Research Article

Cancer Prevention Research

A Phase II Randomized Placebo-Controlled Trial of Oral *N*-acetylcysteine for Protection of Melanocytic Nevi against UV-Induced Oxidative Stress *In Vivo*

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Abstract

Oxidative stress plays a role in UV-induced melanoma, which may arise from melanocytic nevi. We investigated whether oral administration of the antioxidant *N*-acetylcysteine (NAC) could protect nevi from oxidative stress *in vivo* in the setting of acute UV exposure. The minimal erythemal dose (MED) was determined for 100 patients at increased risk for melanoma. Patients were randomized to receive a single dose (1,200 mg) of NAC or placebo, in double-blind fashion, and then one nevus was irradiated (1–2 MED) using a solar simulator. One day later, the MED was redetermined and the irradiated nevus and a control unirradiated nevus were removed for histologic analysis and examination of biomarkers of NAC metabolism and UV-induced oxidative stress. Increased expression of 8-oxoguanine, thioredoxin reductase-1, and γ-glutamylcysteine synthase modifier subunit were consistently seen in UV-treated compared with unirradiated nevi. However, no significant differences were observed in these UV-induced changes or in the pre- and postintervention MED between those patients receiving NAC versus placebo. Similarly, no significant differences were observed in UV-induced changes between subjects with germline wild-type versus loss-of-function mutations in the melanocortin-1 receptor. Nevi showed similar changes of UV-induced oxidative stress in an open-label post-trial study in 10 patients who received NAC 3 hours before nevus irradiation. Thus, a single oral dose of NAC did not effectively protect nevi from UV-induced oxidative stress under the conditions examined. *Cancer Prev Res; 10(1); 36–44. ©2016 AACR.*

Introduction

Melanoma incidence continues to increase rapidly in the United States (1), and despite recent therapeutic advances (2), most patients with metastatic disease will ultimately succumb to it (3). Melanoma may arise from melanocytic nevi (i.e., moles; ref. 4), which frequently are found on sun-exposed areas (5). UV radiation in sunlight (6) or from tanning beds (7) is the major environmental risk factor for melanoma. There is currently no proven effective primary prevention strategy, although education and regular skin screening may facilitate early detection (8), and use of sunscreen may reduce risk (9).

Reactive oxygen species (ROS) are generated in the skin upon UV exposure (10). Oxidative stress caused by elevated ROS can damage intracellular proteins and lipids (11) and lead to the formation of oxidative DNA lesions, such as 8-oxoguanine (8-OG), that can result in oncogenic mutations if not repaired prior to DNA replication (12). Glutathione (GSH) is the major thiol reductant in cells that, along with thioredoxin reductases (TR) and other endogenous antioxidants, can quench ROS and mitigate cellular damage (13). There is a good deal of correlative evidence suggesting that one link between UV radiation and melanoma may lie in the generation of oxidative damage (14), and melanoma has been referred to as a "reactive oxygen–driven tumor" (15). Germline variants in the melanocortin-1 receptor (MC1R), which regulates UV-induced ROS production and metabolism in melanocytes (16), are relatively common, and several are associated with melanoma predisposition independent of UV exposure (17). Finally, we reported that the antioxidant *N*-acetylcysteine (NAC) can delay tumor development in an animal model of UV-induced melanoma (18).

NAC is FDA-approved for acetaminophen toxicity (19) and has subsequently been used to preserve lung function in patients with idiopathic pulmonary fibrosis (20) and to prevent contrast medium–induced nephropathy in patients undergoing angioplasty (21). There are more than 100 studies in progress investigating NAC for other applications, including schizophrenia, heart failure, Parkinson disease, and bipolar disorder (22). NAC is an orally bioavailable antioxidant that is a prodrug form

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of L-cysteine (Cys). Following metabolism of NAC, the enzyme γ-glutamylcysteine synthase (γ-GCS) catalyzes the coupling of Cys via peptide bond to the γ-carboxylate of glutamate, which is the rate-limiting step in GSH synthesis (23). Oxidative stress upregulates two subunits of γ-GCS in cells of the skin: the modifier subunit (Gclm) in melanocytes and the catalytic subunit (Gclc) in keratinocytes (24). Although GSH is found in the plasma and extracellular space, it cannot cross cell membranes and thus, must first be hydrolyzed by γ-glutamyl transferase to release Cys (25), which is transported into cells in its reduced or oxidized forms via the ASCT and X_c- transporters, respectively (Fig. 1). The light subunit of X_c- is encoded by *SLC7A11* and ASCT is encoded by *SLC1A4*.

Topically applied NAC has been used to block UV-induced ROS signaling in human skin (26). We have been investigating its systemic use for the purpose of reducing UV-induced oxidative stress in nevi. Previously, we completed a phase I trial with NAC in 72 patients at increased risk for melanoma (27). We found that an oral dose of 1,200 mg was safe and well tolerated and that nevi could be UV-treated *ex vivo* to evaluate NAC-mediated changes in biomarkers of UV-induced oxidative stress. Here, we present the results of a phase II randomized placebo-controlled trial in 100 patients, designed to test whether oral NAC could protect nevi against UV-induced oxidative stress *in vivo*.

Materials and Methods

Patients

This study was approved by the Institutional Review Board (IRB #50308) of the University of Utah. Patients were recruited from the pigmented lesion clinic at the Huntsman Cancer Institute (Salt Lake City, UT), in which patients with a history of numerous or atypical nevi and/or personal or family history of melanoma are regularly monitored. Patients that were under age 18, critically ill or mentally handicapped, prisoners, pregnant or breastfeeding, non-English speaking, or having history of asthma or allergic reaction to NAC, were excluded. All patients signed a consent form to participate. Patients were not charged for nevus removal or histologic examination, and each was compensated $150 following their participation in the pilot study and $200 following participation in the clinical trial or post-trial study.

