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MENADIONE: SPECTRUM OF ANTICANCER ACTIVITY AND EFFECTS ON NUCLEOTIDE METABOLISM IN HUMAN NEOPLASTIC CELL LINES

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Abstract—The spectrum of cytotoxicity of menadione (MD) was examined in a panel of human cancer cell lines. MD was equipotent against multidrug-resistant and parental leukemia cell lines with IC_{50} values of 13.5 ± 3.6 and 18 ± 2.4 μM respectively. A cervical carcinoma cell line resistant to the antimetabolite, methotrexate (MTX), was as sensitive to MD as its parental cell line. The interactions of fifteen clinically utilized anticancer drugs wit1283MD were examined *in vitro* and the majority were found to be additive, with four agents exhibiting synergism and one agent exhibiting antagonism. MD inhibited the incorporation of radioactive thymidine, uridine and amino acids into DNA, RNA and protein, respectively, in three human cancer cell lines. Some possible reasons for the inhibition of DNA synthesis including effects of MD on intracellular deoxyribonucleoside triphosphate pools were examined and ruled out. Although results from previous studies using rat hepatocytes suggested that mitochondria may be a target of MD, no significant effect of this compound on total intracellular adenosine triphosphate (ATP) pools in human cancer cell lines was observed. Collectively, these *in vitro* results demonstrate that MD possesses a broad spectrum of anticancer activity and suggest the potential utility of this agent in cancer therapy. Future studies directed at elucidation of the mechanism of MD action in human cancer cells are warranted and are under study.

Menadione (MD; 2-methyl-1,4-naphthoquinone) is a synthetic derivative of phylloquinone (vitamin K_1) possessing a simpler structure than clinically utilized anticancer quinones such as doxorubicin, mitoxantrone and mitomycin C (see Fig. 1). MD was shown by others to exhibit anticancer activity in rodent [1–5] and human [6] cancer cell lines *in vitro* and in a variety of patient tumor explants [2, 7] and in animals bearing the Walker 256 carcinosarcoma [8]. Depending on the type of tumor cell line studied, the reported IC_{50} values (i.e. IC_{50} denotes the concentration of drug required to inhibit 50% of cell growth) range from 1 to 50 μM. The drug concentrations required to suppress tumor cell growth *in vitro* are clinically achievable [9, 10]. Experience from a preliminary phase I study of MD indicated a surprisingly low incidence of adverse effects with dosages capable of producing cytotoxic serum concentrations. In particular, cardiac toxicity such as that seen in patients treated with the quinone, doxorubicin, was not observed [9]. Collectively, these factors strongly support the potential of MD as a novel anticancer agent.

Studies of MD action are largely limited to those performed using rat hepatocytes in which this agent was utilized as a model redox-cycling quinone. Several effects of MD in rat hepatocytes have been described including: (a) increased consumption of molecular oxygen (O_2) and generation of superoxide ($O_2^{\cdot}$) [11–13], (b) depletion of glutathione (GSH) pools [11, 13–16], (c) oxidation of protein sulfhydryl groups in cytoskeletal proteins [17], (d) alterations in pyridine nucleotide pools [12, 18–22], (e) decreases in mitochondrial [20, 23] and cellular [21, 22] ATP pools, (f) effects on calcium homeostasis [20, 24, 25] and (g) single-strand DNA breaks [14, 15]. A decrease in cellular GSH pools as a consequence of MD treatment has also been demonstrated using mouse leukemia [3, 4] and human hepatoma [26] cell lines. Results from a single study suggested that MD might inhibit ribonucleotide reductase since deoxyadenosine triphosphate (dATP) and thymidine triphosphate (dTTP) pools were attenuated significantly after MD exposure of Ehrlich-ascites carcinoma cells [27].

A paucity of information is available regarding MD action in human cancer cells. For this reason, in addition to the favorable preliminary clinic trials with MD, we were prompted to investigate the activity of this unique agent in human cancer cell lines. The results presented forthwith suggest that this agent possesses a mechanism(s) of cytocidal action which is independent of direct effects on nucleotide metabolism. The favorable interactions of MD with other clinically used agents and its efficacy against several drug-resistant human cancer cell lines strongly support the potential utility for this agent in cancer treatment.

MATERIALS AND METHODS

Drugs, chemicals and radioisotopes. The water-soluble form of MD, menadione sodium bisulfite (called MD herein), was obtained from the Sigma Chemical Co., St. Louis, MO, U.S.A. Doxorubicin

