CLINICAL STUDY PROTOCOL

Interventional Drug or Biologic

Assessing a Natural Product plus Bioadhesive Nanoparticle (BNP) Sunscreen

Protocol Number

200033876

Protocol Version

February 1, 2023

Version 4

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NCT Number: NCT05736224

Study Phase (1, 2, 3, or 4-postmarketing): Phase 1

REVISION HISTORY:

Include the IRB approved protocol version number and date for each revision of the protocol. All version history should remain in the table and never be deleted. The oldest IRB approved version of the protocol should be listed on the top row. The most recent IRB approved version should be listed on the bottom row.

Revision #	**Version Date**
V1	10/11/2022
1 (V2)	11/30/2022
2 (V3)	12/20/2022
3 (V4)	02/01/2023

Synopsis

Primary Objective The primary objective of this study is to evaluate the effects of a novel sunscreen formulation by assessing the extent of UVR-induced direct and indirect cellular and DNA damage to human skin, in the presence vs absence of the sunscreen, in a population of healthy adults with fair skin (Fitzpatrick Scale type I, II or III).
Secondary Objective (if applicable) N/A
Study Duration 9 months
Study Design This is a phase 1, non-randomized pilot study designed to test the effectiveness of a sunscreen compared to a no treatment control.
Number of Study Sites There will be one study site. Studies will be completed at the 4th floor of the Hunter Radiation Therapy (HRT – 4th floor) building, in the examination room of the YNHH Photopheresis Unit.
Study Population Healthy Caucasian adults Healthy adult volunteers with fair skin. Individuals with fair skin are more susceptible to the UVR-induced DNA damage that we seek to prevent by development of a safer, more effective sunscreen, and thus will comprise the study population.
Number of Participants 30
Primary Outcome Variables Biopsy tissue will be used to assess the effectiveness of the sunscreen by measuring the level of DNA and cellular damage in UVR-exposed vs unexposed, sunscreen treated vs untreated samples by measuring DNA mutation inducing cyclobutene pyrimidine dimers (CPD), DNA strand breaks (γH2AX), and cellular protein damage (3-nitrotyrosine) as biomarkers of response.
Secondary and Exploratory Outcome Variables (if applicable) N/A

Abbreviations

Abbreviation	Explanation
AVO	avobenzone
BNP	bioadhesive nanoparticle
BNP-A/O	bioadhesive nanoparticle encapsulating avobenzone and octocrylene
CPD	cyclobutane pyrimidine dimer
DNA	deoxyribonucleic acid
FDA	United States Food and Drug Administration
gH2AX	gamma (phosphorylated) histone H2AX
GRASE	generally regarded as safe and effective
HPG	hyperbranched polyglycerol
hr	hour
MED	minimal erythema dose
mg	milligram
OCTR	octocrylene
PLA	poly (lactic acid)
PLA-HPG	poly (lactic acid)-hyperbranched polyglycerol
PO	padimate O
PO/BNP	bioadhesive nanoparticles encapsulating padimate O
ROS	reactive oxygen species
SPF	sun protection factor
USP	United States Pharmacopeia
UVA	ultraviolet A
UVB	ultraviolet B
UVR	ultraviolet radiation

Glossary of Terms

Glossary	Explanation

APPROVED BY THE YALE UNIVERSITY IRB 2/21/2023

Table of Contents

1 Introduction

1.1 Introductory Statement

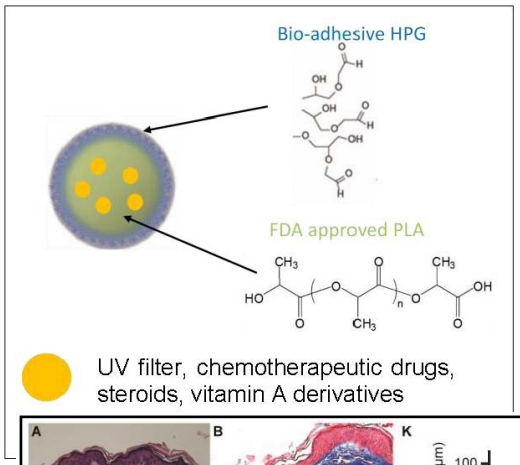
This document is a protocol for a human research study. The purpose of this protocol is to ensure that this study is to be conducted according to ICH GCP guidelines, and according to CFR 21 Part 312, other applicable government regulations and Institutional research policies and procedures.

We have developed a novel bioadhesive nanoparticle technology that aims to make sunscreens safer and longer lasting by encapsulating UVR sunscreen active agents in non-toxic nanoparticles (1, 2). We have strong preclinical and clinical data demonstrating the potential for improved safety, long-duration of retention, and increased effectiveness of bioadhesive nanoparticles (BNPs) loaded with the over-the-counter, FDA GRASE chemical filters avobenzone (AVO) and octocrylene (OCTR). We have additionally identified four naturally occurring non-toxic products (diosmin, ferulic acid, cytisine, trans-resveratrol) that, in our preclinical studies, boost the UVR-absorbing capacity of our BNP sunscreen while also reducing the damaging effects of UVR-induced reactive oxygen species (ROS), to more efficiently prevent UVR-induced cellular and DNA damage. Herein, we propose to test the capacity of our combined natural product plus BNP sunscreen to prevent UV-induced cellular and DNA damage in human skin.

2 Background

2.1.1 Preclinical Experience

Nanoparticle encapsulation, used widely in the field of drug delivery, has demonstrated unique advantages including target specificity and toxicity reduction. Our initial preclinical studies demonstrated that the encapsulation of a model UVR filter, padimate O (PO), in bioadhesive nanoparticles (BNPs) prevents epidermal cellular exposure to UVR filters while enhancing UVR protection (1). We have performed additional studies demonstrating that the BNP platform can also enhance the broad-spectrum protection of UVR filters like avobenzone (AVO) and octocrylene (OCTR). The use of this technology confers bioadhesive characteristics that facilitate tight adherence to the stratum corneum without subsequent intra-epidermal or follicular penetration. As a result, our innovative sunscreen is not only persistently adherent and non-penetrant, but also highly protective against primary UVR damage and secondary ROS toxicity.



We successfully prepared nanoparticles using a biocompatible block copolymer called poly (lactic acid)-hyperbranched polyglycerol (PLA-HPG). These particles have a bioadhesive coating which is tunable for specific topical applications. Additionally, the properties of the coating allow the BNPs to be water-soluble while enabling the suspension of oil-based UVR filters, thereby simplifying the formulation process. We developed a method to load different bioactive agents into the particle (Fig. 1).

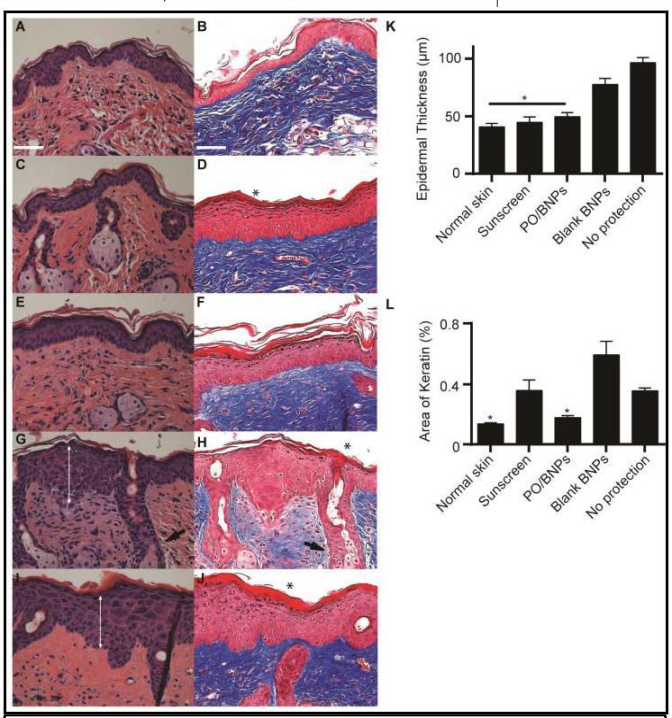


Fig. 2. Histology of dorsal mouse skin sections receiving different topical interventions three days after high dose UVB exposure (2160 J/m^2) Topical interventions included (A-B) normal skin without UV exposure, (C-D) sunscreen, (E-F) PO/BNPs, (G-H) blank BNPs, (I-J) no protection. (K) Epidermal thickness and (L) percent area of keratin within the dorsal skin after receiving topical interventions and UV irradiation. The scale bar represents

We demonstrated that a commercial UVR-filter, padimate O (PO), can be loaded into BNPs and retain a comparable or higher anti-UVR efficiency compared to commercial sunscreen. We compared a commercial sunscreen and PO loaded BNPs (PO/BNPs) on mouse skin and exposed the mice to a high dose of UVB. With the protection of PO/BNPs, the mouse skin showed no sign of sunburn by visual measurement and histopathologic staining (Fig. 2). On the molecular level, PO/BNPs exhibited a

high efficiency in preventing the formation of cyclobutane pyrimidine dimers (CPD), a marker for sunburn, which is comparable to the control sunscreen (data not shown). In all experiments, we used a 20-fold lower dose of UVR-filter in PO/BNPs compared to the control sunscreen, indicating a higher anti-UVR efficiency of PO/BNPs than commercial sunscreen control.

We showed that the PO/BNPs have no penetration into epidermis. We demonstrated that the BNPs themselves are non-penetrating; after applying the particles on pig skin, the BNPs showed substantially high retention, while no penetration of BNPs was observed within pig skin or within follicles (data not shown). When comparing the penetration of free PO and PO-encapsulated BNPs, we found that 6 hours after application on pig skin (a standard mimic for human skin), free PO led to a clear accumulation of the UVR filter in the skin, while the skin treated with BNP-based sunscreen showed no accumulation of PO (data not shown). In addition, the release of PO from BNP was minimal over a 24-hour period, minimizing the possible penetration risk of PO that has been released from the particles. We also demonstrated the non-penetrating property of the BNP-based sunscreen at the molecular level. Free UVR-filters present in the epidermis and dermis can produce reactive oxidation species and cause DNA double strand breaks. In our study, we applied both commercial sunscreen controls and PO/BNPs on mouse skin and treated with UVR irradiation. The samples protected by BNP-based sunscreen showed significantly fewer double strand breaks compared to the samples treated with the commercial sunscreen control, indicating less penetration into skin (data not shown).

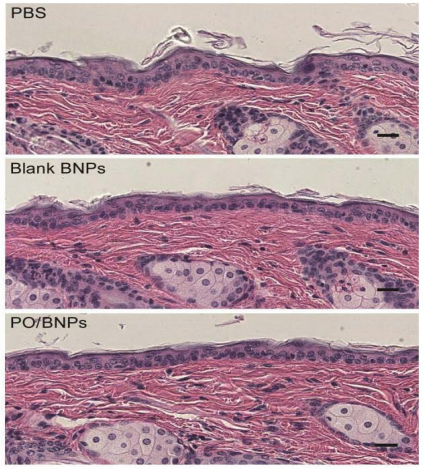


Fig. 3. Evaluation of long term toxicity of PO/BNPs *in vivo*. Representative images of cutaneous histologic sections from FVB/NJ mice treated with PBS, blank BNP, or PO/BNP. Mice received treatment every other day for six total applications. Afterwards, skin treated with PO/BNPs and blank BNPs were indistinguishable from the PBS control. There was no histologic evidence of cutaneous irritancy, toxicity or inflammation. Bar = 20 μm.

BNPs have low toxicity *in vivo* and can reduce the toxicity of reactive oxygen species (ROS) released by UVR filters. In an *in vivo* study aimed at testing the long-term toxicity of BNPs, the particles were applied to dorsal mouse skin every other day for 12 days (six applications). The treated skins showed no evidence of cutaneous irritancy, toxicity, inflammation or damage to skin follicles (Fig. 3). Additionally, photo-induced chemical changes to UVR-filters composition can often produce toxic ROS that damage cells. We demonstrated the BNPs can prevent ROS-induced skin damage by containing the ROS inside the particles (data not shown). Finally, the BNP based sunscreen is long-lasting on the skin. We applied the BNPs on the skin of nude mice and demonstrated that the retention on mouse skin was stable over 24 hours and was water-resistant during this period, but can be readily removed by towel drying (Fig. 4).

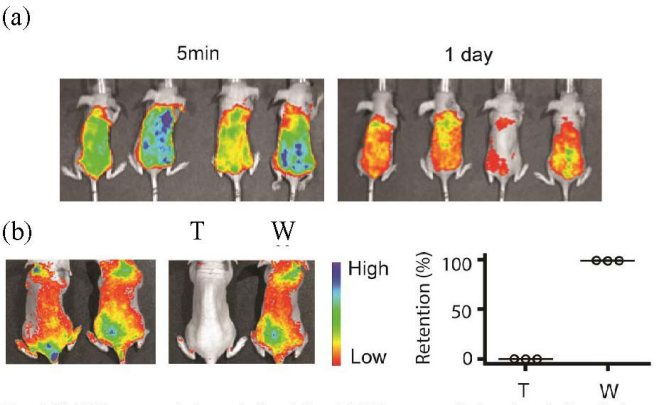


Fig. 4. (a) BNPs encapsulating an infrared dye, IR-780, were applied on dorsal skin of mice and BNP skin retention was imaged with Xenogen at different time points. BNPs showed high retention after 24 hours of application. (b) BNPs encapsulating an infrared dye, IR-780, were applied to the dorsal skin of mice. After wiping with a wet towel (T) or washing with water (W), BNP skin retention was imaged with Xenogen (left) and the fluorescence after wiping or washing was quantified and normalized to the fluorescence intensity at time zero (right).

