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# Isolation and characterization of a mitomycin C-resistant variant of human colon carcinoma HT-29 cells

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**Abstract.** To investigate the resistant mechanisms against MMC in human tumor cells, we isolated an MMC-resistant variant (HT-29/MMC) of HT-29 human colon carcinoma cells. HT-29/MMC cells showed 5-fold resistance to MMC as compared with the parental cell line but did not show cross-resistance to Adriamycin, vincristine, ACNU, bleomycin, or cisplatin. Treatment of the cells with dicoumarol, an inhibitor of DT-diaphorase, reduced the cytotoxicity of MMC in DT-diaphorase proficient HT-29 cells but not in HT-29/MMC cells. HT-29/MMC cells were 5 times more sensitive than HT-29 cells to menadione, which is detoxified by DT-diaphorase. DT-diaphorase was deficient in HT-29/MMC cells as determined by the enzyme activity and immunoblot analysis of the cytoplasmic proteins. Levels of cytochrome P-450 reductase and glutathione S-transferase, however, were comparable in both cell lines. The amount of [3H]-MMC found covalently bound to chromosomal DNA in HT-29/MMC cells was one-fourth that detected in HT-29 cells. Treatment with dicoumarol reduced the DNA-bound MMC in HT-29 cells but not in HT-29/MMC cells. These results indicate that the deficiency in DT-diaphorase, an activating enzyme of MMC, is one of the mechanisms of resistance in HT-29/MMC cells.

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*Abbreviations:* MMC, mitomycin C; DCPIP, 2,6-dichlorophenolindophenol; TEMPOL, 4-hydroxytetramethyl piperidine-1-oxyl; ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl) methyl-3-(2-chloroethyl)-3-nitrosourea; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PMSF, phenyl methyl sulfonyl fluoride; PBS(-), phosphate-buffered saline without calcium or magnesium

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## Introduction

MMC is a clinically useful antitumor antibiotic that has received widespread attention because of its effectiveness against unresponsive solid tumors [1]. To exert its cytotoxic activities, MMC must be activated by bioreduction. Two pathways have been proposed for the reductive activation of MMC. The first metabolic pathway involves the one-electron reduction of MMC to its semiquinone radical, which possesses potent DNA-alkylating activity. Xanthine oxidase, reduced nicotinamide adenine dinucleotide phosphate (NADPH):cytochrome P-450 reductase, and NADPH reductase catalyze this one-electron reduction in biological systems [2–4]. The second metabolic pathway involves a two-electron reduction of MMC to form MMC hydroquinone directly, without an intermediate semiquinone. The MMC hydroquinone also possesses potent DNA-alkylating activities [5]. A major two-electron reductase is NAD(P)H-quinone oxidoreductase, which is known as DT-diaphorase (EC 1.6.99.2). This enzyme can utilize either NADH or NADPH as an electron donor and is specifically inhibited by dicoumarol [6].

The role of DT-diaphorase in the bioreductive activation of MMC has been controversial, and both the cellular and the biochemical data are in conflict. A murine lymphoblast cell line, which exhibits a 24-fold increase in the level of DT-diaphorase as compared with the wild-type cell line, is 5 times more sensitive to MMC than the wild type [7]. Dicoumarol, an inhibitor of DT-diaphorase, reduces aerobic MMC cytotoxicity in a number of cell lines [8–10]. Consistent with these results, MMC has been reported to be metabolized by DT-diaphorase purified from rat liver and HT-29 human colon carcinoma in a pH-dependent manner [11]. Recently, the cytotoxicity of MMC has been reported to be correlated with the activity of DT-diaphorase in colon carcinoma cell lines [12] and non-small cell lung carcinoma [13]. Contrary to these observations, the level of DT-diaphorase detected in three rodent cell lines did not correlate with their MMC cytotoxicity [8], and it has been reported that MMC is not a substrate but rather an inhibitor of purified human kidney DT-diaphorase [14].

The mechanisms of MMC resistance have not been well understood, partly because of the limited reports of isolation of MMC-resistant variants. A reduced level of NADPH:cytochrome P-450 reductase and an increased level of glutathione-S-transferase have been reported to relate to MMC resistance [15, 16]. It has been proposed that DT-diaphorase may be involved in the mechanisms of MMC resistance [10, 12, 17], and Chinese hamster ovary cells deficient in DT-diaphorase have been reported to be resistant to MMC under aerobic conditions [18].

