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Topoisomerase II-dependent novel antitumor compounds merocil and merodantoin induce apoptosis in Daudi cells

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Three photoproducts of merocyanine 540 have been isolated, chemically characterized and synthesized. Two of these photoproducts, merocil and merodantoin, show significant antitumor activity *in vitro* and *in vivo* while demonstrating minimal toxicity to normal cells and tissues. Treatment of lymphoma cells with these compounds resulted in a rapid decline in macromolecular synthesis, DNA fragmentation inhibitable by actinomycin D and cycloheximide, and a marked rise in intracellular calcium. *In vitro* analysis revealed that activity of these compounds is dependent on topoisomerase II. These results are discussed in terms of the novel class of topoisomerase II-dependent compounds for potential use in chemotherapy.

Key words: Apoptosis, calcium, DNA fragmentation, photoproducts, topoisomerase II.

Introduction

Merocyanine 540 (5-[3-sulfopropyl-2(3*H*)benzoxazolylidine)-2-butenylidine]-1,2-dibutyl-2-thiobarbituric acid) is an amphipathic dye that preferentially binds to electrically excitable cells,[1] such as leukemia,[2–4] lymphoma, breast[5] and lung cancer cells,[6] certain classes of immature blood cells, enveloped viruses and virus-infected cells.[4,7] Targets infused with this dye are killed upon subsequent exposure to light. This method of photodynamic killing has been used for *ex vivo* elimination of contaminating tumor cells for the purposes of autologous bone marrow purging. A serious limitation of any photodynamic therapy is that it can only be effective at sites where the appropriate light can penetrate, such as the oral cavity, the bladder, the lungs and any surface where the endoscope can reach. Thus the involvement of lymph nodes with lung cancer, for example, would make the success of photodynamic therapy unlikely.

In previous studies we have demonstrated that one process of photodynamic killing involves light-activated intermediates, and the process of dye irradiation and killing of cells can be separated. We have termed this novel approach *preactivation*, in which an inactive photoactive compound, e.g. merocyanine 540, is pre-illuminated under specific conditions.[8] The singlet oxygen produced attacks the dye molecule itself, resulting in the formation of heretofore unknown compounds that exert their tumoricidal and/or virucidal effect without further requirement of light energy.[9] Therefore, these photoproducts can be administered systemically as therapeutic agents for the treatment of malignancies (metastasized or not) and viral infections. Except for the process of photoproduct generation, the procedure is strictly chemotherapeutic.

We have reported that photoproducts in preactivated MC540 (pMC540) are cytotoxic to cultured tumor cells but very sparing of normal cells and tissues. The lack of toxicity has been determined in *in vivo* models.[8,10–12] More recently, three photoxidization products of MC540 (merocil, meroxazole and merodantoin) (Figure 1) formed under conditions of preactivation have been isolated and characterized.[13] Two of the isolated photoproducts also are cytotoxic to certain tumor cells and are very easily tolerated. For example, *in vivo* treatment of human breast tumors with merodantoin caused a 98% inhibition of tumor growth without observable side effects (in press).[14] In addition, electron microscopy of pMC540-treated cells shows evidence of apoptosis, such as chromatin condensation and blebbing of the plasma membrane and the nuclear envelope.[6] Apoptosis is a physiological 'suicide' process by which unwanted cells are removed from embryonic, developing or somatic tissues without affecting the overall tissue function.[15–18] Apoptosis

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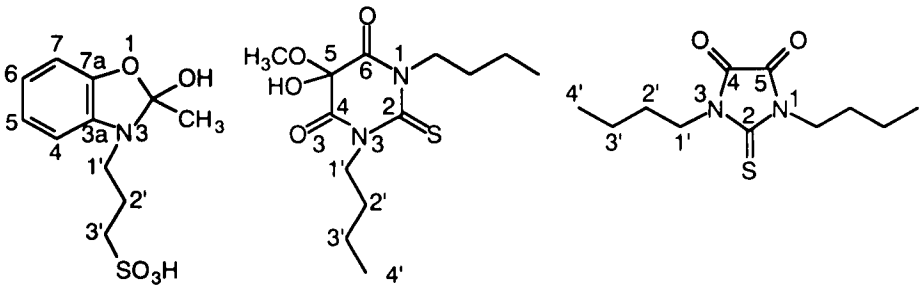

Figure 1. Chemical structures of meroxazole, merocil and merodantoin isolated from preactivated merocyanine 540.

