Identification of Quenchers of Photoexcited States as Novel Agents for Skin Photoprotection

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ABSTRACT

Photooxidative stress is a key mechanism in UVA-induced skin photodamage. Photoexcited states of endogenous UVA chromophores such as porphyrins, melanin precursors, and cross-link-fluorophores of skin collagen exert skin photodamage by direct reaction with substrate molecules (type I photosensitization) or molecular oxygen (type II), leading to formation of reactive oxygen species. Based on our previous research on the role of photoexcited states of endogenous skin chromophores as sensitizers of photooxidative stress, we describe here the identification of a novel class of chemopreventive agents for topical skin photoprotection: quenchers of photoexcited states (QPES). QPES compounds antagonize the harmful excited state chemistry of endogenous sensitizers by physical quenching, facilitating the harmless return of the sensitizer excited state to the electronic ground state by energy dissipation. To identify QPES compounds suitable for development, we designed a primary screening assay based on QPES suppression of photosensitized plasmid cleavage using conditions that exclude antioxidants. This screen is followed with a screen to test for nonsacrificial quenching of dye-sensitized singlet oxygen (1O2) formation by electron paramagnetic resonance detection of 2,2,6,6-tetramethyl-piperidine-1-oxyl, a stable free radical indicative of 1O2 formation. These initial screens identified a pyrrolidine pharmacophore with pronounced QPES activity, and L-proline and other noncytotoxic proline derivatives containing this pharmacophore were then screened for efficacy in cellular models of sensitized photodamage. These compounds showed QPES protection against dye-sensitized and psoralen-UVA-induced apoptosis and suppression of proliferation in cultured human skin keratinocytes and fibroblasts. Furthermore, QPES photoprotection of reconstructed full thickness human skin exposed to solar simulated light has been demonstrated.

The involvement of solar radiation in the pathogenesis of human skin damage is now firmly established. Most of the solar UV energy incident on human skin is in the deeply penetrating UVA region (>95% from 320 to 400 nm), and increasing experimental evidence supports a major role of UVA in skin photoaging and photocarcinogenesis (Kvam and Tyrrell, 1997; Agar et al., 2004). Our previous research has demonstrated a role of photoexcited states of endogenous skin chromophores (Wondrak et al., 2002a,b, 2003) in photooxidative stress, and photoexcited states of skin components and molecular oxygen are rapidly emerging as novel molecular targets for chemoprevention of actinic skin damage as summarized in Fig. 1.

Reactive oxygen species (ROS) and organic free radicals formed in skin during exposure to solar radiation are key mediators of skin photooxidative stress, and numerous interventional approaches, including the topical application of antioxidants, have therefore focused on these agents as potential therapeutic targets for skin photoprotection (Gasparro, 2000). Only recently, some of the molecular mechanisms upstream of ROS formation have been identified, and photosensitization by endogenous skin chromophores has emerged as a mechanism linking initial photon absorption with ROS formation in skin as illustrated in Fig. 1. Many

ABBREVIATIONS: ROS, reactive oxygen species; AGE, advanced glycation endproduct(s); ISC, intersystem crossing; 1O2, singlet oxygen; QPES, quencher of photoexcited states; l-His, L-histidine; BSA, bovine serum albumin; DMEM, Dulbecco’s modified Eagle’s medium; SSL, solar-stimulated light; TB, toluidine blue; OC, open circular; EPR, electron paramagnetic resonance; TEMPO, 2,2,6,6-tetramethyl-piperidine-1-oxyl; TEMP, 2,2,6,6-tetramethyl-piperidine; PBS, phosphate-buffered saline; HBSS, Hanks’ balanced salt solution; PI, propidium iodide; PUVA, psoralen-UVA; 8-MOP, 8-methoxypsoralen; L-Ala, L-alanine; L-Pro, L-proline; 4-OH-L-Pro, 4-hydroxy-L-proline; L-Pro-O-CH3, L-proline methyl-ester; 4-OH-L-Pro-OCH3, 4-hydroxy-L-proline methylester.
skin chromophores, including urocanic acid (Menon and Morrison, 2002), riboflavin, B2 vitamins (Wondrak et al., 2004), melanin precursors (Wenczel et al., 1998), and porphyrins are suspected endogenous photosensitizers (Dalle Carbonare and Pathak, 1992). Extracellular matrix proteins such as collagen and elastin, proteins present in large amount in skin and rich in advanced glycation end products (AGEs) and other cross-link fluorophores, have been identified as potent UVA sensitizers of photooxidative stress (Wondrak et al., 2002a,b, 2003). After initial photon absorption (Fig. 1), excited singlet states can either relax to the ground state without or with light emission (fluorescence) or undergo intersystem crossing (ISC) with formation of highly reactive biradical triplet states. Photoexcited states exert skin photodamage by direct reaction with substrate molecules, including DNA bases (type I photosensitization) and molecular oxygen (type II photosensitization), leading to ROS formation. Singlet oxygen (\(1O_2\)), an electronically excited, highly reactive form of molecular oxygen, is formed after energy transfer between the triplet photoexcited state of the sensitizer and ground state triplet oxygen. 1O2 is a widely accepted example of an excited state mediator of skin photodamage involved in UVA-induced mutagenesis, stress signaling, apoptosis, and remodeling of extracellular matrix components during skin photoaging and carcinogenesis (Klotz et al., 2003; Davies, 2004).

