using topical rapamycin (26), eRapa did not reduce epidermal or whole skin p-mTOR S2448 versus Eudragit (Figs. 2A, B and supplementary Figs. S2A, B). Rapamycin is considered primarily an mTORC1 inhibitor (19), although at high concentrations or long duration, mTORC2 can be inhibited (27). Strikingly, activation of the mTORC1 downstream targets, 4E-BP1 and rpS6, were unaffected by eRapa in epidermis and whole skin (Figs. 2A, B and supplementary Figs. S2A, B). p-Akt S473 is an mTORC2 substrate that unexpectedly was decreased in epidermis and whole skin in eRapa treated mice versus Eudragit (Figs. 2A, B and supplementary Figs. S2A, B). Thus, eRapa-mediated protection against DMBA/TPA-induced dermal carcinogenesis appears not to be through suppressed skin mTORC1 signaling. To test if eRapa inhibited mTORC1 in tumor and not at-risk skin, we assessed papillomas from eRapa and Eudragit control mice, which exhibited similar mTORC1 and mTORC2 signaling versus skin (Figs. 2C, D). These results differ from signaling in other organs in eRapa-treated mice. For instance, in adipose (2) and intestines (4), this eRapa feeding for similar duration reduces p-rpS6 S240/244. Low rapamycin skin delivery could explain these unexpected results. Blood rapamycin was 60–70 ng/ml in eRapa mice, consistent with reports using eRapa for longevity extension (2). Skin and epidermal rapamycin concentrations (10–20 pM) in DMBA/TPA mice were ~4000-fold lower versus blood (Fig. 2E), a concentration that does not inhibit proliferation of a skin cancer cell line or other cancer cell lines *in vitro* (data not shown).

eRapa protection does not require suppression of known pro-inflammatory factors or significant reductions in keratinocyte stem cell numbers, proliferation, or epidermal hyperplasia

Inflammatory factors in the DMBA/TPA model that drive dermal carcinogenesis include IL-17A (17), IL-23 (28), IL-12, IL-1 β , IL-6, TNF- α (29) and phospho(p)-Stat3 (16), but eRapa did not reduce these in epidermis (Figs. 3A, B). CD34⁺CD49f^{mid} suprabasal keratinocyte stem cells in hair follicle bulge region are the likely cancer initiating cells in this model (23, 25). Supporting lack of suppressed pro-inflammatory factors and mTORC1 (Figs. 2A-D), numbers and proliferation (BrdU incorporation) of suprabasal and other keratinocyte stem cells were unaffected (Fig. 3C). As other cells besides keratinocyte stem cells could be targeted by eRapa, the whole epidermal cell compartment was assessed for proliferation (Ki-67) and by epidermal hyperplasia measurements, an indicator of tumor promotion (15). eRapa did not alter the proportion of epidermal cells expressing Ki-67 (Fig. 3D), and although eRapa statistically significantly reduced epidermal hyperplasia in DMBA/TPA-treated mice (Fig. 3E), the magnitude was lower than following topical rapamycin in

this model (26). Thus, eRapa prevents DMBA/TPA-induced skin carcinogenesis without affecting many known pro-carcinogenic factors in this model, suggesting other yet-unidentified mechanisms.

eRapa is not a calorie restriction mimetic

eRapa could reduce food intake, mimicking caloric restriction that reduces cancer and extends lifespan (30), but final weights and weight gain during these studies were similar in Eudragit and eRapa mice (supplementary Fig. S3A). Further, caloric restriction predictably reduces bone mineral density (31), but bone mineral density and content were equivalent in these Eudragit and eRapa mice (supplementary Fig. S3B). Therefore, eRapa-mediated caloric restriction is not a mechanism for cancer prevention here, consistent with a recent report demonstrating that eRapa metabolic effects differ from caloric restriction (32).

