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Identification of a Pyranocoumarin Photosensitizer that is a Potent Inhibitor of Keratinocyte Growth

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ABSTRACT

Photosensitizers are used in the treatment of epidermal proliferation and differentiation disorders such as psoriasis and vitiligo. In the present studies, a ring-expanded carbon homologue of the linear psoralen (furo[3,2-g]benzopyran-7-one) class of photosensitizers, 4,10-dimethyl-*2H,8H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one (NDH2476), was synthesized and analyzed for biological activity. Following activation by ultraviolet light (UVA, 320-400 nm), NDH2476 was found to be a potent inhibitor of keratinocyte growth (IC_{50} = 9 nM). Similar derivatives methylated in the pyrane ring, or containing a saturated pyrane ring structure, were markedly less active or inactive as photosensitizers. NDH2476 was found to intercalate and damage DNA following UVA light treatment as determined by plasmid DNA unwinding and nicking experiments. Taken together, these data demonstrate that an intact furan ring in psoralen photosensitizers is not required for keratinocyte growth inhibition or DNA damage. Our findings that low nanomolar concentrations of a benzopyranone derivative was active as photosensitizer indicates that this or a structurally related

compound may be useful in the treatment of skin diseases involving aberrant epidermal cell growth and differentiation.

INTRODUCTION

Psoralens in combination with ultraviolet light (UVA; 320-400 nm), also referred to as PUVA, are used in the photochemical treatment of a variety of skin diseases including those characterized by abnormal keratinocyte proliferation and differentiation, as well as inflammation such as psoriasis, eczema and chronic actinic dermatitis (1-4). PUVA is also used to treat vitiligo, a skin depigmentational disorder, and certain forms of cancer including cutaneous T cell lymphomas (5-8). The most commonly used psoralen is 8-methoxypsoralen (8-MOP), a furanocoumarin known to intercalate into DNA. Following exposure to UVA light, 8-MOP can modify DNA forming monofunctional and bifunctional adducts. DNA cross-links form via 2+2, cyclobutane-like fusions from double bonds 3-4 and 4'-5' in the psoralen molecule to double bonds in pyrimidine bases (2). A number of naturally occurring and synthetic analogs of linear and angular furanocoumarins (psoralens and angelicins, respectively) have been evaluated in efforts to improve the efficacy of PUVA phototherapy (9-11). One early example is trioxsalen (4, 5', 8-trimethylpsoralen), a lipophilic psoralen derivative that is efficacious against both psoriasis and vitiligo (12, 13).

Our laboratories have been synthesizing and evaluating benzodipyranones as phototherapeutics (14). These compounds are generally described as psoralen analogs in which an unsaturated pyrane ring is substituted for the unsaturated furan ring. Synthetically derived from 7-hydroxycoumarins by acid condensation with propargyl halides, the linear benzo[1,2-b:5,4-b']dipyran-2-ones (Fig. 1, compound 1) and the angular benzo[1,2-b:3,4-b']dipyran-2-ones (Fig. 1, compound 2) are ring-expanded carbon homologues of the linear psoralens (formal name: furo[3,2-g]benzopyran-7-ones) (Fig. 1, compound 3), and their angular angelicin isomers (formal name: furo[2,3-h]benzopyran-2-ones) (Fig. 1, compound 4). A wide variety of methylated and otherwise functionalized variants of these compounds have been synthesized and accessibility to selective

reduction chemistry for the carbon-carbon double bond in the non-pyranone rings has further expanded the compound library (15). In previous studies, we describe convenient synthetic methods for condensing 2-propyn-1-ols and 7-hydroxycoumarins to prepare, isolate, and characterize these compounds; to prepare the reduced pyrane ring analogs, co-reactants for condensation with the 7-hydroxycoumarins are the requisite allyl alcohols (16, 17, 14).

In the present studies we evaluated a number of pyranocoumarins and related analogs as photosensitizers. A linear lipophilic derivative, 4,10-dimethyl-*2H,8H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one (NDH2476), was identified as a potent inhibitor of keratinocyte growth; its activity was dependent on exposure to UVA light. The ability of this pyranocoumarin to unwind and nick plasmid DNA indicates that nuclear DNA is a target for this psoralen mimic which may have clinical potential as a phototherapeutic agent.

