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Photosensitization of Human Leukemic Cells by Anthracenedione Antitumor Agents[1]

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ABSTRACT

1,4-Diamino-substituted anthraquinone antitumor agents (mitoxantrone and ametantrone) and structurally related 1,5- and 1,8-diamino-substituted compounds (AM1 and AM2) were tested for their ability to photosensitize human leukemic cells in culture. Viability was measured using the 3,4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide assay, and DNA and membrane damage were assessed. Following a 1-h exposure to AM2, a dose of drug required to give 50% loss of cell viability (53 μM) was obtained in the dark, which was reduced to approximately 2.4 μM following illumination for 2 min (λ > 475 nm), a dose of light that was completely nontoxic to the cells in the absence of drug. A shift in the cell viability curve was also observed for AM1 but, under identical conditions, the dose modification was only 8.9. In contrast, neither ametantrone nor mitoxantrone gave a decreased viability upon illumination. DNA single-strand breaks as measured by alkaline elution correlated with cell viability. Frank DNA single-strand breaks were produced by AM2 and light, suggesting the production of free radicals. The strand breaks produced by AM2 in the dark and by mitoxantrone (with or without illumination) were protein concealed. No evidence of photo-induced membrane damage, as determined by transport of the model amino acid cycloleucine, could be observed even at supralethal doses.

INTRODUCTION

The 1,4-diamino-substituted anthraquinones mitoxantrone and ametantrone (Fig. 1) were developed in an attempt to produce less cardiotoxic alternatives to the anthracyclines (1, 2). These agents have more negative reduction potentials and, as a result, do not engage readily in intracellular, enzymatically driven, oxidation-reduction processes which can generate cytotoxic free radical species (3-6). Biochemical evidence suggests that nucleic acids are among the principal cellular targets for these agents (7-9). In addition, however, a mechanism involving mitoxantrone and ametantrone as substrates in enzymatic peroxidative oxidation has also been suggested (10, 11).

Anthracenedione-based antitumor agents are intensely colored and since many light-absorbing drugs exert undesirable toxic effects upon exposure to UV or visible light (12), it is important to establish if such agents are capable of sensitizing photochemical reactions involving cellular components. Photosensitization has been demonstrated by certain chemotherapeutic agents including hematoporphyrin derivative (13), anthracyclines (14), and anthrapyrazoles (15-19) and, in the case of hematoporphyrin derivative, has found a practical application in the photodynamic therapy of tumors (13). In the case of mitoxantrone, drug-sensitized decarboxylation of peptides has been observed upon UV (313 nm) irradiation (20), but visible light does not initiate any photosensitized reaction with either mitoxantrone or ametantrone (21). In contrast, however, 1,5- and 1,8-disubstituted aminoanthraquinones structurally related to ametantrone (AM1[3] and AM2, respectively; Fig. 1) have been shown to cause oxygen consumption and formation of superoxide radical and hydrogen peroxide upon illumination of aerated samples in the presence of NADH (21). In addition, these compounds caused formation of DNA single-strand breaks upon exposure to visible light, and a correlation was established between the extent of DNA damage, oxygen consumption, and NADH oxidation in cell-free systems (22).

In the present study AM1, AM2, ametantrone, and mitoxantrone were tested for their ability to photosensitize human leukemic cells in culture. Dark and light toxicities were assessed and compared to the extent of DNA and membrane damage produced.

MATERIALS AND METHODS

Materials. Mitoxantrone and ametantrone were kindly supplied by Dr. K. C. Murdoch (Lederle Laboratories, Pearl River, NY) and AM1 and AM2 by Professor J. R. Brown (Sunderland Polytechnic, Sunderland, England). Stock solutions were prepared in distilled water and stored in the dark.

Cell Culture. K562 human chronic myeloid leukemic cells were maintained in RPM1 1640 medium supplemented with 10% fetal calf serum and 2 mM glutamine at 37°C in a humidified atmosphere containing 5% CO_2 and were tested to be *Mycoplasma* free.

Drug Treatment and Illumination. Cells at a density of 5×10^4/ml were incubated with the specified dose of drug for 1 h at 37°C in the dark. The incubation was terminated by centrifugation (5 min, 300 × *g*), and the cells were washed once with drug-free medium. Cells were either kept in the dark or transferred to Petri dishes for illumination where appropriate. Illumination of dishes on ice with visible light was achieved from a 300 W projector lamp, equipped with a broad band filter giving 50% transmission at 475 nm, clamped above the sample. The incident fluence rate was ~5 W/m^2 as measured using a Model 65A YSI radiometer.