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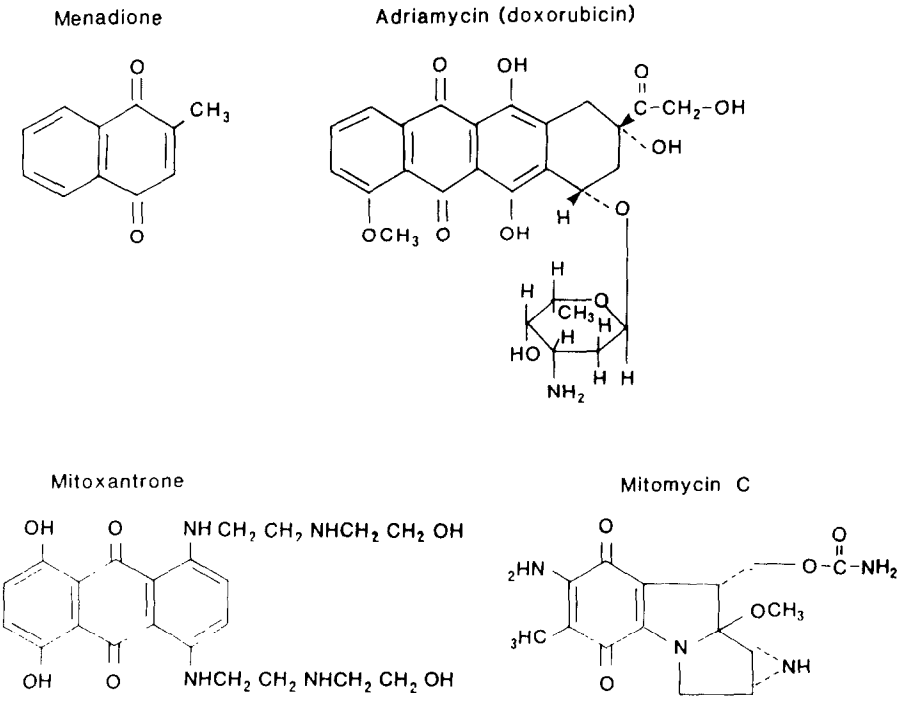

Fig. 1. Structures of menadione (MD) and three clinically used quinone derivatives. MD is the simpler of the four, possessing only a methyl group at the 2-position of the napthoquinone ring.

and *cis*-platinum were obtained from Farmitalia Carlo Erba, Milan, Italy. Vinblastine and vincristine were obtained from Eli Lilly, Indianapolis, IN, U.S.A. Mitoxantrone and thiotepa were obtained from Lederle, Wayne, NJ, U.S.A. Cytosine arabinoside, bleomycin, 5-fluorouracil (5-FU), methotrexate, mitomycin C, dacarbazine (DTIC), actinomycin-D and etoposide (VP-16) were obtained, respectively, from Shinyoku, Kyoto, Japan; Nippon Kayaku, Japan; Hoffmann-LaRoche, Switzerland; Pharmochemie B.V. Haarlem, Holland; Kyowa Hakko, Kogyo, Japan; Miles Pharmaceuticals, West Haven, CT, U.S.A.; Merck Sharp & Dohme International Division of Merck, U.S.A.; and Bristol, F.R.G. Compounds were dissolved in their respective solvents (water or dimethyl sulfoxide) at high concentrations and kept in a −70° freezer. Further dilutions were made before use. New preparations were made every 3 months. A sodium bisulfite control was utilized in all the experiments when water-soluble menadione sodium bisulfite was used. [^{3}H]dATP, [^{3}H]dTTP, [^{3}H]uridine, ^{3}H-labeled amino acids and [^{14}C]thymidine were purchased from the ICN Corp., Costa Mesa, CA, U.S.A.

Cell lines. Human MCF-7 (breast adenocarcinoma), KB (oral epidermoid carcinoma), HeLa (cervical carcinoma) and CEM (T-cell acute lymphoblastic leukemia) cell lines were provided by Dr. Y-C. Cheng. The multidrug-resistant CEM (CEM-VBL) cell line was provided by Dr. William Beck. MCF-7 and KB cell lines were maintained in 5% fetal bovine serum (FBS) in Roswell Park Memorial Institute (RPMI) 1640 medium. CEM and CEM-VBL lines were grown as suspension cultures in RPMI 1640 medium containing 10% FBS. The CEM-VBL cell line was routinely maintained in culture under vinblastine selection (i.e. 100 nM vinblastine). The multidrug-resistant property of the CEM-VBL cell line was verified by 48-hr growth inhibition assays using five anticancer agents (see Table 2). An MTX-resistant HeLa cell line was established by E. O. Ngo in L. M. Nutter's laboratory using increasing increments of MTX in medium as previously described by others [28]. Briefly, HeLa cells were exposed continuously to increasing concentrations of MTX ranging from 80 to 500 nM MTX over a 5-month period. Cell lines grown continuously in the presence of 500 nM MTX were evaluated for their sensitivity to MTX and MD using the growth inhibition assay. The MTX-resistant cell lines were cultured in RPMI 1640 containing 5% FBS and 30 μM thymidine; the latter alleviates MTX-mediated dTTP pool depletion [28]. All medium contained the antibiotic kanamycin (100 μg/mL), and all cell lines were depleted of drugs and thymidine 4 days prior to experimental manipulations.

