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Cell-impermeant pyridinium derivatives of psoralens as inhibitors of keratinocyte growth

Thomas M. Mariano[a], Anna M. Vetrano[a], Shannon L. Gentile[a], Diane E. Heck[b], Marilyn S. Whittemore[c,d], Christophe D. Guillon[c], Ivan Jabin[c], Robert D. Rapp[c], Ned D. Heindel[c], Jeffrey D. Laskin[a,*]

[a]*Department of Environmental and Community Medicine, UMDNJ-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854, USA*
[b]*Department of Pharmacology and Toxicology, Rutgers University, Piscataway, NJ 08854, USA*
[c]*Department of Chemistry, Lehigh University, Bethlehem, PA 18015, USA*
[d]*Buckman Laboratories, Memphis, TN 38108, USA*

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Abstract

Psoralens such as 8-methoxypsoralen and 4,5′,8-trimethylpsoralen (TMP) are used in photochemotherapy for the treatment of a variety of epidermal proliferative diseases. Sequential treatments of the skin with psoralens plus ultraviolet light in the range of 320–400 nm (UVA light), referred to as PUVA therapy, results in the suppression of abnormal keratinocyte growth. With the recognition that the psoralens are phototoxic and carcinogenic, presumably due to their ability to intercalate into DNA and photo cross-link pyrimidine bases following UVA light activation, it is clear that the development of biologically active analogs lacking this activity would be of significant therapeutic benefit. Towards this goal we have characterized active 4′- and 5′-pyridinium derivatives of 4′,5′-dihydro-TMP (H_2TMP), a psoralen analog that does not form DNA cross-links. These analogs, which are charged at physiological pH and cannot penetrate cells, are unique in that they retain biological activity as inhibitors of keratinocyte cell growth when activated by UVA light. However, they do not appear to cross-link or damage DNA as determined by plasmid DNA unwinding and nicking experiments, in intact cells using fluorescent analysis of DNA unwinding assays, and by thymidine uptake studies. Reverse transcription–polymerase chain reaction and western blotting demonstrated that, unlike TMP and H_2TMP, when activated by UVA light, the pyridinium derivatives were not inhibitors of transcription since interferon-γ-inducible nitric oxide synthase mRNA and protein in the keratinocytes were unaffected. Taken together, our data suggest that uptake of the compounds by the cells and DNA cross-link formation are not required for growth inhibition. These findings further support the model that the cell membrane is an important target for the psoralens.

Keywords: Psoralens; PUVA; Photochemotherapy; Growth inhibitors; Ultraviolet light; Psoralen receptor

1. Introduction

Psoralens, also known as furocoumarins, are clinically important drugs used in the treatment of a variety of epidermal proliferative disorders including psoriasis, vitiligo, eczema, and mycosis fungoides [1–3]. The therapeutic effectiveness of these agents is highly dependent upon their activation by UVA light, a treatment referred to as PUVA (*p*soralen plus *UVA*). It is well known that psoralens can enter cells rapidly, intercalate, and subsequently photocross-link DNA, forming both mono- and bis-cyclobutane adducts between the 3,4 and 4′,5′ double bonds of the psoralen and the double bonds in the pyrimidine rings ([3,4]; see Table 1 for psoralen structures). Previous papers from our laboratory have suggested that an alternative site of action for the psoralens, not involving the DNA, is the cell membrane [5–11]. Thus, we demonstrated that the psoralens bind to a specific 22,000 molecular weight membrane receptor protein [9,11]. Moreover, binding of psoralens to this receptor

* Corresponding author. Tel.: +1-732-445-0170; fax: +1-732-445-0119.
E-mail address: jlaskin@eohsi.rutgers.edu (J.D. Laskin).

Abbreviations: BQ, 1,4-benzoquinone; DEPC, diethyl pyrocarbonate; DMEM, Dulbecco's Modified Eagle Medium; EGF, epidermal growth factor; FADU, fluorescent analysis of DNA unwinding; 4′-Pyr-H_2TMP, 4′-pyridinium-4′,5′-dihydro-4,4′,8-trimethylpsoralen; 5′-Pyr-H_2TMP, 5′-pyridinium-4′,5′-dihydro-4,5′,8-trimethylpsoralen; H_2TMP, 4′,5′-dihydro-4,5′,8-trimethylpsoralen; IFN-γ, interferon-γ; NOS2, inducible nitric oxide synthase; LB, Luria-Bertani broth; PUVA, psoralen plus UVA light; RT–PCR, reverse transcription–polymerase chain reaction; TdR, thymidine; TMP, 4,5′,8-trimethylpsoralen; and UVA light, ultraviolet light in the range of 320–400 nm.

