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Effect of Artificial Electron Acceptors on the Cytotoxicity of Mitomycin C and Doxorubicin in Human Lung Tumor Cells

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Abstract—*The cytotoxicities of mitomycin C (MMC) and doxorubicin (DOX) have been proposed to depend on intracellular reduction by reduced flavoproteins. We investigated whether MMC- and DOX-induced cytotoxicity could be inhibited by competing for electrons from reduced flavoproteins by the artificial electron acceptors phenazine methosulfate (PMS), menadione (MEN) and methylene blue (MB). In intact SW-1573 human lung tumor cells these compounds proved to be excellent electron acceptors, as judged from their capacity to induce high levels of cyanide-resistant respiration. In addition, PMS, MEN and MB were found to decrease the cytotoxicity of MMC, by 90, 63 and 29%, respectively, at concentrations that were themselves completely nontoxic. In contrast, DOX cytotoxicity was not detectably affected. These results suggest that in SW-1573 cells flavoprotein-mediated bioreduction is required for the cytotoxic effect of MMC, but not for that of DOX.*

INTRODUCTION

DOX AND MMC are two clinically important quinone anticancer drugs which are effective against a wide variety of solid tumors and leukemias. For both drugs it has been proposed that metabolic reduction of their quinone moieties plays a critical role in their mechanisms of antitumor action [1, 2].

For MMC it is known that only its reduced form is able to act as a bifunctional DNA alkylating agent [3]. Since alkylation of DNA is considered to be a major determinant of cytotoxicity [4], intracellular bioreduction is in all probability required for MMC cytotoxicity. For DOX, enzymatic reduction in cell-free systems has been shown to induce DNA damage [1] and lipid peroxidation [5] and thus might also be involved in the cytotoxicity of this agent. However, solid evidence indicating that such reactions do occur in intact human tumor cells at clinically relevant DOX or MMC concentrations is currently lacking. Therefore, the aim of the present work was to investigate for both MMC and DOX whether, at clinically relevant drug concentrations, bioreduction is required for cytotoxicity.

Our approach was to interfere with the bioreduction process using a set of artificial electron acceptors (AEA) and measure the effect of this interference on the cytotoxicity of MMC and DOX. As schematically illustrated in Fig. 1, flavoproteins use electrons from NADH or NADPH to reduce DOX or MMC. However, in the presence of AEA with high affinity for electrons, electrons will partially be used to reduce these compounds instead of MMC or DOX. Thus if metabolic reduction is obligatory for MMC or DOX cytotoxicity, then high-affinity AEA are expected to interfere with the cytotoxicities of these drugs.

The following AEA were used: phenazine methosulfate (PMS), methylene blue (MB) and menadione (MEN). PMS, MB, and MEN are not specific for only one type of flavoprotein, as these agents accept electrons from a variety of flavoproteins including succinate dehydrogenase [6, 7], lactate dehydrogenase [6], NADH dehydrogenase [8], cytochrome B5 oxidase [8], NADPH cytochrome P450 reductase [8] and DT diaphorase [9]. Since it has not been established which flavoproteins are involved in the bioactivation of quinone anticancer drugs, the wide spectrum substrate character of the AEA used was considered to be an advantage. We

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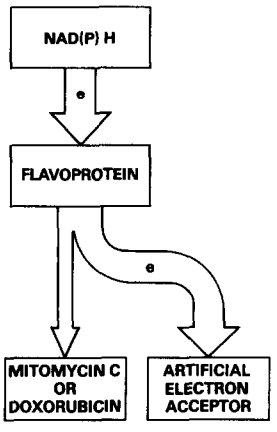

Fig. 1. Scheme illustrating the experimental approach. For explanation, see text.

