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Biochemical Pharmacology 63 (2002) 2001–2009

Biochemical Pharmacology

Mechanisms of growth inhibition in keratinocytes by mercurio-substituted 4′,5′-dihydropsoralens

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Received 5 July 2001; accepted 27 November 2001

Abstract

Psoralens, together with ultraviolet light A (PUVA), are used in the treatment of epidermal proliferative disorders. Although these compounds can enter cells and photo cross-link DNA, lipids and proteins, including a specific membrane receptor, are also potential targets for the psoralens. To better elucidate the site of action of the psoralens, we have synthesized a family of 5′-mercurio-substituted derivatives of 4′,5′-dihydropsoralen. These compounds are identified by their heavy metal content and can be used as a model to deliver thiol reactive psoralen derivatives into keratinocytes. The 5′-mercuriopsoralen derivatives were found to be effective inhibitors of keratinocyte growth without photoactivation. The most active compound, 4,8-dimethyl-5′-iodomercuriomethyl-4′,5′-dihydropsoralen (IC_{50} = 10 μM), was also a potent photosensitizer (IC_{50} = 0.3 μM). Depletion of keratinocyte GSH with buthionine sulfoximine markedly increased their sensitivity to this analog, both with and without UVA light. In contrast, *N*-acetyl-L-cysteine partially protected the cells from growth inhibition, indicating that a sulfhydryl-sensitive site is growth limiting and that this target can be photoactivated. Iodomercurio-4′,5′-dihydropsoralen was found to form adducts with GSH and cysteine, which were not active without UVA light. Thus, these adducts may also contribute to the photosensitization reactions of the parent compound. Using plasmid DNA unwinding assays, iodomercurio-4′,5′-dihydropsoralen was also found to modify DNA, an activity that increased following UVA light treatment. This suggests that DNA damage may contribute to the actions of these psoralens. Taken together, our data demonstrate that there are multiple sites of action for mercuriopsoralens. These compounds may prove useful for understanding the mechanisms of psoralen-induced growth inhibition in the skin. © 2002 Published by Elsevier Science Inc.

Keywords: Psoralens; Keratinocytes; UVA light; PUVA; Photochemotherapy

1. Introduction

A variety of epidermal proliferative disorders including psoriasis, vitiligo, and cutaneous T cell lymphoma can be treated with a class of photosensitizers known as the psoralens [1,2]. The activity of these compounds requires activation by UVA (320–400 nm) in a protocol referred to as PUVA (psoralen plus UVA) photochemotherapy [1,2]. The psoralens are known to intercalate DNA in a dark reaction and form mono- and bifunctional adducts with pyrimidine bases following UVA light treatment [3]. Other targets for the psoralens include membrane receptors, lipids, and proteins [1,4–7]. As a consequence of the DNA-damaging activity of the photoactivated psoralens, these compounds are mutagenic and are known carcinogens in humans [8,9]. A variety of biologically active psoralen derivatives have been synthesized with the aim of reducing phototoxicity. These include angular derivatives with a reduced ability to modify DNA [10] and 4′,5′-dihydro-derivatives, which lack the ability to form bifunctional DNA adducts [11–13].

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Abbreviations: TMP, 4,5′,8-trimethylpsoralen; H_2TMP, 4,8-dimethyl-5′-methyl-4′,5′-dihydropsoralen; Iodomercurio-H_2TMP, 4,8-dimethyl-5′-iodomercuriomethyl-4′,5′-dihydropsoralen; NAc, *N*-acetyl-L-cysteine; UVA, ultraviolet light A (320–400 nm); TCA, trichloroacetic acid; BSO, L-buthionine[*S*,*R*]sulfoximine; DTNB, 5,5′-dithio-bis(2-nitrobenzoic acid); DTT, dithiothreitol.

0006-2952/02/$ – see front matter © 2002 Published by Elsevier Science Inc.
PII: S0006-2952(02)00992-9

In the present studies, we report on the activity of a class of mercurio-substituted 4′,5′-dihydropsoralens synthesized as a model to deliver a reactive moiety directly to rapidly proliferating epidermal cells, and to identify their site of action. These compounds were modeled on the mercurial diuretics, which have been widely used clinically and which display relatively low toxicity in humans [14]. We found that, in the absence of UVA light, mercuriopsoralens are highly effective inhibitors of keratinocyte cell proliferation. However, the activity of some of the analogs can be enhanced by UVA light treatment, indicating that these compounds can act as photosensitizers. In both the presence and absence of UVA light, growth inhibition by mercuriopsoralens is dependent upon cellular GSH, indicating that they act at a sulfhydryl-sensitive site. The most active photosensitizer, iodomercurio-H_2TMP, was found to readily form thiol adducts with GSH or cysteine. These adducts were potent inhibitors of cell growth, but only when activated by UVA light. These derivatives are highly novel and may be useful phototherapeutic agents for the treatment of epidermal proliferative disorders including psoriasis.