Growth inhibition and cytotoxicity assays. Cell growth curves were generated by counting cells 24, 48 and 72 hr after drug additions. Periods of exponential cell growth were used to determine IC_{50} values. The colony-forming assay was also used in this study. For low-density cell plating, cells were plated at a final concentration of 500 cells/60 mm petri dish. After an overnight incubation to allow

cells to attach, drug was then added in fresh culture medium. Following a scheduled time of drug exposure, drug-containing medium was aspirated and drug-free medium was added to the culture. Colonies containing more than 50 cells were scored visually after staining with Giemsa 5–7 days later. For intermediate and high-density cell plating, cells were cultured in flasks at 5×10^4 cells/mL and 5×10^5 cells/mL, respectively, and exposed to drugs for 3 hr. Cells were then harvested and diluted to 500 cells/60 mm petri dish and processed as in low-density plating assays.

Interaction of MD with other anticancer agents. The interaction of MD with fifteen clinically utilized chemotherapeutic agents was analyzed using the median effect approach [29]. This methodology was selected for two major reasons: (1) the interaction of two agents at multiple levels of growth inhibition may be determined, and (2) when used in conjunction with facile cell counting methods, many drugs may be evaluated. The individual IC_{50} values of the fifteen agents and MD were first determined in the KB cell line. KB cells were plated in 96-well plates at 5×10^3 cells/well (N = 3) for each drug concentration. Cells were exposed continuously to a range of different concentrations of MD or the drug under study (i.e. five concentrations) to determine IC_{50} values. Cell numbers were evaluated in triplicate cultures by a tetrazolium-based semi-automated colorimetric assay (MTT) [30] after 3–4 days of culture when cells in drug-free wells reached 80% confluency. After determining the IC_{50} values of all drugs in the KB cell line, seven fixed concentration ratios of MD:drug were tested in the KB cell line (i.e. 25% IC_{50} MD:25% IC_{50} drug; 50% IC_{50} MD:50% IC_{50} drug, etc. up to 200% IC_{50} concentrations). Thus, 105 pairs of drug concentrations using fifteen drugs and MD (i.e. seven fixed concentration ratios per pair) were evaluated for analyses of drug interactions at different fractional levels of growth inhibition (F_a) as compared to MD or the drug alone. The nature of the interactions of MD with other anticancer agents was defined by a combination index (CI) value generated by a computerized program based on a median effect equation [29]. Utilizing this program, synergism is defined as a CI less than 0.8, additive as a CI less than 1.3 but greater than 0.8 and antagonism as a CI greater than 1.3. Those interactions defined as synergistic or antagonistic using this method were confirmed using the clonogenic assay under similar treatment conditions.

Macromolecule synthesis inhibition. KB, MCF-7 and CEM cell lines were grown in log phase at 5×10^5 cells/mL. Incorporation of [^{14}C]thymidine into DNA, [^{3}H]uridine into RNA and a ^{3}H-labeled amino acid mixture into protein was assayed using all three cell lines. After adding radioactive labels and different concentrations of MD, cells were incubated for 3 hr and then harvested. For [^{14}C]thymidine incorporation into DNA and ^{3}H-labeled amino acid mixture into protein, the radioactivities associated with perchloric acid-insoluble fractions were quantitated. Acid-soluble fractions were also counted as an intrinsic control for equilibration of the intracellular pool. For [^{3}H]uridine incorporation into RNA, the acid-insoluble pellets were first denatured by 1 N NaOH, and DNA was precipitated using 4 N perchloric acid; the supernatant containing radioactive ribonucleotides was counted using a Beckman scintillation counter.

Deoxyribonucleotide (dNTP) pools. The CEM cell line was grown in log phase at 5×10^5 cells/mL. Cells were exposed to different concentrations of MD for 3 hr. Perchloric acid-soluble fractions of cell extracts were collected and neutralized to pH 7.2 as described previously [31]. Assay mixtures containing DNA polymerase, [^{3}H]dATP or [^{3}H]dTTP and three other dNTPs were prepared for each dNTP assay [32]. Quantitation of dNTP pools was made using standard curves which were generated using known amounts of dNTPs as previously described [32].

Ribonucleotide pools. Cell extracts were prepared from CEM cells as described under "deoxyribonucleotide pools." Ribonucleotide pools were assayed and quantitated by high pressure liquid chromatography using an ion-exchange Whatman SAX column as previously described [31].

RESULTS

Spectrum of MD anticancer action. The IC_{50} values of MD against four human cancer cell lines as measured by the growth inhibition assay are shown in Table 1. These IC_{50} values are in good agreement with those reported previously for the KB and MCF-7 cell lines (i.e. 50 and 5 μM respectively; [6]) and are within the range of the IC_{50} value determined using murine L1210 leukemia cells (i.e. 20–30 μM MD; [3, 4]).

