Clinical Cancer Research

5-Fluorouracil Enhances Protoporphyrin IX Accumulation and Lesion Clearance during Photodynamic Therapy of Actinic Keratoses: A Mechanism-Based Clinical Trial

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Abstract

Purpose: Actinic keratoses (AK) are precancerous lesions that can progress to squamous cell carcinoma. Photodynamic therapy (PDT) and topical 5-fluorouracil (5FU) are commonly used agents for AK. Empirical reports suggest that combining them can improve the therapeutic response. However, the optimal combined regimen was not clear in terms of proper sequence, timing, and mechanism. This clinical study explored mechanisms of action for neoadjuvantal 5FU and PDT for treatment of AK.

Patients and Methods: A bilaterally controlled trial (17 patients) was performed. One side of the body (face, scalp, forearms) received 5FU pretreatment for 6 days, whereas the other side served as no-pretreatment control. Methylaminolevulinate cream was applied to both sides for 3 hours, and protoporphyrin IX (PpIX) levels were measured by noninvasive fluorimetry and skin biopsy. After red light illumination, lesion clearance was assessed at 3, 6, 9, and 12 months after PDT.

Results: PpIX levels were increased 2- to 3-fold in 5FU-pretreated lesions versus controls. Altered expression of heme-synthetic enzymes (coproporphyrinogen oxidase and ferrochelatase) and induction of p53 were observed, probably accounting for increased PpIX and subsequent cancer cell death. Relative clearance rates after PDT with or without 5FU pretreatment were 75% versus 45% at 3 months, and 67% versus 39% at 6 months, respectively; these differences were statistically significant.

Conclusions: Serial 5FU and PDT improve AK clearance by at least two mechanisms, enhanced photosensitizer accumulation and p53 induction. Because 5FU and PDT are FDA-approved modalities, the combined regimen can be readily employed in clinical practice to reduce AK burden and reduce SCC risk. *Clin Cancer Res; 24(13); 3026–35. ©2018 AACR.*

Introduction

Actinic keratoses (AK) are rough, scaly lesions of dysplastic keratinocytes that arise in chronically sun-exposed skin. AK, which lie at one end of a spectrum of keratinocyte intraepithelial neoplasia (KIN) that includes AK, SCC *in situ*, invasive SCC, and metastatic SCC (1–3), are a strong risk factor for the development of squamous cell carcinoma (SCC; refs. 4, 5). Although some AK spontaneously resolve, others undergo malignant progression, with AK-to-SCC progression rates estimated to be 0.60% at 1 year and 2.57% at 4 years in the Veterans Affairs trial (6). In patients with multiple AK lesions or prior skin cancers, the incidence of SCC may be as high as 20% to 30% (2, 5). The risk of malignant progression is much higher in organ transplant patients; these immunosuppressed individuals have an incidence of SCC 60 to 100 times greater and a much higher rate of metastases than in the normal population, (7–9). Most clinicians agree on the importance of treating AK to prevent the development of invasive SCC (2, 10). As a consequence, and because AK are so common [estimated prevalence, 39.5 million in the United States (11)], the costs to the U.S. healthcare system for managing AK are estimated to be more than 1.2 billion dollars per year (12).

Most of the locally destructive treatments traditionally used for AK and early SCC *in situ* (including surgery, electrocautery, and cryotherapy with liquid nitrogen) are not fully effective over the long term because they fail to account for "field cancerization," the phenomenon in which new foci of neoplasia continually arise within a large area of mutagenized epithelium (13). Photodynamic therapy (PDT) is one of only a few modalities that address the field cancerization problem. To perform PDT, a prodrug called 5-aminoevulinic acid (ALA; ref. 14), or its methyl ester (MAL; ref. 15), is broadly applied onto affected skin. This prodrug is then selectively taken up into neoplastic cells and converted into protoporphyrin IX (PpIX) within mitochondria (16). Subsequent illumination with visible light activates PpIX and initiates oxygen-dependent photochemistry, thereby destroying mitochondrial membranes, activating apoptotic pathways, and leading to tumor

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Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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Translational Relevance

Actinic keratoses (AK) are precancerous lesions that increase the risk of skin cancer in patients with chronic sun damage and/or immunosuppression. A combined approach using 5-fluorouracil (5FU) prior to photodynamic therapy (PDT) was shown in previous clinical reports to be beneficial for AK treatment. Here, we conducted a clinical mechanistic study to determine exactly why the 5FU/PDT combination provides an improved response. We found that 5FU upregulates enzymes in the heme synthesis pathway, increases production of protoporphyrin IX (the active photosensitizer), and increases the expression of proapoptotic p53. Lesion clearance after the combined treatment is better (~2-fold) than after PDT alone, a benefit that remains statistically significant for 6 months. These findings provide a scientific rationale for using 5FU as a neoadjuvant for PDT. Each agent is already FDA-approved for AK, so clinicians can safely employ this practical regimen for their patients with UV-induced field cancerization of the skin.

cell death (17). PDT, using either ALA or MAL, is now widely used to treat AK in many countries (14, 15). However, improvements in PDT efficacy are still needed. Although excellent AK clearance rates (>85%) can be obtained, this is generally only possible if one pretreats the lesions using curettage, microneedling, or fractional laser ablation to increase prodrug absorption into the tissue, thereby adding cost and complexity to the treatment.