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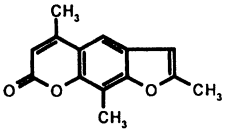
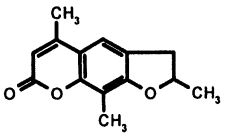
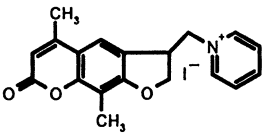
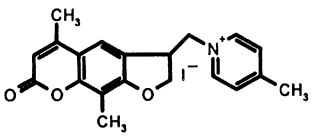
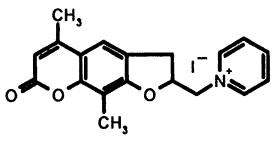
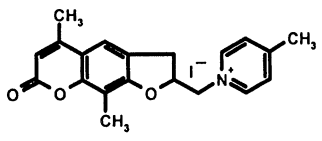
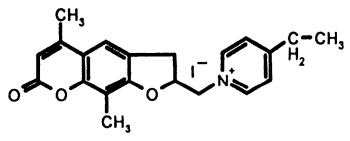
Table 1
Psoralen structures and growth-inhibitory activities

Compound	Structure	IC_{50} (μM)	
		+UVA	−UVA
TMP		0.001	> 10
H_2TMP		1.3	> 100
4′-Pyr-H_2TMP		1.8	> 300
4′-Methyl-Pyr-H_2TMP		2.6	> 300
5′-Pyr-H_2TMP		2.2	> 100
5′-Methyl-Pyr-H_2TMP		> 100	> 300
5′-Ethyl-Pyr-H_2TMP		> 100	> 300

followed by UVA light activation results in phosphorylation of the EGF receptor, inhibition of EGF binding, and reduced EGF receptor tyrosine kinase activity [6,7,10].

Phototoxicity is an important factor limiting the clinical use of the psoralens. Cellular repair of bi-functional DNA cross-links is prone to error [12], and photoactivated psoralens are known to be highly genotoxic, mutagenic, and carcinogenic [13–16]. Indeed, in humans, long-term exposure to high doses of PUVA greatly increases the risk of squamous cell carcinoma and melanoma [13,14]. In studies aimed at developing less phototoxic psoralen derivatives, we synthesized structural analogs of the psoralens that are unable to photocross-link DNA [17]. Importantly, we demonstrated that photobiological activity does not require saturation of the 4′,5′ furan ring in the psoralen. In the present studies, we report on the biological activity of water-soluble

quaternary amines of 4′,5′-dihydropsoralens, which are unable to penetrate the cell. These compounds would be expected to exhibit increased cell surface effects with minimal mutagenic/carcinogenic events [18]. Our findings that these psoralen analogs retain biological activity without inducing DNA damage suggest that they may be useful drugs in photochemotherapy.

2. Materials and methods

2.1. Synthesis of psoralen derivatives

TMP was obtained from the Sigma Chemical Co. H_2TMP was prepared as previously described [17]. A description of the synthesis of all other psoralens used in this study has been published elsewhere [19]. Stock solutions of TMP were prepared in 100% DMSO at a concentration of 4.5 mM; all other psoralens were prepared at 10 mM.

2.2. Cell culture

Mouse PAM 212 keratinocytes were grown in DMEM supplemented with 10% heat-inactivated fetal bovine serum, 100 units/mL of penicillin G, 100 μg/mL of streptomycin sulfate, and 50 μg/mL of gentamicin sulfate (complete medium) at 37° in a humidified incubator with 5% CO_2.

2.3. Growth inhibition of PAM 212 cells by psoralens and UVA light

Growth experiments with psoralens and UVA light were performed as previously described [20]. Briefly, cells were seeded into 6-well tissue culture plates (10,000 cells/well) in 2 mL of complete medium. After 24 hr, the medium was removed and replaced with 1.5 mL of medium containing increasing concentrations of the psoralens. After 30 min at 37°, the covers from the culture plates were removed, and the cells were exposed to UVA light for 10 min at a distance of 9.5 cm from a bank of four Sylvania F40-BL bulbs (1.4 J/cm^2 total UVA irradiation as measured with an International Light UV-radiometer fitted with an IL-SE 115 probe and a 363 UVA pass filter). The cells were then refed with 2 mL of fresh growth medium. After 4 days at 37°, the cells were trypsinized and counted on a model ZBI Coulter Counter. Then the concentration of psoralen required for 50% inhibition of cell growth (IC_{50}) was determined.

2.4. Preparation of plasmid pZeoSV DNA

Chemically competent *Escherichia coli* DH5α cells were transformed with the commercially available 3451 base-pair plasmid pZeoSV (Invitrogen) and plated onto low-salt LB (per L: 10 g Tryptone, 5 g NaCl, 5 g yeast extract, pH 7.5) agar plates containing 25 μg/mL of the antibiotic Zeocin (Invitrogen). Low-salt LB medium (100 mL) containing 25 μg/mL of Zeocin was inoculated with several colonies, and the culture was incubated overnight at 37°. Plasmid DNA was isolated with a Qiagen-tip 500 (Qiagen Corp.) according to the instructions of the manufacturer. The DNA was eluted with TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8) and quantified spectrophotometrically. The 260/280 absorbance ratio of 1.89 indicated very high purity. Agarose gel electrophoresis confirmed high purity plasmid DNA with at least 95% in the supercoiled form. pZeoSV is a vector designed for high-level protein expression of inserted cDNAs in mammalian cells. The Zeocin-resistance gene allows selection of recombinants in bacteria, yeast, insect, and mammalian cells. This vector was chosen because of its ready availability in our laboratory.