is known to occur during a variety of conditions, including exposure to toxins.[17,19–22] It has been reported that most anti-cancer drugs in current use induce apoptosis in target cells.[23,24] The way in which anticancer drugs induce apoptosis is unknown.[23] The key events in apoptosis seem to be the activation of endogenous calcium- and magnesium-dependent endonuclease that cleaves host chromatin into fragments. The occurrence of DNA fragmentation is a characteristic feature of apoptosis in many, but not all, cells.[17,25,26] Other forms of DNA degradation, not involving strand breaks, have been shown to occur in C3HIOT½ cells.[27] Moreover, glucocorticoids that induce apoptosis by a receptor-mediated mechanism[17,28] have been shown to induce production of high-molecular-weight DNA fragments detectable only in pulsed-field gels.[29] These data suggest that high-molecular-weight DNA fragmentation may be a more universal marker for apoptosis.[30] Other pathways, such as perturbation of intracellular pH (pH_i) leading to the activation of deoxyribonucleases, have also been reported.[31] Protein p26 encoded by the *bcl*-2 gene has been shown to regulate cell viability functions in a final common pathway for apoptosis, which can be activated by multiple mechanisms.[32]

Induction of apoptosis in thymocytes by topoisomerase II reactive chemotherapeutic drugs has been reported.[33] Topoisomerases are nuclear enzymes that control the topological states of DNA by catalyzing the concerted breaking and rejoining of DNA strands. These enzymes have been implicated in several important cellular functions, such as replication, transcription, recombination and DNA repair.[34,35] Now, for the first time, the antitumor properties of meroxazole, merocil and merodantoin (photoproducts of MC540) are described to show that induction of apoptosis by these compounds may involve an initial interaction with topoisomerase II.

Materials and Methods

Reagents

Purified merocil (*N,N'*-dibutyl-4,5-dihydro-5-hydroxy-5-methoxy-4-oxo-2-thiouracil), merodantoin (*N,N'*-dibutyl-2-thio-4,5-imidazolindion) and meroxazole (2-hydroxy-2-methyl-3-[3'-sulfopropyl]-benzoxazolin) were provided by Professor B Franck (see Figure 1 for chemical structure). Meroxazole was dissolved in 90% ethanol; merocil and merodantoin were dissolved in dimethyl sulfoxide. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit was obtained from Promega (Madison, WI). [^{3}H] thymidine, [^{3}H]uridine and [^{3}H]alanine were obtained from ICN Radiochemicals (Irvine, CA). Fura-2-AM, BCECF-AM, nigericin and thapsigargin were obtained from Molecular Probes (Eugene, OR).

Cell culture and treatments

All cell lines were obtained from American Type Culture Collection (Rockville, MD) and maintained in recommended growth medium at 37°C in a humidified 5% CO_2/95% air atmosphere. All incubations were performed at 37°C by adding drug in tissue culture media. Typically, 1×10^6 cells/ml were incubated with different concentrations of meroxazole, merocil or merodantoin. After the indicated incubation periods (see Tables 1–3), the cell survival was determined by MTT assay.[36] Vehicle controls were used in all experiments.

Protein, RNA and DNA synthesis

To determine the effect of merocil and merodantoin on the inhibition of protein, RNA and DNA synthesis, Daudi cells (1×10^6 cells/ml) were treated

Table 1. Effects of merocil and merodantoin on different tumor cell lines

Cell Type	Merocil (μM)	Cell kill (%)		Merodantoin (μM)	Cell kill (%)	
		48 h	4 days		48 h	4 days
BT-20 (breast, adenocarcinoma)	2.58	0	15.3 ± 9.3	6.44	0	22.0 ± 6.1
	5.16	0	23.3 ± 1.5	12.93	12.3 ± 12.0	44.0 ± 14.7
	10.36	7 ± 6.2	47.7 ± 18.9	25.81	67.7 ± 11.6	21.0 ± 4.6
	20.69	32.7 ± 6.7	29.3 ± 14.5	51.63	87.0 ± 8.7	14.7 ± 5.5
	41.38	58.7 ± 13.7	15.3 ± 6.4	103.25	94.3 ± 3.5	66.3 ± 8.3
C-33A (carcinoma, cervix, human)	2.58	2	23	6.44	16	11
	5.16	9	26	12.93	29	34
	10.36	35	46	25.81	36	86
	20.69	36	89	51.63	49	63
	41.38	49	97	103.25	56	91
DU 145 (prostate, carcinoma, human)	2.58	0	26	6.44	0	54
	5.16	0	33	12.93	1	85
	10.36	0	71	25.81	8	99
	20.69	5	—	51.63	9	100
	41.38	3	93	103.25	11	100
MS 751 (cervix, carcinoma, human)	2.58	1	10	6.44	8	11
	5.16	7	11	12.93	24	23
	10.36	12	12	25.81	20	66
	20.69	20	29	51.63	28	90
	41.38	20	89	103.25	41	100
JEG-3 (choriocarcinoma, human)	2.58	0	8	6.44	15	18
	5.16	3	11	12.93	53	38
	10.36	32	11	25.81	88	91
	20.69	68	51	51.63	100	100
	41.38	86	86	103.25	100	100

Each cell type (1×10^6 cells/ml; in triplicate) was treated with the indicated concentrations of merocil or merodantoin. After incubation periods for the indicated periods of time, the percent cell kill was determined by MTT assay and calculated as a percentage of untreated controls. For BT-20 cells, mean ± SD of three separate experiments are shown. For other cell types, a representative experiment (carried out in quadruplicate) is shown.