An emerging awareness of the necessity for adequate skin protection against UVA-induced photooxidative stress has lead to the development of topical broad-spectrum UV screens and antioxidants, intervening as depicted in Fig. 1 (Gasparrro, 2000). However, few phosetable chromophores for broadband UVA filtering are available (Maier et al., 2001). Many UVA screens are inefficient, particularly in the near visible UVA and blue visible regions important for skin photosensitization, and rapidly undergo photoisomerization/degradation due to uncontrolled excited state chemistry (Allen et al., 1996; Serpone et al., 2002). Paradoxically, some sunscreens and sun-blockers act as potent triplet state UVA sensitizers, enhancing light-driven formation of ROS and skin cell photodamage (Gulston and Knowland, 1999). Although moderate skin photoprotection by topical application of antioxidants has been demonstrated in many experiments on animal and human skin (Packer and Valacchi, 2002), the therapeutic effectiveness of skin administration of antioxidants is limited by their sacrificial depletion, their pronounced spontaneous redox chemistry, and their negative interference with the highly regulated skin antioxidant network (Meves et al., 2002). Harmful interaction of chemical antioxidants with essential redox signaling in human skin may be anticipated because recent reports point to a significant potential for antioxidant enhanced carcinogenesis in transgenic mice with up-regulated antioxidant responses (Lu et al., 1997).

Based on our previous research on the role of photoexcited states of endogenous skin chromophores as sensitizers of photooxidative stress, we describe here the development of a novel class of chemopreventive agents for topical skin photoprotection. These agents do not act by photon absorption or antioxidant mechanisms, and thus we termed them quenchers of photoexcited states (QPES). QPES are direct molecular antagonists of photoexcited states of endogenous skin chromophores and molecular oxygen as illustrated in Fig. 1. QPES eliminate the harmful excited state chemistry of endogenous skin sensitizers by physical pathways such as energy or reversible charge transfer interactions, facilitating the harmless dissipation of photoexcitation energy. QPES compounds suppress skin photooxidative damage upstream of ROS formation, inhibiting their formation by suppression of photosensitization reactions. Importantly, the use of currently available quencher substances such as NaN3, 1,4-diazabicyclooctane, and L-histidine (L-His) for skin photoprotection does not seem feasible due to their toxicity and/or photostability (Beutner et al., 2000).

In this study, we tested the feasibility of rapid screening and identification of biocompatible physical QPES and present evidence that significant photoprotection of cultured human skin cells and reconstructed human skin can be achieved using newly identified prototype QPES compounds.

Materials and Methods

Chemicals. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Preparation of AGE-Bovine Serum Albumin (BSA). BSA modified with AGE (AGE-BSA) was prepared and characterized as described previously (Wondrak et al., 2002a).

Cell Culture. The established cell line of human epidermal keratinocytes (HaCaT cells), a gift from Dr. Norbert Pusenig (German Cancer Research Center, Heidelberg, Germany), and human dermal fibroblasts (CF3 cells), a gift from Dr. Robert Dell’Orco (Noble Center for Biomedical Research, Oklahoma City, OK), were routinely cul-
tured in DMEM containing 10% fetal bovine serum and kept in a humidified atmosphere containing 5% CO₂ at 37°C.

Irradiation. A kilowatt large area light source solar simulator (Model 91293; Oriel Corporation, Stratford, CT) was used, equipped with a 1000-W Xenon arc lamp power supply (Model 68980) 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 (Model IL1700; International Light Inc., Newburyport, MA), with an SED240 detector for UVB (range 265–310 nm, peak 285 nm) or an 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, solar-stimulated light (SSL) dose was 7.63 mJ/cm²·s·UV A radiation. Using a UVB/C blocking filter, the dose at 365 mm from the source was 5.39 mJ/cm²·s·UV B radiation with a residual UVB dose of 3.16 μJ/cm²·s·UV C.

For toluidine blue (TB) photosensitization, a Sylvania 15-W Cool White light tube was used delivering visible light at an irradiance of 4.29 mW/cm². The irradiance in the visible region (400–700 nm) was determined using spectroradiometer, model 754, from Optronic Laboratories (Orlando, FL). Cells received visible radiation at a distance of 50 mm from the source through the polystyrene lids of cell culture dishes.

Plasmid DNA Cleavage Assay. AGE-sensitized plasmid DNA cleavage was performed essentially as described previously (Wondrak et al., 2002a). DNA strand breakage was measured by the conversion of supercoiled ΦX-174 RP1 double-stranded DNA (SC-DNA) (New England Biolabs, Beverly, MA) to open circular form (OC). A single-strand break in SC-DNA results in the formation open circular DNA (OC-DNA), displaying retarded migration in agarose gel electrophoresis. Inhibitory activity of a compound on AGE-sensitized plasmid DNA cleavage was assessed performing the SSL irradiation (4.58 J/cm² UV A and 0.24 J/cm² UV B) in the presence of the compound using an AGE-BSA preparation glycated for 4 weeks (10 mg/ml). The inhibitory activity was calculated using the following formula:

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\text{Inhibition} = \left(1 - \frac{\% \text{ OC-DNA (AGE/UV/compound) - } \% \text{ OC-DNA (untreated)}}{\% \text{ OC-DNA (AGE/UV) - } \% \text{ OC-DNA (untreated)}}\right) \times 100
\]

with (AGE/UV) indicating the complete DNA cleavage system with sensitizer plus irradiation in the absence of test compound, (untreated) indicating the cleavage system without irradiation, and (AGE/UV/compound) indicating the complete cleavage system in the presence of test compound.