2.5. Preparation of linearized plasmid pZeoSV DNA

Plasmid DNA (10 μg) was linearized by incubation with 100 units of restriction endonuclease *Eco*RI (New England Biolabs) in a reaction volume of 100 μL containing 50 mM Tris–HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM dithioerythritol, pH 7.5, at 37° for 3 hr. The DNA was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, by vol.), twice with chloroform:isoamyl alcohol (24:1, v/v), and then precipitated with ethanol. The DNA was recovered by centrifugation, washed once with 70% ethanol, air-dried, and dissolved in 200 μL of TE buffer.

2.6. Treatment of plasmid pZeoSV DNA with psoralens and UVA light

Each 10-μL reaction mixture contained 75 ng pZeoSV, 0.1 μL psoralen solution (prepared as 100× solutions in DMSO), and TE buffer. Solutions of BQ, which served as a positive control for unwinding and cleavage of DNA, were prepared in 95% ethanol. The reaction volume and components were scaled up several-fold so that multiple aliquots of 10 μL could be removed before and after UVA light treatment for agarose gel analysis. Treatment with UVA light was performed in V-bottomed 96-well plates as described above for the cell cultures.

2.7. Electrophoretic analyses

Samples of DNA (10 μL for native DNA samples or 11.1 μL after denaturation) were mixed with concentrated agarose gel loading buffer [10× = 50% (v/v] glycerol, 4.2 mg/mL of bromophenol blue]. Analyses on neutral gels were performed in 1% agarose in TAE buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA). For analysis of denatured samples, DNA (10 μL) was mixed with 1.1 μL of denaturing solution (2 M NaOH, 0.1 M EDTA), heated in a water bath at 90° for 1 min, and cooled on ice prior to loading on a 1% alkaline agarose gel. Gels were prepared and electrophoresed in alkaline gel buffer (50 mM Tris base,

45 mM boric acid, 30 mM NaOH, 1 mM EDTA). Native and denaturing gels were electrophoresed at 24 V in a Bio-Rad Wide Mini-Subcell GT DNA electrophoresis unit until the bromophenol blue migrated about 8 cm. Alkaline gels were soaked three times in excess distilled water prior to staining. All gels were stained in 0.5 μg/mL of ethidium bromide and photographed with the Eagle Eye II digital documentation system (Stratagene).

2.8. FADU

The extent of DNA modification (unwinding, cleavage, or cross-linking) by psoralens was measured by a modification of the FADU assay [21,22] using adherent keratinocytes. Cells were plated in 24-well plates (125,000 cells/well) in 1 mL of medium. The assay required that the cellular extracts for each concentration of psoralen (or benzoquinone) be treated in three different ways. Thus, three sets of plates were prepared for each compound to be tested. One set of plates was designated as T (total), the second as P (partial), and the third as B (blanks). After incubation overnight, the medium was removed from the plates and replaced with 1 mL of complete DMEM containing psoralen. Cells were incubated at 37° for 30 min and then were exposed to UVA light or maintained at room temperature for 10 min. The plates were placed on ice, medium was removed, and the cells were washed twice with 1 mL of ice-cold PBS. Then ice-cold PBS (0.2 mL) was added to each well, and the assay was performed as described previously [21,22]. The luminescence of the samples was determined on a Perkin-Elmer model LS-5B Luminescence Spectrometer with excitation at 520 nm and emission at 590 nm. A blank was prepared by mixing appropriate volumes of each of the buffers. The percentage of double-stranded DNA (D or D_c for treated or control samples, respectively) is given by 100(P-B)/(T-B). The results are presented as $100(D/D_c)$ or the percentage of control double-stranded DNA versus compound concentration.

2.9. Measurement of [^{3}H]TdR incorporation into cellular DNA

DNA synthesis in keratinocytes was measured by [^{3}H]TdR incorporation as previously described [23]. Briefly, PAM 212 cells were plated in 24-well plates (125,000 cells/well), allowed to adhere overnight, and treated with psoralens as described previously. After UVA light treatment, cultures were incubated in complete medium containing 0.5 mCi/mL of [^{3}H]TdR (New England Nuclear; 20 Ci/mmol) for 6 hr. Cells were washed three times with PBS and then three times with 5% (w/v) trichloroacetic acid. The cells and associated radioactivity were dissolved in 0.2 N NaOH, and aliquots were counted in a liquid scintillation counter (Packard model TRI-CARB 2100TRLiquid Scintillation Analyzer).

