

Photosensitization of Guanine-Specific DNA Damage by a Cyano-Substituted Quinoxaline Di-*N*-oxide

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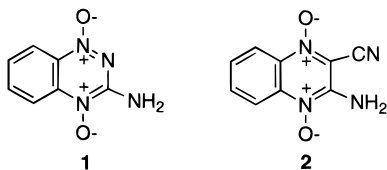
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The cyano-substituted quinoxaline di-*N*-oxide (**2**) is a potential antitumor agent, which selectively kills hypoxic cells. While investigating this drug's potential ability to act as a surrogate for O₂ in DNA damage processes, we discovered that **2** produces alkali-labile lesions selectively at 2'-deoxyguanosine positions upon irradiation in the UV-A ($\lambda_{\text{max}} = 350$ nm) region. Strand damage is induced in single-stranded and double-stranded substrates, with the latter being slightly more susceptible to lesion formation. Alkaline-labile lesions are formed under aerobic and anaerobic conditions. The efficient formation of alkali-labile lesions by **2** suggests that this molecule may exhibit phototoxicity. Subsequent investigation of this process suggests that photoexcited **2** damages DNA via a type I process. The results of experiments aimed at determining the involvement of singlet O₂ are ambiguous and indicate that commonly used experimental tests for this species may be less definitive than often thought. The involvement of other reactive oxygen species in strand damage, such as hydroxyl radical, is ruled out.

Introduction

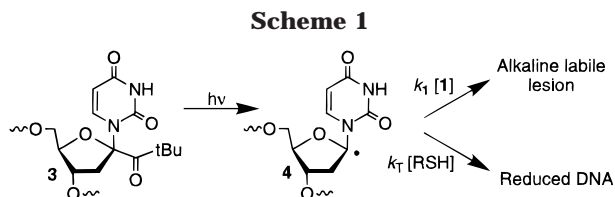
Recently, one of us reported on the light-dependent DNA damage by tirapazamine (**1**), which is the prototypical member of a promising family of antitumor agents (*1*). We now report that photolysis of the structurally related cyano-substituted quinoxaline di-*N*-oxide **2** induces DNA damage much more efficiently, and is selective for 2'-deoxyguanosines.



The benzotriazine and quinoxaline di-*N*-oxides selectively kill the oxygen poor (hypoxic) cells found in tumors (*2–6*). Hypoxic cell selectivities as high as 150 have been measured for substituted quinoxaline di-*N*-oxides. We and others have shown that the prototypical member of this family of molecules, tirapazamine (**1**), exhibits bimodal activity in its DNA damage. Under hypoxic conditions, one-electron reductive activation of **1** initiates radical-mediated DNA strand damage, and in addition, the drug serves as an efficient O₂ surrogate that traps C1'- and C4'-nucleotide radicals in DNA (*7–9*). The rate constant with which tirapazamine traps C1'-nucleotide radicals in single-stranded DNA is approximately 1 order of magnitude lower than the respective rate constant for trapping by O₂, indicating that sub-millimolar concentrations of **1** can compete efficiently with endogenous thiols for reaction with the DNA radical. The cyano-substituted

di-*N*-oxide (**2**) exhibits hypoxia-selective cytotoxic properties similar to those of tirapazamine. Although the mechanism of action of this molecule has not been reported, electrochemical measurements indicate that the nitrile substituent in **2** renders it more susceptible to reductive activation than tirapazamine (**6**). The effect of this substituent on the ability of **2** to act as a surrogate for O₂ is less certain. Consequently, we undertook an investigation aimed at determining the rate constant with which **2** traps photochemically generated C1'-nucleotide radicals in DNA. During the course of these experiments, we observed that UV-A ($\lambda_{\text{max}} = 350$ nm) irradiation of oligonucleotides in the presence of **2** resulted in the efficient formation of alkali-labile lesions localized at 2'-deoxyguanosines. Because photochemical DNA damage mediated by organic molecules can have important toxic and therapeutic consequences, we have characterized light-dependent DNA damage by the potential antitumor agent **2**.