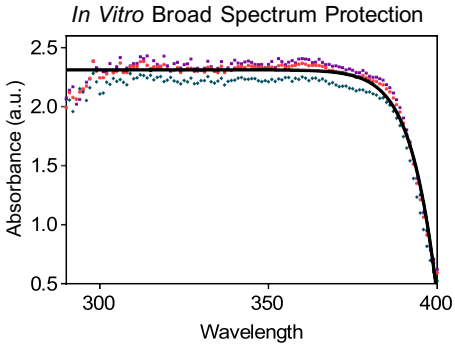
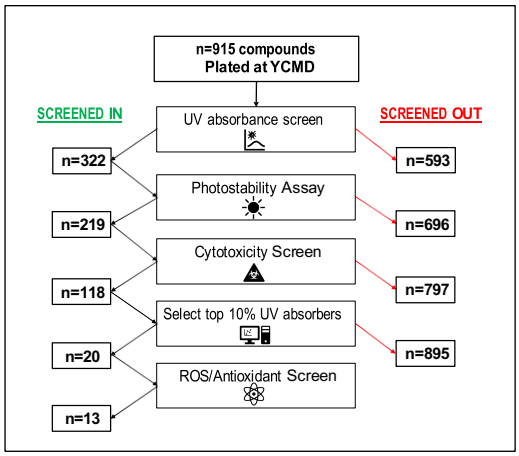


Fig. 5. In vitro broad spectrum protection testing shows uniform absorbance across UVA and UVB wavelengths. Critical wavelength measured: 384 nm.

We have further developed this platform by encapsulating UVR filters that can provide broad spectrum protection (Fig. 5) (2). In vitro testing demonstrates that a BNP sunscreen encapsulating a combination of avobenzone plus octocrylene (BNP-A/O) acts as a neutral density filter capable of absorbing both UVA and UVB wavelengths. This improved BNP formulation has a critical wavelength of 384nm, exceeding the broad spectrum requirements of the FDA monograph (critical wavelength = 370).

In addition to addressing safety concerns regarding current sunscreen agents by engineering the novel BNP technology to enhance performance and prevent penetrance, we have also undertaken a high-throughput screen of naturally occurring compounds to identify safer sunscreen alternatives.

High-throughput screening strategy:



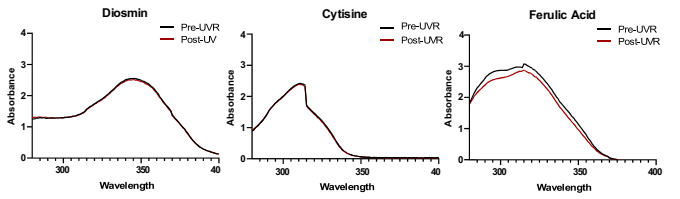
Two small molecule collections at the Yale Center for Molecular Discovery (MicroSource Pure Natural Products and NCI Natural Products Set) containing 915 natural products were sequentially screened for a) their ability to absorb UVA or UVB radiation, b) the degree of photostability they exhibit under solar simulator radiation, c) the extent to which they show minimal cytotoxicity when cultured in vitro with skin cells (keratinocytes, melanocytes), and

d) the extent to which they show minimal ROS generation when exposed to UVR. This resulted in selection of 13 natural compounds that were further scrutinized by additional in vitro testing as well as in vivo (mouse) irritant contact dermatitis testing. Of the 13 natural products resulting from the high-throughput screen, 9 were readily commercially available and passed confirmatory UV absorbance and photostability testing. These 9 then underwent in vitro SPF testing:

Compound	Average SPF (SD)
Cytisine	6.3 (0.1)
Diosmin	2.7 (0.2)
Ferulic Acid	3.1 (0.2)
Gossypin	1.7 (0.1)
Isoliquiritigenin	2.5 (0.3)
Methylxanthoxylin	2.1 (0.1)
Rutin	1.8 (0.0)
Scopoletin	1.6 (0.1)
2/4-dihydroxychalcone	2.2 (0.3)

Three natural compounds, diosmin, ferulic acid, and cytisine, were ultimately chosen. Each displays excellent photostability (Fig 6A) and no in vitro cytotoxicity or ROS generation (Fig 6B) was detected. When formulated as 10% diosmin, 10% ferulic acid, 5% cytosine in FDA P2 cream vehicle, this natural sunscreen gave an in vitro SPF = 48.6 and a high broad spectrum score, λcrit = 373, with no evidence of in vivo irritancy (Fig 6C).

A.



B.

	diosmin	ferulic acid	cytisine
Cytotoxicity (% relative to vehicle control)	-4.33%	-9.67%	-4.61%
ROS generation (relative fluorescence units)	-4670.84	-3167.54	-4441.45

C.

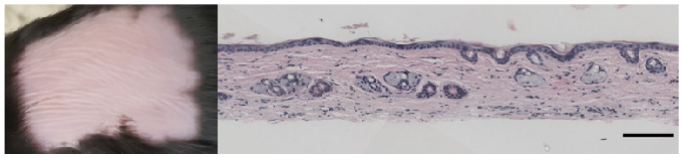


Figure 6. Characteristics of natural product sunscreen compounds. A. UVR absorbance pre- and post- solar simulator radiation exposure shows excellent photostability. B. in vitro culture of selected natural products with three skin cell lines (two keratinocyte lines and one melanocyte line) or vehicle control(0.1% DMSO in culture medium) demonstrates average negative % cytotoxicity – that is, cells cultured with each natural product remained healthier than cells cultured with the control. The fluorescent ROS probe dihydrorhodamine-123 was added to natural products and the mixture exposed to solar simulator radiation. Each natural product generated less ROS than the vehicle control. C. Natural product sunscreen formulated as 10% diosmin, 10% ferulic acid, 5% cytisine in FDA P2 cream vehicle was applied to the shaved dorsal bodywall skin of N=3 C57Bl/6 mice once daily for 5 consecutive days. Throughout treatment, mice were examined daily, and no mice developed erythema, scale, fissuring, or erosions. Histologic findings were indistinguishable from that of the vehicle control group with normal appearing skin without inflammatory infiltrate.

Diosmin is a flavone glycoside found in many citrus fruits. It absorbs UVR primarily in the UVA spectrum. Orally administered diosmin has been used to treat blood vessel disorders and a variety of anti-inflammatory and antioxidant properties have been reported (3). One study also found that diosmin incorporated into a cream and applied to ex vivo human skin samples reduced ROS and CPD following UVB exposure (4). Cytisine is a UVB absorbing alkaloid found in some legumes. It has been marketed as a smoking cessation aid (Tabex™), and has also been suggested to have neuroprotective, anti-diabetic and anti-tumor effects (5). Ferulic acid is a phenolic acid commonly found in many plants. In addition to absorbing UVR (primarily UVB), it is an antioxidant, with excellent free-radical scavenging capacity. It is currently used in a variety of skin care formulations including for photoprotection, anti-aging and skin lightening (6). Trans-resveratrol is another plant derived polyphenolic antioxidant. Although not chosen as one of the final sunscreen candidates in our screen due to its relatively low UVR absorbing capacity, trans-resveratrol has been shown to have significant triplet-state energy quenching capacity (7). UVR-induced chemiexcitation generates high energy reactive oxygen and nitrogen species whose activity can be quenched by trans-resveratrol, thereby reducing subsequent indirect DNA damage. We have therefore included trans-resveratrol in some of our pre-clinical follow-up studies (below).

We have tested BNP-A/O alone or in combination with select natural products in vivo (C57Bl/6 mice) to assess their capacity to block the formation of DNA damaging cyclobutane pyrimidine dimers (CPD) immediately following UVR exposure and found that sunscreen comprising natural products diosmin, ferulic acid, cytisine and BNP-A/O successfully blocked 95.09% of the CPDs (not shown). We also performed a chronic UVR exposure experiment (3 exposures/week for 10 weeks) in which sunscreen was applied to dorsal mouse skin before each UVR exposure (Fig 7). Such chronic UVR exposures cause expansion of p53-mutant keratinocyte clones (called “p53 islands”) that represent microscopic pre-cancerous lesions (8-10). The number of mutant p53 islands was reduced by 92.34% in the mice that received a sunscreen composed of natural products + BNP-A/O, and the area occupied by p53 islands was reduced by 97.61% in these same mice. Also, chronic application of this sunscreen caused no visible skin irritation (data not shown). Together, these data show that addition of select natural products to our BNP-A/O dramatically increases the ability of our sunscreen to block UVR-induced DNA damage.

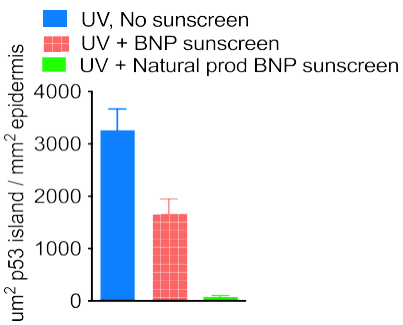


Figure 7. Combined natural product BNP sunscreen is protective in vivo. C57Bl/6 mice underwent chronic solar simulated UVR exposures (1500 J/m^2 UVB + 2700 J/m^2 UVA, 3x/wk, 10wks). 15 minutes before each exposure one group of mice was treated with the FDA monograph recommended amount (2 mg/cm^2) of BNP-A/O containing 3% avobenzone and 9% octocrylene or with sunscreen consisting of 10% ferulic acid, 5% cytisine, 5% trans-resveratrol plus BNP-A/O. Four days following the final exposure, skin was harvested, epidermal sheets prepared and stained for quantification of mutant keratinocyte p53 islands by confocal microscopy. Natural product BNP sunscreen reduced the total area occupied by p53 islands by 97.61%.

Proposed doses and pharmacokinetics:

The FDA monograph recommends applying 2 mg/cm^2 formulated sunscreen. The FDA limits the amount of avobenzone (3%) and octocrylene (10%) that the formulation may contain. Our BNP-A/O will not exceed these limits and provide additional safety by preventing absorbance of the actives into the skin.

Ferulic acid is used in medical cosmetology procedures (microneedling and non-needle mesotherapy, chemical peels, grooming treatments) at concentrations up to 12% (6). Based on our preclinical studies, we propose to use 10% ferulic acid, in a single topical dose equal to 0.9 mg. It has been estimated that ferulic acid intake through food consumption may reach 150-250 mg/day (11). The potential for ferulic acid toxicity in our study, using a dose of 0.9 mg, is exceedingly low. “LD50s equal to 2445 mg/kg and 2113 mg/kg were calculated for male and female rats, respectively (Tada et al., 1999; Ou and Kwok, 2004), whereas an acute LD50 of 3200 mg/kg was calculated in mice (Wang and Ou-Yang, 2005). This low toxicity has been confirmed by numerous experimental studies.” (12-14). For reference, our dose of 0.9 mg applied to a 60 kg individual would equal 0.015 mg/kg, or applied to an 80 kg individual, would equal 0.011 mg/kg. After oral administration, ferulic acid plasma concentrations are maximal at 24 min and the amount of ingested ferulic acid that is absorbed has been estimated to range from 75% (15) to 92% (11). Following topical administration of ferulic acid, one study, using in vivo recovery from hairless mouse skin, reported 16.5 ± 1.92% was absorbed (16), In another study using in vitro human stratum corneum plus epidermis in a Franz cell system, only 2.62% of the applied ferulic acid penetrated through the epidermis (17).

A clinical trial found that daily (oral) resveratrol at doses up to 5 g for 29 days was generally safe (18) and resveratrol (98% trans-resveratrol) has been used to improve lumbar bone mineral density at oral dosing of 75 mg twice daily for 12 months. Based on our preclinical studies, we propose to use a single topical dose of 0.45 mg (5% in formulated sunscreen) which we expect to be non-toxic based on the following studies:.

“Low and high doses of resveratrol, up to 750 mg/kg/day for 3 months, were investigated in vivo in rabbits and rats. The authors concluded that resveratrol is well tolerated and non-toxic and has no effect on reproductive capacity in male or female rats and no embryo- fetal toxicity” (19). “The adverse effects in humans have been investigated in several studies after high-dose resveratrol intake, representing a total of 104 patients (including placebo). The highest doses were 5 g/70 kg for a single intake and 0.9 g/day for iterative administration, corresponding, respectively, to approximately 1/40 and 1/200 of the dose reported to cause nephrotoxicity and 1/4 and 1/20 of the highest dose reported to be safe in rats. No serious

adverse event was detected in any of these studies. Adverse events were mild and only lasted a few days."(19).