2. Materials and methods

2.1. Cells and reagents

PAM 212 keratinocytes were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum as described previously [15,16]. Bovine serum albumin, BSO, NAc, DTT, and Ellman's reagent (DTNB) were purchased from Sigma Chemical Co. [^{3}H]Thymidine (740 GBq/mmol) and [^{3}H]leucine (5.18 TBq/mmol) were obtained from NEN Life Science Products. Preparation of the mercuriopsoralens has been described elsewhere [17]. The compounds used were: P1, iodomercurio-H_2TMP; P2, chloromercurio-H_2TMP; P3, trifluoroacetylmercurio-H_2TMP; P4, acetylmercurio-H_2TMP; P5, 3-fluoro-acetylmercurio-H_2TMP; P6, 3-cyano-acetylmercurio-H_2TMP; P7, iodomercurio-angelicin; and P8, acetylmercurio-angelicin. To prepare the psoralen–cysteine or –GSH conjugates, 10 μL of iodomercurio-H_2TMP (10 mM in DMSO) was reacted with 10 μL GSH (10 mM in H_2O) or cysteine (10 mM in H_2O), and the reactions were incubated at room temperature for 30 min. Compounds were purified by thin layer chromatography [17]. Protein was quantified using the Detergent compatible (Dc) Protein assay (Bio-Rad) with bovine serum albumin as the standard.

2.2. Growth inhibition assays

The effects of the mercuriopsoralens on keratinocyte cell growth were assayed as described previously [18]. Briefly, cells were plated at low density (5×10^3 cells/well) in 6-well tissue culture dishes and allowed to adhere overnight. The medium was then replaced with phenol red-free DMEM supplemented with increasing concentrations of the mercuriopsoralens. Six concentrations and a control were used for each inhibitor, and each concentration was tested in triplicate. After 30 min at 37°, some of the cultures 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) [4–6]. The medium was then replaced with fresh growth medium. After 5 days, cells were removed from the dishes and enumerated using a Coulter Counter (Coulter Electronics, Inc.). The controls and treated samples at each concentration were averaged. Data are presented as the percentage of control growth at each concentration of the mercuriopsoralen. The standard error of each of the triplicate samples was less than 8%. The IC_{50} for growth inhibition was the concentration of each compound that inhibited growth by 50%. In some experiments, cells were incubated for 6 hr with BSO (10 μM) or NAc (3 mM) before treatment with the psoralens.

2.3. GSH assays

A modification of the method of Guaiquil *et al.* [19] was used to quantify keratinocyte GSH. Briefly, 2.5×10^5 cells/well in 6-well dishes were treated for 30 min at 37° with increasing concentrations of the mercuriopsoralens, washed (3×2 mL PBS), and then precipitated with 0.6 mL of ice-cold 5% TCA. After 10 min, 150 μL of the TCA extract was mixed with 50 μL of 1 M $NaHPO_4$ buffer, pH 7.5, containing 0.72 mM DTNB, and 0.4% sulfosalicylic acid. Absorbance was measured 2 min later at 405 nm using a Perkin Elmer HTS7000 Plus microplate reader.

2.4. Psoralen uptake studies

To quantify psoralen uptake, keratinocytes (6×10^6 cells) in 10-cm culture dishes were treated with increasing concentrations of iodomercurio-H_2TMP (P1) in 4 mL of serum-free growth medium. After 30 min at 37°, the cells were washed three times with ice-cold PBS, scraped from the culture dishes into 5 mL of PBS, and centrifuged (10,000 *g*, 10 min, 4°). Cell pellets were transferred to 50-mL conical tubes, and 1 mL of concentrated nitric acid (Fisher Optima) was added. The samples were digested in a microwave oven for 10 min at 60% power (CEM MDS-2000 Microwave, maximum power output 630 W), cooled on ice, and then diluted with distilled water to a final volume of 20 mL. Cell-associated mercury was measured using a Fissons PQ3 inductively coupled plasma mass spectrometer. This method measures total uptake of mercury into the cells and does not distinguish free from bound mercury.

2.5. [^{3}H]Thymidine and [^{3}H]leucine uptake studies

Uptake of [^{3}H]thymidine or [^{3}H]leucine into keratinocytes was monitored as described previously [16,18]. Briefly, cells in 24-well culture dishes (5×10^5/well) were pretreated with iodomercurio-H_2TMP (P1) and UVA light as described above for the growth experiments. Cells were then pulse-labeled with [^{3}H]thymidine (0.5 μCi/mL) for 3 hr or [^{3}H]leucine (0.25 μCi/mL) for 6 hr. The labeling medium was then decanted, and the cells were rinsed with ice-cold PBS (5×1 mL), followed by DNA and protein precipitation with 1 mL of ice-cold 5% TCA. The cells were then rinsed with 5% TCA (5×1 mL), solubilized in 0.5 mL of 0.2 N NaOH, and counted for radioactivity. TCA-soluble material was also evaluated for radioactivity to determine if the iodomercurio-H_2TMP inhibited uptake of the radiolabels. Under these conditions, we found that the mercuriopsoralen had no effect on uptake of either [^{3}H]thymidine or [^{3}H]leucine.

2.6. Preparation of plasmid DNA and treatment with psoralens and UVA light

Plasmid DNA was prepared and assayed for modifications by methods previously described by our laboratories [13]. Briefly, the commercially available 3451 bp plasmid pZeoSV (Invitrogen) was grown in *Escherichia coli* DH5α cells and isolated from 100 mL of an overnight culture using 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. Linear double-stranded plasmid DNA (10 μg) was prepared by incubation with the restriction endonuclease *Eco*RI (New England Biolabs). The DNA was then 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.

Plasmid DNA was treated with psoralens and UVA light in a 10-μL reaction mixture containing 75 ng pZeoSV, 0.1 μL psoralen solution (prepared as 100× solutions in DMSO), and TE buffer. Solutions of iodomercurio-H_2TMP, and of the iodomercurio-H_2TMP–cysteine and iodomercurio-H_2TMP–GSH conjugates 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 for 10 min under the conditions already described for the treatment of cell cultures. Modifications in the treated plasmid samples were analyzed by agarose gel electrophoresis. 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 1.2% agarose at 24 V in neutral gel 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 1.2% 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) 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 washed 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).