The cytotoxic effect of MD on human KB cells was also evaluated using the clonogenic assay. MD

Table 1. Effect of MD on growth of human cancer cell lines

Cell line	Source	IC_{50}* (μM)
CEM	Acute lymphoblastic T-cell leukemia	18.0 ± 2.4
MCF-7	Breast adenocarcinoma	11.2 ± 3.4
KB	Oral epidermoid carcinoma	51.7 ± 5.1
HeLa	Cervical carcinoma	38.0 ± 12.7

* The IC_{50} values were determined at 48 hr (CEM) and 24 hr (MCF-7, KB and HeLa) post-drug addition respectively. Values are averages ± SD (N = 3 experiments performed in duplicate).

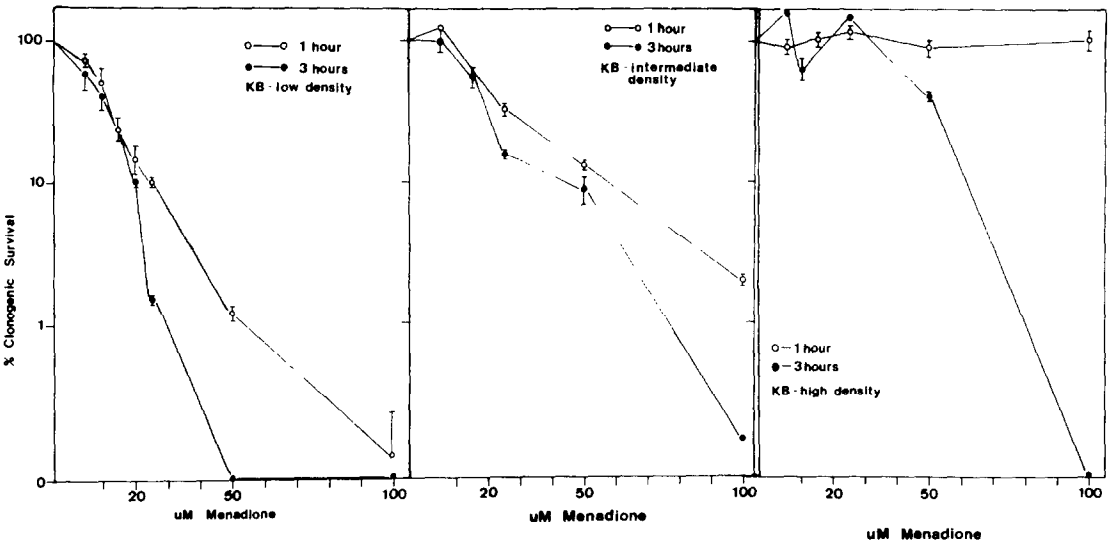

Fig. 2. Clonogenic survival of human KB cells exposed to MD. KB cells were seeded at low (10^2 cells/mL), medium (5×10^4 cells/mL) and high (5×10^5 cells/mL) density. After allowing 24 hr for cell attachment, the cultures were exposed to various concentrations of MD as indicated for 1 (○) or 3 (●) hr. The cells were then seeded at 500 cells/60 mm petri dish and left undisturbed for 8 days, at which time colonies were stained with Giemsa. Only colonies containing more than 50 cells were counted. The data shown are average values (± SD) from two experiments performed in triplicate.

exposure of KB cells resulted in cytotoxicity which was inversely correlated with cell density at the time of drug exposure (Fig. 2). The IC_{50} values of MD (3-hr exposure) in KB cells at low, medium and high (i.e. 10^2 cells/mL, 5×10^4 cells/mL and 5×10^5 cells/mL) cell densities were 20, 23 and 53 μM respectively (Fig. 2). The most striking density-dependent cytotoxicity was observed in cells exposed to MD for 1 hr at high and medium cell densities. At high cell density, a 1-hr exposure of MD had no effect on KB cell survival even using MD concentrations as high as 100 μM. In contrast, the resulting IC_{50} from a 1-hr exposure of cells to MD at medium cell density was 30 μM. It is unlikely that these variations were due to significant differences in the availability of drug since approximately 10^{12}, 10^{10} and 10^9 molecules of MD/cell were present at low, medium and high cell densities respectively. These data suggested to us the possible presence of protective elements at higher cell densities. Previously, it was shown by others that glutathione (GSH) could completely protect mouse L1210 cells from MD-mediated growth inhibition [3]. The protective effect of GSH against MD cytotoxicity was evaluated in the human KB cells. The results shown in Fig. 3 reveal that 1 mM GSH only partially protects against the cytotoxicity of MD in the human KB cell line.

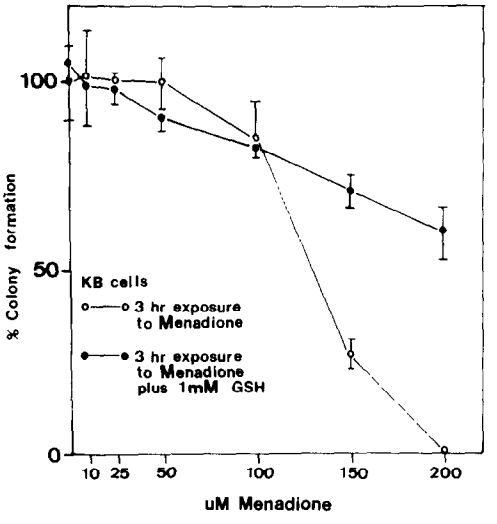

Fig. 3. Partial protection of MD cytotoxicity by GSH. KB cells (5×10^5 cells/mL) were exposed to various concentrations of MD as indicated in the presence (●) or absence (○) of 1 mM GSH. The cells were then processed for the clonogenic assay as described in the legend to Fig. 2. The values shown are averages (± SD) from two experiments performed in triplicate.