Materials and Methods

Cells and reagents. PAM212 mouse keratinocytes were obtained from Dr. Stuart Yuspa (National Institutes of Health) and grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. 4,8-Dimethyl-7-hydroxycoumarin (alternative name 4,8-dimethylumbelliferone) was from AK Scientific (Union City, CA). 4,5',8-trimethyldihydropsoralen (H_2TMP) was prepared as previously described (17). Unless otherwise indicated DMEM and all other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Pyranocoumarins. Methods for the synthesis of the pyranocoumarins and related analogs are described elsewhere (14). For 4,10-dimethyl-*2H,8H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one (NDH2476), 4,8-dimethyl-7-hydroxycoumarin was twice recrystallized from anhydrous ethanol to a purity of >98%. The 7-hydroxyl function was propargylated by 8 hr of reflux in 200 mL of acetone (25 mmol of coumarin and 38 mmol of 3-bromo-1-propyne) containing 10 g of well-stirred suspended anhydrous potassium carbonate. Filtration of the reaction liquid from the carbonate salt yielded an acetone

solution of product which upon concentration *in vacuo* and recrystallization from methanol yielded 3.72 g (64%) of white crystalline 4,8-dimethyl-7-propargylcoumarin (m.p. 139-141^{o}C). This propargyl ether (0.70 g) was cyclized to the above titled benzodipyrane compound (NDH2476) by heating and magnetic stirring at 200^{o}C in 15 mL of N,N-diethylaniline solvent being maintained under a stream of dry nitrogen gas. After heating for 5 hr, the reaction contents were dissolved in 25 mL of ethyl acetate, washed successively with 2 x 25 mL of 6N hydrochloric acid and 2 x 25 mL of water, dried over anhydrous sodium sulfate, evaporated to a brown gum, and purified by preparative thick layer chromatography with a 35:65 ethyl acetate:cyclohexane moving phase on Analtech GF Silica Gel plates. White crystals (0.14 g, 21% yield, from methanol) of product were obtained, m.p. 178-180^{o}C.

The ^{1}H-NMR spectrum in $CDCl_3$ confirmed the structure and the purity of the product with two pendant methyls (3H integration each) at 2.25 and 2.34 ppm; four single 1H integration sp^2 C-H's at 6.11 (C_3-H), 6.97 (C_5-H), 6.43 (C_6-H), and 5.80 (C_7-H); and the unique C_8-CH_2-O- at 4.94 ppm (dd, 2H integration, J = 1.9 coupled to C_6-H, J' = 3.5 Hz coupled to the C_7-H). The NMR spectrum also confirms key structural issues. The fact that both hydrogens in the downfield shifted methylene [-CH_2-O-] are equivalent and each is equally coupled to their adjacent vicinal =C-H at C_7 and to their beta =C-H at C_6 confirms the planarity of this dipyran-2-one system. Basically, the C-H projecting upward at the methylene carbon C_8 is magnetically identical to the C-H projecting downward from that carbon.

Using preparative liquid chromatography, we have also detected and isolated from commercial samples of trioxsalen trace quantities (ca 1%) of 4,10-dimethyl-*2H,8H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one (NDH2476). This is an isomer of trioxsalen which is apparently a byproduct of the manufacturing route for that active pharmaceutical ingredient.