Singlet Oxygen Quenching Assay. The method is based on electron paramagnetic resonance (EPR) detection of 2,2,6,6-tetramethyl-piperidine-1-oxyl (TEMPO), a nitroxide stable free radical (Lion et al., 1976), formed after reaction of photochemically generated O₂⁻ with 2,2,6,6-tetramethylpiperidine (TEMP). TB sensitized formation of a TEMP signal is therefore indicative of O₂⁻ generation, and a quencher is identified by its ability to suppress the formation of the TEMPO signal during dye sensitization. Instrument settings are nominal microwave power, 2 mW; modulation amplitude, 0.4 mT; time constant, 81.92 ms; sweep time, 167.8 s; and number of scans, 4. The complete reaction contained TB, 100 μM; TEMP, 7 mM; and QPES test compound, 20 mM. All solutions were prepared in phosphate-buffered saline (PBS). The reaction tube (quartz capillary tube, 100-μl volume, 1.5 x 90 mm) was irradiated with visible light (1.3 J/cm² total dose, 5-min irradiation time), and the TEMPO signal was measured immediately after irradiation.

Amino Acid Analysis. Amino acid destruction by TB photosensitization was examined after derivatization of a reaction aliquot with 6-aminooquinolyl-N-succinimidylcarbamate using a commercial AccQTag analysis kit according to the manufacturer (Waters, Milford, MA). The derivatized amino acid was chromatographed using a reversed-phase-high-performance liquid chromatography system (C18-μBondapak column, operated isocratically with methanol (25%)/KH₂PO₄, 50 mM, pH 5 (75%/)) equipped with a fluorescence detector set to excitation wavelength 250 nm/emission wavelength 395 nm.

Skin Cell Photoprotection Assay. Photosensitized suppression of skin cell proliferation and its modulation by test compounds was assessed as follows: CF3 fibroblasts or HaCaT keratinocytes were seeded at 5 × 10⁴ cells/dish on 35-mm dishes. After 24 h, cells were washed with Hank's buffer salt solution (HBSS) and immediately treated with the combined action of visible light (0.4 J/cm²) in the presence or absence of TB (3.3 μM in HBSS) as sensitizer dye and various concentrations of QPES test compounds. Total time between addition of QPES test compound/TB sensitizer in the dark and light exposure was kept below 10 s. These minimal preincubation times with sensitizer and QPES test compound before cell irradiation were chosen to reduce the effects of differential cellular uptake of sensitizer and various QPES test compounds on photoprotective efficacy. After 5-min incubation in the dark after irradiation, the cells were washed with HBSS, and fresh culture medium was added. Cell number was determined 72 h later using a Coulter counter.

Cell Death Analysis. Photodynamic induction of cell death was confirmed by annexin-V-fluorescin isothiocyanate/propidium iodide (PI) dual staining of cells followed by flow cytometric analysis. CF3 fibroblasts (200,000) were seeded on 35-mm dishes and 24 h later exposed to visible light (1.3 or 1.8 J/cm²) in the presence of TB (3.3 μM in HBSS) and various concentrations of QPES test compounds as described for the skin cell photoprotection assay above. Cells were harvested 24 h after treatment, and cell staining was performed using an apoptosis detection kit according to the manufacturer’s specifications (APO-AF, Sigma-Aldrich). For psoralen-UVA treatment (PUVA), cultured human HaCaT keratinocytes were exposed to the isolated or combined action of UV A (3.3 J/cm² total dose) and 8-methoxypsoralen (8-MOP, final concentration 0.3 μM in PBS, using a stock solution of 8-MOP in dimethyl sulfoxide) called “PUVA” in the presence or absence of l-Pro (25 mM) called “QPES”. Total time between addition of QPES test compound/8-MOP sensitizer in the dark and light exposure was kept below 10 s. After 20-min incubation in the dark after irradiation, the cells were washed with PBS, and fresh culture medium was added. Photodynamic induction of cell death was examined 24 h after exposure as described above.

