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 ORIGINAL ARTICLE

New Formulations of Acyclothymidine Dinucleosides Reduce Damaging Effects of Ultraviolet Radiation in an Ex Vivo Skin Model

Abbas Raza PhD,[a] Christine Dreis BS,[a] Leon Kircik MD,[b] Naiem T. Issa MD PhD,[c] Robert Vince PhD[a]

[a]Center for Drug Design, Cosmetic Science and Product Innovation, College of Pharmacy, University of Minnesota, Minneapolis, MN
[b]Icahn School of Medicine at Mount Sinai, New York, NY; Indiana University School of Medicine, Indianapolis, IN; Physicians Skin Care, PLLC Louisville, KY; DermResearch, PLLC Louisville, KY; Skin Sciences, PLLC Louisville, KY
[c]Forefront Dermatology, Vienna, Virginia; Dr. Phillip Frost Department of Dermatology & Cutaneous Surgery, University of Miami Miller School of Medicine, Miami, Florida; Department of Dermatology, George Washington University School of Medicine and Health Sciences, Washington, DC

ABSTRACT

The greatest risk factor for skin cancer is exposure to ultraviolet (UV) rays of the sun. Among the 3 types of solar radiation (UVA, UVB, UVC), UVB rays are most commonly associated with skin cancer. UVB exposure promotes the formation of cyclobutane pyrimidine dimers (CPDs) in the DNA of cells in the epidermal skin layers, which can lead to mutations as DNA repair machinery attempts to repair the damage. These mutations can lead directly to skin carcinogenesis. Previous studies in animal and in human ex vivo skin models have shown that topical application of acyclothymidine dinucleosides protects DNA from UV-induced damage by preventing the formation of CPDs and helps initiate repair through the activation of DNA repair enzymes. Here we review the biological evidence leading to the development and formulation of ProteXidine™ (Topix Pharmaceuticals, Inc., Amityville, NY), as a UV protective agent for topical human application. We also provide clinical data pertaining to four ProteXidine™ formulations (test materials 1-4) tested for their abilities to reduce CPDs in an ex vivo human skin tissue model.

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INTRODUCTION

Despite numerous public warnings about protecting the skin against sun's UV exposure and extensive research into topical protective formulations, the incidence of melanoma and non-melanoma skin cancers continues to rise.[1] However, UV exposure to skin can also lead to signs of aging, such as wrinkling and pigmentary disorders, including hyperpigmentation and photocarcinogenesis.[2,3] For decades, researchers have been working on topical skincare products that contain DNA repair enzymes, and collagen or collagen-protecting components to protect skin against UV-induced damage and reduce signs of skin aging and carcinogenesis.[4-6] As DNA repair machinery works to remove UV-induced DNA damage, mutations can be introduced into DNA. If these mutations occur in tumor-suppressive genes such as p53 or others, skin carcinogenesis can occur.[7] Active DNA repair enzymes themselves have been formulated to initiate the DNA repair process more quickly and effectively than the natural repair proteins.[8] There remains a need to explore alternative methods that can potentially mitigate the effects of sun exposure. This involves considering innovative solutions that offer protection against the harmful effects of sunlight, thereby broadening the approach to sun-related issues more comprehensively.

UV-exposed cells generate mutagenic photoproducts, primarily from absorbing radiation in the UVB range (280–320 nm). These include cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) photoproducts within the DNA of cells in the epidermal skin layers. CPDs, being slower to repair compared to (6–4) photoproducts, emerge as the key contributors to mutations in mammals.[9] Current photoprotective agents work by absorbing, reflecting, or scattering UV radiation. However, their effectiveness is constrained by the production of potentially harmful photodegradation products for the skin.[10-13]