Table 2. Effects of merocil and merodantoin in multiple myelomas

Myeloma cell lines	Merocil (μM)	Cell kill (%) 24 h	Merodantoin (μM)	Cell kill (%) 24 h
H.S. Sultan	0.78	22.7 ± 5.5	1.56	20.3 ± 4.0
	1.56	25.0 ± 2.6	3.13	39.3 ± 7.6
	3.13	24.7 ± 1.2	6.25	60.7 ± 9.9
	6.25	53.3 ± 16.7	12.5	72.0 ± 11.4
	12.5	56.0 ± 16.6	25.0	92.0 ± 5.6
8226	0.78	15.7 ± 2.3	1.56	19.7 ± 0.58
	1.56	18.3 ± 6.9	3.13	18.0 ± 2.6
	3.13	23.7 ± 3.2	6.25	25.0 ± 5.0
	6.25	28.0 ± 3.6	12.5	28.3 ± 2.9
	12.5	62.6 ± 6.8	25.0	78.0 ± 8.5
U266B1	0.78	3.3 ± 0.58	1.56	5.7 ± 2.1
	1.56	6.7 ± 1.15	3.13	6.7 ± 3.1
	3.13	13.3 ± 5.7	6.25	9.0 ± 1.0
	6.25	14.0 ± 1.4	12.5	9.7 ± 2.1
	12.5	37.5 ± 3.5	25.0	19.3 ± 6.7

Each myeloma (1×10^6 cells/ml; in triplicate) was treated with the indicated concentrations of merocil or merodantoin. After 24 h of incubation, the percent cell kill was determined by MTT assay and calculated as a percentage of untreated controls. Mean ± SD of three separate experiments, each in quadruplicate.

Table 3. Effect of meroxazole, merocil and merodantoin on L1210 cells

Treatment compound	Cell kill (%) (mean ± SD)
Control	0
Merocil	34 ± 12.4
Merodantoin	25 ± 6.0
Meroxazole	3 ± 1.2
Merocil and meroxazole	33 ± 10.4
Merodantoin and meroxazole	28 ± 3.8
Merocil and merodantoin	78 ± 7.5
Merocil, merodantoin and meroxazole	81 ± 2.7

Mouse leukemia L1210 cells (1×10^6 cells/ml) were treated with different combinations of the indicated compounds. After 24 h of incubation, the percent cell kill was determined by MTT assay. Doses used were merocill = 41.4 μM, merodantoin = 103.3 μM and meroxazole = 91.8 μM. Mean ± SD of three separate experiments, each in quadruplicate.

with merocil (41.4 μM) and merodantoin (103.3 μM) for various lengths of time. These doses were selected because they caused a virtually complete inhibition of cell proliferation in Daudi cells. The cell–drug mixture was gently vortexed to assure homogeneous distribution, and the cells (200-μl volume) were dispensed in 96-well microtiter plates. [^{3}H]Thymidine, [^{3}H]alanine and [^{3}H]uridine (2 μCi/ml) were then added to the wells, followed by different periods of incubation at 37°C for 30 min, and 2, 3, 4, 5, 6 and 24 h. Finally, cells were harvested using a PHD cell harvester and radioactive incorporation of the acid-insoluble fraction was determined using a Beckman LS1701 liquid scintillation counter.

Topoisomerase II-dependent cleavage activity assay

Topoisomerase II-dependent cleavage activity was assayed using the topoisomerase II drug screening kit (TopoGen, Columbus, OH). The standard reaction mixture (20 μl) for the cleavage assay consisted of 30 mM Tris–HCl (pH 7.6), 3 mM ATP, 15 mM mercaptoethanol, 8 mM $MgCl_2$ and 60 mM NaCl. Reactions in the presence and absence of pMC540 (70.5, 140.4 and 210.5 μM) or merodantoin (51.6 and 103.3 μM) were carried out using human topoisomerase II and 0.25 μg of DNA substrate (pYRG) containing a single, high-affinity topoisomerase II cleavage and recognition site.[37] After 30 min of incubation at 37°C, the reaction was terminated by the addition of 2 μl of 10% SDS, 0.3% bromophenol blue and 30% glycerol. Samples were extracted with equal volumes of chloroform:isoamyl alcohol (24:1). Samples were then electrophoresed in 1% agarose in 89 mM Tris-borate, 2 mM EDTA (pH 8.3) at 35 V for 4 h for the analysis of DNA cleavage products. Gels were stained with ethidium bromide (1.0 μg/ml) for 45 min and destained for 2–3 h in water. DNA bands were visualized by trans-illumination with UV and photographed using polaroid type 57 film.

DNA Fragmentation

Following treatment with pMC540 (211.2 μM), merocil (41.4 μM) or merodantoin (103.3 μM), cells were lysed by the addition of a DNA lysis buffer (0.2 M Tris–HCl, pH 8.0, 0.1 M sodium EDTA, 1% sodium dodecyl sulfate and 100 μg/ml proteinase K), and the DNA was extracted by standard phenol:chloroform:isoamyl alcohol extraction procedure.[38] The isolated DNA was analyzed by electrophoresis on 1.0% agarose gels containing 0.2 μg/ml ethidium bromide. A λ DNA *Hin*dIII digest was used as molecular weight standard.

