Inhibition of Akt Enhances the Chemopreventive Effects of Topical Rapamycin in Mouse Skin

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Abstract

The PI3Kinase/Akt/mTOR pathway has important roles in cancer development for multiple tumor types, including UV-induced nonmelanoma skin cancer. Immunosuppressed populations are at increased risk of aggressive cutaneous squamous cell carcinoma (SCC). Individuals who are treated with rapamycin (sirolimus, a classical mTOR inhibitor) have significantly decreased rates of developing new cutaneous SCCs compared with those that receive traditional immunosuppression. However, systemic rapamycin use can lead to significant adverse events. Here, we explored the use of topical rapamycin as a chemopreventive agent in the context of solar-simulated light (SSL)-induced skin carcinogenesis. In SKH-1 mice, topical rapamycin treatment decreased tumor yields when applied after completion of 15 weeks of SSL exposure compared with controls. However, applying rapamycin during SSL exposure for 15 weeks, and continuing for 10 weeks after UV treatment, increased tumor yields. We also examined whether a combinatorial approach might result in more significant tumor suppression by rapamycin. We validated that rapamycin causes increased Akt (S473) phosphorylation in the epidermis after SSL, and show for the first time that this dysregulation can be inhibited in vivo by a selective PDK1/Akt inhibitor, PHT-427. Combining rapamycin with PHT-427 on tumor prone skin additively caused a significant reduction of tumor multiplicity compared with vehicle controls. Our findings indicate that patients taking rapamycin should avoid sun exposure, and that combining topical mTOR inhibitors and Akt inhibitors may be a viable chemoprevention option for individuals at high risk for cutaneous SCC. Cancer Prev Res 9(3): 215–24. ©2016 AACR.

Introduction

Nonmelanoma skin cancer (NMSC) is the most common malignancy worldwide and is rapidly increasing in incidence, representing an expanding public health burden of considerable magnitude (1–3). NMSCs can be categorized as squamous cell carcinomas (SCC) or basal cell carcinomas (BCC), which account for approximately 16% and 80% of all skin cancers, respectively. The remaining 4% are accounted for primarily by melanomas. Cutaneous SCCs account for approximately 2,000 deaths per year in the United States, a number which is likely underreported (4, 5). NMSCs are strongly associated with sun/ultraviolet (UV) light exposure. Natural sunlight contains 90% to 99% UVA (320–400 nm) and 1% to 10% UVB (200–280 nm), depending upon the geographic location. Despite increasing rates of sunscreen use, the effectiveness of this primary prevention approach may not be significant, possibly due to increased exposure times and inefficient re-application of sunscreens (6). NMSCs also represent a major cause of morbidity after organ transplantation. SCCs are the most common cutaneous malignancies seen in this population, with a 65- to 100-fold greater incidence in organ transplant recipients compared with the general population (7). Clearly, more effective prevention and treatment regimes for NMSC are required, especially for individuals at high risk.

Several signaling pathways have been implicated in the UV-mediated carcinogenesis process. Lately, the contribution of the serine/threonine protein kinase mTOR (mammalian target of rapamycin) has attracted attention in several types of cancer due to its ability to integrate stimuli from metabolites, extracellular signals and stressors to regulate translation, proliferation, and autophagy in the cell (8). Phosphoinositide3-kinase (PI3K)/Akt/mTOR signaling is known to be dysregulated in several types of cancer, making the pathway a promising therapeutic target (9). We and others have shown that UV light exposure induces PI3K/Akt/mTOR signaling along with mitogen activated protein kinase (MAPK) signaling in model systems and in human skin (10–15). Dysregulation of PI3Kinase/Akt/mTOR signaling during the progression from normal skin to SCC has also been validated.
The context of solar UV-induced skin tumorigenesis has not been fully characterized. Early studies with rapamycin using adenosarcoma-based xenografts in mice revealed dose-dependent inhibition of tumor growth, metastasis and angiogenesis compared with vehicle controls (21). In contrast, CsA, the most widely used immunosuppressive drug, increased tumor size and angiogenesis (21). Although adverse events can make compliance difficult when rapamycin is administered systemically (20), a retrospective analysis suggests that oral dosing with this drug was an effective long-term therapy for patients suffering from tuberous sclerosis complex skin tumors (22). Collectively, these studies suggest that rapamycin may be a promising preventative agent for populations at risk for certain skin malignancies.