2.10. Effect of psoralens on nitric oxide biosynthesis

For studies on nitric oxide biosynthesis, PAM 212 cells were plated in 24-well plates (125,000 cells/well), allowed to adhere overnight, and treated with psoralens as already described except that psoralen dilutions were made in serum-free, phenol red-free DMEM (phenol red interferes with the subsequent colorimetric assay for nitrite). After UVA light treatment, the medium was removed, and the cells were incubated in serum-free, phenol red-free DMEM containing 1.4 mM l-arginine and 100 units/mL of mouse IFN-γ (IFN-γ was a gift of Pestka Biomedical Laboratories) for 24 hr. The culture medium was assayed for nitrite accumulation (a measure of the nitric oxide released into the medium) using the Greiss reaction [23] with sodium nitrite as the standard. The culture medium (100 μL) or diluted standard was mixed with 50 μL of 1% (w/v) sulfanilamide in 50% phosphoric acid; then 50 μL of 0.1% (w/v) *N*-(1-naphthyl)ethylenediamine dihydrochloride was added. After incubation at room temperature for 30 min, the optical density at 540 nm was read in a Perkin-Elmer model HTS 7000 Plus microplate reader.

Cells were analyzed for NOS2 protein by western blotting as described previously [24]. Briefly, cell proteins (5 μg/lane) were fractionated on 7.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were incubated in rabbit anti-mouse NOS2 antibody (Santa Cruz) followed by horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) as the secondary antibody. Protein was detected by enhanced chemiluminescence (NEN Renaissance Western Blot Chemiluminescence Reagent Plus).

For RT–PCR of the NOS2 mRNA, cells were seeded in 6-well plates (750,000 cells/well), treated with psoralens and IFN-γ as described above, and total RNA was extracted with Trizol reagent (Life Technologies) according to the instructions of the manufacturer. All buffers were prepared with DEPC-treated water. The final RNA pellets from each well were dissolved in 25 μL TE buffer. Samples of the RNAs were diluted 1:200, and the optical densities at 260 and 280 nm were determined. The 260/280 absorbance ratio ranged from 1.91 to 2.00, indicating very high quality RNA. The yield of total RNA ranged from 32 to 60 μg per sample.

RT was performed with a slight modification as described [25]. RNA (0.4 μg) was diluted to 9 μL with DEPC–water, heated at 65° for 5 min, and then chilled on ice. A solution (11 μL**)** of reverse transcription reagents was added to give final reactant components as follows: 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM dNTPs, and 20 μM random hexamers (Roche Molecular Biochemicals), and 200 units SuperScript II RNase H^- reverse transcriptase (Life Technologies). After incubation for 60 min at 37°, 1 μL (2 units) Ribonuclease H (Life Technologies) was added. The ribonuclease was inactivated 20 min later by boiling for 5 min. Then PCR was performed with 2 μL of 1:10 diluted RT reaction plus 8 μL

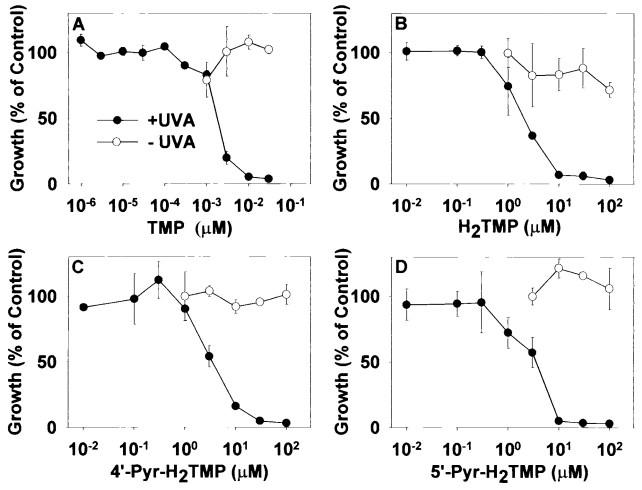


Fig. 1. Growth inhibitory activity of psoralens. Cells were seeded in 6-well plates, treated with psoralen for 30 min at 37°, and then kept for 10 min at room temperature in the absence (○) or presence (●) of UVA light. Four days later, the cells were released from the plates and counted in a Coulter counter. (A) Treatment with TMP; (B) treatment with H_2TMP; (C) treatment with 4′-Pyr-H_2TMP; and (D) treatment with 5′-Pyr-H_2TMP. Untreated cells yielded $1.11 \times 10^6 \pm 1.03 \times 10^5$ cell counts at the end of the incubation period. Values are means ± SEM (N = 3).