Growth inhibition assays. The effects of the pyranocoumarins on keratinocyte growth were assayed in 6-well culture dishes as described previously (18, 19). Briefly, cells were plated in 2 ml of growth medium at low density (5 × 10^3 cells/well). After allowing the cells to adhere overnight, the medium was replaced with DMEM supplemented with either control medium or medium containing

increasing concentrations of the pyranocoumarins. Each concentration was tested in triplicate. After incubating the cells for 30 min at 37°C, 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) (18). 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 IC_{50} for growth inhibition was the concentration of each compound that inhibited growth by 50%. *Treatment of plasmid DNA with pyranocoumarins and UVA light.* Methods previously described by our laboratories were used for plasmid DNA preparation and analysis of modifications (18, 19). Briefly, the 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. TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8) was used to elute the DNA which was quantified spectrophotometrically. The 260/280 absorbance ratio of 1.89 indicated very high purity; plasmid DNA was at least 95% in the supercoiled form as determined by agarose gel electrophoresis. The restriction endonuclease EcoRI (New England Biolabs) was used to prepare linear double-stranded plasmid DNA (10 µg). DNA was purified after extracting twice with phenol:chloroform:isoamyl alcohol (25:24:1, by vol.) and twice with chloroform:isoamyl alcohol (24:1, v/v) followed by precipitation with ethanol. After centrifugation, the DNA was recovered, washed once with 70% ethanol, air dried, and dissolved in 0.2 ml of TE buffer.

Plasmid DNA was treated with the pyranocoumarins and UVA light in a 10-µL reaction mixture containing 75 ng pZeoSV, 0.1 µL of the test compounds (prepared as 100× solutions in DMSO), and TE buffer. The reaction volume and components were scaled up several folds so that multiple aliquots of 10 µL could be removed before and after UVA light treatment for analysis on agarose gels. UVA light treatment was performed in V-bottomed 96-well plates for 10 min under the conditions already described for the treatment of cell cultures. The treated plasmid samples were analyzed for modifications using agarose gel electrophoresis. Analyses of plasmid DNA on neutral

gels were performed in 1.2% agarose in neutral gel buffer (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA). Samples of DNA for analyses were first mixed with concentrated agarose gel loading buffer [10×=50% (v/v) glycerol containing 4.2 mg/mL of bromophenol blue]. Gels were run at 24 V in a Bio-Rad Wide Mini-Subcell GT DNA electrophoresis unit until the bromophenol blue migrated about 8 cm. For analysis of denatured samples, DNA was first mixed with 2 M NaOH supplement with 0.1 M EDTA, heated in a water bath at 90° for 1 min, and cooled on ice prior to loading on 1.2% alkaline agarose gels. Gels were prepared and electrophoresed in alkaline gel buffer (50 mM Tris base, 45 mM boric acid, 30 mM NaOH, 1 mM EDTA). After electrophoresis, gels were washed, stained with ethidium bromide (0.5 μg/mL) and then photographed with an Eagle Eye II digital documentation system (Stratagene, San Diego, CA).

RESULTS AND DISCUSSION

In initial studies, the effects of the pyranocoumarins on keratinocyte growth were assayed (Fig. 2 and Table 1). Of the compounds tested, four displayed growth inhibitory activity (NDH2476, NDH2458, NDH2507 and NDH2457) following activation by UVA light, NDH2476 was the most active (IC_{50} = 13 nM), followed by NDH2458, NDH2507 and NDH2457 (IC_{50}'s = 9,000 nM, 11,000 nM and 50,000 nM, respectively). Two analogs (NDH2441 and NDH2456) had no effect on cell growth at concentrations up to 100 μM. In the absence of ultraviolet light, none of the compounds displayed activity as inhibitors of keratinocyte growth.

To directly evaluate the effects of the compounds on DNA, we performed DNA modification assays. In this assay, supercoiled double-stranded plasmid DNA was treated with the pyranocoumarins. If the compounds nick a strand of DNA, the DNA will unwind, and its migration in neutral agarose gels will decrease significantly (18, 19). Alterations in double-strand migration in denaturing gels are indicative of covalent modifications in the plasmid DNA. Under neutral conditions, we found that none of the pyranocoumarins, either without or with UVA light treatment,

affected plasmid DNA migration (Fig. 3A and B, respectively). Conversely, under denaturing conditions, NDH2476 treated with UVA light, but not NDH2458 or NDH2457 altered the migration of DNA in the gels (Fig. 3C and D, respectively). A small decrease in migration was noted with NDH2507. In both neutral and denaturing agarose gels, 4,5',8-trimethyldihydropsoralen, a positive control, was found to alter migration of the plasmid confirming both DNA nicking and covalent modifications. Taken together, these data indicate that DNA modifications may be an important event leading to growth inhibition by NDH2476.

