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MENADIONE-INDUCED DNA DAMAGE IN A HUMAN TUMOR CELL LINE

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Abstract—The nature and extent of menadione (MD)-induced DNA damage were explored using the human breast cancer cell line MCF-7. Concentration-dependent single-strand (ss) and double-strand (ds) DNA breaks were detected in MD-treated MCF-7 cells using the alkaline- and neutral-elution techniques, respectively. The repair of ss and ds DNA breaks was extensive but not complete after a 6-hr incubation in drug-free medium. Evidence was found for the production of DNA interstrand cross-links in MCF-7 cells treated with the bifunctional alkylating agent, mitomycin C, but not for cells treated with MD. Exposure of MCF-7 cells to etoposide (VP-16), mitoxantrone and camptothecin resulted in the detection of significant amounts of protein-linked DNA breaks, whereas none were found in MD-treated cells. These results support the proposition that MD-induced DNA damage is not likely to be mediated via topoisomerases, nor do significant amounts of protein-linked DNA form in MD-treated cells. Thus, MD serves as a good model for examination of the role of the quinone moiety in DNA damage in relation to redox cycling. Future studies directed at elucidation of the biochemical determinants mediating formation of reactive oxygen species effecting the MD-induced DNA damage are necessary and underway.

Menadione (MD; 2-methyl-1,4-napthoquinone) has been shown to inhibit the growth and clonogenic survival of a variety of animal and human tumor cells *in vitro* and *in vivo* [1–9]. Recently, the efficacy of MD against several drug-resistant cancer cell lines was demonstrated [9], and the potential for this agent in salvage therapy of refractory neoplasms has been suggested [7, 9]. Favorable interactions of MD with ten other clinically important anticancer agents tested *in vitro* were reported, suggesting the possible utility of MD or agents like it in combination chemotherapy [9]. The results from preliminary Phase I studies showed that MD is remarkably well-tolerated, and that MD concentrations required to suppress tumor growth *in vitro* are clinically achievable [10, 11]. In addition to interest in the anticancer action of MD itself, the simple structure of MD provides an ideal model for (1) elucidation of the cytotoxic mechanism(s) imparted by the quinone moiety, which is present in several clinically important antineoplastic agents, and (2) evaluation of the consequences of oxidant stress in human cells. Thus, for multiple reasons, it is important to understand the biochemical pharmacology of this agent in human cells.

Little information is available regarding the mechanism of MD action in human cancer cells. MD has been utilized as a prototype to probe reduction–oxidation (redox) reactions of quinones in rat hepatocytes [12–17]. Quinones may undergo either one- or two-electron reductions resulting in the formation of semiquinone and hydroquinone, respectively. The semiquinone may regenerate the quinone through its reaction with molecular oxygen and in the process produce superoxide $O_2^{\cdot-}$. Similarly, the hydroquinone may produce $O_2^{\cdot-}$ by reaction with molecular oxygen to regenerate the semiquinone [12, 18]. Hence, as depicted in Fig. 1, it is thought that MD and other quinones enter a "redox cycle". The putative redox cycling of MD and the subsequent generation of toxic oxygen species (e.g. hydroxyl radical and singlet oxygen) may culminate in damage to macromolecules [19]. Thus, it is possible that the cytotoxicity of MD in human cancer cells could be a consequence of oxidant stress, specifically, DNA damage. A similar mechanism has, in part, been invoked in the action of other clinically important but more structurally complex anticancer quinone derivatives such as diaziquone (AZQ) [20–22], mitomycin C [19, 23–25], mitoxantrone [26], and doxorubicin [27–30] and model quinone compounds such as benzoquinone mustard [31, 32].

However, as depicted by the question mark in Fig. 1, whether treatment of human cancer cells with MD results in DNA damage has never been explored. To assess this possibility, the nature of MD-induced DNA damage at cytotoxic concentrations has been studied. The results presented forthwith suggest that DNA breaks may play a central role in the cytotoxicity of MD, and that such DNA damage does not appear to be mediated via proteins such as topoisomerases. An abbreviated report of some of the findings of the present study was given previously [33].

