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Synthetically Modified Methoxsalen for Enhanced Cytotoxicity in Light and Dark Reactions

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Abstract

Linear furocoumarins, also known as psoralens, are clinically useful photo-activated pharmaceuticals employed to address hyperproliferative skin diseases. Seven diverse cytotoxic pharmacophores have been synthetically attached to 8-methoxypsoralen via a 5-amino functionality. The resulting unique set of compounds was evaluated for dark and light toxicity against PAM212 keratinocytes in culture.

Graphical Abstract

The natural product furocoumarin, known as methoxsalen or 8-MOP, has a long history as a nonionic reversible DNA intercalator^(1,2) which demonstrates modest anticancer effects in the absence of light^(3,4) but powerful cytotoxic effects upon photoactivation⁽⁵⁻⁷⁾. A photocatalyzed mechanism through dual 2+2 cyclobutane formation between the proximal

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and distal ends of the psoralen and the pyrimidine bases, thereby cross-linking the DNA, has been invoked to explain the cytotoxicity.⁽¹⁾

The synthetic incorporation of traditional anti-cancer pharmacophores onto the furocoumarin's intercalator backbone might be expected to enhance the dark- and/or the light-activated cytotoxicity of the linear three-ring system. The 3,3-dialkyl-1-triazene group^(8,9), the alpha-haloacetamide construct⁽¹⁰⁾, the beta-chloroethylamine moiety⁽¹¹⁻¹³⁾ and the arylazide⁽¹⁴⁻¹⁵⁾ functionality, all have a history of utility as anti-cancer pharmacophores through their incorporation in many other candidate chemotherapeutic platforms. The chemotherapeutic dacarbazine (or DTIC) is the most established drug containing the dialkyltriazene group. A photo-induced enhancement of its activity has recently been reported.⁽¹⁶⁾ Many azide-containing molecules are cytotoxic with the antiviral azidothymidine (Zidovudine) being the most prominent⁽¹⁷⁾ and, of course, the beta-chloroethylamino construct is found in many anticancer agents including Alkeran and Estramustine.^(11, 18)

All of our methoxsalen chemotherapeutic candidates were synthesized in three or four steps from the parent psoralen. Careful nitration of methoxsalen, compound **(2)**, followed by low-pressure hydrogenation of the pendant nitro group, provided a 38% yield of compound **(1)**, the 5-amino-8-methoxypsoralen (a.k.a., 4-amino-9-methoxy-7*H*-furo[3,2-*g*] [1]benzopyran-7-one, or 5-amino-8-MOP or 5-aminomethoxsalen).⁽¹⁹⁾ The 5-amino functionality on this key intermediate constitutes a convenient synthetic handle to which can be attached the indicated cytotoxic pharmacophores: the triazeno group [$-N=N-NR_2$], the alpha-haloacetamido group [$-NH-CO-CH_2Cl$], the beta-chloroethylamino functionality [$-NH-CH_2CH_2-Cl$] and the aryl azido group [$-NH-CO-Ph-N_3-p$]. Synthetic procedures for these attachments are described in the experimental section included in the supporting information and illustrated on Scheme 1.

For biological evaluation, since the linear furocoumarins (psoralens) are most noted for their clinical activity against pathological skin conditions, the PAM212 keratinocyte cell line was chosen as a screening model for both a dark and a light-catalyzed cytotoxicity study with compounds **2** to **9**. The PAM212 line is a murine cutaneous squamous cell carcinoma with a long history of use as a surrogate for human skin hyperproliferative disease.⁽²⁰⁾ Photo- and non photo-biological activity was assayed with this cell line grown in monolayer culture as previously described.^(21, 22)

It is immediately evident, see Table 1, column [B], that only one of the compounds (**9**) demonstrates any cytotoxicity following a short dark exposure. The highly electrophilic alpha-chloroacetamido pharmacophore, incorporated in compound (**9**), is a well-known cytotoxic, genotoxic, and mutagenic construct frequently ranked near the top of similar active-site directed (alkylator) inhibitors. This functionality is one of the most reactive participants in S_N2 reactions and has been incorporated in a large number of active-site directed protein kinase inhibitors.⁽²³⁾ It requires no light activation and it manifests toxicity when incorporated in a wide-variety of structures.^(10, 24, 25)