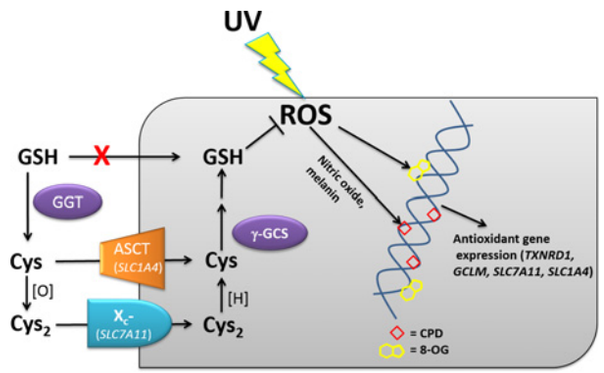

Figure 1.

NAC metabolism and biomarkers of oxidative stress. NAC is metabolized by deacetylation to Cys, which is a GSH precursor. GSH cannot cross cell membranes, but is transformed into Cys and cystine (Cys_2) by γ-glutamyl transpeptidases (GGT). Cys and Cys_2 enter cells via the amino acid transporters ASCT (*SLC1A4*) and X_c- (*SLC7A11*), respectively. Intracellularly, Cys can be reduced by reductases, such as TR-1 (*TXNRD1*), and converted to GSH by γ-glutamylcysteine synthase, the modifier subunit of which is encoded by *Gclm* and upregulated in UV-irradiated melanocytes and nevi. ROS induced by UV radiation can lead to the formation of 8-OG and cyclobutane dimers (CPD, see Discussion) in DNA. ROS also modifies the expression of genes such as *TXNRD1*, *SLC1A4*, *SLC7A11*, and *GCLM* (which encode the proteins TR-1, ASCT, X_c-, and Gclm).

MC1R testing

Determination of *MC1R* germline status was performed under a separate IRB protocol (#73694). Saliva samples were collected using an Oragene Discover Kit (DNA Genotek), and genomic DNA was isolated by the addition of prepIT-L2P solution (DNA Genotek), followed by ethanol precipitation according to the manufacturer's instructions. The coding region of the single exon at the *MC1R* locus was amplified by PCR using primers 5′-GCACCATGAACTAAGCAGGA-3′ and 5′-GGACCAGGGAGG-TAAGGAACT-3′, and the presence of mutations was determined by Sanger DNA sequencing. Subjects were categorized as "low-risk" if no mutations or the polymorphisms V60L or V92M were detected, and "high-risk" if heterozygous or homozygous for the following mutations (associated with MC1R loss of function or increased melanoma risk): D84E, R142H, R151C, I155T, R160W, R163Q, D294H, as described elsewhere (28, 29).

Nevus tissues

Up to two nevi (≥5 mm in diameter to provide sufficient tissue for analysis) on each subject that were not clinically suspicious for melanoma were selected. Following injection of sodium bicarbonate–buffered 1% lidocaine containing 1:100,000 epinephrine, nevi were removed by shave/saucerization technique using a DermaBlade (Medline Industries), and then a representative 1–mm slice was placed in 10% formalin for paraffin embedding and sectioning. The remaining nevus fragments were grossly macrodissected from normal surrounding skin and immediately placed on ice for 8-OG analysis or frozen at −80°C in 700 μL Qiazol reagent (Qiagen) for RNA analysis. A hematoxylin/eosin-stained section was later reviewed by a dermatopathologist (S.R. Florell) to confirm the lesion was a nevus and not melanoma. Additional unstained sections were prepared for IHC.

Solar-simulated irradiation of nevi

A 16S-300-003 SolarLight device was used as described previously (30). Additional information on the device and minimal erythemal dose (MED) testing is provided in Supplementary Information. Nevi were irradiated at doses of 0.5 to 12 MED in the pilot study, or 2 (first 50 patients in the trial) or 1 MED (last 50 patients in the trial and 10 patients in the post-trial study).

Measurement of GSH

Quantification of GSH in nevus fragments was determined by reverse-phase chromatography as described previously (27).

Determination of 8-OG in nevus melanocytes

Nevus fragments were diced with a razor blade, transferred to a 15-mL conical tube containing 1 mL of 0.25% trypsin/EDTA (Thermo Fisher Scientific), and after vortexing were incubated for 45 minutes in a 37°C water bath. Next, 2 mL of Medium 254 (Thermo Fisher Scientific) containing 10% FBS and 1% penicillin–streptomycin–glutamine was added. After vortexing, cells were centrifuged at 4,000 rpm for 5 minutes at room temperature and then resuspended in 1 mL PBS. Cells were separated from

debris, transferred to a new 1.5-mL tube, and recentrifuged. The cell pellet was resuspended in 1 mL PBS containing 4% paraformaldehyde at room temperature. After 15 minutes, cells were centrifuged at 1,000 rpm for 5 minutes and washed once with PBS. Cells were incubated in 1 mL PBS containing 0.2% Triton X-100 (Roche Life Science) for 5 minutes, washed in PBS, and then resuspended in 0.1 mL PBS containing 1:50 dilution of Melan A (mouse IgG, MA1-26245, Thermo Fisher Scientific) and incubated overnight at 4°C. Cells were centrifuged, washed in PBS, then stained for 8-OG using the OxyDNA Fluorometric Assay Kit (Calbiochem) according to the manufacturer's instructions. Briefly, cells were incubated in 0.2 mL of the provided washing solution containing a 1:10 dilution of 8-OG–FITC conjugate and a 1:200 dilution of secondary antibody for Melan-A (goat anti-mouse IgG, A11032 Alexa Fluor 594, Molecular Probes) in the dark for 1 hour at 37°C. Cells were pelleted, washed in PBS, then resuspended in 30 μL PBS and added to a glass slide with one drop of ProLong Gold Antifade Reagent (P36935, Molecular Probes) containing 4,6-diamidino-2-phenylindole (DAPI) and then coverslipped. After drying, slides were stored under foil at 4°C until examined by fluorescence microscopy.