selected PMS, MB and MEN because there is circumstantial evidence that they are indeed able to inhibit drug reduction. MEN has a high affinity for microsomal flavoproteins that reduce both DOX and MMC [8]. Possibly as a consequence of this, equimolar concentrations of MEN strongly exhibit DOX reduction as monitored by electron spin resonance [10]. Furthermore, MEN in submicromolar concentrations, was reported to inhibit microsomal lipid peroxidation [11], a process considered to be dependent on NADPH cytochrome P450 reductase [12]. MB reduces DOX-induced cardiotoxicity in mice [13, 14], a side-effect of DOX treatment in which the metabolic reduction of DOX is thought to play a critical role [15]. PMS is structurally related to MB, but has an even higher affinity for electrons [7]. The rationale for using three different AEA was to minimize the chance that unknown secondary effects on DOX or MMC cytotoxicity would be misinterpreted as being due to inhibition of drug reduction.

In addition to AEA we also evaluated the effect of ellipticine (EL) on the cytotoxicity of MMC and DOX, as this agent inhibits the activity of the flavoprotein NADPH cytochrome P450 reductase [16], an enzyme thought to be involved in the reduction of both drugs [1, 2]. Although the exact mechanism remains to be established, this inhibition is probably due to binding of EL to cytochrome P450. Therefore, EL was expected to inhibit cytochrome P450 reductase activity without interfering with the activity of other flavoproteins.

MATERIALS AND METHODS

Chemicals and drugs

All chemicals used were obtained from Sigma Chemical Company (St. Louis, Missouri), except for DOX, which was manufactured by Laboratoire Roger Bellon (Neuilly sur Seine, France; a gift from Bergel Nederland B.V., Heerhugowaard, The Netherlands), and MMC (clinical grade), which was from Kyowa Hakko Kyogo Co. Ltd., Tokyo, Japan. DOX, PMS, MB and MEN (sodium bisulfite salt of 2-methyl-1,4-naphthoquinone) were stored in physiological salt solution (0.9% NaCl) at −20°C, in the dark. MMC was dissolved in methanol and stored at −20°C, in the dark. Ellipticine was freshly prepared in stock solutions in dimethyl sulfoxide before each experiment.

Cells

Before the present study was undertaken, the human lung tumor cell line SW-1573, originally isolated and characterized as a squamous cell carcinoma by Dr. A. Leibovitz (Scott and White Clinic, Temple, Texas), had been cultured in our laboratories for several years in Ham's F10 medium (Flow Labs Ltd., Irvine, U.K.) supplemented with 10% fetal calf serum (Flow) and 2 mM glutamine (Flow) under an atmosphere of 2% CO_2 in air. Cell cultures were free from mycoplasma contamination, which was checked at monthly intervals by the Hoechst fluorescent dye staining technique, using CV-1 monkey kidney cells as indicators.

Cytotoxicity

Cytotoxicities of DOX and of MMC were determined using clonogenic survival as an endpoint. Exponentially growing cells were treated for 1 h with various concentrations of DOX or MMC. Effects of PMS, MB, MEN and EL on the cytotoxicity of MMC and DOX were determined by addition of the agents 1 h before MMC or DOX were added; the inhibitors continued to be present during subsequent cytostatic drug treatment. After treatment, cells were washed and counted with a Coulter counter. Approximately 500 or 5000 cells in 5 ml of culture medium were seeded in 25 cm^2 Falcon culture flasks and incubated for 8 days at 37°C. Colonies were fixed with 96% ethanol, stained with Giemsa, and counted manually. Cloning efficiency of the cells was usually between 70 and 80%. Solvent-treated controls were identical to untreated controls. Dose modifying factors (DMFs) were calculated as the ratio of the concentration of MMC giving 37% survival in the presence of inhibitor and the concentration of MMC giving 37% survival in the absence of inhibitor. Reduction of cytotoxicity was calculated as 100% − 1/DMF × 100%. Each data point was based on triplicate platings within one experiment; standard deviations of these triplicates are shown in the figures. All experiments were carried out three times, with similar results.