When considering the options for drug delivery to the skin, topical application may be a reasonable approach for mTOR inhibitors in order to achieve local signaling pathway modulation while avoiding the challenges associated with systemic immunosuppression. Clinically, psoriasis and classic Kaposi sarcoma have been successfully treated with topical rapamycin (23, 24). In mouse models, topical pretreatment with rapamycin suppressed acute UVB-induced skin thickening by inhibiting epidermal proliferation (14). However, the effects of topical mTOR inhibition in the context of solar UV-induced skin tumorigenesis has not been reported. Here, we present the first study to examine rapamycin in the context of solar simulated light (SSL) treatment in mice. This study compares the effects of both concurrent rapamycin and SSL treatments (prevention) versus treating with UV to create tumor-prone skin, and then applying rapamycin (intervention). Crucially, the effect of cotreatment with rapamycin and the Akt inhibitor PHT-427 on tumor-prone skin is also presented.

Materials and Methods

Materials

Rapamycin was purchased from LC Laboratories. PHT-427 was donated by PHusis Therapeutics Inc. Antibodies for Western blots were purchased from Santa Cruz Biotechnology (phospho-p70S6 Kinase T389 #sc-11759, total p70S6 #9379), Cell Signaling Technologies (phospho-Akt S473 #4060; phospho-mTOR S2448 #2971; α-tubulin #21285; phospho-S6 Ribosomal Protein #5364), or Sigma-Aldrich (β-actin #A5441). All other reagents were obtained from Sigma-Aldrich.

Solar-simulated light treatment

Female SKH-1 hairless mice (SKH1-Hr6®) at 5 to 8 weeks of age were purchased from Charles River Laboratories for chronic and acute SSL studies. All mouse studies were performed in accordance with protocols approved by the University of Arizona Institutional Animal Care and Use Committee (IACUC). SSL exposure was performed using a bank of 6 UVA340 bulbs (Q-lab Corporation). Fluence intensity was determined using an ILT 1700 Radiometer equipped with UVA and UVB meters (International Light Technologies). Output of the bulbs was determined to be 93% UVA and 7% UVB.

Acute solar UV exposures used a dose of 105 kJ/m² UVA/6.4 kJ/m² UVB. Mice (n = 3) were then sacrificed at the appropriate time points and their back skins were harvested and snap frozen. The frozen epidermis was then scraped, lysed and sonicated as described for Western blot analysis (25). For some acute experiments, mice were treated topically on their backs from the shoulder blades to the top of the hips with agents (rapamycin or PHT-427) dissolved in 200 μL of acetone 48 hours prior, 24 hours prior, and 1 hour prior to the SSL exposure.

For tumorigenesis studies (n = 20), mice were exposed to SSL three times a week. Upon receipt from Charles River Laboratories, mice were randomly assigned to treatment groups. The number of mice per group (20) was calculated to provide enough power to detect a 50% decrease in tumor multiplicity with 95% confidence. UV exposures were initiated at 15.4 kJ/m² UVA/1.2 kJ/m² UVB and increased by 10% weekly until a holding dose of 36.5 kJ/m² UVA/2.8 kJ/m² UVB was reached at week 10. After week 15, UV treatments were stopped at a cumulative dose of 427 kJ/m² UVA/33 kJ/m² UVB. Mice were weighed and observed weekly, and tumors were recorded at first detection, typically after week 15. Tumor burden (area) was calculated by multiplying the length by the width of each tumor in millimeters. Average tumor burden was calculated by dividing the sum of the individual tumor burdens each week by the number of the mice in the treatment group. Multiplicity was defined as the sum of the tumors for each treatment group divided by the number of mice in that group each week. Mice were always sacrificed 24 hours after their final drug treatment. No adverse reactions or changes in group weekly weights due to drug applications were observed.

Rapamycin prevention/intervention tumorigenesis study.

The acute control group received topical applications of 200 μL acetone (vehicle) on the treatment area 1 hour prior to each SSL treatment (15 weeks), which then continued three times a week until the end of the study. The treatment area on each mouse was defined as the back skin from the shoulder blades to the top of the hips. The rapamycin prevention group, which could also be referred to as the “early and protracted treatment” group, received topical rapamycin (50 nmol/back) in 200 ul. acetone 1 hour prior to each SSL exposure and continuing three times a week after UV stopped. The rapamycin intervention group received topical acetone treatments 1 hour prior to each SSL exposure and then switched to topical rapamycin treatments three times a week after UV stopped (i.e., a “post UV” group).