TR-1 expression in nevi

Nevus sections were deparaffinized in CitriSolv Clearing Agent (Thermo Fisher Scientific), then successively washed in 100%, 95%, 80%, and 70% ethanol. After washing in water, slides were placed in antigen retrieval buffer (10 mmol/L sodium citrate, pH 6.0, containing 0.05% Tween 20) and heated for 20 minutes in a microwave. After cooling, slides were washed repeatedly in water and then PBS. Blocking buffer (PBS containing 1% BSA and 0.05% Tween 20) was added to prevent nonspecific binding and drained off after 30 minutes. Primary antibodies against Melan-A (rabbit IgG NBP1-30151, Novus Biologicals) and TR-1 (mouse IgG sc-28321, Santa Cruz Biotechnology) were diluted 1:200 and 1:500, respectively, in blocking buffer and added to sections that were incubated at 4°C in a humidified chamber overnight. After washing with PBS containing 0.05% Tween 20, secondary antibodies for Melan-A (goat anti-rabbit IgG, A11012 Alexa Fluor 594, Molecular Probes) and TR-1 (goat anti-mouse IgG A11001 Alexa Fluor 488, Molecular Probes) were diluted 1:200 in PBS containing 1% normal goat serum, 1% BSA, and 0.05% Tween 20 and added to sections at room temperature for 1 hour. After washing in PBS containing 0.05% Tween 20, one drop of ProLong Gold (Molecular Probes) containing DAPI was added before coverslipping. Slides were stored under foil at −20°C until examination by fluorescent microscopy. For quantification of TR-1 expression, stained slides were viewed on a Nikon Eclipse Ti microscope with a 20× objective, and images were captured using an Andor Clara camera with Nikon NIS Elements software (version 4.30) and subsequently analyzed using ImageJ software (NIH, Bethesda, MD).

Gene expression studies

Nevus samples were disrupted in 0.2 mL Qiazol at 4°C in an Eppendorf tube using a plastic pestle, then stored at −80°C until further processing. RNA was purified using an RNeasy Kit (Qiagen) following the manufacturer's instructions. Eluted RNA was quantified on a Synergy H1 microplate reader (BioTek) using the accompanying Gen5 2.05 software. Further details, including the primer sequences used, are provided in Supplementary Information. A Rotor-Gene Q thermocycler (Thermo Fisher Scientific) was used, and the data were collected using the accompanying software, version 2.0.2. The fold change calculation used the $\Delta\Delta C_t$ method, with the RPLP0 reaction serving as the endogenous normalization control for each sample as described previously (31).

NAC administration

To mask its salty taste, *N*-acetylcysteine solution (NAC, 200 mg/mL solution, American Regent) was diluted approximately 1:4 in Campbell's tomato juice immediately prior to use. Placebo was similarly prepared, substituting normal saline (0.9% sodium chloride) for NAC. The dilutions were prepared behind a curtain by a staff member from our clinical trials office, with a vial of NAC left open on the counter (so the odor of the drug was present in the room), to insure that both the subjects and the investigators were blinded as to which treatment was administered.

Statistical analysis

A biostatistician (K.M. Boucher) constructed the patient randomization scheme and performed all statistical analyses using "R" software, version 2.8.0. P values ≤ 0.05 were considered statistically significant. Patients were randomized to receive NAC or placebo and stratified to insure equal proportions of *MC1R* low- and high-risk genotypes in each arm. Robust analysis of covariance methods (R function "lmrob") was used to analyze relationships between individual categorical variables and change in log MED, with categorical variable and log pretreatment MED as predictors and log posttreatment MED as response. A Wilcoxon test was used to assess significance of differences in the median percent nevus melanocytes with 8-OG expression in the UV-irradiated nevus compared with that in the control (unirradiated) nevus. Secondary analyses were performed comparing the change in UV-induced 8-OG staining of nevi between treatment groups and the modification of treatment effect on the change in 8-OG by high-risk MC1R mutations. The R function lmrob was used to analyze effect ratios (log TR-1 expression in nevus melanocytes in the UV-irradiated nevus compared with that in the control unirradiated nevus), with categorical variable and pretreatment TR-1 staining as predictors and posttreatment TR-1 staining as response. For RNA expression studies, the R function lmrob was used to examine the relationship between individual categorical variables and the change in ΔC_t, with categorical variable and ΔC_t in the unirradiated nevus as predictors and ΔC_t in the irradiated nevus as response. The expression ratio was estimated as $2^{-\Delta C_t}$.

