

Research Article

Dietary Immunosuppressants Do Not Enhance UV-Induced Skin Carcinogenesis, and Reveal Discordance between p53-Mutant Early Clones and Carcinomas

Pieter Voskamp¹, Carolien A Bodmann¹, Gudrun E Koehl², Heggert G. Rebel¹, Marjolein G.E. Van Olderen¹, Andreas Gaumann³, Abdoel El Ghalbzouri¹, Cornelis P. Tensen¹, Jan N. Bouwes Bavinck¹, Rein Willemze¹, Edward K. Geissler², and Frank R. De Gruijij¹

Abstract

Immunosuppressive drugs are thought to cause the dramatically increased risk of carcinomas in sun-exposed skin of organ transplant recipients. These drugs differ in local effects on skin. We investigated whether this local impact is predictive of skin cancer risk and may thus provide guidance on minimizing the risk. Immunosuppressants (azathioprine, cyclosporine, tacrolimus, mycophenolate mofetil, and rapamycin) were assessed on altering the UV induction of apoptosis in human skin models and of p53 mutant cell clones (putative tumor precursors) and ensuing skin carcinomas (with mutant p53) in the skin of hairless mice. Rapamycin was found to increase apoptosis (three-fold), whereas cyclosporine decreased apoptosis (three-fold). Correspondingly, a 1.5- to five-fold reduction ($P = 0.07$) or a two- to three-fold increase ($P < 0.001$) was found in cell clusters overexpressing mutant p53 in chronically UV-exposed skin of mice that had been fed rapamycin or cyclosporine, respectively. Deep sequencing showed, however, that the allelic frequency (~5%) of the hotspot mutations in p53 (codons 270 and 275) remained unaffected. The majority of cells with mutated p53 seemed not to overexpress the mutated protein. Unexpectedly, none of the immunosuppressants admixed in high dosages to the diet accelerated tumor development, and cyclosporine even delayed tumor onset by approximately 15% ($P < 0.01$). Thus, in contrast to earlier findings, the frequency of p53-mutant cells was not predictive of the incidence of skin carcinoma. Moreover, the lack of any accelerative effect on tumor development suggests that immunosuppressive medication is not the sole cause of the dramatic increase in skin cancer risk in organ transplant recipients. *Cancer Prev Res*; 6(2); 129–38. ©2012 AACR.

Introduction

Organ transplant recipients have a high risk of developing squamous cell carcinomas (SCC) of the skin on sun-exposed areas. In the Netherlands, 40% of renal transplant recipients develop skin cancer within 20 years after transplantation (1), and in Australia the risk is substantially greater (70%; ref. 2). These solar-related (3) skin carcinomas in organ transplant recipients tend to be aggressive and often recur more (4).

Because most UV-induced skin carcinomas are thought to be antigenic and targeted by the immune system (5), transplant-related immunosuppression is perhaps an inescapable side effect of organ transplantation treatment. Indeed, some commonly used immunosuppressants directly promote cancer growth or affect antitumorigenic responses, such as apoptosis (6–8). The actions of immunosuppressive drugs used in transplantation practice differ substantially. For example, azathioprine and mycophenolate mofetil, or rather their metabolites 6-mercaptopurine and mycophenolic acid, respectively, inhibit purine synthesis, and thus T-cell proliferation (9). Cyclosporine and tacrolimus block T-cell activation through calcineurin inhibition (9). Rapamycin inhibits mTOR that impairs interleukin (IL)-2 production and therefore T-cell proliferation (9–12). Important for the present discussion is that some of these immunosuppressants also affect skin cells, contributing to carcinoma risk. For instance, azathioprine and cyclosporine adversely affect DNA repair and cyclosporine also impairs apoptosis (6, 7, 13), which are important mechanisms that help protect against UV skin carcinogenesis; azathioprine further enhances DNA damage by photosensitization (8). Indeed, azathioprine and cyclosporine speed

Authors' Affiliations: ¹Department of Dermatology, Leiden University Medical Center, Leiden, the Netherlands; ²Department of Surgery, University of Regensburg, Regensburg; and ³Institute of Pathology, Kaufbeuren, Germany

Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org>).

Corresponding Author: Pieter Voskamp, Department of Dermatology, Leiden University Medical Center, P.O. Box 9600, Leiden 2300 RC, the Netherlands. Phone: +31-715269363; Fax: +31-715248106; E-mail: p.voskamp@gmail.com

doi: 10.1158/1940-6207.CAPR-12-0361

©2012 American Association for Cancer Research.

up UV carcinogenesis in hairless mice (7, 14). However, not all immunosuppressants necessarily promote cancer development. While mycophenolate mofetil has some capacity to reduce tumor cell proliferation (15, 16), rapamycin exhibits impressive inhibitory effects on cancer development. Rapamycin inhibits angiogenesis and tumor cell proliferation (15, 17, 18), leading to impaired inoculated tumor growth in mice and *de novo* tumor development in a p53-null mice (19). Interestingly, azathioprine-treated transplant recipients have more mutant p53-overexpressing cell clusters in precursor skin lesions than immunocompetent patients with skin carcinomas (20); this finding contrasts with results in rapamycin-fed mice that develop fewer mut-p53 cell clusters than control mice (21). Whether this effect is related to the rapamycin-induced increase in apoptotic responses we have observed after UV irradiation (21), is not clear. From a clinical perspective, recent trials do suggest rapamycin treatment decreases the development of skin carcinomas in organ transplant recipients (22, 23).

Here, we compared the tumorigenic properties of an array of commonly used immunosuppressants in experiments using human skin and mouse models. We systematically compared the impact of the immunosuppressants on epidermis and its responses to UV exposure (most specifically, apoptosis) in human skin equivalents (HSE). In addition, we linked these *in vitro* results to local effects of the immunosuppressants on UV-induced early formation of mut-p53 cell clusters, and overall effects on subsequent formation of skin tumors in a hairless mouse model. These experiments allowed us to establish whether local effects of immunosuppressants on UV-induced apoptosis and p53 mutations in the skin are predictive of skin cancer risk. In contrast to our expectations, none of the immunosuppressants enhanced the rate at which skin tumors developed in mice.

Materials and Methods

Human skin equivalents

Generation of HSEs was conducted as described earlier (24). In brief, keratinocytes and fibroblasts were isolated from surplus breast skin obtained from cosmetic surgery of 2 donors (obtained in accordance with the Dutch Law on Medical Treatment Agreement). A total of 80×10^3 fibroblasts were seeded into acetic acid-extracted rat-tail collagen deposited on filter inserts (product no. 3414; Corning). The fibroblast-populated matrices were cultured for a week in standard fibroblast medium. These matrices were subsequently seeded with 5×10^5 low-passage normal human epidermal keratinocytes and incubated with keratinocyte medium. Medium was refreshed twice a week. After 2 weeks of air-exposed culture, the HSEs were processed for analysis.

