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An Assessment of TLR4 and TOPK/PRPK Signaling in Sun Damaged Human Skin Acutely Exposed to Solar Simulated Light

ONGOING PROTOCOL

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1.0 OBJECTIVES

The overall goal of the Skin Cancer Prevention Program Project is to employ novel technologies and develop new therapeutic strategies to prevent intraepithelial neoplasias in the skin (i.e. actinic keratosis, cutaneous squamous cell carcinoma *in situ*). Our objectives in pursuit of this goal are to determine clinical, histopathologic, and protein/phosphoprotein signaling patterns associated with human skin carcinogenesis and to develop safe, highly efficacious intervention strategies for the prevention of skin cancer. The **overall hypothesis of the project** is that Toll-like Receptor 4 (TLR4) and T-LAK cell-originated protein kinase (TOPK) / p53-related protein kinase (PRPK) drive important signaling pathways, with potential synergism to prevent cutaneous squamous cell carcinoma (cSCC), and that TOPK/RPRK and TLR4 signaling pathways are modulated in the skin by acute exposure to solar simulated light (SSL).

The aim of this study is to assess TLR4 and TOPK/PRPK signaling in sun damaged (SD) human skin acutely exposed to solar simulated light (SSL). The purpose of this aim is to assess the effect of acute UV exposure on the signaling pathways of interest in order to validate the acute clinical model prior to intervention with small molecule inhibitors. Acute UV exposure will be evaluated in SD skin to determine the level of activation of the targeted pathways using reverse-phase protein array (LCM-RPPA) analysis and immunohistochemistry (IHC). This translational aim will prospectively analyze TLR4 and TOPK/PRPK signaling pathways activation by acute exposure of human subjects to SSL at twice the minimal erythema dose (MED) for each subject. We will evaluate the modulation of key proposed signaling pathways using RPPA and IHC in acutely irradiated human skin. We will also determine the optimal time points for evaluation of biomarker modulation.

Primary Endpoint: To assess TLR4 and TOPK signaling pathway activation levels pre- and post-SSL exposure in SD skin.

Exploratory Endpoint: To assess the correlation between SD level and the magnitude of SSL-induced pathway activation.

Thirty-six (36) subjects with a broad range of SD on the forearm will be recruited. Based on the standardized clinical photodamage scale [1], we will include mild (N=12), moderate (N=12), and severely (N=12) sun damaged (SD) skin. The level of SD will be scored, using a range of 0-9, where absent SD = 0, mild SD = 1-3, moderate SD = 4-6 and severe SD = 7-9 (see Appendix C).

We will irradiate and biopsy the SD forearm skin of each healthy adult volunteer and collect skin punch biopsies before and after exposure to SSL for analysis using LCM-RPPA and IHC. The results of this study will be used to aid in design of pharmacodynamic SSL exposure studies with small molecule inhibitors in subsequent studies.

2.0 BACKGROUND, SIGNIFICANCE AND PRELIMINARY WORK

One out of three new cancers is a skin cancer, making skin cancer the most common malignancy worldwide. Approximately 4.9 million cases of non-melanoma skin cancer (NMSC) (basal cell carcinoma [BCC] and cutaneous squamous cell cancers [cSCC]) occur annually. As incidence rates for NMSC continue to rise, there is an increasing and substantial impact on morbidity and health care costs that account for $8.1 billion/year in skin cancer treatment with the majority of these lesions representing keratinocytic neoplasms.

New technologies increasingly allow for the capacity to individualize therapeutic prevention strategies based on gene expression patterns and direct, multiplexed cell signaling profiling. Target modulations, pathway activation, skin biology alterations, and other basic science discoveries will be translated into the clinic. Increasingly, our research group has attempted to dissect solar light-induced signal transduction pathways leading to cSCC with the aim of identifying molecular targets for chemopreventive drug development. Essential to this research approach is a requirement to validate molecular targets identified in solar light-induced cSCC carcinogenesis and skin biology models. Strategies to be employed include incorporation of advanced Reverse-Phase Protein Array (RPPA) technology for analysis of protein/phosphoprotein activity and molecular network analysis, and use of immunohistochemistry (IHC) analysis from formalin-fixed paraffin-embedded tissues. These technologies will allow us to detect unique targets for the ultimate individualized treatment of IENs in the skin.

2.1 UV Signal Transduction Pathways

Ultraviolet radiation (UVR), the major etiologic factor in human skin cancer, elicits a number of biological responses in the skin that include pigmentation, erythema, and cell death [2-5]. UVR can be divided by wavelength into UVC (180-280 nm), UVB (280-320 nm), and UVA regions (UVA I, 340-400 nm; UVA II, 320- 340 nm), all of which show strong carcinogenic activity with regard to induction of squamous type tumors in mouse

skin. UVR is a complete carcinogen in that it acts as both an initiator, presumably by causing DNA damage leading to gene mutations, and as a tumor promoter. Because UVC is absorbed by the ozone layer, UVA and UVB are considered to be the major carcinogenic components of solar radiation with relevance for human skin cancer [6-13]. UVA comprises more than 95% of solar UVR and is recognized as a major contributor to carcinogenesis [14-17]. Mechanistically, UVR induces both genotoxic effects such as DNA damage and mutations as well as epigenetic events such as induction of gene expression. DNA damage is induced directly by UVR absorption in the DNA resulting in the induction of cyclobutane pyrimidine dimers (CPD), (6-4) pyrimidine dimers, cytosine photohydrates and purine photoproducts that can yield specific “signature” mutations (C to T and CC to TT) [18,19]. Longer wavelengths can indirectly induce DNA single-strand breaks and DNA-protein crosslinks through reactive oxygen species [15]. Therapeutic prevention agents that demonstrate activity toward these UVR signaling pathways will be evaluated in clinical trials.

2.2 Immunohistochemistry

The use of immunohistochemical biomarkers permits studies with smaller sample size and shorter duration compared to studies that use cancer incidence as an endpoint. To be valid, the quantitative degree and pattern of biomarkers should correlate with carcinogenic transformation or alternatively biomarkers markers may demonstrate the effect of a particular agent on a molecular pathway or biochemical processes (i.e. arachadonic acid/cyclooxygenase pathways, polyamine synthesis). This has been demonstrated by the suppression of polyamine levels in tissue treated with the polyamine synthesis inhibitor, alpha-2-(Difluoromethyl)-dl-ornithine, or DFMO [20,21].