Results

Pilot study of UV-induced markers of oxidative stress *in vivo*

Forty-one subjects were initially recruited for a pilot study (without drug) in which several parameters for UV-induced modulation of biomarkers of oxidative stress in nevi could be evaluated. We used solar-simulated radiation (SSR) to irradiate nevi *in vivo*. Irradiated (but not unirradiated) nevi consistently exhibited erythema clinically, and dyskeratotic cells histologically, 24 to 48 hours following SSR ranging from 0.5 to 12 MED (Supplementary Fig. S1). In our prior *ex vivo* studies, nevus GSH levels was a primary endpoint as NAC would be predicted to support GSH biosynthesis. Previously, we observed GSH depletion in UV-treated nevi (compared with unirradiated nevi on the same subjects) at 24 and 48 hours following UV exposure (27).

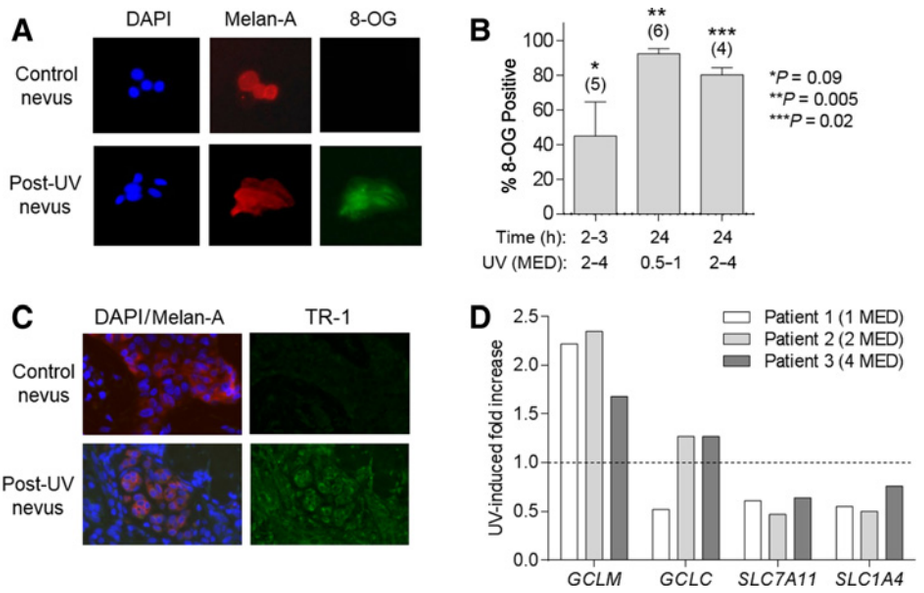

Figure 2.
UV-induced markers of oxidative stress. **A,** Representative staining of dissociated nevus cells for DAPI, Melan-A, or 8-OG. **B,** Shown are percent nevus melanocytes positive for 8-OG, at indicated time and UV dose ranges. Error bars, SEM. Numbers of patients in each group indicated in parentheses. *P* values for comparisons of values for irradiated versus unirradiated tissues at each condition determined by paired *t* tests. **C,** Representative staining of nevus sections for DAPI, Melan-A, and TR-1. **D,** Expression of *GCLM, GCLC, SLC7A11*, and *SLC1A4* genes from RNA isolated from control and UV-irradiated nevi 24 hours after SSR exposure (1–4 MED) in three subjects. Data expressed as ratio of normalized signals from UV-treated to control nevi.

For 27 subjects in this pilot study, one nevus was treated with SSR at doses ranging from 2 to 12 MED and then removed 2 to 48 hours later. A control (unirradiated) nevus was also removed from each subject at the same time, and GSH levels were quantitated in each nevus. We found that GSH levels in SSR-irradiated nevi were consistently higher or the same as in control unirradiated nevi (Supplementary Fig. S2). Thus, in contrast to our prior findings *ex vivo*, we did not observe GSH depletion as a marker of UV-induced oxidative stress in nevi *in vivo*.

In 15 subjects, we assessed the formation of 8-OG in nevi 2 to 24 hours following treatment with SSR (0.5–4 MED). Nevus cells were dissociated and subjected to multicolor immunofluorescence staining (Fig. 2A). Positive staining for 8-OG in UV-treated nevi increased from approximately 45% of the cells at 2 hours to 80% to 100% at 24 hours after irradiation (Fig. 2B). In matched unirradiated nevi from the same patients, 8-OG positivity ranged from 0% to 70% and averaged around 40% (not shown). Although this level of detection may represent background staining or actual oxidative stress associated with nevus manipulation and the staining procedure, we consistently detected a higher percentage of cells expressing 8-OG in each UV-treated nevus compared with the matched unirradiated nevus. Across a range of UV doses, there were significantly ($P = 0.005$, 0.02) more 8-OG–positive cells detected from irradiated versus unirradiated nevi at the 24-hour time point (Fig. 2B). We did not measure ROS in the nevi but have previously reported that 8-OG expression correlates with ROS in UV-irradiated human melanocytes (32). In 10 subjects, we assessed TR-1 expression in fixed sections of unirradiated nevi or nevi 6 or 24 hours following SSR treatment (0.5–4 MED) over a 15-fold concentration range of primary antibody. Staining intensity increased with antibody concentration and dose of SSR, and uniform melanocyte TR-1 staining was consistently observed in SSR-treated compared with unirradiated nevi at SSR doses of $\geq$1 MED at 24 hours (Fig. 2C). Thus, 8-OG and TR-1 proved to be reliable immunohistochemical markers of UV-induced oxidative stress in nevi *in vivo*.