Our group has developed an expertise in the field of applied PDT principles. Using animal tumor models, we identified a new basic principle and treatment approach called *differentiation-enhanced PDT* (18). During the process of prodrug conversion to PpIX within mitochondria, cancer cells can be manipulated to make higher amounts of intracellular PpIX by pretreating them with agents that not only enhance terminal differentiation, but also possess an ability to stimulate PpIX accumulation (18). If done prior to ALA administration, a preincubation with any of three agents identified in our studies [namely, methotrexate (19); calcitriol (20); or 5-fluorouracil (5FU; ref. 21)] causes a 2- to 5-fold increase in intracellular PpIX levels in cultured SCC cells and in SCC tumors grown subcutaneously in mice. In implanted SCC tumors (19, 20) or in early SCC lesions induced by chronic ultraviolet light exposure (22), any of these pretreatments can induce PpIX levels and enhance the therapeutic response. The enhancement of PpIX and tumor cell death is tumor-specific, i.e., normal adjacent tissues are spared (19, 20, 22). Besides their ability to enhance epithelial cancer cell differentiation (i.e., E-cadherin expression), the three agents also regulate certain important enzymes among the eight enzymes present in the heme synthesis pathway. Thus, coproporphyrinogen oxidase (CPO) was upregulated, and ferrochelatase (FC) was downregulated following exposure to the differentiation-promoting agents, with the net result being PpIX accumulation (19, 20). Upregulation of CPO was shown to involve induction of the CPO gene promoter by C/EBP transcription factors, which are well known "master regulators" of terminal differentiation (23).

When attempting to translate these preclinical findings to human skin cancer, we focused attention on 5FU. Unlike methotrexate or calcitriol, 5FU is specifically approved for the treatment of cutaneous AK in humans (24) and can be applied topically. 5FU cream can be effective for AK as a monotherapy, but it must be applied daily for 3 to 5 weeks in order to induce a pronounced inflammatory state (erythema, erosions) that most patients find difficult to tolerate (25). In our studies in murine models of UV-induced skin cancer, a brief period of 5FU application (only 3 days) was sufficient to increase PpIX levels and improve the tumor treatment response. PpIX elevations were accompanied by significant changes in CPO and FC enzyme levels, as well as in E-cadherin (21, 26). In addition, p53 was increased after 5FU pretreatment. Because p53 is a tumor suppressor with proapoptotic functions, p53 appears to be responsible, at least in part, for the increased therapeutic effectiveness of the combined 5FU/PDT regimen (26).

From those preclinical study results, it is reasonable to hypothesize that 5FU pretreatment followed by ALA-based PDT should also be effective in humans with AK. In order to test proof of principle and establish a plausible biochemical mechanism, we designed a clinical pilot study to ask whether previous observations in the murine models might be applicable in human AK. Positive results would provide a solid rationale for anticipating an improved clinical outcome when 5FU and PDT are combined.

Patients and Methods

Clinical study design

The study protocol is summarized in Fig. 1. Enrollment criteria included age >18 years; at least 4 AK lesions on the face, scalp, or forearms; no current use of topical treatments for AK; and a negative history of porphyria. Renal transplant patients could participate if their transplant surgery had occurred at least 2 years before enrollment. The study was conducted in the dermatology department of the Cleveland Clinic between November 2011 and March 2014, under a protocol approved by the Institutional Review Board (IRB), No. 09-1050, and registered at ClinicalTrials.gov (NCT01525329).

Study day 1. Informed consent was obtained. AK lesions were counted, and all sites were carefully photographed as described in "Assessment of Treatment Response" below. Noninvasive fluorescence PpIX measurements were taken from 4 randomly selected lesions (2 on the left side, and 2 on the right) prior to prodrug application (MAL, 16.8% Metvixia cream; Galderma Ltd) and again 3 hours after MAL application, to assess PpIX buildup. PpIX was measured as described in a separate section below.

To decide which side of the body should receive 5FU pretreatment, a two-step, serial block randomization scheme was used. For the first patient in each group, 5FU was assigned by a computer-generated coin toss (for example, the right side). The next patient enrolled was then automatically assigned to the opposite side to assure an equal assignment distribution. The decision not to use a placebo cream was made upon a recommendation from our IRB, whose members felt that the risk of confusion when applying two different creams was unacceptably high in an elderly population. 5FU cream (5%) was prepared by our Research Pharmacy, and patients were instructed to apply it to the assigned side of the body, once daily for 6 days. Also, patients were asked to record symptoms

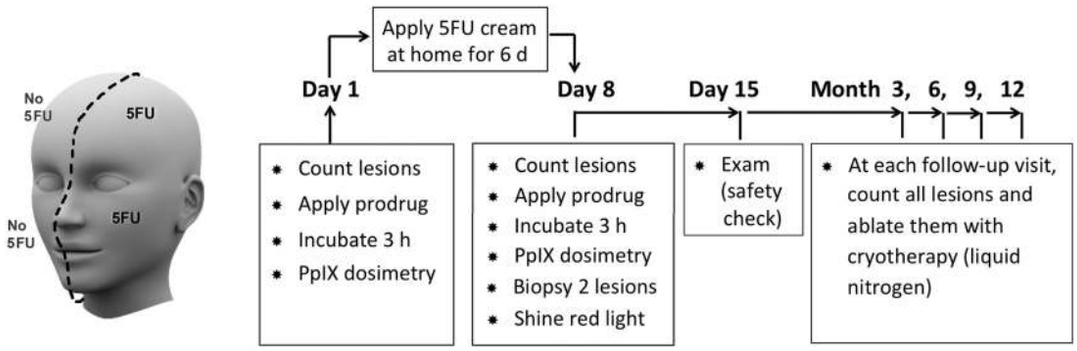

Figure 1.
Schematic of clinical trial protocol. See text for details.

and side effects daily on a daily questionnaire (log sheet) provided to them.

Study day 8. The patient was examined, and lesions counted and photographed. MAL was applied broadly to the entire treatment area. Fluorescence dosimetry was performed on the four assigned AK lesions, at baseline and 3 hours after MAL application. Skin biopsies (4 mm) were performed just prior to illumination; the two largest contralateral lesions were sampled. Specimens were embedded and frozen at the bedside. Biopsy sites were covered with a small circular bandage, and the remaining treatment area was exposed to red light (Aktilite, 635 nm, 37 J/cm^2) using a fan and ice clothes for pain control (27). Patients were told to apply Aquaphor and hydrocortisone cream for comfort at home and to record their symptoms for 6 days on the study questionnaire provided.