eRapa mediates skin neoplasia prevention in the absence of T cells

mTOR signals affect T cell differentiation and function including effects on T cells mediating anti-tumor immunity (33). As we excluded several immune factors important for eRapa efficacy in this model (Figs. 3A, B), we next assessed T cells, as they also play important, but contrasting roles in the DMBA/TPA model: $\gamma\delta$ T cells are protective (20, 34) whereas $\alpha\beta$ T cells generally are pro-tumorigenic (20, 22). eRapa did not significantly alter $\alpha\beta$ or $\gamma\delta$ T cell prevalence or numbers in skin (Figs. 4A, B). To test a role for T cells in eRapa-mediated skin cancer protection in this model, we fed syngeneic $\beta\delta$ TCR knockout mice lacking all T cells with eRapa or Eudragit and induced skin tumors with DMBA/TPA. Despite lack of T cells, eRapa protected mice from DMBA/TPA-induced benign papillomas (Figs. 4C and supplementary Fig. S4). The absence of T cells largely prevented malignant papilloma degenerations in both treatment groups (Fig. 4D), consistent with a detrimental role for T cells in this model (20, 22), but precluding specific assessment of eRapa cancer protection effects.

eRapa protection against tumorigenesis is associated with reduced skin DNA damage and reduction *in vivo* of the specific *hras* mutation that is carcinogenic in this model

DMBA induces well-characterized skin DNA damage (35) required for carcinogenesis in this model, and rapamycin was recently reported to reduce DNA damage in human fibroblasts (36). To test if eRapa's cancer prevention effects included DNA damage protection, we delayed eRapa feeding in WT mice until one week post-DMBA, followed by TPA to promote tumorigenesis. eRapa administration after DMBA abolished tumorigenesis protection as measured by tumor multiplicity ($p = 0.30$ versus Eudragit) and squamous cell carcinoma prevalence ($p = 1.0$ versus Eudragit, Fig. 5A) compared to eRapa given pre-DMBA (Fig. 1). Additionally, protection against general epidermal DNA damage (phospho(p)-H2AX staining for double stranded DNA breaks) was lost when eRapa was given post-DMBA (Fig. 5B). As DMBA in skin predominantly induces a CAA $\rightarrow$ CTA mutation in codon 61 of the *hras* oncogene, we assessed this genetic defect by quantitative PCR and showed that it was unaltered in epidermis of mice given eRapa versus Eudragit post-DMBA (Fig. 5C). Next, we confirmed that eRapa given pre-DMBA reduced general DNA damage in the skin of DMBA/TPA mice by immunofluorescence (Fig. 5D), and

Western blot (Fig. 5E). Strikingly, eRapa given pre-DMBA reduced the *hras* mutation mediating carcinogenesis in this model (Fig. 5F).

Rapamycin *in vitro* at levels achievable in skin protects from DNA damage by DMBA

To test if the low skin rapamycin concentrations in DMBA/TPA mice (Fig. 2E) could reduce DMBA-induced DNA damage, mouse (3T3) and human (HFF) fibroblasts were pre-treated with rapamycin at picomolar concentrations *in vitro* and then induced with DMBA, resulting in reduced DMBA-induced DNA damage in both cell lines (Figs. 6A, B). By contrast, but supporting *in vivo* results, rapamycin after DMBA *in vitro* no longer reduced DMBA-induced DNA damage (Fig. 6C). DMBA is metabolized *in vivo* by Langerhans cells to an active carcinogenic metabolite (21), but eRapa did not alter Langerhans cell prevalence or numbers in DMBA/TPA treated epidermis (Fig. 6D).

Discussion

We previously reported that enterically delivered microencapsulated rapamycin (eRapa) was well tolerated and significantly prevented spontaneous, non-inflammatory neoplasias, including cancers of distinct histologies in various tissues such as intestine and the central nervous system (3, 4). We now show that eRapa also prevents carcinogen-induced, inflammation-driven skin neoplasia and cancer. As many human cancers arise in chronic inflammation (37), our demonstration of eRapa-mediated prevention of inflammation-driven cancers further extends the potential application of rapamycin in human cancer prevention.