As mentioned earlier, MD is a quinone possessing a less complex structure than other clinically utilized quinone derivatives such as doxorubicin (Adriamycin®), mitoxantrone and mitomycin C. Thus, it was of interest to examine the efficacy of MD against a human acute lymphoblastic leukemia (CEM-VBL) cell line exhibiting a multidrug-resistant (MDR) phenotype. As shown in Table 2, and described previously by others [33], this cell line is resistant to several clinically utilized agents including the quinone derivatives Adriamycin and mitoxantrone. However, MD was equally potent against the resistant and parental CEM cell lines with IC_{50} values of 13.5 ± 3.6 and 18 ± 2.4 μM respectively.

The antimetabolite MTX is frequently used

Table 2. IC_{50} Values of various drugs against multidrug-resistant CEM cells*

Drug	IC_{50} CEM	IC_{50} CEM^r	IC_{50} Ratio CEM^r/CEM
Vinblastine	0.59 ± 0.1 nM	505 ± 103 nM	863
Vincristine	3.3 ± 2.9 nM	3077 ± 357 nM	930
Doxorubicin	32.7 ± 4.7 nM	3.6 ± 0.7 μM	110
VP-16	249.4 ± 80.5 nM	1805 ± 359 nM	7.2
Mitoxantrone	8.30 ± 1.3 nM	57.9 ± 14.6 nM	7.0
Menadione	18.0 ± 2.4 μM	13.5 ± 3.6 μM	0.8

* The data shown (averages ± SD; N = 3 experiments) are derived from growth inhibition experiments 48 hr post-drug addition. CEM and CEM^r represent parental and vinblastine-selected multidrug-resistant acute lymphoblastic leukemia cell lines respectively.

Table 3. Effect of MD on MTX-resistant HeLa cell lines

Agent tested	Cell line (IC_{50}) HeLa	HeLa^{MTX}*	IC_{50} Ratio HeLa^{MTX}/HeLa
MD	34 μM	30 μM	0.9
MTX	14 nM	775 nM	55

* HeLa^{MTX} denotes an MTX-resistant HeLa cell line.

clinically in cancer treatment. Apriori, based upon the reported mechanisms of resistance to MTX and structural dissimilarities, cross-resistance with MD was not anticipated. However, the demonstration that a murine L1210 leukemia cell line resistant to the bifunctional alkylating quinone, mitomycin C, was extensively cross-resistant with MTX [34] prompted us to evaluate whether MTX-resistant cells were sensitive to the quinone, MD. As shown in Table 3, MD was equally effective against MTX-resistant (i.e. 55-fold resistant to MTX) and parental HeLa cell lines with IC_{50} values of 30 and 34 μM respectively.

In addition to the efficacy of MD against multidrug-resistant and MTX-resistant human cancer cell lines, MD was found to be as potent against VP-16-resistant human KB* and paraquat-resistant Chinese hamster ovary† cell lines as to the respective parental cells lines. Some possible reasons for this broad spectrum of anticancer action are postulated in the Discussion.

Usually, antineoplastic agents are administered clinically in combination with one or more other agents. To investigate the potential of using MD in combination chemotherapy, the interaction of MD with fifteen clinically useful anticancer agents was investigated *in vitro* as described in Materials and Methods. Due to the extensive amount of data generated from these analyses, a summary of the results, in lieu of the actual isobolograms, is presented in Table 4. The results presented in Table 4 are representative of three separate experiments performed in triplicate and reveal that MD was synergistic with fluorouracil, bleomycin, cisplatin and dacarbazine and antagonistic with methotrexate at the levels of F_a (i.e. F_a = fractional level of growth inhibition) greater than 0 7. The interactions of MD with ten other agents were found to be additive. For drugs where synergistic and antagonistic interactions with MD at an F_a greater than 0.7 were found using the MTT assay, the results were corroborated by the clonogenic assay utilizing the KB cell line (data not shown).

Effect of MD on macromolecule synthesis. It was possible that the cytotoxicity of MD against the human cell lines was a consequence of effects on macromolecule synthesis. To examine this possibility, the incorporations of radioactive thymidine, uridine, and amino acids into DNA, RNA and protein, respectively, were evaluated in the presence and absence of MD in three different human cell lines. MD exposure of human CEM, KB and MCF-7 cells resulted in a concentration-dependent decrease in incorporation of all three radioactive precursors (Fig. 4). The most pronounced effect of MD in all three cell lines was the inhibition of [^{14}C]thymidine incorporation into DNA. The concentrations of MD which produced 50% inhibition of [^{14}C]thymidine incorporation into DNA in the cell lines ranged from 25 to 53 μM (Fig. 4).