Intracellular free Ca^{2+} concentration

Daudi cells (1×10^6 cells/ml) were washed and resuspended in RPMI 1640 plus 2% fetal bovine serum. Fura-2-AM (1 μM) was added to each batch of cells and incubated for 45 min at 37°C. After the incubation period, cells were washed and left untreated or treated with merocil, merodantoin or pMC540 for indicated times, followed by washing three times with Fura-2 buffer (145 mM NaCl, 0.4 mM KCl, 0.1 mM NaH_2PO_4, 0.8 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM glucose and 25 mM HEPES, pH 7.4). Fluorescence of the Fura-2 resuspended cellular suspension was monitored at 37°C with an SLM spectrofluorometer equipped with a circulating water bath for temperature control.[39] Identical sets of experiments were performed in which drug treatment preceded the Fura-2-AM loading of the cells. The cell suspension was excited alternately at 340 and 380 nm, and the fluorescence was measured at 510 nm. Slit width was set at 10 nm wavelength for both excitation and emission. The R_{max} and R_{min} were evaluated by lysing the cells with 1% Triton X-100, followed by the addition of 3 mM EGTA for R_{min}.

Values of $[Ca^{2+}]_i$ were calculated from the ratio of observed fluorescence intensities ($I_{340/380}$) of the intracellular Fura-2 as follows:

$$[Ca^{2+}]_i = K_D \times f_i \times \frac{I_{(340/380)} - I_{(340/380)}\text{min}}{I_{(340/380)}\text{max} - I_{(340/380)}}$$

where K_D = 225 nm for Ca^{2+}/Fura-2 at cytoplasmic ionic conditions.[40] Autofluorescence of unloaded cells was subtracted from the Fura-2-AM loaded values. Fluorescence from Fura-2-AM leaked out of the cells during the course of measurements was determined by reading the fluorescence of the cell-free supernatant. This fluorescence accounted for less than 1–1.5 nM $[Ca^{2+}]_i$ calculated. Data presented are corrected for this value.

Statistical analysis

Results are presented as the arithmetic mean ± SD for each control and experiment group. Differences among the x of groups were determined using the Student's two-tailed t-test (InStat 1.12 computer program; GraphPad for Macintosh) and values of $p < 0.05$ were considered significant.

Results

Cell cytotoxicity

Inhibition of cell proliferative activity by merocil, merodantoin and meroxazole was first evaluated against Daudi cells, because these cells were determined to be among the most sensitive to the effects of pMC540. Results show that following overnight treatment with either merocil (41.4 μM) or merodantoin (103.3 μM), virtually all Daudi cells were killed (Figure 2). However, in a preliminary experiment, meroxazole did not exhibit cytotoxicity against Daudi cells up to a concentration of 458.75 μM and at a concentration of 1.84 mM, only 32% of the cells were killed (results not shown). Therefore, subsequent experiments were done using merocil and merodantoin. The LD_{50} values for merocil and merodantoin were 12.6 and 55.8 μM, respectively.

Cytotoxicity of merocil and merodantoin was evaluated on nine different cancer cell lines (Tables 1 and 2). Results show that JEG-3 human choriocarcinoma, DU 145 human prostate carcinoma and MS 751 human cervix carcinoma were among the most sensitive, whereas myeloma cell lines U266B1, 8226 and H.S. Sultan were the least sensitive to merocil and merodantoin. For slow-growing monolayer cell lines such as BT-20 breast carcinoma, human cervix carcinoma C-33A and MS 751, a longer period of drug treatment was necessary. After 4 days of exposure to drug, C-33A and MS 751 cell lines were virtually eliminated. However, in BT-20 breast adenocarcinoma cells, the percent cell kill on day 4 was significantly less than that observed after 48 h of incubation. This is probably due to the regrowth of surviving cells.

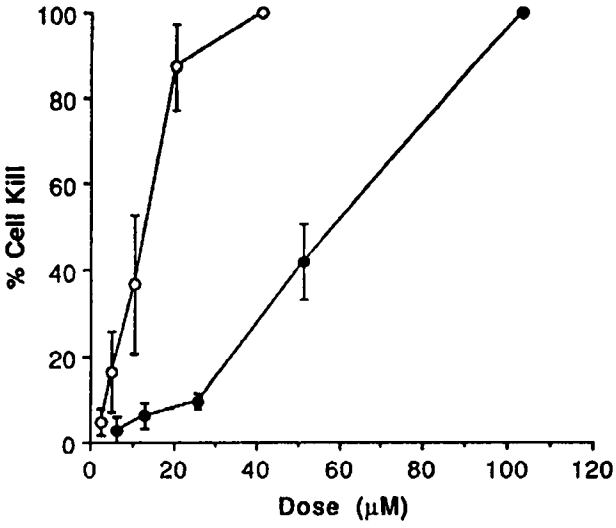

Figure 2. Dose–response curves of merocil and merodantoin. Daudi cells (1 × 10^6 cells/ml) were treated with indicated doses of merocil or merodantoin. After overnight incubation, cell cytotoxicity was determined by MTT assay. Values are the mean ± SD of three separate experiments. ●, Merodantoin; ○, merocil.

