3-Hydroxypyridine Chromophores are Endogenous Sensitizers of Photooxidative Stress in Human Skin Cells

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$⁸$Abbreviations: AGEs, advanced glycation endproducts; BCA, bicinchoninic acid; BSA, bovine serum albumine; BSA-B₆, pyridoxine-BSA conjugate; CAT, catalase; DAB (or DABCO), 1,4-diazabicyclooctan; DES, desmosine; DFO, deferoxamine mesylate; DHR 123, dihydrorhodamine 123; DMEM, Dulbecco’s modified Eagle’s medium; ECM, extracellular matrix; ESI-MS, electrospray-ionization-mass spectrometry; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; HBSS, Hanks’ balanced salt solution; HP, hydroxypyridine; MALDI-TOF-MS, matrix assisted laser desorption-time of flight-mass spectrometry; Man, mannitol; NE-3HP, N-ethyl-3-hydroxypyridinium cation; P, D-penicillamine; PL, pyridoxal; PLP, pyridoxal-5’-phosphate; PM, pyridoxamine; PN, pyridoxine; PI, propidium iodide; PYD, pyridinolin; RNase A, ribonuclease A; ROS, reactive oxygen species; SOD, superoxide dismutase; and SSL, solar simulated light.
Summary

Photocarcinogenesis and photoaging are established consequences of chronic exposure of human skin to solar irradiation. Accumulating evidence supports a causative involvement of UVA irradiation in skin photodamage. UVA photodamage has been attributed to photosensitization by endogenous skin chromophores leading to the formation of reactive oxygen species and organic free radicals as key mediators of cellular photooxidative stress. In this study, 3-hydroxypyridine-derivatives contained in human skin have been identified as a novel class of potential endogenous photosensitizers. A structure activity relationship study of skin cell photosensitization by endogenous pyridinium derivatives (pyridinoline, desmosine, pyridoxine, pyridoxamine, pyridoxal, pyridoxal-5’-phosphate) and various synthetic hydroxypyridine isomers identified 3-hydroxypyridine and N-alkyl-3-hydroxypyridinium cation as minimum phototoxic chromophores sufficient to effect skin cell sensitization towards UVB and UVA, respectively. Photosensitization of cultured human skin keratinocytes (HaCaT) and fibroblasts (CF3) by endogenous and synthetic 3-hydroxypyridine-derivatives led to a dose dependent inhibition of proliferation, cell cycle arrest in G2/M, and induction of apoptosis, all of which were reversible by thiol antioxidant intervention. Enhancement of UVA-induced intracellular peroxide formation and p38 MAPkinase-dependent stress signaling suggest a photooxidative mechanism of skin cell photosensitization by 3-hydroxypyridine derivatives. 3-Hydroxypyridine derivatives were potent photosensitizers of macromolecular damage, effecting protein (RNAse A) photo-crosslinking and peptide (melittin) photooxidation with incorporation of molecular oxygen. Based on these results, we conclude that 3-hydroxypyridine-derivatives comprising a wide range of skin biomolecules, such as enzymatic collagen crosslinks, B6 vitamers, and likely
advanced glycation endproducts in chronologically aged skin constitute a novel class of UVA-photosensitizers, capable of skin photooxidative damage.

INTRODUCTION

Most of the solar ultraviolet (UV) energy incident on human skin is in the deeply penetrating UVA region (> 95% from 320 to 400 nm). Increasing experimental evidence supports a causative involvement of UVA irradiation in photoaging and carcinogenesis of human skin by photooxidative mechanisms (1-5). In contrast to the formation of mutagenic pyrimidine-base photoproducts through direct absorption of UVB (290 - 320 nm) radiation by skin cell DNA (6), UVA radiation results in little photoexcitation of DNA directly, and generation of reactive oxygen species (ROS) and organic free radicals is a widely accepted mechanism of UVA-phototoxicity (reviewed in (7)). The formation of ROS as mediators of photooxidative stress in UV-irradiated skin seems to be dependent on non-DNA chromophores acting as endogenous photosensitizers (2,3,8). Photosensitization occurs as a consequence of initial formation of excited states of chromophores and their subsequent interaction with substrate molecules (type I photoreaction) or molecular oxygen (type II photoreaction) through energy and/or electron transfer (9). Various chromophores contained in human skin, such as urocanic acid (10), riboflavin (11), melanin precursors (12), and advanced glycation endproducts (13,14) have been proposed as endogenous UV-sensitizers of photooxidative stress, but molecular identity and mechanism of action of relevant endogenous skin photosensitizers remain elusive (3,15,16). Recently, we have presented evidence that skin structural proteins such as collagen and elastin and specifically their UVA chromophores represent a novel class of potent endogenous photosensitizers in human skin (13,14,17). Exposure of unirradiated human skin cells to UVA-
irradiated skin extracellular matrix (ECM)-proteins inhibited cell proliferation and induced chromosomal DNA damage proportional to UV-dose that was fully reversed in the presence of catalase. Type I photo-reductive formation of ROS, superoxide and H$_2$O$_2$ in particular, with concomitant light-driven protein oxidation was identified as the key mechanism of ECM-protein-sensitized phototoxicity. Based on our previous identification of the collagen-derived pyridinium crosslink pyridinoline as a candidate photosensitizer contained specifically in ECM-proteins (17), a detailed structure activity relationship study was undertaken to identify the minimum phototoxic chromophore contained in pyridinoline and various other pyridinium compounds from human skin. A photooxidative mechanism of skin cell UVA-photosensitization by all endogenous pyridinium compounds containing the identified minimum chromophore was elucidated. In this study, we present evidence that 3-hydroxypyridine-derivatives comprising a wide range of skin biomolecules, such as enzymatic collagen crosslinks, B$_6$ vitamers, and various advanced glycation endproducts, act as endogenous UVA-photosensitizers.

**MATERIALS AND METHODS**

**Chemicals.** 3-HP and NE-3HP (bromide salt) were purchased from Aldrich (Milwaukee, WI), SB 202190 was from Calbiochem (San Diego, CA), MTS was from Promega (Madison, WI), and desmosine was obtained from Elastin Products Company, Inc. (Owensville, MO). All other chemicals were from Sigma Chemical Co. (St. Louis, MO). Pyridinoline, isolated from acid hydrolysates of bovine bone collagen (18), was kindly provided by Dr. Simon Robins, The Rowett Research Institute, UK. The purity and identity of the preparation employed in sensitization experiments was confirmed by UV-spectroscopy, fluorescence spectroscopy, and
electrospray mass spectrometry (ESI-MS; m/z 429, M⁺ as previously reported (17)) using a LCQ Classic quadrupole ion trap mass spectrometer from Thermo Finnigan (San Jose, CA).