Photoprotection of Full Thickness Human Reconstructed Skin. Full thickness human skin was reconstructed following a published procedure (Schoop et al., 1999). In brief, using acid-soluble collagen, type I, from rat tail tendon in 0.1% acetic acid (BD Biosciences, San Jose, CA), collagen gels were prepared by mixing 12 mm of ice-cold acidic collagen solution (4 mg/ml) with 1.5 ml of 10× concentrated HBSS at 4°C. After neutralization with 270 μl of 1 N NaOH, 1.5 ml of fetal bovine serum containing human dermal fibroblasts (6 × 10⁶ cells) was added, and 2.2-ml aliquots of this solution was poured onto six filter inserts (polycarbonate, 3-μm pore size, Biocoat; BD Biosciences). Then were transferred to a six-well plate. After dermal polymerization, glass rings (20 mm in diameter) were applied onto the gels to prevent epithelial outgrowth to the filter walls. Collagen lattices were then covered with medium consisting of DMEM containing 10% fetal bovine serum and 50 μg/ml ascorbic acid, and the same medium was added to the bottom compartment of the insert. The following day, the top medium was removed and HaCaT keratinocytes (1 × 10⁵/insert) were seeded on top of the dermal reconstruct. After another day, reconstructs were exposed to air by removal of the top medium and then fed by capillarity from the bottom compartment of the insert using DMEM, 10% bovine calf serum supplemented with ascorbic acid (50 μg/ml). The bottom medium was changed every second day. After 20 days, the reconstructs received treatment with test compound followed by...
irradiation. Twenty-four hours later reconstructs were harvested, fixed in formalin, paraffin-embedded, and processed for hematoxylin/eosin staining of this section (4.5 μm in thickness) (Bernerd et al., 2000).

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

Results

QPES Screen 1: Suppression of AGE-Photosensitized DNA Damage by Nonantioxidant Test Compounds. In a recent study on the genotoxicity of glycated proteins as endogenous photosensitizers accumulating in human skin during chronological and actinic aging (Wondrak et al., 2002a), a simple plasmid DNA cleavage assay was established to elucidate the mechanism of phototoxicity of AGE pigments under solar UV irradiation. As demonstrated in Fig. 2 A, photodynamic DNA strand breakage occurred as a result of the combined exposure to glycated bovine serum albumin (AGE-BSA) and solar simulated UV irradiation, measured by the conversion of supercoiled φX 174 plasmid DNA to OC form (approximately 40% of total DNA) that displays retarded mobility in agarose gel electrophoresis. In contrast, no cleavage was observed when DNA was incubated with AGE protein in the absence of irradiation or when irradiation was performed in the absence of sensitizer protein. Importantly, AGE sensitization of DNA damage was shown to proceed equally well under aerobic and nonaerobic conditions, and thus antioxidants such as catalase and superoxide dismutase had little effect on DNA cleavage by AGE sensitization, whereas excited state quenchers such as NaN₃, KI, and organic thiol compounds were strongly protective when present during irradiation, either in the absence or presence of oxygen (Rougee et al., 1988; Beutner et al., 2000). Based on these earlier findings, DNA protection from AGE photosensitization by test compounds present during irradiation was used as a broad initial screen for the identification of agents capable of interfering with sensitized photodamage by mechanisms different from antioxidant or UV screening activity. A typical screening gel obtained from 11 representative test compounds is shown in Fig. 2B. Compound 7 (zinc acetate) interfered with DNA migration and was excluded from further screening. Compound 8 (nicotinamide-N-oxide) increased the observed DNA photodamage beyond the basal level of AGE-sensitized DNA cleavage, providing an example of the screen’s capability to recognize test compounds with intrinsic phototoxicity to be excluded from further screening, thereby meeting a necessary prerequisite of successfully screening photoprotective agents. Compounds 1 (NaCl), 3 (mannitol), 4 (MgCl₂), and 9 [L-alanine (l-Ala)] provided little or no protection, whereas strong protection was observed with test compounds 2 (NaN₃), 5 (D-penicillamine), 6 (NaSCN), 10 (pyrrolidine), and 11 [L-proline (l-Pro)]. DNA protection occurred in a dose-dependent manner as shown in Fig. 2C for compounds 5 and 6. The protective effects observed with pyrrolidine and L-Pro are consistent with earlier reports on quenching of ¹O₂ and other excited states by...
secondary amines, presumably via reversible charge transfer mechanisms (Young et al., 1973). In an attempt to identify biocompatible, nontoxic candidate quencher compounds, a number of proline derivatives were tested for protective activity against AGE-photosensitized DNA damage as summarized in Fig. 2D. In this case, SSL irradiation was performed under strictly anaerobic conditions to further evaluate the ability of the screen to select for test compounds active by nonantioxidant mechanisms of action. However, strict anaerobic conditions were not necessary for identification of active compounds, since equal protective effects were observed under aerobic and anaerobic conditions (as demonstrated for L-proline, active in Fig. 2, B and D), consistent with the documented coexistence of oxygen-dependent and -independent pathways of AGE-sensitized DNA cleavage downstream of AGE photoexcitation (Wondrak et al., 2002a). Consistent with pyrrolidine as the minimum pharmacophore responsible for nonantioxidant suppression of AGE photosensitization, compounds 12 (4-hydroxy-L-proline [4-OH-L-Pro]), 13 (L-proline methylester [L-Pro-O-CH₃]), and 14 (4-hydroxy-L-proline methylester [4-OH-L-Pro-O-CH₃]) were all as protective against AGE photosensitization of DNA damage as L-proline. Based on these data, we conclude that rapid in vitro analysis by high-pressure liquid chromatography was performed with L-Pro, L-Pro-O-CH₃, and L-His before and after prolonged exposure to dye sensitization as shown in Fig. 3C. The probe was stable with all compounds used in Fig. 3, D to G, confirming quenching of O₂ as their likely mechanism of action. To examine the chemical stability of the test compound during quenching, thus demonstrating a physical, nonsacrificial mechanism of action, amino acid analysis by high-pressure liquid chromatography was performed with L-Pro, L-Pro-O-CH₃, and L-His before and after prolonged exposure to dye sensitization as shown in Fig. 3, H to J. Direct evidence for physical quenching was obtained for L-Pro and L-Pro-O-CH₃, which were stable during prolonged dye sensitization, whereas pronounced losses were observed with L-His, consistent with a sacrificial mechanism of action reported previously for this substance (for review, see Davies, 2004). Based on this screen, we conclude that the proline derivatives identified as inhibitors of AGE photosensitization in screen 1 are capable of nonsacrificial physical quenching of photoexcited states.