Rapamycin and other mTOR inhibitors are postulated to suppress tumor growth by directly inhibiting cancer cell mTOR signals (13). Nonetheless, we found that eRapa did not alter epidermal or whole skin p-mTOR S2448 despite significantly reducing skin neoplasia and cancer. mTOR is comprised of two signaling complexes: mTORC1 and mTORC2 (19). In naïve, untreated skin, mTORC1 and mTORC2 activation are noted (38). Rapamycin is considered primarily an mTORC1 inhibitor, but chronic rapamycin also inhibits mTORC2 (27). We found no evidence that eRapa inhibited mTORC1 signals (*e.g.*, p-4E-BP1, p-rpS6) in skin or tumors of DMBA/TPA-treated mice. Reduced p-Akt S473 is consistent with reduced mTORC2 signaling. Our unexpected signaling results are due either to chronic eRapa administration or to oral administration, as four applications of topical rapamycin prior to TPA reduced epidermal p-mTOR and p-rpS6, and increased p-Akt (26), contrasting with our findings. Checkley *et al.* (26), attributed their rapamycin cancer prevention effects to reduction in mTORC1 signaling, and as a result, reducing epidermal hyperplasia. In our studies, lack of mTORC1 suppression with eRapa is supported by a marginal reduction in epidermal hyperplasia with eRapa. More work is required to understand the relevance of these signals to eRapa-mediated tumor suppression, but effects are important as selective mTORC2 inhibition (39) or combined with mTORC1 inhibition (40) are promising cancer therapeutic strategies. mTORC1 could be specifically inhibited in CD34⁺CD49f^{mid} skin cancer initiating cells driving DMBA/TPA carcinogenesis (25), but this is unlikely as mTORC1 was not inhibited in papillomas in eRapa-treated mice, and numbers and proliferation of cancer initiating cells were unaffected. Additional considerations include mTOR inhibition of stromal cells and possible rapamycin effects on stem cell differentiation

into cancer. This latter possibility accords with our finding that eRapa was less able to suppress neoplasia than malignant degeneration, suggesting that rapamycin primarily reduces cancer in this model by limiting the benign to malignant transition. eRapa-mediated DNA damage reduction we now report is also consistent with this concept (41).

mTOR has significant immune effects on mediators of tumorigenesis and anti-tumor immunity (42). eRapa did not reduce pro-carcinogenic IL-17A, IL-23, IL-12, IL-1 β , IL-6, TNF- α or p-Stat3 here, and did not alter detrimental or protective epidermal $\alpha\beta$ or $\gamma\delta$ T cell populations, respectively, consistent with the finding that eRapa-mediated neoplasia protection occurred in the absence of T cells. Nonetheless, T cell effects are not entirely excluded, and these data additionally demonstrate important rapamycin-mediated cancer immune effects that merit further investigations. For instance, rapamycin enhanced memory CD8⁺ T cells that was exploited in a tumor vaccine (43).

Prior studies with rapamycin and DMBA/TPA have either focused on giving rapamycin after DMBA/TPA-induced tumors develop (9) or after DMBA initiation but before TPA promotion (26). The former experimental setup is more relevant to cancer treatment than prevention and both studies omitted possible effects of rapamycin on DMBA-induced DNA damage, which we discovered as a novel eRapa mechanism. DNA damage critical to DMBA/TPA tumorigenesis was reduced by eRapa as evidenced by reduced p-H2AX, indicating double stranded DNA breaks, and by reduced mutated *hras* that specifically promotes carcinogenesis here (35). These observations accord with the possibility that eRapa prevents malignant skin tumor degeneration by reducing DNA damage at initiation. In support, eRapa administration after DMBA abolished tumorigenesis protection. Langerhans cells metabolize DMBA to promote carcinogenesis (21) but eRapa did not alter their numbers. Additional work is required to understand potential Langerhans cell DMBA metabolism effects in this model. Full understanding of metabolic and other cancer prevention mechanisms will help identify at-risk patient populations most likely to benefit from rapamycin cancer prevention and could help improve rapamycin (or similar agents) as cancer prevention drugs.

In our studies in *Apc*^{Min/+} mice, eRapa potently suppressed intestinal neoplasia (4), but intestinal rapamycin concentration was ~7000-fold higher than skin rapamycin concentrations here. Low skin rapamycin concentration suggests that either it is nonetheless sufficient for suppressing tumorigenesis directly in skin, or that higher rapamycin concentrations elsewhere affect other factors (*e.g.*, immunity) that regulate dermal carcinogenesis. eRapa allows better pharmacokinetic control of drug release versus unencapsulated rapamycin and delivers high concentrations to distinct anatomic compartments (4). It could be useful for human trials, especially if high rapamycin tissues concentrations are desired. However our current studies show that high local tissue rapamycin is not necessarily required for effective cancer prevention, and that low rapamycin concentrations effect prevention in specific settings, which could reduce undesired adverse effects. Thus, additional studies of tissue delivery and clinical effects are warranted.