Rapamycin + PHT-427 intervention tumorigenesis study.

In this experiment, all mice received no treatment during the 15 weeks of solar UV exposure, then were split into four groups: acetone (vehicle) control, rapamycin (50 nmol/back), PHT-427 (3.7 μmol/back), or rapamycin + PHT-427 (same doses). All drugs were applied topically in 200 μL three times a week until sacrifice.

Reverse phase protein microarray (RPPA) analysis

Epidermal proteins from naïve female SKH-1 mice treated acutely with SSL were processed for RPPA analysis according to established protocols (16). Briefly, frozen back skin was scraped and ground to powder using a frozen mortar and pestle. The powder was then lysed using a 1:1 mixture of T-PER Tissue Protein...
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Acute SSL treatment activates mTOR/Akt signaling pathways in mouse epidermis

Treatement of SKH-1 hairless mice with an acute dose of SSL resulted in rapid phosphorylation of Akt (S473) and mTOR (S2448), with subsequent phosphorylation of downstream p70S6 kinase. These responses were evident as soon as the acute treatment stopped (0 hour, or about 30 minutes under the bulb), peaking for mTOR at 1 hour after UV and declining by 4 hours after UV (Fig. 1A, 8 hours and 24 hours not shown).

The same epidermal samples were subjected to RPPA protein pathway activation analysis and individually tested for the activation and/or expression of multiple phospho- and total proteins within the Akt–mTOR signaling pathway as kinases and their known substrates (Fig. 1B). Triplicate averages for representative proteins are shown on the heat map, where red

treatments, media were changed to serum-free DMEM including antibiotics and DMSO or drug for the indicated times.

Western blot analysis

Frozen back skin was scraped with a razor blade in order to remove the epidermis and ground to a powder as described for RPPA. The powder was then lysed and sonicated for Western blot analysis (16). Keratinocytes were lysed in RIPA lysis buffer including 1× HALT phosphatase/protease inhibitors (Invitrogen) and briefly sonicated on ice as described (34). Protein concentration was determined using the DC protein assay for both skin and cell culture lysates (BioRad). Lysates were separated by SDS-PAGE, transferred to PVDF membranes, blocked, and probed with primary and secondary antibodies as described (34). For keratinocytes, Western blots shown are representative of n = 3. For back skins, data are representative of n = 2 (3–4 mice/group). All phospho–protein-related images are from fresh blots. Loading controls were performed on relevant stripped blots.

Statistical analysis

Measures, standard deviations, and other summary statistics were calculated by treatment group for all outcome measurements. Primary analysis compared end of study total tumor burden and total tumor counts (multiplicity) among the four treatment groups in the two rapamycin tumorigenesis studies. In the rapamycin prevention/intervention tumorigenesis study, the primary analysis compared end of study total tumor burden and total tumor counts (multiplicity) among the two treatment groups and an acetone control group. Cross-sectional analyses used the Mann–Whitney or Wilcoxon rank-sum test. Uncorrected P values are presented for these cross-sectional analyses.

After appropriate transformations to achieve approximate normality, mixed-effect models were fit for tumor burdens to test for differences over time by treatment groups and to measure subject-specific variability. A random slope was added to account for different growth rates between mice. Tumor counts data were analyzed using a mixed-effects Poisson model to test for differences over time by treatment groups and to measure subject-specific variability. A random slope was added to account for different growth rates between mice. Stata V14 was used for all statistical analyses.

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The same epidermal samples were subjected to RPPA protein pathway activation analysis and individually tested for the activation and/or expression of multiple phospho- and total proteins within the Akt–mTOR signaling pathway as kinases and their known substrates (Fig. 1B). Triplicate averages for representative proteins are shown on the heat map, where red
indicates an increase in signal (phosphorylation or total protein) and green indicates a decrease in signal (a selection of mTOR-related proteins is shown here). With unsupervised clustering, the heat map indicates that the highest phosphorylation/protein levels were at the 0 hour and 30 minutes time points, and the least signal was found in the no-UV control samples. The schema depicted in Fig. 1B represents the linkages between the mTOR/Akt-related proteins shown in the heat map.