In a separate set of experiments, the combined effect of meroxazole, merocil and merodantoin (photoproducts in pMC540) was also investigated against L1210 leukemia cells. Results show (Table 2) that maximum cytotoxicity was obtained when a combination of all three isolates was used, although the contribution by meroxazole when used alone was insignificant. In the presence of one-half concentration of each compound, the observed cytotoxicity was also reduced, suggesting lack of synergy. In previous studies, we have shown that pMC540 is effective in killing L1210 cells *in vitro*.[5]

Inhibition of macromolecular synthesis

The effect of merocil (41.4 μM) and merodantoin (103.3 μM) on DNA, RNA and protein synthesis was investigated. Kinetic studies data show (Figure 3) that the rate of radioactive incorporation in DNA,

RNA and protein continues to decline, starting from 30 min to over a period of approximately 5 h, and was virtually complete after 6 h of incubation in the presence of merocil and merodantoin, suggesting that these compounds prevent mitotic and proliferative activities.

Effect on topoisomerase II

In an attempt to determine whether topoisomerase II is involved in the cytotoxicity mediated by the experimental compounds, topoisomerase II drug screening assay was used. This assay allows the detection of two classes of topoisomerase inhibitors: those that stimulate formation of cleavable complexes and those that antagonize topoisomerase II action on the DNA. Results show (Figure 4) that in the presence of pMC540 and merodantoin, the formation of the cleavable complex (linear DNA species) is clearly visible (lanes 10–12, 14 and 15). The yield of linear DNA species increases with increasing concentration of pMC540 and merodantoin. The conversion of supercoiled DNA substrate (pRYG) was inhibited in the reaction that contained VM-26, an inhibitor of topoisomerase II. These data suggest that the activity of pMC540 and merodantoin is topoisomerase II-dependent and that these compounds do not antagonize its action.

Induction of apoptosis

DNA fractions were isolated from pMC540- or merodantoin-treated lysed Daudi cells and subjected to gel electrophoresis. During the first 30 min or 2 h of exposure to drugs only, high-molecular-weight DNA cleavage patterns without laddering of DNA were observed, indicating that endonucleases were not active at these incubation times and cells have not yet entered apoptosis. In contrast, by 4 and 6 h, there was considerable DNA fragmentation, producing the characteristic DNA ladder on agarose gels (Figure 5) (treatment with pMC540, lanes 2 and 4, 4 and 6 h, respectively, and merodantoin, lanes 3 and 5, 4 and 6 h, respectively). These data suggest that novel compounds do not directly activate the endonuclease and appear to interact with topoisomerase II during the incubation periods to stabilize the DNA cleavage complex. This high-