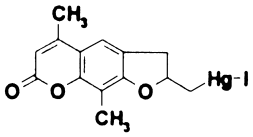
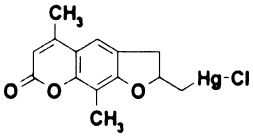
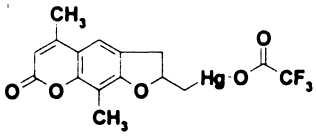
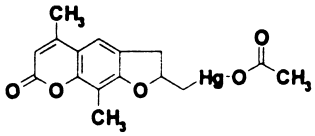
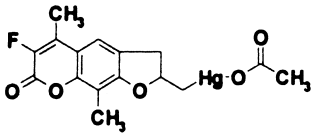
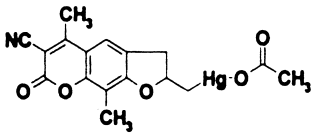
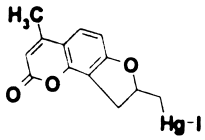
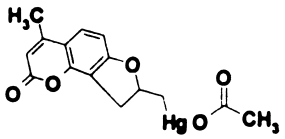
3. Results

3.1. Effects of mercuriopsoralens on keratinocyte growth

In initial studies, we compared the effects of the mercuriopsoralens on keratinocyte growth. All of the analogs were found to be growth inhibitory in the range of 6–32 μM (Table 1). The unsubstituted derivatives were more active than the 3′-substituted mercurio-derivatives (IC_{50} = 6–10 μM vs. 14–30 μM). The methyl angelicins were the least active compounds (IC_{50} = 32 μM). UVA light treatment caused a marked increase (~30-fold) in the sensitivity of the cells to iodomercurio-H_2TMP (P1) (Table 1, Fig. 1), and smaller increases in sensitivity to the other psoralen derivatives. UVA light also increased the activity of the chloromercurio- and trifluoroacetylmercurio-psoralen derivatives (P2 and P3, respectively) approximately 2- to 3-fold. Differences in the sensitivity of the cells to growth inhibition following UVA light treatment were not due to their absorption characteristics. All of the compounds had similar broad absorption spectra in the UVA range with a maximum at 330 ± 5 nm with the exception of the 3-cyano-acetylmercurio-derivative, which had an absorption maximum of 360 nm (Table 1). This is presumably due to conjugation of the cyano-triple bond to the aromatic rings of the psoralen. Thus, it is more likely that differences in sensitivity were due to the ability of the psoralens to form covalent adducts.

The activity of the mercuriopsoralens was found to be dependent upon intracellular levels of GSH. Thus, when cells were depleted of GSH with BSO, a 100- to 200-fold increase in sensitivity to iodomercurio-H_2TMP-induced growth inhibition was observed both in the absence and presence of UVA light (Fig. 1, upper panel). As also shown

Table 1
Effect of mercuriopsoralens on the growth of keratinocytes

Compound	Structure	IC_{50} (μM)		λ_{max}
		+UVA	−UVA	
P1 Iodomercurio-H_2TMP		0.3	10.0	333.1
P2 Chloromercurio-H_2TMP		3.1	8.1	332.2
P3 Trifluoroacetylmercurio-H_2TMP		4.2	7.0	332.7
P4 Acetylmercurio-H_2TMP		5.0	6.0	333.0
P5 3-Fluoro-acetylmercurio-H_2TMP		13	14	331.5
P6 3-Cyano-acetylmercurio-H_2TMP		20	30	360.9
P7 Iodomercurio-angelicin		23	32	325.3
P8 Acetylmercurio-angelicin		23	32	323.8

A growth curve was used to determine the IC_{50} of each compound as described in Section 2. The growth curve experiments were repeated two times with similar results. One representative experiment is shown.

in Fig. 1 (middle panel), under these conditions, BSO readily depleted keratinocytes of GSH. Augmenting intracellular GSH with NAc pretreatment partially protected the cells from growth inhibition by the mercuriopsoralen (Fig. 1, lower panel). Since the sensitivity of the cells to the mercuriopsoralens was dependent upon intracellular GSH, we next determined if intracellular GSH levels changed after mercuriopsoralen uptake. Fig. 2 (upper panel) shows that iodomercurio-H_2TMP (P1) accumulated in the cells in a concentration-dependent manner. This was associated with a decrease in intracellular GSH at concentrations greater than 10 μM (Fig. 2, lower panel). Taken together, these data suggest that the mercuriopsoralens act at a sulfhydryl-sensitive site in keratinocytes. This was supported by our findings that iodomercurio-H_2TMP can react directly with GSH to form a sulfhydryl adduct (unpublished studies). Moreover, in the absence of UVA light, pretreatment of iodomercurio-H_2TMP with GSH completely suppressed its growth inhibitory activity (Fig. 3). Interestingly, the sulfhydryl-derivative was found to retain activity when activated by UVA light, suggesting that sulfhydryl-conjugation reactions do not interfere with the ability of the psoralen to act as a photosensitizer.