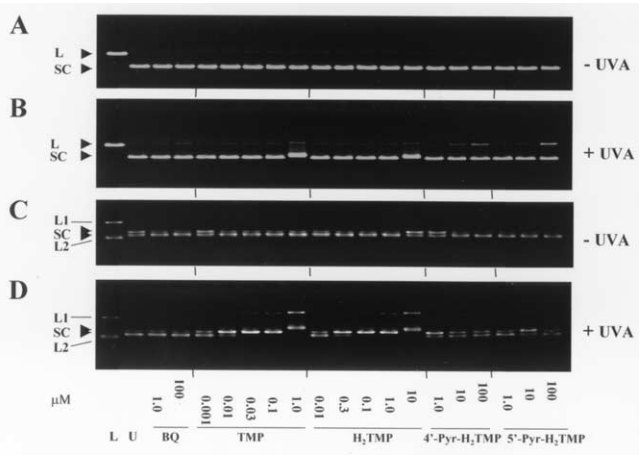


Fig. 2. Treatment of plasmid pZeoSV DNA (75 ng) with psoralens and UVA light. *In vitro* DNA modification assays were performed by treating the supercoiled plasmid DNA with BQ, TMP, H_2TMP, 4′-Pyr-H_2TMP, or 5′-Pyr-H_2TMP in the absence (A and C) or presence (B and D) of 10 min of UVA light. Electrophoretic analysis was performed on 1% neutral (A and B) or alkaline (C and D) agarose gels. Lane L shows products from digestion with restriction endonuclease *Eco*RI to linearize the DNA (100 ng); arrowhead L indicates the position of the linearized DNA, which migrates as one double-stranded band in native gels but is resolved into two well-separated single strands (L1 and L2) in the denaturing gels. Lane U shows untreated DNA; the arrowhead(s) SC indicates the position of the supercoiled plasmid DNA, which migrates as one double-stranded band in native gels but is resolved into two closely migrating single strands in denaturing gels.

of concentrated PCR reagents so that the final reaction contained 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.1 mg/mL of bovine serum albumin, 250 μM dNTPs, 0.4 μM NOS2 primers (Ambion Gene Specific Relative RT–PCR kit; upstream primer sequence is 5′-GCTCATCCGGTACGCTGGCTA-3′; downstream primer sequence is 5′-TCCAATCTCGGTGCCCATGTA-3′), and 0.25 units *Pfu* DNA polymerase (Stratagene). PCR was performed in a Hybaid thermal cycler with a heated lid for 35 cycles of denaturation at 94° for 1 min, annealing at 56° for 30 sec, and extension at 72° for 30 sec with 1 final cycle of extension at 72° for 10 min. Tubes were placed in the thermal cycler when the temperature reached 94°. A PCR control reaction was provided by using 0.5 μL of 1:10 diluted NOS2 target DNA (Ambion Gene Specific Relative RT–PCR kit). Products were analyzed by electrophoresis on 1.4% agarose gels run in TAE buffer as described above.

3. Results

3.1. Growth inhibition of PAM 212 cells by psoralens and UVA light

In initial studies, the biological activity of the psoralens was assessed in cell growth inhibition assays. Representative plots are shown in Fig. 1, and the data are summarized in Table 1. All of the compounds required UVA light for activity. Of the compounds evaluated, TMP was found to be the most reactive with an IC_{50} in the nanomolar concentration range, followed by H_2TMP and the pyridinium compounds. These results demonstrate that, despite the presence of a charged structure, dihydropyridinium psoralens retain activity as inhibitors of cell growth. Moreover, the finding that all of the inhibitors were less active than TMP indicates that the 4′,5′ double bond in TMP is required for maximal activity. Our results also showed that only some of the substituted pyridinium compounds were active in inhibiting keratinocyte growth. Thus, although the 4′-methyl derivative was active, the 5′-methyl and 5′-ethyl derivatives were not. This may be due to structural requirements for binding of the psoralens to cellular targets [11].

3.2. Treatment of plasmid pZeoSV DNA with psoralens and UVA light

We next used a plasmid DNA modification assay to assess the ability of the psoralen derivatives to modify double-stranded circular DNA. 4′-Pyr-H_2TMP and 5′-Pyr-H_2TMP were tested as representative examples of the pyridinium compounds. Native gel analysis indicated that, in the absence of UVA light (Fig. 2A), none of the compounds tested (TMP, H_2TMP, 4′-Pyr-H_2TMP, and 5′-Pyr-H_2TMP) caused nicking or linearization of the supercoiled plasmid DNA as shown by an absence of a shift in band mobility from the faster migrating supercoiled form (SC) to the slower migrating linear forms (L) or open circular (fainter band migrating slightly slower than the linear form). In contrast, in the presence of UVA light (Fig. 2B), slower

migrating bands were observed after treatment of plasmid with high concentrations of either TMP or H_2TMP, indicating extensive modifications in the DNA. These results were confirmed when samples of DNA were electrophoresed in denaturing gels in which the supercoiled and linearized plasmid DNA are resolved into two bands each (Fig. 2, C and D; SC represents the two closely-migrating strands from the supercoiled DNA; L1 and L2 represent the linearized slower- and linearized faster-migrating bands, respectively). Mobility shifts were observed only after UVA light treatment (Fig. 2, B and D). TMP and H_2TMP produced DNA modifications that were concentration-dependent and close to the IC_{50} for growth inhibition. The finding that higher concentrations of H_2TMP were required to damage DNA is consistent with the fact that this compound cannot form DNA cross-links. In contrast to TMP and H_2TMP, 4′-Pyr-H_2TMP and 5′-Pyr-H_2TMP only caused mobility shifts in plasmid DNA bands at very high concentrations that were well above the IC_{50} values for these compounds. Interestingly, at these concentrations, the DNA modification pattern was distinct from that of TMP and H_2TMP. Thus, the 4′-Pyr-H_2TMP and 5′-Pyr-H_2TMP produced bands of lower molecular weight when compared with TMP and H_2TMP, indicating that the extent of DNA modification was less. These data suggest that all of the psoralens tested modified DNA in a manner that did not cause nicks or breaks in the DNA, but rather modified the DNA strands, most likely by forming stable mono-functional adducts.