**QPES Screens 3A and B: Protection of Cultured Human Skin Cells against O₂-Induced Inhibition of Pro-**

Fig. 3. QPES screen 2. TEMPO-EPR assay. The TEMPO free radical signal indicative of O₂ is quantitatively detected by EPR measurement after visible light irradiation (1.3 J/cm² total dose) of TB in PBS in the absence or presence of QPES test compounds. A, positive control, TEMPO signal generated after irradiation of TB in the presence of TEMP. B, standard TEMPO signal obtained from commercial TEMP (2 μM). C, sample (B) after prolonged incubation with L-Pro (20 mM). D to G, suppression of TB-sensitized TEMPO signal in the presence of QPES test compounds (20 mM, each): D, NaN₃; E, L-Pro; F, L-Pro-O-CH₃; G, L-His; and H to J, amino acid analysis before (front tracing) and after (back tracing) prolonged visible light irradiation (7.8 J/cm² total dose) of TB in the presence of L-Pro (H), L-Pro-O-CH₃ (I), and L-His (J). Retention times are given in minutes. i.s. indicates the position of the derivatization reagent used as internal standard.
liferation and Induction of Apoptosis. In an attempt to demonstrate cellular photoprotection by QPES compounds identified in screens 1 and 2, cultured human skin fibroblasts (CF3) were exposed to photooxidative stress from $^{1}\mathrm{O}_2$ formed in situ by dye sensitization in the absence or presence of test compounds and cell proliferation was determined (screen 3A). Fibroblasts were treated with the combined action of light (visible light; 0.4 J/cm² total dose) in the presence or absence of TB (3.3 μM) as the sensitizer dye. For the sake of technical simplicity, screening was performed using minimal preincubation times with QPES test compound and TB sensitizer, known to accumulate in lipophilic cellular compartments (Tremblay et al., 2002), before cell irradiation, thereby reducing the effects of differential cellular uptake of sensitizing and various QPES test compounds on photoprotective efficacy. As shown in Fig. 4A, cell proliferation was strongly suppressed by the combined action of light and sensitizer, whereas the action of light or sensitizer alone did not exert antiproliferative effects.

Protection was observed when cells received photodynamic treatment in the presence of L-Ala, also negative in both screens 1 and 2. In contrast, NaSCN, positive in screens 1 and 2 was not active, demonstrating the importance of viable cell test systems for the validation of photoprotective activities of test compounds obtained in chemical assays. In contrast, significant protection was observed with L-Pro (60% protection), and complete protection against the antiproliferative effects of photodynamic treatment was achieved with L-Pro-O-CH₃, suggesting an enhanced protective potency of the more lipophilic methyl ester derivative. Similar potency was observed with 4-OH-L-Pro-O-CH₃ compared with 4-OH-L-Pro (data not shown).

To examine the relative photoprotective potency of various test compounds in our dye sensitization assay, a comparative dose response for the suppression of $^{1}\mathrm{O}_2$-mediated inhibition of proliferation by doses between 1 and 50 mM was established as shown in Fig. 4B. At a light dose of 0.4 J/cm², the concentration of protective agent necessary to achieve 50% proliferation ($\text{ED}_{50}$ ± S.D.) in photosensitized cells, normalized to 100% proliferation of unirradiated control cells and no proliferation of fully sensitized irradiated cells, was calculated by extrapolation from proliferation-inhibition curves for L-Pro (39.0 ± 6.8 mM), L-Pro-O-CH₃ (1.6 ± 0.5 mM), and L-His (0.8 ± 0.3 mM); whereas no significant protection could be achieved with L-Ala. Similar results were obtained when protection against TB photosensitization was examined in HaCat keratinocytes (data not shown).