Effect of MD on nucleotide pools. It was possible that the inhibition of DNA synthesis observed in MD-treated cells was a consequence of perturbations in nucleotide precursors such as dNTPs. Previously it was reported that MD exposure of mouse tumor cells results in attenuation of dTTP and dATP pools [27], and it was suggested that MD action may be mediated through alterations in nucleotide-metabolizing enzymes such as ribonucleotide- or dihydrofolate-reductases. The impact of MD on intracellular dNTP pools was investigated using the human CEM cell line. As shown in Fig. 5, no significant differences in dTTP, dCTP, dATP or dGTP content was observed in 3 hr-treated CEM cells using MD concentrations which potently inhibited DNA synthesis (Fig. 4).

In the rat hepatocyte system, MD has been reported to decrease significantly mitochondrial [20, 23] and intracellular [21, 22] ATP pools. As

*Dr. Y-C. Cheng, personal communication, cited with permission.

†Dr. T-C. Lee, personal communication, cited with permission.

Table 4. Multiple drug effect analysis*

Drug	Ratio	Synergism	Additive	Antagonism
Fluorouracil	4:5	+		
Mercaptopurine	9:250		+	
Cytarabine	1:200		+	
Hydroxyurea	25:3		+	
Methotrexate	1:1			+
VP-16	1:250		+	
Vincristine	1:5000		+	
Doxorubicin	1:1250		+	
Mitoxantrone	1:300,000		+	
Mitomycin C	217:15,000		+	
Bleomycin	337:20,000	+		
Actinomycin D	1:8200		+	
Cisplatin	1:40	+		
Dacarbazine	1:50	+		
Thiotepa	1:125		+	

* The ratios shown reflect the concentration of a given drug to the concentration of MD employed; synergism is defined as a combination index (CI) less than 0.8; additive is defined as a CI less than 1.3 but greater than 0.8; antagonism is defined as a CI greater than 1.3. The data were generated using the growth inhibition assay and the KB cell line.

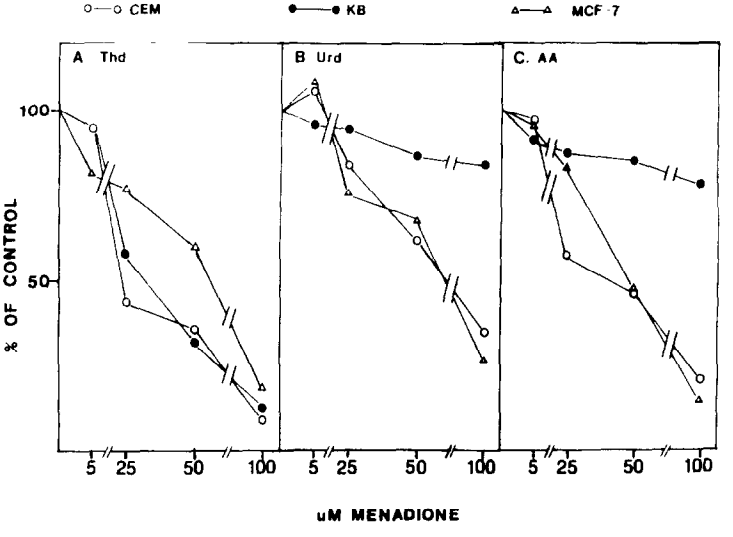

Fig. 4. Impact of MD on macromolecule synthesis. CEM, KB and MCF-7 cells (5×10^5 cells/mL) were exposed to MD at the concentrations indicated for 3 hr in the presence of [^{14}C]thymidine (panel A), [^{3}H]uridine (panel B) or a ^{3}H-labeled amino acid mixture (panel C). After trypsinization and centrifugation to pellet the cells, they were washed in cold phosphate-buffered saline. The neutralized perchloric acid-insoluble and -soluble fractions were prepared as described under Materials and Methods. Acid-insoluble radioactivity was counted in a scintillation counter and the data were expressed relative to control cells which received no MD.

shown in Fig. 4, MD treatment also inhibited RNA synthesis in human cell lines. Thus, it was possible that MD treatment of human cells disturbed ribonucleotide pools. To investigate this possibility, high performance liquid chromatographic analysis of acid-soluble extracts from MD-treated KB cells was employed. As shown in Table 5, a 22% decrease in ATP content relative to untreated KB cells was observed, whereas a 20% increase in the UTP content occurred in KB cells treated with 100 μM MD for 3 hr. Thus, it is unlikely that the inhibition of RNA synthesis in MD-treated KB cells (Fig. 4) is a consequence of depleted ribonucleotide (i.e. UTP) pools. Since the ADP content paralleled the ATP content in MD-treated cells, it is possible that these modest decreases reflect ensuing toxicity rather than direct effects on intracellular pool content. These data are similar to those of Akman *et al.* [4] where a small (i.e. 20%) decrease in ATP content was observed in L1210 cells treated with MD.