3.3. FADU induced by psoralens and UVA light

FADU was used to measure DNA damage in viable cells. The results from this analysis paralleled those from the growth inhibition and *in vitro* DNA experiments. Thus, TMP plus UVA light was the most active combination followed by H_2TMP plus UVA light (Fig. 3A). As observed with the plasmid DNA modification assay, the photoactivated pyridinium derivatives exhibited only a small effect on keratinocyte DNA at very high concentrations. With both TMP and H_2TMP, a marked increase in the percentage of double-stranded DNA was observed, which is indicative of DNA modifications that prevent unwinding of DNA. Thus, these modifications stabilize the DNA, a finding consistent with the ability of these compounds to intercalate and modify DNA. The lower activity for H_2TMP is also consistent with its ability to only form mono-functional DNA adducts. Neither 4′-Pyr-H_2TMP nor 5′-Pyr-H_2TMP was active in inducing DNA unwinding at concentrations that inhibited cell growth. None of the psoralens were active in the absence of UVA light (not shown). In these studies, BQ, a compound known to cause DNA unwinding and cleavage [26], was used as a positive control in intact cells. BQ leads to a decrease in DNA stability as measured by decreased fluorescence associated with double-stranded DNA. In the plasmid DNA modification assay, BQ did not affect supercoiled DNA (Fig. 2). This is likely due to the fact that this quinone requires cellular metabolism for activity [27].

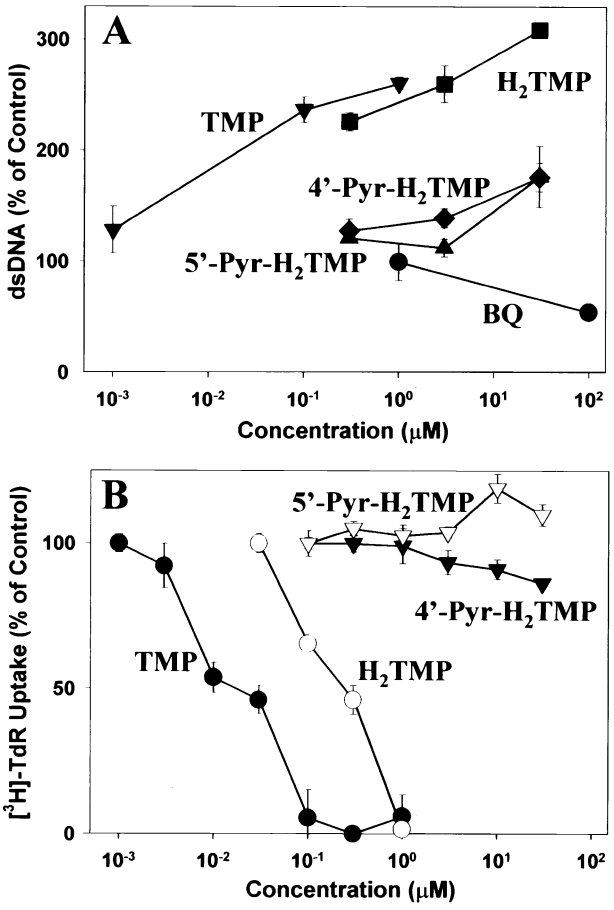


Fig. 3. Effect of psoralens and UVA light treatment on DNA unwinding and synthesis. (A) Fluorescent analysis of DNA unwinding (FADU). Keratinocytes were seeded in 24-well plates and treated with BQ (●), TMP (▼), H_2TMP (■), 4′-Pyr-H_2TMP (◆), or 5′-Pyr-H_2TMP (▲) for 30 min at 37° and exposed to 10 min of UVA light at room temperature. Extracts were prepared and ethidium bromide was added to react with the cellular DNA. The fluorescence associated with the DNA was measured in a Perkin-Elmer model LS-5B Luminescence Spectrometer with excitation at 520 nm and emission at 590 nm. The fluorescence observed was then converted to the percentage of double-stranded DNA and compared with the percentage of double-stranded DNA in untreated samples. Control cells treated only with UVA light had levels of double-stranded DNA equal to 28.3 ± 2.3%. Cells treated with the psoralens but no UVA light gave levels of double-stranded DNA close to controls (not shown). Values are means ± range (N = 2). (B) Inhibition of DNA synthesis. For DNA synthesis studies, cells were treated with TMP (●), H_2TMP (○), 4′-Pyr-H_2TMP (▼), or 5′-Pyr-H_2TMP (▽) and UVA light as described above and then pulse-labeled with [^{3}H]TdR for 6 hr. Cells were then washed with PBS followed by 5% trichloroacetic acid. The cells were solubilized with 0.2 N NaOH, and the associated radioactivity was counted in a liquid scintillation counter. Data are presented as the percentage of control uptake in the absence of the psoralen. Control cells gave 1926.75 ± 60.12 cpm/mg protein. Values are means ± SEM (N = 3).