**Preparation of a covalent bovine serum albumin-vitamin B₆ conjugate (pyridoxylated BSA, BSA-B₆).** BSA-B6 was synthesized by reductive coupling of pyridoxal with protein lysine-residues based on NaCNBH₃ reduction of the initially formed Schiff base as described earlier (19). The reaction mixture contained BSA [350 mg], NaCNBH₃ [58 mg], pyridoxal [64 mg] in a total volume of 1.5 mL 0.25 M phosphate buffer, pH 7.4. The reaction proceeded at 37 °C in the dark overnight. The reaction was terminated by acidification, followed by neutralization and extensive dialysis against water at 4°C for 48 hours under light exclusion. After lyophilization the protein-adduct, called BSA-B₆, was characterized by MALDI-TOF mass spectrometry and fluorescence and UV spectroscopy.

**Irradiation with solar simulated light (SSL) and UVA.** A KW large area light source solar simulator, model 91293, from Oriel Corporation (Stratford, CT) was used, equipped with a 1000 W Xenon arc lamp power supply, model 68920, and a VIS-IR bandpass blocking filter plus either an atmospheric attenuation filter (output 290–400 nm plus residual 650–800 nm, for solar simulated light) or UVB and C blocking filter (output 320–400 nm plus residual 650–800 nm, for UVA), respectively. The output was quantified using a dosimeter from International Light Inc. (Newburyport, MA), model IL1700, with an SED240 detector for UVB (range 265–310 nm, peak 285 nm), or a SED033 detector for UVA (range 315–390 nm, peak 365 nm), at a distance of 365 mm from the source, which was used for all experiments. At 365 mm from the source, SSL dose was 7.63 mJ cm⁻² sec⁻¹ UVA and 0.40 mJ cm⁻² sec⁻¹ UVB radiation. Using UVB/C
blocking filter, the dose at 365 mm from the source was 5.39 mJ cm\(^2\) sec\(^{-1}\) UVA radiation with a residual UVB dose of 3.16 \(\mu\)J cm\(^2\) sec\(^{-1}\).

**Cell culture.** The established cell line of human epidermal keratinocytes (HaCaT cells), a gift from Dr. Norbert Fusenig (German Cancer Research Center, Heidelberg, Germany), and human dermal fibroblasts (CF-3 cells), a gift from Dr. Robert Dell’Orco (Noble Center for Biomedical Research, Oklahoma City, USA) were routinely cultured in DMEM containing 10% fetal bovine serum and kept in a humidified atmosphere containing 5% \(CO_2\) at 37°C (17).

**Assay for photosensitized suppression of skin cell proliferation.** Cells were seeded at 5 x 10\(^4\) cells per dish on 35mm dishes. After 24 h, cells were washed, placed in Hanks’ balanced salt solution (HBSS), and exposed to the combined or isolated action of photodynamic test compound and irradiation (UVA or SSL). Following 30 min incubation after irradiation, the exposure medium was removed and replaced with fresh culture medium. Cell number was determined 72 hr later, and proliferation was compared to cells that received mock-irradiation in HBSS.

**Quantification of \(H_2O_2\) formation.** \(H_2O_2\) formed upon photosensitization was quantified according to a standard procedure using the ferrous iron-xylenol orange assay as reported previously (13).

**Detection of intracellular oxidative stress by flow cytometric analysis.** Intracellular levels of peroxides were analyzed by flow cytometry using dihydorhodamine 123 (DHR 123) as a
specific fluorescent dye probe (17,20,21). To avoid direct photooxidation of the dye probe, cells were first treated with UV irradiation and sensitizer and then loaded with the indicator dye under light exclusion.

**Apoptosis analysis.** Induction of cell death was confirmed by annexin-V-FITC/propidium iodide (PI) dual staining of cells followed by flow cytometric analysis. Cells (200,000) were seeded on 35 mm dishes and received photosensitization 24 hours later. Cells were harvested at various time points after treatment and cell staining was performed using an apoptosis detection kit according to the manufacturer’s specifications (APO-AF, Sigma, St. Louis, MO).

**Cell cycle analysis.** Cells were seeded at 2 x 10^5 per dish on 35mm culture dishes (Sarstedt, USA) and left overnight to attach. After irradiation in the presence or absence of test compound, cells were washed twice with HBSS and fresh culture medium was added. After 24 or 48 h cells were harvested by trypsinization, resuspended in 200 µl PBS, and placed on ice. After addition of 2 ml 70% (v/v) ethanol, 30% (v/v) PBS, cells were incubated for 30 min on ice. The fixed cells were pelleted by centrifugation, resuspended in 800 µl PBS, 100 µl ribonuclease A (1mg/ml PBS), and 100 µl propidium iodide (400 µg/ml PBS), and incubated for 30 min in the dark at 37°C. Cellular DNA content was determined by flow cytometry using the ModFit LT software, version 3.0 (Verity, Topsham, ME).

**p38 MAPkinase Western analysis.** One day before treatment, 200,000 cells were seeded on 35 mm dishes. Cells were washed twice 24 h later with HBSS, followed by addition of 1 mL HBSS and exposure to increasing doses of UVA (up to 9.9 J/cm^2) in the absence or presence of 100 µM
NE-3HP. After irradiation or mock-treatment, cells were incubated for 30 min (37 °C, 5% CO₂), then washed with PBS, lysed in 1x SDS-PAGE sample buffer (200 µl, 0.375 M Tris HCl pH 6.8, 50% glycerol, 10% SDS, 5% β-mercaptoethanol, 0.25% bromophenol blue), heated for 5 min at 95 °C, and placed on ice. Samples (23 µL, containing approximately 45 µg total protein as determined by the BCA assay) were separated by 12% SDS-PAGE followed by immediate transfer to polyvinylidene difluoride membranes (Immobilon, Millipore, Bedford, MA). Equal protein loading and transfer was examined by reversible Ponceau staining and the membrane was then used for p38 immunostaining as published recently (22).

**Sensitization of protein photo-crosslinking and peptide photo-oxidation.** Ribonuclease A (RNAse A, 10 mg/mL PBS) was irradiated with solar simulated light (13.8 J/cm² UVA and 0.72 J/cm² UVB) in the absence or presence of various hydroxypyridine-derivatives (500 µM each, 200 µL total reaction volume). Protein oligomerization as a result of sensitized photocrosslinking was visualized by 15% SDS-PAGE followed by Coomassie-staining and densitometric analysis using an Eagle Eye digital camera (Stratagene, La Jolla, CA). Melittin (1 mg/mL PBS) was irradiated with solar simulated light (13.8 J/cm² UVA and 0.72 J/cm² UVB) in the absence or presence of various hydroxypyridine-derivatives (500 µM each, 200 µL total reaction volume) followed by mass spectrometry.