At light doses approximately 5 to 10 times the dose used for suppression of proliferation, pronounced induction of cell death was observed in CF3 fibroblasts exposed to photodynamic treatment (screen 3B). Photosensitized fibroblasts exhibited pronounced morphological changes characterized by cell rounding, membrane blebbing, and cytoplasmic condensation consistent with photodynamic induction of apoptosis/necrosis, all observed by light microscopy within 24 h after treatment as shown in Fig. 5A. Induction of cellular apoptosis was confirmed by annexin-V-fluorescein isothiocyanate/PI staining followed by flow cytometric analysis performed 24 h after cell photosensitization (Fig. 5A). Cells in early apoptosis [annexinV⁺/PI⁰] and late apoptosis/necrosis [annexinV⁺/PI⁺] were identified starting 6 h after treatment (data not shown), and 24 h later only 35% of cells remained viable. These changes were not observed with administration of photosensitizer in the dark or in the presence of light alone. When the photodynamic treatment of fibroblasts was repeated in the presence of a QPES prototype, NaN₃, an established excited state and $^{1}\mathrm{O}_2$ quencher, significant cell protection allowing over 70% viable cells was observed both by light microscopy and flow cytometry, demonstrating the feasibility of this assay for the identification of compounds for cell protection against photosensitization. Next, the fluence of visible light was adjusted to achieve skin cell viability of 50% 24 h after photodynamic treatment and the assay was used for the screening of potential QPES compounds as shown in Fig. 5B. L-Ala was without significant protective effect even at the highest concentrations. The following compounds showed protective activity with potency decreasing in the order NaN₃ >> L-Pro-O-CH₃ > L-His > L-Pro. NaN₃ provided the strongest photoprotection in the dose range between 1 and 50 mM, and only at 1 mM was there detectable induction of death in a minor fraction of cells. At doses of 10 and 50 mM, L-Pro-O-CH₃ and L-His strongly suppressed photodynamic induction of fibroblast death, with 92% cell viability at 50 mM test compound. At 10 mM test compound, L-Pro-O-CH₃ affected slightly better photoprotection than L-His, probably due to the differential mode of $^{1}\mathrm{O}_2$-quenching and chemical instability of the latter as elaborated above. However, the observed relative photoprotective potency of specific QPES test compounds does only apply to our specific

Fig. 4. QPES screen 3A. Photoprotection of cultured human skin cells against photodynamic inhibition of proliferation. A, cultured human skin fibroblasts were exposed to the combined action of visible light (0.4 J/cm²) in the presence or absence of TB (3.3 μM) as sensitizer and various QPES test compounds (50 mM). Three days after treatment, cell proliferation was quantified by cell counting and normalized to proliferation of untreated controls. B, comparative dose response for the suppression of inhibition of proliferation by QPES at doses between 1 and 50 mM. Photosensitization was performed as in A. Cell proliferation was quantified by cell counting and normalized to 0% proliferation (sensitized cells) and 100% proliferation (untreated controls). *, p < 0.05; **, p < 0.01; and ***, p < 0.001.
cellular TB sensitization assay and will be influenced greatly by many other parameters, such as cell type, chemical nature of the sensitizer, the kinetics of cellular uptake and distribution, and colocalization of sensitizer and test compound.

To provide further evidence for the therapeutic potential of QPES compounds of the pyrrolidine type to antagonize photosensitized skin cell damage, the efficacy of L-Pro to suppress photodynamic cell kill of cultured human skin cells resulting from photosensitization by another sensitizer system, structurally unrelated to TB, was tested. PUVA treatment of HaHaT keratinocytes was chosen as another established model system of skin cell photosensitization (Carraro and Pathak, 1998). In addition to type I anoxic reactions targeting nuclear DNA leading to cross-linking, PUVA treatment is known to induce a type II oxygen-dependent photodynamic reaction with formation of $^1$O$_2$. As shown in Fig. 5C, L-proline, when present during UVA irradiation, strongly suppressed 8-MOP-induced photodynamic elimination of HaCaT keratinocytes, providing experimental evidence that QPES photoprotection using pyrrolidine derivatives can antagonize cellular PUVA damage. Based on these data demonstrating skin cell protection against photodynamic action of diverse sensitizers activated by visible light and UVA, we conclude that the feasibility of achieving cell protection by molecular antagonism of photosensitization using QPES agents has been demonstrated. L-Pro, L-Pro-OCH$_3$, and other L-Pro derivatives that retain the pyrrolidine-pharmacophore are suitable as novel, biocompatible QPES compounds for skin cell photoprotection.

Photoprotection of Reconstructed Full Thickness Human Skin by a Prototype Quencher of Photoexcited States. Next, we sought to obtain proof of principle evidence for the feasibility of achieving skin photoprotection by topical application of a biocompatible quencher of photoexcited states, active in all three QPES screens described above. To this end, a simplified model of full thickness human skin was created by embedding fibroblasts in a collagen matrix followed by epidermal reconstruction (Schoop et al., 1999) using immortalized HaCaT keratinocytes as shown in Fig. 6, left. A moderate dose of solar simulated light, well below the dose known to induce the sunburn response in reconstructs (Bernerd et al., 2000), clearly induced actinic damage in this human skin model as obvious from loss of epidermal cellularity and dermal disintegration 24 h after irradiation (Fig. 6, middle, arrows). Epidermal apoptosis as usually observed during actinic damage in intact human skin was not detected as indicated by the absence of sunburn cells from our irradiated prototype reconstructs exposed to moderate doses of SSL as shown in Fig. 6 (middle). Further experiments measuring cell death by flow cytometry after cell retrieval from skin reconstructs confirmed that photodamage in reconstructs was mainly due to actinic induction of cellular necrosis, which could be suppressed when catalase was included during dermal reconstruction (data not shown). Actinic skin damage in this reconstruct therefore seemed to depend on the induction of photooxidative stress by collagen-mediated UVA/B photosensitization forming H$_2$O$_2$ as observed previously (Wondrak et al., 2003). Consistent with photosensitized pathways of actinic damage, a significant suppression of epidermal and dermal photodamage was observed when solar irradiation of the skin equivalent was performed in the presence of the prototype QPES compound L-Pro, not an antioxidant, as