3.4. DNA synthesis inhibition

The DNA unwinding assay indicated that the pyridinium derivatives of H_2TMP, when activated by UVA light, had no major effect on the DNA of intact cells. As DNA-

modifying agents, psoralens plus UVA light are known to be potent inhibitors of DNA synthesis [28]. We found that photoactivated TMP as well as H_2TMP readily inhibited DNA synthesis in keratinocytes (Fig. 3B). Neither compound inhibited DNA synthesis without UVA light (data not shown). Consistent with its cross-linking activity, TMP was significantly more active than the dihydro-derivative. Despite being growth inhibitory, 4′-Pyr-H_2TMP and 5′-Pyr-H_2TMP did not cause any major alterations in the [^{3}H]TdR uptake (Fig. 3B), indicating that these compounds do not directly modify DNA in intact cells.

3.5. Effects of psoralens on nitric oxide biosynthesis

Compounds that modify or cross-link DNA would be expected to suppress new gene expression in target cells. In keratinocytes, cytokines such as IFN-γ are known to induce NOS2 mRNA, protein synthesis, and subsequent nitric oxide production [23], and this was used as a marker for new gene expression and activity. We found that both photoactivated TMP and H_2TMP inhibited IFN-γ-induced nitric oxide production (Fig. 4A). This correlated with inhibition of NOS2 mRNA and protein expression (Fig. 4, C, D, and E). In the absence of UVA light, these compounds had no effect on nitric oxide production. This activity paralleled their ability to inhibit DNA synthesis (Fig. 3B). Interestingly, at growth inhibitory concentrations, neither 4′-Pyr-H_2TMP nor 5′-Pyr-H_2TMP had any major effect on these activities either with or without UVA light (Fig. 4, compare panels B, D, and E). Taken together, these data provide further support for the idea that a site other than DNA is important in the mechanism by which these compounds inhibit cell growth.

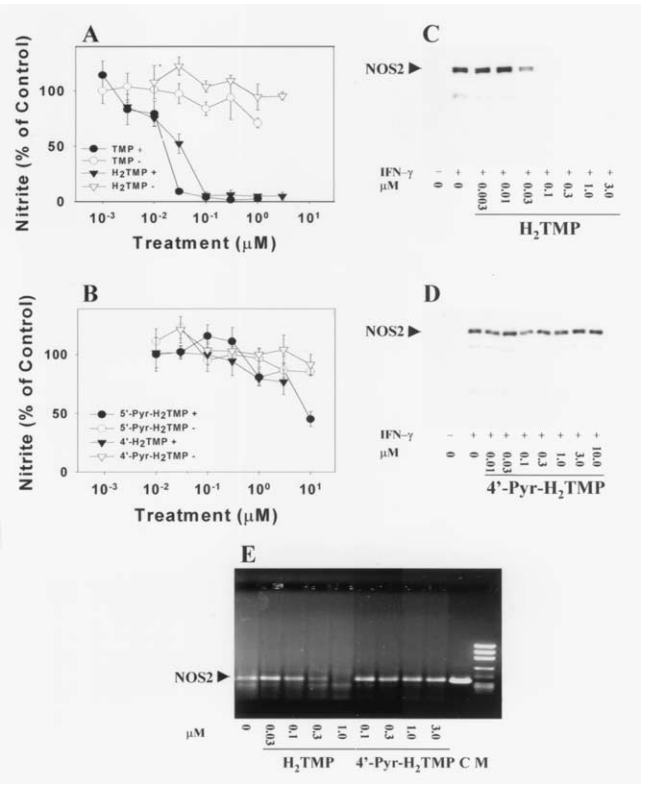


Fig. 4. Effect of psoralens and UVA light on nitric oxide biosynthesis. (A and B) Greiss assay of nitrite production by cells treated with psoralens. Cells were treated with TMP, H_2TMP, 4′-Pyr-H_2TMP, or 5′-Pyr-H_2TMP either in the presence (closed symbols) or absence (open symbols) of UVA light. IFN-γ (100 units/mL) was then added, and 24 hr later the nitrite released into the culture medium was assayed colorimetrically at 540 nm by the Greiss reaction. Control cells treated only with IFN-γ gave 0.166 ± 0.021 O.D. units/mg protein. Values are means ± SEM (N = 3). (C and D) Detection of NOS2 protein by western blotting of cell extracts. Cells were treated with representative psoralens (H_2TMP, panel C, or 4′-Pyr-H_2TMP, panel D) followed by UVA light treatment. IFN-γ was added, and 24 hr later NOS2 expression was analyzed. The position of NOS2 is indicated by the arrowheads. (E) RT–PCR analysis of NOS2 mRNA. Total RNA was extracted from cells treated with H_2TMP or 4′-Pyr-H_2TMP and UVA light. RNA was extracted from the cells and reverse-transcribed. PCR was then performed with NOS2-specific primers, and the products were analyzed on a 1.4% agarose gel. The position of the 349 base-pair NOS2 product is indicated by the arrowhead. Lane C represents a PCR control reaction with NOS2 target DNA; lane M contains ΦX-*Hae*III markers.