In a previous study completed by this group, IHC was performed on samples collected at baseline, 5 min, and 1, 5, and 24 h following 2X MED of SSL on SP skin of 10 subjects; there was a significant increase in pTOPK and pPRPK activity starting as early as 5 min post SSL exposure with a sustained expression up to 24 h (**Fig. 1**). Analysis of TLR4 using the same samples also revealed increased pathway activation primarily at 24 h post-SLL exposure.

IHC assays will include pTLR4, MyD88, p-p65/NFkB, TAB2, pTOPK, p-PRPK, p-Erk, pP38, and pP90RSK. IHC assays previously established will also be performed for validation of SSL-induced changes.

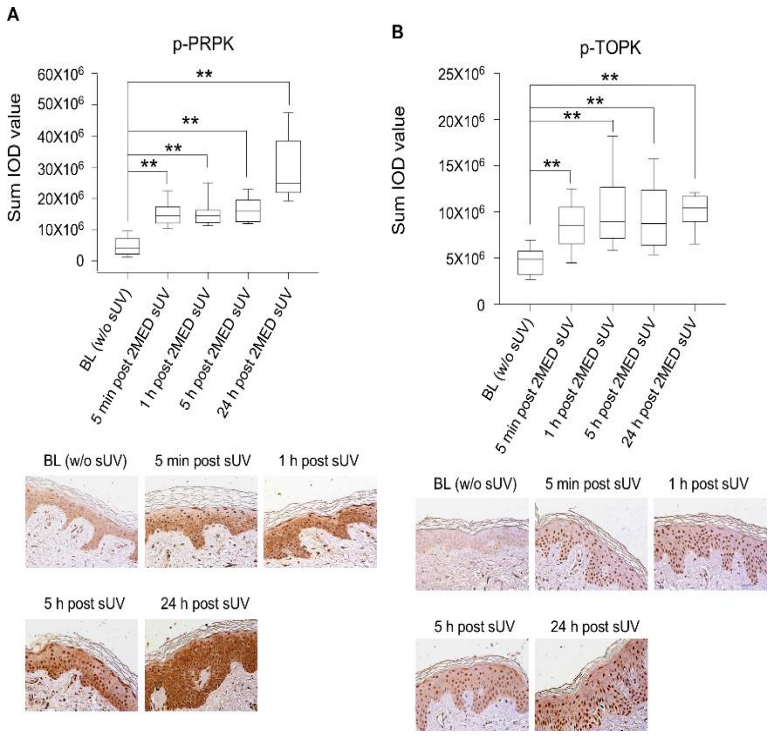


Fig. 1. Comparison of p-PRPK and p-TOPK levels on sUV-exposed human skin harvested in a time-dependent manner. IHC staining was performed and representative data analyzed (30 photos from 10 subjects) for each of the following: normal human skin without sUV, 5 min post 2X MED sUV, 1 h post 2 MED sUV, 5 h post 2 MED sUV and 24 h post 2 MED sUV. Shown is expression of phosphorylated (p)-PRPK (**a**) and p-TOPK (**b**). Magnification: 400x. **Upper panels:** Phosphorylated PRPK, total and p-TOPK levels are presented as sum of the integrated optical density (IOD) values. **Lower panels:** Representative staining (brown color) is shown. The asterisks (*, **) indicate a significant difference *(p* < 0.05 or *p* < 0.001, respectively).

2.3 Mapping of Protein Signaling Networks

Reverse-phase protein array analysis (RPPA), a novel analysis for multiplexed quantitative measurement of multiple signaling proteins from biological specimens represents a novel means of measurement of hundreds of proteins, many of which are phosphoproteins, in a single biopsy specimen. This array technology is a miniaturized calibrated dot-blot immunoassay, and with over 90 peer-reviewed publications supporting the implementation of this platform (60 from the Petricoin/Liotta laboratories), it represents one of the most widely published protein microarray technologies. Moreover, the RPPA platform is now being used within the Petricoin laboratories for clinical trial-based applications since it is run as a CAP/CLIA in-house developed assay, which demonstrates the maturity of the platform. With the RPPA technology, serial dilutions are printed of each sample, control or standard, to maintain sample

concentration. Each spot contains a bait zone measuring only a few hundred microns in diameter. The detection probe can be tagged and signal amplified independently from the immobilized analyte protein. Coupling the detection antibody with highly sensitive amplification systems can yield detection sensitivities to fewer than 1,000 to 5,000 molecules per spot with good linearity (correlation coefficient or R^2 = 0.990-0.999) and inter-experiment precision (R^2= 0.973). Between run and within run analytical precision is between a 3-13% CV (coefficient of variation) [22].

Activation of proteins by phosphorylation of kinase-driven signaling networks contains information regarding the disease process as well as potential therapeutic targets [23]. The phosphorylation state of proteins can be detected and assessed using specific phospho-specific antibodies developed to recognize the phosphorylated isoform of the kinase substrates [23-25]. Each antibody undergoes extensive validation for specificity prior to RPPA analysis using peptide competition and single band detection on Western as well as ligand induction. Currently, Dr. Petricoin has over 300 extensively validated different cell signaling antibodies in his portfolio. Using RPPA, many dozens of kinase substrates can be quantified and measured simultaneously through multiplexed phosphospecific antibody analyses. RPPA has demonstrated sensitivity, precision and accuracy that enable the evaluation of signaling networks from a very small number of cells (e.g. procured using laser capture microdissection) from biopsy material.

3.0 EQUIPMENT INFORMATION

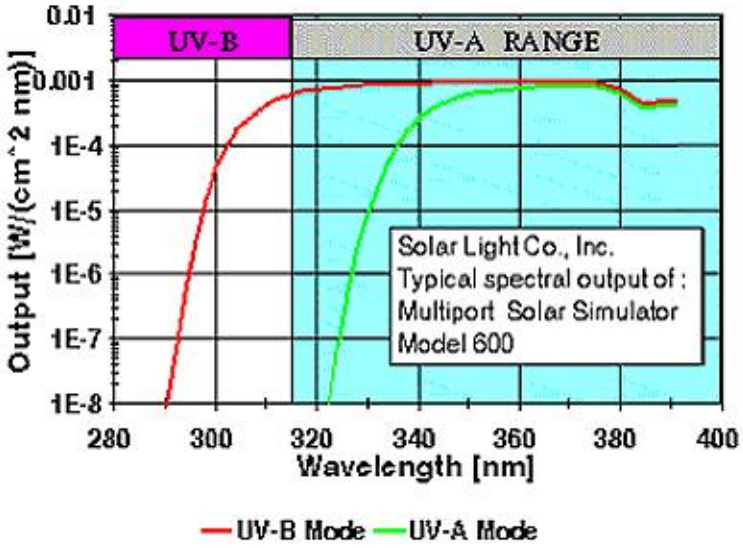
3.1 Solar Simulated Light Source

A Multiport UV Solar Simulator Model 600 (Solar Light Co., Inc., Philadelphia, PA) will be used to administer SSL exposures to the test areas on the skin. The device is equipped with six 8mm liquid light guides (LLG), allowing for 6 simultaneously conducted exposures. A large 3x2 endplate places the LLGs several centimeters apart and is specifically designed for SPF and photopatch testing. The dose of emission from each LLG can be precisely regulated and the spectrum of emission can be limited to UVA (320-390 nm) or UVB+UVA (290-390 nm). For added safety, the device is equipped with a power supply which stabilizes the lamp current. An operator-controlled shutter and dose control system monitor and control the delivered dose.