Our data demonstrate that a pyranocoumarin photosensitizer, 4,10-dimethyl-2H,8H-benzo[1,2-b:5,4-b']dipyran-2-one (NDH2476) is potent inhibitor of keratinocyte growth. The ability of the psoralens to intercalate DNA is due to their linear planar structure. This is an energetically favorable reaction and, following exposure to ultraviolet light, results in the formation of both monofunctional and bifunctional DNA adducts (20). As NDH2476 has a generally similar structure to psoralen, we expected that it would also intercalate and modify DNA following exposure to UVA light. This was found to be the case based on DNA plasmid damage assays. The fact that NDH2476 has the potential to form both monofunctional and bifunctional adducts may account for its high potency in keratinocyte growth inhibition assays. However, similar derivatives either methylated in the pyrane ring or with a saturated pyrane ring structure, including NDH2458, NDH2507 and NDH2457, were markedly less active as photosensitizers in keratinocyte growth assays; this is likely due to a much more limited ability of these compounds to intercalate into DNA and modify DNA bases following treatment with ultraviolet light. This is supported by our observation that these derivatives displayed little or no activity in plasmid DNA unwinding and damage assays. Unlike psoralen or NDH2476, each of the compounds contains a saturated pyrane ring, resulting in a non-planar structure that disrupts π-π^* interactions with pyrimidine bases and hinders intercalation into DNA; it also does not allow for the formation of bifunctional DNA adducts. It should also be noted

that pyranocoumarins with a saturated ring structure have a much more limited ability to absorb UVA light and participate in photosensitization reactions.

The presence of bulky dimethyl groups in the 8 position of the saturated pyrane ring in NDH2458 and NDH2457 also results in steric hindrance for DNA interactions; this significantly reduced activity in keratinocyte growth inhibition assays. Similarly, neither the NDH2476 related trimethyl analog (NDH2441), nor its saturated trimethylderivative (NDH2456) were active keratinocyte growth inhibition or plasmid DNA nicking and damage assays, presumably because of steric hindrance due to the dimethyl group in the 8 position of the pyranocoumarin. It should be noted that NDH2458 is also an angular pyranocoumarin with a saturated pyrane ring. Angelicins are related psoralen derivatives with photosensitizing activity (9, 11). However, they are less active than psoralens since they can only form monofunctional adducts. The limited ability of NDH2458 to inhibit keratinocyte cell growth may also be due to the fact that it only has the capacity to form monofunctional DNA adducts.

In summary, we have identified a photosensitizer, 4,10-dimethyl-*2H,8H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one (NDH2476), that readily intercalates and modifies DNA, and is a potent inhibitor of keratinocyte growth. Unlike the psoralens which contain a furan ring, this pyranocoumarin contains a unsaturated pyrane ring demonstrating that the furan ring structure is not required for photosensitization reactions. Optimal activity of NDH2476 also requires the unsaturated pyrane ring which is likely important for the formation of bifunctional DNA adducts. In contrast to psoralens, which have the capacity to nick DNA and cause strand breakage, NDH2476 only modified DNA, suggesting that it has a unique mechanism of action. The nature of adducts formed by this pyranocoumarin with DNA are not known. It should be noted that photoactivated psoralens can also target other components in cells including proteins and membranes and these may also be targets for NDH2476 that contribute to its mechanism of action. Both psoralen protein adducts and psoralen DNA binding proteins have been identified as well as psoralen-phospholipid adducts (21-28). Alterations in each of these targets has the capacity to activate and/or modify cell signal

transduction pathways that may be important in controlling growth and differentiation pathways leading to therapeutic responses to the photosensitizers (21, 29, 30, 28). At the present time, the precise site of action of pyranocoumarins and their effects on cell signal transduction in epidermal cells are not known. Future studies are needed to determine if these compounds are effective as phototherapeutic agents to treat various skin disorders.