Figure 1.
Solar simulated light (SSL) induces mTOR/Akt-related signaling events in mouse epidermis. SKH-1 hairless mice (n = 3) were acutely irradiated with 105 kJ/m² UVA/6.4 kJ/m² UVB and sacrificed at the indicated times (0 hour refers to mice that were irradiated and immediately sacrificed). Epidermal proteins were analyzed by Western blotting (A) or reverse phase protein microarray (RPPA, B). The heat map represents the average of the three independently analyzed samples per time point (0 hour, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours and 24 hours). The signaling diagram represents some of the proposed signaling pathways activated by SSL based on data obtained through the RPPA analysis.

Blocking SSL-induced mTOR signaling with topical rapamycin causes schedule-dependent outcomes in mouse skin tumorigenesis

We chose to inhibit mTOR function using the immunosuppressant rapamycin in a chemoprevention experiment with three arms: acetone control, rapamycin prevention, and rapamycin intervention. The control group received topical acetone prior to each SSL exposure and then continued the acetone treatments three times a week until sacrifice at week 25, yielding a final tumor
Topical rapamycin treatment causes delayed hyperphosphorylation of Akt (S473), which is blocked by PHT-427 cotreatment in the epidermis

Female SKH-1 mice were pretreated with vehicle or rapamycin (50 nmol/back) three times over the course of 2 days. The mice were then acutely exposed to SSL as in Fig 1 and harvested over a time course of 0 hour to 4 hours after UV for analysis of Akt signaling. Western blot analysis shows that rapamycin pretreatment caused significant inhibition of SSL-induced phosphorylation of S6 Ribosomal Protein, a marker of mTOR activity, at all time points (Fig. 5A). Early time points after SSL treatment indicate that rapamycin caused reduced Akt (S473) phosphorylation compared with vehicle controls, but at 4 hours after SSL, this trend was reversed, and the rapamycin treated skins showed significantly higher Akt (S473) phosphorylation than control skins (Fig. 5C).
slightly greater Akt (S473) phosphorylation than vehicle controls (Fig. 5A). In a second, independent experiment, mice were treated exactly as in Fig. 5A, but harvested at 6 hours and 24 hours after UV in order to verify the previous findings and test their duration. As shown in Fig. 5B, 6 hours after SSL rapamycin pretreatment caused a significant increase in Akt (S473) phosphorylation compared with vehicle plus SSL controls. UV-induced phosphorylation of S6 Ribosomal Protein is still strongly inhibited by rapamycin at this time, indicating that the agent is still functional in the skin. At 24 hours after SSL, rapamycin is still able to inhibit UV-induced phosphorylation of S6 Ribosomal Protein, and Akt (S473) phosphorylation levels are still slightly higher than the vehicle plus SSL control. Notably, at 24 hours the skins treated with rapamycin but no UV have greater Akt (S473) expression than the vehicle no-UV controls (Fig. 5C). A third acute in vivo experiment was performed using rapamycin in the presence of SSL.
to examine whether PHT-427 could inhibit SSL-induced Akt (S473) phosphorylation and possibly the stimulatory effects of rapamycin on this protein in the skin. Mice were treated three times with rapamycin (50 nmol/back) or PHT-427 (3.7 nmol/back), both agents, or vehicle prior to an acute dose of SSL as in Fig. 1. To confirm the delayed hyperphosphorylation of Akt (S473) after SSL in the presence of rapamycin, new mice were treated as described above and harvested at 6 hours and 24 hours after SSL for Western blot analysis of epidermal lysates (B, C). A third acute mouse experiment was performed to test the effects of PHT-427 on p-Akt (S473) in SSL-exposed epidermis. Mice were treated with rapamycin or PHT-427 (3.7 μmol/back) or both as described above and harvested 6 hours post SSL exposure. Epidermal lysates were blotted for p-Akt (S473), p-S6 Ribosomal Protein or β-tubulin as a loading control.