An automatic internal igniter minimizes the electromagnetic interference and a fan provides cooling that increases lamp life. The connection to the power supply is a 5

foot cable equipped with a quick disconnect connector. The operator can select between UVA only and a combined UVA/UVB spectrum by placement of an optical filter.

The source of radiation is a 150 Watt xenon lamp. The spectral output (indicated below) follows the distribution of sunlight from 290 to 390 nm.



(Source: Solar Light Company, Inc)

4.0 SUBJECT ELIGIBILITY

4.1 Inclusion Criteria

- Healthy individuals 18 years of age or older.
- Individuals with mild, moderate, or severe photodamage [1] of the skin on the forearms (also Appendix C) and Fitzpatrick skin type II or III (21 CFR 352.72).
- Females of childbearing potential will need to undergo a pregnancy test at the enrollment visit, after administration of the ICF and before exposure to SSL. Premenopausal female subjects must use an effective method of birth control (such as oral contraceptives, consistent use of barrier contraceptives, IUD, or other proven method of birth control) during study participation. For the purposes of this study, a woman will be considered postmenopausal if any of the following criteria are met: (1) she has had prior bilateral oophorectomy; (2) she is over the age of 60 years; or (3) she is under the age of 60 years and has not had a menstrual period in 12 or more months in the absence of chemotherapy, tamoxifen, toremifene, or ovarian suppression.

- Individuals who are willing to limit sun exposure to the body during the study period and who agree to wear protective clothing when they are outdoors.
- Individuals who have the ability to understand and willingness to sign an informed consent before initiation of study procedures, after the nature of the study is explained to them and they have had the opportunity to ask any questions.

4.2 Exclusion Criteria

- Individuals with any inflammation or irritation of the skin at the test areas, or any skin conditions felt by the study medical provider to contraindicate enrollment. This includes, but is not limited to, psoriasis or atopic dermatitis within the test areas.
    - Test area is defined as the 6 mm areas of skin that is exposed to SSL and will be biopsied.
- Individuals with a history of any untreated skin cancer, melanocytic lesions, or actinic keratoses in the test areas are ineligible. History of such conditions at a body site other than the test areas is not exclusionary if in the opinion of the study medical provider it will not pose a risk to the subject.
- Individuals who have had invasive cancer, chemotherapy or radiation therapy within five years of study enrollment
- Individuals who are immunosuppressed by virtue of medication or disease. This includes AIDS patients, subjects taking oral steroids, and subjects on immunosuppressants/immunomodulators (cyclosporine, chemotherapeutic agents, or biologic therapy), as determined by the examining study medical provider.
- Individuals with serious intercurrent illness including, but not limited to, ongoing or active infection, psychiatric illness, or other situations that in the opinion of the examining study medical provider would limit compliance or interfere with the study regimen.
- Individuals who have used photosensitizing drugs (see Appendix for examples) within 30 days of enrollment, or who will be using a photosensitizing drug during the time of the study, will not be eligible.

Subjects may be reconsidered for eligibility 30 days after the last dose of such medications.

- Individuals who have used any topical medication other than emollients or sunscreen/sunblock on the test area within 30 days prior to study enrollment. If a study participant requires topical medication to the test area during the study, they will be withdrawn from the study.
- Individuals who have used retinoids, steroids, 5-fluorouracil, Levulan, Vaniqua (eflornithine), Solaraze, or Imiquimod (Aldara®) anywhere on the body within 30 days prior to study enrollment. Subjects may be reconsidered for eligibility 30 days after the last topical treatment with such medications.
- Individuals must not take mega-doses of vitamins. Mega-doses are defined as more than 5 capsules of standard multivitamins daily or more than the *Tolerable Upper Intake Levels of Vitamins*, as defined by the Institute of Medicine, National Academy of Sciences. Such vitamin therapy must be discontinued at least 30 days prior to study entry.
- Individuals with a history of deliberate natural or artificial sun exposure (tanning) within 30 days of study enrollment are not eligible.
- Individuals with Fitzpatrick skin type I are ineligible, as the proposed SSL dose could result in a burn of greater than mild severity.
- Individuals with Fitzpatrick skin type IV, V or VI are ineligible, as they are unlikely to exhibit a salient response in the proposed design.
- Individuals currently enrolled in or who plan to enroll in another clinical trial. There must be a 30-day period between completing a previous study and enrolling in this study.
- Individuals with a known allergy to lidocaine are not eligible.
- Females who are pregnant or nursing.

5.0 PRE-STUDY REQUIREMENTS

Pre-study requirements include:

- Administration of the screening consent and HIPAA authorization form;
- Eligibility screening by a study medical provider, including a skin examination and a review of the subject's medical history, concomitant medications, and supplements;

- Subjects must have mild (N=12), moderate (N=12), or severe (N=12) sun damage [1] of the skin on the forearms (Appendix C).

6.0 TREATMENT PLAN

6.1 Subject Recruitment

Thirty-six subjects will be recruited, anticipating that 80% will be fully eligible and will complete the study. Clinic staff will be responsible for study participant recruitment. Male and female individuals will be recruited through the dermatology clinic at the University of Arizona Cancer Center, advertisements, community outreach programs, and from previous study participants who have agreed to be contacted. Subjects will be screened by a medical provider to verify eligibility before enrollment.

6.2 Trial Schema

Informed consent will be administered to all study participants. Female participants of childbearing potential will undergo a urine pregnancy test prior to SSL exposures. If there is a scheduling issue, a pregnancy test will be repeated if ≥ 7 days elapse between MED determination and the administration of SSL at 2X MED for biopsy. Each study participant will act as his/her own control. Areas to be used for baseline biopsies, MED (minimal erythema dose) determination, and study biopsy sites will be marked on the participant's skin with non-permanent surgical marking ink.