Pharmacokinetic studies in humans (20), reveal differences based on route of administration and dose, but generally agree that resveratrol is rapidly absorbed, with peak plasma concentrations 0.8 – 1.5 hr post-dose. There is significant metabolism of resveratrol to multiple forms, mostly excreted in urine (20). The oral absorption of resveratrol in humans is about 75% and is thought to occur mainly by transepithelial diffusion (21). Murakami et al., applied resveratrol orally or topically to mouse skin, and found that both oral and transdermal absorption levels were similar to that described for humans (22). Thus, we expect that approximately 75% of the applied trans-resveratrol will be absorbed.

Cytisine has been used as a smoking cessation aid (e.g. Tabex™), where oral dosing of 1.5 mg every 2 hr is suggested (23). We propose to use a single topical dose of 0.45 mg (5% in formulated sunscreen). We expect this dose to be non-toxic based on reports of clinical trials reviewed by Tzankova and Danchev (24). They report "The clinical trials and observations were carried out on more than 10,000 patients. Smoking cessation was achieved in 55 % - 75 %. Cytisine administration showed no serious adverse reactions during the clinical trials." (24). Toxicology from animal studies is presented in the following table, taken from PubChem (https://pubchem.ncbi.nlm.nih.gov/compound/Cytisine#section=Toxicity):

#	Substance SID	Organism	Test Type	Route	Dose	Effect	Reference
1		rat	LD50	subcutaneous	8750 ug/kg		Farmakologiya i Toksikologiya, 4(1)(34), 1941
2		mouse	LD50	oral	101 mg/kg	BEHAVIORAL: CONVULSIONS OR EFFECT ON SEIZURE THRESHOLD; BEHAVIORAL: REGIDITY	British Journal of Pharmacology., 35(161), 1969 [PMID:4387392]
3		mouse	LD50	intraperitoneal	8550 ug/kg		Zhongcaoyao. Chinese Traditional and Herbal Medicine., 18(214), 1987
4		mouse	LD50	subcutaneous	11764 ug/kg		Farmakologiya i Toksikologiya, 4(1)(34), 1941
5		mouse	LD50	intravenous	1730 ug/kg	BEHAVIORAL: CONVULSIONS OR EFFECT ON SEIZURE THRESHOLD; BEHAVIORAL: REGIDITY	British Journal of Pharmacology., 35(161), 1969 [PMID:4387392]
6		dog	LDLo	intravenous	16 mg/kg		Structure et Activite Pharmacodyanmique des Medicaments du Systeme Nerveux Vegetatif, Bovet, D., and F. ...
7		cat	LD50	intravenous	400 ug/kg	BEHAVIORAL: MUSCLE CONTRACTION OR SPASTICITY)	Izvestiya Akademii Nauk Tadzhikskoi SSR, Otdelenie Biologicheskikh Nauk. Proceedings of the Academy of Sciences of the Tadzhik SSR, Department of Biological Sciences., (2)(104), 1978
8		rabbit	LD50	subcutaneous	5 mg/kg		Farmakologiya i Toksikologiya, 4(1)(34), 1941
9		guinea pig	LDLo	subcutaneous	40 mg/kg		Structure et Activite Pharmacodyanmique des Medicaments du Systeme Nerveux Vegetatif, Bovet, D., and F. ...
10		domestic animals - goat/sheep	LDLo	subcutaneous	500 ug/kg		Veterinary and Human Toxicology., 41(33), 1999 [PMID:9949484]

Cytisine peak plasma concentration is seen 1-2 hr following oral dosing and cytisine is eliminated unchanged, with 18% and 30% of the drug administered orally or intravenously, respectively, found in the urine after 24hr (5). In another study, Klocking et al., found the level of absorption to be 42% following oral administration (25). Internet searches did not identify any studies examining cytisine absorption following topical/skin application.

Diosmin has been used to treat chronic venous insufficiency and hemorrhoid disease, with oral dosing of 1000 mg daily given in 2 divided doses. We propose to use a single topical dose of 0.9 mg (10% in formulated sunscreen), based on our preclinical studies. Diosmin (marketed as "Daflon") has undergone extensive safety evaluation without evidence of toxicity in thousands of patients receiving systemically administered doses of 1000mg daily for 6 weeks to one year (26): "Overall analysis of mid-term and long-term trials: 2850

patients were treated with Daflon 500 mg at the dosage of two tablets per day for six weeks to one year." "Regardless of the treatment duration, the clinical acceptability of Daflon 500 mg was found to be good. The proportion of patients treated with Daflon 500 mg and developing a side effect (10%) was less than that in groups taking placebo (13.9%)."

Following oral administration diosmin is hydrolyzed to diosmetin, which may then be absorbed, however the plasma concentration of diosmetin is low and variable (3), perhaps due to the low aqueous solubility of diosmin. Pharmacokinetic data on topically administered diosmin was not found, however because of the hydrophobic nature of diosmin, and the lipophilic nature of skin, we expect that at least a portion of the applied dose will penetrate the skin.

Based on the single dose we propose to use, compared with doses reported above for other indications, we expect the natural products will not have toxic effects in this study. In addition, we have mixed these natural compounds together in various concentrations in vitro without any apparent chemical reaction (e.g. change in color, pH or temperature). We have also applied the natural products in combination (natural products combined alone or also along with BNP-avobenzone/octocrylene) topically to mouse skin, three times per week for 10 weeks, without any apparent irritation, inflammation or cutaneous toxicity.

Furthermore, many natural sunscreens include plant extracts that contain these compounds. For example, Hawaiian Tropic Sunscreen Powder contains papaya fruit extract and Passiflora extract which have been shown to contain diosmin, and mango extract and Psidium guajava extract which have been shown to contain ferulic acid. Resveratrol is also included in several sunscreens, for example, Paula's Choice Essential Glow SPF30. Cytisine is derived from extracts of the plant family Fabaceae which includes lentils (Lens esculenta) and is included in some sunscreens, for example, Cay Skin Isle Glow SPF45, that uses Lens esculenta extract.

In addition to using only low, non-toxic doses of natural products, and only FDA approved doses of the UV filters avobenzone and octocrylene, we aim to further increase the safety of this study by applying subject selection criterion that will eliminate any individuals with a history of skin cancer or history of any conditions that make you more sensitive to sunlight. The medical history obtained will emphasize the effects of sunlight on their skin, ascertain the general health of the individual, the individual's skin type (I, II, or III), whether the individual is taking medication (topical or systemic) that is known to produce abnormal sunlight responses, and whether the individual is subject to any abnormal responses to sunlight, such as a phototoxic or photoallergic response. In addition, women of child-bearing potential must have negative urine pregnancy test.

We also specifically address whether diosmin, ferulic acid, cytisine, and trans-resveratrol meet the criteria for exemption from an IND:

We reviewed our protocol with Amy Hummel (YCCI, Associate Director fir UND/IDE Management) on November 2, 2022. Based on our exchange and response to questions, we believe that we are exempt from and IND. Our responses included that:

The FDA sunscreen monograph allows us to proceed without an IND – we are using the actives allowed, under the max concentrations permitted. We are not modifying the actives (no covalent changes), just reformulation to improve performance and keep them on the surface of the skin.

The BNP formulation utilizes PLA (which breaks down to lactic acid) and HPG (with breaks down to glycerol) on the surface of the skin. Yale Pharmacy has previously helped us set up GMP conditions for a previous pilot study we conducted and published with Mark Saltzman's lab.

A clinical investigation of a marketed drug is exempt from the IND requirements if all of the criteria for an exemption in § 312.2(b) are met: Are these met for diosmin, ferulic acid, cytisine, and trans-resveratrol? Yes:

• The drug product is lawfully marketed in the United States.

This is correct. Diosmin is marketed under several brand names in the US. For example, Diosmin capsules are available from PipingRock Health Products, Ronkonkoma, NY.

Ferulic acid and resveratrol are included in many cosmetics marketed in the US. For example, The Ordinary Resveratrol 3% + Ferulic acid 3% antioxidant serum for daily skin protection.

Cytisine is available as Tabex or Desmoxan, smoking cessation aids.

• The investigation is not intended to be reported to FDA as a well-controlled study in support of a new indication and there is no intent to use it to support any other significant change in the labeling of the drug.

This is correct.

• In the case of a prescription drug, the investigation is not intended to support a significant change in the advertising for the drug.

This is correct. This is not a prescription drug.

• The investigation does not involve a route of administration, dose, patient population, or other factor that significantly increases the risk (or decreases the acceptability of the risk) associated with the use of the drug product (21 CFR 312.2(b)(1)(iii)).

This is correct. Topical application is far safer than systemic administration.

• The investigation is conducted in compliance with the requirements for review by an IRB (21 CFR part 56) and with the requirements for informed consent (21 CFR part 50).

This is correct.

• The investigation is conducted in compliance with the requirements of § 312.7 (i.e., the investigation is not intended to promote or commercialize the drug product).

This is correct. This is a pilot clinical study funded by the NCI to assess a novel strategy for melanoma prevention.

2.1.2 Clinical Experience

An interventional, nonrandomized trial performed in accordance with a protocol approved by the Yale Human Investigation Committee [HIC#: 1512016909], (N=10, ref. 2) was undertaken to determine the SPF of BNP-A/O (co-encapsulating 3% avobenzone and 9% octocrylene) in healthy volunteers with fair skin (Fitzpatrick skin type I and II), in comparison to FDA approved P2 formulation, which contains 7% padimate O and 3% oxybenzone and has an expected SPF of 16.3 (range 13.7-17.7). BNP-A/O performed comparably to the P2 formulation (2). Skin irritation was not specifically studied in this trial, however no adverse reactions from photosensitivity or photo allergy were observed in subjects receiving BNP-A/O during the duration of the trial.

Rational for proposed doses:

The FDA monograph (21 CFR 201.327) recommends applying 2 mg/cm^2 formulated sunscreen. The FDA limits the amount of avobenzone (3%) and octocrylene (10%) that the formulation may contain. Our BNP-A/O will not exceed these limits and provide additional safety by preventing absorbance of the actives into the skin.

2.2 Background/prevalence of research topic

Nearly 90% of all skin cancers can be attributed to ultraviolet radiation (UVR) from the sun (27). UVR exposure leads to multiple adverse effects including cutaneous phototoxicity (sunburn), photo-aging, and carcinogenesis (27-31). Both UVA and UVB exposure markedly enhance the production of reactive oxidation species (ROS) that damage a variety of cellular components, including genomic DNA, and induce the secretion of immunosuppressive cytokines (32, 33). Conventional sunscreens, though protective against sunburn, have shown the capacity to raise the skin's minimal erythema dose (MED), raising concerns due to their potential to penetrate the skin, enter the bloodstream, and bind hormone receptors. Thus, an innovative sunscreen that offers enhanced protection, while minimizing the potential toxicity induced by its active ingredients, is clearly advantageous over current formulations.

Each year, there are more new cases of skin cancer than the combined incidence of breast, prostate, lung, and colon cancers; and incidence rates continue to rise (34). From 1992-2006, the treatment of non-melanoma skin cancers increased by nearly 77 percent (35). The diagnosis of melanoma is significantly more fatal; 2022 estimates indicate that one person dies of melanoma every hour (34). The economic impact of these high incidence rates also cannot be ignored—the annual cost of treating skin cancers in the United States is estimated at $8.1 B (36). Over the last decade, the average annual cost for skin cancer treatment has increased by more than 126%, compared to 25% for all other cancers (36).

On average, a person's lifetime melanoma risk increases by 80% if they have sustained five or more sunburns during youth (27, 37). Ambient UVR exposure also results in photo-aging; more than 90% of the visible changes in skin are caused by the sun (37). Epidemiologic

studies have proven that sunscreens play an important role in preventing UVR damage and reducing risk. Regular daily use of sunscreen reduces the risk of developing squamous cell carcinoma by 40 percent and the risk of developing melanoma by 50% (29, 30). People who use sunscreen daily also show 24% less skin aging than those who do not (37).

Commercially available sunscreens have incorporated organic and inorganic UVR filters to provide protection, but their overall effectiveness in preventing skin cancer remains controversial (38-40). Both agents have shown the capacity to enhance ROS generation after UVR exposure, suggesting that even small quantities may contribute to cellular damage and ultimately carcinogenesis (38-40). Furthermore, transdermal penetration of these filters through the stratum corneum into epidermal cells, including keratinocytes and Langerhans cells, raises direct toxicity concerns. In addition, the potential for systemic absorption and deposition in adipose tissue may result in additional health risks such as endocrine disruption (38, 41). These adverse effects must be addressed in order to minimize skin cancer risk and minimize potential toxicity.

Nanoparticle encapsulation, used widely in the field of drug delivery, has demonstrated unique advantages including target specificity and toxicity reduction. The incorporation of this technology will eliminate direct skin contact and subsequent epidermal penetration, and thus, provide unprecedented UVR protection and safety. Additionally, the properties of polymer-based nanoparticles may be tuned to offer increased substantivity and better equip individuals for prolonged sun exposure. Hence, we have engineered a safer sunscreen with enhanced UVR protection (1, 2).