**Spectroscopy.** UV-spectra were recorded using a Cary 100 Bio UV-Visible Spectrophotometer from Varian, Inc. (Palo Alto, CA). Fluorescence spectra were recorded using a Spectramax Gemini XS (Molecular Devices, Sunnyvale, CA) 96 well-microtiter plate reader.
**Mass spectrometry.** Mass spectrometry was performed using a Bruker Reflex III MALDI–TOF-Mass Spectrometer (MALDI-TOF-MS) equipped with a nitrogen laser (337 nm). Spectra were recorded in positive ion mode in linear configuration using Ñ-cyano-4-hydroxycinnamic acid as matrix.

**Statistical analysis.** The results are presented as means (± SD) of at least three independent experiments. They were analyzed using the two-sided Student’s t test (*p< 0.05; **p< 0.01; ***p< 0.001).

**RESULTS**

**UVA-photosensitized inhibition of skin cell proliferation by the pyridinium compounds pyridinoline, pyridoxine and other B₆ vitamers, but not desmosine.** In a recent study on the phototoxicity of dermal ECM-proteins and their potential role in sensitized skin photodamage, we identified the collagen-derived pyridinium-crosslink pyridinoline (PYD, see Fig.1) as a photosensitizer of UVA-driven superoxide and H₂O₂ formation (17). To examine the structural requirements for pyridinoline phototoxicity, we measured the sensitized inhibition of proliferation in cultured human fibroblasts (CF3) after cells were exposed to UVA-irradiation (3.3 J/cm²) in the presence or absence of 500 µM pyridinoline or desmosine (DES, see Fig. 1), a structurally related pyridinium-crosslink extracted from elastin. As shown in Fig.2 A, neither pyridinoline nor desmosine displayed any significant dark toxicity. The selected UVA dose in the absence of compound inhibited cell proliferation by 20%. The combined action of pyridinoline and light caused a pronounced inhibition of cell proliferation (approximately 60%). A suppression of cell proliferation by almost 45% was observed, when un-irradiated cells were
exposed to 6 μM H₂O₂, an amount equal to the concentration formed upon UVA irradiation of 500 μM pyridinoline, as quantified in Fig. 2B. Consistent with photosensitized formation of H₂O₂ as the mechanism of inhibition of proliferation by UV-irradiated pyridinoline, complete suppression of photosensitization was achieved when the antioxidants catalase or D-penicillamine were added before irradiation, whereas no protection was achieved using the singlet oxygen quencher sodium azide. No photosensitization of ROS formation or inhibition of skin cell proliferation was observed when cells were irradiated in the presence of desmosine.

Next, we tested pyridoxine (PN, see Fig.1), another endogenous hydroxypyridine-derivative and structural homologue of pyridinoline (23), for photosensitization of H₂O₂ production and inhibition of skin cell proliferation. Pyridoxine was an equally effective sensitizer of UVA-driven H₂O₂ formation as pyridinoline (Fig. 2 B), but sensitized suppression of fibroblast proliferation exceeded the effect of H₂O₂ formed during irradiation (Fig. 2 B). In contrast to pyridinoline sensitization, catalase treatment only weakly reversed pyridoxine sensitization, which suggests a mechanism of pyridoxine-phototoxicity operating in addition to sensitization of H₂O₂ formation. Among various antioxidants including superoxide dismutase, mannitol, and deferoxamine mesylate (data not shown), only penicillamine treatment effectively antagonized pyridoxine-photosensitization (Fig. 2 A). ¹O₂-involvement in pyridoxine-photosensitization was excluded based on the observation that the presence of the singlet oxygen quenchers NaN₃ and DABCO (data not shown) during irradiation was not protective, and irradiation in deuterated PBS did not enhance photosensitization (data not shown). From these initial observations we conclude that the hydroxypyridine-derivatives pyridinoline and pyridoxine are UVA-photosensitizers of light driven ROS formation and that inhibition of skin cell proliferation in the case of pyridoxine depends only partially on ROS formation.
To examine the relative phototoxicity of hydroxypyridine-derivatives, a comparative dose response for the sensitized inhibition of proliferation by increasing doses of the pyridine-derivatives pyridinoline, desmosine, and the B₆ vitamers pyridoxine, pyridoxamine, and pyridoxal (formulas given in Fig.1) was established as shown in Fig. 3 A. No dark toxicity was observed for up to 1 mM of any test compound (data not shown). UVA-phototoxicity increased in the order pyridinoline < pyridoxine < pyridoxamine < pyridoxal. At UVA dose of 3.3 Jcm⁻², the sensitizer concentration necessary to achieve 50% suppression of cell proliferation (IC₅₀ + SD, n=3) observed after light treatment alone (approximately 80% proliferation of unirradiated controls, data not shown), was calculated by extrapolation from proliferation-inhibition curves (shown in Fig. 3 A) for pyridinoline (632 ± 53 µM), pyridoxine (104 ± 17 µM), pyridoxamine (32 ± 7 µM), and pyridoxal (5 ± 1 µM). At concentrations approximately five times the IC₅₀ for suppression of proliferation, pronounced induction of skin cell apoptosis was observed after combined exposure to B₆-vitamers and UVA irradiation as detected by annexinV-FITC/PI staining followed by flow cytometric analysis. Photosensitization of cell kill was particularly pronounced with pyridoxal as shown in Fig. 3 B, with approximately 35% of gated cells in either early or late apoptosis/necrosis at 24 h after irradiation. This induction of cell death was suppressed when the sensitization was performed in the presence of the thiol-antioxidant and free radical scavenger penicillamine. As observed above in Fig. 2 A, NaN₃ did not show photoprotection suggesting that singlet oxygen is not involved in pyridoxal-photosensitized induction of cellular apoptosis (data not shown).