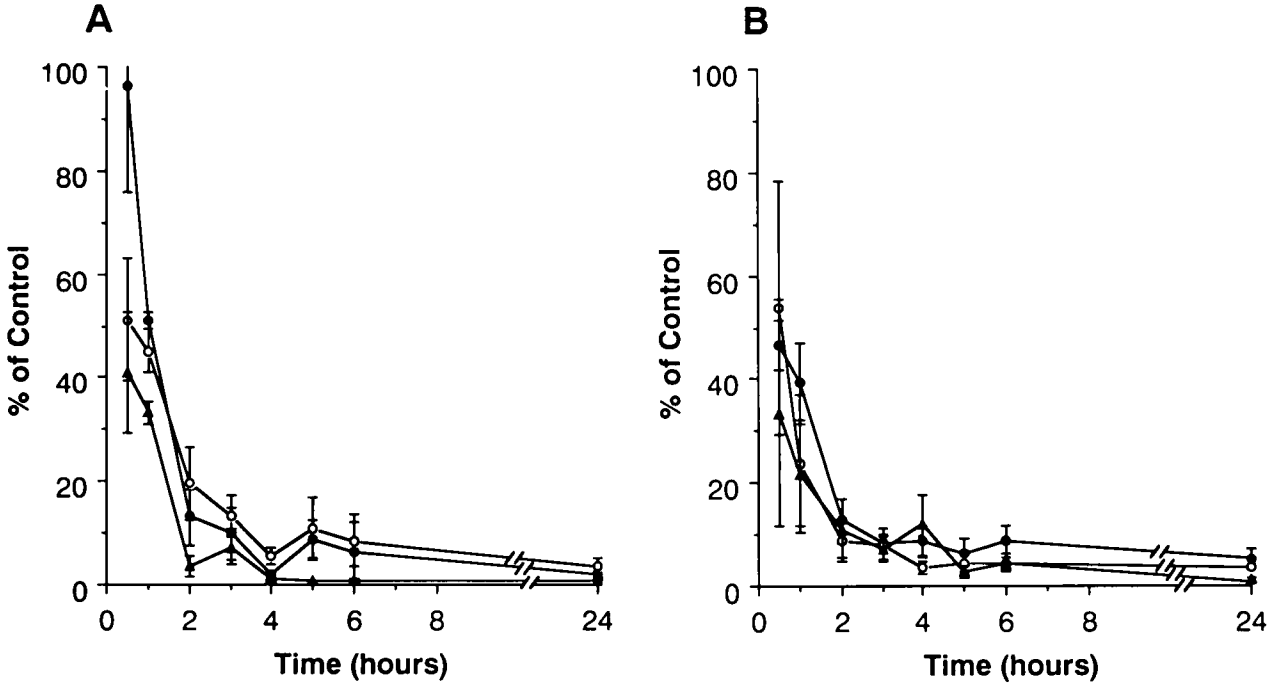

Figure 3. Kinetics of the inhibition of protein, RNA and DNA synthesis in Daudi cells. Daudi cells were treated with 41.4 μM of merocil (A) or 103.3 μM of merodantoin (B) as described in the text. The radioactive isotopes [^{3}H]thymidine, [^{3}H]uridine and [^{3}H]alanine (1 μCi/ml) were added simultaneously, and cells were harvested at the indicated time points followed by the measurement of radioactive incorporation in the acid-insoluble fraction. Values are the mean ±SD of four separate experiments. ●, Alanine; ○, thymidine; ▲, uridine.

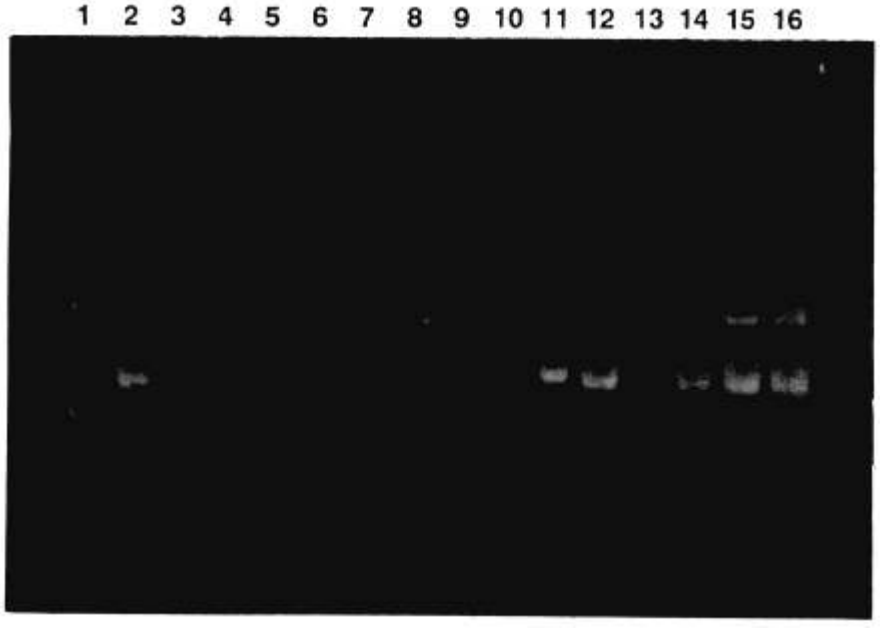

Figure 4. Topoisomerase II-dependent cleavage activity of pMC540 and merodantoin. DNA cleavage reactions were done as described in Materials and methods. Lane 1, pRYGDNA + Topo II (enzyme control); lane 2, supercoiled DNA marker; lane 3, solvent control (2.5% EtOH); lane 4, solvent + Topo II; lane 5, linear DNA marker; lane 6, supercoiled DNA in cleavage buffer; lanes 7, 9 and 13, blank; lane 8, VM-26 + Topo II; lanes 10–12, Topo II + pMC540 (70.2, 140.4 and 210.6 μM); lanes 14 and 15, Topo II + merodantoin (51.6 and 103.3 μM); lane 16, Topo II + dimethyl sulfoxide (solvent used for merodantoin).

molecular-weight DNA fragmentation was inhibited by a 2 h pretreatment of Daudi cells with actinomycin D (1 μg/ml) which inhibits transcription, as well as 100 μM of cycloheximide which inhibits translation of mRNA (data not shown). However, these agents were unable to prevent DNA fragmentation after 4 h of the drug treatment, suggesting that the process of fragmentation has progressed beyond a point of recovery. Treatment of cells with pMC 540, merocil and merodantoin for 1 h in the presence of actinomycin D and cycloheximide (Table 4) also increased the cell viability.