To study the mechanisms of MMC resistance in human tumors, we isolated and characterized an MMC-resistant variant of HT-29 human colon carcinoma cells. We report herein that these MMC-resistant HT-29 human colon carcinoma cells were deficient in DT-diaphorase. We found that the activation of MMC by DT-diaphorase in DT-diaphorase proficient HT-29 cells was a major determinant of the cytotoxicity of MMC under aerobic conditions, whereas in DT-diaphorase deficient HT-29/MMC cells the activation of MMC to exert cytotoxicity was mediated by one-electron reduction.

## Materials and methods

*Chemicals.* MMC, Adriamycin, and [$C_6$-$CH_3$-$^3H_3$] MMC (70.4 Ci/mmol) were generous gifts from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Cisplatin was provided by Bristol Meyers Squibb Co. Ltd., Tokyo, Japan. Bleomycin was a gift from Nippon Kayaku Co. Ltd., Tokyo, Japan. Vincristine was provided by Eli Lilly Co. Ltd., Kobe, Japan. ACNU was provided by Sankyo Co., Ltd., Tokyo, Japan. NADH, NADPH, menadione, DCPIP, PMSF, proteinase K, and reactive blue (Cibacron blue) 2-Sepharose CL-6B were purchased from Sigma Chemical Company. Dicoumarol and TEMPOL were obtained from Wako Pure Chemical Industries, Japan. All other reagents were of analytical grade.

*Cell lines.* An MMC-resistant variant of HT-29 human colon carcinoma (HT-29/MMC) was established in our laboratory as previously described [19]. In brief, HT-29 human colon carcinoma cells were mutagenized with 0.35 mg ethyl methanesulfonate/ml and treated with 50 ng MMC/ml for 3 days. The cells were further treated with gradually increasing concentrations of MMC (up to 100 ng/ml) for 3 months. HT-29/MMC cells did not lose their resistance phenotype over 8 months in MMC-free medium. These cell lines were cultured in a monolayer in RPMI 1640 medium containing 5% fetal bovine serum and 100 mg kanamycin/ml (complete medium) and were kept in a humidified atmosphere (5% $CO_2$, 95% air) at 37° C.

*In vitro cytotoxicity assay.* For the colony-forming-ability assay, HT-29 and HT-29/MMC cells growing in the exponential phase were recovered and seeded into 6-well plates at a density of 1,000 cells/well in 2 ml complete medium and incubated at 37°C overnight. The cells were exposed to drugs at various concentrations for 2 h (MMC) or 6 h (menadione), and the medium was removed. The cells were washed with PBS(-) and further incubated in the complete medium for 7 days (HT-29 cells) and 12 days (HT-29/MMC cells). In the case of pretreatment with dicoumarol, the cells were pretreated with 200 μ*M* dicoumarol for 30 min prior to the addition of MMC. TEMPOL at 10 m*M* was added immediately before the MMC treatment. These concentrations of dicoumarol and TEMPOL did not reduce the colony-forming ability of the cells. The colonies formed were fixed with 10% (v/v) formaldehyde and stained with crystal violet to assess their numbers. For each drug concentration, the experiments were carried out in triplicate and repeated twice.

*Growth inhibition assay.* The growth inhibition assay was performed as previously described [20]. In brief, exponentially growing cells were plated into six-well plates in 2 ml growth medium. HT-29 cells were plated at a density of $2 \times 10^4$ cells/ml, and HT-29/MMC cells were plated at a density of $4 \times 10^4$ cells/ml. After the overnight culture at 37° C, drugs were added at various concentrations and incubated at 37° C for 3 days. Cells were trypsinized and counted with a Coulter counter. The 50% growth-inhibitory drug concentrations ($IC_{50}$ values) were determined as described elsewhere [21].

*Cell cytosol preparation.* HT-29 human colon carcinoma cells and HT-29/MMC cells were grown to subconfluence under standard conditions. The cell layer was washed with PBS(-), scraped into PBS(-) with a cell scraper, and collected by centrifugation at 150 g for 10 min at 4° C. The pelleted cells were then suspended in ice-cold reticulocyte standard buffer (10 m*M* TRIS-HCl, pH 7.4, 10 m*M* NaCl, and 1.5 m*M* $MgCl_2$) containing 0.1 m*M* PMSF and were allowed to stand for 10 min at 4° C. The cells were lysed by 20 strokes of the Dounce homogenizer. The homogenate was centrifuged at 105,000 *g* for 30 min at 4° C to yield a clear cytosol fraction. This cytosol was used as the enzyme source. Protein determination was performed using the method of Bradford [22].