In human skin, B₆ vitamers can occur in free 5’-phosphorylated or unphosphorylated form, and can also be bound covalently to protein (24). As demonstrated in Fig. 3 B, sensitization of UVA-induced skin cell apoptosis observed with pyridoxal-5’-phosphate was
almost as potent as with unphosphorylated pyridoxal. To examine whether vitamin B₆ retains phototoxicity upon covalent coupling with proteins, a simple model protein-vitamin B₆ adduct was synthesized by reductive coupling between pyridoxal and ε-amino groups of protein lysine residues in bovine serum albumin (BSA) performed after initial Schiff base formation (19). The covalent conjugate (BSA-B₆, bearing pyridoxylated lysine residues as shown in Fig. 1) was characterized by MALDI-TOF mass spectrometry. A mass difference \( m \) \([BSA-B₆ - BSA; 66,965 - 65,849]\) of approximately 1100 Da indicated the incorporation of approximately seven pyridoxine moieties per BSA molecule. No residual unmodified BSA or species of intermediate weight were detected in the mass spectrum of BSA-B₆ (data not shown). The UV spectrum of the BSA-B₆ conjugate exhibited a novel absorption maximum at 335 nm (Fig. 3 C), and fluorescence excitation at 335 nm led to strong emission around 400 nm (Fig. 3 D), consistent with B₆-fluorophore incorporation into the protein. BSA-B₆ was devoid of any dark toxicity, since exposure of skin fibroblasts to BSA-B₆ (5 mg/ml PBS) in the absence of UVA-irradiation did not inhibit proliferation as shown in Fig. 3 E. In contrast, pronounced inhibition of cell proliferation resulted from exposure to the combined action of UVA and BSA-B₆, which was not observed upon irradiation in the presence of unmodified or NaCNBH₃-reduced BSA. Based on these data we conclude that unphosphorylated or 5’-phosphorylated B₆-vitamers are UVA photosensitizers and that protein-bound pyridoxine retains phototoxicity.

**Identification of the minimum phototoxic chromophores contained in endogenous hydroxypyridine photosensitizers.** In an attempt to identify the minimum chromophore responsible for the photosensitizer activity of the endogenous hydroxypyridine derivatives pyridinoline and B₆- vitamers, the hydroxypyridine (HP) isomers 2-HP, 3-HP, 4-HP and the N-
alkyl-3-HP-derivative and pyridinoline analogue (25) N-ethyl-3-hydroxypyridinium bromide (NE-3HP, 100 µM each, formulas given in Fig. 1) were tested for photosensitized suppression of HaCaT keratinocyte proliferation by low doses of UVA (see Fig. 4 A). None of the test compounds exhibited significant dark toxicity, and three days after combined treatment with test compound and UVA, only NE-3HP strongly suppressed cell proliferation. Cell counts obtained from samples treated with light and NE-3HP were below seeding density suggesting cell depletion by photosensitized induction of cell death. Induction of apoptosis by the combined action of NE-3HP and UVA was confirmed by annexinV-FITC/PI staining and flow cytometric analysis performed over 24 h after cell treatment (Fig. 4 B). Cells in early [annexinV⁺/PI⁻] and late [annexinV⁺/PI⁺] apoptosis were observed starting 6 h after treatment (data not shown) and 24 h later almost all cells were in late apoptosis/necrosis. These changes were not observed after administration of test compound or light alone. Next, the experiment was repeated using solar simulated light (SSL) instead of UVA to elucidate the possible contribution of UVB wavelengths to UV-photoactivation of the test compounds as shown in Fig. 4 C. With SSL, NE-3HP again induced almost complete inhibition of proliferation of HaCaT keratinocytes, but in this case 3-HP also was cytostatic indicating UVB activation of 3-HP phototoxicity. The differential range of UV-wavelengths effective for the induction of 3-HP and NE-3HP phototoxicity clearly correlate with the distinct UV absorption and fluorescence characteristics of these chromophores: 3-HP is a non-fluorescent compound characterized by broad UV absorption with maxima centered at [nm]: 245 nm (2477), 276 nm (2048), and 312 nm (4435). NE-3HP shows UV absorption maxima centered at [nm]: 248 nm (7506) and 319 nm (5243), and displays intense fluorescence upon excitation at UVB and UVA I (320-340 nm) wavelengths due to broad excitation and emission maxima (ex/em 305/395 nm) very similar to its structural analogue
pyridinoline (25,26). In contrast to the rapid photodegradation of pyridinoline observed with SSL or UVA irradiation described previously (17), prolonged exposure to UVA induced only a minor degradation of NE-3HP (< 5%) as measured by fluorescence and UV spectroscopy (data not shown). To gain further insight into the mechanism of anti-proliferative action of 3-HP sensitization, cell cycle analyses were performed 24 h after photosensitization using the combined action of 100 μM 3-HP and SSL (2.3 J/cm² UVA, 120 mJ/cm² UVB) as shown in Fig. 4 D. The pronounced accumulation of cells with 4n DNA content and depletion of cells with 2n DNA content is consistent with the sensitized induction of a G₂/M block. Treatment with the sensitizer alone did not induce any alterations of the cell cycle, but the isolated action of SSL in the absence of sensitizer induced a moderate accumulation of cells in S and G₂/M phase consistent with the significant suppression of HaCaT cell proliferation by approximately 20% for SSL irradiation alone as shown in Fig.4 C. Next, a comparative dose response was established for the sensitized inhibition of proliferation by 3-HP and NE-3HP. At a SSL dose of 2.3 J/cm² UVA/ 0.12 J/cm² UVB, the sensitizer concentration that caused 50% suppression of cell proliferation (IC₅₀ ± SD, n=3) observed after light treatment alone (80% proliferation of unirradiated controls), was calculated by extrapolation from proliferation-inhibition curves (data not shown) for 3-HP (36 ± 11 μM), and NE-3HP (4 ± 1 μM). Based on this quantitative structure-activity relationship study of skin cell photosensitization by hydroxypyridine-derivatives, 3-hydroxypyridine was identified as the minimum UVB-sensitizer chromophore and N-alkyl-3-hydroxypyridinium cation as the minimum UVA-sensitizer chromophore contained in the endogenous hydroxypyridine photosensitizers pyridoxine, pyridoxamine, pyridoxal, and pyridinoline, respectively.
Photooxidative cellular stress as a result of hydroxypyridine-photosensitization.