Supplements/chemicals

Immunosuppressants dissolved in absolute dimethyl sulfoxide (DMSO) were added to the HSEs at physiologically relevant concentrations (25, 26): rapamycin (Calbiochem) at 10 or 100 nmol/L, 6-mercaptopurine (Sigma-Aldrich) at 50 or 500 nmol/L, cyclosporine (Sigma-Aldrich) at 100 nmol/L or 1 μ mol/L, tacrolimus (Calbiochem) at 100

nmol/L or 1 μ mol/L, and mycophenolic acid (Sigma-Aldrich) at 1 or 10 μ mol/L, at 0.1%(v/v). 6-Mercaptopurine and mycophenolic acid are used as they are the active metabolites of azathioprine and mycophenolate mofetil, respectively (21, 27). For effects on morphology, HSEs were supplemented with immunosuppressants or DMSO only (as vehicle control) during the air-exposed culture. For effects on the response after UVB irradiation, HSEs were supplemented with immunosuppressants for 2 days before UVB irradiation.

The mice

SKH-1 hairless albino mice (Charles River) entered the experiment at 8 to 16 weeks of age; both male and female mice were used equally divided over the groups. The animals were housed individually under a 12-hour light–12-hour dark cycle at 23°C. Experimental chow was supplied in ample amounts (60 g/mouse/wk), and drinking water was available *ad libitum*. Cage enrichment was absent to prevent shielding of the animals from UV exposure. All experiments were carried out in accordance with legislation and approval of the center's ethics committee for animal experiments.

Groups on diets with admixtures of immunosuppressants

To avoid repeated intraperitoneal injections or oral gavage to administer the immunosuppressants, admixtures of the drugs to standard mouse chow were used (Sniff GmbH; ref. 28). For cyclosporine, mycophenolate mofetil and rapamycin dosages in the food were used that resulted in long-term average blood levels in the mice that were similar to the high-end average blood levels in renal transplant patients (cyclosporine at 150 mg/kg in food: 800 ng/mL in blood, mycophenolate mofetil at 660 mg/kg: 2.9 μ g/mL mycophenolic acid, rapamycin at 20 mg/kg: 50 ng/mL; mice of 25–35 g body weight ate 5–7 g per day). Avoiding toxicity, tacrolimus and azathioprine were admixed at maximal long-term tolerable dosages (tacrolimus 20 mg/kg: 3.2 ng/mL, azathioprine 30 mg/kg: subanemic white blood cell count). Six diet groups were formed: rapamycin ($n = 26$), mycophenolate mofetil ($n = 22$), cyclosporine ($n = 24$), tacrolimus ($n = 24$), azathioprine ($n = 26$), and control groups (each $n = 26$) fed the standard chow without admixtures (n includes 12 mice sacrificed to score mut-p53 cell clusters, see later). The tumorigenesis experiment was carried out in 3 parts, (i) rapamycin and cyclosporine, (ii) azathioprine and tacrolimus, and (iii) mycophenolate mofetil, each with a control group on chow without admixture. No apparent differences in food intake and body weights were observed between the diet groups.

UVB irradiation

Mice. The 6 groups of albino hairless SKH1 mice were started on their respective diets 1 week before subjecting them to a regimen of daily UV exposure. TL-12/40W tubes (Philips; 54% output in UVB: 280 to 315 nm—and 46% output in UVA: 315 to 400 nm) were used for daily UV exposure. The lamps were mounted over the cages with grid

covers to allow undisturbed exposure of the mice. The lamps were automatically switched on daily from 12.30 to 12.50 hours. The threshold dose for a sunburn reaction (minimal edemal dose, MED) in the hairless SKH-1 mouse was approximately 500 J/m² UV under these lamps. The lamps were dimmed both electronically and by insertion of perforated metal sheets to expose the mice daily to 250 J/m² of UV radiation (0.5 MED).

HSEs. Skin models were also exposed to 1.4 kJ/m² UV from TL-12/20W tubes at 2.8 W/m².

UVA irradiation of azathioprine-fed mice

Pigmented hairless mice were acquired by 2 rounds of breeding C57BL/6 into SKH1 background. These (F2) mice were put on azathioprine ($n = 12$) or control diet ($n = 13$) 1 week before subjecting them to a regimen of daily UVA exposure. Cleo Professional S R tubes (Philips; 1.4% of output in UVB: 280 to 315 nm—and 98.6% in UVA: 315 to 400 nm) were used for irradiation. MED was determined by exposing mice to 7, 14, 21, or 28 kJ/m² UV (different exposure times were attained by taping off different parts of the dorsal skin). Mice were daily irradiated at 14 kJ/m² (1 MED) in the UVA carcinogenesis experiment.

Tumor assessment

The mice were inspected weekly for tumors, which were registered for each mouse individually on maps (recording number, location, size, and form). For presentation in a single graph of the tumor induction data in Kaplan–Meier plots, the 3 experiments were adjusted to equal median latency times in the control groups (a time shift of maximally 2 weeks, with an average of 8 days). Upon removal of animals from the experiment, tumors and normal skin were isolated for further analysis as described later.

Epidermal sheet preparation and mutant-p53 immunostaining in epidermal sheets

For quantification of mut-p53 cell clusters, 4 SKH1 mice were taken from each diet group after 4, 7, or 10 weeks of UV exposures. Within 24 hours after the last irradiation, mice were killed and pieces of dorsal skin of 11 mm × 34 mm were excised and treated with a 100 µg/mL thermolysin solution, after which the epidermal sheet was separated from the dermis. The procedure of epidermal sheet preparation and subsequent immunostaining and analysis, described elsewhere (29), was followed with 2 modifications: antigen retrieval in 10 mmol/L citrate buffer in an autoclave at 5 minutes, 110°C and mutant-p53 antibody diluted 1:250 (Pab240, Thermo Fisher Scientific). A 1 to 2 mm wide mid-dorsal strip of the epidermal sheets was collected per mouse and used for DNA isolation (QIAamp, Qiagen). Counts of mut-p53–positive cell clusters outside the range of 6-fold over or under the median (in cohorts of 4 mice) were rejected as outliers (with the exception of "0" if the lower limit was less than 1) to produce Fig. 2; this led to exclusion of 9 of 84 measurements from this graph. However, none of the measurements were excluded from statistical tests (see later).

P53 mutation hotspot PCR and deep sequencing

DNA from the murine epidermal strips of 4 mice per group was mixed equimolarly and used in a PCR reaction amplifying the *p53* gene from codon 263 to 276, containing the mutational hotspots at codons 270 and 275. The forward primer contained a sequence-specific part and an adapter sequence at the 5' end of the primer. The reverse primers were unique for each group containing a sequence-specific part, an index sequence, and an adapter sequence (Supplementary Table S1). Primers were high-performance liquid chromatography (HPLC)-purified (Integrated DNA Technologies). PCRs were conducted using high fidelity Phusion enzyme (Finnzymes) in a volume of 40 µL with the following program: 98°C for 30 seconds, then 28 cycles of 98°C for 10 seconds, 64°C for 15 seconds, 72°C for 15 seconds, closing the program with 72°C for 10 minutes. PCR products were run on a 1.8% agarose gel, and stained with SYBR Gold (Invitrogen), after which bands containing the amplicons were excised on a Dark Reader not emitting UV (Clare Chemical Research) and purified using QIAquick (Qiagen) spin columns. Amplicons were analyzed in one lane of an Illumina Sequencer HiSeq2000 (Illumina). Reads were filtered for high-quality reads containing only bases that were called with a minimal fidelity of 99.9% (phred-score ≥ 30). Index sequences were used to distinguish the different samples. For the numbering of the bases, the *p53* transcript with ID ENSMUST00000108658 from Ensembl was used as reference.