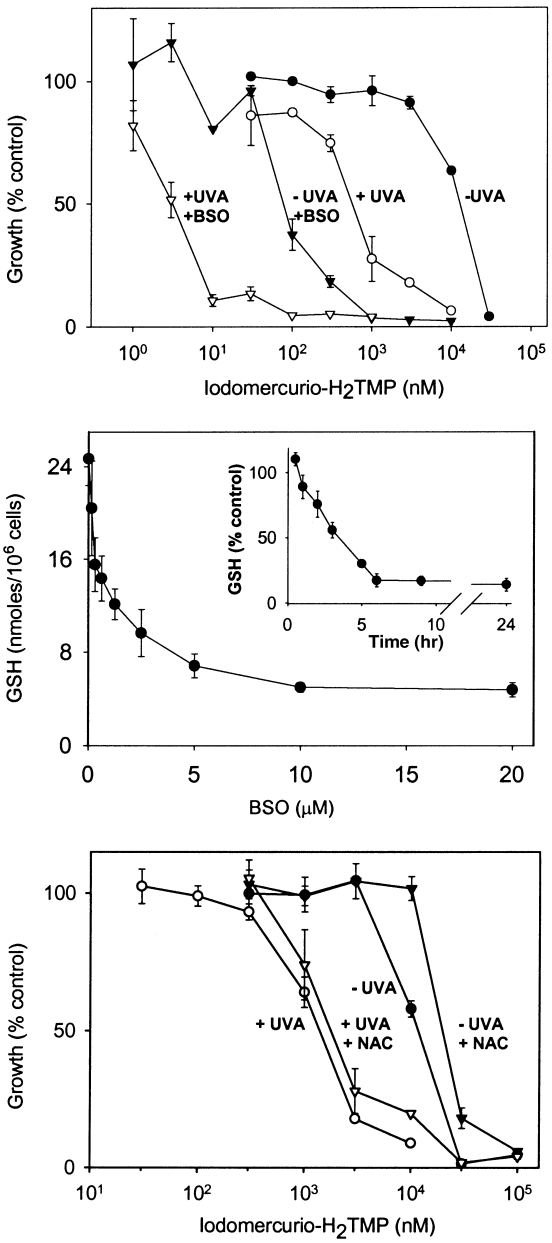


Fig. 1. Effects of iodomercurio-H_2TMP on keratinocyte growth. Upper panel: GSH depletion enhances growth inhibition by psoralens. Cultures were treated with BSO (10 μM) for 6 hr prior to iodomercurio-H_2TMP. Cell growth assays were performed as described in Section 2. Center panel: depletion of keratinocyte GSH by BSO. Cells were treated with increasing concentrations of BSO for 24 hr and then assayed for GSH content. The inset shows the effects of BSO (10 μM) on keratinocyte GSH content over time. Lower panel: effects of NAc on cell growth inhibition by iodomercurio-H_2TMP. Cells were pretreated with 3 mM NAc for 6 hr and then assayed for growth inhibition by iodomercurio-H_2TMP. All data points represent means ± SEM (N = 3). Control plates contained 5.5×10^4 cells.

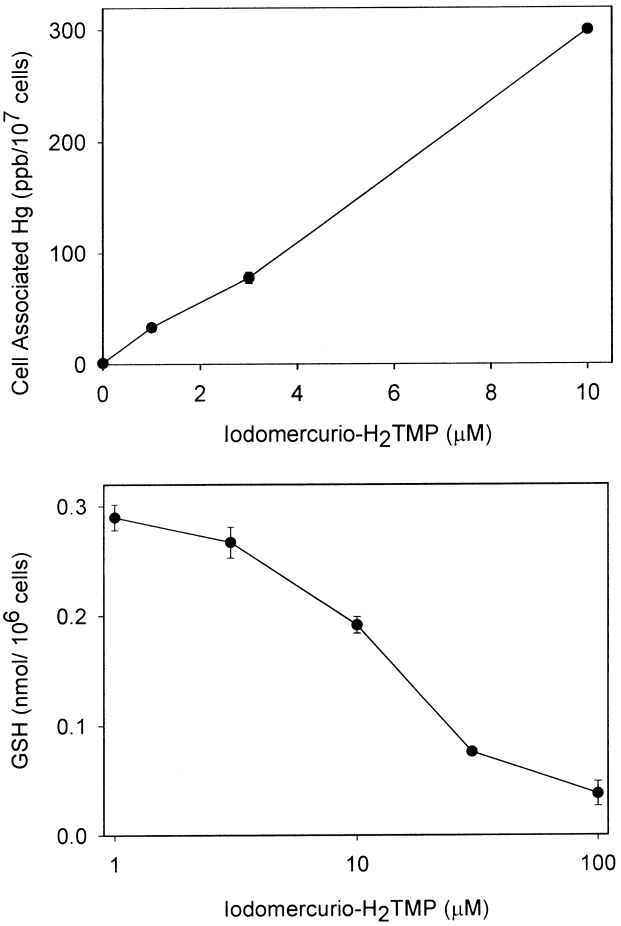


Fig. 2. Accumulation of iodomercurio-H_2TMP in keratinocytes and its effect on intracellular GSH. Upper panel: uptake of iodomercurio-H_2TMP. Keratinocytes were treated with increasing concentrations of the psoralen for 30 min at 37° and then analyzed for Hg content. Lower panel: depletion of intracellular GSH by iodomercurio-H_2TMP. Cells were incubated with increasing concentrations of the psoralen for 30 min at 37°. Unreacted GSH was then assayed. In both panels, each data point represents the mean ± SEM (N = 3).