Skin cell phototoxicity of UVA irradiation depends on the generation of cellular oxidative stress with chemical modification of molecular targets and induction of signaling pathways (27). To examine whether the observed cellular photosensitization by 3-HP-derivatives occurs via the enhancement of known UVA-induced photooxidative mechanisms, generation of intracellular oxidative stress was examined after exposing CF3 fibroblasts to UVA in the presence or absence of pyridoxine followed by loading with the intracellular redox dye DHR 123 in the dark as shown in Fig. 5 A. Upon reaction with intracellular peroxides formed by photooxidation, DHR 123 is irreversibly oxidized and converted to the fluorescent dye rhodamine 123, which allows quantitative analysis of intracellular redox stress by flow cytometric analysis (17,21). Exposure of cells to UVA in the absence of sensitizor induced an approximately three-fold increase over baseline fluorescence observed after loading the cells with the redox dye as shown in Fig. 5 A, which is consistent with UVA-induced intracellular oxidative stress (21). Exposure to sensitizer alone did not induce any enhancement of baseline fluorescence. When cells were exposed to the combined action of UVA and pyridoxine, an additional two-fold increase of rhodamine fluorescence intensity was observed demonstrating the enhancement of UVA-induced photooxidative stress by photosensitization. Similar results were obtained when cells were exposed to the combined action of UVA (3.3 Jcm⁻²) and 3-hydroxypyridine (100 µM), pyridinoline (500 µM) or NE-3-HP (100 µM) (data not shown). These data support the hypothesis that photosensitization of skin cells by 3-HP derivatives enhances intracellular photooxidative stress with formation of reactive species of sufficient longevity, such as protein peroxides (28), capable of oxidizing DHR 123 during cell loading after irradiation.
Activation of mitogen activated protein (MAP) kinases by phosphorylation is an established cellular response to photooxidative stress (27,29) and sensitizer-dependent potentiation of p38 activation by UVA has been used to assess photodynamic effectiveness of therapeutic photosensitizers (30). Using the combination of UVA and NE-3HP, we investigated the photosensitized induction of p38–phosphorylation in cultured human skin cell lines as shown in Fig. 5 B. Cells were irradiated with increasing doses of UVA in the absence or presence of NE-3HP and analysed for p38-phosphorylation by Western blot analysis of protein cell extracts prepared 30 min after irradiation. In CF3 fibroblasts and HaCaT keratinocytes, UVA-induction of dual phosphorylation of p38 was greatly potentiated by NE-3HP. Induction was potentiated at least 10 fold: a low dose of UVA (1.1 J/cm²) administered in the presence of NE-3HP was as effective as a high dose of UVA (9.9 J/cm²) alone. A similar pattern of p38 MAPkinase activation in cultured skin cells occurred, when UVA-photosensitization was performed using pyridoxine (100 µM) or 3-HP (100 µM, each) (data not shown). Based on these results we conclude that biogenic 3-HP-derivatives are potent photosensitizers of UVA-induced MAPkinase stress signaling in cultured human skin cells.

To further examine the involvement of oxidative pathways in the photosensitization of human skin cells by 3-HP-derivatives, antioxidant modulation of sensitized inhibition of skin cell proliferation was examined as depicted in Fig. 5 C. As observed earlier with pyridoxine, D-penicillamine fully reversed the sensitized inhibition of skin cell proliferation resulting from combined treatment with SSL and 3-HP or NE-3HP, when present during irradiation, but was completely ineffective when added immediately after irradiation. Other antioxidants such as DFO, Man, SOD, CAT, and DABCO did not suppress the sensitized inactivation of HaCaT keratinocytes by 3-HP-derivatives. Inhibition of proliferation was not enhanced when the
irradiation was performed in deuterated PBS (data not shown), providing further evidence that singlet oxygen is not involved in the phototoxic action of 3-HP-derivatives on human skin cells.

**Protein damage as a result of hydroxypyridine-photosensitization.** Cellular photosensitization is thought to be triggered by photooxidation of biological macromolecules (28,31). Photosensitization of protein damage by 3-HP-derivatives was examined using a ribonuclease A (RNAse A) photo-crosslinking assay. RNAse A was selected as a model target because it does not contain tryptophan residues, thereby excluding effects of this endogenous UV-sensitizer amino acid on photo-crosslinking (13). RNAse A (monomer, Mw 13,700 Da) was irradiated with SSL in the presence or absence of various hydroxypyridine derivatives and covalent protein-oligomerization (dimer, 28,000 Da; trimer, 42,000 Da, etc.) was examined using reducing SDS-PAGE analysis. As shown in Fig. 6 A and B, sensitized photo-crosslinking occurred in the presence of 3-HP, NE-3HP, pyridoxine, pyridoxamine, and pyridoxal (data not shown), whereas SSL-irradiation in the presence of 2-HP, 4-HP, pyridinoline, and desmosine was ineffective in generating RNAse oligomers. Among the test compounds, sensitization by pyridoxine was most pronounced yielding approximately 35% RNAse-oligomerization. Photo-crosslinking sensitized by the minimum sensitizer-chromophore 3-HP was examined in more detail. Sensitization occurred dose dependently with regard to light dose (data not shown) and sensitizer concentration as shown in Fig. 6 C. Antioxidant effects on pyridoxine-sensitized protein damage were examined next as shown in Fig. 6 D. Antioxidant modulation of pyridoxine-sensitized protein crosslinking clearly paralleled antioxidant inhibition of cell proliferation observed earlier as shown in Fig. 2 A. Among various antioxidants used to test for antioxidant modulation of sensitized protein damage, only the thiol antioxidant, copper ion
chelator, and free radical quencher D-penicillamine (P) fully suppressed pyridoxine-sensitized photo-crosslinking. Hydroxyl radical scavenging using mannitol (Man), iron ion chelation using deferoxamine mesylate (DFO), scavenging of H$_2$O$_2$ and superoxide radical anion using catalase (CAT) and superoxide dismutase (SOD), respectively, and the excited singlet state quencher potassium iodide (KI) (13) did not interfere with pyridoxine sensitization of protein photodamage. In addition, pyridoxine-sensitized photo-crosslinking was not enhanced when the reaction was performed in deuterated PBS, again suggesting that singlet oxygen was not involved (data not shown). Further evidence against the involvement of singlet oxygen in pyridoxine-sensitized RNAse photo-oligomerization was provided by the studies with the singlet oxygen quencher 1,4-diazabicyclo[2.2.2]octane (DAB), which unexpectedly enhanced protein damage, compatible with facilitated electron transfer reactions in the presence of a DABCO radical cation as reported previously (32). Moreover, photo-crosslinking effectively proceeded when the irradiation was performed under argon, demonstrating that pyridoxine photosensitization of protein damage can occur by oxygen-independent reaction pathways (data not shown). Similar data were obtained when photo-crosslinking was sensitized by 3-HP or NE-3HP (data not shown). These data have led us to conclude that 3-HP-derivatives are potent sensitizers of protein photo-crosslinking and that 3-HP-sensitization can proceed in the presence or absence of oxygen by unidentified reaction pathways compatible with type I photoreactions with initial formation of photoexcited states of the sensitizer followed by electron transfer reactions, either of which could effectively be quenched by thiol-compounds.