In recent years, a number of oxidative DNA damage processes have been shown to involve selective modification of 2'-deoxyguanosines (*10*). The agents which induce photochemical DNA damage range from transition metal complexes to molecules employed as sunscreens (*11–13*). The mechanisms of photosensitized damage are typically designated as either direct electron transfer from DNA to the photoexcited agent (type I) or the formation of ¹O₂ (type II), resulting from quenching of the triplet excited state of the agent by O₂. The experiments described in this paper strongly support the involvement of a type I mechanism as the major cause of light-dependent DNA damage induced by **2**. However, the results of experiments designed to examine whether a type II mechanism



(involving 1O_2) contributes to DNA damage by **2** are ambiguous, and our experiments serve as a reminder that commonly used mechanistic chemical probes can provide misleading results, which should be interpreted carefully.

Experimental Procedures

Materials. Oligonucleotides were prepared on an Applied Biosystems Inc. 380B oligonucleotide synthesizer. ^{32}P labeling was carried out with T4 polynucleotide kinase following standard procedures (14). Radiolabeled duplexes were prepared by hybridizing equal amounts of complementary oligonucleotide at 90 °C for 10 min, and then slowly cooling to room temperature. The hybridized material was purified via nondenaturing polyacrylamide gel electrophoresis, and isolated via the crush and soak method, followed by concentration via butanol extraction and desalting via precipitation. Oligonucleotides were sequenced using a reaction specific for 2'-deoxyadenosine and the Maxam-Gilbert A + G reaction (14, 15). $[\gamma\text{-}^{32}P]\text{ATP}$ was from Amersham (Arlington Heights, IL). T4 polynucleotide kinase was from New England Biolabs (Beverly, MA). Superoxide dismutase and catalase were from Boehringer Mannheim (Indianapolis, IN). Phosphorimaging was carried out on a Molecular Dynamics Phosphorimager (Sunnyvale, CA) equipped with ImageQuant software. Quinoxaline di-N-oxide (**2**) was prepared via reported procedures (16).

General Photolysis Procedure. Photolyses were carried out for 20 min in Pyrex tubes (6 mm i.d.) using a Rayonet photoreactor equipped with 16 lamps ($\lambda_{\text{max}} = 350$ nm). Photolysis tubes were loaded with oligonucleotide, **2** (0.2 mM), and other exogenous reagents at the appropriate concentration [e.g., diazobicyclooctane (DABCO),¹ 10 mM] in phosphate buffer (10 mM, pH 7.2) and NaCl (100 mM). After photolysis, the solution was transferred to a microcentrifuge tube, the photolysis tube was washed with H_2O (70 μL), and the combined material was precipitated. The DNA pellets were resuspended in 1 M piperidine (100 μL) and heated at 90–95 °C for 20 min. The samples were then lyophilized, resuspended in H_2O , and lyophilized (2 \times 100 μL). Prior to being loaded on a gel [20% denaturing polyacrylamide gel electrophoresis (PAGE)], the DNA pellets were resuspended in formamide loading buffer by vortexing for 2 min, heating at 55 °C for 5 min, and vortexing again for 1 min. Duplex DNA samples were heated at 95 °C for 5 min, and quickly placed in an ice/water bath, prior to being loaded on a gel.

Procedure for Degassing Samples. Pyrex photolysis tubes were loaded with the appropriate reagents (e.g., **2** and 5'- ^{32}P -labeled **5**). The samples degassed by three freeze-pump-thaw cycles (3 min each) and flame sealed under vacuum.

Results and Discussion

We previously showed that tirapazamine traps 2'-deoxyuridin-1'-yl (**4**) generated photolytically from **3** with a rate constant of $1.0 \times 10^8 \text{ M}^{-1} \text{ s}^{-1}$ in single-stranded DNA (Scheme 1) (9). Although light-dependent DNA damage by tirapazamine has been reported previously (1), we observed no DNA damage photochemically induced by tirapazamine (**1**) during photolysis of the radical precursor **3**. This was despite tirapazamine's highly

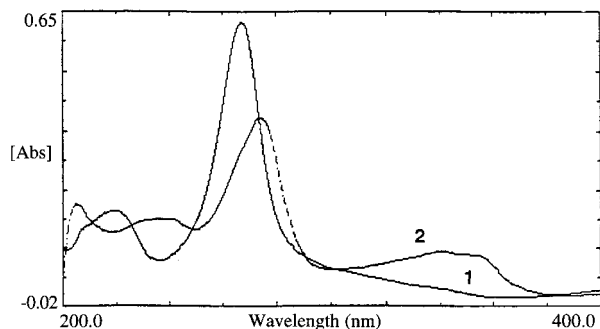


Figure 1. UV/vis spectra of **1** (0.01 mM) and **2** (0.01 mM).