Histology and immunohistochemistry

Biopsies were fixed in 4% formaldehyde, dehydrated, and embedded in paraffin or snap-frozen in liquid nitrogen. Stainings for keratins 10, 16, 17 (K10, K16, and K17), and Ki-67 were conducted on formalin-fixed and paraffin-embedded sections (5 µm) of skin models, mouse skin or tumors. Immunohistochemical analysis of active caspase-3 was conducted on frozen sections (5 µm), which after sectioning were fixed in methanol/acetone (1:1) for 10 minutes. Hematoxylin and eosin (H&E) staining was conducted on deparaffinized sections for tumor staging. Staging of tumors from each group was conducted blindly by a pathologist (A. Gaumann) who is experienced in mouse and human pathology, including diagnosis of actinic keratoses as proper precursors of SCCs. Antigen retrieval for paraffin-embedded sections was conducted by autoclaving the sections in 10 mmol/L citrate buffer (pH 6.0) for 10 minutes at 110°C. The primary antibodies used for immunohistochemistry are listed in the Supplementary Table S2. After overnight incubation with the primary antibody at 4°C, sections were stained using standard protocols with 3-amino-9-ethylcarbazole, substrate of peroxidase.

Estimate of proliferation index

The percentage of Ki-67–positive nuclei in the basal and immediate supra-basal layer was used to determine the proliferation index. A minimum of 100 cells were counted using light-microscopy in 3 different regions in each section.

Statistical tests

Kaplan–Meier plots of tumor-free survival were tested for differences by χ^2 statistics (Graphpad Prism 5.0). Differences in tumor yields were analyzed with randomized block one-way ANOVA, with Bonferroni as *post hoc* test (Graphpad Prism 5.0). Differences in number of mut-p53 cell clusters per treatment (without outlier removal) were calculated in a general linear model multivariate analysis with least significant difference as *post hoc* test. Differences in Ki-67–positive cell percentages in HSEs were calculated with one-way ANOVA and Dunnett as *post hoc* test. Differences in active caspase-3–positive cell fractions in HSEs were calculated with a one-way ANOVA and Bonferroni as *post hoc* test. *P* value less than 0.05 was taken to indicate a significant difference. SPSS 16.0 was used for all statistical analyses of immunohistochemical stainings.

Results

Rapamycin affects epidermis in HSE

From the moment of air exposure, HSEs were cultured with immunosuppressants added to the medium for 2 weeks: rapamycin, tacrolimus, mycophenolic acid, 6-mercaptopurine, cyclosporine, or DMSO (vehicle). Only rapamycin was found to affect the epidermis that developed in the HSE: 3 to 4 epidermal cell layers were

formed instead of 7 to 8 (Fig. 1). Rapamycin treatment resulted in a decreased proliferation index (from 43% to 22% and 11% with 100 and 10 nmol/L rapamycin, respectively; $P < 0.05$; Supplementary Fig. S1). Hyperproliferative markers K16 and K17 showed a decreased expression (Fig. 1), whereas expression of the suprabasal differentiation marker K10 remained unaffected (data not shown).

Cyclosporine decreases and rapamycin increases the active caspase-3–positive cell fraction in HSE after UV irradiation

To examine the effects of immunosuppressants on apoptosis, HSEs from 2 donors were irradiated with UV (1.4 kJ/m^2) 2 days after supplementation of immunosuppressants to the media. The percentage of active caspase-3–positive cells 24 hours after irradiation was determined by immunohistochemistry. Of the 5 immunosuppressants, only rapamycin at 10 and 100 nmol/L increased the percentage of active caspase-3–positive (supra-)basal cells (average 20%; SEM = 4% vs. 7%; SEM = 6% in controls) in skin models of the donor with a low-apoptotic response, whereas only cyclosporine at $1 \mu\text{mol/L}$ was found to decrease the apoptotic percentage (35%; SEM = 10% vs. 100%; SEM = 0% in controls) in skin models of the donor with a high-apoptotic response.

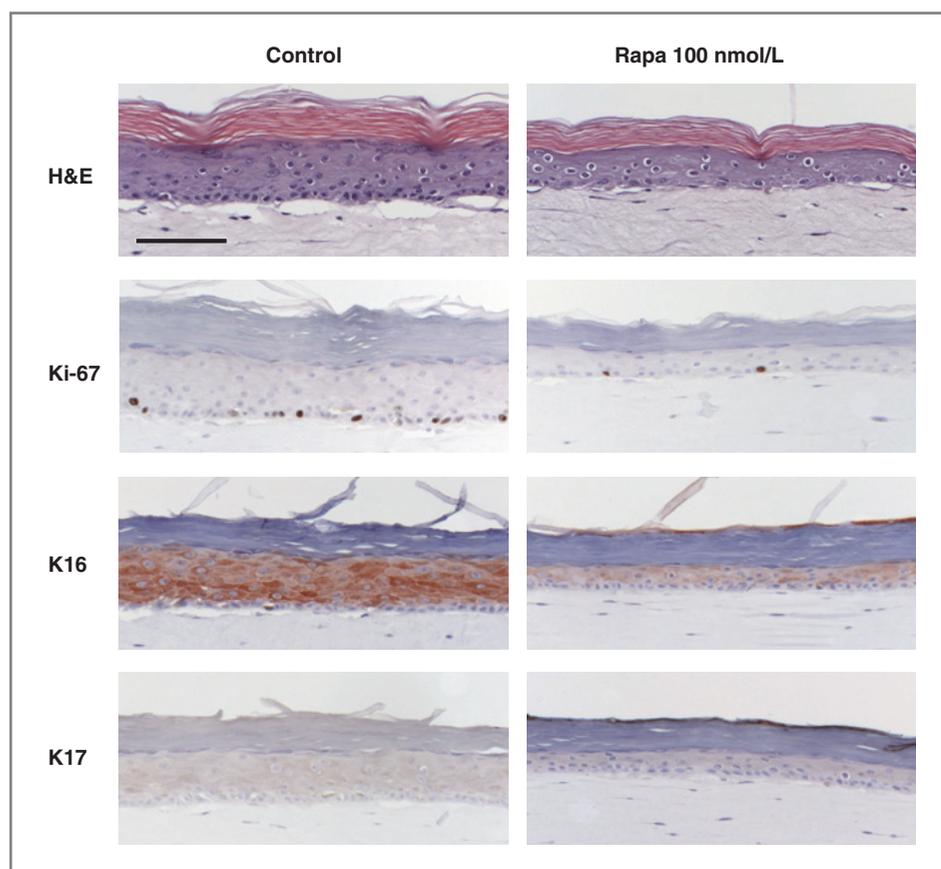


Figure 1. Rapamycin (Rapa) alters epidermal regeneration, proliferation, and K16 and K17 expression in HSEs. Representative pictures are shown. Scale bar: 100 μm .