Previous research has shown that the dinucleotide pTpT, isolated from DNA, offers protection against UV-induced skin tumors in mice, including melanoma and non-melanoma skin cancer.[14-16] However, these molecules have notable drawbacks, including instability, high production cost, and the complexity involved in their preparation and purification. We created a biomimetic

molecule, acyclothymidine dinucleoside, by arranging 2 thymine units on a simplified, non-glycosyl phosphate linkage to mimic a natural DNA segment.[17] This decoy molecule is designed to absorb the desired UV spectrum, protecting natural DNA. Furthermore, the resulting acyclothymidine dinucleoside dimer has the potential to recruit DNA repair enzymes activated by the excised pTpT dimer. These new compounds had the advantage of eliminating the complex and unstable phosphate-linked diribofuranosyl moieties in oligonucleotides. We have demonstrated the protective efficacy of these molecules against UV exposure, preserving both plasmid and cellular DNA. The topical application to hairless mice exposed to UV resulted in reduced CPDs and reduced UV-induced carcinogenesis. After 20 weeks of regular UV exposure, only 40% of the mice treated with acyclothymidine dinucleosides had measurable tumors compared to 80% of the vehicle treated mice.[18]

Acyclothymidine dinucleosides have now been formulated for topical application to human skin (ProteXidine™, Topix Pharmaceuticals Inc., Amityville, NY). Here, we detail trials in human ex vivo skin cultures derived from one individual showing that ProteXidine™ can reduce the formation of CPDs and increase the number of pyknotic epidermal cells, indicating damaged cells exiting the skin after UV damage.

MATERIALS AND METHODS

Skin Tissue Model

The ex vivo human skin tissue (11 mm, NativeSkin®, lot# 202208301, Genoskin Inc., Boston, MA) was obtained from one 34-year-old Caucasian of Fitzpatrick skin type II[19,20] following abdominoplasty. The sample was collected with IRB approval, and informed consent was provided by the individual. Genoskin states in the product manual and has shown in publications that samples have normal skin barrier function (assessed by Lucifer Yellow dye penetration assay and electric impedance spectroscopy), mature stratum corneum, functional basal layer, and all cell types and skin appendages in vivo human skin.[21] After receipt, tissue was placed into culture wells with an equilibration medium provided by Genoskin and equilibrated overnight at 37°C with 5% CO_2 and ~95% relative humidity for 24 hours. The equilibration medium was then removed from each well and replaced with 1 mL fresh maintenance medium (GenoSkin NSA11 Media) prior to performing the experiments.

Test Materials and Application Protocol

Test materials (TMs, Topix Pharmaceuticals, Inc., Amityville, NY) were provided ready to use, stored at room temperature, and protected from light until use. The ProteXidine™ and PRD-Tech™ trademarks are owned by and used under license from the Regents of the University of Minnesota and the UMN Center for Drug Design, US Patent 9,364,406. TMs were TM1 (ProteXidine™ Universal Treatment Complex w/ SPF [Sheer]), TM2 (ProteXidine™ Universal Treatment Complex w/ SPF [Tinted]), TM3 (ProteXidine™ Night Treatment Cream), and TM4 (ProteXidine™ Skin Discoloration Defense). Ex vivo human skin samples were either left untreated, or a total of 15 mL of each TM was pipetted into the center of each ex-vivo culture. A sterile glass spreader was used to distribute the topical material across the surface of each culture. Cultures were visually inspected to ensure even distribution as observed by a lack of pooled liquid or dry spots on the skin. The treated cultures were returned to the incubator at 37°C with 5% CO_2 and ~95% relative humidity for 20 minutes. Cultures were removed, placed in Dulbecco's Phosphate Buffered Saline (DPBS, Sigma-Aldrich, St. Louis, MO), and either positioned on a platform 30 cm from a SOL 500 solar simulator lamp with an H2 filter (Honle UV America Inc., Marlboro, MA) or incubated in DPBS for an equivalent duration at room temperature, outside of the tissue culture incubator not exposed to solar simulated UV light. UV-exposed samples received a UVB dosage of 100 mJ/cm^2 measured with a PMA2106 UVB detector (Solar Light Company, LLC, Glenside, PA, USA) and UVA of 3200-3300 mJ/cm^2. TMs remained on the skin during UV exposure. UV irradiated tissues were returned to the incubator at 37°C with 5% CO_2 and ~95% humidity for 4 or 12 hours. TMs were then washed from the surface of each culture with sterile DPBS. Each washed culture was split in half. One half was fixed in 10% formalin and held in 70% ethanol prior to tissue processing. The other half was snap-frozen and stored at -80°C for future studies. Formalin-fixed tissues were embedded in paraffin and sectioned to 3mm to 5mm size for hematoxylin and eosin (H and E) staining, immunochemistry (IHC) of CPDs and immunofluorescence (IF) of collagen.