MATERIALS AND METHODS

Cell culture. The MCF-7 (breast carcinoma) cell

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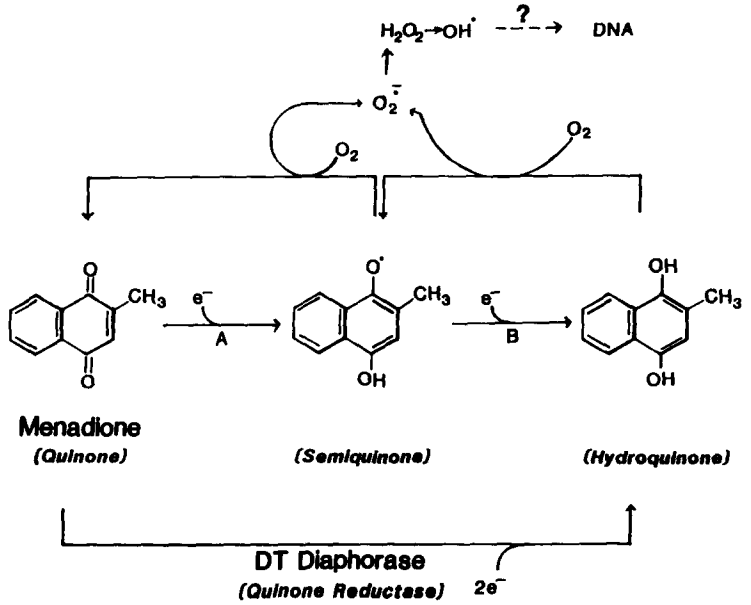

Fig. 1. Redox-cycling of MD. One electron (e^-) reductions denoted by "A" and "B" are thought to be mainly catalyzed by NADPH–cytochrome P450 reductase [12]. Hydroxyl radicals (·OH) formed from reactions between H_2O_2, superoxide ($O_2^{\cdot-}$) and metals (e.g. iron) may damage macromolecules such as DNA [19].

line used in this study was provided by Dr. S. Ramakrishnan. These cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and 100 μg/mL of kanamycin (all tissue culture reagents were from GIBCO, Grand Island, NY). Cytotoxicity was evaluated using the clonogenic assay as described previously [9].

Drug, isotopes and other reagents. The water-soluble form of menadione, menadione sodium bisulfite (Sigma Chemical Co., St. Louis, MO), was prepared immediately prior to use. Mitomycin C, etoposide (VP-16) and camptothecin were obtained, respectively, from Kyowa Hakko, Kogyo, Japan; Merck Sharp & Dohme, International Division, Bristol, Germany; and the Sigma Chemical Co., St. Louis, MO. The compounds were dissolved in their respective solvents and maintained at −70° until used. [2-^{14}C]Thymidine (57 mCi/mmol) and [*methyl*-^{3}H]thymidine (36 Ci/mmol) were obtained from the ICN Corp., Costa Mesa, CA. Polycarbonate filters (2 μm pore size) and proteinase K were obtained from the Nucleopore Corp., Pleasanton, CA, and Boehringer Mannheim Corp., Indianapolis, IN, respectively.

DNA damage studies. Five types of drug-induced DNA damage were evaluated in the present study including: single-strand (ss) DNA breaks, double-strand (ds) DNA breaks, DNA interstrand crosslinks (ISCs), protein-linked DNA breaks and protein-linked DNA. Prior to drug exposures or X-irradiation, exponentially growing cells were radioactively labeled with [^{14}C]thymidine (57 mCi/mmol; 0.2 μCi/mL; 0.2 μM in tissue culture medium) for 24 hr. The cells were washed twice with phosphate-buffered saline (PBS) and suspended to a final density of 2.0×10^5 cells/mL in medium in duplicate for subsequent drug exposures and/or X-irradiation. For quantitation of DNA strand breakage as well as for internal standardization, [^{3}H]thymidine-labeled cells (36 Ci/mmol; 1 μCi/mL; 1 μM) were X-irradiated on ice using a ^{137}Cs source which delivered X-irradiation at a rate of 365 rads/min. The [^{14}C]-thymidine-labeled cells were processed for evaluation of a given type of DNA damage as previously described by others [34, 35] and as briefly outlined ahead: (1) *ss DNA breaks*—cell lysis with proteinase K (0.5 mg/mL) and alkaline elution (pH 12.1); (2) *ds DNA breaks*—cell lysis with proteinase K and neutral elution (pH 9.6); (3) *ISC*—cell lysis with proteinase K and alkaline elution plus and minus X-ray; and (4) *protein-linked DNA and/or protein-linked DNA breaks*—potassium sodium dodecyl sulfate (K^+SDS) precipitation assay [36]. The flow rates and fraction collection time durations used in alkaline and neutral elution experiments were: 0.03 mL/min, 0.5 hr/fraction; and 0.04 mL/min, 2 hr/fraction, respectively. The kinetics of DNA elution from filters was quantitated using the X-irradiated [^{3}H]thymidine-labeled cells under conditions that gave ~50% DNA breakage. All studies were performed in the dark to prevent light-induced DNA damage. Individual drug and X-ray treatments were performed in duplicate in each experiment. In control elution experiments, no effect of MD itself on elution of radioactively labeled DNA from polycarbonate filters was observed.