**Pyridoxine-sensitized peptide photooxidation** Sensitization of macromolecular damage by 3-HP-derivatives was studied in more detail examining peptide photooxidation by MALDI-TOF
mass spectrometry. The peptide melittin ($C_{131}H_{229}N_{39}O_{31}$, Mw 2845.97, monoisotopic peak), previously used as a model target in studies of peptide oxidation and radiation damage (33,34), was SSL-irradiated in the presence or absence of 3-HP derivatives. SSL-irradiation of melittin in the absence of sensitizer did not induce the formation of any reaction products as shown in Fig. 7, but irradiation in the presence of pyridoxine induced the formation of a reaction product in high yields. The detected mass increase of 32 u of the newly formed product [2877.92 u - 2845.91 u, monoisotopic peaks] provided clear evidence for pyridoxine-sensitized introduction of molecular oxygen into the target peptide. Formation of a stable hydroperoxide or endoperoxide product could occur after addition of molecular oxygen to an amino acid side chain or backbone free radical formed by type I photosensitization followed by hydrogen transfer (28,34). As observed with sensitization of RNAse photo-crosslinking, 3-HP and NE-3HP were equally effective sensitizers of melittin photooxidation (data not shown), but pyridinoline was completely inactive. Antioxidant modulation of B$_6$-sensitized photooxidative modification of melittin paralleled the earlier observations with RNAse photo-crosslinking with the exception that irradiation under argon completely suppressed the formation of photooxidized melittin (data not shown). These data provide evidence that under aerobic conditions pyridoxine-sensitized peptide oxidation occurs with incorporation of molecular oxygen.

**DISCUSSION**

UVA-sensitization by endogenous chromophores is rapidly emerging as an important mechanism of skin cell photooxidative stress involved in skin photoaging and carcinogenesis. Therefore, increasing research interest is focused on the elucidation of structure and function of
non-DNA chromophores contained in human skin that might enhance UVA phototoxicity acting as endogenous photosensitizers.

In this study, biogenic 3-hydroxypyridine-derivatives contained in human skin have been identified as a novel class of potential endogenous photosensitizers. Skin cell photosensitization by various endogenous hydroxypyridine-chromophores contained in human skin is demonstrated in Fig. 2 and 3, and 3-HP and N-alkyl-3HP are identified as the minimum chromophores responsible for photosensitization towards SSL and UVA, respectively, as shown in Fig. 4. Photosensitization of cultured human skin cells by 3-HP derivatives resulted in dose dependent inhibition of proliferation, cell cycle arrest, and induction of apoptosis. Multiple lines of experimental evidence for an involvement of photooxidative mechanisms in skin cell phototoxicity of 3-HP derivatives were obtained as presented in Fig. 5: Photosensitization by 3-HP derivatives strongly potentiated UVA-induced intracellular peroxide formation and could be suppressed by thiol antioxidant intervention. UVA-induction of p38 MAPkinase-dependent stress signaling, known to regulate AP-1-activation and matrix metalloproteinase expression in skin fibroblasts (27,29), was strongly enhanced by 3-HP-photosensitization. However, no specific macromolecular target of skin cell photosensitization by biogenic 3-HP-derivatives was identified at this point, although photosensitization of protein and peptide damage was demonstrated in model systems as summarized in Fig. 6 and 7.

The mechanistic basis of hydroxypyridine phototoxicity remains incompletely understood at this point. Particularly, the complex photochemistry of B6-vitamers and their photoproducts has been studied in detail (35-38), but the involvement of specific photoproducts in skin cell photooxidative stress observed in this study remains to be elucidated. Obviously, photosensitization by pyridinoline fully depends on the light-driven formation of superoxide and
H$_2$O$_2$ consistent with earlier observations (17), whereas photosensitization of ROS formation is not essential for skin cell phototoxicity of B$_6$-vitamers, 3-HP, and NE-3HP, which was demonstrated by oxygen independence and a general lack of antioxidant suppression, except inhibition in the presence of penicillamine. Indirect experimental evidence in support of a predominant involvement of type I photosensitization mechanisms initiated by electron transfer reactions between photoexcited 3-HP, NE-3HP, or B$_6$-vitamers and substrate molecules was obtained from [i] the unique inhibitory activity of the thiol-type free radical scavenger penicillamine on sensitized protein and skin cell damage, [ii] the complete ineffectiveness of other antioxidant treatments and anaerobic conditions to block sensitized protein crosslinking, [iii] a lack of enhancement of photosensitization in deuterated buffer, and the ineffectiveness of the singlet oxygen quenchers DABCO and NaN$_3$ to protect cells or suppress protein damage (27,39). Further evidence against involvement of singlet oxygen formation was based on the fact that pyridoxine and 3-HP are known singlet oxygen quenchers (37,38). Obviously, the presence of a phenolic 3-OH substituent is an essential structural requirement for sensitizer activity of all tested pyridine derivatives, since desmosine, devoid of any hydroxyl substituent, and 2-HP and 4-HP, which occur predominantly as the tautomeric pyridone structures in aqueous solutions at neutral pH (40), display no photosensitizer activity. The phenolic character of 3-HP-derivatives may be of crucial importance for the observed sensitization effects, since it is well documented that upon photoexcitation phenolic substances release electrons into aqueous solutions by electron ejection with formation of phenoxytype organic free radicals from the excited triplet state, but conversely act as potent electron scavengers in the ground state (41). Moreover, single electron transfer reactions leading to the formation of 3-hydroxypyridinium (42) and pyridoxine (43) free radicals have been reported previously, and free radical polymerization of synthetic N-
substituted 3-oxypyridinium betaines can be initiated by UV-irradiation (44), consistent with a free radical mechanism of phototoxicity of biogenic 3-HP-derivatives observed in this study. Obviously, the differences in mechanism and potency of photosensitization observed with various 3-HP derivatives are a consequence of substituent effects on hydroxypyridine photochemistry. The enhanced phototoxicity of pyridoxal as compared to the other B₆-vitamers is consistent with the electron withdrawing effects of the carbaldehyde substituent and facilitated hydrogen transfer, known to occur in aromatic compounds with a hydroxyl group in ortho position to a carbonyl group (35). Likewise, the strong difference in photosensitization potency and mechanism between the similar fluorophores N-ethyl-3-HP and pyridinoline must be a consequence of pyridinium ring substitution by bulky, charged alkyl residues that might inhibit type I interaction with larger substrate molecules, but allow interaction with molecular oxygen leading to ROS formation. However, detailed photochemical studies employing laser flash photolysis and electron spin resonance techniques will be necessary to elucidate the excited state chemistry and free radical species involved in photosensitization by members of the 3-HP group of sensitizers. In addition, differential cellular uptake and metabolism likely will contribute to the remarkable differences with regard to phototoxicity and mechanism of sensitization observed with biogenic and synthetic 3-HP-derivatives, which must be elucidated in the future.