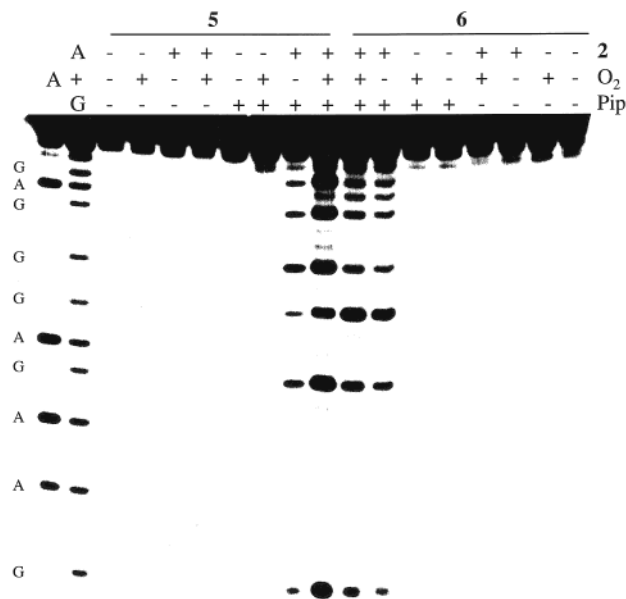


Figure 2. Photochemical damage in **5** and **6** induced by **2** (0.2 mM).

colored nature in solution, which indicates that the molecule absorbs strongly in the UV/vis region. Examination of a UV/vis spectrum of tirapazamine revealed very weak absorption in the region (350 nm) where **3** is irradiated (Figure 1). This weak absorption apparently does not allow the drug to compete with the formation of **4**. In contrast, quinoxaline **2** absorbs more strongly around 350 nm (Figure 1). The strong UV₃₅₀ absorbance of **2** thwarted our efforts to examine the ability of this molecule to trap 2'-deoxyuridin-1'-yl (**4**). However, these experiments led to the interesting observation that **2** causes efficient light-dependent damage to guanine residues in single-stranded and duplex DNA.

Photolysis of 5'- ^{32}P -labeled **5** in the presence of the quinoxaline (**2**) does not produce any detectable direct strand breaks in this substrate. However, piperidine treatment produces fairly uniform strand scission at all 2'-deoxyguanosines in the oligonucleotide (Figure 2). The only site other than a 2'-deoxyguanosine which is labile to piperidine was a deoxyadenosine sandwiched between two 2'-deoxyguanosines near the 3'-terminus of the oligonucleotide. The cleavage selectivity was independent of O_2 concentration and whether the oligonucleotide was hybridized, but the intensity of cleavage was enhanced slightly in the presence of O_2 . Cleavage was also slightly more efficient in double-stranded substrate (**6**) than in the respective unhybridized material. The structure of the alkali-labile lesion(s) is not known at this time. However, the DNA fragments formed upon piperidine

¹ Abbreviations: DABCO, diazabicyclooctane; PAGE, polyacrylamide gel electrophoresis.