We have additionally utilized high-throughput screening to identify naturally occurring non-toxic products for use as additions to or alternatives for existing sunscreen actives. Natural products are compounds produced by living organisms (e.g. tropical plants, algae) that have evolved systems to respond to environmental stressors including UVR (42, 43). Indeed, harnessing the power of natural products for use as sun-protective agents is not a novel concept (44). Some natural compounds can directly interact with UVR to prevent direct DNA damage by absorbing UVR and thus acting as a sunscreen. For example, flavonoids, such as diosmin, contain an aromatic polyphenol backbone and may contain hydroxyl groups that confer excellent UVR absorbing capacity without concerns of paradoxically catalyzing photochemical reactions (45). Such compounds prevent direct formation of mutagenic cyclopyrimidine dimers (CPDs) in DNA.

In addition to their UVR absorbing capacity, some natural products have also been shown to scavenge ROS, for example, ferulic acid and trans-resveratrol have excellent antioxidant properties (6, 7). This is critical for the reduction of indirect DNA damage. Chemiexcitation of electrons in skin chromophores such as melanin results in high-energy triplet states that are responsible for inducing so called “dark” CPDs, that is, indirect DNA damage that occurs after UVR exposure ends (46). Certain antioxidant natural compounds, including trans-resveratrol and ferulic acid, are able to quench triplet state energy and prevent dark CPD formation (7). Thus, the potential “triplet state quencher” capacity of certain natural compounds also makes them attractive candidates as a topical intervention before, during, and after UVR exposure to minimize the formation of dark CPDs.

Our research, thus far, has demonstrated that the bioadhesive nanoparticle (BNP) sunscreen we have developed has demonstrated in an initital investigation superior skin protection from UVR exposure than commercial sunscreens. We have also identified non-toxic, naturally occurring plant-derived compounds that boost the effectiveness of our BNP sunscreen by both increasing the UVR-absorbing capacity and providing antioxidant, triplet state energy quenching capacity. These natural products combined with BNP sunscreen have the potential

to substantially reduce or eliminate both direct and indirect DNA damage caused by UVR exposure, reducing the risk of skin cancer development.

3 Rationale/Significance

3.1 Problem Statement

Skin cancer is the most commonly diagnosed malignancy in the USA and UVR exposure is the major environmental risk factor for skin cancer development. Currently available sunscreens utilize UVR filters that, while absorbing UVR energy, have been shown to induce ROS, resulting in oxidative DNA damage after UVR exposure. Organic sunscreen actives have also been shown to penetrate into the skin, raising direct toxicity, as well as irritant and photoallergic concerns. Further systemic absorption may result in additional health risks such as endocrine disruption. Novel sunscreens that more safely prevent both direct and indirect DNA damage are needed.

3.2 Purpose of Study/Potential Impact

We have produced a bioadhesive nanoparticle (BNP) sunscreen designed to keep organic UVR filters from penetrating into the skin and have incorporated non-toxic natural products into this sunscreen to further safely boost UVR absorbing capacity and reduce oxidative, indirect DNA damage. In this study we propose to test the capacity of this sunscreen to prevent direct and indirect cellular and DNA damage in human skin exposed to UVR.

3.2.1 Potential Risks

UVR exposure: Ultraviolet radiation interacts with light absorbing molecules in different layers of the skin. UVB (280-320nm) is primarily absorbed in the epidermis and may be associated with direct DNA damage and immunosuppression, although narrowband UVB is used as a clinical therapy, and evidence of skin cancer risk is low. UVA (320-400nm) may penetrate into the dermis and is associated with oxidative cellular and DNA damage.

UVR exposure for MED testing and subsequent sunscreen testing may result in erythema (redness).

Biopsy: A total of 5 x 3mm punch biopsies will be taken by standard procedures. A skin biopsy is a generally safe procedure, with risks including: bleeding, bruising, and infection (all rare, <1%), and scarring (limited to <3mm per biopsy site). The resulting minimal erythema on UV exposed skin is not expected to increase risks i.e. over standard skin punch biopsy on non-exposed skin. The dose of UV used is too low and too superficial an exposure to adversely affect wound healing after a punch biopsy. Prior studies on much higher UV-exposed skin with much larger surgical wounds did not show compromised healing (47).

Risk of agents acting in combination: based on the chemical structure of each agent, we do not have any reason to believe that there is any increased toxicity risk to using them in combination. We have mixed these compounds together in various concentrations in vitro

without any apparent chemical reaction (e.g. change in color, pH, or temperature). We have also applied the the natural products in combination (natural products combined alone or also along with BNP-avobenzone/octocrylene) topically to mouse skin (3 times/wk for 10 wks) without any apparent irritation, inflammation, or cutaneous toxicity.

Risks will be minimized by:

1. Using the minimal amount (1 MED) of UVR necessary to obtain the primary objective, and minimizing the area exposed to UVR by using the Multiport 601 solar simulator which limits UVR exposure to 8mm diameter (0.5 cm^2) spots.

2. Using FDA approved UVR filters encapsulated in nanoparticles (avobenzone and octocrylene) and formulating the sunscreen such that FDA approved doses are not exceeded (FDA OTC monograph specifies 3% avobenzone and 10% octocrylene).

3. Using non-toxic natural products (ferulic acid, cytisine, diosmin, trans-resveratrol) and topical doses of these products that are below a) doses considered safe for repeated oral administration for other indications, or b) amounts used in existing cosmetic treatments

4. Trained and experienced personnel will supervise subjects during every phase of participation. Personnel will be instructed to be aware of any warning signs of impending danger and will stop any procedure at that time.

5. Using sterile skin biopsy techniques performed by a board-certified dermatologist, observing sites for 15 minutes after skin biopsy ensure adequate bleeding control, and provided post-biopsy care instructions (change bandage, wash site with soap and water daily, apply ointment and band aid daily; call for any redness, pain, swelling, or discharge; return in 7-10 days for suture removal).

3.2.2 Potential Benefits

The proposed study will enable us to scientifically assess and further develop our nanoparticle sunscreen technology combined with natural products for enhanced UVR protection. While direct benefits to participants are not expected, we believe that society at large would benefit from the translation of this technology for the development of safer more effective sunscreen which may minimize risk for skin cancer development.

4 Study Objectives

4.1 Hypothesis

We hypothesize that our combined natural product plus BNP sunscreen will reduce both direct and indirect UVR-induced cellular and DNA damage to human skin by ≥90%.

4.2 Primary Objective

The primary objective of this study is to evaluate the effectiveness of our novel sunscreen by assessing the extent of UVR-induced direct and indirect cellular and DNA damage to human skin, in the presence vs absence of the sunscreen, in a population of healthy adults with fair skin (Ftizpatrick Scale type I, II or III).

4.3 Secondary Objectives (if applicable)

N/A

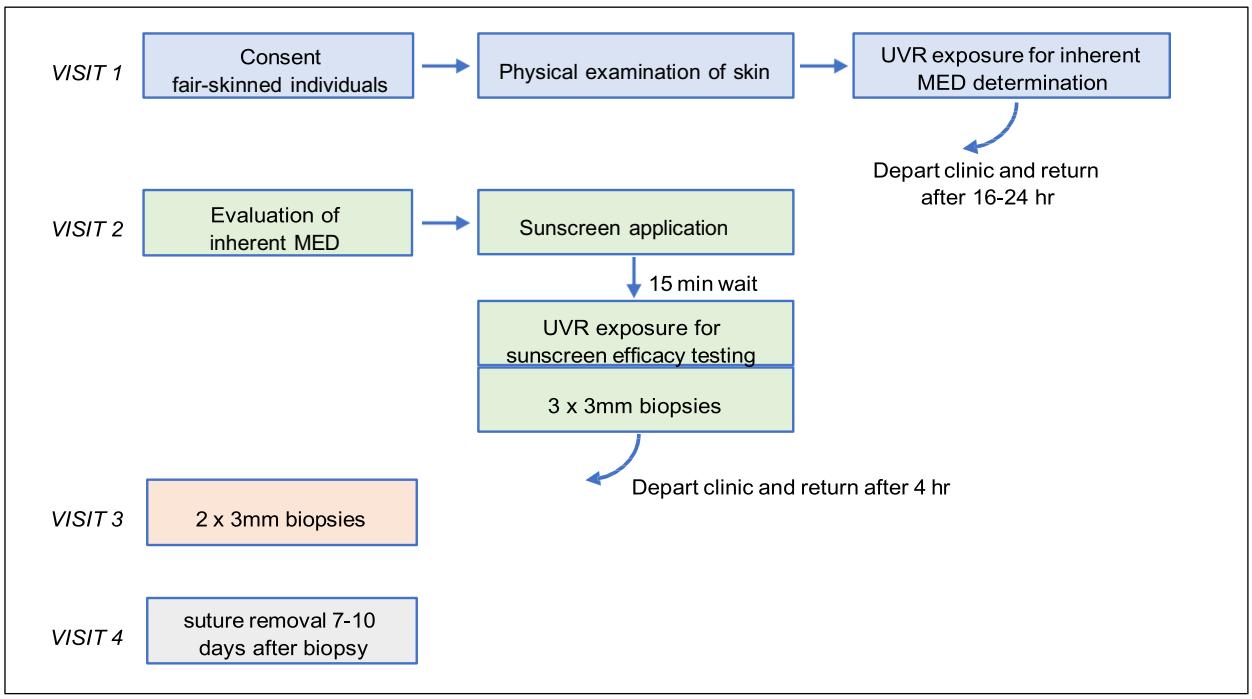
4.4 Exploratory Objectives (if applicable)

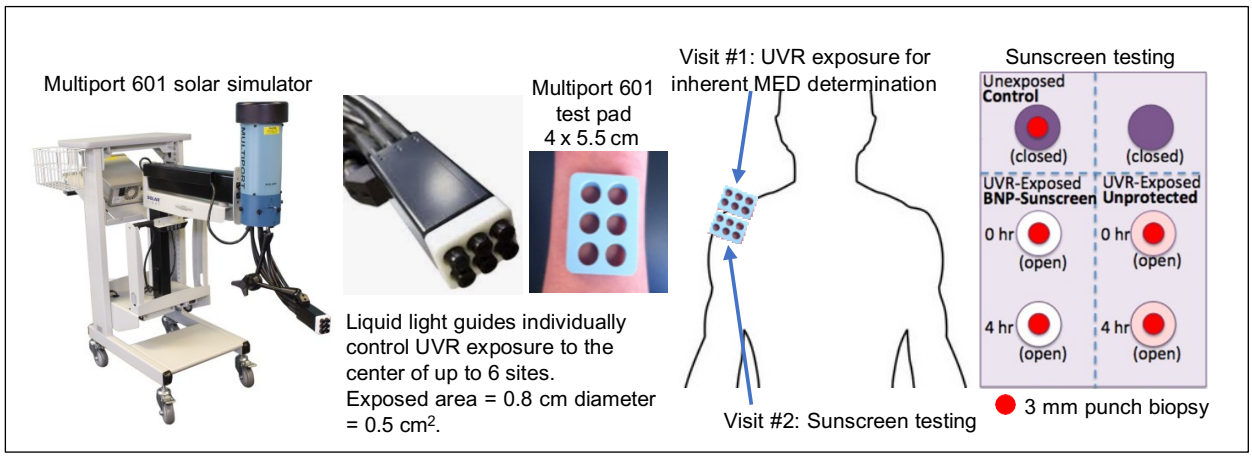
N/A

5 Study Design

5.1 General Design Description

This is a phase 1 non-randomized pilot study designed to test the effectiveness of a sunscreen compared to a no treatment control. Sunscreen application and sample (biopsy) collection will not be blinded, but analysis of the samples will be conducted by laboratory staff blinded to the sample characteristics (to avoid bias). Data obtained from the sample analysis will be de-coded by the PI and used to determine sunscreen efficacy.





The Multiport 601 solar simulator provides highly uniform UVR (290-400nm) and is fully compliant with ISO, FDA, JCIA and COLIPA spectral irradiance standards and all FDA sunscreen testing requirements (21 CFR 201.327). Calibrated probes monitor each exposure and specifically designed ports ensure directed exposure only to the 8mm diameter test sites identified by the blue test pad in the image above. The six black UV ports fit into the 6 openings in the blue patch. Only these six 0.5 cm^2 spots of skin are exposed to UV light. The dose of UV going to each spot can be individually controlled.

The blue test pad is not used for sunscreen delivery; it is used to guide the UV light to six small defined spots (0.5 cm^2 each). The sunscreen will be applied by rubbing the cream/emulsion onto the skin before placement of the blue pad.