4. Discussion

PUVA is known to be a potent inhibitor of keratinocyte growth [1,29,30]. Using cultured keratinocytes, we found that TMP, a clinically efficacious psoralen derivative [31], readily inhibited cell growth when activated by UVA light. The phototoxic actions of TMP are due, in part, to its ability to modify and cross-link DNA [1,32,33]. To produce biologically active psoralens with less phototoxicity, we synthesized a 4′,5′-dihydro-derivative of TMP (H_2TMP) that cannot cross-link DNA [17]. When activated by UVA light, this analog was also an effective inhibitor of keratinocyte growth; however, it was significantly less potent than TMP. Although the reduced potency of H_2TMP as a growth inhibitor may be due to its inability to form DNA cross-links, steric considerations may also limit its activity. In TMP, the methyl group at carbon C-5′ lies in the plane of all the carbon atoms which constitute the three rings. Thus, the entire molecule is a flat surface (see Table 1). In H_2TMP, the two carbons in the furo ring at C-4′ and C-5′ are tetrahedral hybrids, which means that their substituents lie above (and below) the plane of the ring. Because of chirality considerations, the methyl group at C-5′ can be described as being above (50%) and below (50%) the ring plane. This bulky four-atom cluster might be expected to interfere with DNA intercalations. This could lead to reduced monoadduct formation. It should be noted, however, that formation of the saturated derivative of TMP also alters light absorption, which may contribute to decreased activity. Thus, there is altered resonance of the ring structure with reduced aromaticity, which would result in a diminished extinction coefficient for the π-π^* transitions. Aromaticity is also directly related to nucleotide base stacking, and H_2TMP would be expected to have a lower affinity for DNA. These latter considerations might also explain the reduced potency of

H_2TMP in inducing DNA damage in the plasmid DNA unwinding, fluorescence DNA unwinding, and DNA synthesis assays. H_2TMP was also significantly less active than TMP in inhibiting IFN-γ-induced expression of NOS2 and nitric oxide production.

As reported previously by our laboratories, a cell membrane receptor is also a major target for the psoralens [11]. A variety of psoralens including TMP bind with high affinity to a 22,000 molecular weight receptor protein. We found that psoralen receptor activation is directly linked to PUVA-induced inhibition of cell surface binding of EGF to its receptor [6,10]. PUVA induces serine phosphorylation of the EGF receptor, thereby down-regulating its functional activity, in this case, an intrinsic tyrosine kinase activity stimulated by the binding of EGF [7]. We hypothesized that the growth inhibitory effects of the psoralens are caused by, first, binding to the psoralen receptor and activation by UVA light followed by their effects on the EGF receptor [8]. There are structural requirements for the binding of psoralens to their receptors [8,9,11]; for example, both the steric and electronic considerations described above for H_2TMP may limit receptor binding. This, in turn, may regulate the potency of the pyridinium psoralens as inhibitors of keratinocyte growth.

The addition of a pyridinium moiety to the psoralen structure greatly increases water solubility and reduces cellular uptake due to its charge at physiological pH. Accordingly, growth inhibition is not likely due to DNA damage. This is supported by our observations that 4′- and 5′-Pyr-H_2TMP, when activated by UVA light, had no major effects in our DNA damage assays, TdR uptake studies, or gene expression assays. Our results also demonstrate that there are clear structural determinants for biological activity of the pyridinium psoralen analogs. For growth inhibition, 4′- and 5′-Pyr-H_2TMP had activity generally similar to that of H_2TMP. However, 4′-methyl-Pyr-H_2TMP but not 5′-methyl-Pyr-H_2TMP or 5′-ethyl-Pyr-H_2TMP possessed growth inhibitory activity. These data demonstrate that the addition of a charged pyridinium on the psoralen molecule does not reduce biological activity. However, there are constraints to further modification of the 5′-substituted pyridinium analogs. Presumably, the presence of bulky substituents on the 5′-, but not the 4′-pyridinium ring, prevents binding to its cellular target. One could speculate that if the psoralen receptor mediates the growth inhibitory activity of these compounds, then there would be limitations in the recognition of 5′-modified psoralens. Further studies measuring psoralen receptor binding and the effects of these compounds on EGF receptor binding are necessary to evaluate this possibility.

At the present time, the precise mechanisms by which the pyridinium derivatives of H_2TMP inhibit cell growth are not clear, although the psoralen membrane receptors remain a potential target. The fact that these compounds are effective inhibitors of cell growth and yet less phototoxic, i.e. less likely to damage DNA and be genotoxic, when compared with the parent compounds, TMP and H_2TMP, suggests that they may have a high therapeutic index and be useful drugs in photochemotherapy.

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