Fig. 5. QPES screen 3B. Photoprotection of cultured human skin cells against photodynamic induction of apoptosis. A, cultured human skin fibroblasts were exposed to the combined action of visible light (1.8 J/cm$^2$ total dose) in the presence of TB (3.3 μM) called photosensitization and NaN$_3$ (1 mM) called QPES. Twenty-four hours after exposure, cells were examined by light microscopy (left) or flow cytometric analysis of annexin-V-PI-stained cells (right). B, dose-response analysis of suppression of photodynamic induction of apoptosis performed as in A by using a reduced light dose (1.3 J/cm$^2$) by flow cytometric analysis of annexin-V-PI stained cells treated with various QPES test compounds during irradiation. C, cultured human HaCaT keratinocytes were exposed to the isolated or combined action of UVA (3.3 J/cm$^2$ total dose) and 8-MOP (0.3 μM) called PUVA in the presence or absence of L-Pro (25 mM) called QPES. Twenty-four hours after exposure, photodynamic induction of cell death was examined by flow cytometric analysis of annexin-V-PI-stained cells. One representative experiment out of three similar repeats is shown. Numbers indicate percentage of total gated cells per single quadrant.
shown in Fig. 6, right. With l-Pro treatment, preserved epidermal thickness and cellularity and suppression of dermal disintegration were observed 24 h after irradiation of the reconstructs. Based on these prototype skin photoprotection experiments, we conclude that l-Pro and possibly other related QPES compounds may suppress actinic skin cell damage in human skin equivalents exposed to solar simulated UV radiation by suppression of collagen-sensitized photooxidative stress.

### Discussion

Photoexcited states of endogenous photosensitizers and molecular oxygen are promising targets for skin photoprotection (Dalle Carbonare and Pathak, 1992; Gasparro, 2000; Wondrak et al., 2003). Previous studies have mainly focused on the use of experimental $^1O_2$ quenchers as molecular probes for the involvement of $^1O_2$ in cellular photocytotoxicity (Beutner et al., 2000; Klotz et al., 2003), and little effort has been undertaken to develop QPES compounds for skin photoprotection that target triplet photoexcited states upstream of $^1O_2$ (Sima and Kanofsky, 2000). However, lutein and other carotenoid derivatives are currently being explored as $^1O_2$ quenchers for skin photoprotection (Sies and Stahl, 2004), and oral carotenoids are in clinical use for the suppression of photooxidative stress involved in photoaging and carcinogenesis by solar UVA radiation. In QPES screen 1, the suppression of AGE photosensitization of DNA damage depends on a complex mixture of protein-bound sensitizer chromophores mimicking AGE epitopes, suspected key photosensitizers accumulating in chronologically and photoaged human skin (Jeanmaire et al., 2001). Due to the oxygen-independent mechanism of AGE-sensitized DNA damage, compounds positive in this assay most likely interfere with AGE photosensitization by excited state quenching as reported previously (Wondrak et al., 2002a) and compounds that exert photoprotection merely by antioxidant or other physicochemical effects such as osmolytic activity can be excluded.

Compounds positive in QPES screen 1 are further examined in QPES screen 2, which detects the ability of a test compound to suppress the formation of a $^1O_2$ EPR signature signal during dye sensitization of TEMP. TB is used as a sensitizer dye since it is also used in QPES screen 3, but other $^1O_2$ sensitizers such as hematoporphyrin, methylene blue, and rose bengal could also be used in this assay (Ando et al., 1997). QPES screen 2 provides unequivocal evidence that the compound identified in screen 1 antagonizes $^1O_2$ as an excited state molecule with proven involvement in solar UV photodamage. Moreover, as demonstrated with proline derivatives and l-His shown in Fig. 3, physical and chemical quenching can be distinguished if desired. The results of QPES screens 1 and 2 provide combined evidence that a compound is an antagonist of photosensitization (positive in screen 1 and/or screen 2), that a compound acts as $^1O_2$ quencher (positive in screen 2), and whether other mechanisms of excited state antagonism are involved (positive in screen 1, and positive or negative in screen 2). Compounds that are positive in screen 1, but ineffective in screen 2, would still be moved to cellular testing in screen 3, since a compound may interfere with AGE photosensitization, but it may be ineffective as a $^1O_2$ quencher.

