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 mTOR inhibitor rapamycin prevents distinct spontaneous, noninflammatory 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 proinflammatory factors in this model, including p-Stat3, IL17A, IL23, IL12, IL1β, IL6, or TNFα. 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 can prevent cancer, which could lay the foundation for its use as a cancer prevention agent in selected human populations.

Introduction

The U.S. 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 toward 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 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. mTOR signals integrate multiple cues (e.g., growth factors, proinflammatory factors), and helps 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, ref. 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 proinflammatory 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. Because 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, β8 TCR KO) were 8- to 12-week-old male C57BL/6J (BL6, Jackson Laboratories) 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 1 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 ATCC but not authenticated) were cultured in DMEM (Corning Life Sciences) plus 10% FBS. 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 1 week before application of 100 μg DMBA (Sigma) in acetone. Mice were then placed in disposable housing (Taconic) for 1 week, then 12.5 μg 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 × width. At study conclusion, tumors were excised, formalin-fixed, paraffin-embedded, and hematoxylin and eosin 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 qPCR for hras mutation
Genomic DNA (gDNA) was isolated from epidermal scrapings of mice using the Quick-gDNA MiniPrep kit (Zymo Research) per manufacturer’s protocol and tested for purity by NanoDrop (Thermo Scientific). To quantitate the CAA → CITA mutation in hras codon 61, validated primer sequences (21) were used to generate custom TaqMan primers and probe (ABI Life Technologies). qPCR reaction was run on a MyiQ2 thermocycler (Bio-Rad) 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−ΔΔCt. Normalization to naïve skin (no DMBA/TPA) yielded 2−ΔΔCt.

Tissue preparation and immunoblot analysis
Snap-frozen dorsal skin, epidermal scrapings, and tumor tissues were homogenized in RIPA buffer (20 mmol/L Tris-HCl, pH 8.0, 150 mmol/L NaCl, 1 mmol/L disodium EDTA, 1 mmol/L EGTA, 2.5 mmol/L sodium pyrophosphate, 1 mmol/L β-glycerophosphate, 1% triton-X100) plus 1 mmol/L phenylmethylsulphonyl fluoride and Halt protease and phosphatase inhibitor cocktail (Thermo Scientific) and handled as described for the bullet blender protocol (Next Advance). Homogenization was for 5 minutes, speed 10 (skin) and 4 minutes, and speed 9 (tumor) plus beads. Lysates were centrifuged at 14,000 rpm, 4 °C, 10 minutes. Cleared samples were collected into prechilled Eppendorf tubes without disturbing fat. Protein was measured by Bradford method (Thermo Scientific). Of note, 50 μg protein was separated by 4% to 15% SDS polyacrylamide gels (Bio-Rad Hercules), transferred to PVDF membranes (GE water and Process Technologies), blocked in TBS (pH 7.4) plus 0.1% Tween-20 and 5% skim milk, and incubated overnight at 4 °C with 1:1,000 diluted phospho- and/or total antibodies against indicated proteins (Cell Signaling Technology) plus anti-mouse β-actin (Santa Cruz Biotechnology). Membranes were incubated with HRP-conjugated antibodies for 1 hour. Proteins were detected by enhanced chemiluminescence (Pierce). Band quantification was by ImageJ software (NIH).

Cytokine detection
Of note, 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 minutes, speed 10 with beads. Lysates were centrifuged at 14,000 × g at 4 °C for 10 minutes. Supernatants were collected as for immunoblot analysis. A mouse 26-plex kit (Ebioscience) tested skin lysates per the manufacturer’s protocol. Data were 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 digest for general single cell suspensions and specifically for keratinocyte stem cells were done as described (22,
We used validated commercial reagents: LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies), anti-mouse CD3, CD4, Langerin, Ki-67 (Ebioliscience), anti-mouse TCR β chain, CD4, CXCR3 (Biolegend), anti-mouse CD45, CD49f, p-H2AX pS139, CD8, FITC BrdUrd (BD Biosciences), on Becton-Dickinson hardware and software as described (24).

Immunofluorescence and microscopy

Dorsal skin sections (5 μm) were deparaffinized for 2 × 10 minutes then hydrated for 1 × 5 minute each through graded ethanol (100%, 95%, 70%, 0%). Antigen retrieval was by incubation in 10 mmol/L sodium citrate, pH 6.0, for 15 minutes at 95°C, then ambient temperature (in solution) for 20 minutes before water rinse. Endogenous peroxidases were quenched in 3% hydrogen peroxide for 5 minutes. Slides were then blocked for 20 minutes in TBS-T (containing 0.02% Tween 20) + 3% 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 for 2 × 5 minutes in TBS-T, followed by 10-minute incubation in 0.5mg/mL DAPI. Slides were then dehydrated for 1 × 5 minute each through graded ethanol, and then coverslip mounted (Fluoroshield, Sigma). Slides were imaged on a Carl Zeiss Axioplan2 microscope with AxioVision Release 4.5 software.

Statistical analysis

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

Results

eRapa prevents DMBA/TPA-induced dermal neoplasia.