3.2. Effects of iodomercurio-H_2TMP on DNA and protein synthesis

Psoralens are known to intercalate into DNA in a dark reaction. Following UVA light treatment, they effectively inhibit DNA synthesis [18]. Iodomercurio-H_2TMP was found to selectively inhibit DNA synthesis, when compared to protein synthesis, demonstrating that DNA is an important target for this compound (Fig. 4). However, no major changes in DNA or protein synthesis were observed following UVA light treatment (Fig. 4). Since the growth inhibitory activity of iodomercurio-H_2TMP was enhanced by UVA light treatment, sites other than the DNA appear to mediate this activity under these conditions. At the present time, the precise site of action of the mercuriopsoralens is unknown. In accord with the lack of activity of the GSH conjugate of iodomercurio-H_2TMP as an inhibitor of cell growth, this derivative did not inhibit DNA synthesis unless activated by UVA light (Fig. 3, lower panel). These data indicate that the DNA may be a target for this conjugate when photoactivated.

3.3. Effects of iodomercurio-H_2TMP on isolated DNA

To directly evaluate the effects of iodomercurio-H_2TMP on DNA, we performed DNA modification assays. In this

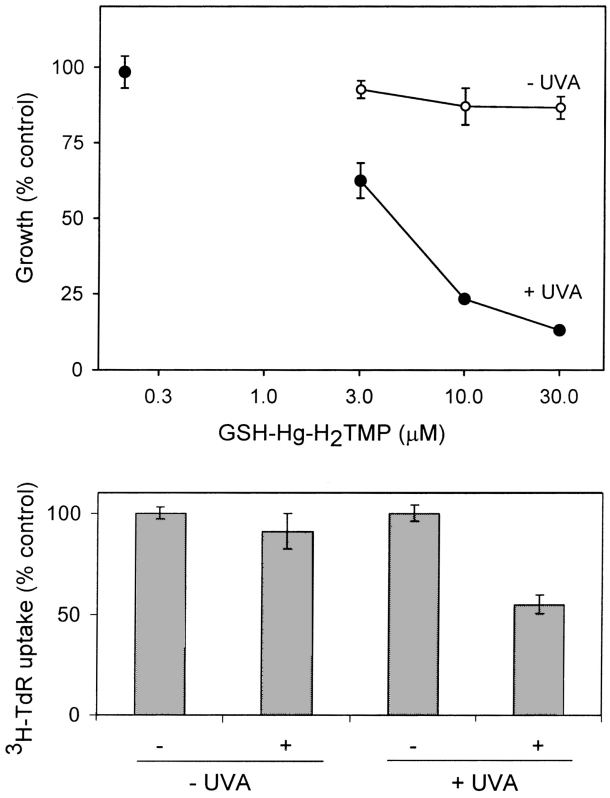


Fig. 3. Effects of the iodomercurio-H_2TMP–GSH conjugate on keratinocyte proliferation and DNA synthesis. Cells were treated with the conjugate followed by UVA light where indicated, and then assayed for growth inhibition (upper panel) or [^{3}H]thymidine ([^{3}H]TdR) uptake (lower panel). In the lower panel, control samples contained 1821 ± 188 dpm of [^{3}H]thymidine/mg protein. Cells were treated with a 10 μM concentration of the mercuriopsoralen conjugate. Each data point represents the mean± SEM (N = 3). Control wells in 24-well dishes contained 5×10^5 cells.

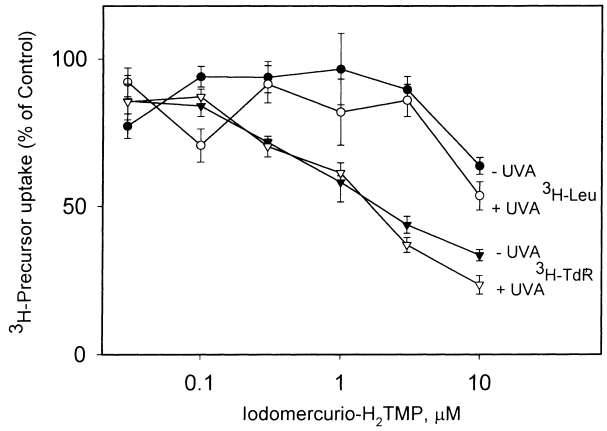


Fig. 4. Effect of iodomercurio-H_2TMP on [^{3}H]precursor uptake. Cells were incubated with the psoralen (30 min) followed by UVA light (open symbols) and then assayed for DNA and protein biosynthesis by [^{3}H]thymidine ([^{3}H]TdR, inverted triangles) or [^{3}H]leucine ([^{3}H]Leu, circles) uptake, respectively. Control samples contained 1103 ± 119 dpm [^{3}H]thymidine/mg protein and 3326 ± 236 dpm of [^{3}H]leucine/mg protein. Each data point represents the mean ± SEM (N = 3).

assay, supercoiled double-stranded plasmid DNA is treated with the psoralen. If the psoralens nick a strand of DNA, the DNA will unwind, and its migration in neutral agarose gels will be decreased greatly. Alterations in double-strand migration in denaturing gels are indicative of covalent modifications in the plasmid DNA. Under neutral conditions, we found that iodomercurio-H_2TMP, either without or with UVA light, did not affect plasmid DNA migration (Fig. 5A and B, respectively). However, under denaturing conditions, iodomercurio-H_2TMP was found to cause a concentration-dependent plasmid modification (Fig. 5C, bands M1 and M2). Moreover, after UVA light treatment, the extent of the modifications to the plasmid were increased (Fig. 5D, bands M1 and M2). These findings are in accord with our studies demonstrating increased growth inhibitory activity following UVA light treatment (Fig. 1 and Table 1). Interestingly, we observed a marked decrease in DNA modification by the psoralen–GSH conjugate in both the absence and presence of UVA light (Fig. 5, panels C and D, bands M1 and M2). Similar results were obtained with a sulfhydryl-containing cysteine adduct (Fig. 5, panels C and D, bands M1 and M2). These data indicate that DNA damage may be an important event leading to growth inhibition, even in the absence of UVA light. Since modification of the iodomercurio-H_2TMP to the mercurio–GSH conjugate leads to decreased DNA alkylation, this may explain the decreased growth inhibition with this compound.