The identification of 3-HP as a potent phototoxic chromophore contained in various human skin molecules must be considered in light of increasing evidence for the involvement of endogenous photosensitizers as key mediators of UVA-induced skin photoaging and photocarcinogenesis (1,3,4). In this study, photoactivation of physiologically relevant concentrations of 3-HP-derivatives occurred by irradiation with doses of UVA and SSL well below the minimal erythemal threshold (45), equivalent to a short exposure of fair human skin to
full spectrum solar UV-irradiation (7). Currently, the occurrence of 3-HP chromophores is established in three groups of ubiquitous tissue biomolecules: (i) the B₆-vitamers pyridoxine, pyridoxamine, pyridoxal, and their respective phosphorylated derivatives and protein conjugates; (ii) the ECM-protein-associated crosslinks pyridinoline and deoxypyridinoline; and (iii) various advanced glycation endproducts (AGEs) that accumulate on long-lived proteins as a result of chronic carbonyl stress during chronological aging (46-48).

(i) Increased photosensitivity is a known consequence of B₆-overdosing in humans (49). Phototoxicity of UV-irradiated pyridoxamine was reported as early as 1947 by Shwartzman and Fisher (50) followed by other reports thereafter (36,51). B₆-vitamers therefore are important as endogenous skin photosensitizers with relevance to human skin in vivo, since human skin contains various B₆-vitamer forms in significant amounts (approximately 100 nmol per g protein (24)), with pyridoxal-5’-phosphate and pyridoxal being the predominant vitamers in vivo. Pyridoxal was clearly the most phototoxic vitamer tested in our assays with pronounced apoptogenicity towards skin fibroblasts after photoactivation at only moderate doses of UVA. In contrast to earlier observations (35), pyridoxal-5’-phosphate was as potent as pyridoxal in photosensitizer-induced skin cell apoptosis, consistent with 3-HP being the minimum chromophore required for sensitizer activity. Our results on B₆-vitamer phototoxicity also point towards a potential risk of photosensitization associated with the use of high doses of pyridoxamine currently in clinical development as a therapeutic intervention for the inhibition of glycation-associated diabetic complications and hyperlipidemia (52). The phototoxic role of B₆-vitamers in human skin as shown by our experiments strongly contrasts but does not contradict a protective role of pyridoxine against singlet oxygen damage observed in a phytotoxic fungus (38).
(ii) Skin collagen pyridinoline content is generally low (approximately 16 mmol per mol collagen (53,54)), but dramatically increases during conditions of wound healing, scar formation, and sclerotic disorganization as referenced in (17). It is tempting to speculate that a significant increase in pyridinoline and deoxypyridinoline content in skin collagen characteristic of scar tissue and sclerotic skin diseases introduces an endogenous UVA-sensitizer that may contribute to the known predisposition of scar tissue towards photocarcinogenesis (55), a hypothesis to be explored in the future.

(iii) AGEs, crosslink chromophores formed by non-enzymatic amino-carbonyl reactions between sugars and protein-bound amino-groups (glycation), accumulate on skin ECM-proteins during conditions of increased carbonyl stress, such as actinic (56) and chronological aging and diabetes (57). The phototoxic activity of AGEs extracted from skin collagen and lens crystallin is well documented (13,58), and photosensitization of skin cell photooxidative stress results from UVA-irradiation of AGEs (14,59). Increasing experimental evidence indicates that 3-HP epitopes are formed during tissue glycation. N-alkyl-3HP derivatives are formed during glycation and lipid peroxidation under physiological conditions (60). Importantly, AGE-fluorophores of the N-alkyl-3HP-type have been isolated in significant amounts from human tissue, such as glycolaldehyde-pyridine from human atherosclerotic lesions (47). Moreover, lys-hydroxy-triosidines, another protein crosslink of the N-alkyl-3-HP-type was isolated from human cornea collagen exposed to the artificial tanning agent dihydroxyacetone (48) and is therefore expected to form in appreciable amounts in human skin exposed to this compound. The phototoxicity of 3-HP-derivatives as described in this study therefore raises the possibility that chemical tanning of human skin places a suspected photosensitizer in direct proximity to sensitive targets and adds to the increasing health concerns associated with the cosmetic use of
dihydroxyacetone preparations (61). Experiments using isolated AGE-products of the 3-HP type will allow testing their phototoxicity and to validate a potential involvement in AGEphtosensitization of human skin cells (14).

Future research will explore a functional involvement of the diverse members of the 3-HP group of endogenous photosensitizers in photodamage and carcinogenesis of UVA-irradiated human skin.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Endogenous and synthetic (hydroxy)-pyridine derivatives examined for phototoxicity. 1: pyridinoline (PYD), 2: desmosine (DES), 3: pyridoxylated lysine residue in a bovine serum albumin-vitamin B₆ conjugate (BSA-B₆), 4: pyridoxine (PN), 5: pyridoxamine (PM), 6: pyridoxal (PL), 7: pyridoxal-5’-phosphate (PLP), 8: 2-hydroxypyridine (2-HP), 9: 3-hydroxypyridine (3-HP), 10: 4-hydroxypyridine (4-HP), 11: N-ethyl-3-hydroxypyridinium cation (NE-3HP).

Figure 2. Pyridinoline and pyridoxine as photosensitizers of UVA-induced inhibition of skin cell proliferation and ROS formation. (A) Human skin fibroblasts (CF3) were exposed to UVA-irradiation (3.3 J/cm²) in the presence or absence of desmosine (DES), pyridinoline (PYD),...
or pyridoxine (PN; 500 µM, each) followed by a 30 min postirradiation incubation. Additionally, mock-irradiated cells were exposed to H₂O₂ (6 µM). After this time the cells were washed with HBSS, fresh DMEM was added, and cell number was determined 72 hr later by cell counting. Proliferation was compared to untreated cells (C). Cell proliferation was also assessed, when the experiment was performed in the presence of catalase (CAT, 400 u/mL), D-penicillamine (P, 10 mM), or NaN₃ (10 mM). (B) Test compounds in HBSS were exposed to UVA-irradiation as described above, but in the absence of cells. Formation of H₂O₂ was quantified in the absence or presence of catalase (CAT) as indicated in Materials and Methods.