Cell Viability Studies. Following the appropriate drug treatment and illumination, cells were transferred to 96-well microtiter plates, 10^4 cells per well, 8 wells per sample. Plates were then kept in the dark at 37°C in a humidified atmosphere containing 5% CO_2. The viability assay is based on the ability of viable cells to reduce a yellow-colored soluble tetrazolium salt, 3,4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide (Sigma Chemical Co., St. Louis, MO), to an insoluble, purple-colored formazan precipitate (23). Following incubation of plates for 5 to 6 days (to allow control cells to increase in number by 10-fold), 20 μl of a 5-mg/ml solution of 3,4,5-dimethylthiazol-2,5-diphenyl tetrazolium bromide in phosphate-buffered saline were added to each well, and the plates were further incubated for 5 h. The plates were then centrifuged for 5 min at 300 × *g*, and the bulk of the medium was pipetted from the cell pellet leaving 10 to 20 μl per well. Two hundred μl of dimethyl sulfoxide were added to each well, and the samples were agitated to ensure complete mixing. The absorbance was then read at a wavelength of 550 nm on a Titertek Multiskan enzyme-linked immunosorbent assay plate reader, and dose-response curves were constructed. For each curve, an ID_{50} value was read as the dose required to reduce the final absorbance to 50% of the control value.

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[3] The abbreviations used are: AM1, 1,5-bis{[(diethylamino)ethyl]amino}-anthraquinone; AM2, 1,8-bis{[(diethylamino)ethyl]amino}anthraquinone; ID_{50}, dose of drug required to give 50% loss of cell viability after a 1-h exposure to drug.

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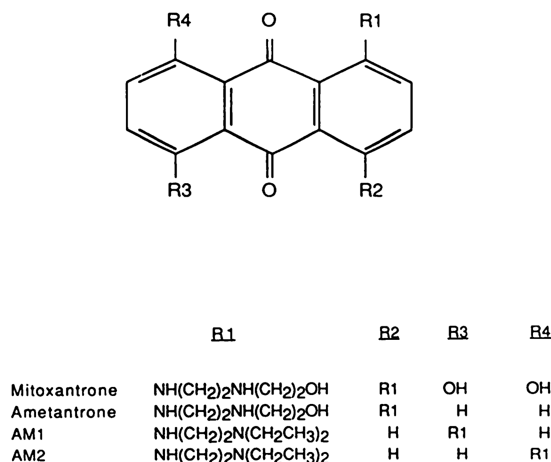


	R1	R2	R3	R4
Mitoxantrone	NH(CH2)2NH(CH2)2OH	R1	OH	OH
Ametantrone	NH(CH2)2NH(CH2)2OH	R1	H	H
AM1	NH(CH2)2N(CH2CH3)2	H	R1	H
AM2	NH(CH2)2N(CH2CH3)2	H	H	R1

Fig. 1. Structures of the drugs used in this study.