*Enzyme activity assay.* DT-diaphorase activity was assayed essentially according to the method of Ernster [6], as modified by Benson et al. [23]. The reaction mixtures contained 25 m*M* TRIS-HCl (pH 7.4), 0.7 mg bovine serum albumin (BSA)/ml, 0.2 m*M* NADH, and 0.04 m*M* DCPIP. Reactions were performed at 25° C in the presence and absence of 20 μ*M* dicoumarol. DT-diaphorase activity was measured as the dicoumarol-sensitive reduction of DCPIP. The reduction of DCPIP was monitored by measuring the absorbance at 600 nm using a Beckman single-beam spectrophotometer. Glutathione S-transferase was assayed according to the method of Hayes and Clarkson [24], and NADPH:cytochrome P-450 reductase activity was measured according to the method of Vermillion and Coon [25].

*Purification of HT-29 DT-diaphorase.* The cytosolic fraction of HT-29 cells was prepared from the cultured HT-29 cells as described above or from the homogenate of the solid HT-29 tumor grown in BALB/c nude mice. Both methods gave essentially the same results. In the latter case, HT-29 cells ($3 \times 10^6$/mouse) were inoculated s.c. into BALB/c nude mice. After 2 weeks, the mice were killed by perivertebral translocation and the globular HT-29 solid tumor mass was removed, freed from the host tissue, minced, and homogenized. The infiltrating host cells in the tumor were minimal.

DT-diaphorase was purified from these cytosolic fractions using Cibacron blue affinity chromatography as previously described [26, 27]. The purified DT-diaphorase was resolved as a single band with a molecular weight of 32–33 kDa on SDS-PAGE and had a specific activity of 84 nmol $min^{-1}$ μg $protein^{-1}$, which was coincident with the previous report [27].

*Immunoblot analysis.* The immunoblot analysis was performed essentially according to the ECL Western blotting protocol (Amersham). The cytosolic proteins from HT-29 and HT-29/MMC cells and the purified DT-diaphorase were subjected to SDS-PAGE (10%–20% gradient). After undergoing electrophoresis, the proteins were transferred to a nitrocellulose membrane. The protein blot was incubated for 2 h with a human DT-diaphorase monoclonal antibody prepared in our laboratory. The monoclonal antibody against DT-diaphorase was prepared as previously described [28]. In brief, DT-diaphorase was purified from HT-29 cells and inoculated into BALB/c mice. A series of monoclonal antibodies against DT-diaphorase were then obtained as previously described [28], and one of these monoclonal antibodies (IgG) was used in the immunoblot analysis. For the detection of P-glycoprotein, cell lysates of HT-29 and HT-29/MMC cells were analyzed by immunoblotting using the C-219 monoclonal antibody as the first antigen.

*Measurement of DNA alkylation.* To compare the extent of MMC activation, we measured the levels of DNA-bound [$^3$H]-MMC, as activated MMC can bind only to DNA. DNA was isolated as previously described [29]. In brief, HT-29 cells and HT-29/MMC cells were incubated with 0.5 μCi [$^3$H]-MMC/ml (70.4 Ci/mmol) for 2 h at 37° C under standard conditions. When dicoumarol was used, cells were treated with 200 μ*M*

**Table 1.** $IC_{50}$ values of various antitumor agents

| Compounds | $IC_{50}$ (ng/ml)[a] | | Resistance ratio[b] |
|---|---|---|---|
| | HT-29 | HT-29/MMC | |
| MMC | 20±3 | 102±6 | 5 |
| MMC + verapamil[c] | 18±2 | 95±4 | 5.3 |
| Adriamycin | 16±2 | 15±3 | 1 |
| Vincristine | 5±0.3 | 2±0.1 | 0.4 |
| Cisplatin | 2±0.4[d] | 2±0.4[d] | 1 |
| Bleomycin | 10±2[d] | 2±0.4[d] | 0.2 |
| ACNU | 43±2[d] | 48±2[d] | 1.1 |

[a] $IC_{50}$ values were obtained from a dose-response curve evaluating five different drug concentrations in triplicate and represent mean values ± SD

[b] Ratio of $IC_{50}$ values for HT-29/MMC versus HT-29 cells

[c] In the presence of 10 μ*M* verapamil

[d] In μg/ml

dicoumarol for 30 min prior to the addition of [$^3$H]-MMC. TEMPOL, however, was added immediately before treatment with [$^3$H]-MMC.