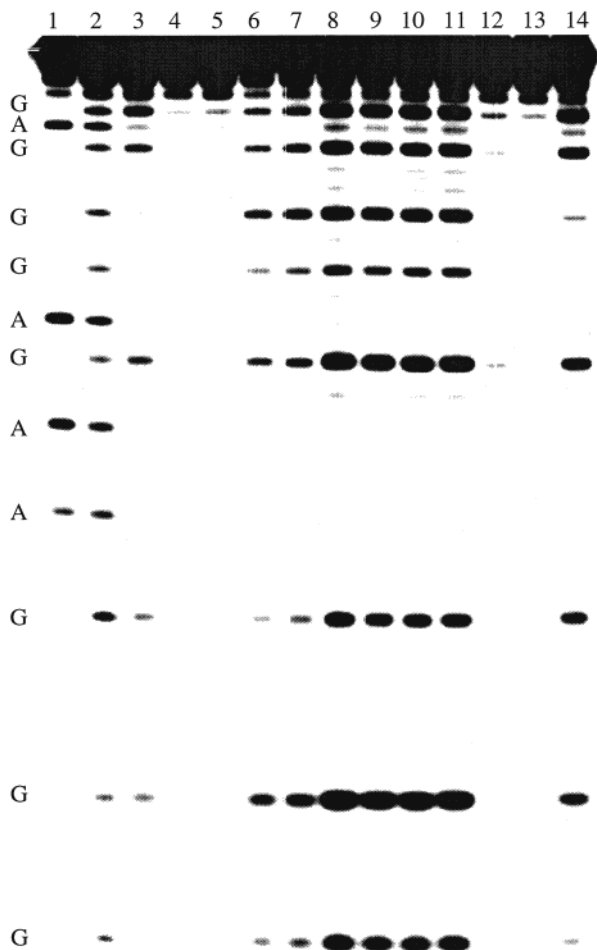


Figure 3. Effects of exogenous agents and O₂ on alkali-labile lesion formation in **5** by **2** (0.2 mM): lane 1, dA sequencing reaction; lane 2, dA + dG sequencing reaction; lanes 3–14, photolysis with **2** (0.2 mM); lanes 3–7, anaerobic conditions; lanes 8–14, aerobic conditions; lane 3, DABCO (10 mM); lane 4, NaN₃ (10 mM); lane 5, β-mercaptoethanol (10 mM); lane 6, no additive; lanes 7 and 8, mannitol (10 mM); lane 9, superoxide dismutase (20 units); lane 10, catalase (30 units); lane 11, no additive; lane 12, β-mercaptoethanol (10 mM); lane 13, NaN₃ (10 mM); and lane 14, DABCO (10 mM).

treatment comigrate with fragments produced via a similar treatment of DNA subjected to glycolysis with formic acid, indicating that the termini contain 3'-phosphates.

5'-d(GTC ACG TGC TGC ATA CGA CGT GCT GAG CCT)
5

5'-d(GTC ACG TGC TGC ATACGACGT GCT GAGCCT)
d(CAG TGC ACG ACG TAT GCT GCA CGA CTC GGA)
6

5'-d(CAT ATG ACG GCA TTC GTT CGC GGG TTA CTG CAA A)
7

5'-d(CAT ATG ACG GCA TTCGT TCGC GGG TTA CTG CAA A)
d(GTA TAC TGC CGT AAGCA AGCG CCC AAT GAC GTT T)
8

Information about the mechanism of alkali-labile lesion formation was inferred by examining the effects of a variety of additives on the efficiency of lesion formation (Figures 3 and 4). Mannitol, superoxide dismutase, and catalase had no effect on alkali-labile lesion formation