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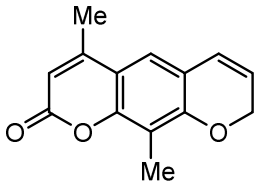
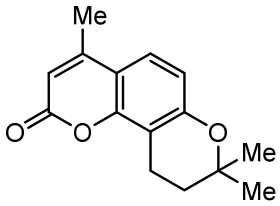
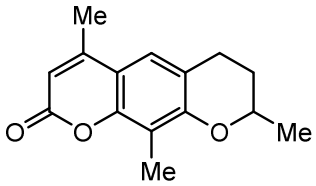
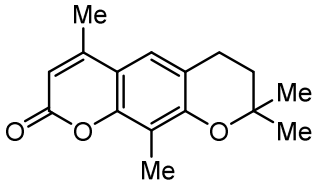
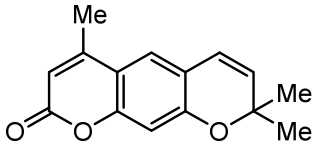
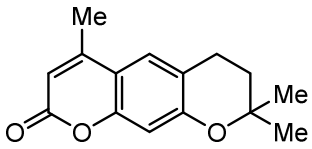
Table 1. Pyranocoumarin structures and growth-inhibitory activities.

Compound[1]	Structure	IC_{50} (nM)	
		+ UVA	– UVA
NDH2476		13	> 100,000
NDH2458		9,000	> 100,000
NDH2507		11,000	> 100,000
NDH2457		50,000	> 100,000
NDH2441		> 100,000	> 100,000
NDH2456		> 100,000	> 100,000

[1] NDH2476: 4,10-dimethyl-*2H,8H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one; NDH2457: 7,8-dihydro-4,8,8,10-tetramethyl-*2H,6H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one; NDH2456: 7,8-dihydro-4,8,8-trimethyl-*2H,6H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one; NDH2441: 4,8,8-trimethyl-*2H,8H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one; NDH2507: 7,8-dihydro-4,8,10-trimethyl-*2H,6H*-benzo[1,2-*b*:5,4-*b'*]dipyran-2-one; NDH2458: 9,10-dihydro-4,8,8,-trimethyl-2H,8H-benzo[1,2-b:3,4-b']dipyran-2-one.

FIGURE LEGENDS

Figure 1. Basic structures of pyranocoumarins, psoralens and angelicins. The panel shows the linear benzo[1,2-b:5,4-b']dipyran-2-ones (compound 1) and the angular benzo[1,2-b:3,4-b']dipyran-2-ones (compound 2) which are ring-expanded carbon homologues of the psoralens (furo[3,2-g]benzopyran-7-ones) (compound 3) and their angular angelicin isomers (formal name: furo[2,3-h]benzopyran-2-ones) (compound 4). The structures of clinically used psoralens [8-methoxypsoralen (8-MOP) and Trioxsalen] are also shown.

Figure 2. Effects of pyranocoumarins on keratinocyte growth. Keratinocyte cultures were treated with control medium or medium containing increasing concentrations of the pyranocoumarins as described in the Materials and Methods. After 30 min at 37°, cells were treated with (closed symbols) or without (open symbols) UVA light. Cells were then refed with fresh growth medium. After 5 days, cells were removed from the dishes and counted on a Coulter Counter. Data are presented as percent inhibition of cell growth relative to controls. Data represent means ± SEM (n = 3). In a typical experiment, control plates contained 5.5×10^4 cells.

Figure 3. Effects of pyranocoumarins and UVA light on plasmid DNA. Plasmid DNA was treated with increasing concentrations of pyranocoumarins. Lane L shows products from the digestion with restriction endonuclease EcoRI 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 S shows untreated plasmid DNA (75 ng); in native gels, the intact plasmid DNA migrates mainly as one supercoiled double-stranded band, whereas, in denaturing gels, it migrates as two closely migrating single strands. 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. H_2TMP was used as a positive control for psoralen-induced plasmid DNA strand breaks.