MED will be determined on the left buttock. After the MED of the subject is determined, three biopsy sites will be outlined on the left forearm. Then SSL at a dose of 2X MED will then be administered at two of the biopsy sites on the left forearm. Skin punch biopsies will then be collected at 1 hour (± 15 minutes) and 24 hours (± 2 hours) post-SSL exposure. In addition, one 6mm skin punch biopsy sample will be collected from the left forearm at baseline to serve as an unexposed (SSL) control sample. The control sample will be collected from a site that is at least 6 cm away from the sites exposed to SSL. All of the biopsy samples will be collected as close to the exact target time as possible. Photographs will be taken of the left forearm prior to skin punch biopsies. Study participants will return for suture removal and follow-up from 10 to 16 days after the final biopsies are collected, and they will be followed for as long as necessary if complications occur. Study participants may have their sutures removed by an outside provider per their preference.

Table 1 Outline of Trial Schema (n = 36)

Screening	Informed Consent Form and HIPAA authorization form administered; Skin examination and medical history reviewed by study provider
Visit 1*	Urine pregnancy test if necessary; SSL exposures administered for MED determination to left buttock.
Visit 2	MED visually determined
Visit 3*	Photograph of left forearm will be taken; SSL to 2 biopsy sites on left forearm at a dose of 2X MED; Biopsy collected from control biopsy site (no SSL) on left forearm; Biopsy collected from 1-hour test site on left forearm.
Visit 4	Photograph of left forearm will be taken; Skin biopsy collected from left forearm at 24 hours after SSL exposure
Visit 5**	Sutures removed 10-16 days after Visit 3

* When possible, Screening Visit may be combined with Visit 1, and Visit 2 may be combined with Visit 3.

**Participant may have their sutures removed by an outside provider.

6.3 Determination of MED (Minimal Erythema Dose)

The MED of each subject will be determined using a solar simulator (Solar Light Co.). MED is defined as the smallest dose of energy necessary to produce confluent erythema with four distinct borders at 22-26 hours post-exposure.

6.3.1 Light Source

A solar simulator will be used. The emission spectrum is from 290 to 390 nm, with <1% energy contributed by wavelengths <290 nm.

6.3.2 Test Site Area

MED will be determined on the left buttock. The test areas will be delineated with a template and skin marker.

6.3.3 Test Sub-sites

Each test area will be subdivided into 6 sub-sites (each 1 cm^2) corresponding to the LLG pattern on the solar simulator.

6.3.4 SSL Irradiation for MED Determination

The solar simulator will be accurately calibrated prior to each use. A series of 6 increasing SSL radiation exposures (expressed as mJ/cm^2) will be administered to the sub-sites on each subject, with LLG 1 emitting the lowest dose of SSL and

LLG 6 emitting the highest dose. All immediate responses to exposure will be recorded. Following exposure, the tests sites will be occluded until evaluation.

6.3.5 Determination of MED

After 22 - 26 hours the test sites will be evaluated for the site and dose producing the MED as defined above.

6.3.6 Evaluation of Test Sites

Evaluation will take place under the same conditions (lighting and position) used when the test sites were irradiated.

7.0 BIOPSY SAMPLE COLLECTION, PROCESSING AND ANALYSES

7.1 Biopsy Procedure

One 6mm skin punch biopsy from the left forearm will be obtained at baseline, at a site at least 6 cm away from SSL exposure, to serve as a control sample (unexposed to SSL). In addition, one 6mm skin punch biopsy sample will be obtained from each biopsy site exposed to a single dose of SSL given at 2X the subject's MED.

Skin will be disinfected with an alcohol preparation, a 30-gauge needle will be used to inject lidocaine 2% with epinephrine, and punch biopsies (6mm in diameter and 0.8mm in depth) followed by suture closure will be obtained for RPPA and IHC analyses.

Table 2. Biopsy samples to be collected.

Bx	Test Site	Collection Timepoint	Initial Processing in Clinic	Analysis
1	Left forearm	control, 0 SSL	½ formalin + ½ snap frozen	Histology/IHC/RPPA
2	Left forearm	1° post 2xMED of SSL	½ formalin + ½ snap frozen	Histology/IHC/RPPA
3	Left forearm	24° post 2xMED of SSL	½ formalin + ½ snap frozen	Histology/IHC/RPPA

7.2 Formalin Fixation and Tissue Preparation

Each 6mm skin biopsy will be bisected immediately, with ½ snap frozen and the other ½ placed into 10% formalin. Fixation times have been standardized to 24 hr in formalin. After 24 hrs samples in formalin will be transferred to 70% ethanol until processing. Processing and paraffin embedding will be done weekly or bi-weekly so that no samples remain unprocessed for more than a week. Additionally, processing times and temperatures have been standardized and a tissue control is processed identically to samples with each run. Once skin samples are paraffin-embedded and mounted in blocks they are stored until they are batched for analyses.

7.3 Immunohistochemistry (IHC) Assays

Tissue sections at 3-5 microns will be cut, followed by deparaffinization and rehydration. All tissue sections will be subjected to antigen retrieval using a citrate buffer in a Decloaking Chamber Pro (Biocare, Walnut Creek, CA). Immunohistochemical staining will be performed using a Universal Alkaline Phosphatase RED biotin-free, multimer-based detection system or a streptavidin-biotin peroxidase system with a DAB chromagen and a hematoxylin counterstain (Ventana Medical Systems, Tucson, AZ) on an automated BenchMark® XT immunostainer (Ventana Medical Systems, Tucson, AZ) or by hand using an appropriate VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA) depending on the primary antibody species and an alkaline phosphatase substrate for detection. In the case where mouse tissue will be stained with mouse primary antibodies, the Mouse on Mouse (M.O.M.™ Vector Laboratories, Burlingame, CA) will be used. Positive controls will be included in each IHC assay and negative controls will include the omission of the primary antibody with replacement by a normal serum from the same species as primary antibody. Biopsies will be batched for all analyses.

Tissue sections will be measured using ImagePro Plus (Media Cybernetics, Silver Springs, MD) software systems and a Leica DMR microscope (Westzlar, Germany), and a Sony 3CCD color video camera (Japan). The percent positive nuclear or cytoplasmic area per 40X field will be determined for each biopsy. A positive control slide for each IHC assay will be included when samples undergo image analysis to assess the daily variability of image analysis. Ten percent of samples will be repeated to assure assay reproducibility in a blinded manner as determined by the Biometry Core.