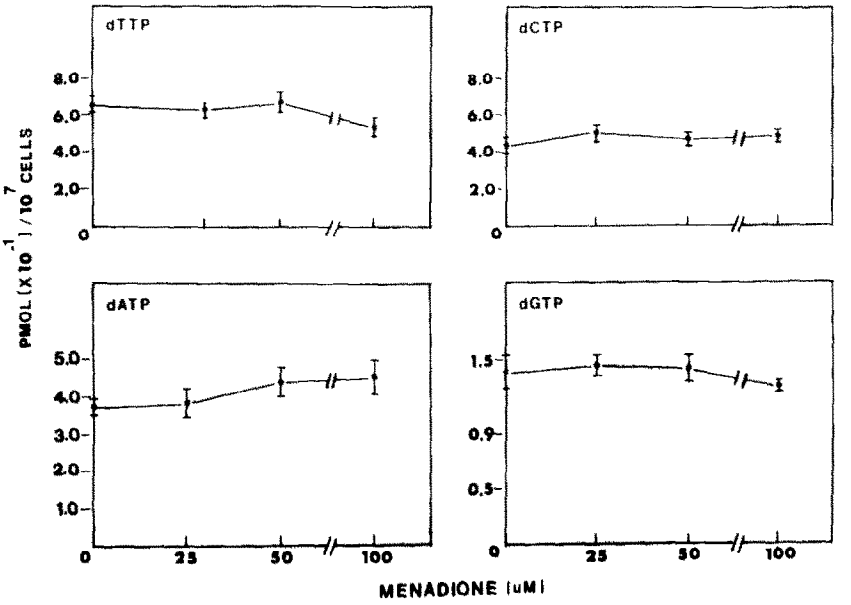

Fig. 5. Effect of MD treatment on deoxynucleoside triphosphate (dNTP) pools in the CEM cell line. CEM cells (5×10^5 cells/mL) were exposed for 3 hr to the concentrations of MD indicated, and neutralized acid-soluble fractions were processed as described under Materials and Methods. The four dNTP pool assays were performed using the acid-soluble CEM fractions in addition to solutions of known amounts of four dNTP standards. The data shown are averages (± SD) from three experiments performed in duplicate.

Table 5. Effect of MD on ribonucleotide pools in KB cells*

Treatment	ADP	ATP	UTP
None	100	100	100
25 μM	95	92	107
50 μM	114	99	116
100 μM	82	78	120

* The data shown are averages from two experiments and are expressed as percent of control cells receiving no MD. The nucleotide contents in untreated KB cell extracts were: 3.6 and 3.5 mM (ATP); 1.5 and 1.4 mM (UTP); and 377 and 354 μM (ADP).

DISCUSSION

In this manuscript the broad spectrum of MD anticancer activity in human cancer cell lines has been demonstrated. The efficacy of MD against different human tumor cell types was variable as evidenced by the IC_{50} (growth inhibition) values shown in Table 1. The reason for this variation may relate to the disposition in cells of the activities of different enzymes thought to be involved in MD metabolism and to the status of intracellular GSH pools. In particular, DT diaphorase is thought to catalyze the two-electron reduction of MD to the dihydroquinone; the dihydroquinone may then be conjugated with substances such as UDP-glucuronic acid for export from the cell [11]. In this manner, putatively toxic oxygen species formed as a consequence of redox-cycling of MD in the presence of molecular oxygen would be attenuated. Thus, differential levels of DT diaphorase activity may represent one biochemical determinant of cellular sensitivity to MD. In studies using rat hepatocytes and the DT diaphorase inhibitor, dicumarol, a detoxifying role of DT diaphorase in MD metabolism was suggested [11, 12]. On the other hand, dicumarol did not enhance oxygen consumption in MD-treated murine L1210 leukemia cells, although synergistic cytotoxicity was observed in the presence of the two agents [4]. There is no information available regarding the role of DT diaphorase in mediation of MD action in human cancer cells. The derivation and characterization of MD-resistant human cancer cells currently ongoing in our laboratory will greatly aid in elucidation of the role of DT diaphorase as a biochemical determinant of cellular sensitivity to MD and to quinones in general.

Cellular GSH and the activities of GSH-metabolizing enzymes have been demonstrated to play an important role in the ultimate efficacy of several chemotherapeutic agents [for reviews see Refs. 35 and 36]. Previously, the protective effect of GSH against MD-induced growth inhibition was demonstrated in mouse L1210 leukemia cells [3]. Evaluation of the protective effect of GSH against MD in human KB cells revealed that GSH could protect against MD cytotoxicity (Fig. 3). However, in contrast to the results found in the mouse leukemia system, the protective effect of GSH on MD toxicity in KB cells was not complete (Fig. 3). Similar results were obtained using the CEM cell line and the growth inhibition assay, namely, partial protection

by GSH against MD action.* Since the entry of GSH into cells is poor, the protection offered by GSH may be at the level of the membrane or by direct conjugation of MD. Our data do not allow us to distinguish between these possibilities.