Assessment of DNA repair. To evaluate DNA repair, MCF-7 cells were exposed to 50 μM MD for 1 hr, after which cells were washed twice with phosphate-buffered saline. These cells were then resuspended in drug-free medium for 0, 2, 3 and

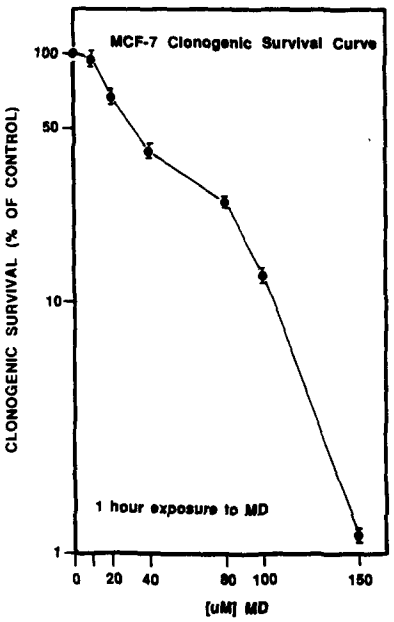

Fig. 2. Cytotoxicity of MD in MCF-7 cells as evaluated by the clonogenic assay. MCF-7 cells were exposed to the indicated concentrations of MD for 1 hr. Cells were then processed as described previously [9]. The IC_{50} of MD in MCF-7 cells measured by this assay was 32 ± 9 μM. Figure 2 is a representation of one experiment done in triplicate. Values are averages ± SD. Cells were seeded at 1500 cells per dish.

6 hr. Alkaline or neutral elution techniques were then immediately performed utilizing these cells.

RESULTS

Induction and repair of MD-induced ss and ds DNA breaks. Prior to the examination of ss and ds DNA breaks induced by MD, the cytotoxicity profile of MD on MCF-7 cells was first determined using the clonogenic assay. Figure 2 shows the clonogenic survival curve of MCF-7 cells exposed for 1 hr to different concentrations of MD. The IC_{50} (concentration of drug that inhibits 50% of clonogenic survival) of MD against MCF-7 cells exposed for 1 hr to drug was 32 ± 9 μM.

The possibility that MD induces ss and ds DNA breaks in the MCF-7 cell line was explored using alkaline and neutral elution techniques. As shown in Fig. 3A, MD treatment of human MCF-7 cells resulted in a concentration-dependent increase in ss DNA breaks as measured by alkaline elution. The extent of ss DNA breaks induced in MCF-7 cells treated with 10 μM MD for 1 hr was equivalent to 134 ± 49 rad equivalents (Table 1). This amount of breakage corresponds to a true break frequency of *0.1206 ss DNA breaks/10^6 nucleotides utilizing the* conversion factor 0.9×10^{-9} ss DNA breaks rad^{-1} $nucleotide^{-1}$ [35].

It was possible that the ss DNA breaks observed after a 1-hr treatment of cells with MD could have resulted from denaturation of ds DNA breaks mediated by MD treatment of cells. This possibility was assessed directly by utilizing the neutral elution technique. As shown in Fig. 3B, a concentration-dependent increase in ds DNA breaks was detected in MCF-7 cells treated with MD. The extent of ds *DNA breakage induced in MCF-7 cells treated with* 15 μM MD for 1 hr was 2578 ± 143 rad equivalents. Calculations of rad equivalents at the different MD concentrations used in the assessment of ss and ds DNA breakage were based on the formula by Zwelling *et al.* [35] and the results are depicted in Table 1.