Cyanide-resistant oxygen consumption

Cellular oxygen consumption was determined using a Clark type electrode (Yellow Springs Instruments Co., Yellow Springs, Ohio) with a Beckman

0260 oxygen analyzer. The reaction chamber volume was 0.89 ml; the medium used was Ham's F10 supplemented with glutamine (2 mM) and 10% FCS, buffered with 25 mM hepes at pH 7.4. After allowing the medium to equilibrate for 10 min at 37°C approx. 5×10^6 cells were added, after which oxygen consumption was recorded. Oxygen consumption was 2.1 ± 0.1 fmoles O_2/cell.min under control conditions. After addition of potassium cyanide (1 mM), which almost immediately decreased the respiration rate to 0.42 ± 0.05 fmoles/cell.min, the effect of addition of AEA on cyanide-resistant respiration was recorded.

ATP

Cells were treated for 2 h at 37°C with AEA at the concentrations indicated. After washing twice with serum-free Ham's F10 medium and lysis in 0.1% (w/v) Triton X-100 in 0.1 M Tris–HCl pH 7.75 containing 2 mM EDTA, ATP was measured in the lyzate using a bioluminescence 3M A.E.C. kit from Lumac (Landgraaf, The Netherlands).

NADPH/NADP$^+$

Cells were treated for 2 h at 37°C with the indicated concentrations of AEA, washed once with serum-free Ham's F10 medium, trypsinized and suspended in physiological salt solution. NADPH and NADP$^+$ were then measured in alkaline and acid cellular extracts, respectively, according to the recycling assay of Jörgensen and Rasmussen [17].

RESULTS

Cytotoxicity of AEA and EL

Cells were treated during 2 h with various concentrations of PMS, MB, MEN or EL, after which clonogenic cell survival was determined. As shown in Fig. 2, the agents became toxic at concentrations exceeding 10, 25, 25 and 7.5 μM respectively. Since free radicals are known to be formed in the metabolism of the AEA used [7, 18, 19], toxicity of high concentrations may be due to excessive oxidative cell damage, or to the disturbance of cellular metabolism by interfering with the reduction of normal substrates of cellular flavoproteins. Toxicity of high EL concentrations is presumably due to intercalation of EL into DNA [20]. In this study, however, only concentrations of AEA or EL were used that were entirely nontoxic in the clonogenic survival assay.

AEA-induced cyanide-resistant respiration

Even though PMS, MB and MEN are known to efficiently accept electrons from flavoproteins in cell-free systems [7, 8], it was important to show that this also occurs in the living tumor cell. Since

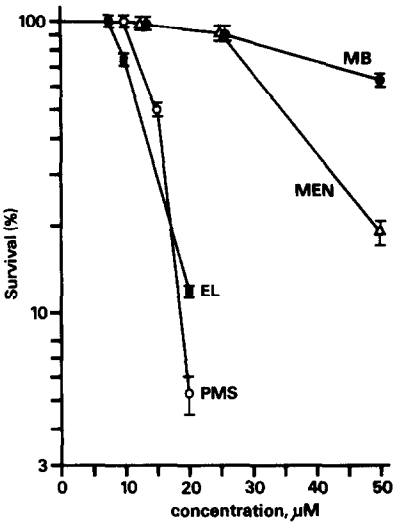

Fig. 2. Cytotoxicity of artificial electron acceptors (AEA) and ellipticine (EL). The figure shows clonal survival of SW-1573 cells after treatment, (2 h, 37°C) with various concentrations of the AEA PMS (○), MEN (△) and MB (●) as well as EL (■).

after reduction, PMS, MB and MEN react with O_2, this reaction can conveniently be monitored in intact cells by measuring the rate of cyanide-resistant oxygen consumption. Cyanide at 1 mM was added to inhibit mitochondrial O_2 consumption in order to uncover AEA reduction, which was not inhibited by this treatment.

As shown in Fig. 3 high rates of cyanide-resistant oxygen consumption were recorded at nontoxic concentrations of PMS, MB and MEN, in the order of 2–6 fmoles O_2/cell.min. These respiration rates were as high or even higher than the normal cellular oxygen consumption, which was 2.1 ± 0.1 fmoles/cell.min. These data indicate that at nontoxic concentrations PMS, MB and MEN act as efficient electron acceptors in intact SW-1573 cells.