MD was found to be equally potent against human multidrug-resistant (MDR) and parental leukemia cells. The MDR leukemia cell line was 110- and 7-fold resistant to the growth-inhibiting effects of the quinone derivatives, doxorubicin and mitoxantrone (Table 2). Since the multidrug-resistant leukemia cell line studied has been shown by others to overexpress the *mdr*-1 encoded 170,000 dalton glycoprotein associated with the active efflux of some chemotherapy agents [33], our data suggests that MD is not a substrate for the *mdr*-1 drug efflux pump. Along these lines, MD was found not to be cross-resistant with VP-16-resistant,† paraquat-resistant,‡ or MTX-resistant (Table 3) cell lines. Noteworthy in this respect are the data from Su *et al.* [7] demonstrating the *in vitro* efficacy of MD against human tumor explants derived from patients with neoplasms which were refractory to other types of chemotherapy. Taken together, these data support the idea that MD may be of utility in salvage therapy in the treatment of some types of drug-resistant cancers [3, 7].

Most chemotherapeutic agents are administered in conjunction with one or more other anticancer agents in order to decrease the chance of survival of drug-resistant tumor cell populations. Thus, it is desirable to investigate the interaction of various agents to gain insight into potentially favorable combinations. The results of drug-interaction studies using fifteen clinically useful agents demonstrated that MD was synergistic with 5-fluorouracil, bleomycin, cisplatin and dacarbazine as measured by growth inhibition of KB cells (Table 4) and confirmed using the clonogenic assay. The synergy between MD and 5-fluorouracil has been reported previously by others using murine cell lines [5, 37]. The reasons for the patterns of synergy shown in Table 4 are not immediately obvious since little is known of MD action in human cells. It has been reported that MD treatment depletes GSH pools in rat hepatocytes and human hepatoma and murine cell lines [11, 13–16] and our preliminary data using three human cancer cell lines showed that MD did decrease intracellular GSH pools.§ Thus, it is possible that MD enhances the efficacy of agents such as cisplatin through the attenuation of cellular GSH, the latter having been shown to play a role in cisplatin action [35]. Ongoing and future studies of the mechanism of MD action in human cancer cells will improve our understanding of these interactions.

In contrast to the synergistic interaction of MD with the above-mentioned agents, MD was antagonistic to MTX in the KB cell line (Table 4). These *in vitro* results using the human KB cell line are in contrast to the synergistic interactions reported between MD and MTX in tumor bearing rats [8] and the L1210 murine leukemia cell line [38]. These apparent discrepancies may relate to differences in tumor cell specific expression of biochemical determinants modulating the response to a given agent (e.g. GSH pools, dTTP pools, and DT-diaphorase and dihydrofolate reductase activities), cell culture conditions and the methodologies employed to assess drug interactions. Furthermore, the *in vivo* pharmacokinetics of a given drug may preclude direct comparisons with *in vitro* findings. The observation that administration of MTX to mice results in reduced hepatic GSH [39] illustrates this point; MTX-mediated GSH reduction has not been reported in cell culture systems to our knowledge.

The mechanism of MD cytotoxicity in human cancer cells is elusive. In accordance with results of others using murine cells [9], MD did inhibit macromolecule synthesis in human cell lines (Fig. 4). Possible reasons for these inhibitions including alterations in deoxyribonucleoside triphosphate (Fig. 5) and ribonucleotide (Table 5) pools were investigated, and no major effects of MD on these parameters were found. That inhibition of DNA synthesis in MD-treated cells is more pronounced than inhibition of RNA and protein synthesis suggests that DNA may be a principal target of MD action. Previously, the induction of single-strand DNA breaks in rat hepatocytes and human fibroblasts treated with MD was demonstrated using chromatographic methods [14, 15]. Further studies regarding the correlation of DNA damage with cytotoxicity and with MD-induced GSH pool changes will enhance our understanding of the mechanism(s) of cytotoxic action of MD. The broad spectrum of MD action against a variety of tumor cell types and drug-resistant cell lines may relate to the multifactorial action of MD against human cancer cells.

In summary, MD possessed a broad spectrum of antitumor activity including multidrug-resistant human cancer cell lines. This agent may not elicit serious toxic side effects in humans and may be a useful candidate in combination chemotherapy. Whether the use of MD in conjunction with other chemotherapeutic agents will result in serious side-effects is not known. MD-induced inhibition of macromolecule biosynthesis is not a consequence of significant changes in deoxyribonucleoside triphosphate or ribonucleotide pools. The mechanism(s) of MD action in human cancer cells is under study and may involve alterations in cellular GSH and DNA damage.

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* E. O. Ngo and L. M. Nutter, unpublished results.

† Dr. Y-C. Cheng, personal communication, cited with permission.

‡ Dr. T-C. Lee, personal communication, cited with permission.

§ E. O. Ngo and L. M. Nutter, unpublished results.

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