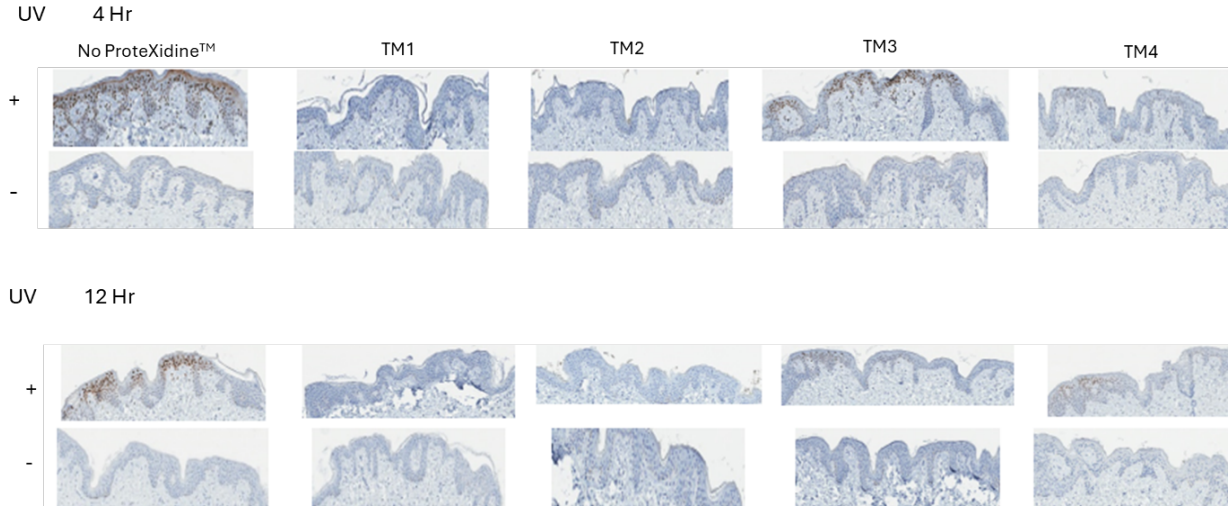
Immunohistochemistry (IHC)

CPD retrieval was performed using a standard protocol (PT Module, Epredia, Seattle, WA) at 98°C for 20 minutes with retrieval solution Dewax and HIER L Buffer (Epredia, TA-999-DHBL). Specimens were incubated with the primary antibody (mouse anti-thymine dimer antibody, H3, Abcam plc, Cat# ab10347, Cambridge, UK, diluted 1:100) and then secondary antibody (Goat Anti-Mouse IgG H&L, Alexa Fluor® 594, Abcam plc, Cat# ab150120, diluted 1:200) for one hour each at room temperature and then mounted in VECTASHIELD® HardSet™ Antifade Mounting Medium with DAPI (Vector Labs, Cat# H-1500-10, Detroit, MI) and protected by a cover slip. Digital images of the stained slides were captured at 40x magnification using a Leica Scanscope CS2 (Leica Biosystems, Deer Park, IL, USA). One tissue section was analyzed per block.

Hematoxylin and eosin staining (H&E)

Tissues were also processed for H and E staining to view the histology of the samples. One tissue section was analyzed per block.

FIGURE 1. CPD Immunohistochemistry 4 hours and 12 hours after UV exposure. Blue indicates cell nuclei and brown indicates CPDs. Fewer CPDs are observed with ProteXidine™ treatment compared to those without ProteXidine™ treatment after UV exposure. TM1-TM4 indicates four formulations of ProteXidine™.