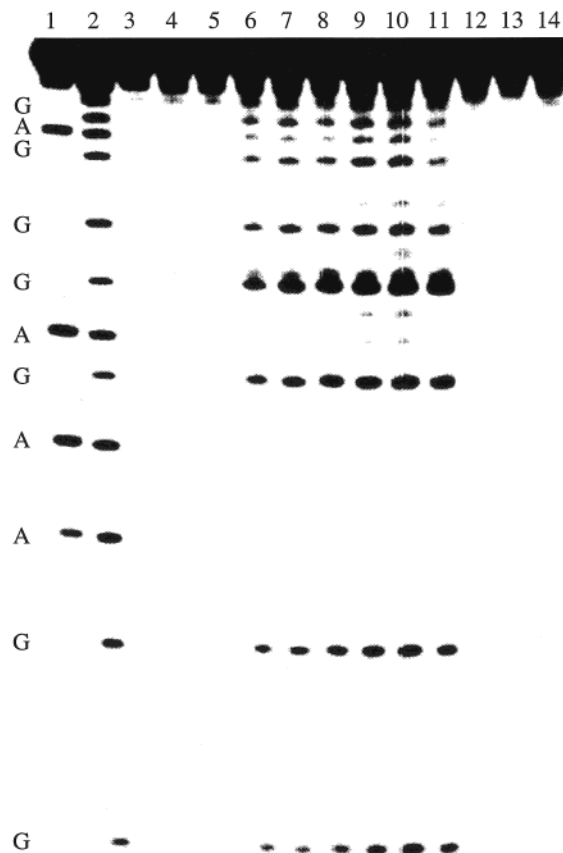


Figure 4. Effects of exogenous agents and O₂ on alkali-labile lesion formation in **6** by **2** (0.2 mM): lane 1, dA sequencing reaction; lane 2, dA + dG sequencing reaction; lanes 3–14, photolysis with **2** (0.2 mM); lanes 3–7, anaerobic conditions; lanes 8–14, aerobic conditions; lane 3, DABCO (10 mM); lane 4, NaN₃ (10 mM); lane 5, β-mercaptoethanol (10 mM); lane 6, no additive; lanes 7 and 8, mannitol (10 mM); lane 9, superoxide dismutase (20 units); lane 10, catalase (30 units); lane 11, no additive; lane 12, β-mercaptoethanol (10 mM); lane 13, NaN₃ (10 mM); and lane 14, DABCO (10 mM).

in single-stranded or double-stranded oligonucleotides, indicating that reactive oxygen species such as hydroxyl radical, superoxide, and hydrogen peroxide were not responsible for the observed strand damage (17, 18). In contrast, sodium azide and β-mercaptoethanol efficiently suppressed lesion formation on **5** and **6** under anaerobic and aerobic conditions. Interestingly, the effects of DABCO on alkali-labile lesion formation strongly depended upon whether the oligonucleotide was hybridized. Alkali-labile lesion formation in single-stranded substrate (**5**) was unaffected by DABCO, but was efficiently inhibited in duplex DNA (**6**). Comparable observations were made in **7** and **8** (data not shown). The cause of this disparate behavior is unknown at this time. One can speculate that DABCO, which will be positively charged at the pH where these reactions are carried out, binds to the duplex more strongly than to single-stranded DNA, and reduces (repairs) oxidized guanines more efficiently. The oxidation potentials of DABCO and 2'-deoxyguanosine are consistent with this possibility (19, 20).

The suppression of alkali-labile lesion formation by azide, thiol, and DABCO has been associated with singlet oxygen-mediated strand damage (11, 21, 22). However, it is clear that, under anaerobic conditions, production of ¹O₂ is not possible. Therefore, we believe that, in the absence of O₂, these additives are competing with the oligonucleotides for reaction with the excited state of **2**.

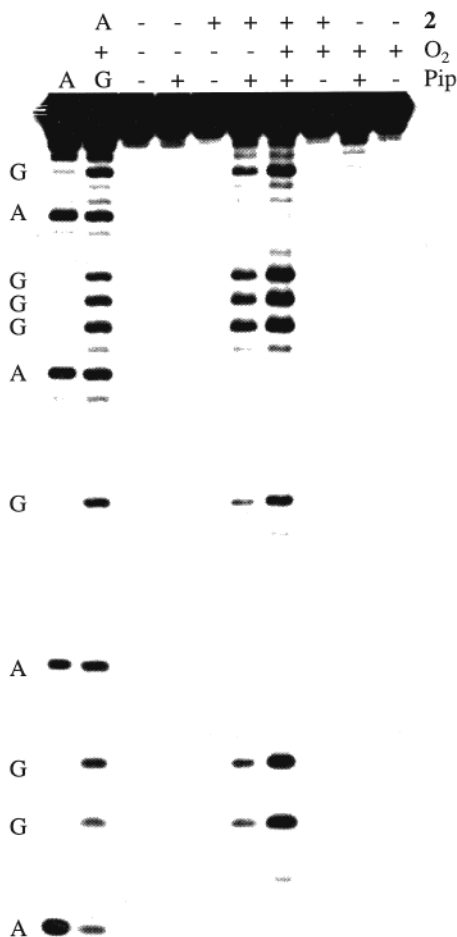


Figure 5. Photochemical damage in **7** induced by **2** (0.2 mM).

Quenching of photoexcited states of organic molecules by amines (such as DABCO), azide, and thiols is well documented (23–26). Our results clearly suggest that DNA cleavage by photoexcited **2** under anaerobic conditions involves a type I mechanism, but these data neither establish nor preclude the involvement of singlet oxygen in the observed labile lesion formation under aerobic conditions. In fact, our results reinforce the concept that suppression of the alkali-labile lesions (Figures 3 and 4) by additives such as sodium azide, thiol, and DABCO is necessary, but insufficient criteria for establishing the involvement of ¹O₂ are available. In addition, the interpretation of such experiments is further complicated by the fact that thiols and amines are excellent reducing agents of cation radicals, such as the guanine cation radical produced upon quenching of the photoexcited state of **2** (27). Finally, it should be noted that strong nucleophiles, such as azide, rapidly trap cation radicals (28).