Table 4. Viability of Daudi cells exposed to pMC540, merocil and merodantoin

Treatment	Viability (%)
Control	89.8
pMC540	65.0
Merocil	65.0
Merodantoin	74.0
Actinomycin D	84.0
pMC540 + actinomycin D	84.0
Merocil + actinomycin D	85.0
Merodantoin + actinomycin D	84.0
Cycloheximide	78.0
pMC540 + cycloheximide	78.0
Merocil + cycloheximide	89.0
Merodantoin + cycloheximide	88.0

Daudi cells were incubated for 24 h in growth medium supplemented with serum.

Effect on intracellular calcium

The increased level of intracellular calcium $[Ca^{2+}]_i$ in response to pMC540, merocil and merodantoin was measured in Fura-2-loaded Daudi lymphoma cells. Results (Figure 6) show that the levels of $[Ca^{2+}]_i$ increased in cells treated with each of these compounds, but only after a 2 h drug exposure. For example, the $[Ca^{2+}]_i$ at 0, 10, 20 and 30 min after treatment with pMC540 was 12.6 ± 1.5, 13.2 ± 1.3, 15.5 ± 5.8 and 11.7 ± 2.0 nmol, respectively, indicating that there was virtually no change at these time points. Maximum change in $[Ca^{2+}]_i$ occurred in pMC540-treated cells. In cells treated with merocil or merodantoin, the elevation in $[Ca^{2+}]$, although significant ($p < 0.001$), was approximately 5-fold less than that of pMC540, suggesting that photoproducts other than merodantoin present in pMC540 also may be involved in modulating $[Ca^{2+}]_i$ levels. Thapsigargin was used to determine whether the observed rise in $[Ca^{2+}]_i$ is due to its release from intracellular stores. Thapsigargin, a sesquiterpene lactone, has been shown to release calcium from intracellular stores by inhibition of the endoplasmic reticulum calcium-ATPase. Addition of 5 μM thapsigargin to merocil- or merodantoin-treated cells stimulated a further rapid increase in the $[Ca^{2+}]_i$, probably by activating the release of Ca^{2+} from intracellular stores. Cell death caused by pMC540 was not preventable under conditions that reduced the extracellular Ca^{2+} to near zero by the addition of

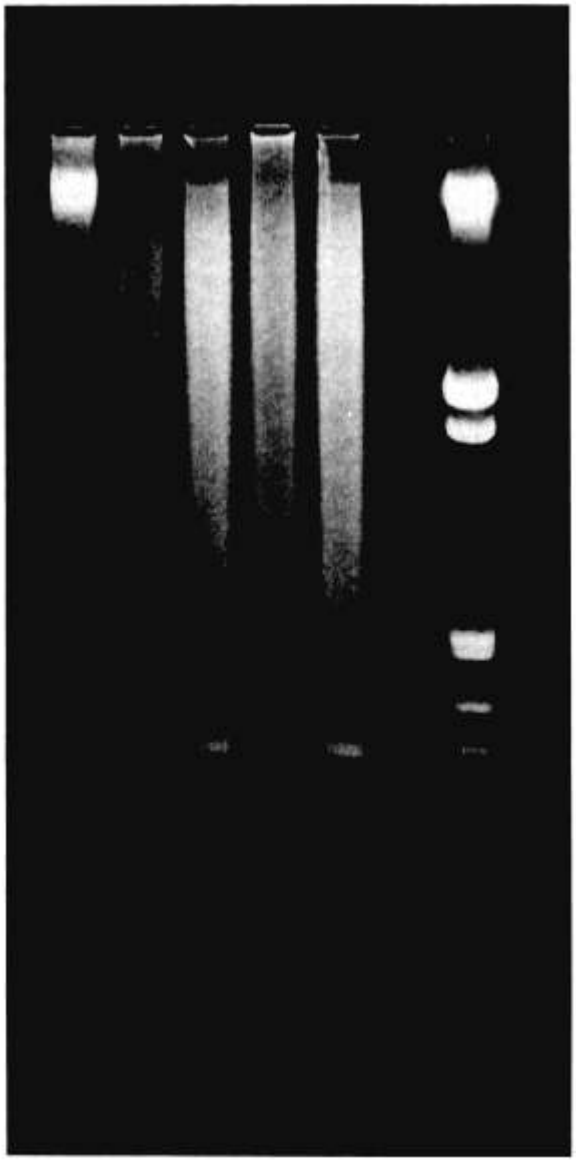

Figure 5. Agarose gel electrophoretic pattern of DNA extracted from Daudi cells following exposure to pMC540 and merodantoin. Lane 1, untreated control; lanes 2 and 4, treated with pMC540; lanes 3 and 5, treated with merodantoin (4 and 6 h, respectively). Lane 6, molecular weight markers (Lambda/*Hin*dIII digest).

3 mM EGTA, suggesting that Ca^{2+} may not be providing the signal for apoptosis, or only a trace amount of $[Ca^{2+}]_i$ is all that is required.