4. Discussion

We have characterized several novel dihydromercuriopsoralens that are potent inhibitors of keratinocyte growth. All of these compounds were active in a concentration-dependent manner in the absence of UVA light. The linear psoralens were found to be more active than the angular derivatives. A generally similar pattern of cellular growth inhibition was observed when the compounds were activated by UVA light. These data indicate that the structure of the psoralen moiety is critical for growth inhibition. However, the psoralen portion of the molecule alone is not sufficient for biological activity. Structural analogs of the mercuriopsoralens lacking mercury such as TMP and 4′,5′-dihydropsoralens-like H_2TMP are inactive unless cells are treated with UVA light [12,18]. Thus, in the absence of UVA light, the mercurial substituent is responsible for the activity of these compounds. Mercury has a high affinity for sulfur, suggesting that cellular sulfhydryls are an important target for the mercuriopsoralens. This is supported by our observations that depleting cells of GSH markedly increases their sensitivity to these compounds, while NAc, which augments GSH levels, partially protects the cells from growth inhibition. It is well recognized that organic mercurials can form complexes with cysteine as well as GSH [14]. Moreover, in some instances, certain organic mercurials contain highly labile carbon–mercury bonds and are excreted as cysteine derivatives [20,21].

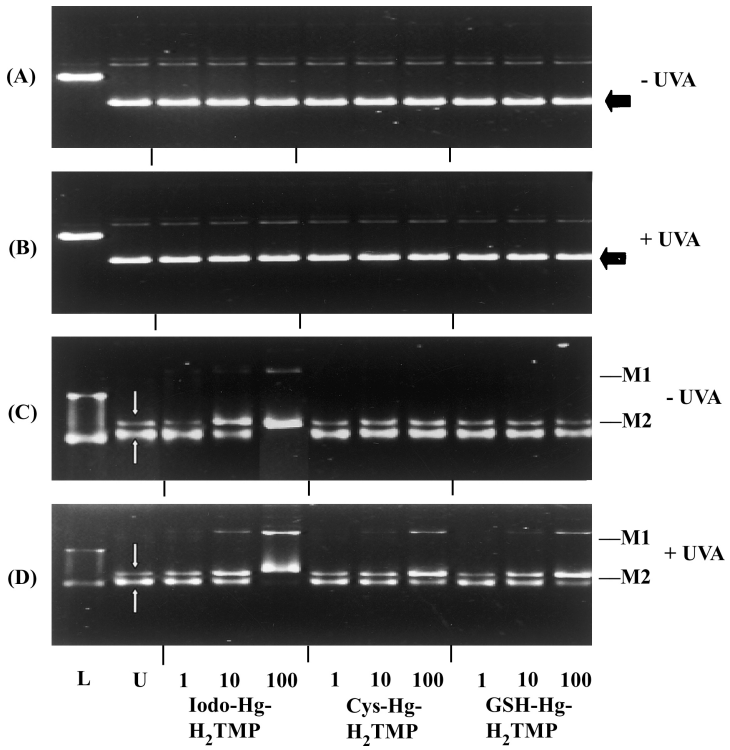


Fig. 5. Effects of psoralens and UVA light on plasmid DNA. Plasmid DNA was treated with 1–100 μM iodomercurio-H_2TMP (iodo-Hg-H_2TMP), iodomercurio-H_2TMP–cysteine conjugate (Cys-Hg-H_2TMP), or iodomercurio-H_2TMP–GSH conjugate (GSH-Hg-H_2TMP). Lane L shows products from the digestion with restriction endonuclease *Eco*RI to linearize the DNA (100 ng); in native gels, the linearized DNA migrates as one double-stranded band, whereas, in denaturing gels, it migrates as two well-separated single strands. Lane U shows untreated plasmid DNA (75 ng); in native gels, the intact plasmid DNA migrates as one supercoiled double-stranded band (indicated by the filled arrows to the right of panels A and B), whereas, in denaturing gels, it migrates as two closely migrating single strands (indicated by the white arrows). Electrophoretic analysis was performed with neutral (panels A and B) or alkaline (panels C and D) agarose gels. Samples were treated with (panels B and D) or without (panels A and C) UVA light. The lines on the right of panels C and D indicate the positions of the slower (M1) and faster (M2) migrating psoralen-modified single strands.

These data indicate that formation of these adducts occurs in cellular systems, and this may be an important biotransformation process.

For biological activity in the absence of UVA light, the mercuriopsoralens presumably form conjugates with critical sulfhydryl-containing peptides or proteins important in growth regulation. Although GSH is one important target, GSH depletion does not appear to be the mechanism by which these compounds act, since iodomercurio-H_2TMP had no major effect on cellular levels of this tripeptide at growth inhibitory concentrations. A GSH–mercuriopsoralen adduct was also found to lack biological activity in the absence of UVA light in growth inhibition assays, in DNA precursor uptake studies, and in plasmid DNA modification assays, indicating that the sulfhydryl conjugates formed in cells are not responsible for the growth inhibitory activity under these conditions. DNA and protein precursor uptake studies suggested that inhibition of DNA synthesis may be important in the actions of mercuriopsoralens. Plasmid DNA modification assays using isolated DNA indicated that the psoralen did not cause strand breaks, but significant DNA modifications such as alkylations occurred. These data suggest that, in addition to sulfhydryls, components of the DNA are targets for compounds such as iodomercurio-H_2TMP. In this regard, mercury(II) is known to stabilize the B structure of DNA and to bind guanine residues, presumably at the electron rich N3 or N7 positions [22].