Visit #1: Subjects meeting eligibility requirements and providing written consent will undergo UVR exposure for inherent minimal erythema dose (MED) determination. An adhesive test pad will be placed on the upper arm to guide UV light delivery to six small defined spots (0.5 cm^2 each). No other skin is exposed to UV light. The doses used depend on the individuals Fitzpatrick Scale skin type:

The Multiport 601 solar simulator will be used to deliver a 1.25x UVR dose series centered around the FDA Standard 1 MED dose for the participant's individual Fitzpatrick Scale skin type. Examples of the UV dose given for different skin types is shown in the table below:

Visit 1						
Test Site # (each site = 0.5 cm^2)	UV dose for MED testing (J/m^2)			Corresponding time at the beach in France on the summer solstice (minutes)		
	Skin Type 1	Skin Type 2	Skin Type 3	Skin Type 1	Skin Type 2	Skin Type 3
1	0	0	0	0	0	0
2	64	128	192	5.91	11.82	17.72
3	80	160	240	7.38	14.77	22.15
4	100	200	300	9.23	18.46	27.69
5	125	250	375	11.54	23.08	34.62
6	156	313	469	14.42	28.85	43.27

The time needed to deliver the maximum UV light dose using our solar simulator ranges from approximately 30 seconds for skin type 1 to 90 seconds for skin type 3.

Visit #2: 16-24 hr after Visit #1 the results of MED testing will be determined by visual inspection and recorded. 1 MED is defined as the smallest UVR dose that produces perceptible redness of the skin with clearly defined borders 16-24 hours after UVR exposure. The area used for MED determination on day 1 will be examined and the UV dose that produced the faintest perceptible redness will be identified. This dose = 1 MED for this individual, and this is the UV dose that will be used to test the sunscreen.

The skin site to be used for sunscreen testing will then be delineated and sunscreen applied (by rubbing the cream/emulsion onto the skin with a gloved fingertip) to the appropriate subsites and allowed to dry for 15 min. An adhesive test pad will be placed over the delineated area to guide the UV light to the correct sites, and the Multiport 610 solar simulator used to deliver 1 MED UVR to the appropriate subsites. Based on our previous study, 1 MED ranged from 64 – 200 J/m^2 for different individuals. The corresponding time at the beach on the summer solstice in France would be 5.91 – 18.46 minutes.

Immediately following UVR exposure 3 x 3mm punch biopsies will be obtained by Dr. Girardi from the appropriate subsites of the test area:

1. Untreated skin, No UVR
2. Untreated skin + 1 MED UVR
3. Sunscreen treated skin + 1 MED UVR

Visit #3: 4 hr after the UVR exposure of visit #2, 2 additional 3 mm punch biopsies will be obtained by Dr. Girardi from the appropriate subsites of the test area:

4. Untreated skin + 1 MED UVR
5. Sunscreen treated skin + 1 MED UVR

Visit #4: 7 – 10 days after biopsies for suture removal only.

5.1.1 Study Date Range and Duration

The expected length of the study is 9 months, beginning in November 2022.

5.1.2 Number of Study Sites

There will be one study site. Studies will be completed at the 4th floor of the Hunter Radiation Therapy (HRT) building, in the Examination Room of the YNHH Photopheresis Unit (Director: Dr. Michael Girardi). Physician Assistant Kacie Carlson, PA-C, has an office on this floor, and will serve as the study coordinator. Nursing staff are available and well-trained. Skin biopsies will be processed in Dr. Girardi's laboratory (HRT618).

5.2 Outcome Variables

Outcome data will be recorded for samples obtained at study visit #2 and #3.

Study visit #2:

The results of MED testing will be determined by visual inspection and recorded.

Samples (3 mm punch biopsies) will be obtained immediately after UVR exposure:

1. Untreated skin with no UVR exposure
2. Untreated skin exposed to 1 MED solar simulator UVR.
3. Sunscreen treated skin exposed to 1 MED solar simulator UVR.

Study visit #3:

Samples (3 mm punch biopsies) will be obtained 4 hr after UVR exposure:

4. Untreated skin exposed to 1 MED solar simulator UVR.
5. Sunscreen treated skin exposed to 1 MED solar simulator UVR.

The study endpoint for participants occurs following collection of 5 biopsy samples, described above. Biopsies will be analyzed to assess the primary objective (below).

Study visit #4: Follow-up of biopsy sites will occur 7-10 days later with suture removal.

5.2.1 Primary Outcome Variables

Each biopsy tissue will be used to assess the effectiveness of the sunscreen by measuring the level of DNA and cellular damage in UVR-exposed vs unexposed and sunscreen treated vs untreated samples by the following 3 methods:

1. DNA will be prepared and assayed by ELISA for quantification of CPDs. CPDs measured in samples obtained immediately after UVR exposure are indicative of direct DNA damage. CPDs will also be measured in samples obtained 4 hr after UVR exposure, at which time any increase in the CPD level, as compared to time 0, is indicative of so called “dark” CPDs that form in response to indirect oxidative DNA damage.

2. Formalin fixed paraffin embedded skin will be stained with anti-gH2AX to identify DNA strand breaks. Indirect, oxidative DNA damage may result in DNA strand breaks that can be quantified by microscopic visualization of gH2AX, which builds up at the site of each strand break.

3. Formalin fixed paraffin embedded skin will be stained with anti-3-nitrotyrosine to identify cellular damage. ROS and high energy triplet state species can result in nitration of tyrosine residues of cellular proteins. This type of damage can be quantified by microscopic visualization of 3-nitrotyrosine.

All testing will be done by laboratory staff blinded to the sample characteristics.

5.2.2 Secondary Outcome Variables (if applicable)

N/A

5.2.3 Exploratory Outcome Variables (if applicable)

N/A

5.3 Study Population

Individuals with fair skin are more susceptible to the UVR-induced DNA damage that we seek to prevent by development of a safer, more effective sunscreen, and thus will comprise the study population.

Healthy adult volunteers will be selected as follows:

(1) Only fair-skin subjects with Fitzpatrick Scale skin types I, II, and III using the following guidelines shall be selected:
Skin Type and Sunburn and Tanning History (Based on first 30 to 45 minutes sun exposure after a winter season of no sun exposure.)

I--Always burns easily; never tans (sensitive).
II--Always burns easily; tans minimally (sensitive).
III--Burns moderately; tans gradually (light brown) (normal).

Do Not Include:
IV--Burns minimally; always tans well (moderate brown) (normal).
V--Rarely burns; tans profusely (dark brown) (insensitive).
VI--Never burns; deeply pigmented (insensitive).

(2) (a) A medical history shall be obtained from all subjects with emphasis on the effects of sunlight on their skin. Ascertain the general health of the individual, the individual's skin type (I, II, or III), whether the individual is taking medication (topical or systemic) that is known to produce abnormal sunlight responses, and whether the individual is subject to any abnormal responses to sunlight, such as a phototoxic or photoallergic response. The subject should not have a history of any conditions that make you more sensitive to sunlight, or a history of skin cancer.

(b) Test site inspection. The physical examination shall determine the presence of sunburn, suntan, scars, active dermal lesions, and uneven skin tones on the areas to be tested. The presence of nevi, blemishes, or moles will be acceptable if in the physician's judgment they will not interfere with the study results.

5.3.1 Number of Participants

We anticipate screening 40 individuals in order to enroll 30 individuals.

5.3.2 Eligibility Criteria/Vulnerable Populations

Eligibility will be determined by study coordinator, Kacie Carlson, PA-C. Dr. Michael Girardi, and Dr. Mark Salzman will not determine subject eligibility or consent subject due to conflicts of interest. If there are any questions regarding eligibility, Dr. Ian Odell will be consulted.

INCLUSION CRITERIA:

In order to be eligible to participate in this study, an individual must meet all of the following criteria:

1. Provision of signed and dated informed consent form
2. Stated willingness to comply with all study procedures and availability for the duration of the study
3. Male or female, aged 18 years old or greater
4. Women of child-bearing potential must have negative urine pregnancy test
5. In good general health as evidenced by medical history
6. Fair skinned with Fitzpatrick Scale skin types I, II or III using the following Skin Type and Sunburn and Tanning History (based on the first 30-45 minutes of sun exposure after a winter season of no sun exposure):
    a. I—always burns easily; never tans (sensitive)
    b. II—always burns easily; tans minimally (sensitive)
    c. III—burns moderately; tans gradually (light brown) (normal)

EXCLUSION CRITERIA:

An individual who meets any of the following criteria will be excluded from participation in this study:

1. Individuals with active or a history of dermatological disorders—psoriasis, rosacea, eczema, vitiligo, lupus, dermatomyositis, etc
2. Individuals known to be subject to any abnormal responses to sunlight, such as phototoxic or photoallergic response.
3. Current use of medication (topical or systemic) that is known to produce abnormal sunlight responses.
4. History of skin cancer (such as basal cell carcinoma, squamous cell carcinoma, melanoma)
5. Family history of melanoma
6. Presence of sunburn, suntan, scars, active dermal lesions or uneven skin tone on the test site.
7. Skin type falling under the Fitzpatrick Scale skin types IV, V or VI using the following Skin Type and Sunburn and Tanning History (based on the first 30-45 minutes of sun exposure after a winter season of no sun exposure):
    - IV—Burns minimally; always tans well (moderate brown) (normal)
    - V—Rarely burns; tans profusely (dark brown) (insensitive)
8. Use of sunscreen within the last week on the test site area (such that UV filter penetration may confound results)
9. Febrile illness within 48 hours.
10. Women with a positive urine pregnancy test

6 Methods

6.1 Treatment

6.1.1 Identity of Investigational Product

The sunscreen to be tested will be administered topically.

The sunscreen contains bioadhesive nanoparticles (BNP) encapsulating avobenzone and octocrylene plus the non-toxic natural products diosmin, ferulic acid, cytisine and trans-resveratrol. Each is described below:

The BNP are composed of a biocompatible block copolymer called poly (lactic acid)-hyperbranched polyglycerol (PLA-HPG). Poly-lactic acid is a degradable polymer with a history of use in medicine, including many FDA-approved medical devices and drug delivery systems, stretching back to the 1970s. For example, PLA microspheres are currently approved as an injectable dermal filler for cosmetic purposes (Sculptra, injectable poly-(lactic acid) microspheres, FDA approval P030050, Aug 3 2004) and a bioabsorbable adhesion barrier (REPEL-CV®, FDA approval P070005, March 6, 2009). PLA has been demonstrated to be safe for human use.

Our nanoparticles include a hyperbranched polyglycerol (HPG) surface modification that enables increased retention on the outer surface of the stratum corneum. This reduces

dermal penetration of the chemical filter, thereby increasing the safety of our sunscreen over currently approved over-the counter sunscreens. Because of this bioadhesion, there is little opportunity for the components of the nanoparticle to interact with cells, tissues and molecules deeper within the skin, or in the rest of the body. HPG is an inert polymer that is currently in advanced preclinical testing. Sitka Biopharma is testing nanoparticles of HPG and docetaxel for intravesicular treatment of bladder cancer, planning to initiate a phase 1 clinical trial (http://www.sitkabiopharma.com). In addition, HPG has been tested in animals as an osmotic agent for use during peritoneal dialysis. In this application, large quantities of HPG are administered into the peritoneal cavity (HPG molecular weight 3 kDa; 2.5% - 15%). In this preclinical testing, exposure of the peritoneum to these large doses of HPG is found to produce less peritoneal injury than control solutions containing glucose as the osmotic agent (48). In addition, HPG appears to be less toxic than alternatives when used as a colloid in cold organ preservation solutions (49). Therefore, HPG appears to be inert, even when exposed to sensitive tissues deep within the body, such as those in the abdominal cavity and the endothelium of harvested organs.

These nanoparticles will encapsulate only FDA-approved active ingredients and combinations as described in the monograph (21 CFR Part 352.1). The proposed human studies will use only approved or lower concentrations of UV filters avobenzone (AVO) and octocrylene (OCTR). We have conducted in vitro and in vivo studies with AVO and OCTR and padimate-O (PO), demonstrating improved safety and decreased penetration in animal models.

We will manufacture the bioadhesive nanoparticles encapsulating avobenzone and octocrylene (BNP-A/O) under sterile conditions in Malone Engineering Center. We will ensure that the nanoparticles in solution (sterile distilled water) are prepared and handled in a sterile environment prior to application to human skin.

USP grade (US Pharmacopoeia) natural products will be purchased. USP grade products meet or exceed the purity requirements of the US Pharmacopoeia and are suitable for food, drug, or medicinal use. USP and NF (National Formulary) are the official standards for all prescription and over-the-counter medicines, dietary supplements, excipients, and other healthcare products manufactured and sold in the US. A single lot of each natural product will be used for the entire study. The natural products will be handled in a sterile environment in Malone Engineering Center for formulation in vehicle (a standard vehicle approved for use in humans, such as Transcutol P or FDA P2 cream vehicle) prior to application to human skin.

6.1.2 Dosage, Administration, Schedule

Each participant will undergo topical UVR exposure one time for inherent minimal erythema dose (MED) determination. The Multiport 601 solar simulator will be used to deliver a 1.25x UVR dose series centered around the FDA Standard 1 MED dose for the participant's individual Fitzpatrick Scale skin type.

Following sunscreen application (below) each participant will undergo a single 1 MED UVR exposure, delivered by the Multiport 601 solar simulator, to test sunscreen efficacy.