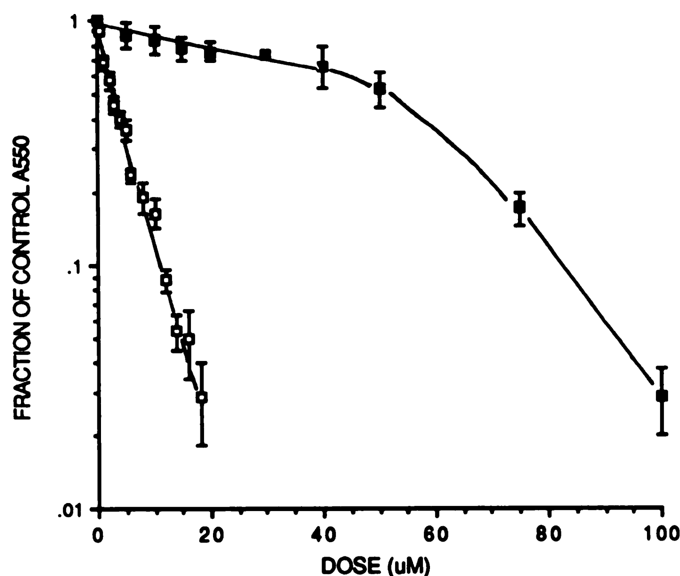
Measurement of DNA Single-Strand Breaks by Alkaline Elution. The basic principles involved in the detection of DNA damage by the alkaline elution assay have been published (24) and the methodology reviewed in detail (25). Cells were radioactively labeled by growing in 0.05 μCi/ml of [^{3}H]thymidine (specific activity, 20 Ci/mmol; Amersham, United Kingdom). After 24 h the label was removed and the cells were grown for an additional 6 h in fresh medium to ensure that the label was incorporated into high-molecular-weight DNA. After appropriate drug treatment/illumination, cells were deposited onto 25-mm polycarbonate filters with a pore size of 2 μm (Nucleopore Corp., Pleasanton, CA). The cells were lysed with a mixture of 2% sodium dodecyl sulfate (BDH Chemicals, Ltd, Poole, England), 0.1 M glycine, and 25 mM EDTA (pH 10). Proteolytic digestion of the lysate was accomplished where appropriate by the addition of proteinase K (0.5 mg/ml; Sigma) dissolved in lysis solution. The eluting solution consisted of 0.1 M tetrapropylammonium hydroxide (Sigma), 2 mM EDTA, and 0.1% sodium dodecyl sulfate (pH 12.1) and was pumped at a rate of 2 ml/h. Fractions were collected at 3-h intervals for 15 h. Radioactivity of the eluted fractions, lysate, filter, and filter holder was determined by scintillation counting using Ecoscint A (National Diagnostics, Manville, NJ) as the scintillation solvent. The amount of DNA damage was quantified as the fraction of the label remaining on the filter after 12 h of alkaline elution.

Amino Acid Uptake. Following drug treatment, resuspension in drug-free medium, and illumination where appropriate, 1 ml of cells was incubated at a concentration of 2×10^6/ml for 10 min with 1 μM [^{14}C]-cycloleucine (50 mCi/mmol; Du Pont-New England Nuclear, Boston, MA). Cells were collected by centrifugation, washed with ice-cold phosphate-buffered saline to remove excess label, and lysed with 1 ml of 0.5% sodium dodecyl sulfate lysis solution, and radioactivity was measured by liquid scintillation counting.

Cytospin Preparations. Following treatment of cells for 1 h at 37°C and resuspension at 5×10^4/ml in drug-free medium, 300-μl aliquots were centrifuged at 600 rpm for 5 min in a Cytospin apparatus (Shandon Southern, Ltd.). Slide preparations were air dried and examined under a Zeiss fluorescent microscope.

RESULTS

The effect of the four diamino-substituted anthraquinones shown in Fig. 1 on the viability of human leukemic K562 cells in culture was investigated both in the dark and following illumination with visible light for 2 min. The band filter used in the illumination (50% transmission at 475 nm) transmitted light being absorbed by all the drugs studied, and the drugs were the only light-absorbing species. Light illumination up to 10 min was completely nontoxic to the cells in the absence of drug (data not shown). Viability curves for AM1 and AM2 are shown in Fig. 2. In the case of both drugs the illumination significantly shifted the viability curve. ID_{50} values were obtained and can be seen in Table 1. AM1 was more toxic in the dark than AM2, but both drugs gave similar ID_{50} values following illumination, resulting in dose modifications under these conditions of 8.85 and 22, respectively. Both the drug and light are necessary to produce the observed effect. Reversing the order and illuminating the cells prior to treatment with drug



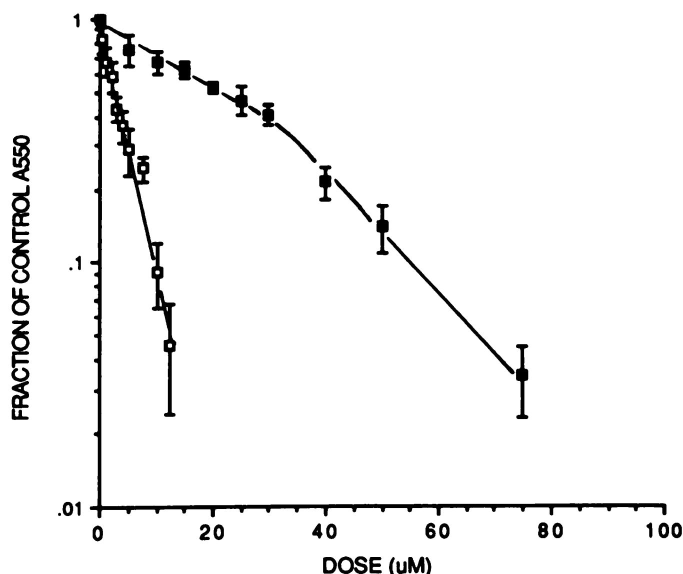


Fig. 2. Cell viability curves for AM1 (*bottom*) and AM2 (*top*) either in the dark (*filled symbols*) or following 2-min illumination (*open symbols*). *Points*, mean of eight individual cell wells; *bars*, SD.