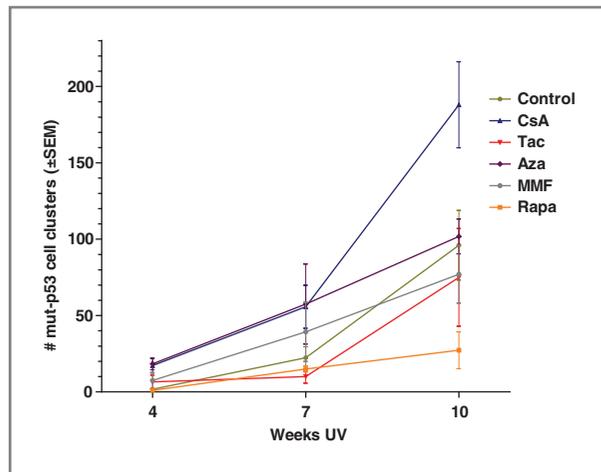


Figure 2. Effects of immunosuppressive drugs on the induction of epidermal mut-P53 cell clusters after 4, 7, and 10 weeks of chronic UV treatment. Cyclosporine (CsA) and azathioprine (Aza)-treated mice developed increased numbers of mut-p53 cell clusters; rapamycin (Rapa)-treated mice developed a lower number of mut-p53 cell clusters compared with control mice. Tac, tacrolimus; MMF, mycophenolate mofetil.

Cyclosporine and azathioprine increase numbers of mut-p53 cell clusters

Because of their limited lifespan, HSEs are not suitable for generating mut-p53 cell clusters. Therefore, we examined the effects of immunosuppressants on generation of mut-p53 cell clusters in mice. Hairless mice were fed with immunosuppressants admixed to the chow at dosages resulting in blood levels that are at the high end of the range of typical blood levels in renal transplant recipients. Because of toxicity of tacrolimus and azathioprine, dosages of these drugs were limited to maximal long-term tolerable levels. The mice were daily irradiated with 0.5 MED UVB for 4, 7, or 10 weeks, at which time numbers of mut-p53-positive cell clusters were determined. Cyclosporine and azathioprine treatment increased the number of mut-p53 cell clusters ($P < 0.001$ and $P < 0.05$, respectively; Fig. 2). Rapamycin tended to inhibit their formation at all time points ($P = 0.07$). The proliferation index of dorsal skin from 4 week-irradiated mice was not affected by any of the immunosuppressants (data not shown). For comparison with *p53* mutational frequencies (described later), we determined in 10 week-irradiated control mice that mut-p53 cell clusters covered 6% of surface area of epidermal sheets bordering the epidermal strip that was removed for mutational analysis.

Frequency of *p53* mutations in UV-exposed skin

The question arose whether the number of mut-p53 cell clusters corresponded with the frequency of mutated alleles in the *p53* gene in the epidermis. To answer this question, DNA from epidermal strips of mice UV-irradiated for 10 weeks and unirradiated control mice was used as template to amplify part of the *p53* gene containing 2 mutational hotspots (codons 270 and 275). PCR products (pooled for

4 mice per group) were analyzed by deep-sequencing, with a sequencing depth between 2 and 8 million for the different samples. Three mutations were common in samples of the UV-irradiated mice: 808C>T = aa270 R>C, 823C>T = aa275 P>S, 824C>T = aa275 P>L, with numbers cumulating to a total between 4.6% and 6.6% of the PCR products; samples from unirradiated mice contained these mutations far less frequently at 0.03% (Fig. 3). There were no clear differences in mutational frequencies between the samples from different immunosuppressive treatments and controls.

Cyclosporine delays tumor induction and rapamycin inhibits tumor growth

To examine the effects of immunosuppressants on UVB-induced tumor formation, mice were fed immunosuppressants in their diets and they were daily exposed to 0.5 MED of UV. Tumor development was assessed weekly. Cyclosporine feeding resulted in increased tumor latency times for tumors more than 1 mm (Fig. 4A). Confirming our earlier results (28), rapamycin-fed mice showed no change in onset of small tumors (diameters around 1 mm) but did show a lowered rate of formation of larger tumors, that is, an inhibition of tumor outgrowth. Rapamycin and cyclosporine groups showed significantly lower yields of tumors more than 4 mm as compared with controls ($P < 0.01$ and $P < 0.001$, respectively; Fig. 4B). Near the end of the experiment, several mice had to be taken out of the different groups because they extensively scratched their tumors. After 155 days, 7 of 12 mice of the

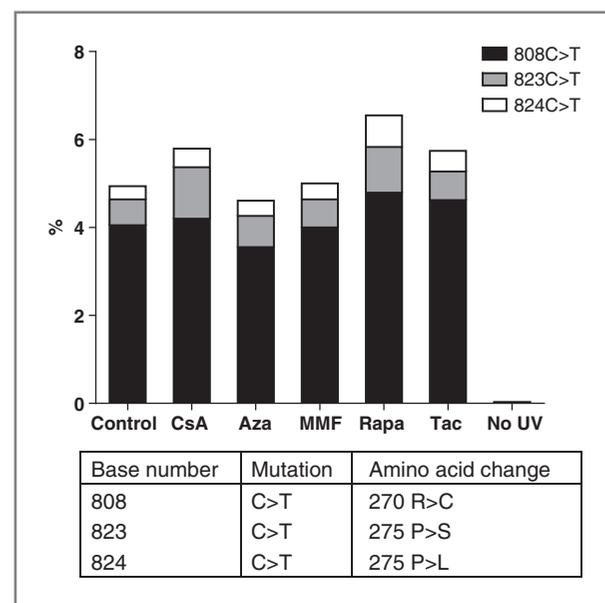


Figure 3. Frequency of hotspot mutations in the *p53* gene. DNA isolated from epidermal strips of mice daily UVB-irradiated for 10 weeks was analyzed by deep-sequencing the fragment-spanning codons 263 to 276 of the *p53* gene. The 3 most commonly occurring mutations are presented in the table and in the graph as percentages of all analyzed PCR products. CsA, cyclosporine; Aza, azathioprine; Rapa, rapamycin; Tac, tacrolimus; MMF, mycophenolate mofetil.