Follow-up visits. Patients returned at 2 weeks and at 3, 6, 9, and 12 months for clinical examination and photographs. All new lesions observed at 3 months and beyond were treated with liquid nitrogen cryosurgery.

Assessment of biochemical endpoints

Noninvasive fluorescent dosimetry of PpIX. The dosimetry technique has been previously described in detail (22, 28). Briefly, a hand-held probe (optical fiber bundle) was used to deliver weak laser pulses of 635 nm wavelength to the skin to excite the PpIX molecules. Fluorescent light from PpIX (>690 nm) was transmitted back up the optical fibers to a detector (Fig. 2A), and the signal was recorded on a laptop computer. In each patient, 4 randomly selected lesions (two 5FU pretreated, 2 controls) were measured. The data were reported in two different ways: (1) difference in fluorescence signal between 5FU-pretreated lesions and nonpretreated lesions; (2) ratio between 5FU-pretreated and nonpretreated lesions (Supplementary Table S2). Full details are in the footnotes of Supplementary Table S2.

Evaluation of PpIX levels in skin biopsies. For every patient, two of the lesions measured previously by fluorimetry (one 5FU-pretreated, one control) were biopsied on day 8. Specimens were frozen in optimum cutting temperature (OCT) embedding compound (Sakura Finetek), cut into 10 μm sections, and placed onto glass slides. For PpIX analysis, the fluorescence generated by excitation of PpIX was measured by confocal microscopy as described (20). Instrument settings on the laser confocal scanning microscope (Leica Microsystems) were chosen so as to generate PpIX-specific fluorescence (excitation wavelength, 635 nm; fluorescence collection at 650–780 nm). The digital images were analyzed with an image-processing program (IPLab, Scanalytics) to integrate fluorescence intensity/pixel over the entire region of interest. Dermal background subtraction was performed. Results from two tissue sections per biopsy were averaged to obtain a PpIX signal for the particular lesion (C, in arbitrary units). Results were reported as the ratio of the 5FU side versus control side ($C_{FU}/C_{control}$).

Histologic staining to evaluate molecular markers in skin biopsies. Frozen sections were evaluated by hematoxylin/eosin staining and by immunofluorescent staining for evaluation of protein marker expression. For immunofluorescence analyses, the primary antibodies were: E-cadherin and p53 (Santa Cruz Biotechnology; 1:100), and Ki-67 (NeoMarkers; 1:250). The secondary antibody was Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch; 1:1,500). Relative expression of marker proteins in immunofluorescently stained sections was measured using digital microscopy (20).

Western blot analysis. Frozen skin biopsies were crushed, dissolved in urea lysis buffer, sonicated, and analyzed on Western blots as described (19). Source and dilutions of primary and secondary antibodies were as follows: ALA dehydratase (ALAD; Abnova; 1:1,000), CPO (custom made, details in ref. 29; 1:5,000), FC (Abnova; 1:1,000), glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz Biotechnology; 1:5,000), porphobilinogen oxidase (PBGD, Abnova; 1:1,000), peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulin G (Jackson Immunoresearch; 1:20,000).

Heme enzyme expression in normal- versus UVB-induced preneoplastic skin in mice. An experiment to ask whether 5FU induced changes in heme enzyme expression might occur selectively in AK, and a mouse model of AK-like lesions was produced as described (22). Briefly, SKH-1 hairless mice were irradiated with a UVB source thrice weekly for 15 weeks. Thickened, preneoplastic areas of skin containing elevated PpIX

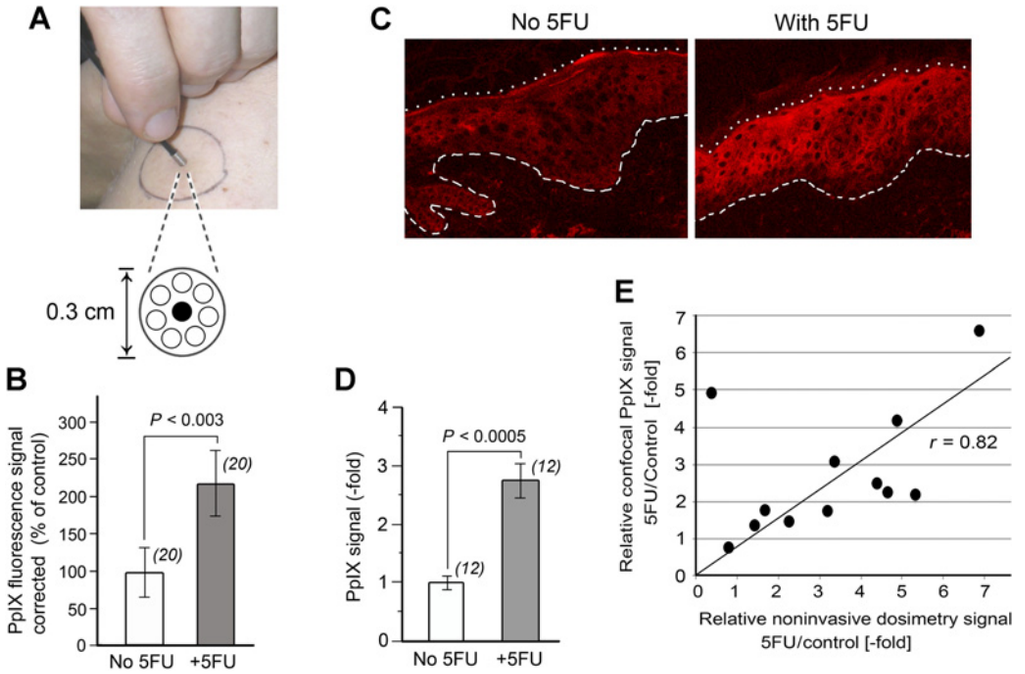

Figure 2.