After their incubation, the cell layers were washed three times with ice-cold PBS(-), trypsinized, and suspended in ice-cold PBS(-). The cells were then washed three times with ice-cold PBS(-) by centrifugation at 150 *g* for 5 min. The pelleted cells were resuspended in 5 ml TNE buffer [10 m*M* TRIS-HCl, pH 7.5, 0.1 *M* NaCl, 1 m*M* ethylenediaminetetraacetic acid (EDTA)], after which 250 μl 10% (w/v) SDS and 50 μl proteinase K (10 mg/ml) were added. The cells were incubated overnight at 55° C, extracted three times with an equal volume of phenol saturated with TE buffer (10 m*M* TRIS-HCl, pH 7.5, 1 m*M* EDTA), and then extracted once with an equal volume of phenol/chloroform (1:1, v/v). The DNA was precipitated by adding 1/50 vol. 5 *M* NaCl and 2.5 vol. cold absolute EtOH. The precipitated DNA was washed with 70% EtOH and dried at room temperature. The purified DNA was dissolved in TE buffer. The yield was 100–130 μg DNA/$10^7$ cells as determined by spectrophotometry. The radioactivity of [$^3$H]-MMC in the isolated genomic DNA was determined by liquid scintillation counting.

## Results

### *MMC-resistant HT-29 cells*

The MMC-resistant variant of HT-29 human colon carcinoma cells (HT-29/MMC) was established by intermittent exposure to MMC. The doubling time of HT-29 cells and HT-29/MMC cells was about 16 and 29 h, respectively. We measured the $IC_{50}$ values in HT-29 cells and HT-29/MMC cells for several antitumor drugs (Table 1). HT-29/MMC cells were 5 times more resistant to MMC than the parental cells, but the resistance was not overcome by the addition of 10 μ*M* verapamil. The resistant cells did not express P-glycoprotein when examined by immunoblot analysis (unpublished data). The responses to Adriamycin, ACNU, and cisplatin were comparable in both cell lines. HT-29/MMC cells were collaterally sensitive to vincristine and bleomycin.

### *DT-diaphorase deficiency in HT-29/MMC cells*

To characterize the mechanisms of the resistance to MMC in HT-29/MMC cells, we measured the effect of TEMPOL, which has superoxide dismutase-like activity [30, 31], on

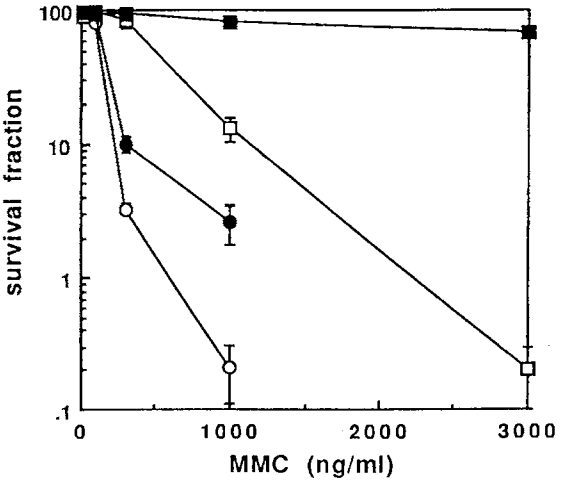


**Fig. 1.** Effect of TEMPOL on the cytotoxicity of MMC in HT-29 and HT-29/MMC cells. Exponentially growing HT-29 (●,○) and HT-29/MMC (■,□) cells were exposed for 2 h to MMC at various concentrations in the presence (●,■) or absence (○,□) of 10 mM TEMPOL. TEMPOL was added immediately before the addition of MMC. Dose-response curves were determined by colony-forming-ability assays. Points represent mean values, and bars indicate the SD of two independent experiments performed in triplicate