Concentrations up to 5 μM of DOX or MMC, which kill practically 100% of the cells within 1 h,

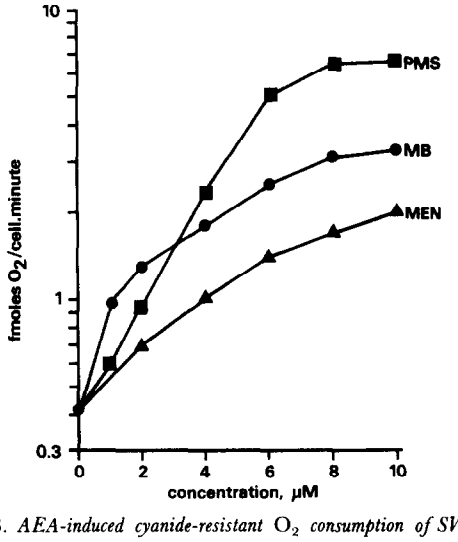

Fig. 3. AEA-induced cyanide-resistant O_2 consumption of SW-1573 cells. ■, PMS; ▲, MEN; ●, MB.

did not induce a measurable cyanide-resistant O_2 consumption, even though the one-electron-reduced semiquinone forms of these drugs are known to readily react with molecular oxygen [21]. This indicates that only a relatively small flux of electrons flows from the flavoproteins to these drugs in intact SW-1573 cells. This situation is schematically depicted in Fig. 1.

Effects of AEA on the cytotoxicity of MMC and DOX

Effects of nontoxic concentrations of PMS, MB and MEN on the cytotoxicity of MMC and DOX were investigated in the clonogenic survival assay. As shown in Figs 4 and 5, 10 μM PMS, 25 μM MB or 10 μM MEN did not alter the cytotoxicity of DOX (Fig. 4) but afforded considerable protection against the cytotoxicity of MMC (Fig. 5). From three independent experiments the dose modifying factors (DMFs) for MMC cytotoxicity were calculated to be 1.4 ± 0.2, 10.0 ± 1.1 and 2.7 ± 0.2 for MB, PMS and MEN, respectively. Figure 6 shows that at the concentrations of AEA used to determine DMFs, maximal protection was achieved. Half-maximal protection occurred at 1.5, 4.0 and 4.0 μM of PMS, MB and MEN, respectively. At maximally protective concentrations the AEA did not cause any detectable depletion of ATP, while also the NADPH/$NADP^+$ ratio was not significantly affected (Table 1). This indicates that the AEA used in this study, in spite of a strongly stimulated cyanide-resistant respiration, apparently did not cause a detectable disturbance of the normal redox status or energy household of the cells.

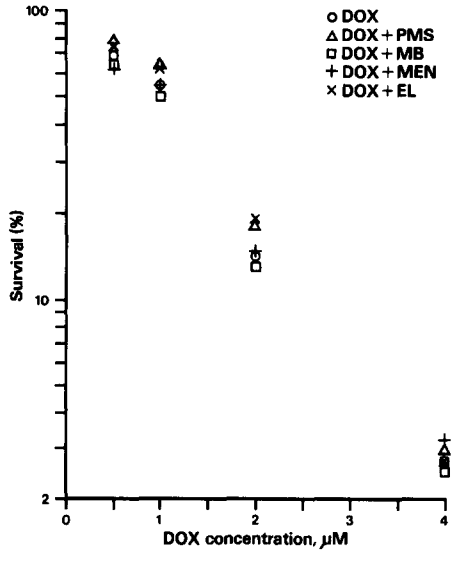

Fig. 4. Effect of 10 μM *PMS* (△), 25 μM *MB* (□), 10 μM *MEN* (+) *and* 7.5 μM *EL* (×) *on the clonal survival of SW-*1573 *cells treated with DOX.*