The reversibility or repair of MD-induced ss and ds DNA breaks was also determined in the MCF-7 cell line. As shown in Fig. 4, by 6 hr after drug removal, 66 ± 7% of the ss DNA breaks formed in 1-hr MD-treated cells were repaired as measured by alkaline elution. Repair of ds DNA breaks in cells after a 1-hr exposure to 50 μM MD was 58 ± 4% by 6 hr post-drug removal. Longer recovery periods could not be evaluated due to dilution of radiolabel as a consequence of cell division or metabolism of the radiolabel thymidine monophosphate in DNA. The incomplete repair of ss and ds DNA breaks in the MCF-7 cells may be due, in part, to a fraction of the drug (i.e. 23% and 11% of [^{3}H]MD still retained in MCF-7 cells 6 and 24 hr after drug removal, respectively) still remaining in the cells as determined by drug retention studies (unpublished results, Ngo EO and Nutter LM). This amount of drug still remaining within the cells may be enough to effect DNA breakage. The latter may account for the fact that repair of the ss and ds DNA breaks was extensive but not complete.

DNA interstrand cross-links and protein associated-links and DNA breaks. Evidence derived from studies using hepatocytes [12–17], mouse leukemia cells [3] and bacteria [37] suggests that MD is a redox cycling agent capable of producing reactive oxygen species (ROS). The results described in this paper suggest that MD does effect ss and ds DNA breaks in human tumor cells. However, no studies have examined the possibility that MD or its metabolites possess the ability to alkylate DNA and/or cause DNA interstrand crosslinks (ISCs).

A modification of the alkaline elution technique as described under Materials and Methods was utilized in order to assess ISC formation in MD-treated MCF-7 cells. As shown in Fig. 5, MD-induced ISCs were not detected in MCF-7 cells, whereas ISCs in cells treated with the bifunctional alkylating quinone, mitomycin C are readily detectable [38]. Since ISCs are thought to mask ss DNA breaks and vice versa [39], the conclusion that MD induces only ss DNA damage must be considered a qualified one.

The formation of protein-linked DNA breaks and protein-linked DNA in cells treated with a number of anticancer agents has been reported. These include, but are not limited to, doxorubicin (DOX) [35, 40], VP-16 [41], mitoxantrone [42] and camptothecin [43]. In the case of DOX, VP-16 and mitoxantrone, the putative identity of the protein in the stabilized DNA–protein complex is topoisomerase II [40, 41], while it is thought that camptothecin action is mediated via topoisomerase I