7.4 Mapping of Protein Signaling Networks

Reverse-Phase Protein Array Construction: RPPAs will be constructed generally as described previously (Section 2.3). Briefly, lysates will be loaded into 384-well plates and serially diluted in lysis buffer for a two-point dilution curve (neat and 1/4). The RPPA technology has been developed and optimized for performance as a fluorescent-based calibrated assay, generally identical in design and analysis to standard ELISA or standard clinical immunoassays. As a calibrated assay, each assay consists of:

a. Experimental patient samples printed in triplicate two-spot dilutions (neat and 1:4)

b. High, medium, and low controls printed in triplicate two-spot dilutions (neat and 1:4)

c. A calibrator, consisting of a 6-10-point curve whereby the analyte of interest is decreasing in concentration in the background of a constant protein concentration.

Image Analysis: The current iteration of the RPPA uses a fluorometric image capture processing system (NovaRay, Alpha Innotech) for image acquisition. The system measures the sample's fluorescence intensity value, subtracts the background, normalizes the result to the total protein, and extrapolates the value to the non-parametrically fit calibration curve to generate a final intensity value. The median of the triplicate values will be reported. Sypro-stained slides will be analyzed with MicroVigene image analysis software, version 2.200 (Vigenetech, North Billerica, MA) and Microsoft Excel 2000 software. Images will be imported into Microvigene, which performed spot finding, local background subtraction, replicate averaging, and total protein normalization to produce a single value for each sample at each endpoint.

8.0 STATISTICAL CONSIDERATIONS

8.1 Sample Size Determination

We will examine the TLR4 and TOPK signaling pathways in acute SSL-exposed SD human skin. Subjects with evidence of SD will be enrolled (N=36); with 85% expected to complete the study (30 evaluable subjects). Primary analysis will compare levels of RPPA analytes in each pathway as stated above in baseline unexposed samples vs. samples collected 1 or 24 h after SSL in SD skin. The power is 93% or above when the correlation between the $\log_2$ fold change of any two different analytes in the same pathway ranges from 0 to 0.4, and the power is 82% if the correlation is 0.6. Exploratory analyses will associate the SD score/severity level with analyte activation in the SD samples. Based on these analyses, the most appropriate post-SSL time point (per pathway) will be selected based on the combination with the largest effect sizes. Furthermore, an exploratory systems biology analysis will be conducted based on these analyses, one post-SSL time point (per pathway) will be selected for future studies based on the combination with the largest effect sizes. Furthermore, an exploratory systems biology analysis will be conducted.

9.0 EARLY STOPPING

9.1 Early Stopping for Futility

Accrual will be halted after five participants have completed SSL exposure. Samples will be evaluated for changes in at least two biomarkers. If no changes are observed in any of the participants, other biomarkers will also be assessed. If no changes in any of the biomarkers are observed in any of the participants in a given group, accrual will stop

and the protocol will be re-evaluated to determine if the SSL doses should be adjusted or the trial should be stopped. Assuming that the response rate for any marker is as low as 50%, there is only a one in 32 (i.e., approximately 0.03) probability of seeing five non-responders, based on the binomial distribution, in a particular marker. The prospect of failing to see response in any of the markers, assuming that two or more are actually valid, is vanishingly small. Thus, choosing a sample size of five for initial assessment runs very little risk of committing a false negative error. This initial process will not assess success, and no inferential statistical analyses will be conducted. Only the presence or absence of expression will be noted.

10.0 TOXICITY MONITORING

No drugs or experimental therapies are included in this study and no toxicities are anticipated.

11.0 DATA AND SAFETY MONITORING

11.1 Identification of the DSMB Obligated for Oversight Responsibilities

The University of Arizona Cancer Center Data Safety and Monitoring Board (DSMB) will provide ongoing oversight for this trial. This study has been assigned a Low Risk level by the DSMB. The DSMB meets monthly to quarterly, depending on the risk level of a clinical trial, to evaluate the safety of the study. A meeting quorum is comprised of 6 voting members, including the Board Chair. The risk level is based on various trial characteristics such as the phase of the study, study endpoints, level of intervention, degree of risk, and the complexity of the trial.

11.2 Identification of the Entity Obligated for Routine Monitoring Duties

Routine study monitoring will be provided by the Quality Assurance/Quality Control (QA/AC) Program to ensure that the study is conducted according to protocol design and regulatory requirements.

This study will also undergo real-time monitoring by the PI and study team, including documentation of any new or ongoing safety issues. For individual participants, adverse events will be assessed at each visit by the study coordinator who will then report the events to the PI at the weekly study staff meeting. A Study Events Form (CRF) will be reviewed and signed by the PI for each subject. Any severe adverse event will be reviewed by the PI within 24 hours of reporting by the subject.

11.3 Monitoring Progress and Data Review Process

Routine monitoring of subject data will be conducted at least quarterly.

The first routine monitoring visit will include at a minimum:

- Informed consent – 50% of cases enrolled;
- Subject eligibility – 10% of cases, up to two subjects;
- Data review – 10% of cases, up to two subjects.

All subsequent monitoring visits will consist of randomly selected subject cases based on current enrollment and include continuing review of previously selected cases, as applicable.

A monitoring visit report and follow-up letter will be completed approximately two weeks after the routine monitoring visit; a copy will be maintained in the study file. The monitor will request additional source documentation, clarification, information, or corrections to the CRF and/or regulatory records from the Clinical Research Coordinator (CRC) or other applicable staff responsible for the study and resolution of queries/findings. Documentation of such a request will be maintained with a copy of the monitor's visit report for follow-up at the next monitoring visit. Electronic records will be available in the institutional database or provided by the QA/QC Program staff.

The Principal Investigator will ensure the accuracy, completeness, legibility and timeliness of the data reported in the Case Report Form (CRF), or other acceptable data formats. Source documentation supporting the study data should indicate the subject's participation in the trial and should document the dates and details of study procedures, adverse events, and patient status.

Case report forms, which include the inclusion/exclusion criteria form, adverse event forms and serious adverse event forms should be completed via the institution database or other acceptable data formats. Trials using paper CRFs will have the data entered with a black ball-point pen or typed. Corrections to the forms should not obscure the original entry and should be made by striking the incorrect information with a single line. Each strike should be accompanied by the initials of the corrector and the correction date. All subject forms and study files will be stored in a secure area limited to authorized staff. Routine monitoring of regulatory documents will be conducted at least annually.

11.4 Process to Implement Study Closure When Significant Risks or Benefits are Identified

The PI will determine if the study at a certain point may need to be closed if significant study related risks or benefits are identified. The PI would then follow up with the DSMB and IRB in a timely manner.