Table 1 *Effect of illumination on the viability of K562 leukemic cells following treatment with anthracenediones*

Drug	ID_{50} (μM) Dark	Light[a]	Light-induced dose modification
AM1	23	2.6	8.85
AM2	53	2.4	22
Ametantrone	50	52	0.96
Mitoxantrone	4.5	4.5	1

[a] Two-min illumination.

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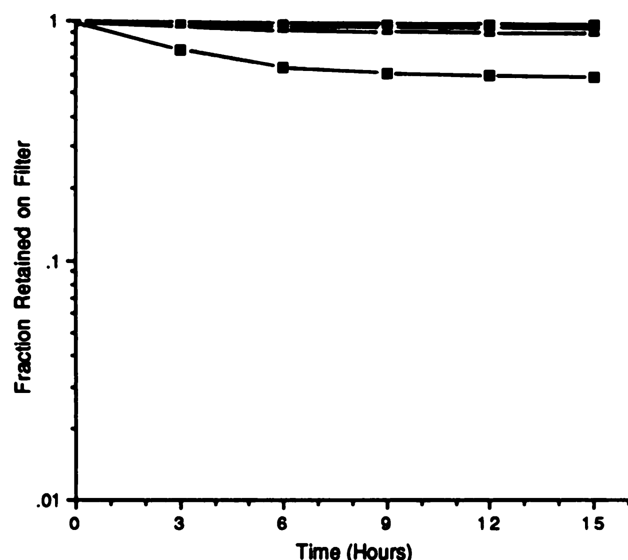
did not shift the dark viability curve (data not shown). In contrast, the 1,4-diamino-substituted anthraquinones (ametantrone and mitoxantrone) gave no significant light-induced dose modifications under identical illumination conditions (Table 1). Mitoxantrone shows the highest dark toxicity among the four drugs studied. A similar light-induced dose modification was obtained for AM2 in a cell growth inhibition assay, confirming the results of the cell viability assay.

Since both AM1 and AM2 have been shown to produce DNA single-strand breaks in plasmid covalently closed, circular DNA upon illumination (22), the production of these lesions in K562 cells was assessed using the technique of alkaline elution (Fig. 3). In the dark no significant level of DNA single-strand breaks was detected up to 10 μM drug, and a low level of breaks was detected only at 50 μM AM2. Following 2-min illumination of cells, however, a large number of single-strand breaks were observed at 2 μM drug, and at 10 μM and above, more than 90% of the DNA was eluted in the first 3-h fraction (Fig. 3). The above assays were performed in the presence of proteinase. When the same experiments were performed in the absence of proteinase, the breaks were observed only in the illuminated samples (data not shown). Illumination of cells in the absence of drug did not produce any detectable single-strand break. The extent of single-strand breaks observed correlated well with the viability of the cells under identical conditions (Table 2). Mitoxantrone-induced strand breaks (with or without illumination) were only observed in the presence of proteinase (data not shown).



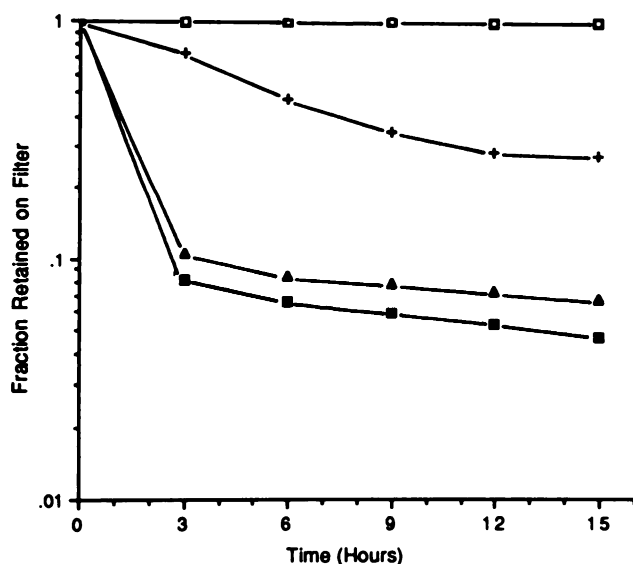


Fig. 3. Alkaline elution profiles of total DNA from K562 cells treated with AM2 at concentrations of 0 (□), 2 (+), 10 (▲), and 50 μM (■). Following 1 h of exposure to drug, cells were either kept in the dark (*top*) or illuminated for 2 min (*bottom*). The alkaline elution assays shown were performed in the presence of proteinase K.