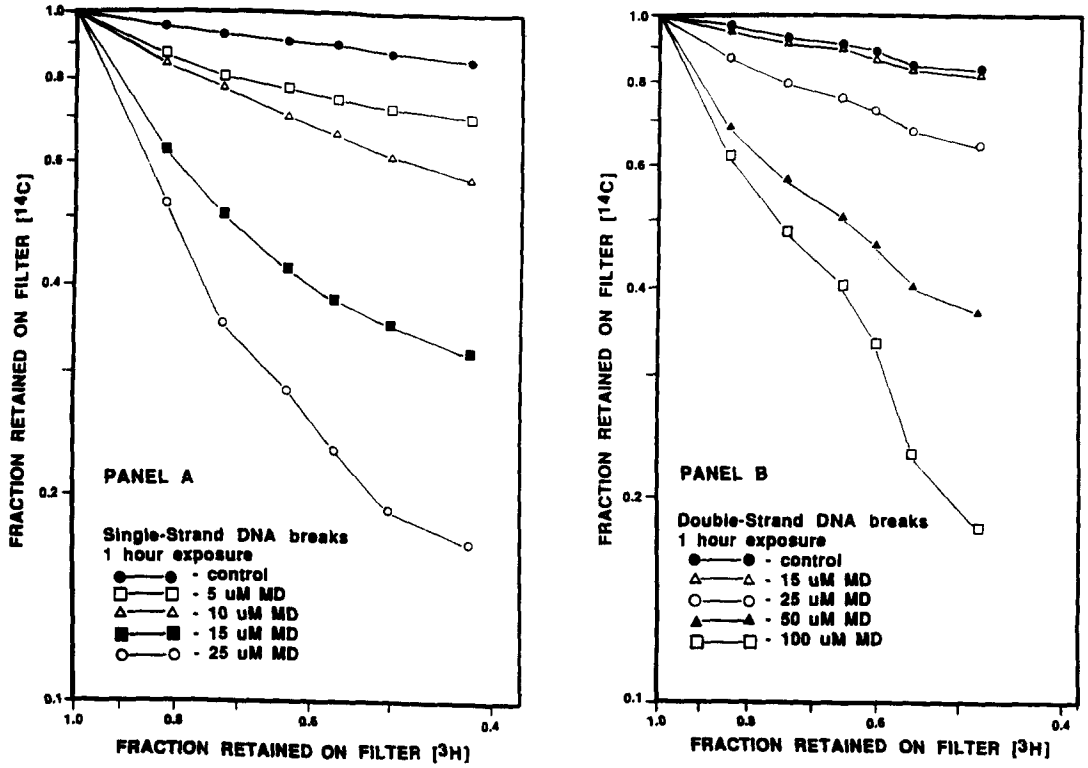

Fig. 3. Induction of single-strand (ss) and double-strand (ds) DNA breaks in human MCF-7 cells treated with MD. The ss (panel A) and ds (panel B) DNA scissions were measured by the alkaline- and neutral-elution techniques, respectively, after exposure of cells to MD for 1 hr (see Materials and Methods for details). The amount of X-ray used in panel A was 270 rads while for panel B, 6000 rads of X-ray was employed for quantitation. In both panels the abcissa scale is drawn 1.5-fold greater than the ordinate scale for diagrammatic clarity.

Table 1. Quantitation of ss and ds DNA breaks in MD-treated MCF-7 cells*

MD [μM]	ss DNA breaks (rad equivalents)	MD [μM]	ds DNA breaks (rad equivalents)
5 (4)†	114 ± 22‡	10 (2)	1,781
10 (6)	134 ± 49	15 (6)	2,578 ± 143
15 (6)	203 ± 40	25 (6)	4,839 ± 745
25 (6)	446 ± 152	50 (6)	8,520 ± 812
50 (6)	763 ± 240	100 (6)	10,660 ± 1,606

* Rad equivalents were determined using a formula by Zwelling *et al.* [35].

† The numbers in parentheses denote the number of experiments performed.

‡ This value represents the range of rad equivalents from two experiments; for the remainder, values are averages ± SD.

inhibition [43]. To gain insight into MD action, it was important to determine whether the ss and ds DNA breaks induced by MD were mediated by or involved protein(s). For this purpose, the potassium sodium dodecyl sulfate (K^+SDS) assay was employed.

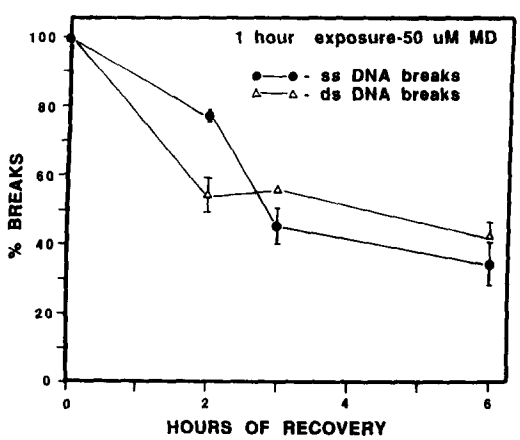

Fig. 4. Repair of ss and ds DNA breaks in MCF-7 cells exposed to MD. After a 1-hr treatment of MCF-7 cells with 50 μM MD, the cells were processed immediately for alkaline (●) or neutral (△) elution (i.e. 0 hr after drug removal) or after 2, 3 and 6 hr of incubation in drug-free medium. Bars represent the range of two separate experiments performed in duplicate.

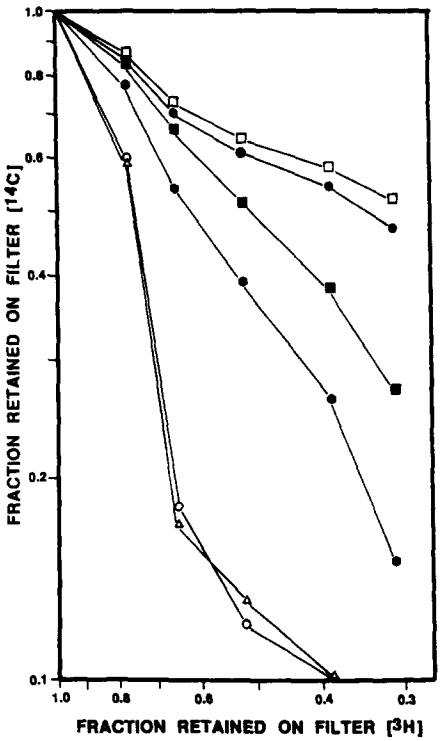

Fig. 5. Formation of interstrand crosslinks (ISCs) in MCF-7 cells. The MCF-7 cells were exposed for 1 hr to either mitomycin C (MMC) or MD. Treatment of cells with X-ray delivered from a ^{137}Cs source was for 0.74 min (365 rads/min = 270 rads of X-ray). The formation of ISCs was then measured utilizing alkaline elution; mitomycin C but not MD treatment of MCF-7 cells resulted in detectable ISCs. Key: control (●); 100 μM MMC (□); 100 μM MMC + 270 rads X-ray (■); 270 rads X-ray (●); 50 μM MD (△); and 50 μM MD + 270 rads X-ray (○).