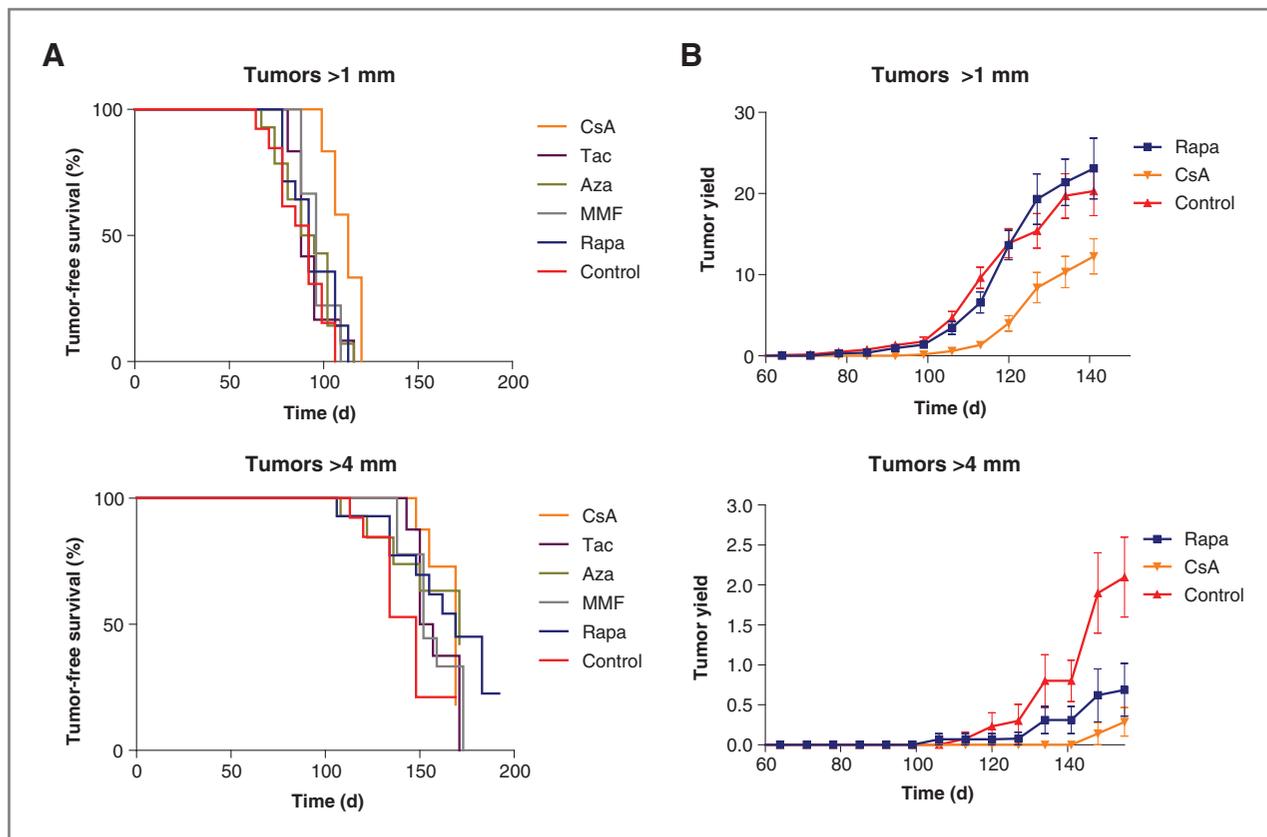


Figure 4. A, tumor-free survival (Kaplan–Meier) plots of the different treatment groups, with different thresholds for tumor detection: diameters more than 1 or 4 mm. B, average tumor yields are shown per mouse for control, rapamycin (Rapa) and cyclosporine (CsA) treatment. Error bars depict SEM. In A and B, CsA vs control >1 mm $P < 0.01$; in B, CsA and Rapa vs control >4 mm $P < 0.001$ and $P < 0.01$, resp. Tac, tacrolimus; Aza, azathioprine; MMF, mycophenolate mofetil.

cyclosporine group were still in the experiment compared with 9 of 14 mice in the control group. None of the other immunosuppressants in the food of the mice seemed to affect the onset or outgrowth of the UV-induced tumors. Histopathology showed all tumors more than 4 mm to be SCCs. No effect of immunosuppressive drugs on tumor type or grading was found.

Azathioprine did not affect UVA-induced tumor induction

To assess whether azathioprine sensitizes mice for UVA carcinogenesis (30), mice were put on an azathioprine-containing diet or control diet for 2 weeks. Besides albino mice, pigmented mice were included because there is evidence that melanin may sensitize skin to UV exposure (31), which could conceivably synergize with azathioprine in photosensitization. No effect of azathioprine was observed on the minimal dose required to raise a perceptible edematous skin reaction 24 hours after irradiation, both in albino hairless SKH1 mice, and in cross-bred (F2) pigmented SKH1/BL6 mice. However, the pigmented mice that were fed azathioprine showed a more extensive skin burning compared with control mice 72 hours after irradiation (Supplementary Fig. S2). UVA carcinogenesis was therefore studied in pigmented mice. In these mice, azathioprine

showed no significant effect on tumor yields for tumors more than 1 or 2 mm (Supplementary Fig. S3). Because some mice started scratching their tumors after 19 weeks in the experiment, they had to be removed from the experiment; as a result, tumor yield data for tumors more than 4 mm could not be measured reliably.

Discussion

In this study, we aimed to establish whether short-term local skin effects of immunosuppressants on UV-induced apoptosis and medium-term effects on *p53* mutations are predictive of the risk of skin cancer development. Of the immunosuppressants tested in this study, rapamycin showed an inhibitory effect on epidermal regeneration and increased UV-induced apoptosis in human skin models, whereas cyclosporine decreased apoptosis. Despite corresponding effects on the formation of clusters of cells overexpressing mutant-*p53* in mice—that is, a decrease by rapamycin, an increase by cyclosporine—the effects on tumor onset were discordant. Cyclosporine was unexpectedly shown to delay tumor onset and rapamycin had no effect on tumor onset but inhibited tumor growth. Remarkably, despite high dietary dosages and blood levels (for rapamycin and cyclosporine adequate to maintain allogeneic heart grafts in mice; ref. 18) none of the dietary-fed

Table 1. Summary of the results obtained for the different immunosuppressants

		Cyclosporine	Tacrolimus	Azathioprine	Mycophenolate mofetil	Rapamycin
Human skin model	Epidermal regeneration	—	—	—	—	↓
	Proliferation	—	—	—	—	↓
	Apoptosis after UV	↓	—	—	—	↑
Mouse model	Proliferation	—	—	—	—	—
	Number of mut-p53 cell clusters	↑	—	↑	—	↓
	P53 mutational frequency in nontumor epidermis	—	—	—	—	—
	Yield of small tumors (>1 mm)	↓	—	—	—	—
	Yield of large tumors (>4 mm)	↓	—	—	—	↓

NOTE: —, no effect; ↓, inhibitory effect; ↑, stimulatory effect.

immunosuppressants increased UV-induced skin carcinogenesis in the mouse model used in this study; an overview of the results is presented in Table 1. Below we discuss the effects of immunosuppressants on UV-responses and the different stages in tumor development, viz. cellular responses in human skin models, mut-p53 cell clusters, p53 mutational frequencies, and tumor development in mice.

In HSEs, we could verify earlier results (6, 32) that rapamycin increased the UV-induced apoptotic response, whereas cyclosporine inhibited this apoptotic response. Epidermal formation in HSE was impaired by rapamycin: resulting in fewer cell layers, a decreased proliferation index (% Ki-67+) and reduced expression of hyperproliferation markers (K16 and K17). This decreased proliferation in HSEs may be related to wound-healing problems associated with rapamycin treatment in transplantation patients (33). In HSEs, cyclosporine pretreatment caused a decrease in the apoptotic response after UV irradiation. This decrease in apoptotic response was evident in HSEs with a high percentage of apoptotic cells, leaving the possibility that cyclosporine exerts this effect only at highly apoptotic UV doses, as suggested by Flockhart and colleagues (34).