PpIX accumulation in AK lesions after 5FU pretreatment, measured using two independent techniques. MAL was applied topically, and the resultant PpIX in each lesion was measured 3 hours later. **A,** Noninvasive dosimeter probe tip placed against the skin; circles indicate optical fiber bundles within the probe tip. **B,** Summary of noninvasive fluorescent measurements of PpIX *in vivo*. $N = 20$ bilaterally matched body sites. **C,** Representative confocal fluorescent images of PpIX in skin biopsies from two bilaterally matched AK lesions from the same patient. One received 5FU pretreatment, the other did not. **D,** Summary of PpIX measurements in skin biopsies *ex vivo*; $N = 12$ pairs of biopsies from bilaterally matched body sites. **E**, Positive correlation between noninvasive measurements (x-axis) and tissue measurements (y-axis) of PpIX within the same lesion; r, correlation coefficient.

levels were identified by Maestro imaging done 4 hours after ALA application (26). Those areas were biopsied for comparison with biopsies of normal skin from the same individuals, to assess levels of individual heme enzymes by Western analysis. These experiments were approved by the Institutional Animal Care and Use Committee of the Cleveland Clinic.

Assessment of clinical endpoints

Treatment response. Global AK lesion counts were determined in two ways: by physical examination and by photography. Examiners counted only visible lesions, i.e., grades 2 and 3 in the classification of Olsen and colleagues (30). All study areas were digitally photographed in studio at every visit, using high-resolution and standardized views (left and right lateral, left and right oblique, and full frontal). The photographs were examined by two independent dermatologists, blinded to the treatment assignment, on a large high-resolution computer monitor, and the two sets of counts averaged. Similar to another recent study that performed clinical and photographic AK counts in a similar manner (31), the concordance between the clinical and photographic counts was good (within 10%), and relative changes were uniformly consistent. However, statistical variability was found to be less with the photographic AK counts, and therefore, the latter were used for the final analyses of treatment response. Any lesion biopsied for the biochemical studies was excluded from the final lesion counts.

Statistical analysis. This study was designed to test three hypotheses: (1) Does 5FU pretreatment lead to higher intralesional PpIX concentrations? (2) Does neoadjuvantal 5FU plus PDT provide better clinical outcomes than PDT alone? (3) Is the 5FU+PDT combination regimen well tolerated by patients?

For the primary endpoint (the effect on PpIX levels), paired t tests were used to compare average PpIX levels between the two treatment arms. To estimate effect sizes and calculate the enrollment target for the clinical trial, PpIX data from our previous animal studies were used. It was determined that 18 patients were needed to detect a 50% increase in mean PpIX change, with 90% power (two-sided test, alpha = 0.05), assuming a common standard deviation of 2.4 and correlation of 0.25. For the secondary endpoint (clinical outcomes), both simple statistical comparisons and a linear mixed-effects model (32) were used to compare the change in lesion counts between the 5FU+PDT combination and PDT alone. The linear mixed-effect model was used in a correlated data structure (Supplementary Table S3). The model for all body sites controlled for baseline lesion count and site, whereas the models for three respective sites only controlled for baseline count. Subgroup analyses were performed at various time points. All models included a random intercept at the subject level. Analyses were conducted using R-studio. Statistical significance was established at two-sided P values < 0.05. Results were not adjusted for multiple comparisons. For the tertiary endpoint (tolerability), a straightforward questionnaire was used by each

subject to record side effects during the week of 5FU pretreatment and during the week after PDT.

Results

5FU pretreatment stimulates increased PpIX accumulation in AK lesions

Seventeen patients were enrolled and completed the 12-month study. In 3 of these patients, 2 different body sites (face and scalp) were analyzed separately, ultimately yielding a total of 20 different body sites for lesion analysis. Demographic characteristics (summarized in Supplementary Table S1) included age >60 years, a significant history of skin cancer, multiple prior sunburns, and extensive outdoor exposure. Of note, the four renal transplant recipients in the study had experienced many more skin cancers (mean of 20 SCC/person) relative to the nontransplant cohort (mean 2.5 SCC/person).

As per the study protocol, the amount of PpIX produced within AK lesions was measured before and 3 hours after application of the prodrug (Fig. 1). This was done on two different days (days 1 and 8). During the week between day 1 and day 8, the patient applied 5FU cream daily to one side of the body, whereas the contralateral side received no pretreatment, thereby allowing us to ask whether 5FU pretreatment exerted a differential effect upon PpIX production (measured via noninvasive PpIX fluorescence measurements on days 1 and 8). Results are summarized in Fig. 2B; the complete dataset is shown in Supplementary Table S2. Even after adjustment for a slight "daylight PDT" effect (33) in some of the patients (no more than 20%, and probably due to unintended sun exposure after the day 1 visit), average PpIX values of 5FU-pretreated lesions were consistently higher than PpIX values of nonpretreated controls (Supplementary Table S2, column 5, difference in ΔS). This was also evident as a relative PpIX induction in 5FU-treated versus control lesions (Supplementary Table S2, column 6, ratio of ΔS). When expressed in relative terms, 5FU pretreatment led to a 2.2-fold increase in PpIX accumulation, a very significant induction (Fig. 2B).

Surface optical measurements can be influenced by many factors that introduce interpatient variability. Therefore, we sought to verify the noninvasive dosimetry results using a second approach. Two AK lesions per patient (one from each side of the body) were biopsied just prior to light exposure and analyzed by confocal microscopy to assess PpIX levels in the tissue (for an example, see Fig. 2C). For 12 patients, both frozen tissue specimens were of sufficient quality to allow a matched comparison. In 11 of these 12 cases, the PpIX fluorescence intensity was higher on the 5FU side (Supplementary Table S2, last column), and the aggregate data showed 2.8-fold higher PpIX levels on the 5FU side (Fig. 2D). To assess reliability of the two different PpIX measurement techniques, noninvasive fluorescence values and confocal PpIX values from each patient were plotted on the same graph (Fig. 2E). The result confirmed a linear relationship and strong positive correlation between the two techniques.