The Multiport 601 solar simulator provides highly uniform UVR (290-400nm) and is fully compliant with ISO, FDA, JCIA and COLIPA spectral irradiance standards and all FDA sunscreen testing requirements (21 CFR 201.327). Calibrated probes monitor each exposure and specifically designed ports ensure directed exposure only to the 8mm diameter test sites.

Formulated sunscreen will be applied topically, one time, on each participant, to a 1.5 x 3 cm area at the FDA monograph (21 CFR 201.327) recommended 2 mg formulated sunscreen per cm^2. Thus, a total of 9 mg of formulated sunscreen will be applied to each participant. The formulated sunscreen will contain:

3% avobenzone (9 mg x 3% = 0.27 mg avobenzone applied)

9% octocrylene (9 mg x 9% = 0.81 mg octocrylene applied)

10% diosmin (9 mg x 10% = 0.9 mg diosmin applied)

10% ferulic acid (9mg x 10% = 0.9 mg ferulic acid applied)

5% cytisine (9mg x 5% = 0.45 mg cytisine applied)

5% trans-resveratrol (9mg x 5% = 0.45 mg trans-resveratrol applied)

Sunscreen preparation:

The sunscreen will be prepared under sterile conditions in Dr. Saltzman's laboratory in the Department of Biomedical Engineering's Malone Engineering Center (MEC). Over 2,000 sq. ft. of this space is solely committed to the laboratory efforts of Dr. Saltzman. This laboratory space includes a wide range of specialized equipment needed for bioengineering research. The sunscreen will be prepared following a written procedure with detailed record-keeping regarding raw ingredients, lot numbers, dates tested/used, measured amounts, and drug yields. All equipment and facilities used in manufacturing, such as biosafety cabinets and chemical hoods, will be clean and properly maintained.

Over the course of the study, one lot of bioadhesive nanoparticle (BNP) sunscreen will be used. We will perform quality assurance testing prior to release. Non-adhesive nanoparticles will be stored at -80° C. Prior to use, these nanoparticles will be incubated in sodium periodate for conversion to the bioadhesive form and resuspended in water for use at a concentration of 250 mg/ml - 750 mg/ml BNP. We will ensure that the final concentration of the active ingredients do not exceed the limits set by the FDA monograph (3% avobenzone, 10% octocrylene). Stability of these nanoparticles will be tested every month over the duration of this study to ensure quality of the product.

Prior to use, USP grade natural products will be stored in the dark at room temperature. These products will be handled in a sterile environment in Malone Engineering Center for formulation in vehicle (a standard vehicle approved for use in humans, such as Transcutol P or FDA P2 cream vehicle) prior to application to human skin. Stability of the formulation will be tested every month over the duration of this study to ensure quality of the product.

6.1.3 Method of Assignment/Randomization

N/A

6.1.4 Blinding and Procedures for Unblinding

Application of the sunscreen and collection of biopsies is not blinded, but all analysis of biopsies (measurement of CPDs, gammaH2AX, and 3-nitrotyrosine) will be done by laboratory staff blinded to the biopsy sample characteristics.

6.1.5 Packaging/Labelling

Nanoparticles used in the pilot study will be produced under sterile conditions in Dr. Saltzman's laboratory in the Department of Biomedical Engineering's Malone Engineering Center (MEC) as a single batch for quality control purposes and monitoring. Previous work has confirmed that these nanoparticles are stable beyond one year. Single lots of each USP grade natural product will also be used and are stable beyond one year. Formulated sunscreen will be produced under sterile conditions in Dr. Saltzman's laboratory using a standard vehicle approved for use in humans, such as Transcutol P or FDA P2 cream vehicle. Formulated sunscreen will be aliquoted under sterile conditions into single use sealed aliquots properly labeled for use in this study. Delivery to the study personnel at HRT 4 will be based upon participant enrollment.

6.1.6 Storage Conditions

Formulated sunscreen will be stored in the dark at 4°C prior to use. Following application of sunscreen on a participant, the remaining sunscreen will be disposed as per Yale OEHS guidelines in Dr. Girardi's laboratory.

6.1.7 Concomitant therapy

Subjects will be excluded from the study if currently taking any photosensitizing drugs (see exclusion criteria). Litt's Drug Eruption & Reaction Manual, Shear, Neil H. 2022

6.1.8 Restrictions

There are no restrictions. Subjects will be given post-biopsy instructions not to remove the band aid for 24 hrs.

6.2 Assessments

6.2.1 Efficacy

Efficacy will be evaluated by analysis of the biopsy tissue:

Each 3 mm punch biopsy will be bisected. Half will be snap frozen in liquid nitrogen and half will be fixed in 10% neutral buffered formalin. The tissue will be used to assess the effectiveness of the sunscreen by measuring the level of DNA and cellular damage in UVR-exposed vs unexposed and sunscreen treated vs untreated samples by the following 3 methods:

1. DNA will be prepared and assayed by ELISA for quantification of CPDs. CPDs measured in samples obtained immediately after UVR exposure are indicative of direct DNA damage. CPDs will also be measured in samples obtained 4 hr after UVR exposure, at which time any increase in the CPD level, as compared to time 0, is indicative of so called "dark" CPDs that form in response to indirect oxidative DNA damage.

2. Formalin fixed paraffin embedded skin will be stained with anti-gH2AX to identify DNA strand breaks. Indirect, oxidative DNA damage may result in DNA strand breaks that can be quantified by microscopic visualization of gH2AX, which builds up at the site of each strand break.

3. Formalin fixed paraffin embedded skin will be stained with anti-3-nitrotyrosine to identify cellular damage. ROS and high energy triplet state species can result in nitration of tyrosine residues of cellular proteins. This type of damage can be quantified by microscopic visualization of 3-nitrotyrosine.

All testing will be done by laboratory staff blinded to the sample characteristics.

6.2.2 Safety and Pregnancy-related policy

The sunscreen will be prepared under sterile conditions in Dr. Saltzman's laboratory in the Department of Biomedical Engineering's Malone Engineering Center (MEC) following written procedures with detailed record-keeping regarding raw ingredients, lot numbers, dates tested/used, measured amounts, and yields. All equipment and facilities used in manufacturing, such as biosafety cabinets and chemical hoods, will be clean and properly maintained. Formulated sunscreen will be aliquoted under sterile conditions into single use sealed aliquots properly labeled for use in this study. Stability of the sunscreen will be tested every month over the duration of this study to ensure quality of the product. For quality control, we will conduct characterization studies to examine nanoparticle size, via dynamic light scattering, and *in vitro* UVR absorbance (for in vitro SPF and critical wavelength), via plate assays. Before proceeding, we will ensure that these parameters are consistent with those previously used in preclinical animal studies.

Risks will be minimized by:

1. Using the minimal amount (1 MED) of UVR necessary to obtain the primary objective, and minimizing the area exposed to UVR by using the Multiport 601 solar simulator which limits UVR exposure to 8mm diameter (0.5 cm^2) spots.

2. Using FDA approved UVR filters encapsulated in nanoparticles (avobenzone and octocrylene) and formulating the sunscreen such that FDA approved doses are not exceeded (FDA OTC monograph specifies 3% avobenzone and 10% octocrylene).

3. Using non-toxic natural products (ferulic acid, cytisine, diosmin, trans-resveratrol) and topical doses of these products that are below a) doses considered safe for repeated oral administration for other indications, or b) amounts used in existing cosmetic treatments

4. Trained and experienced personnel will supervise subjects during every phase of participation. Personnel will be instructed to be aware of any warning signs of impending danger and will stop any procedure at that time.

5. Using sterile skin biopsy techniques performed by a board-certified dermatologist, observing sites for 15 minutes after skin biopsy ensure adequate bleeding control, and provided post-biopsy care instructions (change bandage, wash site with soap and water daily, apply ointment and band aid daily; call for any redness, pain, swelling, or discharge; return in 7-10 days for suture removal).

Since we do not know the teratogenicity of the proposed natural products, women of child-bearing potential will undergo urine pregnancy testing prior to application of topical study

medications. If the test is negative, we will proceed with topical application study agents and UV exposure.

6.2.3 Adverse Events Definition and Reporting

Adverse events (skin irritancy, hypersensitivity, follicular occlusion effects) will be monitored for each participant from the time of the start of the study (first UVR exposure) through the end of intervention.

Adverse events will be monitored by the personnel involved in the testing and administration of the study.

Adverse events will be monitored immediately following UVR exposure (visit #1), 16-24 hr later (start of visit #2), 15 min following application of sunscreen (visit #2), immediately following 1 MED UVR exposure (visit #2), 4 hr post 1 MED UVR exposure (visit #3). Monitoring will include visual inspection and discussion with the participant.

The Principal Investigator will be responsible for determining whether an AE is expected or unexpected. An AE will be considered unexpected if the nature, severity, or frequency of the event is not consistent with the risk information previously described for the study intervention.

Adverse events will be attributed to the study procedures / design by the principal investigator Dr. Michael Girardi as definitely related, probably related, potentially related, unlikely to be related, or not related.

Adverse events will be graded in severity according to the following scale using conditions noted during the study:

- **Mild:** Awareness of sign or symptom, but easily tolerated.
- **Moderate:** Discomfort enough to cause interference with usual activity.
- **Severe:** Incapacitating with inability to work or do usual activity.
- **Not applicable:** In some cases, an adverse event may be an “all or nothing” finding, which cannot be graded.

Reporting:

The principal investigator will report the following types of events to the IRB and all co-investigators listed on the protocol:

Any incident, experience or outcome that meets ALL 3 of the following criteria:

1. Is unexpected (in terms of nature, specificity, severity, or frequency) given (a) the research procedures described in the protocol-related documents, such as the IRB-approved protocol and informed consent document and (b) the characteristics of the subject population being studied; AND

2. Is related or probably related to participation in the research (*probably related* means There is evidence to suggest a causal relationship, and the influence of other factors is unlikely);

AND

3. Suggests that the research places subjects or others at greater risk of harm (including physical, psychological, economic, legal, or social harm) than was previously known or recognized.

Unanticipated Problems Involving Risks to Subjects or Others (UPIRSOs) may be medical or non-medical in nature, and include – but are not limited to – *serious, unexpected, and related adverse events* and *unanticipated adverse device effects*. ***Please note*** that adverse events are reportable to the IRB as UPIRSOs **only** if they meet all 3 criteria listed above.

These UPIRSOs/SAEs will be reported to the IRB in accordance with IRB Policy 710, using the appropriate forms found on the website. All related events involving risk but not meeting the *prompt* reporting requirements described in IRB Policy 710 should be reported to the IRB in summary form at the time of continuing review. If appropriate, such summary may be a simple brief statement that events have occurred at the expected frequency and level of severity as previously documented*.* In lieu of a summary of external events, a current DSMB report can be submitted for research studies that are subject to oversight by a DSMB (or other monitoring entity that is monitoring the study on behalf of an industry sponsor).

The principal investigator, Michael Girardi, will conduct a review of all adverse events upon completion of every study subject. The principal investigator will evaluate the frequency and severity of the adverse events and determine if modifications to the protocol or consent form are required.

6.2.4 Pharmacokinetics (if applicable)

N/A

6.2.5 Biomarkers (if applicable)

N/A

6.3 Study Procedures

All procedures are being performed exclusively for research purposes.

Visit 1: Subjects will first visit the study center (HRT4) for screening, informed consent, and a physical examination with comprehensive medical history. If eligibility requirements are met, the participant will undergo initial evaluation and a test site area equivalent to the Multiport 601 test patch will be delineated in indelible ink on the participant’s upper inner arm. This test site will be used for determination of the minimal erythema dose (MED). The minimal erythema dose (MED) is defined as the smallest UVR dose that produces perceptible redness of the skin with clearly defined borders 16-24 hours after UVR exposure. The Multiport 601 solar simulator will be used to deliver a series of six exposures (to the six subsites of the test pad) to untreated, unprotected skin to determine the subject's inherent MED. The doses selected shall be based on the Standard for the individuals Fitzpatrick Scale skin type and shall be a geometric series represented by (1.25^{n}), wherein each exposure time interval is 25 percent greater than the previous time to maintain the same relative uncertainty (expressed as a constant percentage), independent of the subject's sensitivity to UVR radiation. This inherent MED (Unprotected Skin) shall be used to

determine the dose of UVR (equal to 1 MED) to be administered in subsequent sunscreen efficacy testing. The UVR will be administered by Research Personnel. The participant will be instructed to return to the clinic 16-24 hr post UVR exposure.

Visit 2. 16-24 hr post UVR exposure, the participant will return to the clinic. The MED test area will be digitally photographed and recorded by Research Personnel. Dr. Girardi will read the MED, identifying the lowest dose of UVR that results in perceptible redness of the skin. This dose = 1 MED.