Cyclosporine and azathioprine increased the formation of mut-p53 cell clusters in mice, whereas rapamycin tended to inhibit mut-p53 cell cluster formation. The lower number of UV-induced mut-p53 cell clusters formed in the skin of rapamycin-treated mice is apparently not due to decreased proliferation, as the fraction of Ki-67-positive cells was not decreased in rapamycin-treated mice. A previous study from our group (20) showed no effect of azathioprine on mut-p53 cell cluster formation in mice, but it should be noted that azathioprine in these experiments was administered by intraperitoneal injections, and variations in counts of these clusters were very large.

Immunosuppressants did not affect the mutational frequency of p53 genes in chronically UV-irradiated skin in mice, as measured by deep sequencing, contrasting

with effects on mut-p53 cell cluster formation. On average, 5% of the sequenced alleles were mutated, corresponding to 10% of the cells assuming heterozygous mutations. As the hotspot mutations at codons 270 or 275 comprise about half of the mutations in the p53 gene, the mutational frequency suggests that maximally 20% of the cells in the skin would harbor mutated p53 (if cells harbored more than one mutation in similar percentages as mut-p53 cell clusters, the fraction would come down to about 14%; refs. 29, 35). These results are in agreement with a recent deep sequencing study of human skin from middle-aged individuals revealing that persistent p53 mutations had accumulated in 14% of the epidermal cells (36). The percentage of skin area containing mut-p53 cell clusters in our mice was much lower, approximately 6%. It can therefore be concluded that a minority of the cells harboring a mutated p53 show overexpression of mut-p53 in cell clusters. This indicates that a mutation in p53 is apparently not sufficient to cause overexpression of the p53 protein in mut-p53 cell clusters (21, 37). And tumors may arise from a larger pool of p53-mutated cells than visible in IHC of mutant p53-overexpressing cell clusters. Evidently, the local effects of immunosuppressants in the skin affecting the mut-p53 cell clusters are not paralleled by correspondingly proportional effects on p53 mutations and tumor onset.

None of the immunosuppressants increased UV-induced tumor development, and cyclosporine even delayed tumor onset. Rapamycin had no effect on the development of small tumors, but decreased the outgrowth of tumors. Growth inhibition was reported earlier by our group (28), and the growth inhibition by rapamycin was more prominent in that experiment. Other UV carcinogenesis studies with different treatment schemes have shown either lower tumor yields (38) or higher tumor yields (39) in rapamycin-treated mice, indicating that the precise treatment regimen may be decisive for the outcome of the tumorigenesis experiment. Proliferation of epidermal

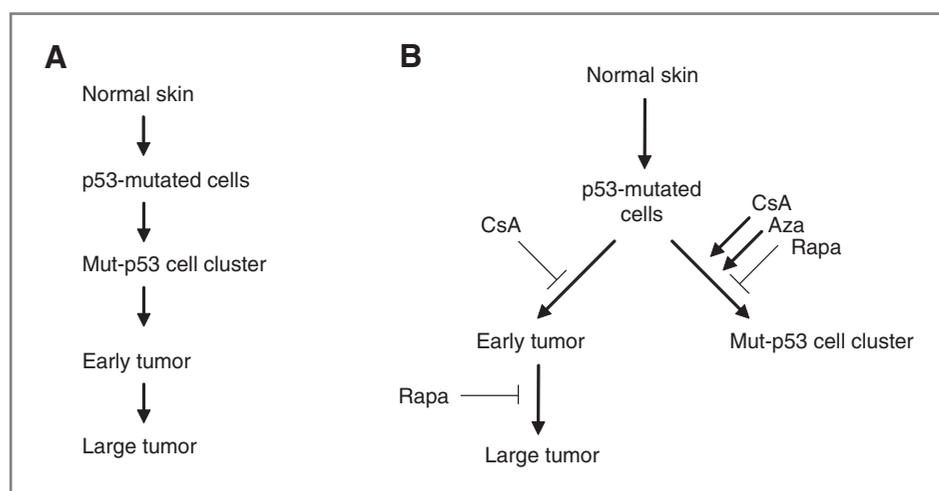


Figure 5. Schematic overview of potential developmental stages in UV-carcinogenesis. A, previous model, scheme adapted from Boukamp (48). B, model based on present results. Effects of immunosuppressants on different stages as described in the article are depicted. CsA, cyclosporine; Rapa, rapamycin; Aza, azathioprine.

keratinocytes was not affected by cyclosporine, making it unlikely that reduced keratinocyte proliferation was causing the tumor delay. Toxicity of cyclosporine on tumors is not very likely either, as a 3-fold lower concentration of cyclosporine in the diet still exerted the same effect (see Supplementary Fig. S4). In contrast to cyclosporine, tacrolimus did not have any measurable effects in this study, possibly due to the low dose of tacrolimus that was used to avoid toxicity problems. Blood levels of tacrolimus were approximately 4 times lower than trough tacrolimus blood levels in organ transplant recipients (40). On the other hand, the effects of cyclosporine on tumor development could be caused by an effect not exerted by tacrolimus (41). A pioneering study of the effect of cyclosporine on UV carcinogenesis (7) showed a (25%) shorter tumor latency time compared with controls. In that experiment, mice were administered cyclosporine in oil by gavage 3 times a week, and 2 to 4 hours after each gavage the mice were exposed to UV, with expected peak drug levels in the blood. The mode of administering cyclosporine in our experiment results in relatively stable cyclosporine blood levels over time; in contrast to gavage. With a different experimental setup, Wulff and colleagues also showed that cyclosporine treatment by repeated intraperitoneal injections reduced the rate of development of new tumors in mice (38). The different ways of administering cyclosporine result in pharmacologically different profiles; with apparently different effects. Further experimentation on the effect of cyclosporine treatment schemes on tumor formation is called for. Like rapamycin, azathioprine was originally developed as an anticancer drug (42) and might exert a cytotoxic effect at high dosages, especially in combination with UV irradiation (43). However, the azathioprine regimen we used did increase the number of mut-p53 cell clusters.

No correlation was found in this study between development of mut-p53 cell clusters and SCCs. Numbers of mut-p53 cell clusters had always correlated with the rate of tumor development in previous studies (5, 29, 44). Mut-p53 cell clusters develop as a clonal expansion of a mutated keratinocyte. Because most SCCs and actinic keratoses

harbor mutations in the *p53* gene, *p53* mutations are considered to be early events in SCC formation, and therefore mut-p53 cell clusters are thought to be precursors of SCCs (45, 46; Fig. 5A). However, an earlier retrospective study did not find any differences in numbers of mut-p53 cell clusters between skin from patients with solitary versus multiple skin carcinomas (47). As we found that compounds (rapamycin vs. cyclosporine) can have opposing effects on mut-p53 cell clusters and SCC formation, it follows that mut-p53 cell cluster formation cannot be used as a simple indicator of tumor risk. These results open up an alternative perspective on the developmental stages of skin carcinomas, one in which SCC development is independent of mut-p53 cell cluster formation (Fig. 5B).