**Figure 3. UVA-photosensitization of cultured human skin fibroblasts by biogenic hydroxy-pyridine derivatives.** CF3 fibroblasts were exposed to the combined action of UVA irradiation (3.3 J/cm²) and test compounds (B6 vitamers, protein-conjugated pyridoxine, desmosine, and pyridinoline, see Fig. 1). (A) Dose response of sensitized inhibition of proliferation using increasing concentrations of the indicated compounds. Proliferation was assessed three days after cell treatment by cell counting and normalized to control proliferation after UVA exposure in the absence of sensitizer. (B) Induction of apoptosis 24 h after exposure to the combined or isolated action of UVA and pyridoxal (PL) or pyridoxal-5’-phosphate (PLP, 100 µM each) in the absence or presence of D-penicillamine (P, 10 mM) as assessed by flow cytometric analysis of annexinV-PI stained cells. One representative experiment out of three similar repeats is shown. Numbers indicate percentage of total gated cells per single quadrant. (C) UV-absorption and (D) fluorescence spectrum of BSA (solid line) and pyridoxylated BSA (BSA-B₆) (broken line, 1 mg/mL PBS, each). (E) Fibroblasts were exposed to UVA (3.3 J/cm²) or mock-irradiation in the presence or absence of BSA-B₆ or control BSA (10 mg/mL, each). Inhibition of proliferation
was assessed by cell counting three days after cell treatment and compared to proliferation of untreated controls.

Figure 4. 3-Hydroxypyridine and N-alkyl-3-hydroxypyridinium cation as minimum sensitizer chromophores contained in pyridoxine and pyridinoline, respectively. Human keratinocytes (HaCaT) were exposed to UVA-irradiation (panels A and B; 2.3 J/cm² UVA) or SSL-irradiation (panel C and D; 120 mJ/cm² UVB and 2.3 J/cm² UVA) in the presence or absence of hydroxypyridine-derivatives (100 µM, each) followed by a 30 min post-irradiation incubation. Cell proliferation was assessed three days after treatment. (A) Inhibition of cell proliferation resulting from UVA-sensitization. (B) Induction of apoptosis, 24 h after exposure to the combined or isolated action of UVA and NE-3HP as assessed by flow cytometric analysis of annexinV-PI stained cells. One representative experiment out of three similar repeats is shown. Numbers indicate percentage of total gated cells per single quadrant. (C) Inhibition of cell proliferation resulting from SSL-sensitization. (D) Cell cycle analysis by flow cytometric analysis of cells stained with propidium iodide performed 24h after treatment. Histograms of a representative experiment are shown. The table summarizes results (% of total gated cells ± SD) from three independent experiments.

Figure 5. Induction of oxidative stress in cultured human skin cells resulting from 3-HP photosensitization. (A) Human keratinocytes (HaCaT) were exposed to UVA-irradiation (3.3 J/cm²) in the presence or absence of pyridoxine (PN, 100 µM) followed by loading with the intracellular redox dye DHR 123. Rhodamine 123-fluorescence intensity indicative of intracellular redox stress was then quantified by flow cytometric analysis. One representative
histogram out of three similar repeats is shown. (B) Photosensitized induction of p38 MAPkinase phosphorylation by the combined action of NE-3HP and UVA on cultured human skin cells. HaCaT keratinocytes and CF3 fibroblasts were treated with increasing doses of UVA (up to 9.9 J/cm²) in the absence or presence of NE-3HP (100 µM). 30 min after irradiation cells were lysed and analyzed by Western blotting using polyclonal anti-phospho p38 and anti-total p38 antibodies as described in Experimental Procedures. (C) Antioxidant suppression of sensitized inhibition of cell proliferation was examined by exposing CF3 fibroblasts to the combined action of 3-HP or NE-3HP (100 µM) and SSL (1.5 J/cm² UVA, 0.06 J/cm² UVB) in the presence or absence of antioxidants: D-penicillamine (P, 10 mM), mannitol (Man, 10 mM), deferoxamine mesylate (DFO, 1 mM), catalase (CAT, 800 u/ml), superoxide dismutase (SOD, 800 u/ml), potassium iodide (KI, 20 mM) and diazabicyclooctane (DABCO, 20 mM). Cell proliferation was assessed three days after treatment by cell counting. Penicillamine (P, 10 mM) was either present during cell irradiation in HBSS (termed ‘co’) or only after irradiation during 30 min post-irradiation incubation (termed ‘post’).

Figure 6. Induction and antioxidant modulation of protein photodamage sensitized by 3-HP-derivatives. Photosensitization of protein damage by 3-HP-derivatives was assessed using an RNase A photo-crosslinking assay. (A) RNase A (10 mg/mL PBS) was irradiated with solar simulated light (13.8 J/cm² UVA and 0.72 J/cm² UVB) in the absence (control, C) or presence of various hydroxypyridine-derivatives (500 µM each) and reaction aliquots were analyzed by 15% SDS-PAGE followed by Coomassie-staining and densitometric analysis of protein oligomerization; migration positions of monomer (M) and dimer (D) are indicated in panels B-D. (B) RNase photo-crosslinking examined as in panel A, comparing desmosine (DES),
pyridinoline (PYD), and 3-HP (500 µM, each). (C) 3-HP-sensitized protein photo-crosslinking was equally examined using increasing sensitizer concentrations (between 0 and 500 µM) activated by a fixed dose of SSL (13.8 J/cm² UVA, 0.72 J/cm²). (D) Pyridoxine-sensitized protein photocrosslinking was performed as in panel A in the presence of various antioxidants used in concentrations as in Figure 5 C. Potassium iodide (KI) was 20 mM.

**Figure 7. Peptide photooxidation sensitized by pyridoxine.** Mass spectrometric analysis of peptide photooxidation sensitized by pyridoxine (PN) and pyridinoline (PYD). The peptide melittin (1 mg/mL PBS) was SSL-irradiated (13.8 J/cm² UVA and 0.72 J/cm² UVB) in the presence or absence of PN or PYD (500 µM, each) followed by MALDI-TOF mass spectrometric analysis. Monoisotopic mass peaks are indicated.
Fig. 1
Fig. 2

A

Proliferation [% control]

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H₂O₂ [μM]

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UVA, AOX, H₂O₂
Fig. 3

A

B

control

PL

PLP

Annexin V-FITC
Fig. 3
**A**

![Graph showing rhodamine 123 counts across PN, UVA, and UVA+PN conditions.](image)

**B**

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| 0  | 1.1 | 3.3 | 9.9 |

**C**

![Bar graph showing proliferation (% control) for various conditions.](image)
3-hydroxypyridine chromophores are endogenous sensitizers of photooxidative stress in human skin cells

Georg T. Wondrak, Michael J. Roberts, Myron K. Jacobson and Elaine L. Jacobson

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