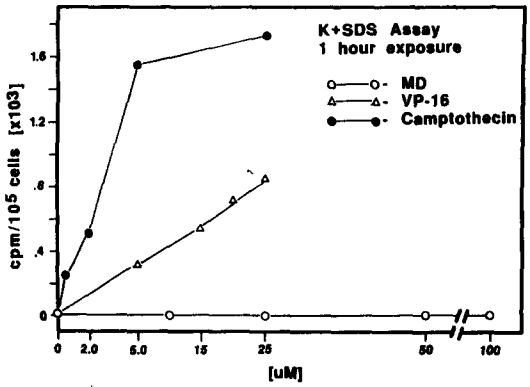

Fig. 6. Absence of protein-linked DNA or protein-linked DNA breaks in MD-treated cells. VP-16 and camptothecin treatment of MCF-7 cells resulted in marked levels of K^+SDS precipitable radioactivity (see Materials and Methods for details).

The K^+SDS assay measures the formation of intracellular protein-linked DNA and/or protein-linked DNA breaks [36]. The use of the K^+SDS assay is advantageous for studying DNA–protein interactions since whole cells are used, thereby alleviating the concerns of requisite cellular metabolism of a compound. As shown in Fig. 6, VP-16 and camptothecin were positive in the K^+SDS assay, whereas MD treatment of MCF-7 cells yielded negligible amounts of precipitable protein-linked radioactivity. MCF-7 cells exposed to mitoxantrone also gave a significant amount of precipitable radioactive counts (data not shown). Collectively, these data suggest that MD-induced DNA damage is not mediated through topoisomerases and does not result in marked levels of protein-linked DNA. In support of the former conclusion, it was observed that VP-16-resistant cells with attenuated levels of topoisomerase II [44] are not cross-resistant with MD*.

DISCUSSION

Several cellular effects have been ascribed to MD in studies mainly utilizing rat hepatocytes including alterations in pyridine nucleotide pools [3, 13], depletion of glutathione (GSH) pools [3, 12, 14–17], oxidation of protein sulfhydryl groups in cytoskeletal proteins [45] and perturbations in calcium homeostasis [46, 47]. Most, if not all of these effects are thought to be related to the redox cycling capacity of MD (Fig. 1). Redox cycling and subsequent generation of ROS have also been implicated in DNA damage vis-à-vis the action of other anticancer quinone agents [19, 27–30].

The induction of ss DNA breaks in MD-treated rodent and human fibroblasts as measured utilizing chromatographic and alkaline sucrose gradient analyses have been reported [15, 16, 48]. However, ss DNA breaks and other types of DNA damage induced by MD in human tumor cells have never been reported, nor has DNA damage been related to the cytotoxicity of this agent. Defining the characteristics of MD-induced DNA damage is not only central toward developing an understanding of the mechanism of anticancer action of this agent, but also to that attributed to the quinone moiety in general. In the present study, the sensitive and quantitative technique of alkaline elution and modifications therein were utilized to define the nature of MD-induced DNA damage in a human breast tumor cell line.

The results shown in Fig. 3A, demonstrating the concentration-dependent induction of ss DNA breaks in MCF-7 cells qualitatively confirm those previously reported using non-neoplastic rodent and human [15, 16, 48] cells. Direct quantitative comparisons are not possible since three distinctly different methodologies were employed using different cells and species. However, from these three independent studies, it is clear that MD is a relatively potent inducer of ss DNA breaks. The extent of ss DNA damage and *in vitro* cytotoxicity [9] is quantitatively comparable to that induced in cells using similar concentrations of the clinically used quinone agent, AZQ [21, 22].