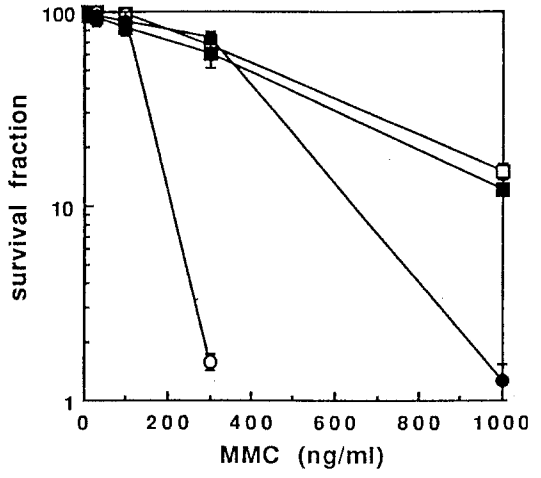


**Fig. 2.** Effect of dicoumarol on the cytotoxicity of MMC in HT-29 and HT-29/MMC cells. Exponentially growing HT-29 (●,○) and HT-29/MMC (■,□) cells were exposed for 2 h to MMC at various concentrations in the presence (●,■) or absence (○,□) of 200 μ*M* dicoumarol. Dicoumarol was added 30 min prior to the addition of MMC. Dose-response curves were determined by colony-forming-ability assays. Points represet mean values, and bars indicate the SD of two independent experiments performed in triplicate

the cytotoxicity of MMC against HT-29/MMC and HT-29 cells. TEMPOL at 10 m*M* almost completely eliminated the cytotoxicity of MMC in HT-29/MMC cells and weakly reduced the cytotoxicity in HT-29 cells, as shown in Fig. 1. These results indicate that the active radical species of MMC was produced to a greater extent in HT-29/MMC cells than in HT-29 cells. Dicoumarol significantly decreased the cytotoxicity of MMC against HT-29 cells, which had high levels of DT-diaphorase [27], whereas the

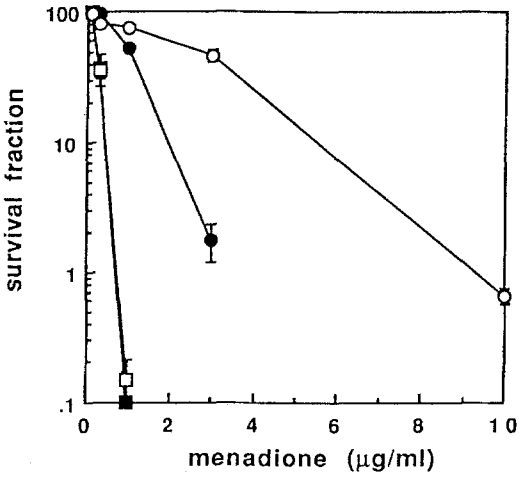


**Fig. 3.** Cytotoxicity of menadione to HT-29 and HT-290/MMC cells. Exponentially growing HT-29 (●,○) and HT-29/MMC (■,□) cells were exposed for 6 h to menadione at various concentratoins in the presence (●,■) or absence (○,□) of 200 μ*M* dicoumarol. Dicoumarol was added 30 min prior to the addition of menadione. Dose-response curves were determined by colony-forming-ability assays. Points represent mean values, and bars indicate the SD of two independent experiments performed in triplicate

**Table 2.** Enzyme activities of HT-29 and HT-29 MMC cells[a]

| Enzymes | HT-29 | HT-29/MMC |
|---|---|---|
| DT-diaphorase | 1800 ±88 | ND[b] |
| NADPH: cytochrome P-450 reductase | 2.7± 0.12 | 2.6± 0.1 |
| Glutathione S-transferase | 105 ±13 | 95 ±10 |

[a] Data represent mean values ± SD for two independent experiments performed in triplicate and are expressed in nmol mg protein$^{-1}$ min$^{-1}$
[b] Not detectable (<5 nmol mg protein$^{-1}$ min$^{-1}$)