A new test site area, near the MED test area, equivalent to the Multiport 601 test patch will be delineated in indelible ink on the subject's arm. Six subsites corresponding to the 6 openings of the test patch will also be delineated in indelible ink. The two subsites in the lower left corner of the total site (two adjacent subsites are equal to 1.5 x 3 cm = 4.5 cm^2) will be treated with sunscreen: Research Personnel will apply the sunscreen in a thin film (2 mg/ cm^2, as described by the FDA) to the delineated 4.5 cm^2 area. The remainder of the area will remain untreated. Fifteen minutes after sunscreen application, the entire test site will be digitally photographed for visual record of irritation. The Multiport 601 solar simulator will be used to deliver a dose = 1 MED UVR to the two lower left (sunscreen treated) and two lower right (untreated) subsites of the test patch. The UVR will be administered by Research Personnel. The top two subsites will not receive UVR.

Immediately following UVR exposure, 3 mm punch biopsies will be obtained by Dr. Girardi from the appropriate subsites of the test area:

1. Untreated skin, No UVR
2. Untreated skin + 1 MED UVR
3. Sunscreen treated skin + 1 MED UVR

Research Personnel will immediately bisect each biopsy, snap freeze half in liquid nitrogen, and fix half in 10% neutral buffered formalin.

The participant will depart with instructions to return 4 hours after their UVR exposure.

Visit #3. 4 hr after the UVR exposure of visit #2, the participant will return to the clinic. 3 mm punch biopsies will be obtained by Dr. Girardi from the appropriate subsites of the test area:

4. Untreated skin + 1 MED UVR
5. Sunscreen treated skin + 1 MED UVR

Research Personnel will immediately bisect each biopsy, snap freeze half in liquid nitrogen, and fix half in 10% neutral buffered formalin.

The participant will depart with instructions on post-biopsy wound care and contact information for study personnel, if there are any concerns about the biopsy sites

The participant will be compensated at the conclusion of visit #3.

6.3.1 Study Schedule

Four total visits are expected.

Schedule of Events:

Visit #1:	Consent
	Medical history and physical examination
	UVR exposure for inherent MED determination
Visit #2:	Evaluate and record inherent MED
	Sunscreen application
	15 min wait
	UVR exposure = 1 MED for sunscreen efficacy testing
	3 x 3mm biopsies collected
Visit #3:	2 x 3mm biopsies collected
	Give instructions for biopsy site care
	Give study personnel contact information if any concerns about skin biopsy sites / healing
Visit #4:	Follow-up 7-10 days after biopsies for suture removal.

6.3.2 Informed Consent

The investigation will be conducted in compliance with the requirements for institutional (HIC) review and with the requirements for informed consent of the FDA regulations (21 CFR Part 50 and 21 CFR Part 56).

All subjects will be of legal age such that parental or surrogate permission is not required. Enrollment is voluntary. Potential subjects interested in enrolling will initiate the process. Research personnel will confirm the subject's consent during the eligibility screening. An informed consent form will be completed prior to the start of the before the research protocol.

We stress that they are under no obligation to sign the consent form or participate in the study if they are uncomfortable with any aspects of it. We also stress that they will not be penalized if they decide not to participate. When the subject arrives for the consent process, we review the protocol with them and indicate that they should ask any questions or express concerns before signing the consent form. In addition, we tell the subjects that they are allowed to withdraw their consent at any time without prejudice and are advised of this fact prior to participating. We also inform the subjects that they may be disqualified based on information gathered on their first visit.

Research personnel will verbally assess the subject's capacity to provide consent. During the initial interview, the potential subject will be able to ask questions and express concerns about participation in conversation. If the subject cannot speak English well enough to understand the protocol, we do not allow them to continue the consent process.

6.3.3 Screening

Eligibility will be determined by personnel involved in the testing and administration of the study: Kacie Carlson, PA-C. Eligibility will be determined by study coordinator, Kacie Carlson, PA-C. Dr. Michael Girardi, and Dr. Mark Salzman will not determine subject

eligibility or consent subject due to conflicts of interest. If there are any questions regarding eligibility, Dr. Ian Odell will be consulted.

Screening will include comprehensive medical history and a physical exam.

(a) A medical history shall be obtained from all subjects with emphasis on the effects of sunlight on their skin. Ascertain the general health of the individual, the individual's skin type (I, II, or III), whether the individual is taking medication (topical or systemic) that is known to produce abnormal sunlight responses, and whether the individual is subject to any abnormal responses to sunlight, such as a phototoxic or photoallergic response. The subject should not have a history of any conditions that make you more sensitive to sunlight, or a history of skin cancer. The subject should not have applied sunscreen to the test area in the past week.

(b) Test site inspection. The physical examination shall determine the presence of sunburn, suntan, scars, active dermal lesions, and uneven skin tones on the areas to be tested. The presence of nevi, blemishes, or moles will be acceptable if in the physician's judgment they will not interfere with the study results.

(c) Women of child-bearing potential will be given a urine pregnancy test. If it is positive, they will not be enrolled.

Subjects who fail screening due to application of sunscreen within the past week may be re-screened one week later.

6.3.4 Enrollment

Participants who have provided informed consent and who have been screened and meet eligibility criteria will be enrolled in the study by Kacie Carlson, PA-C. Eligibility will be determined by study coordinator, Kacie Carlson, PA-C. Dr. Michael Girardi, and Dr. Mark Salzman will not determine subject eligibility or consent subject due to conflicts of interest. If there are any questions regarding eligibility, Dr. Ian Odell will be consulted.

On Study Visits

- Visit #1, in order: consent, screen for eligibility, UVR exposure for MED determination
- Visit #2, in order: read MED, apply sunscreen, UVR exposure = 1 MED, 3 biopsies taken
- Visit #3: 2 additional biopsies taken
- Visit #4: suture removal from biopsy sites

Please refer to Schedule of Events (6.3.1) and procedures (6.3) above for more detail.

6.3.5 End of Study and Follow-up

At the end of the visit #3 participants will receive instructions for biopsy care, study staff contact information, and given an appointment to return in 7-10 days for suture removal.

6.3.6 Removal of subjects

Participants may withdraw voluntarily at any time for any reason.

If a participant experiences an unexpected adverse event immediately following the UVR exposure for MED determination (visit 1), e.g. a photoallergic or phototoxic reaction, assessed by visual inspection and discussion with the participant, the event will be documented and the participant excluded from further study.

If a participant experiences an unexpected reaction to the sunscreen within 15 min of application (visit #2), as assessed by visual inspection and discussion with the participant, the event will be documented, and the participant will be excluded from further study.

6.3.7 Statistical Design

We will initially test the hypotheses using paired t-tests, e.g. comparing the CPDs within each subject, assuming a normal distribution. If needed, we will employ a non-parametric test.

6.3.8 Sample Size Considerations

To test the hypothesis that our sunscreen provides 90% protection over untreated skin, a subject with ≥90% difference within e.g., their immediate CPD values, will be considered a "success" and a less <90% difference will be a "failure." We will summarize each patient's outcome as binary-valued and analyze the data by binomial reference distribution. Analysis of the data as binomial facilitates a study of the power and sample size. The null hypothesis is an equal frequency of successes and failures, corresponding to a binomial parameter of p=0.5. We will assume two-tailed significance tests with (alpha) level 0.1, typical in such small clinical trials. The population p parameters that will exhibit 80% power for different sample sizes is $p < 0.26$ or $p > 0.74$ (25 subjects), $p < 0.28$ or $p > 0.72$ (30 subjects), and $p < 0.29$ or $p > 0.71$ (35 subjects). Values were computed in R using the exact binomial distribution. We anticipate enrolling 30 subjects in the study. These calculations demonstrate only small changes in power for all sample sizes in the range illustrated here.

6.4 Planned Analyses

6.4.1 Primary Objective Analysis

1. Direct DNA damage assessed by the CPD level in biopsies obtained immediately following 1 MED UVR exposure. CPDs will be quantified by ELISA and we will compare the level of CPDs in untreated skin exposed to 1 MED UVR to the level of CPDs in sunscreen treated skin exposed to 1 MED UVR. We hypothesize that sunscreen will reduce UVR-induced CPDs by ≥90% within each participant. The level of CPDs in skin that was not exposed to UVR (negative control in ELISA) may be used to normalize the data between patients. Data will be analyzed by t-test, assuming a normal distribution. A non-parametric test will be used if needed.

2. Indirect DNA damage will be assessed in two ways:

a) by assessing the CPD level in untreated, UVR exposed skin immediately following UVR exposure (T0) vs 4 hr post UVR exposure. The CPD level at T=4hr minus the CPD level at T=0 defines "dark" CPDs that result from indirect DNA damage. This "dark" CPD level will be compared between untreated UVR exposed skin and sunscreen treated UVR exposed skin. We hypothesize that sunscreen will reduce "dark" CPDs by ≥90% within each participant. Data will be analyzed by t-test, assuming a normal distribution. A non-parametric test will be used if needed.

b) by comparing the gH2AX level in untreated UVR exposed skin vs sunscreen treated UVR exposed skin using T0 biopsies and using T=4hr biopsies. gH2AX is detectable at sites of DNA strand breaks, which are associated with indirect DNA damage. gH2AX will

be measured by quantitative immunofluorescent staining with image analysis to determine the integrated density of gH2AX signal within the nuclear area (defined by DAPI stain). We hypothesize that sunscreen will reduce gH2AX signal by ≥90% within each participant. Data will be analyzed by t-test, assuming a normal distribution. A non-parametric test will be used if needed.

3. Indirect cellular damage will be assessed by comparing the 3-nitrotyrosine level in untreated UVR exposed skin vs sunscreen treated UVR exposed skin using T0 biopsies and using T=4hr biopsies. 3-nitrotyrosine is detectable in proteins suffering indirect UVR damage, via ROS. 3-nitrotyrosine will be measured by quantitative immunofluorescent staining with image analysis to determine the integrated density of 3-nitrotyrosine signal. We hypothesize that sunscreen will reduce 3-nitrotyrosine signal by ≥90% within each participant. Data will be analyzed by t-test, assuming a normal distribution. A non-parametric test will be used if needed.

6.4.2 Secondary Objectives Analyses

N/A

6.4.3 Exploratory Objectives Analyses (if applicable)

N/A

6.4.4 Safety

Dermal irritation, allergic hypersensitivity, and follicular occlusion effects will be evaluated by Dr. Girardi. Specifically, clinical evaluation will assess evidence of erythema, edema, xerosis, desquamation, dermatitis, follicular occlusion on a 4-point scale as follows: 0=none; 1=mild; 2=moderate; 3=severe. Subjective assessment of symptoms will be provided by each subject by verbal response to Dr. Girardi's questioning regarding the following: burning, stinging, pruritus, tingling. Assessment will take place 15 min and 4 hr following application of sunscreen.

6.4.5 Analysis of Subject Characteristics

Healthy adult individuals with fair skin are more susceptible to the UVR-induced DNA damage that we seek to prevent by development of a safer, more effective sunscreen, and thus will comprise the study population.

6.4.6 Interim Analysis (if applicable)

N/A

6.4.7 Health economic evaluation

N/A

6.4.8 Other

No other analysis will be done.

6.4.9 Subsets and Covariates

N/A

6.4.10 Handling of Missing Data

If either T=4hr biopsy is missing, only direct DNA damage will be assessed for that participant using the T0 biopsies and CPD quantification by ELISA.

If a T0 biopsy is missing, but both T=4hr biopsies are available, only indirect DNA damage (by gH2AX) and indirect cellular damage (by 3-nitrotyrosine) will be assessed for that participant.

7 Trial Administration

7.1 Ethical Considerations: Informed Consent/Assent and HIPAA Authorization

This study will be conducted in accordance with the ethical principles that have their origin in the Declaration of Helsinki.

There will be no deception; participants are informed of all drugs/procedures.

Because the study does require considerable time and effort (3 timed visits and collection of 5 biopsies), subjects who adhere strictly to the aforementioned research protocols will receive a one-time payment of $200 upon completion of the study.

The subjects will provide private information on a medical history form** to indicate any reason to exclude them from the study. Only Research Personnel Michael Girardi, MD and Kacie Carlson, PA-C will have access to this information. Each subject will have a file that includes their medical history and consent form. These are kept in a locked file cabinet in the study coordinators office (HRT 4096). Only Michael Girardi, MD and Kacie Carlson, PA-C have access to the file cabinet. Each subject will be assigned a code number and all samples collected will be identified by this number. Laboratory staff performing sample testing will only be provided with the code numbers. Data resulting from testing will be stored on a computer and/or a laboratory notebook and will only be identified by sample code number.

**Medical history to be collected from all subjects will focus on the

- effects of sunlight on their skin to ascertain the individual's skin type (I, II, or III)
- whether the individual is taking medication (topical or systemic) that is known to produce abnormal sunlight responses
- whether the individual is subject to any abnormal responses to sunlight, such as a phototoxic or photoallergic response.
- history of skin cancer to include squamous cell carcinoma, basal cell carcinoma or melanoma
- history of skin disease (such as psoriasis, eczema, dermatomyositis, lupus or vitiligo)
- family history of melanoma

7.2 Institutional Review Board (IRB) Review

The protocol will be submitted to the IRB for review and approval. Approval of the protocol must be obtained before initiating any research activity. Any change to the protocol or study team will require an approved IRB amendment before implementation. The IRB will determine whether informed consent and HIPAA authorization are required.