In the present study, the production of ds DNA

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breaks in MD-treated MCF-7 cells was measured using neutral elution (Fig. 3B). Quantitation of the ds DNA breaks utilizing an internal ^{3}H-labeled irradiated control revealed that exposure of MCF-7 cells to 15 μM MD for 1 hr resulted in 2578 ± 143 rad equivalents of ds DNA breakage.

Repair of ss and ds DNA breaks was detected in MCF-7 cells cultured in MD-free medium. It should be noted that the reversibility experiments do not measure fidelity of repair of strand breaks *per se*; rather, they give information regarding DNA size. Thus, it is possible that misincorporated nucleotides are present in the "repaired" DNA; this possibility is not assessible by alkaline elution. Studies are currently underway to examine the fidelity of repair and sequence specificity of MD-induced DNA damage using a bovine papilloma virus episome.

In addition to frank DNA strand breaks, the mode of DNA interaction of some anticancer agents has been shown to involve interstrand DNA cross-links. Notable in this respect is the bifunctional alkylating quinone derivative, mitomycin C [38]. ISCs were detected in MCF-7 cells treated with mitomycin C, but not in those exposed to MD (Fig. 5). Thus, while monoalkylation of DNA has not been ruled out by these experiments, the MD molecule is probably not capable of effecting ISCs in DNA. Use of the episome described above in combination with radiolabeled MD will help address the possibility that MD is capable of monoalkylation of DNA.

The mechanisms of action of several clinically utilized anticancer quinones are thought, in part, to involve proteins such as topoisomerase II [40, 41], and efforts are under way to generate antineoplastic agents which act through topoisomerase I. In addition, it has been demonstrated that treatment of cells with several compounds result in protein-linked DNA. The possibility that MD-induced DNA damage was mediated by or involved proteins had never been explored. The K^+SDS assay was employed to detect possible MD-induced DNA–protein links and/or protein-linked DNA breaks. VP-16 and camptothecin were positive in the K^+SDS assay, consistent with the putative roles of topoisomerases II and I, respectively, in the mechanisms of these DNA-damaging agents. However, MD treatment of MCF-7 cells did not result in production of detectable radioactive DNA–protein complexes. These data, in conjunction with the finding that MD is equally effective against VP-16-resistant and parental KB cells, strongly suggest that MD action is not mediated through topoisomerases, nor does it cause significant amounts of protein-linked DNA in human cancer cells*.

Collectively, the results from this study suggest that the mechanism of MD-induced DNA damage is not qualitatively similar *in toto* to the known mechanisms of DNA damage of any of the clinically used DNA-damaging agents. Although redox cycling of DOX and mitomycin C and subsequent generation of ROS have been suggested, a role for topoisomerases and ISCs has also been invoked in the mechanisms of these agents [38, 40]. The remarkably broad spectrum of anticancer activity of MD against several human cancer cell lines that are resistant to clinically important agents [9] may be a consequence of the unique nature of MD-induced DNA damage. Studies ongoing in our laboratory aimed at identification of MD-related ROS and the determinants modulating MD-induced DNA damage in human neoplastic cells (e.g. the roles of DT diaphorase, catalase, iron and copper in ROS production) will contribute to a more complete understanding of the action of this unique agent.

Distinct advantages to the utility of MD as a model quinone are apparent by the results from the present studies; MD is a structurally simple redox cycling quinone which elicits cytotoxicity at micromolar concentrations and damages DNA in a human cancer cell line. Proteins such as topoisomerases do not appear to play major roles in MD action, and ISCs are not formed in human cancer cells treated with MD. Thus, studies of the quinone moiety using MD are less complicated than those utilizing more structurally and mechanistically complex quinones like DOX. For similar reasons, previous studies aimed at understanding the role of the quinone moiety in antitumor action and resistance in mouse lymphoblastoid cells were performed using the model quinone, hydrolyzed benzoquinone mustard (HBM; [31, 32]).

In summary, results from the present study suggest a role for the quinone moiety in the DNA damage and anticancer action of an agent in the absence of protein and/or topoisomerase involvement and DNA ISC formation. Recently obtained data suggest that the DNA damage in MD-treated MCF-7 cells is directly related to redox cycling and the production of ROS [49]. Current studies in this laboratory directed toward elucidation of the mechanisms underlying resistance to MD in human neoplastic cells will (a) complement our understanding of the mechanistic aspects of MD action, (b) enhance our knowledge of the consequences of oxidant stress in mammalian cells, and (c) reveal determinants of resistance with potentially direct relevance to clinically utilized quinones.

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