A sensitive probe for membrane damage is the transport of the model amino acid cycloleucine (26). Following illumination of cells for 1 h with AM2, no inhibition of [^{14}C]cycloleucine transport could be detected with or without illumination up to 100 μM (data not shown), indicating that no significant membrane damage occurred.

Following treatment of cells with 1 μM AM2, Cytospin preparations were observed under fluorescence microscopy to localize the drug intracellularly (Fig. 4). Although some drug can be seen in the nucleus, the primary intracellular location is in the cytoplasm. Postincubation of cells for up to 4 h following removal of drug did not significantly alter the pattern of localization.

DISCUSSION

This paper demonstrates that diamino-substituted anthraquinones AM1 and AM2, but not the structurally related ametantrone or mitoxantrone, can photosensitize leukemic cells to visible light. This phototoxic effect was observed at drug concentrations which caused insignificant dark toxicity. This demonstrated that the pattern of visible light-initiated photosensitized effects observed for these two compounds (AM1 and AM2) in aerated samples of the drugs, *e.g.*, oxygen consumption, generation of superoxide radical and hydrogen peroxide, etc. (21), is consistent with the generation of phototoxic effects observed in cells.

DNA is among the principal targets for the antracenediones (7), and several lines of evidence indicate intercalation as the major mode of binding (8, 9). It is clear, however, that the photosensitizing properties of AM1 and AM2 are lost upon binding or intercalation into DNA (22). Analysis of DNA damage using the alkaline elution technique from cells treated with drug plus illumination clearly showed the formation of single-strand breaks, the extent of which correlated well with cell viability. This implies that, although DNA damage may contribute to, or be associated with, the cytotoxic mechanism of AM2-induced photosensitization, it is independent of drug binding or intercalation, and the photoxicity is exerted rather

Table 2 *Effect on cell viability and DNA damage of AM2-induced photosensitization of K562 cells*

	Viability[a]		DNA damage[b]	
Dose (μM)	Dark	Light	Dark	Light
0	1	1	0.97	0.97
2	1	0.57	0.96	0.28
10	0.84	0.12	0.92	0.07
50	0.53	<<0.01	0.59	0.05

[a] Expressed as cell viability fraction.
[b] Expressed as fraction of label remaining on the filter after 12 h of alkaline elution. The assays contained proteinase K.

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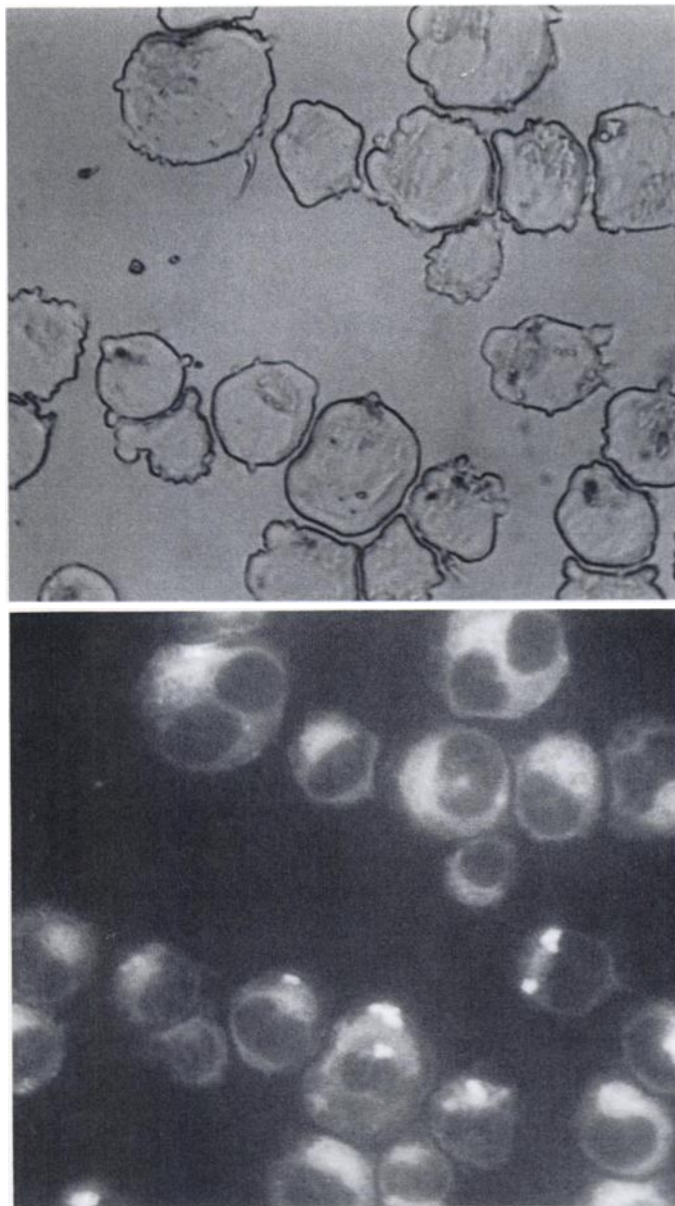