To determine whether eRapa prevents carcinogen (DMBA)-induced and inflammation (TPA)-driven dermal neoplasia, we fed WT C57BL/6 (BL6) male mice eRapa or Eudragit control for one month, then initiated tumors with 100 μg DMBA, and promoted with TPA, 25 μg/week for 24 weeks (21). eRapa significantly increased tumor latency (11 vs. 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;...
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 and B and Supplementary Fig. S2A and S2B). 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 and B and Supplementary Fig. S2A and S2B). p-Akt S473 is an mTORC2 substrate that unexpectedly was decreased in epidermis and whole skin in eRapa-treated mice versus Eudragit (Fig. 2A and B; Supplementary Fig. S2A and S2B). Thus, eRapa-mediated protection against DMBA/TPA-induced dermal carcinogenesis appears not to be through suppressed skin mTORC1 signaling. To test whether 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 and 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 to 70 ng/mL in eRapa mice, consistent with reports using eRapa for longevity extension (2). Skin and epidermal rapamycin concentrations (10–20 pmol/L) in DMBA/TPA mice were approximately 4,000-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 proinflammatory 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 IL17A (17), IL23 (28), IL12, IL1β, IL6, TNFα (29), and phospho(p)-Stat3 (1.6), but eRapa did not reduce these in epidermis (Fig. 3A and B). CD34+CD49+ suprabasal keratinocyte stem cells in hair follicle bulge region are the likely cancer-initiating cells in this model (23, 25). Supporting lack of suppressed proinflammatory factors and mTORC1 (Figs. 2A–D), numbers and proliferation (BrdUrd 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
eRapa protection does not require suppression of known proinflammatory 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 analysis on top. Western blot quantification (N = 10/group, bottom). C, absolute numbers (left) and proliferation (right) by BrdUrd staining of basal (CD34+CD49fmed) keratinocyte stem cells by flow cytometry, N = 8/group. P value, unpaired t test. Data representative of three 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.

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Figure 3.

eRapa protection does not require suppression of known proinflammatory 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 analysis on top. Western blot quantification (N = 10/group, bottom). C, absolute numbers (left) and proliferation (right) by BrdUrd staining of basal (CD34+CD49fmed) keratinocyte stem cells by flow cytometry, N = 8/group. P value, unpaired t test. Data representative of three 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.

model (26). Thus, eRapa prevents DMBA/TPA-induced skin carcinogenesis without affecting many known procarcinogenic 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). Furthermore, 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 antitumor immunity (33). As we excluded several immune factors important for eRapa efficacy in this model (Fig. 3A and B), we next assessed T cells, as they also play important, but contrasting roles in the DMBA/TPA model: γδ T cells are protective (20, 34), whereas αβ T cells generally are protumorigenic (20, 22). eRapa did not significantly alter αβ or γδ T-cell prevalence or numbers in skin (Fig. 4A and B). To test a role for T cells in eRapa-mediated skin cancer protection in this model, we fed syngeneic 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 (Fig. 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 whether eRapa’s cancer prevention effects included DNA damage protection, we delayed eRapa feeding in WT mice (36). To test whether eRapa’s cancer prevention effects included DNA damage protection, we delayed eRapa feeding in WT mice until 1 week after DMBA, followed by TPA to promote tumorigenesis. eRapa administration after DMBA abolished tumorigenesis protection as measured by tumor multiplicity (P = 0.30 vs. Eudragit) and squamous cell carcinoma prevalence (P = 1.0 vs. Eudragit; Fig. 5A) compared with eRapa given pre-DMBA (Fig. 1). In addition, protection against general epidermal DNA damage (phospho[p]-H2AX staining for double-stranded DNA breaks) was lost when eRapa was given after DMBA (Fig. 5B). As DMBA in skin predominantly induces a CAA → CTA mutation in codon 61 of the hras oncogene, we assessed this genetic defect by qPCR and found that it was unaltered in epidermis of mice given eRapa versus Eudragit after 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 analysis (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 whether 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 (Fig. 6A and B). In contrast, but supporting in vitro results, rapamycin after DMBA in vivo 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, noninflammatory 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

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 γδ T cells (CD45+γδ TCR+) and (B) frequency (left) and absolute numbers (right) of γδ T cells (CD45+γδ TCR+). N = 14-15/group. P value, unpaired t test. C, T-cell receptor knockout (β8 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, two-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 exact test.
reducing skin neoplasia and cancer. mTOR is composed of two signaling complexes: mTORC1 and mTORC2 (19). In naïve, untreated skin, mTORC1 and mTORC2 activations 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

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 1 week after DMBA (post-DMBA), then promoted with TPA. P value, two-way ANOVA for tumor multiplicity and the Fisher exact test for SCC prevalence. SCC, squamous cell carcinoma. B, digested epidermis assayed by flow cytometry for p-H2AX. Post-DMBA data compared with 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 qPCR for the CAA → CTA mutation in codon 61 of hras gene. Normalized to naive (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 = 4/group). P value, unpaired t test. F, epidermal genomic DNA from pre-DMBA mice assayed by qPCR for the CAA → CTA mutation in codon 61 of hras gene. Normalized to naive (no DMBA/TPA) epidermis, N = 4/group. P value, unpaired t test.
administration, as four applications of topical rapamycin before TPA reduced epidermal p-mTOR and p-rpS6, and increased p-Akt (26), contrasting with our findings. Checkley and colleagues (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⁺ 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 antitumor immunity (42). eRapa did not reduce procarcinogenic IL17A, IL23, IL12, IL1β, IL6, TNFα, or p-Stat3 here, and did not alter detrimental or protective epidermal ββ or γδ 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 ApcMin/+ mice, eRapa potently suppressed intestinal neoplasia (4), but intestinal rapamycin concentration was approximately 7,000-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 somewhere 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) are inconsistent with rapamycin mediating detrimental immunosuppression and support 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 antiproliferative 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.

Disclosure of Potential Conflicts of Interest

Z.D. Sharp is a consultant/advisory board member for Rapamycin Holdings, Inc. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): V. Dao, S. Pandeswara, D. Callaway, Z.D. Sharp, T.J. Curiel

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