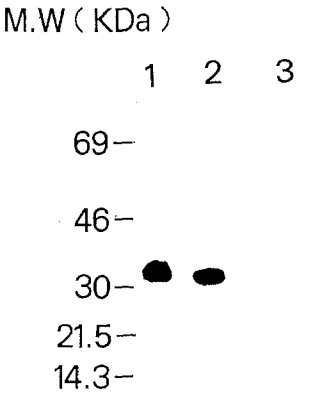


**Fig. 4.** Immunoblot analysis of DT-diaphorase. *Lane 1*, Purified HT-29 DT-diaphorase (50 ng/lane); *lanes 2 and 3*, cytosolic protein (15 μg/lane) from HT-29 and HT-29/MMC cells, respectively

**Table 3.** Amounts of DNA-bound [$^3$H]-MMC detected in HT-29 and HT-29/MMC cells

| | DNA-bound radioactivity (dpm/100 μg genomic DNA) | Index[a] |
|---|---|---|
| HT-29 | 830±22 | 1.0 |
| HT-29 + Di[b] | 489±27 | 0.55 |
| HT-29 + TE[c] | 810±51 | 0.91 |
| HT-29 + Di + TE | 178±15 | 0.20 |
| HT-29/MMC | 214±19 | 0.24 |
| HT-29/MMC + Di | 223±23 | 0.25 |
| HT-29/MMC + TE | 110±15 | 0.12 |
| HT-29/MMC + Di + TE | 115±14 | 0.13 |

DNA-bound [$^3$H]-MMC was measured after the isolation of genomic DNA following a 2-h exposure to 0.5 μCi [$^3$H]-MMC/ml as described in Materials and methods
[a] Relative binding ratio as compared with the parental HT-29 cells
[b] 200 μ*M* dicoumarol was added 30 min prior to the addition of [$^3$H]-MMC
[c] 10 m*M* TEMPOL was added immediately before the addition of [$^3$H]-MMC

sensitivity of HT-29/MMC cells to MMC was not affected by the pretreatment with dicoumarol (Fig. 2). These results suggest that the metabolism of MMC differs between HT-29/MMC cells and HT-29 cells.

We next measured the cytotoxicity of menadione, a physiological substrate of DT-diaphorase, against both cell lines. DT-diaphorase detoxifies menadione by two-electron reduction [32]. HT-29/MMC cells were more sensitive to menadione than were DT-diaphorase proficient HT-29 cells (Fig. 3). Although the sensitivity of HT-29 cells to menadione was increased in the presence of dicoumarol, that of HT-29/MMC cells was not altered.

The lack of a protective effect of dicoumarol against the cytotoxicity of MMC in HT-29/MMC cells and the hypersensitivity to menadione of HT-29/MMC cells under aerobic conditions suggested that the activity of DT-diaphorase in the HT-29/MMC cells could be substantially lower than that in the parental cells. For an investigation of this possibility, the cytosolic fractions were prepared from HT-29/MMC and HT-29 cells and the DT-diaphorase activity was measured as previously described [6, 23]. The activity of DT-diaphorase in HT-29 cells was 1800 nmol mg protein$^{-1}$ min$^{-1}$, which was coincident with the activity shown in the previous report [27], whereas DT-diaphorase activity was not detected in HT-29/MMC cells (Table 2).

To analyze further the deficiency in DT-diaphorase activity in HT-29/MMC cells, we performed an immunoblot analysis of the cytoplasmic proteins from both cell lines. DT-diaphorase was not detected in HT-29/MMC cells (Fig. 4). Furthermore, two-dimensional SDS-PAGE analysis of the cytoplasmic proteins revealed the deficiency of DT-diaphorase in HT-29/MMC cells (data not shown). Thus, HT-29/MMC cells were deficient in DT-diaphorase, which could be deeply involved in the resistance mechanism of HT-29/MMC cells to MMC.

### *DNA alkylation*

We speculated that since HT-29/MMC cells were deficient in DT-diaphorase, the amounts of MMC hydroquinone, which ultimately leads to DNA alkylation, might be decreased in the resistant cells. To prove this, we compared the amounts of the genomic DNA-bound [$^3$H]-MMC in

HT-29 cells and HT-29/MMC cells after treatment of the cells with [3H]-MMC. As shown in Table 3, the DNA-bound radioactivity in HT-29/MMC cells was one-fourth that in HT-29 cells. Treatment of the cells with dicoumarol decreased the DNA-bound radioactivity in HT-29 cells but not inHT-29/MMC cells. These data confirm that MMC was metabolized by DT-diaphorase and that a deficiency in DT-diaphorase prevented the activation of MMC to the hydroquinone form. This resulted in a decrease in the covalent binding of MMC to DNA.