Whether rapamycin is ultimately the best candidate for human cancer prevention is unclear as legitimate concern for its use and use of similar agents in large populations has been raised (44). For instance, rapamycin is considered a potent immunosuppressant. However it is often used in conjunction with other immunosuppressive drugs, and thus its individual effects in humans are incompletely understood. Currently, there is no published study testing rapamycin effects in normal humans. There is also growing evidence that rapamycin and other mTOR inhibitors boost anti-pathogen immunity (45, 46) and improve vaccine responses in elderly humans (47), demonstrating beneficial immune effects. Our findings that eRapa prevents cancer in spontaneous and inflammation driven cancer models, and extends lifespan to normal in *Apc*^{Min/+} mice prone to spontaneous intestinal neoplasia and early death from tumor-associated bleeding (4) is inconsistent with rapamycin mediating detrimental immunosuppression and supports its use as a potential cancer prevention agent

Thus, eRapa is a cancer prevention agent that could be safe and effective in normal hosts. The mechanism for rapamycin-mediated cancer prevention is likely complex and multifactorial. We identified reduced skin DNA damage as one likely mechanism, but cannot exclude other mechanisms, including immune, metabolic or anti-proliferative effects. As mechanistic details are better understood, additional drugs or targets could be identified for cancer prevention. Such information will help determine optimal use of agents, and optimal populations for clinical testing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Supported by AG038048, TL1TR001119 and CA180377 to VD; CA170491, CA54174, The Holly Beach Public Library, The Owens Foundation and the Skinner endowment to TJC.

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Figure 1. eRapa prevents DMBA/TPA-induced dermal neoplasia and malignant degeneration WT BL6 mice on Eudragit or eRapa given DMBA/TPA for 24 weeks. **A.** Tumor prevalence is the proportion of mice with at least 1 visible tumor (>1 mm) lasting > 1 week. p-value, log-rank test. **B.** Tumor multiplicity (average papilloma number/mouse). p-value, 2-way ANOVA. **C.** Total tumor burden (tumor area/mouse). p-value, unpaired *t* test. **D.** Area per tumor is calculated as total tumor burden/number of tumors/mouse. p-value, unpaired *t* test. Mice without tumors were excluded. **E.** Number of mice with squamous cell carcinoma

(SCC) lesions, n=28–29/group. p-value, Fisher’s exact test. **F.** Representative photomicrographs from Eudragit-fed mice. Original magnification, 40x.

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Figure 2. eRapa protection occurs without local mTORC1 suppression or high skin rapamycin concentrations

WT BL6 mice on Eudragit or eRapa given DMBA/TPA for 24 weeks. **A.** Epidermal lysates blotted for indicated proteins. Five mice for Eudragit and eRapa. The numbers refer to the number of tumors per mouse for each lane. **B.** Western blot quantification, N=5/group, from **A.** p-value, unpaired *t* test. **C.** Skin papilloma lysates blotted for indicated proteins. Five mice for Eudragit and eRapa. **D.** Western blot quantification, N=5/group, from **C.** p-value, unpaired *t* test. **E.** Whole skin and epidermis assayed for rapamycin by liquid chromatography/tandem mass spectrometry, N=5/group. p-value, Mann Whitney Test. Dashed line: assay detection limit.

Figure 3. eRapa protection does not require suppression of known pro-inflammatory factors or significant reductions in keratinocyte stem cell numbers, proliferation, or epidermal hyperplasia WT BL6 mice on Eudragit or eRapa given DMBA/TPA for 24 weeks. **A.** Epidermal lysates, N=8/group, assayed by Luminex. p-value, unpaired *t* test. **B.** Epidermal lysates Western blotted for indicated proteins. Five representative mice for Eudragit and eRapa groups in Western blot on top. Western blot quantification (N=10/group, bottom). **C.** Absolute numbers (left) and proliferation (right) by BrdU staining of basal (CD34⁺CD49^{fhi}) and suprabasal (CD34⁺CD49^{fmid}) keratinocyte stem cells by flow cytometry, N=8/group. p-value, unpaired *t* test. Data representative of 3 experiments. Flow cytometry gating on left. p-value, unpaired *t* test. **D.** Epidermal cells stained for Ki-67 and assessed by flow cytometry, N=8/group. p-value, unpaired *t* test. **E.** >300 epidermal thickness measurements from hematoxylin and eosin sections, N=13–14/group. p-value, unpaired *t* test.