The IRB will conduct continuing review at intervals appropriate to the degree of risk, but not less than once per year.

A study closure report will be submitted to the IRB after all research activities have been completed.

Other study events (e.g. data breaches, protocol deviations) will be submitted per Yale's IRB policies.

7.3 Subject Confidentiality

Subject confidentiality is held in strict trust by the research team. Confidentiality of all information in the study will be maintained by identifying subjects by unique code numbers. No subjects are identified by name in any of the published literature and only by code in data storage areas, to which access is limited to Research Personnel. The individual subject files are the only place where names are noted and linked to their unique code number, and these files are kept in a locked file cabinet in the office of the PI. Access to this file cabinet is limited to Michael Girardi and Kacie Carlson. To date, we have never had a violation of confidentiality. The Yale Human Investigation Committee may inspect all study records.

7.4 Deviations/Unanticipated Problems

If the study team becomes aware of an anticipated problem (e.g. data breach, protocol deviation), the event will be reported to the IRB by PI Michael Girardi.

7.5 Data Collection

At visit #1 the subjects will provide private information on a medical history form to indicate any reason to exclude them from the study. Only Research Personnel Michael Girardi, MD and Kacie Carlson, PA-C will have access to this information. Each subject will have a file that includes their medical history and consent form. These are kept in a locked file cabinet in the study coordinators office (HRT4096) office. Only Michael Girardi, MD and Kacie Carlson, PA-C will have access to the file cabinet. Each subject will be assigned a code number and all samples collected will be identified only by this number. Laboratory staff performing sample testing will only be provided with the deidentified code numbers. Data resulting from sample testing will be stored on a computer and/or in a laboratory notebook and will only be identified by sample code number.

7.6 Data Quality Assurance

Dr. Girardi will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified frequency, which must be conducted at a minimum of every 6 months (including when reapproval of the protocol is sought). During the review process, the principal investigator (monitor) will evaluate whether the study should continue unchanged, require modification/amendment, or close to enrollment.

Due to Conflict of Interests declared by Dr. Michael Girardi, Dr. Mark Salzman and Julia Lewis, PhD, Dr. Jeffrey Gehlhausen, Assistant Professor of Dermatology – YUSM, will provide and independent review of the raw data and data analysis.

A single medical history form will be used throughout the study.

All sample testing will be performed in Dr. Girardi's laboratory by staff who have been trained in all test procedures and who will follow a single standardized testing protocol.

7.7 Study Records

The study protocol, consent forms, subject medical records and code numbers, and data generated from subject samples will be considered study records.

7.8 Access to Source Documents

The subject's medical history form will be used to determine study eligibility. Only Research Personnel Michael Girardi and Kacie Carlson will have access to these forms / documents.

7.9 Data or Specimen Storage/Security

De-identified specimens will be stored in Dr. Girardi's locked research laboratory (HRT 618).

Data collected from specimen testing will be stored on a computer and/or a laboratory notebook and will be identified only by the specimen code number.

7.10 Retention of Records

Study documents should be retained for a minimum of 2 years after the last approval of a marketing application in an International Conference on Harmonisation (ICH) region and until there are no pending or contemplated marketing applications in an ICH region or until at least 2 years have elapsed since the formal discontinuation of clinical development of the study intervention. These documents should be retained for a longer period, however, if required by local regulations. No records will be destroyed without the written consent of the sponsor, if applicable. It is the responsibility of the sponsor to inform the investigator when these documents no longer need to be retained.

Study documents will be maintained for a minimum of 3 years, as required by the funding organization (NIH).

7.11 Study Monitoring

Dr. Girardi and Kacie Carlson, PA-C will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews. Monitoring will occur throughout the entire study, after each patient completes the study visits. Modifications will be made if needed based on any issues that may arise. The IRB will be notified of any modifications.

7.12 Data Safety Monitoring Plan

Dr. Girardi will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified frequency, which must be conducted at a minimum of every 6 months (including when reapproval of the protocol is sought). During the review process, the principal investigator (monitor) will evaluate whether the study should continue unchanged, require modification/amendment, or close to enrollment.

Due to Conflict of Interests declared by Dr. Michael Girardi, Dr. Mark Salzman and Julia Lewis, PhD, Dr. Jeffrey Gehlhausen, Assistant Professor of Dermatology – YUSM, will provide and independent review of the raw data and data analysis.

Either the principal investigator, or the Yale HIC shall have the authority to stop or suspend the study or require modifications.

The risks associated with the current study are deemed greater than minimal for the following reasons:

(1) pre-clinical testing showed no evidence of skin irritancy or hypersensitivity for the natural products or BNP-sunscreen, with or without UV exposure; (2) our BNP technology does not

require covalent modifications to encapsulate the UV filter, and instead physically entraps these agents with excipients, such as PLA, which are generally recognized as safe (GRAS); (3) per the FDA OTC Monograph that details the active ingredients and concentrations permissible without need for additional approval (Code for Federal Regulations-Part 352), we will adhere to these guidelines and formulate the active ingredients accordingly, for which avobenzone and octocrylene have been approved for use at up to 3% and 10%, respectively. 4) Since we do not know the teratogenicity of the proposed natural products, women of child-bearing potential will undergo urine pregnancy testing prior to application of topical study medications. If the test is negative, we will proceed with topical application study agents and UV exposure.

This protocol presents greater than minimal risks to the subjects and Unanticipated Problems Involving Risks to Subjects or Others (UPIRSOs), including adverse events, are not anticipated. In the unlikely event that such events occur, Reportable Events (which are events that are serious or life-threatening and unanticipated (or anticipated but occurring with a greater frequency than expected) and possibly, probably, or definitely related) or Unanticipated Problems Involving Risks to Subjects or Others that may require a temporary or permanent interruption of study activities will be reported immediately (if possible), followed by a written report within 5 calendar days of the Principal Investigator becoming aware of the event to the IRB (using the appropriate forms from the website) and any appropriate funding and regulatory agencies. The investigator will apprise fellow investigators and study personnel of all UPIRSOs and adverse events that occur during the conduct of this research project through regular study meetings, and via email as they are reviewed by the principal investigator.

The protocol's research monitor(s), e.g., Data and Safety Monitoring Boards, study sponsors, funding and regulatory agencies, and regulatory and decision-making bodies will be informed of adverse events within 5 days of the event becoming known to the principal investigator.

Although we have assessed the proposed study as one of greater than minimal risk, the potential exists for anticipated and/or unanticipated adverse events, serious or otherwise, to occur since it is not possible to predict with certainty the absolute risk in any given individual or in advance of first-hand experience with the proposed study methods. Therefore, we provide a plan for monitoring the data and safety of the proposed study as follows:

Attribution of Adverse Events:

Adverse events will be monitored for each subject participating in the study and attributed to the study procedures / design by the principal investigator (*Insert Investigator Name*) according to the following categories:

a.) Definite: Adverse event is clearly related to investigational procedures(s)/agent(s).

b.) Probable: Adverse event is likely related to investigational procedures(s)/agent(s).

c.) Possible: Adverse event may be related to investigational procedures(s)/agent(s).

d.) Unlikely: Adverse event is likely not to be related to the investigational procedures(s)/agent(s).

e.) Unrelated: Adverse event is clearly not related to investigational procedures(s)/agent(s).

Plan for Grading Adverse Events:

The following scale will be used in grading the severity of adverse events noted during the study:

1. Mild adverse event
2. Moderate adverse event
3. Severe

Plan for Determining Seriousness of Adverse Events:

Serious Adverse Events:

In addition to grading the adverse event, the PI will determine whether the adverse event meets the criteria for a Serious Adverse Event (SAE). An adverse event is considered serious if it results in any of the following outcomes:

1. Death;
2. A life-threatening experience in-patient hospitalization or prolongation of existing hospitalization;
3. A persistent or significant disability or incapacity;
4. A congenital anomaly or birth defect; OR
5. Any other adverse event that, based upon appropriate medical judgment, may jeopardize the subject's health and may require medical or surgical intervention to prevent one of the other outcomes listed in this definition.

An adverse event may be graded as severe but still not meet the criteria for a Serious Adverse Event. Similarly, an adverse event may be graded as moderate but still meet the criteria for an SAE. It is important for the PI to consider the grade of the event as well as its "seriousness" when determining whether reporting to the IRB is necessary.

Plan for reporting UPIRSOs (including Adverse Events) to the IRB

The principal investigator will report the following types of events to the IRB:

Any incident, experience or outcome that meets ALL 3 of the following criteria:

1. Is unexpected (in terms of nature, specificity, severity, or frequency) given (a) the research procedures described in the protocol-related documents, such as the IRB-approved protocol and informed consent document and (b) the characteristics of the subject population being studied; AND
2. Is related or possibly related to participation in the research (*possibly related* means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); AND
3. Suggests that the research places subjects or others at greater risk of harm (including physical, psychological, economic, legal, or social harm) than was previously known or recognized.

Unanticipated Problems Involving Risks to Subjects or Others (UPIRSOs) may be medical or non-medical in nature, and include – but are not limited to – *serious, unexpected, and related adverse events* and *unanticipated adverse device effects*. ***Please note*** that adverse events are reportable to the IRB as UPIRSOs **only** if they meet all 3 criteria listed above.

These UPIRSOs/SAEs will be reported to the IRB in accordance with IRB Policy 710, using the appropriate forms found on the website. All related events involving risk but not meeting the *prompt* reporting requirements described in IRB Policy 710 should be reported to the IRB in summary form at the time of continuing review. If appropriate, such summary may be a simple brief statement that events have occurred at the expected frequency and level of severity as previously documented*.* In lieu of a summary of external events, a current DSMB report can be submitted for research studies that are subject to oversight by a DSMB (or other monitoring entity that is monitoring the study on behalf of an industry sponsor).

Plan for reporting adverse events to co-investigators on the study, as appropriate the protocol's research monitor(s), e.g., industrial sponsor, Yale Cancer Center Data and Safety Monitoring Committee (DSMC), Protocol Review Committee (PRC), DSMBs, study sponsors, funding and regulatory agencies, and regulatory and decision-making bodies.

For the current study, the following individuals, funding, and/or regulatory agencies will be notified (choose those that apply):

X All Co-Investigators listed on the protocol.

X Yale IRB

The principal investigator Michael Girardi, MD will conduct a review of all adverse events upon completion of every study subject. The principal investigator will evaluate the frequency and severity of the adverse events and determine if modifications to the protocol or consent form are required.

7.13 Study Modification

Dr. Girardi will be responsible for monitoring the data, assuring protocol compliance, and conducting the safety reviews at the specified frequency, which must be conducted at a minimum of every 6 months (including when reapproval of the protocol is sought). During the review process, the principal investigator will evaluate whether the study should continue unchanged, require modification/amendment, or close to enrollment.

All proposed modifications will be submitted to the IRB as protocol modifications and changes will be implemented upon IRB approval.

7.14 Study Discontinuation

If the study is prematurely terminated or suspended, the Principal Investigator (PI) will promptly inform study participants and the Institutional Review Board (IRB). Circumstances regarding the termination or suspension will be provided. Study participants will be contacted, as applicable, and be informed of changes to study visit schedule.

Circumstances that may warrant termination or suspension include, but are not limited to:

- Determination of unexpected, significant, or unacceptable risk to participants
- Demonstration of efficacy that would warrant stopping
- Insufficient compliance to protocol requirements
- Data that are not sufficiently complete and/or evaluable
- Determination that the primary endpoint has been met
- Determination of futility

7.15 Study Completion

The study will be complete when subjects have been enrolled and data analyzed. The IRB will be notified when subjects are enrolled, and data analyzed. At this time, the study will be closed.

7.16 Conflict of Interest Policy

The independence of this study from any actual or perceived influence, such as by the pharmaceutical industry, is critical. Therefore, any actual conflict of interest of persons who have a role in the design, conduct, analysis, publication, or any aspect of this trial will be disclosed and managed. Furthermore, persons who have a perceived conflict of interest will be required to have such conflicts managed in a way that is appropriate to their participation in the trial. The study leadership in conjunction with the appropriate conflict of interest review committee has established policies and procedures for all study group members to disclose all conflicts of interest and will establish a mechanism for the management of all reported dualities of interest.

All investigators will follow the applicable conflict of interest policies. And, as stated above, due to Conflict of Interests declared by Dr. Michael Girardi, Dr. Mark Salzman and Julia Lewis, PhD, Dr. Jeffrey Gehlhausen, Assistant Professor of Dermatology – YUSM, will provide and independent review of the raw data and data analysis.

7.17 Funding Source

This study is funded by the Yale SPORE in Skin Cancer, NCI P50CA121974.

7.18 Publication Plan

Published manuscripts generated from this work will be made available to the public free of charge, per NIHPA guidelines and we will follow the Final NIH Statement on Sharing Research Data (Notice NOT-OD-03-032) issued February 26, 2003. Dr. Girardi holds primary responsibility for publishing the study results.

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