12.0 ADVERSE EVENTS

12.1 Evaluation for Adverse Events

The safety of all participants enrolled in this study will be monitored throughout the study. At baseline a study events form will be completed. Study personnel will review study events with the study participant at each visit. Any reportable adverse experience (i.e., greater than baseline level), whether considered study related or not, will be recorded in an study events report form, along with the dates of onset, resolution, severity and remedial measures taken. In all cases, study participants will be followed until resolution of the event. In addition, patients will be instructed to notify the study staff by telephone of any problems that occur between visits and, if necessary, be evaluated by the Investigator or study staff at an unscheduled interim visit.

12.2 Adverse Event (AE) Definitions

The following definitions of terms guided by the International Conference on Harmonization and the US Code of Federal Regulations apply to this section:

- **Adverse event** is any untoward medical occurrence in a patient or clinical investigation subject that does not necessarily have a causal relationship with the study procedures.
- **Serious adverse event** is any adverse event that is fatal or life-threatening, is permanently disabling, requires or prolongs inpatient hospitalization, is a congenital anomaly, is a nonmelanoma skin cancer on the test area but not at more distant sites, or overdose.
- **Life-threatening**, for the purpose of reporting adverse events, refers to an event in which the patient was, in the view of the initial reporter, at **immediate** risk of death at the time of the event as it occurred (i.e., it does not include an event that, had it occurred in a more serious form, might have caused death).
- **Associated with the study procedures** means that there is a reasonable possibility that the experience (event) may have been caused by the study procedures.
- **Unexpected adverse experience** (event) means any adverse experience (event) that is inconsistent with the procedures described in this protocol.

12.3 Reporting of Adverse Events

The approving Institutional Review Board (IRB)/Ethics Committee (EC) must be notified of any fatal, life-threatening and/or serious adverse events, regardless of cause, on a timely basis and must be apprised of all adverse events by written report on a periodic and timely basis, at least annually.

A written report of all serious adverse events and deaths will be submitted by the Investigator to the IRB/EC. In this report, the Investigator will advise whether or not the adverse event is judged to be related to the study procedures. All such patients with adverse events should be followed clinically and by the appropriate diagnostic evaluations.

All adverse events, regardless of severity, and whether or not ascribed to the study procedures will be recorded in the appropriate section of the Case Report Form. The Investigator will follow patients withdrawn from the study due to adverse events until the outcome is determined and, when appropriate, additional written reports and documentation will be provided.

12.4 Data Safety and Monitoring Board

The University of Arizona Cancer Center Data and Safety Monitoring Board (DSMB) will provide ongoing oversight for this trial. Routine monitoring will be provided by the Quality Assurance/Quality Control (QA/QC) Program to ensure that the investigation is conducted according to protocol design and regulatory requirements.

This trial will also undergo real-time monitoring by the PI and study team, including documentation of real-time monitoring of any new or ongoing safety issues. For individual participants, adverse events will be assessed by the principal investigator and the study coordinator as they occur.

12.5 Classification of Adverse Events by Severity

The severity of local skin reactions will be classified according to the criteria below. For systemic effect, the investigator must categorize the severity of each adverse event according to the following guidelines:

Mild: Grade 1 NCI CTCAE; or if not found in the NCI CTCAE table, an adverse event that is asymptomatic or barely noticeable to the patient; not interfering with patient's daily activity performance or functioning; generally not requiring alteration or cessation of study procedures; and/or ordinarily not needing therapeutic intervention.

Moderate: Grade 2 NCI CTCAE; or if not found in the NCI CTCAE table, an adverse event of sufficient severity as to possibly make the patient moderately

uncomfortable; possibly influencing the patient's daily activity performance or functioning; generally not impairing the patient ability to continue in the study; and/or possibly needing therapeutic intervention.

Severe: Grade 3 NCI CTCAE; or if not found in the NCI CTCAE table, an adverse event generally causing severe discomfort; significantly influencing the patient's daily activity performance or functioning; generally requiring alteration or cessation of study procedures; and/or generally requiring therapeutic intervention.

Life-threatening or disabling: Grade 4 NCI CTCAE; or if not found in the NCI CTCAE table, an adverse event that is considered to be life-threatening; resulting in significant disability or incapacity; and/or representing the worst possible occurrence of that event.

Death related to AE: Grade 5 NCI CTCAE to be used only in the unlikely event that death is determined to be related to the study procedures.

12.6 Classification of Adverse Events by Relationship to Study Procedures

The Investigator will assess the relationship of each adverse event to the study procedures after careful consideration, and according to the following guidelines:

NO, NOT RELATED: This category is applicable to those adverse events that are clearly due to extraneous causes (concurrent drugs, environment, etc.) and do not meet the criteria for study procedure relationship listed under PROBABLY NOT; POSSIBLY; PROBABLY; AND YES, RELATED.

PROBABLY NOT RELATED: This category applies to those adverse events that are judged to be unlikely to be related to the study procedures. An adverse event may be considered to be PROBABLY NOT RELATED when it meets at least two (2) of the following criteria:

a) It does not follow a reasonable temporal sequence from administration of the study procedures.

b) It could readily have been produced by the patient's clinical state, environmental or toxic factors, or other modes of therapy administered to the patient.

c) It does not follow a known or expected response pattern to the study procedures.

d) It does not reappear or worsen when the study procedures are re-administered.

POSSIBLY RELATED: This category applies to those adverse events that are judged to be perhaps related to the study procedures. An adverse event may be considered POSSIBLY RELATED when it meets at least one (1) of the following criteria:

a) It follows a reasonable temporal sequence from administration of the study procedures.
b) It could not readily have been produced by the subject's clinical state, toxic or environmental, or other modes of therapy administered to the subject.
c) It follows a known or expected response pattern to the study procedures.

PROBABLY RELATED: This category applies to those adverse events that are felt with a high degree of certainty to be related to the study procedures. An adverse event may be considered PROBABLY RELATED if it meets at least two (2) of the following criteria:
a) It follows a reasonable temporal sequence from administration of the study procedures.
b) It could not be reasonably explained by the known characteristics of the patient's clinical state, environmental or toxic factors, or other modes of therapy administered to the patients.
c) It disappears or decreases on cessation or reduction in study procedures. There are exceptions when an adverse event does not disappear upon discontinuation of the procedures, yet a relationship clearly exists.
d) It follows a known or expected response pattern to the study procedures.

YES, RELATED: This category applies to those adverse events that are incontrovertibly related to study procedures. An adverse event may be assigned to this category if it meets at least the first three (3) of the following criteria:
a) It follows reasonable temporal sequence from administration of the study procedures.
b) It could not be reasonably explained by the known characteristics of the patient's clinical state, environmental or toxic factors, or other modes of therapy.
c) It disappears or decreases on cessation or reduction in study procedures. There are exceptions when an adverse event does not disappear upon discontinuation of the procedures, yet a relationship clearly exists
d) It follows a known or expected response pattern to the study procedures.
e) It reappears or worsens when the study procedures are re-administered.