5FU pretreatment affects molecular biomarkers of differentiation and apoptosis in AK lesions

In our previous work with murine SCC models, a striking and consistent effect after pretreatment with methotrexate, Vitamin D, or 5FU was the induction of terminal differentiation markers in tumors (18, 26). To assess the effect of 5FU on cellular differentiation in human AK, frozen tissue sections from our study patients were analyzed. The proliferation marker Ki-67 served as a positive control; Ki-67 expression in 5FU-pretreated lesions dropped to 30% of levels in the control lesions (Fig. 3A and A′), an expected finding because 5FU is known to inhibit cellular proliferation (34). To assess the differentiation status of AK lesions, a marker of epithelial differentiation was examined. E-cadherin expression was induced 3.6-fold in the 5FU-pretreated lesions (Fig. 3B and B′), in agreement with the earlier observations in murine AK/SCC (26). We also examined p53 expression; p53 protein levels were approximately 5-fold higher in 5FU-pretreated lesions (Fig. 3C and C′).

Heme pathway enzymes are altered by 5FU pretreatment

In our preclinical studies of differentiation-inducing enhancers of ALA-PDT, the expression of enzymes in the heme-synthetic pathway was changed in a manner that should favor enhanced production of PpIX (19, 20, 26, 29). To ask whether 5FU pretreatment causes similar changes in heme-synthetic enzyme in human AK lesions, protein levels of the four enzymes most frequently cited as potentially rate-limiting for heme synthesis (ALAD, PBGD, CPO, and FC) were analyzed in lesional tissues (Fig. 4 and Supplementary Fig. S1). This analysis was performed in 15 patients, but due to the technical challenges of obtaining sufficient material from 4 mm skin biopsies, matched analyses (tissue available from both left and right) were possible in only 11 patients. Nevertheless, results were informative. 5FU pretreatment of AK lesions caused reproducible changes in the expression of two enzymes that lie immediately upstream and downstream of PpIX, namely CPO and FC (see Fig. 6). For *CPO*, expression is induced by 5FU (Fig. 4A), whereas for FC, expression is decreased (Fig. 4B). Other enzymes (ALAD and PBGD) were highly variable, so that only changes in CPO and FC were significant (Fig. 4C and D). Among 11 patients with a bilaterally matched dataset, 100% displayed a relative increase in CPO expression with 5FU, and 75% showed decreased FC expression with 5FU. The net effect of these changes was an enhanced accumulation of PpIX, because higher amounts of upstream enzymes should enhance flow of substrate through the pathway, whereas a decrease in FC (the last enzyme, which catalyzes incorporation of iron into heme) should lead to the accumulation of PpIX that is blocked from completing the final step in the pathway.

We had shown in our previous work (26) that 5FU's effect upon PpIX accumulation is specific for tumor cells, but the question remained whether this tumor-specificity can be accounted for at the heme enzyme level. To address this, we compared the expression of CPO after 5FU administration in a mouse model of UVB-induced AK; this permitted the ethical analysis of normal skin as well as AK lesions in the same individual animals. The data shown in Supplementary Fig. S2 confirm that 5FU specifically induces CPO expression in AK lesions, whereas CPO is only minimally affected in normal, nondysplastic skin. Other enzymes were examined, but showed no significant changes.

The clinical response of AK lesions to PDT is improved by 5FU pretreatment

A secondary goal of this human pilot trial was to measure clearance rates of AK lesions after PDT, with or without 5FU pretreatment. Typical changes induced by 5FU and PDT are illustrated for one AK lesion in Fig. 5A–D. Compared with the pretreatment baseline (Fig. 5A), lesions that received 6 days of topical 5FU cream became slightly more erythematous (Fig. 5B).

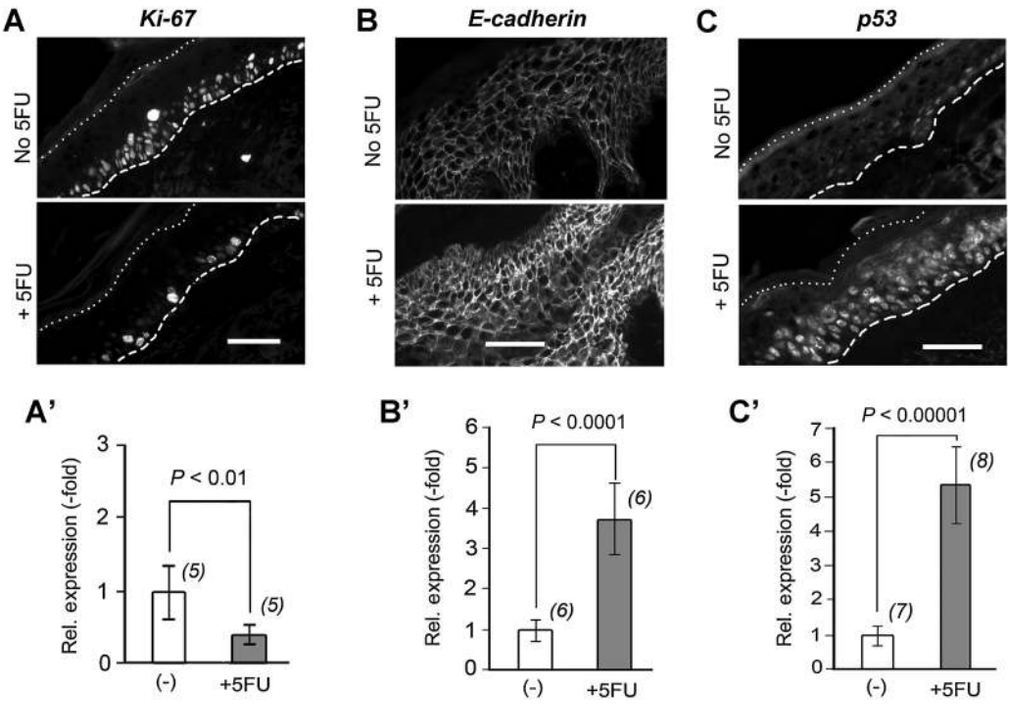

Figure 3.