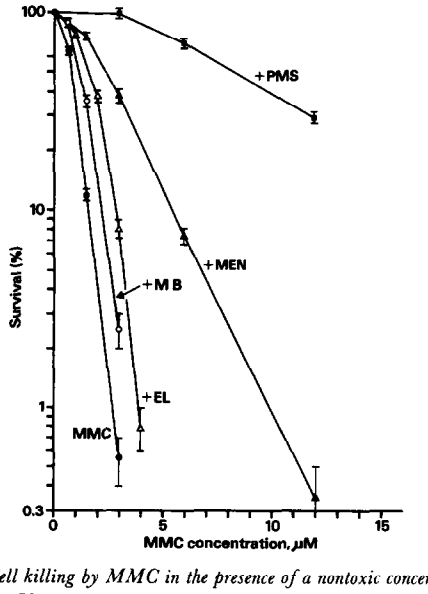

Fig. 5. Cell killing by MMC in the presence of a nontoxic concentration of AEA or EL. Effects are shown of 25 μM *MB* (○), 10 μM *MEN* (▲), 10 μM *PMS* (■) *and* 7.5 μM *EL* (△) *on clonal survival of MMC treated SW-*1573 *cells* (●).

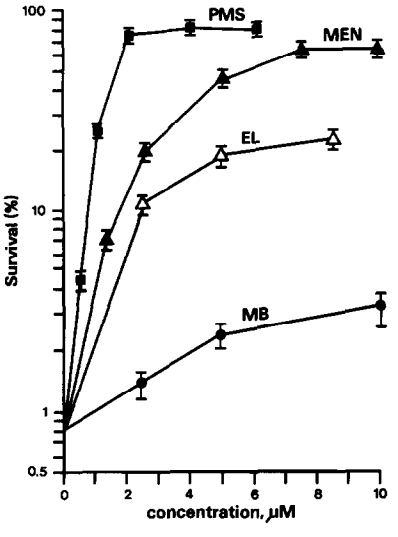

Fig. 6. Cellular protection against MMC by AEA/EL. The figure shows dose–response curves for the protection afforded by MB (●), *MEN* (▲), *PMS* (■) *and EL* (△) *against the cytotoxicity of a fixed concentration of MMC.*

Table 1. *Effect of AEA on NADPH/$NADP^+$ ratio and ATP levels in SW-*1573 *cells**

Treatment	Concentration (μM)	NADPH/$NADP^+$	ATP (%)
None	—	13.1 ± 1.5	100 ± 9
PMS	2	ND	98 ± 16
	10	11.0 ± 0.2	68 ± 6
MEN	10	11.3 ± 0.9	98 ± 11
MB	10	13.7 ± 0.9	99 ± 2

*Measurements were carried out after a 2 h incubation period with the indicated concentrations of each of the AEA. ND, not done. The ATP signal in untreated cells was set at 100 arbitrary units.

Effects of tBOOH and EL on the cytotoxicity of MMC and DOX

The data presented thus far confirm the proposed importance of metabolic reduction for the cytotoxicity of MMC. However, the AEA have the property in common that they might induce some form of sublethal oxidative damage resulting from the formation of free radicals. Such damage might trigger cellular adaptive responses possibly involved in the repair of MMC-induced DNA damage.

Tert-butylhydroperoxide (tBOOH), an agent that is not considered to be an efficient electron acceptor, but known to cause oxidative damage to cells [22], was utilized as a negative control. We found that treatment of SW-1573 cells with 25 μM tBOOH, causing 20% clonogenic cell death, did not afford any measurable protection against the cytotoxicity of MMC (results not shown). This suggests that a cellular response to induction of sublethal oxidative cell damage is not likely to be involved in the protective effects of PMS, MB and MEN against the cytotoxicity of MMC.