13.0 CLINIC PERSONNEL

13.1 Recruiting study subjects
13.2 Scheduling appointments and screening clinics
13.3 Administering informed consent and HIPAA authorization
13.4 Preparation and completion of subject study records

13.5 Motivating and evaluating compliance to the study protocol
13.6 Clinical safety monitoring
13.7 Responding to current and potential subject requests and concerns.
13.8 Assisting study medical providers with the collection of tissue samples
13.9 Quality control and assurance of data collection and study procedures

14.0 HUMAN SUBJECTS

14.1 Up to 36 healthy volunteers will participate in this clinical trial. Based on the distribution of the skin cancer population, subject populations responding to recruitment efforts are predominantly Caucasian as the target population consists primarily of people with moderate to heavy sun damage. This study targets individuals with Fitzpatrick skin types II and III. Every effort will be made to recruit minority subjects, commensurate with study objectives and project goals.

14.2 In order to protect human subjects in this study, we will recruit only women who have a negative pregnancy test. Women who are pregnant or nursing are not eligible to participate. Every attempt will be made to include eligible women and minority subjects in these trials.

14.3 Human Tissue Samples (HTS) obtained from these human volunteers will be utilized strictly for research purposes. HTS to be obtained include epidermal tissue samples. The security of HTS will be protected by detailed Standard Operating Procedures for processing, sharing and tracking the chain of custody of HTS.

14.4 All information and specimens obtained from these human volunteers will be utilized strictly for research purposes. Information to be collected includes medical history, physical exam, adherence evaluations and adverse event monitoring. Information will be treated according to HIPAA regulations. Study participants will be asked to read and sign a Research Authorization (HIPAA consent form) in order to participate in the study.

14.5 Subjects will be recruited through the dermatology clinic at the Arizona Cancer Center, newspaper, advertisements, community outreach programs, and from previous study participants who have agreed to be contacted.

14.6 After the initial eligibility screening, subjects will have a detailed discussion of the study requirements and procedures with the study medical provider or coordinator. All information regarding the study procedures will be provided to the subjects in understandable laymen terms on an IRB-approved informed consent form and a

HIPAA Authorization form. All subjects will receive copies of the signed forms for their records.

14.7 Potential risks from the number or intensity of light exposures proposed are small. The light exposures given to determine the MED may result in a mild sun-burn, and tanned areas approximately the size of a pencil eraser, which will fade over time. Side effects associated with the use of subcutaneous lidocaine are rare, particularly when given in a site such as the forearm. Skin biopsies are expected to result in permanent scars. These potential effects are clearly outlined in the informed consent and subjects will be carefully monitored during the study. Subjects will be identified only by subject number in all communications.

14.8 The important information to be obtained in this study is critical to the optimal design of future skin cancer chemoprevention trials. The risks to subjects participating are well described in the consent form and are reasonable given the study objectives.

14.9 Subjects will receive financial compensation for participation (Appendix D).

14.10 Subjects may not participate in the study if they are enrolled in any other clinical trial using an investigational drug or device. A waiting period of at least 30 days must be observed between participation in another clinical trial and enrollment in this study. If a subject chooses to join another clinical trial after they have enrolled in this study, they must be dropped from this study.

14.11 Subjects must avoid sun exposure to the treatment areas until their study participation has been completed and all biopsy sites have healed.

15.0 REFERENCES

1. McKenzie NE. Development of a photographic scale for consistency and guidance in dermatologic assessment of forearm sun damage. Arch Dermatol 2011;147(1):31-36. doi:10.1001/archdermatol 2010.392.
2. Epstein JH. Photocarcinogenesis, skin cancer, and aging. J Am Acad Dermatol 1983;9(4):487-502.2
3. IARC. Solar and ultraviolet radiation. IARC Monograph Evaluating Carcinogenic Risks to Humans 1992:55:1-316.
4. Ley RD. Photoreactivation in humans.Proc Natl Acad Sci USA 1993;90(10):4337.
5. Setlow RB. The wavelengths in sunlight effective in producing skin cancer: a theoretical analysis. Proc Natl Acad Sci U S A 1974;71 (9):3363-6.
6. Fitzpatrick T, Sober AJ, Pearson BJ, Lew R. Cutaneous carcinogenic effects of sunlight in humans. New York: Plenum Publishing Corp; 1976.
7. Forbes P, Davis RJ, Urbach F. Experimental ultraviolet carcinogenesis. New York: Plenum Publishing Corp; 1976.
8. Fry R, Ley RD. Ultraviolet radiation-induced skin cancer. New York: Raven Press; 1989.
9. Matsui MS, Laufer L, Scheide S, DeLeo V. Ultraviolet-B (290-320 nm)-irradiation inhibits epidermal growth-factor binding to mammalian cells. J Invest Dermatol 1989;92(4):617-22.
10. de Gruijl FR. Photocarcinogenesis: UVA vs UVB. Methods in Enzymology. 2000;319:359-66.
11. de Laat A, van der Leun JC, de Gruijl FR. Carcinogenesis induced by UVA (365-nm) radiation: the dose-time dependence of tumor formation in hairless mice. Carcinogenesis 1997;18(5):1013-20.
12. Elmets C. Cutaneous photocarcinogenesis. Boca Raton: CRC Press; 1992.
13. Kelfkens G, de Gruijl FR, van der Leun JC. The influence of ventral UVA exposure on subsequent tumorigenesis in mice by UVA or UVB irradiation. Carcinogenesis 1992;13(11):2169-74.
14. Boutwell RK. The function and mechanism of promoters of carcinogenesis. CRC Crit Rev Toxicol 1974;2(4):419-43.
15. Danpure HJ, Tyrrell RM. Oxygen-dependence of near UV (365 NM) lethality and the interaction of near UV and X-rays in two mammalian cell lines. Photochem Photobiol 1976;23(3):171-7.
16. Strickland PT. Photocarcinogenesis by near-ultraviolet (UVA) radiation in Sencar mice. J Invest Dermatol 1986;87(2):272-5.
17. Willis I, Menter JM, Whyte HJ. The rapid induction of cancers in the hairless mouse utilizing the principle of photoaugmentation. J Invest Dermatol 1981;76(5):404-8.
18. Tessmann L. Mechanism of UV-reactivation. Boca Raton: CRC Press; 1976.