Using the present mouse model and experimental setup, the effect of the immunosuppressive drugs on tumor risk seems not to be in agreement with the increased skin cancer risk in transplant recipients. Moreover, we did not find consistent links between effects on UV-induced apoptosis, frequency of *p53* mutations and the ultimate formation of skin tumors. These discrepancies between the effects on mut-p53 cell clusters and tumors, and the striking overall lack of enhanced UV carcinogenesis in the present mouse experiments imply that the mouse model does not properly emulate skin cancer development in organ transplant recipients. Elucidation of these discrepancies, whether they are caused by an incomplete experimental model (not fully representing organ transplantation with complex immunosuppressive regimens) or physiologic differences between mice and humans, should contribute to a better understanding of the risk of skin carcinogenesis in organ transplant recipients.

Disclosure of Potential Conflicts of Interest

E.K. Geissler has honoraria from Speakers Bureau and is a consultant/advisory board member of Pfizer. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: P. Voskamp, H.G. Rebel, C.P. Tensen, J.N.B. Bavinck, R. Willemze, E.K. Geissler, F.R. De Gruij

Development of methodology: P. Voskamp, G.E. Koehl, H.G. Rebel, F.R. De Gruijl

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.A. Bodmann, H.G. Rebel, A. Gaumann

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): P. Voskamp, H.G. Rebel, A. Gaumann, C. P. Tensen, F.R. De Gruijl

Writing, review, and/or revision of the manuscript: P. Voskamp, G.E. Koehl, A. Gaumann, A. El Ghalbzouri, C.P. Tensen, J.N.B. Bavinck, R. Willemze, E.K. Geissler, F.R. De Gruijl

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Voskamp, C.A. Bodmann, G.E. Koehl, M.G.E. Van Olderen

Study supervision: C.P. Tensen, J.N.B. Bavinck, R. Willemze, E.K. Geissler, F.R. De Gruijl

Grant Support

This research was supported by the Dutch Cancer Society, grant number 2007-3910.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 21, 2012; revised November 26, 2012; accepted November 29, 2012; published OnlineFirst December 11, 2012.

References

- Hartevelt MM, Bavinck JNB, Kootte AMM, Vermeer BJ, Vandenbroucke JP. Incidence of skin-cancer after renal-transplantation in the Netherlands. *Transplantation* 1990;49:506-9.
- Bouwes Bavinck JN, Hardie DR, Green A, Cutmore S, MacNaught A, O'Sullivan B, et al. The risk of skin cancer in renal transplant recipients in Queensland, Australia. A follow-up study. *Transplantation* 1996;61:715-21.
- Bavinck JN, De Boer A, Vermeer BJ, Hartevelt MM, van der Woude FJ, Claas FH, et al. Sunlight, keratotic skin lesions and skin cancer in renal transplant recipients. *Br J Dermatol* 1993;129:242-9.
- Euvrard S, Kanitakis J, Claudy A. Skin cancers after organ transplantation. *N Engl J Med* 2003;348:1681-91.
- Kripke ML, Fisher MS. Immunologic aspects of tumor induction by ultraviolet radiation. *Natl Cancer Inst Monogr* 1978:179-83.
- Yarosh DB, Pena AV, Nay SL, Canning MT, Brown DA. Calcineurin inhibitors decrease DNA repair and apoptosis in human keratinocytes following ultraviolet B irradiation. *J Invest Dermatol* 2005;125:1020-5.
- Kelly GE, Meikle W, Sheil AG. Effects of immunosuppressive therapy on the induction of skin tumors by ultraviolet irradiation in hairless mice. *Transplantation* 1987;44:429-34.
- O'Donovan P, Perrett CM, Zhang X, Montaner B, Xu Y-Z, Harwood CA, et al. Azathioprine and UVA light generate mutagenic oxidative DNA damage. *Science* 2005;309:1871-4.
- Allison AC. Immunosuppressive drugs: the first 50 years and a glance forward. *Immunopharmacology* 2000;47:63-83.
- Guertin DA, Sabatini DM. The pharmacology of mTOR inhibition. *Sci Signal* 2009;2:pe24.
- Sengupta S, Peterson TR, Laplante M, Oh S, Sabatini DM. mTORC1 controls fasting-induced ketogenesis and its modulation by ageing. *Nature* 2010;468:1100-4.
- Geissler EK, Schlitt HJ, Thomas G. mTOR, cancer and transplantation. *Am J Transplant* 2008;8:2212-8.
- Kelly GE, Meikle W, Sheil AG. Scheduled and unscheduled DNA synthesis in epidermal cells of hairless mice treated with immunosuppressive drugs and UVB-UVA irradiation. *Br J Dermatol* 1987;117:429-40.
- Reeve VE, Greenoak GE, Gallagher CH, Canfield PJ, Wilkinson FJ. Effect of immunosuppressive agents and sunscreens on UV carcinogenesis in the hairless mouse. *Aust J Exp Biol Med Sci* 1985;63 (Pt 6):655-65.
- Tressler RJ, Garvin LJ, Slate DL. Anti-tumor activity of mycophenolate mofetil against human and mouse tumors *in vivo*. *Int J Cancer* 1994;57:568-73.
- Engl T, Makarevic J, Relja B, Natsheh I, Muller I, Beecken WD, et al. Mycophenolate mofetil modulates adhesion receptors of the beta1 integrin family on tumor cells: impact on tumor recurrence and malignancy. *BMC Cancer* 2005;5:4.
- Guba M, von Breitenbuch P, Steinbauer M, Koehl G, Flegel S, Hornung M, et al. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. *Nat Med* 2002;8:128-35.
- Koehl GE, Andrassy J, Guba M, Richter S, Kroemer A, Scherer MN, et al. Rapamycin protects allografts from rejection while simultaneously attacking tumors in immunosuppressed mice. *Transplantation* 2004;77:1319-26.
- Koehl GE, Gaumann A, Zuelke C, Hoehn A, Hofstaedter F, Schlitt HJ, et al. Development of *de novo* cancer in p53 knock-out mice is dependent on the type of long-term immunosuppression used. *Transplantation* 2006;82:741-8.
- de Graaf YG, Rebel H, Elghalbzouri A, Cramers P, Nellen RG, Willemze R, et al. More epidermal p53 patches adjacent to skin carcinomas in renal transplant recipients than in immunocompetent patients: the role of azathioprine. *Exp Dermatol* 2008;17:349-55.
- Voskamp P, Bodmann CA, Rebel HG, Koehl GE, Tensen CP, Bouwes Bavinck JN, et al. Rapamycin impairs UV induction of mutant-p53 overexpressing cell clusters without affecting tumor onset. *Int J Cancer* 2012;131:1267-76.
- Salgo R, Gossman J, Schöfer H, Kachel HG, Kuck J, Geiger H, et al. Switch to a sirolimus-based immunosuppression in long-term renal transplant recipients: reduced rate of (pre-)malignancies and nonmelanoma skin cancer in a prospective, randomized, assessor-blinded, controlled clinical trial. *Am J Transplantation* 2010;10:1385-93.
- Euvrard S, Morelon E, Rostaing L, Goffin E, Brocard A, Tromme I, et al. Sirolimus and secondary skin-cancer prevention in kidney transplantation. *N Engl J Med* 2012;367:329-39.
- El-Ghalbzouri A, Gibbs S, Lamme E, Van Blitterswijk CA, Ponc M. Effect of fibroblasts on epidermal regeneration. *Br J Dermatol* 2002;147:230-43.
- Krotz F, Keller M, Derflinger S, Schmid H, Gloe T, Bassermann F, et al. Mycophenolate acid inhibits endothelial NAD(P)H oxidase activity and superoxide formation by a Rac1-dependent mechanism. *Hypertension* 2007;49:201-8.
- Odlind B, Hartvig P, Lindström B, Lönnnerholm G, Tufveson G, Grefberg N. Serum azathioprine and 6-mercaptopurine levels and immunosuppressive activity after azathioprine in uremic patients. *Intl J Immunopharmacol* 1986;8:1-11.
- Allison AC, Eugui EM. Mycophenolate mofetil and its mechanisms of action. *Immunopharmacology* 2000;47:85-118.
- de Gruijl FR, Koehl GE, Voskamp P, Strik A, Rebel HG, Gaumann A, et al. Early and late effects of the immunosuppressants rapamycin and mycophenolate mofetil on UV carcinogenesis. *Int J Cancer* 2010;127:796-804.
- Rebel H, Kram N, Westerman A, Banus S, van Kranen HJ, de Gruijl FR. Relationship between UV-induced mutant p53 patches and skin tumours, analysed by mutation spectra and by induction kinetics in various DNA-repair-deficient mice. *Carcinogenesis* 2005;26:2123-30.
- Attard NR, Karran P. UVA photosensitization of thiopurines and skin cancer in organ transplant recipients. *Photochem Photobiol Sci* 2012;11:62-8.
- Takeuchi S, Zhang W, Wakamatsu K, Ito S, Hearing VJ, Kraemer KH, et al. Melanin acts as a potent UVB photosensitizer to cause an atypical mode of cell death in murine skin. *Proc Natl Acad Sci U S A* 2004;101:15076-81.
- Sugie N, Fujii N, Danno K. Cyclosporin-A suppresses p53-dependent repair DNA synthesis and apoptosis following ultraviolet-B