In separate studies using human melanocytes that were UV treated *in vitro*, we evaluated RNA expression of antioxidant-related genes and found several that were consistently modulated by UV exposure. Genes relevant to NAC metabolism included subunits of the γ-GCS and the Cys transporters *SLC1A4* and *SLC7A11*. In 3 patients, we assessed expression levels for these four genes in SSR-treated nevi (1–4 MED) at 24 hours and in matched unirradiated nevi. As shown in Fig. 2D, *GCLM* (but not *GCLC*) was consistently upregulated by UV treatment, and *SLC1A4* and *SLC7A11* were downregulated. Thus, we used these three genes going forward as relevant biomarkers of response to SSR in nevi *in vivo*.

Phase II trial

A total of 100 subjects were recruited for a randomized placebo-controlled trial to examine whether NAC could modulate these markers in nevi in the setting of acute UV exposure. The trial design is depicted in Fig. 3, and Supplementary Table S1 details the characteristics of the subjects who were randomized to receive NAC or placebo. For all subjects, germline *MC1R* status was evaluated by DNA sequencing, and this was factored into the randomization scheme, so that subjects with low- and high-risk MC1R alleles would be equally distributed into the drug and placebo arms. After determination of the MED, subjects were given NAC or placebo. Immediately thereafter, one nevus was SSR treated (at a dose based on each subject's MED), and the next day (approximately 24 hours later), both the irradiated nevus and an unirradiated nevus were removed for analysis. Histologic examination of a representative fragment from each lesion revealed that all were benign neoplasms.

The MED results are presented in Table 1. Although the ratio of posttreatment MED to pretreatment MED was 5% lower in the NAC versus placebo group, this difference was not statistically significant ($P = 0.068$). We also did not observe a difference in the ratio of MEDs between subjects with low-risk and high-risk MC1R alleles. We tested for a potential interaction between treatment and MC1R status variables, but did not find one ($P = 0.745$). Analysis of 8-OG and TR-1 staining in UV-irradiated nevi is summarized in Table 2. All SSR-treated nevi exhibited a high percentage of melanocyte 8-OG staining (~95%) compared with unirradiated nevi (~33%), which was

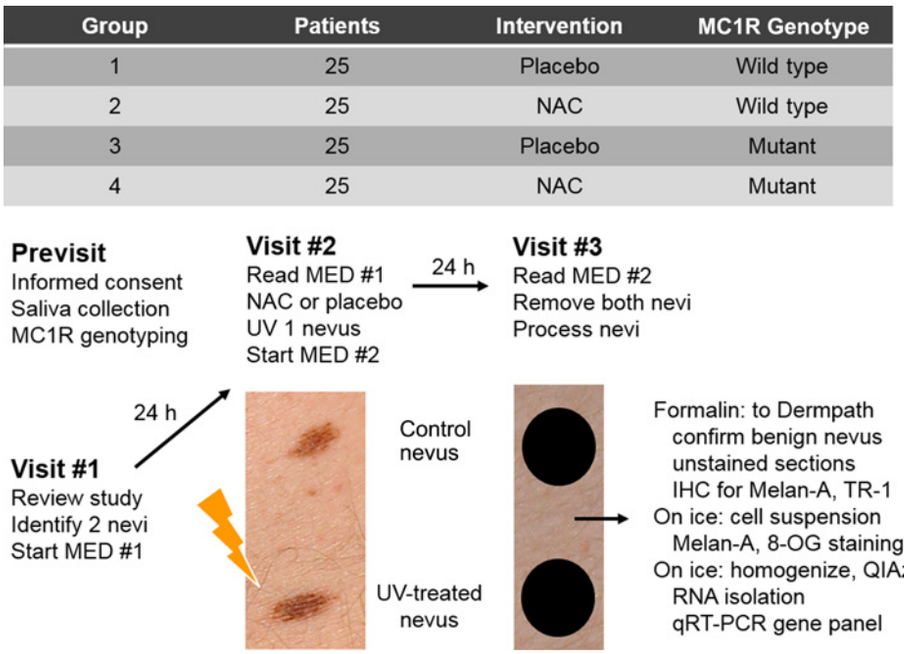

Figure 3.
Subject randomization and clinical trial design. Individuals were randomized to one of four groups such that half were scheduled to receive drug and half to receive placebo. On the basis of sequencing of the *MC1R* gene, approximately half the subjects were wild type (WT) and half had at least one germline high-risk allele (mutant). At the first visit, MED testing was initiated. After 24 hours, the MED was determined and either 1,200 mg NAC or placebo was administered. One nevus was UV irradiated (1–2 MED), and a second round of MED testing was initiated. After 24 hours, the postintervention MED was determined, and the UV-irradiated nevus and a control unirradiated nevus were removed. Each nevus was fragmented for histologic, immunohistochemical, and molecular analyses.

statistically significant ($P < 0.001$ for placebo group). However, there was not a significant difference between samples from patients given NAC or placebo ($P = 0.65$). Similarly, we did not observe a difference in 8-OG staining between subjects with low-risk and high-risk MC1R alleles ($P = 0.48$). We did not find a significant interaction between treatment and MC1R status variables (not shown). Melanocyte staining for TR-1 was increased in SSR-treated compared with unirradiated nevi ($P = 0.003$ for placebo group), but we did not observe a significant difference between samples from patients given NAC or placebo ($P = 0.43$). Similarly, we did not observe a difference in TR-1 staining between subjects with low-risk and high-risk MC1R alleles ($P = 0.54$). We did not find a significant interaction between treatment and MC1R status variables (not shown). Finally, we examined the expression levels of three genes from RNA isolated from the nevi, which are summarized in Table 3. Levels of *GCLM* were increased (ΔC_t decreased) in irradiated nevi compared with unirradiated nevi ($P < 0.001$ for placebo group), but we did not observe a significant difference between samples from patients given NAC or placebo ($P = 0.76$). Similarly, we did not observe a difference in *GCLM* expression changes between subjects with low-risk and high-risk MC1R alleles ($P = 0.52$). Levels of *SLC1A4* and *SLC7A11* were decreased in irradiated nevi compared with unirradiated nevi (not significant, $P = 0.12$, 0.62 for placebo groups, respectively), and we did not observe a significant difference between samples from patients given NAC or placebo ($P = 0.63$, 0.73, respectively). Similarly, we did not observe a difference in expression changes of *SLC1A4* or *SLC7A11* between subjects with low-risk and high-risk MC1R alleles ($P = 0.27$, 0.40, respectively). We did not find a significant interaction between treatment and MC1R status variables for any of these three genes (not shown). Thus UV-induced modulation of markers of oxidative stress in nevi was not significantly different in subjects receiving NAC compared with those receiving placebo.