With UVA exposure (column [A]) compounds **2**, **5**, **6**, **7**, and **9** displayed phototoxicity. Of course, methoxsalen **2** as an established photo-activated DNA cross-linker, displayed its expected effect.^(1,2, 5-7) Compound (**9**), the chloroacetamido which possesses dark-reaction cytotoxicity, was slightly more cytotoxic with brief UVA exposure than it had been in the brief dark exposure (column [B]) this confirming the photosensitization effects also seen in the other methoxsalen analogs **5**, **6**, and **7**. This photosensitized behavior is not necessarily a reflection of the parent tri-cyclic aromatic (methoxsalen) molecule's light-induced post-intercalation crosslinking. An alternative explanation is possible for the IC₅₀ of 33 µM compound (**7**). Independent of any presumed sensitization deriving from the methoxsalen core, organic azides can independently photolyze to nitrenes, a highly electrophilic DNA-alkylator species.⁽²⁶⁾ In addition, as noted above, the dialkyltriazene has its own unique photolability contributing to its cytotoxicity.⁽¹⁶⁾

Table 1 reports the keratinocyte inhibitory effects with UVA activation, column [A]; with brief dark exposure of the agents to the keratinocyte cultures, column [B]; and with five-day dark incubation of all agents and keratinocytes, column [C].

The pharmacological relationship of DNA intercalators to the inhibition of cell growth is, of course, well established. Across any similarly structured family of intercalators, the most DNA-affinic members often seem to possess the highest cellular toxicity.^(27, 28)

The structural relationships, however, which make molecules good intercalators are more complex, many and varied. In addition to being flat, fused three (or more) ring molecules, successful DNA intercalators frequently exhibit a large area of π electron delocalization. Few of them contain loci of high charge concentration within the zone of intercalation although charged moieties attached peripherally onto the intercalator core can enhance avidity (if positively charged) or reduce avidity (if negatively charged). The best intercalators are often good electron acceptors since the AT and GC base pairs with which they associate are good electron donors. This donor-acceptor feature, if present, results in very favorable aromatic stacking interactions between these two systems. Favorable intercalation characteristics have been reviewed.^(27, 29)

In whole cell toxicity studies, any given structural class of intercalators often shows a correlation of cytotoxicity to lipophilicity. Whether the enhanced lipophilicity is linked to facilitated membrane permeation or an enhanced DNA-binding has never been determined but many examples of this lipophilic correlation are known.⁽³⁰⁻³²⁾ In our study of the five-day, dark exposure of the candidate chemotherapeutics (see column [C] of Table 1), compounds (**3**) to (**6**) constitute a narrow structural class with only minor variations in the dialkyltriazeno sidechain. These do show such a correlation with lipophilicity. The computed logPs (cLogPs) obtained from the ChemDraw Professional, version 17.0.0.206 (121), are 4.47 for the most lipophilic compound, (**6**), whose IC₅₀ is 7 µM and 2.88 for the least lipophilic analog, (**3**), whose IC₅₀ was 20 µM. Compound (**4**) with cLogP of 3.94 had an IC₅₀ of 6 µM and the methyl/ethyl triazeno analog, (**5**), with cLogP of 3.41 had IC₅₀ of 10 µM. Here again compounds (**7**) and (**8**) were inactive and the highly electrophilic active site inhibitor (**9**) was best-in-class.