RESULTS AND DISCUSSION

Following successful testing of the acyclothymidine dinucleosides in vitro and in vivo in animal models[17,18] we formulated ProteXidine™ for topical delivery to human skin. For proof of principle, we tested the formulations on an ex vivo skin model derived from one individual to determine whether we observed reduced signs of damaging effects of UV exposure upon application of ProteXidine™. Figure 1 shows CPDs in ex vivo skin 4 and 12 hours after UV exposure with and without each ProteXidine™ formulation (TM1-TM4). Reduced detection of CPDs was observed in the ProteXidine™ treated samples at both time points, although CPDs remained with the TM3 formulation.

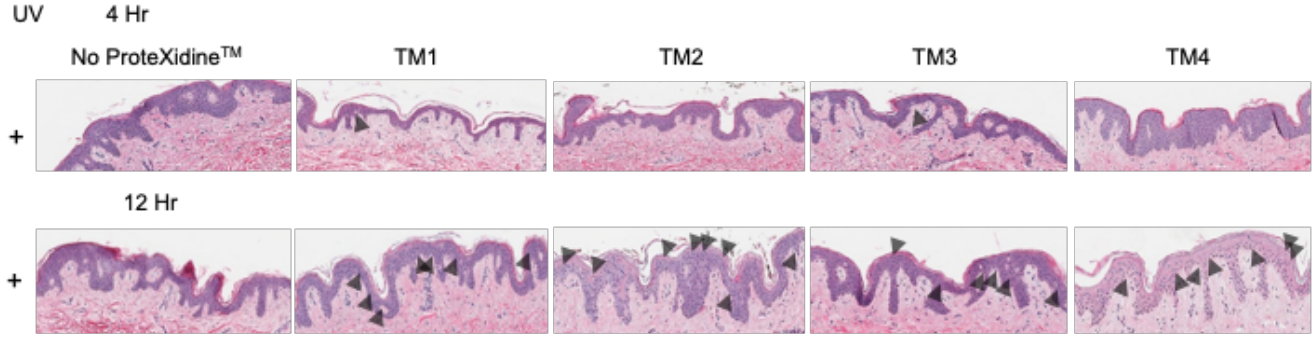
We next examined single histological sections from each sample and observed increased pyknotic nuclei in the UV-exposed ex vivo skin models treated with ProteXidine™, indicating damaged cells being removed from the skin structure (Figure 2). The effect was more pronounced at the 12-hour time point than the 4-hour time point, with all four formulations implicating that ProteXidine™ induced selective apoptosis of the UV-damaged cells.

The proof of principle study indicates that ProteXidine™ treatment of an ex vivo skin model reduced the effects of UV damage compared to samples without ProteXidine™.

CONCLUSION

ProteXidine™ formulated for topical application to human skin and tested on an ex vivo skin model resulted in less detectable CPDs following UV exposure compared to the UV-exposed sample without ProteXidine™. All four formulations of ProteXidine™reduced the detection of CPDs to various extents. Examination of histological sections revealed higher numbers of pyknotic nuclei in UV-exposed ProteXidine™ treated samples compared to the UV-exposed sample without ProteXidine™.

FIGURE 2. Hematoxylin and Eosin stain 4 hours and 12 hours after UV exposure. Pyknotic nuclei are marked by black arrows indicating damaged cells exiting the skin structure. TM1-TM4 indicates four formulations of ProteXidine™.



Finally, one formulation of ProteXidine™ appeared to reduce collagen damage after UV exposure. Together, the data support that topical ProteXidine™ delivery to the skin may be useful to reduce several damaging effects of UV exposure.

DISCLOSURES

AR, CD, and RV are inventors of Protexidine. NTI has received honorarium as consultant and speaker for Topix Pharmaceuticals. LK has received honorarium as consultant and speaker for Topix Pharmaceuticals.

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AUTHOR CORRESPONDENCE

Naiem T Issa MD PhD

E-mail:..... naiem.issa@gmail.com