In our studies, iodo-, chloro- and various acetyl-mercurial-derivatives of the dihydropsoralens were analyzed. In the absence of UVA light, the growth inhibitory activity of the compounds was generally similar, except for the 3-cyano-derivatized psoralen and, as already indicated, the angelicins. Taken together, these data suggest that, under these conditions, biological activity is not dependent upon the type of mercurial substitution. Psoralens are linear furocoumarins, and the precise function of their structure in the activity of the compounds examined in the present studies is not clear. Although the furocoumarin is likely to regulate the uptake and subcellular distribution of the compounds, it is also important in the interaction of the psoralen derivatives with specific intracellular targets. Psoralens intercalate into DNA, and the linearity of the furocoumarin is an important structural requirement for this activity [3]. Angelicins are angular furocoumarins, intercalate DNA poorly, and display low activity as photosensitizers, which may account for the low activity of the angular mercuriopsoralens [10]. A cyano-substitution at the 3 position alters the resonance structure of the psoralen ring as shown by our observation of an altered absorbance

spectrum of this 3-cyano-acetylmercurio-H_2TMP (P6), when compared with other psoralens, and this presumably alters its ability to intercalate DNA and bind other cellular targets [3–6]. This may also contribute to the reduced activity of this analog. In the presence of UVA light, the 3-cyano substitution also blocks DNA adduct formation at the C3 position of the coumarin ring.

Another important intracellular target for the psoralens is a specific 22,000 molecular weight membrane and cytoplasmic receptor [4,5]. This receptor is known to be important in the regulation of epidermal cell growth. Activation of the receptor by photoactivated psoralens down-regulates receptors for epidermal growth factor (EGF), reduces its intrinsic tyrosine kinase activity, and stimulates an EGF receptor serine kinase [6,23], processes that can restrict the growth of epidermal cells in diseases such as psoriasis. Linear furocoumarins bind to the psoralen receptor with high affinity, and the lower activity of the 3-cyano-acetylmercurio-H_2TMP, as well as the angelicins, may be due to weak receptor binding [4]. One could speculate that covalent modification of the psoralen receptor by the mercuriopsoralens results in its activation, leading to growth inhibition. At the present time, we cannot exclude the possibility that both DNA binding and psoralen receptor activation, as well as other intracellular targets, are important in the mechanism by which the mercuriopsoralens cause keratinocyte growth inhibition.

The mercuriopsoralens varied significantly with respect to their activities as photosensitizers with many of the compounds displaying little or no additional responses to UVA light. Based on the structure of the furocoumarin, it is not clear why the compounds vary in activity. As already indicated, for the angelicin- and 3-cyano-derivatives, low activity may be due to an inability of these compounds to bind to sensitive intracellular targets. Iodomercurio-H_2TMP was the most active photosensitizer when compared with the other psoralen derivatives. This analog is taken up rapidly by keratinocytes. However, the extent to which it complexes with cellular material including DNA, the psoralen receptor, and/or other sulfhydryl-containing proteins, peptides, or amino acids remains to be determined. The striking differences in activity between the iodomercurio- and chloromercurio-derivatives may be due to differences in uptake, distribution, and metabolism of these compounds. Of interest was our finding that UVA light failed to enhance iodomercurio-H_2TMP-induced inhibition of DNA synthesis. At the present time, it is not known if inhibition of DNA synthesis, in fact, represents psoralen–DNA modifications. Multiple mechanisms can cause inhibition of DNA synthesis including inactivation of proteins regulating cell cycle transit [24,25] and an imbalance in cell growth [26]. Our finding that iodomercurio-H_2TMP conjugated to GSH retains activity as a photosensitizer suggests that the photosensitization reactions may depend upon the formation of these adducts. As observed with the parent compound, these adducts may also function by binding to DNA and/or the psoralen receptor; however, other sites of action for these derivatives cannot be excluded. With iodomercurio-H_2TMP, the greatest biological activity was observed in GSH-depleted cells, indicating that the photosensitization reactions for growth inhibition were also dependent on a sulfhydryl-sensitive site, possibly independent of the DNA. This is in accord with our findings of a lack of an effect of UVA light on DNA synthesis inhibition by iodomercurio-H_2TMP.

Currently, it is uncertain if the mercuriopsoralens will be of therapeutic value in the treatment of skin diseases since there is concern that mercury-containing compounds are toxic. However, mercury diuretics, a class of related organic compounds containing similar mercurio-reactive moieties, have been in wide use clinically with few reports of toxicity [14]. This may be due to the fact that little, if any, ionic mercury is detectable in tissue or urine after injection of the organic compound [14]. Similarly, the mercuriopsoralens may not be metabolized to a toxic form when administered to patients topically for the treatment of skin diseases. Further studies on the disposition of the mercury contained in the psoralens are required. Nevertheless, these compounds are useful in understanding the mechanism by which chemical photosensitizers regulate epidermal proliferation.

Acknowledgments

This work was supported by NIH Grants ES06897, ES05022, and ES03647. We thank Willie Johnson for assistance in performing the mercury analysis.