Fig. 4. Cytospin preparation of K562 cells treated for 1 h with 1 μM AM2 and visualized under phase-contrast (*top*) or red fluorescence (*bottom*) microscopy.

by that portion of the drug that is not bound to DNA. The strand breaks produced by AM2 plus light are not concealed by protein, consistent with their being frank breaks resulting from the production of free radicals. In contrast, the strand breaks produced by AM2 in the dark are only observed in the presence of proteinase K. This is the case for mitoxantrone (with or without illumination), where the breaks are protein concealed and protein associated and is consistent with the cleavage being mediated by topoisomerase II (27).

Other workers have shown, using diaminoanthraquinones analagous to those studied in this work, that the quantum yield of triplet state formation for 1,5- and 1,8-isomers is significantly higher ($\phi T > 0.69$) than for 1,4-isomers ($\phi T < 0.029$) (28–30). In accord with this dependence between structure and ϕT (quantum yield of triplet state formation) is the photoreactivity of those isomers assessed as the capability for participation in electron transfer processes (21) and singlet oxygen formation (28, 31). For example, illumination of AM1 or AM2 in aerated solutions in the presence of NAD(P)H generates superoxide radical (21). This species, although not very reactive, can lead to the production of hydrogen peroxide, and in the Fenton cycle generate highly reactive hydroxyl radicals which are known to damage DNA (32). Since the presence of a suitable electron donor such as NADH or ascorbic acid was required to produce DNA damage in the presence of AM1 and significantly increased the damage in the presence of AM2 as photosensitizer in experiments on isolated DNA (22), the present study indicates that both drugs, or at least AM1, can utilize a pool of endogenous electron donors. Photoreactive diaminoanthraquinones were shown to be able to generate singlet oxygen upon illumination in organic solvents (28, 31). It is unknown, however, if this capacity will be retained in intracellular milieu.

DNA damage has been observed for the photosensitizer hematoporphyrin derivative (33). Distinctly different mechanisms of phototoxicity to those observed here for the anthracenediones are operating, however, since the more hydrophobic prophyrins photosensitize mainly at sites of amino acid and nucleoside transport (26, 34). In the present study no evidence of photodamage affecting transport of the model amino acid cycloleucine could be observed even at supralethal doses.

Both the novel photosensitizing drugs (AM1 and AM2) appear to be more efficient in the leukemic cell killing than the clinically useful agent mitoxantrone, as well as being significantly less toxic to the cells in the dark (Table 1). This higher dark tolerance, especially for AM2, creates an opportunity for improvement of the dose modification factor.

In conclusion, the 1,5- and 1,8-disubstituted aminoanthraquinones are capable of photosensitizing human leukemic cells, whereas the structurally related 1,4-analogues mitoxantrone and ametantrone do not. While it is likely that there are other intracellular targets in addition to DNA subject to diaminoanthraquinone-photosensitized attack, the present study clearly shows that the degree of photosensitized degradation of DNA correlated well with the viability of the cells. Whether such properties will contribute to unnecessary phototoxic side effects of such agents remains to be established. Alternatively, the magnitude of the photosensitization observed suggests a possible application in the photodynamic therapy of cancer. The potential for this approach as well as attempts to chemically modulate the light absorption characteristics of such agents is presently under investigation.

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