When inhibition of flavoprotein-mediated reduction of MMC by AEA is indeed responsible for the decreased MMC cytotoxicity by AEA, then inhibition of NADPH cytochrome P450 reductase, which is considered to be an important flavoprotein involved in the bioactivation of MMC [2], should also provide some degree of protection. As shown in Figs 5 and 6, EL, an agent known to inhibit the activity of NADPH cytochrome P450 reductase, indeed protected against the cytotoxicity of MMC, with a DMF of 1.8 ± 0.2 at 7.5 μM and with half-maximal protection at 2.5 μM. Since similar concentrations of EL were required in cell-free systems to inhibit NADPH cytochrome P450 reductase activity [14], these data confirm NADPH cytochrome P450 reductase being a flavoprotein involved in the bioreductive activation of MMC.

As expected from the lack of effect of AEA on the cytotoxicity of DOX, EL failed to protect against DOX when used at concentrations providing maximal protection against MMC. Only at much higher, i.e. strongly cytotoxic concentrations (15 μM) 20% survival), was a relative protection against DOX observed (DMF = 1.4).

DISCUSSION

Our data show that at completely nontoxic concentrations PMS, MB and MEN act as efficient electron acceptors in intact SW-1573 human lung tumor cells. Furthermore, these compounds did not decrease cellular ATP levels nor significantly altered the cellular NADPH/$NADP^+$ ratio (Table 1), indicating that the agents used behave as high-affinity electron acceptors in SW-1573 cells without seriously disturbing the normal redox status or energy household of the cells.

PMS, MB and MEN reduced the cytotoxicity of MMC by 90 ± 1, 29 ± 11 and 63 ± 3%, respectively, suggesting that these agents interfere with a major metabolic pathway leading to MMC cytotoxicity. Since the agents have the capacity in common to oxidize flavoproteins, it is plausible that this metabolic pathway involves the reduction of MMC by cellular flavoproteins. This was further indicated by the observations that EL, an inhibitor of NADPH cytochrome P450 reductase activity, also protected against the cytotoxicity of MMC.

A crude correlation seems to exist between the cytotoxicities of the AEA and the extents of protection against MMC cytotoxicity, suggesting that at high concentrations interference with the normal physiological functioning of flavoproteins might occur, which may even become lethal. Although being indicative for reduction of PMS, MB and MEN occurring in the cells, the cyanide-resistant respiration induced by these agents did not appear to correlate with the extent of protection against MMC cytotoxicity. It should be pointed out, however, that the AEA used are able to oxidize a wide variety of flavoproteins, which are unlikely to be all involved in the reduction of MMC.

Since metabolic reduction activates MMC into a bifunctional alkylating agent able to bind to proteins and DNA, interference with this activating step by AEA might reduce cellular MMC accumulation. However, since we cannot distinguish between a direct effect on in- or efflux of MMC and decreased drug binding due to decreased metabolic activation, the effect of AEA on MMC accumulation was not investigated.

Contrary to the effect on the cytotoxicity of MMC, all inhibitors used in this study failed to afford protection against the cytotoxicity of DOX. One possible explanation for this result could be that different flavoproteins are involved in the metabolic activation of MMC and of DOX. However, as far as we know, no flavoproteins have been described which reduce DOX but which do not donate electrons to one of the AEA used here. Therefore, this possibility seems unlikely.

More likely explanations for the lack of effect of PMS, MB, MEN and EL on DOX cytotoxicity may be (1) that these agents do inhibit the metabolic reduction of DOX, but that DOX does not need flavoprotein-mediated bioreduction for its cytotoxicity, or (2) that DOX is actually not reduced to a significant extent in the intact tumor cell. Presently we cannot discriminate between these two possibilities. On the one hand, flavoprotein-*in*dependent mechanisms of DOX cytotoxicity have been suggested [23, 24]; on the other hand, DOX is known to rapidly intercalate into the nuclear DNA, after which it can no longer be reduced by cellular flavoproteins [25].

Summarizing, our results are consistent with the notion that clonogenic killing of SW-1573 cells by MMC largely depends on metabolic reduction by cellular flavoproteins probably including NADPH cytochrome P450 reductase. However, the results are not readily compatible with a similar mechanism for DOX cytotoxicity.

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