Discussion

The present study demonstrates that two photoproducts (merocil and merodantoin) derived from pMC540 were cytotoxic to several tumor cell lines. The degree of cytotoxicity varied with the

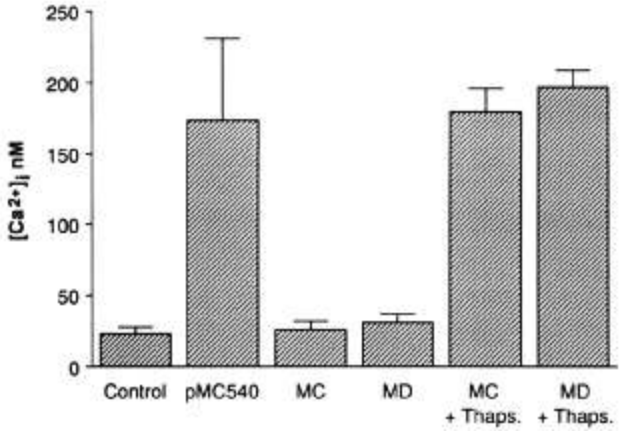

Figure 6. Washed and Fura-2-AM-loaded Daudi cells (1×10^6 cells/ml) were treated with pMC540, merocil (MC), merodantoin (MD), MC plus thapsigargin (Thaps) or MD plus thapsigargin followed by washing and resuspension in Fura-2-AM buffer. Fluorescence of cellular suspension was measured alternatively at 340 nm_{ex}, 380 nm_{ex} and 510 nm_{em}. Intracellular calcium concentration was calculated from the ratio of observed fluorescence intensities. Values are the mean ±SD of four separate experiments.

compound and the cell types used. The third photoproduct, meroxazole, was found to be virtually ineffective in inducing cell death. Both merocil and merodantoin were effective in causing a rapid (starting within 30 min and continuing over a period of 5 h) inhibition of macromolecular synthesis, suggesting that they inhibit both mitotic and proliferative activities. The presence of a precipitous early decline in [^{3}H]thymidine incorporation into drug-treated cells demonstrates that cells do not progress into the S phase of the cell cycle.

Despite their apparent structural diversity, many antitumor drugs have been reported to have the common property of stabilizing a key covalent reaction intermediate of topoisomerase II, termed the cleavable complex.[41] Drug screening assay for topoisomerase II inhibitors revealed that *in vitro* treatment of purified DNA substrate (pRYG) with pMC540 or merodantoin caused a dose-dependent formation of the cleavable complex. The cleavable complex formation was inhibited in the presence of VM-26, a drug which specifically inhibits the resealing reaction of topoisomerase II, suggesting that the activity of novel compounds is dependent on topoisomerase II. Further evidence in support of these results is based on our preliminary experiments demonstrating that the catalytic activity of topoisomerase II correlates with the susceptibility of cells to the cytotoxic action of pMC540 and merodantoin as determined by the decatenation of kDNA network. The topoisomerase II activity is

3- to 4-fold lower in crude cellular and nuclear extracts from cells that are not susceptible to the cytotoxic action of these compounds (unpublished data). However, the role of other topoisomerases has not been ruled out and studies are under way to determine the role of topoisomerase II *in vivo*. It has been reported that topoisomerase II-reactive chemotherapeutic drugs induce apoptosis.[29,33] A number of anticancer drugs have been shown to induce apoptosis in susceptible cells. The fact that the structurally different agents, which interact with different targets, can induce cell death with some common features suggests that cytotoxicity is determined by the ability of cells to engage the programmed cell death.[23]

We have reported that pMC540 induces apoptosis in Daudi cells as determined by electron microscopy of treated cells.[8] To try to determine whether these novel compounds induce apoptosis, their effect on DNA fragmentation and classical DNA ladder formation was investigated. pMC540 (a mixture of photoproducts), as well as its chemically synthesized active isolate, was effective in causing the characteristic internucleosomal ladder formation which could be observed clearly after 4 h of the drug treatment. At earlier time points, e.g. 30 min and 2 h, only high-molecular-weight DNA fragmentation was seen. Cleavage of DNA into large fragments prior to internucleosomal fragmentation has been reported recently for Dexamethasone-induced apoptosis in thymocytes.[42] DNA fragmentation observed at 2 h was inhibited in the presence of RNA synthesis inhibitor actinomycin D and protein synthesis inhibitor cycloheximide. However, at later time points (4 and 6 h), this inhibition was not observed, indicating that cell death had progressed beyond a point of recovery. Actinomycin D and cycloheximide are known to cause DNA fragmentation at high concentrations, but they cannot induce program cell death.[33,43]

Taken together, these results suggest that novel compounds specifically interact with topoisomerase II and induce apoptosis in target cells. This effect of the topoisomerase II-dependent compounds requires protein and RNA synthesis, indicating that they are acting at an early step of apoptosis preceding the requirement for transcription and not as the result of non-specific DNA damage.

The data presented here add a new dimension to the cytoxic effects and therapeutic value of these compounds that are proving to be effective against solid tumors without the attendant side effects in *in vivo* models.[14] Further research is under way towards enhancing our understanding of the underlying mechanism of action of these novel compounds.

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