Figure 4. eRapa mediates skin neoplasia prevention in the absence of T cells

WT BL6 mice on Eudragit or eRapa and given DMBA/TPA for 24 weeks. Digested epidermis assayed by flow cytometry for **A.** frequency (left) and absolute numbers (right) of $\alpha\beta$ T cells (CD45⁺ β TCR⁺) and **B.** frequency (left) and absolute numbers (right) of $\gamma\delta$ T cells (CD45⁺ $\gamma\delta$ TCR⁺), N=14–15/group. p-value, unpaired *t* test. **C.** T cell receptor knockout ($\beta\delta$ TCR KO, lacking all T cells) and syngeneic WT BL6 (data from Fig. 1 as control) mice on Eudragit or eRapa given DMBA/TPA for 24 weeks. Left: tumor multiplicity. p-value, 2-way ANOVA. Right: total tumor burden. p-value, unpaired *t* test. **D.** Number of mice with squamous cell carcinoma (SCC, n=8–9/group). p-value, Fisher's exact test.

Figure 5. eRapa protection against tumorigenesis is associated with reduced skin DNA damage and reduction *in vivo* of the specific *hras* mutation that is carcinogenic in this model

A. Tumor multiplicity (left) and SCC prevalence (right) in WT BL6 mice fed Eudragit or eRapa one week after DMBA (post-DMBA), then promoted with TPA. p-value, 2-way ANOVA for tumor multiplicity and Fisher's exact test for SCC prevalence. SCC, squamous cell carcinoma. **B.** Digested epidermis assayed by flow cytometry for p-H2AX. Post-DMBA data compared to WT BL6 mice fed Eudragit or eRapa for 1 month before DMBA (pre-DMBA). p-value, unpaired *t* test. **C.** Epidermal genomic DNA from post-DMBA mice assayed by quantitative PCR for the CAA → CTA mutation in codon 61 of *hras* gene.

Normalized to naïve (no DMBA/TPA) epidermis, N=4–5/group. p-value, unpaired *t* test. **D.** Representative immunofluorescence staining of whole skin from pre-DMBA mice using anti phospho-H2AX (p-H2AX) and DAPI. Secondary antibody: anti-mouse IgG Alexa Fluor 488. Three eRapa or Eudragit mice representative of N=10/group for immunofluorescence studies are shown in the bottom 3 rows. **E.** Epidermal lysates blotted for indicated proteins. Five mice shown for Eudragit and eRapa (left). Western blot quantification (right, N=5/group). p-value, unpaired *t* test. **F.** Epidermal genomic DNA from pre-DMBA mice assayed by quantitative PCR for the CAA → CTA mutation in codon 61 of *hras* gene. Normalized to naïve (no DMBA/TPA) epidermis, N=4/group. p-value, unpaired *t* test.

Figure 6. Rapamycin *in vitro* at levels achievable in skin protects from DNA damage by DMBA
A. Mouse fibroblasts (3T3) and **B.** human foreskin fibroblasts (HFF) pre-treated with indicated doses of rapamycin for 24 hours before DNA damage was induced with 16.4 $\mu\text{g/ml}$ DMBA, then assessed by flow cytometry for phospho-H2AX (p-H2AX) staining. p-value, unpaired *t* test. **C.** 3T3 mouse fibroblasts pre-incubated with DMBA for 24 hours to induce DNA damage, then treated with 200 pM rapamycin without continuing DMBA for 24–72 hours. DNA damage assessed by flow cytometry p-H2AX staining. p-value, unpaired *t* test. **D.** Digested epidermis assayed by flow cytometry for prevalence (left) and numbers (right) of Langerhans cells (CD11b⁺Langerin⁺). p-value, unpaired *t* test.