References

[1] Bethea D, Fullmer B, Syed S, Seltzer G, Tiano J, Rischko C, Gillespie L, Brown D, Gasparro FP. Psoralen photobiology and photochemotherapy: 50 years of science and medicine. J Dermatol Sci 1999;19:78–88.
[2] Parrish JA, Stern RA, Pathak MA, Fitzpatrick TB. Photochemotherapy of skin diseases. In: Regan JD, Parrish JA, editors. The science of photomedicine. New York: Plenum Press, 1982. p. 595–624.
[3] Song PS, Tapley KJ. Photochemistry and photobiology of the psoralens. Photochem Photobiol 1979;29:1177–91.
[4] Laskin JD, Lee E, Yurkow EJ, Laskin DL, Gallo MA. A possible mechanism of psoralen phototoxicity not involving direct interaction with DNA. Proc Natl Acad Sci USA 1985;82:6158–62.
[5] Yurkow EJ, Laskin JD. Characterization of a photoalkylated psoralen receptor in HeLa cells. J Biol Chem 1987;262:8439–42.
[6] Laskin JD, Lee E. Psoralen binding and inhibition of epidermal growth factor binding by psoralen/ultraviolet light (PUVA) in human epithelial cells. Biochem Pharmacol 1991;41:125–32.
[7] Caffieri S, Zarebska Z, Dall'Acqua F. Psoralen photosensitization: damage to nucleic and membrane lipid components. Acta Biochim Pol 1996;43:241–6.
[8] Gasparro FP, Felli A, Schmitt IM. Psoralen photobiology: the relationship between DNA damage, chromatin structure, transcription, and immunogenic effects. Recent Results Cancer Res 1997;143:101–27.

[9] Stern RS. The risk of melanoma in association with long-term exposure to PUVA. J Am Acad Dermatol 2001;44:755–61.
[10] Dall'Acqua FD, Vedaldi S, Caffieri A, Guiotto P, Rodighiero F, Baccichetti F, Carlassare F, Bordin F. Methylangelicins: new potential agents for the photochemotherapy of psoriasis. Structure–activity studies on the dark and photochemical interactions with DNA. J Med Chem 1981;24:806–11.
[11] Rodighiero G, Dall'Acqua F. Present aspects concerning the molecular mechanism of photochemotherapy with psoralens. Drugs Exp Clin Res 1986;12:507–15.
[12] Jetter MM, Heindel ND, Laskin JD. Novel synthesis of dihydroxanthyletin and dihydrosesselin derivatives. J Heterocyclic Chem 1990;27:995–7.
[13] Mariano TM, Vetrano AM, Gentile SL, Heck DE, Whittemore MS, Guillon CD, Jabin I, Rapp RD, Heindel ND, Laskin JD. Cell-impermeant pyridinium derivatives of psoralens as inhibitors of keratinocyte growth. Biochem Pharmacol 2002;63:31–9.
[14] Cafruny EJ. The site and mechanism of action of mercurial diuretics. Pharmacol Rev 1968;20:89–116.
[15] DeGeorge GL, Heck DE, Laskin JD. Arginine metabolism in keratinocytes and macrophages during nitric oxide biosynthesis: multiple modes of action of nitric oxide synthase inhibitors. Biochem Pharmacol 1997;54:103–12.
[16] Heck DE, Laskin DL, Gardner CR, Laskin JD. Epidermal growth factor suppresses nitric oxide and hydrogen peroxide production by keratinocytes. Potential role for nitric oxide in the regulation of wound healing. J Biol Chem 1992;267:21277–80.
[17] Whittemore MS. Synthesis, structure–activity relationships and photochemical studies of novel coumarins, dihydropsoralens and dihydroangelicins as photoactivated agonists of the psoralens receptor. Ph.D. Dissertation. Lehigh University, Bethlehem, PA, 1998.
[18] Yurkow EJ, Laskin JD. Mechanism of action of psoralens: isobologram analysis reveals that ultraviolet light potentiation of psoralen action is not additive but synergistic. Cancer Chemother Pharmacol 1991;27:315–9.
[19] Guaiquil VH, Farber CM, Golde DW, Vera JC. Efficient transport and accumulation of Vitamin C in HL-60 cells depleted of glutathione. J Biol Chem 1997;272:9915–21.
[20] Weiner IM, Levy RI, Mudge GH. Studies on mercury diuresis: renal excretion, acid stability and structure–activity relationships of organic mercurials. J Pharmacol Exp Ther 1964;138:96–112.
[21] Weiner IM, Müller OH. A polarographic study of mersalyl(salyrgan)-thiol complexes and of the excreted products of mersalyl. J Pharmacol Exp Ther 1955;113:241–9.
[22] Gruenwedel DW, Cruikshank MK, Smith GM. Effect of Hg(II) on d(GCGCATATGCGC)$_2$ conformation: UV absorption and circular dichroism studies. J Inorg Biochem 1993;52:251–61.
[23] Mermelstein FH, Abidi TF, Laskin JD. Inhibition of epidermal growth factor receptor tyrosine kinase activity in A431 human epidermoid cells following psoralen/ultraviolet light treatment. Mol Pharmacol 1989;36:848–55.
[24] Coradini D, Pellizaro C, Marimpietri D, Abolafio G, Daidone MG. Sodium butyrate modulates cell cycle-related proteins in HT29 human colonic adenocarcinoma cells. Cell Prolif 2000;33:139–46.
[25] Taylor WR, Stark GR. Regulation of the G2/M transition by p53. Oncogene 2001;20:1803–15.
[26] Kamen B. Folate and antifolate pharmacology. Semin Oncol 1997; 24(Suppl 18):30–9.