Immunohistochemical analysis of markers of proliferation, differentiation, and proapoptotic potential in human AK lesions ± 5FU pretreatment. Biopsies of contralaterally matched AK lesions (one receiving 5FU pretreatment and the other not) were labeled using immunofluorescence. Examples of immunohistochemical staining for (**A**) Ki-67; (**B**) E-cadherin; and (**C**) p53 are shown. Dotted lines, stratum corneum; dashed lines, basement membrane. Scale bar, 50 μm. (**A′–C′,** Quantitation of the immunofluorescence intensity from all specimens; *n*, number of patient biopsies analyzed.

The subsequent MAL-PDT treatment caused marked inflammation within the target lesion, as intended (Fig. 5C). This inflammation eventually resolved, with substantial improvement and/or complete lesion clearance noted by 3 months after PDT (Fig. 5D).

The time course of change in the number of AK lesions (both absolute and relative counts) following PDT ± 5FU was recorded at all body sites, as documented in Supplementary Fig. S3. An example illustrating the data for 1 patient is presented in Fig. 5E. Typical for the majority of patients, AK counts declined more on the body site receiving 5FU+PDT than on the site receiving PDT alone. To analyze all the data in aggregate, a linear mixed-effects model was employed to calculate relative lesion clearance rates (CR) following PDT and to examine relationships between various groups of interest as described in Supplementary Table S3. This analysis showed that CR in the absence versus presence of 5FU pretreatment was 45% versus 75% at 3 months and 39% versus 67% at 6 months after PDT. The advantage provided by neoadjuvantal PDT was statistically significant at 3 and 6 months, and showed a trend toward relative benefit at 9 and 12 months (Supplementary Table S3, and Fig. 5F).

To analyze safety and side effects, patients filled out a questionnaire (symptom log). Results collated in Supplementary Table S4 show that the 5FU/PDT combination treatment was well tolerated, with no major side effects other than the local inflammatory reaction typically associated with PDT treatment.

Discussion

PDT is now a popular treatment for AK as well as SCC *in situ*, and many investigators are exploring ways to improve its clinical efficacy. Recent case reports and clinical series have shown that improved therapeutic benefit can be obtained by combining PDT with topical 5FU (35–39). Although a better outcome might seem obvious when combining two well-known therapeutic modalities, deeper inspection is required to truly understand why a particular 5FU/PDT combination actually works, and why it is beneficial to apply 5FU beforehand (as a neoadjuvant) rather than simultaneously or afterward to achieve the desired benefit. In the current study, we have described why 5FU is useful as a neoadjuvant to increase the effectiveness of aminolevulinate-based PDT for AK in human skin.

In our clinical study, biomarkers that had offered mechanistic insights in murine tumor models (19, 20, 26) were employed. In a bilaterally controlled clinical trial, half of each patient's AK lesions were pretreated with topical 5FU, whereas the other half were not. Then after applying the prodrug, levels of PpIX synthesized in the lesions were measured using two independent techniques. We found that 5FU pretreatment induces a 2- to 3-fold increase in intralesional PpIX levels, an important biochemical finding that correlates with significant improvement in the therapeutic response (nearly 2-fold better with the combined 5FU/PDT regimen). These findings make sense, given that a higher concentration of target photosensitizer (PpIX) should lead to greater

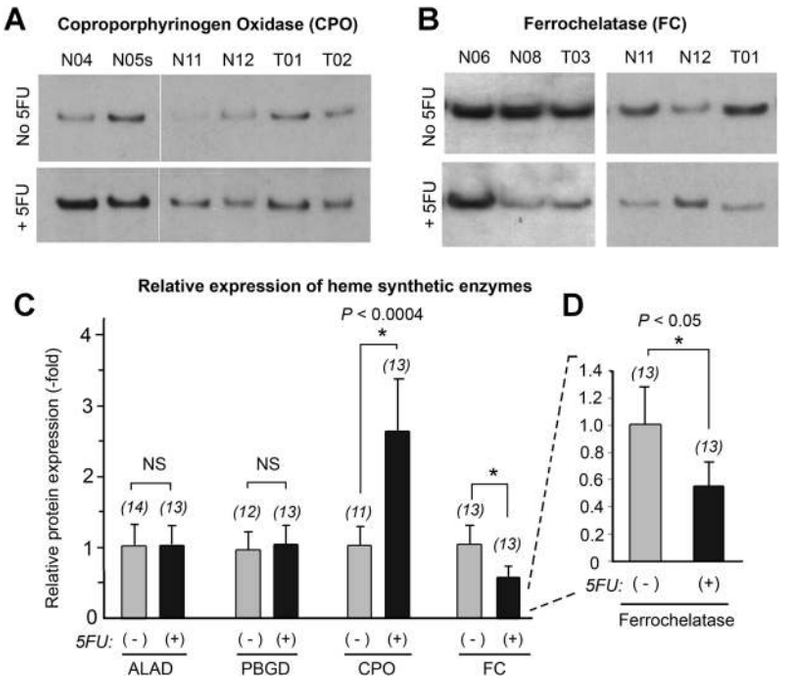

Figure 4.
Relative changes in protein expression of the major heme pathway enzymes in AK lesions, in bilaterally matched lesions ± 5FU pretreatment. Matched AK skin lesion specimens (one from the control side, the other from the 5FU-pretreated side) were dissolved in lysis buffer and subjected to Western analysis. Western blots from six representative patients among at least 12 analyzed for each enzyme are shown for CPO (**A**) and FC (**B**), showing typical differences between contralateral biopsies (matched in the same patient). Quantification is shown for all the patient samples (**C**), after normalization to GAPDH. Note the highly significant difference in CPO. **D,** Enlargement to emphasize the significant change in FC expression. *P* values are from two-sided Student *t* test. See Supplementary Fig. S1 for compilation of all Western blot data including the GAPDH controls.