## Discussion

We established an MMC-resistant variant (HT-29/MMC) of the HT-29 human colon carcinoma cell line. The HT-29/MMC cells were 5 times more resistant to MMC than were the parental HT-29 cells. The doubling time of the HT-29/MMC cells was about twice that of the parental HT-29 cells. A part of the resistance of HT-29/MMC cells might be due to their longer doubling time; however, the sensitivity of HT-29/MMC cells to other antitumor agents such as Adriamycin and cisplatin was comparable with that of the parental HT-29 cells. In fact, the MMC-DNA adduct was remarkably reduced in the HT-29/MMC cells. Therefore, the difference in doubling time could not be deeply involved in the resistance to MMC of HT-29/MMC cells.

MMC requires reductive activation to exert its cytotoxic effect. The newly established HT-29/MMC cells are deficient in DT-diaphorase, which could result in several changes in the metabolism of MMC. One metabolic change might be the reduction of MMC hydroquinone, an activated form of MMC, which ultimately leads to DNA alkylation. In fact, the amount of DNA-bound [3H]-MMC was lower in HT-29/MMC cells than in HT-29 cells (Table 3). Since the treatment of the cells with dicoumarol decreased the DNA-bound radioactivity of MMC in HT-29 cells but not in HT-29/MMC cells, the reduction in the amounts of alkylated DNA in HT-29/MMC cells can at least in part be attributed to the inability of the cells to form MMC hydroquinone because of their deficiency in DT-diaphorase. DT-diaphorase purified from rat liver has been unequivocally shown to bioactivate mitomycin C [33]. Also, the role of DT-diaphorase in the mechanism of MMC resistance has been discussed in human cell strains [17] and in human tumor cells [10, 13]. In accordance with these reports, our results indicate a high likelihood that the deficiency in DT-diaphorase is involved in the mechanism of MMC resistance in HT-29/MMC cells.

Another possible metabolic change of MMC in HT-29/MMC cells would be that MMC is metabolized only by one-electron reduction and that the amount of radical species, including the MMC semiquinone radical, may thus be increased in the resistant cells. In such a case, the effects of radical scavengers, such as TEMPOL, could be expected to be more effective in HT-29/MMC cells than in the parental HT-29 cells. The aerobic cytotoxicity of MMC in HT-29/MMC cells was almost completely eradicated by TEMPOL, but the cytotoxicity against HT-29 cells was only slightly inhibited by TEMPOL (Fig. 1). Also, the DNA-bound radioactivity of [3H]-MMC in HT-29/MMC cells was reduced by treatment with TEMPOL but was not evident in HT-29 cells (Table 3). MMC could be metabolized by both one- and two-electron reduction in HT-29 cells. When HT-29 cells were pretreated with dicoumarol, the MMC could be metabolized only by one-electron reduction, as the DT-diaphorase had been inactivated. Therefore, the addition of TEMPOL after dicoumarol pretreatment could result in a significant reduction in the amounts of DNA-bound [3H]-MMC.

These results suggest that in DT-diaphorase proficient HT-29 cells, two-electron reduction of MMC may play an important role in the aerobic cytotoxicity of MMC and that the deficiency of DT-diaphorase in HT-29/MMC cells causes resistance to MMC. MMC was metabolized only by one-electron reduction in DT-diaphorase deficient HT-29/MMC cells, and in the resistant cells, radical species of MMC including semiquinone, which are inactivated by TEMPOL, may be a critical determinant in the aerobic cytotoxicity of MMC.

In summary, the present results suggest that the resistance of HT-29/MMC cells to MMC under aerobic conditions is caused by their deficiency in DT-diaphorase, which results in a reduction in the levels of MMC hydroquinone ultimately leading to DNA alkylation. DT-diaphorase deficient HT-29/MMC cells metabolize MMC to its semiquinone radical only by one-electron reduction, which may be the critical determinant in the aerobic cytotoxicity of MMC against the resistant cells. On the other hand, in DT-diaphorase proficient HT-29 cells, two-electron reduction of MMC catalyzed by DT-diaphorase may be the predominant determinant of the aerobic cytotoxicity of MMC.

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