None of the compounds is as toxic with light (Table 1, Column [A]) as is the parent methoxsalen for which the dual 2+2 cyclobutane formation with pyrimidine bases requires pre-intercalation and photoactivation.^(1, 2) One would not expect a significant electronic perturbation of the photo-adduct proclivities arising as a result of substitution at C5 (compared to methoxsalen as the reference compound). All these side chain pharmacophores have insignificant electronic interaction with the furanocoumarin platform, as measured by their Hammett constants. For example the σ_p values for $-N=N-NMe_2$ (-0.03), $-NHCOCH_2Cl$ (-0.03), and $-NH-CO-Ph$ (-0.06) are practically indistinguishable from $-H$ (0.00) as in the parent methoxsalen.⁽³³⁾ Also, in 1H -NMR the chemical shifts of the respective protons on C3 – a position vinylogously linked to the C5 substituent – are isochronous at 6.38 ± 0.11 ppm thus implying very similar electron donations.

However, photo-toxicity – as manifest in bis-adduct formation arising from the psoralen double bonds – requires much more than just suitable electronic features. It requires a good intercalative fit and in our case attachments on compounds (**3**) to (**9**) are likely inhibitors of such binding. Ramaiah showed that in a series of six photoactive acridine intercalators, with varying-sized bulky groups at carbon-9, that DNA-binding decreased with the steric bulk of the substituents.⁽³⁴⁾ In a pyranocoumarin system we, too, showed that a single strategically-placed methyl at the edge of an intercalatable molecule thwarted DNA binding and substantially reduced phototoxicity.⁽³⁵⁾ The conclusion is that the phototoxicity enhanced effects seen in compounds (**5**), (**6**) and (**7**), compared to their dark-reaction toxicities arise from the functional group itself at C5 and not from the intercalation of its carrier psoralen.

For compound (**8**), the beta-chloroethylurea – a pharmacophore found in drugs such as cyclophosphamide and estramustine – is known to require a metabolic cleavage at the carbonyl, usually effected in the liver, before the chloroethylamine alkylating species is released.^(36, 37) In our cell culture model such hydrolysis is apparently not possible and no cytotoxic activity is seen. There are no reports in the literature of photoactivation of beta-chloroethyl amides/ureas and no such activity was seen in our study.

The highly toxic alpha-chloroacetamido moiety (Compound **9**) needs no UVA activation (compare columns [A] and [B]) and given sufficient time (column [C]) can manifest an impressive inhibition of keratinocyte growth. That functionality damages proteins by multiple pathways, at room temperature, and in the dark. Mass spectral studies have demonstrated that chloroacetamides alkylate at cysteine and the nitrogen termini of aspartate, glutamate, histidine, and lysine as well as perform oxidations at methionine, tyrosine, and tryptophan.⁽³⁸⁾

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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(1) R = NH₂ (amino-8-MOP), (2) R = H (8-MOP)

Figure 1.

Core furocoumarin structure and the most common numbering system:

Scheme 1.
Syntheses of Compounds (3) to (9):

Table 1.

Effect of 8-methoxypsoralen derivatives on keratinocyte growth*:

Cpd	R-group	Purity [#] & Physical Aspect mp (°C)	[A] IC ₅₀ with UVA light (30 min contact)	[B] IC ₅₀ w/o UVA light (30 min contact)	[C] IC ₅₀ w/o UVA (5 day contact)
(2)	H (8-MOP)	Pale yellow crystals 148-149	0.1 µM	>100 µM	>100 µM
(3)	-N=N-NMe ₂	Yellow crystals 187-189	>100 µM	>100 µM	20 µM
(4)	-N=N-NEt ₂	Orange crystals 123-124	>100 µM	>100 µM	6 µM
(5)	-N=N-N(Me)(Et)	Yellow crystals 145-147	>70 µM	>100 µM	10 µM
(6)	-N=N-N(Me)(nBu)	Dark yellow crystals 66-67	4 µM	>100 µM	7 µM
(7)	-NH-CO-(4-N ₃ -Ph)	Light tan solid 185-187	33 µM	>100 µM	>300 µM
(8)	-NH-CO-NH(CH ₂) ₂ Cl	White crystals 242-244	>100 µM	>100 µM	>300 µM
(9)	-NHCOCH ₂ Cl	Beige crystals 270-272	40 µM	50 µM	4 µM

* IC₅₀ = concentration of psoralen inhibiting growth by 50%[#] All compounds had combustion analyses (C,H,N) within ± 0.3% of theoretical.