target cell death when the light is turned on. More interesting, however, may be our data regarding the likely mechanisms by which 5FU pretreatment induces higher PpIX levels. This is illustrated in Fig. 6. In principle, the concentration of each type of porphyrin intermediate present during heme synthesis is dependent upon the relative levels and activity of the catalytic enzymes in the heme synthetic pathway. We have shown that after 5FU pretreatment, the levels of CPO (which lies upstream of PpIX) are increased, whereas the levels of FC (located immediately downstream of PpIX) are decreased (Fig. 4). The net result is higher accumulation of PpIX. Interestingly, the same two enzymes were similarly altered in our preclinical studies with murine SCC tumors (18–20, 26). Two additional enzymes, ALAD and PBGD, also showed alterations in expression after differentiation-

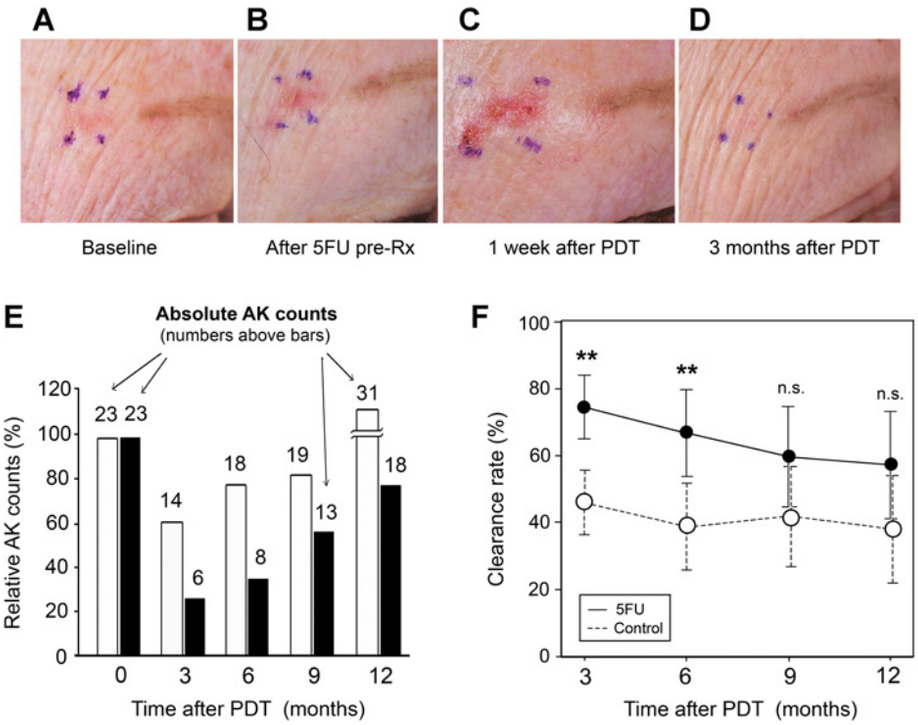

Figure 5.
Clinical response of AK lesions to combined 5FU/PDT treatment. Lesions in bilaterally matched skin regions were treated with MAL-PDT after a 1-week pretreatment ± 5FU. **A–D,** Photographs to show how a typical AK lesion, near the right eyebrow, responded after PDT. **E,** AK lesion counts on the control side (white bars) or the 5FU-pretreated side (black bars) at the end of each 3-month interval. This shows just one patient example, to illustrate changes in absolute lesion counts, and in relative counts (percent change). Note: Supplementary Fig. S2 is a compilation of all the lesion count data. PDT was performed only once, prior to the 3-month visit. **F,** Relative clearance rates, i.e., percent reduction of lesions from baseline, calculated using the linear mixed-effects model, as described in Supplementary Table S3. **, the difference is statistically significant, $P < 0.001$.

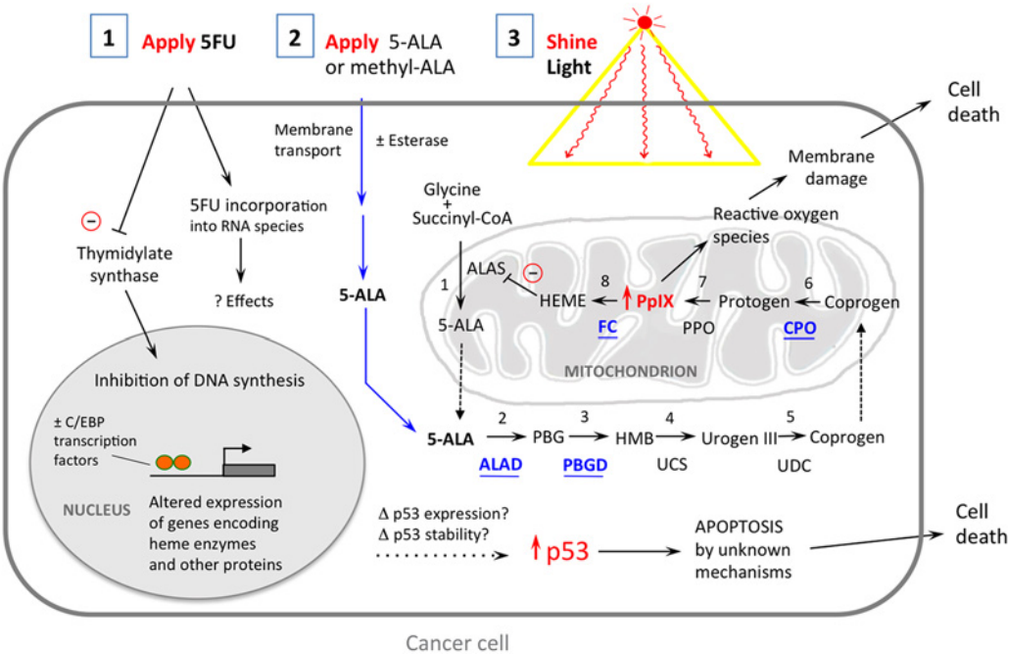

Figure 6.