QPES screen 3 evaluates the potential of a test compound to protect cultured human skin cells against photosensitized damage by suppression of inhibition of proliferation and induction of cell death after photodynamic treatment using the cationic phenothiazinium-type $^1O_2$ sensitizer TB, previously shown to induce apoptosis upon photodynamic treatment of Jurkat cells (Tremlay et al., 2002). Quantitative evaluation of photoprotective activity is limited to screen 3, since positive activity in screens 1 and 2 does not necessarily translate into skin cell protection in screen 3, as exemplified by the screening results obtained with NaSCN.
Using this screening method, pyrrolidine and the pyrrolidine derivatives L-Pro, 4-OH-L-Pro, L-Pro-O-CH₃, 4-OH-L-Pro-O-CH₃, and L-Pro-NH₂, all containing a free secondary amine pharmacophore, were identified as photostable QPES compounds, active in screens 1 and 2 as shown in Fig. 2 and 3. Our results obtained with L-Pro in screen 2 are consistent with another report describing ¹O₂ antagonism by L-Pro in vitro (Alia and Matysik, 2001). Moreover, strong photoprotection of cultured human skin cells and human skin reconstructs was achieved using millimolar concentrations of pyrrolidine derivatives as shown in Figs. 4 to 6, suggesting their potential usefulness as biocompatible, photoprotective agents in topical formulations. However, due to the structural variety, complex mechanistic nature, and heterogeneous localization of endogenous skin photosensitizers, various QPES optimized to target and antagonize specific sensitizers may be needed in parallel to ensure effective skin photoprotection against photosensitization. The suppression of PUVA-induced photodynamic kill of HaCaT keratinocytes by L-Pro strongly suggests that the identified pyrrolidine pharmacophore effectively antagonizes the phototoxic activity of various structurally unrelated photosensitizers, such as TB and 8-MOP. It remains to be determined whether the differential photoprotective efficacy observed with various QPES compounds as described in Fig. 4B results from chemical differences affecting QPES potency or from differential cellular uptake and distribution affecting colocalization of sensitizers and QPES compound.

Many organic secondary and tertiary amines are established state quenchers (Young et al., 1973) known to interfere with ¹O₂ in cellular systems: 1,4-diazabicyclooctane is an established molecular probe for ¹O₂ involvement in biological processes (Beutner et al., 2000), and cellular polyamines such as spermine have been proposed as endogenous antioxidants and ¹O₂ quenchers (Khan et al., 1992). However, cellular toxicity and chemical instability of these agents precludes their therapeutic use for skin photoprotection. For pyrrolidine, the quencher pharmacophore contained in L-Pro and other active derivatives identified in this study, appreciable singlet oxygen quenching rates (reaching 1.4 × 10⁸ M⁻¹ s⁻¹ in a rose bengal/acetonitrile system) have been reported (Clennan et al., 1989). According to the accepted mechanism of ¹O₂ deactivation by organic amines, the radiationless deactivation of the initially formed singlet encounter complex is enhanced by the formation of a singlet exciplex, which is stabilized by the transfer of electric charge from the quencher to the oxygen molecule (Schweitzer and Schmidt, 2003). This species decays mainly by intersystem crossing to a triplet charge transfer ground state complex, which finally dissociates to the ground state amine and oxygen without charge separation in most cases. Indeed, nonsacrificial physical deactivation of ¹O₂ by the pyrrolidine QPES test compounds L-Pro and L-Pro-O-CH₃ is strongly suggested by the results obtained in QPES screen 2 as summarized in Fig. 3. In secondary amines, low ionization potential and hydrogen bond donating activity correlate with higher quenching activities, but the question of whether the enhanced photoprotective potency of L-Pro-O-CH₃ compared with L-Pro is due to changes in these parameters cannot be answered at this point and will require comparative determination of ¹O₂ quenching rate constants in various solvents (Clennan et al., 1989). Additionally, the enhanced cellular photoprotection achieved with L-Pro-O-CH₃ may result from increased lipophilicity and membrane partitioning of the compound, since TB sensitization of ¹O₂ formation occurs primarily in lipophilic compartments such as cell membranes (Tremblay et al., 2002). Indeed, methyl-esterification of L-Pro increases the log p partition coefficient of the compound by two orders of magnitude as calculated by Krippen’s fragmentation (Casas and Batlle, 2002; data not shown). Importantly, the finding that L-Pro-alkyl-esterification enhances the photoprotective bioactivity of L-Pro opens an opportunity for the molecular design of proline ester derivatives optimized for topical application to skin with prolonged residence time and/or penetration into deeper layers of skin based on lipophilic partitioning. Lipophilic targeting based on partition coefficient modulation by compound esterification circumvents the delivery problems associated with topical application of many strongly hydrophilic nutrients and therapeutics to skin, as successfully performed with enhanced topical delivery of nicotinic acid (Jacobson et al., 2000) and 5-aminovalinic acid (Casas and Batlle, 2002).

The role of photoexcited states in skin photodamage and carcinogenesis suggests that direct molecular antagonism of photooxidative stress by physical QPES compounds has the potential to reduce skin photocarcinogenesis and photoaging. According to the accepted importance of UVA irradiation in sensitized skin photodamage, QPES are predicted to be most efficacious against photodamage caused by UVA irradiation and are therefore intended for combinatorial use with existing agents for skin photoprotection, especially antioxidants and UV screens. Thus QPES could be functionally synergistic additives in existing sunscreen formulas and may provide other beneficial effects such as enhancement of photostability of sunscreens (Chatelain and Gabard, 2001). Moreover, topical QPES substances could limit the detrimental effects of long-lasting systemic photosensitizers used in photodynamic therapy of malignancies (Rougee et al., 1988).

The rapid identification of biocompatible QPES antagonists of AGE sensitization and singlet oxygen presented here provides a prototype approach for the future development of QPES into a novel class of chemopreventive agents for targeted skin photoprotection.

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References


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