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Figure 5.
Topical treatment with rapamycin prior to SSL causes hyperphosphorylation of Akt (S473) in mouse epidermis which is inhibited by PHT-427. Female SKH-1 hairless mice (n = 3) were pretreated three times (48 hours, 24 hours, and 1 hour) with rapamycin (50 nmol/back) prior to acute SSL treatment at the dose used in Fig. 1. Mice were then sacrificed along a time course up to 4 hours and epidermal proteins were extracted for Western blot analysis (0 hour indicates sacrifice immediately after UV exposure, A). To confirm the delayed hyperphosphorylation of Akt (S473) after SSL in the presence of rapamycin, new mice were treated as described above and harvested at 6 hours and 24 hours after SSL for Western blot analysis of epidermal lysates (B, C). A third acute mouse experiment was performed to test the effects of PHT-427 on p-Akt (S473) in SSL-exposed epidermis. Mice were treated with rapamycin or PHT-427 (3.7 μmol/back) or both as described above and harvested 6 hours post SSL exposure. Epidermal lysates were blotted for p-Akt (S473), p-S6 Ribosomal Protein or β-tubulin as a loading control.

Discussion
In the current study, we examine the effects of topical application of rapamycin in the context of SSL-induced skin tumorigenesis using both naïve and "tumor-prone" mouse models. The results of this direct comparison strikingly delineate a schedule-dependent outcome on tumor burden and multiplicity in SSL-exposed SKH-1 mice in which cotreatment is detrimental while post-treatment is protective. These dualistic findings corroborate those of previous trials in which SKH-1 mice were exposed to UIVB and treated with rapamycin by i.p. injection (38, 39). However, the cotreatment protocol was in fact protective when rapamycin was used in dietary form during UV exposure or topically during two-stage chemical carcinogenesis (40, 41). This raises the possibility that dietary effects and tumor stimulus may contribute to the outcome of rapamycin treatment in the skin. However, our results confirm that topical application of nanomolar amounts of rapamycin have significant effects on epidermal response to UV/tumorigenesis on the same order as that found in i.p. models (2 mg/kg) in the same strain of mice. Topical application of rapamycin may therefore be a treatment option for some immunosuppressed patients at high risk for cutaneous SCC yet unable or unwilling to switch their current systemic regimen. Imaging results in vivo show that rapamycin treatment caused a substantial potentiation of acute SSL-induced apoptotic...
The combination of rapamycin and PHT-427 was also found to have a significant additive effect on inhibition of tumor multiplicity in SSL-treated tumor-prone skin. It should be noted that the trends in the data near the end of the measurements suggest that, given time, these tumors could have developed resistance to the action of the agents being tested. Increasing tumor volume could also affect the ability of the topical agent(s) to penetrate into the larger tumors. Future studies may benefit from a higher dose of both rapamycin and PHT-427, because neither agent caused any observed toxicities or disturbances in weight (data not shown). Notably, the current data provide evidence that blocking both mTOR and PDK/Akt signaling in UV-induced tumor-prone skin causes significant delays and decreases in tumorogenesis. Topical combinatorial therapy with agents such as these should therefore be considered for possible clinical use in immunosuppressed individuals at high risk for cutaneous SCCs.

The observation that treatment with rapamycin concurrently with solar UV caused increased tumor multiplicity and burden in our topical model (the "prevention" arm of Fig. 2) and in the literature with i.p. injections suggests not only that patients taking rapamycin may need to dramatically reduce their sun exposure. It evokes several more questions that require careful clinical testing. For instance, the possibility that UV may catalyze the conversion of topical rapamycin to tumorigenic metabolites should be addressed. In addition, the fact that immunosuppressed patients who switched from CsA to rapamycin dramatically reduced their risk of developing future SCCs (as long as they did not have multiple SCCs before conversion) is in alignment with the "intervention" model findings in Fig. 2, which is supported by other treatment regimes (39, 41). Additional studies in SKH-1 mice have found that dietary CsA exposure during UV protected against skin tumorogenesis, while post-UV dietary CsA had no effect and bolus treatments with the agent during UV increased tumor burden (46). It is therefore possible that continuous versus intermittent dosing with rapamycin may also have differing effects on tumor outcomes. In addition, recent studies with mice have discovered that circadian rhythm can affect immune responses (47, 48). Overall, the schedule-dependent effect found in Fig. 2, which is supported by other treatment regimes (39, 41), and the additive inhibition of tumorigenesis seen with rapamycin-PHT-427 treatment support confidence that future clinical evaluations will identify optimal treatment schedules/combinations on an individual basis to support immunosuppression and reduce the risk of developing skin lesions in high-risk populations.

**Disclosure of Potential Conflicts of Interest**

E. Perchin III is co-founder of Theranostics Health, Inc., co-founder, chief science officer of Perthera, Inc., and has ownership interest in a patent from George Mason University. No potential conflicts of interest were disclosed by the other authors.
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