In view of the ambiguous nature of the quenching of strand damage by the exogenous reagents listed above, D₂O, which increases the lifetime of singlet O₂, was employed as an alternative probe for the generation of this reactive oxygen species (29–32). The level of strand damage was increased by ~67% in D₂O solvent compared to that in H₂O under aerobic conditions. This is a considerably smaller increase than that observed in systems where singlet oxygen is generated from dyes (29). Furthermore, control experiments carried out in the same solvents under anaerobic conditions showed an ~40% increase in D₂O. Taken together, these experiments

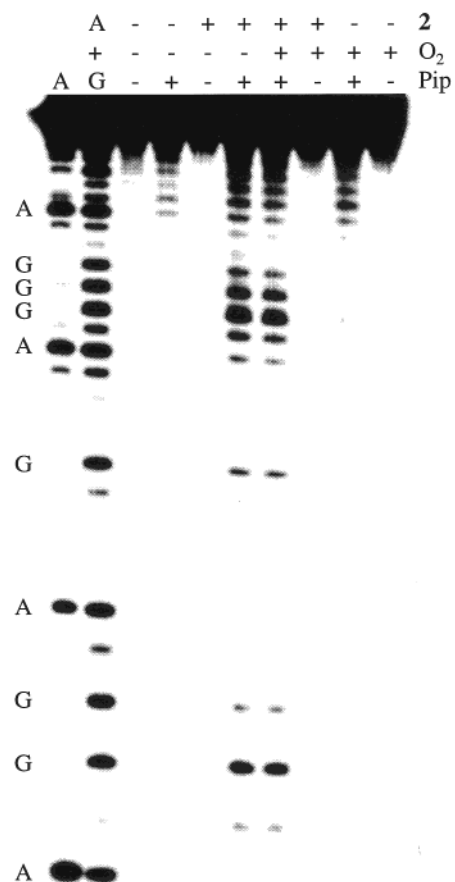


Figure 6. Photochemical damage in **8** induced by **2** (0.2 mM).

suggest that if involved, singlet O₂ is a minor player in the observed strand damage. We hypothesize that D₂O increases the lifetime of the excited state of **2**, which in turn gives rise to an increased level of strand damage. This has been observed in other systems (33–35).

The proposed involvement of a type I cleavage process, as well as the selectivity for alkali-labile lesion formation at 2'-deoxyguanosine, suggests that this nucleobase undergoes one-electron oxidation. The viability of this proposed route for strand scission was probed by exploiting the well-established effect of local sequence on oxidation potentials of 2'-deoxyguanosines in double-stranded DNA (36–38). Consequently, irradiation of **2** in the presence of 5'-³²P-labeled **7** yielded a strand damage pattern comparable to that observed for **5** in terms of sequence selectivity and the effect of O₂ (Figure 5). Similar photolysis of solutions containing hybridized substrate (**8**) and **2** resulted in preferential alkali-labile lesion formation at the 2'-deoxyguanosine closest to the 5'-oligonucleotide terminus of a 2'-deoxyguanosine triplet (Figure 6). Strand damage at this site was clearly more pronounced than at the 2'-deoxyguanosine closest to the 5'-oligonucleotide terminus of a 2'-deoxyguanosine doublet, which was in turn more intense than that at isolated 2'-deoxyguanosines.

Conclusions

The cyano-substituted quinoxaline (**2**) provides an efficient method for introducing alkali-labile lesions selectively at 2'-deoxyguanosines. Damage is introduced into the biopolymer using relatively long wavelength light (350 nm). The majority of the lesions produced under both

aerobic and anaerobic conditions are believed to occur via electron transfer from the nucleobase to photoexcited **2**, although under aerobic conditions there may be some contribution from singlet oxygen. Our work emphasizes that qualitative effects of exogenous reagents are sometimes insufficient for determining the intermediacy of singlet oxygen in DNA strand damage (33). In addition, the proclivity of **2** for producing alkali-labile lesions as a result of UV-A irradiation suggests that this potential antitumor agent may exhibit phototoxicity.

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