Table 1. Analysis of MED

Variable	Patients	Univariate analysis Effect ratio[a] (95% CI), *P*
Treatment		
Placebo	50 (50%)	1.00
Drug	50 (50%)	0.95 (0.91–1.00), 0.068
MC1R status		
Low-risk	50 (50%)	1.00
High-risk	50 (50%)	1.00 (0.95–1.05), 0.941

NOTE: Additional analyses of age, gender, height, weight, hair color, and eye color as covariates did not reveal any significant predictors (not shown). We did not find a significant interaction between treatment and MC1R status variables ($P = 0.745$).
[a]Effect ratio is the ratio of posttreatment MED to pretreatment MED.

Post-trial study

We considered the possibility that NAC (or its metabolites) needed to be present in nevi at the time of UV exposure to have a greater effect. In our prior study, we detected metabolites of NAC in nevi 3 hours after ingestion (27). We did not attempt to detect NAC metabolites, such as Cys, in the nevus samples in this study, which were all obtained 24 hours following drug ingestion, as we had previously found that these metabolites were undetectable in nevi after 6 hours (27). We recruited 10 subjects for a post-trial open-label study in which all received NAC and nevi were SSR treated (1 MED) 3 hours after NAC ingestion. Half of the subjects (5/10) had a high-risk MC1R allele. Both unirradiated and irradiated nevi were removed approximately 24 hours after SSR exposure and then analyzed for 8-OG and TR-1 staining, and expression of the *GCLM*, *SLC1A4*, and *SLC7A11* genes. For these analyses, SSR responses for these 10 patients who received drug were compared with the patients (49–50) who received placebo in the randomized trial above. The data are presented in Supplementary Table S2. The SSR-induced responses in nevi were not significantly different between the patients who received NAC 3 hours before SSR treatment and those who received placebo.

Table 2. Immunohistologic staining for markers of UV-induced oxidative stress

Variable	Patients[a]	Percent 8-OG (mean ± SD) Control	Percent 8-OG (mean ± SD) UV irradiated	Univariate analysis P[b]
Treatment				
Placebo	49 (51%)	33.1 ± 24.2	96.2 ± 7.5[c]	
Drug	48 (49%)	33.6 ± 25.1	94.6 ± 12.3	0.65
MC1R status				
Low-risk	48 (49%)	35.9 ± 26.9	95.8 ± 11.0	
High-risk	49 (51%)	30.8 ± 21.9	95.0 ± 9.3	0.48

		Log (x100) TR-1 (mean ± SD) Control	Log (x100) TR-1 (mean ± SD) UV irradiated	Univariate analysis Effect ratio[d] (95% CI), P
Treatment				
Placebo	50 (51%)	1.05 ± 0.76	1.26 ± 0.70[e]	1.00
Drug	49 (49%)	1.16 ± 0.58	1.28 ± 0.55	0.94 (0.81–1.10), 0.43
MC1R status				
Low-risk	49[c] (49%)			1.00
High-risk	50 (51%)			0.95 (0.82–1.11), 0.54

NOTE: We tested for potential interaction between treatment and MC1R status for both analyses and did not find significance for either (not shown).
[a]One subject with low-risk MC1R randomized to drug was excluded from both analyses because the lesions removed were seborrheic keratoses and not nevi, and 2 subjects (one high-risk MC1R randomized to drug and one low-risk MC1R randomized to placebo) were excluded from analysis of 8-OG because there was insufficient tissue.
[b]Wilcoxon test was used to assess significance of differences in the median percent nevus melanocytes with 8-OG expression in the UV-irradiated nevus compared with that in the control (unirradiated) nevus. Additional analyses of 8-OG response with gender, height, weight, hair color, and eye color as covariates did not reveal any significant predictors (not shown).
[c]For placebo group, difference between control and UV-irradiated was significant ($P < 0.001$, Wilcoxon test).
[d]The effect ratio is the ratio of log TR-1 expression in nevus melanocytes in the UV-irradiated nevus compared with that in the control (unirradiated) nevus. Additional analyses of TR-1 response with age, gender, height, weight, hair color, and eye color as covariates did not reveal any significant predictors (not shown).
[e]For placebo group, difference between control and UV-irradiated was significant ($P = 0.003$, Wilcoxon test).