- irradiation. *Photodermatol Photoimmunol Photomed* 2002;18:163–8.
33. Dean PG, Lund WJ, Larson TS, Prieto M, Nyberg SL, Ishitani MB, et al. Wound-healing complications after kidney transplantation: a prospective, randomized comparison of sirolimus and tacrolimus. *Transplantation* 2004;77:1555–61.
 34. Flockhart RJ, Diffey BL, Farr PM, Lloyd J, Reynolds NJ. NFAT regulates induction of COX-2 and apoptosis of keratinocytes in response to ultraviolet radiation exposure. *FASEB J* 2008;22:4218–27.
 35. Kramata P, Lu Y-P, Lou Y-R, Singh RN, Kwon SM, Conney AH. Patches of mutant p53-immunoreactive epidermal cells induced by chronic UVB irradiation harbor the same p53 mutations as squamous cell carcinomas in the skin of hairless SKH-1 mice. *Cancer Res* 2005;65:3577–85.
 36. Stahl PL, Stranneheim H, Asplund A, Berglund L, Ponten F, Lundeborg J. Sun-induced nonsynonymous p53 mutations are extensively accumulated and tolerated in normal appearing human skin. *J Invest Dermatol* 2011;131:504–8.
 37. King P, Craft AW, Malcolm AJ. p53 Expression in three separate tumours from a patient with Li-Fraumeni's syndrome. *J Clin Pathol* 1993;46:676–7.
 38. Wulff BC, Kusewitt DF, VanBuskirk AM, Thomas-Ahner JM, Duncan FJ, Oberyszyn TM. Sirolimus reduces the incidence and progression of UVB-induced skin cancer in SKH mice even with co-administration of cyclosporine A. *J Invest Dermatol* 2008;128:2467–73.
 39. Duncan FJ, Wulff BC, Tober KL, Ferketich AK, Martin J, Thomas-Ahner JM, et al. Clinically relevant immunosuppressants influence UVB-induced tumor size through effects on inflammation and angiogenesis. *Am J Transplantation* 2007;7:2693–703.
 40. Therapeutic Drug Monitoring of Tacrolimus. Dutch Society of Hospital Pharmacist; 2005. Available from: http://www.2nvza.nl/uploaddb/download_object.asp?atoom=5858&VolgNr=1
 41. Norman KG, Canter JA, Shi M, Milne GL, Morrow JD, Sligh JE. Cyclosporine A suppresses keratinocyte cell death through MPTP inhibition in a model for skin cancer in organ transplant recipients. *Mitochondrion* 2010;10:94–101.
 42. Lash AA. Anticancer drugs for noncancer diseases. *Medsurg Nurs* 1996;5:177,180–4,190.
 43. Brem R, Karran P. Oxidation-mediated DNA cross-linking contributes to the toxicity of 6-thioguanine in human cells. *Cancer Res* 2012;72:4787–95.
 44. Rebel H, Mosnier LO, Berg RJW, Westerman-de Vries A, van Steeg H, van Kranen HJ, et al. Early p53-positive foci as indicators of tumor risk in ultraviolet-exposed hairless mice: kinetics of induction, effects of DNA repair deficiency, and p53 heterozygosity. *Cancer Res* 2001;61:977–83.
 45. Jonason AS, Kunala S, Price GJ, Restifo RJ, Spinelli HM, Persing JA, et al. Frequent clones of p53-mutated keratinocytes in normal human skin. *Proc Natl Acad Sci U S A* 1996;93:14025–9.
 46. Berg RJ, van Kranen HJ, Rebel HG, de Vries A, van Vloten WA, Van Kreijl CF, et al. Early p53 alterations in mouse skin carcinogenesis by UVB radiation: immunohistochemical detection of mutant p53 protein in clusters of preneoplastic epidermal cells. *Proc Natl Acad Sci* 1996;93:274–8.
 47. le Pelletier F, Soufir N, de La Salmoniere P, Janin A, Basset-Seguin N. p53 Patches are not increased in patients with multiple nonmelanoma skin cancers. *J Invest Dermatol* 2001;117:1324–5.
 48. Boukamp P. Non-melanoma skin cancer: what drives tumor development and progression? *Carcinogenesis* 2005;26:1657–67.

Cancer Prevention Research

Dietary Immunosuppressants Do Not Enhance UV-Induced Skin Carcinogenesis, and Reveal Discordance between p53-Mutant Early Clones and Carcinomas

Pieter Voskamp, Carolien A Bodmann, Gudrun E Koehl, et al.

Cancer Prev Res 2013;6:129-138. Published OnlineFirst December 11, 2012.

Updated version	Access the most recent version of this article at: doi: 10.1158/1940-6207.CAPR-12-0361
Supplementary Material	Access the most recent supplemental material at: http://cancerpreventionresearch.aacrjournals.org/content/suppl/2012/12/11/1940-6207.CAPR-12-0361.DC1

Cited articles	This article cites 45 articles, 10 of which you can access for free at: http://cancerpreventionresearch.aacrjournals.org/content/6/2/129.full#ref-list-1
-----------------------	--

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, use this link http://cancerpreventionresearch.aacrjournals.org/content/6/2/129 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.