Cartoon to summarize the findings and proposed mechanisms in this study. See text for explanation. Abbreviations for the substrates and enzymes of the eight steps in the heme synthesis pathway were defined in our previous publication (Sinha and colleagues, ref. 29).

promoting therapy, here and in the preclinical studies, but those changes were less consistent. Given the many differences between previous murine studies and this clinical trial (e.g., implanted SCC cell lines vs. native AK lesions; systemic 5FU vs. topical 5FU), the consistency of changes in CPO and FC expression across diverse experimental systems further strengthens our hypothesis that CPO and FC are the main drivers of PpIX accumulation following 5FU pretreatment.

Enhanced expression of p53 appears to be another mechanism at play during 5FU pretreatment. Induction of p53 protein is expected to enhance apoptosis in AK lesions after PDT-initiated cellular damage (40). In the patient samples, 5FU pretreatment led to a 5-fold increase in p53 protein levels. We recently published a detailed study examining 5FU and PDT in murine SCC tumors and found that 5FU significantly elevates p53 protein expression. Increased p53 levels were seen after 3 days of exposure to 5FU and lasted for at least 24 hours after PDT (26). Interestingly, the proapoptotic effects of 5FU in this setting may not depend upon conventional functions of p53, because 5FU-enhanced PDT efficacy was also observed in tumors harboring a mutant p53 allele, as well as in p53-null tumors (although to a lesser extent; ref. 26). Therefore, elevated p53 levels observed in our patients' 5FU-pretreated lesions probably do have a role in augmenting PDT-induced cell death, regardless of their mutational status. Surveys of human AK have shown variable frequency of p53 mutations, ranging from 7% to 53% (41–43).

Other molecular events after 5FU pretreatment may also be important (Fig. 6). 5FU exerts inhibitory effects on both DNA and RNA syntheses (34, 44). 5FU also enhances terminal differentiation (45). We have shown that differentiation-promoting agents can affect the expression of C/EBP transcription factors, "master regulators" of differentiation (46), and thereby affect genes that regulate heme enzyme expression (23). Our study of the murine CPO gene showed that Vitamin D and methotrexate alter the expression of C/EBP isoforms, which can then differentially bind to enhancer sites in the CPO gene promoter (23). Here in the clinical trial, we measured heme enzyme protein levels because changes in proteins are likely to have a functional impact. Significant changes in the CPO and FC proteins were observed after 5FU, but the molecular level at which gene regulation occurs (transcriptional, translational, or protein stability) remains unknown. It might nevertheless be intriguing to ask whether regulation of the heme enzymes occurs as part of a regulated transcriptional network, e.g., a "transcriptome" regulated by C/EBPs.

The clinical results of this study have major implications for patient care. Despite a relatively small sample size, the bilaterally controlled data strongly support previous clinical reports showing that serial 5FU and PDT can significantly improve AK lesion clearance. Clearance rates in our study were only modest because the trial was intentionally designed to increase the possibility of demonstrating a significant difference in PDT outcomes ± 5FU. Suboptimal conditions (no debridement, no occlusion) were chosen to provide a suboptimal lesion clearance after PDT alone, leaving room to detect any improvement due to 5FU. Clinicians in the United States may also wonder about our choice of red light for this study. At the time that the trial was initiated, our goal was to replicate our earlier studies in mice, which were performed using red light PDT. We therefore chose MAL and red light, the regimen preferred in Europe and also available in the United States at that time. Although MAL is no longer sold in the United States, it is important to recognize that MAL is readily de-esterified to ALA upon entry into cells, and beyond that point, the metabolic events of heme synthesis are the same whether MAL or ALA was

used as the prodrug (47). Therefore, the 5FU-related mechanisms reported here should apply to all PDT regimens that depend upon the conversion of ALA to PpIX. Once PpIX is formed, a number of different wavelengths of light can activate the protoporphyrin, including blue (400 nm), red (635 nm), and a number of others (48). For this reason, not only red light but also blue light and broad-spectrum daylight PDT should benefit from neoadjuvantal 5FU, an assertion supported by results of recent clinical studies (37–39).

Regarding the goal of preventing neoplastic progression, studies have shown that PDT by itself can delay the onset of new AK lesions in field-cancerized skin (49). Other studies indicate that topical 5FU monotherapy can have an AK-preventive effect (50). Therefore, we speculate that a 5FU/PDT combination will further enhance the overall prophylactic benefit, although this needs to be formally tested. This would be especially important for organ transplant patients at high risk for developing SCC, a population in whom capecitabine (Xeloda), an oral precursor of 5FU, is sometimes used because of its high efficacy and relative tumor specificity. A subgroup analysis of the four renal transplant recipients in our study showed that their AK clearance rates were statistically indistinguishable from those of immunocompetent patients, indicating that the 5FU/PDT combination is also helpful in the organ transplant group (Supplementary Table S3). Finally, as another important endpoint, patients recorded their symptoms and side effects during 5FU application and after PDT; the results show that this combination treatment is very well tolerated (Supplementary Table S4).

In conclusion, this report describes a serial regimen of topical 5FU and aminolevulinate-based PDT and establishes a mechanistic rationale for why this particular combination is effective for AK. With the combination, lesion clearance is significantly improved and side effects are well tolerated. Clinical translational impact should be immediate. Each therapy is already approved by regulatory agencies in most countries, and therefore the combined regimen can be used now to eliminate neoplastic precursors and reduce cutaneous SCC development in high-risk patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Study supervision: E.V. Maytin, M. Riha, A. Vidimos

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