Table 3. Analysis of transcriptional markers of UV-induced oxidative stress

Variable	Patients[a]	ΔC_t[b] *GCLM* (mean ± SD) Control	ΔC_t[b] *GCLM* (mean ± SD) UV irradiated	Univariate analysis Analysis of covariance Expression ratio[c] (95% CI), P
Treatment				
Placebo	50 (51%)	9.74 ± 0.69	9.14 ± 0.75[d]	1.00
Drug	49 (49%)	9.42 ± 0.98	8.99 ± 0.83	0.97 (0.82–1.16), 0.76
MC1R status				
Low-risk	49 (49%)			1.00
High-risk	50 (51%)			1.07 (0.88–1.30), 0.52

		ΔC_t *SLC1A4* (mean ± SD) Control	ΔC_t *SLC1A4* (mean ± SD) UV irradiated	
Treatment				
Placebo	50 (51%)	9.21 ± 0.83	9.40 ± 1.06[e]	1.00
Drug	49 (49%)	9.28 ± 0.89	9.29 ± 0.87	1.06 (0.84–1.34), 0.63
MC1R status				
Low-risk	49 (49%)			1.00
High-risk	50 (51%)			1.04 (0.83–1.31), 0.73

		ΔC_t *SLC7A11* (mean ± SD) Control	ΔC_t *SLC7A11* (mean ± SD) UV irradiated	
Treatment				
Placebo	50 (51%)	8.97 ± 1.04	9.03 ± 0.95[f]	1.00
Drug	49 (49%)	8.62 ± 0.96	8.73 ± 1.13	1.16 (0.90–1.49), 0.27
MC1R status				
Low-risk	49 (49%)			1.00
High-risk	50 (51%)			1.11 (0.87–1.43), 0.40

NOTE: We tested for potential interaction between treatment and MC1R status for each analysis and did not find significance for any of them (not shown).
[a]One subject with low-risk MC1R randomized to drug was excluded from the analyses because the lesions removed were seborrheic keratoses and not nevi.
[b]ΔC_t is the difference in C_t between the gene of interest and RPLP0.
[c]Analysis of covariance was used to examine the relationship between individual categorical variables and the change in ΔC_t, with categorical variable and ΔC_t in the unirradiated nevus as predictors and ΔC_t in the irradiated nevus as response. Additional analyses of age, gender, height, weight, hair color, and eye color as covariates did not reveal any significant predictors (not shown). The expression ratio is estimated as $2^{-\Delta C_t}$.
[d]For placebo group, difference between control and UV irradiated was significant ($P < 0.001$, Wilcoxon test).
[e]For placebo group, difference between control and UV irradiated was significant ($P = 0.012$, Wilcoxon test).
[f]For placebo group, difference between control and UV irradiated was not significant ($P = 0.062$, Wilcoxon test).

Table 4. Summary of prior and current studies

Conditions	UV	Study type	Biomarker	Results	Reference
Mouse model	FS20T12	Controlled	Free thiols	↓ After UV	Cotter et al. (18)
			Free thiols	↓ UV depletion after NAC	
			8-OG	↑ After UV	
			8-OG	↓ UV-induced 8-OG after NAC	
			Melanoma	NAC delays tumor onset	
Ex vivo nevi	FS20T12	Open label, phase Ib	GSH	↓ After UV	Goodson et al. (27)
			ROS	↑ After UV	
			GSH	↑ 3 h after NAC	
			Cys	↑ 3 h after NAC	
			GSH	↓ UV depletion after NAC (∼ half of subjects)	
In vivo nevi	SSR	Pilot study (no drug)	GSH	not ↓ after UV	Current study
			8-OG	↑ After UV	
			TR-1	↑ After UV	
			GCLM	↑ After UV	
			SLC1A4	↓ After UV	
			SLC7A11	↓ After UV	
In vivo nevi	SSR	Phase II, blinded, controlled[a]	8-OG	↑ After UV, no NAC effect	Current study
			TR-1	↑ After UV, no NAC effect	
			GCLM	↑ After UV, no NAC effect	
			SLC1A4	↓ After UV, no NAC effect	
			SLC7A11	↓ After UV, no NAC effect	
In vivo nevi	SSR	Open-label, post-trial study[b]	8-OG	↑ After UV, no NAC effect	Current study
			TR-1	↑ After UV, no NAC effect	
			GCLM	↑ After UV, no NAC effect	
			SLC1A4	↓ After UV, no NAC effect	
			SLC7A11	↓ After UV, no NAC effect	

[a]NAC given immediately prior to UV treatment.
[b]NAC given 3 hours prior to UV treatment.

Discussion

We have now examined the chemoprotective effects of NAC on UV-induced oxidative stress in three studies, ranging from preclinical to human trials. Our prior and current findings are summarized in Table 4. First, we showed that NAC reduced intracellular ROS and 8-OG in UV-treated melanocytes and that oral delivery of NAC restored GSH levels and blocked 8-OG formation in UV-treated skin (18). In melanoma-prone mice (33), oral NAC significantly delayed melanoma tumor onset (18). Second, we completed a Phase Ib trial in which we established the safety of a single 1,200 mg oral NAC in human subjects and showed that NAC could protect against UV-induced depletion of GSH in nevi irradiated *ex vivo* (27). In the pilot phase of the third study described here, however, we did not observe GSH depletion in nevi irradiated *in vivo*. This difference may indicate that we do not fully understand the dynamics of GSH metabolism *in vivo*. In the subsequent phase II trial reported here, we examined the effects of NAC on endpoints that were modulated by UV *in vivo*, namely 8-OG formation, TR-1 expression, and mRNA levels of *GCLM*, *SLC1A4*, and *SLC7A11*.