19. Brash DE, Rudolph JA, Simon JA, Lin A, McKenna GJ, Baden HP, et al. A role for sunlight in skin cancer: UV-induced p53 mutations in squamous cell carcinoma. Proc Natl Acad Sci U S A 1991;88(22):10124-8.

20. Boone CW, Kelloff GJ, Steele VE. Natural history of intraepithelial neoplasia in humans with implications for cancer chemoprevention strategy. Cancer Research 1992;52(7):1651-9.

21. Alberts DS, Dorr RT, Einspahr JG, Aickin M, Saboda K, Xu MJ, et al. Chemoprevention of human actinic kerotoses by topical 2-(difluoromethyl)-dl-ornithine. Cancer Epidemiol. Biomarkers Prev. 2000;9:1281-1286.

22. Rapkiewicz A, Espina V, Zujewski JA, Lebowitz PF, Filie A, Wulfkuhle J, et al. The needle in the haystack: application of breast fine-needle aspirate samples to quantitative protein microarray technology. Cancer 2007;111(3):173-84.

23. Petricoin EF, 3rd, Espina V, Araujo RP, Midura B, Yeung C, Wan X, et al. Phosphoprotein pathway mapping: Akt/mammalian target of rapamycin activation is negatively associated with childhood rhabdomyosarcoma survival. Cancer Res 2007;67(7):3431-40.

24. Liotta LA, Espina V, Mehta AI, Calvert V, Rosenblatt K, Geho D, et al. Protein microarrays: meeting analytical challenges for clinical applications. Cancer Cell 2 003;3(4):317-25.

25. Sheehan KM, Gulmann C, Eichler GS, Weinstein JN, Barrett HL, Kay EW, et al. Signal pathway profiling of epithelial and stromal compartments of colonic carcinoma reveals epithelial-mesenchymal transition. Oncogene 2007.

26. Fullerton A, Fischer, T, Lahti A, et al. Guidelines for measurement of skin colour and erythema. A report from the standardization group of the european society of contact dermatitis. Contact Dermatitis, 1996;35:1-10.

27. Takiwaki H. Measurement of skin color: practical application and theoretical considerations. J Med Invest 1998;44:121-6.

28. Healy ZR, Dinkova-Kostova AT, Wehage SL, et al. Precise determination of the erythema response of human skin to ultraviolet radiation and quantification of effects of protectors. Photodermatology, Photoimmunology & Photomedicine, 2009;25:45-50.

APPENDIX A

LIST OF ABBREVIATIONS

AEs adverse events

AK actinic keratosis

BX biopsy

CFR Code of Federal Regulations

CPD **cyclobutane pyrimidine dimers**

cSCC cutaneous squamous cell carcinoma

CTCAE Common Terminology Criteria for Adverse Events

DFMO difluormethylornithine

DNA Deoxyribonucleic acid is a molecule that carries the genetic instructions used in the growth, development, functioning and reproduction of all known living organisms and many viruses.

FDA Food and Drug Administration

HIPAA Health Insurance Portability and Accountability Act

IHC or Immunohistochemistry refers to the process of detecting antigens (e.g., proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens.

IRB Internal Review Board

MED Minimal Erythema Dose, MED is defined as the smallest dose of energy necessary to produce confluent erythema with four distinct borders at 22-26 hours post-exposure.

NCI National Cancer Institute

NMSC non-melanoma skin cancer

p53 Tumor protein p53, also known as p53, is a protein that in humans is encoded by the *TP53* gene. The p53 protein is crucial in multicellular organisms, where it regulates the cell cycle and functions as a tumor suppressor, preventing cancer.

PRPK p53-related protein kinase

RPPA Reverse-phase protein array analysis is a protein array designed as a micro- or nano- scaled dot-blot platform that allows measurement of protein expression

levels in a large number of biological samples simultaneously in a quantitative manner.

SCC squamous cell carcinoma
SD sun damaged

SP sun protected

SSL solar simulated light

TLT treatment limiting toxicity

TOPK T-LAK cell-originated protein kinase

TLR4 Toll-like Receptor 4

TUNEL or TdT assayterminal deoxyribonucleotide transferase

UVR Ultraviolet radiation

APPENDIX B

Common Photosensitizing Drugs

Brand Name	Generic Name	Therapeutic Class
Cordarone	amiodarone	antiarrhythmic
Bactrim	trimethoprim	antimicrobial
Diabinese	chlorpropamide	antidiabetic (oral)
Vibramycin	doxycycline	antimicrobial
Phenergan	promethazine	antihistamine
Gris-Peg	griseofulvin	antimicrobial
Thorazine	chlorpromazine	Antipsychotic
Aldoril	hydrochlorothiazide	Diuretic
Orinase	tolbutamide	antidiabetic

Additional classes of drugs with photosensitizing potential are: acne medications, antibiotics (esp. tetracyclines and nalidixic acid), antihistamines, phenothiazines, sulfa drugs, tricyclic antidepressants, thiazide diuretics, sulfonylureas, quinidine.

APPENDIX C

ASSESSMENT OF PHOTODAMAGE*

CLINICAL SIGN	ABSENT	MILD	MODERATE	SEVERE
Fine Wrinkling Visible creases or folds < 1 mm* in width and depth	0	1 2 3	4 5 6	7 8 9
Coarse Wrinkling Thickening of skin causing visible creases or folds ≥ 1 mm* in width and depth	0	1 2 3	4 5 6	7 8 9
Abnormal Pigmentation Abnormally increased or decreased coloration of skin; may be localized or diffuse	0	1 2 3	4 5 6	7 8 9
Global Overall assessment of sun damage	0	1 2 3	4 5 6	7 8 9

*Development of a Photographic Scale for Consistency and Guidance in Dermatologic Assessment of Forearm Sun Damage. McKenzie NE, Saboda KL, Hu C, Curiel-Lewandrowski C. Archives of Dermatology, 2011; 147(1):31-36.

APPENDIX D

SCHEDULE OF SUBJECT COMPENSATION

Subjects will receive financial compensation for their participation in the study.

If a subject discontinues participation for any reason, they will receive a pro-rated amount of compensation for the visits they did complete.

The compensation schedule is as follows:

Screening		**no compensation**
Visit 1	**enrollment, assessments, SSL for MED determination**	**$ 40**
Visit 2	**Determine MED***	**$ 25**
Visit 3	**SSL exposures, collection of control & 5° biopsies***	**$110**
Visit 4	**Collection of 24° biopsies**	**$ 70**
Visit 5	**Suture removal - final visit****	**$ 30**
Total		**$275**

***Visits 2 and 3 may be combined when scheduling allows.**
****Patient may have their sutures removed by another provider, per their preference.**