Several differences in the experimental designs of our phase I and II trials are important to consider. First is the elapsed time between NAC administration and UV treatment, which was 3 hours in the phase I study as we could detect Cys in nevi 3 hours after NAC ingestion (27). In the phase II trial, we irradiated nevi immediately after NAC administration, largely for practical considerations. We believed that this small change would be inconsequential given the evidence from both *in vitro* and *in vivo* models that elevated ROS levels and DNA damage appear almost immediately after UV exposure and last for at least 3 to 5 hours (16, 18, 32, 34). In fact, a recent study described the production of cyclobutane dimers in melanocytes 3 hours after UV exposure (so-called dark CPDs) that were suppressed by NAC (34). However, when we failed to see an effect of NAC on any of our endpoints, we did consider the possibility that Cys itself may act directly to quench ROS, instead of primarily serving as a source for the resynthesis of GSH. Under this scenario, the absence of supplemental Cys in the tissues at the time of irradiation might decrease the therapeutic effect of NAC. Thus, we did perform a small (post-trial) study in which nevi were SSR-treated 3 hours after NAC administration, but unfortunately did not see protection by the drug. Therefore, we do not believe that timing of NAC ingestion relative to UV treatment was the major (or only) reason that we failed to see any effect of the drug on biomarkers of UV-induced oxidative stress and damage.

A second consideration is the UV light sources that were used. Although the FS20T12 bulbs (27) used for the phase I trial and the SSR device (30) used here cause the same spectrum of mutations and have a similar effect on melanoma development in transgenic mice (35, 36), they differ significantly in the relative proportion of UVA and UVB (FS20T12: 20% UVA/80% UVB; SolarLight: 92% UVA/8% UVB) emitted. These UV sources may have differential effects on human skin, including modulation of the innate and cellular immune systems and UV-induced production of nitric oxide and ROS (37), which could potentially affect the endpoints that we evaluated as well as the effects of NAC.

A third consideration is the conditions under which the nevi were irradiated. In the phase I trial, nevi were irradiated *ex vivo* and then incubated at 37°C for 24 to 48 hours in room air supplemented with 5% CO_2. In the phase II trial, nevi were irradiated *in vivo* and removed 24 hours later for analysis. The *ex vivo* nevi were likely significantly hyperoxic compared with those treated *in vivo*, as the pO_2 is approximately 3 to 4 times greater in an incubator than *in vivo* (38). This situation is analogous to that encountered

during ischemia and reperfusion, where the redox buffering capacity of the tissues is significantly altered by fluctuations in oxygen levels (39). It is exactly this GSH-dependent redox capacity that we are seeking to support by supplementing the tissue with NAC; therefore, it is not unreasonable to speculate that changes in the conditions under which the tissues were treated could significantly influence the outcomes of our two studies.

In addition to UV exposure, mutations in *MC1R* (28) and *CDKN2A* (32) are also implicated in elevated oxidative stress in melanocytes. These and other genetic factors could potentially influence the acute responses of individual nevi to UV irradiation and the capacity for NAC-mediated protection. In both of our studies, each patient served as their own control because one nevus was removed prior to NAC administration (phase I study) or was not irradiated (phase II study). The *ex vivo* design of the phase I study allowed us to divide nevi in half and analyze both irradiated and unirradiated nevi, before and after NAC. Efforts to control for intersubject biological variation in this phase II study included incorporation of MED determination so that we could administer a biologically equivalent dose of SSR to each participant (while UV dosing was not MED-based in the phase I study). In the phase I trial, we did see a trend toward greater protection with NAC in subjects with high-risk MC1R alleles (40) and thus took this factor into account as we ensured that equal numbers of high- and low-risk mutation carriers were in each arm of the phase II study.

Although animal studies from our group (18) and others (41) suggest potential utility of NAC in skin cancer prevention, there are reasons to be cautious about the chronic use of antioxidants in this context. Selenium, an antioxidant micronutrient that can support the metabolism of ROS via the selenoproteins TR and glutathione peroxidase (42), has actually been found to increase risk for nonmelanoma skin cancer in high-risk populations (43). Using mice transgenic for hepatocyte growth factor, we previously reported that although a single topical treatment of selenomethionine to neonatal mice 24 hours prior to UV irradiation delayed melanoma formation, continued application of selenomethionine to the skin of these mice promoted the growth of early-stage tumors (44). Recent studies in other mouse models have raised similar concerns for NAC. Daily administration of NAC increased metastases in a $BRAF^{V600E}$-driven transgenic model (45) and in patient-derived melanoma xenografts (46). Thus, although antioxidants have the potential to lower melanoma risk when utilized at the initiation stage in a primary prevention setting, providing antioxidants to tissue harboring initiated cells or early-stage tumors may accelerate tumor growth and/or metastasis. This would be of particular concern in clinical prevention trials based on chronic antioxidant use, where study participants might be melanoma survivors harboring subclinical metastases or early-stage tumors at the time of enrolment.

Nevertheless, we believe that the administration of antioxidants in the acute setting (i.e., during periods of UV exposure) has the potential to decrease oxidative stress in epidermal tissues while avoiding the potential risks of chronic antioxidant administration. It is possible that our inability to detect any effect of NAC on biomarkers of oxidative stress and damage was related to difficulties inherent in attempting to evaluate the dynamics of these systems in tissues by analyses that must be made at a single time point. It was not practical for participants to undergo repeated nevus biopsies to detect changes in biomarkers as a function of time. To overcome this difficulty, we are working to develop noninvasive methods that can be used to make repeated measurements of the effects of UV-induced oxidative stress that will allow us to follow changes in redox status as a function of time. For example, it is now possible to measure antioxidant status in tissues using fluorescence lifetime imaging microscopy of NADH and NADPH (47).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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